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# Molecular phylogeny of two lineages of Leuciscinae cyprinids (*Telestes* and *Scardinius*) from the peri-Mediterranean area based on cytochrome *b* data

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**Abstract**

We examined phylogenetic relationships in two lineages of Leuciscinae cyprinid fishes based on the sequence data of the complete mitochondrial DNA region coding for the cytochrome *b* gene (1140 bp). *Telestes* includes obligate riverine, moderately cold water-adapted species whereas *Scardinius* comprises warm-adapted species living in lowland lakes and still waters of rivers and streams. We also analysed selected representatives of *Leuciscus* and *Phoxinellus* because the taxonomic status of some species belonging to these genera is dubious and they could be placed in the genus *Telestes*. The study includes 18 species, 43 populations, and 111 individuals from 9 of the 14 peri-Mediterranean ichthyogeographic districts. Clades recovered from the phylogenetic analyses do not support previous taxonomic assumptions based on morphology. *Telestes*, *Leuciscus*, and *Phoxinellus* do not form monophyletic assemblages; phylogenetic analyses suggest that *L. polylepis*, *L. turskyi*, *P. croaticus*, and *P. metohiensis* should be included in *Telestes*. Similarly, populations of *Scardinius erythrophthalmus* do not cluster together and the endangered *S. scardafa*, endemic to central Italy and surviving in a single locality, is nested within them. The radiations of *Telestes* and *Scardinius* occurred in different time periods. A major diversification of *Telestes* is consistent with a sea dispersal during the freshwater Messinian "Lago Mare" phase of the Mediterranean Sea. Cladogenetic events within *Scardinius* are likely related to the extension and confluence of river drainages in lowlands following multiple lowering of the sea level during the Quaternary glaciations.


*Keywords:* Biogeography; Freshwater fishes; Mediterranean area; Mitochondrial DNA; Cytochrome *b*; *Telestes*; *Scardinius*

## 1. Introduction

In southern Europe, cyprinids are extremely rich in number of endemic species (about 77 species), especially when compared with the relatively uniform cyprinid fauna of central and northern Europe (Kottelat, 1997). This particular diversification led to the recognition of 14 peri-Mediterranean ichthyogeographic districts (Bianco, 1990; see Fig. 1). Basically, two alternative hypotheses have been proposed to explain the present high level of endemism in the freshwater fish fauna of the area. According to Banarescu (1960, 1992) peri-Mediterranean rivers were colonised via river captures from Central Europe during a long time span (from the Oligocene, 35 myr until late Pliocene, 1.7 myr ago) following the hydrogeographic and geotectonic history of European area. Bianco (1990) proposed an alternative hypothesis suggesting that the colonisation of the peri-Mediterranean area by primary freshwater fishes occurred during the freshwater phase of the Mediterranean Sea (termed by Bianco as "Lago Mare"), when the Mediterranean Sea almost completely dried up (Messinian Crisis) and was refilled with fresh water from the Paratethys. This lacustrine phase of the Mediterranean

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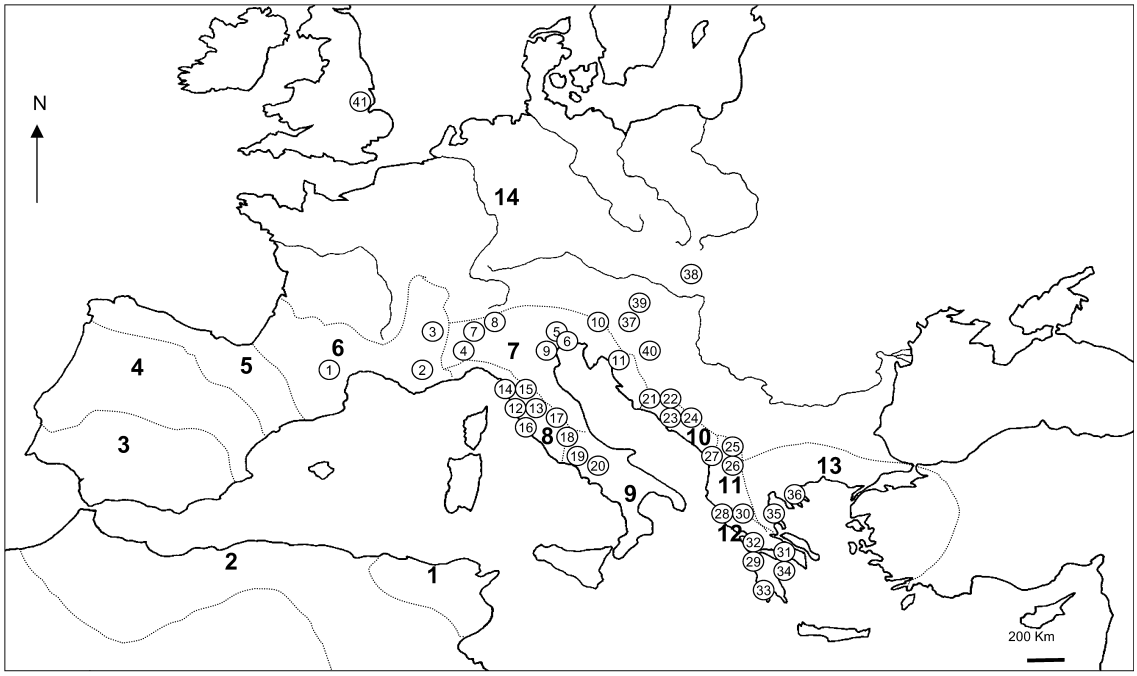


Fig. 1. Schematic map of the peri-Mediterranean ichthyogeographic districts (redrawn from Bianco, 1990) and localities from which the different populations and species of Leuciscinae cyprinids were collected. Dotted lines indicate district boundaries; bold numbers identify different districts. Circled numbers correspond to those in the last column of Table 1.

Sea acted as the main route of dispersal of most freshwater species. The opening of the Strait of Gibraltar filled the Mediterranean basin with marine water; this represented a strong vicariant event responsible for the present pattern of diversification of southern European freshwater fish fauna. Such a debated scenario stimulated several phylogenetic studies based on molecular approaches, which led to useful insights into the local evolution and diversification at the species, genus, and even subfamily level of the world's second largest fish family (Berrebi, 1995; Briolay et al., 1998; Brito et al., 1997; Durand et al., 1999, 2000, 2002, 2003; Gilles et al., 1998a; Hänfling and Brandl, 1998, 2000; Ketmaier et al., 1998, 2003; Tsingenopoulos and Berrebi, 2000; Tsingenopoulos et al., 2002; Zardoya and Doadrio, 1998, 1999; Zardoya et al., 1999).

We choose to investigate the phylogenetic relationships within populations and species of two genera of cyprinids with widespread distributions in southern Europe: *Telestes* and *Scardinius*. Since these two lineages comprise a relatively low number of species, this allowed us to focus on a relatively small number of species and to test alternative biogeographic hypotheses within a well-defined phylogenetic framework (Zardoya and Doadrio, 1999). *Telestes* includes obligate riverine, moderately cold water-adapted species. These species have not been subjected to human-induced faunal translocations because of their lack of economic value (Bianco, 1995a,b; Chappaz and Brun, 1993). Thus, their present distribution should reflect their natural evolutionary history. On allozymic and morpho-ecological bases, Gilles et al. (1998b) and Ketmaier et al. (1998) suggested that *Telestes* should be considered a different genus from *Leuciscus* and include the following species: *T. souffia* (with a relatively wide distribution north of the Alps from southern France to Slovenia), *T. muticellus* (endemic to most of the Italian peninsula), and *T. pleurobipunctatus* (endemic to several rivers in Greece). Two other species are presently placed in the genus: *T. montenigrinus* (endemic to a single river in Montenegro, Yugoslavia) and *T. beoticus* (endemic to a few localities in Greece). All these species have been included in the present study with multiple populations for *T. souffia* and *T. muticellus*. We also analysed four other species belonging to two distinct genera (*Phoxinellus metohiensis*, *P. croaticus*, *Leuciscus turskyi*, *L. polylepis*) because morphological and ecological evidence suggests that they should be included in the genus *Telestes* (Banarescu and Herzig-Straschil, 1998; Ketmaier et al., 1998).

The genus *Scardinius* comprises five warm-adapted species (*S. erythrophthalmus*, *S. scardafa*, *S. acarnanicus*, *S. graecus*, and *S. racovitzai*) living in lowland lakes and still waters of rivers and streams. All but *S. racovitzai* are included in this study. We examined a single populations for both *S. acarnanicus* and *S. graecus*, which are endemic to small geographic areas (western-central and eastern-central Greece, respectively; Ketmaier et al., 2003). We analysed 14 populations of *S. erythrophthalmus*, the most widespread species of the genus distributed throughout Europe except the Iberian peninsula.

This species, originally distributed north of the Apennines, was extensively transplanted into the original distribution range of *S. scardafa* (central Italy, district 8; Fig. 1), leading to the extinction of this species in almost all its original range (Bianco, 1993; Bianco and Ketmaier, 2001). *S. scardafa* is apparently limited to a single population (Bianco, 1993; Ketmaier et al., 2003).

The aim of this study was to produce a molecular phylogeny of the *Telestes* and *Scardinius* lineages by analysing sequence variation of the complete mitochondrial cytochrome *b* gene (cyt*b*, 1140 bp). The inferred phylogeny was then used to clarify the taxonomy of these genera and to help understand their pattern and timing of colonisation of the Mediterranean area.

## 2. Materials and methods

### 2.1. Sampling, DNA sources, PCR amplification, and sequencing

Total cellular DNA was extracted from muscle, fins, and scales of 111 individuals of the populations and species listed in Table 1. Voucher specimens for each tissue sample used in the study are available in the corresponding GenBank record. Three specimens per population/species were analysed, except for a few cases (see Table 1). As outgroups, we used *Cyprinus carpio*, a species belonging to the subfamily Cyprininae (Chang et al., 1994; GenBank Accession No. X61010) and *Pachychilon pictus* which belongs to the subfamily Leuciscinae but it is sufficiently distant from *Telestes* and *Scardinius* to avoid any background noise resulting from introgression (Zardoya and Doadrio, 1999). The complete cyt*b* sequences of three cyprinid species were retrieved from GenBank (see Table 1 for details) and analysed together with the sequences collected for this study. In particular, to test the hypothesis of the placement of *P. metohiensis*, *P. croaticus*, *L. turskyi*, *L. polylepis* within *Telestes*, we included *Leuciscus leuciscus*, *L. cephalus*, and *Phoxinellus prespensis* in all analyses. We also included *Tropidophoxinellus spartiaticus* because morphological and previous molecular work (Bianco, 1988; Zardoya and Doadrio, 1999) suggest that *Tropidophoxinellus* is the sister taxon of *Scardinius*. In addition, the Museum of the Academy of Natural Sciences (Philadelphia, USA) provided us with a tissue sample of *S. scardafa* from the ethanol-preserved specimen number 6212 (in the Academy of Natural Sciences Database); this specimen belongs to the type series used to describe the species and was collected in 1837 prior to any translocations of fishes that altered the original species distribution of freshwater fishes in Italy.

DNA was extracted using the Easy-DNA extraction kit from Invitrogen. For the historical sample of *S. scardafa* we used the mouse-tail protocol of the Easy-DNA extraction kit (Invitrogen); extractions from this sample were carried out together with blank extractions to aid in the detection of contamination. PCR amplifications of the entire cyt*b* were carried out using the protocols and the mitochondrial cyt*b* primers L15267 and H16526 reported in Brito et al. (1997). Because DNA extracted from historical samples is generally degraded, we were able to amplify only about 70% of the entire cyt*b* (786 bp) in overlapping fragments using several primer pairs designed from the sequences obtained from the fresh tissues (primers available from the first author). PCR fragments were purified with the GenElute PCR DNA Purification Kit from Sigma. Sequences were determined with an automated sequencer (Applied Biosystems 373A) following the manufacturer's protocols. Strands were sequenced in both directions for each individual. Internal primers for sequencing were L15267, L15516a, L15592, H15891, and H16461 (Brito et al., 1997); the historical sample was sequenced with these primers plus specifically designed primers (see above). Sequences have been submitted to GenBank (Accession Nos. AY509823–AY509862).

### 2.2. Phylogenetic analysis

Sequences were edited using Sequencher 3.1.1 (Gene Code Corporation, Ann Arbor, MI) and aligned by eye. Saturation of the sequences was investigated by plotting the absolute number of transitions (Ti) and transversions (Tv) against the Tamura and Nei (1993) gamma corrected distances ($D_{T\&N}$) for all codon positions and for each codon position separately.

Aligned sequences were analysed by the maximum parsimony (MP; heuristic searches, ACCTRAN character-state optimisation, 100 random stepwise additions, TBR branch-swapping algorithm) (Farris, 1970; Hendy and Penny, 1982), maximum likelihood (ML; heuristic searches, 100 random stepwise additions, TBR branch-swapping algorithm) (Felsenstein, 1981), neighbour-joining (NJ) (Saitou and Nei, 1987), and Bayesian methods (Huelsenbeck, 2000; Larget and Simon, 1999; Mau and Newton, 1997; Mau et al., 1999; Rannalla and Yang, 1996). MP, ML, and NJ analyses were performed using PAUP* 4.0β10 (Swofford, 2002); Bayesian analysis was carried out using MrBayes (Huelsenbeck, 2000). MP searches were carried out giving equal weight to all substitutions and down weighting transitions three times transversions (Tv3 × Ti). We ran the ML analyses on PAUP* 4.0β10 after having determined the best evolutionary model for our data set using the program ModelTest (Posada and Crandall, 1998). According to the results of this program we ran all our ML analyses using the GTR + Γ model (variable rates, shape parameter $\alpha = 0.358$). NJ analyses were carried out on $D_{T\&N}$ distances calculated with the same empirically

Table 1
Ichthyogeographic districts (according to Bianco, 1990; see Fig. 1), taxa included in the study, sampling drainages, abbreviations used for each populations (only when multiple populations for a given species were analysed), and the sample size (*N*)

| Ichth. district | Taxa | Drainage/lake (L.) | Pop. code | *N* | Map |
|---|---|---|---|---|---|
| 6 | *Leuciscus cephalus* | Herault | Lc1 | 2 | 1 |
| | *Telestes souffia* | Herault | Ts1 | 3 | 1 |
| | *Telestes souffia* | Var | Ts2 | 3 | 2 |
| | *Scardinius erythrophthalmus* | Rhone | Se1 | 3 | 3 |
| 7 | *Telestes muticellus* | Po | Tm1 | 3 | 4 |
| | *Telestes muticellus* | Bacchiglione | Tm2 | 3 | 5 |
| | *Telestes muticellus* | Isonzo | Tm3 | 3 | 6 |
| | *Scardinius erythrophthalmus* | Po | Se2 | 3 | 7 |
| | *Scardinius erythrophthalmus* | L. Maggiore | Se3 | 3 | 8 |
| | *Scardinius erythrophthalmus* | Brenta | Se4 | 3 | 9 |
| | *Scardinius erythrophthalmus* | Krka | Se12 | 3 | 10 |
| | *Leuciscus polylepis* | Stream near Jospidol | — | 1 | 11 |
| 8 | *Telestes muticellus* | Ombrone | Tm4 | 3 | 12 |
| | *Telestes muticellus* | Tiber | Tm5 | 3 | 13 |
| | *Scardinius erythrophthalmus* | L. Massaciuccoli | Se5[a] | 3 | 14 |
| | *Scardinius erythrophthalmus* | L. Monte Doglio | Se6[a] | 3 | 15 |
| | *Scardinius erythrophthalmus* | L. Vico | Se7[a] | 3 | 16 |
| | *Scardinius erythrophthalmus* | L. Salto | Se8[a] | 3 | 17 |
| | *Scardinius scardafa* | L. Scanno | — | 10 | 18 |
| 9 | *Telestes muticellus* | Volturno | Tm6 | 3 | 19 |
| | *Telestes muticellus* | Sabato | Tm7 | 3 | 20 |
| 10 | *Telestes souffia* | Cetina | Ts4 | 3 | 21 |
| | *Leuciscus tursky* | Cikola | — | 1 | 22 |
| | *Phoxinellus metohiensis* | Nevesinjsko Polje | — | 1 | 23 |
| | *Phoxinellus croaticus* | Ricica | — | 1 | 24 |
| 11 | *Telestes montenigrinus* | Moraca | — | 1 | 25 |
| | *Pachychilon pictus* | Vjose | — | 1 | 26 |
| | *Scardinius erythrophthalmus* | L. Skadar | Se13 | 3 | 27 |
| 12 | *Telestes pleurobipunctatus* | Thiamis | Tp1 | 3 | 28 |
| | *Telestes pleurobipunctatus* | Alfios | Tp2 | 3 | 29 |
| | *Phoxinellus prespensis* | L. Prespa | — | [b] | 30 |
| | *Scardinius graecus* | L. Yliki | — | 3 | 31 |
| | *Scardinius acarnanicus* | L. Trichonis | — | 3 | 32 |
| | *Tropidophoxinellus spartiaticus* | Evrotas | — | [b] | 33 |
| | *Telestes beoticus* | Kifissos | — | [c] | 34 |
| 13 | *Leuciscus cephalus* | Pinios | Lc2 | 3 | 35 |
| | *Scardinius erythrophthalmus* | L. Volvi | Se14 | 3 | 36 |
| 14 | *Scardinius erythrophthalmus* | L. Cerknica | Se9 | 3 | 37 |
| | *Scardinius erythrophthalmus* | Danube | Se10 | 3 | 38 |
| | *Scardinius erythrophthalmus* | L. Bacica | Se11 | 3 | 39 |
| | *Telestes souffia* | Sava | Ts3 | 3 | 40 |
| | *Leuciscus leuciscus* | Trent | — | 3 | 41 |

Numbers in the last column (Map) correspond to collecting sites in Fig. 1.
[a] Allochthnous anthropogenic populations.
[b] Data from Zardoya et al. (1999): GenBank Accession Nos. AF090763 and AF090777.
[c] Data from Zardoya and Doadrio (1999): GenBank Accession Nos. AF090770 and AF090772.

determined gamma parameters used for ML analyses. For the Bayesian approach we employed the same models of sequence evolution as in the ML searches. Site-specific rate variation was partitioned by codon positions. MrBayes was run for 2 million generations with a sampling frequency of 100 generations. We run one cold and three heated Markov chains. From the 20,000 trees found, we discarded the first 10% in order to include only trees for which convergence of the Markov chains had been reached. The remaining trees were used to construct a 50% majority rule consensus tree using PAUP* 4.0β10.

The bootstrap method (Felsenstein, 1985) was employed to test the robustness of the phylogenetic hypotheses generated by MP, ML, and NJ analyses (1000 bootstrap replicates for MP and NJ and 100 bootstrap

replicates for ML). For the unweighted MP analyses we also calculated the Bremer support index (Bremer, 1988) with the AutoDecay 4.0 program (Eriksson, 1999). The reliability of nodes in Bayesian trees was determined by calculating the percentage of trees found by the program that contained that grouping.

Competing phylogenetic hypotheses were tested using the Templeton (1983) test, two-tailed Wilcoxon rank-sum test (Larson, 1994) and Shimodaira–Hasegawa log-likelihood (SH) test (Shimodaira and Hasegawa, 1999) as implemented in PAUP* 4.0β10. The resampling estimated log-likelihood (RELL) technique was used in SH tests. MP and ML trees were compared with trees obtained with the other phylogenetic methods and with constrained trees constructed to test alternative phylogenetic hypotheses. We also carried out a parametric bootstrap (PB) (Hillis et al., 1996; Huelsenbeck and Crandall, 1997) only for those comparisons where alternative topologies did not significantly differ at the SH (Goldman et al., 2000). PB was carried out by generating 100 simulated data sets with Seq-Gen (Rambaut and Grassly, 1997) and then using PAUP* 4.0β10 to compute a likelihood score for the constrained tree topology for each of the 100 data sets and to compute a likelihood score for the best tree of each of the 100 data sets. We calculated the difference between each pair of likelihood scores to check where the difference between the unconstrained and the constrained tree falls into this distribution.

The molecular clock hypothesis was tested with two methods. First, we used the likelihood ratio test (Goldman, 1993) which compares the log-likelihood of the ML trees with and without assuming a molecular clock. To check in detail the rate heterogeneity within two lineages (given an outgroup), we employed the two-cluster test of Takezaki et al. (1995) using Kimura's (1980) distance with gamma parameter as implemented in Phyltest (Kumar, 1996). This test is different from the others because it is possible to include multiple sequences in each of the lineages. Those taxa that showed significantly different substitutions rates (at the 5% level) were excluded from further analyses. Divergence times ($T$) were calculated per lineage according to the formula $T = D/2r$, where $D$ is the average divergence between two clusters (calculated from $D_{T\&N}$ values on all positions) and $r$ is the evolutionary rate. Previously published cyt*b* molecular clock rates for freshwater fishes range between 0.76 and 1% substitutions per million years (Dowling et al., 2002; Zardoya and Doadrio, 1999). We used the evolutionary rate of 1% per million years (myr) because it has been widely employed in recent studies on peri-Mediterranean cyprinids (i.e., Durand et al., 2002) including a phylogeographic study of *T. souffia* (Salzburger et al., 2003). Furthermore, this rate takes into account the problem of past hybridisation.

## 3. Results

### 3.1. Sequence variation and phylogenetic analysis

We sequenced the entire cyt*b* gene (1140 bp; both strands) for each of the 111 individuals included in the study, totalling about 253 kb of new cyt*b* sequences. Different individuals of the same population always had identical sequences; hence we used a single sequence per population for our analyses. All mutational changes were base substitutions; no indels were found. Most of the detected substitutions are in third codon positions (47.2%). The average percentage of A + T is 54.4% in all positions; as usual for mtDNA coding genes we found a deficit of guanine in third codon positions. Ti's by far outnumber Tv's, even in comparisons between the ingroup and the outgroup. Inspection of saturation plots for each position (not shown) suggests that Ti's are approaching saturation in third codon positions but not in first and second codon positions.

Fig. 2 shows the ML tree and summarises the results of the other phylogenetic methods employed in the study. The MP trees (unweighted and with Tv's weighted three times more than Ti's) were statistically different from the ML, NJ, and Bayesian trees with the Templeton (1983) test (Table 2) because of the position of *T. spartiaticus*, *L. leuciscus*, and *P. prespensis* in the MP trees; these positions were not supported by bootstrap values (trees not shown). MP, ML, NJ, and Bayesian topologies were statistically indistinguishable with the Shimodaira and Hasegawa (1999) test (Table 2). Relationships within *Scardinius* and *Telestes* were always largely congruent in the different phylogenetic analyses. Bayesian analyses always gave supports higher than those obtained by bootstrap probabilities in ML, MP, and NJ analyses; our results are in line with the considerations of Suzuki et al. (2002) that bootstrap probabilities are slightly conservative, whereas posterior probabilities can be too liberal.

All the *Telestes* species plus *L. turskyi*, *L. polylepis*, *P. croaticus*, and *P. metohiensis* are placed in a well-supported clade. *Telestes*, *Leuciscus*, and *Phoxinellus* never form monophyletic clades; constraining these genera to form monophyletic assemblages produced significantly worse trees than the one presented in Fig. 2 (Templeton and Shimodaira–Hasegawa tests, $p < 0.003$; Table 2). At the intraspecific level, multiple populations of *T. souffia*, *T. muticellus*, and *T. pleurobipunctatus* always form strongly supported monophyletic clades; relationships within *T. souffia* and *T. muticellus* are always well resolved and supported, and generally follow a geographic pattern (populations are placed in the trees mainly according to the proximity of the collecting sites).

*Scardinius* species form a strongly supported monophyletic group; *S. scardafa* is embedded within the

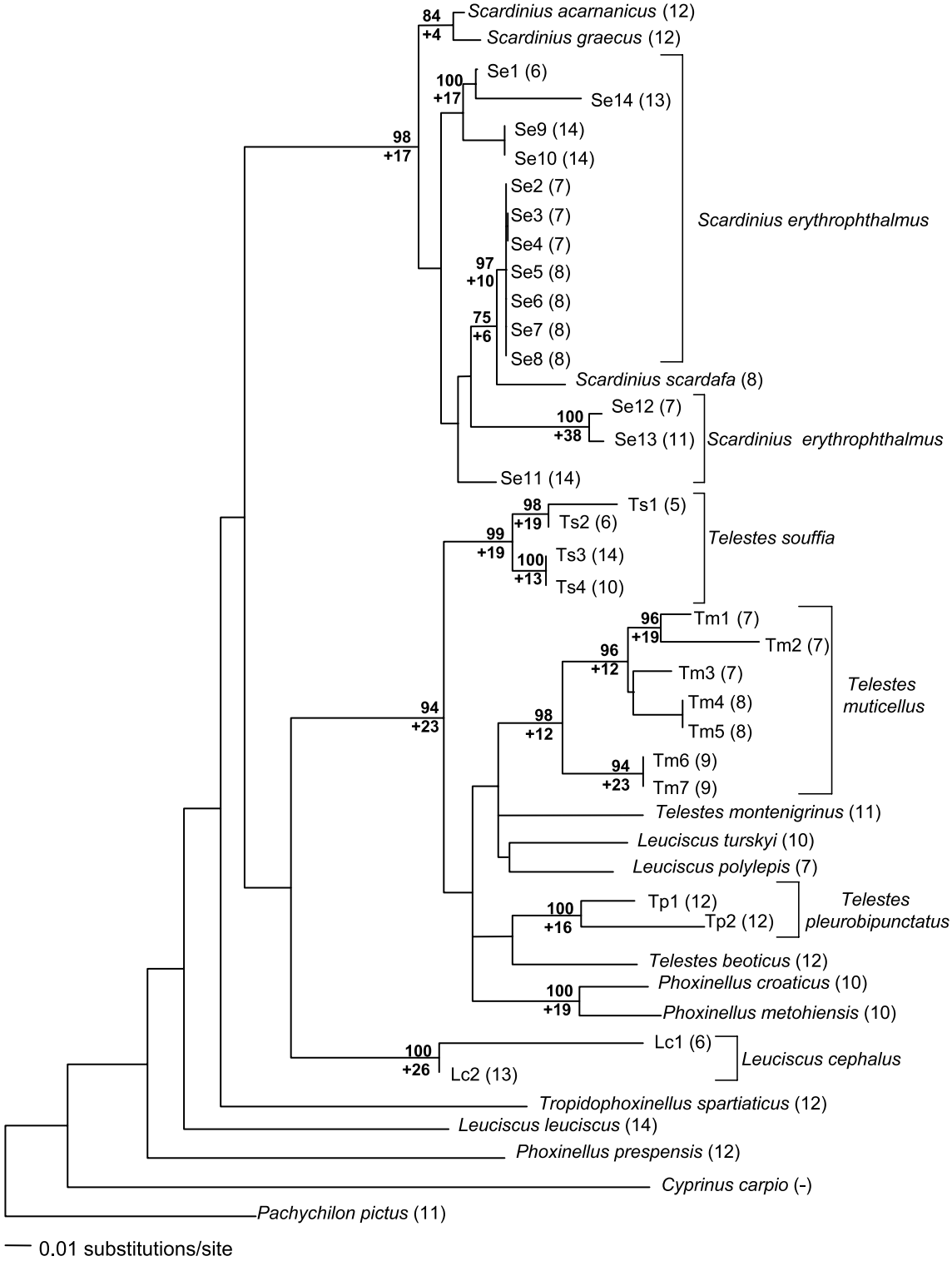


Fig. 2. ML estimate of the phylogenetic relationships of species included in the study using the GTR + Γ model (−ln = 9851.57; shape parameter = 0.358). Numbers at nodes are bootstrap values for ML (100 replicates; top) and decay indexes for MP (bottom). For the labelled nodes we always obtained a bootstrap support $\geqslant$90% for MP and NJ (1000 replicates) and a statistical support of 100% in the Bayesian analysis (2 million generations). Numbers after population/species names refer to ichthyogeographic districts in Table 1 and Fig. 1.

*S. erythrophthalmus* populations, which thus do not form a monophyletic assemblage. The monophyly of *S. erythrophthalmus* was statistically rejected by the Templeton (1983) and the PB tests ($p = 0.032$ and $p < 0.02$, respectively; Table 2). The fragment of the cyt*b* gene, we were able to amplify and sequence (about

Table 2
Summary of $p$ values for Templeton (1983) (T), Shimodaira and Hasegawa (1999) (SH), and parametric bootstrap (PB) (Hillis et al., 1999; Huelsenbeck and Crandall, 1997) tests for the four different phylogenetic methods employed in the study (ML, MP, NJ, and Bayesian)

| | T | SH | PB |
|---|---|---|---|
| *Tree* | | | |
| ML | 0.026* | 0.536 | — |
| $MP_{unweighted}$ | Best | 0.965 | — |
| $MP_{Tv3 \times Ti}$ | 0.874 | 0.935 | — |
| NJ | 0.047* | 0.137 | — |
| Bayesian | 0.012* | Best | — |
| *Constraints* | | | |
| *Telestes* monophyletic | <0.0001* | 0.000* | — |
| *Leuciscus* monophyletic | <0.0001* | 0.000* | — |
| *Phoxinellus* monophyletic | <0.0001* | 0.003* | — |
| *S. erythrophthalmus* monophyletic | 0.002* | 0.131 | <0.02** |

Constraint trees were constructed to test alternate phylogenetic hypotheses. Data were alternatively constrained such that genera *Telestes*, *Leuciscus*, *Phoxinellus*, and *S. erythrophthalmus* populations constitute monophyletic clades. PB was carried out only for the constraint enforcing the monophyly of *S. erythrophthalmus* (see text).

* Tree topology is significantly different from the best tree for $p \leqslant 0.05$.

** The observed difference between the log-likelihood of the best tree vs the alternative tree is larger than the values of all 100 replicates of the null distribution.

70% of the entire cyt*b*) from the historical sample of *S. scardafa* collected prior to any translocations of fishes in Italy, is identical to sequences from the 10 specimens of *S. scardafa* from Lake Scanno and contains all the 23 substitutions fixed between *S. scardafa* and all the 42 *S. erythrophthalmus* specimens included in the study.

### 3.2. Rate tests and divergence time estimates

We used two tests to check whether rate constancy holds among different lineages. The likelihood ratio statistic ($\delta$; calculated using likelihoods from the GTR + Γ model) was much greater ($\delta = 301.44$) than the critical $\chi^2$ values ($\delta = 55.76$; $p = 0.05$) for the given degrees of freedom (df = 41). According to these results the molecular clock hypothesis must be rejected for all taxa. The two-cluster test of Takezaki et al. (1995) using Kimura's (1980) distance with gamma parameter showed that four taxa (*L. cephalus*, *T. spartiaticus*, *L. leuciscus*, and *P. prespensis*) had substitution rates significantly different from the average (at a 5% level) and were excluded from further analyses. According to the two-cluster test rate constancy was not rejected at a 5% level among the remaining taxa. We also conducted the likelihood ratio test excluding *L. cephalus*, *T. spartiaticus*, *L. leuciscus* and *P. prespensis*. In this case, $\delta$ was 38.16, which is less than the critical $\chi^2$ value (at a 5% level) of 52.19 for df = 37. Indeed, Zardoya and Doadrio (1999) showed that *P. prespensis* has a substitution rate significantly faster than average. Assuming an evolutionary rate of 1% per million years we estimated divergence times for the statistically supported splits within *Telestes* and *Scardinius*. Our data suggest that diversification within the genus *Telestes* and its closely allied species took place between 3.25 and 7.9 myr ago, while the radiation within *Scardinius* occurred more recently, between 0.5 and 3.6 myr ago.

## 4. Discussion

### 4.1. Phylogeny

Our analyses bring new insights into the phylogeny and diversification of two lineages of cyprinids and give further support to phylogenies presented in recent studies using both allozymes and mtDNA. At the same time, we are aware that this study is based on a single mtDNA gene, which can only provide a maternal perspective of the real history of the groups here considered. However, we want to emphasise that most of the phylogenetic studies on cyprinids are based on cyt*b* sequences; consequently a large amount of data is available as a framework in which to adequately place our results. *Telestes*, *Leuciscus*, and *Phoxinellus* do not form monophyletic assemblages, and alternative hypotheses enforcing the monophyly of these genera were statistically rejected. *Leuciscus* shows the most entangled situation, with at least three maternal lineages that do not share a common ancestor. According to our results, *L. leuciscus* and *L. cephalus*, together with other 15 species that constitute the monophyletic *L. cephalus*-group (Zardoya and Doadrio, 1999; Zardoya et al., 1999), should be separated into two genera. This is in agreement with previous findings calling into question the monophyly of *Leuciscus* and suggesting the placement of the *L. cephalus* -group in the genus *Squalius* (Briolay et al., 1998; Gilles et al., 1998a, 2001; Hänfling and Brandl, 2000; Machordom et al., 1999; Sanjur et al., 2003). In all our phylogenetic analyses, *L. polylepis*, *L. turskyi*, *P. croaticus*, and *P. metohiensis* are embedded within the genus *Telestes*. This supports the suggestion of Banarescu and Herzig-Straschil (1998) to include these four species in *Telestes* on the basis of morphology and ecological data. Nevertheless, the analysis of a larger DNA data set is needed to resolve the phylogenetic relationships within *Telestes*. The cyt*b* does not allow a clear resolution between the species. This lack of resolution could be due to the rapid radiation that these species underwent (see below in Section 4.2). Alternatively, analysis using a faster evolving mtDNA fragment could be able to resolve these relations.

In the *Scardinius* phylogeny, the Greek species are basal to the other congeneric species. The genetic data on *S. erythrophthalmus* in Italy reflect the altered

situation due to the man-mediated fish transfer from northern to central Italy (districts 7 and 8). Allochthonous populations (Se5, Se6, Se7, and Se8; district 8) are fixed for the same haplotype, which is the haplotype found in the autochthonous population from the Po River (Se2; district 7). Also for this lineage we obtained some unexpected results with respect to the traditional taxonomy of the group. In particular, populations of *S. erythrophthalmus* are not clustered in a monophyletic clade because *S. scardafa* is nested within them. Our data suggest a complex phylogeography for *S. erythrophthalmus* in southern Europe and raise the possibility of a taxonomic revision of the taxon. Phylogenetic analyses revealed the existence of at least three divergent mitochondrial lineages and that *S. scardafa* is sister to the northern Italian one (plus the four central Italian populations of allochthonous origin). The cyt*b* sequence of the museum specimen of *S. scardafa* and the data on the extant population of this species compared with the other *S. erythrophthalmus* populations analysed suggest that no mtDNA gene flow occurred between these taxa. Thus we would recommend that there be no further introductions of *S. erythrophthalmus* into central Italy and that a proper management strategy be adopted for the single surviving population of *S. scardafa*.

We propose two different (but not mutually exclusive) hypotheses to account for the discrepancies observed in the two lineages between classical taxonomy based on morphology and molecular phylogeny. First, ichthyologists may have been misled by some morphological characters that do not trace the evolutionary history of the groups but are shaped by adaptations to environmental conditions. As already noted by several authors (Agnèse et al., 1990; Durand et al., 2002) the morphological characters used to infer phylogenetic relationships within cyprinids are often not informative and this is especially true at and below the species level. Second, mitochondrial introgression between different lineages could have played an important role, given that this phenomenon is particularly frequent in cyprinids (Gerber et al., 2001). Gilles et al. (1998b) and Salzburger et al. (2003) demonstrated recent mtDNA gene flow between *T. souffia* and *T. muticellus* in two areas where these two lineages meet after post-glacial expansion. One of our *T. souffia* populations (Ts2) was from one of these areas. We did not find any trace of hybridisation in Ts2 or in any of the 11 populations of *T. souffia* and *T. muticellus* we sequenced, rather populations of the two species are grouped in highly supported monophyletic clades. This could be due to the relatively low number of individuals per populations we sequenced. Nevertheless, we cannot reject a priori the hypothesis of genetic exchanges among lineages included in the study. The contradictory signals from morphology and genetics could be explained if the evolution of mtDNA and the morphological characters used to diagnose them are decoupled, with the former basically reflecting adaptation to environmental conditions and the latter tracing genetic exchanges among lineages. However, our data cannot support or reject this interpretation at the moment, as the analysis of independent (nuclear) loci is necessary to properly address the issue.

### 4.2. Biogeography and molecular dating

Our data suggest that the diversification of *Telestes* preceded that of *Scardinius*. Given the sequence divergence, a first split separated several species of *Telestes* (including *L. polylepis*, *L. turskyi*, *P. croaticus*, and *P. metohiensis*) from *T. souffia* between 5.6 and 7.9 myr ago. We cannot provide single datings for the emergence of *T. pleurobipunctatus*, *T. beoticus*, *T. muticellus*, *T. montenigrinus*, *L. turskyi*, and *L. polylepis* because our sequences were not able to unambiguously resolve their phylogenetic position. However, if we consider that the separation between *P. croaticus* and *P. metohiensis* occurred about 3.25 myr ago and that intraspecific divergences within *T. muticellus* and *T. souffia* started more or less in the same period, we can conclude that cladogenetic events among *T. pleurobipunctatus*, *T. beoticus*, *T. muticellus*, *T. montenigrinus*, *L. turskyi*, and *L. polylepis* likely took place between 5 and 3 myr ago. Our results closely match the dating of the split between *T. souffia* and *T. muticellus* by Gilles et al. (1998b) and Salzburger et al. (2003) based on two other mtDNA genes (16S rRNA and control region, respectively). All these estimates are not in agreement with the Banarescu hypothesis, rather they generally agree with the hypothesis of a peri-Mediterranean dispersion of freshwater fishes during the "Lago Mare" phase of the Mediterranean Sea (Bianco, 1990; Hsü, 1987). When the Mediterranean Sea was refilled with water from the Atlantic Ocean (after the opening of the Strait of Gibraltar) the peri-Mediterranean hydrographical systems became isolated from each other. The re-establishment of marine salinity in the Mediterranean Sea occurred quite rapidly. Thus, it is reasonable to assume that a number of different lineages originated almost simultaneously by vicariance. Our tree topology is consistent with the "Lago Mare" hypothesis. A long and gradual colonisation, as postulated by Banarescu hypothesis, would translate in a tree with deep dichotomies, whereas a colonisation followed by a rapid diversification would produce a tree with many short branches, which is what we observed.

This biogeographic scenario does not hold for *Scardinius*; time estimates for this genus place all splitting events in the Quaternary between 0.5 and 3.6 myr ago. During glaciations, lowering of the sea level determined confluences of water between rivers flowing into the epicontinental area of the Mediterranean Sea. This is particularly well documented for the

Po River, whose basin extended as far as the border of the meso-Adriatic ditch, capturing water from a large number of rivers on both sides of the Adriatic Sea; similar phenomena occurred repeatedly around the Mediterranean area (Bartolini and Pranzini, 1988; Bianco, 1990). One can thus easily envision a major dispersal event of this genus (and presumably of other warm-adapted lineages) via river confluences in lowlands.

Also intraspecific divergence took place at different times in *Telestes* and *Scardinius* ($3.15 \pm 1.55$ to $1.45 \pm 0.95$ myr and $1.35 \pm 1$ myr, respectively). A likely explanation for this difference takes into account the ecology of dispersal of the representatives of the two lineages. *Telestes* species, typical of the upper reaches of rivers, could cyclically move up and down along the same river course following the movements of their typical habitat during interglacial and glacial times because of the world-wide changes in temperature (Salzburger et al., 2003). However, it is unlikely that long-distance dispersal events were as common in *Telestes* as in *Scardinius*. Characteristics of *Telestes* species, such as a short life-span, the tendency to form sedentary populations, and to lay only a few thousand eggs in each spawning season (Kottelat, 1997), are all conducive to a low potential of long-distance dispersal (Tibbets and Dowling, 1996). On the other hand, long-lived species such as *Scardinius* (maximum reported age = 19 years), which forms high-density populations and produces many thousands of eggs (Crivelli, 1996), could have a higher probability of maintaining a certain degree of gene exchange when dispersal routes are available. In this regard, the coalescence of rivers during the Quaternary occurred exclusively on lowlands, creating the habitat of election for rudds (see Section 1). *Telestes* species could probably descend to the lower part of river courses during glacial maxima, but it is also likely that they experienced sub-optimal environmental conditions there. Garner et al. (1998) found that the minnow *Phoxinus phoxinus* (another cold-adapted rheophilic species) can change habitat, moving from deep running (and colder) to shallow still (and warmer) waters to escape predators, but the authors also showed that the minnows are inactive and do not eat in shallow waters. Long-distance dispersal seems unlikely or, at least, occasional in such conditions. These considerations and our genetic data are also in line with the findings of Ibrahim et al. (1996), who showed that genetic patchiness is more pronounced when dispersal is leptokurtic, that is when the vast majority of individuals of a given species disperse in short distances but only a few in intermediate or long distances. It should also be noted that our data match those of Tsingenopoulos et al. (2002), which reported a similar pattern of genetic differentiation in their comparison of rheophilic and lacustrine groups of barbs (genus *Barbus*).

## 5. Conclusions

This study provides insights into the phylogeny of two widespread lineages of cyprinids in the southern European area and can serve as a guide in revising their taxonomy at the species and genus level. Our analyses also show that the *Telestes* and *Scardinius* lineages radiated in different time periods, corresponding to different paleogeographic scenarios. Quaternary events had different effects on the two lineages, probably due to differences in the ecology (capability) of dispersal between riverine, moderately cold water-adapted species (*Telestes*) and lacustrine warm-adapted ones (*Scardinius*). Primary freshwater fishes have traditionally been viewed in a vicariance context. However, it is evident from our data that, at least for the geographic scale considered, the present distribution pattern of these organisms can be most effectively explained by a dispersal–vicariance approach.

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