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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.

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).

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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,b), whereas fluvial types complete their life cycle in running freshwaters (Mizuno, 1960; Nishijima, 1968; Iwata, 2001a,b). 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 (Katoh 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