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Morphological and Molecular Variation in *Rhinogobius rubromaculatus* (Pisces: Gobiidae) in Taiwan

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Hui-Ling Cheng, Shong Huang, and Sin-Che Lee (2005) Morphological and molecular variation in *Rhinogobius rubromaculatus* (Pisces: Gobiidae) in Taiwan. *Zoological Studies* **44**(1): 119-129. A new color type was discovered in the Linbian River of southern Taiwan while investigating morphological and genetic variations of *Rhinogobius rubromaculatus*. The technical approaches we used included allozyme starch gel electrophoresis and partial mitochondrial DNA sequence analysis. Specimens from the Linbian River were characterized by smaller body and egg sizes, and indicated a significant negative correlation with temperature. None of the alleles was fixed among the new color type and *R. rubromaculatus* populations, with an allozyme genetic distance of from 0.001 to 0.078, which greatly differed from those of *R. candidianus* (0.523~0.687) and 2 other undetermined *Rhinogobius* species from the Ryukyus, Japan (0.326~0.464). The complete sequences of the cytochrome b gene, tRNA genes, and the control region of mtDNA revealed substitution differences within *R. rubromaculatus* at 207 base pairs (bp) and among the 3 species at 363 bp. The sequence diversity within the population was from 0.000 to 0.004. Among local populations of *R. rubromaculatus*, the sequence mean distance diversities were from 0.005 to 0.074, which is smaller than that between *R. rubromaculatus* and another *Rhinogobius species* (0.094~0.113). Both markers were congruent in revealing significant differences between samples of *R. rubromaculatus* (global F_{ST} = 0.404 for the allozyme and Φ_{ST} = 0.986 for the mitochondrial DNA data). The molecular trees of *R. rubromaculatus* constructed based on both allozyme and mtDNA data revealed a closer relationship between central and southern populations than with a northern population. Discrete phenotypes could have been produced by phenotypic plasticity.
http://www.sinica.edu.tw/zool/zoolstud/44.1/119.pdf

Key words: Allozyme, Mitochondrial DNA, Population differentiation, *Rhinogobius rubromaculatus*.

The fluviatile goby, *Rhinogobius rubromaculatus*, from central Taiwan was first nominated in 1996 based on both morphometric and allozymic variations (Lee and Chang 1996). This species is an endemic freshwater fish distributed in tributaries of rivers throughout Taiwan at low and mid-elevations ranging from sea level to 1200 m except on the eastern side of the Central Mountain Range (Chen and Shao, 1996). The fish is generally pale brown with dusky transverse bands, and numerous red spots scattered over the entire body except the anal fin which is uniformly dark brownish. It also possesses the largest egg, higher vertebrae numbers, a shorter snout, and fewer pectoral rays among the Taiwanese members of the *Rhinogobius* genus. Nevertheless, little is known about its population structure.

During the course of field trips to extensively collect freshwater gobies throughout the island, a surprising new color type, which externally resembles *R. candidianus* and *R. rubromaculatus*, was obtained from a small river, the Linbian, in southwestern Taiwan. The body size of the Linbian form is the smallest among the *Rhinogobius* species in Taiwan. No other form has red spots scattered over the body. It also differs from 2 fluviatile species, *Rhinogobius* sp. 1 (yellow belly medium-egg type) and *Rhinogobius* sp. 2 (blue

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belly medium-egg type) (Kawanabe and Mizuno 1989), of the Ryukyu Is., Japan. Chen et al. (1999) described a new species with a higher vertebral count, *R. xianshuiensis*, from Fujian Province, China. In a comparison with other species of *Rhinogobius* using morphological characters with higher vertebrae numbers, *R. xianshuiensis* is most similar to *R. rubromaculatus* (Chen et al. 1999). We surveyed morphometric characters, the cephalic sensory system, and coloration of samples from the Linbian River, and found that these could not be categorized into any of the known species.

The *Rhinogobius brunneus* species complex is extremely variable in color and is thought to consist of many morphs (Akihito et al. 1984, Kawanabe and Mizuno 1989, Chen and Shao 1996). Some authors have shown that some of the color morphs are genetically differentiated from each other on the basis of allozyme data, egg size, and clutch size (Masuda et al. 1989, Katoh and Nishida 1994, Kon and Yoshino 2003). Color polymorphism is an intriguing evolutionary phenomenon. Gene flow may promote adaptations by making genetic nobilities available throughout the species range, but can also restrict differentiation among local populations. In some species and populations of fish, resource polymorphism is primarily due to genetic polymorphism (Hori 1993), but in some others species, morphs may result primarily from phenotypic plasticity (Meyer 1987, Robinson and Wilson 1996).

As an isolated island, Taiwan lies on the western fringe of the Pacific Ocean and is under the influence of subtropical weather regimes. The island is ~90 km from Fujian Province, China on the Asian continental land mass across the Taiwan Strait, and is situated between the East China Sea to the northeast and the South China Sea in the southwest, resulting in a unique freshwater fish fauna on the island. The study of the freshwater fishes in Taiwan is considered to have tremendous value for confirming the relationships between the island and adjacent areas. According to species distributions, some authors found that the freshwater fish fauna of western Taiwan can be divided into 2 main groups: (i) the Tsengwen and Kaoping Rivers of southwestern Taiwan and (ii) all of the rivers located on the western side of the Central Mountain Range but north of the Choshui River (Oshima 1919, Tzeng 1986). Such a hypothesis has been demonstrated in the endemic minnow, *Acrossocheilus paradoxus* (Wang et al. 2000), and *Varicorhinus barbatulus* (Wang et al. 2004), but this was not borne out by another endemic minnow, *Zacco pachycephalus* (Wang et al. 1999).

The aims of the present study were to make morphological comparisons among the Linbian form and another 4 *Rhinogobius* species. We attempted to confirm their reproductive isolation by examining bases by allozyme analysis and using mtDNA as markers to estimate the genetic structure, in order to infer if the resource polymorphism was primarily due to genetic polymorphism or phenotypic plasticity, and to reconstruct the phylogeographical patterns of *R. rubromaculatus*.

MATERIALS AND METHODS

In total, 193 individuals used in this study were collected from 7 sites: 5 in Taiwan and 2 in the Ryukyu Is., Japan (Fig. 1). Details of sampling localities and numbers of specimens for allozyme and mtDNA analyses are given in table 1.

Morphology

The specimens used in the present study are described in the following section. Descriptions of

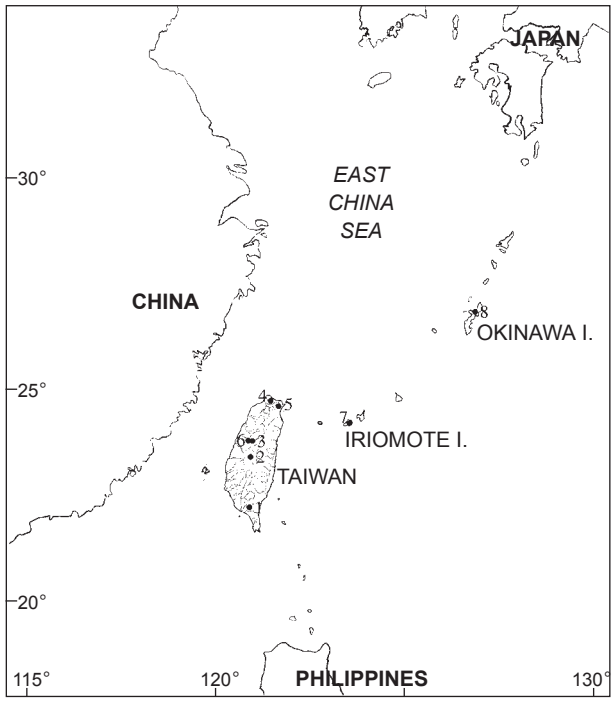

Fig. 1. Collection localities of the 4 *Rhinogobius* species in this study. 1. Linbian River; 2. Chingshui River; 3. Lileng River; 4. Masu River; 5. Pinglin River; 6. Dachia River; 7. Urauchigawa River; 8. Genka River.

coloration patterns were all based on fresh specimens. All counts and measurements were made from specimens preserved in 70% ethanol. Counting methods followed Lee and Chang (1996). The measurements and terminology of the cephalic sensory system followed Akihito et al. (1984). Fin rays (except the pectoral fin) and vertebrae were counted using x-ray radiography. Mature females with a gonadosomatic index (GSI) of larger than 12.0 were used for analysis of egg size. Ovaries were removed, weighed, and preserved in 70% ethanol. Diameters were measured for 20 eggs randomly selected from the ovaries of a female.

Allozyme electrophoresis

Specimens collected were stored at -70°C until the experiments. Protein extracts were prepared from skeletal muscle, eyes, and liver tissue. The kinds of enzymes, buffer systems, and tissues used were modified from those of Lee and Chang (1996) and are shown in table 2. Staining techniques followed Shaklee and Keenan (1986) and Pasteur et al. (1988). Locus nomenclature followed Shaklee et al. (1990).

The BIOSYS-1 program (Swofford and Selander 1989) was adopted to estimate genetic variability (heterozygosity (*H*), percent of polymorphic loci under the 0.95 criterion (P_{95}), and *F*-statistics), quantify divergence among and within collecting sites, test the conformance with Hardy-Weinberg equilibrium expectations, perform hierarchical analysis of the population differentiation (Wright, 1978), and calculate the genetic similarity (I) and genetic distance (D) (Nei 1978). A phylogenetic tree among relevant samples was constructed using the Neighbor-joining method (Saitou and Nei 1987) in the PHYLIP software package (Felsenstein 1995).

Mitochondrial DNA (mtDNA)

For the mtDNA studies, specimens of each species were randomly chosen from those used in the allozyme analysis. Crude DNA was extracted from 0.05 g of skeletal muscle tissues by the GENTRA genomic DNA purification kit (Gentra, Minneapolis, MN, USA). A fragment of approximately 2124 bp of mitochondrial DNA, including the complete cytochrome b gene, 2 tRNA genes, and the D-loop region was amplified. Primers CY1 (5'-YYTAACCRRGACYAATGACTTGA-3') and D2 (5'-CCGGAGTATGTAGGGCATTCTCAC-3') were designed. Each 100-(l PCR reaction included 10 ng of template DNA, 20 pmol of each primer (CY1 and D2), 8 µL dNTP (2.5 mM each), 10 µL 10X buffer, and 2.5 U *Taq* polymerase (Takara, Shiga, Japan). The amplification conditions were as follows: 35 cycles of 94°C for 1 min for denaturation, 50°C for 1 min for annealing, and 72°C for 105 s for elongation; this was followed by a 72°C extension for 10 min and finally storage at 4°C. PCR products were purified using Viogene spin columns (Viogene, Taipei, Taiwan). The amplified DNA was directly sequenced on an automated DNA sequencer (ABI PRISM 377; Applied Biosystem/Perkin Elmer, Boston, MA, USA) using a fluorescence dye terminator cycle sequencing kit (Bigdye, Boston, MA, USA).

Sequences were aligned using published sequences of *R. giurinus* and *R. maculafasciatus* from GenBank with Lasergene software (DNASTAR, Madison, WI, USA) and the BioEdit program

Table 1. Sampling sites of 4 members of the *Rhinogobius* species complex used in this study including the numbers of fish screened using allozyme electrophoresis (AE) and mtDNA analyses, respectively

Species	Locality			Code	AE	mtDNA
		River system	Local Branch			
Rhinogobius rubromaculatus	1. Pingtung, Taiwan	Linbian R.		LB	19	8
	2. Nantou, Taiwan	Choshui R.	Chingshui R.	CS	24	8
	3. Taichung, Taiwan	Dachia R.	Lileng R.	LL	24	8
	4. Taipei, Taiwan	Masu R.		MS	24	7
	5. Taipei, Taiwan	Shuangshi R.	Pinglin R.	PL	20	8
Rhinogobius candidianus	6. Taichung, Taiwan	Dachia R.		DC	30	7
Rhinogobius sp. 1 (yellow belly)	7. Iriomote I., Japan	Urauchigawa R.		YB	30	8
Rhinogobius sp. 2 (blue belly)	8. Okinawa I., Japan	Genka R.		BB	22	8

(vers. 5.0.9) (Hall 1999). Haplotype diversity, nucleotide diversity, pair-wise genetic distances, and Tajima's D statistic (Tajima 1989a) were computed using the program ARLEQUIN 2.0 (Schneider et al. 2000) and were tested for significance with the permutation test using 1000 replicates. Subsequently, samples were grouped and subjected to a hierarchical analysis of variance (AMOVA) (Exoffier et al. 1992) in ARLEQUIN to ascertain whether the observed group structure was significant. Φ_{ST} values from the pairwise analyses were estimated as measures of population differentiation, also computed using ARLEQUIN. A phylogenetic tree was constructed using the Neighbor-joining (NJ) method with 1000 bootstraps in the PHYLIP package (Felsenstein 1995).

RESULTS

Morphology

There is a dark blotch on the 1st dorsal fin of the new color type, *Rhinogobius* sp., caught from the Linbian River that is distinct within the members of the *R. brunneus* complex. The color pattern of *R. rubromaculatus* in northern and central Taiwan is similar to the blue belly type found in the Ryukyus of Japan (Fig. 2). Sensory papillae numerous with patterns resemble those of most species of the *R. brunneus* species complex. The standard length of mature females (range, 28.8~38.5 mm; mean, 32.4 mm) and egg size (mean, 1.32 ± 0.05 mm) in the Linbian River were significantly smaller than those of *R. rubromaculatus* from 4 other rivers ($p < 0.001$), and these showed significantly negative correlations with temperature ($R^2 = 0.806$) (Fig. 3). The head/body length ratio ranged from 25.3% to 30.8% (average, 28.4%) between *R. candidianus* (27.3%) and *R. rubromaculatus* (30.2%~30.9%). The snout/head ratio was from 20.2% to 30.4% (average, 27.4%) between *R. rubromaculatus* (23.8%~24.3%) and *R. candidianus* (33.3%). The 1st dorsal fin commences at the anterior 1/3 of the pectoral fin, and is moderately high with 6 spines; the 2nd dorsal fin inserts exactly opposite the anus, and has 1 spine and 7~9 rays. The anal fin has 1 spine, and the rays vary from 7 to 8. The number of pectoral fin rays varies from 15 to 19. The ventral fin is in the

Table 2. List of enzyme systems, tissues, and buffers used in the analyses

Enzyme	E.C. no.	Locus	Tissue	Buffer
Aspartate aminotransferase	2.6.1.1	*AAT-1**	muscle	TC8.0
		*AAT-2**	muscle	TC8.0
Adenylate kinase	2.7.4.3	*AK**	muscle	TC8.0
Creatine kinase	2.7.3.2	*CK-A**	muscle	LiOH
		*CK-B**	eye	LiOH
Esterase	3.1.1.-	*EST-1**	liver	LiOH
Fumarase	4.2.1.2	*FUM**	eye	TC7.0
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	*GAPDH**	eye	TC7.0
Glucose-6-phosphate isomerase	5.3.1.9	*GPI-A**	liver	LiOH
		*GPI-B**	muscle	LiOH
Isocitrate dehydrogenase ($NADP^+$)	1.1.1.42	*IDHP-A**	liver	TC8.0
		*IDHP-B**	muscle	TC8.0
Lactate dehydrogenase	1.1.1.27	*LDH-A**	muscle	TC7.0
		*LDH-B**	eye	TC7.0
		*LDH-C**	eye	TC7.0
Malate dehydrogenase	1.1.1.37	*MDH-A**	liver	TC8.0
		*MDH-B**	muscle	TC8.0
Malic enzyme ($NADP^+$)	1.1.1.40	*MEP-1**	muscle	TC8.0
		*MEP-2**	muscle	TC8.0
Mannose-6-phosphate isomerase	5.3.1.8	*MPI**	muscle	LiOH
6-Phosphogluconate dehydrogenase	1.1.1.14	*6-PGDH**	eye	TC7.0
Phosphoglucomutase	2.7.5.1	*PGM-1**	muscle	TVB
		*PGM-2**	muscle	TVB
Sorbitol dehydrogenase	1.1.1.14	*SDH**	muscle	TC8.0
Xanthine dehydrogenase	1.2.1.37	*XDH**	liver	LiOH

disc with the frenum concave posteriorly in the middle and has 1 spine and 5 rays. The caudal fin is rounded with 14~16 branched rays. The vertebrae number varies from 26 to 28. Detailed data are summarized in table 3.

Allozyme electrophoresis

In total, 25 loci with 55 alleles were encoded for 16 enzymes. Among the 25 loci examined, 6 loci were monomorphic including *AAT-2**, *CK-B**, *EST-1**, *GAPDH**, *IDH-A**, and *LDH-C**. The rest had polymorphic allelic frequencies, and these are shown in table 4. The mean numbers of alleles per locus were 1.1 (LB, CS, LL, and BB), 1.2 (MS, DC, and YB), and 1.4 (PL). The percentage of polymorphic loci varied from 8.0% (LB, LL, BB, and YB) to 20% (MS and PL). Estimates of genetic variability were low, with observed heterozygosities ranging from 0.016 (BB) to 0.082 (PL), and expected heterozygosities ranging from 0.021 (BB) to 0.089 (PL) (Table 5). Nei's distance (Table 6) calculated from data in table 4 indicated that the Linbian form is closer to the typical *R. rubromaculatus* (D = 0.028~0.073) than to *R. candidianus* (D = 0.523) or *Rhinogobius* sp. 1 and 2, of the Ryukyus (0.326~0.372).

None of the fixed alleles was observed between the Linbian form and the typical *R. rubromaculatus*. *AAT-1**, *GPI-B**, *IDH-2**, *LDH-B**, *MEP-1**, *MEP-2**, *PGM-2**, *SDH**, and *XDH** were invariant in the population of *R. rubromaculatus*. *F*-statistics were calculated for all specimens of *R. rubromaculatus*, including samples from the Linbian River. F_{ST} was significantly larger than 0 at 6 loci (AK, FUM, GPI-A, LDH-A, MDH-A, and 6-PGDH) with an overall F_{ST} of 0.404 ($p < 0.001$), which indicates evident differentiation between populations. The Chi-square test for the inbreeding index (F_{IS} = -0.007) did not significantly differ for any loci, so that the estimated F_{IT} (0.400) could largely be explained by differentiation among populations.

The genetic distances, D (Nei, 1978), among populations of *R. rubromaculatus* varied in a range of from 0.001 (MS and PL) to 0.078 (CS and MS). The Neighbor-joining tree constructed (Fig. 4) based on Nei's distances showed that populations of northern Taiwan clustered together and were differentiated from populations of west-central and

Fig. 2. (A) *Rhinogobius rubromaculatus* (southern Taiwan), 38.0 mm SL; (B) *R. rubromaculatus* (northern and central Taiwan), 42.3 mm SL; (C) yellow-belly type, 42.1 mm SL; (D) blue-belly type, 43.1 mm SL.

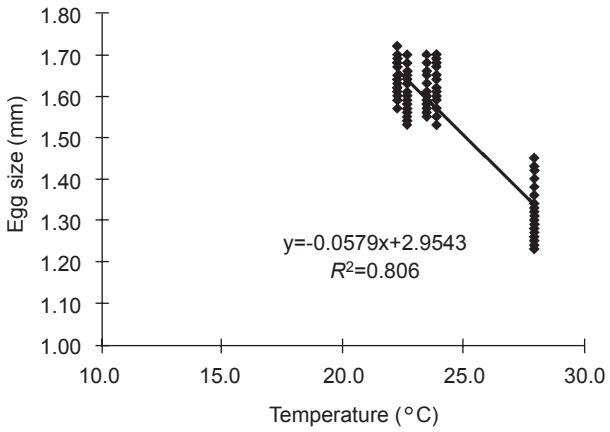

Fig. 3. Plots of regression lines of egg size (Y) on temperature (X) for *Rhinogobius rubromaculatus*.

Table 3. Comparisons of some selected body measurements and meristic counts among 4 *Rhinogobius* species

	R. rubromaculatus				
	Taiwan				
	Linbian R.	Chingshui R.	Lileng R.	Masu R.	Pinglin R.
Body length (mm SL)	28.8~38.5 (32.4)	31.5~4.05 (35.3)	31.0~42.3 (35.7)	31.8~41.3 (36.2)	31.0~40.5 (35.0)
Percent of SL					
Head	25.3%~30.8% (28.4%)	26.8%~33.0% (30.4%)	27.3%~34.2% (30.9%)	26.5%~33.7% (30.5%)	27.0%~33.3% (30.2%)
Body depth	17.1%~21.7% (19.1%)	17.1%~21.5% (19.1%)	17.0%~23.6% (20.8%)	16.8%~22.2% (19.9%)	16.8%~22.9% (20.2%)
Percent of HL					
Snout	20.2%~30.4% (27.1%)	20.8%~26.2% (23.8%)	21.4%~26.9% (24.0%)	20.8%~26.0% (24.3%)	21.1%~26.6% (24.3%)
Orbit	20.5%~26.1% (23.4%)	19.8%~26.7% (22.8%)	20.1%~26.5% (23.4%)	20.4%~26.0% (23.5%)	19.9%~26.4% (23.0%)
Fin ray formulae					
Second dorsal	I, 7~9	I, 7~10	I, 7~10	I, 7~9	I, 7~10
Pectoral	15~19	15~18	15~18	15~18	15~18
Anal	I, 7 or 8	I, 7~9	I, 8 or 9	I, 7~9	I, 7~9
Branched caudal	14~16	14~16	14~16	14~16	14~16
Vertebrae	26~28	27	27	27	27
Egg size (mm SL)	1.32 ± 0.05	1.62 ± 0.04	1.64 ± 0.03	1.60 ± 0.04	1.62 ± 0.04
Mean water temperature (°C)*	27.9	23.5	22.3	22.7	23.9

	R. candidianus	*Rhinogobius* sp. 1 (yellow belly)	*Rhinogobius* sp. 2 (blue belly)
		Iriomote I., Japan	Okinawa I., Japan
	Dachia R.	Urauchigawa R.	Genka R.
Body length (mm SL)	32.8~63.8 (49.6)	32.9~47.6 (40.0)	32.1~48.3 (39.8)
Percent of SL			
Head	24.2%~30.3% (27.3%)	24.6%~30.2% (27.5%)	24.0%~30.8% (27.4%)
Body depth	16.8%~20.5% (19.3%)	16.6%~20.5% (18.9%)	17.3%~22.9% (19.6%)
Percent of HL			
Snout	31.5%~35.8% (33.3%)	30.5%~35.3% (32.8%)	29.8%~35.1% (32.0)
Orbit	20.5%~26.3% (24.5%)	20.0%~26.4% (24.0%)	19.7%~26.8% (23.3%)
Fin ray formulae			
Second dorsal	I, 8~10	I, 8 or 9	I, 8 or 9
Pectoral	17~19	18~20	16~19
Anal	I, 8~10	I, 7 or 8	I, 7~9
Branched caudal	14~16	14~16	14~16
Vertebrae	26	26	26`27
Egg size (mm SL)	1.19 ± 0.05	—	—
Mean water temperature (°C)*	22.7	—	—

*Source: Modified from the Water Quality Monitoring Network of the Environmental Protection Administration of Taiwan on-line.

southern Taiwan. Genetic differentiation among populations is clearly reflected in the spatial distribution of the allelic frequencies, which varied among samples of *R. rubromaculatus*. Substantial differences were found at LDH-A*, MDH-A*, and 6-PGDH*. A hierarchical analysis of genetic variation revealed that most variation was distributed among different localities (76.76%).

Table 4. Allelic frequencies in 5 populations of *Rhinogobius rubromaculatus* and the 3 other *Rhinogobius* species

		Population							
Locus	Allele	1. LB	2. CS	3. LL	4. MS	5. PL	6. DC	7. YB	8. BB
*AAT-1**	a						0.059		
	b	1.000	1.000	1.000	1.000	1.000	0.941	1.000	1.000
*AK**	a	1.000	1.000	0.771	0.667	0.775			
	b			0.229	0.333	0.225	1.000	1.000	1.000
*CK-A**	a					0.025			
	b	1.000	1.000	1.000	1.000	0.975		0.986	1.000
	c						1.000	0.014	
*FUM**	a	1.000	0.667	1.000	1.000	1.000		1.000	1.000
	b		0.333				1.000		
*GPI-A**	a					0.050			
	b	0.105			0.708	0.625	0.088		
	c	0.895	0.333	0.875	0.292	0.325	0.912	1.000	1.000
	d		0.667	0.125					
*GPI-B**	a							1.000	1.000
	b						1.000		
	c	1.000	1.000	1.000	1.000	1.000			
*IDH-2**	a								1.000
	b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
*LDH-A**	a							1.000	1.000
	b	1.000	1.000	1.000	0.833	0.850	1.000		
	c				0.167	0.150			
*LDH-B**	a	1.000	1.000	1.000	1.000	1.000			0.136
	b						1.000	1.000	0.864
*MDH-A**	a				0.104	0.125			
	b	1.000	1.000	1.000	0.167	0.400	1.000	1.000	1.000
	c				0.729	0.475			
*MDH-B**	a	0.026					1.000	1.000	1.000
	b	0.974	1.000	1.000	1.000	1.000			
*MEP-1**	a						0.015		
	b						0.985	0.042	
	c	1.000	1.000	1.000	1.000	1.000		0.819	1.000
	d							0.139	
*MEP-2**	a						0.074	0.889	1.000
	b	1.000	1.000	1.000	1.000	1.000	0.926	0.111	
*MPI**	a							0.014	0.841
	b							0.986	0.114
	c						0.985		0.045
	d	1.000	1.000	1.000	1.000	1.000	0.015		
*6-PGDH**	a				0.750	0.750			
	b	0.789	0.250	0.429			1.000	1.000	1.000
	c	0.211	0.750	0.571	0.250	0.250			
*PGM-1**	a	1.000	1.000	1.000	1.000	0.975	1.000	1.000	1.000
	b					0.025			
*PGM-2**	a						0.044		
	b	1.000	1.000	1.000	1.000	1.000	0.956	1.000	1.000
*SDH**	a						1.000		
	b	1.000	1.000	1.000	1.000	1.000		1.000	1.000
*XDH**	a	1.000	1.000	1.000	1.000	1.000		1.000	1.000
	b						1.000		

Site codes are given in table 1.

mtDNA sequencing

Total complete sequences from 2122 to 2127 bp in length of mtDNA including 1141 bp of the cytochrome b gene, 72 bp of the threonine tRNA gene, 71 (or 72) bp of the proline tRNA gene, and 840 to 844 bp of the control region, revealed 31 putative mtDNA haplotypes from 62 individuals including the outgroup, *R. candidianus*, *Rhinogobius* sp. 1, and *Rhinogobius* sp. 2. Sequences were deposited in GenBank under the accession numbers AY645686~AY645716. Differences between haplotypes were caused by substitutions (with a transition/transversion ratio of 2.5) and 8 indels included in the sequences. Haplotypes were unique in each of 2 populations. The genetic variability shown in table 5 indicates that haplotype diversity was remarkably high within most populations and ranged from 0.679 (PL) to 0.929 (YB and BB) with the exception of DC (0.286). Nucleotide diversity was low and ranged from 0.0001 (DC) to 0.0037 (YB). Similar to the results of the allozyme analysis, a closely related species complex on the NJ tree constructed (Fig. 5) based on pair-wise genetic distances (Table 6) indicated that specimens of the Linbian form were also closer to *R. rubromaculatus* (D = 0.035~0.059) than to *R. candidianus* (D = 0.113) or *Rhinogobius* sp. 1, 2, of the Ryukyus (0.0966~0.099).

The overall sequence distances among local populations including the Linbian River of *R. rubromaculatus* were from 0.005 to 0.074 (Table 6). According to the NJ dendrogram (Felsenstein 1995) constructed based on genetic distances, 5 populations fell into 2 clusters, which included a northern group (MS and PL) and a south-central group (LL, CS, and LB). The Φ_{ST} value calculated for all *R. rubromaculatus* populations, including samples from the Linbian River was 0.986. The hierarchical analysis of variation (AMOVA) revealed that the largest amount of variation was derived from the higher differences between groups (55.70%), as well as those among populations within groups (42.88%), in contrast to a very low variation (1.42%) within populations.

Tests of the goodness-of-fit revealed that the model of a sudden population expansion for the pairwise distribution of *R. rubromaculatus* haplotypes could not be rejected. Tajima's D-statistical test was performed to determine departures from

neutrality of the mtDNA data. We obtained a D value for the population in the Linbian River of 1.764, which significantly differed from 0 ($p < 0.05$), indicating a deviation from neutrality. Furthermore, this supports the model of a sudden population expansion after a bottleneck (Tajima 1989b).

An estimate of the average nucleotide substitutions (K) between haplotypes was 0.004 ± 0.001. Assuming evolutionary rates of between 1% and 5% changes per 10^6 years for fishes as a reference (Penzo et al. 1998, Dawson et al. 2002), the range of divergence times among *R. rubromaculatus* populations was estimated to be 90 000~428 000 years before the present. On average, haplotypes in Taiwan shared a most-recent common ancestor 0.26 Ma.

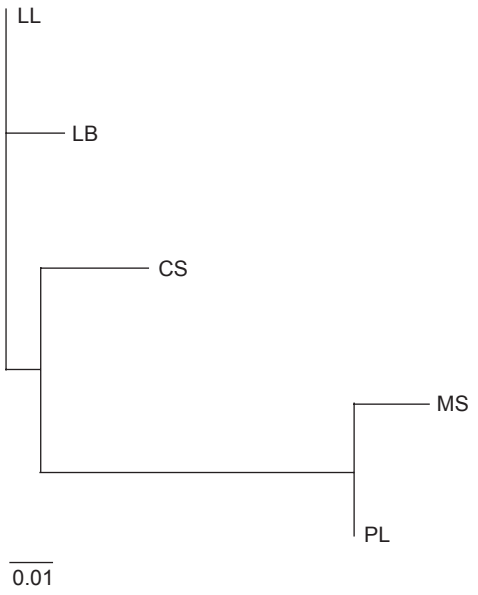

Fig. 4. Neighbor-joining tree based on Nei's unbiased genetic distances calculated from allozyme data in table 4. CS, Chingshui River; LB, Linbian River; LL, Lileng River; MS, Masu River; PL, Pinglin River.

DISCUSSION

With the exception of color patterns, members of *Rhinogobius* have only minor distinguishable morphological differences, but they exhibit distinct segregation of ecological niches, such as currents and still water, large and small rivers, upstream and downstream areas, fast and slow currents, and deep and shallow areas, as well as showing egg-size diversity (Akihito et al. 1984). Comparing data on egg diameters with Masaya (1994), the present results indicate that *R. rubromaculatus* is a new fluviatile goby in *Rhinogobius* with large eggs.

The present study records the new color type for the first time in the *Rhinogobius brunneus* species complex. Specimens from the Linbian River were characterized by significantly smaller body and egg sizes. Egg size is important in determining life history strategies because it affects fitness traits such as larval size, growth rates, survival, and adult fecundity. Although *R. rubromaculatus* does not show latitudinal clinal patterns in body or egg size, it does show a negative correlation with temperature. However, body size has been shown to increase not only with latitude, but also with elevation (Berven 1982). One explanation why body size evolves in response to temperature is that persistently higher nutrient availability during growth at lower temperatures may have driven an evolutionary reallocation of

Table 5. Summary of genetic variability for 8 sites of *Rhinogobius* species used in this study (with standard errors in parentheses)

Site	Mean no. of alleles per locus	Percent of polymorphic loci	Mean heterozygosity		No. of mtDNA haplotypes	Haplotype diversity	Nucleotide diversity	Percent of the most common haplotype
			Direct-count	Expected				
LB	1.1 (0.1)	8.0	0.020 (0.012)	0.030 (0.021)	3	0.750 (0.097)	0.0013 (0.0008)	0.375
CS	1.1 (0.1)	12.0	0.047 (0.030)	0.052 (0.029)	4	0.750 (0.139)	0.0006 (0.0004)	0.500
LL	1.1 (0.1)	8.0	0.028 (0.021)	0.023 (0.017)	3	0.750 (0.097)	0.0010 (0.0007)	0.375
MS	1.2 (0.1)	20.0	0.078 (0.035)	0.079 (0.033)	4	0.857 (0.102)	0.0009 (0.0007)	0.286
PL	1.4 (0.1)	20.0	0.082 (0.033)	0.089 (0.036)	3	0.679 (0.122)	0.0005 (0.0004)	0.500
DC	1.2 (0.1)	12.0	0.021 (0.009)	0.022 (0.010)	2	0.286 (0.196)	0.0001 (0.0001)	0.857
YB	1.2 (0.1)	8.0	0.016 (0.011)	0.021 (0.015)	6	0.929 (0.084)	0.0037 (0.0022)	0.250
BB	1.0 (0.1)	8.0	0.019 (0.012)	0.023 (0.014)	6	0.929 (0.084)	0.0021 (0.0013)	0.250

Site codes are given in table 1.

resources to growth. This reallocation would in turn lead to evolutionarily increased growth efficiency, growth rates, and adult body size. The population of central Taiwan collected from a medium elevation of about 450~550 m, is located at higher elevations than those of the population in northern Taiwan, so the temperatures of those localities are similar and are lower than those found in southern Taiwan. Although distributions of this species are similar, there are some differences in microhabitat use. Therefore, the greater coloration and morphometric variations which exist in the population of southern Taiwan may have primarily resulted from phenotypic plasticity.

A similar topology of the dendrogram constructed from both allozyme data and DNA sequencing indicates an allied clustering between the new color type and the typical *R. rubromaculatus* rather than with *R. candidianus*. Genetic distances calculated with the allozyme data between the new color type and the typical *R. rubromaculatus* were 0.000 to 0.007, which are lower than the 0.025 indicated for the population level, and values between the new color type and *R. candidianus* fell within the range (0.025~0.609) set for the species level in freshwater fish (Shaklee et al. 1982). A similar result was also found using the mtDNA data (the maximum sequence divergences among populations were 0.070 ± 0.006). In this paper, the NJ tree constructed using mtDNA data (Fig. 5) indicated that *R. rubromaculatus* populations of central and southern regions are closely related, and this result is consistent with findings of Wang et al. (2000, 2004). This phylogenetic relationship suggests a rather-recent divergence of *R. rubromaculatus*, which may be traced back to the time of separation of rivers in central and northern regions about 10^5 years ago (Tzeng 1986, Ota 1998), consistent with times estimated based on mtDNA.

Table 6. Genetic distances based on allozyme (above) and pair-wise genetic distances based on mtDNA sequences (below) for 8 populations of *Rhinogobius*

	1. LB	2. CS	3. LL	4. MS	5. PL	6. DC	7. YB	8. BB
1. LB	----	0.032	0.028	0.073	0.053	0.523	0.326	0.372
2. CS	0.035	----	0.021	0.078	0.057	0.562	0.398	0.448
3. LL	0.042	0.043	----	0.070	0.052	0.566	0.361	0.409
4. MS	0.059	0.070	0.061	----	0.001	0.641	0.412	0.464
5. PL	0.058	0.070	0.062	0.005	----	0.624	0.400	0.451
6. DC	0.113	0.113	0.111	0.108	0.109	----	0.422	0.503
7. YB.	0.099	0.101	0.097	0.097	0.098	0.055	----	0.075
8. BB	0.096	0.101	0.094	0.097	0.099	0.059	0.019	----

Site codes are given in table 1.

In the mtDNA sequence assays, 17 different haplotypes were detected among 39 *R. rubromaculatus* specimens, but all of the genotypes proved to be closely related and spatially localized. The implication is that contemporary gene flow has been low enough in relation to the population size to permit lineage sorting and random drift to promote genetic divergence among populations that nonetheless were in recent historical contact. The mtDNA pattern in *R. rubromaculatus* probably reflects a relatively recent separation from an ancestral stock during the species' colonization of western Taiwan, as well as pronounced restrictions on contemporary gene flow between small, scattered local populations.

According to deviation from neutrality by Tajima's *D* test, no statistical test values significantly differed from 0 except for the LB population ($D = 1.764$, $p < 0.005$). If a population has recently experienced a bottleneck, a negative value of *D* can also be obtained. Since the values of *D* observed in the case of mtDNA sequence polymorphisms are positive, the bottleneck effect cannot explain the result. A possible explanation is that the population has not yet reached equilibrium. Under the neutral mutation hypothesis, the probability that *D* is positive is less than 1/2 (Tajima 1989a). If a selectively neutral site is linked to a site at which natural selection is operating, then the selected site might affect the value of *D* for the neutral site. Therefore, there may be a site at which natural selection, which increases genetic variation, is operating.

Intraspecific morphs differ in various respects: behavior and life history characteristics such as adult body size, body shape, and color. Some species of fish exhibit latitudinal variations in body size, with larger individuals found in populations at higher latitudes (Schltz et al. 1996, Billerbeck et al. 2000). The fluviatile goby does not show reduced diversity with latitude; nonetheless, the loss of genetic variation within local populations may have been affected by glacial and/or postglacial events. Hierarchical analyses based on allozyme and mtDNA data showed that most variation was distributed among different localities. Lower genetic variability within each locality is usually attributed to historical factors due to founder events during range expansion following deglaciation. Given the unambiguous congruence of 2 independent mark-

ers in the fluviatile goby, we favor the hypothesis that neutral variation provides the main explanation, and we suggest that genetic variation was lost during the postglacial expansion of *R. rubromaculatus* into formerly glaciated areas.

The results of our study reveal the large morphological variation with little genetic difference among populations of *Rhinogobius rubromaculatus*. After discovery of a new color type in the Linbian River, analysis indicated that it resulted from phenotypic plasticity. A series of studies suggested that most of the color morphs of the *R. brunneus* species complex are discrete species based on allozyme data, egg size, and life history variations. Certainly, a more-encompassing study across the entire species complex of *R. brunneus* would reveal greater biodiversity. Further investigations into the phylogeny of *R. brunneus*, a widespread and highly subdivided species complex, will make important contributions to understanding the evolution of *Rhinogobius* of East Asia.

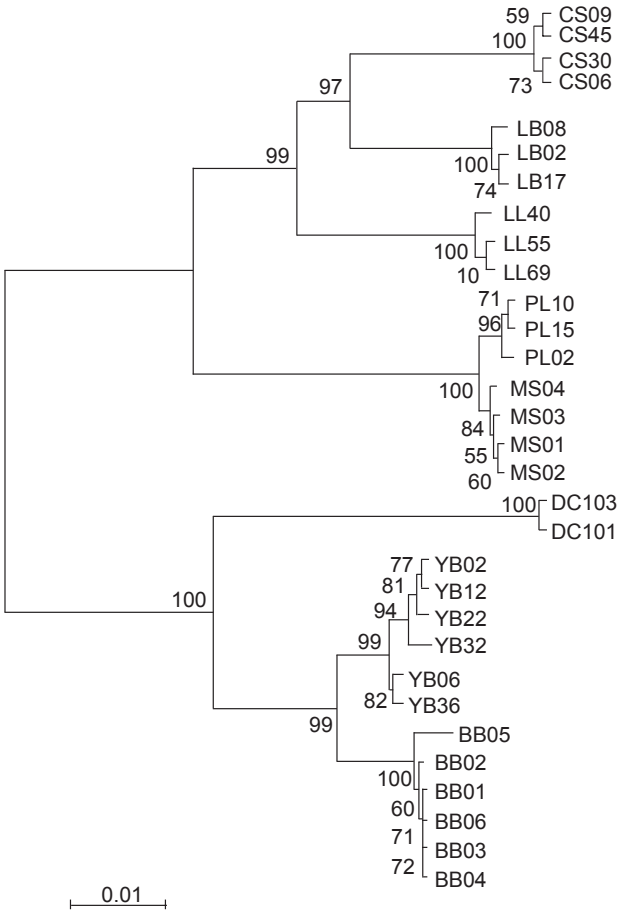

Fig. 5. Neighbor-joining tree constructed based on comparisons of the sequence alignment of the complete sequence of the cytochrome b gene, tRNA genes, and the control region of mtDNA. Bootstrapping values were estimated from 100 replicates; only numbers exceeding 50% are shown for each node. BB, blue belly, Genka River; CS, Chingshui River; DC, Dachia River; LB, Linbian River; LL, Lileng River; MS, Masu River; PL, Pinglin River. YB, yellow belly, Urauchigawa River.

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