Occurrence of Two Types of *Hynobius naevius* in Northern Kyushu, Japan (Amphibia: Urodela)

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ABSTRACT—A survey to examine genetic variation among *Hynobius naevius* from four localities of Fukuoka Pref., northern Kyushu, Japan, resulted in the detection of two, sympatric, genetic types (A and B) that are clearly different in the allelic frequencies of four loci (*ACOH-A*, *ACOH-B*, *ADH-A*, and *SOD-A*) in each locality. Morphological investigations between the two genetic types also proved that they are clearly discriminated; the type A is about 75 mm in SVL, lacks mottling pattern on bluish purple dorsum, and possesses relatively short vomerine teeth series, while the type B is about 60 mm in SVL, and has light mottling on reddish purple ground color. These results strongly suggest that reproductive isolation occurs between these two types, and that they could be regarded as separate species. Populations from Toyotacho, western Honshu, and Yabe-machi, central Kyushu, both close to Fukuoka Pref., were very similar to the types A and B, respectively. From these results, we consider that two evolutionary lineages that first evolved allopatrically in western Honshu and southern Kyushu secondarily contacted and became sympatric in the region of northern Kyushu.

Key words: Amphibia, Urodela, *Hynobius naevius*, cryptic species, Japan

INTRODUCTION

Hynobius naevius, described by Schlegel (1838) as *Salamandra naevia*, is one of the earliest known Japanese small salamanders and is currently considered to occur widely in western Japan, including southwestern Honshu, Shikoku, and Kyushu (Matsui and Misawa, 1996). Presence of geographic variations in this species has long been known, and Oyama (1940), on the basis of morphological observation on specimens from Kyushu, regarded the populations from Aso, Kumamoto Pref., central Kyushu, as a distinct subspecies *H. n. yatsui*, although the name has never been used thereafter. Sato (1943), on the other hand, studied morphological variation of this species throughout its range of distribution and recognized four morphotypes, i.e., standard type (for the populations from Chugoku and northern Kyushu districts) and three local types (southern Kyushu, Shikoku, and Kinki). He (Sato, 1943), however, did not consider them taxonomically distinct from each other.

More recently, Sato *et al.* (1994), by analyzing color patterns and body sizes of specimens from various localities

in Kyushu, distinguished three allopatric morphotypes of *H. naevius* within Kyushu (northern, central, and southern Kyushu types) and delimited their ranges of distribution (Fig. 1). On the basis of diagnostic characteristics given by Sato *et al.* (1994), especially of color patterns, we tried to classify specimens at hand. We, however, found that the specimens of all the northern or central and southern Kyushu types of Sato *et al.* (1994) were included in our samples from each of several localities of Fukuoka Pref., northern Kyushu (Fig. 1). Because the character states diagnostic between northern and central Kyushu types of Sato *et al.* (1994) were continuous, it was difficult to discriminate these two morphotypes. Sympatric occurrence of more than one morphotypes was so surprising that we made intensive field investigations in these areas. As a result, we could detect many breeding sites of the northern Kyushu type, but failed to find those of the southern Kyushu type.

Results of these field observations prompted us to investigate whether or not each of these two sympatric types is actually reproductively independent entity. We, therefore, surveyed genetic relationships of these two types using protein electrophoresis that is a powerful tool to distinguish cryptic species within morphologically conservative amphibians (e.g., Good, 1989; Highton, 1999; Matsui *et al.*, 2000).

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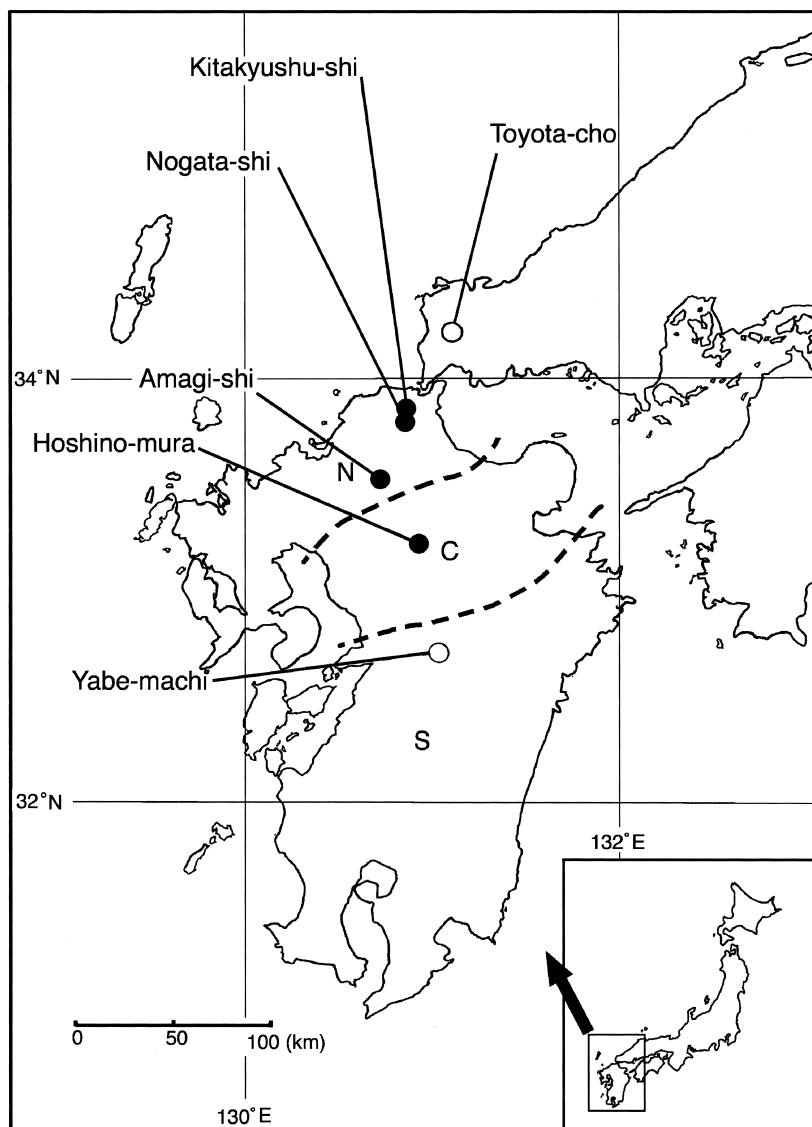
Fig. 1. A map of western Japan, showing sample localities in the present study. Closed and open circles, respectively, indicate localities of specimens used in the allozyme and morphological variation analyses, and those used in the morphological variation analyses only. Broken lines are borderlines of three allopatric morphotypes: northern (N), central (C) and southern (S) Kyushu types (Sato *et al.,* 1994).

We also made a detailed morphological survey and clarified genetic and morphological relationships of the two sympatric types of *H. naevius.*

MATERIALS AND METHODS

Genetic survey

Salamanders collected from the field were anesthetized with Chloretone saturated solution, and their livers were removed and maintained frozen at −80°C until using for electrophoresis. Voucher specimens were fixed in 10% formalin, later preserved in 70% ethanol, and stored at Kyoto University or Mr. Tanabe's private collection (Appendix 1).

Homogenized tissue extracts were subjected to standard horizontal starch gel electrophoresis (Shaw and Prasad, 1970; Ayala *et al.,* 1972) using Starch Art (Otto Hiller, Madison, Wisconsin, USA) and Connaught starch (Connaught Lab., Willowdale, Ontario, Can-

ada) mixed in a 4:1 ratio and then suspended in buffer at a concentration of 12%.

Genetic interpretations of zymograms were based on criteria developed by Selander *et al.* (1971). Enzyme nomenclature, E. C. numbers, and the notation of loci, electromorphs, and alleles mainly follow Murphy *et al.* (1996).

At first, in order to select loci that include large intrapopulational variations, we used 13 specimens from AMAGI, Fukuoka Pref., that included both the northern and southern types of Sato *et al.* (1994). We analyzed 18 loci encoding 12 enzymes (Table 1) that were commonly used in genetic investigations of salamanders (Matsui *et al.,* 2000, 2001).

As a result, we detected large amount of genetic variations at nine loci controlling six enzymes (*ADH-A, ACOH-A, ACOH-B, LDH-A, MDH-A, MDH-B, PGM-A, PGM-C,* and *SOD-A*). On the basis of the preliminarily result, we conducted a larger scale of survey to assay these loci for all the specimens from four localities in Fukuoka Pref. [AMAGI (N=96 including above 13 specimens), KITAKYUSHU (N=65), NOGATA (N=17), and HOSHINO (N=6)], collected

Table 1. Enzyme, presumptive loci, and buffer systems used in the analyses of allozyme variations among samples.

Enzyme	E.C.number	Locus	Buffer systems*
Aspartate aminotransferase	2.6.1.1	*AAT-A*	CAPM6
Aspartate aminotransferase	2.6.1.1	*AAT-B*	CAPM6, TC7
Aconitate hydratase	4.2.1.3	*ACOH-A*	TC7, TC8
Aconitate hydratase	4.2.1.3	*ACOH-B*	TC7, TC8
Alcohol dehydrogenase	1.1.1.1	*ADH-A*	TBE8.7, TC7
Fumarate hydratase	4.2.1.1	*FUM-A*	TBE8.7
Glucose-6-phosphate isomerase	5.3.1.9	*GPI-A*	CAPM6
Glutamate dehydrogenase	1.4.1.3	*GTDH-A*	TC8
Isocitorate dehydrogenase	1.1.1.42	*IDH-A*	TC7
L-Lactate dehydrogenase	1.1.1.27	*LDH-A*	CAPM6, TC7
L-Lactate dehydrogenase	1.1.1.27	*LDH-B*	CAPM6, TC7
Malate dehydrogenase	1.1.1.37	*MDH-A*	CAPM6, TC7
Malate dehydrogenase	1.1.1.37	*MDH-B*	CAPM6, TC7
Malic enzyme	1.1.1.40	*MDHP-A*	CAPM6, TC7
Malic enzyme	1.1.1.40	*MDHP-B*	CAPM6, TC7
Phosphoglucomutase	5.4.2.2	*PGM-A*	TC7
Phosphoglucomutase	5.4.2.2	*PGM-C*	TC7
Superoxide dismutase	1.15.1.1	*SOD-A*	TBE8.7, TC7

*CAPM6: citrate-aminopropylmorphorine, pH 6.0 (Clayton and Tretiak, 1972); TC7: tris-citrate, pH 7.0 (Shaw and prasad, 1970); TC8: tris-citrate, pH 8.0 (Clayton and Tretiak, 1972); TBE8.7: tris-borate-EDTA, pH 8.7 (Boyer *et al.*, 1963).

between April 1992 and August 2001 (Fig. 1).

We calculated the expected number of each genotype in specimens from a given locality using Levene's (1949) formula for small sample size. First, we checked variable loci by Chi-square goodness-of-fit tests to determine whether or not they are in Hardy-Weinberg equilibrium ($P>0.05$). We calculated all these statistics using the BIOSYS-1 computer program (Swofford and Selander, 1981). Second, we classified all specimens into different genotypes if they had any different alleles in each locus. We then calculated similarities of each genotype with the standard genotype (see "RESULT" section).

Morphological survey

We examined morphology of a total of 172 preserved specimens [AMAGI (N=91); KITAKYUSHU (N=58); NOGATA (N=17); HOSHINO (N=6)], each of which has been assigned to either of the two genetic types (A and B) by the genetic analyses (see result). These genetic types from each sample locality will be hereafter referred to as sub-samples. Some of very small specimens used for genetic analyses could not be included in the morphological analyses. We pooled specimens from NOGATA and KITAKYUSHU in the following statistical analyses (referred as "KITAKYUSHU"), because these localities are geographically very close and each of the two types from there were genetically very close with each other (see below). We excluded the HOSHINO sub-samples from statistical analyses because of their small sample sizes. For comparisons, we also incorporated specimens from two additional areas adjacent to Fukuoka, i.e., Toyota-cho, Yamaguchi Pref. (TOYOTA, N=12) and Yabe-machi, Kumamoto Pref. (YABE, N=12; see Appendix 1 and Fig. 1). From each of these two localities, only one genetic type has been known from our preliminary surveys.

We first separated male, female, and young by observing gonads, and measured 22 morphometric characters for all of them: SVL, HL, HW, LJL, SL, IND, IOD, UEW, UEL, AGD, TRL, TAL, TAW, TAH, FLL, HLL, 2FL, 3FL, 3TL, 5TL, VTW, and VTL (see

Appendix 2 for farther details). All measurements were made to the nearest 0.1 mm with dial calipers, and under a stereoscopic binocular microscope where necessary.

We first investigated sexual and ontogenetic variations within a sub-sample. One of the two genetic types from AMAGI was chosen for this analysis because it included the largest number of specimens (total N=57). We applied analysis-of-variance (ANOVA) with Tukey range test for SVL variation. Allometric relationships to SVL of each of the remaining characters were also examined in this sub-sample using analysis-of-covariance (ANCOVA). All measurements were log-transformed in this analysis.

We then analyzed variations among sub-samples in SVL and its allometric relationships to the remaining characters. Based on the results of analyses for the AMAGI-B sub-sample, we analyzed SVL variations separately for each sex. On the other hand, we separated young and adults in the analyses of allometric variations among sub-samples. We set the significant level at 0.05 in all the above analyses.

For multivariate analyses, PRINCOMP and CANDISC programs (SAS, 1985) were used for the principal component analysis and the canonical discriminant analysis, respectively, through the facilities of Data Processing Centre, Kyoto University. In the multivariate analyses, we used all the 22 morphological characters by log-transforming all measurements and separately treated females and males. Young specimens were not included since there was great variation in SVL even within a sub-sample. For specimens whose tail tip was damaged or regenerated, we used an estimated value from the line of regressions.

RESULTS

Allozymic variation

The most variable locus was *PGM-C* with seven alleles while the least variable locus was *MDH-B* with only two alleles. Samples from all the four localities deviated significantly from Hardy-Weinberg expectation at most of polymorphic loci, all showing deficiency or complete absence of heterozygotes. Therefore, we divided these samples into two types as follows: by the differences in allele compositions, 184 specimens examined were divided into 89 genotypes of which we regarded the one, represented by the largest number of specimens (N=58), as the standard genotype. We assessed the degree of similarity of each of other genotypes to the standard genotype, by simply counting the number of alleles (maximum = 18 for the total nine loci) shared between them. As a result we recognized bimodal distributions of specimens against the genotypic similarities with complete lack of intermediates (Fig. 2). When we applied the same procedure for each local sample, we also obtained similar bimodal distributions. From these results, we expected that each sample actually consists of two genetic types.

These results strongly indicated that each specimen examined actually represents one of the two genetic types, and that the two types coexist with each other in all localities examined. We, therefore, grouped specimens into two separate sub-samples (hereafter referred as locality name plus A or B) and compared allelic frequencies between them for each locality. The two types demonstrably differed from each other in frequencies of dominant alleles at four loci
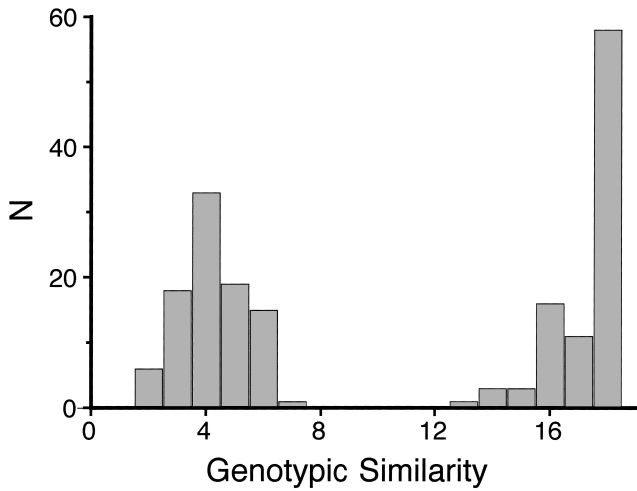
Fig. 2. Frequency distribution of specimens against genotypic similarities relative to the standard (most common) genotype.

(*ADH-A*, *ACOH-A*, *ACOH-B*, and *SOD-A*) (Fig. 3 and Table 2). Namely, the type A is genetically characterized by possessing alleles a, b, and d at *ADH-A* locus, c at *ACOH-A*, a, c, and d at *ACOH-B*, a at *SOD-A*, while the type B is characterized by possessing alleles c at *ADH-A*, b and e at *ACOH-A*, a at *ACOH-B*, c at *SOD-A*. HOSHINO-B sub-sample differed from other type B sub-samples at *ACOH-A* locus, but allelic compositions of other loci of HOSHINO-B

sub-sample were similar to those of other sub-samples. These results indicated that the above four loci are reliable characters to diagnose each type and that there is little genetic exchange between the two types. After we split samples from each of four localities into two genetic types, we made statistical tests for genotype frequencies in each of the eight sub-samples with the Hardy-Weinberg expectation. The results clearly indicated better fitness to the expectation of genotype frequencies than in calculations made for undivided original samples. Of 72 genotype frequency data (8 sub-samples × 9 loci), 44 proved to be monomorphic, and of the remaining 28 polymorphic combinations, 25 (89%) did not differ significantly from the expectation values ($P > 0.05$). Rather high percentage (11%) of deviations from the expectation was ascribed to significant heterozygote deficiencies in a few loci in two sub-samples (*ADH-A* and *MDH-B* in AMAGI-A; *PGM-A* in KITAKYUSHU-A).

Morphological variation

In AMAGI-B, preliminary analyzed for variation within a sub-sample, females were slightly, but significantly larger than males. We, therefore, separately analyzed SVL variations among four sub-samples from KITAKYUSHU and AMAGI. In both sexes, the sub-samples of the type A or those of the type B from different localities did not differ significantly in any combination compared, but sub-samples of the two genetic types were collectively significantly different
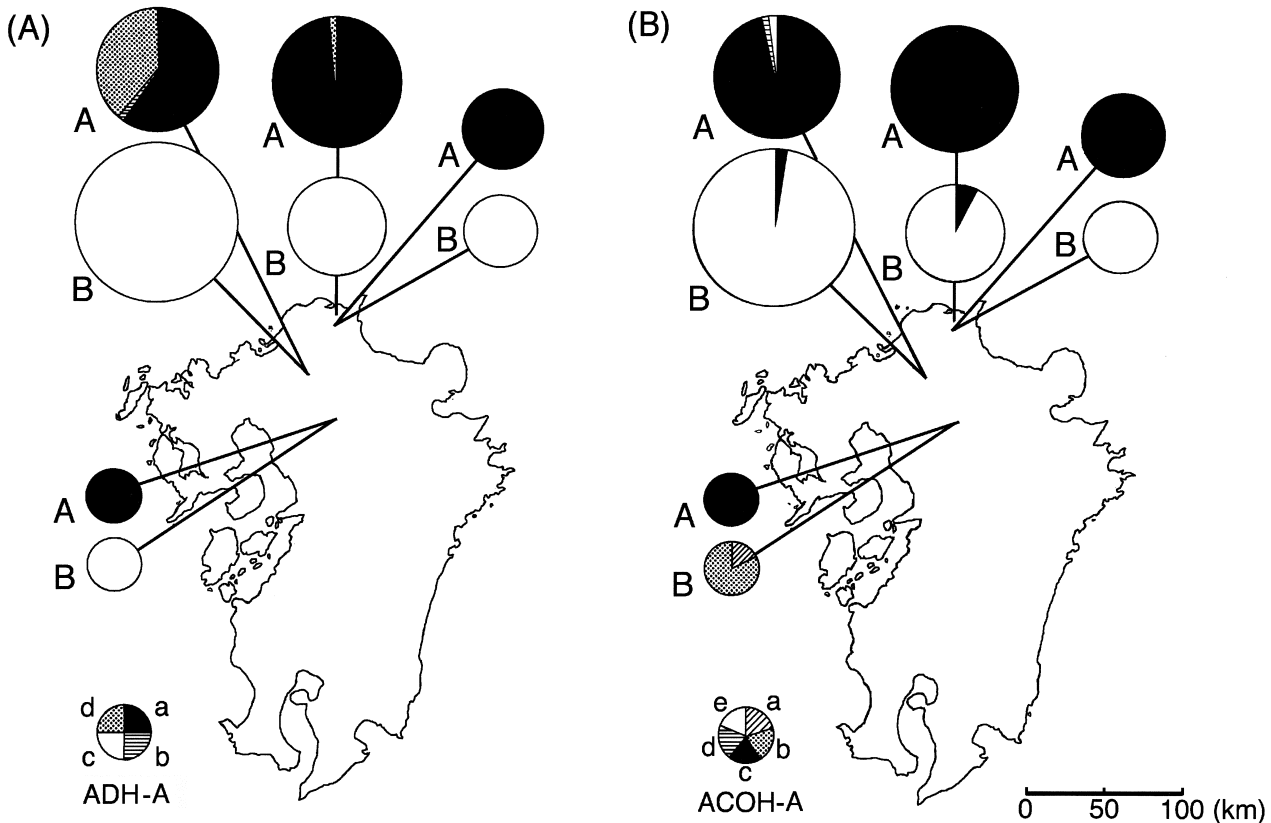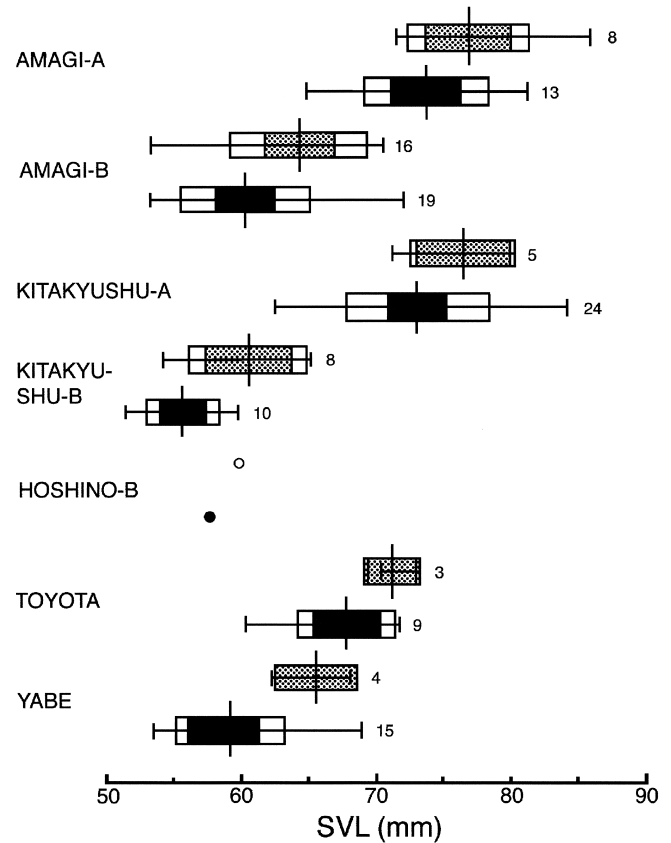

Fig. 3. Distribution of allelic frequencies at *ADH-A* (A) and *ACOH-A* (B) loci in two genotypes from four localities of Fukuoka Pref. Upper circle: Type A, lower circle: Type B. Letter near the circle indicates the genetic type (A or B).

Table 2. Genetic distributions for polymorphic loci resolved, and genetic variability at nine loci among *Hynobius naevius*. A=mean number of alleles per locus; P=percentage of loci polymorphic; H=mean hetrozygosity by direct count.

	Sample							
	AMAGI		KITAKYUSHU		NOGATA		HOSHINO	
Type	A	B	A	B	A	B	A	B
Locus (N)	(37)	(59)	(41)	(24)	(11)	(6)	(3)	(3)
ADH-A	a0.60	d1.00	a0.99	d1.00	a1.00	d1.00	a1.00	d1.00
	b0.01		c0.01					
	c0.39							
ACOH-A	c0.98	c0.03	c1.00	c0.06	c1.00	e1.00	c1.00	a0.17
	d0.01	e0.97		e0.94				b0.83
	e0.01							
ACOH-B	a0.01	a1.00	a0.04	a0.94	a0.09	a1.00	c0.83	a1.00
	c0.95		b0.34	c0.06	c0.91		d0.17	
	d0.04		c0.62					
LDH-A	b1.00	c1.00	b1.00	b0.62	b1.00	b0.42	b1.00	a0.50
				c0.38		c0.58		b0.50
MDH-A	a0.07	b0.02	b0.48	b0.06	b0.50	c1.00	b1.00	c1.00
	b0.84	c0.98	c0.52	c0.94	c0.50			
	c0.09							
MDH-B	a0.78	a0.99	a1.00	a1.00	a1.00	a1.00	a0.33	a1.00
	b0.22	b0.01					b0.67	
PGM-A	a0.01	b1.00	a0.38	b1.00	a0.50	b1.00	b1.00	b1.00
	b0.92		b0.62		b0.45			
	c0.07				c0.05			
PGM-C	a0.03	d1.00	c0.91	b0.10	b0.05	d1.00	f1.00	d1.00
	b0.05		e0.09	c0.08	c0.68			
	c0.15			d0.82	d0.27			
	d0.19							
	e0.34							
	f0.20							
	g0.04							
SOD-A	a0.95	c1.00	a1.00	c1.00	a1.00	c1.00	a1.00	c1.00
	b0.01							
	c0.04							
A	3.11	1.33	1.67	1.67	1.67	1.11	1.22	1.22
P	88.9	33.3	55.6	55.6	44.4	11.1	22.2	22.2
H	0.183	0.013	0.133	0.111	0.172	0.056	0.111	0.074

Fig. 4. A comparison of ranges, means, standard deviations, and standard errors of snout vent length (SVL) in males (black bar) and females (dotted bar) of seven populations, showing the difference between the two types within one locality. Specimens from NOGATA are included in KITAKYUSHU-A or -B (see text).

For the reference samples from TOYOTA and YABE, the following results were obtained. In each sex, the YABE sample (62.2–68.1 mm in females, 53.4–68.8 mm in males) differed significantly from the large group but insignificantly from the small group in SVL. Males of the TOYOTA sample (60.3–71.7 mm) were intermediate between the two groups, with significant differences from all other sub-samples. On the other hand, females of the TOYOTA sample (69.8–73.3 mm) differed significantly only from females of the KITAKYUSHU-B among all sub-samples compared.

For allometric comparisons among sub-samples, we combined male and female adults for 10 morphological characters (HW, LJL, SL, IND, UEL, TRL, TAL, HLL, VTW, and VTL), because these characters showed no significant differences in either slope or position of regression lines against SVL between the sexes in the AMAGI-B sub-sample preliminarily analyzed. Young specimens were separately analyzed from adults because they significantly differed in some of above 10 characters in the AMAGI-B sub-sample.

We compared the above 10 characters among each of two sub-samples from AMAGI and KITAKYUSHU and samples from TOYOTA and YABE using ANCOVA. As shown in Table 3, there were significant differences in many charac-

in SVL (Tukey range test, $P<0.05$). In females, specimens assigned to the type A were larger (SVL=71.2–85.9 mm) than the type B specimens (53.3-70.4 mm). Similarly, males of the type A (62.4–84.2 mm) were larger in SVL than males of the type B (51.3–72.0 mm; Fig. 4). Thus, the types A and B could be regarded as the large and small groups, respectively.

Sub-samples from HOSHINO were not included in the above statistic analyses, because of the insufficient number of specimens (only one adult for each sex of the type B and no adult specimens for the type A). Nonetheless, SVLs of the type B specimens (a female with 59.8 mm and a male with 57.7 mm SVL) fell within the ranges of the small group.

ters in the position of the regression lines between the types A and B in AMAGI and KITAKYUSHU both in adults and young. TOYOTA differed more significantly in the position from the type B than from the type A, while YABE differed more from the type A and TOYOTA than from the type B (Table 3). When all these sub-samples or samples were clustered by UPGMA using the total number of differences in slope and position (Table 3), two discrete groups were recognized in both of adults and young (specimens of TOYOTA and YABE not available). Irrespective of localities, four sub-samples formed two groups each of which included only

Table 3. The number of morpholological characters that differed significantly (*P*<0.05) in either slope (upper right) or position (lower left) of regression lines among four sub-samples from Fukuoka Pref. (AMA=AMAGI, KIT=KITAKYUSHU) and two additional samples from adjacent area (TOY=TOYOTA, YAB=YABE). The first and second numerals indicate young and adults, respectively (young specimens are lacking in TOY and YAB). A and B following locality abbreviations indicate genetic types.

|	AMA-A	AMA-B	KIT-A	KIT-B	TOY	YAB
AMA-A		0, 1	1, 0	2, 2	–, 0	–, 1
AMA-B	10, 10		3, 1	2, 1	–, 1	–, 0
KIT-A	4, 4	9, 10		4, 2	–, 3	–, 1
KIT-B	9, 9	4, 2	5, 10		–, 1	–, 1
TOY	–, 1	–, 10	–, 4	–, 9		–, 2
YAB	–, 10	–, 4	–, 10	–, 4	–, 10	
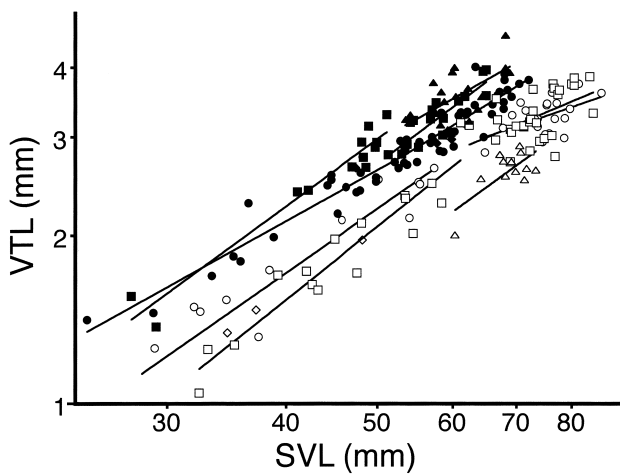
Fig. 5. Relationship between SVL and length of vomerine teeth series (VTL) in eight populations. Open circle: AMAGI-A (regression equation: log VTL=0.592log SVL-0.595, r=0.514 in adult, and log VTL=1.193log SVL-1.677, r=0.939 in young); Closed circle: AMAGI-B (regression equation: log VTL=1.047log SVL-1.363, r=0.835 in adult, and log VTL=0.930log SVL-1.159, r=0.944 in young); Open square: KITAKYUSHU-A (regression equation: log VTL=0.719log SVL-0.826, r=0.508 in adult, and log VTL=1.358log SVL-1.989, r=0.948 in young); Closed square: KITAKYUSHU-B (regression equation: log VTL=1.274log SVL-1.734, r=0.869 in adult, and log VTL=1.244log SVL-1.640, r=0.972 in young); Open diamond: HOSHINO-A; Closed diamond: HOSHINO-B; Open triangle: TOYOTA (regression equation: log VTL=1.235log SVL-1.852, r=0.706 in adult); Closed triangle: YABE (regression equation: log VTL=0.957log SVL-1.154, r=0.664 in adult).

one genetic type. TOYOTA belonged to the group including sub-samples of the type A from Fukuoka Pref., while YABE was a member of another group that encompassing the type B sub-samples.

Specimens of the type A and TOYOTA were characterized by a shorter vomerine teeth series than those of the type B and YABE, and specimens of TOYOTA had the shortest vomerine teeth series in those of all sub-samples or samples examined (Fig. 5).

Results from the PRINCOMP of adult males are presented in Fig. 6A. The first principal component (PRN1) had an eigenvalue of 18.33 (proportion: 0.83) and second (PRN2) had a value of 1.19 (proportion: 0.09). In the first component, all but VTL of 22 variables had eigenvectors between 0.181 and 0.229. In the second component VTL had large vectors (0.869). As a whole, two major groups were separated in the first and second axes; one group consisted of the type B and YABE, and the other consisted of the type A and TOYOTA.

Fig. 6B shows results for adult females. Eigenvalues of the first and second principal components were nearly sim-
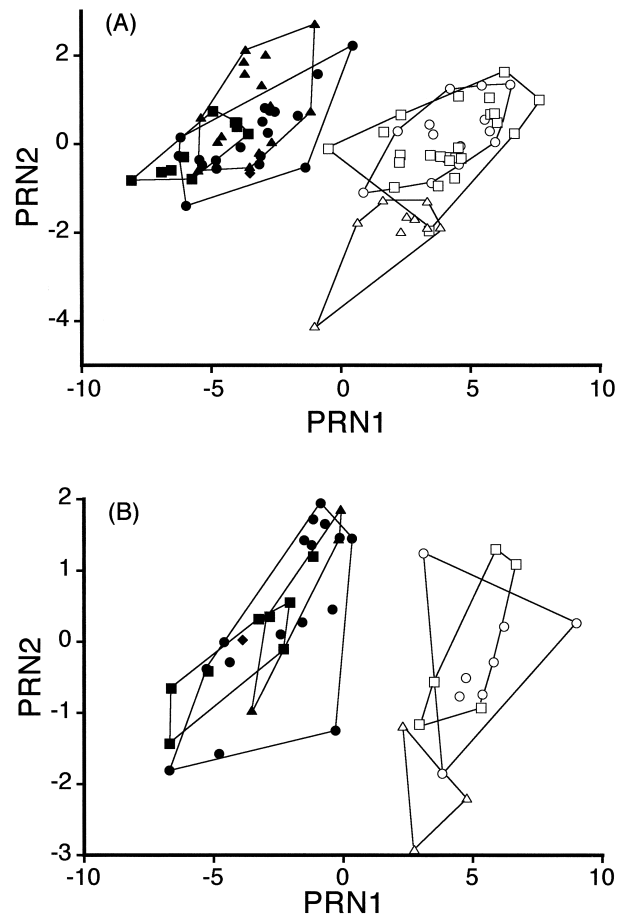
Fig. 6. Plot of first against second principle scores from PCA for seven populations. Open circle: AMAGI-A; Closed circle: AMAGI-B; Open square: KITAKYUSHU-A; Closed square: KITAKYUSHU-B; Closed diamond: HOSHINO-B; Open triangle: TOYOTA; Closed triangle: YABE. A=males; B=females.

ilar to those of males [16.95 (proportion: 0.77) and 1.44 (proportion: 0.07), respectively]. In the first component, all variables had similar eigenvectors (0.176–0.236) except for VTL and TAW that had relatively large vectors (0.869 and 0.515, respectively) in the second component. Like in males, the identical two major groups were recognized in the first and second axes.

Fig. 7A shows a scatter diagram of CANDISC of adult males. The eigenvalues of the first (CAN1) and second (CAN2) axes accounted for 35.93 (proportion: 0.82) and 3.12 (proportion: 0.07), respectively. On the first axis, the highest absolute magnitude of the standardized canonical discriminant coefficients was -6.24 of SVL, followed by HL (3.50) and TRL (2.58). On the second axis, SVL (–7.75), TRL (4.79), FLL (–2.38), and HLL (–1.86) were high contributors. As in results from the PRINCOMP, we recognized two groups (type B and YABE, vs. type A and TOYOTA) in the results of CANDISC.

A scatter diagram of CANDISC of adult females is shown in Fig. 7B. Both of the first (CAN1) and second (CAN2) axes had the eigenvalues similar to those of males

[38.73 (proportion: 0.76) and 5.23 (proportion: 0.10), respectively]. Absolute magnitude of the standardized canonical discriminant coefficients was one fold higher than in males on the first axis (–68.39 of SVL), but as in males, higher magnitude was found in TRL (52.57), and HL (15.49). Similarly, SVL (–12.53), TRL (11.21), HLL (–6.29), and FLL (4.08) were high contributors on the second axis like males. Two groups recognized included members also similar to those found in males. From above analyses, we confirmed again that the two genetic types from Fukuoka Pref. also differ from each other in morphometric characters.

Further morphological difference between the two genetic types also resides in the color patterns. Specimens belonging to the type A had bluish purple ground color without mottling on the dorsal surface of head and body, but with pale white mottling ventrally. Specimens from TOYOTA differed from the type A, having the grayish purple ground color with the grayish to yellowish white mottling. Color pattern of the type B and YABE specimens more conspicuously differed from that of the type A. The ground color was reddish purple, and was covered by white to brownish white mottling on whole body surface.

DISCUSSION

Results from this study revealed that salamanders currently recognized as *H. naevius* is actually comprised of two genetically and morphologically distinct types (A and B) that occur sympatrically in a wide range of Fukuoka Pref., northern Kyushu. Most of adults from this region are easily classified into either of the two types from their body size and pattern of coloration; Adults of the type A are large, reach to 75 mm or more in SVL, and lack mottling pattern on bluish purple dorsum of head and body, while adults of the type B are small, usually less than 70 mm in SVL, and have white to brownish white mottling pattern on reddish purple ground color of body. Further, specimens of the type A are characterized by relatively short vomerine teeth series compared with that of the type B. More importantly, there is a large genetic differentiation between the two types, and they are clearly discriminated from each other by checking alleles they possess; the two types from each locality showed fixed differences at *ADH-A* locus. This implies that these two sympatric types are isolated reproductively at least in the localities examined by us. Because each of the two types is regarded as being consisted of genetically and morphologically unique entity, we consider that each of them is regarded as a good biological species. However, since *H. naevius* (sensu lato) is distributed in a wide area of western Japan, further studies including samples from other regions are necessary for taxonomic conclusions.

In our continuous field observations including supposed breeding seasons, we could find only adults of the type A, some near the egg sacs beneath stones in the streams. We randomly collected these egg sacs and reared hatched larvae to metamorphosis (SVL=23.4–26.4 mm). They proved
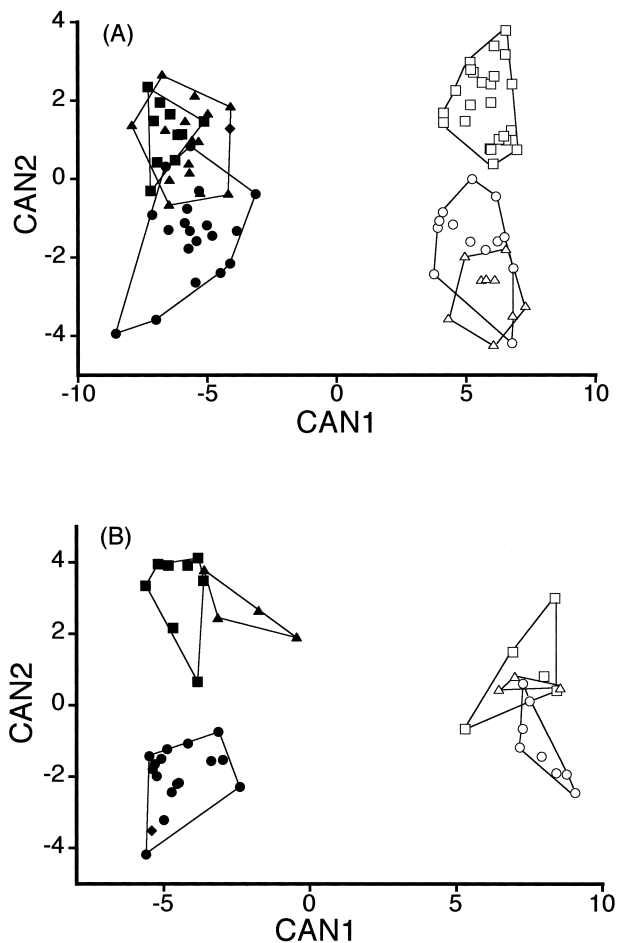
Fig. 7. Plot of first against second canonical variates from CAN for six populations. Open circle: AMAGI-A; Closed circle: AMAGI-B; Open square: KITAKYUSHU-A; Closed square: KITAKYUSHU-B; Closed diamond: HOSHINO-B; Open triangle: TOYOTA; Closed triangle: YABE. A=males; B=females.

to belong to the type A by later allozyme analyses. By contrast, none of the adults or egg sacs of type B have been found in the streams, but we got just metamorphosed young individuals (SVL=19.3–19.7 mm) under the ground far from the stream, that were genetically identified as the type B. We, therefore, suspect that the two types have different breeding habits and such reproductive differences might play major roles as premating isolation mechanisms (Mayr, 1963) between the two types.

Sato *et al.* (1994) recognized three allopatric morphotypes in adults of this species in Kyushu (northern Kyushu type: no white mottling on the dorsal surface of head and trunk; central Kyushu type: white mottling on the surface of large body; southern Kyushu type: white mottling covering over whole surface of small body).

Some of our specimens from localities within the range of the northern Kyushu type of Sato *et al.* (1994) had white mottling on dorsum, a feature of the central Kyushu type, and the contrary case also held. In genetic analyses, both of the northern and central Kyushu types of Sato *et al.* (1994) were identified as the type A. We surmise that dorsal white mottling varies continuously, and is actually not diagnostic to differentiate the northern and central types of Sato *et al.* (1994). Although only young individual available prohibited morphological assignment, samples from HOSHINO, a locality within the range of the central Kyushu type of Sato *et al.* (1994), were assigned to either of the two types A or B in our genetic analysis. HOSHINO-B had unique alleles in *ACOH-A* (Fig. 3), but we consider that the degree of this difference is too small to regard this population as another group. Thus, we at present consider the northern and central types of Sato *et al.* (1994) form a single group and correspond to our type A, while the southern Kyushu type (Sato *et al.*, 1994) corresponds to our type B.

When populations from Fukuoka Pref. were compared morphologically with those from adjacent regions, the type A formed a group with a population from western Honshu (TOYOTA) in the body proportion, although they slightly differed in SVL and coloration. From our own results and available published reports, we think that the type A represents a lineage now distributed in an area from northern Kyushu to Chugoku district [the standard type of Sato (1943)], while the type B represents another one now known from northern to southern Kyushu. Most Japanese *Hynobius* species are considered to have diverged as a result of geographic isolations, probably due to their low dispersal abilities (Matsui *et al.*, 2000; Nishikawa *et al.*, 2001). We surmise that ancestors of these two lineages were also once isolated geographically, then diverged genetically and morphologically in each area, and newly shared an area of sympatry in northern Kyushu by their secondary contact.

Other than *H. naevius*, there are two species of lotic breeding salamanders, *H. boulengeri* and *H. stejnegeri*, in Kyushu. These two species have a large body size and, until now, each of them has been thought to coexist with *H. naevius* in central to southern parts. In the light of results of this study to split *H. naevius* into the types A and B, we consider that only the type B coexists with *H. boulengeri* or *H. stejnegeri*, and that the type A is allopatric with these two species (Fig. 8; data partly from Nishikawa *et al.*, 2001). We suspect that it would be probably difficult for the type A to coexist with *H. boulengeri* or *H. stejnegeri,* because these salamanders similarly have large body sizes and their ecological niche would overlap. On the contrary, the small body size in the type B seems to have allowed this salamander to coexist with larger salamanders.

Generally, in Japanese small salamanders, lotic breeders oviposit eggs that were represented by larger sizes and smaller number than in lentic breeders. In case of lotic breeders from Kyushu, the type A of *H. naevius*, *H. boulengeri* and *H. stejnegeri,* all with large adult body size, usually lay eggs in montane streams, and larvae hibernate there and become relatively large before metamorphosis. Although we still have not yet discovered egg sacs or larvae of the type B of *H. naevius* in spite of our intensive field survey, we suspect that the breeding site of the type B might be not in open streams where larger forms utilize, but in very small streams under the ground where larvae, hatched from large eggs with large amount of yolks, might develop without feeding and metamorphose at a small body size (SVL=19.3–19.7 mm compared with 23.4–26.4 mm in type A). This type of unique larval development and metamor-
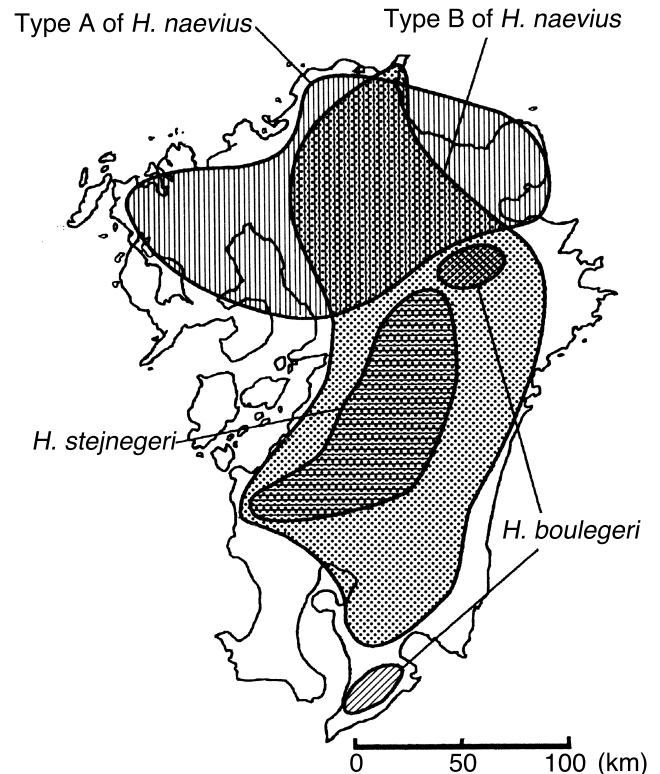
Fig. 8. A map of Kyushu, showing ranges of distributions of the type A and type B of *H. naevius*, *H. boulengeri*, and *H. stejnegeri*. Data for distributional ranges of *H. boulengeri*, and *H. stejnegeri* were taken from Nishikawa *et al.* (2001)

phosis has been reported for a Shikoku population of *H. naevius* (Tanabe, 2002).

Misawa and Matsui (1997, 1999) reported that the body size at metamorphosis, but neither growth rate nor age at sexual maturity, strongly affects the adult size in *H. kimurae*, a lotic breeding species from Honshu with ecological traits similar with that of *H. naevius*. We suspect that the size at metamorphosis is a factor to affect the adult size also in *H. naevius*. If this is the case, the body size difference in the two types in the area of sympatry would reflect the difference in size at metamorphosis.

Character displacement in body size has been reported in several animals (e.g., beetles: Kawano, 2002; salamanders: Jaeger *et al.*, 2002; frogs: Matsui, 1994; lizards: Roughgarden *et al.*, 1983). These previous reports predict that body size of the related species overlaps in allopatric situations, and that one species becomes larger while the other becomes smaller in sympatric situations (but see Roughgarden *et al.*, 1989). The type A from northern Kyushu was larger than that from western Honshu in our result, and this seems to support the idea that the type A lineage diverged toward larger body in Kyushu. However, allopatric populations of the type B from southern Kyushu seem to have a body size similar to those from northern Kyushu where they are sympatric with the type A (Tominaga, unpublished data). Thus, it is not easy at present to discuss the body size difference between the two types with relation to character displacement, and further survey in larger area of Kyushu is badly needed.

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Appendix 1 Voucher specimens used for this study are stored at Graduate School of Human and Environmental Studies, Kyoto University (KUHE), Osaka Museum of Natural History (OMNH), Mr. Tanabe's private collection (T), and Mr. Sato's private collection (S).

Type A: AMAGI (Amagi-shi, Fukuoka Pref.): KUHE 25997, 26165-26167, 26641-26642, 26857-26858, 26860-26861, 27282-27284, 27807, 27883-27885, 27899-27900, 27904-27905, 28372-28374, 28377, 28487-28488, 28577-28578, 28673-28674, 28955-28957, 29364, 29367, 29369; KITAKYUSHU (Kitakyushu-shi, Fukuoka Pref.): KUHE 12984-12988, 16746-16752, 22798-22801, 27128, 27133-27134, 27267, 27271-27272, 27281, 27909-27910, 28202, 28204-28207, 28379-28383, 28496, 28579-28582, T 2400; NOGATA (Nogata-shi, Fukuoka Pref.): KUHE 26713-26715, 27160, 27163-27164, 27200, 27303-27304, 27306-27307; HOSHINO (Hoshino-mura, Fukuoka Pref.): KUHE 27299-27301; TOYOTA (Toyota-cho, Yamaguchi Pref.): KUHE 28599-28610.

Type B: AMAGI: KUHE 26092, 26168-26179, 26635-26640, 26859, 26862, 27143, 27808, 27886-27898, 27901-27903, 27906-27908, 28375-28376, 28485-28486, 28489-28493, 28675, 29254-29258, 29365-29366; KITAKYUSHU: KUHE 26706-26707, 27127, 27130-27132, 27268-27270, 27911, 28201, 28203, 28384-28391, 28497-28500, S 3 unnumbered specimens; NOGATA: KUHE 26716-26717, 27161-27162, 27201, 27305; HOSHINO: KUHE 26622-26623, 27298; YABE (Yabe-machi, Kumamoto Pref.): KUHE 28007-28015, OMNH Am 10141-10143, 10145-10148, 10150-10151, 10155.

Appendix 2 Dimensions of characters used in this study.

SVL (snout-vent length): from tip of snout to anterior tip of vent; HL (head length): from tip of snout to wrinkle of throat; HW (head width): measured at angle anterior to parotoid grand; LJL (lower jaw length): from tip of lower jaw to the angle of jaw; SL (snout length): from tip of snout to anterior tip of upper eyelid; IND (internarial distance): the minimum distance between the external nares; IOD (interorbital distance): the minimum distance between the paired upper eyelids; UEW (upper eyelid width): the greatest width of the upper eyelid; UEL (upper eyelid length): the greatest eye diameter of the eye, including upper eyelid; AGD (axilla-groin distance): the minimum distance between axilla and groin; TRL (trunk length): from wrinkle of throat to anterior tip of vent; TAL (tail length): from anterior tip of vent to tip of tail; TAW (tail width): tail width measured at middle of tail; TAH (tail height): tail height measured at middle of tail; FLL (forelimb length): distance from the axilla to the tip of the longest finger; HLL (hindlimb length): distance from the groin to the tip of the longest toe; 3FL (third finger length): distance from the base point between second and third fingers to tip of the third finger; 3TL (third toe length): distance from the base point between third and forth toes to tip of the third toe; 5TL (fifth toe length): distance from the base point between fourth and fifth toes to tip of the fifth toe; VTW (vomerine teeth series width): the greatest width of vomerine teeth series; VTL (vomerine teeth series length): the greatest length of vomerine teeth series