Quaternary climatic cycles promoted (re)colonization and diversification events in Adriatic sand gobies

Running title: Adriatic sand goby phylogeny

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Abstract

The Adriatic Sea and Adriatic Sea drainage system (or Adriatic region) is characterized by high levels of species richness. This especially applies to endemic fishes, as exemplified by the sand gobies. However, the diversity of this group is probably still underestimated. Several keys for gobiid identification, notably of the Adriatic region, have been proposed on the basis of morphological characters, but difficulties persist in species identification. Molecular phylogenies support the monophyly of the sand gobies, but relationships between genera and species remain poorly resolved. In this work, the diversity and evolutionary history of the Adriatic sand gobies (*Pomatoschistus* and *Knipowitschia*) were investigated by applying phylogenetic reconstructions, species delimitation tests and molecular dating based on mitochondrial *cytochrome b* and nuclear *rhodopsin 1* genes. The concatenation of both genes allowed, for the first time, the creation of a highly resolved phylogeny at the interspecific level. The generic diversity within the sand gobies is probably considerably higher than formerly assumed. Our results highlight cryptic diversity, and suggest that new species remain to be described within *Knipowitschia* and *Pomatoschistus*. Most of all, our results emphasized the urgent need of a taxonomic revision of the sand gobies. Several past events were at the origin of sand goby diversification in the Adriatic region. It seems that glacial periods promoted the intraspecific diversification of the endemic species, whereas interglacial periods promoted the (re)colonization of the Adriatic Sea by more widespread species.

1. INTRODUCTION

The Mediterranean Sea is considered a hotspot of biodiversity (e.g., Bianchi et al., 2012; Coll et al., 2010; Cuttelod, García, Malak, Temple, & Katariya, 2009; Médail & Quézel, 1999; Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000). While it represents only 0.82% and 0.32% of the global oceanic surface area and volume, respectively (Bianchi & Morri, 2000), it hosts at least 7% ($\approx 17,000$ species) of the known global marine biodiversity (Coll et al., 2010, 2012), and is characterized by a high level of endemism (Bianchi et al., 2012; Coll et al., 2010). This biodiversity was fostered by the complex and dynamic geological history of the area, from the closure of the Tethys via the Messinian Salinity Crisis (MSC), to the Last Pleistocene Glaciation (Bianchi & Morri, 2000; Bianchi et al., 2012). These past events separated the Mediterranean Sea into several biogeographic regions, including the Adriatic bioregion (Bianchi & Morri, 2000; Coll et al., 2010; Hattab et al., 2015). The Adriatic Sea is a semi-enclosed shallow sea connected to the Ionian Sea via the Strait of Otranto. This narrow connection with the rest of the Mediterranean Sea favours isolation, which may help explain the high levels of species richness, especially for endemic fishes (Coll et al., 2010, 2012). Unfortunately, the Adriatic Sea is also one of the most impacted by human activities (pollution, eutrophication, habitat degradation, and exploitation of marine resources, and climate change) on its coastal areas and shelves (Ben Rais Lasram & Mouillot, 2009; Coll, Santojanni, Palomera, Tudela, & Arneri, 2007; Ben Rais Lasram, Guilhaumon, Albouy, Somot, Thuiller, & Mouillot, 2010; Coll et al., 2012; Lotze, Coll, & Dunne, 2011).

The most diversified group of fish in the Adriatic region is the family Gobiidae (Gobiidae+Gobionellidae sensu Thacker, 2009). The recognition of gobiid fishes in this region started in the middle of the 19th century with the description of four species (Steindachner, 1861, 1863, 1870; Verga, 1841), and 52 species occupying marine, brackish and freshwater habitats have so far been identified (Kottelat & Freyhof, 2007; Kovačić & Šanda, 2007; Kovačić, Lipej, & Dulčić, 2020; Kovačić, Šanda, Kirinčić, & Zanella 2012; Lipej & Dulčić, 2004; Miller, 2009; Miller & Šanda, 2008). The level of endemism is also high in the Adriatic gobiids, especially within the sand gobies (sensu McKay & Miller, 1997: *Pomatoschistus*, *Knipowitschia*, *Gobiusculus*, and *Economidichthys*). Among the 35 recognized species, 14 live in the Adriatic region (genera *Pomatoschistus* Gill, 1863 and *Knipowitschia* Iljin, 1927), and at least eight are endemic (Table 1; Froese & Pauly, 2019). This diversity is probably underestimated, especially in light of recent new descriptions (e.g., Kovačić, 2005a; Kovačić & Šanda, 2007; Miller, 2009; Miller & Šanda, 2008).

Unfortunately, Adriatic sand gobies are under cumulative threats (Coll et al., 2012; Franco, Fiorin, Franzoi, & Torricelli, 2005; Miller, 1990; Smith & Darwall, 2006; Spinelli, De Matteo, Costagliola, Giacobbe, & Kovačić, 2017). Several keys for gobiid identification, notably gobiids of the Adriatic Sea, have been proposed on the basis of morphological characters, but difficulties persist in the identification of some species (Miller, 1986; Kovačić, 2008). The molecular phylogeny of the sand gobies has been partially investigated (Agorreta et al., 2013; Huyse, Van Houdt, & Volckaert, 2004; Thacker, Gkenas, Triantaphyllidis, Malavasi, & Leonardos, 2019; Vanhove et al., 2012), and has supported monophyly. However, relationships between genera and species are poorly resolved, and based on mitochondrial markers, the monophyly of both *Pomatoschistus* and *Knipowitschia* (Geiger et al., 2014; Huyse et al., 2004; Penzo, Gandolfi, Bargelloni, Colombo, & Patarnello, 1998; Thacker et al., 2019) is doubted.

A sound assessment of species diversity and speciation processes is important for conservation issues, notably for threatened fishes playing a major role in the food web, such as sand gobies (Lafaille, Feunteun, & Lefeuvre, 1999; Pasquaud, Girardin, & Elie, 2004; Pockberger, Kellnreitner, Ahnelt, Asmus, & Asmus, 2014; Souza, Dias, Marques, Antunes, & Martins, 2014). Here, the diversity and evolutionary history of the Adriatic sand gobies (*Pomatoschistus* and *Knipowitschia*) were investigated by applying phylogenetic reconstructions, species delimitation tests, and molecular dating, and based on mitochondrial *cytochrome b* (*cytb*) and nuclear *rhodopsin 1* (*rh1*) genes. Particular attention was paid to interspecific molecular phylogeny to address the following questions: Are the Adriatic endemic sand gobies monophyletic? Is there cryptic diversity? Which past events did influence their diversification?

2. MATERIAL AND METHODS

2.1 Sampling and DNA analyses

The gobies were either caught by professional fishermen with fishing nets (no ethical approval required), or collected during scuba diving by hand net or electrofished in freshwaters (sampling permits provided by governmental or competent authorities, and available upon request).

Tissue samples of sand gobies (genera *Pomatoschistus* and *Knipowitschia*) were taken from 62 specimens corresponding to 19 morphologically identified species (or morphospecies; Cronquist, 1978) (Table 1). Most of the studied species are either endemic to

the Adriatic region or their range includes the Adriatic Sea (Figure 1). Following the phylogeny of Agorreta et al. (2013) based on mitochondrial and nuclear markers, this dataset was complemented by two closely related species, *Gobiusculus flavescens* (Fabricius, 1779) and *Pseudaphya ferreri* (de Buen & Fage, 1908), which was used as an outgroup (Table 1).

DNA extractions were performed from 96% ethanol-preserved tissue samples using standard procedures (Sambrook, Fritsch, & Maniatis, 1989). Complete *cytb* and partial *rh1* were PCR-amplified ($T_m = 50^\circ\text{C}$ and 49°C , respectively) using L15267 5'-AATGACTTGAAGAACCACCGT-3' (Briolay, Galtier, Brito, & Bouvet, 1998) and GobCB2R 5'-GGGAGGRDTTTAACCTCCG-3' (this study) as external primers, and when necessary, PomCB1F 5'-TTCCCCTTTGTAGTACTAGCAG-3' (this study) and GobCB3R 5'-GGGTGRAAGGGRATYTTGTC-3' (this study) as internal primers for *cytb*. Finally, PminRh1F 5'-GCGCCTACATGTTCTTCCTT-3' (Larmuseau, Raeymaekers, Ruddick, Van Houdt, & Volckaert, 2009) and Rh1039r 5'-TGCTTGTTTCATGCAGATGTAGA-3' (Chen, Bonillo, & Lecointre, 2003) were used for *rh1*. Amplicon sizes were 1220 bp and 820 bp for *cytb* and *rh1*, respectively. Direct sequencing was carried out in both directions with the technical facilities of the genotyping and sequencing platform (<https://genseq.umontpellier.fr/WordPress/>) of the “Institut des Sciences de l’Evolution de Montpellier” (ISEM, Montpellier, France). Sequences were aligned by hand using MEGA v7 (Kumar, Stecher, & Tamura, 2016).

2.2 Phylogenetic reconstructions and species delimitation

Phylogenetic analyses were based on DNA nucleotide sequences, and they were conducted on both separate (*cytb* and *rh1*) and concatenated (*cytb* sequences + *rh1* sequences) gene datasets with the ISEM “Montpellier Bioinformatics Biodiversity” platform (<http://mbb.univ-montp2.fr/MBB/index.php>). Phylogenetic trees were reconstructed with two probabilistic methods: the maximum likelihood (ML) approach using PhyML v3.0 (Guindon, Dufayard, Lefort, Anisimova, Hordijk, & Gascuel, 2010) and a Bayesian inference (BI) with MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003). Appropriate models of sequence evolution were determined using MrModeltest v2.3 (Nylander, 2004). ML reconstructions were conducted under the model GTR (Yang, 1994) with a proportion of invariable sites (I) and a gamma distribution (G) for all datasets. Node robustness was estimated by bootstrap percentage values (BP) after 1000 pseudo-replicates. A mixed model analysis was applied in BI according to codon positions: SYM (Zharkikh, 1994) +I+G for the first position, and HKY (Hasegawa, Kishino, & Yano, 1985) +I for the second position of *cytb*, and GTR+I+G for the

third *cytb* position and all *rh1* positions. Five independent runs of five Markov chain Monte-Carlo chains (MCMC) were simultaneously carried out for 5,000,000 generations. Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of trees sampled every 100 generations after a burn-in stage of 25,000 generations. Alternative hypotheses of sand goby relationships were compared with the Shimodaira-Hasegawa Test (Shimodaira & Hasegawa, 1999) as implemented in PAUP* 4.010b (Swofford, 2002).

DNA-sequence-based species delimitation methods were performed to test whether their outcome would be in accordance with present taxonomy. Several species trees were thus considered as initial hypotheses: a tree reconstructed from consensus *cytb+rh1* sequences obtained for each genetically identified species; a tree reconstructed from consensus *cytb* sequences obtained for putative species identified with bPTP (<http://species.h-its.org/ptp/>; Zhang, Kapli, Pavlidis, & Stamatakis, 2013); a tree reconstructed from consensus *cytb* sequences obtained for putative species identified with bGMYC (<https://sites.google.com/site/noahmreid/software>; Reid & Carstens, 2012). These hypotheses were thus tested with the multi-gene coalescent-based method implemented in BPP v3.3 (Yang, 2015; Yang & Rannala, 2010). Several combinations of priors for ancestral population size (θ s: $\alpha = 1, 2, 10, 20, 100, 200$; $\beta = 10, 20, 100, 200, 1000, 2000$) and root age (τ_0 : $\alpha = 1, 2, 10, 20, 100, 200$; $\beta = 10, 20, 100, 200, 1000, 2000$) were tested. For each test, the other priors were the following: speciesdelimitation = 1, speciestree = 1, speciesmodelprior = 1, algorithm = 0, finetune $\epsilon = 2$, usedata = 1, locusrate = 1, heredity = 2 (scalar values = 0.25 for mitochondrial marker and 1 for nuclear marker) and cleandata = 0. Finetune variables were automatically adjusted, and swapping rates for each parameter were checked for recommended values (0.10-0.80) (Yang, 2016). Each analysis was run twice to confirm consistency between runs. In all cases, the analyses were based on the *cytb+rh1* dataset.

Intra- and interspecific genetic distances were estimated by the uncorrected *p*-distance with MEGA.

2.3 Divergence time estimates

To estimate the time to the most recent common ancestor (TMRCA) of the genetically identified species of Adriatic sand gobies, Bayesian coalescent analyses were performed for two speciation models (Yule and birth-death) with three molecular clocks (strict, relaxed uncorrelated log-normal and relaxed uncorrelated exponential) using *BEAST2 (Ogilvie, Bouckaert, & Drummond, 2017). These analyses were also conducted on the “Montpellier Bioinformatics Biodiversity” platform. To test which speciation models and clocks best fit

our data, they were compared using the Akaike's information criterion through MCMC (AICM; Raftery, Newton, Satagopan, & Krivitsky, 2007) with Tracer v1.6 (Rambaut, Suchard, Xie, & Drummond, 2014). For sand gobies, otoliths are the most common fossil remains, while skeletal remains are scarce (Bratishko, Schwarzhans, Reichenbacher, Vernyhorova, & Coric, 2015; Gierl & Reichenbacher, 2017; Schwarzhans, 2014, Schwarzhans et al., 2017). For this reason, some authors performed otolith-based dating using the ages of *P. bunyatovi* and *K. suavis* as calibration points. These species are considered the earliest *Pomatoschistus* (16 Myr) and *Knipowitschia* (13 Myr), respectively (Thacker et al., 2019). Phylogenetic relationships of these extinct species with the extant *Pomatoschistus* and *Knipowitschia* species are not yet established. Associating fossil sand goby otoliths with extant species is difficult because of potentially multiple homoplasies emerging from the shaping of the otolith morphology (Schwarzhans, 2014; Schwarzhans et al., 2017), even if the combination of qualitative (morphology) and quantitative (morphometry) approaches seem promising (Gierl, Liebl, Šanda, Vukić, Esmaceli, & Reichenbacher, 2018; Gut, Vukić, Šanda, Moritz, & Reichenbacher, 2020). Moreover, several previously published studies (Huyse et al., 2004; Penzo et al., 1998; Thacker et al., 2019; Vanhove et al., 2012) demonstrated that these sand goby groups are not monophyletic, and that some *Pomatoschistus* and *Knipowitschia* species belong to different genera (respectively, *Ninnigobius* and *Orsinigobius*; Geiger et al., 2014; Thacker et al., 2019). It thus seems very difficult to properly locate these calibration points in a sand goby molecular phylogeny if phylogenetic relationships between extant and extinct species are not clearly identified. For these reasons, fossils were not used for calibration, but instead two scenarios were investigated on the basis of the *cytb* mutation rate. Rocha, Robertson, Rocha, Van Tassell, Craig, & Bowens (2005) estimated this rate from *cytb* sequences of two sister species of the goby *Evorthodus*. These species are distributed allopatrically on both sides of the Isthmus of Panama. According to Rocha et al. (2005), these species were presumed to have diverged because of the final closure of this isthmus around 3.5 - 3.1 Myr. However, recent studies based on biogeographical and oceanic data, as well as molecular dating suggest that the isthmus formed millions of years earlier, between 15 – 10 Myr (Erkens & Hoorn, 2016; O'Dea et al., 2016; Stange, Sánchez-Villagra, Salzburger, & Matschiner, 2018). One rate (scenario 1) was thus fixed to $1.025 \pm 0.060\%$ / site / Myr (i.e., 1.97 – 2.17% substitution / Myr, following Rocha et al., 2005; Keith, Lord, & Lorion, 2011) and a log-normal distribution prior was used with $M = 0.01025$ and $S = 0.036$, while the other rate (scenario 2) was fixed to $0.280 \pm 0.060\%$ / site / Myr (i.e., 0.23 – 0.34% substitution / Myr, following Erkens & Hoorn, 2016; Stange et al., 2018) and a log-normal distribution

prior was used with $M = 0.0028$ and $S = 0.115$. *BEAST2 analyses were performed with five independent runs of 300 million generations with the first 10% discarded as burn-in. Markov chain convergence was checked visually by inspection of the traces, while the run stability was measured using the effective sample size ($ESS > 200$ for all parameters) using Tracer. Results of the independent convergent runs were combined with LogCombiner v2.4.6 (Bouckaert et al., 2014) to estimate TMRCA and 95% confidence intervals. A consensus tree was then generated using TreeAnnotator v2.4.6. (Bouckaert et al., 2014) with maximum clade credibility and mean node heights.

3. RESULTS

3.1 Phylogenetic analyses and species limits

Full alignments (Alignments S1-S2) were 1140 and 735 nucleotides long for *cytb* and *rh1*, with 454 (40%) and 94 (13%) phylogenetically informative sites, respectively. All new sequences were deposited in the GenBank database under the accession numbers MK619412-71 for *cytb* and MK625102-67 for *rh1* (Table S1).

ML and BI analyses provided similar tree topologies (Figures 2 and S1-S2). The Adriatic sand gobies, and especially the endemic species, are not a monophyletic group. Indeed, *Pomatoschistus* and *Knipowitschia* appear as paraphyletic genera since *P. quagga* (Heckel, 1837) and *P. bathi* Miller, 1982 are grouped together with all the *Knipowitschia* species ($BP = 99\%$; $PP = 1.00$), except *K. punctatissima* (Canestrini, 1864) and *K. croatica* Mrakovčić, Kerovec, Mišetić, & Schneider, 1994 that are grouped with *P. montenegrensis* Miller & Šanda, 2008 and *P. canestrinii* (Ninni, 1883) but with low support ($BP = 57\%$; $PP < 0.80$) (Figure 2). *Pomatoschistus knerii* (Steindachner, 1861) is the first branch of the whole group in our analysis. *Knipowitschia panizzae* (Verga, 1841), *K. radovici* Kovačić, 2005 and *K. mrakovcici* Mrakovčić, Schneider, & Kerovec, 1994 formed a single clade ($BP = 100\%$; $PP = 1.00$). *Pomatoschistus canestrinii* is split into three lineages, one of them clustered with *P. montenegrensis* Miller & Šanda, 2008 ($BP = 89\%$; $PP = 1.00$). Three specimens of *P. minutus* (Pallas, 1770) from the Venice lagoon (G0554, G1532 and G2011) were included in the dataset: one grouped with *P. minutus* from Spain and France ($BP = 100\%$; and $PP = 1.00$), while the two other specimens formed a separate clade ($BP = 100\%$; $PP = 1.00$). *Pomatoschistus marmoratus* (Risso, 1810) is the sister group to *K. croatica* + *K. punctatissima* / *P. canestrinii* + *P. montenegrensis* but with low support ($BP < 50\%$; $PP < 0.80$). Because this node is the weakest in the tree, other alternative positions of *P.*

marmoratus were investigated with the test of Shimodaira and Hasegawa (1999): (1) *P. marmoratus* as the sister group of all the Adriatic sand gobies except *P. knerii* and *P. minutus*; (2) *P. marmoratus* as the sister group of *P. montenegrensis* and *P. canestrinii*; (3) *P. marmoratus* as the sister group of *K. punctatissima* and *K. croatica*. The best ML tree was found to be tree (2), but the other tree topologies, including the one in Figure 2, are not significantly worse at the 5% confidence level ($0.076 < p < 0.797$).

Four hypotheses were considered in the frame of the species delimitation test. Two hypotheses are based on the tree topology (Figure 2): phylogenetic hypothesis 1 considers 20 genetically identified species (*K. panizzae* + *K. radovici* + *K. mrakovcici* = one species; the three lineages of *P. canestrinii* = three species; the Venice lineage and the Spain+France+Venice lineage of *P. minutus* = two species), and phylogenetic hypothesis 2 considers 16 genetically identified species (*K. panizzae* + *K. radovici* + *K. mrakovcici* = one species; *P. montenegrensis* + *P. canestrinii* = one species; the Venice lineage and the Spain+France+Venice lineage of *P. minutus* = one species). Results would have taxonomic implications mainly for two species complexes: *K. radovici* and *K. mrakovcici* could be considered as junior synonym of *K. panizzae*, while *P. montenegrensis* could be considered as junior synonym of *P. canestrinii*. The bPTP approach identified 25 putative species (estimated number between 19 and 35), while the bGMYC analysis identified 21 putative species with a posterior probability > 0.95. These last two hypotheses were not validated by the multi-gene coalescent-based method with BPP from the concatenated dataset (Table S2). With highest posterior probabilities obtained for large ancestral population sizes and deep divergence among species, the BPP analyses rather supported the phylogenetic hypotheses, though without favouring either one of them (Table S2).

Genetic distances were estimated within and between species (phylogenetic hypothesis 1) of *Pomatoschistus*, *Knipowitschia* and the outgroup (Tables S3 to S5 for the *cytb+rh1*, *cytb* and *rh1* datasets, respectively). In *Pomatoschistus* and *Knipowitschia* species, intragroup distances were $0.0 \pm 0.0\%$ to $4.2 \pm 0.4\%$ for the concatenated dataset. In the outgroup, these *cytb+rh1* distances were $0.8 \pm 0.2\%$ for *G. flavescens* and $1.6 \pm 0.3\%$ for *Pseudaphya ferreri*. Intergroup distances were $1.6 \pm 0.3\%$ to $14.7 \pm 0.8\%$ for the concatenated dataset. These *cytb+rh1* distances between the Adriatic sand gobies and the outgroup were $11.2 \pm 0.7\%$ to $14.0 \pm 0.8\%$ for *G. flavescens* and $14.0 \pm 0.9\%$ to $15.7 \pm 0.9\%$ for *Pseudaphya ferreri*. Note that *cytb+rh1* genetic distances between *P. montenegrensis* and the three *P. canestrinii* lineages were from $3.6 \pm 0.4\%$ to $7.8 \pm 0.6\%$, while these distances between the three *P. canestrinii* lineages were from $1.6 \pm 0.3\%$ to $6.4 \pm 0.5\%$. Lastly, the *cytb+rh1* distance

between the Venice lineage and the Spain+France+Venice lineage of *P. minutus* was $6.8 \pm 0.5\%$.

3.2 Divergence dates among Adriatic sand gobies

Bayesian coalescent analyses were conducted under the GTR+I+G model for both *cytb* and *rh1* dataset. The AICM suggested that the birth-death speciation model and relaxed uncorrelated exponential clock provided the statistically best fit for our dataset in scenario 1, while the Yule speciation model and relaxed uncorrelated exponential clock provided the statistically best fit for our dataset in scenario 2. Results from three out of five independent runs converged and were thus combined with LogCombiner for further analyses (Figures 3 and S3). Estimated clock rate for *rh1* was $0.968 \pm 0.7\%$ / site / Myr in the scenario 1 and $0.17 \pm 0.02\%$ / site / Myr in the scenario 2.

In the scenario 1, times to the most recent common ancestor for the populations of extant species included in our dataset are between 1.54 Myr for *P. bathi* and 0.09 Myr for *K. montenegrina* Kovačić & Šanda, 2007, *K. thessala* (Vinciguerra, 1921), *K. mermere* Ahnelt, 1995 and one lineage of *P. canestrinii*. In the scenario 2, these times are between 6.57 Myr for *P. bathi* and 0.40 Myr for *K. montenegrina*, *K. thessala* and *K. mermere*.

4. DISCUSSION

4.1 Phylogenetic relationships of Adriatic sand gobies

Discrepancies exist between the previously published results and the present one (see below for details). These discrepancies can be explained by the diversity of evolutionary proxies used for phylogenetic reconstructions, such as mitochondrial (*cytb*, *cytochrome c oxidase 1*, and/or *12S* and *16S rRNA* genes) and/or nuclear (*rh1*, first intron of the ribosomal protein *s7* gene or Internal Transcribed Spacer 1) markers, and/or morphological (meristic and morphometric characters), behavioural (for instance, bioacoustics and courtship) and life history (for instance, age at sexual maturity or longevity) traits (Huyse et al., 2004; Knebelsberger & Thiel, 2014; Larmuseau, Huyse, Vancampenhout, Van Houdt, & Volckaert, 2010; Malavasi, Gkenas, Leonardos, Torricelli, & McLennan, 2012; Thacker et al., 2019; Vanhove et al., 2012; Vukić, Kovačić, Zogaris, & Šanda, 2016; Vukić, Ulqini, & Šanda, 2017). However, the concatenation of *cytb* and *rh1* provided here a highly resolved phylogeny at the interspecific level.

Several molecular genetic studies have pointed to monophyly of the sand gobies sensu lato (Agorreta et al., 2013; Huyse et al., 2004; Thacker et al., 2019; Vanhove et al., 2012), with *Gobiusculus* Duncker, 1928 as a junior synonym of *Pomatoschistus* (Huyse et al., 2004; Knebelsberger & Thiel, 2014; Larmuseau et al., 2010; Thacker et al., 2019; Vanhove et al., 2012). In the present study, the dataset, including the genera *Pomatoschistus*, *Knipowitschia* and *Gobiusculus*, allowed us to confirm this monophyly (Figure 2), where *Gobiusculus* appeared as the sister species to the rest of the sand gobies. Obviously, these results should be taken with caution since *Economidichthys* Bianco, Bullock, Miller, & Roubal, 1987 was not included in our dataset.

As already reported previously (Huyse et al., 2004; Malavasi et al., 2012; Penzo et al., 1998; Thacker et al., 2019; Vanhove et al., 2012), our data show that the genera *Pomatoschistus* and *Knipowitschia* are not monophyletic. *Knipowitschia* would be monophyletic if the genus *Orsinigobius* Gandolfi, Marconato, & Torricelli, 1986 is recognized for *K. punctatissima* and *K. croatica* as proposed by Geiger et al. (2014) and Thacker et al. (2019). These authors also proposed to recognize the genus *Ninnigobius* Whitley, 1951 for *P. montenegrensis* and *P. canestrinii* (*N. montenegrensis* and *N. canestrinii*). In Huyse et al. (2004), Vanhove et al. (2012), and the present study, the clade including *K. punctatissima* and *K. croatica* formed a monophyletic group with the clade including *P. montenegrensis* and *P. canestrinii*, although this group is moderately supported in ML (BP = 57%) in the present study. A phylogenetic analysis based on behavioural and life history traits produced a tree suggesting a close relationship between *P. canestrinii* and *K. punctatissima* (Malavasi et al., 2012). Even if several morphological features (mainly colouration with small, intense, black spots) differ markedly in *P. canestrinii* and *P. montenegrensis* compared to other *Pomatoschistus* species, the tendency for canal reduction suggests that *P. canestrinii* might be a sister species of *Knipowitschia* species from the Adriatic, Aegean and Ponto-Caspian catchments (Kovačić, 2005b; Miller, 2004; Miller & Šanda, 2008). At this point, it is not possible to include with certainty *K. punctatissima* and *K. croatica* in *Orsinigobius*, and *P. montenegrensis* and *P. canestrinii* in *Ninnigobius*, or even consider that these species belong to a single genus.

Morphological variations are observed in populations of *P. canestrinii* in particular (Kovačić, 2005b). Our results split *P. canestrinii* into three lineages corresponding to three geographic areas (Figure 2; Table 1), possibly corresponding to different ecological conditions. As observed previously (Geiger et al., 2014; Thacker et al., 2019), *P. canestrinii* appears paraphyletic with respect to *P. montenegrensis*. *Pomatoschistus montenegrensis*

might thus be considered as a junior synonym of *P. canestrinii*. However, both species are distinguishable by three main morphological characters: (1) the presence of scales only on the axilla, behind the pectoral fin in *P. montenegrensis*; (2) the occurrence of dark spots mainly on the head in *P. montenegrensis*, and (3) the consistent loss of the pre-opercular and posterior oculo-scapular head canals in *P. montenegrensis* (Miller and Šanda, 2008). Species delimitation tests also supported the recognition of several species within this group (at least four; $0.99 < PP < 1.00$). Genetic distances are relatively high between *P. montenegrensis* and the three lineages of *P. canestrinii*: $5.5 \pm 0.7\%$ (D1744 and D1746), $5.7 \pm 0.7\%$ (D1747, D1748 and D1749) and $12.4 \pm 0.9\%$ (G1594 and G1595) for *cytb* (Table S4). These distances are similar to those observed between *K. thessala* and *K. mermere* ($5.0 \pm 0.6\%$) or *K. mermere* and *K. goerneri* Ahnelt, 1991 ($5.5 \pm 0.6\%$) for the lowest values, and even superior to the values observed between *K. panizzae* (including *K. radovici* and *K. mrakovcici*) and *K. montenegrina* ($4.1 \pm 0.5\%$) or between *K. milleri* (Ahnelt & Bianco, 1990) and *K. goerneri* ($3.1 \pm 0.5\%$). These genetic distances fall within the range observed for congeneric species of fishes, and especially for pairs of sister species (mean *cytb* genetic distance = 12%; Johns & Avise, 1998). From a morphological and genetic standpoint, the three lineages of *P. canestrinii* should be considered sibling species. The type material of *P. canestrinii* is from two geographically distinct localities (Venice, Italy and Jadro River near Split, Croatia, central Adriatic), and no holotype was specifically designated (Ninni, 1883). It is thus difficult to assign one of these lineages to this species. Careful revision of this species complex is necessary to resolve its taxonomy.

Among the endemic Adriatic *Knipowitschia* species, *K. panizzae*, *K. radovici* and *K. mrakovcici* were genetically extremely similar in our analyses (Figures 2 and S1-S2; Geiger et al., 2014; Thacker et al., 2019), although they are regarded as distinct morphological species (Kovačić, 2005a; Miller, 2009). *Knipowitschia mrakovcici* shows the closest morphological resemblance with *K. radovici*, but differs in adult male coloration and interorbital canals (Miller, 2009). In *K. mrakovcici*, squamation is extensively reduced compared to *K. panizzae* (Miller, 2009). The morphological differences observed between these three *Knipowitschia* species could be seen as phenotypic plasticity. On the other hand, the polytomy observed for these species could be the result of a recent speciation event, where the local freshwater populations (*K. radovici* from the Neretva river basin and *K. mrakovcici* from the Krka river basin) diversified quickly morphologically from the widespread euryhaline species *K. panizzae*. However, based on the high genetic similarity and species delimitation tests (BPP

PP = 1.00), *K. radovici* and *K. mrakovcici* were here considered junior synonyms of *K. panizzae*.

A dichotomy between populations of *P. minutus* was already proposed in the early 1980s (Wallis & Beardmore, 1983), and subsequently repeatedly underlined (Boissin, Hoareau, & Berrebi, 2011; Gysels, Hellemans, Patarnello, & Volckaert, 2004; Huyse et al., 2004; Stefanni & Thorley, 2003). The Venice lineage is regarded as a separate Evolutionarily Significant Unit (sensu Moritz, 1994) by Stefanni and Thorley (2003) and as a cryptic species of *Pomatoschistus* by Gysels et al. (2004). The present study confirms this dichotomy but also suggests a further split between the Venice specimens: one lineage consisting of the specimens from Venice, France and Spain, and another consisting of only specimens from Venice. Nothing is known about the ecological requirements of the two lineages from Venice, such as if they occupy different depths, microhabitats, reproduction period or have different feeding habits. The genetic distance on *cytb* ($10.5\% \pm 0.8\%$; Table S4) between the Venice lineage and the other *P. minutus* is similar to those from Gysels et al. (2004). Stefanni, Gysels, Volckaert and Miller (2003), and Gysels et al. (2004) also reported that the genetic distance between the Adriatic and other *P. minutus* is similar to that between *P. minutus* and its closest relative species (*P. lozanoi* and *P. norvegicus*). These genetic distances fall within the range observed for congeneric species of fishes (Johns & Avise, 1998). Species delimitation tests supported the existence of two clades within *P. minutus* (BPP PP = 1.00 for both clades). Because of large genetic distance, results of species delimitation tests and reciprocal monophyly between the Venice lineage and other *P. minutus*, the Venice lineage should be considered an undescribed *Pomatoschistus* species. This conclusion was already suggested by Boissin et al. (2011), but deserves further investigation.

Based on morphological characters, Economidis and Miller (1990) synonymized the genus *Orsinigobius* with *Knipowitschia*, while Miller (1973, 1986, 2004) synonymized the genus *Ninnigobius* with *Pomatoschistus*. Recently, some authors (Geiger et al., 2014; Thacker et al., 2019) resurrected the genera *Ninnigobius* and *Orsinigobius* based on the *cytochrome c oxidase I* gene. However, these authors did not justify the adopted taxonomy, even if it seems that they rely on the phylogenetic species concept (Cracraft, 1989). Based on this concept, other genera could be resurrected based on the present results. For instance, *P. quagga* and *P. bathi* are a monophyletic group closely related to most *Knipowitschia* species, while *P. marmoratus* is a sister species of the clade including *K. punctatissima* and *K. croatica* as well as *P. montenegrensis* and *P. canestrinii*. Iljin (1930) considered *P. quagga* distinct from other sand gobies, and placed this species in its own genus, *Engrauligobius* Iljin, 1930, while

Syrrhothonus charrieri Chabanaud, 1933 was proposed as a junior synonym of *P. marmoratus*. However, in this latter case, alternative names exist, such as *Iljinia* de Buen, 1930, with its type species *P. microps* (Krøyer, 1838), a closely related species of *P. marmoratus* (Geiger et al., 2014; Huyse et al., 2004; Thacker et al., 2019; Vanhove et al., 2012). Since several key taxa are lacking in the present study, it seems premature to resurrect some genera. A more integrative approach of this taxonomy should be promoted by including appropriate taxon sampling (including type species of the genera and available type material), morphological and molecular genetic proxies, as well as ecological and life history traits.

4.2 Evolutionary history of Adriatic sand gobies

The Adriatic sand gobies, and in particular the endemic species among them, are not monophyletic. This suggests that the diversification of these gobies was seemingly related to different past events. Several scenarios have been proposed to explain the evolutionary history of the sand gobies. Thacker et al. (2019) suggested that episodes of diversification among sand gobies are correlated to the Middle Miocene Tethys closure / Paratethys isolation (18-12 Myr; Rögl, 1999) and the drawdown of the Mediterranean Sea during the Messinian Salinity Crisis (5.97 – 5.33 Myr; Manzi et al., 2013). These authors excluded the possibility that intra- or interspecific differentiation may correspond to Pleistocene glaciations. According to Thacker et al. (2019), diversification in the freshwater lineages of sand gobies (*P. montenegrensis*, *P. canestrinii*, *K. punctatissima*, *K. croatica*, *K. panizae* and *K. montenegrina*) occurred partly or totally in the Adriatic Sea during the MSC. In contrast, other authors (Huyse et al., 2004; Penzo et al., 1998; Vanhove et al., 2012) considered that the diversification of sand goby genera occurred during the Lago Mare phase (i.e., period of environmental fluctuations due to repeated connections between the Mediterranean Sea and the Paratethys; Roveri et al., 2014) of the MSC, while interspecific diversification coincided with Pleistocene glaciations. According to Huyse et al. (2004), the Adriatic populations of *Pomatoschistus* were then isolated after the sea-level drop in the Mediterranean Sea. Several studies have demonstrated the importance of Pleistocene glaciations on population differentiation and speciation events of sand gobies (e.g., Boissin et al., 2011; Gysels et al., 2004; Larmuseau et al., 2009; Tougaard et al., 2014) and other Atlantic and Mediterranean fish species (Jenkins et al., 2018; Patarnello et al., 2007).

In the present study, two scenarios were investigated based on two different *cytb* mutation rates ($1.025 \pm 0.060\%$ / site / Myr and $0.28 \pm 0.060\%$ / site / Myr). Although some discrepancies exist between scenario 1 and some previously published studies (Huyse et al.,

2004; Vanhove et al., 2012), this scenario remains consistent with the temporal framework provided by these studies and the fossil record (Schwarzahns et al., 2017) for the evolutionary history of the sand gobies. On the other hand, scenario 2 suggests a much older origin for the sand gobies (> 45 Myr), while the fossil record suggests an origin around 20 Myr (Schwarzahns et al., 2017). Thus, scenario 2 seems unrealistic, and for this reason it will not be discussed further. Scenario 1 provided estimates of divergence times close to those from Vanhove et al. (2012) with a mutation rate of 0.5% / Myr for the *12S* and *16S rRNA* genes. For instance, TMRCA of the clade *K. punctatissima* and *P. canestrinii* was $\approx$ 6.5 Myr in the present study and $\approx$ 5 Myr in Vanhove et al. (2012) and the origin of *Knipowitschia* was $\approx$ 4 Myr and $\approx$ 3 Myr, respectively. In Thacker et al. (2019), TMRCA for *Knipowitschia* was $\approx$ 14 Myr, and in general, TMRCA obtained with otolith-based dating are older than in scenario 1, but younger than in scenario 2. The fossil record is the most reliable source of independent information for the calibration of phylogenetic trees. However, fossil dating, the phylogenetic placement of the fossil, and the incompleteness of the fossil record are potential sources of error (Forest, 2009). The differences in divergence time estimates observed between the present study and previously published studies can be due to the way the trees were time-calibrated (*cytb* mutation rates versus *12S* and *16S rRNA* gene mutation rates, otolith-based dating, past geological event used as calibration point), but also due to the use of different time estimate methods (Bayesian coalescent analyses versus likelihood-ratio test; Huyse et al., 2004; Thacker et al., 2019; Vanhove et al., 2012).

Since the present dataset does not include all species of sand gobies, it would be speculative to argue on their origin, or on the impact of the MSC in their evolutionary history. However, our molecular dating does not support the idea that MSC played a role in the diversification of extant Adriatic sand goby species. Species diversification of these gobies was rather related to the Pleistocene climatic cycles, between 1.54 and 0.09 Myr. Divergence time estimates for most endemic Adriatic sand goby species (*K. montenegrina*, *K. sp.*, *P. montenegrensis*, *P. canestrinii*, *K. punctatissima*, *K. croatica* and *P. cf. minutus* “Venice”) suggest that intraspecific diversification occurred in the Adriatic basin at different glacial periods. The progressive drop in sea level (up to -115 m; Sikora, Mihanović, & Vilibić, 2014) and the subsequent disappearance of marine habitats might have forced some isolated populations of euryhaline sand gobies to adapt to a fluvio-lacustrine environment rather than tracking marine habitats. This would explain why most of the endemic Adriatic sand gobies are freshwater species. Contrary to Huyse et al. (2004) who considered the freshwater lifestyle in sand gobies monophyletic, Penzo et al. (1998) suggested that adaptation to

freshwater habitats evolved several times independently in some goby lineages (*Knipowitschia* and *Padogobius*) from euryhaline ancestors. Our results support this latter view since each clade concerned had a euryhaline sister group: *P. quagga* and *P. bathi* for *Knipowitschia*; *P. marmoratus* for the clade including *K. punctatissima* and *K. croatica* as well as *P. montenegrensis* and *P. canestrinii*.

A scenario to explain the differentiation event related to *P. cf. minutus* “Venice” and *P. minutus* was proposed by Stefanni and Thorley (2003). *Pomatoschistus minutus* is a temperate fish adapted to cold conditions currently distributed from the North Sea to the Mediterranean and Black seas (Rochard & Elie, 1994). In the Mediterranean Sea, it is restricted to cooler northern waters (Boissin et al., 2011). Stefanni and Thorley (2003) suggested that, because of more boreal conditions in the Mediterranean Sea during the last glacial maximum (0.026 – 0.019 Myr; Clark et al., 2009), this species might have formed a single widespread population. After this glacial period, *P. minutus* might have retreated northwards in response to temperature and sea-level rise. In the Adriatic Sea, a population was probably trapped in the north because it was not able to cross the warmer waters of the southern part. From the present molecular dating, however, it seems that intraspecific diversification would not be related to the last glacial maximum, but rather to the Elsterian glacial period (0.478 – 0.424 Myr; Lisiecki & Raymo, 2005), an older period in which the sea-level was among the lowest during the Pleistocene glaciations (Harff et al., 2017).

If the Pleistocene glacial periods are at the origin of the endemic Adriatic sand goby species, the occurrence of more widespread species, such as *P. marmoratus*, seems more related to interglacial periods (Figure 3). These are Mediterranean species and thus more adapted to warm waters. During warmer Pleistocene periods, they probably mirrored the movement of the (paleo)coastline and expanded their distribution northwards when water temperature and sea level rose (Marino, Pujolar, & Zane, 2011; Pujolar, Marceta, Saavedra, Bressan, & Zane, 2010). Since only Adriatic specimens of *P. marmoratus* were included in our dataset, the origin of the Adriatic populations remains unclear. Several studies on marine species (Astolfi, Dupanloup, Rossi, Bisol, Faure, & Congiu, 2005; Cavraro et al., 2017; Congiu, Rossi, & Colombo, 2002; Debes, Zachos, & Hanel, 2008; Maltagliati, Di Giuseppe, Barbieri, Castelli, & Dini, 2010; Marino et al., 2011) suggested a population expansion from the adjacent Ionian Sea because of extensive gene flow between the Adriatic and Ionian populations. Phylogeographic studies of sand gobies at a Mediterranean scale could therefore provide an answer to this hypothesis.

5. CONCLUSIONS

The aims of the present study were, first, to investigate the phylogenetic relationships and cryptic species diversity among Adriatic sand goby species (genera *Pomatoschistus* and *Knipowitschia*), and second, to better understand their evolutionary history in order to determine which past events were at their origin.

Even if the present study is focused on Adriatic species only, the concatenation of mitochondrial and nuclear markers allows us to propose, for the first time, a highly resolved phylogeny at the interspecific level. This phylogeny emphasizes the urgent need of taxonomic revision based on a complete dataset of sand goby species and a combination of multi-locus sequences and morphological approaches. Within endemic Adriatic sand gobies, the phylogeny, species delimitation tests, and genetic distances highlight cryptic diversity, and suggest that some putative new species remain to be described within the sand goby group.

The Adriatic sand gobies are not a monophyletic group, not even the endemic species, and several past events were at their origin. It seems that Pleistocene climatic cycles impacted their evolutionary history in two ways: glacial periods promoted the intraspecific diversification of the endemic species, whereas interglacial periods promoted the (re)colonization of the Adriatic Sea by more widespread species.

Mediterranean biodiversity is currently threatened by human activities, climate change, and biological invasions (Bianchi et al., 2012). In particular, climate warming is expected to have a significant influence on Mediterranean endemic fish species, especially those from the Adriatic Sea (Ben Rais Lasram et al., 2010). Endemic species, effectively trapped, are eventually threatened with extinction. Moreover, climate warming may also induce the fragmentation of suitable habitat, accelerating population decline and the extinction of more widespread species (Ben Rais Lasram et al., 2010). Consequently, extinction of the endemics and habitat fragmentation for widespread species would have a major impact on ecosystem structure and functioning (Lotze et al., 2011), especially if species playing a key role in the food web, such as sand gobies, are involved.

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DATA AVAILABILITY STATEMENT

Cytochrome b and *rhodopsin 1* gene sequences that support the findings of this study are openly available in GenBank at <https://www.ncbi.nlm.nih.gov/genbank/> under the following accession numbers: MK619412-71 and MK625102-67, respectively. Full alignments for both genes are available in Supporting Information of this article.

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Figure legends

Figure 1. Map showing localities of *Pomatoschistus* and *Knipowitschia* species considered in this study. Locality numbers and samples are listed in Table 1. Major seas other than the Adriatic Sea are labelled (WMS: Western Mediterranean Sea; IS: Ionian Sea; AS: Aegean Sea).

Figure 2. Maximum likelihood tree reconstructed from the concatenated dataset of the mitochondrial *cytochrome b* and nuclear *rhodopsin1* genes for Adriatic *Pomatoschistus* and *Knipowitschia* species. Samples are detailed in Table1. Names of the morphospecies are listed on the right. Species with “•” are endemic species of the Adriatic Sea. Numbers at the nodes indicate bootstrap percentages ($\geq 50\%$) and posterior probabilities (≥ 0.80). Black circles indicate nodes with BP = 100% and PP = 1.00. The white circle is for a node with BP < 50% and PP < 0.80, while nodes with “-“ are weakly supported either in maximum likelihood or in Bayesian approach.

Figure 3. Species tree chronogram for Adriatic *Pomatoschistus* and *Knipowitschia* species estimated by *BEAST2 based on the *cytochrome b* and *rhodopsin 1* datasets (scenario 1). Values at nodes indicate the time (in Myr) to the most recent common ancestor, and values in brackets the 95% confidence intervals. Main past events that could have had an impact on the evolutionary history of sand gobies are indicated in grey (Tethys closure and Paratethys isolation, and Messinian Salinity Crisis from Thacker et al., 2019; MSC and Pleistocene climatic cycles from Huyse et al., 2004; Penzo et al., 1998; Vanhove et al., 2012). Only glacial periods of the Pleistocene are in grey.

SUPPORTING INFORMATION

Figure S1. Maximum likelihood tree reconstructed from the *cytochrome b* gene dataset for Adriatic *Pomatoschistus* and *Knipowitschia* species. Samples are detailed in Table 1. Names of the morphospecies are listed on the right. Species with “•” are endemic species of the Adriatic Sea. Numbers at nodes are for bootstrap percentages ($\geq 50\%$) and posterior probabilities (≥ 0.80). Black circles indicate nodes with BP = 100% and PP = 1.00. White circles are for nodes with BP < 50% and PP < 0.80, while nodes with “-” are weakly supported either in maximum likelihood or in the Bayesian approach.

Figure S2. Maximum likelihood tree reconstructed from the *rhodopsin 1* gene dataset for Adriatic *Pomatoschistus* and *Knipowitschia* species. Samples are detailed in Table 1. Names of the morphospecies are listed on the right. Species with “•” are endemic species of the Adriatic Sea. Numbers at nodes are for bootstrap percentages ($\geq 50\%$) and posterior probabilities (≥ 0.80). Black circles indicate nodes with BP = 100% and PP = 1.00. White circles are for nodes with BP < 50% and PP < 0.80, while nodes with “-” are weakly supported either in maximum likelihood or in the Bayesian approach.

Figure S3. Species tree chronogram for Adriatic *Pomatoschistus* and *Knipowitschia* species estimated by *BEAST2 based on the *cytochrome b* and *rhodopsin 1* datasets (scenario 2). Values at nodes indicate the time (in Myr) to the most recent common ancestor, and in brackets, the 95% confidence intervals. Past events that could have had an impact on the evolutionary history of sand gobies and reported on Figure 3 are indicated in grey (Tethys closure and Paratethys isolation, and Messinian Salinity Crisis from Thacker et al., 2019; MSC and Pleistocene climatic cycles from Huyse et al., 2004; Penzo et al., 1998; Vanhove et al., 2012).

Table S1. Detailed list of sand goby species taken into account in the present study. Sampled localities, latitude and longitude coordinates, GenBank accession numbers for the *cytochrome b* and *rhodopsin 1* genes and tissue providers are indicated. Locality numbers mirror those of Figure 1.

Table S2. Initial hypotheses for species delimitation analysis using BPP. All tested combinations of priors for ancestral population size (θ_s) and root age (τ_0) are presented.

1017 Number of putative species for each hypothesis is indicated in brackets. Values in grey are for
1018 posterior probability > 0.95 obtained for both runs.

1019

1020 **Table S3.** Intraspecific (in the diagonal with standard error in brackets) and interspecific
1021 (below the diagonal with standard error above the diagonal) genetic distances for Adriatic
1022 *Pomatoschistus* and *Knipowitschia* species and the outgroup from the concatenated
1023 (*cytochrome b*+*rhodopsin 1*) dataset.

1024

1025 **Table S4.** Intraspecific (in the diagonal with standard error in brackets) and interspecific
1026 (below the diagonal with standard error above the diagonal) genetic distances for Adriatic
1027 *Pomatoschistus* and *Knipowitschia* species and the outgroup from the *cytochrome b* gene
1028 dataset.

1029

1030 **Table S5.** Intraspecific (in the diagonal with standard error in brackets) and interspecific
1031 (below the diagonal with standard error above the diagonal) genetic distances for Adriatic
1032 *Pomatoschistus* and *Knipowitschia* species and the outgroup from the *rhodopsin 1* gene
1033 dataset.

1034

Table legends

Table 1. List of sand goby species analysed in the present study. Locality numbers mirror those of Figure 1. Species with “•” are endemic species of the Adriatic Sea. GenBank accession numbers for the *cytochrome b* and *rhodopsin 1* genes, and tissue providers are listed in Table S1. FSJF: Jörg Freyhof’s fish collection, Zoologische Forschungsmuseum Alexander Koenig, Bonn, Germany; ISEM: Institut des Sciences de l’Evolution, Montpellier, France; LBEG: Laboratory of Biodiversity and Evolutionary Genomics, Leuven, Belgium; MNHN: Museum National d’Histoire Naturelle, Paris, France; NMP: Czech Národní Muzeum, Prague, Czech Republic; PMR: Prirodoslovni Muzej, Rijeka, Croatia.

Taxon	Voucher	Lab ID	Locality
Order Gobiiformes			
Family Gobiidae			
Subfamily Gobiinae			
Genus <i>Knipowitschia</i> Iljin, 1927			
<i>K. caucasica</i> (Berg, 1916) – Caucasian dwarf goby			
	FSJF 1987	C3	1. Ulubat river, Turkey
	FSJF 40	C6	2. Golubitzkaya village, Russia
	NMP uncatalogued	HL22	3. Akcakoca, Black Sea, Turkey
	NMP uncatalogued	R19	4. Lake Babadag, Romania
<i>K. croatica</i> Mrakovčić, Kerovec, Mišetić & Schneider, 1994v- Neretva dwarf goby•			
	NMP P6V 84588	A2020	5. Trebižat river, Teskera, Bosnia and Herzegovina
	PMR VP3261	D1761	6. Lake Polje, Croatia
	NMP uncatalogued	Norin2	7. Norin river, Croatia
<i>K. goerneri</i> Ahnelt, 1991 – Corfu dwarf goby			
	NMP uncatalogued	Butr4	8. Butrint lagoon, Albania
	NMP uncatalogued	Rreza2	8. Butrint lagoon, Rreza Lake, Albania
	NMP uncatalogued	Tricho1	9. Lake Trichonis, Acheloos basin, Greece
<i>K. mermere</i> Ahnelt, 1995 – Marmara goby			
	FSJF 1846	M4	10. Tekelioğlu, Turkey
	FSJF 2319	M5	11. Gediz river, North of Adala, Turkey
<i>K. milleri</i> (Ahnelt & Bianco, 1990) – Acheron spring goby			
	NMP P6V 142642	KMIL1	12. Acheron river, Greece
	NMP P6V 142646	KMIL5	12. Acheron river, Greece
<i>K. montenegrina</i> Kovačić & Šanda, 2007•			
	NMP P6V 89688	D67	33. Buna, Albania
	NMP P6V 89681	D163	14. Lake Skadar, Albania
	PMR VP2462	D1325	13. Morača river, Ponari, Montenegro
	PMR VP2462	D1326	13. Morača river, Ponari, Montenegro
	NMP P6V 89874	D1327	14. Morača river, Ponari, Montenegro
<i>K. mrakovcici</i> Mrakovčić, Schneider & Kerovec, 1994•			
	NMP P6V 142650	MRA3	15. Lake Visovacko, Croatia
	NMP P6V 142652	MRA5	15. Lake Visovacko, Croatia
<i>K. panizzeae</i> (Verga, 1841) – Adriatic dwarf goby•			
	PMR VP1985	C1188	16. Virski most, Privlaka, Croatia
	PMR VP1905	C1205	17. Cetina river, Radmanove mlinice, Croatia
	ISEM uncatalogued	G1544	18. Venice, Italy
<i>K. punctatissima</i> (Canestrini, 1864) – Italian spring goby•			
	NMP P6V 140772	D1768	19. Stella river basin, Ariis di Rivignano, channel, Italy
	NMP P6V 140773	D1769	19. Stella river basin, Ariis di Rivignano, channel, Italy
	PMR VP2718	D1770	19. Stella river basin, Ariis di Rivignano, channel, Italy
	NMP P6V 140771	D1771	19. Stella river basin, Ariis di Rivignano, channel, Italy
<i>K. radovici</i> Kovačić, 2005 – Norin goby•			
	NMP P6V 85634	C1037	20. Hutobo blato wetland, Bosnia and Herzegovina
	NMP uncatalogued	Norin1	7. Norin river, Croatia
<i>K. thessala</i> (Vinciguerra, 1921) – Thessaly goby			
	NMP P6V 141422	D2006	21. Litheos river, Pinios Basin, Greece
	NMP P6V 141418	D2008	21. Litheos river, Pinios Basin, Greece
<i>K. sp.</i> •			

NMP P6V 89682	D733	22. Gjadrit river, Vau Dejes, Albania
NMP P6V 89683	D734	22. Gjadrit river, Vau Dejes, Albania

Genus *Pomatoschistus* Gill, 1863

P. bathi Miller, 1982

NMP uncatalogued	CG089A	23. Kamenovo, Montenegro
PMR VP2418	D1734	24. Unije, Croatia

P. canestrinii (Ninni, 1883) - Canestrini's goby•

PMR VP3255	D1744	25. Raša, Croatia
PMR VP3255	D1746	25. Raša, Croatia
PMR VP3254	D1747	26. Baćinska lakes, Croatia
PMR VP3254	D1748	26. Baćinska lakes, Croatia
PMR VP3254	D1749	26. Baćinska lakes, Croatia
ISEM uncatalogued	G1594	18. Venice, Italy
ISEM uncatalogued	G1595	18. Venice, Italy

P. knerii (Steindachner, 1861) – Kner's goby

PMR VP2460	D865	27. Krk Island, Croatia
NMP uncatalogued	D1410	27. Vanjsko Šilo, Krk Island, Croatia
NMP P6V 89860	D1412	27. Vanjsko Šilo, Krk Island, Croatia

P. marmoratus (Risso, 1810) – Marbled goby

NMP P6V 86948	A1120	28. Jaz beach, Montenegro
NMP P6V 87086	A1613	29. Radoviči, Montenegro
NMP P6V 140006	D1478	30. Sv. Marak, Krk Island, Croatia
ISEM uncatalogued	G1580	18. Venice, Italy

P. minutus (Pallas, 1770) – Sand goby

LBEG uncatalogued	A1	30. Guadalquivir estuary, Spain
ISEM uncatalogued	G0003	31. Mauguio, Gulf of Lion, France
ISEM uncatalogued	G0554	18. Venice, Italy
ISEM uncatalogued	G1532	18. Venice, Italy
ISEM uncatalogued	G1976	32. Gironde estuary, France
ISEM uncatalogued	G2011	18. Venice, Italy

P. montenegrensis Miller & Šanda, 2008•

NMP P6V 89686	D70	33. Buna, Albania
NMP P6V 89668	D152	14. Lake Skadar, Albania
NMP P6V 89878	D1260	13. Morača river, Montenegro
NMP P6V 89884	D1261	13. Morača river, Montenegro

P. quagga (Heckel, 1837) – Quagga goby

PMR VP2388	D1740	34. Kraljevica, Croatia
PMR VP2388	D1741	34. Kraljevica, Croatia

Genus *Gobiusculus* Duncker, 1928

G. flavescens (Fabricius, 1779) - Two-spotted goby

MNHN uncatalogued	BPS-0246	Concarneau, Brittany, France
NMP P6V 142728	D1825	Galicia, Spain

Genus *Pseudaphya* Iljin, 1930

P. ferrerii (de Buen & Fage, 1908) – Ferrer's goby

NMP P6V 89049	D835	Selce, Croatia
NMP uncatalogued	D1428	Selce, Croatia

Supporting Information

Quaternary climatic cycles promoted (re)colonization and diversification events in Adriatic sand gobies

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Supporting Figures

Figure S1. Maximum likelihood tree reconstructed from the *cytochrome b* gene dataset for Adriatic *Pomatoschistus* and *Knipowitschia* species. Samples are detailed in Table 1. Names of the morphospecies are listed on the right. Species with “•” are endemic species of the Adriatic Sea. Numbers at nodes are for bootstrap percentages ($\geq 50\%$) and posterior probabilities (≥ 0.80). Black circles indicate nodes with BP = 100% and PP = 1.00. White circles are for nodes with BP < 50% and PP < 0.80, while nodes with “-” are weakly supported either in maximum likelihood or in the Bayesian approach.

Figure S2. Maximum likelihood tree reconstructed from the *rhodopsin 1* gene dataset for Adriatic *Pomatoschistus* and *Knipowitschia* species. Samples are detailed in Table 1. Names of the morphospecies are listed on the right. Species with “•” are endemic species of the

Adriatic Sea. Numbers at nodes are for bootstrap percentages ($\geq 50\%$) and posterior probabilities (≥ 0.80). Black circles indicate nodes with BP = 100% and PP = 1.00. White circles are for nodes with BP < 50% and PP < 0.80, while nodes with “-“ are weakly supported either in maximum likelihood or in the Bayesian approach.

Figure S3. Species tree chronogram for Adriatic *Pomatoschistus* and *Knipowitschia* species estimated by *BEAST2 based on the *cytochrome b* and *rhodopsin 1* datasets (scenario 2). Values at nodes indicate the time (in Myr) to the most recent common ancestor, and in brackets, the 95% confidence intervals. Past events that could have had an impact on the evolutionary history of sand gobies and reported on Figure 3 are indicated in grey (Tethys closure and Paratethys isolation, and Messinian Salinity Crisis from Thacker et al., 2019; MSC and Pleistocene climatic cycles from Huyse et al., 2004; Penzo et al., 1998; Vanhove et al., 2012).

Supporting Tables

Table S1. Detailed list of sand goby species taken into account in the present study. Sampled localities, latitude and longitude coordinates, GenBank accession numbers for the *cytochrome b* and *rhodopsin 1* genes and tissue providers are indicated. Locality numbers mirror those of Figure 1.

Table S2. Initial hypotheses for species delimitation analysis using BPP. All tested combinations of priors for ancestral population size (θ_s) and root age (τ_0) are presented. Number of putative species for each hypothesis is indicated in brackets. Values in grey are for posterior probability > 0.95 obtained for both runs.

Table S3. Intraspecific (in the diagonal with standard error in brackets) and interspecific (below the diagonal with standard error above the diagonal) genetic distances for Adriatic *Pomatoschistus* and *Knipowitschia* species and the outgroup from the concatenated (*cytochrome b*+*rhodopsin 1*) dataset.

Table S4. Intraspecific (in the diagonal with standard error in brackets) and interspecific (below the diagonal with standard error above the diagonal) genetic distances for Adriatic *Pomatoschistus* and *Knipowitschia* species and the outgroup from the *cytochrome b* gene dataset.

Table S5. Intraspecific (in the diagonal with standard error in brackets) and interspecific (below the diagonal with standard error above the diagonal) genetic distances for Adriatic

Pomatoschistus and *Knipowitschia* species and the outgroup from the *rhodopsin 1* gene dataset.

Supporting Alignments

Alignment S1. Full alignment of *cytochrome b* gene sequences.

Alignment S2. Full alignment of *rhodopsin 1* gene sequences.

FIGURE S1

FIGURE S2

FIGURE S3

TABLE S1 Detailed list of sand goby species taken into account in the present study. Sampled localities, latitude and longitude coordinates, GenBank accession numbers for the *cytochrome b* and *rhodopsin 1* genes and tissue providers are indicated. Locality numbers mirror those of Figure 1.

Species	Common Name	Label	Locality	Latitude	Longitude	Accession Number		Source
						<i>cytochrome b</i> gene	<i>rhodopsin 1</i> gene	
Order Gobiiformes								
Family Gobiidae								
Subfamily Gobiinae								
Genus <i>Knipowitschia</i>								
<i>K. caucasica</i>	Caucasian dwarf goby	C3	1 Ulubat river, Turkey	N 40°12'19.5"	E 28°25'58.1"	MK619412	MK625102	FSJF§
		C6	2 Golubitzkaya village, Russia	N 45°18'34.2"	E 37°18'12.6"	MK619413	MK625103	FSJF
		HL22	3 Akcakoca, Black Sea, Turkey	N 41°1'38.1"	E 31°9'26.6"	KF214252*‡	MK625104	NMP
		R19	4 Lake Babadag, Romania	N 44°53'34.5"	E 28°43'16"	KF214254*	MK625105	NMP
<i>K. croatica</i> •†	Neretva dwarf goby	A2020	5 Trebižat river, Teskera, Bosnia and Herzegovina	N 43°11'0.44"	E 17°31'22.59"	MK619414	MK625106	NMP
		D1761	6 Lake Polje, Croatia	N 45°8'22.8"	E 14°38'47.6"	MK619415	MK625107	PMR
		Norin2	7 River Norin, Croatia	N 43°4'3.7"	E 17°36'41.3"	MK619416	MK625108	NMP
<i>K. goerneri</i>	Corfu dwarf goby	Butr4	8 Butrint lagoon, Albania	N 39°48'48"	E 20°1'0"	KY462220**	MK625109	NMP
		Rreza2	8 Butrint lagoon, Lake Rreza, Albania	N 39°44'34"	E 20°3'56"	KY462223**	MK625110	NMP
		Tricho1	9 Lake Trichonis, Achelooos basin, Greece	N 38°35'20"	E 21°28'2"	KY462228**	MK625111	NMP
<i>K. mermere</i>	Marmara goby	M4	10 Tekelioğlu, Turkey	N 38°35'41.9"	E 27°59'44.7"	MK619417	MK625112	FSJF
		M5	11 Gediz river, North of Adala, Turkey	N 38°35'37.6"	E 28°16'18.8"	MK619418	MK625113	FSJF
<i>K. milleri</i>	Acheron spring goby	KMIL1	12 Acheron river, Greece	N 39°14'34"	E 20°28'39"	MK619419	MK625114	NMP
		KMIL5	12 Acheron river, Greece	N 39°14'34"	E 20°28'39"	MK619420	MK625115	NMP
<i>K. montenegrina</i> •	-	D67	33 Buna, Albania	N 42°18'36"	E 19°11'55"	MK619421	MK625116	NMP
		D163	14 Lake Skadar, Albania	N 42°4'5.3"	E 19°30'43.7"	MK619422	MK625117	NMP
		D1325	13 Morača river, Ponari, Montenegro	N 42°18'36"	E 19°11'55"	MK619423	MK625118	PMR
		D1326	13 Morača river, Ponari, Montenegro	N 42°18'36"	E 19°11'55"	MK619424	MK625119	PMR
		D1327	13 Morača river, Ponari, Montenegro	N 42°18'36"	E 19°11'55"	MK619425	MK625120	NMP
<i>K. mrakovcici</i> •	-	MRA3	15 Lake Visovacko, Croatia	N 43°51'28.2"	E 15°58'43.9"	MK619426	MK625121	NMP
		MRA5	15 Lake Visovacko, Croatia	N 43°51'28.2"	E 15°58'43.9"	MK619427	MK625122	NMP
<i>K. panizae</i> •	Adriatic dwarf goby	C1188	16 Virski most, Privlaka, Croatia	N 44°15'12.8"	E 15°9'30"	MK619428	MK625123	PMR
		C1205	17 Cetina river, Radmanove mlinice, Croatia	N 43°39'37.4"	E 16°42'51.2"	MK619429	MK625124	PMR
		G1544	18 Venice, Italy	N 45°26'13.9"	E 12°20'4.5"	MK619430	MK625125	ISEM
<i>K. punctatissima</i> •	Italian spring goby	D1768	19 Stella river basin, Ariis di Rivignano, channel, Italy	N 45°52'20.4"	E 13°5' 14.3"	MK619431	MK625126	NMP
		D1769	19 Stella river basin, Ariis di Rivignano, channel, Italy	N 45°52'20.4"	E 13°5' 14.3"	MK619432	MK625127	NMP
		D1770	19 Stella river basin, Ariis di Rivignano, channel, Italy	N 45°52'20.4"	E 13°5' 14.3"	MK619433	MK625128	PMR
		D1771	19 Stella river basin, Ariis di Rivignano, channel, Italy	N 45°52'20.4"	E 13°5' 14.3"	MK619434	MK625129	NMP
<i>K. radovici</i> •	Norin goby	C1037	20 Hutovo blato wetland, Bosnia and Herzegovina	N 43° 3'41.59"	E 17°47'6.67"	MK619435	MK625130	NMP
		Norin1	7 Norin river, Croatia	N 43°4'3.7"	E 17°36'41.3"	MK619436	MK625131	NMP
<i>K. thessala</i>	Thessaly goby	D2006	21 Litheos river, Pinios Basin, Greece	N 39°33'14"	E 21°46'8"	KF214247*	MK625132	NMP
		D2008	21 Litheos river, Pinios Basin, Greece	N 39°33'14"	E 21°46'8"	MK619437	MK625133	NMP
<i>K. sp.</i> •	-	D733	22 Gjadrit river, Vau Dejes, Albania	N 42°1'26.1"	E 19°41'44.2"	MK619438	MK625134	NMP

		D734	22 Gjadrit river, Vau Dejes, Albania	N 42°1'26.1"	E 19°41'44.2"	MK619439	MK625135	NMP
Genus <i>Pomatoschistus</i>								
<i>P. bathi</i>	-	CG089A	23 Kamenovo, Montenegro	N 42°16'29.6"	E 18°53'23.6"	MK619440	MK625136	NMP
<i>P. canestrinii</i> •	Canestrini's goby	D1734	24 Unije, Croatia	N 44°38'48.8"	E 14°14'45.2"	MK619441	MK625137	PMR
		D1744	25 Raša, Croatia	N 45°4'58.9"	E 14°5'7.9"	MK619442	MK625138	PMR
		D1746	25 Raša, Croatia	N 45°4'58.9"	E 14°5'7.9"	MK619443	MK625139	PMR
		D1747	26 Bačinska lakes, Croatia	N 43°5'39.7"	E 17°27'18.2"	MK619444	MK625140	PMR
		D1748	26 Bačinska lakes, Croatia	N 43°5'39.7"	E 17°27'18.2"	MK619445	MK625141	PMR
		D1749	26 Bačinska lakes, Croatia	N 43°5'39.7"	E 17°27'18.2"	MK619446	MK625142	PMR
		G1594	18 Venice, Italy	N 45°26'13.9"	E 12°20'4.5"	MK619447	MK625143	ISEM
<i>P. knerii</i>	Kner's goby	G1595	18 Venice, Italy	N 45°26'13.9"	E 12°20'4.5"	MK619448	MK625144	ISEM
		D865	27 Krk Island, Croatia	N 45°5'24.7"	E 14°32'58.8"	MK619449	MK625145	PMR
		D1410	27 Vanjsko Šilo, Krk Island, Croatia	N 45°8'46.1"	E 14°39'49.1"	MK619450	MK625146	NMP
		D1412	27 Vanjsko Šilo, Krk Island, Croatia	N 45°8'46.1"	E 14°39'49.1"	MK619451	MK625147	NMP
<i>P. marmoratus</i>	Marbled goby	A1120	28 Jaz beach, Montenegro	N 42°17'26.9"	E 18°51'1.6"	MK619452	MK625148	NMP
		A1613	29 Radovići, Montenegro	N 42°23'49.3"	E 18°40'18.7"	MK619453	MK625149	NMP
		D1478	27 Sv. Marak, Krk Island, Croatia	N 45°6'24.02"	E 14°40'3.45"	MK619454	MK625150	NMP
		G1580	18 Venice, Italy	N 45°26'13.9"	E 12°20'4.5"	MK619455	MK625151	ISEM
<i>P. minutus</i>	Sand goby	A1	30 Guadalquivir estuary, Spain	N 36°47'49.86"	E 6°20'35.78"	MK619456	MK625152	LBEG
		G0003	31 Mauguio, Gulf of Lion, France	N 43°36'55.7"	E 4°0'35.4"	MK619457	MK625153	ISEM
		G0554	18 Venice, Italy	N 45°26'13.9"	E 12°20'4.5"	MK619458	MK625154	ISEM
		G1532	18 Venice, Italy	N 45°26'13.9"	E 12°20'4.5"	MK619459	MK625155	ISEM
		G1976	32 Gironde estuary, France	N 45°31'58.7"	E 0°59'52"	MK619460	MK625156	ISEM
		G2011	18 Venice, Italy	N 45°26'13.9"	E 12°20'4.5"	MK619461	MK625157	ISEM
		D70	33 Buna, Albania	N 41°57'36.58"	E 19°23'23.13"	MK619462	MK625158	NMP
<i>P. montenegrensis</i> •	-	D152	14 Lake Skadar, Albania	N 42°4'5.3"	E 19°30'43.7"	MK619463	MK625159	NMP
		D1260	13 Morača river, Montenegro	N 41°57'46.8"	E 18°24'19.7"	MK619464	MK625160	NMP
		D1261	13 Morača river, Montenegro	N 41°57'46.8"	E 18°24'19.7"	MK619465	MK625161	NMP
		D1740	34 Kraljevica, Croatia	N 45°16'25.1"	E 14°34'13"	MK619466	MK625162	PMR
<i>P. quagga</i>	Quagga goby	D1741	34 Kraljevica, Croatia	N 45°16'25.1"	E 14°34'13"	MK619467	MK625163	PMR
Genus <i>Gobiusculus</i>								
<i>G. flavescens</i>	Two-spotted goby	BPS-0246	Concarneau, Brittany, France	N 47°52'32.5"	E 3°55'20.6"	MK619468	MK625164	MNHN
		D1825	Galicia, Spain	N 42°37'10.1"	E 7°51'47.2"	MK619469	MK625165	NMP
Genus <i>Pseudaphya</i>								
<i>P. ferreri</i>	Ferrer's goby	D835	Selce, Croatia	N 45°9'29.6"	E 14°43'14.4"	MK619470	MK625166	NMP
		D1428	Selce, Croatia	N 45°9'29.6"	E 14°43'14.4"	MK619471	MK625167	NMP

† •Endemic sand gobies of the Adriatic Sea

‡ * Šanda R., unpublished; ** Vukić et al. (2017)

§ FSJF: Jörg Freyhof's fish collection, Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany; NMP: Národní Muzeum, Prague, Czech Republic; ISEM: Institut des Sciences de l'Evolution de Montpellier, Montpellier, France; LBEG: Laboratory of Biodiversity and Evolutionary Genomics, Leuven, Belgium; MNHN: Museum National d'Histoire Naturelle, Paris, France; PMR: Prirodoslovni Muzej, Rijeka, Croatia.

TABLE S2 Initial hypotheses for species delimitation analysis using BPP. All tested combinations of priors for ancestral population size (θ s) and root age (τ_0) are presented. Number of putative species for each hypothesis is indicated in brackets. Values in grey are for posterior probability > 0.95 obtained for both runs.

θ ($\alpha - \beta$) and τ ($\alpha - \beta$) priors	Phylogenetic hypothesis 1 (20)	Phylogenetic hypothesis 2 (16)	bPTP hypothesis (25)	bGMYC hypothesis (21)
1- 10/ 1 - 100	0.71 / 0.72	0.84 / 0.86	0.00 / 0.00	0.00 / 0.00
1 - 100 / 1 - 1000	0.95 / 0.97	0.98 / 0.98	0.02 / 0.01	0.03 / 0.03
2 - 20 / 2 - 200	0.42 / 0.40	0.70 / 0.73	0.00 / 0.00	0.00 / 0.00
2 - 200 / 2 - 2000	0.99 / 0.96	0.99 / 0.94	0.02 / 0.03	0.00 / 0.08
1 - 10 / 2 - 200	0.74 / 0.73	0.86 / 0.84	0.00 / 0.00	0.00 / 0.01
1 - 100 / 2 - 2000	0.97 / 0.98	0.99 / 0.99	0.00 / 0.00	0.00 / 0.01
1 - 1000 / 2 - 2000	1.00 / 0.99	1.00 / 1.00	0.00 / 0.02	0.03 / 0.05
1 - 1000 / 1 - 1000	0.99 / 1.00	1.00 / 1.00	0.00 / 0.03	0.03 / 0.07
2 - 2000 / 2 - 2000	1.00 / 1.00	1.00 / 1.00	0.01 / 0.03	0.00 / 0.00

TABLE S3 Intraspecific (in the diagonal with standard error in brackets) and interspecific (below the diagonal with standard error above the diagonal) genetic distances for Adriatic *Pomatoschistus* and *Knipowitschia* species and the outgroup from the concatenated (cytochrome b+rhodopsin 1) dataset.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1 <i>K. caucasica</i>	0.003 (0.001)	0.008	0.006	0.006	0.005	0.005	0.005	0.007	0.006	0.005	0.007	0.007	0.007	0.007	0.008	0.007	0.007	0.007	0.007	0.006	0.007	0.008
2 <i>K. croatica</i>	0.130	0.003 (0.001)	0.008	0.008	0.008	0.008	0.008	0.006	0.008	0.007	0.007	0.008	0.008	0.008	0.008	0.008	0.007	0.008	0.008	0.007	0.008	0.009
3 <i>K. goerneri</i>	0.067	0.130	0.005 (0.001)	0.005	0.003	0.005	0.005	0.008	0.005	0.005	0.007	0.007	0.007	0.007	0.007	0.008	0.008	0.007	0.008	0.006	0.008	0.008
4 <i>K. nemere</i>	0.064	0.130	0.038	0.001 (0.001)	0.005	0.005	0.004	0.008	0.004	0.005	0.006	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.006	0.007	0.008
5 <i>K. milleri</i>	0.066	0.129	0.020	0.042	0.005 (0.002)	0.005	0.005	0.008	0.005	0.005	0.007	0.007	0.007	0.007	0.007	0.007	0.008	0.008	0.007	0.007	0.008	0.008
6 <i>K. montenegrina</i>	0.058	0.130	0.050	0.050	0.054	0.001 (0.000)	0.004	0.008	0.005	0.003	0.006	0.007	0.007	0.007	0.008	0.007	0.008	0.007	0.008	0.006	0.008	0.008
7 <i>K. panitzsae</i>	0.057	0.131	0.054	0.052	0.057	0.026	0.006 (0.001)	0.007	0.005	0.004	0.006	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.008	0.006	0.007	0.008
8 <i>K. punctatissima</i>	0.125	0.069	0.128	0.130	0.127	0.126	0.128	0.003 (0.001)	0.008	0.007	0.007	0.007	0.008	0.007	0.007	0.007	0.007	0.007	0.008	0.008	0.007	0.008
9 <i>K. thesada</i>	0.064	0.132	0.049	0.035	0.048	0.053	0.136	0.001 (0.000)	0.005	0.006	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.008	0.008
10 <i>K. sp.</i>	0.055	0.127	0.051	0.051	0.051	0.017	0.029	0.124	0.054	0.001 (0.001)	0.006	0.007	0.007	0.007	0.007	0.008	0.007	0.008	0.006	0.007	0.008	0.008
11 <i>P. bathi</i>	0.098	0.131	0.108	0.109	0.113	0.108	0.109	0.131	0.110	0.109	0.042 (0.004)	0.006	0.007	0.007	0.007	0.007	0.007	0.006	0.007	0.006	0.008	0.008
12 <i>P. conestrinii</i> 2	0.126	0.126	0.131	0.128	0.130	0.127	0.130	0.120	0.132	0.129	0.123	0.025 (0.003)	0.005	0.005	0.008	0.006	0.007	0.007	0.006	0.007	0.007	0.007
13 <i>P. conestrinii</i> 1	0.120	0.116	0.115	0.118	0.117	0.120	0.125	0.118	0.123	0.122	0.123	0.057	0.000 (0.000)	0.003	0.008	0.006	0.008	0.008	0.004	0.007	0.008	0.008
14 <i>P. conestrinii</i>	0.118	0.117	0.115	0.114	0.117	0.119	0.124	0.114	0.121	0.119	0.123	0.064	0.016	0.002 (0.001)	0.008	0.006	0.007	0.008	0.004	0.007	0.008	0.008
15 <i>P. knerii</i>	0.134	0.147	0.127	0.130	0.131	0.135	0.134	0.138	0.138	0.137	0.124	0.124	0.133	0.130	0.001 (0.001)	0.008	0.007	0.008	0.008	0.007	0.007	0.009
16 <i>P. marmoratus</i>	0.122	0.141	0.124	0.126	0.125	0.126	0.124	0.136	0.126	0.125	0.128	0.102	0.125	0.124	0.129	0.010 (0.001)	0.007	0.007	0.006	0.007	0.007	0.008
17 <i>P. minutus</i>	0.124	0.133	0.120	0.122	0.122	0.127	0.127	0.135	0.127	0.127	0.119	0.122	0.124	0.125	0.118	0.125	0.014 (0.002)	0.005	0.008	0.007	0.007	0.008
18 <i>P. minutus</i> "Venice"	0.125	0.135	0.123	0.126	0.126	0.130	0.130	0.137	0.127	0.130	0.125	0.114	0.122	0.125	0.118	0.125	0.068	0.009 (0.002)	0.008	0.007	0.008	0.007
19 <i>P. montenegrensis</i>	0.123	0.114	0.122	0.121	0.124	0.120	0.126	0.115	0.125	0.123	0.123	0.078	0.036	0.036	0.133	0.126	0.126	0.123	0.002 (0.001)	0.007	0.008	0.008
20 <i>P. quagga</i>	0.100	0.130	0.104	0.105	0.110	0.102	0.103	0.135	0.105	0.105	0.095	0.124	0.125	0.126	0.130	0.123	0.121	0.121	0.125	0.001 (0.001)	0.008	0.007
21 <i>Gobiusculus</i>	0.129	0.140	0.131	0.133	0.133	0.131	0.130	0.135	0.140	0.130	0.131	0.127	0.130	0.127	0.112	0.129	0.115	0.121	0.130	0.133	0.008 (0.002)	0.007
22 <i>Pseudaphya</i>	0.149	0.157	0.148	0.149	0.156	0.156	0.157	0.152	0.155	0.154	0.143	0.142	0.146	0.144	0.140	0.145	0.147	0.144	0.145	0.144	0.137	0.016 (0.003)

TABLE S4 Intraspecific (in the diagonal with standard error in brackets) and interspecific (below the diagonal with standard error above the diagonal) genetic distances for Adriatic *Pomatoschistus* and *Knipowitschia* species and the outgroup from the *cytochrome b* gene dataset.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1 <i>K. caucasica</i>	0.005 (0.001)	0.012	0.009	0.009	0.009	0.008	0.008	0.011	0.008	0.009	0.010	0.010	0.011	0.010	0.010	0.010	0.010	0.011	0.011	0.010	0.010	0.011
2 <i>K. croatica</i>	0.200	0.005 (0.002)	0.011	0.011	0.012	0.011	0.011	0.008	0.011	0.010	0.012	0.011	0.010	0.009	0.011	0.012	0.010	0.011	0.010	0.011	0.011	0.011
3 <i>K. goerneri</i>	0.100	0.196	0.007 (0.002)	0.006	0.005	0.008	0.007	0.010	0.006	0.008	0.010	0.011	0.010	0.010	0.012	0.010	0.010	0.009	0.010	0.009	0.010	0.012
4 <i>K. mermere</i>	0.094	0.194	0.055	0.001 (0.001)	0.007	0.007	0.007	0.010	0.006	0.008	0.009	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.009	0.011	0.012
5 <i>K. milleri</i>	0.100	0.196	0.031	0.062	0.009 (0.003)	0.008	0.007	0.011	0.007	0.008	0.011	0.011	0.010	0.011	0.012	0.010	0.010	0.010	0.011	0.010	0.011	0.012
6 <i>K. montenegrina</i>	0.086	0.196	0.080	0.073	0.087	0.001 (0.001)	0.005	0.011	0.008	0.004	0.011	0.010	0.010	0.010	0.012	0.010	0.010	0.010	0.011	0.009	0.011	0.012
7 <i>K. panizae</i>	0.084	0.197	0.086	0.077	0.091	0.041	0.010 (0.002)	0.011	0.007	0.005	0.010	0.009	0.010	0.010	0.010	0.010	0.009	0.009	0.012	0.009	0.010	0.011
8 <i>K. punctatissima</i>	0.186	0.101	0.185	0.187	0.186	0.182	0.186	0.004 (0.001)	0.010	0.011	0.011	0.011	0.010	0.010	0.012	0.011	0.010	0.011	0.010	0.011	0.010	0.011
9 <i>K. thessala</i>	0.096	0.197	0.076	0.050	0.075	0.084	0.082	0.199	0.000 (0.000)	0.008	0.009	0.010	0.009	0.010	0.010	0.010	0.010	0.009	0.011	0.009	0.010	0.011
10 <i>K. sp.</i>	0.081	0.191	0.081	0.076	0.083	0.027	0.045	0.178	0.084	0.002 (0.001)	0.010	0.010	0.010	0.010	0.010	0.011	0.010	0.009	0.011	0.009	0.011	0.012
11 <i>P. bathi</i>	0.150	0.202	0.163	0.164	0.174	0.164	0.164	0.195	0.165	0.165	0.064 (0.007)	0.011	0.010	0.010	0.009	0.009	0.010	0.010	0.010	0.010	0.010	0.012
12 <i>P. canestrinii</i> 2	0.193	0.195	0.195	0.191	0.197	0.190	0.195	0.183	0.196	0.193	0.188	0.040 (0.006)	0.008	0.008	0.010	0.008	0.011	0.010	0.009	0.010	0.010	0.011
13 <i>P. canestrinii</i> 1	0.183	0.180	0.170	0.175	0.175	0.177	0.186	0.179	0.182	0.182	0.189	0.092	0.000 (0.000)	0.005	0.011	0.009	0.010	0.010	0.007	0.009	0.010	0.011
14 <i>P. canestrinii</i>	0.180	0.182	0.170	0.169	0.175	0.177	0.185	0.173	0.180	0.178	0.188	0.103	0.025	0.003 (0.001)	0.011	0.009	0.009	0.010	0.007	0.010	0.009	0.011
15 <i>P. knerii</i>	0.192	0.215	0.180	0.185	0.188	0.194	0.192	0.201	0.198	0.196	0.179	0.179	0.193	0.189	0.002 (0.001)	0.011	0.010	0.010	0.011	0.011	0.011	0.011
16 <i>P. marmoratus</i>	0.187	0.220	0.186	0.188	0.190	0.189	0.187	0.205	0.189	0.189	0.197	0.155	0.193	0.191	0.185	0.015 (0.003)	0.010	0.010	0.011	0.008	0.010	0.010
17 <i>P. minutus</i>	0.177	0.192	0.170	0.174	0.176	0.182	0.182	0.188	0.180	0.181	0.174	0.175	0.177	0.180	0.165	0.182	0.017 (0.003)	0.008	0.011	0.009	0.010	0.012
18 <i>P. minutus</i> "Venice"	0.179	0.196	0.175	0.182	0.181	0.187	0.187	0.191	0.181	0.186	0.184	0.163	0.175	0.180	0.166	0.181	0.105	0.015 (0.003)	0.010	0.009	0.010	0.011
19 <i>P. montenegrensis</i>	0.190	0.177	0.181	0.180	0.186	0.179	0.188	0.174	0.185	0.184	0.188	0.124	0.055	0.057	0.194	0.194	0.182	0.177	0.004 (0.001)	0.010	0.010	0.012
20 <i>P. quagga</i>	0.150	0.198	0.154	0.156	0.166	0.152	0.153	0.198	0.156	0.156	0.148	0.187	0.187	0.190	0.187	0.187	0.176	0.178	0.188	0.002 (0.001)	0.010	0.011
21 <i>Gobiusculus</i>	0.187	0.209	0.192	0.193	0.197	0.192	0.190	0.198	0.204	0.192	0.194	0.187	0.191	0.187	0.162	0.190	0.163	0.173	0.193	0.193	0.011 (0.003)	0.009
22 <i>Pseudaphya</i>	0.208	0.227	0.208	0.211	0.221	0.220	0.221	0.212	0.218	0.218	0.203	0.201	0.206	0.205	0.196	0.205	0.204	0.201	0.206	0.204	0.195	0.022 (0.004)

TABLE S5 Intraspecific (in the diagonal with standard error in brackets) and interspecific (below the diagonal with standard error above the diagonal) genetic distances for Adriatic *Pomatoschistus* and *Knipowitschia* species and the outgroup from the *rhodopsin 1* gene dataset.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1 <i>K. caucasica</i>	0.001 (0.001)	0.005	0.004	0.004	0.004	0.004	0.004	0.005	0.004	0.004	0.004	0.005	0.005	0.005	0.007	0.005	0.007	0.007	0.005	0.004	0.007	0.007
2 <i>K. croatica</i>	0.018	0.000 (0.000)	0.006	0.006	0.006	0.006	0.006	0.005	0.006	0.006	0.005	0.004	0.004	0.004	0.008	0.004	0.007	0.007	0.004	0.006	0.007	0.007
3 <i>K. goerneri</i>	0.014	0.025	0.003 (0.002)	0.003	0.001	0.001	0.002	0.007	0.003	0.001	0.005	0.006	0.006	0.006	0.007	0.005	0.007	0.007	0.006	0.006	0.006	0.007
4 <i>K. mermere</i>	0.016	0.027	0.010	0.000 (0.000)	0.003	0.004	0.004	0.006	0.004	0.004	0.005	0.006	0.006	0.006	0.007	0.005	0.007	0.007	0.006	0.005	0.007	0.007
5 <i>K. milleri</i>	0.012	0.023	0.002	0.010	0.000 (0.000)	0.001	0.001	0.007	0.002	0.001	0.005	0.006	0.006	0.006	0.007	0.005	0.007	0.007	0.006	0.005	0.006	0.007
6 <i>K. montenegrina</i>	0.013	0.024	0.002	0.011	0.001	0.000 (0.000)	0.002	0.007	0.003	0.000	0.005	0.006	0.007	0.006	0.007	0.006	0.007	0.007	0.007	0.006	0.007	0.007
7 <i>K. panizae</i>	0.014	0.024	0.004	0.012	0.002	0.003	0.001 (0.001)	0.006	0.003	0.002	0.005	0.006	0.007	0.006	0.007	0.006	0.007	0.007	0.007	0.005	0.006	0.007
8 <i>K. punctatissima</i>	0.027	0.017	0.037	0.038	0.034	0.036	0.033	0.001 (0.001)	0.006	0.007	0.006	0.004	0.004	0.004	0.007	0.005	0.008	0.008	0.004	0.006	0.007	0.008
9 <i>K. thessala</i>	0.012	0.024	0.006	0.011	0.004	0.004	0.005	0.033	0.001 (0.001)	0.003	0.005	0.006	0.006	0.006	0.007	0.005	0.007	0.007	0.006	0.005	0.007	0.007
10 <i>K. sp.</i>	0.013	0.024	0.002	0.011	0.001	0.000	0.003	0.036	0.004	0.000 (0.000)	0.005	0.006	0.007	0.006	0.007	0.006	0.007	0.007	0.007	0.006	0.007	0.007
11 <i>P. bathi</i>	0.015	0.017	0.019	0.020	0.017	0.018	0.019	0.029	0.018	0.018	0.006 (0.003)	0.005	0.005	0.005	0.007	0.004	0.006	0.006	0.005	0.003	0.007	0.007
12 <i>P. canestrinii</i> 2	0.018	0.013	0.027	0.026	0.025	0.026	0.026	0.019	0.026	0.026	0.018	0.001 (0.001)	0.001	0.001	0.007	0.004	0.007	0.007	0.002	0.005	0.007	0.006
13 <i>P. canestrinii</i> 1	0.018	0.014	0.028	0.027	0.025	0.027	0.027	0.020	0.027	0.027	0.018	0.001	0.000 (0.000)	0.001	0.007	0.004	0.007	0.007	0.002	0.005	0.007	0.007
14 <i>P. canestrinii</i>	0.017	0.013	0.026	0.025	0.024	0.025	0.026	0.019	0.025	0.025	0.017	0.001	0.001	0.000 (0.000)	0.007	0.004	0.007	0.007	0.002	0.005	0.007	0.006
15 <i>P. knerii</i>	0.040	0.038	0.042	0.041	0.040	0.041	0.041	0.036	0.038	0.041	0.037	0.036	0.037	0.035	0.000 (0.000)	0.008	0.007	0.007	0.007	0.007	0.007	0.007
16 <i>P. marmoratus</i>	0.017	0.014	0.024	0.026	0.022	0.023	0.023	0.025	0.023	0.023	0.016	0.016	0.017	0.015	0.039	0.002 (0.001)	0.006	0.007	0.004	0.005	0.007	0.007
17 <i>P. minutus</i>	0.038	0.038	0.040	0.038	0.038	0.039	0.039	0.049	0.039	0.039	0.031	0.037	0.038	0.036	0.042	0.034	0.008 (0.003)	0.003	0.007	0.006	0.007	0.008
18 <i>P. minutus</i> "Venice"	0.037	0.037	0.039	0.035	0.037	0.038	0.038	0.048	0.038	0.038	0.030	0.036	0.037	0.035	0.041	0.033	0.007	0.000 (0.000)	0.007	0.006	0.007	0.008
19 <i>P. montenegrensis</i>	0.017	0.013	0.026	0.025	0.024	0.025	0.026	0.019	0.025	0.025	0.017	0.004	0.004	0.003	0.034	0.015	0.036	0.035	0.000 (0.000)	0.006	0.006	0.006
20 <i>P. quagga</i>	0.019	0.021	0.024	0.023	0.021	0.023	0.023	0.033	0.022	0.023	0.010	0.023	0.024	0.023	0.037	0.020	0.033	0.030	0.023	0.000 (0.000)	0.007	0.007
21 <i>Gobiusculus</i>	0.035	0.029	0.033	0.036	0.030	0.032	0.032	0.035	0.035	0.032	0.031	0.030	0.030	0.029	0.032	0.031	0.038	0.037	0.029	0.035	0.001 (0.001)	0.007
22 <i>Pseudaphya</i>	0.053	0.045	0.053	0.049	0.051	0.052	0.052	0.057	0.051	0.052	0.047	0.047	0.048	0.047	0.049	0.047	0.055	0.054	0.047	0.048	0.044	0.007 (0.003)