Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular Phylogenetics and Evolution 32 (2004) 324–336
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Paleoclimatic history and vicariant speciation in the "sand goby" group (Gobiidae, Teleostei)☆

Tine Huyse,* Jeroen Van Houdt, and Filip A.M. Volckaert

Laboratory of Aquatic Ecology, Katholieke Universiteit Leuven, Ch. de Bériotstraat 32, B-3000 Leuven, Belgium

Received 11 September 2003; revised 5 November 2003
Available online 31 January 2004

Abstract

Vicariant and climatic cycling speciation hypotheses of the 'sand gobies' belonging to the genera *Pomatoschistus*, *Gobiusculus*, *Knipowitschia*, and *Economidichthys* are tested using molecular phylogenies constructed of nuclear DNA (ITS1 locus) and mitochondrial DNA (12S and 16S fragments). These gobies are among the most abundant in the Eastern Atlantic-Mediterranean region, and play an important role in the ecosystem. Considerable ITS1 length differences, primarily due to the presence of several tandem repeats, were found between species and even within individuals. Therefore, phylogenetic analyses focused on fragments of the 12S and 16S mtDNA region that have been sequenced for 16 goby taxa. The 'sand gobies' clustered as a monophyletic group as proposed on morphological grounds. However, *G. flavescens*, *E. pygmaeus*, and *K. punctatissima* clustered within the *Pomatoschistus* species, pointing to a paraphyletic origin of these genera. Furthermore, the genetic divergence between *P. minutus* from the Adriatic Sea *versus* the Atlantic-Mediterranean region was as high as the divergence within the *P. minutus* complex, suggesting that *P. minutus* from the Adriatic Sea should be considered as a distinct species. The "star" phylogeny might suggest that these gobies evolved in a very short time period, possibly linked to the drastic alterations in the Mediterranean Sea during and immediately after the Messinian salinity crisis at the end of the Miocene. The freshwater life-style appeared monophyletic; equating its origin with the salinity crisis resulted in a molecular clock estimate of 1.4% divergence per million years. The last common ancestor probably occupied sandy bottoms and a coastal niche while several species subsequently adapted to new habitats (pelagic, freshwater or stenohaline). The origin of the shallowest clades dated back to the glacial cycling during the Pleistocene epoch.

Keywords: ITS1 rRNA; Mediterranean Sea; Messinian salinity crisis; Vicariance; Miocene; Pleistocene

1. Introduction

Climatic changes and geological events play a key role in speciation events. The Mediterranean Sea offers a great natural study system for vicariant speciation studies. It underwent substantial changes from the Oligocene/Miocene boundary onwards, inducing speciation in e.g., European cyprinids (Zardoya and Doadrio, 1999), Eurasian killifishes (Hrbek and Meyer, 2003), and Paleartic brown frogs (Veith et al., 2003). In the Miocene, the proto-Mediterranean was connected through seaways to the Atlantic and Indian Oceans as well as to the Paratethys (Fig. 1), enabling a free exchange of fauna between these regions. At that time, subtropical marine faunas extended up to Eastern Europe and the Middle East (Néraudeau et al., 2001). About 15–14 million years ago (mya), the Eastern Mediterranean seaway closed when the Arabian and Anatolian plates collided (Rögl, 1999; Seidenkrantz et al., 2000) and from this point on the fauna of the modern Mediterranean Sea and the modern Indo-West Pacific Region evolved independently from each other. The isolation of the Paratethys from the Mediterranean occurred in the mid-Miocene (12–10 mya), while the Mediterranean-Atlantic connection became constricted at the end of the Miocene. The subsequent desiccation of the Mediterranean basins is known as the Messinian salinity crisis (MSC) and started about 5.96 mya (Hsü et al., 1977; Krijgsman

☆ Nucleotide sequence data reported in this paper are available in the GenBank database under the Accession Nos. AJ616333–AJ616343; AJ616806–AJ616837.

* Corresponding author.
E-mail address: Tine.Huyse@bio.kuleuven.ac.be (T. Huyse).

1055-7903/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2003.11.007

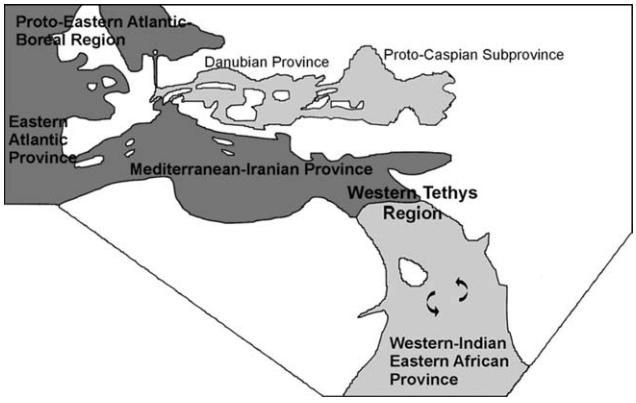

Fig. 1. Marine biogeography of the circum-Mediterranean area in the Oligocene–early Miocene. The Western Tethys Region is divided into the Mediterranean-Iranian Province and the Western Indian-Eastern African Province (after Harzhauser et al., 2002).

et al., 1999), leading to the origin of several hyper- and hyposaline lakes. Canyon incision in the Aegean region for example, most likely caused the transition to 'Lago Mare' (sea-sized lake) conditions by capturing freshwater of the Black Sea drainage (Krijgsman et al., 1999), supposedly allowing the subsequent spreading of fresh water fish (Bianco, 1990). This period lasted almost 700,000 years, and most of the ancient Indo-Pacific ancestral fauna became extinct. However, according to Por and Dimentman (1985), the gobies are considered as possible Messinian fish relicts, together with the cyprinodont fish genus *Aphanius*. Pliocene flooding that ended the MSC isolated populations and triggered endemic speciation on newly isolated Mediterranean islands, with a possible example of *Hydrobia* sp. (Wilke, 2003).

The Gobiidae represents one of the most diverse families of fish, occupying marine, brackish, and freshwater habitats in the tropical and temperate seas of the world (Hoese, 1984; Miller, 1986). Among the Eastern Atlantic-Mediterranean gobioid fishes, a so-called 'sand goby' group consisting of four phenetic genera has been recognized: *Pomatoschistus* (Gill) *Gobiusculus* (Duncker) *Knipowitschia* (Ljin), and *Economidichthys* (Bianco, Bullock, Miller, and Roubal). In the past, systematic difficulties have arisen, especially in the genus *Pomatoschistus*, due to their small body size and superficial resemblance to each other (Webb, 1980). Several allozyme studies have been carried out (Mckay and Miller, 1991, 1997; Wallis and Beardmore, 1984a), resulting in conflicting phylogenies.

Pomatoschistus is the dominant gobiid genus of the Atlantic and Mediterranean coasts of Europe, comprising about 11, mainly marine species (Miller, 1986). The *Pomatoschistus minutus* complex, including *P. minutus*, *P. lozanoi*, and *P. norvegicus*, is thought to have speciated only recently; occasionally the former two species hybridise (Fonds, 1973; Wallis and Beardmore, 1980). Despite the morphological similarity, the karyological differentiation is substantial, with differences in the number of chromosomes and chromosome arms (Webb, 1980). The monotypic boreal genus *Gobiusculus* is represented by the marine and pelagic *G. flavescens*. It is recorded from the eastern Atlantic excluding the southern region; Mediterranean records remain to be confirmed (Miller, 1986). The genus *Knipowitschia* contains an assemblage of freshwater and euryhaline gobies occurring in the Black and Caspian basins; two freshwater species are endemic to the Mediterranean. According to Miller (1990), many *Knipowitschia* species evolved following the draining of the Western Paratethys into the Adriatic Sea during the MSC. Its systematic status has been under debate: it embraces a number of species otherwise placed in the separate genera *Orsinigobius* created by Gandolfi et al. (1985) and *Hyrcanogobius* (Ljin) (Miller, 1990). The West Balkanian genus *Economidichthys*, characterized by a unique perianal organ (Bianco et al., 1987), is thought to share common ancestry with *Knipowitschia*. It comprises two endemic species: *E. pygmaeus* and *E. trichonis*, the latter being Europe's smallest freshwater fish (Economidis and Miller, 1990).

The systematic relationships of the sand gobies with other gobioids remains unclear from morphology but the most likely sister group to the sand gobies has to be looked for in the Indo-Pacific region (Mckay and Miller, 1997). The mid-Miocene isolation of the Paratethys from the Mediterranean Tethys is believed to have induced the vicariant separation of the sand gobies and other Atlantic-European gobies (Mckay and Miller, 1991). Besides the pelagic *G. flavescens*, *P. quagga*, and *P. knerii*, all other sand gobies are usually found on sandy bottoms. Most of them are marine or euryhaline, with the exception of *P. canestrinii* (brackish and freshwater), *K. punctatissima*, and *Economidichthys*, suggesting a polyphyletic origin of a freshwater life style. The only other Mediterranean freshwater gobiids are found in the genus *Padogobius*: *P. martensii* and *P. nigricans*. All freshwater species have a fragmented distribution (Fig. 2), suggesting a vicariant speciation caused by the MSC (Miller, 1990). However, the phylogenetic relationships between *Padogobius* spp. and the sand gobies (Mckay and Miller, 1997; Penzo et al., 1998) suggest that both groups acquired the freshwater lifestyle independently of each other.

In order to to link speciation events to paleoclimate and test the following hypotheses, fragments of the 12S and 16S mtDNA region and the nuclear ITS1 region have been sequenced for 16 goby taxa (*Pomatoschistus*, *Knipowitschia*, Economidichthys, and *Gobiusculus*). The main goal was to construct a robust phylogeny, that could be used as a basis (1) to test if the morphological phylogeny correspondents with the molecular phylogeny, e.g., to test the monophyly of the "sand goby"

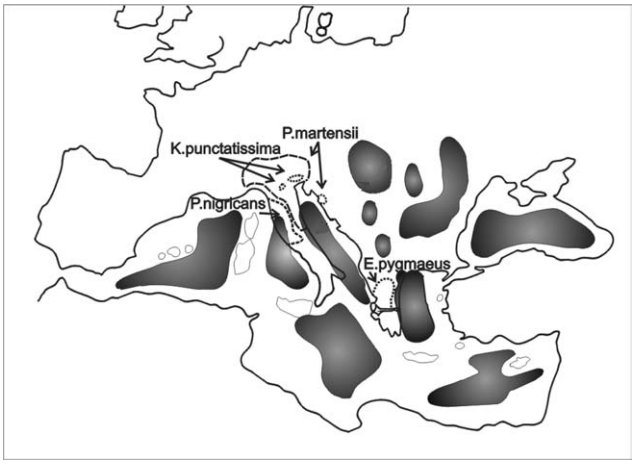

Fig. 2. Geographical distribution of the Mediterranean freshwater gobies (after Miller, 1990) and the Miocene geography of the Mediterranean and Paratethys during the Messinian salinity crisis (after Banarescu, 1992).

group and its respective genera, (2) to study the evolution of niche occupation, and (3) to study the origin and evolution of this goby group in the light of the paleoclimatic and geological history of its habitat. This latter encompasses the search for a sister group, testing the monophyly of the freshwater species and the link between the origin of the freshwater lifestyle and the MSC.

2. Materials and methods

2.1. Collection of material

In total, 45 individuals belonging to fourteen species and four genera were collected along the North-Eastern Atlantic continental shelf, the Adriatic and the Mediterranean Sea. *Economidichthys pygmaeus* specimens were collected from the Acheron River in Greece. Fish species, collection site, geographic distribution and habitat preference are shown in Table 1. The geographic distribution of the freshwater goby species is shown in Fig. 2. All specimens were preserved in 85% ethanol.

2.2. Amplification and sequencing of the ITS 1 rDNA, 12S and 16S mtDNA

DNA was extracted following the NucleoSpin Tissue protocol (BD Biosciences). The complete ITS1 region was analysed for 2 specimens per species. The primers MD1F: 5′-CTT GAC TAT CTA GAG GAA GT-3′ and 5.8SR: 5′-AGC TTG GTG CGT TCT TCA TCG A-3′ (Sajdak and Phillips, 1997) were used to amplify the ITS1 fragment. The total reaction volume (25 μl) consisted of: 1× PCR buffer, 0.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer, 1 μl template, 1 U *Taq* polymerase and mQ-H_2O. The mixtures were layered with mineral oil, heated for 4 min at 97 °C and subjected to 35 cycles as follows: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and then cooled at 4 °C. The PCR products were purified by means of GFX columns according to the manufacturer's instructions (Amersham Biosciences). These products were used for cloning following manufacturer's instructions (TA cloning kit, Invitrogen). The PCR products of the cloned products were purified and directly sequenced in both directions. Sequencing was done following the protocol of SequiTherm EXCEL II (Epicentre Technologies); 5% DMSO was added to overcome sequencing difficulties related to G/C rich templates. The reaction products were separated and visualised on a LICOR 4200 system using a 6% Long Ranger gel (FMI BioProducts). For each specimen two clones were sequenced. The 12S and 16S mtDNA was amplified and sequenced for two to six specimens per species using the following primers: 16SH 5′-CCGGTCTGAACTCAGATCACGT-3′, 16SL 5′-CGCCTGTTTATCAAAAACAT-3′ (Palumbi et al., 1991), 12SH 5′-TGACTGCAGAGGGTGACGGGGC GGTGTGT-3′, 12SL 5′-AAAAAGCTTCAAACTGGG ATTAGATACCCCACTAT-3′ (Kocher et al., 1989), with an annealing temperature of 64 and 54 °C, respectively.

2.3. Datasets and alignment of sequences

The first 12S–16S mtDNA dataset consisted exclusively of sand goby species (see Table 1); *Gobius paganellus*, *Padogobius nigricans*, and *P. martensi* were taken as outgroup since these were most closely related to the sand gobies (Penzo et al., 1998). In a second dataset the following GenBank sequences were added: *Gobius niger*, *G. bucchichi*, *G. auratus*, *Zebrus zebrus*, *Zosterisessor ophiocephalus*, and *Bovichtus variegatus* (see Table 1 for GenBank Accession Numbers). The latter species was used as outgroup (Penzo et al., 1998). The ITS1 rDNA dataset consisted of a limited set of goby sequences and no suitable outgroup was available. Sequences were aligned using the program SOAP (Löytynoja and Milinkovitch, 2001). It generates alternative CLUSTAL W alignments by using all possible combinations of gap opening penalty values ranging from e.g., 7–15 and gap extension penalty values ranging from e.g., 3–7. The program then identifies the "unstable-hence-unreliable" characters by comparing the different alignments and excludes these particular regions. Afterwards, those characters can be re-included in the PAUP analyses and the impact of unstable sites on phylogeny reconstruction can be evaluated.

2.4. Phylogenetic analyses

The 12S and 16S fragments were combined in a single dataset since the incongruence-length difference test (Farris et al., 1995) implemented in PAUP* v. 4.01b (Swofford, 2001) provided no evidence for significant

Table 1
Goby species used in this study, including collection site, number of specimens sequenced (12S and 16S mtDNA) or GenBank Accession number, habitat, and natural distribution range

Species	Collection site	Country	*N* sequences/ Accession No.	Distribution
Pomatoschistus minutus (Pallas)	Ostend	B	3	Eastern Atlantic, northern
	Trondheim	N	1	Mediterranean and Black Sea
	Venice lagoon	I	3	
P. lozanoi (de Buen)	North Sea	B	2	Eastern Atlantic (North sea to north-western Spain + Portugal
	Texel	NL	2	
P. pictus (Malm)	Bergen	N	5	Eastern Atlantic: Norway to Spain and Canary Is.
P. norvegicus (Collet)	Bergen	N	4	Eastern Atlantic (Lofotens to western English Channel) + Mediterranean
P. microps (Kroyer)	Ostend, North Sea	B	3	Eastern Atlantic, Baltic Sea, north-western Mediterrenean and Atlantic Marocco
P. marmoratus (Risso)	Venice lagoon	I	1	Mediterranean, Black Sea, Sea of Azov, Suez Canal, Iberian Peninsula
	Chioggia	I	1	
P. marmoratus sp. 1	Venice lagoon	I	2	
	Chioggia	I	2	
P. marmoratus sp. 2	Venice lagoon	I	2	
P. knerii (Steindachner)	Venice lagoon	I	1	Adriatic and Toscanic archipelago, Tyrrhenian Sea (?)
P. quagga (Heckel)			AF067277 AF067264	Western Mediterranean and Adriatic
P. canestrinii (Ninni)	Dalmatia	CRO	3	Mediterranean (Adriatic)
Gobiusculus flavescens (Fabricius)	Trondheim,	N	3	Eastern Atlantic, from western Baltic to north-west Spain, Mediterranean (Sicily and the Adriatic)
	Bergen		2	
Economidichthys pygmaeus (Holly)	Acheron river	GR	3	rivers and streams of western Greece north Albania to be confirmed.
Knipowitschia panizzae (Verga)	Po-Delta	I	1	Adriatic and Tyrrhenian brackish waters; lake Trasimeno, Italy (introd)
K. panizzae sp.	Venice lagoon	I	1	
K. punctatissima (Canestrini)			AF067273 AF067260	Northeastern Italy, west Slovenia, north Dalmatia
Padogobius nigricans (Canestrini)			AF067270 AF067257	Only in rivers of west central Italy
P. martensii (Günther)			AF067274 AF067261	Italian rivers of the northern Adriatic, Dalmatian rivers Zrmanje and Krka
Gobius paganellus (Linnaeus)			AF067271 AF067258	Eastern Atlantic, Mediterranean and Black Sea

G, Belgium; N, Norway; NL, The Netherlands; I, Italy; GR, Greece; CRO, Croatia; F, France.

difference in the phylogenetic signal of both regions ($P = 0.65$). Plotting transitions and transversions against divergence of the complete dataset using DAMBE v. 4.0.75 (Xia and Xie, 2001) did not show saturation. ModelTest v. 3.06 (Posada and Crandall, 1998) was used to estimate the optimal model of molecular evolution in a likelihood-testing framework. These parameters were used in the maximum likelihood (ML) method using PAUP*; trees were statistically tested by calculating *P* values for the individual branches. This likelihood-ratio test assesses whether branch-lengths are significantly different from zero by optimizing all branch lengths under the constraint that one of the branches is zero, for each branch tested. For maximum parsimony (MP) the exhaustive search method was performed using the branch and bound algorithm. In these analyses gaps were treated both as missing data and fifth character since indels might be phylogenetically informative; all sites were equally weighted. The Neighbor-joining (NJ) search was conducted (1000 replicates of tree-bissection reconnection branch swapping) from a matrix of ML genetic distances calculated under the optimised model. Bayesian inference (BI) using MrBayes v. 3.0b4 (Huelsenbeck and Ronquist, 2001) was performed, using the model previously optimized using ML search. Posterior probabilities were estimated over 2.0×10^6 generations, sampling the Markov chains at an interval of 100 generations. In addition, four incrementally heated Markov chains were used. Sample points generated before reaching stationary were

discarded as "burn in" samples. The base composition for all sequences was compared using a 5% χ^2 test on the average composition (TREE-PUZZLE v. 5.0, Schmidt et al., 2002). The molecular-clock hypothesis was tested assuming the HKY model and γ-distributed rates across sites, with the likelihood ratio test (LRT) for the clock hypothesis implemented in TREE-PUZZLE. In order to test for rate constancy among the different goby lineages, the two-cluster and branch length test were performed using Lintree (Takezaki et al., 1995).

Conflicting phylogenetic signal was evaluated with the split decomposition method in the program SplitsTree v. 3.1 (Huson, 1998). This method does not attempt to force data onto a tree, providing an indication of how tree-like the data are. The phylogenetic content of a sequence alignment can also be visualized by the likelihood mapping analysis implemented in TREE-PUZZLE (Strimmer and von Haeseler, 1997). This method distinguishes between phylogenetic signal producing treelike topologies and phylogenetic noise, producing star- and/or netlike topologies. Using constraint analyses in PAUP*, different topological constraints were constructed and compared using the Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999). A first topological constraint was based on the proposed relationships by Miller (1986) and Mckay and Miller (1997); other constrains enforced the monophyly of the "sand goby" group, of its respective genera and of the freshwater species.

3. Results

3.1. Characteristics of the ITS1, 12S, and 16S sequences

The amplified ITS1 region varied considerably in length between the *Pomatoschistus* spp. The smallest fragment was found in *P. pictus* (694 bp), followed by *P. microps* (729/691 bp), *P. knerii* (734 bp), *P. marmoratus* sp. 1 and sp. 2 (752/748 bp, respectively), *Gobiusculus flavescens* (781 bp), *P. lozanoi* (819 bp), and *P. minutus* (811 bp). For *P. norvegicus* only about 600 bp were sequenced but the total PCR fragment was about 800 bp long. The sequences have been submitted to the GenBank nucleotide database under the following Accession No.: AJ616333–AJ616343. These interspecific length differences are due to two large insertions of 29 and 39 bp, respectively, besides smaller insertions of eight and nine basepairs in the latter three species. *G. flavescens* showed a similar pattern of (smaller) insertions, while *P. microps* and *P. marmoratus* showed only insertions of about two to five basepairs. In the sequence of *P. marmoratus* GA_{4-7} tandem repeats were found. Also intra-individual length differences were found: two clones sequenced from one *P. lozanoi* specimen differed in 371 bp, due to a complete deletion of the central part of the sequence. The beginning and end of the fragment was identical in the two clones. Also *P. microps* showed intra-individual variation; the *p*-distance between the two clones was about 2%, resulting from point mutations and an insertion/deletion of a $(GAGAGGGAGA)_2$ repeat.

The amplified 12S and 16S fragments were about 400 and 580 bp, respectively (GenBank Accession Nos. AJ616806–AJ616837). The specimens from *P. minutus* collected in the Adriatic Sea (Chioggia) differed in two transitions from the sequence of Penzo et al. (1998). In comparison with the specimens sequenced from the North Sea, 12 substitutions, two transversions and one insertion/deletion event (12S and 16S) were found.

The sample of the Venice lagoon appeared to be a mixture of species. One of the sequences was, apart from one transition, identical to the *P. marmoratus* sequence of Penzo et al. (1998) and will be referred to as *P. marmoratus*. Although morphologically not distinguishable from *P. marmoratus*, two other genotypes were found, here referred to as *P. marmoratus* 1 and *P. marmoratus* 2, differing in 0.4–1.0% sequence variation. Another specimen was morphologically determined as *K. panizzae* and showed 0.4% difference in the 16S fragment compared to the *K. panizzae* sequence of Penzo et al. (1998); it will be referred to as *K. panizzae* sp.

3.2. Phylogenetic analyses

Excluding all ambiguous regions in the ITS1 alignment resulted in a 614 bp fragment. The base composition of that fragment was biased towards [GC], with a percentage of 69.1% (base composition *p*-value of 63–99%; with γ-shape parameter = 0.3; transition:transversion ratio = 1.6; clock not rejected). Likelihood mapping analysis showed 7.6% of all quartet-topologies to be star-like. The pairwise distances between sister taxa were comparable to those generated with the 12S and 16S sequences, but the ITS1 distances between the *P. minutus* and *P. microps* complex were considerably higher (about 12%) resulting in larger branch lengths. The topology (Fig. 3) is well resolved and all treebuilding methods produced the same tree. Since no ITS sequences were available for other goby species, more extensive analyses apply to the 12S and 16S mtDNA dataset (see below).

Likelihood mapping analysis on the 12S and 16S dataset showed 14.2% of all quartet-topologies to be star-like. A homogenous base composition was found for all members and the [GC] content was 50.3%. Modeltest selected the GTR + I + G model as the most appropriate model of molecular evolution under the Akaike Information Criterion, with the γ-shape parameter estimated at 0.6 and the proportion of invariable sites at 0.6. According to the LRT performed using

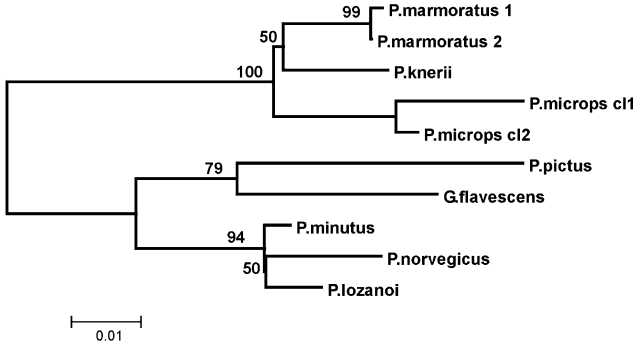

Fig. 3. Neighbor-joining phylogram constructed with the ITS1 sequences of *Pomatoschistus* spp.; the numbers represent the bootstrap values (1000 replicates).

TREE-PUZZLE all sequences behaved in a clock-like fashion. The NJ and MP bootstrap values were rather low; the posterior probabilities were moderate (Fig. 4). The best supported cluster is the *P. minutus* complex comprising *P. minutus*, *P. lozanoi*, *P. norvegicus*, and *P. minutus* from the Adriatic Sea. Based on the pairwise distance matrix (see the table in Appendix A), the genetic differentiation within this cluster was rather low (about 1.3%). *P. microps*, *P. marmoratus*, and *P. knerii* clustered parallel to the *P. minutus* complex. Three branches of the maximum-likelihood phylogram were not significantly different from zero as shown by the LRT in PAUP*: (1) the branch separating the *P. quagga* and *Knipowitschia pannizae* clade from the remaining clade ($P = 0.07$), (2) the branch leading to the *K. punctatissima* and *P. canistrinii* clade ($P = 0.15$), and (3) the branch leading to *Gobius paganellus* and *Padogobius nigricans* ($P = 0.08$). These are the branches that were collapsed in the linearized tree (Fig. 5). The linearized tree was constructed using *Bovichtus variegatus* as outgroup.

When performing an analysis including all other goby sequences and excluding unstable regions with SOAP, a 750 bp fragment was obtained. TREE-PUZZLE showed a deviating base composition for *G. niger* and *Z. zebrus*; the clock hypothesis was rejected. The split graph (Fig. 6) represents the 'sand goby' group as a closely related monophyletic group, quite distant from all other gobiids, and the phylogeny resembled a star phylogeny. According to the two-cluster test, *K. panizzae* evolved significantly slower while *G. niger*, *G. bucchichi*, and *Z. ophiocephalus* evolved significantly faster.

3.3. Topological constraints

The genus *Pomatoschistus* is not monophyletic: enforcing a monophyly of all *Pomatoschistus* species decreased the likelihood scores significantly at a 5% level

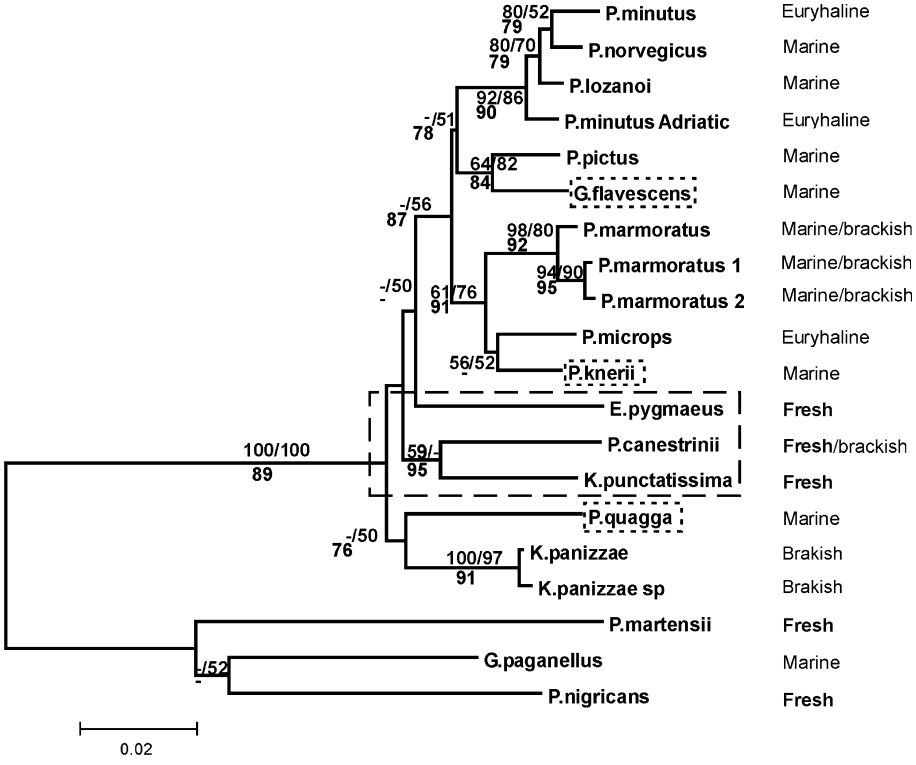

Fig. 4. Maximum-likelihood phylogram of 12S and 16S mtDNA sequences (800 bp) of the 'sand goby' group (ln $L = -3023.04$; rmatrix = (2.9 10.5 2.0 0.9 10.5); $\alpha = 0.6$; pinvar = 0.6). Bootstrap values are shown for the maximum parsimony/Neighbor-joining analyses above the branches and posterior probabilities below. (MP: 142 parsimony informative sites; tree length = 376; C.I. = 0.56; R.I. = 0.60). Sand gobies with a freshwater and a pelagic life style are boxed; salinity tolerances are indicated.

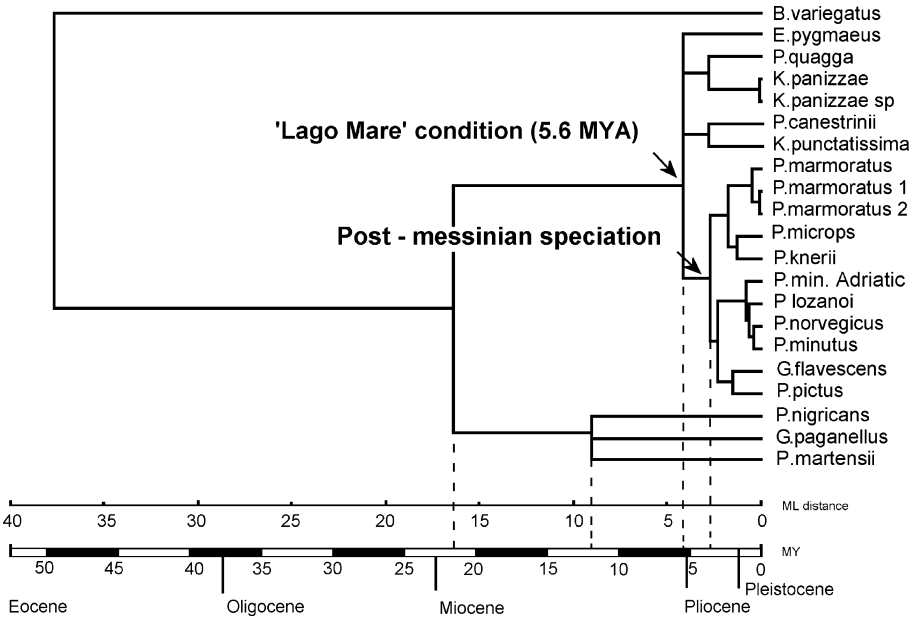

Fig. 5. Clock-constrained maximum likelihood tree constructed of 12S and 16S mtDNA sequences (800 bp) of the 'sand goby' group. The scale bars show the patristic distances and the time scale which resulted from a clock calibration of 1.4%/my as based on the Messinian salinity crisis (see text).

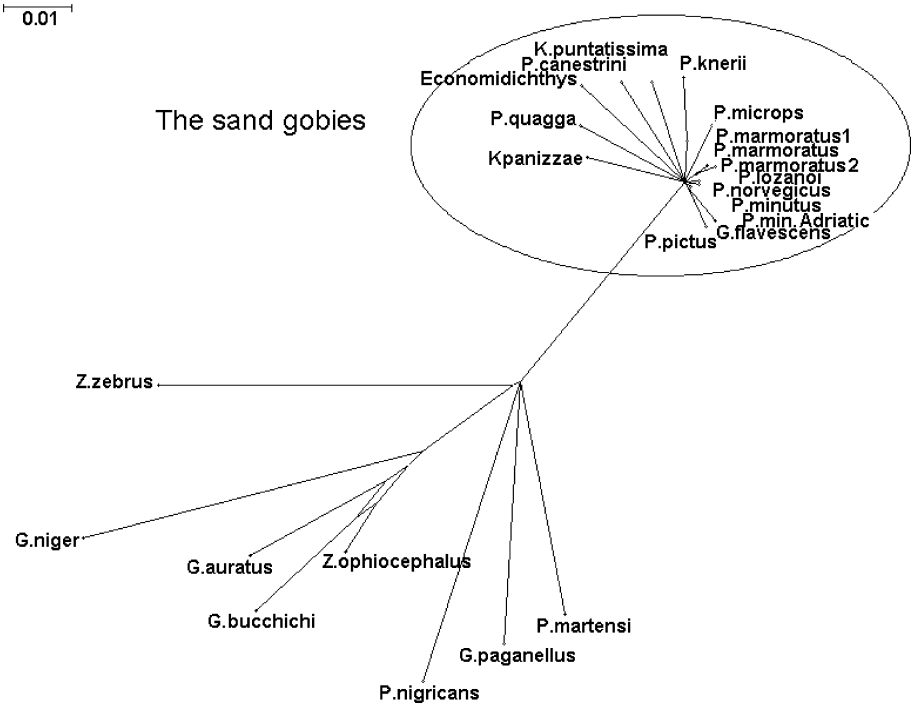

Fig. 6. Splits graph obtained from the 12S and 16S mtDNA sequences (750 bp) of the 'sand goby' group and related gobiids; Fit = 60.3; 1000 bootstrap samples.

(SH test: $P = 0.02$). Also *Knipowitschia* appeared polyphyletic: *K. punctatissima* clustered with *P. canestrinii* while *K. pannizae* clustered with *P. quagga* although the bootstrap values were rather low. A topological constraint based on the morphological diagnostic characters as defined by Miller (1986) and Mckay and Miller (1997) decreased the likelihood significantly ($P < 0.01$). Forcing the three freshwater species in a monophyletic group required one extra step in the parsimony analysis; a SH test showed that this actually significantly increased the

likelihood score ($P < 0.01$). The three species with a pelagic lifestyle (*G. flavescens*, *P. quagga*, and *P. knerii*) clustered in three distinct clades, indicating a polyphyletic origin of this trait.

4. Discussion

We obtained a consistent phylogeny that allowed for testing various speciation hypotheses. The 'sand goby' group appeared as a closely related monophyletic group, fairly distant from all other gobiids. The sister-group relationships were well supported and independent of the treebuilding methods used, whereas the more basal relationships linking the different genera were less certain. Since all sand goby sequences evolved in a clock-like fashion, a molecular clock could be calibrated and a linearized tree was constructed. The link between speciation events and the paleoclimatic history is discussed below.

4.1. Phylogenetic relationships: nDNA versus mtDNA

The ITS1 phylogeny based on a sub-sample of nine species was in agreement with the 12S–16S phylogeny, which reinforces the reliability of the inferred phylogeny. Since the ITS1 rDNA region showed intra-individual differences for some species, we abandoned sequencing the remaining goby species. However, the differentiation so far detected within *Pomatoschistus microps* spp. seems to have accumulated after the speciation between *P. microps* and *P. marmoratus*. As such, ITS1 remained useful for phylogenetic reconstruction. Intraspecific and intra-individual length differences in ITS1 were mainly due to the presence of microsatellites. Such regions are prone to mutation by slippage mechanisms both in vitro and in vivo (Schlötterer and Tautz, 1992). Different ITS1 types found in single *P. microps* individuals may either result from interchromosomal dispersal of the nucleolar organizer regions (Vogler and Desalle, 1994) as reported for *Gobius niger* (Caputo, 1998), or from interbreeding with sibling species. This might allow new alleles of the ITS1 to be introduced into a species at a level high enough to partially counteract the effects of concerted evolution (Tang et al., 1996). Since hybridisation has been reported in *Tridentiger* and *Pomatoschistus* gobies (Mukai et al., 1997; Wallis and Beardmore, 1980) it might be also the case with *P. microps*. Further research should test this possibility.

4.2. Phylogenetic relationships: morphology and ecology versus DNA

The molecular phylogeny agrees with the morphological criteria in the sense that the 'sand goby' group forms a monophyletic group of genetically closely related species, morphologically characterized by a distinctive head sensory papillae pattern. The overall grouping was also confirmed by allozyme studies (Mckay and Miller, 1997). However, with respect to the interrelationships within this group, some conflicts arose. Both *Pomatoschistus* and *Knipowitschia* appeared paraphyletic. Studies by Mckay and Miller (1997) and Penzo et al. (1998), using allozymes and mtDNA sequencing, respectively, reached the same conclusion. *G. flavescens*, which is thought to belong to another genus (Miller, 1986), clustered within the *Pomatoschistus* clade, suggesting either that it actually belongs to this genus, or that *Pomatoschistus* is paraphyletic. Its grouping with *P. pictus*, although with moderate bootstrap values, was confirmed in the ITS1 phylogeny. The position of *Economidichthys pygmaeus* could not be resolved; as such, its sister relationship with *Knipowitschia* (Miller, 1990) could not be confirmed. Miller (pers. comm.), supported by the allozyme study of Mckay and Miller (1997), placed *P. marmoratus* with the *P. minutus* complex, based on a shared character: the villi on the pterygoid membrane. However, the present phylogenetic analyses suggest that *P. microps* and *P. marmoratus* are the most closely related, in congruence with the allozyme study of Wallis and Beardmore (1984a) who placed them within the *P. microps* complex. No allozyme results of *P. knerii* are available, but according to Miller (pers. comm.) it is supposed to cluster with *Knipowitschia* while it grouped together with *P. microps* and *P. marmoratus* in this study. *P. microps* would join a separate group together with *P. tortonesii* and *P. bathi*, but it could not be confirmed due to the lack of specimens. Due to the low bootstrap values, no final conclusions can be made regarding the precise basal relationships. The short branch lengths leading to the *Knipowitschia*, *Economidichthys*, and *Pomatoschistus* genera and the SplitsTree analysis suggest that these groups might have evolved within a relatively short time period. This might also explain the low bootstrap levels and the fairly low consistency index. Similar observations were made in a phylogenetic study of Asian gobioid groups (based on cyt *b*) and explained by successive adaptive radiation events (Akihito et al., 2000). In an American group of gobiids, molecular studies have shown that the high species diversity has arisen during periods of adaptive radiation linked with major habitat shifts (Rüber et al., 2003).

Independent of the bootstrap values, topologies can still be tested using constraint analyses. The backbone constraint analysis showed that the morphological grouping as discussed above was significantly worse than the phylogeny obtained in this study. This indicates that parallel evolution might have played an important role in the sand goby group. For example the adaptation to a pelagic lifestyle, implying a slightly emarginated caudal fin with black caudal spot, big lateral situated

eyes, and reduced transverse *c* rows, occurred in *G. flavescens*, *P. quagga*, and *P. knerii* three times independently from each other. Although the freshwater species belong to different genera, the molecular data indicated a single origin of the freshwater adaptation in the sand gobies. The last common ancestor probably occupied sandy bottoms in a coastal niche while several species subsequently adapted to new habitats. Examples are the stenohaline species found scattered throughout the tree: *P. norvegicus*, *P. pictus*, and *P. quagga*. The same is true for the euryhaline species, *K. panizzae*, *P. microps*, and *P. minutus*, the latter two being also the most widely distributed.

4.3. Miocene origin of the goby ancestral fauna

The present study confirmed that sand gobies represent a distinct assemblage without an obvious sister group. The evolutionary gap between this line and the other Atlantic-Mediterranean gobiines is substantial (Fig. 6). Based on morphological characters, Mckay and Miller (1997) conclude that the most likely sister group is the Indo-Pacific genus *Nesogobius*. Although no published sequences are available for these species, the 12S region has been sequenced in the closely related genus *Tridentiger*, another putative sister group. Based on the γ corrected pair-wise genetic distances (data not shown), the gobies *T. brevispinis* and *T. kuroiwa* (GenBank Accession Nos. AB022900 and AB022902), both euryhaline and freshwater gobies from Japan and Korea, appeared closely related to the sand gobies. A preliminlary phylogeny reconstruction (300 bp of the 12S region) clustered *Tridentiger* together with the sand gobies; more genes have to be sequenced to confirm this outcome. Based on 600 bp of 16S mtDNA, the Eastern Pacific goby *Gillichthys mirabilis* (GenBank Accession No. AF266165) clustered with the sand gobies. These preliminary findings might support the view of Mckay and Miller (1997) that closure of the Atlantic-Mediterranean part of the early Tethys could be the major vicariant event that irrevocably separated the sand-goby and *Nesogobius-Tridentiger* stocks. A similar example of such vicariant speciation has been described for the killifish genus *Aphanius* (Hrbek and Meyer, 2003).

There seems to be no obvious geological or hydrographic event that might account for the separation of the sand gobies from the other Atlantic-Mediterranean gobiid lines (*Padogobius* and *Gobius* spp.), which apparently occurred before the separation of the Atlantic-Mediterranean region from the Indo Pacific region (Fig. 5). According to Miller (1990), the divergence of the freshwater Mediterranean (*Padogobius*) and West Balkanian (*Economidichthys*) lines from the Ponto-Caspian sister groups (*Knipowitschia*) was a consequence of the late Miocene MSC. However, as can be inferred from the linearized tree (Fig. 5), the divergence of the *Padogobius* line from the 'sand goby' line occurred before the divergence of the *Economidichthys* and *Knipowitschia* lines. Taking the separation of the *Padogobius* lines as a calibration point, this results in a relatively high molecular clock of 3.3%/my. Therefore it is more reasonable to equate the origin of the freshwater lifestyle in gobies with the MSC.

4.4. The Messinian salinity crisis: trigger for speciation in the 'sand goby' group?

Taking the isolation of the Mediterranean Sea and subsequent origin of the Lago Mare system at 5.59 mya (Krijgsman et al., 1999) as a calibration point for the origin of the freshwater lifestyle, the patristic distances of the linearized tree (Fig. 5) would be translated into a rate of 1.43%/my. This estimate is lower than the standard mtDNA clock for birds and mammals (2%/my) but higher than the clock for poikilothermic vertebrates, which is assumed to tick several-fold slower (Cantatore et al., 1994; Martin et al., 1992). Many controversies exist regarding the molecular clock. However, it might be postulated that the fast generation time (about 1–2 years) of gobies and their small body size (*Economidichthys* comprises the smallest European freshwater fish) would compensate for the slower clock generally assumed for fish (Martin and Palumbi, 1993). Penzo et al. (1998) invoked a rate of 4.7 times faster than in other vertebrates to reconcile the history of the gobiids with the paleogeological history of the Mediterranean. However, applying this rate would imply that the separation of sand-goby and *Tridentiger* stocks occurred only 3–4 mya, when the Mediterranean was already isolated from the Indo-Pacific region.

Our clock estimate would corroborate the view of Miller (1990) with respect to the origin of the freshwater lifestyle in the sand gobies. During the MSC, Paratethyan *Knipowitschia* spp. invaded the Adriatic Sea and subsequently spread during the Lago Mare phase. The origin from various Lago Mare systems and subsequent isolation by the Atlantic marine transgression might explain the discontinuous distribution of the freshwater species (Fig. 2). With the opening of the Straits of Gibraltar (5.33 mya) and subsequent re-flooding of the Mediterranean basins, the ancestral population of *K. punctatissima* and *P. canestrinii* might have split up; one population leading to *K. punctatissima* retained the freshwater lifestyle while the other population, leading to the euryhaline *P. canestrinii*, had to adapt to the marine environment. At the same time, gobiine ancestors from the eastern Atlantic re-colonized the newly formed Mediterranean Sea. Adaptation to new and free ecological niches might have lead to a radiation resulting in the present day ichthyofauna (Ahnelt et al., 1995). The timing of the second speciation burst is estimated at

3.9 mya (Fig. 5) and could be regarded as a post-Messinian speciation event.

It would be useful to include the freshwater species *Knipowitschia thessala* found in the Thessaly river system in Greece and the widespread euryhaline Ponto-caspian *Knipowitschia caucasica* in the phylogenetic analyses. According to Miller (1990) the latter species is closely related to *K. panizzae*, whereas allozyme studies (Wallis and Beardmore, 1984a) point to a common ancestry with *P. microps* and *P. marmoratus*. Molecular sequencing might clarify this issue and provide more 'test material' for the above scenario.

Hrbek and Meyer (2003) tested vicariant speciation hypotheses for Eurasian killifishes and estimated a molecular clock of about 0.86%/my. Applying this clock resulted in an estimation of 9.8 my for the origin of the freshwater lifestyle, after the separation of the Paratethys from the Mediterranean Sea. After this separation the Paratethys freshened gradually, possibly triggering the origin of the freshwater lifestyle. The origin of the *P. microps–P. marmoratus* clade and the *P. minutus* complex—*G. flavescens* with *P. pictus* clade corresponds with 4.2 and 5.3 my, respectively, close to the re-opening of the Straits of Gibraltar that ended the MSC.

4.5. The Pleistocene epoch as a trigger for speciation in the Pomatoschistus minutus complex

The shortest branch lengths were found within the *Pomatoschistus minutus* complex and between the *P. marmoratus* genotypes sampled in the Adriatic Sea. Our clock estimates of 0.86 and 1.4%/my both pointed into the direction of Pleistocene speciation, about 1.94–1.18 mya, respectively. The Pleistocene glaciations were the most significant historical events during the evolutionary lifespan of most Holarctic species and are believed to have sped up the speciation process in the present day sister taxa (Avise et al., 1998). During glaciation, populations were forced into separated refugia, initiating allopatric speciation. This scenario might account for the speciation within the *P. minutus* complex. Examples of refugia were suspected in the Bay of Biscay and the Atlantic drainages of the Western Iberian Peninsula (Garcia-Marin et al., 1999; Gysels et al., in press). However, an alternative explanation might invoke sympatric speciation by ecological specialization to different niches that came available during the interglacial phases. According to Wallis and Beardmore (1984b), speciation and habitat diversification might have been linked. Whether the karyological transformations were cause or consequence of the origin of these species remains questionable (Webb, 1980).

The difference between the *P. minutus* specimens sampled from the Adriatic and the rest of the Mediterranean Sea and Atlantic Ocean was of the same magnitude as the interspecific differences between *P. minutus*, *P. lozanoi*, and *P. norvegicus*. Also at the allozyme level distinct differences were found (Stefanni et al., 2003; Wallis and Beardmore, 1983). Recently, Stefanni and Thorley (2003) assigned *P. minutus* from the Adriatic Sea and elsewhere to separate Evolutionary Significant Units based on the mtDNA haplotypes of the control region. They claimed that the separation dated about 5000 to 10,000 years ago. However, our findings indicate that *P. minutus* from the Adriatic Sea should be regarded as a distinct species, analogous to *P. minutus*, *P. lozanoi*, and *P. norvegicus*.

This leads to a third speciation scenario in addition to the hypotheses described above. During the Pleistocene, a subpopulation of the ancestor of the *P. minutus* complex might have become isolated in the Adriatic, as a consequence of a sudden drop in sea level, followed by an independent evolution. At the same time, speciation of the *P. minutus* complex gave rise to *P. minutus*, *P. norvegicus*, and *P. lozanoi* in the Mediterranean Sea followed by subsequent (post-glacial) colonization of the Eastern Atlantic Ocean. This scenario is corroborated by the cyt *b* haplotype distribution and the clustering of endemic Mediterranean haplotypes of *P. minutus* (Gysels et al., in press). A second species pair described in the literature is *P. pictus pictus* and *P. pictus adriaticus* (Miller, 1986). Unfortunately, no specimens were available for genetic analyses. The smallest divergence between *P. marmoratus* genotypes sampled in the Mediterranean and Adriatic (0.4–1.0%, *p*-distances 16S) might be induced by the isolation of the Adriatic during the Last Ice Age. The fall in sea level narrowed the Siculo-Tunisian Strait, isolating the Adriatic from the rest of the Mediterranean Sea. Today this area is still isolated by a topologically controlled cyclonic gyre in the South Adriatic pit (Magoulas et al., 1996), possibly limiting dispersal of pelagic larvae. This isolation is also reflected by the anchovy distribution in the Adriatic (Magoulas et al., 1996).

In conclusion, there are presently two scenarios on the origin of the freshwater lifestyle in the sand gobies: (1) under a molecular clock of 1.4%/my the Messinian salinity crisis appeared responsible for this adaptation; (2) applying a general fish mtDNA clock of 0.85%/my suggests that the gradual freshening of the Paratethys after its separation of the Mediterranean (10–12 mya) might have been the historical trigger. In that case, the speciation of the *P. marmoratus* complex and the *P. minutus* complex coincide with the re-flooding of the Mediterranean that ended the MSC. Thus, in either way the salinity crisis had a major impact on sand goby speciation. Sequences of the 12S and 16S mtDNA of *Tridentiger* and *Nesogobius* lineages should provide an ultimate test of their sister-group relationship to the sand gobies. Complemented with the remaining sand goby species, this will allow a refined calibration of the molecular clock and an objective test of the

here-proposed hypotheses. If they approximate the actual history, than it must be concluded that the present taxonomy does not reflect the evolutionary history of the sand gobies.

Acknowledgments

T.H. has been supported by a scholarship of the Institute of Scientific and Technological research (IWT-Vlaanderen). Sampling at the Marine Biological Station in Bergen has been supported by the Improving Human Potential Programme from the European Union (Contract No. HPRI-1999-CT-00056). Sampling at the Trondheim Biological Station has been supported by Training and Mobility - Large Scale Facility programme of the EC. We thank A. J. Crivelli, B. Dezfuli, P. Franzoi, E. Karlsbakk, C. Mazzoldi, P. Torricelli, C. Pampoulie, and F. Bonhomme for assistance with sampling, A. Vadori for help with sequencing, P. Miller for providing *Economidichthys* material, determination of the Adriatic material and fruitful discussions. Special thanks to E. Gysels and L. Bargelloni for inspiring discussions and R. Zardoya for critical reading of an earlier draft of this paper. This study is supported by the Belgian government (Federal Services of Scientific, Technological and Cultural Research, contract MN/DD/42).

Appendix A

Total number of substitutions (upper right triangle) and Kimura-2 parameter distances with γ correction ($\alpha = 0.6$; pinvar = 0.6; lower left triangle), based on the 12S and 16S mtDNA fragments of the sand gobies

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 *P. microps*	0	18	21	34	33	33	32	30	31	42	40	41	42	43	43	102	106	111
2 *P. knerii*	0.025	0	24	34	30	28	29	26	29	40	42	40	38	39	41	100	105	111
3 *P. marmoratus*	0.030	0.035	0	33	31	27	24	26	31	42	47	43	37	38	46	103	109	112
4 *P. minutus*	0.053	0.053	0.051	0	10	11	14	27	30	40	47	46	42	42	51	107	110	115
5 *P. norvegicus*	0.051	0.046	0.047	0.014	0	8	12	29	30	40	44	43	41	41	48	104	104	112
6 *P. lozanoi*	0.051	0.042	0.041	0.015	0.011	0	8	23	28	42	43	41	36	36	45	104	108	111
7 *P. minutus* Adriatic.	0.049	0.044	0.036	0.019	0.016	0.011	0	23	30	41	44	41	36	38	45	99	104	107
8 *P. pictus*	0.045	0.039	0.039	0.041	0.044	0.034	0.034	0	18	40	43	39	38	38	46	105	107	114
9 *G. flavescens*	0.046	0.043	0.046	0.045	0.045	0.042	0.045	0.025	0	39	40	41	34	34	42	106	109	118
10 *E. pygmaeus*	0.071	0.066	0.070	0.066	0.066	0.070	0.067	0.066	0.063	0	46	43	44	44	48	107	110	116
11 *P. canestrinii*	0.068	0.071	0.082	0.082	0.075	0.073	0.075	0.072	0.065	0.077	0	36	43	43	49	105	111	117
12 *K. puntatissima*	0.068	0.066	0.072	0.077	0.070	0.067	0.067	0.063	0.066	0.072	0.057	0	42	44	44	101	103	111
13 *K. panizzae*	0.070	0.061	0.060	0.070	0.067	0.057	0.057	0.062	0.053	0.073	0.072	0.069	0	2	35	99	105	104
14 *K. panizzae* sp.	0.072	0.063	0.062	0.070	0.067	0.057	0.061	0.062	0.053	0.073	0.072	0.073	0.003	0	37	101	107	106
15 *P. quagga*	0.072	0.067	0.078	0.089	0.081	0.075	0.075	0.078	0.068	0.081	0.084	0.074	0.055	0.058	0	106	113	114
16 *G. paganellus*	0.266	0.257	0.267	0.284	0.264	0.269	0.251	0.275	0.273	0.283	0.280	0.250	0.249	0.256	0.29	0	63	74
17 *P. martensii*	0.292	0.291	0.300	0.313	0.279	0.300	0.284	0.298	0.300	0.305	0.318	0.261	0.275	0.282	0.326	0.122	0	81
18 *P. nigricans*	0.295	0.304	0.297	0.310	0.294	0.296	0.281	0.309	0.325	0.330	0.344	0.289	0.273	0.281	0.319	0.154	0.175	0

References

Ahnelt, H., Bianco, P.G., Schwammer, H., 1995. Systematics and zoogeography of *Knipowitschia caucasica* (Teleostei: Gobiidae) based on new records from the Aegean Anatolian area. Ichthyol. Explor. Freshw. 6, 49–60.

Akihito Iwata, A., Kobayashi, T., Ikeo, K., Imanishi, T., Ono, H., Umehara, Y., Hamamatsu, C., Sugiyama, K., Ikeda, Y., Sakamoto, K., Fumihito, A., Ohno, S., Gojobori, T., 2000. Evolutionary aspects of Gobioid fishes based upon a phylogenetic analysis of mitochondrial cytochrome *b* genes. Gene 259, 5–15.

Avise, J.C., Walker, D., Johns, G.C., 1998. Speciation durations and pleistocene effects on vertebrate phylogeography. Proc. R. Soc. London B 265, 1707–1712.

Banarescu, P., 1992. Zoogeography of fresh waters. Distribution and dispersal of fresh water animals in North America and Eurasia. Aula-Verlag, Wiesbaden.

Bianco, P.G., 1990. Vanishing fresh-water fishes in Italy. J. Fish. Biol. 37, 235–237.

Bianco, P.G., Bullock, A.M., Miller, P.J., Roubal, F.R., 1987. A unique teleost dermal organ in a new European genus of fishes (Teleostei, Gobioidei). J. Fish. Biol. 31, 797–803.

Cantatore, P., Roberti, M., Pesole, G., Ludovico, A., Milella, F., Gadaleta, M.N., Saccone, C., 1994. Evolutionary analysis of cytochrome *b* sequences in some Perciformes—evidence for a slower rate of evolution than in mammals. J. Mol. Evol. 39, 589–597.

Caputo, V., 1998. Nucleolar organizer (NOR) location and cytotaxonomic implications in six species of gobiid fishes (Perciformes, Gobiidae). Ital. J. Zool. 65, 93–99.

Economidis, P.S., Miller, P.J., 1990. Systematics of freshwater gobies from Greece (Teleostei:Gobiidae). J. Zool. 221, 125–170.

Farris, J.S., Kallersjo, M., Kluge, A.G., Bult, C., 1995. Constructing a significance test for incongruence. Syst. Biol. 44, 570–572.

Fonds, M., 1973. Sand gobies in the Dutch Wadden Sea (*Pomatoschistus*, Gobiidae, Pisces). Neth. J. Sea Res. 6, 417–478.

Gandolfi, G., Marconato, A., Torricelli, P., 1985. Posizione sistematica e biologia de un ghiozzo delle acque dolci italiane: *Orsinigobius* (gen. nov.) *punctatissimus* (Canestrinii, 1864) (Pisces, Gobiidae),

Bollettino del Museo civico della Storia naturale di Verona. 12, 367–380.

Garcia-Marin, J.L., Utter, F.M., Pla, C., 1999. Postglacial colonization of brown trout in Europe based on distribution of allozyme variants. Heredity 82, 46–56.

Gysels, E.S., Hellemans, B., Patarnello, T., Volckaert, F.A.M., Recurrent and historic gene flow of the sand goby *Pomatoschistus minutus* on the European Continental Shelf and in the Mediterranean Sea. Biol. J. Linn. Soc. in press.

Harzhauser, M., Piller, W.E., Steiniger, F.F., 2002. Circum-Mediterranean Oligo-Miocene biogeographic evolution—the gastropod's point of view. PALAEO 183, 103–133.

Hoese, D.F., 1984. Gobioidei relationships. In: Moser, H.G., Richards, W.J., Cohen, D.M., Fahay, M.P., Kendall Jr., A.W., Richardson, S.L. (Eds.) Ontogeny and systematics of, fishes. Am. Soc. Ichthyol. Herpetol. Spec. Pub. No. 1. pp. 588–591.

Hrbek, T., Meyer, A., 2003. Closing of the Tethys Sea and the phylogeny of Eurasian killifishes (Cyprinodontiformes: Cyprinodontidae). J. Evol. Biol. 16, 17–36.

Hsü, K.J., Montadert, L., Bernoulli, D., Biancacita, M., Erickson, A., Garrison, R.E., Kidd, R.B., Melieres, F., Muller, C., Wright, R., 1977. History of Mediterranean salinity crisis. Nature 267, 399–403.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES. Bayesian inference of phylogeny. Bioinformatics 17, 754–755.

Huson, D.H., 1998. SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics 14, 68–73.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X., Wilson, A.C., 1989. Dynamics of mitochondrial DNA evolution in animals—amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86, 6196–6200.

Krijgsman, W., Hilgen, F.J., Raffi, I., Sierro, F.J., Wilson, D.S., 1999. Chronology, causes and progression of the Messinian salinity crisis. Nature 400, 652–655.

Löytynoja, A., Milinkovitch, M.C., 2001. SOAP: cleaning multiple alignments from unstable blocks. Bioinformatics 17, 573–574.

Magoulas, A., Tsimenides, N., Zouros, E., 1996. Mitochondrial DNA phylogeny and the reconstruction of the population history of a species: the case of the European anchovy (*Engraulis encrasicolus*). Mol. Biol. Evol. 13, 178–190.

Martin, A.P., Naylor, G.J.P., Palumbi, S.R., 1992. Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. Nature 357, 153–155.

Martin, A.P., Palumbi, S.R., 1993. Body size, metabolic-rate, generation time, and the molecular clock. Proc. Natl. Acad. Sci. USA 90, 4087–4091.

Mckay, S.I., Miller, P.J., 1991. Isozyme criteria in the testing of phyletic relationships between species of *Gobius* and related Eastern Atlantic—Mediterranean genera (Teleostei, Gobiidae). J. Fish. Biol. 39, 291–299.

Mckay, S.I., Miller, P.J., 1997. The affinities of European sand gobies (Teleostei: Gobiidae). J. Nat. Hist. 31, 1457–1482.

Miller, P.J., 1986. Gobiidae. In: Whitehead, P.J.P., Bauchot, M.L., Hureau, J.C., Nielsen, J., Tortonese, E. (Eds), Fishes of the Northeastern Atlantic and the Mediterranean. Vol. 3. UNESCO, Paris, pp. 1019-1085.

Miller, P.J., 1990. The endurance of endemism—the Mediterranean fresh-water gobies and their prospects for survival. J. Fish. Biol. 37, 145–156.

Mukai, T., Naruse, K., Sato, T., Shima, A., Morisawa, M., 1997. Multiregional introgressions inferred from the mitochondrial DNA phylogeny of a hybridizing species complex of Gobiid fishes, genus *Tridentiger*. Mol. Biol. Evol. 14, 1258–1265.

Néraudeau, D., Goubert, E., Lacour, D., Rouchy, J.M., 2001. Changing biodiversity of Mediterranean irregular echinoids from the Messinian to the present-day. PALAEO 175, 43–60.

Palumbi, S.R., Martin, A., Romano, S., Mcmillan, W.O., Stice, L., Grabowski, G., 1991. The simple fool's guide to PCR. University of Hawaii Press, Honolulu.

Penzo, E., Gandolfi, G., Bargelloni, L., Colombo, L., Patarnello, T., 1998. Messinian salinity crisis and the origin of freshwater lifestyle in Western Mediterranean gobies. Mol. Biol. Evol. 15, 1472–1480.

Por, F.D., Dimentman, C., 1985. Continuity of Messinian biota in the Mediterranean basin. In: Stanley, D.J., Wezel, F.C. (Eds.), Geological evolution of the Mediterranean basin. Springer-Verlag, Berlin, pp. 545–557.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Rögl, F., 1999. Mediterranean and Paratethys. Facts and hypotheses of an Oligocene to Miocene paleogeography (Short Overview). Geol. Carpath. 50, 339–349.

Rüber, L., Van Tassell, J.L., Zardoya, R., 2003. Rapid speciation and ecological divergence in the American seven-spined gobies (Gobiidae, Gobiosomatini) inferred from a molecular phylogeny. Evolution 57, 1584–1598.

Sajdak, S.L., Phillips, R.B., 1997. Phylogenetic relationships among *Coregonus* species inferred from the DNA sequence of the first internal transcribed spacer (ITS1) of ribosomal DNA. Can. J. Aquat. Sci 54, 1494–1503.

Schlötterer, C., Tautz, D., 1992. Slippage synthesis of simple sequence DNA. Nucleics Acids Res. 20, 211–215.

Schmidt, H.A., Strimmer, K., Vingron, M., von Haeseler, A., 2002. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics 18, 502–504.

Seidenkrantz, M.S., Kouwenhoven, T.J., Jorissen, F.J., Shackleton, N.J., Van Der Zwaan, G.J., 2000. Benthic foraminifera as indicators of changing Mediterranean—Atlantic water exchange in the Late Miocene. Mar. Geol. 163, 387–407.

Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114–1116.

Stefanni, S., Thorley, J.L., 2003. Mitochondrial DNA phylogeography reveals the existence of an Evolutionarily Significant Unit of the sand goby *Pomatoschistus minutus* in the Adriatic (Eastern Mediterranean). Mol. Phyl. Evol. 28, 601–609.

Stefanni, S., Gysels, E., Volckaert, A.M., Miller, P.J., 2003. Allozyme variation and genetic divergence in the sand goby, *Pomatoschistus minutus* (Teleostei: Gobiidae). J. Mar. Biol. Ass. UK 83, 1143–1149.

Strimmer, K., von Haeseler, A., 1997. Likelihood-mapping: a simple method to visualize phylogenetic content of a sequence alignment. Proc. Natl. Acad. Sci. USA 94, 6815–6819.

Swofford, D.L., 2001. PAUP*: Phylogenetic Analysis Using Parsimony (and other methods), Version 4.0. Sinauer Associates, Sunderland, MA.

Takezaki, N., Rzhetsky, A., Nei, M., 1995. Phylogenetic test of the molecular clock and linearized trees. Mol. Biol. Evol. 12, 823–833.

Tang, J., Toe, L., Back, C., Unnasch, T.R., 1996. Intra-specific heterogeneity of the rDNA internal transcribed spacer in the *Simulium damnosum* (Diptera: Simuliidae) complex. Mol. Biol. Evol. 13, 244–252.

Veith, M., Kosuch, J., Vences, M., 2003. Climatic oscillations triggered post-Messinian speciation of Western Palearctic brown frogs (Amphibia, Ranidae). Mol. Phyl. Evol. 26, 310–327.

Vogler, A.P., DeSalle, R., 1994. Evolution and phylogenetic information—content of the ITS1 region in the tiger beetle *Cicindela dorsalis*. Mol. Biol. Evol. 11, 393–405.

Wallis, G.P., Beardmore, J.A., 1980. Genetic evidence for naturally occuring fertile hybrids between two goby species, *Pomatoschistus*

minutus and *P. lozanoi* (Pisces, Gobiidae). Marine Ecology Progress Series 3, 309–315.

Wallis, G.P., Beardmore, J.A., 1983. Genetic differentiation between populations of *Pomatoschistus minutus* from the Bristol Channel and the Adriatic. Genetica 62, 75–80.

Wallis, G.P., Beardmore, J.A., 1984a. An electrophoretic study of the systematic relationships of some closely related goby species (Pisces, Gobiidae). Biol. J. Linn. Soc. 22, 107–123.

Wallis, G.P., Beardmore, J.A., 1984b. Genetic variation and environmental heterogeneity in some closely related goby species. Genetica 62, 223–237.

Webb, C.J., 1980. Systematics of the *Pomatoschistus minutus* complex (Teleostei: Gobiidae). Philos. Trans. R. Soc. Lond., Ser. B 291, 201–241.

Wilke, T., 2003. *Salenthydrobia* gen. nov (Rissooidea : Hydrobiidae): a potential relict of the Messinian salinity crisis. Zool. J. Linn. Soc. 137, 319–336.

Xia, X., Xie, Z., 2001. DAMBE: software package for data analysis in molecular biology and evolution. J. Hered. 92, 371–373.

Zardoya, R., Doadrio, I., 1999. Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. J. Mol. Evol. 49, 227–237.