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

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Abstract

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

Keywords

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

1. Introduction

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

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

The family Gobiidae (Order Gobiifromes; Betancur-R et al., 2013, 2014) is one of the most divergent groups among teleost fishes in terms of the number of species and ecology, and it has interested researchers in the fields of ecology and evolutionary biology (Yamada et al., 2009; Rüber and Agorreta, 2011). The genus *Rhinogobius* is widely distributed in freshwaters from East to Southeast Asia. It consists of more than 85 species, and it is the largest genus of freshwater gobies (Suzuki et al., 2004; Oijen at al., 2011). The species of this genus are classified into three types based on migration-related life history. The amphidromous type is the most general form, in which larval fish flow down to the sea immediately after hatching in the river. This is followed by early feeding and growth at the sea, and then a return the river at the juvenile stage for subsequent growth and reproduction (Mizuno, 2001; Keith and Lord, 2011). Lentic types complete their life cycle in standing freshwaters such as lakes and marshes (Takahashi and Okazaki, 2002; Tsunagawa et al., 2010a, 2010b), whereas fluvial types complete their life cycle in running freshwaters (Mizuno, 1960; Nishijima, 1968; Iwata, 2001a, 2001b). Corresponding to these life history types, there is a large interspecific variation in egg size in *Rhinogobius* species (Mizuno, 1960; Nishida, 2001; Tamada, 2001; Closs et al., 2013). Amphidromous and lentic species produce small eggs (0.6–0.9 mm in the major axis; Katoh and Nishida, 1994; Tsujimoto, 2008; Takahashi and Okazaki, 2002), whereas fluvial species spawn larger eggs (1.1–2.1 mm; Mizuno, 1960; Katoh and Nishida, 1994). The fluvial species also exhibit egg size variation, i.e., species on the mainland of Japan produce larger eggs (1.4–2.1 mm; Mizuno, 1960) than several species in the Ryukyu Archipelago (1.1–1.5 mm; often called "middle-sized eggs"; Katoh and Nishida, 1994).

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

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

Given the considerable confusion regarding *Rhinogobius* taxonomy and the lack of ecological information for large numbers of *Rhinogobius* species (Suzuki and Chen, 2011), exhaustive analysis, particularly with the inclusion of species outside Japan, is difficult to conduct at present. Therefore, this study focused mainly on the Japanese *Rhinogobius* species, most of which are endemic to Japan and whose taxonomic and ecological information are relatively well understood. First, we estimated the phylogenetic relationships among all known Japanese species by using multiple nuclear gene sequences. We also estimated their mtDNA-based phylogeny. The objectives of this study were as follows: (1) to reveal the patterns of phylogenetic diversification of Japanese *Rhinogobius* gobies; (2) to reveal the spatiotemporal patterns of interspecific hybridization causing mitochondrial introgression; (3) to test whether the freshwater species, including fluvial and lentic types, evolved independently from amphidromous species; and (4) to reveal the relationships between life history and egg size evolution.

2. Materials and Methods

2.1. Sampling

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

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

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

2.2. DNA extraction, PCR, and sequencing

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

PCR amplification was performed in a 15-μl volume containing 8 μl ultrapure water, 1.5 μl 2.5 mM dNTP mix, 1.5 μl Ex-Taq buffer, 1.5 μl of each 5 μM primer, 0.3 μl Ex-Taq DNA polymerase (Takara, Shiga, Japan), and 1 μl (ca. 10–100 ng) of DNA template. We also used KOD FX or KOD Plus Neo (Toyobo, Osaka, Japan) for specimens in which the amplification was difficult. The PCR using KOD FX was performed in a 15-μl volume containing 2.4 μl ultrapure water, 3 μl 2.0 mM dNTP mix, 7.5 μl buffer, 0.9 μl of each 5 μM primer, 0.3 μl KOD FX DNA polymerase, and 1 μl of DNA template. The PCR using KOD Plus Neo was performed in a 15-μl volume containing 8.0 µl ultrapure water, 1.5 µl 2.0 mM dNTP mix, 1.5 µl buffer, 0.9 µl 25 mM $MgCl_2$, 0.9 µl of each 5 µM primer, 0.3 µl KOD Plus Neo DNA polymerase, and 1 µl of DNA template. The settings for PCR using Ex-Taq consisted of the first step (denature, 94 °C, 2 min), 35 cycles of the second step (denature, 94 °C, 30 s; annealing, 48–62 °C, 30 s; extension, 72 °C, 1 min), and the last step (extension, 68 °C, 7 min); and those for PCR using KOD FX or KOD Plus Neo consisted of the first step (denature, 94 °C, 2 min), 35 cycles of the second step (denature, 98 °C, 10 s; annealing, 55–62 °C, 30 s; extension, 68 °C, 30–40 s), and the last step (extension, 68 °C, 7 min). Nested PCR was performed using two primer pairs described by Li et al. (2007) in some samples; PCR products were diluted 20–100 times with water, and PCR was performed again using these diluted PCR products as templates.

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

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

2.3. Inference of molecular phylogeny

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

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

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

2.4. Estimation of divergence time

We estimated the divergence time from concatenated data of mtDNA regions and nuclear genes, independently. Because to date, no fossil records are available that could be used to construct the tree for *Rhinogobius* and its relatives, we used the following two geographic events and previously estimated molecular evolutionary rates for divergence time calibration. First, the Bonin Islands are oceanic islands that have never been connected to continents. They were formed 1.8 million years ago (Mya) or later, and it is assumed that their endemic fauna was formed 0.9–1.8 Mya (Kaizuka, 1977; Imaizumi and Tamura, 1984; Chiba, 2002). The time of the most recent common ancestors (tMRCA) of *R. ogasawaraensis* (endemic species to the Bonin Islands) and its sister species, or its clade was constrained following a normal prior distribution within the limit of 0–2.0 million years (Myr) (mean = 1.8, standard deviation (SD) = 0.4; calibration 1, CA1). Since it is possible that interspecific hybridization occurred after this divergence (see Discussion), we did not use this constraint in the mtDNA-based phylogenetic tree. Secondly, we used the opening time of the Tokara Gap, which divided the Japanese Archipelago and the Ryukyu Archipelago (1.55 ± 0.154 Mya; Osozawa et al., 2012). Migration of amphidromous *Rhinogobius* species between the mainland and Ryukyu Archipelago just after the opening of the gap was probably not restricted because their larvae can disperse through the coastal areas. With further extension of the gap, their possible migration across the gap gradually decreased; finally, the isolation of populations at either side of the gap was complete. Therefore, we used the opening time of the Tokara Gap as the upper limit of the divergence time between mutually monophyletic groups distributed in the mainland and in the Ryukyu Archipelago. The tMRCA of the mutually monophyletic groups was constrained following a normal prior distribution within the limit of 0–1.7 Myr (mean = 1.55, SD = 0.4; calibration 2, CA2). Since the mtDNA phylogeny revealed the existence of mainland and Ryukyu groups beyond species boundaries (see Results), we applied CA2 to their tMRCA. On the other hand, in the nuclear DNA analysis, we used this constraint only for clear intraspecific divergence because a previous study suggested that another older geographic event could have caused a similar vicariance pattern between mainland and Ryukyu species in some gobiid groups (Mukai, 2010). Finally, we used the molecular evolutionary rate of 3.0%/Myr (pairwise) (95% highest posterior density (HPD), 0.7–4.8%/Myr) for cytb, which was estimated for the genus *Gymnogobius* (Tabata and Watanabe 2013), phylogenetically relatively close to *Rhinogobius* (Agorreta et al., 2013). We assumed that the evolutionary rate of cytb followed a lognormal prior distribution ranging from 0.0 to 10^{100} per Mya (initial value = 0.015, mean = 0.015, SD = 0.7, offset = 0.0, mean in real space = yes).

We used BEAST v1.7.5 (Drummond et al., 2012) to estimate the divergence time of trees inferred using mtDNA and nuclear genes, respectively. Originally, we attempted to construct a species tree by using *BEAST (Heled and Drummond, 2010) for nuclear genes using several prior settings. However, the parameters did not converge and we abandoned this analysis. Later, we attempted to conduct the phylogenetic analysis with different evolutionary models for respective nuclear genes, but the parameters did not converge well either. Although the reason for this was not clear, it might be possible that low sequence variation and introgression in a part of the loci might influence the analyses. Finally, we concatenated sequences from the six nuclear genes and estimated a dated nuclear gene tree. In order to date the tree, we adopted the random local clock model, which assumes one or more independent rates on different branches (Drummond and Suchard, 2010). We did not specify partition by codon position, because the parameters did not converge in mtDNA or nuclear DNA data when partitioned. We used jModelTest v2.1.3 (Darriba et al., 2012) to select the evolutionary model without partitioning and selected the best model based on BIC. We selected the speciation tree prior (Yule process; Yule, 1925; Gernhard, 2008) and estimated a starting tree using the UPGMA method. We conducted MCMC analysis four times independently. For each MCMC, we performed a run of 50 and 100 million generations (for mtDNA and nuclear genes, respectively), sampling every 1000th generation. The first 10% of the trees were discarded as burn-in for each run. We assessed whether parameter values for individual runs had reached equilibrium and convergence by visually assessing their trace plots in Tracer 1.5. Individual chains were combined using the LogCombiner v1.7.5. Finally, we analyzed combined runs using Tree Annotator v1.7.5.

2.5 Reconstruction of ancestral states

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

3. Results

3.1. Phylogeny and divergence time estimates based on mtDNA sequences

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

The calibration point CA2 was applied for the divergence between the M-5+M-6 (most samples collected from the mainland of Japan) and M-7 (collected from the Ryukyu Archipelago) haplotypes. The tMRCA of these was estimated at 1.17 Myr (95% HPD, 0.62–1.70 Myr in Table 2). The tMRCA of all the Japanese *Rhinogobius* species was estimated at1.59 Myr (0.73–2.39 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 (0.47–1.44 Myr), respectively.

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

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

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

In the N-1 clade, *Rhinogobius* sp. TO formed a monophyletic group with a part of *R. flumineus* samples (BP 57%, PPM 0.93, PPB 0.90). Although statistical support was weak, *R. brunneus* and *Rhinogobius* sp. YB were each polyphyletic in N-3-1 (AU test, *Rhinogobius* sp. YB, p = 0.42; *R. brunneus*, p = 0.335). In N-3-2 clade, *R. fluviatilis* and *R. ogasawaraensis* constituted a sister group with *Rhinogobius* sp. DL (BP 83%, PPM 1.0, PPB 1.0). In N-3-3, *Rhinogobius* sp. MO and *Rhinogobius* sp. BB formed a weakly supported monophyletic group in the ML tree (BP 52%, PPB 0.54; not supported by MrBayes analysis). The resolution of the relationships among other specimens, including *Rhinogobius* sp. OR, *Rhinogobius* sp. BW, *Rhinogobius* sp. BF, *Rhinogobius* sp. OM, *Rhinogobius* sp. KZ, and *R. kurodai* was low. *Rhinogobius* sp. BW, a species endemic to Lake Biwa, the largest lake in Japan, formed a monophyletic group with *Rhinogobius* sp. BF, distributed in western area of the mainland of Japan, and *Rhinogobius* sp. OM, distributed in the coastal area and rivers flowing into Lake Biwa, but the statistical support was weak (BP 70%, PPM 0.53, PPB 0.64).

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

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

3.3 Ancestral state reconstruction

The best-fit model among the MuSSE sub-models was the simplest model for both life history and egg size, i.e., speciation, extinction, and transition rates were independent to state (Table S6). In the *Rhinogobius* species of Japan, the life history and egg size transformations were estimated to have occurred at least five and four times, respectively, although the proportional likelihood values were not very high for parts of the ancestral states (59.6–99.9%; Fig. 4a). The estimated egg size transformations always occurred together with life history transformation. The evolutionary change from amphidromous to fluvial life, accompanied by an increase in egg size, was estimated to have occurred in at least three independent lineages (*R. flumineus*, *Rhinogobius* sp. BB, and *Rhinogobius* sp. YB). The evolutionary change from amphidromous to lentic life was estimated to have occurred in one lineage (*Rhinogobius* sp. BW and *Rhinogobius* sp. BF). There was no clear change in egg size in this lineage (Tables 1, S1). In the *R. flumineus* and *Rhinogobius* sp. TO lineage, it was inferred that the lentic life most likely evolved from the fluvial life of *R. flumineus*, accompanied by a decrease in egg size.

4. Discussion

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

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

The inferred phylogenetic relationships suggest several biogeographic scenarios that explain the divergence among the species. Among the freshwater species of *Rhinogobius*, the close relationship between *R. flumineus* (fluvial) and *Rhinogobius* sp. TO (lentic) was revealed in this study. Although statistical support was not strong, the latter being derived from a clade within the former in the early to middle Pleistocene (1.43 Mya; 95% HPD, 0.48–2.44 Mya). *Rhinogobius flumineus* is distributed widely in western Japan, including the Ise Bay area, in which *Rhinogobius* sp. TO has a restricted distribution (Suzuki and Sakamoto, 2005; Fig. 4b). The latter species occurs in marsh habitats of hilly areas with rich spring waters (Suzuki and Mukai, 2010). This area has been rich in lentic environments since the Pliocene; e.g., a large wetland area known as the Paleo-Lake Tokai existed from the Pliocene to the middle Pleistocene (Yoshida, 1990; Makinouchi, 2001). The distribution, habitat, and speciation of *Rhinogobius* sp. TO may be associated with such past lentic environments.

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

The inferred phylogenetic relationships and geographic distributions suggest the patterns of allopatric speciation in amphidromous species. *Rhinogobius ogasawaraensis*, endemic to the Bonin Islands, oceanic islands 1000 km south of the mainland of Japan (Suzuki et al., 2011), was estimated to be the sister species of *R. fluviatilis*, which is widely distributed on the mainland of Japan (Fig. 4d). Prior to the present study, *R. ogasawaraensis* was considered closely related to *R. brunneus* in body color characteristics (Suzuki, 1992). However, our results suggest that *R. ogasawaraensi*s was established by colonization of the common ancestor with *R. fluviatilis* from the mainland of Japan after the separation from *Rhinogobius* sp. DL, which is distributed in the Ryukyu Archipelago. Although amphidromous species may extend their geographic range through coastal or marine habitats (McDowall, 2001), juveniles of amphidromous *Rhinogobius* species are restricted to the coastal zone (Oshiro and Nishijima, 1978; Kondo et al., 2013) and probably do not migrate across the open ocean. Therefore, the dispersal from the mainland to the Bonin Islands would be an exceptional event.

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

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

4.2 The history of hybridization inferred from mtDNA introgression

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

The mtDNA phylogeny, along with the nuclear gene phylogeny and divergence time estimations, provides information on historical patterns of hybridization in *Rhinogobius*. First, *Rhinogobius ogasawaraensis* was resolved as distant to other Japanese *Rhinogobius* species (except for *R. giurinus*) in the mtDNA phylogeny; nevertheless, *R. ogasawaraensis* was included in the clade N-3-2 of the nuclear gene phylogeny. This conflict indicates that a large-scale mtDNA introgression involving almost all species in both Japanese and Ryukyu archipelagos started with a common haplotype (or close haplotypes) after the divergence of *R. ogasawaraensis*. Second, the mtDNA and nuclear DNA analyses gave similar estimation of the divergence time of *R. ogasawaraensis* (1.59 and 1.55 Myr, respectively) although different calibration sets were used (Table 2). This agreement probably validates the estimation that the large-scale introgression occurred around that time (the early to middle Pleistocene). Third, even within the mainland of Japan or the Ryukyu region, mtDNAs resolved a part of species polyphyletic (e.g., the mainland *R. fluviatilis* and Ryukyu *Rhinogobius* sp. BB). This also supports the limited but ubiquitous hybridization in the *Rhinogobius* fishes, although incomplete lineage sorting in mtDNA is an alternative explanation in this scale. Fourth, it should be noted that freshwater species, such as *R. flumineus*, *Rhinogobius* sp. TO, *Rhinogobius* sp. BW (the mainland), and a part of *Rhinogobius* sp. BB (Ryukyu), branched out earlier and have retained their independent lineages in the mtDNA phylogeny. This tendency suggests that the reproductive isolation between amphidromous and freshwater species is stronger than that between amphidromous species and has prohibited the freshwater species from hybridizing with other species.

What type of historical process has constructed the above patterns? Interspecific hybridization often occurs in the conditions of enforced syntopic occurrence, which is caused by, for instance, reduction of habitats and secondary contact of populations following disappearance of geographic or ecological barriers (Seehausen, 2004; Toews and Brelsford, 2012). Although the *Rhinogobius* species show similar reproductive habits that include oviposition under a stone and male parental care for the eggs, reproductive isolation among sympatric species is achieved by micro- or mesoscale differences of spawning habitat in the river course (Mizuno, 1982; Tamada, 2000; Hirashima and Tachihara, 2006), assortative mating based on nuptial coloration and courtship behavior (Mizuno, 1987), or both. However, some conditions causing disturbance of such reproductive isolation would have occurred since the early to middle Pleistocene, in which the large part of Japanese *Rhinogobius* would have experienced large-scale hybridization. Freshwater habitat reduction by the rise in sea level with global climate fluctuations might be one such potential situation causing the overlap of spawning habitats among species. Mukai et al. (2012) reported that introgressive hybridization has occurred contemporarily among some freshwater *Rhinogobius* species following artificial transplantation. This example demonstrates that environmental distribution or changes can easily disturb reproductive isolation among *Rhinogobius* fishes. Furthermore, if some types of mtDNA were favored by natural selection, large-scale introgression of the particular mtDNA would rapidly progress through interspecific hybridization (Ballard and Whitlock, 2004). The *Rhinogobius* fishes may provide a model system to study the mechanisms of maintenance and decay of reproductive isolation, such as the Lake Victoria cichlids that suffered a collapse of assortative mating based on visual sense with the increase of turbidity (Seehausen et al., 1997). This group may also be useful as a model system for studying mitochondrial introgression and replacement through interspecific hybridization (Mukai and Takahashi, 2010).

4.3 Life history and egg size evolution and speciation

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

The evolutionary changes of the life history type and egg size are closely associated with each other in *Rhinogobius* species. The reconstructed patterns in the phylogeny included three series of transformations, i.e., from an amphidromous to a fluvial type, from an amphidromous to a lentic type, and from a fluvial to a lentic type. Although statistical support of the ancestral reconstruction associated with the last transformation was not very strong, the pattern is supported by the geographical distribution of the relevant species (see below).

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

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

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

The present wetland environment in the Ise Bay area is inhabited by several highly endemic species, including freshwater fishes (*Pseudorasbora pugnax*, *Cobitis minamorii tokaiensis*, as well as *Rhinogobius* sp. TO; Kawamura, 2006; Nakajima, 2012; Kawase and Hosoya, 2015), aquatic hemipteran insects *Nepa hoffmanni*, and plants (e.g., magnoliacean *Magnolia stellata*; Ueda, 2002); these suggest that such environment has been maintained for a long period. The small egg size and body size in *Rhinogobius* sp. TO are likely the characters representing this adaptation. The dwarf morphology of *Rhinogobius* sp. TO is very similar to that of other lentic species (*Rhinogobius* sp. BF and *Rhinogobius* sp. BW), and these three forms were treated as a single species until recently (Akihito et al., 2002; Suzuki and Mukai, 2010). However, the present study clarified that *Rhinogobius* sp. TO belongs to a different lineage from *Rhinogobius* sp. BF and *Rhinogobius* sp. BW, indicating that their lentic life history and dwarf morphology were the result of convergent evolution. The evolutionary change suggested in the freshwater *R. flumineus* and *Rhinogobius* sp. TO lineage emphasizes the adaptive flexibility of *Rhinogobius* fishes.

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

In summary, the considerable species diversity of *Rhinogobius* has been promoted and maintained by parallel life history divergence through colonization of and adaptation to various freshwater habitats, including flowing waters, marshes, and lakes. The life history divergence involving the changes in migration patterns and egg size probably functions as a direct mechanism of reproductive isolation among the divergent populations. To elucidate the whole picture of life history evolution in *Rhinogobius*, further phylogenetic analysis using comprehensive taxon sampling from East/Southeast Asia is necessary.

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Figure legends

Fig. 1.

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

Fig. 2.

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

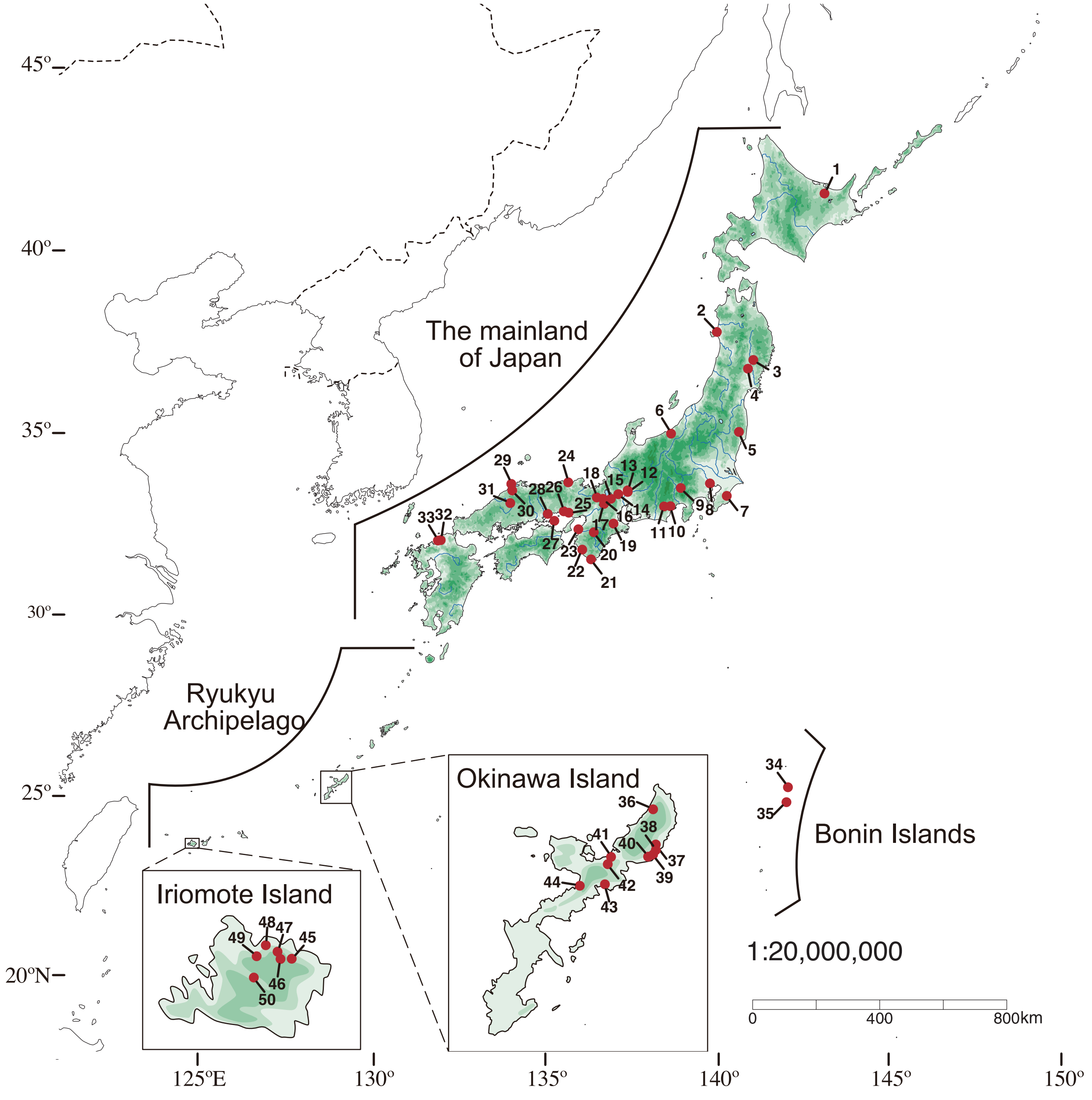
Fig. 3.

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

Fig. 4.

(a) The Bayesian phylogenetic tree of the selected *Rhinogobius* species with ancestral state reconstruction and divergence time inferred from concatenated sequences of six nuclear genes. Each bar plot indicates a height of 95% HPD of the corresponding node. Calibration points were indicated as CA1 (formation of the Bonin Islands) and CA2 (formation of the Tokara Gap). Pie graphs of each node indicate the proportional likelihood of the states at the node inferred by the MuSSE model. Statistical support values (> 0.9) are indicated next to the major nodes. The proportional likelihood of the nodes indicated by an asterisk is inconsistent when another sample of *Rhinogobius* sp. BF was used. (b)–(e) Phylogenetic relationships among selected species with their present distribution patterns. See the Discussion section for details.

Fig. 1



45°
40°
35°
30°
25°
20°N
125°E
130°
135°
140°
145°
150°
The mainland of Japan
Ryukyu Archipelago
Okinawa Island
Iriomote Island
Bonin Islands
1:20,000,000
0
400
800km
1
2
3
4
5
6
7
8
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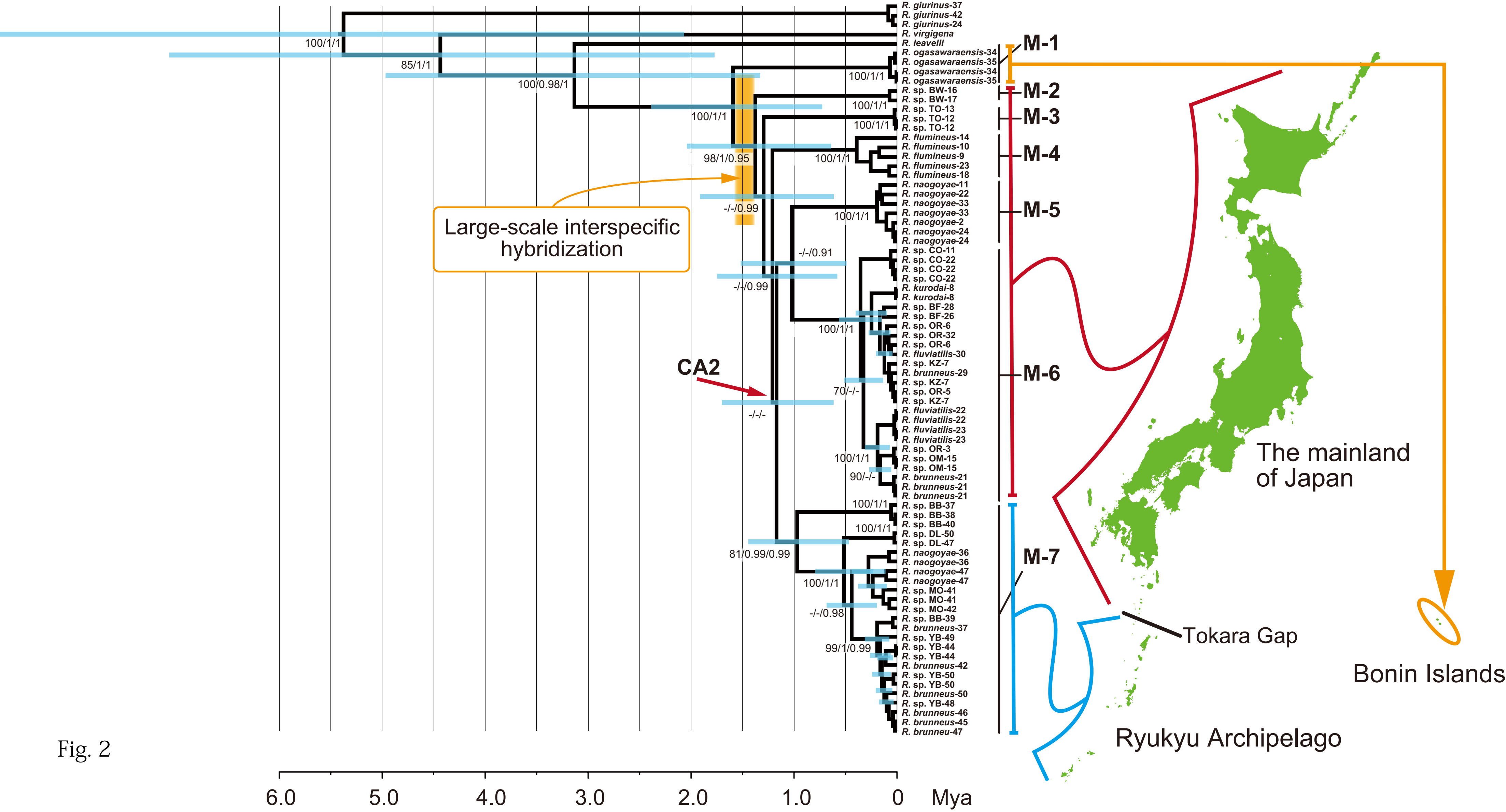
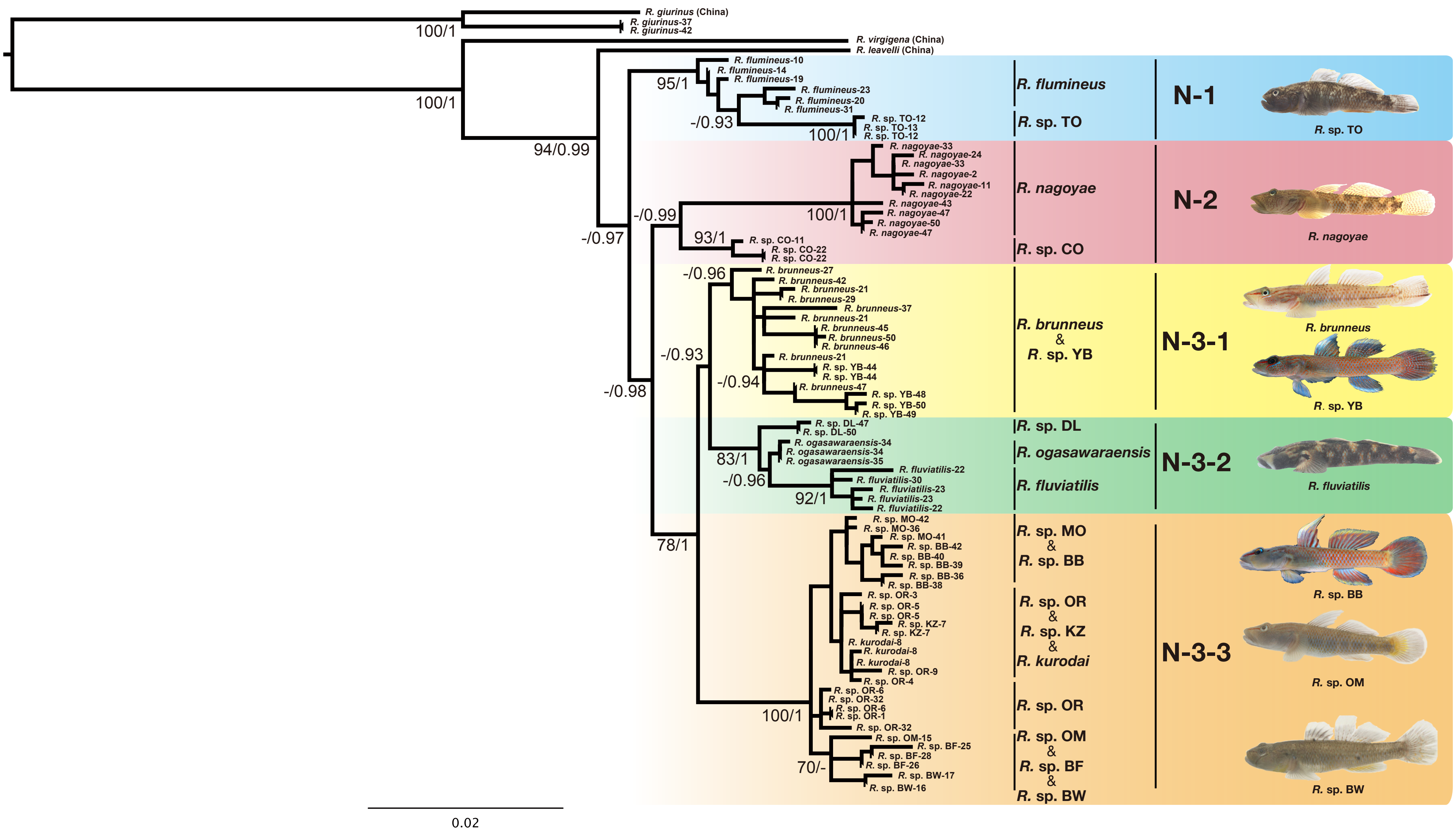

R. giurinus-37
R. giurinus-42
R. giurinus-24
R. virgigena
R. leavelli
R. ogasawaraensis-34
R. ogasawaraensis-35
R. ogasawaraensis-34
R. ogasawaraensis-35
R. sp. BW-16
R. sp. BW-17
R. sp. TO-13
R. sp. TO-12
R. sp. TO-12
R. flumineus-14
R. flumineus-10
R. flumineus-9
R. flumineus-23
R. flumineus-18
R. naogoyae-11
R. naogoyae-22
R. naogoyae-33
R. naogoyae-33
R. naogoyae-2
R. naogoyae-24
R. naogoyae-24
R. sp. CO-11
R. sp. CO-22
R. sp. CO-22
R. sp. CO-22
R. kurodai-8
R. kurodai-8
R. sp. BF-28
R. sp. BF-26
R. sp. OR-6
R. sp. OR-32
R. sp. OR-6
R. fluviatilis-30
R. sp. KZ-7
R. brunneus-29
R. sp. KZ-7
R. sp. OR-5
R. sp. KZ-7
R. fluviatilis-22
R. fluviatilis-22
R. fluviatilis-23
R. fluviatilis-23
R. sp. OR-3
R. sp. OM-15
R. sp. OM-15
R. brunneus-21
R. brunneus-21
R. brunneus-21
R. sp. BB-37
R. sp. BB-38
R. sp. BB-40
R. sp. DL-50
R. sp. DL-47
R. naogoyae-36
R. naogoyae-36
R. naogoyae-47
R. naogoyae-47
R. sp. MO-41
R. sp. MO-41
R. sp. MO-42
R. sp. BB-39
R. brunneus-37
R. sp. YB-49
R. sp. YB-44
R. sp. YB-44
R. brunneus-42
R. sp. YB-50
R. sp. YB-50
R. brunneus-50
R. sp. YB-48
R. brunneus-46
R. brunneus-45
R. brunneu-47
100/1/1
85/1/1
100/0.98/1
100/1/1
100/1/1
100/1/1
100/1/1
98/1/0.95
100/1/1
-/-/0.99
100/1/1
-/-/0.91
-/-/0.99
100/1/1
70/-/-
-/-/-
100/1/1
90/-/-
100/1/1
100/1/1
81/0.99/0.99
100/1/1
-/-/0.98
99/1/0.99
Large-scale interspecific hybridization
CA2
M-1
M-2
M-3
M-4
M-5
M-6
M-7
The mainland of Japan
Tokara Gap
Bonin Islands
Ryukyu Archipelago
6.0
5.0
4.0
3.0
2.0
1.0
0
Mya

Fig. 2



R. giurinus (China)
R. giurinus-37
R. giurinus-42
100/1
100/1
R. virgigena (China)
R. leavelli (China)
94/0.99
R. flumineus-10
R. flumineus-14
R. flumineus-19
95/1
R. flumineus-23
R. flumineus-20
R. flumineus-31
-/0.93
R. sp. TO-12
R. sp. TO-13
R. sp. TO-12
100/1
R. flumineus
R. sp. TO
N-1
R. sp. TO
R. nagoyae-33
R. nagoyae-24
R. nagoyae-33
R. nagoyae-2
R. nagoyae-11
R. nagoyae-22
R. nagoyae-43
R. nagoyae-47
R. nagoyae-50
R. nagoyae-47
100/1
-/0.99
-/0.97
93/1
R. sp. CO-11
R. sp. CO-22
R. sp. CO-22
R. nagoyae
R. sp. CO
N-2
R. nagoyae
R. brunneus-27
R. brunneus-42
R. brunneus-21
R. brunneus-29
R. brunneus-37
R. brunneus-21
R. brunneus-45
R. brunneus-50
R. brunneus-46
R. brunneus-21
R. sp. YB-44
R. sp. YB-44
R. brunneus-47
R. sp. YB-48
R. sp. YB-50
R. sp. YB-49
-/0.96
-/0.93
-/0.94
-/0.98
R. brunneus
&
R. sp. YB
N-3-1
R. brunneus
R. sp. YB
R. sp. DL-47
R. sp. DL-50
R. ogasawaraensis-34
R. ogasawaraensis-34
R. ogasawaraensis-35
R. fluviatilis-22
R. fluviatilis-30
R. fluviatilis-23
R. fluviatilis-23
R. fluviatilis-22
83/1
-/0.96
92/1
R. sp. DL
R. ogasawaraensis
R. fluviatilis
N-3-2
R. fluviatilis
78/1
R. sp. MO-42
R. sp. MO-36
R. sp. MO-41
R. sp. BB-42
R. sp. BB-40
R. sp. BB-39
R. sp. BB-36
R. sp. BB-38
R. sp. OR-3
R. sp. OR-5
R. sp. OR-5
R. sp. KZ-7
R. sp. KZ-7
R. kurodai-8
R. kurodai-8
R. kurodai-8
R. sp. OR-9
R. sp. OR-4
R. sp. OR-6
R. sp. OR-32
R. sp. OR-6
R. sp. OR-1
R. sp. OR-32
R. sp. OM-15
R. sp. BF-25
R. sp. BF-28
R. sp. BF-26
R. sp. BW-17
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N-3-3
R. sp. BB
R. sp. OM
R. sp. BW
0.02

Fig. 3

Fig. 4

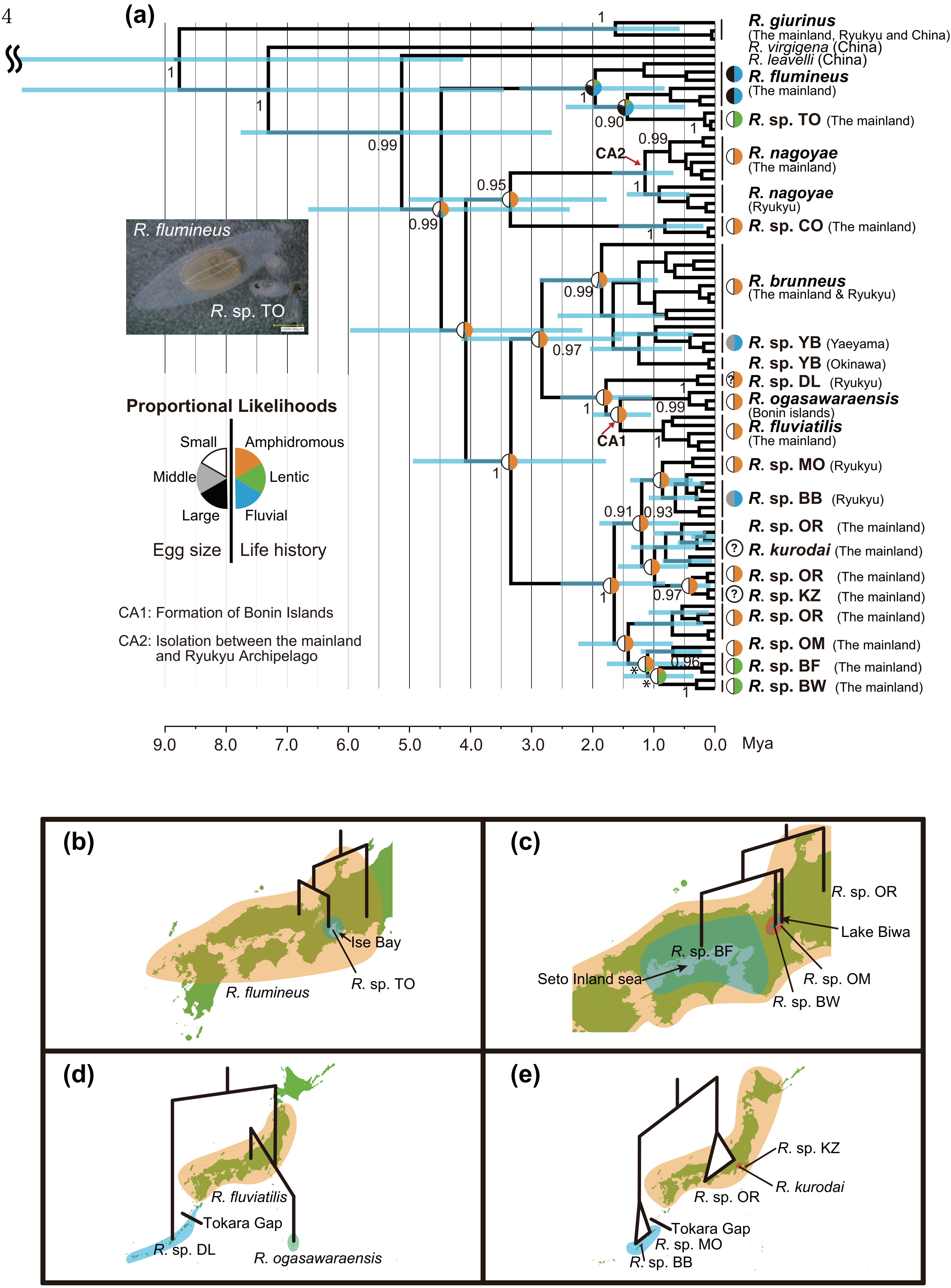

(a)
R. giurinus (The mainland, Ryukyu and China)
R. virgigena (China)
R. leavelli (China)
R. flumineus (The mainland)
R. sp. TO (The mainland)
R. nagoyae (The mainland)
R. nagoyae (Ryukyu)
R. sp. CO (The mainland)
R. brunneus (The mainland & Ryukyu)
R. sp. YB (Yaeyama)
R. sp. YB (Okinawa)
R. sp. DL (Ryukyu)
R. ogasawaraensis (Bonin islands)
R. fluviatilis (The mainland)
R. sp. MO (Ryukyu)
R. sp. BB (Ryukyu)
R. sp. OR (The mainland)
R. kurodai (The mainland)
R. sp. OR (The mainland)
R. sp. KZ (The mainland)
R. sp. OR (The mainland)
R. sp. OM (The mainland)
R. sp. BF (The mainland)
R. sp. BW (The mainland)
CA1
CA2
R. flumineus
R. sp. TO
Proportional Likelihoods
Small
Middle
Large
Amphidromous
Lentic
Fluvial
Egg size
Life history
CA1: Formation of Bonin Islands
CA2: Isolation between the mainland and Ryukyu Archipelago
9.0 8.0 7.0 6.0 5.0 4.0 3.0 2.0 1.0 0.0 Mya
(b)
Ise Bay
R. flumineus
R. sp. TO
(c)
R. sp. OR
Lake Biwa
R. sp. BF
Seto Inland sea
R. sp. OM
R. sp. BW
(d)
R. fluviatilis
Tokara Gap
R. sp. DL
R. ogasawaraensis
(e)
R. sp. KZ
R. kurodai
R. sp. OR
Tokara Gap
R. sp. MO
R. sp. BB

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.
***Rhinogobius flumineus* : RF**	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
***Rhinogobous* sp. YB: YB**	Fluvial	Middle	YB–OK111211–3	Sukuta R. / Sukuta R. (Okinawa Island, Okinawa)	44
			YB–OK111211–4	Sukuta R. / Sukuta R. (Okinawa Island, Okinawa)	44
			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
***Rhinogobius* sp. BB: BB**	Fluvial	Middle	BB–OK121111–1	Sate R. / Sate R. (Okinawa Island, Okinawa)	36
			BB–OK110924–15	Aha R. / Aha R. (Okinawa Island, Okinawa)	37
			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
***Rhinogobius* sp. OM: OM**	Amphidromous	Small	OM–SG110725–1	Sakura R. / Lake Biwa and Yodo R. (Higashioumi, Shiga)	15
			OM–SG110725–2*	Sakura R. / Lake Biwa and Yodo R. (Higashioumi, Shiga)	15
***Rhinogobius* sp. OR: OR**	Amphidromous	Small	OR–HO120408–1	Abashiri R. / Abashiri R. (Memanbetsu, Hokkaido)	1
			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–NI110816–1*	Seki R. / Seki R. (Joetsu, Niigata)	6
			OR–NI110816–2	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
***Rhinogobius brunneus* : DA**	Amphidromous	Small	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
			DA–OK110923–3	Genka R. / Genka R. (Okinawa Island, Okinawa)	42
			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
***Rhinogobius fluviatilis* : LD**	Amphidromous	Small	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
***Rhinogobius* sp. CO: CO**	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
***Rhinogobius nagoyae* : CB**	Amphidromous	Small	CB–AK111003–3*	Nomura R. / Nomura R. (Oga, Akita)	2
			CB–SZ110911–6	Seto R. / Seto R. (Fujieda, Shizuoka)	11
			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
***Rhinogobius* sp. MO: MO**	Amphidromous	Small	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
			MO–OK110923–7*	Genka R. / Genka R. (Okinawa Island, Okinawa)	42
***Rhinogobius* sp. DL: DL**	Ampnidromous	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
***Rhinogobius ogasawaraensis* : BI**	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
***Rhinogobius* sp. BW: BW**	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
***Rhinogobius* sp. TO: TO**	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
***Rhinogobius* sp. BF: BF**	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
***Rhinogobius* sp. KZ: KZ**	Unknown	Unknown	KZ–CB100418–1	Mizusawa R. / Ichimiya R. (Chousei, Chiba)	7
			KZ–CB100418–2*	Mizusawa R. / Ichimiya R. (Chousei, Chiba)	7
			KZ–CB100418–3	Mizusawa R. / Ichimiya R. (Chousei, Chiba)	7
***Rhinogobius kurodai* : KU**	Unknown	Unknown	KU–TK100705–1*	Shinjuku gyoen (Shinjuku, Tokyo)	8
			KU–TK100705–2	Shinjuku gyoen (Shinjuku, Tokyo)	8
			KU–TK100705–3	Shinjuku gyoen (Shinjuku, Tokyo)	8
Rhinogobius leavelli	Amphidromous	Unknown	R. leavelli	Fangcheng, Guangxi, China	
Rhinogobius virgigena	Unknown	Unknown	R. virgigena	Fangcheng, Guangxi, China	
Outgroup					
***Rhinogobius giurinus* : RG**	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(Mya, 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 (*R. fluviatilis* + *R. ogasawaraensis*)	1.55	1.05–2.00
CA2 (*R. nagoyae* 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
R. flumineus + *R*. sp. TO	1.43	0.49–2.44
R. sp. OR + *R*. sp. KZ + *R. kurodai* vs. *R*. sp. MO +	1.20	0.59–1.89
R. sp. BW + *R*. sp. BF + *R*. sp. OM	1.10	0.49–1.77
R. sp. BW + part of *R*. sp. BF	0.90	0.35–1.51
All Japanese species	4.48	2.38–6.66

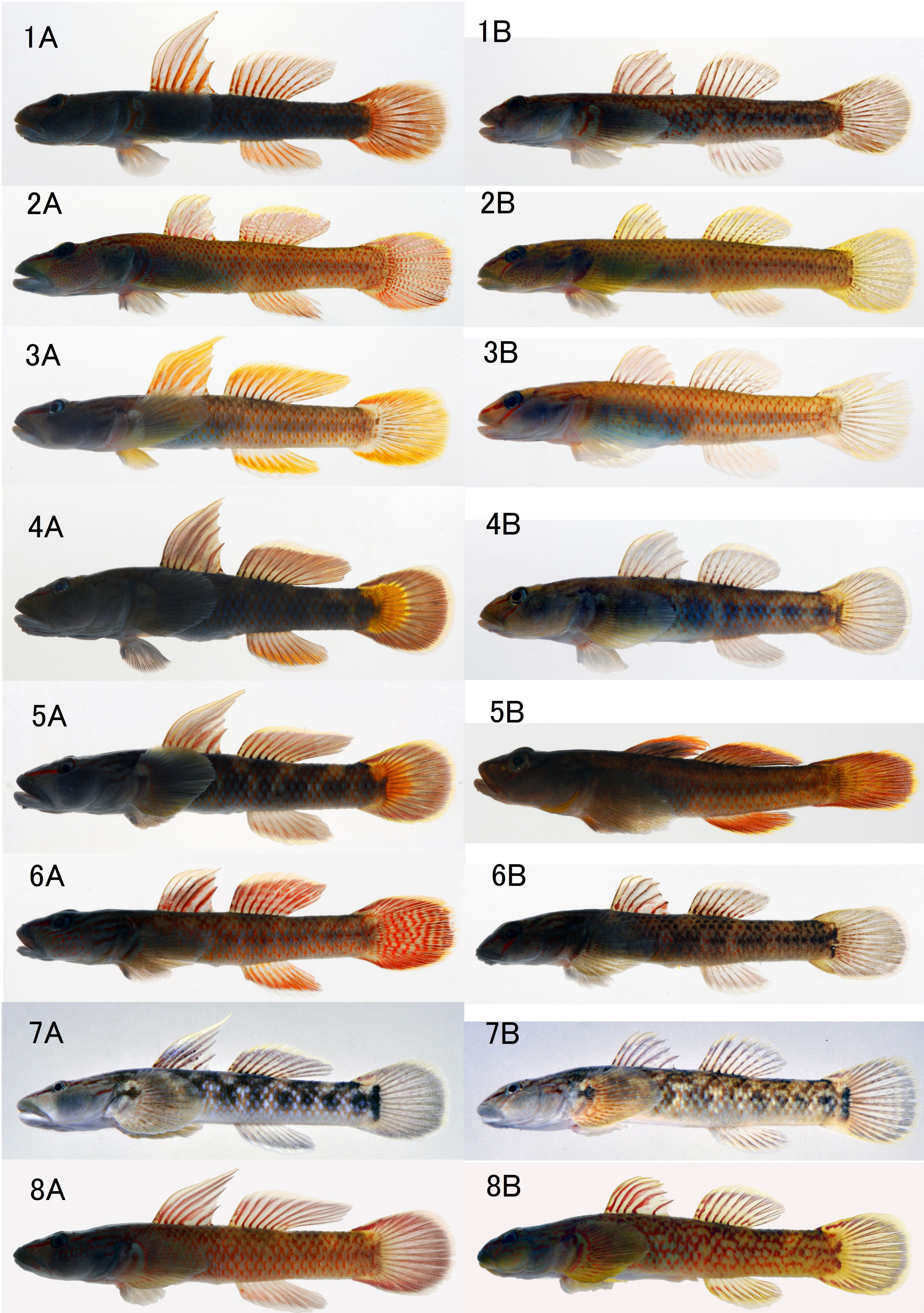


Fig. S1 Photographs of all *Rhinogobius* species distributed in Japan.

1: *Rhinogobius flumineus* (Mizuno, 1960), Nagaragawa River, Gifu Pref. A: OMNH-P 40703, male, B: OMNH-P 40704, female.
2: *Rhinogobous* 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.

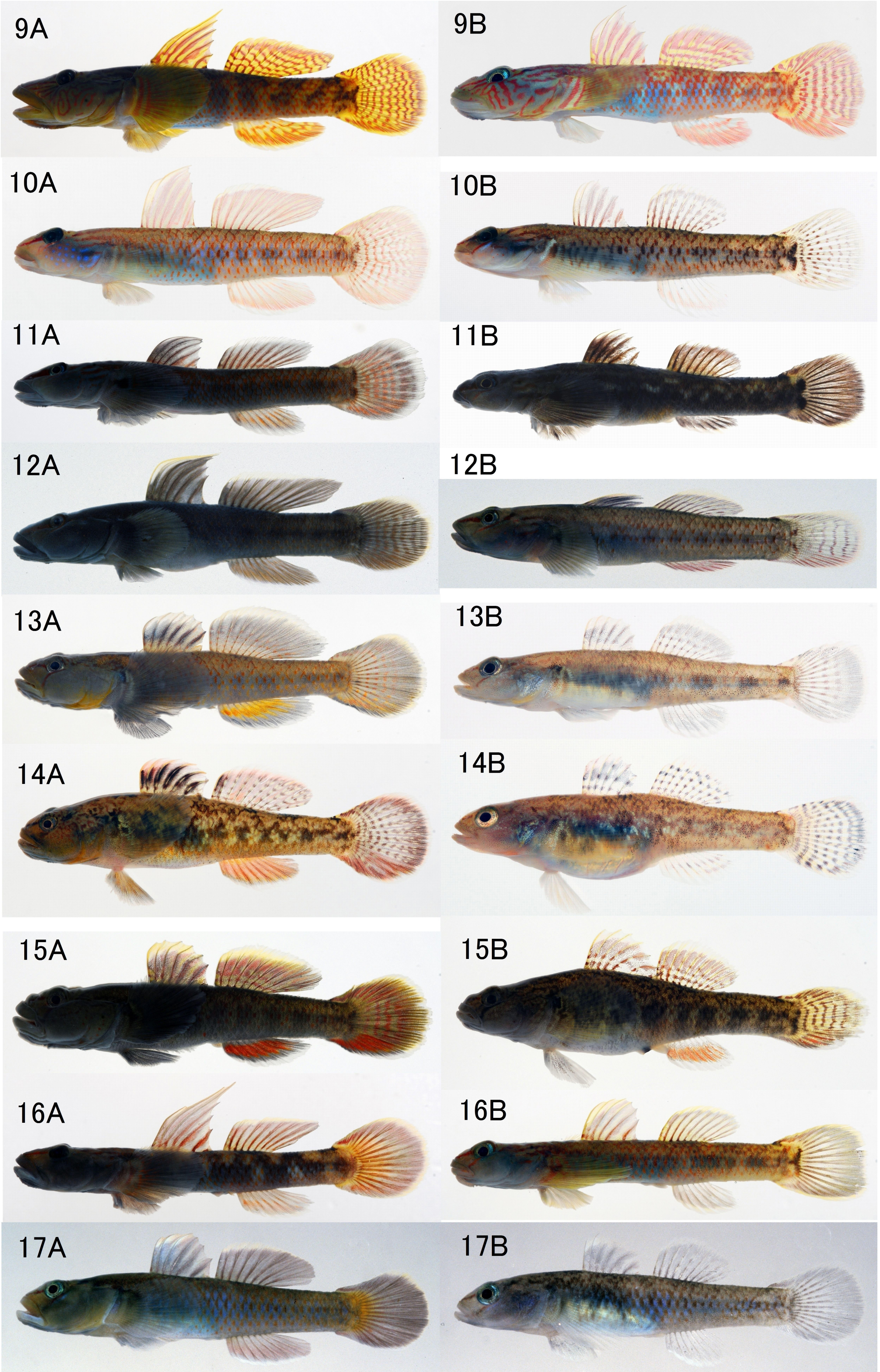
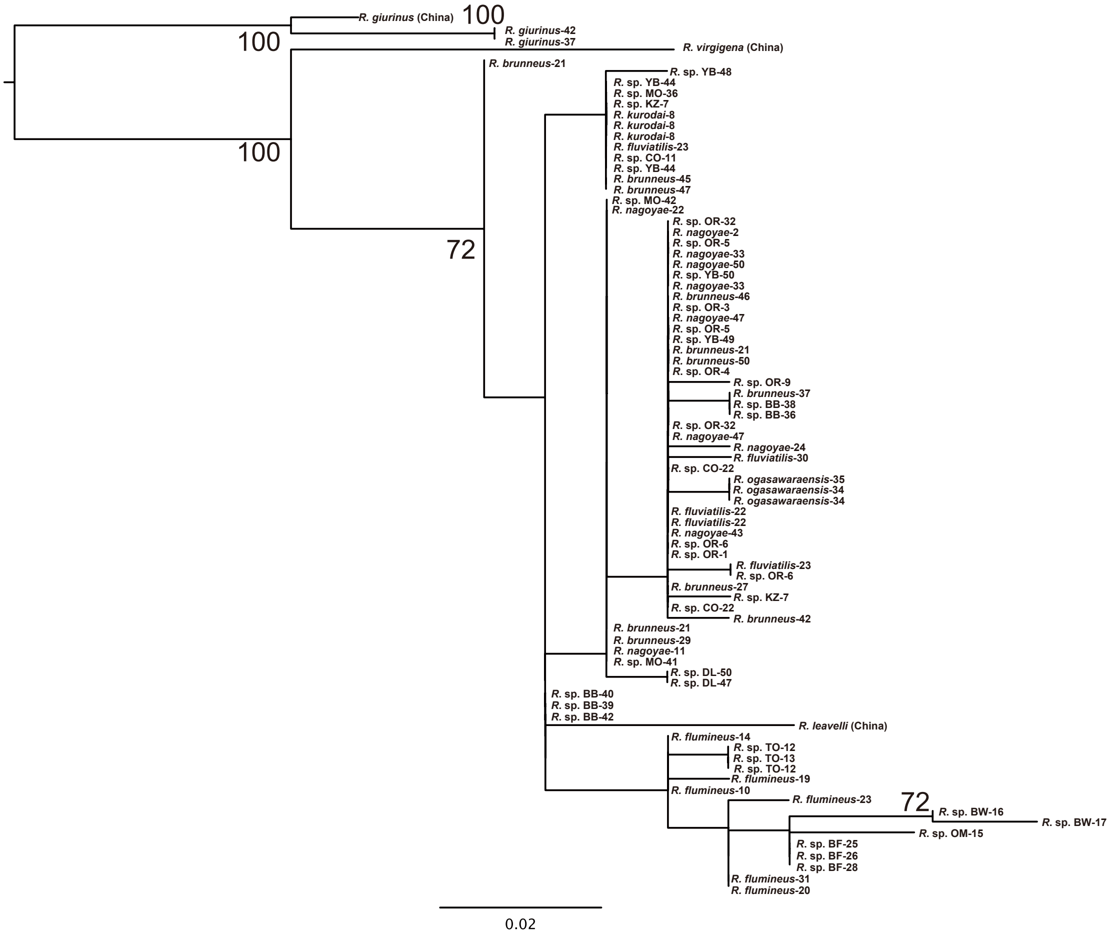


Fig. S1 Continued.

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: OMNHP 40656, male, B: OMNHP 40663, female.
17: *Rhinogobius kurodai* (Tanaka. 1908), Moat, Chiyouda, Tokyo. A: OMNH-P 21132, male, B: OMNH-P 21136, female.

(a) myh6



(b) RYR3

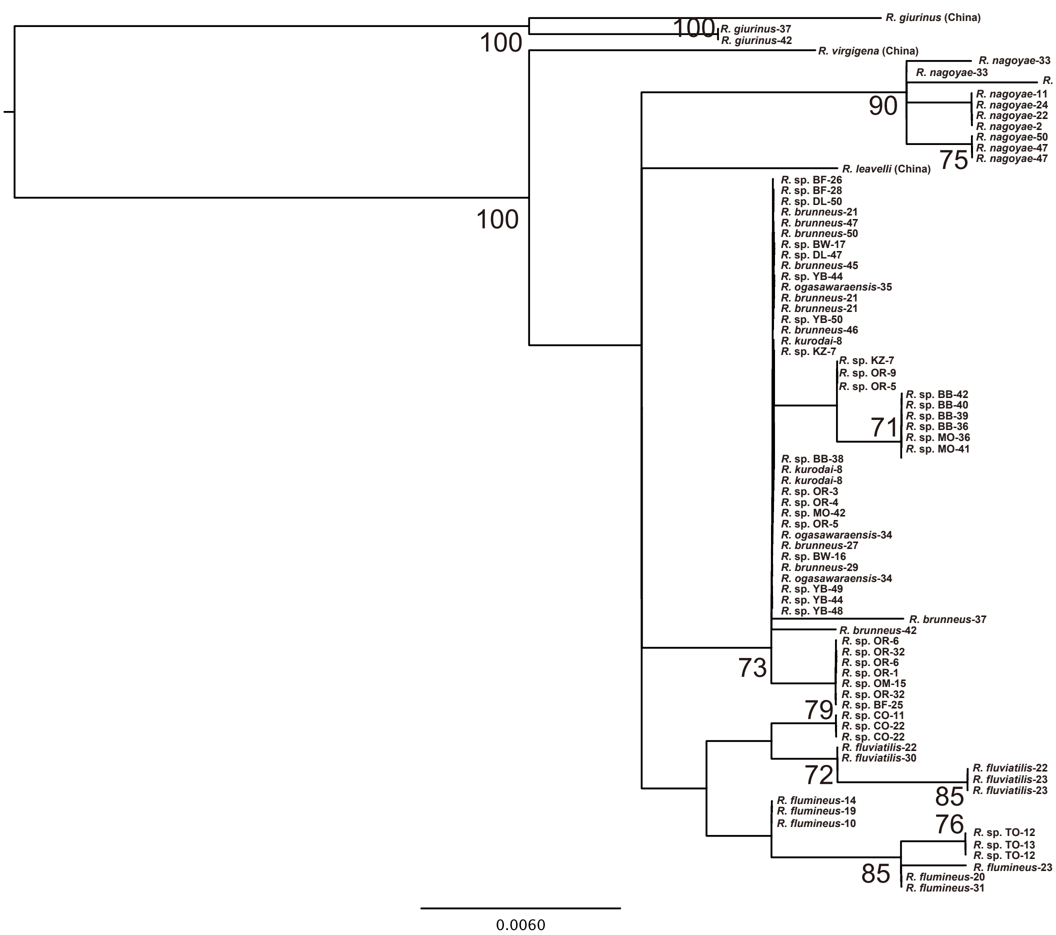
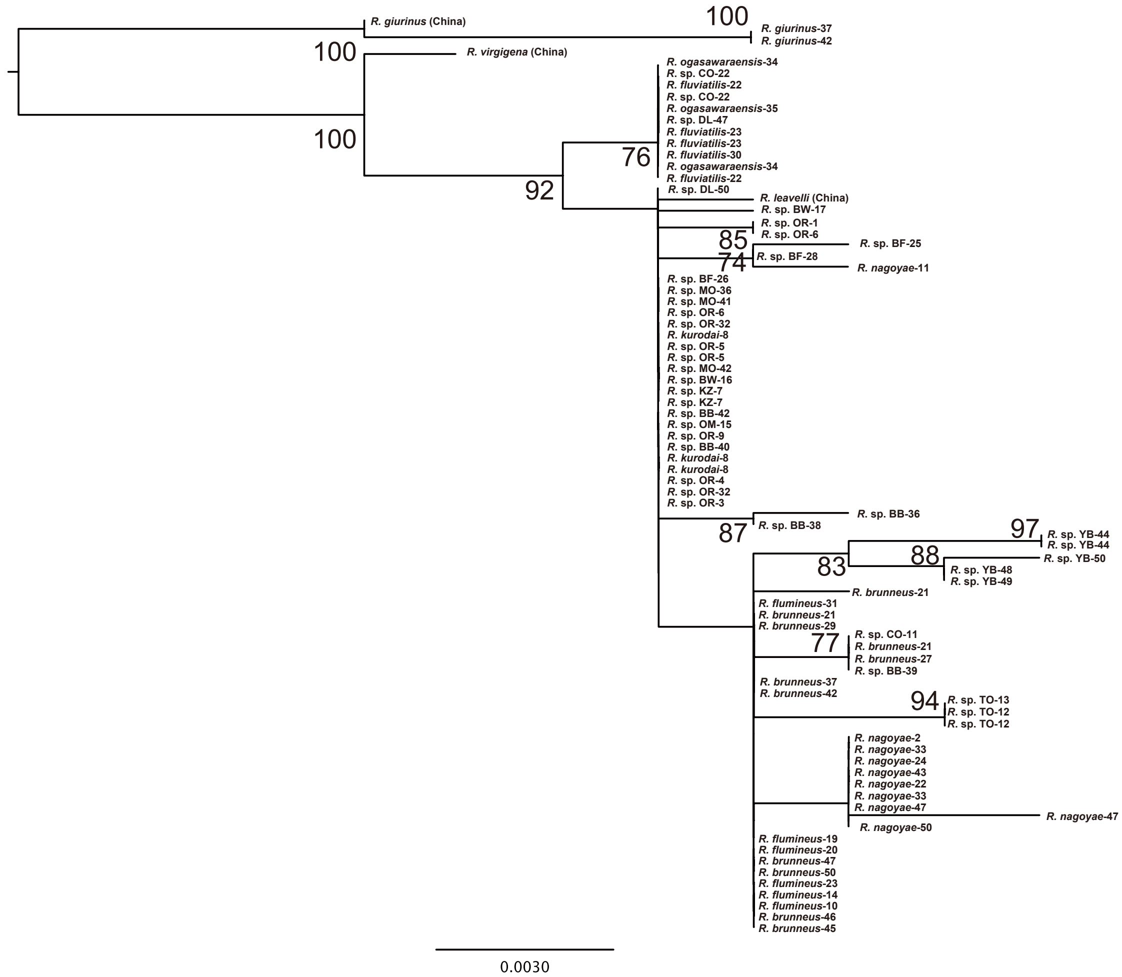


Fig. S2 Maximum likelifood trees of the selected Rhinogobius species based on respective nuclear gene sequences. Bootstrap values of >70% are indicated.

(c) Ptr



(d) RAG2

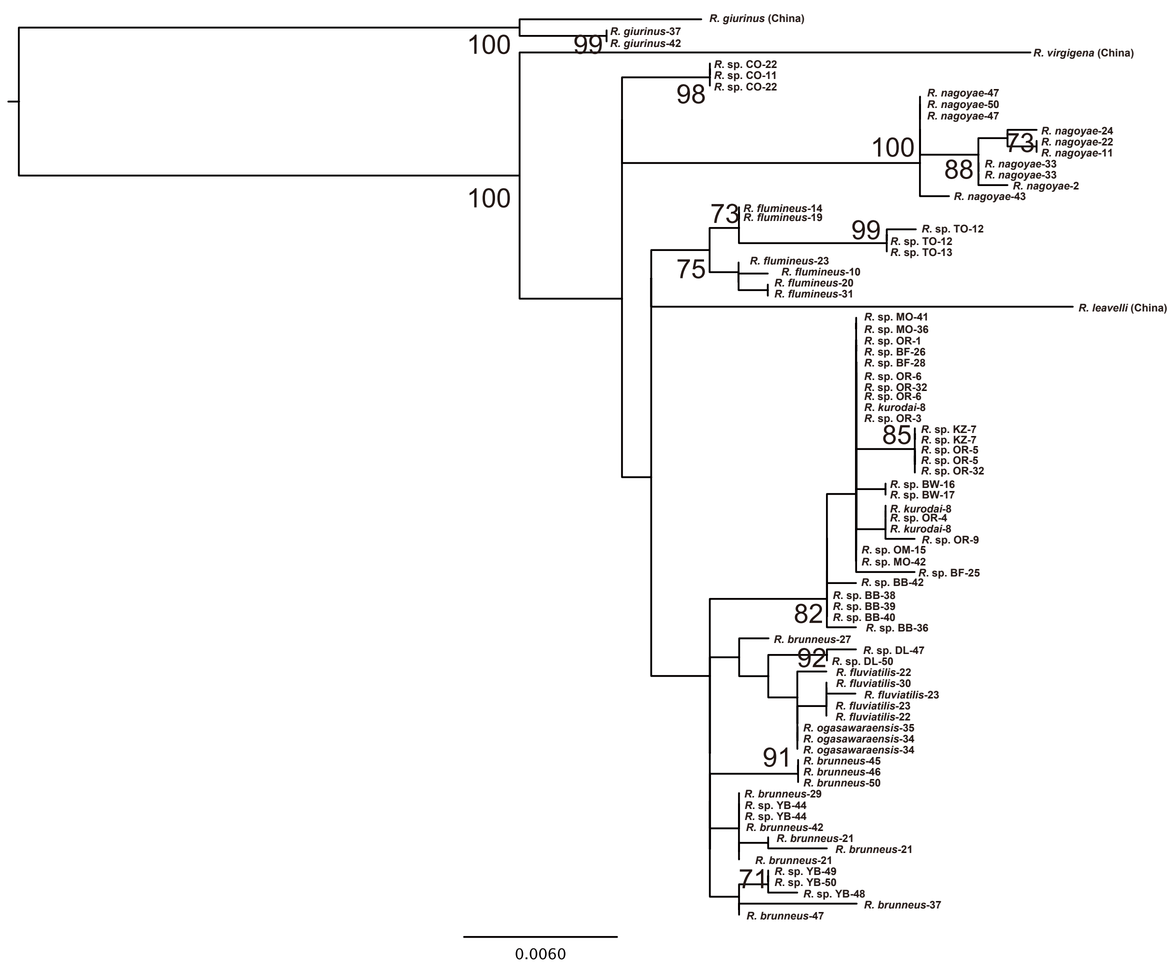


Fig. S2 Continued.

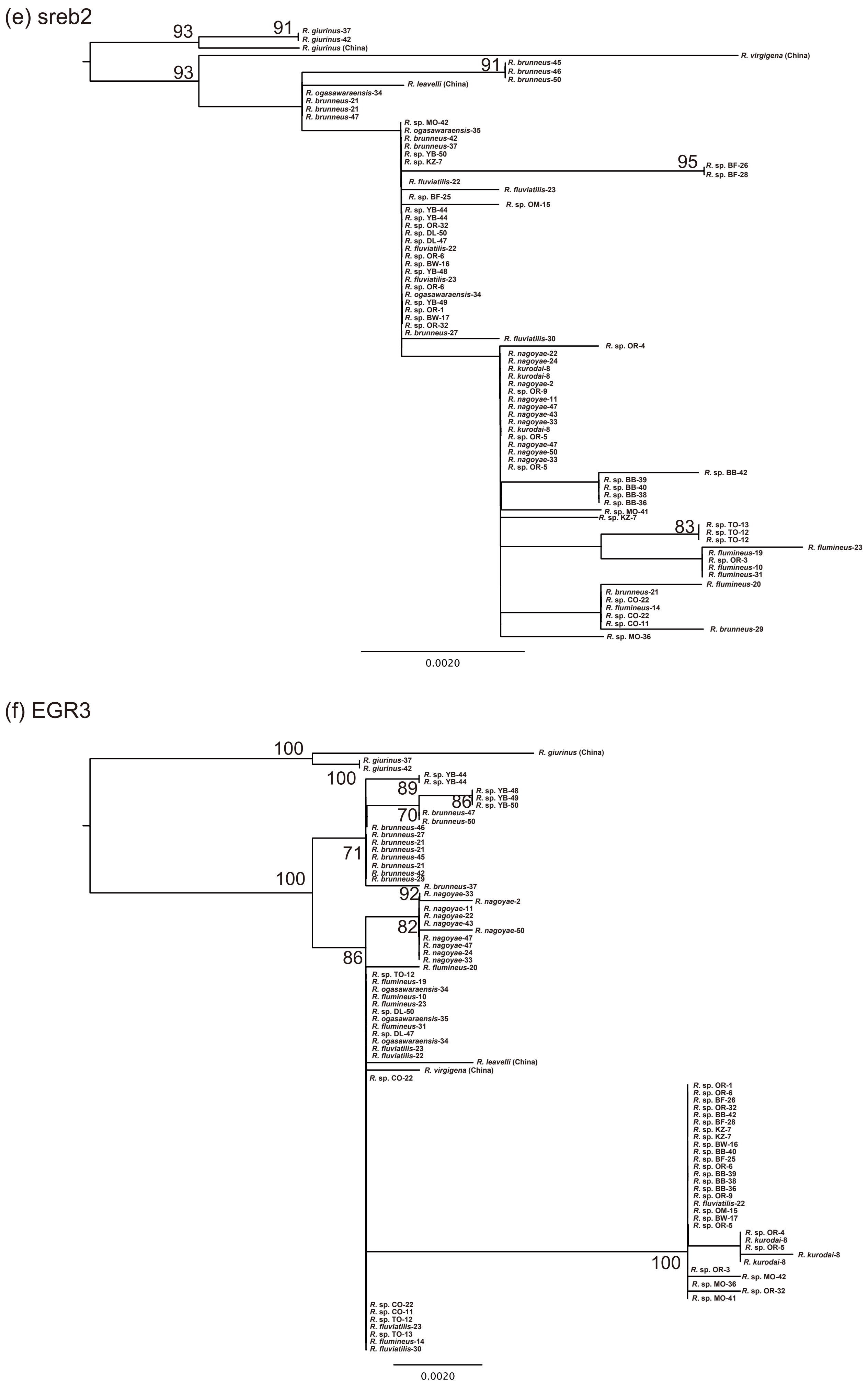


Fig. S2 Continued.

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

[a] Akihito et al. (2013) newly separated six morphological species (*Rhinogobius* sp. OM, *Rhinogobius* sp. KZ, *R . kurodai* , *Rhinogobius* sp. BW, *Rhinogobius* sp. TO, *Rhinogonius* 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 sicientific 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.

[b]*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/CO1	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.
R.leavelli	NSMT–P 120835	AB988324	AB988406	AB988488	AB988570	AB988652	AB988734	AB988820	AB988898	AB988976

R.virgigena	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)[a]	Number of Parsimony informative site (bp)[a]	Annealing temp. (°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	GGAACTATYGGTAAGCARATGG	RYR3_F22	TCGGTAAGCARATGGTGGACA	Li et al. (2007)
					RYR3_R968	TGGAAGAAKCCAAAKATGATGC	RYR3_R931	AGAATCCRGTGAAGAGCATCCA	
Ptr	636	30/22	24/14	55	R-PtrF	TGTATCTCATCTATGCCTCTTTTTCA			Li et al. (2007); this study
					R-PtrR	AGAGGTGACCGTCAGGATGAG			
RAG2	882	115/87	79/50	55	R-RAG2F	GTCGAACCCCAAACAATGAG			Lovejoy et al. (2001); this stud
					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	GGYTTCTTGTCCTTCTGTTTSAG			
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	GGTCTTAGGAACCAAAAACTCTTGGTGCAA			Miya and Nishida (2000)
					H13366-ND5-Rhi	AAGGCTGTGAATGATGGAGC			Mukai et al. (2012)
cytb	1178	351/309	297/225	48	L14724	TGACTTGAARAACCAYCGYYG			Palumbi et al. (1991)
					H16500-CR	GCCCTGAAATAGGAACCAGA			Inoue et al. (2000)

[a] With outgroup / without outgroup

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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+1
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.

mtDNA	
CO1	TrN+G
ND5	TrN+I+G
cytb	GTR+I+G
nuclear DNA	
Concatenated	HKY+I+G

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 (λ), extinction (μ), and transition rates (q).

Model	DF	AIC	λ1	λ2	λ3	μ1	μ2	μ3	q12	q21	q13	q31	q23	q32
Life History														
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 λ	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 μ	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 λ and μ	8	111.29	0.429	-	-	5.19E-09	-	-	0.0679	1.83E-07	0.0845	7.67E-08	0.513	0.318
Equal λ 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 μ 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 λ,μ, q (reversal transition rates are equal)	5	106.02	0.429	-	-	2.70E-08	-	-	0.0567	-	0.1057	-	0.447	-
Equal λ and q (all transition ratse are equal)	5	106.71	0.462	-	-	3.75E-08	0.444	0.149	0.1249	-	-	-	-	-
Equal μ and q (all transition rates are equal)	5	105.80	0.496	0.304	0.198	3.88E-08	-	-	0.1099	-	-	-	-	-
Equal λ,μ, q (all transition rates are equal)	3	103.06	0.429	-	-	1.58E-08	-	-	0.0983	-	-	-	-	-
Egg size														
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 λ	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 μ	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 λ and μ	8	98.88	0.429	-	-	9.15E-07	-	-	0.3955	2.304	5.66E-07	0.348	2.15E-09	7.88E-10
Equal λ 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 μ 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 λ,μ, q (reversal transition rates are equal)	5	98.17	0.429	-	-	2.62E-07	-	-	0.0758	-	0.0651	-	1.28E-07	-
Equal λ and q (all transition rates are equal)	5	97.50	0.476	-	-	3.70E-08	0.855	0.387	0.1022	-	-	-	-	-
Equal μ and q (all transition rates are equal)	5	96.41	0.481	1.06E-05	0.211	2.90E-07	-	-	0.0725	-	-	-	-	-
Equal λ,μ, q (all transition rates are equal)	3	94.45	0.429	-	-	2.26E-07	-	-	0.0675	-	-	-	-	-