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1 Title: Phylogeny, hybridization, and life history evolution of *Rhinogobius* gobies in Japan, inferred  
2 from multiple nuclear gene sequences

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24

25 Abstract

26 *Rhinogobius* fishes (Gobiidae) are distributed widely in East and Southeast Asia, and represent  
27 the most species-rich group of freshwater gobies with diversified life histories (i.e.,  
28 amphidromous, fluvial, and lentic). To reveal their phylogenetic relationships and life history  
29 evolution patterns, we sequenced six nuclear and three mitochondrial DNA (mtDNA) loci from 18  
30 species, mainly from the mainland of Japan and the Ryukyu Archipelago. Our phylogenetic tree  
31 based on nuclear genes resolved three major clades, including several distinct subclades. The  
32 mtDNA and nuclear DNA phylogenies showed large discordance, which strongly suggested  
33 mitochondrial introgression through large-scale interspecific hybridization in these regions. On the  
34 basis of the molecular dating using geological data as calibration points, the hybridization  
35 occurred in the early to middle Pleistocene. Reconstruction of the ancestral states of life history  
36 traits based on nuclear DNA phylogeny suggests that the evolutionary change from  
37 amphidromous to freshwater life, accompanied by egg size change, occurred independently in at  
38 least three lineages. One of these lineages showed two life history alterations, i.e., from  
39 amphidromous (small egg) to fluvial (large egg) to lentic (small egg). Although more inclusive  
40 analysis using species outside Japan should be further conducted, the present results suggest the  
41 importance of the life history evolution associated with high adaptability to freshwater  
42 environments in the remarkable species diversification in this group. Such life history divergences  
43 may have contributed to the development of reproductive isolation.

44

45 Keywords

46 *Rhinogobius*, Life history, Introgressive hybridization, Speciation, Nuclear gene, Adaptation to a  
47 novel environment

48

49

50 1. Introduction

51 Colonization of a novel environment is one of the important factors promoting species  
52 diversification (Schluter, 2000; Yoder et al., 2010; Betancur-R. et al., 2012). It is predicted that  
53 new selective pressure occurring in novel habitats generates divergent natural selection between  
54 the ancestral population and the newly colonized population and promotes adaptation and  
55 subsequent ecological speciation (Schluter, 2009; Nosil, 2012). This process is thought to be one  
56 of the fundamental mechanisms of adaptive radiation (Schluter, 2000; Losos, 2009).

57 Migration is a key process for species diversification through colonization of a novel  
58 environment (Winker, 2000; McDowall, 2001). Migration promotes gene flow among populations,  
59 whereas it also leads to colonization of favorable habitats and consequent ecological divergence  
60 (Winker, 2000). Diadromous fishes provide representative cases for such a diversification  
61 mechanism along with life history evolution. Diadromous fishes that spawn in rivers are often  
62 isolated in freshwaters (i.e., landlocked), which leads to ecological diversification followed by  
63 speciation, as suggested in anadromous sticklebacks (McKinnon et al., 2004), amphidromous  
64 gobies (Katoh and Nishida, 1994), sculpins (Goto and Andoh, 1990), and galaxias (Waters et al.,  
65 2010). These evolutionary processes that accompany the diversification of freshwater–diadromous  
66 species, as well as those seen in lakes (e.g., Seehausen, 2006; Bernatchez et al., 2010), are the  
67 most remarkable examples of adaptive radiation in fishes (Lee and Bell, 1999; Vega and Wiens,  
68 2012; Betancur-R. et al., 2012, 2015).

69 The family Gobiidae (Order Gobiiformes; Betancur-R et al., 2013, 2014) is one of the most  
70 divergent groups among teleost fishes in terms of the number of species and ecology, and it has  
71 interested researchers in the fields of ecology and evolutionary biology (Yamada et al., 2009;  
72 Rüber and Agorreta, 2011). The genus *Rhinogobius* is widely distributed in freshwaters from East  
73 to Southeast Asia. It consists of more than 85 species, and it is the largest genus of freshwater  
74 gobies (Suzuki et al., 2004; Oijen et al., 2011). The species of this genus are classified into three

75 types based on migration-related life history. The amphidromous type is the most general form, in  
76 which larval fish flow down to the sea immediately after hatching in the river. This is followed by  
77 early feeding and growth at the sea, and then a return the river at the juvenile stage for subsequent  
78 growth and reproduction (Mizuno, 2001; Keith and Lord, 2011). Lentic types complete their life  
79 cycle in standing freshwaters such as lakes and marshes (Takahashi and Okazaki, 2002;  
80 Tsunagawa et al., 2010a, 2010b), whereas fluvial types complete their life cycle in running  
81 freshwaters (Mizuno, 1960; Nishijima, 1968; Iwata, 2001a, 2001b). Corresponding to these life  
82 history types, there is a large interspecific variation in egg size in *Rhinogobius* species (Mizuno,  
83 1960; Nishida, 2001; Tamada, 2001; Closs et al., 2013). Amphidromous and lentic species  
84 produce small eggs (0.6–0.9 mm in the major axis; Katoh and Nishida, 1994; Tsujimoto, 2008;  
85 Takahashi and Okazaki, 2002), whereas fluvial species spawn larger eggs (1.1–2.1 mm; Mizuno,  
86 1960; Katoh and Nishida, 1994). The fluvial species also exhibit egg size variation, i.e., species on  
87 the mainland of Japan produce larger eggs (1.4–2.1 mm; Mizuno, 1960) than several species in the  
88 Ryukyu Archipelago (1.1–1.5 mm; often called “middle-sized eggs”; Katoh and Nishida, 1994).

89 The egg size variation in *Rhinogobius* species has been explained as an adaptation to feeding  
90 and swimming ability during the larval period (Nishida, 1994, 2001; McDowall, 2007), and to  
91 increasing larval survival in rivers, which is a relatively harsh and unproductive environment  
92 (Closs et al., 2013). The large number of small eggs produced by amphidromous and lentic species  
93 are adaptive to standing waters with abundant small-sized plankton, such as bays, lakes, and  
94 marshes, where strong swimming ability is not necessary. On the other hand, fluvial species may  
95 need to produce larger eggs and larvae that can persist in running waters, and that utilize large  
96 prey items in rivers. Interspecific variation in egg size within fluvial species has been explained by  
97 the presence or absence of coexisting predators; larger larvae would be preferred under stronger  
98 predation pressure (Nishida, 2001).

99 These large variations in life history and egg size in *Rhinogobius* provide a profitable case for

100 pursuing adaptive radiation via colonization of novel habitats associated with the ecology of  
101 migration (Nishida, 1994, 2001). For the purpose of reconstructing such evolutionary patterns,  
102 including those of life histories and the related traits such as egg size, a reliable interspecific  
103 phylogeny is essential (Harvey and Pagel, 1991; Schluter, 2000; Losos, 2009). To date, genetic  
104 relationships among some Japanese *Rhinogobius* species have been examined based on allozyme  
105 polymorphisms (Masuda et al., 1989; Katoh and Nishida, 1994; Sakai et al., 2000), in which some  
106 landlocked species producing large eggs were inferred to have evolved in parallel to different  
107 amphidromous species that produce small eggs (Kato and Nishida, 1994; Nishida, 1994, 2001).  
108 However, the previous studies targeted only a small set of species and failed to obtain a robust  
109 phylogeny; hence, the frequency and generality of the life history changes and evolutionary  
110 patterns of related traits have not been well understood. Furthermore, some molecular  
111 phylogenetic studies using mitochondrial DNA (mtDNA) sequence data have been conducted for  
112 the *Rhinogobius* species (Aonuma et al., 1998; Mukai et al., 2005). However, mtDNA showed  
113 remarkable trans-species polymorphisms, in which the sympatric species tended to form  
114 monophyletic clades beyond species boundaries; this strongly suggests interspecific introgressive  
115 hybridization (Mukai et al., 2005). In cases involving introgression of organellar genomes via  
116 hybridization, phylogenetic information from multiple nuclear genes is necessary to estimate  
117 species phylogenetic relationships (Maddison, 1997; Bossu and Near, 2009; Waters et al., 2010;  
118 Near et al., 2011).

119 Given the considerable confusion regarding *Rhinogobius* taxonomy and the lack of ecological  
120 information for large numbers of *Rhinogobius* species (Suzuki and Chen, 2011), exhaustive  
121 analysis, particularly with the inclusion of species outside Japan, is difficult to conduct at present.  
122 Therefore, this study focused mainly on the Japanese *Rhinogobius* species, most of which are  
123 endemic to Japan and whose taxonomic and ecological information are relatively well understood.  
124 First, we estimated the phylogenetic relationships among all known Japanese species by using

125 multiple nuclear gene sequences. We also estimated their mtDNA-based phylogeny. The  
126 objectives of this study were as follows: (1) to reveal the patterns of phylogenetic diversification  
127 of Japanese *Rhinogobius* gobies; (2) to reveal the spatiotemporal patterns of interspecific  
128 hybridization causing mitochondrial introgression; (3) to test whether the freshwater species,  
129 including fluvial and lentic types, evolved independently from amphidromous species; and (4) to  
130 reveal the relationships between life history and egg size evolution.

131

## 132 2. Materials and Methods

### 133 2.1. Sampling

134 A total of 18 species of *Rhinogobius* are known from Japanese waters, 15 of which are endemic  
135 to Japan (Suzuki and Chen, 2011; Akihito et al. 2013, but see below; Fig. S1; Table S1). Although  
136 their species status has been evidenced by morphological, ecological, and genetic studies,  
137 scientific names of more than half of the species have yet to be determined or provided mainly  
138 because of their morphological similarity, insufficient taxonomic description, and poor condition  
139 of type specimens (Mizuno, 2001; Suzuki et al., 2011). Specific codes consisting of two alphabet  
140 characters have been commonly used for such species (e.g., BW, BB, DL; Mizuno, 2001; Akihito  
141 et al., 2002, 2013) and are also used in this paper. We basically followed the classification of  
142 Akihito et al. (2013), which includes 17 Japanese species. However, since one (or more) species  
143 classified into *Rhinogobius* sp. OR (sensu Akihito et al., 2002) was not included in Akihito et al.  
144 (2013), we tentatively use the name *Rhinogobius* sp. OR for the unclassified species.

145 A total of 96 specimens of 18 Japanese species were collected from the mainland of Japan, the  
146 Ryukyu Archipelago, and the Bonin Islands from 2001 to 2013 (Tables 1 and S2; Fig. 1).  
147 Specimens of wide-ranging species were sampled from two to eight geographically distant  
148 locations, if possible. These specimens, along with three specimens of the three continental  
149 species (*R. giurinus*, *R. leavelli*, and *R. virgigena*), were used in the analyses.

150 We took photographs of live specimens whenever possible and identified the species according  
151 to references (Akihito et al., 2002, 2013; Suzuki et al., 2004; Chen and Kottelat, 2005). Specimens  
152 were anesthetized using 2-phenoxyethanol, and their right pectoral fin or muscle tissue was  
153 preserved in 100% ethanol for DNA extraction. The specimens were fixed in 10% formalin,  
154 transferred to 70% ethanol, and deposited in the National Museum of Nature and Science, Tokyo,  
155 as voucher specimens (NSMT-P 65160, 65165, 120783–120861).

156

## 157 2.2. DNA extraction, PCR, and sequencing

158 Total genomic DNA was extracted using a Genomic DNA Purification Kit (Promega, Madison,  
159 Wisconsin, USA). PCR amplification targeted three regions of mtDNA [cytochrome c oxidase  
160 subunits 1 (CO1), NADH dehydrogenase subunits 5 (ND5), and cytochrome b (cytb); total of  
161 2781 bp] and six regions of nuclear DNA [myosin heavy polypeptide 6 (myh6), a novel protein  
162 similar to vertebrate ryanodine receptor 3 (RYR3), hypothetical protein LOC564097 (Ptr), super  
163 conserved receptor expressed in brain 2 (sreb2), recombinase-activating proteins 2 (RAG2), early  
164 growth response gene family 3 (EGR3); total of 4755 bp]. The primer sets and annealing  
165 temperature settings followed those in previous studies (see Table S3). New primer sets for Ptr and  
166 RAG2 were designed for a part of specimens based on the sequences determined for *Rhinogobius*  
167 specimens using Primer3 (Rozen and Skaletsky, 2000).

168 PCR amplification was performed in a 15- $\mu$ l volume containing 8  $\mu$ l ultrapure water, 1.5  $\mu$ l 2.5  
169 mM dNTP mix, 1.5  $\mu$ l Ex-Taq buffer, 1.5  $\mu$ l of each 5  $\mu$ M primer, 0.3  $\mu$ l Ex-Taq DNA polymerase  
170 (Takara, Shiga, Japan), and 1  $\mu$ l (ca. 10–100 ng) of DNA template. We also used KOD FX or  
171 KOD Plus Neo (Toyobo, Osaka, Japan) for specimens in which the amplification was difficult.  
172 The PCR using KOD FX was performed in a 15- $\mu$ l volume containing 2.4  $\mu$ l ultrapure water, 3  $\mu$ l  
173 2.0 mM dNTP mix, 7.5  $\mu$ l buffer, 0.9  $\mu$ l of each 5  $\mu$ M primer, 0.3  $\mu$ l KOD FX DNA polymerase,  
174 and 1  $\mu$ l of DNA template. The PCR using KOD Plus Neo was performed in a 15- $\mu$ l volume



175 containing 8.0 µl ultrapure water, 1.5 µl 2.0 mM dNTP mix, 1.5 µl buffer, 0.9 µl 25 mM MgCl<sub>2</sub>,  
176 0.9 µl of each 5 µM primer, 0.3 µl KOD Plus Neo DNA polymerase, and 1 µl of DNA template.  
177 The settings for PCR using Ex-Taq consisted of the first step (denature, 94 °C, 2 min), 35 cycles  
178 of the second step (denature, 94 °C, 30 s; annealing, 48–62 °C, 30 s; extension, 72 °C, 1 min), and  
179 the last step (extension, 68 °C, 7 min); and those for PCR using KOD FX or KOD Plus Neo  
180 consisted of the first step (denature, 94 °C, 2 min), 35 cycles of the second step (denature, 98 °C,  
181 10 s; annealing, 55–62 °C, 30 s; extension, 68 °C, 30–40 s), and the last step (extension, 68 °C, 7  
182 min). Nested PCR was performed using two primer pairs described by Li et al. (2007) in some  
183 samples; PCR products were diluted 20–100 times with water, and PCR was performed again  
184 using these diluted PCR products as templates.

185 The PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA)  
186 or Illustra ExoStar (GE Healthcare, Little Chalfont, Buckinghamshire, UK) at 37 °C. They were  
187 sequenced using an automated DNA sequencer (ABI 3130xl, Applied Biosystems, Foster City, CA,  
188 USA) with the above amplification primers and using the BigDye Terminator Cycle Sequencing  
189 FS Ready Reaction Kit ver. 3.1 (Applied Biosystems).

190 Nucleotide sequences were edited in MEGA5 (Tamura et al., 2011) or MacClade 4.08a  
191 (Maddison and Maddison, 2000). The DNA sequences were aligned using ClustalW (Larkin et al.,  
192 2007) or manually. Heterozygous sites within individual specimens were rare (0–1.1% for genes);  
193 these were coded as IUPAC degenerate nucleotide symbols and directly used in the analyses. The  
194 obtained sequences were deposited in DDBJ/GenBank/EMBL (accession nos. AB988263–  
195 AB988988).

196

### 197 2.3. Inference of molecular phylogeny

198 Gene trees were constructed using maximum likelihood (ML) and Bayesian methods. We chose  
199 *Rhinogobius giurinus* as the outgroup because it is the most distant species within the genus

200 (Masuda et al., 1989; Mukai et al., 2005). Maximum likelihood analyses were carried out using  
201 RAxML-7.2.6 (Stamatakis, 2006). We analyzed three types of data sets, i.e., concatenated data of  
202 the three mtDNA regions, six sets of each nuclear gene, and concatenated data of the six nuclear  
203 genes. Model selections based on Akaike information criterion (AIC) and optimal partition setting  
204 analysis were performed using PartitionFinder v1.0.0 (Lanfear et al., 2012) (Table S4). We treated  
205 each gene as a single locus and partitioned it by codon position. The partition scheme was  
206 searched using the “greedy” algorithm. The evolutionary model was selected from GTR, GTR+I,  
207 GTR+G, or GTR+I+G models based on the AIC. The credibility of clades was evaluated by 1000  
208 bootstrap replicates. Bayesian analyses were implemented using MrBayes 3.2.1 (Ronquist and  
209 Huelsenbeck, 2003) for two data sets (concatenated three mtDNA regions and concatenated six  
210 nuclear genes). Model selection based on the Bayesian information criterion (BIC) and optimal  
211 partition setting analysis were performed using PartitionFinder with the “greedy” algorithm. We  
212 treated each gene as a single locus and partitioned it by codon position. In MrBayes, the analysis  
213 was run for 20 and 50 million generations (for mtDNA and nuclear DNA, respectively), with two  
214 independent runs of four Markov chain Monte Carlo (MCMC) chains and sampling every 100  
215 generations. The trace files were checked in Tracer 1.5 (Rambaut et al., 2013) to ensure that the  
216 chains had reached convergence and the first 25% of trees were discarded as burn-in. Trees were  
217 visualized using FigTree v1.3.1 (Rambaut, 2009).

218 When a maximum likelihood tree or Bayesian tree topology did not support the monophyly of  
219 some morphological species, we conducted a statistical test of monophyly of the species as  
220 described below. First, we estimated the maximum likelihood tree under monophyletic constraints  
221 of the species by RAxML. Second, we conducted the approximate unbiased (AU) tests  
222 (Shimodaira, 2002) in CONSEL (Shimodaira and Hasegawa, 2001) for the two trees that were  
223 constructed under constraints or no constraints (i.e., maximum likelihood tree) and confirmed  
224 whether the monophyly of the species was statistically rejected or not.

225

226 2.4. Estimation of divergence time

227 We estimated the divergence time from concatenated data of mtDNA regions and nuclear genes,  
228 independently. Because to date, no fossil records are available that could be used to construct the  
229 tree for *Rhinogobius* and its relatives, we used the following two geographic events and previously  
230 estimated molecular evolutionary rates for divergence time calibration. First, the Bonin Islands are  
231 oceanic islands that have never been connected to continents. They were formed 1.8 million years  
232 ago (Mya) or later, and it is assumed that their endemic fauna was formed 0.9–1.8 Mya (Kaizuka,  
233 1977; Imaizumi and Tamura, 1984; Chiba, 2002). The time of the most recent common ancestors  
234 (tMRCA) of *R. ogasawaraensis* (endemic species to the Bonin Islands) and its sister species, or its  
235 clade was constrained following a normal prior distribution within the limit of 0–2.0 million years  
236 (Myr) (mean = 1.8, standard deviation (SD) = 0.4; calibration 1, CA1). Since it is possible that  
237 interspecific hybridization occurred after this divergence (see Discussion), we did not use this  
238 constraint in the mtDNA-based phylogenetic tree. Secondly, we used the opening time of the  
239 Tokara Gap, which divided the Japanese Archipelago and the Ryukyu Archipelago ( $1.55 \pm 0.154$   
240 Mya; Osozawa et al., 2012). Migration of amphidromous *Rhinogobius* species between the  
241 mainland and Ryukyu Archipelago just after the opening of the gap was probably not restricted  
242 because their larvae can disperse through the coastal areas. With further extension of the gap, their  
243 possible migration across the gap gradually decreased; finally, the isolation of populations at either  
244 side of the gap was complete. Therefore, we used the opening time of the Tokara Gap as the upper  
245 limit of the divergence time between mutually monophyletic groups distributed in the mainland  
246 and in the Ryukyu Archipelago. The tMRCA of the mutually monophyletic groups was  
247 constrained following a normal prior distribution within the limit of 0–1.7 Myr (mean = 1.55, SD  
248 = 0.4; calibration 2, CA2). Since the mtDNA phylogeny revealed the existence of mainland and  
249 Ryukyu groups beyond species boundaries (see Results), we applied CA2 to their tMRCA. On the

250 other hand, in the nuclear DNA analysis, we used this constraint only for clear intraspecific  
251 divergence because a previous study suggested that another older geographic event could have  
252 caused a similar vicariance pattern between mainland and Ryukyu species in some gobiid groups  
253 (Mukai, 2010). Finally, we used the molecular evolutionary rate of 3.0%/Myr (pairwise) (95%  
254 highest posterior density (HPD), 0.7–4.8%/Myr) for cytb, which was estimated for the genus  
255 *Gymnogobius* (Tabata and Watanabe 2013), phylogenetically relatively close to *Rhinogobius*  
256 (Agorreta et al., 2013). We assumed that the evolutionary rate of cytb followed a lognormal prior  
257 distribution ranging from 0.0 to  $10^{100}$  per Mya (initial value = 0.015, mean = 0.015, SD = 0.7,  
258 offset = 0.0, mean in real space = yes).

259 We used BEAST v1.7.5 (Drummond et al., 2012) to estimate the divergence time of trees  
260 inferred using mtDNA and nuclear genes, respectively. Originally, we attempted to construct a  
261 species tree by using \*BEAST (Heled and Drummond, 2010) for nuclear genes using several prior  
262 settings. However, the parameters did not converge and we abandoned this analysis. Later, we  
263 attempted to conduct the phylogenetic analysis with different evolutionary models for respective  
264 nuclear genes, but the parameters did not converge well either. Although the reason for this was  
265 not clear, it might be possible that low sequence variation and introgression in a part of the loci  
266 might influence the analyses. Finally, we concatenated sequences from the six nuclear genes and  
267 estimated a dated nuclear gene tree. In order to date the tree, we adopted the random local clock  
268 model, which assumes one or more independent rates on different branches (Drummond and  
269 Suchard, 2010). We did not specify partition by codon position, because the parameters did not  
270 converge in mtDNA or nuclear DNA data when partitioned. We used jModelTest v2.1.3 (Darriba  
271 et al., 2012) to select the evolutionary model without partitioning and selected the best model  
272 based on BIC. We selected the speciation tree prior (Yule process; Yule, 1925; Gernhard, 2008)  
273 and estimated a starting tree using the UPGMA method. We conducted MCMC analysis four times  
274 independently. For each MCMC, we performed a run of 50 and 100 million generations (for

275 mtDNA and nuclear genes, respectively), sampling every 1000th generation. The first 10% of the  
276 trees were discarded as burn-in for each run. We assessed whether parameter values for individual  
277 runs had reached equilibrium and convergence by visually assessing their trace plots in Tracer 1.5.  
278 Individual chains were combined using the LogCombiner v1.7.5. Finally, we analyzed combined  
279 runs using Tree Annotator v1.7.5.

280

## 281 2.5 Reconstruction of ancestral states

282 We conducted ancestral state reconstruction for life history and egg size traits under the multiple  
283 state speciation and extinction (MuSSE) model (FitzJohn, 2012). If speciation, extinction, and  
284 character transition rates are different associated with traits, assuming equal rates for these  
285 parameters under ML framework will be a major violation of the ancestral state reconstruction  
286 (Maddison, 2006; Goldberg and Igić, 2008; Pyron and Burbrink, 2014). The MuSSE model is one  
287 of the models accounting for such state-dependent diversification and a generalized version of  
288 binary state speciation and extinction (BiSSE) model (Maddison et al., 2007) to allow the use of  
289 multistate characters. The MuSSE analysis was applied to the smallest monophyletic group  
290 including all Japanese species, for which taxon sampling was dense. We chose one sample for  
291 every species randomly and excluded the other samples from the nuclear DNA tree inferred by  
292 BEAST using the ‘ape’ package (Paradis et al., 2004) in R (R Development Core Team, 2014).  
293 Exceptionally, two samples were used for *R. flumineus* and *Rhinogobius* sp. OR because they were  
294 suggested to be non-monophyletic. We then conducted a model comparison between the full  
295 MuSSE model and 11 parameter-constrained sub-models. We selected the best model using AIC  
296 scores. Finally, we conducted an ancestral state reconstruction under the selected model in the ML  
297 framework. The model selection and ancestral state reconstruction under the MuSSE model were  
298 conducted using the R package ‘diversitree’ v 0.9-7 (FitzJohn, 2012). The data for life history and  
299 egg size of each species were obtained from previous studies (see Tables 1, S1).

300

### 301 3. Results

#### 302 3.1. Phylogeny and divergence time estimates based on mtDNA sequences

303 The maximum likelihood and Bayesian analyses based on three mtDNA regions revealed six  
304 clades in the Japanese *Rhinogobius* species (Fig. 2): M-1 (*R. ogasawaraensis*, bootstrap  
305 probability inferred by RAxML [BP] 100%, posterior probability inferred by MrBayes [PPM]  
306 100%, posterior probability inferred by BEAST [PPB] 1.0); M-2 (*Rhinogobius* sp. BW, BP 100%,  
307 PPM 1.0, PPB 1.0); M-3 (*Rhinogobius* sp. TO, BP 100%, PPM 1.0, PPB 1.0); M-4 (*R. flumineus*,  
308 BP 100%, PPM 1.0, PPB 1.0); M-5 (*R. nagoyae* collected from the mainland of Japan; BP 100%,  
309 PPM 1.0, PPB 1.0); M-6 (other samples collected from the mainland of Japan; BP 100%, PPM 1.0,  
310 PPB 1.0); and M-7 (other samples collected from the Ryukyu Archipelago; BP 81%, PPM 0.99,  
311 PPB 0.99). *Rhinogobius nagoyae* and *R. brunneus*, which are distributed both on the mainland of  
312 Japan and the Ryukyu Archipelago, were each polyphyletic, with M-5 and M-7 haplotypes  
313 according to the locality (AU test, *R. nagoyae*,  $p < 0.001$ ; *R. brunneus*,  $p < 0.001$ ). In the clade  
314 M-6, *Rhinogobius* sp. OR and *R. fluviatilis* were not monophyletic (AU test, *Rhinogobius* sp. OR,  
315  $p < 0.001$ ; *R. fluviatilis*,  $p < 0.001$ ). In the clade M-7, *R. brunneus* and *Rhinogobius* sp. BB were  
316 also not monophyletic (AU test, *R. brunneus*,  $p < 0.001$ ; *R. sp.* BB,  $p < 0.001$ ).

317 The calibration point CA2 was applied for the divergence between the M-5+M-6 (most samples  
318 collected from the mainland of Japan) and M-7 (collected from the Ryukyu Archipelago)  
319 haplotypes. The tMRCA of these was estimated at 1.17 Myr (95% HPD, 0.62–1.70 Myr in Table  
320 2). The tMRCA of all the Japanese *Rhinogobius* species was estimated at 1.59 Myr (0.73–2.39  
321 Myr), and the tMRCAs of M-5+M-6 and M-7 were 1.02 Myr (0.49–1.52 Myr) and 0.97 Myr  
322 (0.47–1.44 Myr), respectively.

323

#### 324 3.2. Gene trees and divergence time estimation based on nuclear gene sequences

325 The variable sites and parsimony informative sites of the nuclear genes for *Rhinogobius* fishes,  
326 excluding *R. giurinus*, were 2.5% (EGR3)–9.9% (RAG2), and 1.3% (sreb2)–5.7% (RAG2),  
327 respectively. Although the resolution and statistical support of the phylogenetic trees based on  
328 each nuclear DNA data set were low (Figure S2), they were much improved in the ML and  
329 Bayesian trees based on the concatenated dataset (Fig. 3). The descriptions and analyses hereafter  
330 are based on ML and Bayesian trees inferred from the six nuclear genes.

331 In contrast to the result of mtDNA analyses, most of the respective species were resolved as  
332 monophyletic in the nuclear gene trees. The Japanese species were monophyletic in the current  
333 dataset, and they were divided into three clades with strong or moderate statistical supports (Fig.  
334 3): clade N-1 (*R. flumineus* and *R. sp. TO*; BP 95%, PPM 1.0, PPB 1.0); N-2 (*R. nagoyae* and *R.*  
335 *sp. CO*; BP 68%, PPM 0.99, PPB 0.95); and N-3 (the remaining 13 species; BP 78%, PPM 1.0,  
336 PPB 1.0). The N-3 clade was further divided into three subclades: N-3-1 (*R. brunneus* and  
337 *Rhinogobius sp. YB*; BP 65%, PPM 0.96, PPB 0.99); N-3-2 (*R. fluviatilis*, *R. ogasawaraensis*, and  
338 *Rhinogobius sp. DL*; BP 83%, PPM 1.0, PPB 1.0); and N-3-3 (*Rhinogobius sp. MO*, *Rhinogobius*  
339 *sp. BB*, *Rhinogobius sp. OR*, *Rhinogobius sp. BW*, *Rhinogobius sp. BF*, *Rhinogobius sp. OM*,  
340 *Rhinogobius sp. KZ*, and *R. kurodai*; BP 100%, PPM 1.0, PPB 1.0). None of the analyses resolved  
341 the relationships among N-1, N-2, and N-3 clades.

342 In the N-1 clade, *Rhinogobius sp. TO* formed a monophyletic group with a part of *R. flumineus*  
343 samples (BP 57%, PPM 0.93, PPB 0.90). Although statistical support was weak, *R. brunneus* and  
344 *Rhinogobius sp. YB* were each polyphyletic in N-3-1 (AU test, *Rhinogobius sp. YB*,  $p = 0.42$ ; *R.*  
345 *brunneus*,  $p = 0.335$ ). In N-3-2 clade, *R. fluviatilis* and *R. ogasawaraensis* constituted a sister  
346 group with *Rhinogobius sp. DL* (BP 83%, PPM 1.0, PPB 1.0). In N-3-3, *Rhinogobius sp. MO* and  
347 *Rhinogobius sp. BB* formed a weakly supported monophyletic group in the ML tree (BP 52%,  
348 PPB 0.54; not supported by MrBayes analysis). The resolution of the relationships among other  
349 specimens, including *Rhinogobius sp. OR*, *Rhinogobius sp. BW*, *Rhinogobius sp. BF*, *Rhinogobius*

350 sp. OM, *Rhinogobius* sp. KZ, and *R. kurodai* was low. *Rhinogobius* sp. BW, a species endemic to  
351 Lake Biwa, the largest lake in Japan, formed a monophyletic group with *Rhinogobius* sp. BF,  
352 distributed in western area of the mainland of Japan, and *Rhinogobius* sp. OM, distributed in the  
353 coastal area and rivers flowing into Lake Biwa, but the statistical support was weak (BP 70%,  
354 PPM 0.53, PPB 0.64).

355 We applied the calibration point CA1 (< 2 Myr) for the tMRCA of *R. fluviatilis* (distributed  
356 widely in the mainland of Japan) and *R. ogasawaraensis* (endemic to the Bonin Islands) and CA2  
357 (< 1.7 Myr) for the tMRCA of *R. nagoyae* on the mainland of Japan and the Ryukyu Archipelago  
358 (Fig. 4). As a result of these calibration points, the divergence time of *R. ogasawaraensis* and *R.*  
359 *fluviatilis* was estimated at 1.55 Myr (95% HPD, 1.05–2.00 Myr), and that of *R. nagoyae* on the  
360 mainland of Japan and the Ryukyu Archipelago was estimated at 1.14 Myr (0.68–1.69 Myr).

361 The tMRCAs among the species that are geographically isolated from each other whose  
362 distributions were geographically separated were estimated as follows (Table 2): tMRCA of N-3-2  
363 (*R. fluviatilis* [the mainland], *R. ogasawaraensis* [Bonin Islands], and *Rhinogobius* sp. DL  
364 [Ryukyu Archipelago]) was 1.78 Myr (1.03–2.53 Myr); tMRCA of a part of N-3-3 (*Rhinogobius*  
365 sp. OR + *Rhinogobius* sp. KZ + *Rhinogobius kurodai* [the mainland] and *Rhinogobius* sp. MO +  
366 *Rhinogobius* sp. BB [Ryukyu Archipelago]) was 1.20 Myr (0.59–1.89 Myr); and tMRCA of  
367 *Rhinogobius* sp. BW (Lake Biwa) + *Rhinogobius* sp. BF (western area of the mainland of Japan) +  
368 *Rhinogobius* sp. OM was 1.10 Myr (0.49–1.77 Myr). The tMRCA of the Japanese *Rhinogobius*  
369 species, except for *R. giurinus*, was estimated at about 4.48 Myr (2.38–6.66 Myr).

370

### 371 3.3 Ancestral state reconstruction

372 The best-fit model among the MuSSE sub-models was the simplest model for both life history  
373 and egg size, i.e., speciation, extinction, and transition rates were independent to state (Table S6).  
374 In the *Rhinogobius* species of Japan, the life history and egg size transformations were estimated



375 to have occurred at least five and four times, respectively, although the proportional likelihood  
376 values were not very high for parts of the ancestral states (59.6–99.9%; Fig. 4a). The estimated  
377 egg size transformations always occurred together with life history transformation. The  
378 evolutionary change from amphidromous to fluvial life, accompanied by an increase in egg size,  
379 was estimated to have occurred in at least three independent lineages (*R. flumineus*, *Rhinogobius*  
380 sp. BB, and *Rhinogobius* sp. YB). The evolutionary change from amphidromous to lentic life was  
381 estimated to have occurred in one lineage (*Rhinogobius* sp. BW and *Rhinogobius* sp. BF). There  
382 was no clear change in egg size in this lineage (Tables 1, S1). In the *R. flumineus* and *Rhinogobius*  
383 sp. TO lineage, it was inferred that the lentic life most likely evolved from the fluvial life of *R.*  
384 *flumineus*, accompanied by a decrease in egg size.

385

## 386 4. Discussion

### 387 4.1 Phylogenetic relationships of Japanese *Rhinogobius* fishes and their biogeography

388 Our analysis using multiple nuclear gene data from all known Japanese *Rhinogobius* species  
389 revealed the existence of three major clades and resolved the overall relationships among the  
390 species. Previously, morphological studies have not been conducted to infer the phylogenetic  
391 relationships of *Rhinogobius* fishes because of their generally high level of morphological  
392 similarity. Although phylogenetic hypotheses for selected species from the mainland of Japan have  
393 been inferred from the allozyme data, consistent and reliable results have not been obtained  
394 (Masuda et al., 1989; Sakai et al., 2000). The present study is the first to examine the phylogenetic  
395 relationships of *Rhinogobius* using dense taxonomic sampling, although it was mostly restricted to  
396 Japanese species.

397 The inferred phylogenetic relationships suggest several biogeographic scenarios that explain  
398 the divergence among the species. Among the freshwater species of *Rhinogobius*, the close  
399 relationship between *R. flumineus* (fluvial) and *Rhinogobius* sp. TO (lentic) was revealed in this

400 study. Although statistical support was not strong, the latter being derived from a clade within the  
401 former in the early to middle Pleistocene (1.43 Mya; 95% HPD, 0.48–2.44 Mya). *Rhinogobius*  
402 *flumineus* is distributed widely in western Japan, including the Ise Bay area, in which *Rhinogobius*  
403 sp. TO has a restricted distribution (Suzuki and Sakamoto, 2005; Fig. 4b). The latter species  
404 occurs in marsh habitats of hilly areas with rich spring waters (Suzuki and Mukai, 2010). This area  
405 has been rich in lentic environments since the Pliocene; e.g., a large wetland area known as the  
406 Paleo-Lake Tokai existed from the Pliocene to the middle Pleistocene (Yoshida, 1990; Makinouchi,  
407 2001). The distribution, habitat, and speciation of *Rhinogobius* sp. TO may be associated with  
408 such past lentic environments.

409 Other freshwater species, *Rhinogobius* sp. BF, *Rhinogobius* sp. BW, and *Rhinogobius* sp. OM,  
410 formed a monophyletic clade, but with weak statistical support. They are distributed in marshes  
411 and ponds around the Seto Inland Sea, the pelagic zone of Lake Biwa, and coastal areas and rivers  
412 flowing into Lake Biwa (Fig. 4c). Their common ancestor was estimated to have diverged from a  
413 part of *Rhinogobius* sp. OR, which is amphidromous and distributed widely on the mainland of  
414 Japan. The common ancestor probably adapted to freshwater environments around the Seto Inland  
415 Sea area, in which there existed a large-scale freshwater system (known as the Second Seto Inland  
416 Lake/River system), including the Paleo-Lake Biwa at the eastern edge (Yokoyama and Nakagawa,  
417 1991). The estimated tMRCA of *Rhinogobius* sp. BW and part of *Rhinogobius* sp. BF (0.90 Myr;  
418 0.35–1.51 Myr; Table 2) is roughly congruent with the time when the large, deep offshore  
419 environment of Lake Biwa started to develop (0.4 Mya; Yokoyama, 1984; Meyers et al., 1993;  
420 Kawabe, 1994). This suggests that *Rhinogobius* sp. BW has derived from the ancestral lentic form  
421 with and adapted to the pelagic environment of Lake Biwa.

422 The inferred phylogenetic relationships and geographic distributions suggest the patterns of  
423 allopatric speciation in amphidromous species. *Rhinogobius ogasawaraensis*, endemic to the  
424 Bonin Islands, oceanic islands 1000 km south of the mainland of Japan (Suzuki et al., 2011), was

425 estimated to be the sister species of *R. fluviatilis*, which is widely distributed on the mainland of  
426 Japan (Fig. 4d). Prior to the present study, *R. ogasawaraensis* was considered closely related to *R.*  
427 *brunneus* in body color characteristics (Suzuki, 1992). However, our results suggest that *R.*  
428 *ogasawaraensis* was established by colonization of the common ancestor with *R. fluviatilis* from  
429 the mainland of Japan after the separation from *Rhinogobius* sp. DL, which is distributed in the  
430 Ryukyu Archipelago. Although amphidromous species may extend their geographic range through  
431 coastal or marine habitats (McDowall, 2001), juveniles of amphidromous *Rhinogobius* species are  
432 restricted to the coastal zone (Oshiro and Nishijima, 1978; Kondo et al., 2013) and probably do  
433 not migrate across the open ocean. Therefore, the dispersal from the mainland to the Bonin Islands  
434 would be an exceptional event.

435 Marine environments at smaller spatial scales also affect gene flow and probably the  
436 allopatric speciation of *Rhinogobius* species. The opening of the Tokara Gap, which isolated the  
437 terrestrial biota between the Japanese and Ryukyu archipelagos (Ota, 1998), probably also caused  
438 divergence in some species groups of the amphidromous *Rhinogobius*. There were three sets of  
439 mutually monophyletic lineages distributed in the mainland of Japan (+ the Bonin Islands) and the  
440 Ryukyu Archipelago; i.e., *R. nagoyae* (the mainland) vs. *R. nagoyae* (Ryukyu) in N-2; *R.*  
441 *fluviatilis* (the mainland) + *R. ogasawaraensis* (Bonin) vs. *Rhinogobius* sp. DL (Ryukyu) in N-3-2;  
442 *Rhinogobius* sp. OR + *Rhinogobius* sp. KZ + *R. kurodai* (the mainland) vs. *Rhinogobius* sp. MO +  
443 *Rhinogobius* sp. BB (Ryukyu) in N-3-3. The estimated divergence time of each pair was 1.14  
444 (95% HPD, 0.68–1.69; this used as a calibration point), 1.78 (95% HPD, 1.03–2.53), and 1.20  
445 (95% HPD, 0.59–1.89) Myr, respectively (Table 2), showing similar values among the pairs. This  
446 roughly supports the hypothesis that the opening of the Tokara Gap caused the divergence in these  
447 groups. The distribution of *Rhinogobius* sp. DL extends to north of the Tokara Gap. This might  
448 have resulted from secondary dispersal and should be examined based on detailed population  
449 structures in future studies.

450 The opening of the Tokara Gap similarly explains the divergence between the mainland of  
451 Japan and Ryukyu subspecies of the osmeriform *Plecoglossus altivelis* (ca. 1 Myr divergence;  
452 Nishida, 1985, 1986), which is the representative of amphidromous species distributed widely in  
453 East Asia, similarly to *Rhinogobius*. In contrast to the above three *Rhinogobius* groups and other  
454 taxa such as *P. altivelis*, *R. brunneus* did not show any significant differentiation between the  
455 Japanese and Ryukyu archipelagos. The different dispersal abilities of their larvae may explain the  
456 presence or absence and the extent of the genetic differentiation among the species pairs.

457

#### 458 4.2 The history of hybridization inferred from mtDNA introgression

459 The inconsistency between our mtDNA and nuclear DNA inferred phylogenies suggests a  
460 large-scale introgressive hybridization involving multiple *Rhinogobius* species within Japan. In  
461 the mtDNA phylogeny, the mainland and Ryukyu populations of two widely distributed species (*R.*  
462 *nagoyae* and *R. brunneus*) formed a group with other species in those regions. Overall, specimens  
463 of nine species from the mainland formed a monophyletic group (M-5 + M-6), although statistical  
464 support was not strong, and those of six species from the Ryukyu Archipelago formed a  
465 monophyletic group (M-7). However, such polyphyletic relationships were statistically rejected by  
466 the nuclear DNA data; morphologically defined species basically formed a monophyletic group.  
467 This pattern of geographical sorting of the mtDNA lineages is explained by interspecific  
468 introgressive hybridization rather than incomplete lineage sorting (Toews and Brelsford, 2012)  
469 because the latter is expected to cause more random distribution of mtDNA haplotypes.

470 The mtDNA phylogeny, along with the nuclear gene phylogeny and divergence time  
471 estimations, provides information on historical patterns of hybridization in *Rhinogobius*. First,  
472 *Rhinogobius ogasawaraensis* was resolved as distant to other Japanese *Rhinogobius* species  
473 (except for *R. giurinus*) in the mtDNA phylogeny; nevertheless, *R. ogasawaraensis* was included  
474 in the clade N-3-2 of the nuclear gene phylogeny. This conflict indicates that a large-scale mtDNA

475 introgression involving almost all species in both Japanese and Ryukyu archipelagos started with a  
476 common haplotype (or close haplotypes) after the divergence of *R. ogasawaraensis*. Second, the  
477 mtDNA and nuclear DNA analyses gave similar estimation of the divergence time of *R.*  
478 *ogasawaraensis* (1.59 and 1.55 Myr, respectively) although different calibration sets were used  
479 (Table 2). This agreement probably validates the estimation that the large-scale introgression  
480 occurred around that time (the early to middle Pleistocene). Third, even within the mainland of  
481 Japan or the Ryukyu region, mtDNAs resolved a part of species polyphyletic (e.g., the mainland *R.*  
482 *fluviatilis* and Ryukyu *Rhinogobius* sp. BB). This also supports the limited but ubiquitous  
483 hybridization in the *Rhinogobius* fishes, although incomplete lineage sorting in mtDNA is an  
484 alternative explanation in this scale. Fourth, it should be noted that freshwater species, such as *R.*  
485 *flumineus*, *Rhinogobius* sp. TO, *Rhinogobius* sp. BW (the mainland), and a part of *Rhinogobius* sp.  
486 BB (Ryukyu), branched out earlier and have retained their independent lineages in the mtDNA  
487 phylogeny. This tendency suggests that the reproductive isolation between amphidromous and  
488 freshwater species is stronger than that between amphidromous species and has prohibited the  
489 freshwater species from hybridizing with other species.

490       What type of historical process has constructed the above patterns? Interspecific hybridization  
491 often occurs in the conditions of enforced syntopic occurrence, which is caused by, for instance,  
492 reduction of habitats and secondary contact of populations following disappearance of geographic  
493 or ecological barriers (Seehausen, 2004; Toews and Brelsford, 2012). Although the *Rhinogobius*  
494 species show similar reproductive habits that include oviposition under a stone and male parental  
495 care for the eggs, reproductive isolation among sympatric species is achieved by micro- or  
496 mesoscale differences of spawning habitat in the river course (Mizuno, 1982; Tamada, 2000;  
497 Hirashima and Tachihara, 2006), assortative mating based on nuptial coloration and courtship  
498 behavior (Mizuno, 1987), or both. However, some conditions causing disturbance of such  
499 reproductive isolation would have occurred since the early to middle Pleistocene, in which the

500 large part of Japanese *Rhinogobius* would have experienced large-scale hybridization. Freshwater  
501 habitat reduction by the rise in sea level with global climate fluctuations might be one such  
502 potential situation causing the overlap of spawning habitats among species. Mukai et al. (2012)  
503 reported that introgressive hybridization has occurred contemporarily among some freshwater  
504 *Rhinogobius* species following artificial transplantation. This example demonstrates that  
505 environmental distribution or changes can easily disturb reproductive isolation among  
506 *Rhinogobius* fishes. Furthermore, if some types of mtDNA were favored by natural selection,  
507 large-scale introgression of the particular mtDNA would rapidly progress through interspecific  
508 hybridization (Ballard and Whitlock, 2004). The *Rhinogobius* fishes may provide a model system  
509 to study the mechanisms of maintenance and decay of reproductive isolation, such as the Lake  
510 Victoria cichlids that suffered a collapse of assortative mating based on visual sense with the  
511 increase of turbidity (Seehausen et al., 1997). This group may also be useful as a model system for  
512 studying mitochondrial introgression and replacement through interspecific hybridization (Mukai  
513 and Takahashi, 2010).

514

#### 515 4.3 Life history and egg size evolution and speciation

516 We successfully reconstructed the patterns of life history and egg size evolution of *Rhinogobius*  
517 species in Japan. Because of our taxon sampling bias toward Japanese species, careful  
518 consideration is needed for reconstruction and explanation of evolutionary patterns of ecological  
519 traits. However, since all non-amphidromous and some amphidromous species used in this study  
520 are endemic to Japan and some are inferred to have derived around Japan, estimation of the trait  
521 transformation based on our phylogeny should provide meaningful insights into ecological and  
522 species diversification in *Rhinogobius* fishes.

523 The evolutionary changes of the life history type and egg size are closely associated with each  
524 other in *Rhinogobius* species. The reconstructed patterns in the phylogeny included three series of

525 transformations, i.e., from an amphidromous to a fluvial type, from an amphidromous to a lentic  
526 type, and from a fluvial to a lentic type. Although statistical support of the ancestral reconstruction  
527 associated with the last transformation was not very strong, the pattern is supported by the  
528 geographical distribution of the relevant species (see below).

529 The first pattern, from amphidromous to fluvial, is inferred to have occurred independently in  
530 the *R. flumineus*, *Rhinogobius* sp. YB, and *Rhinogobius* sp. BB lineages, when they diverged from  
531 their ancestors. In all three fluvial species, their egg size became larger than that of the  
532 amphidromous species. The reconstruction remains ambiguous for *R. flumineus* due to its basal  
533 phylogenetic position in Japanese species and the presence of several unexamined fluvial/large  
534 egg species in the continent (Chen et al., 2008). On the other hand, the other two fluvial species  
535 (*Rhinogobius* sp. YB and *Rhinogobius* sp. BB) occurring in the Ryukyu Archipelago most likely  
536 originated directly from *R. brunneus* and *Rhinogobius* sp. MO, respectively, based on their  
537 phylogenetic relationships, recent divergence times, and restricted geographic distribution of the  
538 fluvial species. This means that convergent evolution of the increase in egg size occurred at least  
539 in these two lineages. This conclusion agrees with the inference from previous studies based on  
540 allozyme polymorphism (Kato and Nishida, 1994; Nishida, 1994). Based on the restricted  
541 occurrence of *Rhinogobius* sp. YB in the upper reaches of waterfalls, Nishida (1994, 2001) and  
542 Kano et al. (2012) suggested that this species evolved through parallel evolution from the  
543 amphidromous ancestor (*R. brunneus*) as a result of population isolation following the formation  
544 of waterfalls. On the other hand, since *Rhinogobius* sp. BB is usually found in rivers without  
545 waterfalls, Kondo et al. (2013) inferred that this species derived from the ancestral *Rhinogobius* sp.  
546 MO population that invaded the upper reaches of rivers when the sea level decreased and rivers  
547 increased in length. To test these hypotheses for evolutionary processes of the fluvial species,  
548 further study from multiple aspects, including population genetics with highly sensitive multilocus  
549 markers, is needed.

550 The second pattern of life history transformation, from amphidromous (*Rhinogobius* sp. OR  
551 or OM) to lentic (*Rhinogobius* sp. BF + *Rhinogobius* sp. BW), involved a slight decrease in egg  
552 size (Takahashi and Okazaki, 2002). The difference in their egg size is not as large as that between  
553 fluvial (large egg) and amphidromous (small egg) species (Table S1; Takahashi and Okazaki,  
554 2002). Since larvae of both the amphidromous and lentic species grow in a plankton-rich  
555 environment (i.e., the sea near a river mouth and lakes or ponds), such similar feeding  
556 environments may not cause remarkable differences in the adaptive sizes of their hatched larvae  
557 and eggs in relation to feeding efficiency. However, the lentic species are possibly released from  
558 the lower limit of egg size needed for enduring starvation during their flow down the river just  
559 after hatching (Moriyama et al., 1998; Iguchi and Mizuno, 1999; Tamada, 2008, 2009). The  
560 smaller body size at maturation in lentic species (Takahashi and Okazaki, 2002) probably favors  
561 smaller eggs for increased fecundity. These factors may explain the smaller egg size in lentic  
562 species.

563 The third pattern of life history transformations, from fluvial (*R. flumineus*) to lentic  
564 (*Rhinogobius* sp. TO) type, involved a possible reversal evolution in egg size, i.e., egg size  
565 changed from a small size (ancestral amphidromous species) to a small size (*Rhinogobius* sp. TO)  
566 through a large state (*R. flumineus*) in this lineage. Although the ancestral reconstruction for this  
567 lineage was not very strongly supported by the MuSSE analysis, this pattern is supported by the  
568 following considerations: (a) *Rhinogobius* sp. TO derived most likely from a lineage of *R.*  
569 *flumineus*, and (b) the very restricted distribution of *Rhinogobius* sp. TO (around the Ise Bay area)  
570 is included within the wide range of *R. flumineus*. These suggest that the former species is the one  
571 that colonized from fluvial to lentic environments, such as the lake or marsh that existed around  
572 the present Ise Bay area (the Paleo-Lake Tokai), as discussed above.

573 The present wetland environment in the Ise Bay area is inhabited by several highly endemic  
574 species, including freshwater fishes (*Pseudorasbora pugnax*, *Cobitis minamorii tokaiensis*, as well



575 as *Rhinogobius* sp. TO; Kawamura, 2006; Nakajima, 2012; Kawase and Hosoya, 2015), aquatic  
576 hemipteran insects *Nepa hoffmanni*, and plants (e.g., magnoliacean *Magnolia stellata*; Ueda,  
577 2002); these suggest that such environment has been maintained for a long period. The small egg  
578 size and body size in *Rhinogobius* sp. TO are likely the characters representing this adaptation.  
579 The dwarf morphology of *Rhinogobius* sp. TO is very similar to that of other lentic species  
580 (*Rhinogobius* sp. BF and *Rhinogobius* sp. BW), and these three forms were treated as a single  
581 species until recently (Akihito et al., 2002; Suzuki and Mukai, 2010). However, the present study  
582 clarified that *Rhinogobius* sp. TO belongs to a different lineage from *Rhinogobius* sp. BF and  
583 *Rhinogobius* sp. BW, indicating that their lentic life history and dwarf morphology were the result  
584 of convergent evolution. The evolutionary change suggested in the freshwater *R. flumineus* and  
585 *Rhinogobius* sp. TO lineage emphasizes the adaptive flexibility of *Rhinogobius* fishes.

586 The reconstructed life history changes did not include the changes from freshwater (fluvial or  
587 lentic) to amphidromous types. The amphidromous life history requires adaptations to migrating  
588 behavior and salinity tolerance, which are not required for freshwater fish (McDowall, 2004). In  
589 fact, some freshwater *Rhinogobius* species have weakened or completely lost their salinity  
590 tolerance (*Rhinogobius* sp. YB and *Rhinogobius* sp. BB; Hirashima and Tachihara, 2000). Loss of  
591 standing genetic variation through purifying selection or bottleneck during colonization of  
592 freshwater environment may have prevented the freshwater species from re-acquiring the  
593 amphidromous life. Competition or hybridization with prior amphidromous residents may also  
594 prevent this reversal in evolution.

595 In summary, the considerable species diversity of *Rhinogobius* has been promoted and  
596 maintained by parallel life history divergence through colonization of and adaptation to various  
597 freshwater habitats, including flowing waters, marshes, and lakes. The life history divergence  
598 involving the changes in migration patterns and egg size probably functions as a direct mechanism  
599 of reproductive isolation among the divergent populations. To elucidate the whole picture of life

600 history evolution in *Rhinogobius*, further phylogenetic analysis using comprehensive taxon  
601 sampling from East/Southeast Asia is necessary.

602

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614

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911 Figure legends

912 Fig. 1.

913 A map of the collection sites. The location codes correspond to those in Table 1.

914

915 Fig. 2.

916 The Bayesian tree of the selected *Rhinogobius* species with estimated divergence time based on  
917 partial mtDNA sequence data (2781 bp) inferred with BEAST. Support values are indicated  
918 beside the branches (RAxML BP/ MrBayes PPM/ BEAST PPB). Only support values >70% in  
919 ML, and >0.9 in PPM and PPB are indicated. Some support values for intraspecific relationships  
920 are not shown. Each bar plot indicates 95% HPD height of the node. CA2 indicates the position of  
921 calibration point 2 (formation of the Tokara Gap).

922

923 Fig. 3.

924 The maximum likelihood tree of the selected *Rhinogobius* species inferred from concatenated  
925 sequences of six nuclear genes (myh6, RYR3, Ptr, RAG2, sreb2, and EGR3; 4755bp). Maximum  
926 likelihood bootstrap values (>70%) and Bayesian posterior probability (>0.9) are indicated  
927 (BP/BPP). Some support values for intraspecific relationships are not shown.

928

929 Fig. 4.

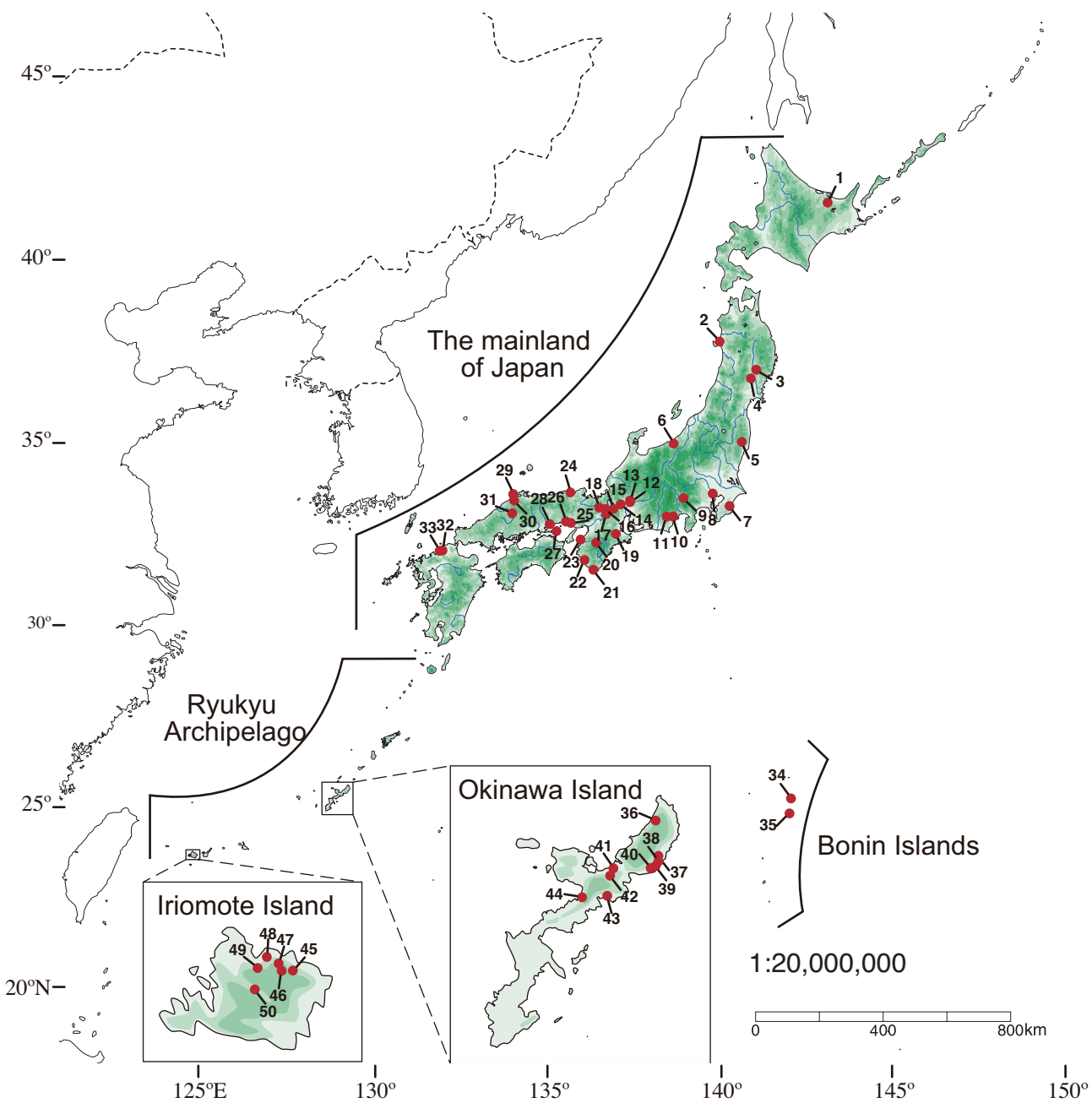
930 (a) The Bayesian phylogenetic tree of the selected *Rhinogobius* species with ancestral state  
931 reconstruction and divergence time inferred from concatenated sequences of six nuclear genes.  
932 Each bar plot indicates a height of 95% HPD of the corresponding node. Calibration points were  
933 indicated as CA1 (formation of the Bonin Islands) and CA2 (formation of the Tokara Gap). Pie  
934 graphs of each node indicate the proportional likelihood of the states at the node inferred by the  
935 MuSSE model. Statistical support values (> 0.9) are indicated next to the major nodes. The

936 proportional likelihood of the nodes indicated by an asterisk is inconsistent when another sample  
937 of *Rhinogobius* sp. BF was used. (b)–(e) Phylogenetic relationships among selected species with  
938 their present distribution patterns. See the Discussion section for details.

939



Fig. 1



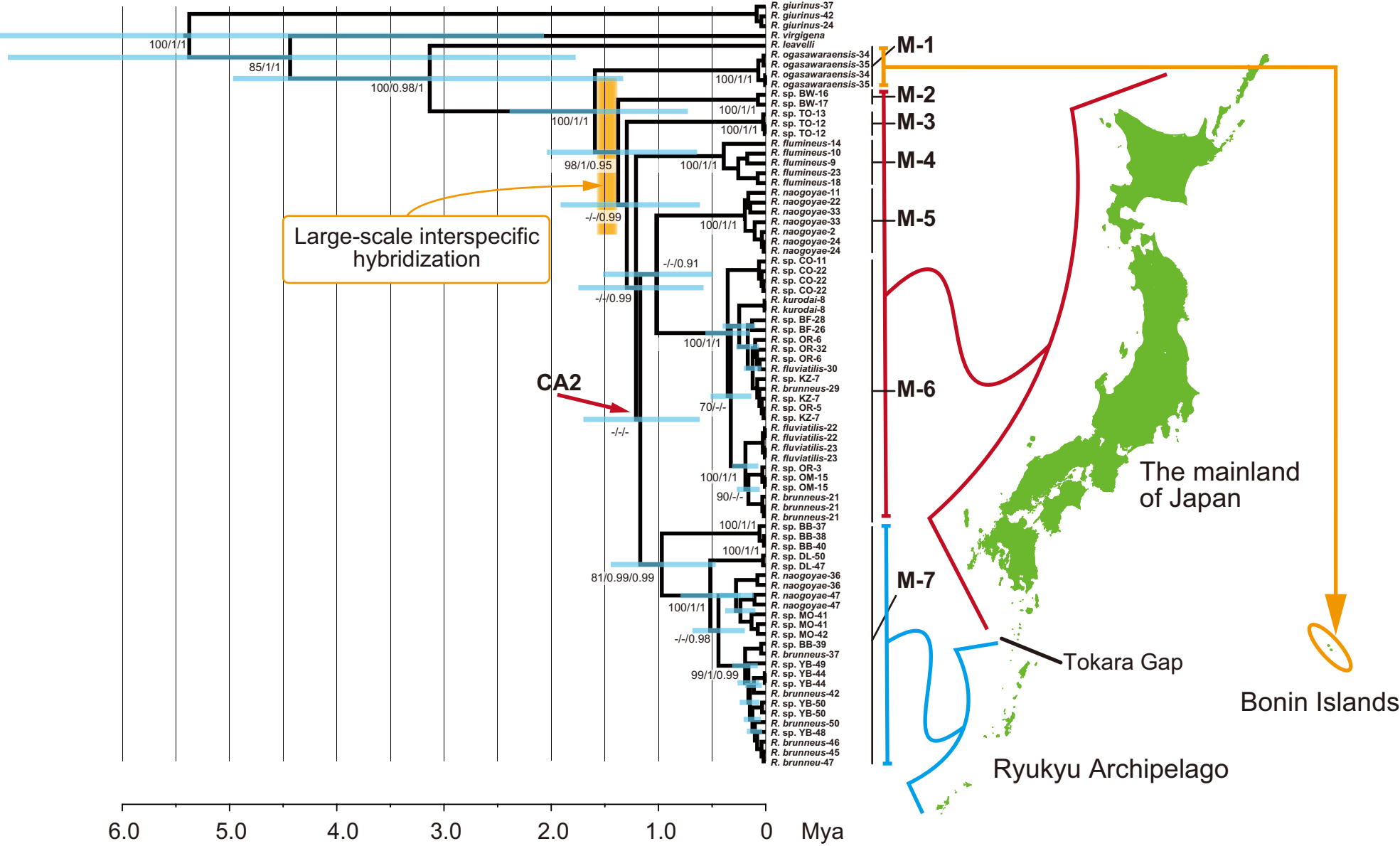


Fig. 2

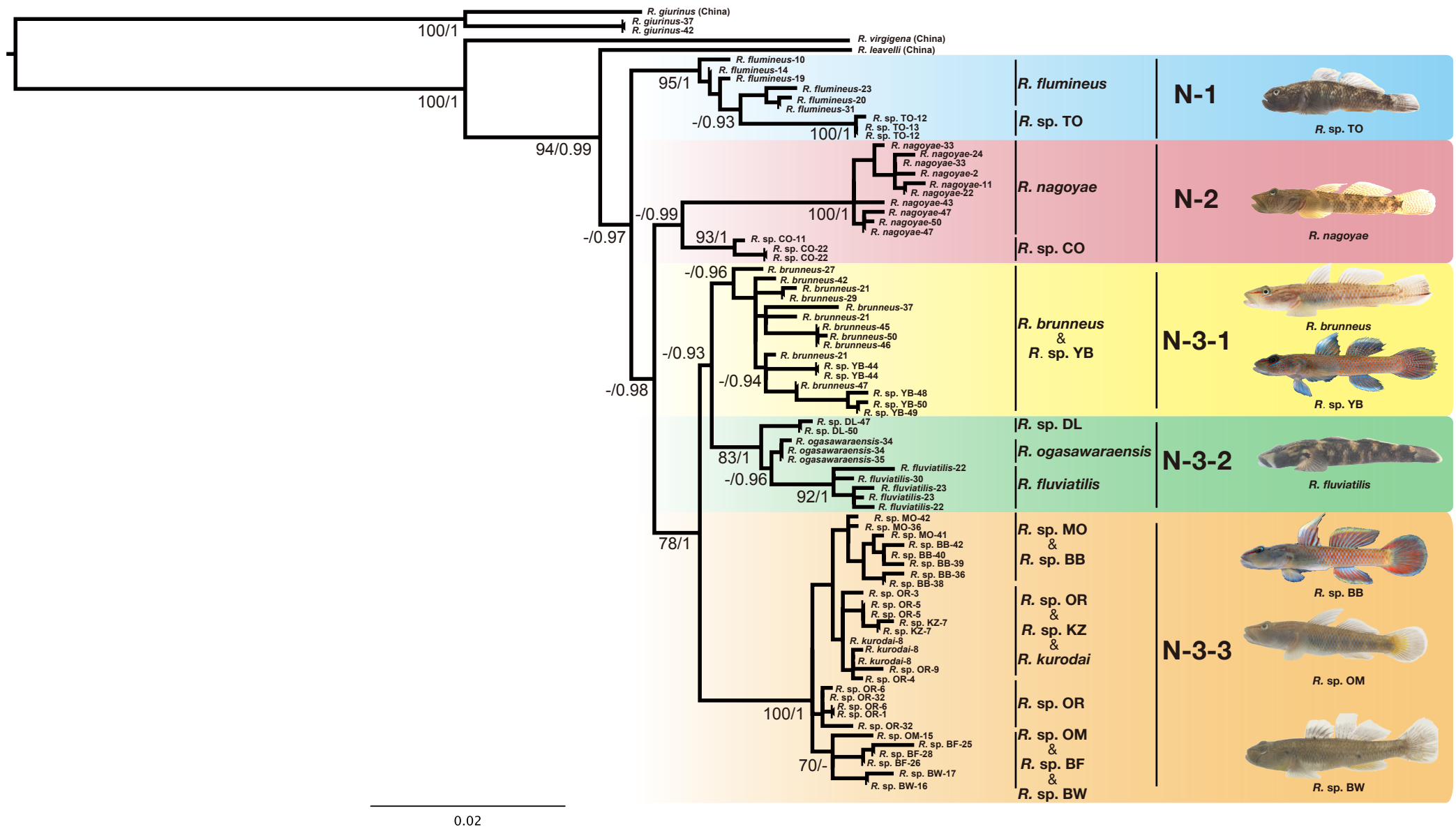
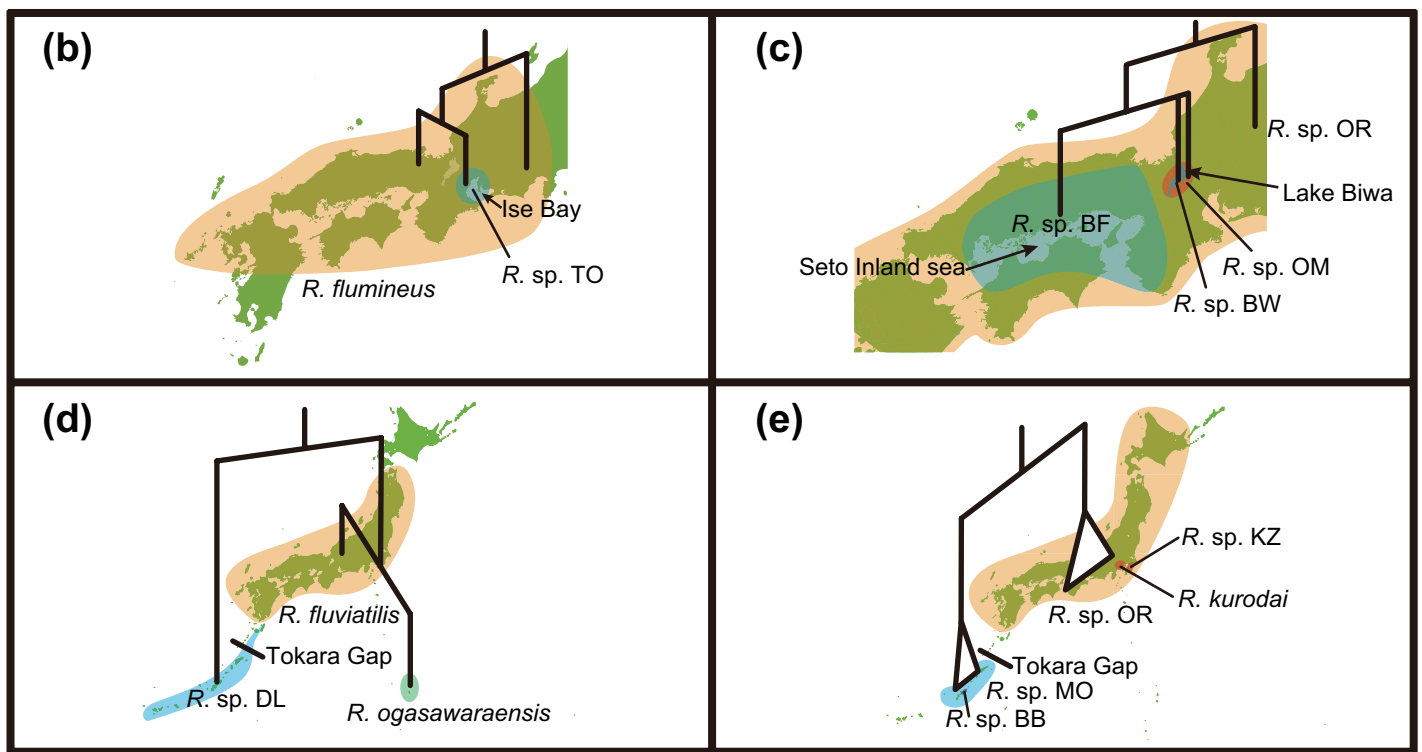
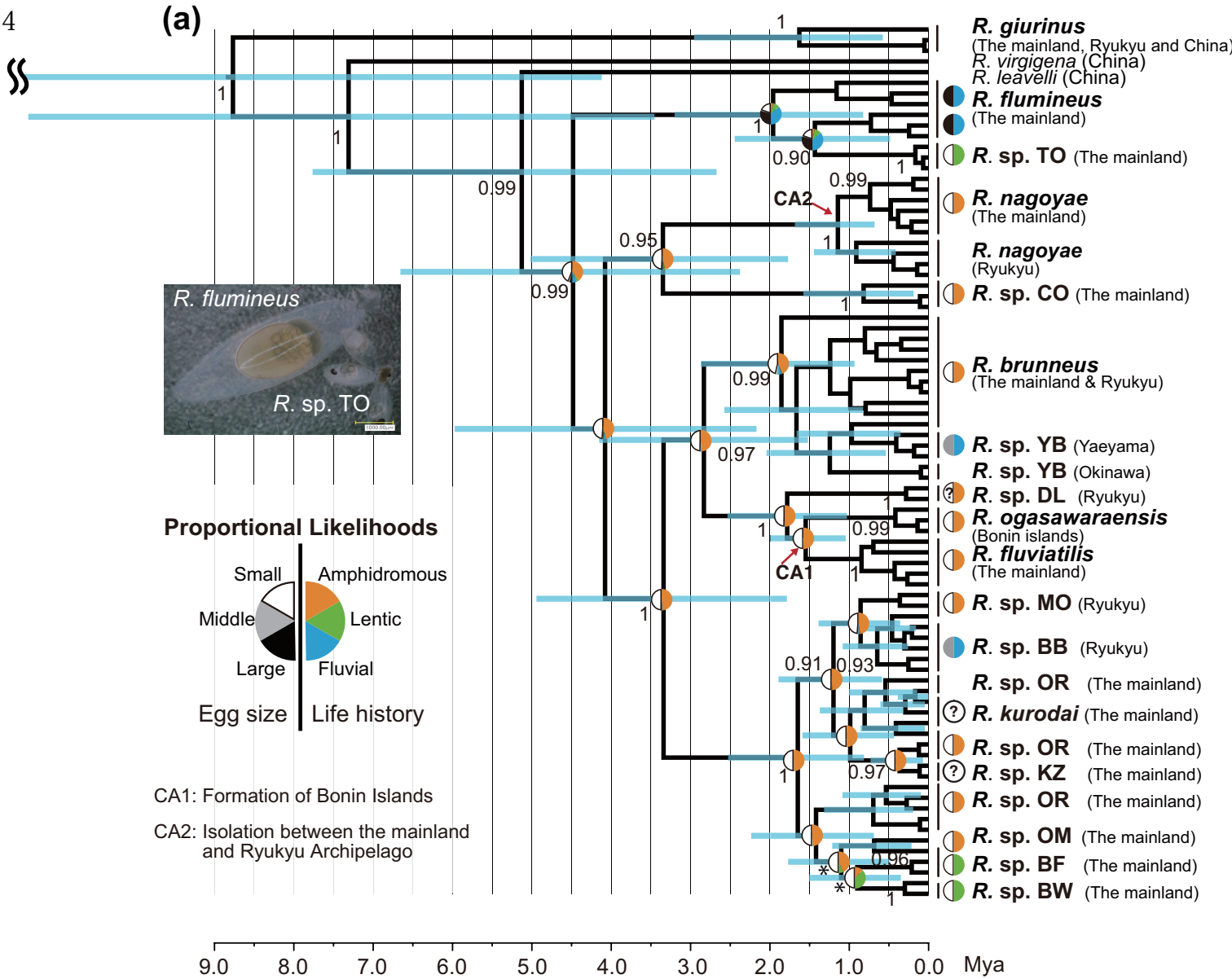


Fig. 3

Fig. 4



**Table 1**

List of samples used in this study. Locality numbers correspond to those in Fig. 1. Asterisks indicate specimens used in the MuSSE analysis (see Fig. 4a).

Species: species code	Life history	Egg size	Specimen ID	River / River system (Locality)	Locality No.
<b>Rhinogobius flumineus : RF</b>	Fluvial	Large	RF-YN120714-1	Fuefuki R. / Fujigawa R. (Koufu, Yamanashi)	9
			RF-SZ110911-28*	Ichiba R. / Seto R. (Fujieda, Shizuoka)	10
			RF-GF110816-1	Irrigation channel / Ibi R. (Anpachi, Gifu)	14
			RF-KY120402-1	Kamo R. / Lake Biwa and Yodo R. (Kyoto, Kyoto)	18
			RF-ME120905-1	Nakamura R. / Kumozu R. (Matsuzaka, Mie)	19
			RF-NR120930-1	Yumitehara R. / Shingu R. (Nosegawa, Nara)	20
			RF-OS110805-6*	Yamanaka R. / Onosato R. (Hannan, Osaka)	23
			RF-HI120512-1	Tabusa R. / Gounokawa R. (Shobara, Hiroshima)	31
			RF-OK111211-3	Sukuta R. / Sukuta R. (Okinawa Island, Okinawa)	44
			RF-OK111211-4	Sukuta R. / Sukuta R. (Okinawa Island, Okinawa)	44
<b>Rhinogobius sp. YB: YB</b>	Fluvial	Middle	YB-KR120624-1	Kura R. / Kura R. (Iriomote Island, Okinawa)	48
			YB-PN120622-5	Hinai R. / Hinai R. (Iriomote Island, Okinawa)	49
			YB-IR120328-3	Urauchi R. / Urauchi R. (Iriomote Island, Okinawa)	50
			YB-IR120328-4*	Urauchi R. / Urauchi R. (Iriomote Island, Okinawa)	50
			BB-OK121111-1	Sate R. / Sate R. (Okinawa Island, Okinawa)	36
			BB-OK110924-15	Aha R. / Aha R. (Okinawa Island, Okinawa)	37
<b>Rhinogobius sp. BB: BB</b>	Fluvial	Middle	BB-OK120305-1	Uka R. / Uka R. (Okinawa Island, Okinawa)	38
			BB-OK110924-32*	Tsurasaku (Okinawa Island, Okinawa)	39
			BB-OK121111-4	Shinkawa R. / Shinkawa R. (Okinawa Island, Okinawa)	40
			BB-OK121111-5	Shinkawa R. / Shinkawa R. (Okinawa Island, Okinawa)	40
			BB-OK120305-2	Genka R. / Genka R. (Okinawa Island, Okinawa)	42
			OM-SG110725-1	Sakura R. / Lake Biwa and Yodo R. (Higashiomi, Shiga)	15
			OM-SG110725-2*	Sakura R. / Lake Biwa and Yodo R. (Higashiomi, Shiga)	15
			OR-HO120408-1	Abashiri R. / Abashiri R. (Memanbetsu, Hokkaido)	1
<b>Rhinogobius sp. OM: OM</b>	Amphidromous	Small	OR-IW090528-1	Channel / Kitakami R. (Oshu, Iwate)	3
			OR-IW081011-2	Pond / Kitakami R. (Ichinoseki, Iwate)	4
			OR-FS091026-1*	Ootakine R. / Abukuma R. (Tamura, Fukushima)	5
			OR-FS091026-2	Ootakine R. / Abukuma R. (Tamura, Fukushima)	5
			OR-NII10816-1*	Seki R. / Seki R. (Joetsu, Niigata)	6
			OR-NII10816-2	Seki R. / Seki R. (Joetsu, Niigata)	6
			OR-YN120714-5	Fuefuki R. / Fujigawa R. (Koufu, Yamanashi)	9
			OR-HK110724-5	Irrigation channel / Saigou R. (Fukutsu, Fukuoka)	32
			OR-HK110724-6	Irrigation channel / Saigou R. (Fukutsu, Fukuoka)	32
			DA-WK110825-1	Esuno R. / Esuno R. (Nishimuro, Wakayama)	21
			DA-WK110825-2	Esuno R. / Esuno R. (Nishimuro, Wakayama)	21
			DA-WK110825-3	Esuno R. / Esuno R. (Nishimuro, Wakayama)	21
			DA-KW120730-1*	Yoshida R. / Yoshida R. (Shodoshima Island, Kagawa)	27
			DA-SM120511-1	Karakawa R. / Karakawa R. (Izumo, Shimane)	29
			DA-OK110924-23	Aha R. / Aha R. (Okinawa Island, Okinawa)	37
			<b>Rhinogobius sp. OR: OR</b>	Amphidromous	Small
DA-IR120626-1	Yuchin R. / Yuchin R. (Iriomote Island, Okinawa)	45			
DA-IR120625-1	Geda R. / Geda R. (Iriomote Island, Okinawa)	46			
DA-IR120622-4	Omija R. / Omija R. (Iriomote Island, Okinawa)	47			
DA-IR120327-2	Urauchi R. / Urauchi R. (Iriomote Island, Okinawa)	50			
LD-WK110824-23	Migi-Aizu R. / Aizu R. (Tanabe, Wakayama)	22			
LD-WK110824-24	Migi-Aizu R. / Aizu R. (Tanabe, Wakayama)	22			
LD-OS110805-1	Yamanaka R. / Onosato R. (Hannan, Osaka)	23			
LD-OS110805-2*	Yamanaka R. / Onosato R. (Hannan, Osaka)	23			
LD-SM120511-5	Ono R. / Hii R. (Izumo, Shimane)	30			
<b>Rhinogobius brunneus : DA</b>	Amphidromous	Small	CO-SZ110911-1	Seto R. / Seto R. (Fujieda, Shizuoka)	11
			CO-WK110824-2	Migi-Aizu R. / Aizu R. (Tanabe, Wakayama)	22
			CO-WK110824-17*	Migi-Aizu R. / Aizu R. (Tanabe, Wakayama)	22
			CO-WK110824-21	Migi-Aizu R. / Aizu R. (Tanabe, Wakayama)	22
			CB-AK111003-3*	Nomura R. / Nomura R. (Oga, Akita)	2
			CB-SZ110911-6	Seto R. / Seto R. (Fujieda, Shizuoka)	11
<b>Rhinogobius sp. CO: CO</b>	Amphidromous	Small	CB-WK110824-6	Migi-Aizu R. / Aizu R. (Tanabe, Wakayama)	22
			CB-HY110809-1	Satsu R. / Satsu R. (Mikata, Hyogo)	24
			CB-HY110809-2	Satsu R. / Satsu R. (Mikata, Hyogo)	24
			CB-HK110724-1	Saigo R. / Saigo R. (Fukutsu, Fukuoka)	33
			CB-HK110724-2	Saigo R. / Saigo R. (Fukutsu, Fukuoka)	33
			CB-OK130630-1	Sade R. / Sade R. (Okinawa Island, Okinawa)	36
			CB-OK130630-2	Sade R. / Sade R. (Okinawa Island, Okinawa)	36
			CB-OK110923-1	Genka R. / Genka R. (Okinawa Island, Okinawa)	42
			CB-OK110925-2	Teima R. / Teima R. (Okinawa Island, Okinawa)	43
			CB-IR120622-1	Omija R. / Omija R. (Iriomote Island, Okinawa)	47
			CB-IR120622-3	Omija R. / Omija R. (Iriomote Island, Okinawa)	47
			CB-IR120327-1	Urauchi R. / Urauchi R. (Iriomote Island, Okinawa)	50
			MO-OK121111-3	Sate R. / Sate R. (Okinawa Island, Okinawa)	36
			MO-OK111211-1	Hiranami R. / Hiranami R. (Okinawa Island, Okinawa)	41
MO-OK111211-2	Hiranami R. / Hiranami R. (Okinawa Island, Okinawa)	41			
<b>Rhinogobius sp. DL: DL</b>	Amphidromous	Unknown	DL-IR120622-2	Omija R. / Omija R. (Iriomote Island, Okinawa)	47
			DL-IR020223-1	Urauchi R. / Urauchi R. (Iriomote Island, Okinawa)	50
			DL-IR120328-1*	Urauchi R. / Urauchi R. (Iriomote Island, Okinawa)	50
			DL-IR120622-3	Urauchi R. / Urauchi R. (Iriomote Island, Okinawa)	50
<b>Rhinogobius ogasawaraensis : BI</b>	Amphidromous	Small	BI-CC011116-1*	Yatsuse R. / Yatsuse R. (Chichijima, Ogasawara)	34
			BI-CC011116-2	Yatsuse R. / Yatsuse R. (Chichijima, Ogasawara)	34
			BI-HH011124-1	Oki harbor (Hahajima, Ogasawara)	35
			BI-HH011124-2	Oki harbor (Hahajima, Ogasawara)	35
<b>Rhinogobius sp. BW: BW</b>	Lentic	Small	BW-SG110623-3	Lake Biwa, Imajuku / Lake Biwa and Yodo R. (Otsu, Shiga)	16
			BW-SG130523-1*	Lake Biwa, Imajuku / Lake Biwa and Yodo R. (Otsu, Shiga)	16
			BW-SG130601-1	Lake Biwa, Moriyama / Lake Biwa and Yodo R. (Moriyama, Shiga)	17
<b>Rhinogobius sp. TO: TO</b>	Lentic	Small	TO-GF110820-1*	Ogase pond / Kiso R. (Kakamigahara, Gifu)	12
			TO-GF110820-2	Ogase pond / Kiso R. (Kakamigahara, Gifu)	12
			TO-GF120415-7	Kandou pond / Kiso R. (Kakamigahara, Gifu)	13
<b>Rhinogobius sp. BF: BF</b>	Lentic	Small	BF-HY110605-1	Kakogawa R. / Kakogawa R. (Kakogawa, Hyogo)	25
			BF-HY110913-1*	Pond (Takasago, Hyogo)	26
			BF-OY110722-1	Uryu R. / Yoshii R. (Okayama, Okayama)	28
			KZ-CB100418-1	Mizusawa R. / Ichimiya R. (Chousei, Chiba)	7
<b>Rhinogobius sp. KZ: KZ</b>	Unknown	Unknown	KZ-CB100418-2*	Mizusawa R. / Ichimiya R. (Chousei, Chiba)	7
			KZ-CB100418-3	Mizusawa R. / Ichimiya R. (Chousei, Chiba)	7
			KU-TK100705-1*	Shinjuku gyoen (Shinjuku, Tokyo)	8
<b>Rhinogobius kurodai : KU</b>	Unknown	Unknown	KU-TK100705-2	Shinjuku gyoen (Shinjuku, Tokyo)	8
			KU-TK100705-3	Shinjuku gyoen (Shinjuku, Tokyo)	8
			R. leavelli	Amphidromous	Unknown
R. virgigena	Unknown	Unknown	R. virgigena	Fangcheng, Guangxi, China	
Outgroup					
<b>Rhinogobius giurinus : RG</b>	Amphidromous	Small	RG-HY110809-18	Satsu R. / Satsu R. (Mikata, Hyogo)	24
			RG-OK110924-19	Aha R. / Aha R. (Okinawa Island, Okinawa)	37
			RG-OK110925-17	Genka R. / Genka R. (Okinawa Island, Okinawa)	42
			RG-CH990405-1	Fangcheng, Guangxi, China	

**Table 2.**Estimated divergence time of major clades of *Rhinogobius* gobies.

mtDNA	tMRCA(My, hight mean)	95% HPD (Mya)
CA2 (M-5+M-6 vs. M-7)	1.17	0.62–1.70
M-5+M-6	1.02	0.49–1.52
M-7	0.97	0.47–1.44
All Japanese species	1.59	0.73–2.39
nuclear DNA		
CA1 ( <i>R. fluviatilis</i> + <i>R. ogasawaraensis</i> )	1.55	1.05–2.00
CA2 ( <i>R. nagoyae</i> in mainland + Ryukyu)	1.14	0.68–1.69
N-3-2	1.78	1.03–2.53
N-3-3	1.65	0.81–2.53
<i>R. flumineus</i> + <i>R.</i> sp. TO	1.43	0.49–2.44
<i>R.</i> sp. OR + <i>R.</i> sp. KZ + <i>R. kurodai</i> vs. <i>R.</i> sp. MO +	1.20	0.59–1.89
<i>R.</i> sp. BW + <i>R.</i> sp. BF + <i>R.</i> sp. OM	1.10	0.49–1.77
<i>R.</i> sp. BW + part of <i>R.</i> sp. BF	0.90	0.35–1.51
All Japanese species	4.48	2.38–6.66

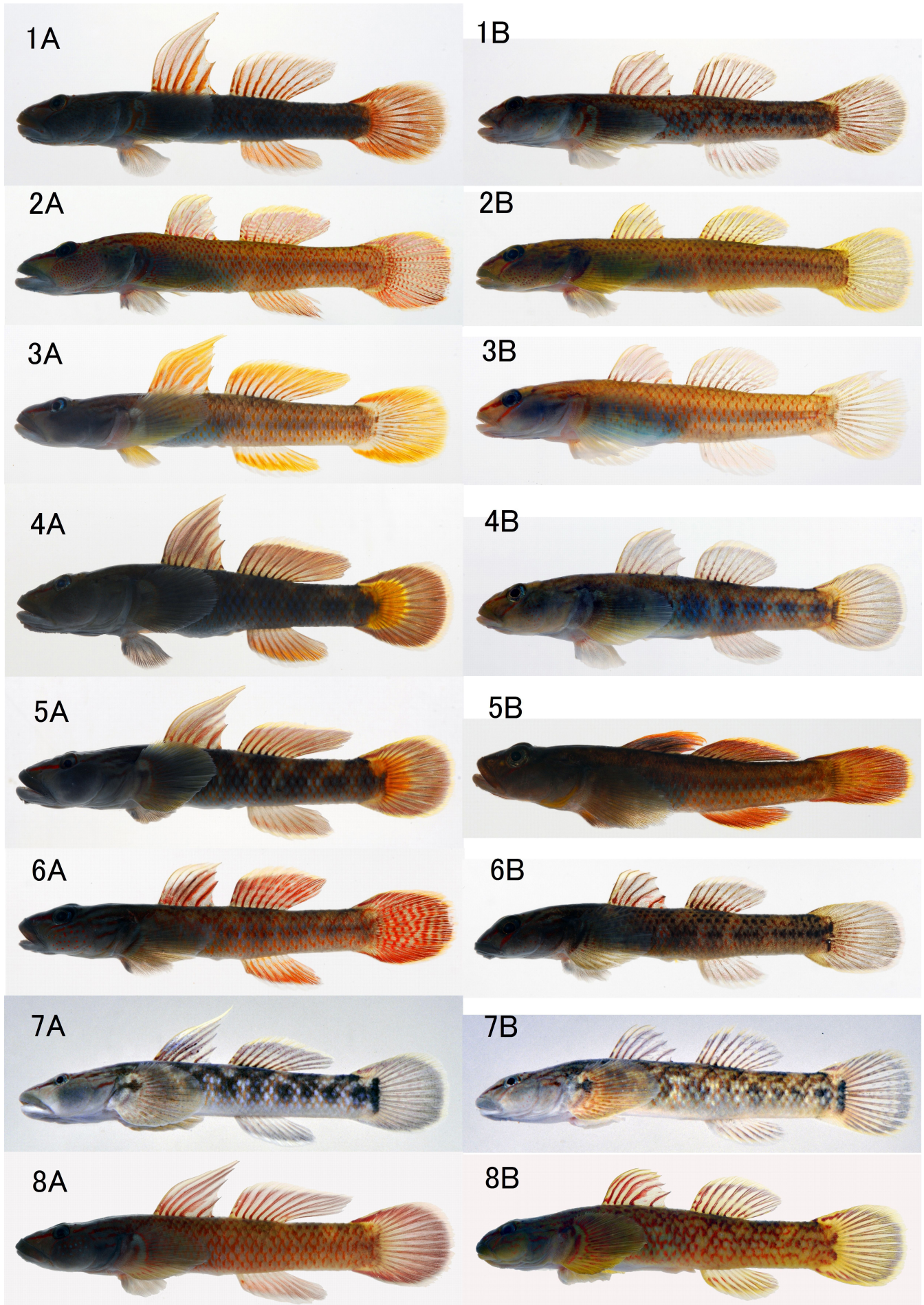


Fig. S1 Photographs of all *Rhinogobius* species distributed in Japan.

- 1: *Rhinogobius flumineus* (Mizuno, 1960), Nagaragawa River, Gifu Pref. A: OMNH-P 40703, male, B: OMNH-P 40704, female.
- 2: *Rhinogobius* sp. YB, Hinaigawai River, Iriomote Island, the Ryukyu Islands. A: OMNH-P 40256, male, B: OMNH-P 40255, female.
- 3: *Rhinogobius* sp. BB, Ooigawa River, Okinawa Island, the Ryukyu Islands. A: OMNH-P 40303, male, B: OMNH-P 40302, female.
- 4: *Rhinogobius* sp. OM. A: OMNH-P 40605, male, Yasugawa River, Shiga Pref., B: OMNH-P 40609, female, Ukawa-river, Shiga Pref.
- 5: *Rhinogobius* sp. OR. A: OMNH-P 40721, male, Maruyamagawa River, coast of Japan Sea of Hyogo Pref., B: OMNH-P 34814, male, pond, Ichinoseki, Iwate Pref.
- 6: *Rhinogobius brunneus* (Temminck and Schlegel, 1845), Aikawagawa River, Nagasaki Pref. A: OMNH-P 35063, male, B: OMNH-P 35064, female.
- 7: *Rhinogobius fluviatilis* Tanaka, 1925, Tairagawa River, Kagoshima Pref. A: OMNH-P 18393, male, B: OMNH-P 18392, female.
- 8: *Rhinogobius* sp. CO, Inouzawagawa River, Shizuoka Pref. A: OMNH-P 40616, male, B: OMNH-P 40618, female.

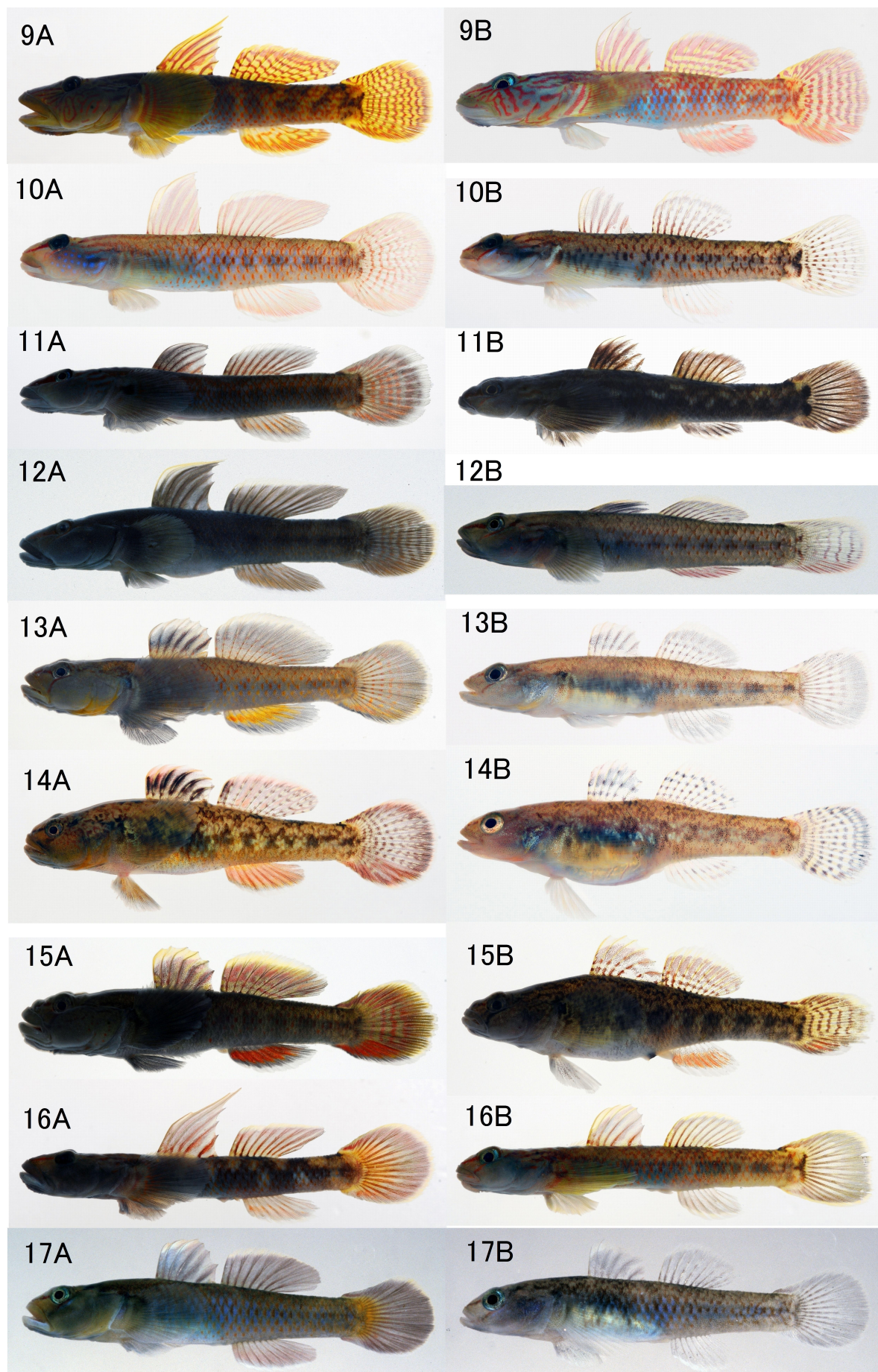
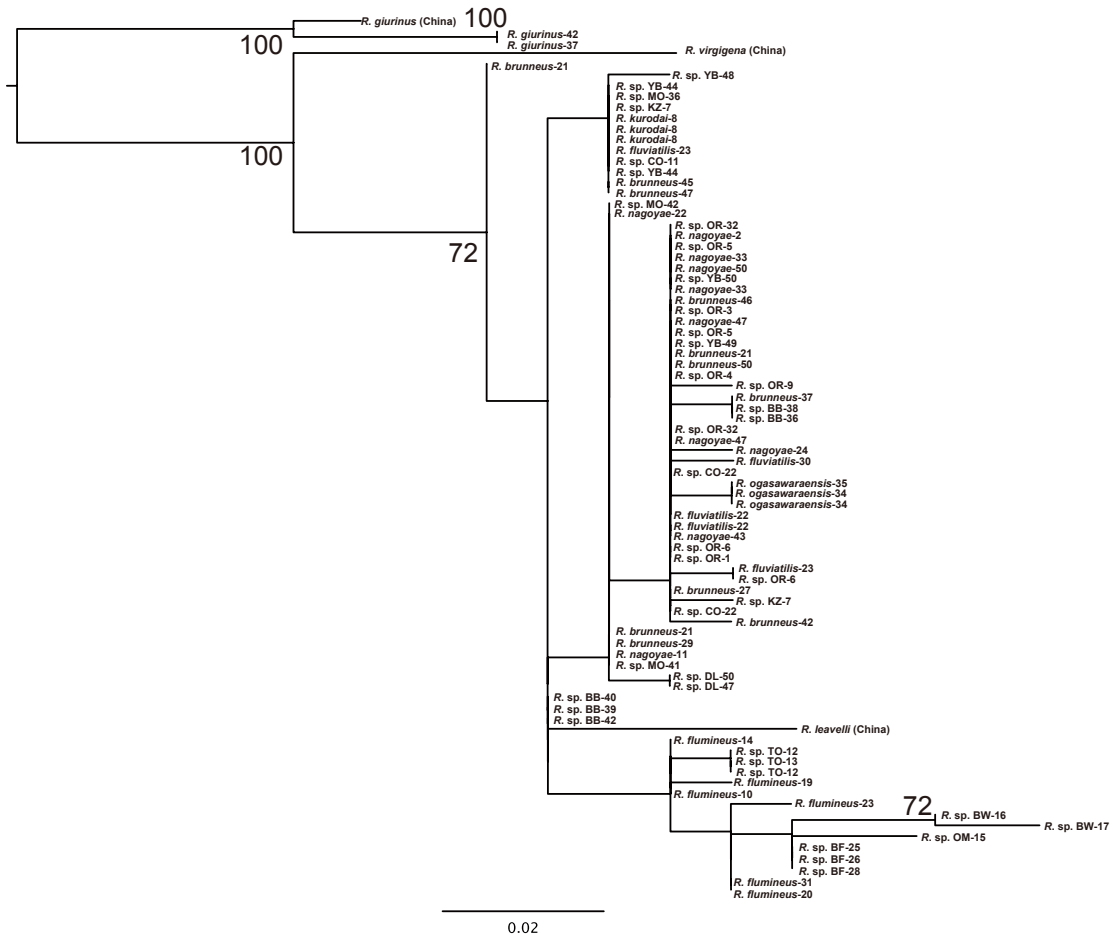


Fig. S1 Continued.

- 9: *Rhinogobius nagoyae* Jordan and Seale, 1906. A: OMNH-P 37667, male, Maruyamagawa River, coast of Japan Sea of Hyogo Pref., B: OMNH-P 38182, male, Teimagawa River, Okinawa Island, the Ryukyu Islands.
- 10: *Rhinogobius* sp. MO, Sate River, Okinawa Island, the Ryukyu Islands. A: OMNH-P 40281, male, B: OMNH-P 40282, female.
- 11: *Rhinogobius* sp. DL, Urauchi River, Iriomote Island, the Ryukyu Islands. A: OMNH-P 40035, male, B: OMNH-P 40036, female.
- 12: *Rhinogobius ogasawaraensis* Suzuki, Chen and Senou, 2011, Chichi Island, Ogasawara Is. A: OMNH-P 8262, male, Yatsusegawa River, B: OMNH-P 8257, female, Oomura.
- 13: *Rhinogobius* sp. BW, Ukawa, Lake Biwa, Shiga Pref. A: OMNH-P 23928, male, B: OMNH-P 23929, female.
- 14: *Rhinogobius* sp. TO, Kanaregawa River, Aichi Pref. A: OMNH-P 40705, male, B: OMNH-P 40708, female.
- 15: *Rhinogobius* sp. BF, Maruyamagawa River, coast of Japan Sea of Hyogo Pref. A: OMNH-P 37710, male, B: OMNH-P 37711, female.
- 16: *Rhinogobius* sp. KZ, Isumigawa River, Chiba Pref. A: OMNH-P 40656, male, B: OMNH-P 40663, female.
- 17: *Rhinogobius kurodai* (Tanaka, 1908), Moat, Chiyouda, Tokyo. A: OMNH-P 21132, male, B: OMNH-P 21136, female.



(a) myh6



(b) RYR3

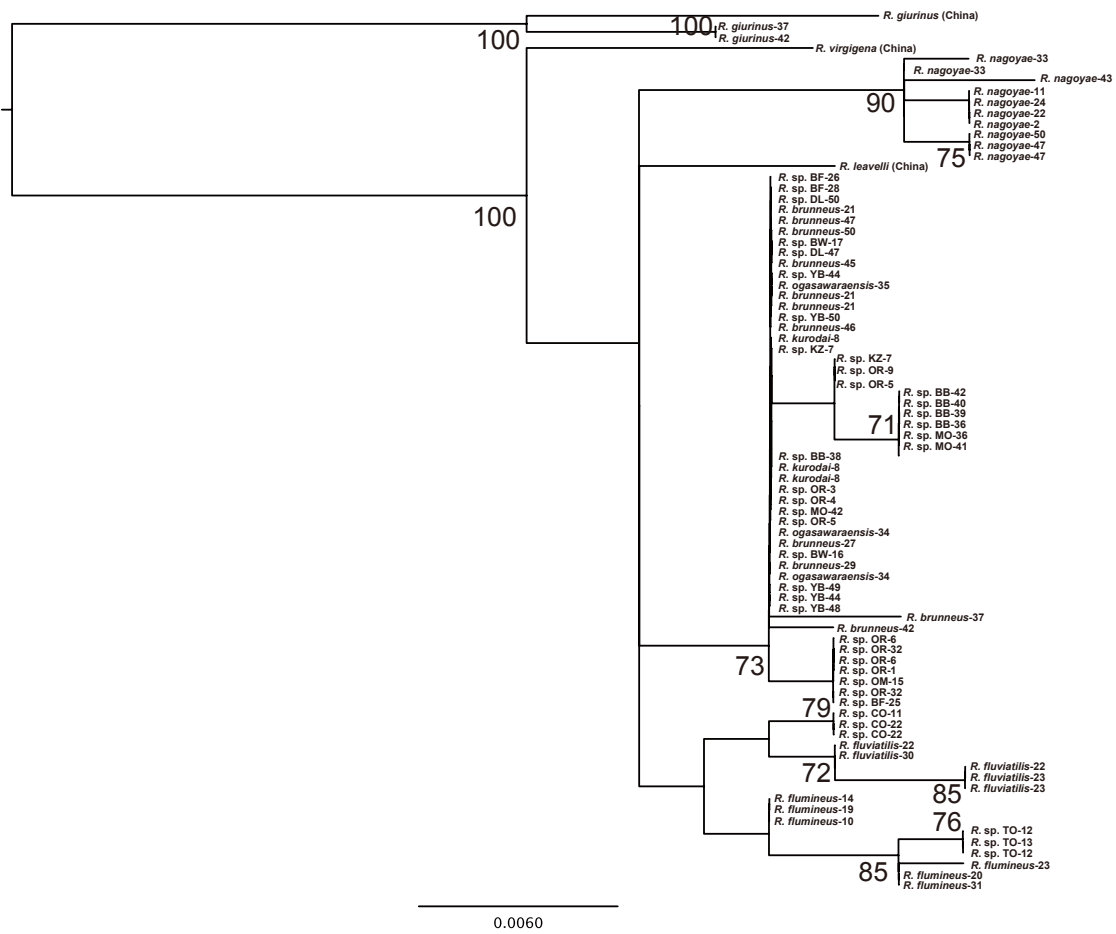
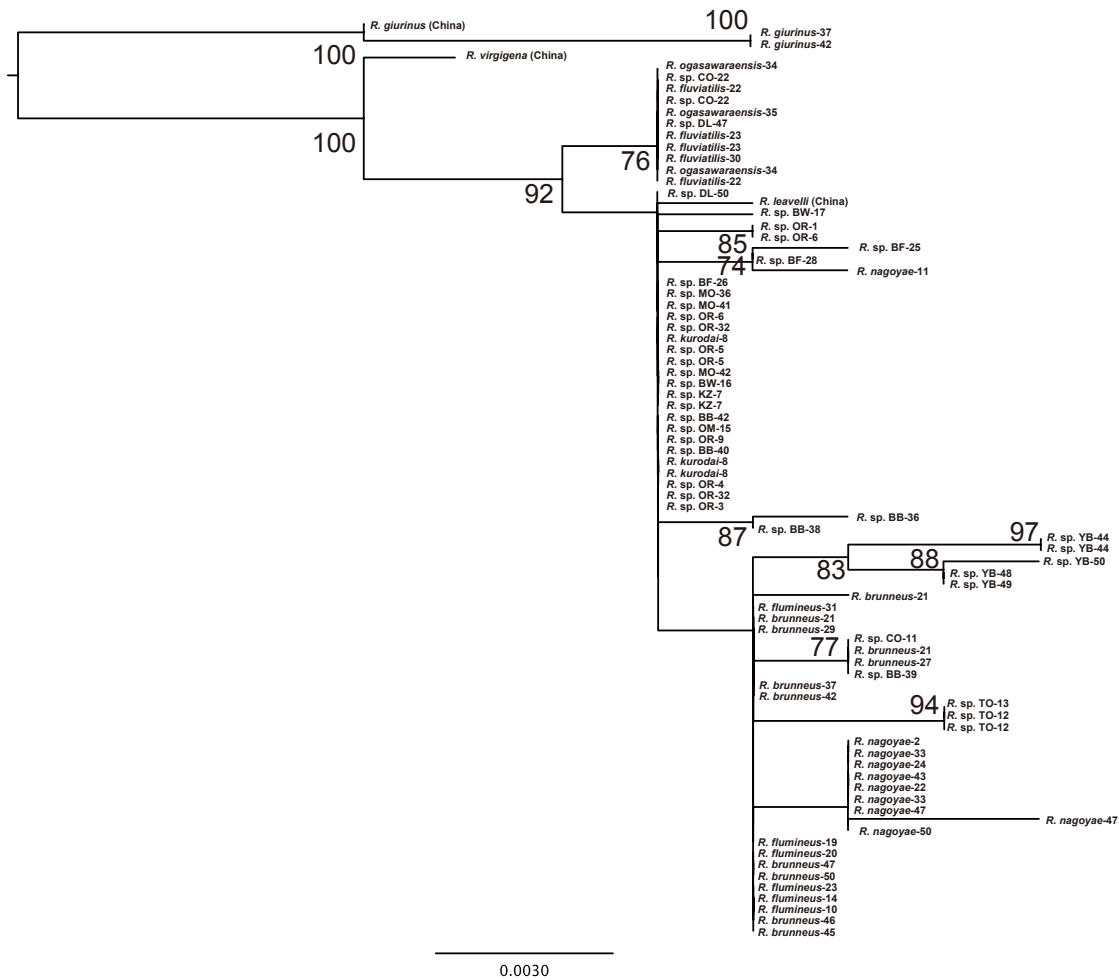


Fig. S2 Maximum likelihood trees of the selected *Rhinogobius* species based on respective nuclear gene sequences. Bootstrap values of >70% are indicated.

(c) Ptr



(d) RAG2

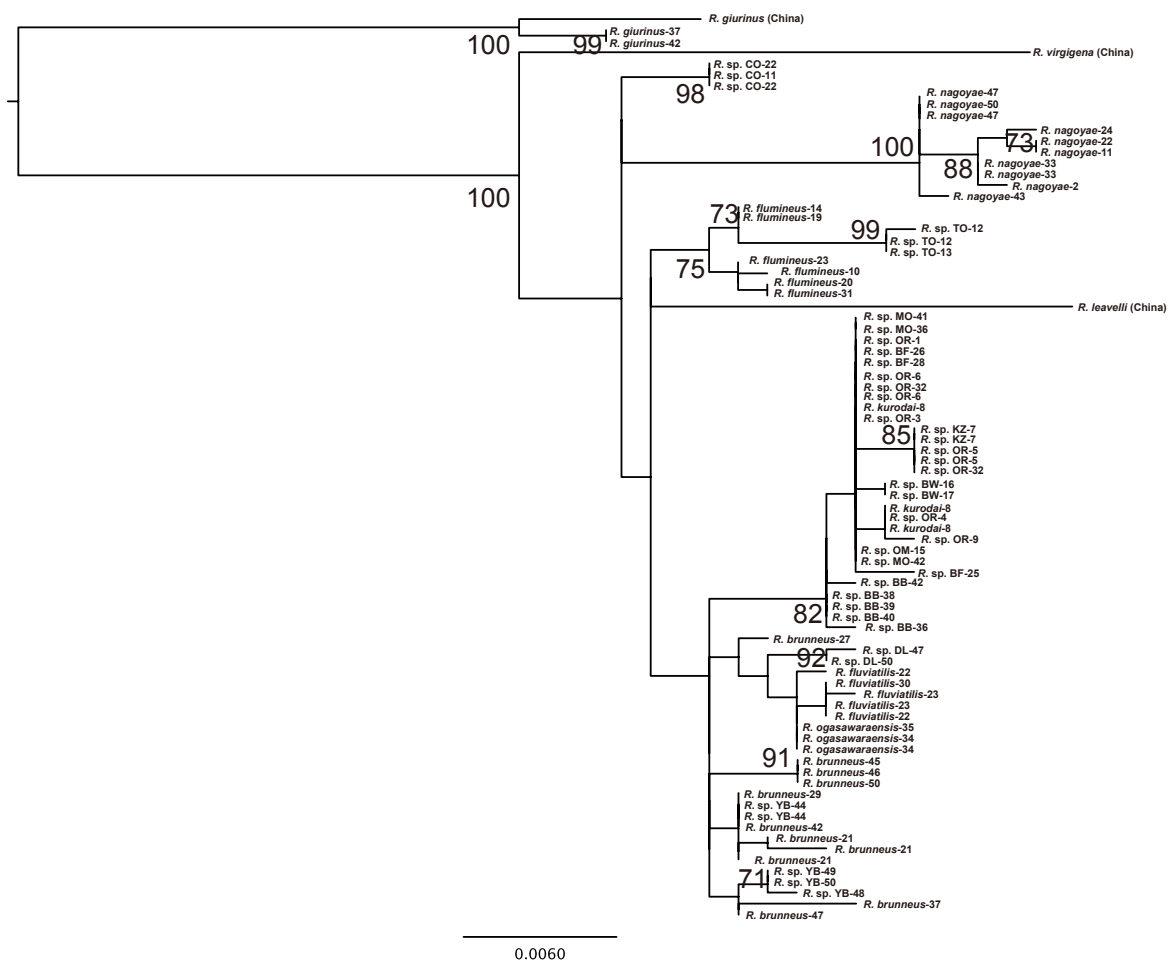
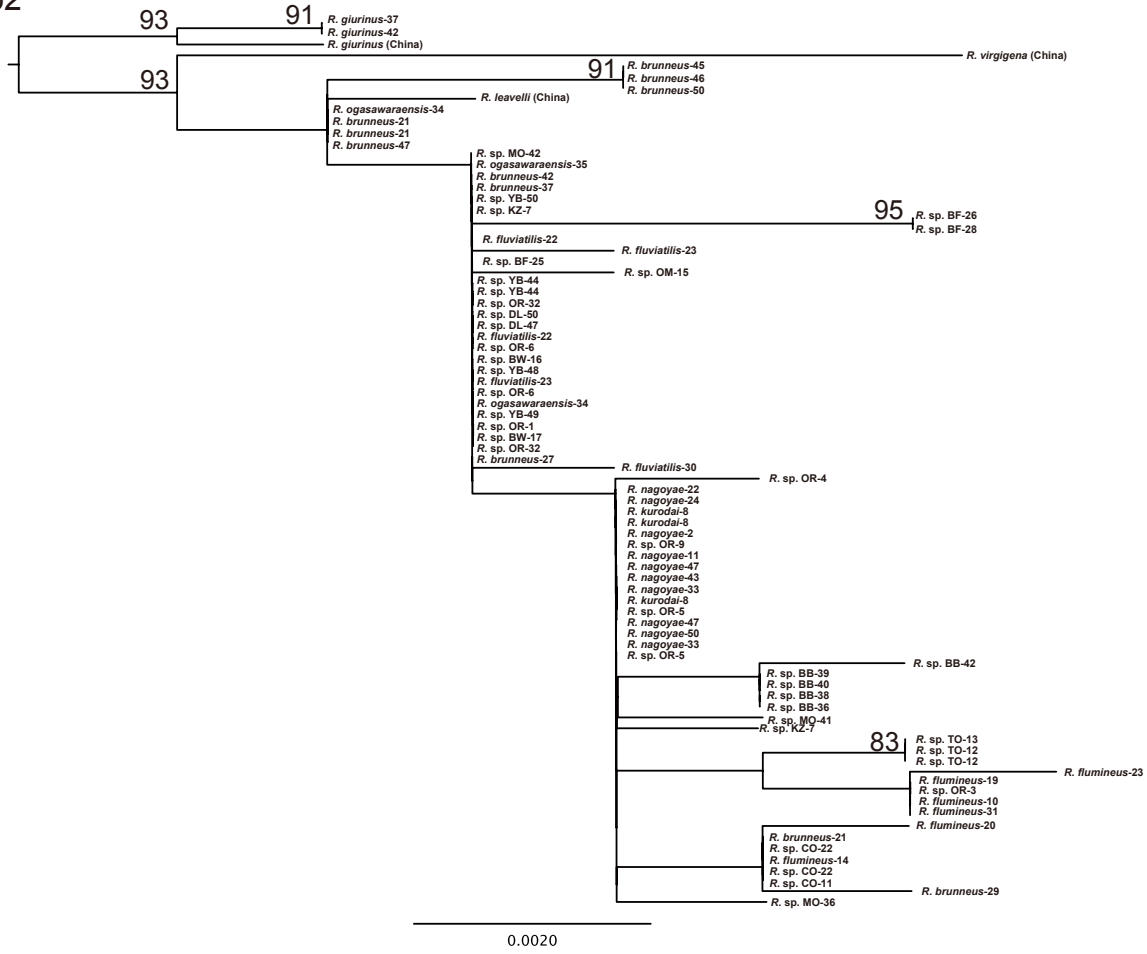


Fig. S2 Continued.

(e) sreb2



(f) EGR3

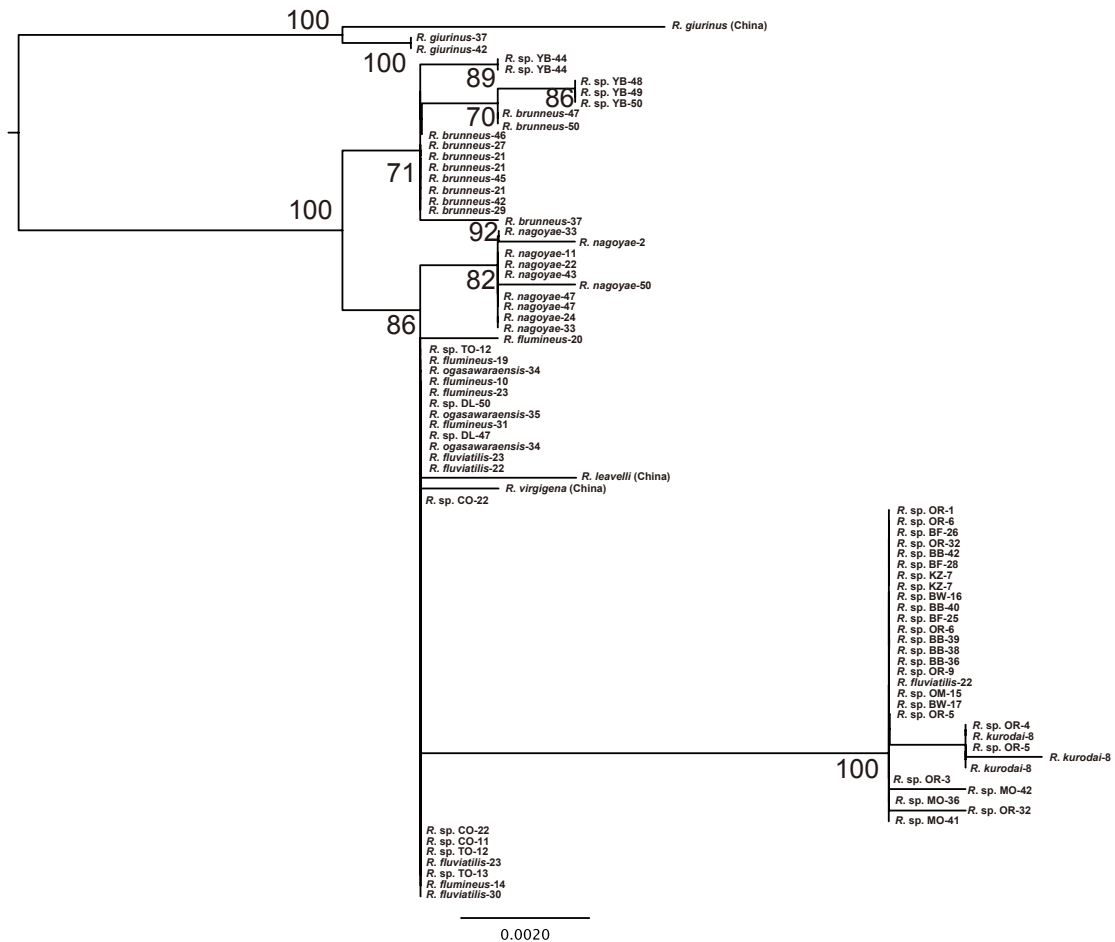


Fig. S2 Continued.

**Table S1.**Distribution and life history characteristics of Japanese *Rhinogobius* species.

Scientific name	Japanese name	Distribution in Japan (the mainland of Japan/Ryukyu Archipelago)	Distribution outside Japan	Life history	Egg size	References
<i>Rhinogobius flumineus</i>	Kawa-Yoshinobori	Yamanashi–Kyushu/—	—	Fluvial	Large	Mizuno (1960)
<i>Rhinogobius</i> sp. YB	Kibara-Yoshinobori	—/Ryukyu Archipelago	—	Fluvial	Middle	Shinomiya et al. (2005); Nishida (2001); Kon and Hirashima and Tachihara (2000) Kato and Nishida (1994); Nishida (2001)
<i>Rhinogobius</i> sp. BB	Aobara-Yoshinobori	—/North area of Okinawa Island	—	Fluvial	Middle	Maruyama et al. (2004); Takahashi and Okazaki
<i>Rhinogobius</i> sp. OM	Oumi-yoshinobori	Shiga, Lake Biwa/—	—	Amphidromous <sup>b</sup>	Small	Akihito et al. (2002)
<i>Rhinogobius</i> sp. OR <sup>a</sup>	Tou-Yoshinobori	Hokkaido–Kyushu/—	—	Amphidromous	Small	Tsunagawa and Arai (2008); Kato and Nishida (1994); Tamada (2005a)
<i>Rhinogobius brunneus</i>	Kuro-Yoshinobori	Chiba–Kyushu/Yakushima–Iriomote Island	—	Amphidromous	Small	Tsunagawa and Arai (2008); Tamada (2001)
<i>Rhinogobius fluviatilis</i>	Oo-Yoshinobori	Honshu–Kyushu/—	—	Amphidromous	Small	Tsunagawa and Arai (2008); Tamada (2001)
<i>Rhinogobius</i> sp. CO	Ruri-Yoshinobori	Hokkaido-Kyushu/—	Korea	Amphidromous	Small	Tsunagawa and Arai (2008); Tamada (2001); Kato and Nishida (1994); Tsunagawa and Arai (2008); Wu et al. (2008)
<i>Rhinogobius nagoyae</i>	Shima-Yoshinobori	Mainlands/Ryukyu Archipelago	Korea, Taiwan, China	Amphidromous	Small	Kondo et al. (2013)
<i>Rhinogobius</i> sp. MO	Aya-Yoshinobori	—/Amamioshima-Kume-jima Island	—	Amphidromous	Small	Akihito et al. (2013)
<i>Rhinogobius</i> sp. DL	Hira-Yoshinobori	—/Yakushima-Iriomote-jima Island	—	Amphidromous	no data	Suzuki et al. (2011)
<i>Rhinogobius ogasawaraensis</i>	Ogasawara-Yoshinobori	Bonin Islands	—	Amphidromous	Small	Takahashi and Okazaki
<i>Rhinogobius</i> sp. BW	Biwa-Yoshinobori	Lake Biwa/—	—	Lentic	Small	Tsunagawa et al. (2010a); Tsujimoto (2008); Yamasaki (personal observation)
<i>Rhinogobius</i> sp. TO	Tokai-Yoshinobori	Aichi, Mie, Gifu/—	—	Lentic	Small	Tsunagawa et al. (2010b); Tsujimoto (2008); Tsujimoto et al. (2003); Hirashima and Nakamura (2014)
<i>Rhinogobius</i> sp. BF	Shimahi-Yoshinobori	Kinki and Setouchi district/—	—	Lentic	Small	Akihito et al. (2013)
<i>Rhinogobius</i> sp. KZ	Kazusa-yoshinobori	Chiba/—	—	no data	no data	Akihito et al. (2013)
<i>Rhinogobius kurodai</i>	Kuroda-haze	Tokyo, Kanagawa, Shizuoka/—	—	no data	no data	Akihito et al. (2013)
<i>Rhinogobius giurinus</i>	Gokurakuhaze	Mainlands/Ryukyu Archipelago	Korea, Taiwan, China	Amphidromous	Small	Wu et al. (2008); Akihito et al. (2013); Tamada (2005b)

<sup>a</sup> Akihito et al. (2013) newly separated six morphological species (*Rhinogobius* sp. OM, *Rhinogobius* sp. KZ, *R. kurodai*, *Rhinogobius* sp. BW, *Rhinogobius* sp. TO, *Rhinogobius* sp. BF) from *Rhinogobius* OR in Akihito et al. (2002), and proposed discarding the name "*Rhinogobius* sp. OR". However, there exist one or more other species in *Rhinogobius* sp. OR (sensu Akihito et al., 2002) that are not included in the six morphological species, for which no scientific names/codes are given in Akihito et al. (2013). We hence use the name "*Rhinogobius* sp. OR" for the remaining species in the present paper.

<sup>b</sup> *Rhinogobius* sp. OM mainly inhabits the middle to lower reaches of rivers flowing into Lake Biwa and its shore. It exhibits amphidromous life history using the lake instead of the ocean, and is treated as the amphidromous type.

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Table S2

Accession and voucher numbers of samples used in this study.

Specimen ID	Voucher	Nuclear/myh6	RYR3	Ptr	RAG2	sreb2	EGR3	mtDNA/COI	ND5	cytb
RF-OS110805-6	NSMT-P 120783	AB988263	AB988345	AB988427	AB988509	AB988591	AB988673	AB988755	AB988833	AB988911
RF-GF110816-1	NSMT-P 120784	AB988264	AB988346	AB988428	AB988510	AB988592	AB988674	AB988756	AB988834	AB988912
RG-HY110809-18	NSMT-P 120785	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988757	AB988835	AB988913
BF-HY110605-1	NSMT-P 120786	AB988265	AB988347	AB988429	AB988511	AB988593	AB988675	N.D.	N.D.	N.D.
LD-OS110805-1	NSMT-P 120787	AB988266	AB988348	AB988430	AB988512	AB988594	AB988676	AB988758	AB988836	AB988914
LD-OS110805-2	NSMT-P 120788	AB988267	AB988349	AB988431	AB988513	AB988595	AB988677	AB988759	AB988837	AB988915
LD-WK110824-23	NSMT-P 120789	AB988268	AB988350	AB988432	AB988514	AB988596	AB988678	AB988760	AB988838	AB988916
LD-WK110824-24	NSMT-P 120790	AB988269	AB988351	AB988433	AB988515	AB988597	AB988679	AB988761	AB988839	AB988917
OM-SG110725-1	NSMT-P 120791	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988762	AB988840	AB988918
OM-SG110725-2	NSMT-P 120792	AB988270	AB988352	AB988434	AB988516	AB988598	AB988680	AB988763	AB988841	AB988919
OR-NI110816-1	NSMT-P 120793	AB988271	AB988353	AB988435	AB988517	AB988599	AB988681	AB988764	AB988842	AB988920
OR-NI110816-2	NSMT-P 120794	AB988272	AB988354	AB988436	AB988518	AB988600	AB988682	AB988765	AB988843	AB988921
CB-HK110724-1	NSMT-P 120795	AB988273	AB988355	AB988437	AB988519	AB988601	AB988683	AB988766	AB988844	AB988922
CB-HK110724-2	NSMT-P 120796	AB988274	AB988356	AB988438	AB988520	AB988602	AB988684	AB988767	AB988845	AB988923
CB-HY110809-1	NSMT-P 120797	AB988275	AB988357	AB988439	AB988521	AB988603	AB988685	AB988768	AB988846	AB988924
CB-HY110809-2	NSMT-P 120798	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988769	AB988847	AB988925
CB-WK110824-6	NSMT-P 120799	AB988276	AB988358	AB988440	AB988522	AB988604	AB988686	AB988770	AB988848	AB988926
TO-GF110820-1	NSMT-P 120800	AB988277	AB988359	AB988441	AB988523	AB988605	AB988687	AB988771	AB988849	AB988927
TO-GF110820-2	NSMT-P 120801	AB988278	AB988360	AB988442	AB988524	AB988606	AB988688	AB988772	AB988850	AB988928
CO-WK110824-2	NSMT-P 120802	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988773	AB988851	AB988929
CO-WK110824-17	NSMT-P 120803	AB988279	AB988361	AB988443	AB988525	AB988607	AB988689	AB988774	AB988852	AB988930
CO-WK110824-21	NSMT-P 120804	AB988280	AB988362	AB988444	AB988526	AB988608	AB988690	AB988775	AB988853	AB988931
DA-WK110825-1	NSMT-P 120805	AB988281	AB988363	AB988445	AB988527	AB988609	AB988691	AB988776	AB988854	AB988932
DA-WK110825-2	NSMT-P 120806	AB988282	AB988364	AB988446	AB988528	AB988610	AB988692	AB988777	AB988855	AB988933
DA-WK110825-3	NSMT-P 120807	AB988283	AB988365	AB988447	AB988529	AB988611	AB988693	AB988778	AB988856	AB988934
BW-SG110623-3	NSMT-P 120808	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988779	AB988857	AB988935
CO-SZ110911-1	NSMT-P 120809	AB988284	AB988366	AB988448	AB988530	AB988612	AB988694	AB988780	AB988858	AB988936
CB-SZ110911-6	NSMT-P 120810	AB988285	AB988367	AB988449	AB988531	AB988613	AB988695	AB988781	AB988859	AB988937
RF-SZ110911-28	NSMT-P 120811	AB988286	AB988368	AB988450	AB988532	AB988614	AB988696	AB988782	AB988860	AB988938
BF-HY110913-1	NSMT-P 120812	AB988287	AB988369	AB988451	AB988533	AB988615	AB988697	AB988783	AB988861	AB988939
DA-OK110923-3	NSMT-P 120813	AB988288	AB988370	AB988452	AB988534	AB988616	AB988698	AB988784	AB988862	AB988940
DA-OK110924-23	N.D.	AB988289	AB988371	AB988453	AB988535	AB988617	AB988699	AB988785	AB988863	AB988941
MO-OK110923-7	NSMT-P 120815	AB988290	AB988372	AB988454	AB988536	AB988618	AB988700	AB988786	AB988864	AB988942
BB-OK110924-15	NSMT-P 120816	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988787	AB988865	AB988943
BB-OK110924-32	NSMT-P 120817	AB988291	AB988373	AB988455	AB988537	AB988619	AB988701	AB988788	AB988866	AB988944
RG-OK110924-19	NSMT-P 120818	AB988292	AB988374	AB988456	AB988538	AB988620	AB988702	AB988789	AB988867	AB988945
RG-OK110925-17	NSMT-P 120819	AB988293	AB988375	AB988457	AB988539	AB988621	AB988703	AB988790	AB988868	AB988946
CB-AK111003-3	NSMT-P 120820	AB988294	AB988376	AB988458	AB988540	AB988622	AB988704	AB988791	AB988869	AB988947
BI-CC011116-1	NSMT-P 65160	AB988295	AB988377	AB988459	AB988541	AB988623	AB988705	AB988792	AB988870	AB988948
BI-CC011116-2	NSMT-P 65160	AB988296	AB988378	AB988460	AB988542	AB988624	AB988706	AB988793	AB988871	AB988949
BI-HH011124-1	NSMT-P 65165	AB988297	AB988379	AB988461	AB988543	AB988625	AB988707	AB988794	AB988872	AB988950
BI-HH011124-2	NSMT-P 65165	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988795	AB988873	AB988951
DL-IR020223-1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988796	AB988874	AB988952
RF-KY120402-1	NSMT-P 120821	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988797	AB988875	AB988953
OR-HK110724-5	NSMT-P 120822	AB988298	AB988380	AB988462	AB988544	AB988626	AB988708	AB988798	AB988876	AB988954
OR-HK110724-6	NSMT-P 120823	AB988299	AB988381	AB988463	AB988545	AB988627	AB988709	N.D.	N.D.	N.D.
CB-IR120327-1	NSMT-P 120824	AB988300	AB988382	AB988464	AB988546	AB988628	AB988710	AB988799	AB988877	AB988955
DA-IR120327-2	NSMT-P 120825	AB988301	AB988383	AB988465	AB988547	AB988629	AB988711	AB988800	AB988878	AB988956
MO-OK111211-1	N.D.	AB988302	AB988384	AB988466	AB988548	AB988630	AB988712	AB988801	AB988879	AB988957
MO-OK111211-2	NSMT-P 120826	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988802	AB988880	AB988958
BB-OK120305-1	NSMT-P 120827	AB988303	AB988385	AB988467	AB988549	AB988631	AB988713	AB988803	AB988881	AB988959
BB-OK120305-2	NSMT-P 120828	AB988304	AB988386	AB988468	AB988550	AB988632	AB988714	N.D.	N.D.	N.D.
DL-IR120328-1	N.D.	AB988305	AB988387	AB988469	AB988551	AB988633	AB988715	N.D.	N.D.	N.D.
YB-OK111211-3	N.D.	AB988306	AB988388	AB988470	AB988552	AB988634	AB988716	AB988804	AB988882	AB988960
YB-OK111211-4	N.D.	AB988307	AB988389	AB988471	AB988553	AB988635	AB988717	AB988805	AB988883	AB988961
YB-IR120328-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988806	AB988884	AB988962
YB-IR120328-4	NSMT-P 120829	AB988308	AB988390	AB988472	AB988554	AB988636	AB988718	AB988807	AB988885	AB988963
BF-OY110722-1	N.D.	AB988309	AB988391	AB988473	AB988555	AB988637	AB988719	AB988808	AB988886	AB988964
DA-SM120511-1	NSMT-P 120830	AB988310	AB988392	AB988474	AB988556	AB988638	AB988720	AB988809	AB988887	AB988965
LD-SM120511-5	NSMT-P 120831	AB988311	AB988393	AB988475	AB988557	AB988639	AB988721	AB988810	AB988888	AB988966
TO-GF120415-7	NSMT-P 120832	AB988312	AB988394	AB988476	AB988558	AB988640	AB988722	AB988811	AB988889	AB988967
CB-OK110925-2	NSMT-P 120814	AB988313	AB988395	AB988477	AB988559	AB988641	AB988723	N.D.	N.D.	N.D.
DA-IR120622-4	N.D.	AB988314	AB988396	AB988478	AB988560	AB988642	AB988724	AB988812	AB988890	AB988968
DA-IR120625-1	N.D.	AB988315	AB988397	AB988479	AB988561	AB988643	AB988725	AB988813	AB988891	AB988969
DA-IR120626-1	N.D.	AB988316	AB988398	AB988480	AB988562	AB988644	AB988726	AB988814	AB988892	AB988970
DL-IR120622-2	NSMT-P 120833	AB988317	AB988399	AB988481	AB988563	AB988645	AB988727	AB988815	AB988893	AB988971
CB-IR120622-1	NSMT-P 120834	AB988318	AB988400	AB988482	AB988564	AB988646	AB988728	AB988816	AB988894	AB988972
CB-IR120622-3	N.D.	AB988319	AB988401	AB988483	AB988565	AB988647	AB988729	N.D.	N.D.	N.D.
YB-PN120622-5	N.D.	AB988320	AB988402	AB988484	AB988566	AB988648	AB988730	AB988817	AB988895	AB988973
YB-KR120624-1	N.D.	AB988321	AB988403	AB988485	AB988567	AB988649	AB988731	AB988818	AB988896	AB988974
OR-FS091026-1	N.D.	AB988322	AB988404	AB988486	AB988568	AB988650	AB988732	AB988819	AB988897	AB988975
OR-FS091026-2	N.D.	AB988323	AB988405	AB988487	AB988569	AB988651	AB988733	N.D.	N.D.	N.D.
<i>R. leavelli</i>	NSMT-P 120835	AB988324	AB988406	AB988488	AB988570	AB988652	AB988734	AB988820	AB988898	AB988976

<i>R. virgigena</i>	NSMT-P 120836	AB988325	AB988407	AB988489	AB988571	AB988653	AB988735	AB988821	AB988899	AB988977
RG-CH990405-1	NSMT-P 120837	AB988326	AB988408	AB988490	AB988572	AB988654	AB988736	N.D.	N.D.	N.D.
OR-IW081011-2	N.D.	AB988327	AB988409	AB988491	AB988573	AB988655	AB988737	N.D.	N.D.	N.D.
OR-IW090528-1	NSMT-P 120838	AB988328	AB988410	AB988492	AB988574	AB988656	AB988738	AB988822	AB988900	AB988978
OR-HO120408-1	N.D.	AB988329	AB988411	AB988493	AB988575	AB988657	AB988739	N.D.	N.D.	N.D.
RF-HI120512-1	N.D.	AB988330	AB988412	AB988494	AB988576	AB988658	AB988740	N.D.	N.D.	N.D.
RF-YN120714-1	NSMT-P 120839	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988823	AB988901	AB988979
OR-YN120714-5	NSMT-P 120840	AB988331	AB988413	AB988495	AB988577	AB988659	AB988741	N.D.	N.D.	N.D.
RF-ME120905-1	NSMT-P 120841	AB988332	AB988414	AB988496	AB988578	AB988660	AB988742	N.D.	N.D.	N.D.
RF-NR120930-1	NSMT-P 120842	AB988333	AB988415	AB988497	AB988579	AB988661	AB988743	N.D.	N.D.	N.D.
DA-KW120730-1	NSMT-P 120843	AB988334	AB988416	AB988498	AB988580	AB988662	AB988744	N.D.	N.D.	N.D.
KU-TK100705-1	NSMT-P 120844	AB988335	AB988417	AB988499	AB988581	AB988663	AB988745	AB988824	AB988902	AB988980
KU-TK100705-2	NSMT-P 120845	AB988336	AB988418	AB988500	AB988582	AB988664	AB988746	AB988825	AB988903	AB988981
KU-TK100705-3	NSMT-P 120846	AB988337	AB988419	AB988501	AB988583	AB988665	AB988747	N.D.	N.D.	N.D.
BW-SG130523-1	NSMT-P 120849	AB988338	AB988420	AB988502	AB988584	AB988666	AB988748	N.D.	N.D.	N.D.
BW-SG130601-1	NSMT-P 120850	AB988339	AB988421	AB988503	AB988585	AB988667	AB988749	AB988826	AB988904	AB988982
BB-OK121111-1	NSMT-P 120851	AB988340	AB988422	AB988504	AB988586	AB988668	AB988750	N.D.	N.D.	N.D.
BB-OK121111-4	NSMT-P 120852	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988827	AB988905	AB988983
BB-OK121111-5	NSMT-P 120853	AB988341	AB988423	AB988505	AB988587	AB988669	AB988751	N.D.	N.D.	N.D.
MO-OK121111-3	NSMT-P 120854	AB988342	AB988424	AB988506	AB988588	AB988670	AB988752	N.D.	N.D.	N.D.
KZ-CB100418-1	NSMT-P 120855	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988828	AB988906	AB988984
KZ-CB100418-2	NSMT-P 120856	AB988343	AB988425	AB988507	AB988589	AB988671	AB988753	AB988829	AB988907	AB988985
KZ-CB100418-3	NSMT-P 120857	AB988344	AB988426	AB988508	AB988590	AB988672	AB988754	AB988830	AB988908	AB988986
CB-OK130630-1	NSMT-P 120860	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988831	AB988909	AB988987
CB-OK130630-2	NSMT-P 120861	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	AB988832	AB988910	AB988988

**Table S3.**

Information of loci and primers used in this study

Locus name	Length (bp)	Number of variable sites (bp) <sup>a</sup>	Number of Parsimony informative sites (bp) <sup>a</sup>	Ann eali ng tem p. (° C)	1st primer name	primer 1 (5'-3')	2nd primer name	primer 2 (5'-3')	Reference of primers
Nuclear									
myh6	719	38/29	22/12	55	myh6_F459	CATMTTYTCCATCTCAGATAATGC	myh6_F507	GGAGAATCARTCKGTGCTCATCA	Li et al. (2007)
					myh6_R1325	ATTCTCACCACCATCCAGTTGAA	myh6_R1322	CTCACCACCATCCAGTTGAACAT	
RYR3	806	45/32	31/19	55	RYR3_F15	GGAACATYGGTAAGCARATGG	RYR3_F22	TCGGTAAGCARATGGTGGACA	Li et al. (2007)
					RYR3_R968	TGGAAGAAKCCAAAKATGATGC	RYR3_R931	AGAATCCRGTAAGAGCATCCA	
Ptr	636	30/22	24/14	55	R-PtrF	TGTATCTCATCTATGCCTCTTTTCA			Li et al. (2007); this study
					R-PtrR	AGAGGTGACCGTCAGGATGAG			
RAG2	882	115/87	79/50	55	R-RAG2F	GTCGAACCCCAAACAATGAG			Lovejoy et al. (2001); this study
					R-RAG2R	GCTGTCGTCCAATTCATGTG			
sreb2	870	31/27	14/11	55	sreb2_F10	ATGGCGAACTAYAGCCATGC	sreb2_F27	TGCAGGGGACCACAMCAT	Li et al. (2007)
					sreb2_R1094	CTGGATTTTCTGCAGTASAGGAG	sreb2_R1082	CAGTASAGGAGCGTGGTGCT	
EGR3	842	33/21	21/13	55	E3 F161	AATATCATGGACYTGGGNATGG			Chen et al. (2008)
					E3 1136R	GGYTTCTTGTCTTCTGTTTTSAG			
mtDNA									
CO1	638	171/154	125/87	48	FishCO1F	TCAACCAACCACAAAGACATTGGCAC			Ward et al. (2005)
					FishCO1R	TAGACTTCTGGGTGGCCAAAGAATCA			
ND5	965	319/286	270/209	48	L12329-Leu	CTCTTGGTGCAAMTCCAAGT			Miya and Nishida (2000)
					H13396-ND5	CCTATTTTTCGGATGTCTTG			
					L12321-Leu	GGTCTTAGGAACCAAAAACCTTGGTGCAA			Miya and Nishida (2000)
					H13366-ND5-Rhi	AAGGCTGTGAATGATGGAGC			Mukai et al. (2012)
cytb	1178	351/309	297/225	48	L14724	TGACTTGAARAACCA YCGYYG			Palumbi et al. (1991)
					H16500-CR	GCCCTGAAATAGGAACCAGA			Inoue et al. (2000)

<sup>a</sup> With outgroup / without outgroup

Reference

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**Table S4.**

Models selected by Partitionfinder, using AIC for RAxML and BIC for MrBayes.

Subset	Subset partition	Best model
RAxML/mtDNA		
	1 CO1-1st, CO1-2nd, ND5-1st, ND5-2nd, cytb-1st, cytb-2nd	GTR+I+G
	2 CO1-3rd, ND5-3rd, cytb-3rd	GTR+I+G
MrBayes/mtDNA		
	1 CO1-2nd, ND5-2nd, cytb-2nd	GTR+I
	2 CO1-3rd, ND5-3rd, cytb-3rd	GTR+I+G
	3 CO1-1st, ND5-1st, cytb-1st	K80+I+G
RAxML/nuclear		
	1 myh6-1st, myh6-2nd, RYR3-1st, RYR3-3rd, Ptr-1st, Ptr-2nd, RAG2-2nd, RAG2-3rd, sreb2-1st, sreb2-2nd, EGR3-1st, EGR3-2nd	GTR+I+G
	2 myh6-3rd, RYR3-2nd, Ptr-3rd, RAG2-1st, sreb2-3rd, EGR3-3rd	GTR+I+G
MrBayes/nuclear		
	1 myh6-2nd, RYR3-1st	F81+I
	2 myh6-3rd, RYR3-2nd, Ptr-3rd, sreb2-3rd, EGR3-3rd	GTR+I+G
	3 myh6-1st, RYR3-3rd, Ptr-1st, RAG2-2nd, RAG2-3rd, sreb2-1st	HKY+I
	4 Ptr-2nd, sreb2-2nd	F81
	5 RAG2-1st	HKY+I
	6 EGR3-1st, EGR3-2nd	F81

**Table S5**

Models for BEAST analysis selected by  
BIC implemented by jModelTest v 2.1.3.

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mtDNA

CO1	TrN+G
ND5	TrN+I+G
cytb	GTR+I+G

nuclear DNA

Concatenated	HKY+I+G
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**Table S6**

Estimated parameters by state dependent diversification analysis under the MuSSE model. Character state codes (1-3) indicate amphidromous, lentic, and fluvial in life history analysis, and small, middle, large in egg size analysis respectively. These models estimated speciation ( $\lambda$ ), extinction ( $\mu$ ), and transition rates (q).

Model	DF	AIC	$\lambda_1$	$\lambda_2$	$\lambda_3$	$\mu_1$	$\mu_2$	$\mu_3$	q12	q21	q13	q31	q23	q32
<b>Life History</b>														
Full	12	118.18	0.485	0.379	0.145	7.48E-09	1.28E-06	2.40E-05	0.0830	4.03E-07	0.0744	3.67E-06	0.550	0.286
Equal $\lambda$	10	115.21	0.447	-	-	2.72E-11	9.36E-10	0.255	0.0783	1.12E-07	0.0938	1.19E-06	0.589	0.280
Equal $\mu$	10	114.18	0.485	0.379	0.145	6.29E-07	-	-	0.0830	9.68E-09	0.0744	1.50E-05	0.550	0.286
Equal q	9	112.77	0.492	0.354	0.140	0	3.45E-07	0	0.0749	-	0.1035	-	0.489	-
Equal $\lambda$ and $\mu$	8	111.29	0.429	-	-	5.19E-09	-	-	0.0679	1.83E-07	0.0845	7.67E-08	0.513	0.318
Equal $\lambda$ and q (reversal transition rates are equal)	7	109.98	0.439	-	-	4.32E-09	0.189	0.00857	0.0698	-	0.1065	-	0.444	-
Equal $\mu$ and q (reversal transition rates are equal)	7	108.77	0.492	0.354	0.140	8.86E-09	-	-	0.0749	-	0.1035	-	0.489	-
Equal q (all transition rates are equal)	7	109.53	0.512	0.359	0.204	6.87E-06	0.363	1.34E-05	0.1274	-	-	-	-	-
Equal $\lambda, \mu, q$ (reversal transition rates are equal)	5	106.02	0.429	-	-	2.70E-08	-	-	0.0567	-	0.1057	-	0.447	-
Equal $\lambda$ and q (all transition rates are equal)	5	106.71	0.462	-	-	3.75E-08	0.444	0.149	0.1249	-	-	-	-	-
Equal $\mu$ and q (all transition rates are equal)	5	105.80	0.496	0.304	0.198	3.88E-08	-	-	0.1099	-	-	-	-	-
Equal $\lambda, \mu, q$ (all transition rates are equal)	3	103.06	0.429	-	-	1.58E-08	-	-	0.0983	-	-	-	-	-
<b>Egg size</b>														
Full	12	106.22	0.511	1.21E-06	0.335	4.91E-06	1.97E-11	5.54E-06	0.2125	1.085	5.12E-11	0.334	3.21E-09	3.58E-06
Equal $\lambda$	10	102.86	0.438	-	-	6.44E-09	1.63E-05	0.102	0.3941	2.298	6.15E-07	0.311	7.26E-08	5.95E-08
Equal $\mu$	10	102.22	0.511	1.75E-06	0.335	6.04E-07	-	-	0.2125	1.085	5.04E-07	0.334	1.91E-09	1.07E-06
Equal q	9	103.62	0.506	4.39E-09	0.222	1.39E-07	0.592	1.42E-06	0.1372	-	0.0661	-	5.19E-07	-
Equal $\lambda$ and $\mu$	8	98.88	0.429	-	-	9.15E-07	-	-	0.3955	2.304	5.66E-07	0.348	2.15E-09	7.88E-10
Equal $\lambda$ and q (reversal transition rates are equal)	7	101.05	0.484	-	-	6.76E-07	1.023	0.330	0.1470	-	0.0783	-	7.07E-09	-
Equal $\mu$ and q (reversal transition rates are equal)	7	100.19	0.482	2.78E-07	0.225	2.76E-05	-	-	0.0803	-	0.0695	-	3.55E-05	-
Equal q (all transition rates are equal)	7	100.07	0.498	2.42E-09	0.212	8.33E-08	0.429	0.132	0.0929	-	-	-	-	-
Equal $\lambda, \mu, q$ (reversal transition rates are equal)	5	98.17	0.429	-	-	2.62E-07	-	-	0.0758	-	0.0651	-	1.28E-07	-
Equal $\lambda$ and q (all transition rates are equal)	5	97.50	0.476	-	-	3.70E-08	0.855	0.387	0.1022	-	-	-	-	-
Equal $\mu$ and q (all transition rates are equal)	5	96.41	0.481	1.06E-05	0.211	2.90E-07	-	-	0.0725	-	-	-	-	-
Equal $\lambda, \mu, q$ (all transition rates are equal)	3	94.45	0.429	-	-	2.26E-07	-	-	0.0675	-	-	-	-	-