

Messinian Salinity Crisis and the Origin of Freshwater Lifestyle in Western Mediterranean Gobies

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The present paper reports on a molecular study based on 12S rRNA and 16S rRNA mitochondrial genes partly sequenced in 13 species of western Mediterranean gobies, three of which are strictly freshwater-dwelling. A total of 867 bp were aligned and used for the phylogenetic reconstruction. Two major lineages were identified, one clustering the sand gobies in a monophyletic clade. Relationships among taxa based on sequence analysis only partly match those based on morphological criteria, suggesting that the latter are somehow insufficient to correctly establish phylogenetic relationships within this family. The results provide evidence for a multiple independent evolution of the freshwater lifestyle in *Knipowitschia* and *Padogobius* lineages. On the basis of the present results, it is uncertain whether the freshwater preference within the genus *Padogobius* originated twice independently in *P. nigricans* and *P. martensii* or only once in their common ancestor. Estimation of the ages of the two major lineages of this group of fish with a molecular clock (in combination with the construction of a linearized tree) suggests that they are much older (at least 40 Myr) than previously thought. Thus, there should be no correlation between their diversification and the Miocene–Pliocene geological events, including the so-called Messinian salinity crisis, which occurred about 10 MYA and is believed to have played a role in their evolution. Alternatively, these gobies would have an evolutionary rate at least fourfold faster than those of other vertebrates.

Introduction

The family Gobiidae is represented by several thousand species, mostly distributed in the tropical and temperate regions. Gobies underwent an extensive ecological radiation, with species occupying marine, brackish, and freshwater habitats. Forty-four species of gobiids, belonging to 18 genera, have been described for the western Mediterranean basin, many of them inhabiting Italian waters. Only three of these are strictly freshwater species, namely *Padogobius martensii* (Günther), *Padogobius nigricans* (Canestrini), and *Knipowitschia punctatissima* (Canestrini). Although all three breed in freshwater, they show clear differences in habitat preference. *Knipowitschia punctatissima* inhabits spring waters in which the temperature is constantly low and the bottom substrate is sandy or muddy with a rich vegetation; its geographic distribution is restricted to northeastern Italy (fig. 1). *Padogobius martensii* displays a strong preference for stony substrates, it is not restricted to spring waters, and its distribution, wider than that of the sympatric *K. punctatissima*, covers the drainage area that the Po River occupied during the last glaciation. The third freshwater species, *P. nigricans*, exhibits a habitat preference very similar to that of *P. martensii* but is geographically isolated from the other two gobies, being distributed on the Tyrrhenian side of central Italy (Gandolfi et al. 1991) (fig. 1). Bianco (1987) suggested that the origin of these three endemic Italian freshwater species dates back to the Miocene–Pliocene period, when the Messinian salinity crisis led to the formation of a lake-sized sea (“Lago-Mare”). According to this

hypothesis, the progressive evaporation of Mediterranean waters and the consequent disappearance of marine habitats might have forced some species to adapt to a freshwater environment. Following the reflooding of the Mediterranean Basin by the Atlantic Ocean through the Gibraltar Strait, these species retained their freshwater habitats and thus remained isolated from the euryhaline Ponto-Caspian group of species. Such isolation probably gave rise to the freshwater endemisms existing today in the Mediterranean and Balkan area (Miller 1990).

The relationships among some species of Italian gobies, including the three freshwater species, are controversial. Recent revisions of their systematics has resulted in a reconsideration of the status of *P. martensii* (Gandolfi and Tongiorgi 1974), assignment of *Gobius nigricans* (Canestrini) to the genus *Padogobius* (Bianco and Miller 1990), and synonymization of the genus *Orsinigobius* with *Knipowitschia* (Economidis and Miller 1990).

The relationship between freshwater and marine-euryhaline gobies represents a long-standing problem with this group of species. The difficulty in identifying morphological characters suitable for unambiguously establishing the phylogeny among taxa leaves open the question of whether the adaptation to the freshwater habitats evolved only once or several times independently. Previous studies, based on cladistic analyses of morphological characters and allozyme data, have suggested a polyphyletic origin of freshwater inhabitation among Italian gobies (Miller et al. 1994). In particular, they have indicated a close relationship between the genera *Knipowitschia* and *Pomatoschistus*, which apparently form a monophyletic clade together with the other sand gobies (McKay and Miller 1997). Conversely, the relationship between the two freshwater species of *Padogobius* and the sand gobies remains unclear. In fact, although allozyme studies have indicated that the

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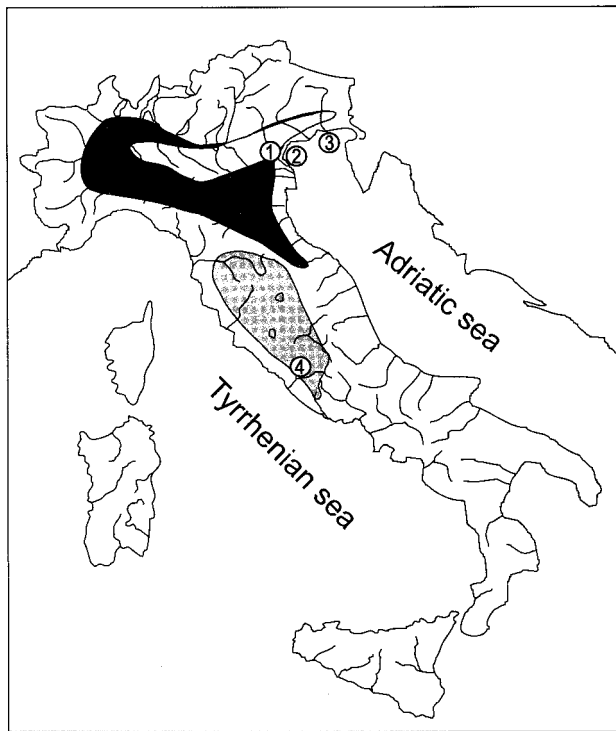


FIG. 1.—Geographic distribution of the three freshwater species endemic to Italy: *Padogobius martensii* (heavy gray area), *Knipowitschia punctatissima* (light gray area), and *Padogobius nigricans* (gray area). Numbers indicate sampling areas: 1 = Veneto rivers; 2 = Lagoon of Venice; 3 = Bay of Trieste; 4 = Tevere River.

P. martensii + *P. nigricans* clade is separated from the sand goby lineage (McKay and Miller 1997), some meristic features (namely a dorsal pterygiophore polymorphism and vertebral count) have suggested a possible relationship between them (Bianco and Miller 1990). In addition, the strictly freshwater habit exhibited by some species of both *Padogobius* and *Knipowitschia* genera could be considered further evidence for this relationship. Given these conflicting data, the systematic position of *P. martensii* and *P. nigricans* is intriguing, and the evolutionary process leading to the freshwater lifestyle of these species remains unclear.

The goal of the present study is to establish the phylogenetic relationships among some species of western Mediterranean gobies, including the three freshwater species endemic to Italy, by means of nucleotide sequence analyses of 12S rRNA and 16S rRNA mitochondrial genes. The investigation is particularly aimed at resolving the relationships between *P. martensii*, *P. nigricans*, and *K. punctatissima* and tracing the evolution of their freshwater habit in relation to the geological history of the Mediterranean region.

Materials and Methods

Species Under Investigation

We collected a total of 13 species of Gobiidae with different habitat preferences. Two of the freshwater species, namely *K. punctatissima* and *P. martensii*, were collected in the catchment area of Veneto Streams,

whereas *P. nigricans* was sampled in the drainage of the Tevere River (fig. 1). Brackish water and marine species were obtained from lagoons and inshore waters of the Northern Adriatic Sea (fig. 1); their habitat preferences and distributions are reported in table 1. *Bovichthys variegatus* (Notothenioidea), *Lycodichthys pearborni*, and *Pachychara brachycephalum* (Zoarcoidea) were used in the phylogenetic analyses as outgroups. Once collected, all samples were preserved in 70% ethanol until the analysis.

Amplification and Sequencing of 12S and 16S rRNA Genes

Total genomic DNA was extracted from white muscle by proteinase K/SDS dissolution and purified by phenol:chloroform extraction and ethanol precipitation according to standard procedure (Sambrook, Fritsch, and Maniatis 1989). The amplification of the target genes by the polymerase chain reaction (PCR) (Saiki et al. 1988) was performed. The PCR primers used were 12Sa and 12Sb (Kocher et al. 1989) and 16Sa and 16Sb (Palumbi et al. 1991), which amplified DNA fragments of about 370 and 500 bp, respectively. PCR products were directly sequenced by means of the dideoxy cycle sequencing technique, using 5' end-labeled primers (^{33}P).

Phylogenetic Analysis and Rate Constancy Test

The orthologous DNA sequences were entered in a multiple-sequence editor (ESEE) (Cabot and Beckembach 1989) and aligned using the computer program CLUSTAL W (Thompson, Higgins, and Gibson 1994) with default settings; this alignment was inspected and adjusted by eye. We inferred the relationships among the investigated taxa by two different methods of phylogenetic analysis: the neighbor-joining (NJ) analysis was performed as implemented by the program MEGA (Kumar, Tamura, and Nei 1993), while maximum-parsimony (MP) reconstructions were performed with PAUP (version 3.1.1; Swofford 1993). In the NJ analysis, genetic distances were measured with correction for multiple hits according to Kimura's (1980) two-parameter model. MP reconstruction was performed by an exhaustive search of the most parsimonious tree(s), with transitions (ts): transversions (tv) weighted 2:1 (see *Results*). Robustness of both MP and NJ trees was assessed by bootstrap analysis (Felsenstein 1985) with 1,000 replications.

Rate constancy was assessed according to Takezaki, Rzhetsky, and Nei (1995); this method was implemented in a computer program made available by the authors. The test can be performed following production of the tree topology with a tree-building method (without the assumption of rate constancy) and after rooting of the tree using one or more outgroups. In our analyses, we used the program's "two-cluster test" for estimating the equality of the average substitution rate between two clusters (each cluster may comprise one or more taxa) that are created by an internal node in a given tree. The difference between the mean observed numbers of substitutions per site from a given node of the tree to the tip of each of the two clusters bifurcating from that node

Table 1
Synoptic Table Showing a List of the Investigated Species, with Their Common Names, Habitat Preferences, and Geographic Distributions

Species Name	Common Name	Habitat Preferences	Geographic Distribution
<i>Gobius auratus</i> Risso	Golden goby	Inshore, among stones	Eastern Atlantic (Spain) and Mediterranean
<i>Gobius bucchichi</i> Steindachner	Bucchich's goby	Inshore, on sand or mud	Mediterranean and Black Sea
<i>Gobius niger</i> Linnaeus	Black goby	Estuaries, lagoons, and inshore waters, on sand or mud	Eastern Atlantic and Baltic Sea, Mediterranean and Black Sea
<i>Gobius paganellus</i> Linnaeus	Rock goby	Inshore and intertidal, under stones and on rocky shores with weed cover	Eastern Atlantic, Mediterranean and Black Sea
<i>Knipowitschia panizzae</i> Verga	Lagoon goby	Brackish estuaries and lagoons	Adriatic and Tyrrhenian brackish waters; Lake Trasimeno, Italy (introduced)
<i>Knipowitschia punctatissima</i> Canestrini	Orsini's goby	Spring waters, on sand or mud among reach vegetation	Northeastern Italy, west Slovenia, north Dalmatia
<i>Padogobius martensii</i> Günther	Martens' goby	Freshwater on stony substrates	Northern Italy, Po River drainage
<i>Padogobius nigricans</i> Canestrini	Arno goby	Freshwater on stony substrates	West-central Italy
<i>Pomatoschistus marmoratus</i> Risso	Marbled goby	Inshore sand, estuaries and brackish lagoons	Mediterranean and Black Sea
<i>Pomatoschistus minutus</i> Pallas	Sand goby	Estuaries and lagoons (juveniles), inshore sand and muddy bottom (adults)	Eastern Atlantic, northern Mediterranean and Black Sea
<i>Pomatoschistus quagga</i> Heckel	Quagga goby	Inshore	Western Mediterranean and Adriatic
<i>Zebrus zebrus</i> Risso	Zebra goby	Inshore, lagoons, and intertidal pools; under stones and among algae and sea grass	Mediterranean
<i>Zosterisessor ophiocephalus</i> Pallas	Grass goby	Inshore, brackish waters of estuaries and lagoons, among grass roots on mud	Mediterranean and Black Sea

is expressed as d . Under the assumption of rate constancy, the expectation of d between the two clusters is zero. The deviation of d from zero can be tested by the two-tailed normal test (for further details, see Takezaki, Rzhetsky, and Nei 1995).

In order to generate a "linearized tree" (LT) (in which branch lengths leading from one taxon to any other in the tree are proportional to their genetic distance), we have eliminated all taxa showing substitution rates significantly different from average, as indicated in Takezaki, Rzhetsky, and Nei (1995).

Results

A total of 867 bp (505 bp for 16S rRNA and 362 bp for 12S rRNA) were aligned for all 16 species investigated. Sequences were deposited in GenBank under the accession numbers AF067254–AF067266 for 12S rRNA and AF067267–AF067279 for 16S rRNA. Gaps and ambiguous alignments were excluded from the analysis. Genetic distances were calculated on both transitions and transversions as well as on transversions only (data not shown). Tv+ts distances ranged between 0.0262 (*P. minutus*–*P. marmoratus*) and 0.1941 (*Gobius niger*–*Pomatoschistus quagga*). In order to test whether the transitional mutations were saturated, for each pairwise comparison we plotted the absolute number of transitions against the absolute number of transversions. The tv/ts linear relationship ($y = 0.521x + 4.79$; $R^2 = 0.960$) suggested that transitions are not saturated and that the ts:tv ratio is close to 2:1.

In the phylogenetic analysis, both MP and NJ analyses provide strong support for the existence of two dis-

tinct clades, one clustering together all five species of sand gobies and the other grouping the remaining eight gobies species (fig. 2). In the sand gobies clade, *Knipowitschia panizzae* seems to have branched off earlier from the ancestral lineage than the other congeneric freshwater species, *K. punctatissima*, which is instead clustered into the *Pomatoschistus* group. Within this lineage, *Pomatoschistus minutus* and *Pomatoschistus marmoratus* (both euryhaline species) are the closest species, and MP analysis suggested *K. punctatissima* as their sister group.

With regard to the cluster grouping the other eight species of Gobiidae, two subclusters were identified by both MP and NJ phylogenetic reconstructions. Interestingly, one of the two subclusters grouped together all those marine species (relatively euryhaline) inhabiting muddy or sandy substrates, with *G. niger* clearly separated from *Gobius auratus*, *Gobius bucchichi*, and *Zosterisessor ophiocephalus*. In the second subcluster, the two freshwater species of the genus *Padogobius* (*P. nigricans* and *P. martensii*) were grouped in a single clade together with *Gobius paganellus*, which came out as the sister group to *P. nigricans*. The position of the marine species *Zebrus zebrus* was not resolved by either the NJ or the MP reconstructions, as indicated by the low bootstrap values for this branch in both trees (fig. 2a and b).

We also tested the homogeneity of the substitution rates within and between lineages by means of the two-cluster test; rate constancy was assessed by tv+ts and by tv only. Using tv+ts, *G. niger*, *G. auratus*, *Z. ophiocephalus*, *P. minutus*, and *K. panizzae* showed substitution rates significantly different from the average; in

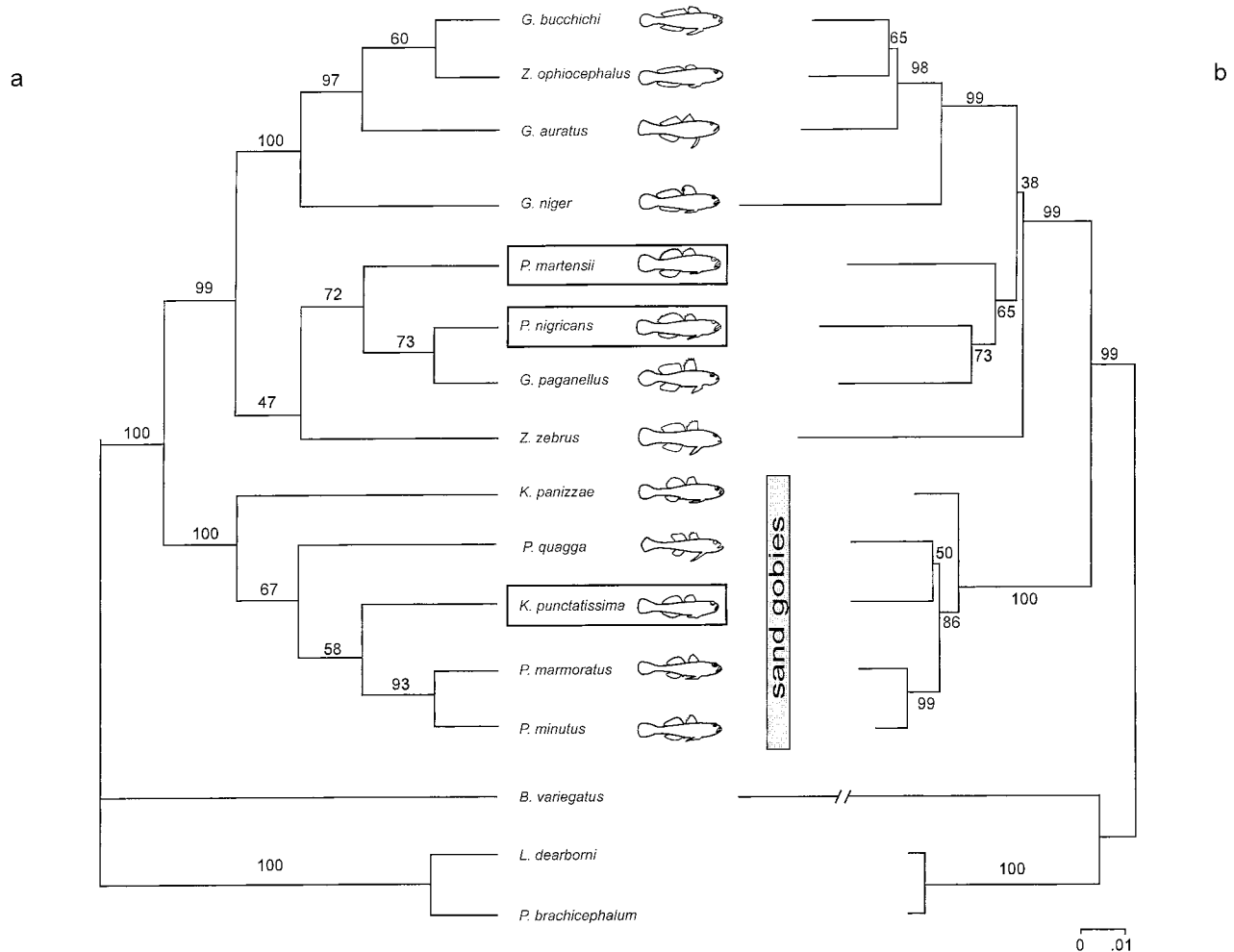


FIG. 2.—*a*, Neighbor-joining tree (right side) representing the phylogenetic relationships of the 13 gobiid species included in the present study: *Bovichthys variegatus* (Notothenioidea), *Lycodichthys pearborni*, and *Pachychara brachycephalum* (Zooarchoidea) were used as outgroups. Pairwise distances between taxa were calculated according to the two-parameter Kimura (1980) model. The scale bar indicates 0.01 sequence distance. Numbers at branches refer to bootstrap values after 1,000 replicates. *b*, Majority-rule consensus tree (left side) after 1,000 bootstrap replicates using maximum parsimony; bootstrap values are reported for each node. Transversions were given a higher weight than transitions (2:1) according to the observed bias (see text). A total of 233 sites were informative for parsimony. A heuristic search (with stepwise random addition of taxa, 100 replicates) with all changes equally weighted yielded a single most-parsimonious tree (tree length: 806 steps) with a consistency index (excluding uninformative sites) of 0.616.

contrast, consideration of only tv shows a single species, namely *K. panizzae*, with a substitution rate statistically slower than those of all the other taxa. Since tv and ts showed a linear relationship, we decided to construct an LT based on tv only, in order to include the largest possible number of species (fig. 3). The creation of an LT provides a genetic distance between clades which, assuming a molecular clock model, can be used to make tentative datings for the major evolutionary events described in the tree.

Discussion

Phylogenetic Relationships

The phylogenetic analysis based on mitochondrial sequences suggests a polyphyletic origin of freshwater habitat among western Mediterranean gobies, with *K. punctatissima* being derived independently from the other two freshwater species belonging to the genus *Pa-*

dogobius. The latter are clustered together in a single clade with the species of the genus *Gobius*. In this context, it is worth noting that *G. paganellus*, a moderately euryhaline marine species, is the sister group to the freshwater *P. nigricans*. With few exceptions, the two major clusters identified by the molecular phylogeny overlap with the subdivision of species based on the presence/absence of the suborbital papillae and longitudinal rows (and their various interconnections), which represent an important morphological criterion in the classification of Gobiidae. According to these two key characters, *K. punctatissima* was originally classified as a species of the monotypic genus *Orsinigobius*. Further investigations recognized the presence of a number of characters shared between this species and the genus *Knipowitschia*, thereby justifying the synonymization of *Orsinogobius* with *Knipowitschia* (Gandolfi, Torricelli, and Cau 1985; Economidis and Miller 1990). The ab-

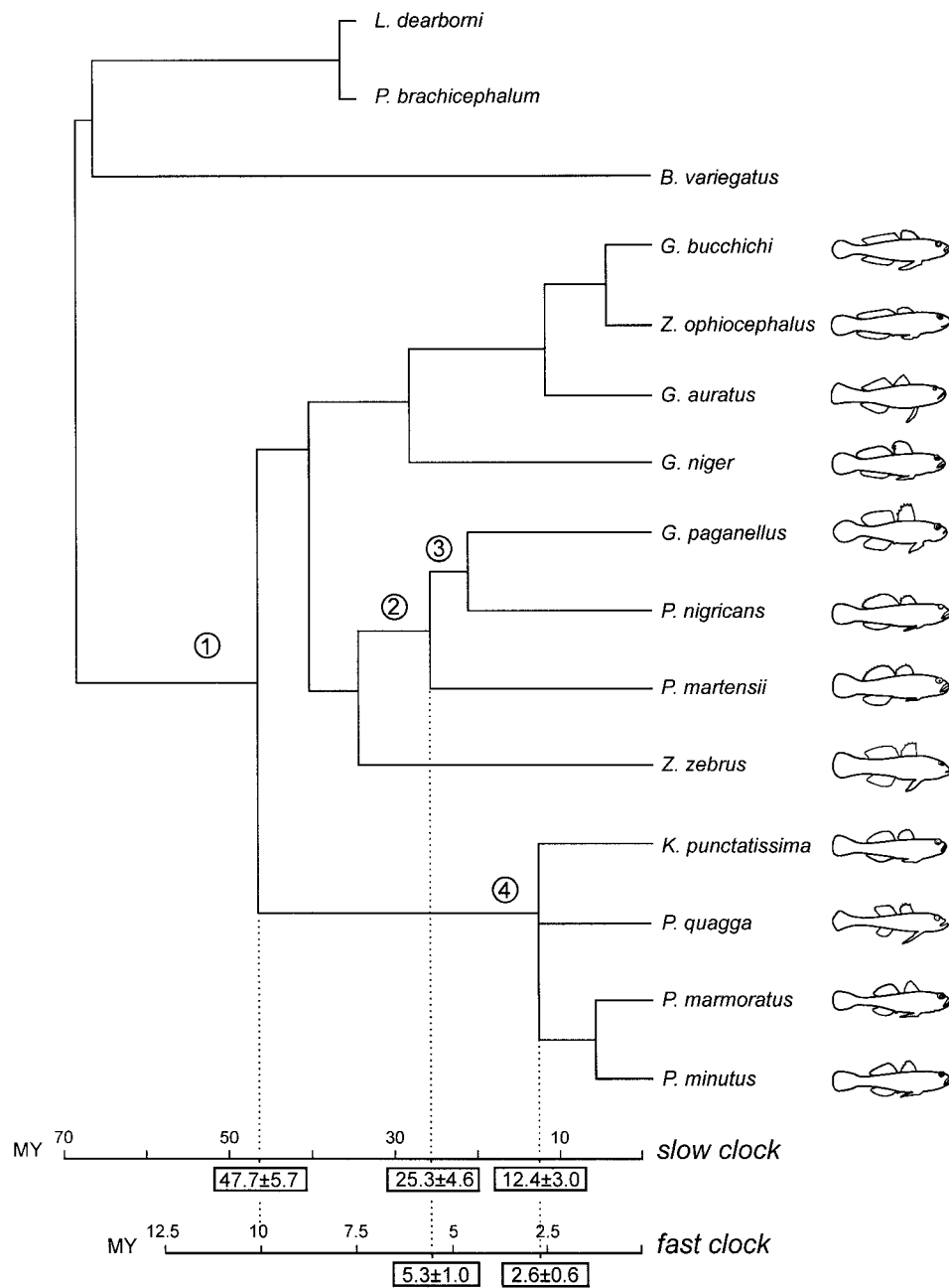


FIG. 3.—Linearized tree according to Takezaki, Rzhetsky, and Nei (1995), based on pairwise distances calculated on transversions only. The tree is based on the assumption of a homogeneous rate of sequence divergence among taxa. All of the ingroup species are included except for *K. panizzae*, which has a significantly different evolutionary rate according to the present analysis. Branch length connecting each pair of taxa is proportional to their distance calculated on transversions. Scale bars below the tree show two alternative timescales according to the different calibrations of molecular clock: “slow clock,” calibrated assuming a divergence rate of 0.14% transversions per million years, and “fast clock,” calibrated assuming a rate four times as high (0.65% tv/Myr). Circled numbers indicate nodes of the tree describing relevant events in the evolution of this group of species (for details, see text). Boxed numbers reported on each of the two timescales refer to the alternative time estimate specific to the node connected by the dashed line. Standard errors (SEs) of the time estimate reflect the error associated with the re-estimation of branch lengths along the linearized tree under the assumption of rate constancy. SEs for the distance of each interior node from the external nodes are computed following equation 15 in Takezaki, Rzhetsky, and Nei (1995) and are part of the output of the Cluster Test program.

sence of the longitudinal row and suborbital papillae in *K. punctatissima* (characters which are distinctive of the genus *Knipowitschia*) was therefore considered a secondary loss (Bianco et al. 1987; Economidis and Miller 1990).

The molecular data support the grouping in a single clade of the genera characterized by the presence of a longitudinal row and suborbital papillae, namely *Knipowitschia* (the absence of these characters in *K. punctatissima* is therefore to be considered a derived state,

as discussed above) and *Pomatoschistus*. Further evidence for the *Knipowitschia*–*Pomatoschistus* association is provided by allozyme investigations, which grouped sand gobies in a single clade (Miller et al. 1994; McKay and Miller 1997). Within this lineage, the phylogenetic reconstruction based on mtDNA indicates that the genus *Knipowitschia* is paraphyletic, since *K. punctatissima* is placed in the *Pomatoschistus* clade as sister group to *P. minutus* and *P. marmoratus*, whereas *K. panizzae* forms the basal branch to the cluster of all sand gobies (fig. 2). This tree topology suggests that *K. panizzae* is part of a more ancient lineage, possibly including other Ponto-Caspian species (e.g., *Knipowitschia caucasica* and *Knipowitschia longicaudata*, sharing many morphological and ecological features with *K. panizzae*), that branched off earlier from the lineage of the sand gobies. The position of *P. quagga* (which did not turn out to be closely related to the *P. minutus* + *P. marmoratus* lineage) indicates that the genus *Pomatoschistus* is also paraphyletic. Allozyme data were somewhat contradictory in regard to the monophyletic status of *Pomatoschistus* and *Knipowitschia* genera, although a recent study supported the paraphyly of *Pomatoschistus* (Miller et al. 1994; McKay and Miller 1997). A clearer picture of the evolution of the sand goby lineage might be achieved with the inclusion of additional taxa thought to be related to those considered in the present work: for example, *Pomatoschistus canestrini* (which is endemic to Adriatic marine waters but is more easily adaptable to low salinity than any of the other congeneric species) (Gandolfi, Torricelli, and Cau 1982), gobies inhabiting the eastern Mediterranean area, such as *Knipowitschia thessala*, and/or species belonging to the genus *Economidichthys*.

Among the species grouped in a lineage separated from the sand gobies, excluding *Z. zebrus*, whose position is unresolved, the molecular phylogeny identifies two subclusters that group the remaining taxa according to their substrate preferences. In fact, *G. niger*, *G. buccichichi*, *G. auratus*, and *Z. ophiocephalus*, which are species typical of muddy or sandy substrates, are all clustered together. It is also worth noting that the genus *Zosterisessor*, monotypically represented by *Z. ophiocephalus*, is included in this clade as the sister group to *G. buccichichi*. The other subgroup includes the remaining species, all inhabiting rocky bottoms, among which are the two Italian freshwater species belonging to the genus *Padogobius*. *Padogobius martensii* and *P. nigricans*, although genetically very close, do not appear as sister species, because *P. nigricans* was clustered with *G. paganellus*. Interestingly, the latter species is one of the very few marine gobies inhabiting rocky and/or stony bottoms like the two freshwater *Padogobius* spp. The geographic separation observed between *P. martensii* and *P. nigricans*, in combination with the genetic relationships revealed by the phylogenetic reconstruction (*P. nigricans* more closely related to *G. paganellus* than to *P. martensii*), might suggest an independent origin of the freshwater condition within the genus *Padogobius*. However, the bootstrap value supporting the *P. nigricans* + *G. paganellus* grouping is relatively low

(73%); thus, we cannot rule out alternative hypotheses. In fact, the possibility also exists that the freshwater lifestyle originated only once, in the ancestor of *P. martensii*, *P. nigricans*, and *G. paganellus*; secondarily the latter species might have recolonized the marine habitat.

The phylogenetic position of *G. paganellus*, which is placed outside the clade grouping the rest of the *Gobius* species, as well the inclusion in this clade of *Z. ophiocephalus*, indicates that the genus *Gobius* is paraphyletic. Similar conclusions were also reached in the allozyme investigations of McKay and Miller (1991). In that study, however, the monophyly of this genus was challenged by the “aberrant” position of *G. auratus*, which, in our study, is grouped together with *G. niger*, *G. buccichichi*, and *Z. ophiocephalus*. The fact that *Z. ophiocephalus* (which is the only representative of its genus) turns out to be genetically very close to *Gobius* species, as also indicated by protein polymorphism (McKay and Miller 1991, 1977; Miller et al. 1994), suggests the opportunity to revise its systematic status and to eventually include it in the genus *Gobius*.

Geological History, Molecular Clock, and Evolution of Freshwater Lifestyle

Using the LT constructed on transversional mutations, after excluding *K. panizzae*, which has a different rate of substitution, it is possible to approximately date the major evolutionary events described in the LT, i.e., every bifurcation generated by a node of the tree. Assuming that the use of a molecular clock is legitimated by the exclusion of taxa with a different mutation rate, an absolute time estimate requires a clock calibration. This is actually a difficult task. Previous investigations tackling this issue indicated that a reliable mutation rate for mitochondrial ribosomal genes in vertebrates should be 0.14% tv/Myr (Hillis and Dixon 1991; Allard et al. 1992; Caccone et al. 1997 and references therein). This value seems to fit a large variety of organisms, including fish (Meyer 1993; Ritchie et al. 1996; Bargelloni et al. 1997).

The deeper node of the LT (node 1 in fig. 3) separates the *Knipowitschia* + *Pomatoschistus* clade (the sand gobies) from the *Gobius* + *Zosterisessor* + *Padogobius* lineage. This split is believed to be a vicariant separation caused by the mid-Miocene isolation of the Paratethys (Sarmatic) basin from the Mediterranean Tethys sea (McKay and Miller 1991) (fig. 4a). According to geological evidence, this event occurred about 10–12 MYA (Steininger and Rögl 1984). However, assuming a rate of 0.14% tv/Myr, the majority of the nodes in the LT tree appear to be much older than 10–12 Myr, apparently predating the separation of Paratethys from the Mediterranean region. In particular, the split of the Sarmatic and Mediterranean goby lineages seems to have occurred more than 40 MYA (fig. 3), i.e., at least four times as early as previously thought.

Focusing our attention on the evolution of the freshwater forms within each of these two major clades, it was suggested that adaptation to freshwater originated during the Messinian salinity crisis and the formation of the “Lago-Mare” in the Mediterranean Basin about 5.5

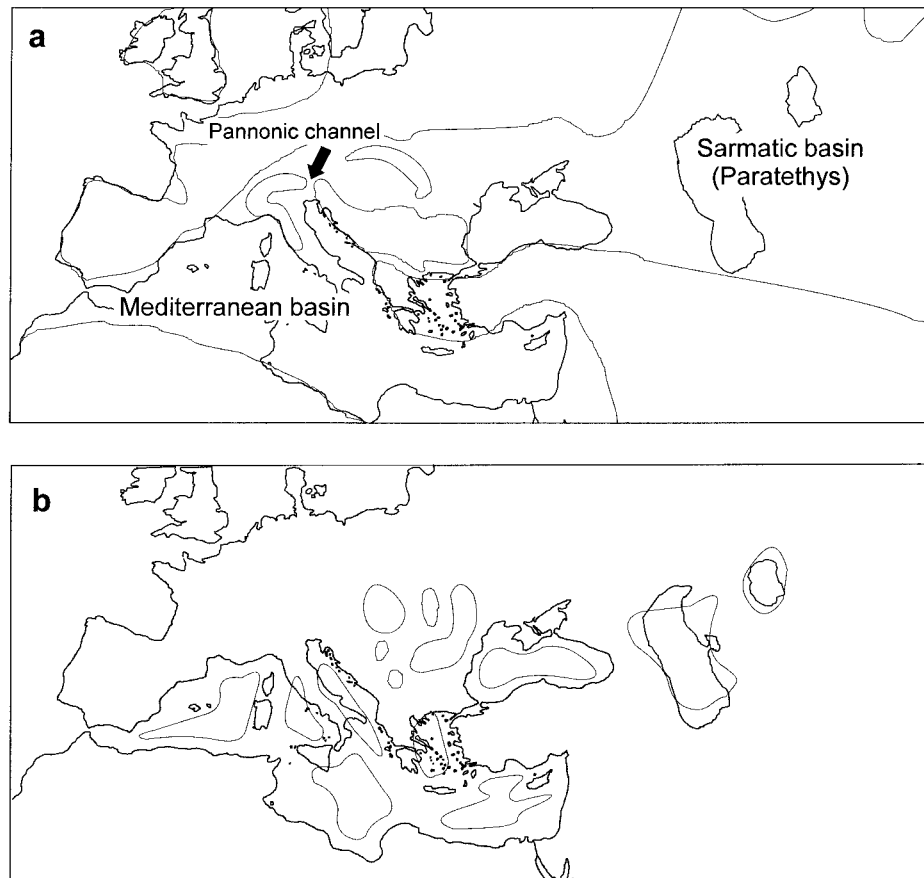


FIG. 4.—*a*, Extension of land (gray shadow) during the early/middle Miocene (24–15 MYA) before the complete split of residual Tethys in Paratethys (Sarmatic), and the Mediterranean Basin. Arrows indicate the “Pannonic channel,” a potential route of ichthyofauna dispersal from Asia/central Europe to the Mediterranean. *b*, Expansion of land (gray shadow) during the Messinian salinity crisis (5.5 MYA) with extensive desiccation of the Mediterranean sea.

MYA at the Miocene–Pliocene boundary (Hsü et al. 1977) (fig. 4*b*). These dramatic environmental modifications were also suggested to be responsible for the origin of the freshwater habit in species, such as the blennid *Salaria fluviatilis* and the salmonid *Salmo trutta marmoratus*, belonging to primarily marine fish families (Banareescu 1973; Bianco 1987). Similarly, the Messinian salinity crisis was hypothesized to have promoted the colonization of freshwater inland by euryhaline ancestors in each of the two goby lineages inhabiting the western Mediterranean (Economidis and Miller 1990; McKay and Miller 1991; Miller et al. 1994). However, the calculation inferred from our LT for the branches leading to the three freshwater taxa (nodes 2–4 in fig. 3) indicates that their origin could be much older than 5.5 Myr; i.e., *K. punctatissima* branched off from the common ancestral lineage of the other Ponto-Caspian species (node 4) about 12 MYA, whereas the clade which includes *P. martensii* and *P. nigricans* (nodes 2 and 3) seems to be as old as 20–25 Myr. If these estimates are correct, the proposed correlation between the evolution of these groups of fish and the Miocene–Pliocene geological and paleoclimatic events occurring in the Mediterranean region should be rejected. Rather, this scenario would be in agreement with the hypothesis of an Asiatic/central European origin of the Mediterranean

fish fauna (Banareescu 1973). According to this view, freshwater gobies inhabiting circum-Mediterranean areas should be regarded as relicts of a westward dispersal of Asiatic ichthyofauna through the Pannonic channel (fig. 4*a*) which occurred during early Miocene (24 MYA) prior to the complete separation between the Paratethys and the Mediterranean Basin (Banareescu 1992, pp. 838–847).

An alternative explanation can be envisaged if we assume that the gobies’ molecular clock runs faster than those of other species investigated so far. In such a case, we could try to recalibrate the clock to considering the vicariant separation between the two major lineages of the tree, which is hypothesized to have occurred at the mid-Miocene Sarmatic/Mediterranean separation 10–12 MYA (Bianco 1987; McKay and Miller 1991; Miller et al. 1994). According to this recalibration, the mutation rate for the mitochondrial ribosomal genes in gobies would be 0.67% tv/Myr (which is 4.7 times as fast as those in other vertebrates). This new calibration supports an early Pliocene origin of the two freshwater species of the genus *Padogobius*. However, on the basis of the present data, it is uncertain whether the evolution of this habitat preference occurred independently in the two species of *Padogobius* (as their allopatric distribution seems to indicate), or only once in their ancestor as the

consequence of the Messinian salinity crisis 5.5 MYA (fig. 3). In the latter case, this ancestral species should have been present on both western and eastern sides of Italy in residual marine Tethys habitats.

Using a rate of 0.67% TV/Myr, *K. punctatissima* seems to have a more recent origin (about 2.6 Myr) than to the two *Padogobius* species. If this is true, then the paleoclimatic events that promoted the freshwater condition in this sand goby are different from those for *P. martensii* and *P. nigricans* and, in any case, unlinked to the Messinian salinity crisis. However, the LT omits *K. panizzae*, which, according to our phylogeny, is a sister taxon to the rest of the sand gobies (fig. 3). This absence may affect the estimation of the time of divergence for the taxa belonging to this clade.

Considering the small body size of gobies (*Pandaka pygmaea*, the smallest vertebrate known, is a goby) (Miller 1996), the possibility of a faster accumulation of mutations would be in agreement with the “metabolic rate” theory, which postulates an inverse relationship between mutation rate and body mass (Rand 1994). In addition, gobies have a short generation time, which tends to favor a faster accumulation of mutational changes (Martin and Palumbi 1993). However, in the absence of specific data on the metabolism of gobies, this remains a speculation that needs to be investigated experimentally.

In conclusion, our data provide strong support for a multiple independent origin of the freshwater habit in the three endemic Italian species of gobies. Moreover, our phylogenetic analysis suggests that the morphological characters currently used in systematic analyses of Gobiidae are likely to be insufficient to correctly infer the phylogenetic relationships among the members of this family.

The molecular clock approach, based on both protein-coding and ribosomal mitochondrial genes, turned out to be very useful in studying the evolution of various fish taxa in association with major geological events. For instance, this allowed a better understanding of the present-day biogeographic pattern of marine and freshwater tropical fishes inhabiting both sides of the Panama Isthmus (Bermingham, McCafferty, and Martin 1997). Estimates of divergence time based on molecular data also suggested a tight link between the remarkable radiation displayed by the suborder Notothenioidei in the Southern Ocean and ice formation in the Antarctic waters (Bargelloni et al. 1994, 1997; Ritchie et al. 1996). The use of the molecular clock to estimate the timing of evolutionary events occurring in the western Mediterranean gobies suggests two alternative hypotheses: (1) the examined gobies species are much older than previously thought, and thus the Miocene–Pliocene geological and paleoclimatic modifications have played no role in their evolution; or (2) these fishes have a mitochondrial substitution rate much faster than those of other vertebrates.

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