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Cryptic diversity of *Rhinogobius rubromaculatus* species complex (Gobiidae) in Taiwan: Mitogenomes reveal their evolutionary history

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ABSTRACT: *Rhinogobius rubromaculatus* species complex is a group of freshwater gobies endemic to Taiwan. To understand the cryptic species diversity of *R. rubromaculatus* species complex, this study collected 501 specimens of the genus *Rhinogobius* from 34 rivers in Taiwan and used the sequences of mitochondrial DNA cytochrome b gene (1140 bp) as a DNA barcoding tool to identify species. In total, four allopatric lineages were found within *R. rubromaculatus* species complex, and large genetic divergences (p-distances > 0.043) support that they are distinct species. To better understand their evolutionary history, the complete mitochondrial genomes (mitogenomes) of these four lineages were sequenced. These results showed that (1) the mitogenomes characteristics of these four lineages were similar to those of other *Rhinogobius* species; (2) the mitogenome genetic distances (p-distances > 0.045) also supported that these four lineages are different species; (3) the results of the Approximate Bayesian Computation analyses support that these four species colonized Taiwan through four different colonization routes; (4) the *R. rubromaculatus* species complex reveal an evolutionary history similar to other Taiwanese freshwater fish such as *Squalidus*, *Cobitis sinensis* and *Acrossocheilus paradoxus*; and (5) the complex geological history of Taiwan has shaped the colonization history and distribution patterns of *R. rubromaculatus* species complex. These results have important implications for the conservation, population genetics and evolution of freshwater fishes in Taiwan.

KEY WORDS: Approximate Bayesian Computation, cryptic species, mitogenome, *Rhinogobius yangminshanensis*.

INTRODUCTION

Taiwan island is located off the southeastern coast of the Asian continent and in the south of the Japanese archipelago. This island is a small island, with an area of only 35,570 square kilometers, but it has diverse environmental conditions, diverse topography, and complex geological history. Geological evidence reveals that the land-bridges connected Taiwan to the Japanese archipelago and Asian continent during glaciations (Hsu, 1990; Kimura, 2000). Previous phylogeographic studies have documented that the freshwater species migrated to Taiwan from the Asian continent and the Japanese archipelago via multiple routes during multiple glaciations (Gascoyne *et al.,* 1979; Fairbanks, 1989; Ota, 1998; Chiang *et al.,* 2013; Lin *et al.,* 2016; Chiu *et al.,* 2017). Moreover, many studies have documented that the complex geological history and topography shaped the distribution patterns of freshwater species and their population structures (Chiang *et al.,* 2010; Hsu *et al.,* 2014; Han *et al.,* 2019; Ju *et al.,* 2021, 2024; Kang *et al.,* 2022). Although there are many studies on freshwater species in Taiwan, there are still many unresolved issues.

Among the freshwater fishes in Taiwan, the maximum number of species in the same genus is four (e.g., genus *Hemimyzon*), but the genus *Rhinogobius* includes at least nine species (Fricke *et al.,* 2024; Froese and Pauly, 2024). *Rhinogobius* is a genus of gobies native to tropical and temperate regions of eastern Asia. Among *Rhinogobius* species in Taiwan, *R. rubromaculatus* is as a new species proposed by Lee and Chang (1996) from western Taiwan. Cheng *et al.* (2005) found that this species was widely distributed in northern, northwestern, western, southwestern and southern Taiwan and exhibited a significant population structure. Recently, Chen *et al.* (2022) described a new species *R. yangminshanensis* from northern Taiwan, and described *R. yangminshanensis* and *R. rubromaculatus* as *R. rubromaculatus* species complex. Moreover, Chen *et al.* (2022) suggested that *R. rubromaculatus* might exist cryptic species in the different basins from western Taiwan.

Previous studies have found that some congeneric freshwater fishes colonized Taiwan from one single origin, e.g., *Candidia barbata* and *C. pingtungensis* (Chen *et al.,* 2009), and *Opsariichthys kaopingensis* and *O. pachycephalus* (Lin *et al.,* 2016). The ancestral populations of these genera colonized Taiwan from one single origin, and then the populations spread. When geological barriers formed, populations were isolated and evolved to become distinct species (Lin *et al.,* 2016). Besides, some studies have found that the congeneric species in Taiwan did not form a monophyletic group, and suggested that they originated from different sources, and even colonized Taiwan through different routes at different times, e.g., *Aphyocypris kikuchii* and *A. moltrechti* (Ju *et*

Table 1. PCR primers (F: forward primer, R: reverse primer) used in this study. The location of primers and the lengths of the PCR products are also displayed.

Names	Location	length	sequences
RrMT_F1:	12S rRNA		5'-AAGAGGGCCGGTAAAACTCG-3'
RrMT_R1:	16S rRNA	1750	5'-GGCGATGTTTTTGGTAAACA-3'
RrMT_F2:	16S rRNA		5'-TCGCCTGTTTACCAAAAACA-3'
RrMT_R2:	tRNAGln	1950	5'-GGAAGCACTAAGAGTTTTGA-3'
RrMT_F3:	tRNAGln		5'-AAGAGATCAAAACTCTTAGT-3'
RrMT_R3:	COI	1600	5'-TGCCAATGTCTTTATGGTTG-3'
RrMT_F4:	COI		5'-TCTACCAACCATAAAGACAT-3'
RrMT_R4:	COII	1700	5'-AGGTGATGCTGCGTCTTGAA-3'
RrMT_F5:	COII		5'-TAGGATTTCAAGACGCAGCA-3'
RrMT_R5:	COIII	1600	5'-AAGGGCTGGGGTCTACTATG-3'
RrMT_F6:	COIII		5'-TACCACATAGTAGACCCCAG-3'
RrMT_R6:	tRNAArg	1250	5'-GGGTCATTAGGTAATTGTGG-3'
RrMT_F7:	tRNAArg		5'-AGTCCACAATTACCTAATGA-3'
RrMT_R7:	tRNASer	1780	5'-ACCGAGGGTACGAGGGTTAG-3'
RrMT_F8:	tRNASer		5'-TGCTAACCCTCGTACCCTCG-3'
RrMT_R8:	ND5	900	5'-TTGAGTGAAGTAGGGCAGAG-3'
RrMT_F9:	ND5		5'-CGGTCTCTGCCCTACTTCAC-3'
RrMT_R9:	tRNAGlu	1700	5'-TGTAGTTGAATTACAACGGT-3'
RrMT_F10:	tRNAGlu		5'-AAAAACCACCGTTGTAATTC-3'
RrMT_R10:	d-loop	1700	5'-AAATAGGAACCAAATGCCAG-3'
RrMT_F11:	d-loop		5'-ACTATTCCTGGCATTTGGTT-3'
RrMT_R11:	12S rRNA	800	5'-TGGCACGAGTTTTACCGGCC-3'

al., 2021), and *Microphysogobio alticorpus* and *M. brevirostris* (Chang *et al.,* 2016). Their ancestors had differentiated before colonizing Taiwan (Chang *et al.,* 2016).

Many studies have suggested that the discovery of cryptic species diversity is an important factor influencing future conservation decisions (Bickford *et al.,* 2006; Delić *et al.,* 2017; Hending, 2024). Struck *et al.* (2018) proposed that revealing the evolutionary history of cryptic species can help understand biodiversity and contribute to conservation efforts. Accordingly, the major aim of this study is to find out whether cryptic species exist within *R. rubromaculatus* species complex. If so, what is their evolutionary history?

To achieve the above objectives, genetic variability was analyzed using the mitochondrial DNA (mtDNA) cytochrome *b* gene (cyt *b*) and the complete mitochondrial genome (mitogenome). Mitochondrial DNA Sequences are often analyzed in studies of animal phylogeography (Yang *et al.,* 2016; Chiu *et al.,* 2017; Han *et al.,* 2019). Among all the mtDNA genes, the cyt *b* gene is widely accepted marker for assessing the levels of genetic diversity and differentiation (Zhao *et al.,* 2018; Kang *et al.,* 2022; Ju *et al.,* 2024). Additionally, the cyt *b* gene has proven to be effective in identifying fishes, and many other animal groups (Ghouri *et al.,* 2020; Deconinck *et al.,* 2023; Gómez-Lépiz *et al.,* 2024). In this study, cyt *b* gene sequences were used to identify the species of *R. rubromaculatus* species complex. In addition, mitogenome is also one of the most widely used molecular tools in phylogenetic analyses (Sun *et al.,* 2022; Tang *et al.,* 2023; Li *et al.,* 2024). To better understand the evolutionary history of *R. rubromaculatus* species complex, this study used the variations in the entire mitogenomes to test their colonization history.

MATERIALS AND METHODS

Specimens collection and species identification

A total of 501 specimens of *Rhinogobius* species were collected from 34 localities (rivers) across Taiwan. All specimens are housed in the laboratory of Yu-Min Ju, National Museum of Marine Biology and Aquarium. Fish were collected from field sites with seines and fatally anesthetized with MS-222 (Sigma). The samples were fixed and stored in 100% ethanol.

Genomic DNA was extracted from muscle tissue using a genomic DNA purification kit (Gentra Systems, Valencia, CA). The entire cyt *b* gene from all specimens and complete mitogenome from *R. rubromaculatus* species complex were amplified by polymerase chain reaction (PCR). In this study, the primers were developed based on the mitogenomes of the genus *Rhinogobius* in NCBI (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov) (Table 1). Each 50 µl PCR reaction mixture contained 5 ng of template DNA, 5 µl of 10x reaction buffer, 4 µl of dNTP mix (10 mM), 5 pmol of each primer and 2U of Taq polymerase (TaKaRa, Taq polymerase). The PCR was programmed on an MJ Thermal Cycler as one cycle of denaturation at 94°C for 4 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 48 - 52°C for 1 min 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, U.S.A.). The chromatograms were assessed using the software CHROMAS (Technelysium), and the sequences were manually edited using BIOEDIT 6.0.7 (Hall, 1999). The nucleotide sequences were aligned with Clustal X 1.81 (Thompson *et al.,* 1997).

Population and mitogenome analyses

To avoid misidentification of species, this study used morphological characteristics and DNA barcode dataset (cyt *b* dataset) to identify the *Rhinogobius* species. All cyt *b* sequences were identified by a Neighbor Joining (NJ) tree estimation within the maximum composite likelihood substitution model by MEGA-X (Kumar *et al.,* 2018) and BLAST analysis in NCBI to ensure that all species were not misidentified.

The levels of intra-population genetic diversity of *R. rubromaculatus* species complex were estimated based on the haplotype diversity (Hd) and nucleotide diversity ($\theta\pi$) indices in DnaSP 5.10 (Librado and Rozas, 2009). The most appropriate nucleotide substitution model was

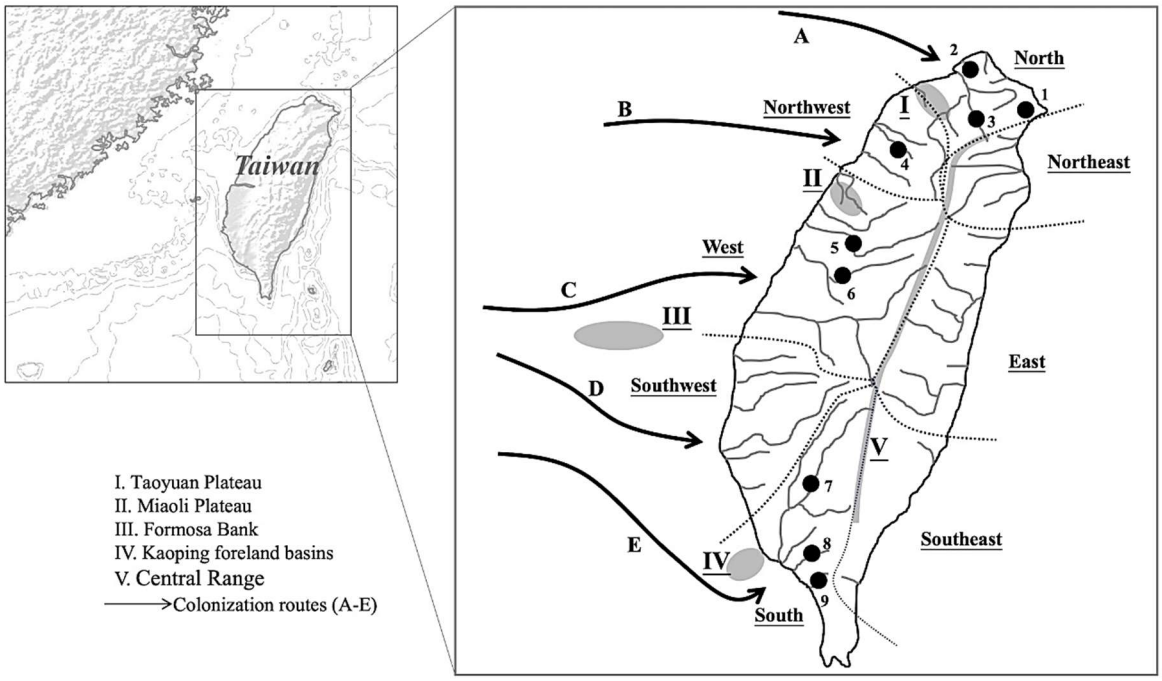


Fig. 1. The sampling locations of the ***Rhinogobius rubromaculatus*** and ***R. yangminshanensis*** are indicated by •. Possible colonization routes are displayed by arrows, as reported by Liao *et al.* (2008), Chiang *et al.* (2013) and Ju *et al.* (2018).

estimated using the Bayesian information criterion (BIC) in jmodelTest 2.0 (Darriba *et al.,* 2012). The phylogenetic analysis was performed using a maximum likelihood (ML) estimation with MEGA-X. Bootstrapping was performed with 1000 replications. The p-distance values implemented by MEGA-X were used to examine the genetic variations within and between species.

Protein-coding genes (PCGs), tRNA-coding genes and ribosome-coding genes (rRNA) were identified by BLAST compared with the corresponding complete mitogenome sequences of the genus *Rhinogobius*. Base composition was determined using MEGA-X. The AT skewing and GC skewing of the nucleotide composition were measured according to the following formulas: AT skew = (A – T)/(A + T) and GC skew = (G – C)/(G + C) (Perna and Kocher, 1995).

Finally, the Approximate Bayesian Computation (ABC) framework was used to determine the evolutionary history using the software DIYABC ver. 2.0 (Cornuet *et al.,* 2014). Reference table was built using all statistics for 1,000,000 simulated data sets per scenario. We adopt a uniform prior for all scenarios and do not impose any restrictions on the population sizes and coalescent times. Posterior probabilities were compared by logistic regression.

RESULTS

Molecular identification and diversity

The sequences of the mtDNA cyt *b* (1140 bp) from 501 specimens of *Rhinogobius* species were obtained. After morphological identification, NJ tree and BLAST analyses, a total of eleven *Rhinogobius* species were found. The genetic distance (p-distance) between *R. rubromaculatus* and *R. similis* (0.175) was larger than other comparisons, while the genetic distance between *R. candidianus* and *R. henchuenensis* was the smallest (0.027) (Table 2). Divergences within these *Rhinogobius* species ranged from 0.001 (*R. gigas* and *R. henchuenensis*) to 0.022 (*R. rubromaculatus*) (Table 2). The p-distance within *R. rubromaculatus* (0.022) and *R. yangminshanensis* (0.021) were higher than those within other species (Table 2). In addition, this study also downloaded all cyt *b* sequences of all *Rhinogobius* species in Japan, China and Taiwan from NCBI. A total of 990 sequences were obtained, and the results supported the clustering of *R. yangminshanensis* and *R. rubromaculatus* as a monophyletic (Fig. S1).

A total of 12 specimens of *R. yangminshanensis* were found from four populations in the northern and northwestern regions, and 30 specimens of *R. rubromaculatus* were found from five populations in western and southern regions (Table 3; Fig. 1). The total nucleotide diversities within the two species were similar (*R. yangminshanensis* = 2.053×10^{-2}; *R. rubromaculatus* = 2.109×10^{-2}). Within *R. yangminshanensis*, the population DS had the highest haplotype and nucleotide diversities (Hd = 0.933 and $\theta\pi = 0.672 \times 10^{-2}$); and within *R. rubromaculatus*, the populations WU and FG had the highest haplotype diversity (Hd = 1.000), but the population KP had the highest nucleotide diversity ($\theta\pi = 0.839 \times 10^{-2}$) (Table 3). The ML phylogenetic displayed

Table 2. Matrix of the pairwise nucleotide divergences (p-distance) based on mtDNA cyt *b* gene among ***Rhinogobius*** species in Taiwan. The underline indicated the divergence within species.

	RF	RG	RD	RH	RC	RL	RY	RR	RM	RN	RS
***R. formosanus* (RF)**	0.006										
***R. gigas* (RG)**	0.034	0.001									
***R. delicatus* (RD)**	0.044	0.042	0.002								
***R. henchuenensis* (RH)**	0.035	0.032	0.030	0.001							
***R. candidianus* (RC)**	0.031	0.029	0.037	0.027	0.017						
***R. lanyuensis* (RL)**	0.075	0.070	0.080	0.076	0.074	0.005					
***R. yangminshanensis* (RY)**	0.122	0.117	0.123	0.118	0.121	0.112	0.021				
***R. rubromaculatus* (RR)**	0.121	0.114	0.121	0.115	0.120	0.113	0.071	0.022			
***R. maculafasciatus* (RM)**	0.134	0.131	0.139	0.141	0.136	0.134	0.135	0.135	0.010		
***R. nantaiensis* (RN)**	0.143	0.138	0.142	0.144	0.142	0.139	0.139	0.139	0.047	0.005	
***R. similis* (RS)**	0.160	0.154	0.161	0.151	0.158	0.161	0.167	0.175	0.154	0.162	0.007

Table 3. Samples of ***Rhinogobius yangminshanensis*** and ***R. rubromaculatus*** used for analysis, location code and summary statistics, including sample size (*N*), haplotype list, haplotype diversity (Hd) and nucleotide diversity (θπ) based on the mtDNA cytochrome *b* dataset.

River (code)	N	Hd	θπ (x10^{-2})
R. yangminshanensis	**12**	**0.924**	**2.053**
Lineage RY1	9	0.000	0.000
1. Shuangsi (SS)	1	-	-
2. Datun (DT)	2	0.000	0.000
3. Danshuei (DS)	6	0.933	0.672
Lineage RY2	3	0.000	0.000
4. Touqian (TC)	3	0.000	0.000
R. rubromaculatus	**30**	**0.922**	**2.109**
Lineage RRa	6	0.933	0.308
5. Tajia (TJ)	3	0.667	0.252
6. Wu (WU)	3	1.000	0.357
Lineage RRb	24	0.880	0.661
7. Kaoping (KP)	12	0.621	0.839
8. Linben (LB)	10	0.844	0.323
9. Fongkan (FG)	2	1.000	0.089

Table 4. Nucleotide compositions of the lineages of ***Rhinogobius yangminshanesis*** (RY1 and RY2) and ***R. rubromaculatus*** (RRa and RRb).

		Genome[1]	PCGs[2]	Light tRNAs[3]	Heavy tRNAs[4]	2 rRNA	d-loop
length	RY1	16506	11428	1066	489	2643	835
	RY2	16497	11425	1066	490	2636	845
	RRa	16496	11428	1067	488	2635	843
	RRb	16492	11430	1065	489	2630	843
AT%	RY1	52.7	51.8	53.2	50.7	53.6	58.7
	RY2	52.9	51.7	53.5	52.5	53.8	59.5
	RRa	53.4	52.9	54.0	51.9	53.0	59.3
	RRb	52.2	51.0	53.9	51.6	52.8	58.6
AT skew	RY1	0.0215	-0.0252	0.1252	0.1223	0.2195	-0.0068
	RY2	0.0170	-0.0327	0.1248	0.1455	0.2279	-0.0185
	RRa	0.0343	-0.0008	0.1307	0.1407	0.2156	-0.0185
	RRb	0.0153	-0.0312	0.1138	0.1588	0.2173	-0.0444
GC skew	RY1	-0.2849	-0.3652	-0.1131	-0.1847	-0.1178	-0.1548
	RY2	-0.2806	-0.3639	-0.1199	-0.1990	-0.1090	-0.1407
	RRa	-0.3093	-0.1047	-0.1196	-0.1943	-0.1174	-0.1319
	RRb	-0.2845	-0.3655	-0.1076	-0.1983	-0.1165	-0.1111

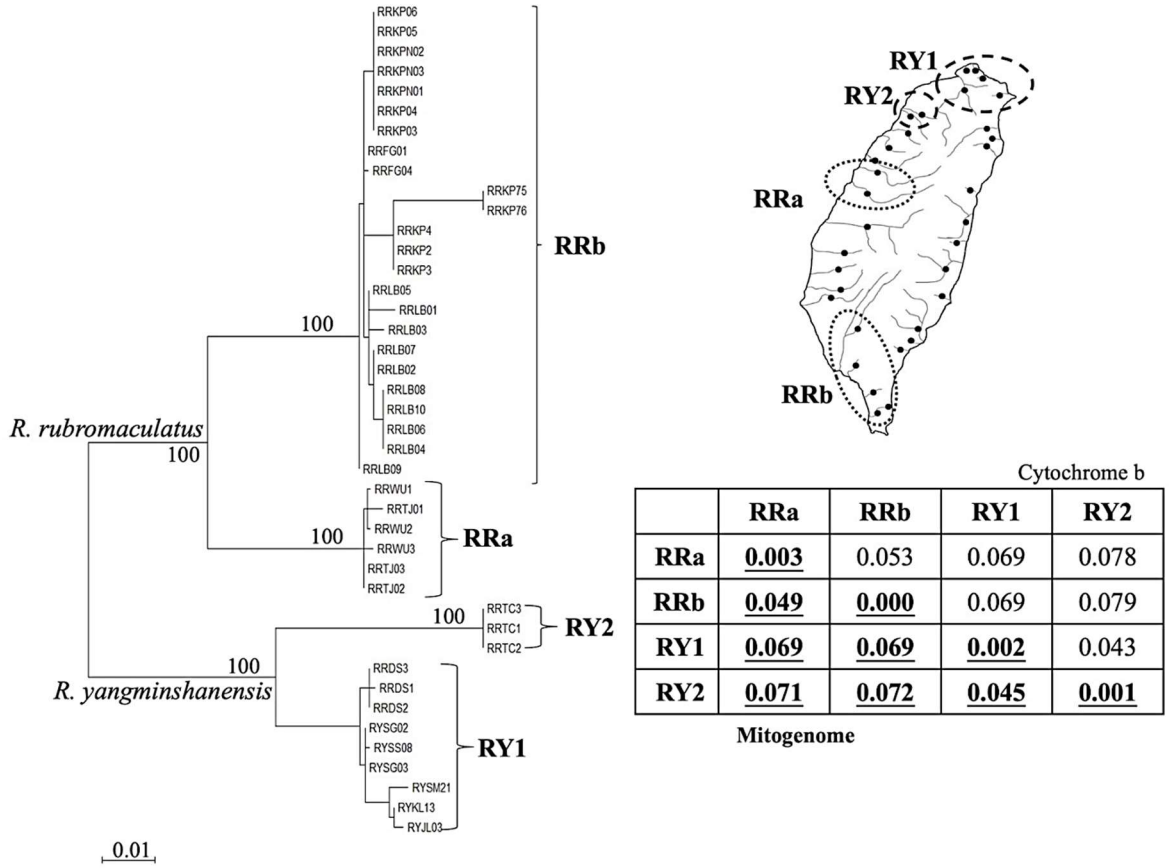
AT% = [A+T]/[A+T+G+C], AT skew = [A-T]/[A+T], GC skew = [G-C]/[G+C].
[1] whole genome. [2] PCGs are protein-coding genes. [3] Light tRNAs are those transcribed from the heavy strand mitochondrial DNA, including Phe, Val, Leu, Ile, Met, Trp, Asp, Lys, Gly, Arg, His, Ser, Leu, Thr, Pro. [4] Heavy tRNAs are those transcribed from the light strand, including Gln, Ala, Asn, Cys, Tyr, Ser, Glu.

that all *R. yangminshanensis* divided into two lineages (RY1 and RY2), and all *R. rubromaculatus* divided into two lineages (RRa and RRb) (Fig. 2). These lineages were all distributed allopatrically. The p-distance between these lineages ranged from 0.043 (lineages RY1 and RY2) to 0.079 (RY2 and RRb) (Fig. 2).

Mitogenomic characteristics

To obtain more genetic information, the complete mitogenome sequences of these four lineages (RY1, three specimens from population DS, RY2, three specimens from population TC, RRa, three specimens from population WU and RRb, three species in population KP) were sequenced. The lengths of the mitogenomes within lineages were identical. The total lengths of the complete mitogenomes of these four lineages ranged from 16492 bp (lineage RRb) to 16506 bp (lineage RY1) (Table 4). The ML tree reconstructed based on complete mitogenomes displayed the same topology as that based on cyt *b* data (data not shown). The p-distance between these lineages ranged from 0.045 (lineages RY1 and RY2) to 0.072 (RY2 and RRb) (Fig. 2).

The mitogenomes of these four lineages all contained 37 mitochondrial genes (13 PCGs, 22 tRNA-coding genes and 2 rRNA-coding genes) and two noncoding regions (OL and d-loop, control region) (Table S1). ND5 and ATP8 were the largest and smallest genes, respectively (Table S1). One of the PCGs (ND6), eight tRNA-coding genes (Gln, Ala, Asn, Cys, Tyr, Ser, Glu, Pro), and one noncoding region (OL) are encoded on the L-strand, and the other 28 genes (12 PCGs, 14 tRNA-coding genes, and 2 rRNA-coding genes) and d-loop are encoded on the H-strand (Table S1).



Cytochrome b

	RRa	RRb	RY1	RY2
RRa	**0.003**	0.053	0.069	0.078
RRb	**0.049**	**0.000**	0.069	0.079
RY1	**0.069**	**0.069**	**0.002**	0.043
RY2	**0.071**	**0.072**	**0.045**	**0.001**

Mitogenome

Fig. 2. The maximum likelihood tree of the mtDNA cytochrome *b* sequences of ***Rhinogobius rubromaculatus*** and ***R. yangminshanensis*** in Taiwan. The number at the nodes are bootstrap values. The most appropriate nucleotide substitution model was TrN (Tamura-Nei). The p-distances between haplogroups were estimated based on the cytochrome b gene (above diagonal) and the complete mitogenome (below diagonal).

The total length of PCGs in these four lineages ranged from 11425 bp (lineage RY2) to 11430 (lineage RRb), accounting for 69.24%–69.30% of the entire mitogenome (Table 4). The average AT nucleotide content of these complete mitogenomes were similar (52.7% in lineage RY1, 52.9% in lineage RY2, 53.4% in lineage RRa and 52.2% in lineage RRb; Table 4). All genes had high A + T content: 51.0%–52.9% for PCGs, 53.2%–54.0% for light tRNA genes, 50.7%–52.5% for heavy tRNA genes, 52.8%–53.8% for rRNA genes, and 58.6%–59.5% for d-loop. The overall AT skews in these entire mitogenomes were 0.0215 (lineage RY1), 0.0170 (lineage RY2), 0.0343 (lineage RRa) and 0.0153 (lineage RRb), and the overall GC skews were -0.2849 (lineage RY1), -0.2806 (lineage RY2), -0.3093 (lineage RRa) and -0.2845 (lineage RRb) (Table 4). The AT skew and GC skew values of PCGs in these four lineages were negative, indicating that the bases T and C were more abundant than A and G (Table 4). Among PCGs, three genes [COII, ATP8 and ND5 (only in lineage RRa)] had positive AT skew values, and the remaining genes had negative AT skew values; and the GC skew values of all genes were negative except ND6 (Table S2).

Colonization history

To better understand the relationships among these four lineages (RY1, RY2, RRa and RRb), six historical scenarios were proposed and examined using the program DIYABC. In the first scenario (scenario I), the four lineages arrived in Taiwan via four divergent colonization routes (Fig. 3A). Under scenario II, lineages RY1 and RY2 originated from the same colonization route, while the other lineages colonized from two other routes (Fig. 3B). In scenario III, lineage RRa and RRb originated from the same colonization route, and the other lineages colonized from the other two routes (Fig. 3C). Scenario IV: Based on the phylogenetic analysis (Fig. 2), these four clades arrived in Taiwan via two different colonization routes (Fig. 3D). Under scenario V, these four clades colonized Taiwan through a single colonization route and then became distinct species (Fig. 3E). Finally, scenario VI showed that the species "*R. rubromaculatus*" (lineages RY2, RRa and RRb) colonized via one route, while "*R. yangminshanensis*" (lineage RY1) colonized via another route (Fig. 3F).

All four data sets (cyt *b*, complete mitogenome, rRNAs and PCGs) supported scenario I with the highest posterior probability, which was much higher than other

Table 5. Relative posterior probabilities for each scenario (Fig. 3) and their 95 % confidence intervals (lower-upper) based on the logistic estimate by DIYABC.

	Scenario I	Scenario II	Scenario III	Scenario IV	Scenario V	Scenario VI
Cytochrome b	0.9982 (0.9972-0.9992)	0.0010 (0.0003-0.0017)	0.0008 (0.0003-0.0014)	0.0000 (0.0000-0.0000)	0.0000 (0.0000-0.0000)	0.0000 (0.0000-0.0000)
mitogenome	0.9996 (0.9992-0.9999)	0.0001 (0.0000-0.0002)	0.0003 (0.0000-0.0007)	0.0000 (0.0000-0.0000)	0.0000 (0.0000-0.0000)	0.0000 (0.0000-0.0000)
rRNAs	0.9986 (0.9981-0.9991)	0.0006 (0.0003-0.0009)	0.0008 (0.0004-0.0011)	0.0000 (0.0000-0.0000)	0.0000 (0.0000-0.0000)	0.0000 (0.0000-0.0000)
PCGs	1.0000 (1.0000-1.0000)	0.0000 (0.0000-0.0000)	0.0000 (0.0000-0.0000)	0.0000 (0.0000-0.0000)	0.0000 (0.0000-0.0000)	0.0000 (0.0000-0.0000)

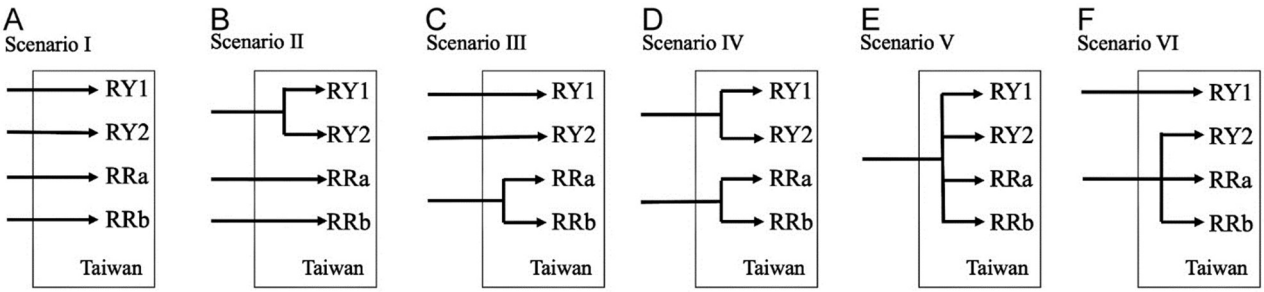


Fig. 3. The six colonization scenarios tested in DIYABC. **A**: Scenario I: Four lineages (RY1, RY2, RRa and RRb) colonized via four independent routes. **B**: Scenario II: Lineages RY1 and RY2 colonized via a single route, and the other two lineages colonized via two independent routes. **C**: Scenario III: Lineages RY1 and RY2 colonized via two independent routes, while the other lineages colonized via one single route. **D**: Scenario IV: Lineages RY1 and RY2 colonized via one route and lineages RRa and RRb colonized via another route. **E**: Scenario V: Four lineages colonized via one route. **F**: Scenario VI: Lineage RY1 colonized from one route and others colonized via another route.

scenarios (cyt *b*: 0.9982, 95% CI: 0.9972–0.9992; mitogenome: 0.9996, 95% CI: 0.9992–0.9999; rRNAs: 0.9986, 95% CI: 0.9981–0.9991; PCGs: 1.0000, 95% CI: 1.0000–1.0000), and the 95% CI of scenario I did not overlap with the 95% CI of other scenarios (Table 5). Therefore, the four lineages may have colonized Taiwan from mainland China through four different routes.

DISCUSSION

Mitogenomic features of *R. rubromaculatus*

The mitogenomes of *R. rubromaculatus* species complex encode 37 typical mitochondrial genes (13 protein coding genes, 2 ribosomal RNA-coding genes, and 22 transfer RNA-coding genes) and two typical noncoding control regions, the d-loop and origin of the light strand (OL) (Table S1). Overall, the mitogenomes of *R. rubromaculatus* species complex from these four lineages in the present study have the same gene order and composition as other *Rhinogobius* mitogenomes in previous studies (Wang *et al.,* 2019; Maeda *et al.,* 2021; Hu *et al.,* 2023). The gene order of the mitogenomes of *Rhinogobius* is similar to that of most teleost, although the loss of the ND6 gene in the mitogenomes of Antarctic fish (Papetti *et al.,* 2007), the loss of $tRNA^{pro}$ in genus *Trichiurus* (Yi *et al.,* 2022), and different types of the rearrangements in Pleuronectiformes (Gong *et al.,* 2015) and Stomiiformes (Arrondo *et al.,* 2020) have been observed.

Cryptic diversity of *R. rubromaculatus*

The taxonomy of the genus *Rhinogobius* remains unresolved. Some species do not currently have a valid name such as *Rhinogobius* sp. MO and *Rhinogobius* sp. YB (Yamasaki *et al.,* 2015). The species diversity of the genus *Rhinogobius* is also unfathomable. Until recently, new species such as *R. tandikan* (Maeda *et al.,* 2021) and *R. lianchengensis* (Wang and Chen, 2022) have been proposed. Chen *et al.* (2022) described a new species, *R. yangminshanensis*, from northern Taiwan. However, this study found that *R. yangminshanensis* also exists in northwestern Taiwan based on the morphological characteristics and DNA barcode analyses (Figs 1 & S1; Table 3). Further analyses revealed that the populations in northern region (populations SS, DT and DS) and that in northwestern region (population TC) divided into different lineages, RY1 and RY2, respectively (Fig. 2). The p-distance based on the cyt *b* dataset between these two lineages was 0.043 (Fig. 2). However, the p-distance between *Rhinogobius* species in Taiwan based on the cyt *b* dataset ranged from 0.027 (between *R. henchuenensis* and *R. candidianus*) to 0.175 (between *R. rubromaculatus* and *R. similis*) (Table 2). Moreover, the genetic distance between RY1 and RY2 based on complete mitogenomes was 0.045 (Fig. 2). Li *et al.* (2012) proposed that the genetic distances between mitogenome sequences of *Hemibarbus maculatus* and *H. labeo* and between *H. maculatus* and *H. barbus* were 0.021 and 0.024, respectively. Accordingly, the highly divergent mtDNA

sequences revealed the existence of cryptic species within the morphospecies *R. yangminshanensis.* Chen *et al.* (2022) described the holotype and paratypes of *R. yangminshanensis* from northern Taiwan. Accordingly, this study identified lineage RY1 as *R. yangminshanensis* and lineage RY2 as *Rhinogobius* sp. RY2. More information is needed to provide a valid taxonomic name for *Rhinogobius* sp. RY2 in future studies.

Likewise, Chen *et al.* (2022) suggested that *R. rubromaculatus* might exist cryptic species in the different basins from western Taiwan. This study also found that the *R. rubromaculatus* divided into two clades (RRa and RRb, Fig. 2). Based on the cyt *b* and mitogenome datasets, the p-distance between these two lineages were 0.053 and 0.049, respectively (Fig. 2). These results suggest the existence of cryptic species within morphospecies *R. rubromaculatus.* Lee and Chang (1996) described the holotype and paratypes of *R. rubromaculatus* from Tadu River and Tsoshui River, respectively. Both rivers are located in western Taiwan. Therefore, this study identified these two lineages as *R. rubromaculatus* (RRa), and *Rhinogobius* sp. RRb. More information is needed to provide a valid taxonomic name for *Rhinogobius* sp. RRb in future studies.

Colonization history of *R. rubromaculatus*

Previous studies have revealed that the distribution patterns and population structures of freshwater species in Taiwan are shaped by the topography of this island, and five phylogeographic breaks have been discussed (I-V; Fig. 1) (Han *et al.,* 2019; Kang *et al.,* 2022; Ju *et al.,* 2024). Taoyuan Plateau restricted the distribution range of *Sinibrama macrops*, and shaped the population structure of *R. candidianus* (Kang *et al.,* 2022); Miaolo Plateau restricted the distribution range of *M. alticorpus* and *M. brevirostris*, and shaped the population structure of *C. barbata* (Ju *et al.,* 2024); and Kaoping foreland basins restricted the distribution range of *Onychostoma alticorpus* and *R. candidianus*, and shaped the population structure of *Cobitis sinensis* (Chiang *et al.,* 2013). Previous studies have also proposed that the freshwater species colonized Taiwan through multiple routes (A-E; Fig. 1) (Liao *et al.,* 2008; Chiang *et al.,* 2013; Lin *et al.,* 2016; Ju *et al.,* 2018). For example, Chiang *et al.* (2010, 2013) reported that *C. sinensis* colonized Taiwan island via routes B, D and E.

According to the results of morphological characteristics, phylogenetic analysis and genetic distances, this study identified these four lineages in *R. rubromaculatus* species complex into two species groups, *R. yangminshanensis* group, including *R. yangminshanensis* and *Rhinogobius* sp. RY2, and *R. rubromaculatus* group, including *R. rubromaculatus* and *Rhinogobius* sp. RRb. The distribution patterns of these two species groups are similar to those of *M. alticorpus* and *M. brevirostris* (Chang *et al.,* 2016) and clades I and II of *Acrossocheilus paradoxus* (Ju *et al.,* 2018). These species were isolated by the Miaoli Plateau (phylogeographic break II; Figs 1, 2). Chang *et al.* (2016) found that these two *Microphysogobio* species originated from different continental populations through different routes during different glacial periods, and Ju *et al.,* (2018) also suggested that these two clades of *A. paradoxus* colonized Taiwan through different routes. The DIYABC analyses results also supported that *R. yangminshanensis* group and *R. rubromaculatus* group colonized Taiwan via different routes, e.g., *Microphysogobio* species and *A. paradoxus* (Fig. 3; Table 5). Although the uplift of the Miaoli Plateau did not lead to the speciation of the *R. yangminshanensis* group and *R. rubromaculatus* group, it restricted their dispersal as other freshwater fishes.

Within *R. yangminshanensis* group, *R. yangminshanensis* and *Rhinogobius* sp. RY2 were separated by the Taoyuan Plateau (phylogeographic break I; Figs 1, 2). This study found that the Taoyuan Plateau restricted the distribution areas of some freshwater fishes such as *Squalidus argentatus*, *Sinibrama macrops*, and *H. labeo*; and shaped population structures of some freshwater fishes such as *M. brevirostris* (Chang *et al.,* 2016), *R. candidianus* (Kang *et al.*, 2022), and *C. barbata* (Ju *et al.,* 2024). The DIYABC results revealed that *R. yangminshanensis* and *Rhinogobius* sp. RY2 colonized Taiwan via different routes (Fig. 3; Table 5). The results did not support that *R. yangminshanensis* and *Rhinogobius* sp. RY2 became distinct species due to the uplift of the Taoyuan Plateau. This study found that the distribution patterns of *S. argentatus* and *S. iijmae* were the same as those of *R. yangminshanensis* and *Rhinogobius* sp. RY2. To understand whether species in the north and south of the Taoyuan Plateau might have originated from different ancestors, the phylogeny of the genus *Squalidus* was analyzed based on the complete mitogenomes downloaded from NCBI. The results displayed that *S. argentatus* and *S. iijmae* did not form a monophyletic group (Fig. S2). The results suggest that *S. argentatus* and *S. iijmae* did not originate from the same ancestral populations in mainland China. Accordingly, it is possible that *R. yangminshanensis* and *Rhinogobius* sp. RY2 might originate from different origins, and the results of the DIYABC analyses also support this hypothesis (Fig. 3; Table 5).

In the southeastern Taiwan Strait, the Kaoping foreland basins (phylogeographic break IV, Fig. 1) was formed in 2–3 mya, and the depth is up to 200 m within 3 km of the shoreline (Boggs *et al.,* 1979; Chen *et al.,* 1999). Wang *et al.* (1999, 2011) reported that this sea trench interrupted the extension of the Kaoping River toward the land bridge during ice ages. Even during ice ages, freshwater species in south of Kaoping River could not migrate northward cross the Kaoping foreland basins to reach the land bridge. Many phylogeographic studies of

the freshwater fishes have displayed that the Kaoping foreland basins did block migration between the north and south of Kaoping River indeed [*O. pachycephalus* (Wang *et al.,* 1999); *Spinibarbus hollandi* and *O. alticorpus* (Chiang *et al.,* 2017); *R. candidianus* (Kang *et al.,* 2022); *C. barbata* (Ju *et al.,* 2024)]. Actually, the ichthyofauna of the freshwater fishes in the north and south of the Kaoping River were different (Chiang *et al.,* 2017; Kang *et al.,* 2022; Ju *et al.,* 2024). Although the *C. sinensis* was distributed in both north and south of the Kaoping River, the populations on both sides originated from different continental origins and through different colonization routes (Chiang *et al.,* 2010, 2013). Therefore, it is possible that the *R. rubromaculatus* and *Rhinogobius* sp. RRb originated from different origins and colonized through different routes.

Ancestor of *R. rubromaculatus* in mainland China

The DIYABC analyses supported that the four species of *R. rubromaculatus* species complex colonized Taiwan via four different colonization routes from mainland China (Fig. 3 and Table 5). Previous studies (Chiang *et al.,* 2013; Chang *et al.,* 2016; Ju *et al.,* 2018) and the phylogeny of the genus *Squalidus* (Fig. S2) support the possibility that these four species originated from different sources. Our study suggests that they may have originated from a single species, the same species but with population differentiation, or a group of sister species. Even if they originated from a single species, the populations migrated along different routes and then diverged.

However, although we have downloaded all available sequences from NCBI, we did not find any possible ancestral lineage from mainland China (Fig. S1). Previous studies have reported that the species pools of Taiwan are most likely located in Zhejiang and Fujian Provinces (e.g., Chiang *et al.,* 2013; Lin *et al.,* 2016, 2023). The dataset from NCBI only includes 13 freshwater *Rhinogobius* species from mainland China, which only 7 species are from Zhejiang and Fujian Provinces. Wang (2022) reported that they found 10 freshwater *Rhinogobius* species from Fujian Province, including 3 undescribed species, and Li *et al.* (2018) described a new species from the Qiantang River, Zhejiang Province. This study found that except for *R. changitinensis*, the mtDNA cyt *b* gene information of above 10 species had not been uploaded to NCBI. This study suggests that there are still some undescribed species in mainland China, and the genetic information of some species has not been examined and uploaded to NCBI. Therefore, this study cannot find any possible ancestral lineage from mainland China. However, rapid urban expansion has led to loss of natural habitat in some areas. Therefore, our results provide important resources for the further researches.

CONCLUSION

This study confirmed four allopatrically distributed species within *R. rubromaculatus* species complex, *R. yangminshanensis, Rhinogobius* sp. RY2, *R. rubromaculatus* and *Rhinogobius* sp. RRb (Fig. 2). The results of the DIYABC analyses and the phylogeographic patterns of other freshwater fishes supported that they colonized Taiwan via different routes. This study suggests that the four species diverged before they colonized Taiwan, although their ancestors have yet to be discovered. The complex geologic history of Taiwan island has shaped their colonization history and distribution patterns. In the future, their morphological characteristics are needed to give them valid taxonomic names. Our current results can provide more information on the diversity of *Rhinogobius*. Future studies should collect more species in mainland China to examine their phylogenetic relationships and evolutionary history.

The nucleotide diversity of *R. yangminshanensis*, *Rhinogobius* sp. RY2, *R. rubromaculatus* and *Rhinogobius* sp. RRb ($\theta\pi$ = 0.005, 0.000, 0.003 and 0.007, respectively; Table 3) were lower than those of some freshwater fish in Taiwan (0.009 for *A. kikuckii* see Lin *et al.,* 2008; 0.009 for *M. brevirostris* see Chang *et al.,* 2016; 0.016 for *R. candidianus* see Kang *et al.,* 2022). Thus, this study suggests that the existence of low levels of genetic diversity support the need to development management strategies for these four species. The results of this research are of great value for future assessments of the conservation and resource management of *R. rubromaculatus* species complex.

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