

# Phylogeny, systematics and biogeography of the European sand gobies (Gobiiformes: Gobionellidae)

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The sand gobies are a group of 30 species in five genera (*Knipowitschia*, *Pomatoschistus*, *Economidichthys*, *Ninnigobius* and *Orsinigobius*) native to the seas and fresh waters of Europe and the Ponto-Caspian region. We construct a phylogeny incorporating new DNA sequence data (mitochondrial *COI* gene) for species in Greece and the Adriatic (Venetian Lagoon) with existing sequences sampled from throughout European waters. Our comprehensive analysis confirms the distinctness of *Orsinigobius* and *Ninnigobius*, supports that *Gobiusculus* is part of *Pomatoschistus* and supports the monophyly of *Economidichthys* and *Knipowitschia*. We then calibrate the phylogeny using fossils, interpret the phylogenetic biogeography of sand gobies throughout Europe and the Mediterranean and estimate the historical divergence patterns in the group. We infer the origin of the sand goby clade at 33.0 Mya, near the Eocene–Oligocene boundary, and show that radiations among marine *Pomatoschistus* clades in the Miocene are correlated with the closure of the Tethys seaway. Younger freshwater clades of *Ninnigobius*, *Orsinigobius* and subclades of *Knipowitschia* diversified in the late Miocene and early Pliocene, centred in the Adriatic region and concordant with the Lago Mare phase of the Messinian salinity crisis. *Economidichthys* is the exception to this pattern; it inhabits freshwater but originated and diversified during the mid-Miocene.

ADDITIONAL KEYWORDS: DNA barcode, *Economidichthys* – Europe – Gobiidae – *Gobiusculus* – *Knipowitschia* – Lago Mare, Mediterranean – *Orsinigobius* – *Pomatoschistus* – Tethys.

## INTRODUCTION

The seas and freshwaters of Europe host a diverse and complex goby fauna, including wide-ranging marine and euryhaline species in addition to geographically restricted freshwater endemics. They are familiar and well known, and include several species of conservation concern (Miller, 1990, 2004; Vanhove *et al.*, 2012). Among the European species are a group of five genera

collectively known as sand gobies (*Knipowitschia* Iljin, 1927; *Pomatoschistus* Gill, 1863; *Economidichthys* Bianco, Bullock, Miller & Roubal, 1987; the genera *Ninnigobius* Whitley, 1951 and *Orsinigobius* Gandolfi, Marconato & Torricelli, 1986 have also been used). Together, those genera contain 30 species and are distributed from the coastal Atlantic of western Europe, throughout the Mediterranean and many of its associated freshwaters, and into the drainages of the Black, Aral and Caspian seas. Taxonomic and phylogenetic work on the group has been extensive and sometimes contradictory but has yielded a wealth of data bearing

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on relationships both within sand goby genera and among sand gobies and their more distant relatives (Penzo *et al.*, 1988; Huyse, Van Houdt & Volckaert, 2004; Larmuseau *et al.*, 2010; Malavasi *et al.*, 2012; Vanhove *et al.*, 2012).

Sand gobies are well supported as a monophyletic group, based on both molecular and morphological data [mitochondrial 12S/16S: Penzo *et al.*, 1988; Huyse *et al.*, 2004; Larmuseau *et al.*, 2010 (also rhodopsin); Vanhove *et al.*, 2012; morphology: Economidis & Miller, 1990; Malavasi *et al.*, 2012). The placement of sand gobies within Gobioidae has been more obscure, but the higher level works of Thacker & Roje (2011), Thacker (2011, 2013) and, in particular, Agorreta *et al.* (2013) have shown that sand gobies are part of the family Gobionellidae, sister to European marine genera such as *Buenia* Iljin, 1930, *Deltentosteus* Gill, 1863 and *Lebetus* Winther, 1877, rather than the more distantly related *Gobius* Linnaeus, 1758, *Padogobius* Iljin, 1933, *Zebrus* (deBuen, 1930) and *Zosterisessor* Whitley, 1935 in the family Gobiidae (complete generic composition of gobiid and gobiionellid lineages is given by Thacker, 2015). This placement explains the observation in earlier phylogenetic studies that sand gobies are deeply divided from common European marine gobies, as noted by McKay & Miller (1997) and Penzo *et al.* (1998) and as depicted in the phylogenies of Huyse *et al.* (2004), Larmuseau *et al.* (2010) and Vanhove *et al.* (2012).

Among sand gobies, the two largest genera (*Knipowitschia* and *Pomatoschistus*) are distinct, both in ecology and in distribution. *Pomatoschistus* species inhabit marine or occasionally estuarine waters and are distributed along the coasts of the Baltic, northeastern Atlantic, Mediterranean and Adriatic seas. They are found in inshore mud, sand or rubble habitats (Miller, 1986). *Knipowitschia* prefers estuarine to fresh waters. It is known from the streams and coastal lagoons of the Adriatic, but from there its distribution stretches to the east, including the eastern Mediterranean, the Aegean and the shores of the Black, Caspian and Aral seas. A few *Knipowitschia* are landlocked freshwater endemics with very restricted ranges (Ahnelt, 2011; Vukić *et al.*, 2016). *Economidichthys* is a genus of two species that is known only from coastal lakes and rivers in western Greece (Miller, 2004; Kottelat & Freyhof, 2007; Freyhof, 2011).

Phylogenetic studies focusing on sand gobies have varied in their sampling and in the details of their recovered relationships, but all have shown that *Knipowitschia* and *Pomatoschistus* are not monophyletic as traditionally construed. Much of the nonmonophyly is alleviated if two smaller genera, *Orsinigobius* and *Ninnigobius*, are recognized. *Orsinigobius* [including species *Orsinigobius punctatissimus* (Canestrinii, 1864) and *Orsinigobius croaticus* (Mrakovčić, Kerovec,

Misetic & Schneider, 1996)] and *Ninnigobius* [including *Ninnigobius canestrinii* (Ninni, 1883) and *Ninnigobius montenegrensis* (Miller & Šanda, 2008)] are distinct from *Knipowitschia* and *Pomatoschistus*, respectively (Ahnelt & Bianco, 1990; Huyse *et al.*, 2004; Miller & Šanda, 2008; Vanhove *et al.*, 2012; Geiger *et al.*, 2014). There is also agreement across several molecular phylogenetic analyses that *Gobiusculus flavescens* Fabricius, 1779, a common species in coastal marine habitats from the Atlantic coast of Spain northward into Norway, is nested within *Pomatoschistus* (Huyse *et al.*, 2004; Larmuseau *et al.*, 2010; Vanhove *et al.*, 2012; Knebelberger & Thiel, 2014). Within *Pomatoschistus* and *Knipowitschia*, interrelationships of some species have been investigated (Geiger *et al.*, 2014; Knebelberger & Thiel, 2014; Vukić *et al.*, 2016), but there has not been a comprehensive phylogenetic hypothesis inferred among and within sand goby genera.

Significant sources of inter- and intraspecific sequence information for sand gobies are the various recent efforts to obtain DNA barcodes for European fishes. The studies of Costa *et al.* (2012), Geiger *et al.* (2014), Knebelberger & Thiel (2014) and Landi *et al.* (2014) have generated abundant data for the mitochondrial *COI* locus and demonstrated its usefulness in species identification and application of conservation. Those data are also suitable for phylogenetic analysis and are among the most reliable mitochondrial genes for that purpose (Zardoya & Meyer, 1996). Although the *COI* locus generally does not provide sufficient phylogenetic resolution for large-scale analyses (Rubinoff & Holland, 2005), here the taxonomic scope is narrow enough that *COI* is useful. In the present study, we combine existing *COI* data with new sequences for several species of sand gobies sampled from around mainland Greece and the Adriatic (Venetian Lagoon). We use this combined data set to infer a comprehensive sand goby phylogeny and evaluate how well current generic and species assignments concur with phylogenetic patterns. We then use the fossil record of sand gobies, along with a legacy calibration for Gobionellidae, to calibrate the phylogeny, and we consider the ages and biogeographical patterns of various sand goby radiations in the context of the geological history of Europe.

## MATERIAL AND METHODS

### SPECIMEN COLLECTION AND DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

We collected 71 specimens either by hand net or by electrofishing gear from 18 geographically distinct localities throughout the sand gobies' distributional range in Greece. To expand the sample size and geographical

breadth, we also included 12 samples from three species from the Venetian Lagoon in the analysis (Table 1, Fig. 1). The specimens were killed by immersion in tricaine methanesulphonate (MS-222), placed on ice, and fixed later in the laboratory in 70–100% ethanol. Information on sample codes, species and sampling localities is given in Table 1, and a map of localities is shown in Figure 1.

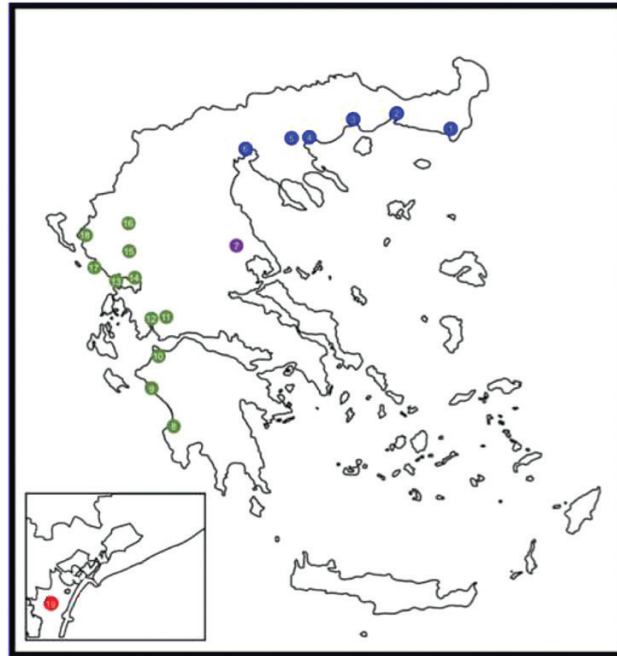
We extracted total genomic DNA from muscle tissues by standard sodium dodecyl sulphate proteinase-K digestion, phenol:chloroform:isoamylalcohol extraction and ethanol precipitation. We amplified a partial segment of the mitochondrial protein encoding the *COI* gene using the forward primer FishF1 5'-TCAACCAACCACAAAGACATTGGCAC-3' and

the reverse primer FishR1 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3' (Ward *et al.*, 2005). An alternative primer pair, FishF2 5'-TCGACTAATCATAAAGATATCGGCAC-3' and FishR2 5'-ACTTCAGGGTGACCGAAGAATCAGAA-3', was used (Ward *et al.*, 2005) for *N. canestrinii*. We performed PCR amplifications in a final volume of 25 µL containing template DNA (10–100 ng), 25 mM MgCl<sub>2</sub>, 0.4 µM of each primer and Taq DNA polymerase (Qiagen Inc.). The cycling profile of the PCR included an initial denaturation of 95 °C for 2 min, 35 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 60 s, with a final extension of 92 °C for 10 min. The PCRs were checked on 2% agarose gels stained with ethidium bromide, and excess

**Table 1.** List of the species examined from each sampling area, categorized by region

| Site         | Locality                  | Species                            | GenBank number |
|--------------|---------------------------|------------------------------------|----------------|
| Aegean coast |                           |                                    |                |
| 1            | Drana Lagoon              | <i>Knipowitschia caucasica</i>     | KX673961-63    |
| 2            | Lake Vistonis             | <i>Knipowitschia caucasica</i>     | KX673957-60    |
| 3            | Vassova Lagoon            | <i>Knipowitschia caucasica</i>     | KX673953–56    |
|              |                           | <i>Pomatoschistus marmoratus</i>   | KX673972       |
| 4            | Rihios River              | <i>Knipowitschia caucasica</i>     | KX673948–49    |
| 5            | Lake Volvi                | <i>Knipowitschia caucasica</i>     | KX673950–52    |
| 6            | Axios River               | <i>Knipowitschia caucasica</i>     | KX673944–47    |
| Thessaly     |                           |                                    |                |
| 7            | Lake Karla                | <i>Knipowitschia thessala</i>      | KX673940-43    |
| Ionian coast |                           |                                    |                |
| 8            | Lake Kaifa                | <i>Knipowitschia milleri</i>       | KX673937-39    |
| 9            | Kotyhi Lagoon             | <i>Knipowitschia milleri</i>       | KX673932-36    |
| 10           | Kalogria Lagoon           | <i>Knipowitschia milleri</i>       | KX673926-31    |
| 11           | Trichonis Lake            | <i>Economidichthys trichonis</i>   | KX673893       |
|              |                           | <i>Economidichthys pygmaeus</i>    | KX673908-12    |
| 12           | Agios Dimitrios River     | <i>Economidichthys pygmaeus</i>    | KX673913-15    |
| 13           | Louros River              | <i>Economidichthys pygmaeus</i>    | KX673902-04    |
| 14           | Arachthos River           | <i>Economidichthys pygmaeus</i>    | KX673897       |
| 15           | Ziros Lake                | <i>Economidichthys pygmaeus</i>    | KX673898-01    |
| 16           | Pamvotis Lake             | <i>Economidichthys pygmaeus</i>    | KX673894-96    |
|              |                           | <i>Knipowitschia milleri</i>       | KX673916-18    |
| 17           | Acheron River             | <i>Knipowitschia milleri</i>       | KX673923-25    |
| 18           | Kalamas River             | <i>Economidichthys pygmaeus</i>    | KX673905-07    |
|              |                           | <i>Knipowitschia milleri</i>       | KX673919-22    |
| Adriatic     |                           |                                    |                |
| 19           | Venetian Lagoon           | <i>Knipowitschia panizae</i>       | KX673964-67    |
|              |                           | <i>Ninnigobius canestrinii</i>     | KX673968-71    |
|              |                           | <i>Pomatoschistus marmoratus</i>   | KX673973–75    |
|              | Towuti Lake (Indonesia)   | <i>Mugilogobius adeia</i>          | KM887159       |
|              | Towuti Lake (Indonesia)   | <i>Mugilogobius latifrons</i>      | KM887161       |
|              | Towuti Lake (Indonesia)   | <i>Mugilogobius hitam</i>          | KM887169       |
|              | Tyligal Estuary (Ukraine) | <i>Zosterisessor ophiocephalus</i> | EU444698       |
|              | Israel                    | <i>Gobius niger</i>                | KM538356       |
|              | Baltic Sea                | <i>Gobius niger</i>                | KM077839       |

Site number refers to the location of each sampling site as shown in Figure 1.



**Figure 1.** Map of sampling localities in Greece and the Venetian Lagoon (inset). Sites indicated in blue are on the Aegean coast; purple indicates Thessaly; green localities are on the Ionian coast; and the Adriatic (Venetian Lagoon) is indicated in red. Locality numbers correspond to those given in Table 1.

primers and unincorporated nucleotides removed from successful reactions with spin column purification kits (Nucleospin Extract II; Macherey-Nagel). The amplified fragments were then sent out for bidirectional sequencing at the VBC Biotech Service GmbH (Wien, Austria).

We generated *COI* sequence data from 83 representatives of the species *N. canestrinii*, *Pomatoschistus marmoratus* (Risso, 1810) and *Knipowitschia panizae* (Verga, 1841) from Venice; *Economidichthys trichonis* Economidis & Miller, 1990, *Economidichthys pygmaeus* (Holly, 1929) and *Knipowitschia milleri* (Ahnelt & Bianco, 1990) from western Greece (Ionian sea); and *Knipowitschia thessala* (Vinciguerra, 1921), *Knipowitschia caucasica* (Berg, 1916) and *P. marmoratus* (Risso, 1810) from northern Greece (Thessaly on the northern Aegean coast). Sequences were 625 bp in length and are deposited in GenBank under the accession numbers KX673893–KX673975. We also sequenced five outgroup taxa, including single exemplars of *Mugilogobius latifrons* (Boulenger, 1897), *Mugilogobius adeia* Larson & Kottelat, 1992, *Mugilogobius hitam* Larson, Geiger, Hadiaty & Herder, 2014 (Gobionellidae, *Mugilogobius* lineage) and one individual of *Zosterisessor ophiocephalus* (Pallas, 1814) and two of *Gobius niger* Linnaeus, 1785 (Gobiidae, *Gobius* lineage).

In addition to the newly determined sequences from Greece and the Venetian Lagoon, we used archived

DNA barcode sequences for sand goby species obtained from GenBank and largely derived from the works of Costa *et al.* (2012), Geiger *et al.* (2014), Kneibelsberger & Thiel (2014) and Landi *et al.* (2014). GenBank numbers of archived DNA barcode sequences used in the analysis are given in Table 2. We assembled a total of 271 individual sequences from 24 nominal sand goby species, with sampling that girds the European continent, from the Baltic Sea in the north, throughout the North Sea, Northeastern Atlantic and the Mediterranean, Aegean and Ionian seas and many coastal freshwater drainages. Following the taxonomy given by Froese & Pauly (2017), we lack the two species of *Knipowitschia* [*Knipowitschia longicaudata* (Kessler, 1877) and *Knipowitschia iljini* Berg, 1931] known from the Black, Azov and Caspian seas, *Knipowitschia bergi* (Iljin, 1928) from the Caspian sea, one of the two sympatric species known from a brackish coastal lake near the southern coast of Turkey (*Knipowitschia caunosi* Ahnelt, 2011); and five *Pomatoschistus*: *Pomatoschistus bathi* Miller, 1982 from the Adriatic and Eastern Mediterranean, *Pomatoschistus knerii* (Steindachner, 1861) from the coast of Croatia, *Pomatoschistus nanus* Engin & Seyhan, 2017 and *Pomatoschistus anatoliae* Engin & Innal, 2017 from the coast of Turkey, and *Pomatoschistus quagga* (Heckel, 1839) from the Western Mediterranean. In particular, *P. quagga* is of interest because earlier phylogenetic studies

**Table 2.** Archived *COI* sequences from GenBank used in this study

|          |                     |
|----------|---------------------|
| KJ553311 | <i>E. pygmaeus</i>  |
| KJ553316 | <i>E. pygmaeus</i>  |
| KJ553364 | <i>E. pygmaeus</i>  |
| KJ553482 | <i>E. pygmaeus</i>  |
| KJ553547 | <i>E. pygmaeus</i>  |
| KJ553595 | <i>E. pygmaeus</i>  |
| KJ553631 | <i>E. pygmaeus</i>  |
| KJ553325 | <i>E. trichonis</i> |
| KJ553425 | <i>E. trichonis</i> |
| KJ553450 | <i>E. trichonis</i> |
| KJ553457 | <i>E. trichonis</i> |
| KJ553322 | <i>K. byblisia</i>  |
| KJ553368 | <i>K. byblisia</i>  |
| KJ553411 | <i>K. byblisia</i>  |
| KJ553412 | <i>K. byblisia</i>  |
| KJ553454 | <i>K. byblisia</i>  |
| KJ553539 | <i>K. byblisia</i>  |
| KJ553570 | <i>K. byblisia</i>  |
| KJ553602 | <i>K. byblisia</i>  |
| KJ553641 | <i>K. byblisia</i>  |
| HQ600736 | <i>K. caucasica</i> |
| KJ553350 | <i>K. caucasica</i> |
| KJ553351 | <i>K. caucasica</i> |
| KJ553376 | <i>K. caucasica</i> |
| KJ553407 | <i>K. caucasica</i> |
| KJ553419 | <i>K. caucasica</i> |
| KJ553420 | <i>K. caucasica</i> |
| KJ553455 | <i>K. caucasica</i> |
| KJ553557 | <i>K. caucasica</i> |
| KJ553566 | <i>K. caucasica</i> |
| KJ553584 | <i>K. caucasica</i> |
| KJ553633 | <i>K. caucasica</i> |
| KJ553390 | <i>K. ephesi</i>    |
| KJ553459 | <i>K. ephesi</i>    |
| KJ553609 | <i>K. ephesi</i>    |
| KJ553345 | <i>K. mermere</i>   |
| KJ553363 | <i>K. mermere</i>   |
| KJ553410 | <i>K. mermere</i>   |
| KJ553448 | <i>K. mermere</i>   |
| KJ553452 | <i>K. mermere</i>   |
| KJ553480 | <i>K. mermere</i>   |
| KJ553611 | <i>K. mermere</i>   |
| KJ553365 | <i>K. milleri</i>   |
| KJ553395 | <i>K. milleri</i>   |
| KJ553398 | <i>K. milleri</i>   |
| KJ553444 | <i>K. milleri</i>   |
| KJ553465 | <i>K. milleri</i>   |
| KJ553512 | <i>K. milleri</i>   |
| KJ553527 | <i>K. milleri</i>   |
| KJ553530 | <i>K. milleri</i>   |
| KJ553558 | <i>K. milleri</i>   |
| KJ553576 | <i>K. milleri</i>   |
| KJ553591 | <i>K. milleri</i>   |
| KJ553658 | <i>K. milleri</i>   |

**Table 2.** *Continued*

|          |                          |
|----------|--------------------------|
| KJ553378 | <i>K. montenegrina</i>   |
| KJ553384 | <i>K. montenegrina</i>   |
| KJ553417 | <i>K. montenegrina</i>   |
| KJ553451 | <i>K. montenegrina</i>   |
| KJ553543 | <i>K. montenegrina</i>   |
| KJ553352 | <i>K. mrakovcici</i>     |
| KJ553370 | <i>K. mrakovcici</i>     |
| KJ553375 | <i>K. mrakovcici</i>     |
| KJ553514 | <i>K. mrakovcici</i>     |
| KJ553521 | <i>K. mrakovcici</i>     |
| KJ553537 | <i>K. mrakovcici</i>     |
| KJ553555 | <i>K. mrakovcici</i>     |
| KJ553594 | <i>K. mrakovcici</i>     |
| KJ552355 | <i>K. panizzae</i>       |
| KJ553310 | <i>K. panizzae</i>       |
| KJ553342 | <i>K. panizzae</i>       |
| KJ553355 | <i>K. panizzae</i>       |
| KJ553377 | <i>K. panizzae</i>       |
| KJ553400 | <i>K. panizzae</i>       |
| KJ553423 | <i>K. panizzae</i>       |
| KJ553424 | <i>K. panizzae</i>       |
| KJ553437 | <i>K. panizzae</i>       |
| KJ553468 | <i>K. panizzae</i>       |
| KJ553469 | <i>K. panizzae</i>       |
| KJ553491 | <i>K. panizzae</i>       |
| KJ553511 | <i>K. panizzae</i>       |
| KJ553579 | <i>K. panizzae</i>       |
| KJ553613 | <i>K. panizzae</i>       |
| KJ553643 | <i>K. panizzae</i>       |
| KJ553652 | <i>K. panizzae</i>       |
| KJ553466 | <i>K. radovici</i>       |
| KJ553481 | <i>K. radovici</i>       |
| KJ553552 | <i>K. radovici</i>       |
| KJ553599 | <i>K. radovici</i>       |
| KJ553636 | <i>K. radovici</i>       |
| KJ553656 | <i>K. radovici</i>       |
| KJ553439 | <i>K. thessala</i>       |
| KJ553464 | <i>K. thessala</i>       |
| KJ553503 | <i>K. thessala</i>       |
| KJ553809 | <i>N. canestrinii</i>    |
| KJ554046 | <i>N. canestrinii</i>    |
| KJ554069 | <i>N. canestrinii</i>    |
| KJ553666 | <i>N. montenegrensis</i> |
| KJ553703 | <i>N. montenegrensis</i> |
| KJ553759 | <i>N. montenegrensis</i> |
| KJ554044 | <i>N. montenegrensis</i> |
| KJ554070 | <i>N. montenegrensis</i> |
| KJ553751 | <i>O. croaticus</i>      |
| KJ553780 | <i>O. croaticus</i>      |
| KJ553811 | <i>O. croaticus</i>      |
| KJ553834 | <i>O. croaticus</i>      |
| KJ553854 | <i>O. croaticus</i>      |
| KJ554032 | <i>O. croaticus</i>      |
| KJ554053 | <i>O. croaticus</i>      |
| KJ553912 | <i>O. punctatissimus</i> |

Table 2. Continued

|          |                          |
|----------|--------------------------|
| KJ553993 | <i>O. punctatissimus</i> |
| KJ128503 | <i>P. flavescens</i>     |
| KJ128504 | <i>P. flavescens</i>     |
| KM077830 | <i>P. flavescens</i>     |
| KM077831 | <i>P. flavescens</i>     |
| KM077832 | <i>P. flavescens</i>     |
| KM077833 | <i>P. flavescens</i>     |
| KM077834 | <i>P. flavescens</i>     |
| KM077835 | <i>P. flavescens</i>     |
| JQ775029 | <i>P. lozanoi</i>        |
| JQ775030 | <i>P. lozanoi</i>        |
| JQ775031 | <i>P. lozanoi</i>        |
| JQ775032 | <i>P. lozanoi</i>        |
| JQ775033 | <i>P. lozanoi</i>        |
| KM077847 | <i>P. lozanoi</i>        |
| KM077848 | <i>P. lozanoi</i>        |
| KM077849 | <i>P. lozanoi</i>        |
| KJ554258 | <i>P. marmoratus</i>     |
| KJ554272 | <i>P. marmoratus</i>     |
| KJ554281 | <i>P. marmoratus</i>     |
| KJ554294 | <i>P. marmoratus</i>     |
| KJ554300 | <i>P. marmoratus</i>     |
| KJ554461 | <i>P. marmoratus</i>     |
| KJ709583 | <i>P. marmoratus</i>     |
| KJ709584 | <i>P. marmoratus</i>     |
| KJ128585 | <i>P. microps</i>        |
| KJ128586 | <i>P. microps</i>        |
| KJ554119 | <i>P. microps</i>        |
| KJ554147 | <i>P. microps</i>        |
| KJ554336 | <i>P. microps</i>        |
| KJ554517 | <i>P. microps</i>        |
| KJ768285 | <i>P. microps</i>        |
| KJ768286 | <i>P. microps</i>        |
| KJ768287 | <i>P. microps</i>        |
| KM077850 | <i>P. microps</i>        |
| KM077851 | <i>P. microps</i>        |
| KM077852 | <i>P. microps</i>        |
| KM077853 | <i>P. microps</i>        |
| KM077854 | <i>P. microps</i>        |
| KM077855 | <i>P. microps</i>        |
| KM077856 | <i>P. microps</i>        |
| KJ128587 | <i>P. minutus</i>        |
| KJ128588 | <i>P. minutus</i>        |
| KM077857 | <i>P. minutus</i>        |
| KM077858 | <i>P. minutus</i>        |
| KM077859 | <i>P. minutus</i>        |
| KM077860 | <i>P. minutus</i>        |
| KM077861 | <i>P. minutus</i>        |
| KM077862 | <i>P. minutus</i>        |
| KM077863 | <i>P. minutus</i>        |
| KM077864 | <i>P. minutus</i>        |
| KM077865 | <i>P. minutus</i>        |
| KM077866 | <i>P. minutus</i>        |
| KJ128589 | <i>P. norvegicus</i>     |
| KJ128590 | <i>P. norvegicus</i>     |

Table 2. Continued

|          |                      |
|----------|----------------------|
| KM077867 | <i>P. norvegicus</i> |
| KM077868 | <i>P. norvegicus</i> |
| KM077869 | <i>P. norvegicus</i> |
| KM077870 | <i>P. norvegicus</i> |
| KM077871 | <i>P. norvegicus</i> |
| KM077872 | <i>P. norvegicus</i> |
| KM077873 | <i>P. norvegicus</i> |
| KM077874 | <i>P. norvegicus</i> |
| KJ128591 | <i>P. pictus</i>     |
| KM077875 | <i>P. pictus</i>     |
| KM077876 | <i>P. pictus</i>     |
| KM077877 | <i>P. pictus</i>     |
| KM077878 | <i>P. pictus</i>     |
| FJ751920 | <i>P. tortonesi</i>  |
| FJ751921 | <i>P. tortonesi</i>  |
| FJ751922 | <i>P. tortonesi</i>  |
| KJ709585 | <i>P. tortonesi</i>  |
| KJ709586 | <i>P. tortonesi</i>  |

have placed it outside *Pomatoschistus* and suggested a closer relationship to *Knipowitschia*, specifically *K. panizzae* (Penzo *et al.*, 1988; Huyse *et al.*, 2004; Vanhove *et al.*, 2012).

#### PHYLOGENETIC ANALYSIS AND CALIBRATION

We assembled the data matrix using Geneious (Biomatters, Ltd) version 10.1.3, aligned the sequences based on the amino acid translation, and adjusted and trimmed the alignment by eye. We used MrBayes version 2.0.9 (Geneious plugin) to infer phylogeny, specifying a general time reversible, with gamma-distributed rate variation and invariant sites (GTR+G+I) substitution model, as chosen by the R module *phangorn* (Schliep, 2011). We ran the analysis for  $10.0 \times 10^7$  generations, with four simultaneous chains, sampling every 1000 replications, discarding the first 10% of trees as burn-in and then constructing a 50% majority-rule consensus phylogeny of the remaining trees. In order to calibrate the phylogeny, we reduced the data set to two exemplars of each sand goby species (three for *P. marmoratus*, in order to represent the geographical range) to avoid loss of computational efficiency as a result of the intraspecific polytomies. We inferred a Bayesian tree as outlined above and performed the calibration analysis on a matrix of 49 individuals (including the six outgroup exemplars) with Beast 1.7.5 (Drummond *et al.*, 2012), run with an uncorrelated lognormal relaxed clock model and a birth/death speciation prior. We assigned a calibration of 48.7 Mya, applied as a normal prior, to the node subtending the family Gobionellidae, including *Mugilogobius* in addition to the sand gobies. This calibration was derived from the analysis of Thacker (2015). We also applied calibrations to each of the genera *Economidichthys*, *Pomatoschistus* and

*Knipowitschia*. Dates for the origins of these sand goby genera were derived from Schwarzahns *et al.* (2017). We applied the ages of the earliest known *Economidichthys* (15.0 Mya), *Pomatoschistus* (16.0 Mya) and *Knipowitschia* (13.0 Mya) as exponential priors. We ran the Beast analysis for  $10.0 \times 10^7$  generations, with trees sampled every 1000 generations, and discarded the first 10% as burn-in. At the end of the analyses, estimated effective sample sizes (ESS) for all parameters well exceeded 200, and we constructed maximum clade credibility consensus tree using TreeAnnotator 1.7.5 (Drummond *et al.*, 2012) and visualized this tree using FigTree 1.3.1 (Rambaut, 1999).

## RESULTS

The Bayesian hypothesis of relationships is shown in Figure 2. The tree is rooted with *Gobius* and *Zosterisessor* (*Gobius* lineage, family Gobiidae) as distal outgroups, with representatives of *Mugilogobius* (*Mugilogobius* lineage, family Gobionellidae) serving as the more proximal outgroup. In total, the hypothesis includes a novel sequence for 83 sand goby individuals and six outgroup individuals (five species), combined with 182 *COI* barcode sequences from GenBank. In addition to the five outgroup species, the hypothesis includes all species of *Ninnigobius*, *Orsinigobius* and *Economidichthys* (two each) plus eight *Pomatoschistus* (including *Pomatoschistus flavescens*). Within *Knipowitschia*, eight species-level clades are resolved, but they contain 12 species names. This is the most comprehensive sand goby analysis to date, and the first to include a gobionellid outgroup, in keeping with the placement of sand gobies in Gobionellidae rather than Gobiidae (Thacker & Roje, 2011; Thacker, 2013).

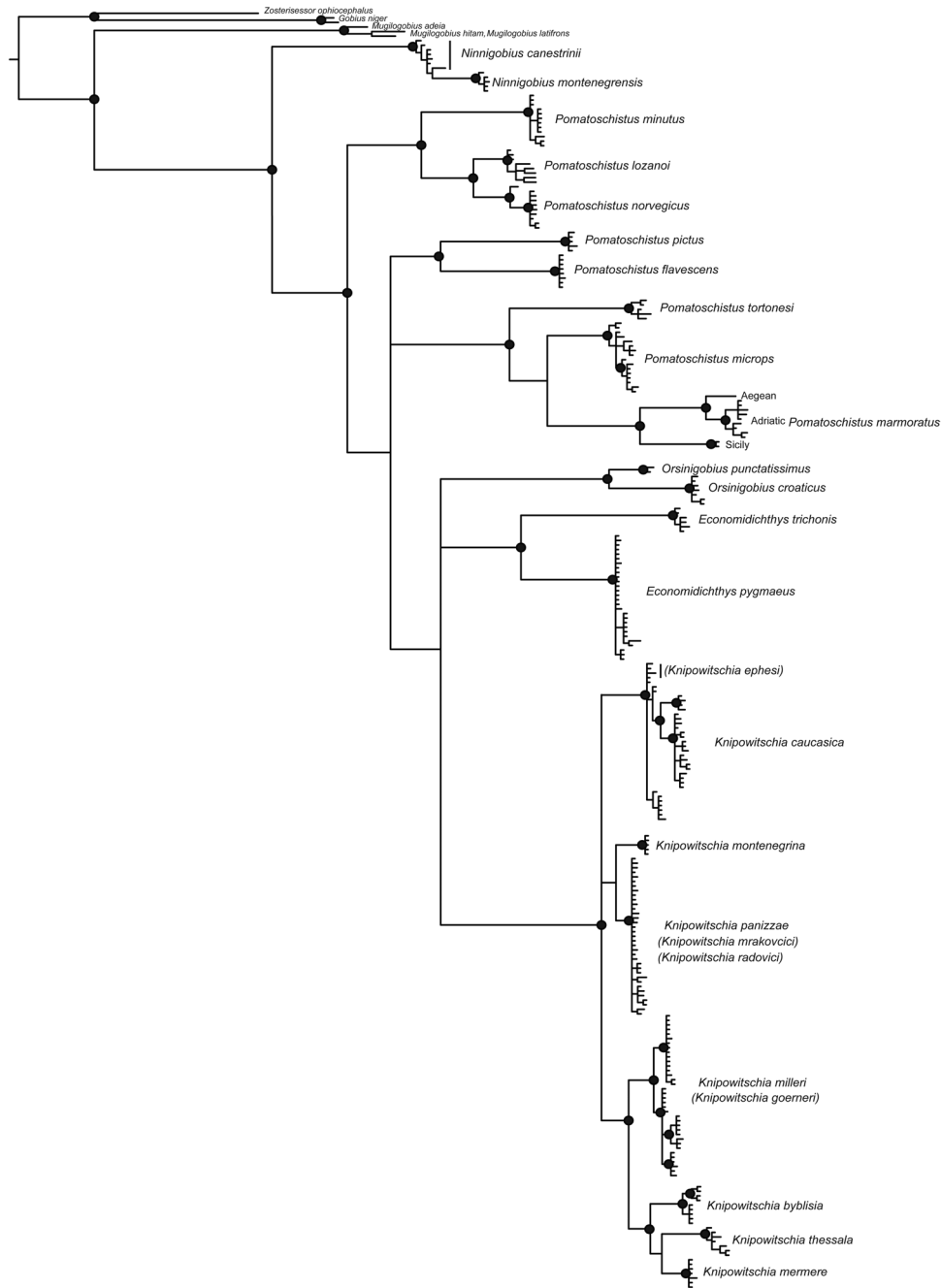
Across the topology, posterior support values are weakest at the intergeneric nodes, but much stronger for individual species and smaller species groups. Support for the monophyly of sand gobies is strong, at 99%. Within sand gobies, the deepest split is between *Ninnigobius* and the remainder of the genera. Miller & Šanda (2008), in their description of *N. montenegrensis*, discuss the differences between that species and *N. canestrinii*, relative to *Pomatoschistus*; *Ninnigobius* species have reductions in head pores and scalation, a unique spotted pigment pattern and a freshwater habitat, rather than marine. They concluded that the only unique character among those was the colour pattern, which did not in itself justify a separation of those two species into a separate genus. Our analysis concurs with Geiger *et al.* (2014) in supporting the recognition of *Ninnigobius* based on molecular data. Our hypothesis differs in recovering *N. canestrinii* as paraphyletic with respect to *N. montenegrensis*. Although the *N. montenegrensis* individuals are distinct, the

internodes among *N. canestrinii* are short and may simply be poorly resolved.

Outside *Ninnigobius*, the genus *Pomatoschistus* is resolved as three lineages that are sequential sister taxa to a clade including *Knipowitschia*, *Economidichthys* and *Orsinigobius*. The lineages include *Pomatoschistus pictus* (Malm, 1865) and *P. flavescens*; the ‘*Pomatoschistus minutus* complex’ consisting of *P. minutus* (Pallas, 1770), *Pomatoschistus norvegicus* (Collett, 1902) and *Pomatoschistus lozanoi* (deBuen, 1923); and a clade including *Pomatoschistus tortonesi* Miller, 1969, *P. marmoratus* (Risso, 1810) and *Pomatoschistus microps* (Kroyer, 1838). Support for each of these three clades is high, at 99%, but their placement relative to each other, and to the other sand goby taxa, is poorly resolved. These same three clades (less *P. tortonesi* and *P. marmoratus*) were also recovered by Kneibelsberger & Thiel (2014), with similar weak support for their interrelationships. The clades reflect biogeographical and ecological patterns; most *Pomatoschistus* are distributed in the Northeastern Atlantic and are nearshore but exclusively marine (*P. minutus* in the Adriatic, which we did not sample, is genetically distinct and might represent a new species; Stefanni & Thorley, 2003; Gysels *et al.*, 2004). *Pomatoschistus microps*, *P. marmoratus* and *P. tortonesi* are tolerant of lower salinities, inhabiting estuaries and marshes, and range into both the Mediterranean and the Black Sea.

In a separate clade from the *Pomatoschistus* lineages, the genera *Knipowitschia*, *Orsinigobius* and *Economidichthys* are resolved as a polytomy, but support for the monophyly of each genus is strong (99%). *Orsinigobius* includes two species (*O. punctatissimus* and *O. croaticus*) known from Adriatic freshwater drainages of Italy and Croatia that have been separated from *Knipowitschia* owing to their reductions in head pores. Another species, *K. milleri*, known from the Acheron river in western Greece, was originally described in *Orsinigobius* (Ahnelt & Bianco, 1990), but later studies (Miller, 2004; Kottelat & Freyhof, 2007; Freyhof, 2011) included it in *Knipowitschia*, a placement that we confirm. The two *Economidichthys* species (*E. pygmaeus* and *E. trichonis*) from freshwaters in western Greece are grouped together, as are all of the *Knipowitschia*.

Within *Knipowitschia*, we recover three lineages. The first contains *K. caucasica*, sampled from eastern Greece and Turkey and including four individuals classified as *Knipowitschia ephesi* Ahnelt, 1995, a species endemic to a brackish coastal lake near Marmara, on the Aegean coast of Turkey. There is quite a bit of structure within *K. caucasica*, and it is possible that there are cryptic or unidentified species within its wide range and that further sampling of *K. ephesi* will resolve that taxon as distinct. Our sampling did not include *K. caucasica* from the Adriatic, so we were



**Figure 2.** Bayesian phylogenetic hypothesis of sand gobies, including 265 individuals plus six outgroups. Black circles at nodes indicate 95–100% posterior probability, and localities of *Pomatoschistus marmoratus* individuals are indicated. *Ninnigobius canestrinii* is paraphyletic with respect to *Ninnigobius montenegrensis*, and *Pomatoschistus* is paraphyletic in this hypothesis, although support values along the phylogenetic backbone are not strong. Individuals identified as *Knipowitschia ephesi* are indicated; they are recovered among *Knipowitschia caucasica*, although there is structure within *K. caucasica* that might warrant recognition as distinct species. Within *Knipowitschia panizzae* are individuals identified as *Knipowitschia mrakovcici* and *Knipowitschia radovici*, and here there is little intraspecific structure. Individuals identified as *Knipowitschia goerneri* are included within *Knipowitschia milleri*. Two clades are evident within *Knipowitschia byblisia*, one of which might correspond to the sympatric *Knipowitschia caunosii*.



unable to determine whether or not that population is a distinct species, as suggested by Economidis & Miller (1990) and Kovačić & Pallaoro (2003). A second clade resolves *Knipowitschia montenegrina* Kovačić & Sanda, 2008 as sister to a large assemblage with little phylogenetic structure, consisting of individuals assigned to *Knipowitschia mrakovcici* Miller, 2009, *Knipowitschia radovici* Kovačić, 2005 and *K. panizzae*; all those are known from the Adriatic drainages of Croatia and Montenegro. Finally, a third clade includes three clades, one of which contains individuals from that same lake, classified as *Knipowitschia byblisia* Ahnelt, 2011, but including two subgroups that may coincide with *K. byblisia* and similar endemic species, *K. caunosi* [those sequences derived from the study of Geiger *et al.* (2014) and are vouchered in the collection of Jörg Freyhof; we did not examine them]. That clade is sister to two other species, *K. thessala* from Greece and *Knipowitschia mermere* Ahnelt, 1995 from the same Turkish lake, and that clade is sister to *K. milleri*, also from Greece. Within the *K. milleri* clade were included several individuals provisionally classified as *Knipowitschia goerneri* (Ahnelt, 1991), a species from the Ionian coast of Greece, including Corfu Island. These individuals were mixed among specimens of *K. milleri*; therefore, we consider *K. goerneri* a synonym.

The calibrated Bayesian phylogeny is shown in Figure 3. Although the sampling is much reduced, the species-level topology is equivalent to the complete analysis, except that *Pomatoschistus* is recovered as monophyletic (although poorly supported). In the calibrated analysis, Beast has also resolved the polytomy among *Knipowitschia*, *Orsinigobius* and *Economidichthys*. We infer a crown age of 32.6 Mya (95% highest posterior density range 24.9–41.5 Mya) for the sand gobies, slightly after the Eocene/Oligocene boundary. The stem ages of genera (except *Ninnigobius* at 32.6 Mya) range from 22.3 to 26.3 Mya in the late Oligocene/early Miocene, a narrow span that is consistent with the weak resolution along that phylogenetic backbone and, possibly, with a rapid radiation of genera. Given the uncertainty among those nodes, the dates (particularly the sequence of divergences) should be interpreted cautiously.

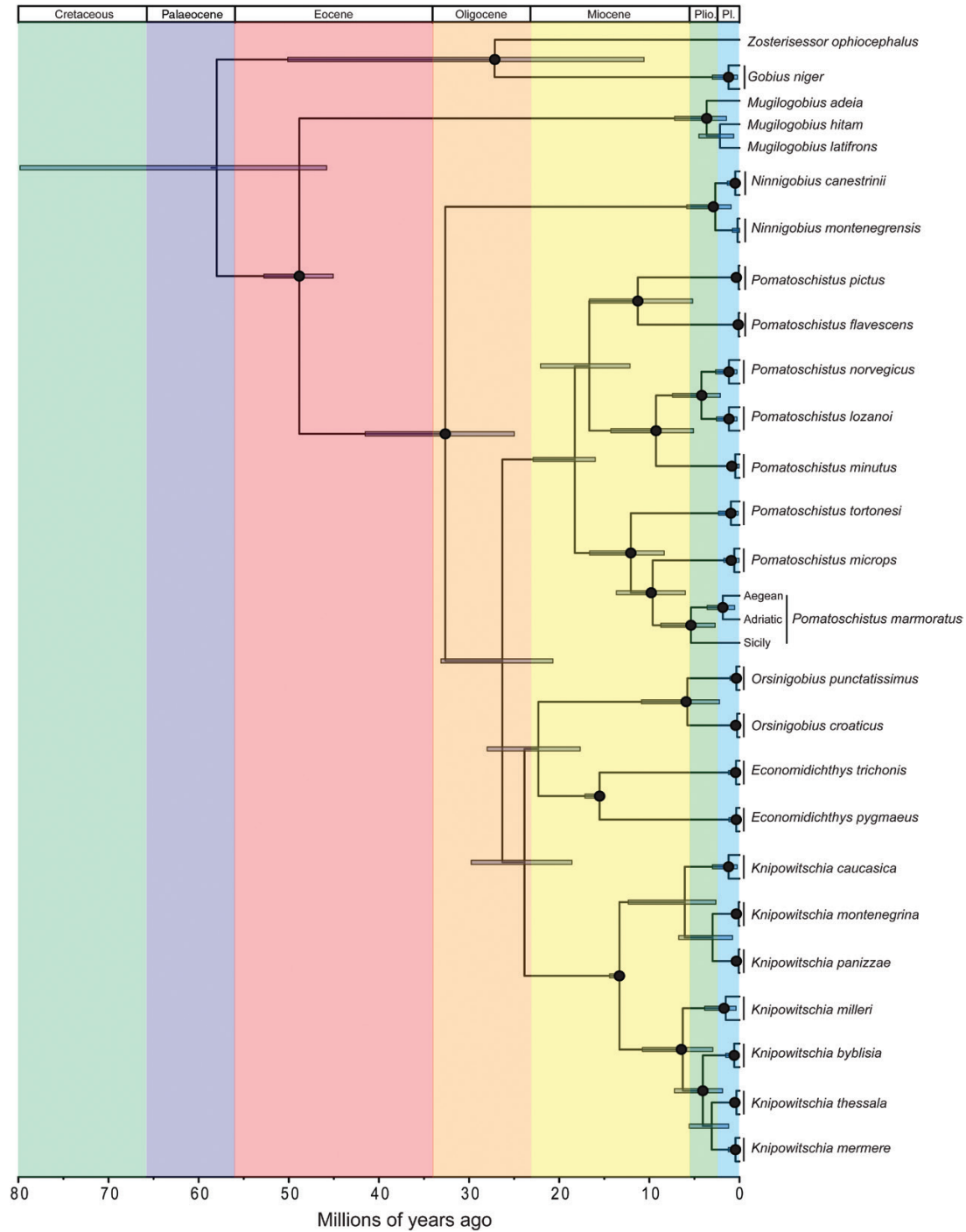
The genera and subgeneric lineages are all well supported, at 100% (except *Pomatoschistus*). Inferred crown ages and confidence intervals for sand goby genera and subgroups are given in Table 3, and Kimura two parameter (K2P) distances among taxa are provided in the Supporting Information (Table S1). The youngest ages (2.7–5.8 Mya) are in the clades distributed in the Adriatic, including *Ninnigobius*, *Orsinigobius* and the *K. panizzae*/*K. montenegrina* lineage. Although the stem lineages of those clades are much older, the crown age indicates the period of most recent diversification.

## DISCUSSION

Our phylogeny incorporates and unites data for sand gobies from across their geographical range in the northeastern Atlantic, Mediterranean, Balkanian and Ponto-Caspian regions. This is the most comprehensive phylogenetic hypothesis yet proposed for sand gobies, and it provides clarification of species and genus boundaries that, in turn, informs the taxonomy. We confirm that the smaller genera *Ninnigobius* and *Orsinigobius* are distinct, that the Atlantic species formerly distinguished as *G. flavescens* is part of *Pomatoschistus*, and that *Orsinigobius*, *Economidichthys* and *Knipowitschia* form a clade. Within *Knipowitschia*, we show that one of the species from the lake near Marmara (*K. ephesi*) is nested within *K. caucasica*, but recover the other lake species as distinct, including two clades that correspond to *K. byblisia* and, possibly, also *K. caunosi*, sympatric endemics. In contrast, and as first determined by Geiger *et al.* (2014), we show that *K. mrakovcici*, *K. radovici* and *K. panizzae* are not distinct; therefore, the older name, *K. panizzae*, should be used for this entire group. We also confirm that *K. goerneri* from western Greece is included within *K. milleri*. This comprehensive analysis clarifies sand goby relationships and allows assessment of the current taxonomy. Relationships of species within both *Pomatoschistus* and *Knipowitschia* also agree well (where they overlap) with the earlier studies of Huyse *et al.* (2004), Kneibelsberger & Thiel (2014) and Vukić *et al.* (2016).

In the context of our phylogeny, we can interpret the geographical distribution of sand goby lineages. The most obvious distinction is between the generally Atlantic/Western Mediterranean marine species of *Pomatoschistus* and the euryhaline- to freshwater-dwelling *Knipowitschia* in the Eastern Mediterranean, Black and Caspian seas. These genera both include a few wide-ranging generalists in addition to a suite of species with more restricted distributions. The smaller genera *Ninnigobius*, *Orsinigobius* and *Economidichthys* are all restricted to drainages of the Adriatic and Ionian seas, and some lineages of *Knipowitschia* and populations of *Pomatoschistus* also inhabit that area, making it the centre of diversity for sand gobies, with a total of 17 species.

The Mediterranean basin has undergone a series of complex hydrographic and geological changes over the past 20 Myr. The terminal Tethyan Event, the collision of Africa with Asia that separated the contiguous Tethys seaway, took place gradually in the Miocene, 12–18 Mya. This event isolated the goby lineages in the Mediterranean and Atlantic from those in the tropical Indian Ocean (Thacker, 2015); what is now the Mediterranean is a remnant of the western Tethys sea. To the north and east of the Alps, encompassing the Black and Caspian Sea basins, was an



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**Figure 3.** Phylogeny of sand gobies, based on a reduced data set of 43 individuals plus outgroups. The hypothesis is calibrated with a legacy date of 48.7 Mya at the base of Gobionellidae (from Thacker, 2015), and fossil calibrations for the origins of *Economidichthys* (15.0 Mya), *Pomatoschistus* (16.0 Mya) and *Knipowitschia* (13.0 Mya) derived from Schwarzahns *et al.* (2017). Error bars indicate 95% highest posterior density, and black circles at nodes indicate 95–100% posterior probability. Localities of different *Pomatoschistus marmoratus* individuals are indicated.

inland sea known as the Paratethys throughout the Miocene. The Paratethys was isolated over most of the period of Tethys closure, except for occasional marine

transgressions (Orszag-Sperber, 2006; Schwarzahns *et al.*, 2017). It was brackish to freshwater habitat, owing to the inflow of several large rivers, with

**Table 3.** Inferred crown ages (in millions of years ago) with 95% confidence intervals (in parentheses), and geographical ranges and habitat for sand goby clades

| Clade                                                                                 | Age (Mya)        | Range                                                         |
|---------------------------------------------------------------------------------------|------------------|---------------------------------------------------------------|
| Sand goby clade                                                                       | 32.6 (24.9–41.5) | Europe seas and rivers                                        |
| <i>Ninnigobius</i>                                                                    | 2.7 (0.9–5.8)    | Adriatic drainages, FW                                        |
| <i>Pomatoschistus</i>                                                                 | 18.3 (16.0–22.9) | NE Atlantic, Western Mediterranean, Adriatic marine           |
| <i>P. minutus</i> / <i>P. lozanoi</i> / <i>P. norvegicus</i> clade                    | 9.3 (5.1–14.3)   | NE Atlantic, North Sea, Baltic Sea, marine                    |
| <i>P. pictus</i> / <i>P. flavescens</i> clade                                         | 11.3 (5.2–16.7)  | NE Atlantic, North Sea, Baltic Sea, marine                    |
| <i>P. tortonesi</i> / <i>P. marmoratus</i> / <i>P. microps</i> clade                  | 12.1 (8.3–16.6)  | NE Atlantic, Western Mediterranean, Adriatic, marine          |
| <i>Knipowitschia</i>                                                                  | 13.3 (13.0–14.4) | Eastern Mediterranean, Adriatic, Black, Caspian, FW, brackish |
| <i>K. panizae</i> / <i>K. montenegrina</i> clade                                      | 3.0 (0.8–6.7)    | Adriatic drainages, FW                                        |
| <i>K. milleri</i> / <i>K. thessala</i> / <i>K. mermere</i> / <i>K. byblisia</i> clade | 6.3 (3.0–10.8)   | Eastern Mediterranean, Adriatic, Black, Caspian, FW, brackish |
| <i>Orsinigobius</i>                                                                   | 5.8 (0.9–5.8)    | Adriatic drainages, FW                                        |
| <i>Economidichthys</i>                                                                | 15.5 (15.0–17.1) | Greece (Ionian Sea drainages), FW                             |

Abbreviations: FW, freshwater; NE, northeastern.

particularly acute reductions in salinity after its final separation from the Tethys at ~12 Mya (Rögl, 1998, 1999b).

During the Pliocene and Pleistocene, the Paratethys gradually shrank to its current remnants: the Black, Caspian and Aral seas. In the same period, between 5 and 6 Mya, the strait of Gibraltar closed, resulting in the gradual drying of the Mediterranean known as the Messinian Salinity Crisis (MSC; Hallam, 1994; Krijgsman *et al.*, 1999; Duggen *et al.*, 2003; Orszag-Sperber, 2006). Various hypotheses on goby speciation in the Mediterranean region (Penzo *et al.*, 1998; Stefanni & Thorley, 2003; Huyse *et al.*, 2004; Vanhove *et al.*, 2012) have been put forward, including different interpretations of the role of tectonic and sea-level changes during the MSC vs. those caused by Pleistocene glaciation cycles. The MSC is characterized by a drastic drop in sea level and the establishment of brackish lakes throughout the Mediterranean region, a period known as the Lago Mare phase (Penzo *et al.*, 1998; Huyse *et al.*, 2004). One possibility is that sand gobies evolved in these fresh and brackish environments and then radiated during subsequent phases characterized by higher sea levels, reflooding and salinity increases (Huyse *et al.*, 2004; Malavasi *et al.*, 2012). It is also possible that much later, during the Pleistocene, a series of glaciations and consequent additional fluctuations in sea levels triggered population subdivision and bursts of speciation (Stefanni & Thorley, 2003). Either or both (or neither) of these periods of tectonic change may have facilitated diversification among sand gobies, but distinguishing among those theories requires a time scale for sand goby diversification. A calibrated phylogenetic study of European Cyprinidae, a group also thought to have diversified in concert with the

Lago Mare phase, showed instead that their diversification occurred as early as the Oligocene, well predating the Miocene and Pliocene events of the MSC (Levy, Doadrio & Almada, 2009).

Previous phylogenetic works on sand gobies have used molecular clocks, with or without a biogeographical calibration, as a source of evolutionary timing estimates for sand gobies. Huyse *et al.* (2004) used mitochondrial ribosomal genes to infer sand goby phylogeny, which showed a rapid divergence (short internodes) among genera along the backbone of the tree. They calibrated this divergence with a biogeographical date of 5.6 Mya, on the assumption that the diversification of genera, and the adoption of freshwater lifestyle in *Economidichthys*, *Ninnigobius* and *Orsinigobius*, corresponded to the Lago Mare phase of the MSC. Their resultant clock estimate of 1.4% pairwise divergence (0.7% lineagewise divergence) per million years is comparable to mitochondrial divergence rates in other vertebrates. They then further postulated that more recent species diversification within *Pomatoschistus* (in particular, the *P. minutus* complex) was related to Pleistocene glaciation between 1.94 and 1.18 Mya that constrained and fragmented the ranges available to species in the northeastern Atlantic. Populations of *Pomatoschistus* in the Adriatic would also have been isolated by sea-level regression in the Mediterranean at this time. In contrast, Penzo *et al.* (1998), also based on mitochondrial ribosomal genes, included four sand goby taxa in a larger phylogeny of Mediterranean gobies and placed the diversification of sand gobies at either 2.6 or 12.4 Mya, as calibrated by alternate transversion-only clocks. The older date is derived from a 0.14% lineagewise transversion change per million year vertebrate clock estimate, and

the younger from assuming that the split between sand gobies and the more distantly related genera *Gobius*, *Zosterisessor* and *Padogobius* was related to the Lago Mare phase. Vanhove *et al.* (2012), also using mitochondrial ribosomal genes, examined the eastern Mediterranean lineages and applied two clock calibrations, 0.5 and 1.5% lineage-wise divergence per million years. These calibrations yield upper and lower bounds on the origin of sand gobies at 2.9 and 9.0 Mya, and diversification within the genera at 2.0–6.0 Mya. These estimates correlate well with the Lago Mare phase at 5.9 Mya, followed by a diversification within genera somewhat later, concordant with Pleistocene glaciation.

Our calibrated phylogeny (Fig. 3; ages of major clades given in Table 3) uses fossil calibrations (rather than a molecular clock or biogeographical calibration) and indicates that crown ages for sand goby genera are much older than previous estimates, ranging from the mid-Miocene (*Pomatoschistus* at 18.3 Mya; *Economidichthys* at 15.5 Mya; *Knipowitschia* at 13.3 Mya) to the late Miocene/early Pliocene (*Orsinogobius* at 5.8 Mya; *Ninnigobius* at 2.7 Mya). These crown radiations in freshwater clades (*Ninnigobius*, *Orsinogobius* and the two clades within *Knipowitschia*) are generally younger than the marine radiations (*Pomatoschistus* clades), although the comparatively old *Economidichthys* is an exception (Table 3). The older, mid-Miocene radiations are correlated with the closure of the Tethys seaway, the isolation and decreasing salinity in the Paratethys, and the establishment of cooler environments in the Mediterranean after its separation from the Indian Ocean (Rögl, 1998, 1999b); the origin of *Economidichthys* occurred at roughly the same time as the emergence of Greece owing to sea-level regression (Rögl, 1999a). *Pomatoschistus*, *Economidichthys* and *Knipowitschia* had become established long before the MSC, and their diversification is more likely to be related to the isolation and fluctuations in the Tethys and Paratethys.

In contrast, the younger (late Miocene/early Pliocene) diversification events in the freshwater sand goby lineages *Ninnigobius*, *Orsinogobius* and the two *Knipowitschia* clades all occurred exclusively or partly in the Adriatic region, and all occurred during the MSC, a period of dramatically reduced aquatic habitats and overall cooler temperatures in the Mediterranean region (Rögl, 1998, 1999b). The eastern Mediterranean would have been reduced to brackish lakes (Lago Mare), with the Adriatic exposed except for rivers draining from the Balkans and the Alps. Marine species that could persist in the Atlantic would potentially reoccupy the Mediterranean after the MSC, but freshwater species would be isolated and fragmented, probably promoting speciation. *Orsinogobius*, *Ninnigobius*

and *Knipowitschia* clades have widely varying stem ages (*Ninnigobius* is the oldest among sand gobies, dating back to the early Oligocene), but all experienced their crown diversification around the time of the MSC.

By the Pleistocene, all the sand goby species were established, and only diversification within species was occurring. Structure within species is generally shallow, with the exception of some unusually deep structure within *P. marmoratus*. In that species, the individuals sampled from Sicily are distinct from another clade including one individual from the Aegean coast of Greece and a large Adriatic clade from the Venetian Lagoon, the Po River drainage and the Cetina River drainage in Croatia (similar patterns were recovered in *P. marmoratus* by Mejri *et al.*, 2011 and Vanhove *et al.*, 2012, and in *P. minutus* by Stefanni & Thorley, 2003 and Gysels *et al.*, 2004). This Pliocene and Pleistocene diversification could be old enough to be related to the MSC and could even indicate the presence of cryptic species within *P. marmoratus*. Neither intraspecific nor interspecific differentiation in sand gobies is young enough to be correlated with Pleistocene glaciation-induced changes in sea level. Rather, we correlate episodes of diversification among sand gobies with both the Miocene Tethys closure/Paratethys isolation and the diminution of the Mediterranean during the Miocene/Pliocene MSC. We show that the sand gobies have occupied European waters for much longer than previously assumed and that they have diversified in concert with major tectonic events in the region.

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## SUPPORTING INFORMATION

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**Table S1.** Kimura two parameter genetic distances among sand goby taxa used in BEAST analysis