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

3

4 Running title: Adriatic sand goby phylogeny

5

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28

29 **Keywords**

30 Cryptic diversity, endemism, *Knipowitschia*, *Pomatoschistus*, phylogeny

31

32 **Abstract**

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

52

53 1. INTRODUCTION

54 The Mediterranean Sea is considered a hotspot of biodiversity (e.g., Bianchi et al.,
55 2012; Coll et al., 2010; Cuttelod, García, Malak, Temple, & Katariya, 2009; Médail &
56 Quézel, 1999; Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000). While it
57 represents only 0.82% and 0.32% of the global oceanic surface area and volume, respectively
58 (Bianchi & Morri, 2000), it hosts at least 7% (\approx 17,000 species) of the known global marine
59 biodiversity (Coll et al., 2010, 2012), and is characterized by a high level of endemism
60 (Bianchi et al., 2012; Coll et al., 2010). This biodiversity was fostered by the complex and
61 dynamic geological history of the area, from the closure of the Tethys via the Messinian
62 Salinity Crisis (MSC), to the Last Pleistocene Glaciation (Bianchi & Morri, 2000; Bianchi et
63 al., 2012). These past events separated the Mediterranean Sea into several biogeographic
64 regions, including the Adriatic bioregion (Bianchi & Morri, 2000; Coll et al., 2010; Hattab et
65 al., 2015). The Adriatic Sea is a semi-enclosed shallow sea connected to the Ionian Sea via
66 the Strait of Otranto. This narrow connection with the rest of the Mediterranean Sea favours
67 isolation, which may help explain the high levels of species richness, especially for endemic
68 fishes (Coll et al., 2010, 2012). Unfortunately, the Adriatic Sea is also one of the most
69 impacted by human activities (pollution, eutrophication, habitat degradation, and exploitation
70 of marine resources, and climate change) on its coastal areas and shelves (Ben Rais Lasram &
71 Mouillot, 2009; Coll, Santojanni, Palomera, Tudela, & Arneri, 2007; Ben Rais Lasram,
72 Guilhaumon, Albouy, Somot, Thuiller, & Mouillot, 2010; Coll et al., 2012; Lotze, Coll, &
73 Dunne, 2011).

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

87 Unfortunately, Adriatic sand gobies are under cumulative threats (Coll et al., 2012; Franco,
88 Fiorin, Franzoi, & Torricelli, 2005; Miller, 1990; Smith & Darwall, 2006; Spinelli, De
89 Matteo, Costagliola, Giacobbe, & Kovačić, 2017). Several keys for gobiid identification,
90 notably gobiids of the Adriatic Sea, have been proposed on the basis of morphological
91 characters, but difficulties persist in the identification of some species (Miller, 1986; Kovačić,
92 2008). The molecular phylogeny of the sand gobies has been partially investigated (Agorreta
93 et al., 2013; Huyse, Van Houdt, & Volckaert, 2004; Thacker, Gkenas, Triantaphyllidis,
94 Malavasi, & Leonardos, 2019; Vanhove et al., 2012), and has supported monophyly.
95 However, relationships between genera and species are poorly resolved, and based on
96 mitochondrial markers, the monophyly of both *Pomatoschistus* and *Knipowitschia* (Geiger et
97 al., 2014; Huyse et al., 2004; Penzo, Gandolfi, Bargelloni, Colombo, & Patarnello, 1998;
98 Thacker et al., 2019) is doubted.

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

110

111 **2. MATERIAL AND METHODS**

112

113 **2.1 Sampling and DNA analyses**

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

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

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

125 DNA extractions were performed from 96% ethanol-preserved tissue samples using
126 standard procedures (Sambrook, Fritsch, & Maniatis, 1989). Complete *cytb* and partial *rh1*
127 were PCR-amplified ($T_m = 50^\circ\text{C}$ and 49°C , respectively) using L15267 5'-
128 AATGACTTGAAGAACCACCGT-3' (Briolay, Galtier, Brito, & Bouvet, 1998) and
129 GobCB2R 5'- GGGAGGRDTTTAACCTCCG-3' (this study) as external primers, and when
130 necessary, PomCB1F 5'- TTCCCCTTTGTAGTACTAGCAG-3' (this study) and GobCB3R
131 5'- GGGTGRAAGGGRATYTTGTC-3' (this study) as internal primers for *cytb*. Finally,
132 PminRh1F 5'- GCGCCTACATGTTCTTCCTT-3' (Larmuseau, Raeymaekers, Ruddick, Van
133 Houdt, & Volckaert, 2009) and Rh1039r 5'- TGCTTGTTTCATGCAGATGTAGA-3' (Chen,
134 Bonillo, & Lecointre, 2003) were used for *rh1*. Amplicon sizes were 1220 bp and 820 bp for
135 *cytb* and *rh1*, respectively. Direct sequencing was carried out in both directions with the
136 technical facilities of the genotyping and sequencing platform
137 (<https://genseq.umontpellier.fr/WordPress/>) of the "Institut des Sciences de l'Evolution de
138 Montpellier" (ISEM, Montpellier, France). Sequences were aligned by hand using MEGA v7
139 (Kumar, Stecher, & Tamura, 2016).

140

141 **2.2 Phylogenetic reconstructions and species delimitation**

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

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

161 DNA-sequence-based species delimitation methods were performed to test whether
162 their outcome would be in accordance with present taxonomy. Several species trees were thus
163 considered as initial hypotheses: a tree reconstructed from consensus *cytb+rh1* sequences
164 obtained for each genetically identified species; a tree reconstructed from consensus *cytb*
165 sequences obtained for putative species identified with bPTP (<http://species.h-its.org/ptp/>;
166 Zhang, Kapli, Pavlidis, & Stamatakis, 2013); a tree reconstructed from consensus *cytb*
167 sequences obtained for putative species identified with bGMYC
168 (<https://sites.google.com/site/noahmreid/software>; Reid & Carstens, 2012). These hypotheses
169 were thus tested with the multi-gene coalescent-based method implemented in BPP v3.3
170 (Yang, 2015; Yang & Rannala, 2010). Several combinations of priors for ancestral population
171 size (θ s: $\alpha = 1, 2, 10, 20, 100, 200$; $\beta = 10, 20, 100, 200, 1000, 2000$) and root age (τ_0 : $\alpha = 1,$
172 $2, 10, 20, 100, 200$; $\beta = 10, 20, 100, 200, 1000, 2000$) were tested. For each test, the other
173 priors were the following: speciesdelimitation = 1, speciestree = 1, speciesmodelprior = 1,
174 algorithm = 0, finetune $\epsilon = 2$, usedata = 1, locusrate = 1, heredity = 2 (scalar values = 0.25 for
175 mitochondrial marker and 1 for nuclear marker) and cleandata = 0. Finetune variables were
176 automatically adjusted, and swapping rates for each parameter were checked for
177 recommended values (0.10-0.80) (Yang, 2016). Each analysis was run twice to confirm
178 consistency between runs. In all cases, the analyses were based on the *cytb+rh1* dataset.

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

181

182 **2.3 Divergence time estimates**

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

189 our data, they were compared using the Akaike's information criterion through MCMC
190 (AICM; Raftery, Newton, Satagopan, & Krivitsky, 2007) with Tracer v1.6 (Rambaut,
191 Suchard, Xie, & Drummond, 2014). For sand gobies, otoliths are the most common fossil
192 remains, while skeletal remains are scarce (Bratishko, Schwarzhans, Reichenbacher,
193 Vernyhorova, & Coric, 2015; Gierl & Reichenbacher, 2017; Schwarzhans, 2014,
194 Schwarzhans et al., 2017). For this reason, some authors performed otolith-based dating using
195 the ages of *P. bunyatovi* and *K. suavis* as calibration points. These species are considered the
196 earliest *Pomatoschistus* (16 Myr) and *Knipowitschia* (13 Myr), respectively (Thacker et al.,
197 2019). Phylogenetic relationships of these extinct species with the extant *Pomatoschistus* and
198 *Knipowitschia* species are not yet established. Associating fossil sand goby otoliths with extant
199 species is difficult because of potentially multiple homoplasies emerging from the shaping of
200 the otolith morphology (Schwarzhans, 2014; Schwarzhans et al., 2017), even if the
201 combination of qualitative (morphology) and quantitative (morphometry) approaches seem
202 promising (Gierl, Liebl, Šanda, Vukić, Esmaceli, & Reichenbacher, 2018; Gut, Vukić, Šanda,
203 Moritz, & Reichenbacher, 2020). Moreover, several previously published studies (Huysse et
204 al., 2004; Penzo et al., 1998; Thacker et al., 2019; Vanhove et al., 2012) demonstrated that
205 these sand goby groups are not monophyletic, and that some *Pomatoschistus* and
206 *Knipowitschia* species belong to different genera (respectively, *Ninnigobius* and *Orsinigobius*;
207 Geiger et al., 2014; Thacker et al., 2019). It thus seems very difficult to properly locate these
208 calibration points in a sand goby molecular phylogeny if phylogenetic relationships between
209 extant and extinct species are not clearly identified. For these reasons, fossils were not used
210 for calibration, but instead two scenarios were investigated on the basis of the *cytb* mutation
211 rate. Rocha, Robertson, Rocha, Van Tassell, Craig, & Bowens (2005) estimated this rate from
212 *cytb* sequences of two sister species of the goby *Evorthodus*. These species are distributed
213 allopatrically on both sides of the Isthmus of Panama. According to Rocha et al. (2005), these
214 species were presumed to have diverged because of the final closure of this isthmus around
215 3.5 - 3.1 Myr. However, recent studies based on biogeographical and oceanic data, as well as
216 molecular dating suggest that the isthmus formed millions of years earlier, between 15 – 10
217 Myr (Erkens & Hoorn, 2016; O'Dea et al., 2016; Stange, Sánchez-Villagra, Salzburger, &
218 Matschiner, 2018). One rate (scenario 1) was thus fixed to $1.025 \pm 0.060\%$ / site / Myr (i.e.,
219 1.97 – 2.17% substitution / Myr, following Rocha et al., 2005; Keith, Lord, & Lorion, 2011)
220 and a log-normal distribution prior was used with $M = 0.01025$ and $S = 0.036$, while the other
221 rate (scenario 2) was fixed to $0.280 \pm 0.060\%$ / site / Myr (i.e., 0.23 – 0.34% substitution /
222 Myr, following Erkens & Hoorn, 2016; Stange et al., 2018) and a log-normal distribution

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

231

232 **3. RESULTS**

233

234 **3.1 Phylogenetic analyses and species limits**

235 Full alignments (Alignments S1-S2) were 1140 and 735 nucleotides long for *cytb* and
236 *rh1*, with 454 (40%) and 94 (13%) phylogenetically informative sites, respectively. All new
237 sequences were deposited in the GenBank database under the accession numbers MK619412-
238 71 for *cytb* and MK625102-67 for *rh1* (Table S1).

239 ML and BI analyses provided similar tree topologies (Figures 2 and S1-S2). The
240 Adriatic sand gobies, and especially the endemic species, are not a monophyletic group.
241 Indeed, *Pomatoschistus* and *Knipowitschia* appear as paraphyletic genera since *P. quagga*
242 (Heckel, 1837) and *P. bathi* Miller, 1982 are grouped together with all the *Knipowitschia*
243 species (BP = 99%; PP = 1.00), except *K. punctatissima* (Canestrini, 1864) and *K. croatica*
244 Mrakovčić, Kerovec, Mišetić, & Schneider, 1994 that are grouped with *P. montenegrensis*
245 Miller & Šanda, 2008 and *P. canestrinii* (Ninni, 1883) but with low support (BP = 57%; PP <
246 0.80) (Figure 2). *Pomatoschistus knerii* (Steindachner, 1861) is the first branch of the whole
247 group in our analysis. *Knipowitschia panizzae* (Verga, 1841), *K. radovici* Kovačić, 2005 and
248 *K. mrakovcici* Mrakovčić, Schneider, & Kerovec, 1994 formed a single clade (BP = 100%;
249 PP = 1.00). *Pomatoschistus canestrinii* is split into three lineages, one of them clustered with
250 *P. montenegrensis* Miller & Šanda, 2008 (BP = 89%; PP = 1.00). Three specimens of *P.*
251 *minutus* (Pallas, 1770) from the Venice lagoon (G0554, G1532 and G2011) were included in
252 the dataset: one grouped with *P. minutus* from Spain and France (BP = 100%; and PP = 1.00),
253 while the two other specimens formed a separate clade (BP = 100%; PP = 1.00).
254 *Pomatoschistus marmoratus* (Risso, 1810) is the sister group to *K. croatica* + *K.*
255 *punctatissima* / *P. canestrinii* + *P. montenegrensis* but with low support (BP < 50%; PP <
256 0.80). Because this node is the weakest in the tree, other alternative positions of *P.*

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

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

280 Genetic distances were estimated within and between species (phylogenetic hypothesis
281 1) of *Pomatoschistus*, *Knipowitschia* and the outgroup (Tables S3 to S5 for the *cytb+rh1*, *cytb*
282 and *rh1* datasets, respectively). In *Pomatoschistus* and *Knipowitschia* species, intragroup
283 distances were $0.0 \pm 0.0\%$ to $4.2 \pm 0.4\%$ for the concatenated dataset. In the outgroup, these
284 *cytb+rh1* distances were $0.8 \pm 0.2\%$ for *G. flavescens* and $1.6 \pm 0.3\%$ for *Pseudaphya ferreri*.
285 Intergroup distances were $1.6 \pm 0.3\%$ to $14.7 \pm 0.8\%$ for the concatenated dataset. These
286 *cytb+rh1* distances between the Adriatic sand gobies and the outgroup were $11.2 \pm 0.7\%$ to
287 $14.0 \pm 0.8\%$ for *G. flavescens* and $14.0 \pm 0.9\%$ to $15.7 \pm 0.9\%$ for *Pseudaphya ferreri*. Note
288 that *cytb+rh1* genetic distances between *P. montenegrensis* and the three *P. canestrinii*
289 lineages were from $3.6 \pm 0.4\%$ to $7.8 \pm 0.6\%$, while these distances between the three *P.*
290 *canestrinii* lineages were from $1.6 \pm 0.3\%$ to $6.4 \pm 0.5\%$. Lastly, the *cytb+rh1* distance

291 between the Venice lineage and the Spain+France+Venice lineage of *P. minutus* was $6.8 \pm$
292 0.5% .

293

294 **3.2 Divergence dates among Adriatic sand gobies**

295 Bayesian coalescent analyses were conducted under the GTR+I+G model for both *cytb*
296 and *rh1* dataset. The AICM suggested that the birth-death speciation model and relaxed
297 uncorrelated exponential clock provided the statistically best fit for our dataset in scenario 1,
298 while the Yule speciation model and relaxed uncorrelated exponential clock provided the
299 statistically best fit for our dataset in scenario 2. Results from three out of five independent
300 runs converged and were thus combined with LogCombiner for further analyses (Figures 3
301 and S3). Estimated clock rate for *rh1* was $0.968 \pm 0.7\%$ / site / Myr in the scenario 1 and 0.17
302 $\pm 0.02\%$ / site / Myr in the scenario 2.

303 In the scenario 1, times to the most recent common ancestor for the populations of
304 extant species included in our dataset are between 1.54 Myr for *P. bathi* and 0.09 Myr for *K.*
305 *montenegrina* Kovačić & Šanda, 2007, *K. thessala* (Vinciguerra, 1921), *K. mermere* Ahnelt,
306 1995 and one lineage of *P. canestrinii*. In the scenario 2, these times are between 6.57 Myr
307 for *P. bathi* and 0.40 Myr for *K. montenegrina*, *K. thessala* and *K. mermere*.

308

309 **4. DISCUSSION**

310

311 **4.1 Phylogenetic relationships of Adriatic sand gobies**

312 Discrepancies exist between the previously published results and the present one (see
313 below for details). These discrepancies can be explained by the diversity of evolutionary
314 proxies used for phylogenetic reconstructions, such as mitochondrial (*cytb*, *cytochrome c*
315 *oxidase 1*, and/or *12S* and *16S rRNA* genes) and/or nuclear (*rh1*, first intron of the ribosomal
316 protein *s7* gene or Internal Transcribed Spacer 1) markers, and/or morphological (meristic and
317 morphometric characters), behavioural (for instance, bioacoustics and courtship) and life
318 history (for instance, age at sexual maturity or longevity) traits (Huysse et al., 2004;
319 Knebelsberger & Thiel, 2014; Larmuseau, Huysse, Vancampenhout, Van Houdt, & Volckaert,
320 2010; Malavasi, Gkenas, Leonardos, Torricelli, & McLennan, 2012; Thacker et al., 2019;
321 Vanhove et al., 2012; Vukić, Kovačić, Zogaris, & Šanda, 2016; Vukić, Ulqini, & Šanda,
322 2017). However, the concatenation of *cytb* and *rh1* provided here a highly resolved phylogeny
323 at the interspecific level.

324 Several molecular genetic studies have pointed to monophyly of the sand gobies sensu
325 lato (Agorreta et al., 2013; Huyse et al., 2004; Thacker et al., 2019; Vanhove et al., 2012),
326 with *Gobiusculus* Duncker, 1928 as a junior synonym of *Pomatoschistus* (Huyse et al., 2004;
327 Knebelsberger & Thiel, 2014; Larmuseau et al., 2010; Thacker et al., 2019; Vanhove et al.,
328 2012). In the present study, the dataset, including the genera *Pomatoschistus*, *Knipowitschia*
329 and *Gobiusculus*, allowed us to confirm this monophyly (Figure 2), where *Gobiusculus*
330 appeared as the sister species to the rest of the sand gobies. Obviously, these results should be
331 taken with caution since *Economidichthys* Bianco, Bullock, Miller, & Roubal, 1987 was not
332 included in our dataset.

333 As already reported previously (Huyse et al., 2004; Malavasi et al., 2012; Penzo et al.,
334 1998; Thacker et al., 2019; Vanhove et al., 2012), our data show that the genera
335 *Pomatoschistus* and *Knipowitschia* are not monophyletic. *Knipowitschia* would be
336 monophyletic if the genus *Orsinigobius* Gandolfi, Marconato, & Torricelli, 1986 is
337 recognized for *K. punctatissima* and *K. croatica* as proposed by Geiger et al. (2014) and
338 Thacker et al. (2019). These authors also proposed to recognize the genus *Ninnigobius*
339 Whitley, 1951 for *P. montenegrensis* and *P. canestrinii* (*N. montenegrensis* and *N.*
340 *canestrinii*). In Huyse et al. (2004), Vanhove et al. (2012), and the present study, the clade
341 including *K. punctatissima* and *K. croatica* formed a monophyletic group with the clade
342 including *P. montenegrensis* and *P. canestrinii*, although this group is moderately supported
343 in ML (BP = 57%) in the present study. A phylogenetic analysis based on behavioural and life
344 history traits produced a tree suggesting a close relationship between *P. canestrinii* and *K.*
345 *punctatissima* (Malavasi et al., 2012). Even if several morphological features (mainly
346 colouration with small, intense, black spots) differ markedly in *P. canestrinii* and *P.*
347 *montenegrensis* compared to other *Pomatoschistus* species, the tendency for canal reduction
348 suggests that *P. canestrinii* might be a sister species of *Knipowitschia* species from the
349 Adriatic, Aegean and Ponto-Caspian catchments (Kovačić, 2005b; Miller, 2004; Miller &
350 Šanda, 2008). At this point, it is not possible to include with certainty *K. punctatissima* and *K.*
351 *croatica* in *Orsinigobius*, and *P. montenegrensis* and *P. canestrinii* in *Ninnigobius*, or even
352 consider that these species belong to a single genus.

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

358 might thus be considered as a junior synonym of *P. canestrinii*. However, both species are
359 distinguishable by three main morphological characters: (1) the presence of scales only on the
360 axilla, behind the pectoral fin in *P. montenegrensis*; (2) the occurrence of dark spots mainly
361 on the head in *P. montenegrensis*, and (3) the consistent loss of the pre-opercular and
362 posterior oculo-scapular head canals in *P. montenegrensis* (Miller and Šanda, 2008). Species
363 delimitation tests also supported the recognition of several species within this group (at least
364 four; $0.99 < PP < 1.00$). Genetic distances are relatively high between *P. montenegrensis* and
365 the three lineages of *P. canestrinii*: $5.5 \pm 0.7\%$ (D1744 and D1746), $5.7 \pm 0.7\%$ (D1747,
366 D1748 and D1749) and $12.4 \pm 0.9\%$ (G1594 and G1595) for *cytb* (Table S4). These distances
367 are similar to those observed between *K. thessala* and *K. mermere* ($5.0 \pm 0.6\%$) or *K.*
368 *mermere* and *K. goerneri* Ahnelt, 1991 ($5.5 \pm 0.6\%$) for the lowest values, and even superior
369 to the values observed between *K. panizzae* (including *K. radovici* and *K. mrakovcici*) and *K.*
370 *montenegrina* ($4.1 \pm 0.5\%$) or between *K. milleri* (Ahnelt & Bianco, 1990) and *K. goerneri*
371 ($3.1 \pm 0.5\%$). These genetic distances fall within the range observed for congeneric species of
372 fishes, and especially for pairs of sister species (mean *cytb* genetic distance = 12%; Johns &
373 Avise, 1998). From a morphological and genetic standpoint, the three lineages of *P.*
374 *canestrinii* should be considered sibling species. The type material of *P. canestrinii* is from
375 two geographically distinct localities (Venice, Italy and Jadro River near Split, Croatia,
376 central Adriatic), and no holotype was specifically designated (Ninni, 1883). It is thus
377 difficult to assign one of these lineages to this species. Careful revision of this species
378 complex is necessary to resolve its taxonomy.

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

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

393 A dichotomy between populations of *P. minutus* was already proposed in the early
394 1980s (Wallis & Beardmore, 1983), and subsequently repeatedly underlined (Boissin,
395 Hoareau, & Berrebi, 2011; Gysels, Hellemans, Patarnello, & Volckaert, 2004; Huyse et al.,
396 2004; Stefanni & Thorley, 2003). The Venice lineage is regarded as a separate Evolutionarily
397 Significant Unit (sensu Moritz, 1994) by Stefanni and Thorley (2003) and as a cryptic species
398 of *Pomatoschistus* by Gysels et al. (2004). The present study confirms this dichotomy but also
399 suggests a further split between the Venice specimens: one lineage consisting of the
400 specimens from Venice, France and Spain, and another consisting of only specimens from
401 Venice. Nothing is known about the ecological requirements of the two lineages from Venice,
402 such as if they occupy different depths, microhabitats, reproduction period or have different
403 feeding habits. The genetic distance on *cytb* ($10.5\% \pm 0.8\%$; Table S4) between the Venice
404 lineage and the other *P. minutus* is similar to those from Gysels et al. (2004). Stefanni,
405 Gysels, Volckaert and Miller (2003), and Gysels et al. (2004) also reported that the genetic
406 distance between the Adriatic and other *P. minutus* is similar to that between *P. minutus* and
407 its closest relative species (*P. lozanoi* and *P. norvegicus*). These genetic distances fall within
408 the range observed for congeneric species of fishes (Johns & Avise, 1998). Species
409 delimitation tests supported the existence of two clades within *P. minutus* (BPP PP = 1.00 for
410 both clades). Because of large genetic distance, results of species delimitation tests and
411 reciprocal monophyly between the Venice lineage and other *P. minutus*, the Venice lineage
412 should be considered an undescribed *Pomatoschistus* species. This conclusion was already
413 suggested by Boissin et al. (2011), but deserves further investigation.

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

425 *Syrrhothonus charrieri* Chabanaud, 1933 was proposed as a junior synonym of *P.*
426 *marmoratus*. However, in this latter case, alternative names exist, such as *Iljinia* de Buen,
427 1930, with its type species *P. microps* (Krøyer, 1838), a closely related species of *P.*
428 *marmoratus* (Geiger et al., 2014; Huyse et al., 2004; Thacker et al., 2019; Vanhove et al.,
429 2012). Since several key taxa are lacking in the present study, it seems premature to resurrect
430 some genera. A more integrative approach of this taxonomy should be promoted by including
431 appropriate taxon sampling (including type species of the genera and available type material),
432 morphological and molecular genetic proxies, as well as ecological and life history traits.

433

434 **4.2 Evolutionary history of Adriatic sand gobies**

435 The Adriatic sand gobies, and in particular the endemic species among them, are not
436 monophyletic. This suggests that the diversification of these gobies was seemingly related to
437 different past events. Several scenarios have been proposed to explain the evolutionary
438 history of the sand gobies. Thacker et al. (2019) suggested that episodes of diversification
439 among sand gobies are correlated to the Middle Miocene Tethys closure / Paratethys isolation
440 (18-12 Myr; Rögl, 1999) and the drawdown of the Mediterranean Sea during the Messinian
441 Salinity Crisis (5.97 – 5.33 Myr; Manzi et al., 2013). These authors excluded the possibility
442 that intra- or interspecific differentiation may correspond to Pleistocene glaciations.
443 According to Thacker et al. (2019), diversification in the freshwater lineages of sand gobies
444 (*P. montenegrensis*, *P. canestrinii*, *K. punctatissima*, *K. croatica*, *K. panizzae* and *K.*
445 *montenegrina*) occurred partly or totally in the Adriatic Sea during the MSC. In contrast,
446 other authors (Huyse et al., 2004; Penzo et al., 1998; Vanhove et al., 2012) considered that the
447 diversification of sand goby genera occurred during the Lago Mare phase (i.e., period of
448 environmental fluctuations due to repeated connections between the Mediterranean Sea and
449 the Paratethys; Roveri et al., 2014) of the MSC, while interspecific diversification coincided
450 with Pleistocene glaciations. According to Huyse et al. (2004), the Adriatic populations of
451 *Pomatoschistus* were then isolated after the sea-level drop in the Mediterranean Sea. Several
452 studies have demonstrated the importance of Pleistocene glaciations on population
453 differentiation and speciation events of sand gobies (e.g., Boissin et al., 2011; Gysels et al.,
454 2004; Larmuseau et al., 2009; Tougard et al., 2014) and other Atlantic and Mediterranean fish
455 species (Jenkins et al., 2018; Patarnello et al., 2007).

456 In the present study, two scenarios were investigated based on two different *cytb*
457 mutation rates ($1.025 \pm 0.060\%$ / site / Myr and $0.28 \pm 0.060\%$ / site / Myr). Although some
458 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 (Schwarzhan 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 (Schwarzhan 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

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

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

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

526

527 **5. CONCLUSIONS**

528

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

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

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

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

555

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569

570 **DATA AVAILABILITY STATEMENT**

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

575

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- 954
- 955
- 956

957 **Figure legends**

958

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

963

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

972

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

981

982

983 **SUPPORTING INFORMATION**

984

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

992

993 **Figure S2.** Maximum likelihood tree reconstructed from the *rhodopsin 1* gene dataset for
994 Adriatic *Pomatoschistus* and *Knipowitschia* species. Samples are detailed in Table1. Names
995 of the morphospecies are listed on the right. Species with “•” are endemic species of the
996 Adriatic Sea. Numbers at nodes are for bootstrap percentages ($\geq 50\%$) and posterior
997 probabilities (≥ 0.80). Black circles indicate nodes with BP = 100% and PP = 1.00. White
998 circles are for nodes with BP < 50% and PP < 0.80, while nodes with “-“ are weakly
999 supported either in maximum likelihood or in the Bayesian approach.

1000

1001 **Figure S3.** Species tree chronogram for Adriatic *Pomatoschistus* and *Knipowitschia* species
1002 estimated by *BEAST2 based on the *cytochrome b* and *rhodopsin 1* datasets (scenario 2).
1003 Values at nodes indicate the time (in Myr) to the most recent common ancestor, and in
1004 brackets, the 95% confidence intervals. Past events that could have had an impact on the
1005 evolutionary history of sand gobies and reported on Figure 3 are indicated in grey (Tethys
1006 closure and Paratethys isolation, and Messinian Salinity Crisis from Thacker et al., 2019;
1007 MSC and Pleistocene climatic cycles from Huyse et al., 2004; Penzo et al., 1998; Vanhove et
1008 al., 2012).

1009

1010 **Table S1.** Detailed list of sand goby species taken into account in the present study. Sampled
1011 localities, latitude and longitude coordinates, GenBank accession numbers for the *cytochrome*
1012 *b* and *rhodopsin 1* genes and tissue providers are indicated. Locality numbers mirror those of
1013 Figure 1.

1014

1015 **Table S2.** Initial hypotheses for species delimitation analysis using BPP. All tested
1016 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

1035 **Table legends**

1036

1037 **Table 1.** List of sand goby species analysed in the present study. Locality numbers mirror
1038 those of Figure 1. Species with “•” are endemic species of the Adriatic Sea. GenBank
1039 accession numbers for the *cytochrome b* and *rhodopsin 1* genes, and tissue providers are listed
1040 in Table S1. FSJF: Jörg Freyhof’s fish collection, Zoologische Forschungsmuseum Alexander
1041 Koenig, Bonn, Germany; ISEM: Institut des Sciences de l’Evolution, Montpellier, France;
1042 LBEG: Laboratory of Biodiversity and Evolutionary Genomics, Leuven, Belgium; MNHN:
1043 Museum National d’Histoire Naturelle, Paris, France; NMP: Czech Národní Muzeum, Prague,
1044 Czech Republic; PMR: Prirodoslovni Muzej, Rijeka, Croatia.

1045

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

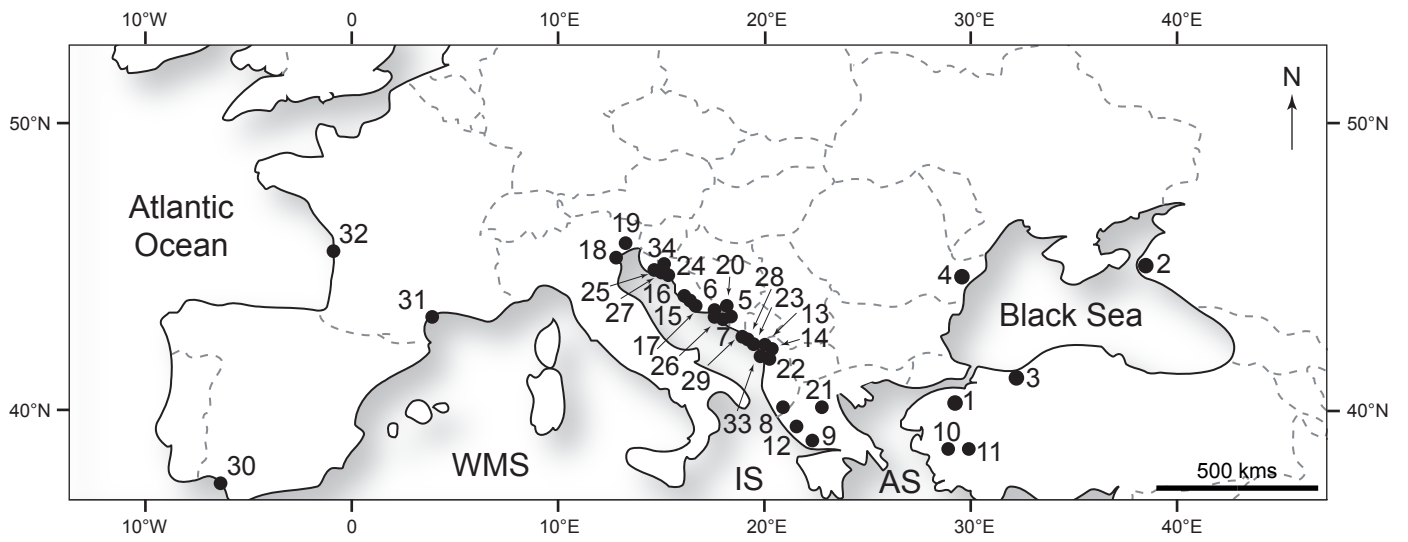
G. flavescens (Fabricius, 1779) - Two-spotted goby

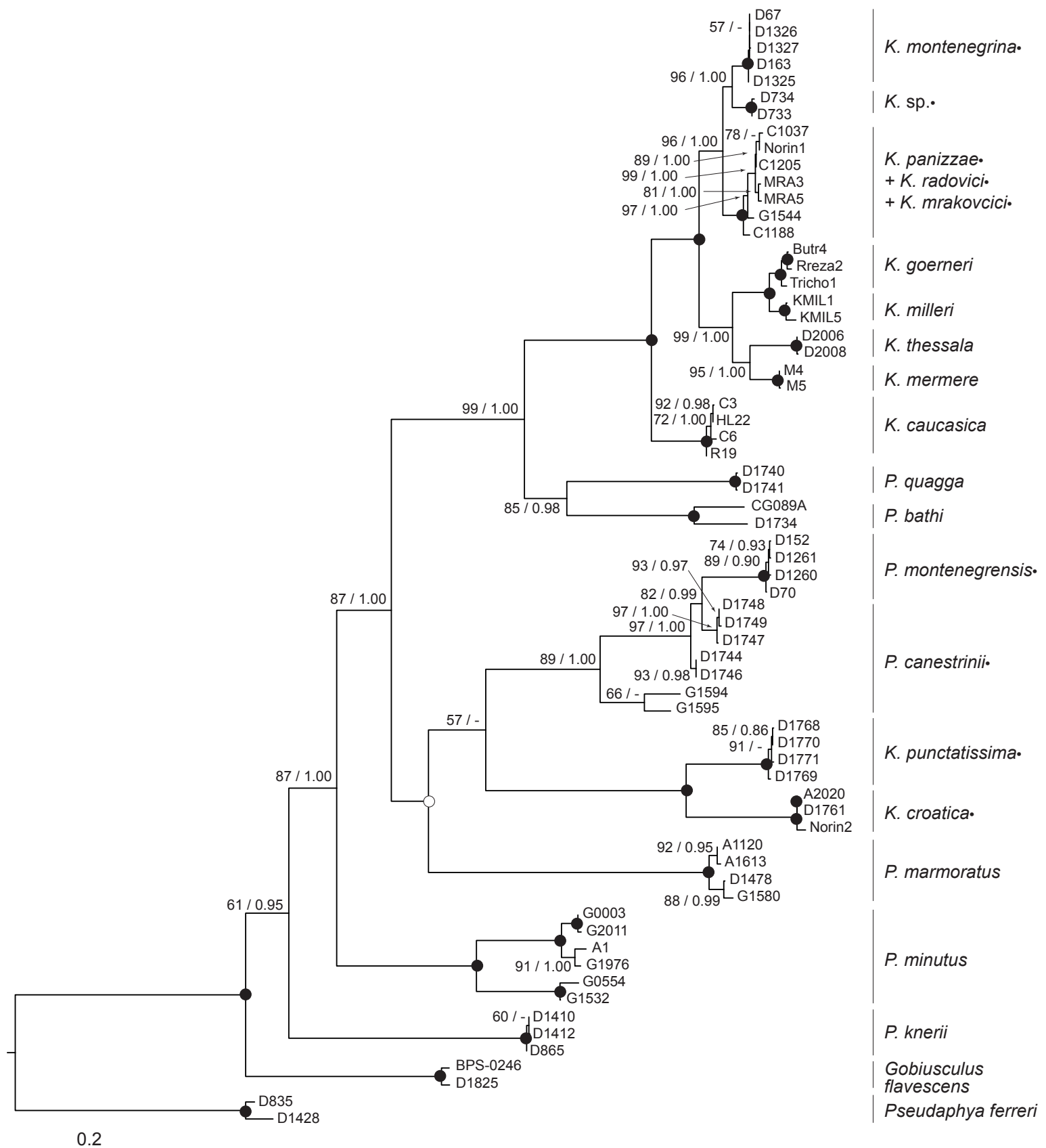
MNHN uncatalogued	BPS-0246	Concarneau, Brittany, France
NMP P6V 142728	D1825	Galicia, Spain

Genus *Pseudaphya* Iljin, 1930

P. ferreri (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

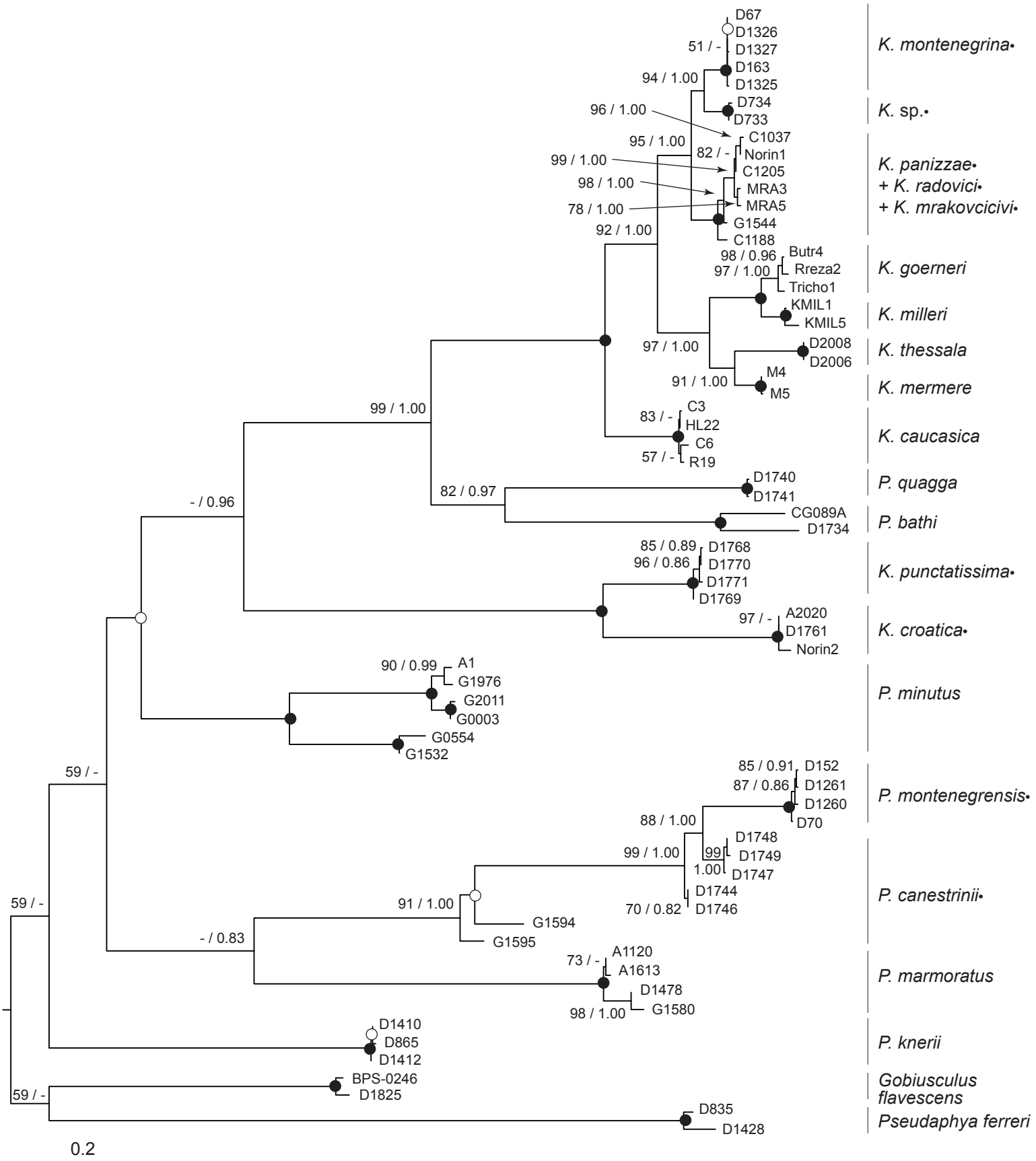


FIGURE S2

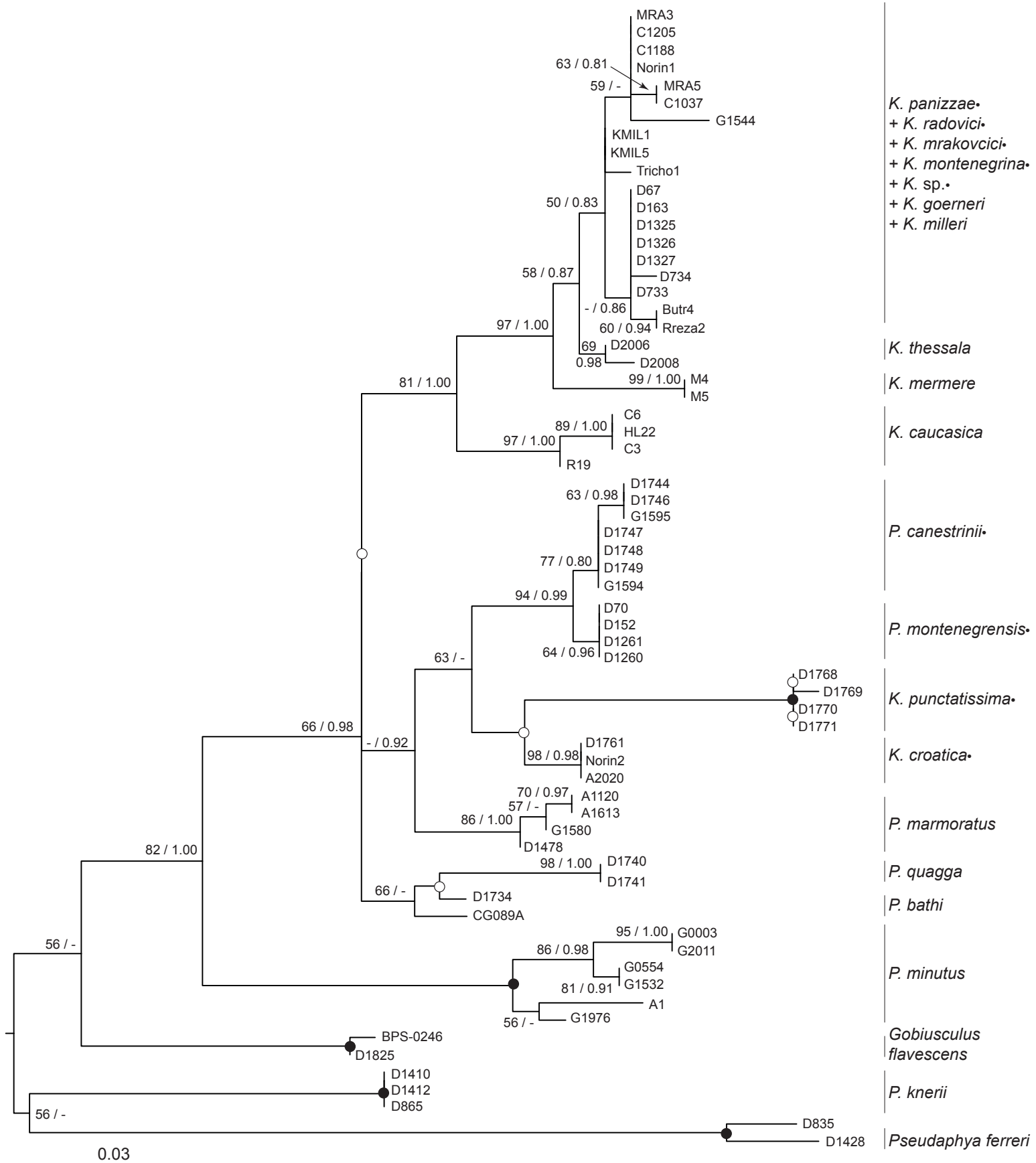


TABLE S1 Detailed list of sand goby species taken into account in the present study. Sampled localities, latitude and longitude coordinates, GenBank accession numbers for the *cytochrome b* and *rhodopsin 1* genes and tissue providers are indicated. Locality numbers mirror those of Figure 1.

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
<i>K. croatica</i> •†	Neretva dwarf goby	R19	4 Lake Babadag, Romania	N 44°53'34.5"	E 28°43'16"	KF214254*	MK625105	NMP
		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
<i>K. goerneri</i>	Corfu dwarf goby	Norin2	7 River Norin, Croatia	N 43°4'3.7"	E 17°36'41.3"	MK619416	MK625108	NMP
		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
<i>K. mermere</i>	Marmara goby	Tricho1	9 Lake Trichonis, Achelooos basin, Greece	N 38°35'20"	E 21°28'2"	KY462228**	MK625111	NMP
		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. panizzae</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, Piniios Basin, Greece	N 39°33'14"	E 21°46'8"	KF214247*	MK625132	NMP
		D2008	21 Litheos river, Piniios 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 Pomatoschistus								
<i>P. bathi</i>	-	CG089A	23 Kamenovo, Montenegro	N 42°16'29.6"	E 18°53'23.6"	MK619440	MK625136	NMP
		D1734	24 Unije, Croatia	N 44°38'48.8"	E 14°14'45.2"	MK619441	MK625137	PMR
<i>P. canestrinii</i> •	Canestrini's goby	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
		G1595	18 Venice, Italy	N 45°26'13.9"	E 12°20'4.5"	MK619448	MK625144	ISEM
<i>P. knerii</i>	Kner's goby	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
<i>P. montenegrensis</i> •	-	D70	33 Buna, Albania	N 41°57'36.58"	E 19°23'23.13"	MK619462	MK625158	NMP
		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
<i>P. quagga</i>	Quagga goby	D1740	34 Kraljevica, Croatia	N 45°16'25.1"	E 14°34'13"	MK619466	MK625162	PMR
		D1741	34 Kraljevica, Croatia	N 45°16'25.1"	E 14°34'13"	MK619467	MK625163	PMR
Genus Gobiussculus								
<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 Pseudaphya								
<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 *Kaipovitschia* species and the outgroup from the concatenated (cytochrome *b*+*rhdopsin 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	0.007	0.007	0.008	0.008	0.007	0.007	0.009
3 <i>K. goernerii</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	0.008	0.007	0.006	0.008	0.008
4 <i>K. menmere</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	0.008	0.007	0.007	0.007	0.007	0.007	0.007	0.008	0.008	0.007	0.008
9 <i>K. thessala</i>	0.064	0.132	0.049	0.035	0.048	0.054	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
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	0.007	0.006	0.007	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. constrictus</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. constrictus</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. constrictus</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	0.119	0.122	0.124	0.125	0.118	0.125	0.014 (0.002)	0.005	0.008	0.007	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	0.105	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>Pseudophya</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. panizzae</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	0.010	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	0.011	0.010	0.009	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	0.193	0.189	0.002 (0.001)	0.011	0.010	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. panizzae</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)