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Systematic Studies on the Freshwater Goby, *Rhinogobius* Species (Perciformes, Gobiidae) III. Geographic Variation and Subspecific Differentiation in *Rhinogobius giurinus*, with a Comment on Genetic Relationships among Four Species of the Genus *Rhinogobius* in Korea

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Key Words:
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Genetic and morphological variations of *Rhinogobius giurinus* were surveyed. Populations of *R. giurinus* were clearly divided into two forms (Form-A and Form-B). Starch gel electrophoresis was used to study genetic variation of this species. Three loci (*Aco, Mpi, α Gpd*) out of 27 showed fixed allelic differences between Form-A and Form-B and they are well differentiated from each other genetically (Rogers' S=0.871). These two forms, moreover, are found to be allopatric in distribution and morphologically different in body length and caudal fin color pattern. Therefore, they are considered as two distinct taxa of subspecific rank. In addition, the genetic relationships among 5 taxa within 4 species of the genus *Rhinogobius* were investigated. Three species of the *Rhinogobius brunneus* complex (*R.* sp. OR, *R.* sp. CB and *R.* sp. CO) are well differentiated from each other genetically and two taxa of *R. giurinus* are genetically divergent from three species of the *Rhinogobius brunneus* complex (average Nei's D=0.603, average Rogers' S=0.534).

The gobiid fishes (Pisces, Gobiidae), which are distributed throughout the tropical and temperate waters of the world, have adapted to various environments and have acquired various life histories. A group of them, the genus *Rhinogobius,* is a common group of freshwater fishes in Korea, Japan and China (Tzeng, 1986; Akihito et al., 1993; Jeon and Aonuma, 1995; Kim, 1995; Kim and Yang, 1996a, b). In Japan, the taxonomic status of *R. brunneus* has been well studied and it was reported that this species consisted of eight color types (*R.* sp. OR, *R.* sp. CB, *R.* sp. CO, *R.* sp. DL, *R.* sp. DA, *R.* sp. MO, *R.* sp. BB, *R.* sp. YB and *R.* sp. LD) of specific rank (Akihito et al., 1993). However, since there is no detailed study on geographic variation of *R. giurinus,* the taxonomic status of this species is not clear.

Only two species, *R. giurinus* (Rutter) and *R. brunneus* (Temminck and Schlegel), were recognized in Korea as valid species until 1992 (Kim et al., 1986, 1987; Chyung, 1973). Kim et al. (1992) surveyed geographic variation of *R. brunneus* and found that this species consisted of two distinct specific taxa on the basis of the color and genetic differentiation. Most recently, it was reported that Korean *R. brunneus* includes three or four color types (*R.* sp. OR, *R.* sp. CB, *R.* sp. CO and *R.* sp. LD; Jeon and Aonuma, 1995; Kim, 1995; Kim and Yang, 1996a, b). Kim and Yang (1996b) showed that three types (*R.* sp. OR, *R.* sp. CB, *R.* sp. CO) are genetically distinct and reproductively isolated from each other and they have some differences in microhabitat preference.

In this paper, we report the result of isozymic and morphological analyses designed to calibrate the extent of divergence among the *R. giurinus* populations and elucidate their taxonomic status. We also report the phylogenetic relationships and the degrees of genetic differentiation among the *Rhinogobius* species.

Materials and Methods

Collection

Specimens of 12 populations of *Rhimogobius* sp. OR, *R.* sp. CB, *R.* sp. CO and *R. giurinus*, in the genus *Rhinogobius* were collected from 9 streams in Korea and Japan (Table 1). Each specimen was identified according to color pattern and external morphological

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Table 1. Collection localities, collection dates, and sample sizes for electrophoresis of the *Rhinogobius* species examined in this study

Collection locality	Collection date	No. of specimen
Rhinogobius giurinus		
1. Namhan River: Hansu-myun, Cheweon-gun, Chungcheongbuk-do, Korea	June 30, 1991	20
2. Keum River: Yangchon-myun, Nonsan-gun, Chungcheongnam-do, Korea	Oct. 28, 1992	20
3. Dongjin River: Heungdeok-myun, Kochang-gun, Jeollabuk-do, Korea	Oct. 29, 1992	10
4. Yeongsan River: Yeongam-myun, Yeongam-gun, Jeollanam-do, Korea	May 4, 1993	2
5. Chungmun Stream: Seogwipo-shi, Cheju Isl., Korea	Oct. 29, 1993	20
6. Choho Stream: Amami-Isl., Japan	Oct. 11, 1991	14
Rhinogobius sp. OR		
7. Bukhan River: Kapyeong-eup, Kapyeong-gun, Kyeongki-do, Korea	May 5, 1991	32
8. Maeup Stream: Keundeok-eup, Samcheok-gun, Kangwon-do, Korea	May 20, 1994	19
9. Buk Stream: Kanseong-eup, Koseong-gun, Kangwon-do, Korea	July 21, 1993	8
Rhinogobius sp. CB		
10. Maeup Stream: Keundeok-eup, Samcheok-gun, Kangwon-do, Korea	May 20, 1994	20
11. Buk Stream: Kanseong-eup, Koseong-gun, Kangwon-do, Korea	July 21, 1993	40
12. Choho Stream: Amami-Isl., Japan	June 25, 1993	20
Rhinogobius sp. CO		
13. Akkeun Stream: Seogwipo-shi, Cheju Isl., Korea	July 31, 1992	20
14. Buk Stream: Kanseong-eup, Koseong-gun, Kangwon-do, Korea	July 21, 1993	11

characters described by Akihito et al. (1993), Kim (1995), Jeon and Aonuma (1995) and Kim and Yang (1996b).

Protein electrophoresis

Live specimens were transported to the laboratory and were stored at -70℃ until use. In the laboratory, the skeletal muscle from each specimen was removed and homogenized by glass homogenizer in half volume of distilled water and were centrifugated at 18,000 rpm for 30 min at 4℃ to obtain the supernatant for electrophoresis. Voucher specimens were fixed in 10% formalin, preserved in 70% ethanol, and deposited in Yang's collection at Inha University. The supernatant was subjected to horizontal starch-gel (12%) electrophoresis and histochemical staining procedures (Selander *et al*, 1971; Yang et al., 1994; Buth, personal communication; Table 2). Multiple loci were numbered sequentially, and alleles were designated alphabetically with "a" being the fastest migrant. Individual genotypes were used to calculate allele frequencies for each population, these in turn were used to estimate the degree of genic variability and to calculate matrices of genetic similarity (Rogers, 1972) and genetic distance (Nei, 1972). Nei's (1972) distance coefficients were then clustered by the unweighted pair group method using arithmetic averages linkage (UPGMA: Sneath and Sokal, 1973) to provide a general estimate of the overall genetic relationships among species or populations.

Morphological analysis

Body length and color pattern of the caudal fin were compared among the specimens of *R. giurinus* to detect morphological differences between two genetic groups of this species.

Results

Geographic variation of Rhinogobius giurinus

In the *Rhinogobius giurinus* (Fig. 1, populations 1-6;

Table 2. Collection localities, collection dates, and sample size for electrophoresis of the *Rhinogobius* species examined in this study

Buffer system	E. C. No.*	Enzyme	Condition
Continuous tris citrate II (pH 8.0)	2. 6. 1. 2	Alanine aminotransferase (*Alat-1, 2*)	100 V/3 h
	1. 1. 1.37	Malate dehydrogenase (*Mdh-1, 2*)	
	1. 1. 1.42	Isocitrate dehydrogenase (*Idh*)	
	1. 1. 1.40	Malic enzyme (*Me-1, 2*)	
	2. 7. 5. 1	Phosphoglucomutase (*Pgm*)	
	4. 2. 1. 3	Aconitate hydratase (*Aco*)	
	2. 7. 3. 2	Creatine kinase (*Ck-1, 2*)	
LiOH (pH 8.1)	5. 3. 1. 9	Phosphoglucose isomerase (*Pgi-1, 2*)	300 V/3 h
	N. S.**	General protein (*Gp-1, 2, 3, 4, 5*)	
	2. 6. 1. 1	Glutamate oxaloacetate isomerase (*Got-1, 2*)	
	3. 4.11.11	Peptidase (*Pept-1*)	
	1. 1. 1.27	Lactate dehydrogenase (*Ldh-1, 2*)	
	1. 1. 1.29	Glycerate dehydrogenase (*Glydh*)	
Tris maleic EDTA (pH 7.4)	5. 3. 1. 8	Mannose phosphate isomerase (*Mpi*)	100 V/5 h
	1. 1. 1.43	6-Phosphogluconate dehydrogenase (*6Pgd*)	
	1. 1.99. 5	αGlycerophosphate dehydrogenase (*αGpd*)	

* E. C. No.: Enzyme commission number
** N. S.: Non specific

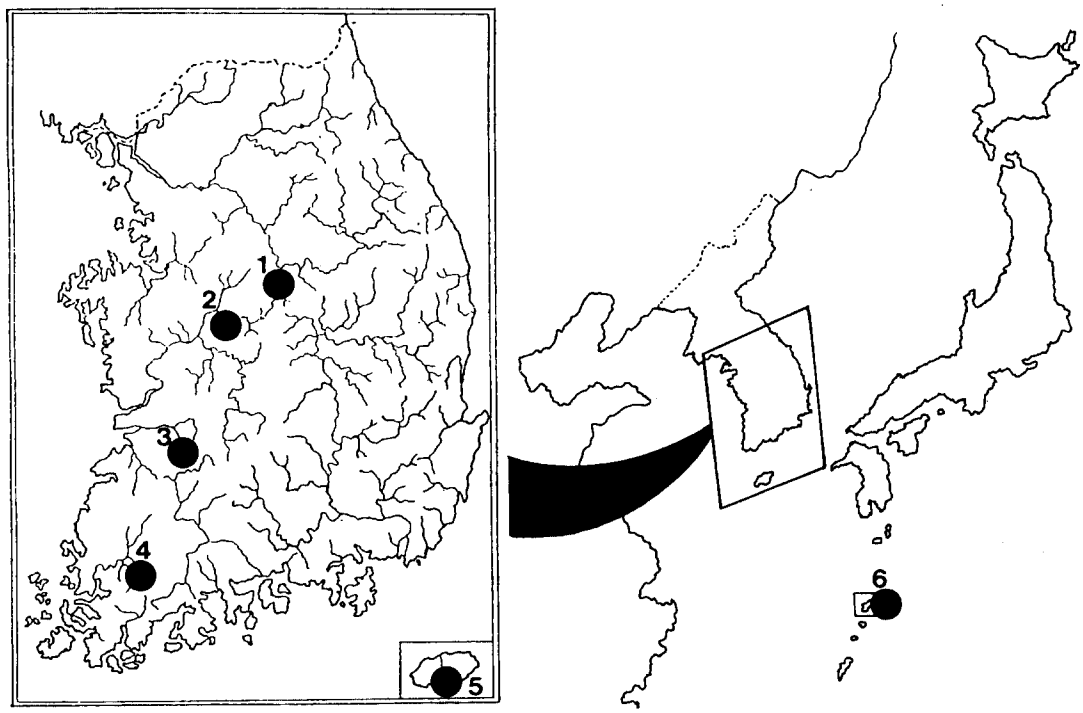

Fig. 1. Collection localities of *Rhinogobius giurinus* in Korea and Japan. Population numbers refer to Table 1.

Table 3), 19 loci (*Idh, Me-1, Alat-1, 2, Gp-1, 2, 3, 4, 5, Pgi-1, Got-2, Glydh, Mdh-2, 6Pgd-1, Ck-1, 2, Ldh-1, 2, Pept-1*) out of the 27 presumptive loci scored were monomorphic across all populations and the remaining 8 loci were polymorphic at the $P_{0.99}$ criterion level (Table 3). As shown in Table 3, 4 and Fig. 2 genetic assays revealed that *R. giurinus* was clearly divided into two genetic groups (Form-A and Form-B) in Korea and Japan. Genetic dissimilarities between Form-A (pops. 1-4; Table 3) and Form-B (pops. 5-6) include completely different alleles at *Mpi* and *Aco* loci and diagnostic difference at the 95% confidence level (Ayala and Powell, 1972) at *αGpd*. Since these two forms have allelic differences at the loci mentioned above we are convinced that these loci are significant diagnostic loci to discriminate two forms of *R giurinus*.

Based on allelic frequencies listed in Table 3 average genetic distances and similarities among populations of the *R. giurinus* forms were estimated (pops. 1-6; Table

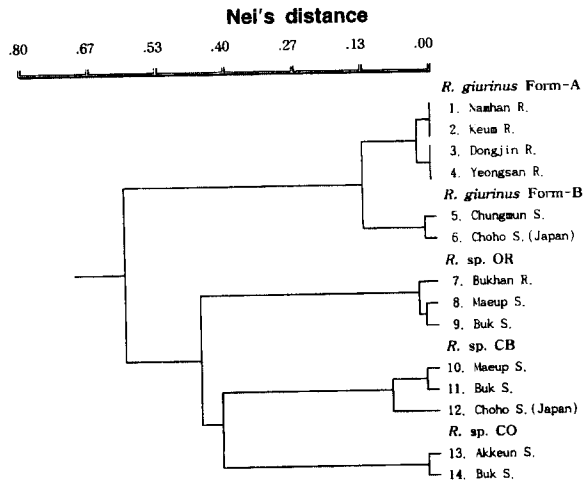

Fig. 2. A dendrogram of 14 populations in the *Rhinogobius* species based on Nei's genetic distance coefficients.

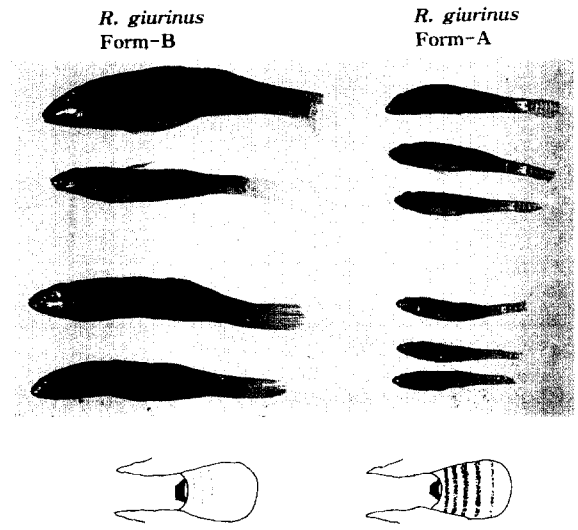

Fig. 3. Morphological diagnostic characters between Form-A and Form-B of *Rhinogobius giurinus*. Note band patterns on the caudal fin and body size.

Table 3. Allele frequencies of 16 populations in the *Rhinogobius* species

Locus	*R. giurinus* Form-A				*R. giurinus* Form-B		*R.* sp. OR			*R.* sp. CB			*R.* sp. CO	
	1*	2	3	4	5	6	7	8	9	10	11	12	13	14
Mpi	a**	a	a	a	d	d(0.64) e(0.36)	a(0.47) b(0.53)	a(0.50) b(0.50)	a(0.37) b(0.63)	a(0.37) b(0.63)	a(0.47) b(0.53)	b	a(0.55) b(0.45)	a(0.96) b(0.04)
Aco	a	a	a	a	b	b	b	b	b	b	b	c	b	b
αGpd	b(0.93)*** c(0.07)	b(0.88) c(0.12)	c	c	d	c(0.07) d(0.93)	b(0.25) c(0.75)	a(0.03) b(0.42) c(0.44) d(0.11)	a(0.13) b(0.31) c(0.56)	b	b	b	b(0.98) c(0.02)	b
Idh	b	b	b	b	b	b	b	b	b	b	b	b	c	c
Me-1	c	c	c	c	c	c	c	c	c	b	b	b	a(0.35) b(0.65)	a
Me-2	e	e	e	e	b(0.40) e(0.60)	e	a(0.72) b(0.28)	a(0.45) b(0.50) c(0.05)	b	d	d	d	b	b
Alat-1	c	c	c	c	c	c	a(0.98) b(0.02)	a	a	b	a(0.01) b(0.99)	b	d	d
Alat-2	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Pgm	a	a	a	a	a	a(0.89) c(0.11)	a(0.55) b(0.45)	a(0.79) b(0.21)	a(0.81) b(0.19)	a	a	a(0.98) c(0.02)	a	a(0.96) b(0.04)
Gp-1	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Gp-2	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Gp-3	a	a	a	a	a	a	b	b	b	b	b	b	b	b
Gp-4	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Gp-5	c	c	c	c	c	c	a(0.98) b(0.02)	a	a	b	b	b	d	d
Pgi-1	b	b	b	b	b	b	a(0.06) b(0.38) c(0.56)	b(0.74) c(0.26)	b(0.75) c(0.25)	b	b	b(0.88) c(0.12)	b(0.95) c(0.05)	a(0.05) b(0.77) c(0.18)
Pgi-2	c	c	c	c	c	b(0.04) c(0.96)	b	b	a(0.12) b(0.88)	a(0.02) b(0.60) c(0.38)	b(0.60) c(0.40)	b(0.35) c(0.65)	b	a(0.27) b(0.73)
Got-1	b	b	b	b	b(0.78) c(0.22)	b(0.61) c(0.39)	a(0.22) b(0.78)	b	b	b	b	a(0.05) b(0.93) c(0.02)	b	b
Got-2	c	c	c	c	c	c	a(0.73) c(0.27)	a(0.87) c(0.13)	a	b	b	b	b	b
Glydh	a	a	a	a	a	a	a	a	a	b	b	b	a	a
Mdh-1	a	a(0.98) b(0.02)	a	a	a	a	a	a	a	a	a	a	a	a
Mdh-2	a	a	a	a	a	a	a	a	a	a	a	a	a	a
6Pgd	c	c	c	c	c	c	a(0.97) b(0.03)	a	a	a	a(0.95) b(0.05)	a	a(0.98) c(0.02)	a(0.68) b(0.32)
Ck-1	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Ck-2	c	c	c	c	c	c	a	a	a	b	b	b	b	b
Ldh-1	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Ldh-2	b	b	b	b	b	b	a	a	a	b	b	c	b	b
Pept	a	a	a	a	a	a	b	b	b	b	b	b	c	c

* Population number, ** Allele, *** Allele frequency

4). Rogers' genetic similarities (S) between regional populations of the same nominal form were high (Form-A: $S \geq 0.966$, Form-B: S=0.958), whereas the average genetic similarities between two forms were notably lower (S=0.871). The UPGMA clustering based on Nei's genetic distances shows the level of dissimilarities among two forms as well as the similarities among populations within the same nominal forms (Fig. 2).

Korean Form-A which is only found in the upper streams on inland shows far lower genic variation (A=1.0, P=1.90, Ho=0.003, He=0.004) than that of Form-B(A=1.07, P=4.93, Ho=0.040, He=0.041) which is found in the river-mouth on Cheju Isl. and Japan (Fig. 1, Table 5).

For morphological comparisons of these two genetic forms, a sum of 186 adult specimens from 6 popula-

Table 4. Rogers' (1972) genetic similarities (below diagonal) and Nei's (1972) genetic distances (above diagonal) for 14 populations of the *Rhinogobius species*

Species & Locality	1	2	3	4	5	6	7	8	9	10	11	12	13	14
R. giurinus Form-A														
1. Namhan R.	-	.997	.966	.966	.867	.876	.516	.550	.535	.544	.549	.495	.541	.550
2. Keum R.	.000	-	.967	.967	.867	.876	.517	.551	.535	.541	.546	.492	.538	.547
3. Dongjin R.	.032	.029	-	1.000	.866	.876	.532	.548	.541	.510	.515	.461	.506	.516
4. Yeongsan R.	.032	.029	.000	-	.866	.876	.532	.548	.541	.510	.515	.461	.506	.516
R. giurinus Form-B														
5. Chungmun S.	.126	.125	.129	.129	-	.958	.540	.566	.558	.534	.536	.461	.534	.525
6. Choho S. (Jpn.)	.112	.111	.112	.112	.013	-	.538	.558	.547	.527	.529	.455	.517	.509
R. sp. OR														
7. Bukhan R.	.612	.609	.581	.581	.586	.591	-	.943	.916	.619	.623	.566	.610	.577
8. Maeup S.	.563	.562	.564	.564	.539	.556	.015	-	.961	.655	.657	.597	.656	.621
9. Buk S.	.598	.596	.584	.584	.553	.583	.036	.012	-	.652	.646	.594	.659	.634
R. sp. CB														
10. Maeup S.	.589	.592	.657	.657	.612	.622	.439	.391	.411	-	.993	.894	.703	.649
11. Buk S.	.578	.581	.645	.645	.608	.617	.439	.391	.412	.001	-	.889	.706	.654
12. Choho S.	.685	.689	.760	.760	.761	.773	.523	.479	.492	.089	.093	-	.601	.554
R. sp. CO														
13. Akkeun S.	.602	.605	.668	.668	.609	.645	.458	.396	.392	.331	.330	.477	-	.931
14. Buk S.	.586	.588	.654	.654	.622	.660	.491	.434	.432	.404	.398	.577	.031	-

tions were used, and significant differences between two forms were detected (see Fig. 3, Table 6). The specimens of Form-A (<45 mm) had relatively shorter bodies than Form-B (>50 mm). Distinct differences between the two forms, moreover, were found in the banding pattern on the caudal fin (Fig. 3).

Genetic relationships among the Rhinogobius species

To estimate the degree of genetic differentiation among 5 taxa of the genus *Rhinogobius* including two forms of *R. giurinus*, Nei's genetic distance and Rogers' genetic similarity among 14 populations of these taxa were calculated from the allelic frequencies listed in Table 3 (Table 4). The genetic distances among populations within each taxa were very close (0.000 to 0.093), whereas the genetic distances among taxa were from 0.111 (Form-A and Form-B of *R. giurinus*) to 0.773 (*R.* sp. CB and *R. giurinus* Form-B).

The UPGMA clustering based on Nei's genetic distances shows the level of dissimilarities among 5 taxa as well as the similarities among populations within the same taxa (Fig. 2).

Discussion

Rhinogobius giurinus was first described by Rutter (1897) in the genus *Gobius*. Mori (1952) regarded *Ctenogobius hadropterus* by Jordan and Snyder (1901) and *Rhinogobius hadropterus* by Mori (1928) as synonym of *Gobius giurinus* Rutter, 1897 and he, at the same time, recombined *Gobius giurinus* into *Rhinogobius giurinus,* which has been generally followed by many authors (Kawanabe and Mizuno, 1989; Akihito et al., 1993; Jeon and Aonuma, 1995; Kim and Yang, 1996a).

On the basis of morphological descriptions and ecological studies of the *Rhinogobius* species, Jordan and Snyder (1901) and Oshima (1919) described "*R. giurinus* have ..... body size of fifty (mm) and over with a few very indistinct vertical wavy bands on the caudal fin ....." and this species inhabits estuaries or lower reaches of streams in Japan (Kawanabe and Mizuno, 1989; Akihito et al., 1993). In this study, *R. giurinus* Form-B (Japan and Cheju Isl.) showed morphological and ecological characteristics of typical *R. giurinus* (Rutter) mentioned above. However, *R. giurinus* Form-

Table 5. Genetic variation of two *Rhinogobius giurinus* forms

Population	N	Mean No. of alleles (A)	% Polymorphism (P)	Mean heterozygosity	
				Observed (Ho)	Expected (He)
R. giurinus Form-A					
1. Namhan River	20	1.0	3.7	.002	.005
2. Keum River	20	1.1	3.7	.011	.010
3. Dongjin River	10	1.0	0	.000	.000
4. Yeongsan River	2	1.0	0	.000	.000
Mean		1.03	1.85	.003	.004
R. giurinus Form-B					
5. Chungmun stream	20	1.1	7.4	.035	.031
6. Choho Stream(Japan)	14	1.2	14.8	.045	.051
Mean		1.15	11.1	.040	.041

Table 6. In the 6 localities of *R. giurinus*, habitats, sample size for morphological analysis, and body size of specimen

Locality	Habitat	Sample size	Body size (range, mm)
R. giurinus Form-A			
1. Namhan River	the upper reaches	51	38.41-43.33
2. Keum River	the upper reaches	44	36.19-42.63
3. Dongjin River	the upper reaches	26	27.87-38.41
4. Yeongsan River	the upper reaches	4	38.79-41.35
R. giurinus Form-B			
5. Chungmun Stream (Cheju Isl.)	the lower reaches	45	52.23-77.20
6. Choho Stream (Japan)	the lower reaches	16	57.65-73.52

A were all collected on the upper reaches of 4 rivers in Korea, and differs from Form-B in the morphological characters. Form-A (<45 mm) is distinctly smaller than Form-B (>50 mm) in overall body size (see Table 6): Form-A has 4-5 distinct speckles on the caudal fin, whereas Form-B shows very indistinct bands (see Fig. 3). Moreover, *R. giurinus* Form-A are well differentiated genetically from Form-B with three significant diagnostic loci (*Mpi, Aco* and *αGpd*). Genetic and morphological evidences made us to conclude that it seems reasonable to assign *R. giurinus* Form-A as a new taxon of subspecific rank.

In addition, the genetic relationships among 5 taxa within 4 species of the genus *Rhinogobius* were investigated. Three species of the *Rhinogobius brunneus* complex (*R.* sp. OR, *R.* sp. CB and *R.* sp. CO) are well differentiated from each other genetically and two taxa of *R. giurinus* are genetically divergent from three species of the *Rhinogobius brunneus* complex (average Nei's D=0.603). Divergent time estimation (Nei, 1975) of *R. giurinus* and the *Rhinogobius brunneus* complex indicates that they diverged during about 3.0 million years before present (MYBP).

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