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Phylogenetics and biogeography of the Balkan 'sand gobies' (Teleostei: Gobiidae): vulnerable species in need of taxonomic revision

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Within the Atlantic–Mediterranean region, the 'sand gobies' are abundant and widespread, and play an important role in marine, brackish, and freshwater ecosystems. They include the smallest European freshwater fish, *Economidichthys trichonis*, which is threatened by habitat loss and pollution, as are several other sand gobies. Key to good conservation management is an accurate account of the number of evolutionary significant units. Nevertheless, many taxonomic and evolutionary questions remain unresolved within the clade, and molecular studies are lacking, especially in the Balkans. Using partial 12S and 16S mitochondrial ribosomal DNA sequences of 96 specimens of at least eight nominal species (both freshwater and marine populations), we assess species relationships and compare molecular and morphological data. The results obtained do not support the monophyly of *Economidichthys*, suggesting the perianal organ to be a shared adaptation to hole-brooding rather than a synapomorphy, and urge for a taxonomic revision of *Knipowitschia*. The recently described *Knipowitschia montenegrina* seems to belong to a separate South-East Adriatic lineage. *Knipowitschia milleri*, an alleged endemic of the Acheron River, and *Knipowitschia* cf. *panizzae*, are shown to be very closely related to other western Greek *Knipowitschia* populations, and appear conspecific. A distinct Macedonian–Thessalian lineage is formed by *Knipowitschia thessala*, whereas *Knipowitschia caucasica* appears as an eastern lineage, with populations in Thrace and the Aegean. The present study combines the phylogeny of a goby radiation with insights on the historical biogeography of the eastern Mediterranean, and identifies evolutionary units meriting conservation attention. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **105**, 73–91.

ADDITIONAL KEYWORDS: *Economidichthys* – ichthyology – *Knipowitschia* – Mediterranean – *Pomatoschistus*.

INTRODUCTION

The Balkans are considered as Europe's prime centre of endemism and a hotspot of biodiversity (Kryštufek & Reed, 2004). Their glacial refugium role renders the Balkans, as well as other regions (e.g. the Iberian and Italian peninsulae), particularly interesting both in a biogeographical and a conservational framework, leading to a highly diverse fauna (Durand, Persat & Bouvet, 1999a). Nevertheless, their biogeographical history and the processes leading to this high

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diversity are poorly investigated or understood (Kryštufek & Reed, 2004). An important mechanism underlying species richness, and hence a useful target in evolutionary research and biogeography, is adaptive radiation, as exemplified by phylogenetic work on fishes such as the Neotropical monophyletic goby tribe Gobiosomatini (seven-spined gobies) (Rüber, Van Tassell & Zardoya, 2003; Taylor & Hellberg, 2005; Larmuseau *et al.*, 2011). The goby family (Teleostei, Perciformes, Gobiidae) represents one of the most (if not the most) speciose fish families worldwide, with about 1950 described species (Nelson, 2006). Gobies have adapted to a wide range of habitats, including marine, brackish, and freshwater, and show an impressive range of mating behaviours (Miller, 1986; Kottelat & Freyhof, 2007). The eastern Mediterranean, in particular the Balkans and the Black and Caspian basins, hosts the highest diversity of gobies in Europe, including many endemic species (Kottelat & Freyhof, 2007; Kovačić & Patzner, 2011), dominated in freshwater by the so-called 'sand gobies'. The Balkan 'sand gobies' present a useful framework for studying patterns of diversity and evolution because of: (1) their species richness and degree of endemicity; (2) the importance of species radiations in evolutionary research; (3) the understudied biogeography of the eastern Mediterranean in general (Zogaris, Economou & Dimopoulos, 2009); and (4) the usefulness of freshwater fishes as biogeographical tools (Hugueny, 1989; Heino, 2001). Additional intrinsic features (e.g. perianal organ; conservation importance) render them scientifically interesting. Surprisingly, considering their prevalence, abundance and potential with respect to biogeographical studies in the Balkans, molecular studies on these species are still rare.

The monophyletic sand goby clade (Huyse, Van Houdt & Volckaert, 2004), morphologically characterized by features of the head canals and vertebrae, includes the genera *Pomatoschistus* Gill, 1863, *Knipowitschia* Iljin, 1927, *Economidichthys* Bianco, Bullock, Miller & Roubal, 1987, and *Gobiusculus* Duncker, 1928. *Orsinigobius* Gandolfi, Marconato & Torricelli, 1986, and possibly *Hyrcanogobius* Iljin, 1928, are synonymous with *Knipowitschia* (Miller, 2004b). These small benthic species occupy parts of the eastern Atlantic Ocean, the Mediterranean Sea, and the Balkan and Ponto-Caspian continental regions (Miller, 2004c). The diversity of sand gobies is underestimated, as illustrated by numerous recent descriptions (Kovačić, 2005; Kovačić & Šanda, 2007; Miller & Šanda, 2008; Miller, 2009) and new records (Van Neer *et al.*, 1999; Stefanni, 2000; Turan *et al.*, 2005; Ozen, Irmak & Bilecenoglu, 2007; Mejri *et al.*, 2009). Several sand gobies deserve conservation attention as a result of anthropogenic threats, such as wetland degradation, water extraction, land reclamation, structural changes of water bodies, introduction of predators, and pollution (Economidis, 1995; Maitland & Crivelli, 1996; Kottelat & Freyhof, 2007; Özcan, 2009). Furthermore, because endangered species are important for environmental assessment and for prioritization in conservation management (Smith & Darwall, 2006), the freshwater sand gobies, with their wide habitat range and high level of endemism, might be considered as useful indicator species for monitoring the conservation of Mediterranean inland aquatic ecosystems. Scientific attention could perhaps render them attractive enough to be regarded as flagship species for the Mediterranean ichthyofauna and ambassadors for wetlands in the region.

Biodiversity surveys and conservation measures are seriously hampered by ambiguities in species delineations and assignments (Bickford *et al.*, 2006; Economou *et al.*, 2007). Based on morphology and morphometrics, many controversies exist about sand goby taxonomy, such as the systematic validity of taxa diagnosed by characters that may exhibit intraspecific phenotypic plasticity (e.g. reduction in head canals and squamation; Kovačić, 2005). The status of nominal genera currently recognized is likely to change (Kottelat & Freyhof, 2007). Molecular studies could help to clarify the relationships and evolutionary history of sand gobies (Penzo *et al.*, 1998; Huyse *et al.*, 2004; Miller & Šanda, 2008). Phylogenetic analyses allow us to pinpoint some issues of taxonomic interest, and to identify evolutionary significant units (ESUs) for conservation. Although the definition of this concept, which should represent the minimal unit to address conservation matters, is somewhat problematic (Vogler & DeSalle, 1994; Crandall *et al.*, 2000; Fraser & Bernatchez, 2001), ESUs coupled with historical biogeography have already been defined in Mediterranean sand gobies (Stefanni & Thorley, 2003).

MATERIAL AND METHODS

Specimen collection

We sampled Economidis' goby *Economidichthys pygmaeus* (Holly, 1929), Trichonis goby *Economidichthys trichonis* Economidis & Miller, 1990, Caucasian dwarf goby *Knipowitschia caucasica* (Kawrajsky *in* Berg 1916), Miller's dwarf goby *Knipowitschia milleri* (Ahnelt & Bianco, 1990), Thessaly goby *Knipowitschia thessala* (Vinciguerra, 1921), Adriatic dwarf goby *Knipowitschia pannizae* (Verga, 1841) (*sensu* the Evinos population identified by Ahnelt & Bianco, (1990); hereafter referred to as *Knipowitschia* cf. *panizzae*), Montenegro dwarf goby *Knipowitschia*

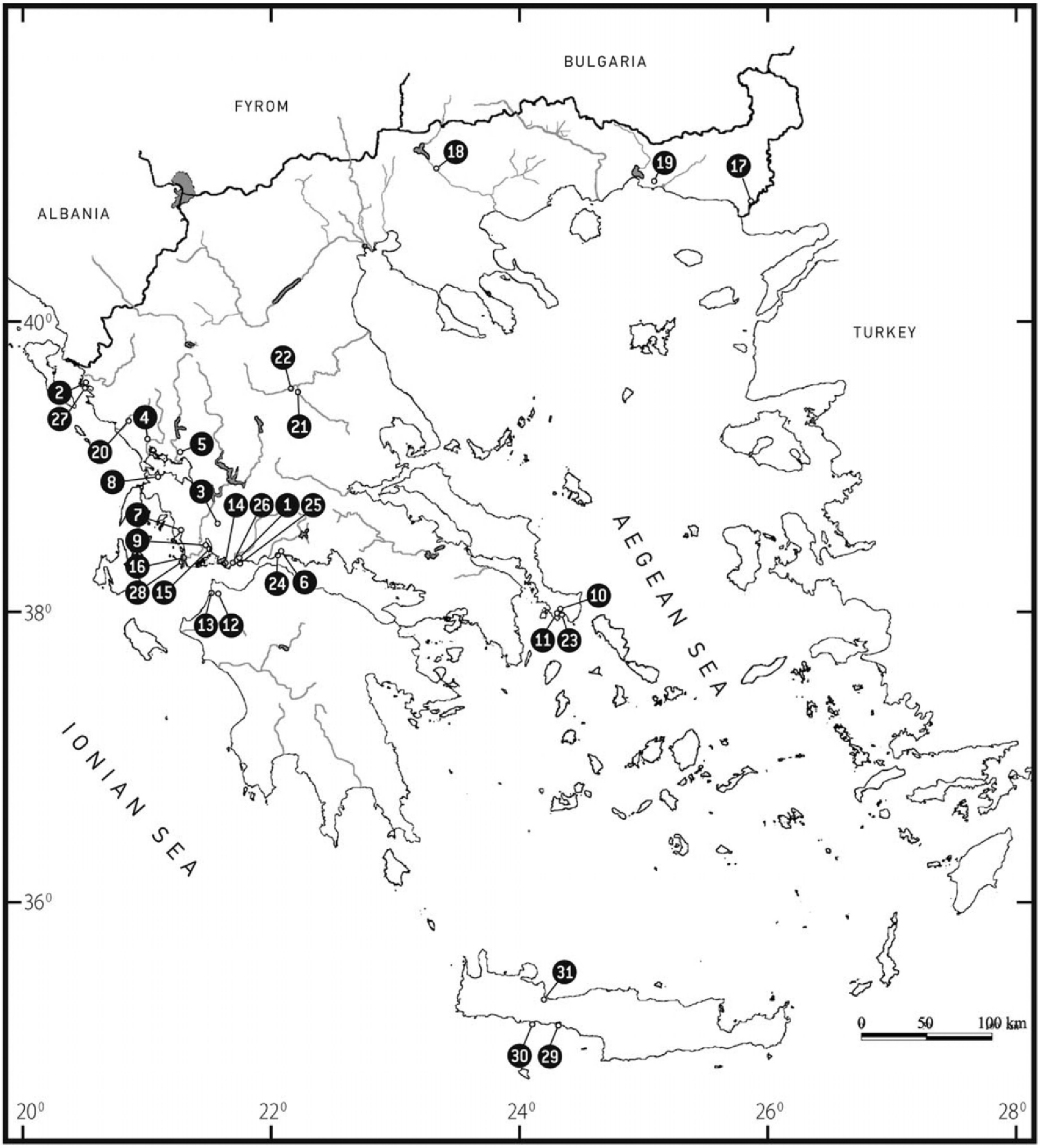

Figure 1. Map showing sand goby sampling localities, except for the location on the River Morača. More details on sampling sites and species are provided in Table 1.

montenegrina Kovačić and Šanda, 2007 and marbled goby *Pomatoschistus marmoratus* (Risso, 1810). Valid taxon names (according to currently accepted descriptions and identifications), status, sampling locations, habitats, number of individuals sequenced, and GenBank accession numbers are shown in Figure 1 and Table 1. Taxon and author names are used *sensu* Eschmeyer (2010). Gobies were collected using hand and seine nets and backpack electrofishing in a range of freshwater and brackish habitats. Specimens were

Table 1. Populations included in the phylogenetic analyses, including their species ascription, collection location, hydrographic drainage basin, habitat description, IUCN Red List category (Smith & Darwall, 2006), and number of sequenced specimens

Species	Site number	Location	Drainage basin	Habitat description	Number of sequences	GenBank accession number
Economidichthys pygmaeus	*1*	Kryoneri Springs	Evinos	Freshwater karstic springs with reedbed	3	JN388369-72
(freshwater)	*2*	Kalamas Delta	Kalamas	Deltaic freshwater channel	2	JN388373-5
Least concern	*3*	Lake Trichonis	Acheloos	Large freshwater lake-shore	4	JN388376-7
	4	Skala Springs	Louros	Spring-fed freshwater channel	2	JN388378-80
	5	Agios Georgios Springs	Arachthos	Spring-fed freshwater stream	2	JN388381-2
	6	Chiliadou Springs	Mornos	Spring-fed freshwater stream	4	JN388383-4
	7	Astakos Springs	Astakos	Spring-fed brackish stream	2	JN388385-6
	8	Vlychos Springs, Vonitsa Lagoon	Vlychos	Spring-fed brackish channel	2	JN388387-8
	9	Aitoliko Stream	Acheloos	Spring-fed freshwater creek	8	GQ398131-2 Larmuseau *et al.* (2010)
Economidichthys trichonis (freshwater) Endangered	*3*	Lake Trichonis, site Myrtias	Acheloos	Large deep freshwater lake -shore	4	JN388364-8
Knipowitschia caucasica (euryhaline)	*10*	Lake Vidra (Karystos Wetland)	Rigia	Semi-natural freshwater lake	2	JN388321-2
	11	Rigia Stream	Rigia	Brackish river-mouth	2	JN388321-2
Knipowitschia sp.	*12*	Kalogria Laspoloutra	Prokopos	Artificial pool beside lagoon	2	JN388306-7
(brackish water)	*13*	Kalogria Prokopos Lagoon	Prokopos	Brackish marsh	2	JN388308-9
Knipowitschia cf. *panizzae* (freshwater, brackish)	*14*	Evinos Lagoon (Klisova)	Evinos	Brackish lagoon	2	JN388310-2
Knipowitschia sp.	*15*	Aitoliko Lagoon	Acheloos	Brackish lagoon	2	JN388313-5
(brackish water)	*16*	Acheloos Estuary	Acheloos	Brackish lagoon	1	JN388316, JN399059
Knipowitschia caucasica (euryhaline)	*17*	Evros River, site Feres	Evros	Freshwater canalized river	2	JN388323-4
	18	Strymon River, Strymoniko bridge	Strymon	Riparian riverine marshes	2	JN388319-20

	19	Vosvozis Stream near Lake Mitrikou	Filiouris	Lowland freshwater river	2	JN388325-7
Knipowitschia milleri (freshwater to slightly brackish) Critically endangered	*20*	Acheron Delta	Acheron	Brackish channel	12	GQ398133-7 Larmuseau *et al.* (2010) JN388303-5
Knipowitschia thessala (freshwater) Endangered	*21*	Enipeas tributary	Pinios	Small lowland river	1	JN388317-8
	22	Pinios River, main course, near Farkadona bridge	Pinios	Large lowland river	4	JN388317-8
Knipowitschia montenegrina	*32*	Morača River, site Ponari (Montenegro)	Morača	Deltaic channel, near estuary	3	JN388301-2
Pomatoschistus marmoratus	*23*	Livadaki	Karystos Bay	Marine beach	2	JN388331-3
(marine and brackish water)	*24*	Chiliadou estuary	Mornos	Marine beach	2	JN388357-8
	25	Kryoneri estuary	Evinos	Marine beach	2	JN388353-6
	26	Evinos estuary	Evinos	River-mouth	2	JN388359-60
	27	Drepano beach	Kalamas	Marine beach	2	JN388361-3
	28	Acheloos estuary	Acheloos	Marine beach, outflow of brackish lagoon	8	JN388342-52
	29	Plakias Bay	Southern Crete	Marine sandy beach	2	JN388334-7
	30	Frangokastello cove	Southern Crete	Marine sandy beach	2	JN388338-41
	31	Giorgiopoulis stream-mouth	Kourna	Brackish spring-fed stream; near stream-mouth	2	JN388328-30

Site numbers correspond to the map (Fig. 1), excluding number 32, which is the only site outside Greece. For details on the hydrographic basins of most sites, see Economou *et al.* (2007). Note that *K. caucasica* is not mentioned in Smith & Darwall (2006) and that *K. montenegrina* was only described subsequently; hence, no conservation status is mentioned.

preserved in 99% ethanol until further processing. Voucher specimens are part of the reference collection of the Hellenic Centre for Marine Research (Institute of Inland Waters, Anavyssos, Greece) and are available from the authors on request.

EXTRACTION AND AMPLIFICATION OF MITOCHONDRIAL 12S AND 16S rDNA

DNA was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel) in accordance with the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using a GeneAmp PCR system 2700 thermocycler (Applied Biosystems). The reaction volume of 25 μL contained 1 × PCR buffer (Eurogentec), 200 μM of dNTPs (Amersham Pharmacia Biotech), 2 mM of $MgCl_2$ (50 mM) (Eurogentec), 0.5 U of *Taq*-polymerase (Eurogentec), 0.8 μM of each primer (Eurogentec), 1 μL of template DNA, topped up with milli-Q water (Millipore). Primers used were 16SH 5′-CCGGTCTGAACTCAGATCACGT-3′, 16SL 5′-CGCCTGTTTATCAAAAACAT-3′ (Palumbi *et al.*, 1991), 12SH 5′-TGACTGCAGAGGGTGACGGGGCG GTGTGT-3′ and 12SL 5′-AAAAAGCTTCAAACTGG GATTAGATACCCCACTAT-3′ (Kocher *et al.*, 1989).

After an initial denaturation of 3 min at 95 °C, samples were subject to 35 cycles of 30 s at 95 °C, 30 s at 52 °C (16S) or 64 °C (12S) and 50 s at 72 °C. After a final elongation of 7 min at 72 °C, samples were cooled down to 4 °C. PCR products were purified with NucleoFast (Macherey-Nagel) in accordance with the manufacturer's instructions. They were sequenced using the same primers as above, with the Big Dye Terminator 3.1 kit (Applied Biosystems), applying a 1/8 dilution of the Big Dye Terminator sequencing protocol. Finally the products were run on an ABI PRISM 3130 Avant Genetic Analyser automated sequencer (Applied Biosystems).

SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS

Sequences available from other sand goby species were retrieved from GenBank (Table 2). *Padogobius bonelli* (Bonaparte, 1846) (synonymous with *Padogobius martensii*) and *Padogobius nigricans* (Canestrini, 1867) were selected as outgroup taxa. As has been extensively shown, these species belong to the same clade as, for example, *Gobius* Linnaeus, 1758 and *Millerigobius* Bath, 1973 and are firmly distinct from the sand gobies (Penzo *et al.*, 1998; Huyse *et al.*, 2004; Giovannotti

Table 2. GenBank sequences of sand gobies with habitat description and sampling location

Species	Location	GenBank accession number	Reference
Pomatoschistus quagga (Heckel, 1837) (marine)	Northern Adriatic	AF067264 and –77	Penzo *et al.* (1998)
Knipowitschia punctatissima (freshwater)	Veneto Streams basin (Italy)	AF067260 and –73	
Pomatoschistus minutus (Pallas, 1770) (brackish, marine)	Venice Lagoon (Italy)	AJ616816 and –33	Huyse *et al.* (2004)
	Ostend (North Sea, Belgium)	AJ616813 and –30	
Pomatoschistus microps (Krøyer, 1838) (brackish, marine)	Ostend (North Sea, Belgium)	AJ616811 and –28	
Pomatoschistus marmoratus (brackish, marine)	Venice Lagoon (Italy)	AJ616809 and –26	
Pomatoschistus canestrinii (freshwater, brackish)	Dalmatia (Croatia)	AJ616818 and –35	
Pomatoschistus lozanoi (de Buen, 1923) (brackish, marine)	North Sea (Belgium)	AJ616815 and –32	
Pomatoschistus norvegicus (Collett, 1902) (marine)	Bergen (Norway)	AJ616814 and –31	
Pomatoschistus pictus (Malm, 1865) (marine)	Bergen (Norway)	AJ616817 and –34	
Knipowitschia panizzae (freshwater, brackish)	Po Delta and Venice Lagoon (Italy)	AJ616806 and –22–4	
Gobiusculus flavescens (Fabricius, 1779) (brackish, marine)	Trondheim (Norway)	AJ616836 and –19	
Padogobius bonelli (freshwater)	Sentino River (Italy)	EF218637 and –52	Giovannotti *et al.* (2007)
Padogobius nigricans (freshwater)	Tevere River (Italy)	AF067257 and –70	Penzo *et al.* (1998)

et al., 2007; Vanhove *et al.*, 2011). Following the incongruence-length difference test (Farris *et al.*, 1995) carried out for the same regions by Huyse *et al.* (2004), 12S and 16S rDNA fragments were concatenated into a single dataset. Sequences were aligned by MUSCLE, version 3.8 (Edgar, 2004) using default distance measures and sequence weighting schemes. The resulting alignment was trimmed with trimAl, version 1.2b (Capella-Gutiérrez, Silla-Martínez & Gabaldón, 2009), making use of an automated trimming algorithm optimized for subsequent maximum likelihood (ML) phylogenetic tree reconstruction. jModelTest, version 0.1.1 (Guindon & Gascuel, 2003; Felsenstein, 2005; Posada, 2008) was used to estimate the optimal model of molecular evolution, using a ML optimized starting tree. The TPM2uf+I+Γ model was selected based on the corrected Akaike information criterion (AICc) (Hurvich & Tsai, 1989). For subsequent implementation in phylogenetic software, we chose the GTR+I+Γ model (gamma shape parameter: 0.48; proportion of invariable sites: 0.58) (Tavaré, 1986; Rodriguez *et al.*, 1990), representing the commonly available one with the highest AICc score. A model-averaged phylogeny demonstrated that hardly any nodes displayed phylogenetic uncertainty because of model selection. Hence, we feel confident that the slight overparametrization will not influence our analyses. Under this model, a ML search was carried out in PhyML, version 3.0 (Guindon & Gascuel, 2003), assessing nodal support through 1000 bootstrap samples using the nearest-neighbour interchange branch swapping algorithm. The model was also used in Bayesian analysis, implemented in MrBayes, version 3 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Posterior probabilities were calculated over 4×10^6 generations. At that point, stationarity of the Markov chain was reached, as indicated by a standard deviation of split frequencies of 0.005, by the absence of a trend in the log probabilities plotted over generations, and by a potential scale reduction factor (Gelman & Rubin, 1992) converging to 1. The Markov chain was sampled every other 100 generations. We discarded 25% of the samples as 'burn-in'. For calculation of ML genetic distances according to the optimized model, and for phylogeny reconstruction using the maximum parsimony (MP) method, PAUP* 4.01b (Swofford, 2001) was used, with the PaupUp interface (Calendini & Martin, 2005). A heuristic search was performed using 1000 replicates of nearest-neighbour interchange branch swapping; gaps were treated as a fifth character.

Because the monophyly of *Economidichthys* is under discussion, we performed constraint analyses to assess whether enforcing monophyly on this genus significantly changed the likelihood of the tree. We used the Shimodaira–Hasegawa (Shimodaira & Hasegawa, 1999) and Kishino–Hasegawa topological tests (Kishino & Hasegawa, 1989) (one-sided, in view of the inclusion of the ML tree; Goldman, Anderson & Rodrigo, 2000; Schmidt, 2009), implemented in PAUP*.

To assess the phylogenetic content of the dataset, a likelihood-mapping analysis (Strimmer & von Haeseler, 1997) was carried out in TREE-PUZZLE, version 5.2 (Schmidt *et al.*, 2002). Using the same software, a likelihood-ratio-test (LRT) was performed to test the molecular clock hypothesis, and a clock-constrained tree was constructed by quartet puzzling, with the parameters resulting from jModelTest. Based on mutation rates of mitochondrial rDNA of $0.5–0.56\% \times 10^{-6}$ years (sicydiine gobies; Keith *et al.*, 2011), $0.6\% \times 10^{-6}$ years (*Mugilogobius* Smitt, 1900 and *Pandaka* Herre, 1927 gobies; Mukai, Suzuki & Nishida, 2004), $0.7\% \times 10^{-6}$ years (the cyprinid *Telestes souffia* (Risso, 1827); Costedoat & Gilles, 2009), $1\% \times 10^{-6}$ years (Centropominae; Tringali *et al.*, 1999), $1.11\% \times 10^{-6}$ years (*Syngnathus* Linnaeus, 1758 pipefish; Wilson, 2006), $1.4\% \times 10^{-6}$ years ('sand gobies'; Huyse *et al.*, 2004) and $1.3–1.6\% \times 10^{-6}$ years (Blenniidae; Almada *et al.*, 2009), we used constant mutation rates of 0.5% and $1.5\% \times 10^{-6}$ years to draw a calibrated tree. 12S and 16S are relatively conservative markers compared to several other mitochondrial (mt)DNA genes (Wan *et al.*, 2004) and poikilotherm animals often display relatively slow evolutionary rates (Johns & Avise, 1998). However, because we are dealing (over a smaller phylogenetic range) with small-bodied taxa with a short generation time, relatively higher mutation rate estimations are more realistic (Martin & Palumbi, 1993; Gillooly *et al.*, 2005; Wilson, 2006). Hence, we did not take unconventionally low values (such as $0.23\% \times 10^{-6}$ years for gymnotiforms and mormyriforms; Alves-Gomes, 1999) into account. To check whether there was any noise left in the dataset as a result of unstable characters, we partitioned the data in ten mutation rate categories using TIGER, version 1.0 (Cummins & McInerney, 2011) and removed the fastest evolving category with the StripBin script. Likelihood-mapping was subsequently performed on the resulting alignment. Alignment files were converted using ALTER, version 1.2 (Glez-Peña *et al.*, 2010) and trees were drawn with FigTree, version 1.3 (http://tree.bio.ed.ac.uk/software/figtree) and MEGA, version 5 (Tamura *et al.*, 2011).

RESULTS

Ninety-six specimens were sequenced, belonging to at least eight nominal species: *K. montenegrina*, *K.* cf. *panizzae*, *K. caucasica*, *K. milleri*, *K. thessala*, *E. pygmaeus*, *E. trichonis*, and *P. marmoratus*. The length of

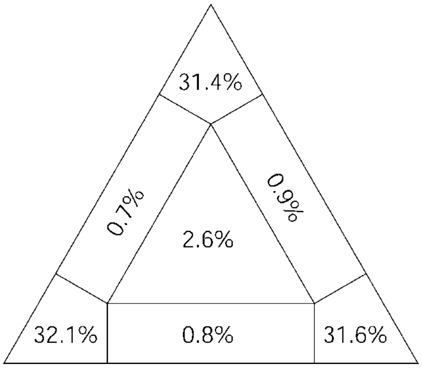

Figure 2. Likelihood-mapping analysis of the dataset (815 bp sequences of 12S and 16S rDNA for all sand gobies) demonstrating the strong phylogenetic content.

the amplified products was approximately 444 and 584 bp for 12S and 16S rDNA, respectively. After trimming, a total of 815 bp were retained in the dataset. This data matrix contained 204 variable sites of which 145 were identified as parsimony-informative. Fifty-five unique haplotypes were identified. Conspecific individuals originating from distinct geographical populations often differed in one to four positions (except for rather 'distant' *P. marmoratus* populations from the Adriatic, Euboea, and Crete). The MP tree had a consistency index of 0.56, a retention index of 0.81 and a tree length of 501. Likelihood-mapping showed that 95.1% of quartets were fully ('tree-like') and 2.4% partly ('network-like') resolved, whereas 2.6% remained unresolved (Fig. 2). After removal of the fastest evolving 30 nucleotide positions, this changed to 91.3%, 3.7%, and 5%, respectively. Hence, the resolution of the dataset did not decrease as a result of fast evolving sites. The LRT showed that a clock-like evolution of the sequences should not be rejected at a significance level of 0.05. A clock-constrained quartet puzzling tree with timescales corresponding to two various mutation rates is shown in Figure 3. Using the upper (1.5% × 10^{-6} years) and lower (0.5% × 10^{-6} years) assumed mutation rates, the first divergence event, leading to the major lineages of sand gobies, took place between 2.9 and 9 Mya). A first diversification within the different genera is estimated to date back to 2–6 Mya.

Knipowitschia appear paraphyletic in all analyses (Fig. 4). However, except for *Knipowitschia punctatissima* (Canestrini, 1864), which did not cluster with its alleged congeners but rather with *Pomatoschistus canestrinii* (Ninni, 1883), all taxa belonging to the genus are consistently grouped together. This *Knipowitschia* cluster is estimated to have diverged between 0.6 and 2.8 Mya. It is subdivided into distinct lineages with appreciable statistical support (posterior probability of at least 95 under Bayesian inference, or a bootstrap value of at least 75 in all other analyses): (1) *K. montenegrina* from the Morača River in Montenegro, perhaps being part of a south-eastern Adriatic lineage; (2) *K. panizzae* from the Adriatic basin (Po Delta and Venice Lagoon); (3) an Ionian clade: *Knipowitschia* sp. (Aitoliko Lagoon, Acheloos Lagoon, Prokopos Lagoon at Kalogria), *K.* cf. *panizzae* (Evinos Lagoon) and *K. milleri* (Acheron Delta); (4) the Macedonian–Thessalian *K. thessala* from the Pinios River; and (5) the Thracian–Aegean *K. caucasica* (Strymon, Evros and Vosvozis Rivers) and *K. caucasica* from the island of Euboea (Rigia Stream and Lake Vidra). Within the Ionian *Knipowitschia* lineage, the Aitoliko, Acheloos, Kalogria, and Evinos (*K.* cf. *panizzae*) populations never differed by more than 0.6% from one another, and never more than 1.3% from *K. milleri* (see the Supporting information, Table S1). Populations of *K. caucasica* did not differ more than 0.8% gamma-corrected GTR distance from each other. *Knipowitschia montenegrina* was separated by 2.1–2.9% from the other representatives in the main *Knipowitschia* cluster, Adriatic *K. panizzae* by 1.8–2.9%, and *K. thessala* by 1.3–2.8%.

Monophyly of *Economidichthys* is not supported in any of the analyses. Although enforcing monophyly of the genus significantly reduced the length of the MP tree to 487, the Kishino–Hasegawa test proved the constraint tree to be significantly worse at $P < 0.001$ ($P < 0.08$ for the Shimodaira–Hasegawa test). The genetic distance between its two species is 4.4–6.0%, which exceeds the distance between the various *Knipowitschia* species (except for *K. punctatissima*). *Economidichthys pygmaeus* sequences are very similar (and, after alignment trimming, mostly identical) throughout the range, never exceeding a 0.3% GTR+I+Γ distance, except for the population from Chiliadou Springs (Mornos Delta, Corinthian Gulf) which differed by 1.5–1.7%. The split between *E. pygmaeus* from the Mornos Basin and its conspecifics is timed at 0.8–2.3 Mya.

Although *Pomatoschistus* is paraphyletic, all *Pomatoschistus* spp. cluster together in all analyses, apart from *Pomatoschistus quagga* and *P. canestrinii*. *Gobiusculus flavescens* is a member of the main *Pomatoschistus* clade (confirming Huyse *et al*., 2004). Within *P. marmoratus*, three lineages are apparent: (1) an Euboean–Cretan cluster; (2) the representatives from the Venice Lagoon and from Drepano beach, the northernmost Greek location (located near the Greek–Albanian border); and (3) all other *P. marmoratus* samples from mainland western Greece. The most recent common ancestor of eastern Mediterranean *P. marmoratus* dates back 0.7–2.1 Mya. *Pomatoschistus marmoratus* from Venice Lagoon differed by up to 2.4% from its Greek conspecifics, with the minimal distance being 1.3–1.4% (to gobies from

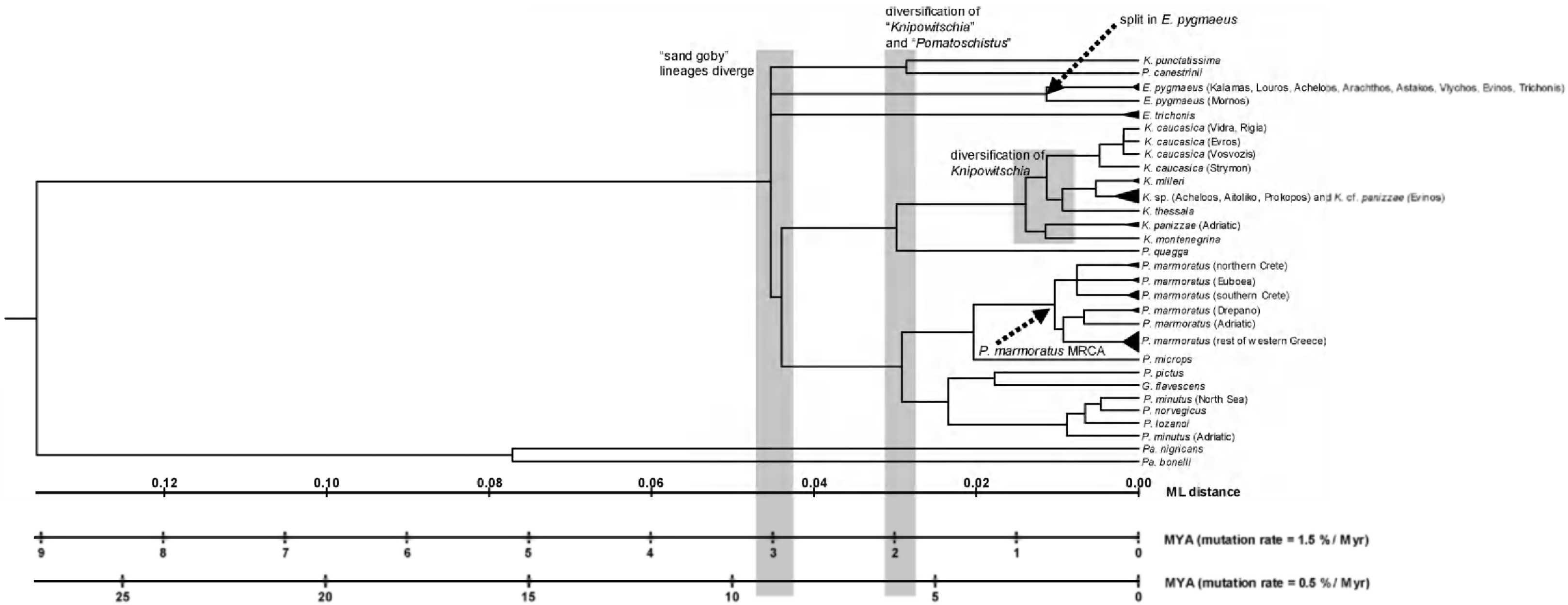

Figure 3. Clock-constrained quartet puzzling tree based on 815 bp of 12S and 16S rDNA sequences for the sand gobies under study. Maximum likelihood distances are indicated, and a timescale is provided, calibrated assuming a strict molecular clock of 1.5% and 0.5% × 10^{-6} years, respectively. For additional explanation regarding the evolutionary events indicated, see text.

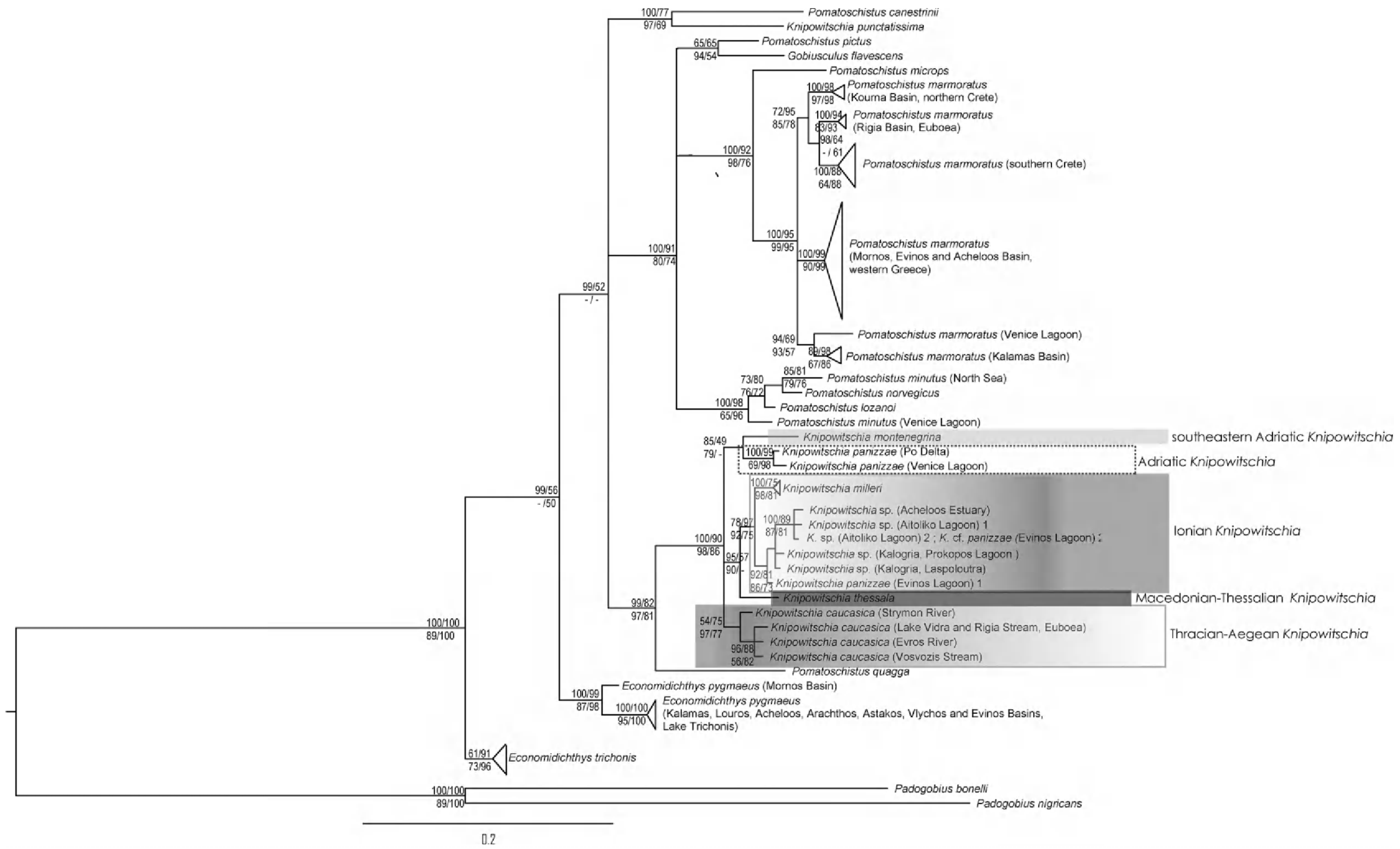

Figure 4. Cladogram constructed for the 12S and 16S rDNA sequences (815 bp) of the sand gobies. Statistical support for each node is shown: BI posterior probability/ML bootstrap/puzzle support/MP bootstrap. Clades not recovered in a particular analysis are marked with '–'. Branch lengths indicate the expected number of substitutions per site under Bayesian inference.

Drepano). Most Greek *P. marmoratus* sequences were less than 0.5% apart, except for the Euboean (Livadaki, Karystos Bay) and Cretan (Plakias, Frangokastello, Giorgiopoulis) populations, which were more similar to each other than to specimens from mainland western Greece.

DISCUSSION

The sand gobies are a monophyletic clade within the Gobiidae (Huyse *et al.*, 2004), including many endemics in the eastern Mediterranean basin. Many species are threatened by man and, for *E. pygmaeus* and *K. thessala*, the type localities have been destroyed and their populations lost (Economidis, 1991; Maitland & Crivelli, 1996). Despite the urgent need for their protection, conservation efforts are hindered by a lack of taxonomic knowledge (Economou *et al.*, 2007). Indeed, complexes of so-called cryptic species can be problematic in conservation policy (Wan *et al.*, 2004; Bickford *et al.*, 2006). Larmuseau *et al.* (2008) have shown that molecular work may assist in identifying morphologically cryptic sand gobies. Apart from species identification, molecular phylogeny/phylogeography is necessary to identify conservation units allowing management of the genetic diversity within species (Wan *et al.*, 2004) and to demarcate biogeographical boundaries in aquatic biomes (Riddle *et al.*, 2008; Costedoat & Gilles, 2009), with both representing crucial steps in conservation planning and resource management. We can pinpoint some lineages that merit additional taxonomic and conservational scrutiny because genetic distances can be compared to those between recognized species (for an example in *Rutilus rutilus* (Linnaeus, 1758), see Larmuseau *et al.*, 2009), although this of course depends on the quality of current morphological taxonomy (Johns & Avise, 1998). Based on previous work on gobies using mitochondrial rDNA (Harada *et al.*, 2002; Huyse *et al.*, 2004; Mukai *et al.*, 2004), corrected pairwise distances between closely-related species appear to be 2–4% at least. With this cut-off in mind, we might suggest some sand gobies whose taxonomy merits further scrutiny. Because a thorough revision is hence essential for these taxa, we will focus on lineages rather than species to see how major sand goby lineages fit into the historical biogeography of the Balkans.

Partial 12S and 16S mitochondrial ribosomal DNA sequences were used to clarify the phylogenetic relationships of taxa belonging to *Economidichthys* and *Knipowitschia*. The various analyses show a similar picture. *Economidichthys* spp. appear as a basal, paraphyletic group within the consistently monophyletic sand gobies. Most taxa assigned to *Knipowitschia* and *Pomatoschistus*, respectively, strongly cluster together. The only exceptions are the well-supported clade formed by *P. canestrinii* and *K. punctatissima*, and *P. quagga* as sister to the remaining representatives of *Knipowitschia*.

PHYLOGENETIC STATUS OF THE GENUS *ECONOMIDICHTHYS*

Economidichthys is endemic to western Greece. The widespread *E. pygmaeus* occurs in rivers, lowland streams, and freshwater bodies along the western Greek mainland, the island of Lefkas, and Lake Trichonis. This lake and the adjacent Lake Lysimachia are the only water bodies hosting *E. trichonis*. This is the smallest European freshwater fish and, in its larval stage, the smallest free-living European vertebrate (Miller, 2004a; Kottelat & Freyhof, 2007). The position of the genus *Economidichthys* based on molecular data was not resolved in the study of Huyse *et al.* (2004), which only included *E. pygmaeus*. The genus appears to be ancestral within the sand gobies, although many basal nodes are unresolved. Gamma-corrected genetic distances situate *E. trichonis* closest to its alleged congener. However, none of the phylogenetic analyses support monophyly of the genus. The two species of the genus differ more from each other than do species in other sand goby genera (a minimum of 4.4% pairwise genetic distance between *E. pygmaeus* and *E. trichonis* versus a maximum of 2.9% between the species in the *Knipowitschia* clade).

The most remarkable autapomorphy of *Economidichthys* is the perianal organ, a highly-derived organ unique among teleosts (Miller, 1990). However, both species exhibit substantial embryological and developmental differences, which might be adaptations to different lifestyles. Embryonic and larval developmental patterns have long been suggested to be evolutionarily conservative (and phylogenetically informative) compared to highly progressive adult patterns (Ahlstrom & Moser, 1976; Moser *et al.*, 1984; Strauss, 1992; Strathmann, 2000). Because of the dissimilar development of both *Economidichthys* species, Daoulas *et al.* (1993) hypothesized the perianal organ to be a shared adaptation (rather than a synapomorphy) to hole-brooding, with an antiseptic role. Although eggs are always more vulnerable to infections when deposited in sheltered locations (Potts, 1984), it is argued that both *Economidichthys* spp. experience this risk to a higher extent than other hole-brooders. They are almost unique among teleosts in using reed cavities as spawning sites, which are poorly aerated holes of organic origin. Many gobies possess accessory testis-associated structures, most often termed seminal vesicles or sperm duct glands, whose species-specific structure has been hypothesized to depend on ecological or physiological conditions rather than on phylogeny (Fishelson, 1991). These are mostly present in males only (Cole & Hoese, 2001), in contrast to the perianal organ. However, both sexes in *Gobiodon* Bleeker, 1856 and *Paragobiodon* Bleeker, 1873 exhibit seminal vesicles that are suggested to include an antiseptic function. Species in both of these genera brood on corals (Mazzoldi, Petersen & Rasotto, 2005), and hence, similar to *Economidichthys*, on organic substrates.

Aphia minuta (Risso, 1810) and *Crystallogobius linearis* (Düben, 1845) are Atlantic–Mediterranean gobies known to be similar to *E. trichonis* with respect to biology and egg morphology (Caputo *et al.*, 2003; La Mesa *et al.*, 2005). Comparing the 12S and 16S rDNA sequences of those species (GenBank accession numbers EF218623, EF218635, EF218638, and EF218650; Giovannotti *et al.*, 2007), however, showed a Kimura-two-parameter (Kimura, 1980) genetic distance of approximately 16% and 13%, respectively, to *E. trichonis*, refuting sister-taxa affinities between those taxa (and showing that developmental characters can be homoplasious as well). Hence, to the best of our current knowledge, there are no known sister taxa to *Economidichthys*. This fact, together with the existence of only two lineages belonging to this genus (and this despite our thorough sampling) suggests that (past) extinction events might have been important in this group.

An exception to the generally very low genetic diversity within *E. pygmaeus* are the specimens caught in the Chiliadou Springs, off the Mornos River, at its easternmost distribution and less than 100 km from the nearest *E. pygmaeus* sampling location. They differ from the other *E. pygmaeus* populations by 1.5–1.7%, which is, by approximately two-fold, the highest pairwise distance found within the Thracian–Aegean *K. caucasica* or within the Ionian *Knipowitschia*. A difference between Chiliadou and western Greek populations was also found in *Valencia letourneuxi* (Sauvage, 1880) (Cyprinodontiformes, Valenciidae) (Vogiatzi *et al.*, 2009). Although this does not suffice to raise this population to the species level (see above), it is remarkable that it also deviates phenotypically from other *E. pygmaeus* [e.g. in (head) size parameters and in a colour pattern with reduced vertical stripes; A. N. Economou, S. Zogaris, pers. observ.] to the extent that it might have erroneously been assigned to *Knipowitschia* several times (Economou *et al.*, 1999). A geological event possibly isolating the Mornos basin from other drainages home to *E. pygmaeus* is the alternation between marine and freshwater (the so-called Corinth Lake) conditions in the Corinthian Gulf during the past million years (Moretti *et al.*, 2003; Zelilidis, 2003; Lykousis *et al.*, 2007; Lykousis, 2009). Approximately 0.9 Mya, the then lacustrine Gulf was flooded by the sea (Westaway, 1996). This corresponds to the estimated timing of the separation of the Mornos population (0.8–2.3 Mya) because the actual mutation rate of the genes is probably towards the higher edge of the range used (see above). In view of the relatively high risk of anthropogenic extinction of often unique local sand goby populations (Miller, 1990), this Mornos population deserves to be treated with extra conservational focus as an ESU because it represents the most phylogenetically distinct *E. pygmaeus* population.

NEED FOR TAXONOMIC REVISION OF THE PARAPHYLETIC *KNIPOWITSCHIA*

Knipowitschia has representatives in the Caspian, Black, Aegean, Ionian, and Adriatic basins, many of which are local freshwater endemics. Based on 12S and 16S rDNA sequence data, Penzo *et al.* (1998) and Huyse *et al.* (2004) demonstrated the paraphyly of the genus. The additional species sequenced here fall within the *Knipowitschia* clade, so the genus appears valid, with the exception of *K. punctatissima* that clusters with *P. canestrinii* in all analyses. It should be noted that the affiliation of *K. punctatissima* has been under discussion before, and the absence of the longitudinal row and suborbital papillae led to its classification under the newly-described *Orsinigobius*. Subsequently, morphological similarities with *Knipowitschia* led to the synonymization of *Orsinigobius* with *Knipowitschia* (which appears to be unjustified based on the present results), and the absent characters were considered as secondary losses (Economidis & Miller, 1990; Penzo *et al.*, 1998). The genetic divergence among the various *Knipowitschia* species is limited (2.9% at most). Furthermore, the genetic distances within the Ionian populations are almost equal to those between Ionian populations and *K. thessala* or *K. caucasica*. The fact that the level of inter- and intraspecific distances is similar, confusing current species delineation, indicates the existence of a cryptic species complex (Nelson, 1999), and *Knipowitschia* is certainly in need of a revision.

The recently described *Knipowitschia montenegrina* clusters with its Adriatic congener *K. panizzae*. Before being raised to species status, its representatives were assigned to *K. panizzae*, although it is morphologically not its most reminiscent species (Kovačić & Šanda, 2007). In view of the genetic distance of at least 2.1% to its congeners, the morphological species is genetically confirmed. It is, however, difficult to explain how isolation, either through geographical distance or vicariant barriers, could have provoked speciation here (Skoulikidis *et al.*, 2009; Zogaris *et al.*, 2009). Perhaps *K. montenegrina* and *K. pannizae* stem from separate Danubian colonization events. Mostly on the basis of mtDNA markers or allozyme genetics, other Balkan fish populations were suggested to stem from Pleistocene river capture from the Danube to the southern (Durand *et al.*, 1999b, 2003; Šanda, Lusková & Vukić, 2005) and northern Adriatic (Tsigenopoulos & Berrebi, 2000; Šlechtová *et al.*, 2004; Papa & Schreiber, 2005).

The 0.5–1.3% genetic distance of *K. milleri* to its only other Ionian congeners *Knipowitschia* sp. and *K.* cf. *panizzae* is low, indicative of intraspecific rather than interspecific variation. Their respective larvae cannot be reliably distinguished using morphology (Miller, Madurell & Economou, 2004). This could suggest that divergence was indeed recent enough for genetic mixing to occur. Bearing in mind that few Ionian *Knipowitschia* populations are adequately studied morphologically, and that phenotypic variation is observed in *Knipowitschia* spp. (Miller, 2004b; Kovačić, 2005), it is uncertain whether the distinctive features of the Acheron *K. milleri* are real or merely represent intraspecific variation. In view of the low genetic distances, all Ionian representatives of *Knipowitschia* appear to be conspecific with *K. milleri*. River confluences as a result of recent marine regression in the Ionian region (Chronis, Piper & Anagnostou, 1991), and a drop of salinity in the coastal zone, provided occasions for sympatry and could explain the homogeneity in Ionian *Knipowitschia* (and *E. pygmaeus*) populations (e.g. for chub; Durand *et al.*, 1999b). Because Kovačić (2008) asserts that *K. panizzae* in the Adriatic cannot be distinguished from *K. caucasica*, a thorough study of *Knipowitschia* in the Adriatic and Ionian regions is necessary to determine whether there are different species present, and whether certain endemics are limited in range and should as such be treated as ESUs. Caution is still required before forging species entities (or in this case, lumping taxa) in the Ionian and South-East Adriatic ecoregions. It would be interesting to include other poorly known *Knipowitschia* spp. from these regions in the analysis, such as *Knipowitschia croatica* Mrakovic, Kerovec, Misetic & Schneider, 1996, *Knipowitschia mrakovcici* Miller, 2009, *Knipowitschia radovici* Kovačić, 2005, and *Knipowitschia goerneri* Ahnelt, 1991, an endemic to the island of Corfu.

Biogeographical and historical implications for the Balkans

Five lineages of *Knipowitschia* are recognized in the present study: south-eastern Adriatic, Adriatic, Ionian, Macedonian–Thessalian and Thracian–Aegean. These insights provide support for the historical view that the south-eastern Adriatic, Ionian, Macedonian–Thessalian, and Thracian–Aegean (or northern Aegean) regions have a distinct evolutionary history reflecting ichthyogeographical boundaries (Bianco, 1986; Bănărescu, 2004; Economou *et al.*, 2007; Zogaris *et al.*, 2009). As such, each region requires separate conservation planning strategies. We reiterate that more work, such as in the Western and Eastern Aegean, may reveal the historical affinities and biogeographies among these and the Thracian ecoregions. *Knipowitschia* spp., being tiny fish, survive in small populations in minute wetlands on several Aegean islands. They may represent one of the few widespread genera, suitable as biogeographical indicators, in the remarkably species-depauperate insular realm of these ecoregions.

Several studies have invoked Lago Mare-phases during the Messinian Salinity Crisis (MSC) to explain the presence of Paratethyan *Knipowitschia* in the Mediterranean basin (Miller, 1990; Huyse *et al.*, 2004). If the genus is this old, a multitude of 'diversification routes' could have led to its current diversity: two Lago Mare phases (Clauzon *et al.*, 2005), independent Paratethyan connections (Sonnenfeld, 1974; Sakınç & Yaltırak, 2005), and several colonization routes for Danubian freshwater fishes towards the Adriatic or Aegean (Doadrio & Carmona, 2003; Levy, Doadrio & Almada, 2009). However, even the slowest molecular clock dates *Knipowitschia* diversification in this region well after the MSC or Lago Mare phases. We suggest that Pleistocene glaciation cycles drove diversification within *Knipowitschia*, as has been proposed for *Pomatoschistus* (Gysels *et al.*, 2004; Huyse *et al.*, 2004), and allowed the spread and diversification of freshwater fishes in the region (Miller & Šanda, 2008). However, this alone does not explain the range of the various *Knipowitschia* species. Indeed, clustering of *K. thessala* with the Ionian lineage appears to favour Pleistocene river capture events rather than a spread through the Adriatic and Ionian seas (Durand *et al.*, 1999b). Both Thracian–Aegean *K. caucasica* and Macedonian–Thessalian *K. thessala* could stem from originally Paratethyan river captures (Economidis & Bănărescu, 1991). Geographically, *K. thessala* interrupts northern and southern Thracian–Aegean *K. caucasica*. This might result from relatively later Danubian colonization via the Axios River (Durand *et al.*, 1999b). Lastly, concerning the Thracian–Aegean *Knipowitschia* lineage, clustering of the Euboean and Thracian–Aegean specimens could be the result of dispersal through a freshened Aegean Sea approximately 0.2 Mya, which corresponds to the molecular dating. Affinities between Euboea and Thrace have also been found for two cyprinid genera (Zardoya, Economidis & Doadrio, 1999; Tsigenopoulos & Berrebi, 2000).

The presence of both of the *Economidichthys* spp. and an endemic *Knipowitschia* lineage in the Ionian region leads to a higher local biodiversity compared to the other study areas. This supports the general agreement that Ionian drainages have long been isolated from other European (including Greek) drainages. Together with the glacial refugium role of the western Balkans and with the presence of ancient lakes in the Acheloos drainage (e.g. Lake Trichonis: Economou *et al.*, 1994), this isolation shaped a unique freshwater ichthyofauna with a high degree of endemism (Economou *et al.*, 2007; Skoulikidis *et al.*, 2009) deserving special attention for conservation.

COMPARISON WITH MARINE TAXA: *P. MARMORATUS*

The genetic clusters of the marine *P. marmoratus*, namely (1) the islands of Euboea and Crete; (2) Drepano and the Adriatic; and (3) western Greece, confirm the biogeographical boundary between the Ionian and Aegean in the eastern Mediterranean Sea (Spalding *et al.*, 2007). In view of the timing, Pleistocene events must have played a role in structuring the region's marbled goby populations. Separate monophyletic clusters of Aegean and Adriatic marine sand gobies, as a result of glaciation events, were predicted by Stefanni & Thorley (2003) based on *Pomatoschistus minutus* molecular data. The fact that the Drepano specimens (near the Kalamas Basin and the Greek–Albanian border) cluster with the Adriatic *P. marmoratus* could point to a correlation between genetic and geographical distances. The genetic distances found in *P. marmoratus* throughout the sampling area do not exceed those found in freshwater gobies on a much smaller spatial scale (e.g. within *Knipowitschia*), suggesting that dispersal barriers in the marine realm are not as sharp as in freshwater systems.

PERSPECTIVES

The results obtained in the present study indicate that the natural range, phylogeny, and phylogeography of Balkan sand gobies, both marine and freshwater, reflect ichthyogeographical boundaries proposed on the basis of fish assemblage composition (Bianco, 1986; Bănărescu, 2004; Economou *et al.*, 2007; Zogaris *et al.*, 2009). Taxonomic uncertainties were highlighted in *Knipowitschia* and *Economidichthys*, and ancient fish dispersal pathways previously mentioned in the literature were recognized in the goby radiation by linking its phylogeny to the region's historical biogeography. On the scale of single or adjacent river drainages, *Knipowitschia* is understudied, necessitating further molecular and morphological work to formally describe species demarcations. With the exception of one taxon, it appears as a monophyletic genus. Its low within-clade genetic variability might represent recent or ongoing speciation in a cryptic species complex, and its phylogeny reflects the Balkan aquatic ecoregions. Because the geographical resolution of our sampling design was rather high, additional molecular work should focus on other regions, such as the northern Balkans along the Adriatic coastline, and the Ponto-Caspian basin. Indeed, approximately 12 endemic sand gobies remain to be phylogenetically positioned (Eschmeyer, 2010). The retrieval of a basal polytomy in several studies on this system, current higher taxon coverage, the fast divergence in other gobies (Akihito *et al.*, 2000), and the high phylogenetic content of the dataset point to a fast radiation process. In view of classical problems with mitochondrial gene trees (Shaw, 2002; Rubinoff, 2006), additional morphological work is recommended (Avise, 2000), together with nuclear sequences to assess phenomena such as hybridization, and for a more reliable assignment of ESUs *sensu* Moritz (1994).

To increase resolution, parasite molecular data might be useful because parasites are known to sometimes complement (genetic) host data (Nieberding & Olivieri, 2007; Blakeslee, Byers & Lesser, 2008; Barson *et al.*, 2010). Preliminary molecular results on *Gyrodactylus* von Nordmann, 1832 flatworms on various *E. pygmaeus* populations show a higher genetic diversity than their host, pointing to a Pleistocene isolation event in the Kalamas River (the northernmost border of its range) (M. P. M. Vanhove, B. Geens, pers. observ.). As such, this approach looks very promising for generating additional information on the current study system.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. GTR+I+Γ pairwise genetic distances (in %) between the haplotypes included in the phylogenetic analysis, for a fragment of 815 bp of 12S and 16S rDNA. The number of times a haplotype was found on a given location is provided in parenthesis. Haplotypes preceded by an asterisk (*) were retrieved from GenBank. Sequences were available for *Knipowitschia milleri* (GQ398133-7) and *Economidichthys pygmaeus* (GQ398131-2), of which additional specimens were sequenced.