<table>
<thead>
<tr>
<th>Title</th>
<th>Phylogeny, hybridization, and life history evolution of Rhinogobius gobies in Japan, inferred from multiple nuclear gene sequences.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Yamasaki, Yo Y; Nishida, Mutsumi; Suzuki, Toshiyuki; Mukai, Takahiko; Watanabe, Katsutoshi</td>
</tr>
<tr>
<td>Citation</td>
<td>Molecular phylogenetics and evolution (2015), 90: 20-33</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2015-04-27</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/2433/198127">http://hdl.handle.net/2433/198127</a></td>
</tr>
<tr>
<td>Right</td>
<td>© 2015 Elsevier Inc. Licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International <a href="http://creativecommons.org/licenses/by-nc-nd/4.0/">http://creativecommons.org/licenses/by-nc-nd/4.0/</a>. NOTICE: this is the author’s version of a work that was accepted for publication in ‘Molecular Phylogenetics and Evolution’. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in ‘Phylogeny, hybridization, and life history evolution of Rhinogobius gobies in Japan, inferred from multiple nuclear gene sequences’, VOL90, 2015, doi:10.1016/j.ympev.2015.04.012.; 許諾条件により本文ファイルは2016-04-27に公開.; This is not the published version. Please cite only the published version. この論文は出版社版ではありません。引用の際には出版社版をご確認ご利用ください。</td>
</tr>
<tr>
<td>Type</td>
<td>Journal Article</td>
</tr>
<tr>
<td>Textversion</td>
<td>author</td>
</tr>
</tbody>
</table>
Title: Phylogeny, hybridization, and life history evolution of *Rhinogobius* gobies in Japan, inferred from multiple nuclear gene sequences

Authors: Yo Y. YAMASAKI\textsuperscript{a}, Mutsumi NISHIDA\textsuperscript{b,c}, Toshiyuki SUZUKI\textsuperscript{c}, Takahiko MUKAI\textsuperscript{d}, Katsutoshi WATANABE\textsuperscript{a}

Author’s affiliation:

\textsuperscript{a} Graduate School of Science, Kyoto University, Kitashirakawa-oiwake-cho, Sakyo, Kyoto 606-8502, Japan

\textsuperscript{b} Atmosphere and Ocean Research Institute, The University of Tokyo, Kashiwa, Chiba 277-8564, Japan

\textsuperscript{c} Kawanishi-midoridai Senior High School, 1-8 Kouyoudai, Kawanishi, Hyogo 666-0115, Japan

\textsuperscript{d} Faculty of Regional Studies, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

\textsuperscript{e} Current address: University of the Ryukyus, 1 Senbaru, Nishihara-cho, Okinawa 903-0213, Japan

Corresponding author:

Katsutoshi Watanabe
Graduate School of Science, Kyoto University, Kitashirakawa-oiwake-cho, Sakyo, Kyoto 606-8502, Japan.
Tel.: +81-75-753-4079

e-mail: watanak@terra.zool.kyoto-u.ac.jp
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 Gobiiformes; 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 et 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 *Rhinogobius* 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), superconserved 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 MgCl₂, 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. fluviatilis*, 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 at 1.59 Myr (0.73–2.39 Myr), and the tMRCA 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 tMRCA's 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 (*Rhinogobius* sp. OR + *Rhinogobius* sp. KZ + *Rhinogobius kurodai* [the mainland] and *Rhinogobius* sp. MO + *Rhinogobius* sp. BB [Ryukyu Archipelago]) was 1.78 Myr (1.03–2.53 Myr); tMRCA of a part of N-3-3 (*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 et 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. ogasawaraensis* 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.

Acknowledgments

We thank M. Hosokawa, N. Oseko, M. Aizawa, T. Abe, M. Kumazawa, Y. Miyazaki, Y. Kanzaki, T. Tsunagawa, S. Nakamura, R. Tabata, H. Nakagawa, T. Mishina, T. Nakano, Y. Kano, C.-G. Zhang, R. Nishiguchi, and Hakuryo Junior and Senior High School Biology Club for collecting samples, A. Iwata, M. Kondo and K. Maeda for giving valuable comments and precious literature, E. Kawaguchi for helping sequencing analysis, T. Fujisawa for giving helpful comments for phylogenetic analysis, and the two anonymous reviewers, the handling editor and members of Laboratory of Animal Ecology of Kyoto University for giving helpful comments. This study was partially supported by JSPS KAKENHI (nos. 21370035, 23370041, 26291079 and 26250044) and “Formation of a Strategic Base for Biodiversity and Evolutionary Research: from Genome to Ecosystem” of the GCOE.

References


Nishijima, S., 1968. Two forms of the gobiod fish *Rhinogobius brunneus* from Okinawa–Jima,


a redescription of Gobius brunneus Temminck and Schlegel, 1845. J. Natn. Taiwan Mus. 64,
1–17.

Oshiro, N., Nishijima, S., 1978. An observation on the juveniles of “Yoshinobori” (Gobiidae) in

Palaeogeographic reconstruction of the 1.55 Ma synchronous isolation of the Ryukyu Islands,
Japan, and Taiwan and inflow of the Kurishio warm current. Int. Geol. Rev. 54, 1369–1388.

Ota, H., 1998. Geographic patterns of endemism and speciation in amphibians and reptiles of the
Ryukyu Archipelago, Japan, with special reference to their Paleogeographical implications.


Pyron, R.A., Burbrink, F.T., 2014. Early origin of viviparity and multiple reversions to oviparity in


Rambaut, A., 2009. Fig Tree v1.3.1 < http://tree.bio.ed.ac.uk/software/figtree/>

Rambaut, A., Suchard, M.A., Xie, D., Drummond, A.J., 2013. Tracer v1.5.0
http://beast.bio.ed.ac.uk/Tracer


Suzuki, T., 1992. Ogasawara shotou chichi jima de erareta yoshinobori no 1 shu–Ogasawara yoshinobori (shinshou)–(yohou) (One of the Yoshinobori species found in Chichijima in Bonin

(in Japanese)


Tamada, K., 2009. Variation in clutch and egg sizes in the amphidromous goby *Rhinogobius* sp. CB along a river course and within a spawning season. Ichthyol. Res. 56, 69–75.


Yokoyama, T., Nakagawa, Y., 1991. Setonaikai no utsurikawari (Transition of the Seto Inland Sea), Sanwashobou, Hyogo, Japan. (in Japanese)


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, Prr, 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.
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
R. virgigena
R. leavelli
R. ogasawaraensis
R. sp. TO-12
R. flumineus
R. naogoyae
R. sp. CO-22
R. brunneus
R. fluviatilis
R. sp. OR-32
R. kurodai
R. sp. BB-37
R. giurinus

The mainland of Japan
Tokara Gap
Bonin Islands
Ryukyu Archipelago

Large-scale interspecific hybridization

Fig. 2
Fig. 3
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).

<table>
<thead>
<tr>
<th>Species name</th>
<th>Location</th>
<th>Specimen ID</th>
<th>Genus</th>
<th>Species code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhinogobius fluviatilis</td>
<td>Lake Biwa, Moriyama</td>
<td>Bi-IR120622-4</td>
<td>R. fluviatilis</td>
<td>RF</td>
</tr>
<tr>
<td>Rhinogobius leavelli</td>
<td>Lake Biwa, Moriyama</td>
<td>Bi-IR120622-4</td>
<td>R. leavelli</td>
<td>RL</td>
</tr>
</tbody>
</table>

Note: The table includes various species of Rhinogobius, with their respective locations and specimen IDs. The study used samples from different localities and species codes to analyze the genetic diversity and population structure of Rhinogobius species.
Table 2.
Estimated divergence time of major clades of *Rhinogobius* gobies.

<table>
<thead>
<tr>
<th>mtDNA</th>
<th>tMRCA (Mya, high mean)</th>
<th>95% HPD (Mya)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA2 (M-5+M-6 vs. M-7)</td>
<td>1.17</td>
<td>0.62–1.70</td>
</tr>
<tr>
<td>M-5+M-6</td>
<td>1.02</td>
<td>0.49–1.52</td>
</tr>
<tr>
<td>M-7</td>
<td>0.97</td>
<td>0.47–1.44</td>
</tr>
<tr>
<td>All Japanese species</td>
<td>1.59</td>
<td>0.73–2.39</td>
</tr>
</tbody>
</table>

nuclear DNA

<table>
<thead>
<tr>
<th></th>
<th>tMRCA (Mya, high mean)</th>
<th>95% HPD (Mya)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1 (<em>R. fluviatilis</em> + <em>R. ogasawaraensis</em>)</td>
<td>1.55</td>
<td>1.05–2.00</td>
</tr>
<tr>
<td>CA2 (<em>R. nagoyae</em> in mainland + Ryukyu)</td>
<td>1.14</td>
<td>0.68–1.69</td>
</tr>
<tr>
<td>N-3-2</td>
<td>1.78</td>
<td>1.03–2.53</td>
</tr>
<tr>
<td>N-3-3</td>
<td>1.65</td>
<td>0.81–2.53</td>
</tr>
<tr>
<td><em>R. flumineus</em> + <em>R. sp. TO</em></td>
<td>1.43</td>
<td>0.49–2.44</td>
</tr>
<tr>
<td><em>R. sp. OR</em> + <em>R. sp. KZ</em> + <em>R. kurodai</em> vs. <em>R. sp. MO</em></td>
<td>1.20</td>
<td>0.59–1.89</td>
</tr>
<tr>
<td><em>R. sp. BW</em> + <em>R. sp. BF</em> + <em>R. sp. OM</em></td>
<td>1.10</td>
<td>0.49–1.77</td>
</tr>
<tr>
<td><em>R. sp. BW</em> + part of <em>R. sp. BF</em></td>
<td>0.90</td>
<td>0.35–1.51</td>
</tr>
<tr>
<td>All Japanese species</td>
<td>4.48</td>
<td>2.38–6.66</td>
</tr>
</tbody>
</table>
Fig. S1 Photographs of all *Rhinogobius* species distributed in Japan.


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.


8: *Rhinogobius* sp. CO, Inouzawagawa River, Shizuoka Pref. A: OMNH-P 40616, male, B: OMNH-P 40618, female.
Fig. S1 Continued.


10: *Rhinogobius* sp. MO, Sate River, Okinawa Island, the Ryukyu Islands. A: OMNH-P 40281, male, B: OMNH-P 40282, female.


14: *Rhinogobius* sp. TO, Kanaregawa River, Aichi Pref. A: OMNH-P 40705, male, B: OMNH-P 40708, female.


16: *Rhinogobius* sp. KZ, Isumigawa River, Chiba Pref. A: OMNH 40656, male, B: OMNH 40663, female.

Fig. S2 Maximum likelihood trees of the selected Rhinogobius species based on respective nuclear gene sequences. Bootstrap values of >70% are indicated.
Fig. S2 Continued.
Fig. S2 Continued.
<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Japanese name</th>
<th>Distribution in Japan (the mainland of Japan/Ryukyu Archipelago)</th>
<th>Distribution outside Japan</th>
<th>Life history</th>
<th>Egg size</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhinogobius flumineus</td>
<td>Kawa-Yoshinobori</td>
<td>Yamanashi–Kyushu/—</td>
<td>—</td>
<td>Fluvial</td>
<td>Large</td>
<td>Mizuno (1960)</td>
</tr>
<tr>
<td>Rhinogobius sp. YB</td>
<td>Kibara-Yoshinobori</td>
<td>—/Ryukyu Archipelago</td>
<td>—</td>
<td>Fluvial</td>
<td>Middle</td>
<td>Shinomiya et al. (2007); Kon and Hiroshima and Tachihara (2000)</td>
</tr>
<tr>
<td>Rhinogobius sp. BB</td>
<td>Aobara-Yoshinobori</td>
<td>—/North area of Okinawa Island</td>
<td>—</td>
<td>Fluvial</td>
<td>Middle</td>
<td>(1994); Nishida (2001);</td>
</tr>
<tr>
<td>Rhinogobius sp. OM</td>
<td>Oumi-yoshinobori</td>
<td>Shiga, Lake Biwa/—</td>
<td>—</td>
<td>Amphidromous</td>
<td>Small</td>
<td>Maruyama et al. (2004); Takahashi and Okaizaki (2002)</td>
</tr>
<tr>
<td>Rhinogobius sp. OR*</td>
<td>Tou-Yoshinobori</td>
<td>Hokkaido–Kyushu/—</td>
<td>—</td>
<td>Amphidromous</td>
<td>Small</td>
<td>Akihito et al. (2002)</td>
</tr>
<tr>
<td>Rhinogobius brunneus</td>
<td>Kuro-Yoshinobori</td>
<td>Chiba–Kyushu/Yakushima–Iriomote Island</td>
<td>—</td>
<td>Amphidromous</td>
<td>Small</td>
<td>Kato and Nishida (1994);</td>
</tr>
<tr>
<td>Rhinogobius fluviatilis</td>
<td>Oo-Yoshinobori</td>
<td>Honshu–Kyushu/—</td>
<td>—</td>
<td>Amphidromous</td>
<td>Small</td>
<td>Takahashi and Okazaki (2000); Nishida (2001)</td>
</tr>
<tr>
<td>Rhinogobius sp. CO</td>
<td>Ruri-Yoshinobori</td>
<td>Hokkaido–Kyushu/—</td>
<td>—</td>
<td>Amphidromous</td>
<td>Small</td>
<td>Akihito et al. (2002)</td>
</tr>
<tr>
<td>Rhinogobius nagoyae</td>
<td>Shima-Yoshinobori</td>
<td>Mainlands/Ryukyu Archipelago</td>
<td>Korea, Taiwan, China</td>
<td>Amphidromous</td>
<td>Small</td>
<td>Nishida (1994); Tsunagawa and Arai (2008); Wu et al.</td>
</tr>
<tr>
<td>Rhinogobius sp. MO</td>
<td>Aya-Yoshinobori</td>
<td>—/Amamioshima-Kume-jima Island</td>
<td>—</td>
<td>Amphidromous</td>
<td>Small</td>
<td>Kondo et al. (2013)</td>
</tr>
<tr>
<td>Rhinogobius sp. DL</td>
<td>Hirayoshi-Yoshinobori</td>
<td>Ogasawara-Yoshinobori</td>
<td>—</td>
<td>Amphidromous</td>
<td>Small</td>
<td>Akihito et al. (2013); Suzuki et al. (2011)</td>
</tr>
<tr>
<td>Rhinogobius sp. BW</td>
<td>Shima-Yoshinobori</td>
<td>Lake Biwa/—</td>
<td>—</td>
<td>Lentic</td>
<td>Small</td>
<td>Tsunagawa et al. (2010a);</td>
</tr>
<tr>
<td>Rhinogobius sp. TO</td>
<td>Tokai-Yoshinobori</td>
<td>Aichi, Mie, Gifu/—</td>
<td>—</td>
<td>Lentic</td>
<td>Small</td>
<td>Tsujimoto (2008); Yamasaki (personal observation)</td>
</tr>
<tr>
<td>Rhinogobius sp. BF</td>
<td>Shimahire-Yoshinobori</td>
<td>Kinki and Setouchi district/—</td>
<td>—</td>
<td>Lentic</td>
<td>Small</td>
<td>Tsujimoto et al. (2010b);</td>
</tr>
<tr>
<td>Rhinogobius sp. KZ</td>
<td>Kazusa-yoshinobori</td>
<td>Chiba/—</td>
<td>—</td>
<td>Lentic</td>
<td>Small</td>
<td>Tsujimoto et al. (2003); Hirashima and Nishida (2010)</td>
</tr>
<tr>
<td>Rhinogobius kurodai</td>
<td>Kuroda-haze</td>
<td>Tokyo, Kanagawa, Shizuoka/—</td>
<td>—</td>
<td>Lentic</td>
<td>Small</td>
<td>Akihito et al. (2002);</td>
</tr>
<tr>
<td>Rhinogobius giurinus</td>
<td>Gokurakuhaze</td>
<td>Mainlands/Ryukyu Archipelago</td>
<td>Korea, Taiwan, China</td>
<td>Amphidromous</td>
<td>Small</td>
<td>Wu et al. (2008); Akihito et al. (2013); Tamada (2005b)</td>
</tr>
</tbody>
</table>

* 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.

† 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.

References


<table>
<thead>
<tr>
<th>Species</th>
<th>Accession</th>
<th>Date</th>
<th>Location</th>
<th>Host</th>
<th>Collection</th>
<th>Accession</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. virgigena</td>
<td>NSMT–P 120836</td>
<td>AB988325</td>
<td>AB988407</td>
<td>AB988489</td>
<td>AB988571</td>
<td>AB988653</td>
<td>AB988735</td>
</tr>
<tr>
<td>BG–CH990405–1</td>
<td>NSMT–P 120837</td>
<td>AB988326</td>
<td>AB988408</td>
<td>AB988490</td>
<td>AB988572</td>
<td>AB988654</td>
<td>AB988736</td>
</tr>
<tr>
<td>OR–IW081011–2</td>
<td>N.D.</td>
<td>AB988327</td>
<td>AB988409</td>
<td>AB988491</td>
<td>AB988573</td>
<td>AB988655</td>
<td>AB988737</td>
</tr>
<tr>
<td>OR–IW090528–1</td>
<td>N.D.</td>
<td>AB988328</td>
<td>AB988410</td>
<td>AB988492</td>
<td>AB988574</td>
<td>AB988656</td>
<td>AB988738</td>
</tr>
<tr>
<td>OR–IW100512–1</td>
<td>N.D.</td>
<td>AB988329</td>
<td>AB988411</td>
<td>AB988493</td>
<td>AB988575</td>
<td>AB988657</td>
<td>AB988739</td>
</tr>
<tr>
<td>OR–IW110512–1</td>
<td>N.D.</td>
<td>AB988330</td>
<td>AB988412</td>
<td>AB988494</td>
<td>AB988576</td>
<td>AB988658</td>
<td>AB988740</td>
</tr>
<tr>
<td>OR–IW120512–1</td>
<td>N.D.</td>
<td>AB988331</td>
<td>AB988413</td>
<td>AB988495</td>
<td>AB988577</td>
<td>AB988659</td>
<td>AB988741</td>
</tr>
<tr>
<td>OR–IW130512–1</td>
<td>N.D.</td>
<td>AB988332</td>
<td>AB988414</td>
<td>AB988496</td>
<td>AB988578</td>
<td>AB988660</td>
<td>AB988742</td>
</tr>
<tr>
<td>OR–IW140512–1</td>
<td>N.D.</td>
<td>AB988333</td>
<td>AB988415</td>
<td>AB988497</td>
<td>AB988579</td>
<td>AB988661</td>
<td>AB988743</td>
</tr>
<tr>
<td>OR–IW150512–1</td>
<td>N.D.</td>
<td>AB988334</td>
<td>AB988416</td>
<td>AB988498</td>
<td>AB988580</td>
<td>AB988662</td>
<td>AB988744</td>
</tr>
<tr>
<td>OR–IW160512–1</td>
<td>N.D.</td>
<td>AB988335</td>
<td>AB988417</td>
<td>AB988499</td>
<td>AB988581</td>
<td>AB988663</td>
<td>AB988745</td>
</tr>
<tr>
<td>OR–IW170512–1</td>
<td>N.D.</td>
<td>AB988336</td>
<td>AB988418</td>
<td>AB988500</td>
<td>AB988582</td>
<td>AB988664</td>
<td>AB988746</td>
</tr>
<tr>
<td>OR–IW180512–1</td>
<td>N.D.</td>
<td>AB988337</td>
<td>AB988419</td>
<td>AB988501</td>
<td>AB988583</td>
<td>AB988665</td>
<td>AB988747</td>
</tr>
<tr>
<td>OR–IW190512–1</td>
<td>N.D.</td>
<td>AB988338</td>
<td>AB988420</td>
<td>AB988502</td>
<td>AB988584</td>
<td>AB988666</td>
<td>AB988748</td>
</tr>
<tr>
<td>OR–IW200512–1</td>
<td>N.D.</td>
<td>AB988339</td>
<td>AB988421</td>
<td>AB988503</td>
<td>AB988585</td>
<td>AB988667</td>
<td>AB988749</td>
</tr>
<tr>
<td>OR–IW210512–1</td>
<td>N.D.</td>
<td>AB988340</td>
<td>AB988422</td>
<td>AB988504</td>
<td>AB988586</td>
<td>AB988668</td>
<td>AB988750</td>
</tr>
<tr>
<td>OR–IW220512–1</td>
<td>N.D.</td>
<td>AB988341</td>
<td>AB988423</td>
<td>AB988505</td>
<td>AB988587</td>
<td>AB988669</td>
<td>AB988751</td>
</tr>
<tr>
<td>OR–IW230512–1</td>
<td>N.D.</td>
<td>AB988342</td>
<td>AB988424</td>
<td>AB988506</td>
<td>AB988588</td>
<td>AB988670</td>
<td>AB988752</td>
</tr>
<tr>
<td>OR–IW240512–1</td>
<td>N.D.</td>
<td>AB988343</td>
<td>AB988425</td>
<td>AB988507</td>
<td>AB988589</td>
<td>AB988671</td>
<td>AB988753</td>
</tr>
<tr>
<td>OR–IW250512–1</td>
<td>N.D.</td>
<td>AB988344</td>
<td>AB988426</td>
<td>AB988508</td>
<td>AB988590</td>
<td>AB988672</td>
<td>AB988754</td>
</tr>
<tr>
<td>OR–IW260512–1</td>
<td>N.D.</td>
<td>AB988345</td>
<td>AB988427</td>
<td>AB988509</td>
<td>AB988591</td>
<td>AB988673</td>
<td>AB988755</td>
</tr>
<tr>
<td>OR–IW270512–1</td>
<td>N.D.</td>
<td>AB988346</td>
<td>AB988428</td>
<td>AB988510</td>
<td>AB988592</td>
<td>AB988674</td>
<td>AB988756</td>
</tr>
<tr>
<td>Locus name</td>
<td>Length (bp)</td>
<td>Number of variable sites (bp)</td>
<td>Ann. alignment p. (%)</td>
<td>1st primer name</td>
<td>primer 1 (5'-3')</td>
<td>2nd primer name</td>
<td>primer 2 (5'-3')</td>
</tr>
<tr>
<td>------------</td>
<td>------------</td>
<td>-------------------------------</td>
<td>----------------------</td>
<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>myh6</td>
<td>719 38/29</td>
<td>22/12</td>
<td>55</td>
<td>myh6_F459</td>
<td>CATMTTYTCATCTCAGATAATGC</td>
<td>myh6_F507</td>
<td>GGGAATACRTCKGTCATCATCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>myh6_R1325</td>
<td>ATTCCTACCACTCCAGTGAAG</td>
<td>myh6_R1322</td>
<td>CTCACCACCATCCAGTGGACAT</td>
</tr>
<tr>
<td>RYR3</td>
<td>806 45/32</td>
<td>31/19</td>
<td>55</td>
<td>RYR3_F15</td>
<td>GGAACATYTGGTAAGCARATTTGG</td>
<td>RYR3_F22</td>
<td>TCAGTAAACGARATGTTGGACAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RYR3_R968</td>
<td>TGGAAAGAACKCAAKATGATG</td>
<td>RYR3_R931</td>
<td>AGAATCRRGTGAAAGACATCCA</td>
</tr>
<tr>
<td>Ptr</td>
<td>636 30/22</td>
<td>24/14</td>
<td>55</td>
<td>R-PtrR</td>
<td>TGTATCCTCATCTGCTCTTCTCT</td>
<td>AGAGTGACCGCTAGGAG</td>
<td>Li et al. (2007); this study</td>
</tr>
<tr>
<td>RAG2</td>
<td>882 115/87</td>
<td>79/50</td>
<td>55</td>
<td>R-RAG2F</td>
<td>ATGGCAGACTAYAGCATGCA</td>
<td>RYR3_F22</td>
<td>TCAGGAGGAGGCAMCAT</td>
</tr>
<tr>
<td>sreb2</td>
<td>870 31/27</td>
<td>14/11</td>
<td>55</td>
<td>sreb2_F10</td>
<td>ATGGCGAATAYAGCATGCA</td>
<td>sreb2_F27</td>
<td>TGGCAGGAGGAGGCAMCAT</td>
</tr>
<tr>
<td>EGR3</td>
<td>842 33/21</td>
<td>21/13</td>
<td>55</td>
<td>E3_F161</td>
<td>AATATCATGACAYGCTGGAGNATG</td>
<td>E3_1136R</td>
<td>GGGTCTCTCTGCTTGTTSAG</td>
</tr>
<tr>
<td>CO1</td>
<td>638 171/154</td>
<td>125/87</td>
<td>48</td>
<td>FishCO1F</td>
<td>TCAACCAACCAAAAGGAGATGGC</td>
<td>FishCO1R</td>
<td>TCCAGCCGCCATCGCAAGAAATCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H13396-ND5</td>
<td>GGTCTTAGGAACCAAAAAATCTG</td>
<td>H13366-ND5-Rhi</td>
<td>GGTCTTAGGAACCAAAAAATCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L13321-Leu</td>
<td>GGTCTTAGGAACCAAAAAATCTG</td>
<td>H13366-ND5-Rhi</td>
<td>GGTCTTAGGAACCAAAAAATCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H14724</td>
<td>TGACTTTGAARACCAAYCYGGY</td>
<td>H16500-CR</td>
<td>GGCCTGAAATAGGACAGCA</td>
</tr>
</tbody>
</table>

*With outgroup / without outgroup

Reference


<table>
<thead>
<tr>
<th>Subset</th>
<th>Subset partition</th>
<th>Best model</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAxML/mtDNA</td>
<td>1 CO1-1st, CO1-2nd, ND5-1st, ND5-2nd, cytb-1st, cytb-2nd</td>
<td>GTR+I+G</td>
</tr>
<tr>
<td></td>
<td>2 CO1-3rd, ND5-3rd, cytb-3rd</td>
<td>GTR+I+G</td>
</tr>
<tr>
<td>MrBayes/mtDNA</td>
<td>1 CO1-2nd, ND5-2nd, cytb-2nd</td>
<td>GTR+I</td>
</tr>
<tr>
<td></td>
<td>2 CO1-3rd, ND5-3rd, cytb-3rd</td>
<td>GTR+I+G</td>
</tr>
<tr>
<td></td>
<td>3 CO1-1st, ND5-1st, cytb-1st</td>
<td>K80+I+G</td>
</tr>
<tr>
<td>RAxML/nuclear</td>
<td>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</td>
<td>GTR+I+G</td>
</tr>
<tr>
<td></td>
<td>2 myh6-3rd, RYR3-2nd, Ptr-3rd, RAG2-1st, sreb2-3rd, EGR3-3rd</td>
<td>GTR+I+G</td>
</tr>
<tr>
<td>MrBayes/nuclear</td>
<td>1 myh6-2nd, RYR3-1st</td>
<td>F81+1</td>
</tr>
<tr>
<td></td>
<td>2 myh6-3rd, RYR3-2nd, Ptr-3rd, sreb2-3rd, EGR3-3rd</td>
<td>GTR+I+G</td>
</tr>
<tr>
<td></td>
<td>3 myh6-1st, RYR3-3rd, Ptr-1st, RAG2-2nd, RAG2-3rd, sreb2-1st</td>
<td>HKY+I</td>
</tr>
<tr>
<td></td>
<td>4 Ptr-2nd, sreb2-2nd</td>
<td>F81</td>
</tr>
<tr>
<td></td>
<td>5 RAG2-1st</td>
<td>HKY+I</td>
</tr>
<tr>
<td></td>
<td>6 EGR3-1st, EGR3-2nd</td>
<td>F81</td>
</tr>
</tbody>
</table>
**Table S5**
Models for BEAST analysis selected by BIC implemented by jModelTest v 2.1.3.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtDNA</td>
<td></td>
</tr>
<tr>
<td>CO1</td>
<td>TrN+G</td>
</tr>
<tr>
<td>ND5</td>
<td>TrN+I+G</td>
</tr>
<tr>
<td>cyt b</td>
<td>GTR+I+G</td>
</tr>
<tr>
<td>nuclear DNA</td>
<td></td>
</tr>
<tr>
<td>Concatenated</td>
<td>HKY+I+G</td>
</tr>
</tbody>
</table>
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).

<table>
<thead>
<tr>
<th>Model</th>
<th>DF</th>
<th>AIC</th>
<th>λ1</th>
<th>λ2</th>
<th>λ3</th>
<th>µ1</th>
<th>µ2</th>
<th>µ3</th>
<th>q_12</th>
<th>q_21</th>
<th>q_13</th>
<th>q_31</th>
<th>q_23</th>
<th>q_32</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Life History</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>12</td>
<td>118.18</td>
<td>0.485</td>
<td>0.379</td>
<td>0.145</td>
<td>7.48E-09</td>
<td>1.28E-06</td>
<td>2.40E-05</td>
<td>0.0830</td>
<td>0.0744</td>
<td>3.67E-06</td>
<td>0.550</td>
<td>0.286</td>
<td></td>
</tr>
<tr>
<td>Equal λ</td>
<td>10</td>
<td>115.21</td>
<td>0.447</td>
<td>-</td>
<td>-</td>
<td>2.72E-11</td>
<td>9.36E-10</td>
<td>0.255</td>
<td>0.0783</td>
<td>1.12E-07</td>
<td>0.0938</td>
<td>1.19E-06</td>
<td>0.589</td>
<td>0.280</td>
</tr>
<tr>
<td>Equal µ</td>
<td>10</td>
<td>114.18</td>
<td>0.485</td>
<td>0.379</td>
<td>0.145</td>
<td>6.29E-07</td>
<td>-</td>
<td>-</td>
<td>0.0830</td>
<td>9.68E-09</td>
<td>0.0744</td>
<td>1.50E-05</td>
<td>0.550</td>
<td>0.286</td>
</tr>
<tr>
<td>Equal q</td>
<td>9</td>
<td>112.77</td>
<td>0.492</td>
<td>0.354</td>
<td>0.140</td>
<td>-</td>
<td>3.45E-07</td>
<td>0.0749</td>
<td>-</td>
<td>0.1035</td>
<td>-</td>
<td>0.489</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Equal λ and µ</td>
<td>8</td>
<td>111.29</td>
<td>0.429</td>
<td>-</td>
<td>-</td>
<td>5.19E-09</td>
<td>-</td>
<td>-</td>
<td>0.0679</td>
<td>1.83E-07</td>
<td>0.0845</td>
<td>7.67E-08</td>
<td>0.513</td>
<td>0.318</td>
</tr>
<tr>
<td>Equal λ and q (reversal transition rates are equal)</td>
<td>7</td>
<td>109.98</td>
<td>0.439</td>
<td>-</td>
<td>-</td>
<td>4.32E-09</td>
<td>0.189</td>
<td>0.00857</td>
<td>0.0698</td>
<td>-</td>
<td>0.1065</td>
<td>-</td>
<td></td>
<td>0.444</td>
</tr>
<tr>
<td>Equal µ and q (reversal transition rates are equal)</td>
<td>7</td>
<td>108.77</td>
<td>0.492</td>
<td>0.354</td>
<td>0.140</td>
<td>8.66E-09</td>
<td>-</td>
<td>-</td>
<td>0.0749</td>
<td>-</td>
<td>0.1035</td>
<td>-</td>
<td></td>
<td>0.489</td>
</tr>
<tr>
<td>Equal λ,µ, q (reversal transition rates are equal)</td>
<td>7</td>
<td>109.53</td>
<td>0.512</td>
<td>0.359</td>
<td>0.204</td>
<td>6.87E-06</td>
<td>0.363</td>
<td>1.34E-05</td>
<td>0.1274</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equal λ µ and q (all transition rates are equal)</td>
<td>5</td>
<td>106.02</td>
<td>0.429</td>
<td>-</td>
<td>-</td>
<td>2.70E-08</td>
<td>-</td>
<td>-</td>
<td>0.0567</td>
<td>-</td>
<td>0.1057</td>
<td>-</td>
<td></td>
<td>0.447</td>
</tr>
<tr>
<td>Equal λ and µ and q (all transition rates are equal)</td>
<td>5</td>
<td>106.71</td>
<td>0.462</td>
<td>-</td>
<td>-</td>
<td>3.75E-08</td>
<td>0.444</td>
<td>0.149</td>
<td>0.1249</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equal µ and q (all transition rates are equal)</td>
<td>5</td>
<td>105.80</td>
<td>0.496</td>
<td>0.304</td>
<td>0.198</td>
<td>3.88E-08</td>
<td>-</td>
<td>-</td>
<td>0.1099</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equal λ,µ, q (all transition rates are equal)</td>
<td>3</td>
<td>103.06</td>
<td>0.429</td>
<td>-</td>
<td>-</td>
<td>1.58E-08</td>
<td>-</td>
<td>-</td>
<td>0.0983</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Egg size</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>12</td>
<td>106.22</td>
<td>0.511</td>
<td>1.21E-06</td>
<td>0.335</td>
<td>4.91E-06</td>
<td>1.97E-11</td>
<td>5.54E-06</td>
<td>0.2125</td>
<td>1.085</td>
<td>5.12E-11</td>
<td>0.334</td>
<td>3.21E-09</td>
<td>3.58E-06</td>
</tr>
<tr>
<td>Equal λ</td>
<td>10</td>
<td>102.86</td>
<td>0.438</td>
<td>-</td>
<td>-</td>
<td>6.44E-09</td>
<td>1.63E-05</td>
<td>0.102</td>
<td>0.3941</td>
<td>2.298</td>
<td>6.15E-07</td>
<td>0.311</td>
<td>7.26E-08</td>
<td>5.95E-08</td>
</tr>
<tr>
<td>Equal µ</td>
<td>10</td>
<td>102.22</td>
<td>0.511</td>
<td>1.75E-06</td>
<td>0.335</td>
<td>6.04E-07</td>
<td>-</td>
<td>0.2125</td>
<td>-</td>
<td>1.085</td>
<td>5.04E-07</td>
<td>0.334</td>
<td>1.91E-09</td>
<td>1.07E-06</td>
</tr>
<tr>
<td>Equal q</td>
<td>9</td>
<td>103.62</td>
<td>0.506</td>
<td>4.39E-09</td>
<td>0.222</td>
<td>1.39E-07</td>
<td>0.592</td>
<td>1.42E-06</td>
<td>0.1372</td>
<td>-</td>
<td>0.0661</td>
<td>-</td>
<td>5.19E-07</td>
<td>-</td>
</tr>
<tr>
<td>Equal λ and µ</td>
<td>8</td>
<td>98.88</td>
<td>0.429</td>
<td>-</td>
<td>-</td>
<td>9.15E-07</td>
<td>-</td>
<td>0.3955</td>
<td>2.304</td>
<td>5.66E-07</td>
<td>0.348</td>
<td>2.15E-09</td>
<td>7.88E-10</td>
<td></td>
</tr>
<tr>
<td>Equal λ and q (reversal transition rates are equal)</td>
<td>7</td>
<td>101.05</td>
<td>0.484</td>
<td>-</td>
<td>-</td>
<td>6.76E-07</td>
<td>1.023</td>
<td>0.330</td>
<td>0.1470</td>
<td>-</td>
<td>0.0783</td>
<td>-</td>
<td>7.07E-09</td>
<td>-</td>
</tr>
<tr>
<td>Equal µ and q (reversal transition rates are equal)</td>
<td>7</td>
<td>100.19</td>
<td>0.482</td>
<td>2.78E-07</td>
<td>0.225</td>
<td>2.76E-05</td>
<td>-</td>
<td>0.0803</td>
<td>-</td>
<td>0.0695</td>
<td>-</td>
<td>3.55E-05</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Equal q (all transition rates are equal)</td>
<td>7</td>
<td>100.07</td>
<td>0.498</td>
<td>2.42E-09</td>
<td>0.212</td>
<td>8.33E-08</td>
<td>0.429</td>
<td>0.132</td>
<td>0.0929</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Equal λ,µ, q (reversal transition rates are equal)</td>
<td>5</td>
<td>98.17</td>
<td>0.429</td>
<td>-</td>
<td>-</td>
<td>2.62E-07</td>
<td>-</td>
<td>0.0758</td>
<td>-</td>
<td>0.0651</td>
<td>-</td>
<td>1.28E-07</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Equal λ and q (all transition rates are equal)</td>
<td>5</td>
<td>97.50</td>
<td>0.476</td>
<td>-</td>
<td>-</td>
<td>3.70E-08</td>
<td>0.855</td>
<td>0.387</td>
<td>0.1022</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Equal µ and q (all transition rates are equal)</td>
<td>5</td>
<td>96.41</td>
<td>0.481</td>
<td>1.06E-05</td>
<td>0.211</td>
<td>2.90E-07</td>
<td>-</td>
<td>0.0725</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Equal λ,µ, q (all transition rates are equal)</td>
<td>3</td>
<td>94.45</td>
<td>0.429</td>
<td>-</td>
<td>-</td>
<td>2.26E-07</td>
<td>-</td>
<td>0.0675</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>