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Mitochondrial genetic diversity of *Rhinogobius giurinus* (Teleostei: Gobiidae) in East Asia

Yu-Min Ju [a], Jui-Hsien Wu [b], Po-Hsun Kuo [c], Kui-Ching Hsu [c], Wei-Kuang Wang [d], Feng-Jiau Lin [e], Hung-Du Lin [f, *]

[a] National Museum of Marine Biology and Aquarium, Pingtung, 944, Taiwan
[b] Eastern Marine Biology Research Center of Fisheries Research Institute, Council of Agriculture, Taitung, 950, Taiwan
[c] Department of Industrial Management, National Taiwan University of Science and Technology, Taipei, 106, Taiwan
[d] Department of Environmental Engineering and Science, Feng Chia University, Taichung, 407, Taiwan
[e] Tainan Hydraulics Laboratory, National Cheng Kung University, Tainan, 709, Taiwan
[f] The Affiliated School of National Tainan First Senior High School, Tainan, 701, Taiwan

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ABSTRACT

Rhinogobius giurinus (Gobiidae: Gobionellinae) is an amphidromous goby that is widely distributed in East Asia. However, little is known about its population structure. In this study, *R. giurinus* from Japan, Taiwan and mainland China were used to evaluate the population genetic structure using mitochondrial DNA cytochrome *b* gene sequences (1122 bp). In total, 123 sequences were analyzed from seventeen populations. All 44 mtDNA haplotypes were identified as belonging to ten haplogroups, and four haplogroups were only distributed in the upstream of the Yangtze River and Hainan Island. The phylogeny and geography did not have a significant relationship. Our results (1) found that the mtDNA genetic diversity of this species was less than that of the freshwater goby; (2) showed that the lack of a population genetic structure might result in its amphidromous life cycle and high migrating potentiality; (3) indicated that although it is an amphidromous species, *R. giurinus* could not migrate across a deep sea, such as the South China Sea and Pacific Ocean; and (4) considered that the Kuroshio Current might act as a barrier for gene flows between East Japan and East China.

1. Introduction

Rhinogobius is a genus of the freshwater goby that is native to tropical and temperate parts of East Asia. There are 64 valid species within this genus (Froese and Pauly, 2015). Among them, most species are primarily freshwater fish, and very few species are amphidromous (Froese and Pauly, 2015). *Rhinogobius giurinus* (Rutter, 1897) is an amphidromous species. This species inhabits marine, brackish and fresh waters and feeds on aquatic insects, invertebrates, small fish, zooplankton, phytoplankton and plant detritus. This species' native range includes the east part of China, Taiwan, the Korean Peninsula, Japan, the Ryukyu Islands and North Vietnam (Tzeng et al., 2006). Although this species is widely distributed in East Asia, its population structure is unknown.

* Corresponding author.
E-mail addresses: yumine@nmmba.gov.tw (Y.-M. Ju), ymj561725@gmail.com (J.-H. Wu), phkuo@mail.ntust.edu.tw (P.-H. Kuo), joekchsu@yahoo.com.tw (K.-C. Hsu), weikuangwang@gmail.com (W.-K. Wang), fjlin@mail.ncku.edu.tw (F.-J. Lin), varicorhinus@hotmail.com (H.-D. Lin).

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Geologically, Taiwan island, the Ryukyu archipelago and Japanese islands have been defined as the East Asian islands or East Asian land bridge islands. Previous phylogeographical studies proposed that the East Asia islands were connected to the surrounding landmasses more than once, which allowed various organisms to expand onto the islands via south and north sources (to Korea from Japan and to the south of mainland China from Taiwan) (Ota, 1998; Zhang et al., 2008; Chiang et al., 2010). However, when the East Asian land bridge islands connected together, this long island arc (East Asia island arc) might have functioned as a dispersal barrier between the East China Sea and North Pacific Ocean (Fig. 1; Hu et al., 2011). In other words, during glaciation events, the East Asia island arcs assisted in the migration of freshwater biota but opposed the dispersal of saltwater fauna (Liu et al., 2007; Hu et al., 2011).

Previous studies of the population genetic structures in the North Pacific Ocean sometimes noted the effects of the Kuroshio Current (Chang et al., 2012; Ju et al., 2013; Lord et al., 2015). The Kuroshio Current is a northward current that emerges from the Philippines and flows along the east coast of Taiwan, the Ruykyu archipelago and the east coast of the Japanese mainland (Fig. 1). Lord et al. (2015) proposed that the Kuroshio Current zone could act as a barrier to larval dispersal between the western and eastern parts of the Asia amphidromous Sicydiinae goby's distribution. Our study hypothesized that the Kuroshio Current could potentially act as a barrier to dispersal between the East China Sea and Pacific Ocean as well as the East Asia island arcs. However, Chang et al. (2012) proposed that the Kuroshio Current facilitated the migrations of *Hirundichthys oxycephalus* (bony flying fish). Ju et al. (2013) found that an Asian amphidromous Sicydiinae goby (*Sicyopterus japonicus*) lacked a geographic structure and proposed that the life history of oceanic planktonic larvae and the Kuroshio Current were the important factors that facilitated the migration of *S. japonicus* in Taiwan. Lord et al. (2015) examined the population structure of another Sicydiinae goby (*Stiphodon percnopterygionus*) and found results similar to those reported for *S. japonicas*. These two studies proposed that the larvae were probably transported by the Kuroshio Current in the northwest Pacific Ocean. Accordingly, our study investigated whether the Kuroshio Current acted as a barrier or a facilitator of the *R. giurinus* population structure.

Additionally, *R. giurinus* can survive and reproduce in a completely closed river system, and some researchers have considered this taxon to be a landlocked freshwater goby (Tzeng et al., 2006). Previous phylogeographical studies (*e.g.*, Chen et al., 2007; Chiang et al., 2013; Yang et al., 2016) suggested that primary freshwater fish had limited opportunities for gene flow. In China, the Yangtze River, Pearl River, southeast coastal districts, Taiwan and the Hainan Islands were defined as different zoogeographical regions based on fish assemblages (Li, 1981). Thus, our study examined whether the geological events in East Asia (Chiang et al., 2013; Yang et al., 2016) contributed to the differentiation of *R. giurinus*. To address the above problems, our study used the mitochondrial DNA (mtDNA) cytochrome *b* (cyt *b*) gene to evaluate the population genetic structure and infer the migrating potentiality of *R. giurinus*.

2. Materials and methods

A total of 120 *R. giurinus* specimens were collected from 14 localities in Taiwan Island, Hainan Island and mainland China (Table 1; Fig. 1). The fish were collected from field sites with seines and fatally anesthetized with MS-222 (Sigma, St. Louis,

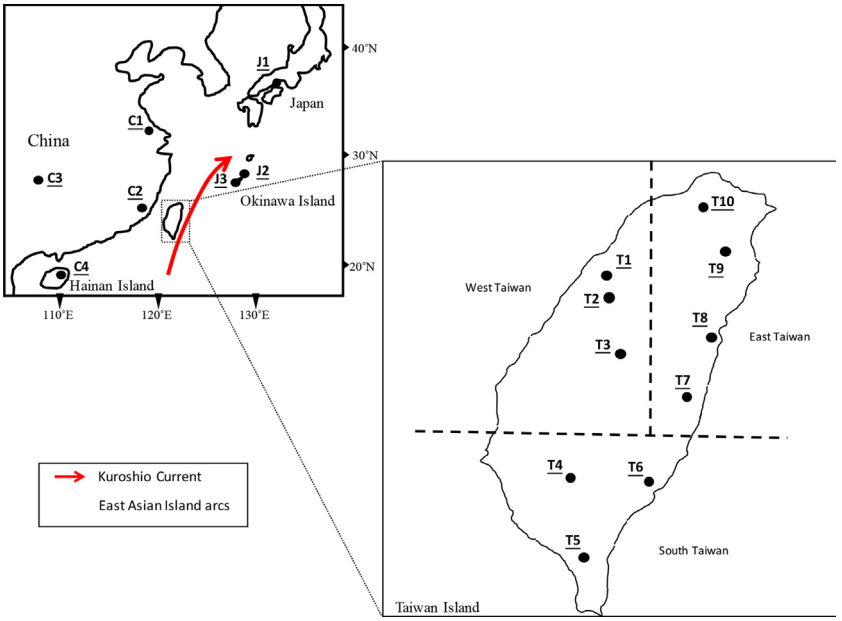

Fig. 1. *Rhinogobius giurinus* sample locations in East Asia. All localities used in this study are indicated by •. The blue line and red arrow indicated the East Asia island arcs and the Kuroshio Current, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MO). The samples were fixed and stored in 100% ethanol. Genomic DNA was extracted from muscle tissue using the Genomic DNA Purification Kit (Gentra Systems, Valencia, CA, USA). The entire cyt *b* gene was amplified by polymerase chain reaction (PCR) using the primers GcytbL (5′- GACTTGAAAAACCACCGTTG -3′) and GcytbH (5′- CTCCGATCTCCGGATTACAAGAC -3′). Each 50 μl PCR reaction mixture contained 5 ng of template DNA, 5 μl of 10x reaction buffer, 5 μl of dNTP mix (10 mM), 5 pmol of each primer and 2U of Taq polymerase (Promega, Madison, WI, USA). The PCR was programmed on a MJ Thermal Cycler as one cycle of denaturation at 95 °C for 4 min, 30 cycles of denaturation at 94 °C for 45 s, annealing at 48 °C for 1 min 15 s and extension at 72 °C for 1 min 30 s, followed by a 72 °C extension for 10 min and 4 °C for storage. The purified PCR products were sequenced using an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). The chromatograms were checked with the CHROMAS software (Technelysium), and the sequences were manually edited using BIOEDIT 6.0.7 (Hall, 1999). Sequences of three specimens from Japan were directly obtained from GenBank for a joint analysis (AB988913, AB988945 and AB988946; Table 1; Fig. 1). In total, 123 sequences from seventeen populations were analyzed. These populations were sorted into eight groups defined based on their ichthyofaunal characteristics as follows (Oshima, 1923; Li, 1981): Japan (J1-J3), West Taiwan (T1-T3), South Taiwan (T4-T6), East Taiwan (T7-T10), and four groups in China (C1-C4) (Table 1; Fig. 1).

Nucleotide sequences were aligned in Clustal X 1.81 (Thompson et al., 1997). The phylogenetic analyses were performed using the maximum likelihood (ML) and Bayesian inference (BI) methods. Selection of the best-fit nucleotide substitution models was performed using the Bayesian information criterion (BIC) in jmodeltest 2.0 (Darriba et al., 2012). The most appropriate nucleotide substitution model was TrN (TN93, Tamura and Nei, 1993). The ML analysis was performed using the PhyML 3.0 program (Guindon et al., 2010) and started with a neighbor-joining tree. Bootstrapping was performed with 1000 replications. The BI analysis was performed using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). The posterior probability values were used as support for the Bayesian topology. Log-likelihood stability was reached after approximately 500,000 generations. The first 350 trees were excluded as burn-in values, and the remaining trees were used to compute a 50% majority rule consensus. The haplotype network was constructed using Arlequin 3.5 (Excoffier and Lischer, 2010).

The intra-population genetic diversity levels were estimated using haplotype diversity (*h*) (Nei and Tajima, 1983) and nucleotide diversity (θ_π and θ_ω) indices (Jukes and Cantor, 1969) in DnaSP 4.10.8 (Rozas et al., 2003). The current genetic diversity estimates (θ_π) are based on pairwise differences between sequences, and the historical diversity estimates (θ_ω) are based on the number of segregating sites among sequences. Comparing the estimates generated by these two indices provides insight into population dynamics over recent evolutionary history (Templeton, 1993). The existence of a phylogeographic structure was examined following the method of Pons and Petit (1996) by calculating two genetic differentiation indices (G_{ST} and N_{ST}) in DnaSP. The nucleotide divergence was calculated with the p-distance in MEGA 6 (Tamura et al., 2013).

3. Results

A total of 44 haplotypes (1122 bp, 80 variable sites and 51 phylogenetic informative sites; GenBank accession numbers KX578172-212) were obtained for 123 sequences from the 17 populations analyzed (Table 1; Fig. 1). The nucleotide sequences

Table 1
Samples of used for mtDNA analyses, location, code and summary statistics. Haplogroup recoved in the phylogenetic analysis (Fig. 2). Haplotypes indicated the number of private haplotype (P) and the number of individual of shared haplotypes (S1-S4) in each population.

Locality	Code	Sample size	Haplotype diversity (h)	Nucleotide diversity		Haplogroup	Haplotypes				
				θ_π (%)	θ_w (%)		P	S1	S2	S3	S4
Japan		3	1.00	0.71	0.71						
1. Hyogo, Sats River	J1	1	–	–	–	2	1				
2. Okinawa, Aha River	J2	1	–	–	–	2	1				
3. Okinawa, Genka River	J3	1	–	–	–	2	1				
Taiwan		87	0.88	0.46	0.57						
4. Houlong River	T1	9	0.78	0.67	0.53	1, 9, 10	3	2			
5. Daan River	T2	9	0.67	0.15	0.10	10	1		1	4	
6. Sun Moon Lake	T3	10	0.36	0.03	0.03	10	0	2	8		
7. Kaoping River	T4	10	–	–	–	10	1		6		
8. Sichong River	T5	8	–	–	–	10	1				
9. Dapochih Wetland	T6	9	0.58	0.22	0.33	3, 10	0	1	1	6	1
10. Xiuguluan River	T7	8	0.79	0.31	0.41	3, 10	3	4			1
11. Liyu Lake	T8	9	0.69	0.43	0.30	1, 10	2		2	1	
12. Dongshan River	T9	7	0.81	0.09	0.07	9	4				
13. Tamsui River	T10	8	0.48	0.38	0.33	1, 8, 10	2		2		
China		33	0.95	0.80	1.06						
14. Dalong River	C1	7	0.29	0.15	0.22	8	2				
15. Jiulong River	C2	9	0.89	0.42	0.43	1, 3, 9	6				
16. Wujiang River	C3	9	0.75	0.56	0.43	4, 6	4				
17. Hainan Island	C4	8	1.00	0.65	0.62	1, 5, 7	8				
Total		123	0.94	0.68	1.34		40				

were A + T (53.9%) rich. The haplotype diversities within populations varied, ranging between 0.29 and 1.00 (Table 1). The nucleotide diversity (θ_π) in the region was the highest in the China mainland (0.80) and the lowest in Taiwan Island (0.46). Estimates of the current (θ_π) and historical (θ_ω) genetic diversity per site for each sample indicated that most samples showed a pattern of growth ($\theta_\pi > \theta_\omega$; Table 1). However, populations in Taiwan Island, mainland China, and the total sample displayed a pattern of decline ($\theta_\pi < \theta_\omega$; Table 1).

Among the 44 unique haplotypes, 40 haplotypes were private haplotypes; the remaining four haplotypes (S1-S4) were shared by more than two populations (Table 1). Our study found that most of the mainland China populations had more private haplotypes, whereas none of the Japan and mainland China populations had shared haplotypes (Table 1). In Taiwan Island, all populations, excluding Sun Moon Lake (T3) and Dapochih Wetland (T6), had at least one private haplotype, the Dapochih Wetland population (T6) had the most shared populations, and the populations in Sichong River (T5) and Dongshan River (T9) did not have a shared haplotype.

A comparison of the fixation N_{ST} (0.567) and G_{ST} (0.369) indices revealed that phylogeny and geography did not have a significant relationship. In the phylogenetic analyses, the topologies of the ML and BI trees were different. Our study found that all haplotypes in both the ML and BI trees fell into ten haplogroups (Fig. 2), although the relationships among these ten haplogroups were not supported by bootstrap values in these two phylogenetic trees. Thus, our study used the haplotype network to examine the relationships among haplogroups. The haplotype network (Fig. 3) was consistent with the topology of the phylogenetic tree (Fig. 2), with all haplotypes falling into ten haplogroups. Three haplogroups (1, 7 and 9) were located at internal positions, whereas the others were located at tip positions. These three internal haplogroups were distributed in mainland China (excluding C1 & C3) and East and West Taiwan (Fig. 3). Furthermore, the distribution patterns of internal haplogroups (Fig. 3) did not consist with the geological groups (Fig. 1; Table 1). All connections between haplogroups were fewer than five mutation changes.

The pairwise divergence among these ten haplogroups ranged from 0.006 to 0.015 (p-distance). The largest distance existed between haplogroups 6 and 10, but the differentiation between these haplogroups was only seventeen base pairs (17/1122). Among these ten haplogroups, haplogroup 2 was only distributed in Japan; haplogroups 4–7 were only distributed in China populations C3 and C4; haplogroup 10, which was the most widespread haplogroup, was only distributed in the Taiwan populations (excluding population T9); and haplogroups 1, 3, 8, 9 were distributed in China and Taiwan (Tables 1 and 2; Figs. 2 and 3). Most populations had only one haplogroup (Table 2).

4. Discussion

A comparison of the N_{ST} (0.567) and G_{ST} (0.369) fixation indices showed that phylogeny and geography did not have a significant relationship. The mtDNA genealogy and network were not consistent with the groups defined based on their

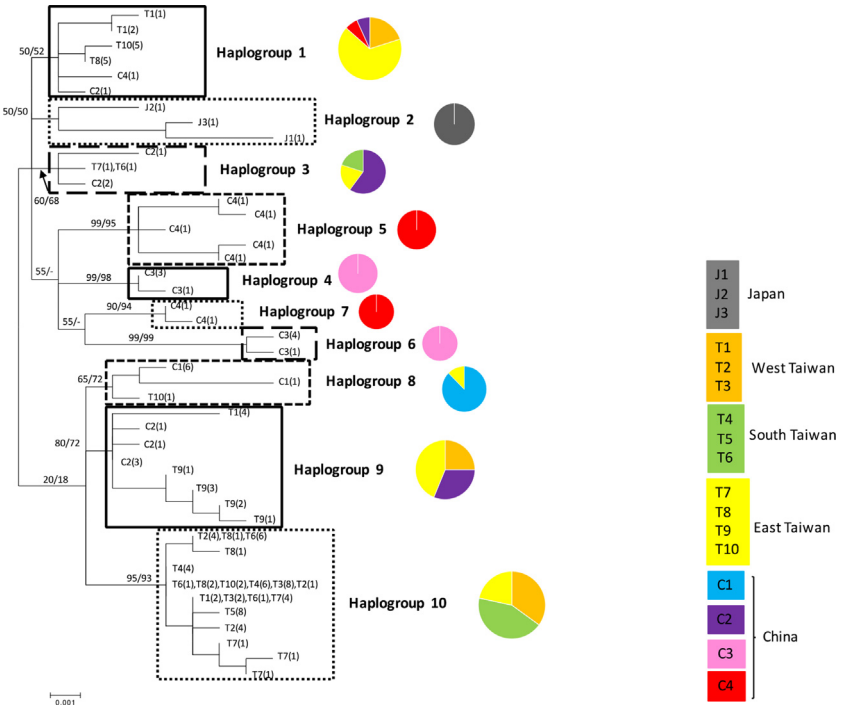

Fig. 2. The *Rhinogobius giurinus* maximum-likelihood (ML) tree. The numbers at the nodes are bootstrap values of ML and Bayesian inference (BI). Pie charts represent the relative frequencies of specimens for the sampling populations.

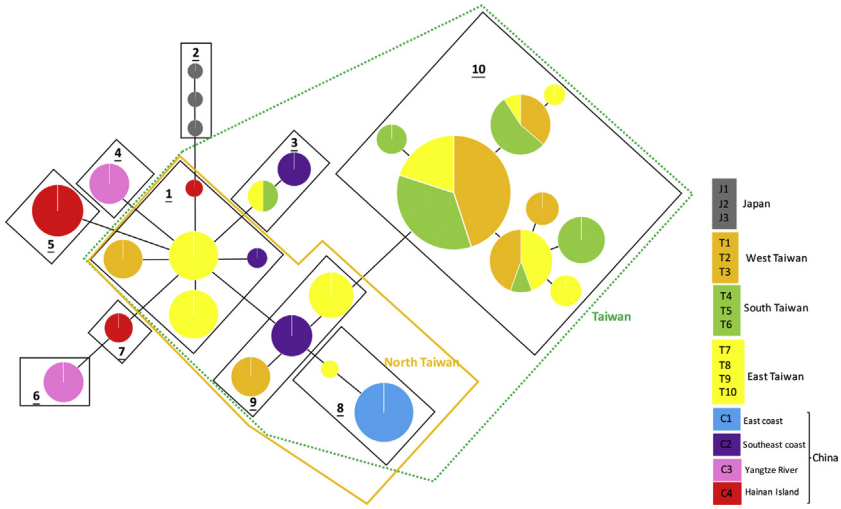

Fig. 3. The haplotype network of *Rhinogobius giurinus*. The sizes of the circles are roughly proportional to the individual frequencies, and the pie charts represent the relative frequencies of specimens for the sampling populations. All connections between haplogroups were less than five mutation changes.

ichthyofauna (Figs. 1 and 3; Table 1). These results supported the finding that *R. giurinus* lacked a population structure. Compared with other goby, the mtDNA genetic diversity of *R. giurinus* ($\theta_\pi = 0.679\%$) was smaller than that of *R. maculafasciatus* ($\theta_\pi = 2.27\%$) (Cheng et al., 2005). However, *R. maculafasciatus* is an endemic freshwater fish in Taiwan. Previous studies (Allibone and Wallis, 1993; Doherty et al., 1995) proposed that freshwater species with a diadromous life cycle exhibited lower inter-population structures than did primary freshwater species. Accordingly, our study suggested that the lack of a population genetic structure for *R. giurinus* might result in its amphidromous life cycle.

Tzeng et al. (2006) described *R. giurinus*is as a landlocked freshwater goby. Indeed, *R. giurinus*can survives and reproduces in closed freshwater systems. In our study, the C3 population (Wujiang River) may be a landlocked population because the Wujiang River is located upstream of the middle Yangtze River, and the distance between the Wujiang River and the coast is more than 1000 km (Fig. 1). Additionally, the second largest freshwater lake in mainland China (Dongting Lake) is located between the Wujiang River and the other coastal populations. Thus, these complex landforms might limit its migrations. Our results showed that the C3 population did not share haplotypes and haplogroups with the other sampling populations (Tables 1 and 2). These results imply that its migrating potentiality might be limited. However, our study found that not all individuals of the C3 population fell into a monophyletic haplogroup instead of two private haplogroups (4 and 6; Tables 1 and 2). The haplotype network (Fig. 3) demonstrated that these two haplogroups (haplogroups 4 and 6) in population C3 were not sister groups and that the haplogroups 1 and 7 were located between haplogroups 4 and 6. Haplogroups 1 and 7 were distributed in Taiwan, Hainan and mainland China (Tables 1 and 2). Accordingly, our study found that the C3 population might be connected to other populations more than once and that all connections between haplogroups were due to fewer than five mutation changes. Thus, our study suggested that *R. giurinus* had high migrating potentiality.

In our study, the populations in mainland China (C1-C4) and Japan (J1-J3) were all connected to East Taiwan (Fig. 3). However, among these populations, population C4 in Hainan Island was closer to South Taiwan than East Taiwan geographically (Fig. 1). Moreover, our study found that the depth of the sea around South and East Taiwan was deeper than the depth in West Taiwan (Taiwan Strait). Thus, our study suggested that *R. giurinus* could not cross a deep sea, such as the South China Sea and Pacific Ocean, and instead was dispersed through a shallow sea, such as the Taiwan Strait and continental shelf. Furthermore, our study found that haplogroup 10 was widely present in Taiwan Island (Table 1). This result supported the finding that the gene flows among populations in Taiwan were unlimited. Previous studies (*e.g.,* Tzeng, 1986; Wang et al., 2004) proposed that Taiwan Island was divided into three major geographical regions based on ichthyofaunal and phylogeographical studies of freshwater species and that the rivers in these regions were almost impossible to exchange with one another. Thus, our study suggested that the wide distribution of haplogroup 10 in Taiwan Island was likely due to the coastal current around the island.

The spawning season of *R. giurinus* is from July to October. During this season, the Kuroshio Current flows from the Philippines through Taiwan to Japan. Previous studies (Chang et al., 2012; Ju et al., 2013; Lord et al., 2015) suggested that this ocean current in the Pacific might facilitate the dispersal of fish. Our study also considered that the lack of a population genetic structure of *R. giurinus* was due to the Kuroshio Current. However, populations C1 and C2 were close to Japan geographically but were close to East Taiwan (Figs. 1 and 3). Lord et al. (2015) reported that the Kuroshio Current might act as a barrier between the East China Sea and North Pacific Ocean. Additionally, our study suggested *R. giurinus* could not cross the deep sea. Thus, the Kuroshio Current might break the migration of *R. giurinus* between East China and East Japan.

Table 2
The distributions of haplogroups. The bold indicated the haplogroup only displayed in single population, and underlined indicated the population only had single haplogroup.

	J1	J2	J3	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	C1	C2	C3	C4
Haplogroup 1				3							5		5		1		1
Haplogroup 2	1	1	1														
Haplogroup 3									1	1					3		
Haplogroup 4																4	
Haplogroup 5																	5
Haplogroup 6																5	
Haplogroup 7																	2
Haplogroup 8													1	7			
Haplogroup 9				4								7			5		
Haplogroup 10				2	9	10	10	8	8	7	4		2				

The geological history indicated that the migrations of freshwater species in mainland China, Japan and Taiwan Island occurred freely during glaciation events. Previous phylogeographic studies of freshwater species (Chiang et al., 2013; Chiu et al., 2016) showed that the populations in Taiwan were close to the populations in South China and that the populations in Japan were close to the populations in North Taiwan. In our study, the structure of *R. giurinus* (Fig. 3) seemingly matched this pattern. Moreover, as shown above, *R. giurinus* migrated via the shallow sea and did not cross the deep sea. Thus, our study demonstrated that although *R. giurinus* was an amphidromous fish, the landform changes in the glaciations might have facilitated its dispersions as well as freshwater fish. Future studies need to perform more sampling to confirm this hypothesis.

Acknowledgements

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