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Molecular Evidence on the Evolutionary and Biogeographical Patterns of European Cyprinids

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Abstract. The phylogenetic relationships of 106 European cyprinid taxa were determined based on the complete nucleotide sequence (1140 bp) of the mitochondrial cytochrome *b* gene. The molecular phylogeny was used (1) to revise the current systematics of European cyprinids, (2) to establish the phylogenetic utility of traditional morphological characters that are widely used in Cyprinidae systematics, and (3) to discuss alternative hypotheses on the biogeography of the family in Europe. The age of the major lineages within European cyprinids was tentatively estimated with a molecular clock and showed full agreement with the fossil record of the group. Moreover, the results provided unambiguous evidence for a close phylogenetic affinity of some Caucasian and Greek endemic cyprinid taxa (e.g., *B. capito* and *B. brachycephalus* and *Leuciscus keadicus*, *Barbus graecus*, and *B. albanicus*, respectively) to Iberian and North African, but not Central European, cyprinids. The existence of such unexpected phylogenetic relationships refutes the classical hypothesis on the biogeography of European cyprinids, which assumes a dispersal of the cyprinid fauna from central Europe to southern Europe and northern Africa during the Miocene (and, hence, predicts a close phylogenetic relationship of all Caucasian, Greek, Iberian, and North African cyprinids to central European taxa). Instead, the existence of a Mediterranean realm independent of the central European route seems plausible based on the molecular evidence. It is likely that the new biogeographical scenario proposed here might apply to other primary freshwater European animals with low dispersal abilities, including fish, amphibians, and invertebrates.

Key words: Molecular phylogeny — Cytochrome *b* — Biogeography — Cyprinids

Introduction

Cyprinids, the largest and most successful family of primary freshwater fish in Eurasia, Africa, and northern America, are a good model for comprehending the evolutionary mechanisms driving the diversification and distribution of species. Primary freshwater fish are restricted to river and lake drainage systems and show little capacity for transwatershed dispersal. Thus, their distribution closely reflects their biogeographical history.

In particular, European cyprinids have an interesting pattern of distribution, in which numerous endemic species are found on the Iberian Peninsula and in southern Greece, with a relatively uniform fauna in Central Europe. Traditionally, it is believed that European cyprinids originated in eastern Asia and subsequently spread to Siberia during the Eocene (Banarescu 1989, 1992). However, because Europe was separated from Siberia by the shallow Ob Sea, their dispersal to Europe was impeded. During the Oligocene, cyprinids were able to colonize Europe because of the uplift of the Urals but ended once these mountains were formed. Three alternative hypotheses have been proposed to explain the further dispersion of cyprinids across Europe during the Miocene.

The first, classical, hypothesis proposes that cyprinids spread across central Europe via river connections to the

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southern part of the continent and northern Africa (across the Gibraltar strait) until the Pliocene (Banarescu 1960, 1989, 1992; Almaça 1976, 1988). Subsequent isolation of the Iberian Peninsula and southern Greece from the rest of the continent would have been responsible for their rich endemic fauna. During most of the Miocene, southern Italy was below sea level (Steininger and Rögl 1984), and therefore, its cyprinid fauna would have a more recent origin (Plio-Pleistocene) (Bianco 1990). Furthermore, the uniform cyprinid fauna of central Europe was seriously depleted during the glacial periods and replaced mainly with Danubian cyprinid fauna during interglacial and postglacial periods (Banarescu 1989, 1992).

The second hypothesis (Doadrio 1990, 1994) is based on the close affinities of Asian, North African, southern Greek, and Iberian barbels. According to this hypothesis, the formation of the actual North African coast by landmass movements across the Mediterranean Sea in the early Pliocene favored the South Mediterranean vicariance of barbels and the subsequent colonization of Northern Africa and the Iberian Peninsula (Doadrio 1990, 1994).

Finally, the third hypothesis (Bianco 1990) proposes that a major dispersal of cyprinids occurred around the circum-Mediterranean region during the Messinian salinity crisis (6–5 MYA), when the Mediterranean basin almost dried up and was subsequently refilled with fresh water from the Sarmatic Sea (Paratethys) (Hsü et al. 1977). According to this hypothesis, the so-called Lago Mare phase of the Mediterranean would have allowed the dispersal of freshwater fish across the Mediterranean and would be responsible for the actual high level of endemics on the Iberian Peninsula and in Southern Greece (Bianco 1990). However, the existence of the Lago Mare phase of the Mediterranean is highly controversial, and no fossil data support the complete desiccation of the Mediterranean basin (Steininger and Rögl 1984).

The above three hypotheses proposed to explain the colonization of Europe by cyprinids during the Miocene are based mainly on the actual distribution patterns, fossil record, and morphological similarities of European cyprinid taxa rather than on their phylogenetic relationships (but see Doadrio 1990, 1994). However, to discern alternative hypotheses on the biogeographical origin of European cyprinids, it is necessary to understand their phylogenetic relationships. The disjunct distribution of European cyprinids can be interpreted only within a phylogenetic framework that establishes monophyletic groups within the taxon.

In the present study we have examined the phylogenetic relationships of representatives of most of the cyprinid genera occurring in Europe to revise the systematics of the family, to evaluate the phylogenetic utility of the morphological characters that have traditionally been used in inferring phylogenetic relationships within Cyprinidae, and to test among the three alternative biogeographical hypotheses. Complete mitochondrial cytochrome *b* sequences (1140 bp) of 89 cyprinid taxa from the Iberian Peninsula, Greece, and the Caucasus were determined (see Table 1) and analyzed with the currently used methods of phylogenetic inference. Cytochrome *b* sequences of 15 cyprinids from central Europe (Briolay et al. 1998) (see Table 1), *Cyprinus carpio* (Chang et al. 1994), and *Carassius auratus* (Zardoya and Doadrio 1998) were also included in the phylogenetic analyses.

Materials and Methods

Laboratory Procedures

Fish samples representing 89 taxa of European cyprinids (one specimen per taxon) were collected on the Iberian Peninsula (Zardoya and Doadrio 1998), in Greece (Zardoya et al. 1999), and in the Caucasus (this paper) (Table 1). Total cellular DNA was extracted from muscle following standard phenol/chloroform protocols (Towner 1991). Primers specifically designed for cyprinids (Schmidt and Gold 1993; Zardoya and Doadrio 1998) were used to amplify, via PCR, the entire cytochrome *b* gene. Thirty-five to forty cycles of PCR (denaturing at 94°C for 60 s, annealing at 45–50°C for 60 s, and extension at 72°C for 105–180 s) were performed in 25-μl reactions containing 67 m*M* Tris–HCl, pH 8.3, 1.5 m*M* $MgCl_2$, a 0.4 m*M* concentration of each dNTP, a 2.5 μ*M* concentration of each primer, template DNA (10–100 ng), and Taq DNA polymerase (1 U; Promega). PCR products were cloned using the pGEM-T vector (Promega) into *E. coli* JM109 and sequenced using the FS-Taq Dye Deoxy Terminator cycle-sequencing kit (Applied Biosystems Inc.) on an automated DNA sequencer (Applied Biosystems 377) following the manufacturer's instructions. DNA sequences of both strands were obtained using M13 universal (forward and reverse) sequencing primers.

Phylogenetic Analyses

The new sequences were aligned with the complete cytochrome *b* sequences of 15 French (Briolay et al. 1998) cyprinids, *Cyprinus carpio* (Chang et al. 1994), and *Carassius auratus* (Zardoya and Doadrio 1998). One Characidae (*Astyanax fasciatus*) (Zardoya and Doadrio 1998) and one Balitoridae (*Crossostoma lacustre*) (Tzeng et al. 1992) were used as outgroup taxa. Alignment was based on the inferred amino acid sequence. No ambiguous alignments were found and no gaps were postulated. All codon positions were included in the phylogenetic analyses.

Maximum-parsimony (MP) analyses [PAUP* version d64 (Swofford 1998)] were performed using heuristic searches (TBR branch swapping; MULPARS option in effect) with 10 random stepwise additions of taxa. Transversions (Tv) were given 4 or 10 times the weight of transitions (Ts). The ancestral character-state reconstruction of the evolution of the number of pharyngeal tooth rows was performed using MacClade version 3.06 (Maddison and Maddison 1992). Neighbor-joining (NJ) (Saitou and Nei 1987) analyses based on HKY85 corrected distance matrices (using empirical Ts/Tv ratios and base frequencies) were performed with PAUP* version d64 (Swofford 1998). The robustness of the inferred MP and NJ trees was tested by bootstrapping (Felsenstein 1985) (as implemented in PAUP*, with 100 and 500 pseudoreplications, respectively). Maximum-likelihood (ML) analyses (based on the HKY85 model with empirical Ts/Tv ratios and base

Table 1. Cyprinid taxa, sampling drainages, and GenBank accession numbers

Taxa	Drainage	Genbank No.
Greece		
Alburnoides bipunctatus ohridanus	Aoos	AF090740
Alburnoides b. ohridanus	Prespa	AF090741
Alburnoides b. strymonicus	Strymon	AF090742
Alburnus alburnus macedonicus	Doirani	AF090743
Alburnus a. strumicae	Strymon	AF090745
Alburnus a. thessalicus	Pinios	AF090744
Barbus albanicus	Arachthos	AF090779
Barbus barbus macedonicus	Axios	AF090780
Barbus b. thessalus	Pinios	AF090781
Barbus cyclolepis cyclolepis	Evros	AF090782
Barbus c. sperchiensis	Sperchios	AF090783
Barbus c. strumicae	Agiaki	AF090784
Barbus euboicus	Manikiotiko	AF090785
Barbus graecus	Kifissos	AF090786
Barbus peloponnesius	Alphios	AF090787
Barbus petenyi	Aliakmon	AF090788
Barbus petenyi	Vegoritis	AF090789
Barbus prespensis	Prespa	AF090790
Barbus rebeli	Aoos	AF090791
Chalcalburnus belvica	Prespa	AF090746
Chondrostoma prespensis	Prespa	AF090747
Chondrostoma vardarensis	Aoos	AF090748
Chondrostoma vardarensis	Aoos	AF090749
Gobio banarescui	Aliakmon	AF090751
Gobio gobio balcanicus	Gallikos	AF090750
Leuciscus cephalus macedonicus	Nestos	AF090752
Leuciscus c. prespensis	Prespa	AF090753
Leuciscus c. vardarensis	Sperchios	AF090754
Leuciscus cephalus	Manikiotiko	AF090755
Leuciscus peloponnensis	Thyamis	AF090756
Leuciscus peloponnensis	Alphios	AF090757
Leuciscus p. moreoticus	Stymphalia	AF090758
Leuciscus borysthenicus	Fotolivos	AF090759
Leuciscus keadicus	Evrotas	AF090760
Pachychilon macedonicus	Axios	AF090761
Pachychilon pictus	Aoos	AF090762
Phoxinellus prespensis	Prespa	AF090763
Pseudophoxinus stymphalicus	Stymphalia	AF090766
Pseudophoxinus stymphalicus	Trichonis	AF090767
Pseudophoxinus st. marathonicus	Kifissos	AF090768
Pseudophoxinus st. thesproticus	Louros	AF090769
Rutilus prespensis	Prespa	AF090771
Rutilus rutilus	Strymon	AF090772
Rutilus ylikiensis	Kifissos	AF090773
Rutilus ylikiensis	Trichonis	AF090774
Scardinius acarnanicus	Trichonis	AF090775
Telestes beoticus	Kifissos	AF090770
Telestes pleurobipunctatus	Arachthos	AF090764
Telestes p. alfiensis	Alphios	AF090765
Tropidophoxinellus hellenicus	Pinios	AF090776
Tropidophoxinellus spartiaticus	Evrotas	AF090777
Vimba melanops	Strymon	AF090778
Portugal		
Chondrostoma lusitanicum	Arade	AF045986
Chondrostoma macrolepidotus	Mondego	AF045980
Spain		
Anaecypris hispanica	Guadiana	AF045978
Barbus bocagei	Duero	AF045969
Barbus comizo	Tajo	AF045967
Barbus steindachneri	Guadiana	AF045968
Barbus graellsii	Ebro	AF045973
Barbus guiraonis	Buyent	AF045972
Barbus haasi	Ebro	AF045976
Barbus meridionalis	Tordera	AF045977
Barbus microcephalus	Guadiana	AF045971
Barbus sclateri	Guadalquivir	AF045970
Chondrostoma arcasii	Duero	AF045979
Chondrostoma lemmingii	Guadiana	AF045987
Chondrostoma lemmingii	Guadiana	AF045988
Chondrostoma lemmingii	Guadalquivir	AF045989
Chondrostoma polylepis	Tajo	AF045982
Chondrostoma p. duriensis	Duero	AF045983
Chondrostoma p. willkommii	Guadalquivir	AF045984
Chondrostoma toxostoma	Ebro	AF045985
Gobio gobio	Tajo	AF045996
Iberocypris palaciosi	Guadalquivir	AF045990
Leuciscus carolitertii	Duero	AF045994
Leuciscus cephalus	Ebro	AF045995
Leuciscus alburnoides	Guadiana	AF045992
Leuciscus pyrenaicus	Guadiana	AF045991
Algeria		
Barbus callensis	Kebir	AF045974
France		
Abramis brama	Saone	Y10441
Alburnoides bipunctatus	Saone	Y10445
Alburnus alburnus	Rhone	Y10443
Barbus barbus barbus	Durance	Y10450
Blicca bjoerkna	Saone	Y10442
Chondrostoma nasus	Rhone	Z75109
Gobio gobio	Rhone	Y10452
Leucaspius delineatus	Rhone	Y10447
Leuciscus cephalus	Rhone	Y10446
Phoxinus phoxinus	Rhone	Y10448
Pseudorasbora parva	Rhone	Y10453
Rutilus rutilus	Saone	Y10440
Scardinius erythropthalmus	Rhone	Y10444
Telestes souffia	Saone	Y10439
Tinca tinca	Saone	Y10451
The Caucasus		
Alburnus filippii	Samur	AF095602
Barbus brachycephalus	Terek	AF095603
Barbus capito	Terek	AF045975
Barbus ciscaucasicus	Kuma	AF095604
Barbus tauricus	Kuban	AF095605
Chondrostoma oxyrhynchum	Samur	AF095606
Gobio ciscaucasicus	Uluchaj	AF095607
Hemiculter leucisculus	Sulak	AF095608
Leuciscus cephalus orientalis	Rubas	AF095609
Rutilus caspicus	Samur	AF095610

frequencies) were performed using PUZZLE version 4.0 (Strimmer and von Haeseler 1996) and 10,000 pseudo-replications.

A rate constancy test (two-cluster test) was performed with LINTREE (Takezaki et al. 1995) using the HKY85 distance. Those taxa that showed significantly different substitution rates (at the 5% level) were excluded from further analyses. The remaining taxa were reanalyzed in PUZZLE version 4.0 (Strimmer and von Haeseler 1996) with ML using the clock-like option to obtain a clock-constrained tree (in which all root-to-tip distances have equal value). ML analyses were based on the HKY85 model with empirical Ts/Tv ratios and base frequencies.

European cyprinid biogeography was analyzed in a cladistic framework using Diva 1.1. (Ronquist 1997). To establish a putative biogeographical scenario, the inferred molecular phylogeny as well as the

actual distribution of the taxa was analyzed, and ancestral distributions were optimally reconstructed taking into account both vicariant and dispersal events.

Results

Phylogenetic Relationships of European Cyprinids

A total of 1140 positions was analyzed, of which 513 were constant sites and 500 were phylogenetically informative sites using the parsimony criterion. An overall Ts/Tv ratio of 4.6 was estimated for this data set. Pairwise sequence divergence between taxa varied from 0.3 to 25%. Variability among sequences was detected mainly in third codon positions. Substitutions showed some level of saturation in third codon positions (between 25 and 55% sequence divergence) but not in first and second codon positions (not shown).

The phylogenetic analysis of the European cyprinid data set with NJ, using *Astyanax fasciatus* (Characidae) (Zardoya and Doadrio 1998) and *Crossostoma lacustre* (Balitoridae) (Tzeng et al. 1992) as outgroup taxa, recovered the tree shown in Fig. 1. The robustness of this tree was confirmed by bootstrapping (Felsenstein 1985) (Fig. 1). Two main clades corresponding to the subfamilies Cyprininae and Leucisicinae (Cavender and Coburn 1992) were found (Fig. 1). Moreover, at least two lineages of *Barbus* (*Barbus* s.s. and *Luciobarbus*), one of *Hemiculter*, one of *Tinca*, one of *Gobio*, one of *Phoxinus*, one of *Pseudophoxinus* + *Phoxinellus*, and nine of Leuciscinids (including *Pachychilon, Pseudophoxinus, Scardinius, Tropidophoxinellus, Abramis* + *Vimba* + *Blicca, Anaecypris* + *Leucaspius* + *Chalcalburnus* + *Alburnus, Alburnoides, Leuciscus, Rutilus* s.str., and *Telestes* s.l. + *Chondrostoma*) were identified (Fig. 1). Interestingly, *Hemiculter, Tinca, Gobio, Phoxinus*, and *Alburnus* (and relatives), which have been traditionally classified in their own subfamilies (for a review see Howes 1991), are recovered as distinct, more or less basal lineages within Leuciscinae (Fig. 1). Moreover, at least two origins for the actual European cyprinid fauna were found. Some taxa, Cyprininae as well as Leuciscinae, clearly revealed a central European origin, whereas others (e.g., *Barbus graecus, B. albanicus, B. brachycephalus,* Iberian barbels, Iberian *Leuciscus*, and *L. keadicus*) were found to have Mediterranean ties (Fig. 1). MP analyses using Ti:Tv ratios of 4:1 (12 MP trees of 8513 steps) and 10:1 (12 MP trees of 14,564 steps) recovered similar and congruent topologies (not shown).

Phylogenetic Utility of Morphological Traits

Cyprinids have traditionally been diagnosed by a protrusile mouth, toothless jaws, and pharyngeal teeth (Howes 1991). However, these characters have recently been considered plesiomorphic (see Cavender and Coburn 1992; Fink and Fink 1996). Some Cyprinids, mainly cyprinins (with the exception of *Carassius*), are also characterized by the presence of barbels. The original (noncladistic) diagnoses of cyprinid subfamilies were based largely on the number of pharyngeal tooth rows and the presence or absence of barbels (Howes 1991). To evaluate the phylogenetic utility of such morphological traits, they were mapped onto the molecular phylogeny based on cytochrome *b* nucleotide sequence data. The number of rows of pharyngeal teeth (Rutte 1962) was found to be a fairly good phylogenetic character at the genus level, in which different states could be unambiguously associated (with few exceptions) with different monophyletic assemblages (Fig. 2). On the other hand, the presence or absence of barbels was found to be a poor phylogenetic trait (not shown).

Cladogenic Events Within European Cyprinids

A total of 49 taxa representing the main European cyprinid lineages was subjected to ML analyses. Rate constancy among these taxa was assessed with the two-cluster test (Takezaki et al. 1995) using HKY85 distances. Three taxa, namely, *Pseudophoxinus stymphalicus maratonicus, Phoxinellus prespensis,* and *Alburnus filippi,* showed a substitution rate significantly faster (at a 5% level) than the average. These taxa were excluded from subsequent analyses. A clock-constrained tree was constructed by ML based on HKY85 distances (Fig. 3), in which branch lengths can be used to estimate tentative divergence dates.

The molecular clock was calibrated (0.76% per MY) using two important and well-dated geological events: the formation of the strait of Korinthos in the late Pliocene (2.5 MYA), which separates the Peloponnesus from the mainland (Dermitzakis 1990), and the opening of the Gibraltar Strait after the Messinian salinity crisis (5 MYA), which separates North Africa from the Iberian Peninsula. *L. peloponnensis* from the Thyamis and Alphios rivers (4.5% nucleotide sequence divergence) and *Telestes pleurobipunctatus* from the Arachthos River and *T. p. alfiensis* from the Alphios River (4.6%) are cyprinid taxa that were separated by the formation of the Strait of Korinthos. On the other hand, the flooding of the Gibraltar Strait prompted the separation of North African barbs such as *Barbus callensis* from Iberian barbs such as *B. bocagei* (7.4%), *B. graellsii* (8.3%), *B. guiraonis,* (8.3%), *B. steindachnerii* (7.5%), *B. comizo* (7.6%), *B. microcephalus* (8.2%), and *B. sclateri* (8.5%). Tentative minimum divergence dates for the main cladogenic events affecting European cyprinids are shown in Table 2. The standard errors associated with these estimates were calculated using the clock-constrained ML tree. According to the results, cyprinids may have originated in the Eocene (Fig. 3 and Table 2). The two main subfamilies, i.e., Cyprininae and Leuciscinae, may have appeared

Fig. 1. Phylogenetic relationships of European cyprinids based on cytochrome *b* sequence data. A 50% majority-rule consensus bootstrap tree obtained with NJ (using HKY85 distances using empirical base composition and transition/transversion ratio), based on 500 pseudo-replications, is depicted. *Astyanax fasciatus* (Characidae) and *Crossostoma lacustre* (Balitoridae) were used as outgroup. Nodes with bootstrap values below 50% were forced to collapse and yield polytomies. MP analyses arrived at similar and congruent trees (see text).

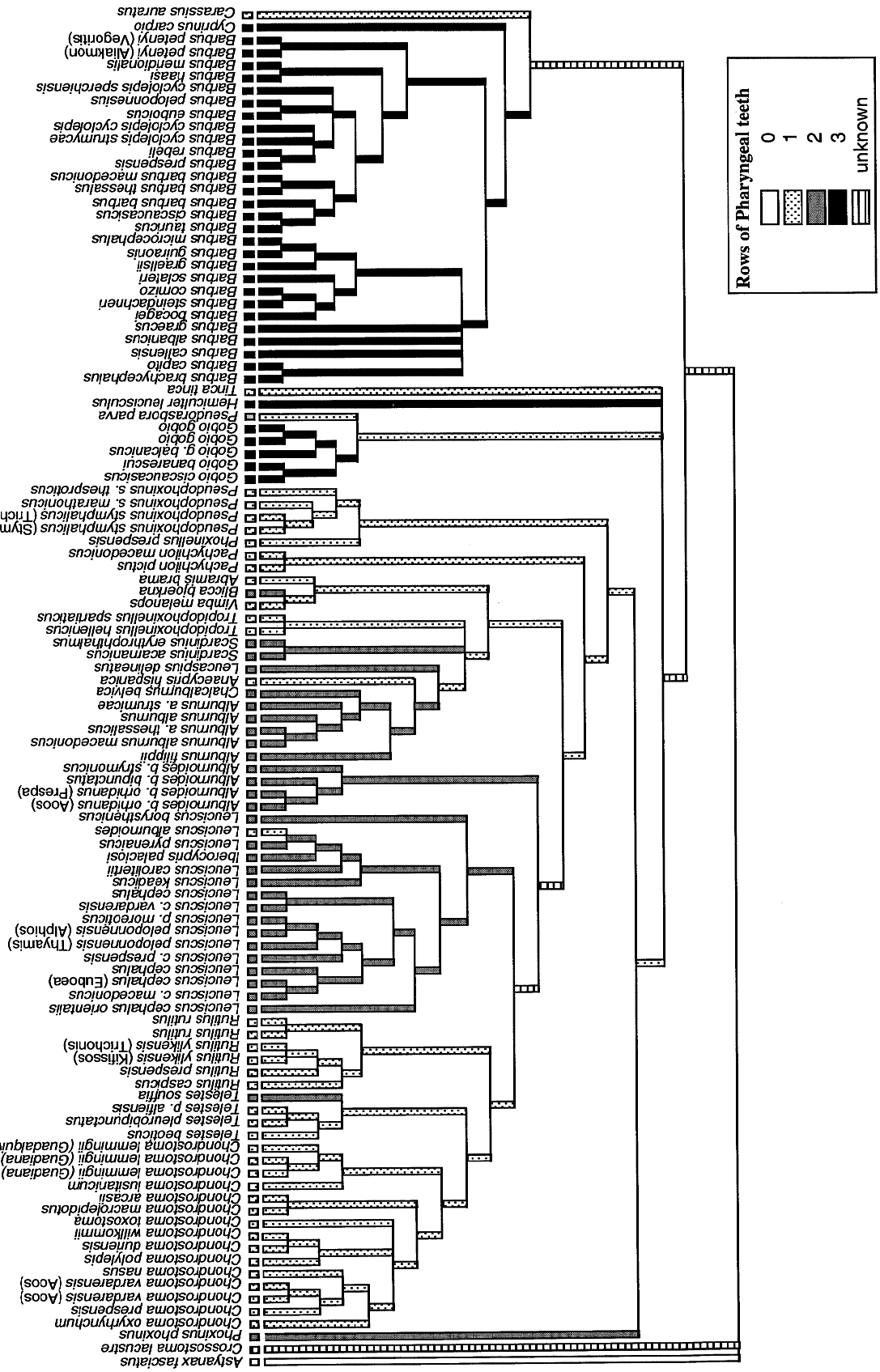

Fig. 2. Evolution of the number of rows of pharyngeal teeth in European cyprinids. The number of rows (zero to three) of pharyngeal teeth of each taxa were mapped onto the cytochrome *b* molecular phylogeny. Ancestral states were reconstructed with the maximum-parsimony method using MacClade (Maddison and Maddison 1992).

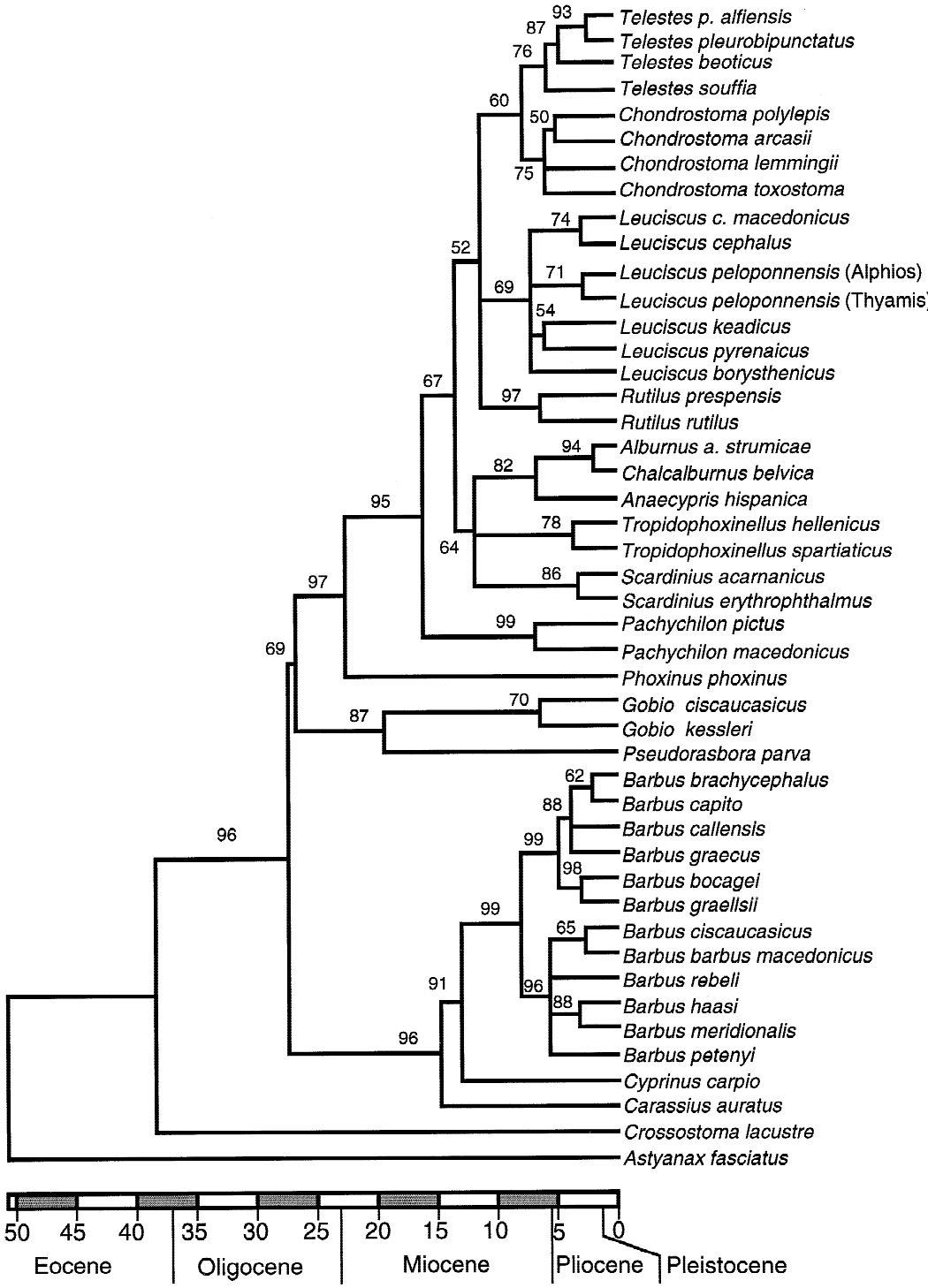

Fig. 3. Clock-constrained ML tree showing the major cladogenetic events in the history of European cyprinids. The tree was constructed on the assumption of a homogeneous rate of sequence divergence among taxa [as tested by the two-cluster test (Takezaki et al. 1995)]. The *scale bar* below the tree shows the time scale resulting from a calibration of the molecular clock (0.76% per MY) based on the formation of the Strait of Korinthos and the opening of the Gibraltar Strait (see text). *Numbers in nodes* are quartet puzzling (Strimmer and von Haeseler 1996) support values based on 10,000 pseudo-replications.

in the mid-Oligocene and radiated during the late Oligocene and Miocene. Major speciation events resulting in the current European cyprinid fauna occurred largely during the Pliocene (Fig. 3 and Table 2).

Discussion

The phylogenetic analysis of the cytochrome *b* nucleotide sequence data supports the traditional subdivision of

Table 2. Estimated dates of separation of the major European cyprinid lineages

Cladogenetic event	Date (MYA)	(±SE)
Separation of North African, Greek, and Caucasian *Luciobarbus*	3.8	(0.3)
Separation of Iberian *Luciobarbus*	5.0	(0.3)
Separation of *L. keadicus* from *L. pyrenaicus*	6.0	(0.4)
Separation of *Anaecypris*	6.8	(0.6)
Radiation of *Leuciscus* (*Squalius*)	7.2	(0.3)
Separation of Iberian *Chondrostoma*	7.8	(0.3)
Separation of *Barbus* and *Luciobarbus*	8.1	(0.4)
Origin of *Leuciscus* (*Squalius*)	11.4	(0.4)
Major radiation within leuciscins	13.6	(0.5)
Separation of *Pachychilon*	16.4	(0.8)
Separation of phoxinins	23.0	(1.0)
Separation of gobionins (including *Pseudorasbora*)	27.2	(0.9)
Separation of Cyprininae and Leuciscinae	27.7	(0.9)
Origin of cyprinids	38.9	(2.5)

European Cyprinidae into two subfamilies: Cyprininae (including barbins) and Leuciscinae (including cultrins, tincins, gobionins, phoxinins, and alburnins + leuciscins) (Zardoya and Doadrio 1998; Zardoya et al. 1999). This result is in agreement with previous phylogenies based on osteological data (Chen et al. 1984; Cavender and Coburn 1992). Two recent molecular phylogenies based on complete cytochrome *b* (Briolay et al. 1998) and partial 16S rRNA and cytochrome *b* (Gilles et al. 1998) nucleotide sequence data have also shown that alburnins should be included within leuciscins and that phoxinins are the sister group of alburnins + leuciscins (as in Fig. 1). However, in both studies, the relative positions of gobionins and tincins were unresolved due to low bootstrap support (Briolay et al. 1998; Gilles et al. 1998). In general, the phylogenetic relationships reported by Briolay et al. (1998), although supported by low bootstrap values, are in agreement with ours (Zardoya and Doadrio 1998; Zardoya et al. 1999; this paper). On the other hand, the phylogenetic relationships (particularly within Leuciscinae) recovered by Gilles et al. (1998) are not supported by our results. As Gilles et al. (1998) point out, the lack of resolution of their analyses is due mainly to the design of the data set (too high a number of taxa with respect to the number of informative characters) and the unfortunate selection of hybrid specimens as representatives of some of the species (with the associated artifactual results due to introgression processes).

The cyprinid groupings reported here imply either that the absence of barbels is an ancestral cyprinid state, and, hence, that tincin and gobionin barbels are not homologous to those of barbins (Fink and Fink 1996), or that the presence of barbels is the ancestral cyprinid state and that they were lost by the common ancestor of phoxinins, alburnins, and leuciscins (Howes 1991). Taking into account that the barbel structure and pattern of innervation of *Cyprinus* and *Barbus* are more complex than those of *Tinca* and *Gobio* (Howes 1991), we consider the first hypothesis to be more reliable. In any case, the phylogenetic utility of this trait is rather limited. Conversely, the number of rows of pharyngeal teeth showed reduced homoplasy and proved to be a more reliable phylogenetic marker (at least at the genus level) when mapped onto the cytochrome *b* molecular phylogeny (Fig. 2). According to the mapping of this trait, the common ancestor of leuciscins, phoxinins, and alburnins had a single row of pharyngeal teeth. The acquisition of two rows of pharyngeal teeth seems to have occurred independently in *Phoxinus*, *Scardinius*, *Blicca*, *Alburnus*, *Alburnoides*, *Telestes*, and *Leuciscus* [with the exception of *Leuciscus alburnoides*, a hybridogenetic species (Carmona et al. 1997), which has reverted to the ancestral state], whereas *Pseudophoxinus*, *Pachychilon*, *Tropidophoxinellus*, *Rutilus*, and *Chondrostoma* retained the ancestral state (Fig. 2). *Anaecypris hispanica* exhibits a single row of pharyngeal teeth, whereas *Leucaspius* and *Alburnus* spp. (in the same monophyletic group) show two rows. Hence, according to the NJ phylogeny, it seems that *A. hispanica* has reverted to the ancestral character. However, in the MP analyses, the position of *A. hispanica* was basal to that of *Leucaspius* and *Alburnus* spp. (Zardoya et al. 1999), suggesting that, in fact, the single row of *A. hispanica* is a retention of the ancestral state (Fig. 2). *Barbus* and *Cyprinus* are characterized by three rows of pharyngeal teeth. This character state is shared with *Gobio* and *Hemiculter* (Fig. 2).

The calibration of the molecular clock to 0.76 %/MY is highly congruent with the widely accepted rate of substitution of 0.7% per MY for mitochondrial DNA of poikilothermic vertebrates (Martin and Palumbi 1993; see also Johns and Avise 1998). The estimated divergence dates are in full agreement with the fossil record of Eurasia (Cavender 1991) (although slightly underestimated due to the inherent biases associated to the calibration of a molecular clock). However, caution should be taken with the oldest estimates, e.g., the cypriniform–characiform split, in which the error is considerably higher due to saturation in the cytochrome *b*. According to the estimated dates (Fig. 3 and Table 2), cyprinids likely originated in the Eocene (about 39 MYA). This tentative dating supports the hypothesis, based on the fossil record, of an Asian origin of the family (Banarescu 1989; Cavender 1991).

The two subfamilies, Cyprininae and Leuciscinae, which may have originated in the mid-Oligocene (around 27 MYA) (Fig. 3 and Table 2), show different evolutionary and biogeographical patterns. The data suggest a very early radiation of Leuciscinae and a later radiation of Cyprininae. However, the latter result is probably an artifact due to the absence of key basal lineages within the Cyprininae data set. Cyprininae includes at least two

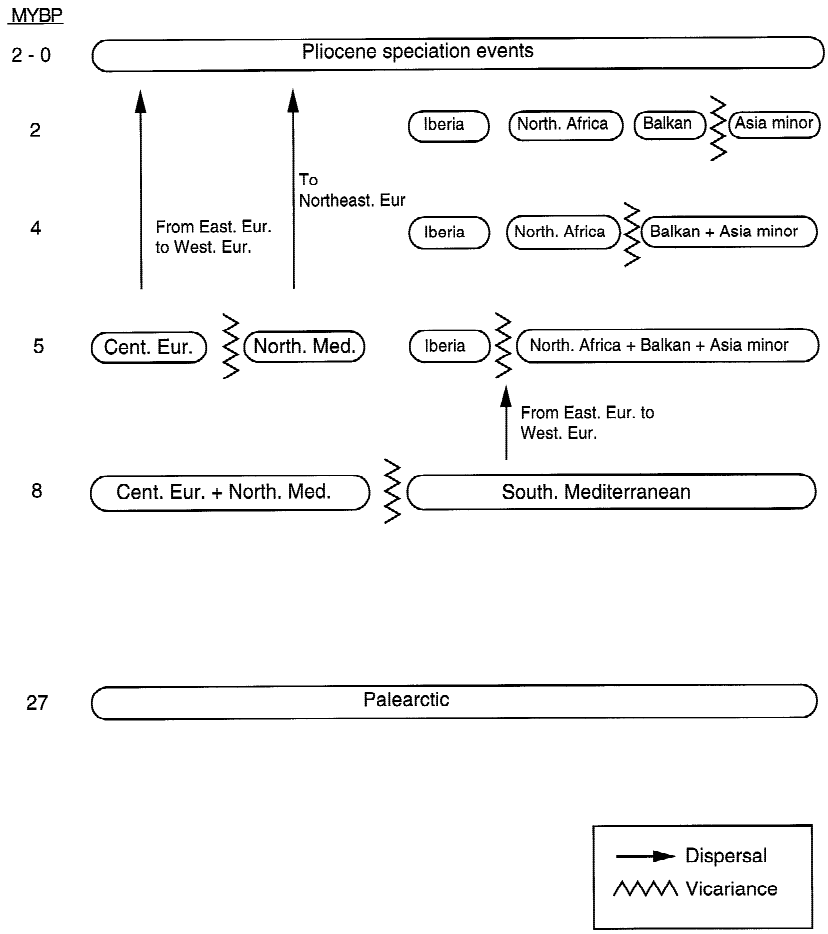

Fig. 4. Paleogeographical hypothesis of European Cyprininae evolution. Major dispersal and vicariance events of the Cyprininae are shown. Cyprininae may have crossed from Asia to Europe in the mid-Oligocene (27 MYA) while the Urals were forming. The Alpine orogenesis (10–8 MYA) may lead to a vicariant split of barbins into two subgenera, *Barbus* and *Luciobarbus*, which inhabited central Europe + northern Mediterranean and southern Mediterranean, respectively. In the late Miocene, cladogenesis in *Luciobarbus* followed the successive plate tectonic movements that occurred in the southern Mediterranean region. The subgenus *Barbus* was likely divided into two subgroups (*B. meridionalis* and related species, *B. barbus* and related species) during the regression of the Sarmatic Sea, in the late Miocene (5 MYA). In the Pliocene (2 MYA), the establishment of the actual drainage systems led to a major speciation event in both *Barbus* and *Luciobarbus*.

monophyletic groups (subgenera *Barbus* and *Luciobarbus*) with unrelated allopatric patterns (Figs. 1 and 3). Actually, *Luciobarbus* (Doadrio 1990) occurs on the Iberian Peninsula and in northern Africa, southwestern Greece, and Asia Minor (up to the Caucasus), whereas *Barbus* is distributed in central Europe, north Mediterranean, and the Caucasus. Therefore, our results are in clear disagreement with the classical hypothesis of a unique origin of European barbels and further dispersal through central Europe during the Oligocene and Miocene (Banarescu 1960; Almaça 1976, 1988; Banarescu 1989, 1992). Based on the dating of the main cladogenetic events of European Cyprininae (Fig. 3 and Table 2), and their actual distribution patterns, it is possible to postulate a paleobiogeographical scenario that reconstructs the vicariant and dispersal events that participated in the evolution of these taxa (summarized in Fig. 4) using a dispersal–vicariance analysis (Ronquist 1997) (not shown). According to this scenario, the lineage leading to modern European *Barbus* originated in the mid-Miocene (around 13 MYA) (Fig. 3 and Table 2). The oldest fossil record of *Barbus* in Europe is also mid-Miocene (15–11 MYA) (Quenstedt 1852), supporting our dating. Moreover, the major cladogenetic event in the *Barbus* lineage was the separation of the South Mediterranean barbins from the central European and North Mediterranean taxa (about 8 MYA) (Fig. 4) (Doadrio 1990, 1994). This event might be directly related to the Alpine orogenesis in the late Miocene (10–8 MYA), which created a stretch of mountain chains from the Alps through the Dinarides and the Hellenides to Anatolia (Maldonado 1985), separating the above-mentioned regions.

On the other hand, and according to the dispersal–vicariance analysis (Ronquist 1997), European Leuciscinae have suffered numerous and complex cladogenetic events which are more difficult to interpretate and correlate with paleogeographical events. According to the fossil record, by the late Oligocene–early Miocene, leuciscins had reached the Iberian Peninsula (Cabrera and Gaudant 1985). Around that time (about 16 MYA), the *Pachychilon* lineage may have originated (Fig. 3 and Table 2). Hence, the Greek endemic *Pachychilon* may be a relict genus reminiscent of the fossil leuciscin fauna that inhabited Europe in the early to mid-Miocene [e.g., "*Rutilus*" *antiquus* and "*Rutilus pachecoi*" (Cabrera and Gaudant 1985), *Palaeoleuciscus* (Gaudant 1977)]. To test this hypothesis further, it would be interesting to find apomorphies between *Pachychilon* and the above-mentioned fossil taxa. The first major radiation within leuciscins occurred in the mid-Miocene (13.6 MYA), and extant lineages such as those leading to, e.g., *Scardi-*

nius, *Rutilus*, *Leuciscus*, and *Alburnus* were originated (Cavender 1991). The well-known fossil record of *Leuciscus* on the Iberian Peninsula dates back to the middle Miocene (De la Peña 1995), supporting these datings. Finally, extant species of European Leuciscinae originated mostly during the Pliocene (2.5–1.8 MYA), when the configuration of the actual European drainages was set up (Banarescu 1989).

In conclusion, our data provide a molecular phylogenetic framework which turns out to be very useful in revising the systematics of European cyprinids and the phylogenetic utility of morphological characters currently used to infer phylogenetic relationships within Cyprinidae. Furthermore, molecular evidence strongly supports biogeographical hypotheses (Bianco 1990; Doadrio 1990, 1994) that highlight the importance of the southern Mediterranean realm in the evolution of some European cyprinid taxa. The distribution of primary freshwater fish is directly related to paleobiogeography. Therefore, it is expected that the relationships reported here may also be found in other freshwater animals such as fish [e.g., *Valencia* and *Cobitis* (Banarescu 1989), gobies (Penzo et al. 1998)], amphibians [e.g., *Rana* and *Triturus* (Oosterbroek and Arntzen 1992; Beerli et al. 1996)], *Salamandra* (Veith et al. 1998), and invertebrates [e.g., decapods (Albrecht 1982), mollusks] living in southern Europe.

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