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# Morphological Variation in a Japanese Salamander, *Hynobius kimurae* (Amphibia, Caudata)

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We studied variation in morphometric and meristic characters and color pattern in the salamander *Hynobius kimurae*, examining 282 males from 24 localities encompassing the whole distributional range of the species in Honshu, the mainland of Japan. Multivariate analyses of 24 morphometric characters resulted in the separation of two groups, (1) eastern populations from the Kanto District to Shizuoka Prefecture of the Chubu District, and (2) central-western populations from Aichi Prefecture of the Chubu District westwards. Similar groups were recognized in meristic characters and color pattern. These geographic patterns of morphological variation coincided with the pattern of genetic differentiation inferred from allozymes in this species, except for the position of one population from the intermediate region. Some of the morphometric and meristic characters significantly correlated with environmental parameters of sampling sites, and suggested effects of differential habitat conditions among populations on the geographic morphological variation in this species.

**Key words:** morphometry, geographic variation, morphocline, biogeography, genetic variation

## INTRODUCTION

Small salamanders of the genus *Hynobius* Tschudi, 1838 are remarkably diversified in Japan, where 17 endemic species have been recognized (Matsui et al., 2004; Tominaga and Matsui, 2008), and several additional, cryptic species are now under study (e.g., Matsui et al., 2006; Nishikawa et al., 2007). *Hynobius kimurae* Dunn, 1923 is a lotic breeder widely occurring in montane regions of Honshu, the main island of Japan, from the Kanto to the Chugoku Districts. Sato (1933) examined the external morphology of 123 adults of a population from the Tango Peninsula, Kyoto Prefecture, Kinki District, and clarified that the species is sexually dimorphic and is fairly variable in dorsal color pattern, degree of development of the fifth toe, and shape of the vomerine tooth series. In addition to high morphological variation even within a population, some individuals of this species superficially resemble *H. naevius* (Temminck and Schlegel, 1838) and *H. yatsui* Oyama, 1947, which is also a lotic breeder widely occurring in western Japan (Sato, 1933; Tominaga et al., 2005b). For this reason, *H. kimurae* was once treated as a subspecies of *H. naevius* sensu lato (Nakamura and Uéno, 1963). However, later discovery of a syntopic distribution (Matsui, 1979, 1981; Okada et al., 1997), as well as results of allozyme electrophoresis (Matsui et al., 2000; Tominaga et al., 2005a) and mitochon-

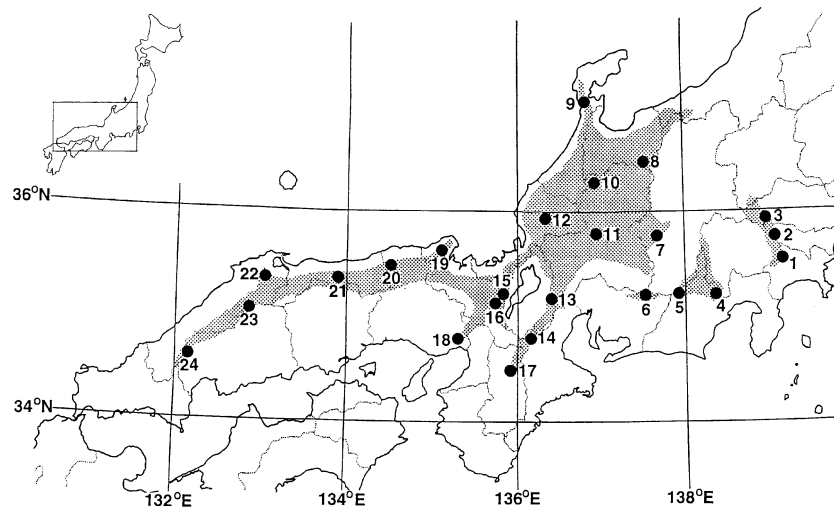
drial DNA analyses (Matsui et al., 2007), of these species clarified their heterospecific and even remote phylogenetic relationship.

According to information available on *H. kimurae*, populations from eastern and western Honshu differ in body size and dorsal markings (Matsui, 1981), karyotype (Ikebe et al., 1986), clutch size and larval life history (Misawa and Matsui, 1997), and growth pattern (Misawa and Matsui, 1999). Results of allozyme analysis of 21 populations (Matsui et al., 2000) indicated this species to include two groups of populations that are highly divergent genetically. However, no detailed morphological analysis of this species has been conducted throughout its wide distributional range. In this study, we analyzed morphological variation in *H. kimurae*, using many specimens from representative localities covering the whole species range, to examine whether or not the patterns of genetic and morphological variation are congruent with each other.

## MATERIALS AND METHODS

A total of 282 specimens representing 24 populations (Pop.), including those from the type locality (Mt. Hieizan, Otsu City, Shiga Pref.), were used in this study. Briefly, they were from three localities in the Kanto District (Pops. 1–3), nine localities in the Chubu District (Pops. 4–12), eight localities in the Kinki District (Pops. 13–20), and four localities in the Chugoku District (Pops. 21–24) (Fig. 1, Table 1); a specimen list is available from the authors on request. All but populations 1, 14, and 23 have been studied electrophoretically for allozyme variation (Matsui et al., 2000). Collections were made between 1989 and 1998, mainly between November and April, when males congregate under stones in small streams where breeding occurs. We used only adult males, because some

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**Fig. 1.** Map of Honshu, Japan, showing the locations of 24 populations of *Hynobius kimurae* included in this study. For population names, refer to Table 1 (Pops. 1–3, Kanto District; Pops. 4–12, Chubu District; Pops. 13–20, Kinki District; Pops. 21–24, Chugoku District). Hatched area indicates known distribution range of *H. kimurae*.

**Table 1.** Populations of *Hynobius kimurae* studied.

Pop. no.	Locality	n	Latitude (°N)	Longitude (°E)	Altitude (m)	Temperature (°C)	Precipitation (mm)
1	Kiyokawa-mura, Kanagawa Pref.	7	35°28'	139°14'	550	11.9	1540
2	Hachioji-shi, Tokyo Pref.	21	35°42'	139°13'	450	11.9	1111
3	Yokoze-machi, Saitama Pref.	15	35°56'	139°07'	740	9.8	1083
4	Shizuoka-shi, Shizuoka Pref.	13	35°01'	138°24'	980	9.9	2218
5	Hamamatsu-shi, Shizuoka Pref.	14	35°16'	137°57'	1100	9.0	1846
6	Toyota-shi, Aichi Pref.	12	35°14'	137°35'	1100	8.5	1573
7	Kiso-machi, Nagano Pref.	10	35°05'	137°41'	1080	7.6	1683
8	Toyama-shi, Toyama Pref.	7	36°29'	137°26'	1200	6.3	1934
9	Hodatsushimizu-cho, Ishikawa Pref.	8	36°47'	136°05'	480	10.6	1912
10	Shirakawa-mura, Gifu Pref.	7	36°16'	136°57'	1020	6.5	1876
11	Gujyo-shi, Gifu Pref.	5	35°44'	137°00'	460	10.7	2241
12	Echizen-shi, Fukui Pref.	15	35°52'	136°12'	420	11.3	1963
13	Koga-shi, Shiga Pref.	15	35°00'	136°22'	620	9.2	1522
14	Iga-shi, Mie Pref.	5	34°42'	136°17'	760	10.7	1327
15	Otsu-shi, Shiga Pref.	12	35°04'	135°51'	720	10.8	1645
16	Kyoto-shi, Kyoto Pref.	21	35°08'	135°45'	550	11.4	1600
17	Sakurai-shi, Nara Pref.	13	34°28'	135°53'	720	10.5	1239
18	Kobe-shi, Hyogo Pref.	15	34°46'	135°15'	680	11.0	1301
19	Kyotango-shi, Kyoto Pref.	15	35°33'	135°02'	320	12.5	1865
20	Shiso-shi, Hyogo Pref.	16	35°19'	134°31'	1040	7.8	2092
21	Misasa-cho, Tottori Pref.	10	35°24'	134°01'	580	10.8	1890
22	Unnan-shi, Shimane Pref.	11	35°19'	133°03'	380	12.2	1558
23	Shobara-shi, Hiroshima Pref.	8	35°05'	132°52'	1040	7.6	1330
24	Akiota-cho, Hiroshima Pref.	7	34°36'	132°11'	960	8.5	1747

*Hynobius* species are known to exhibit sexual dimorphism in some morphological characters (e.g., Sato, 1933, 1943; Tominaga et al., 2005b; Nishikawa et al., 2007), and it is more difficult to collect females than males.

For specimens fixed in 10% formalin and later preserved in 70% ethanol, we took 26 body measurements (Appendix 1) to the nearest 0.1 mm with dial calipers, and when necessary, under a binocular dissecting microscope. Tail length (TAL) and fifth toe length (5TL) were omitted from all analyses because many individuals had damaged or regenerated tails, and some individuals completely lacked their fifth toes.

In the univariate analyses among samples, SVLs were compared by the Tukey-Kramer test (Sokal and Rohlf, 1995). We then calculated the percentage ratio (R) of each morphometric character to SVL, and made comparisons using Dunn's multiple comparisons (Sokal and Rohlf, 1995). Additionally, ratios considered to be taxonomically important [vomerine tooth series width to length (VTW/VTL) and width to height of the tail in its middle region (MTAW/MTAH)] were also compared among samples.

In the multivariate analyses, we used log-transformed raw values as input variables. We adopted multiple-group principal component analysis (MGPCA) (e.g., Thorpe and Leamy, 1983; Thorpe,

1988; Overton et al., 1997), because the results of simple multivariate analyses are sometimes strongly affected by absolute body size (Carr, 1996; Adams and Beachy, 2001). MGPCA was conducted based on all 24 morphometric characters. To assess the relationship between genetic and morphometric differentiation, we examined correlations of Mahalanobis distances thus obtained to Nei's (1978) genetic distances among samples reported by Matsui et al. (2000). We used a statistical package (SAS, 1990) and ran analyses through the facilities of Data Processing Center of Kyoto University.

We counted the numbers of teeth on the upper and lower jaws and vomer under a binocular dissection microscope, and used Dunn's multiple comparisons (Sokal and Rohlf, 1995) for interpopulation comparisons. For comparisons of dorsal body color, we first divided specimens into seven types (A–G) by the amount of yellow spots on the brown dorsum, based on Sato's (1943) illustration. However, no specimen examined had a pattern similar to type A (no yellow spots) of Sato (1943). On the contrary, we found many individuals with more yellow spots than in type G (most widely spotted by yellow) shown by Sato (1943). We, therefore, added type H to accommodate specimens with more spots than type G. Because types B to E of Sato (1943) vary continuously, it was sometimes difficult to classify individuals according to this system. We therefore defined the following four types: Type 1=types A to C of Sato (1943), with dorsal yellow spots at most several in number; Type 2=types D and E of Sato (1943), with dorsal yellow spots fewer than 10 per cm<sup>2</sup>; Type 3=type F of Sato (1943), with 10 or more dorsal yellow spots per cm<sup>2</sup>; Type 4=type G of Sato (1943), with dorsal yellow spots continuous, forming yellow marbling; and newly designated type H, with dorsal ground color yellow (see Fig. 5). We used Fisher's exact test implemented in the software R 2.3.1 (R Development Core Team, 2006) to compare interpopulation variation in the frequency of these four color types.

To estimate relationships between environmental factors (X) and morphological variation (Y), we first analyzed correlations by simply regressing Y linearly with X. Mean SVL and medians of morphometric variables, and averages of geographic (latitude, longitude, and altitude) and climatological (mean annual temperature and annual precipitation) parameters for collecting sites, were calculated for each population sample. Using R 2.3.1, we then performed partial regression analyses (Zar, 1996) by regressing Y with only one environmental factor and controlling the other factors. In this procedure, all 24 populations were combined first, and then five eastern (Pops. 1–5) and 18 western populations (Pops. 7–24) were analyzed separately. This was because the results of MGPCA indicated significant morphological differentiation between these two groups. Population 6 was omitted because of its ambiguous placement in the two groups (see Results). However, the small number of eastern populations made it impossible to conduct the analysis, and hence only western populations were considered. Climatological parameters were obtained from the Japan Meteorological Agency (URL: <http://www.data.kishou.go.jp>). Because in-situ data were rarely taken exactly where we collected specimens, we chose from the source above available sites surrounding the actual collection sites and averaged the data from them; see methods in Matsui (1984) and Tominaga et al. (2005b). All comparisons were made using  $P < 0.05$  as significant.

## RESULTS

### Morphometric variation

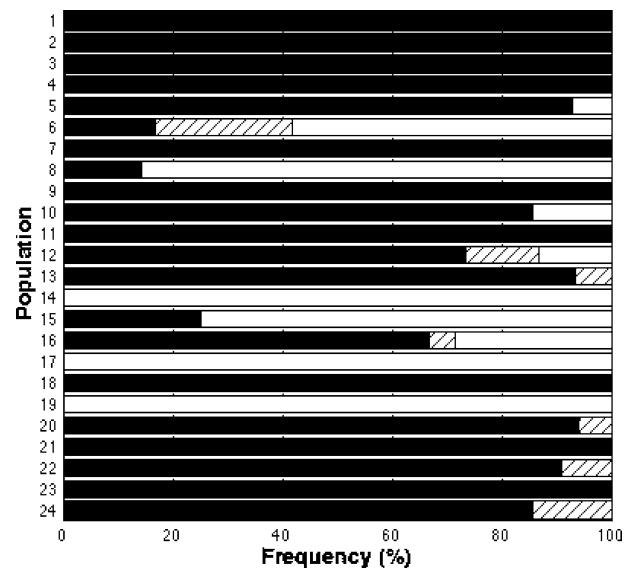
In 282 males examined, SVL ranged from 57.1 to 95.0 mm, with a mean ( $\pm 2SE$ ) of  $71.6 \pm 0.94$  mm (Table 2). Four populations (Pops. 4 and 5 [Chubu] and Pops. 22 and 24 [Chugoku]) were significantly larger than the remaining 20 populations ( $P < 0.01$ ), and mean SVL tended to be larger in the eastern (Pops. 1, 4, 5, Kanto and part of Chubu) and western (Pops. 21–24, Chugoku) populations than in some

geographically intermediate populations (Pops. 7, 8, 11, 13, 14, 16, 19).

Tail length (TAL) was much more unstable than other characters, because 15% (43/282) of males examined had tails damaged or apparently regenerated. Similarly, degree of development of the fifth toe (5TL) was variable, and 23% (65/282) of males lacked the toe on both sides and 3.5% (10/282) lacked it on either side (Fig. 2). A higher occur-

**Table 2.** Variation in snout-vent length (SVL, in millimeters) in male *Hynobius kimurae*.

Pop. no.	Mean	2SE	Range
1	73.7	2.26	70.3–79.1
2	71.7	1.46	65.7–80.4
3	71.7	1.93	65.0–76.8
4	83.5	2.98	76.5–93.1
5	86.2	2.45	77.8–91.6
6	72.0	2.04	64.6–77.6
7	63.7	2.13	58.1–67.5
8	61.9	1.36	58.4–63.8
9	71.0	2.81	65.2–78.6
10	71.6	2.06	67.4–75.4
11	63.0	1.83	60.8–66.3
12	68.9	2.14	62.0–76.4
13	65.1	1.80	58.2–70.5
14	65.5	5.23	58.5–73.1
15	68.1	1.53	63.3–72.5
16	63.8	2.09	57.1–78.5
17	67.6	1.85	63.4–73.3
18	68.0	1.22	64.3–71.6
19	66.7	2.74	58.7–76.1
20	68.8	1.89	62.1–76.6
21	74.3	3.24	65.8–82.0
22	85.3	3.91	75.2–93.7
23	76.0	2.93	68.0–78.9
24	82.6	5.85	75.7–95.0



**Fig. 2.** Variation in the development of the fifth toe in 24 populations of *Hynobius kimurae*. Rectangles show the frequency: filled, 5 toes on both sides; hatched, 4 toes on one side and 5 on the other; open, 4 toes on both sides.

rence (>75%) of individuals with only four toes was observed in populations from part of Chubu (Pop. 8) and most of Kinki (Pops. 14, 15, 17, 19). In contrast, all individuals from Kanto (Pops. 1–3), part of Chubu (Pops. 4, 7, 9, 11), part of Kinki (Pop. 18), and part of Chugoku (Pops. 21, 23) had well-developed fifth toes on both hindlimbs.

Table 3 shows variation in ratios of morphometric characters relative to SVL. Interpopulation comparisons showed the greatest variation in RVTL, for which 47 combinations were significantly different, and RMXTAH was also variable (significant difference in 31 combinations) among populations. Of these, RVTL tended to be smaller in easternmost populations (smallest in Pop. 1) than in more western populations, but RMXTAH showed the reverse tendency (larger in Pops. 1–3, 5). The least variable characters were RTRL and RHL (significant difference in one combination, each), and RAGD (significant difference in seven combinations) was also less variable than the others. The vomerine tooth series from eastern populations (Pops. 1–3, 5) was shallow (median of VTW/median of VTL>110.6%), whereas that from some central-western populations (Pops. 10, 13, 19) was very deep (VTW/VTL<79.4%). The middle region of the tail was most compressed (i.e., small MTAW/MTAH, <64.8%) in easternmost populations (Pops. 1, 2), whereas individuals from some central-western populations (Pops. 6, 10, 19, 20, 22) had the least compressed tail (MTAW/MTAH>86.9%).

**MGPCA**

Because the results of MGPCA indicated the first eigenvector to represent size, where each coefficient had a similar sign and magnitude, this vector was omitted in conducting the “size out” CANDISC (Fig. 3). The proportions of eigenvalues of the first three axes of “size out” CANDISC, respectively, accounted for 56.4%, 12.4% and 9.7% (Wilks’s lambda value, 0.000368; *P*<0.01). Two groups, one contain-

ing Kanto and part of Chubu (=eastern group, Pops. 1–5), and another encompassing part of Chubu to Chugoku (=central and western groups, Pops. 6–24), were completely separated by the first and second axes (Fig. 3). In the central-western group, Pop. 6 (part of Chubu) was positioned close to the eastern group, but largely overlapped with other populations, while each of Pop. 22 and Pop. 24 (both Chugoku) only slightly overlapped with the remaining populations.

**Relationship between morphometric and genetic variation**

Because Pop. 6 positioned in the central-western group by MGPCA had been grouped genetically in the eastern group (Matsui et al., 2000), we did not separate the two groups and examined morphological and genetic relationships. As a result, Mahalanobis distances derived from CANDISC positively correlated to Nei’s (1978) genetic distances (*r*=0.612, *P*<0.01) (Fig. 4).

**Meristic character variation**

Medians and ranges of variation in UJTN are shown in Table 4. Population 16 (Kinki) had the smallest and Pop. 2 (Kanto) had the largest median. Populations from the eastern part of the distribution range (Pops. 1–6, Kanto and part of Chubu) had large medians, while the values tended to be small in the western populations (Pops. 15, 16, Kinki). Of 55 combinations showing a significant difference, Pop. 2 was remarkable, with a larger UJTN value than in the other 12 populations (*P*<0.05). By contrast, Pops. 15 and 16 had significantly smaller UJTN values than the other eight and 12 populations, respectively (*P*<0.05). As with UJTN, LJTJN tended to be large in eastern populations (Pops. 1–6, Kanto and part of Chubu), but small in western populations (Pops. 13–24, Kinki and Chugoku). However, differently from UJTN, Pop. 11 (Chubu) had a large LJTJN value, like the

**Table 3.** Medians of percentage ratios of morphometric characters to SVL, and an additional three ratios, in *Hynobius kimurae*. For character abbreviations, refer to Appendix 1, and for population numbers, refer to Table 1.

Pop. no.	RHL	RHW	RMX HW	RLJL	RSL	RIND	RIOD	RUE W	RUEL	ROL	RAG D	RTRL	RBT AW	RMT W	RMX TAH	RMT AH	RFL	RHLL	R2FL	R3FL	R3TL	RVT W	RVTL	VTW/VTL	MTAW/MXTAH	MTAW/MTAH
1	23.7	17.6	18.2	13.2	6.9	6.0	5.6	3.4	5.0	3.0	5.15	76.3	10.7	7.2	13.6	11.1	24.6	29.6	4.8	4.2	7.0	5.2	4.7	110.6	52.9	64.9
2	23.4	17.5	19.1	13.3	6.8	6.6	6.2	3.4	5.4	2.8	5.16	76.6	10.6	8.0	15.3	12.4	24.8	29.0	4.9	4.3	6.9	5.9	5.2	113.5	52.3	64.5
3	23.7	17.2	18.3	13.3	6.9	6.6	6.3	3.6	5.3	2.9	5.18	76.3	10.9	8.3	13.2	10.6	24.3	29.0	4.3	3.8	6.5	5.6	5.0	112.0	62.9	78.3
4	23.5	16.7	17.9	13.2	7.0	6.2	6.0	3.4	5.3	2.8	5.18	76.5	10.7	7.6	12.2	10.4	24.4	28.4	4.0	3.5	5.9	5.8	5.7	101.8	62.3	73.1
5	23.7	17.0	17.9	13.4	7.0	6.2	6.1	3.3	5.0	2.9	5.29	76.3	10.9	8.2	13.1	10.2	24.5	28.8	4.3	3.6	6.1	5.5	5.2	105.8	62.6	80.4
6	23.6	16.9	17.6	13.1	6.7	6.0	5.9	3.2	5.2	3.3	5.17	76.4	10.3	8.5	12.1	9.6	24.3	28.0	4.3	3.7	6.5	5.2	5.4	96.3	70.2	88.5
7	24.0	16.2	17.4	13.2	6.9	6.3	6.2	3.5	5.4	3.0	5.10	76.0	9.9	6.4	10.5	7.9	25.2	29.3	4.4	4.0	6.7	5.6	5.9	94.9	60.9	81.0
8	22.9	17.5	18.2	13.8	6.7	5.9	6.0	3.0	5.5	3.5	5.27	77.1	11.0	7.9	10.7	9.5	22.9	27.3	4.6	4.2	7.2	5.5	6.0	91.7	73.8	83.2
9	23.6	16.9	18.8	12.8	6.8	5.7	5.9	3.4	5.1	3.0	5.31	76.4	11.9	8.4	12.1	11.4	23.6	27.7	4.1	3.4	6.0	5.3	6.2	85.5	69.4	73.7
10	23.2	16.4	17.3	12.7	6.8	5.7	5.4	3.0	5.1	3.0	5.31	76.8	10.3	7.3	9.5	8.4	24.3	29.1	4.1	3.6	6.7	5.0	6.3	79.4	76.8	86.9
11	24.1	16.8	18.5	13.7	6.9	5.8	5.8	3.5	5.8	3.4	5.16	75.9	10.4	7.0	9.8	8.3	24.3	27.4	4.2	3.7	6.2	5.5	6.3	87.3	71.4	84.3
12	23.5	17.0	18.1	13.1	6.8	5.4	5.4	3.5	5.3	3.0	5.31	76.5	11.8	9.0	12.4	10.6	22.6	26.6	4.0	3.5	6.3	4.9	6.1	80.3	72.6	84.9
13	24.2	16.9	18.9	13.3	6.9	5.5	5.8	3.5	5.4	3.3	5.12	75.8	11.2	8.2	11.7	10.4	24.8	28.9	4.7	4.0	6.9	5.3	6.7	79.1	70.1	78.8
14	23.4	16.8	18.2	13.2	6.9	5.7	5.6	3.2	5.2	2.9	5.27	76.6	11.6	8.5	11.7	10.5	22.7	28.3	4.4	3.7	7.2	4.9	5.5	89.1	72.6	80.9
15	23.6	16.9	18.2	13.2	6.8	5.7	5.7	3.1	5.1	2.9	5.34	76.4	11.2	8.0	12.1	10.4	24.0	27.3	4.6	3.8	7.0	4.8	6.0	80.0	66.1	76.9
16	24.1	17.7	18.6	13.4	6.8	5.8	5.6	3.4	5.7	2.9	5.08	75.9	10.3	7.7	12.0	11.2	24.8	27.8	4.5	3.8	6.6	5.6	7.0	80.0	64.2	68.8
17	23.6	16.6	17.8	13.1	6.9	5.9	5.9	3.1	5.2	3.2	5.28	76.4	10.4	7.5	11.7	9.8	22.9	28.0	4.3	3.8	7.2	5.1	5.0	102.0	64.1	76.5
18	23.9	16.9	18.5	13.1	6.9	5.8	5.7	3.4	5.2	2.9	5.29	76.1	11.2	7.9	11.0	9.7	23.8	28.0	4.4	4.1	6.9	5.5	6.3	87.3	71.8	81.4
19	23.8	17.4	18.9	13.1	6.9	6.2	6.0	3.6	5.3	3.0	5.26	76.2	12.1	9.8	11.1	11.0	23.9	28.4	4.3	3.8	7.1	4.9	6.4	76.6	88.3	89.1
20	23.5	16.4	17.5	13.0	6.8	6.1	6.0	3.1	5.2	3.2	5.32	76.5	9.9	7.9	10.6	8.9	23.7	28.0	4.0	3.5	6.5	5.5	6.2	88.7	74.5	88.8
21	23.7	17.4	18.2	13.0	6.7	5.9	5.7	3.3	5.1	2.9	5.23	76.3	11.0	8.1	11.5	10.2	23.7	28.2	4.5	4.1	6.7	5.3	6.0	88.3	70.4	79.4
22	23.8	18.0	18.8	13.1	6.6	5.9	5.9	3.5	5.0	2.7	5.24	76.2	11.8	9.1	11.5	10.2	23.3	28.1	4.2	3.6	6.0	5.3	6.4	82.8	79.1	89.2
23	23.8	17.0	18.4	12.9	6.7	5.9	5.9	3.0	4.9	3.0	5.28	76.2	10.6	8.3	11.5	10.4	24.0	28.4	4.2	3.8	6.5	5.5	6.3	87.3	72.2	79.8
24	23.5	16.7	18.3	13.0	6.7	6.0	5.9	3.1	4.9	2.8	5.22	76.5	11.5	8.5	11.9	10.4	23.0	27.7	4.0	3.4	6.2	5.7	5.9	96.6	71.4	81.7

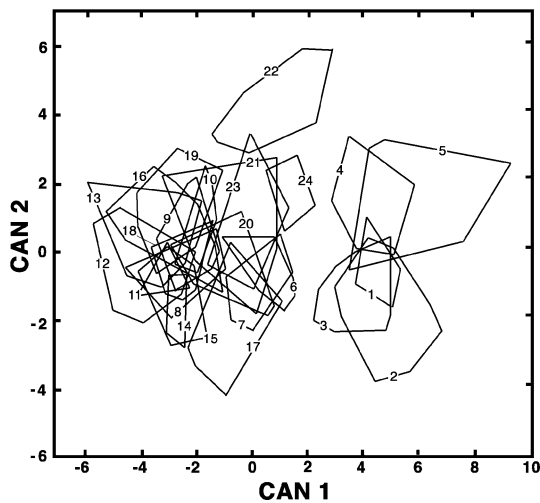


Fig. 3. Plot of first against second canonical variables for 24 populations of *Hynobius kimurae*.

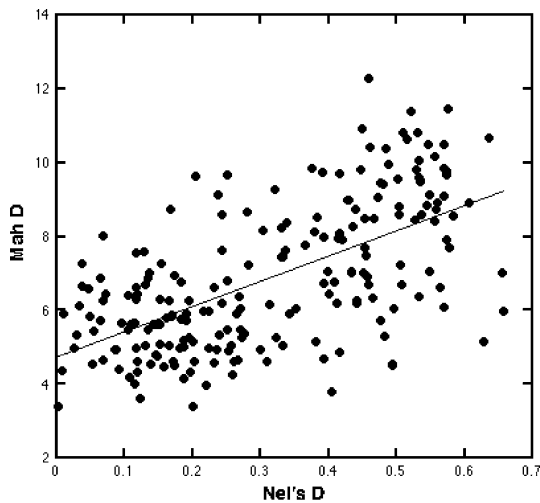


Fig. 4. Correlation between genetic and morphological variation in *Hynobius kimurae*. Genetic (x) and morphological (y) variation are shown by Nei's (1978) genetic distance (Matsui et al., 2000) and Mahalanobis distance obtained from the CANDISC procedure, respectively. There is a significantly positive correlation ( $y=6.84x+4.74$ ,  $r=0.612$ ,  $P<0.01$ ).

eastern populations. The smallest and the largest LJTN values were observed in Pop. 16 (Kinki) and Pop. 5 (Chubu), respectively. Significant differences were found in 56 combinations, and Pops. 15 and 16 (Kinki) had significantly smaller LJTN values than the other 10 and 13 populations, respectively ( $P<0.05$ ). VTN exhibited a geographical pattern different from those found in UJTN and LJTN. Significant differences were observed in 29 combinations, but were much smaller than for UJTN or LJTN. Population 10 (Chubu) had the largest median, which was larger than for the other five populations ( $P<0.05$ ). Among populations from the eastern part of the distributional range (Pops. 1–6), only Pop. 4 had a large median, whereas Pops. 1–3 had much smaller values. However, just as for UJTN and LJTN, Pops. 15 and 16 (Kinki) had significantly smaller VTN values than the other nine and seven populations, respectively ( $P<0.05$ ).

Table 4. Variation in the number of teeth in populations of *Hynobius kimurae*.

Pop. no.	Upper jaw teeth		Lower jaw teeth		Vomerine teeth	
	Median	Range	Median	Range	Median	Range
1	75	71–79	71	68–74	56	47–63
2	78	67–88	68	54–77	54	44–66
3	76	70–80	72	70–77	55	48–68
4	75	70–84	72	64–81	70	62–90
5	74	70–80	73.5	68–81	60	50–71
6	74	68–80	73	63–79	58	45–79
7	65	51–75	58.5	50–71	52	41–62
8	64	54–71	59	55–71	59	58–67
9	72.5	63–79	69.5	56–75	64.5	49–72
10	69	63–73	65	60–68	72	59–83
11	73	68–75	73	68–77	71	63–70
12	70	65–75	67	56–77	68	55–86
13	66	54–75	65	53–71	64	53–83
14	64	60–70	63	62–64	57	56–63
15	56	50–63	54.5	45–61	49.5	43–55
16	53	46–61	46	35–57	52	34–66
17	67	59–74	67	60–75	58	48–64
18	61	57–69	61	56–70	58	48–67
19	65	53–72	63	50–68	63	53–77
20	62.5	59–71	62	51–69	59	48–68
21	61.5	56–67	59	56–67	55	47–66
22	60	54–66	57	55–64	63	51–79
23	60	56–65	54	51–58	62.5	56–67
24	61	51–66	56	53–59	52	50–65

**Relationships between environmental parameters and external morphology**

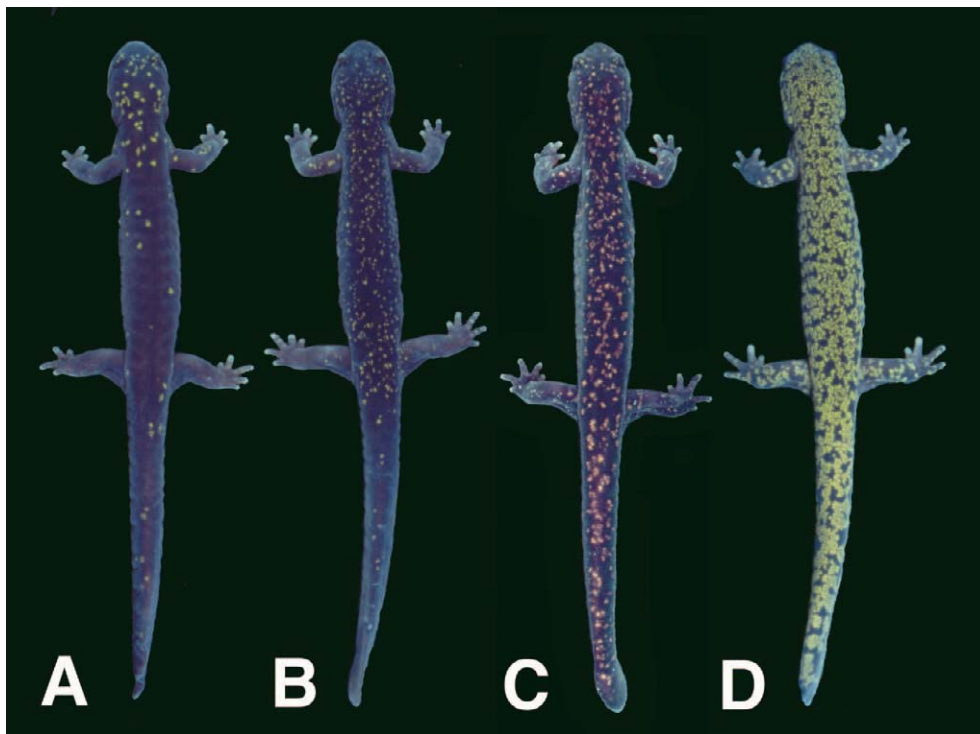
The sampling sites ranged from 36°47'–34°28'N, 139°14'–132°11'E, and 320–1200 m a.s.l. in latitude, longitude, and altitude, respectively, and from 6.3–12.2°C and 1083–2241 mm in mean annual temperature and annual precipitation, respectively. Except for longitude, parameters for the two groups recognized by MGPCA (Pops. 1–5 and Pops. 6–24) largely overlapped.

In the simple regression analysis, mean SVL was not significantly ( $P<0.05$ ) correlated with latitude, longitude, altitude, mean annual temperature, or annual precipitation, when all populations were included (Table 5). However, RLJL, RSL, RFLL, and RHLL were positively correlated with longitude, while RMTAW and RVTL were negatively correlated with longitude. Five ratio variables (RHW, RMXHW, RUEW, RBTAW, and RMTAH) showed negative correlations with altitude. RHW, RMXHW, RUEW, RBTAW, RMXTAH, and RMTAH were positively correlated, and ROL negatively correlated, with mean annual temperature, while RMXTAH, RMTAH, R2FL, R3FL, and R3TL were negatively correlated with annual precipitation. None of the tooth numbers correlated with altitude or mean annual temperature. However, UJTN and VTN were positively correlated with latitude, UJTN and LJTN were positively correlated with longitude, and VTN was positively correlated with annual precipitation.

The results of the partial regression analysis were moderately different from those of the simple regression analysis. When all 24 populations were included, 12 variables were

**Table 5.** Coefficients of correlation of morphological characters with environmental variables. Only characters significantly correlated with at least one variable are shown. For character abbreviations, refer to Appendix 1 (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

	Latitude	Longitude	Altitude	Temperature	Precipitation
Morphometric					
RHW	-0.015	-0.037	-0.652**	0.698**	-0.312
RMXHW	-0.011	-0.125	-0.718**	0.612**	-0.269
RLJL	-0.005	0.411*	0.006	0.056	0.061
RSL	0.035	0.722**	0.099	0.029	-0.022
RUEW	0.304	0.386	-0.498*	0.439*	0.051
ROL	0.219	0.190	0.214	-0.399*	0.260
RBTAW	0.044	-0.257	-0.457*	0.408*	0.011
RMTAW	-0.086	-0.403*	-0.373	0.360	-0.087
RMXTAH	-0.176	0.380	-0.307	0.509*	-0.482*
RMTAH	-0.098	0.089	-0.526**	0.627**	-0.400*
RFL	0.325	0.532**	0.188	-0.191	-0.009
RHLL	0.137	0.465*	0.252	-0.212	-0.325
R2FL	-0.176	0.369	-0.313	0.390	-0.586**
R3FL	-0.159	0.286	-0.262	0.301	-0.536**
R3TL	-0.263	0.086	-0.103	0.114	-0.482*
RVTL	0.151	-0.555**	-0.186	-0.068	0.375
Meristic					
UJTN	0.455*	0.765**	-0.024	0.058	0.138
LJTN	0.378	0.667**	-0.047	0.099	0.267
VTN	0.418*	0.060	-0.047	-0.100	0.568**



**Fig. 5.** Examples of body-color variation in *Hynobius kimurae*. **(A)** Type 1, dorsal yellow spots at most several in number. **(B)** Type 2, dorsal yellow spots fewer than 10 per  $\text{cm}^2$ . **(C)** Type 3, dorsal yellow spots 10 or more per  $\text{cm}^2$ . **(D)** Type 4, dorsal yellow spots continuous, forming yellow marbling, or dorsal ground color yellow. For details of the types, refer to the text. Not to scale.

correlated ( $P < 0.05$ ) with longitude, five with temperature, four each with altitude and latitude, and two with precipitation. Among these, the relationships of RFL, RHLL, UJTN, and LJTN with longitude; RHW, RMXTAH, RMTAH, and ROL with temperature; and RMXTAH and RMTAH with pre-

cipitation were identical with those observed in the simple regression analysis.

When only 18 western populations were analyzed, significant correlations were found in only six variables with longitude, four with latitude, three with altitude, one with

temperature, and none with precipitation. Significant correlations commonly observed in both analyses were as follows: RUEL and RSL were positively correlated with longitude, RMTAW negatively with longitude, RHL negatively with latitude and altitude, RTRL positively with latitude and altitude, and RAGD positively with latitude.

### Color variation

Frequencies of occurrence of the four color types (Fig. 5) are shown in Fig. 6. Types 1 and 2 (few dorsal yellow spots) were found in high frequency among the eastern populations (Pops. 1–4), and type 4 (many yellow spots) was not found in these populations. In the remaining 20 populations, type 4 (continuous dorsal yellow spots forming yellow marbling) occurred more frequently (>50%) in 13 populations, and all individuals of Pops. 17 and 18 (part of Kinki) were of this type.

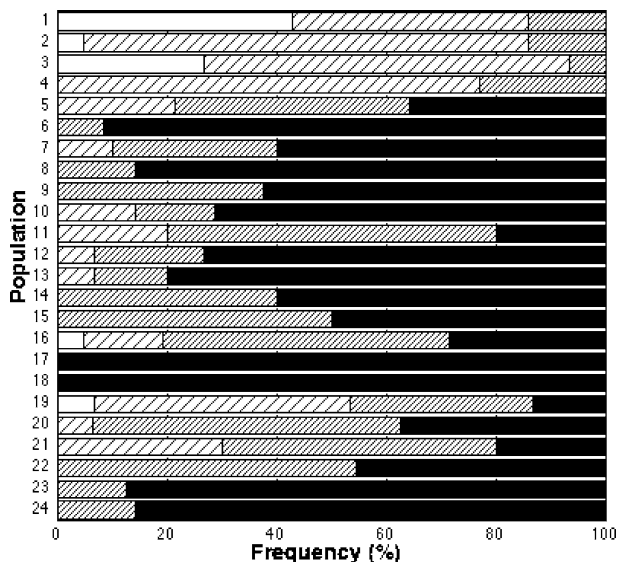


Fig. 6. Body-color variation in 24 populations of *Hynobius kimurae*. Rectangles show the frequency: open, Type 1; coarsely hatched, Type 2; finely hatched, Type 3; filled, Type 4.

Fisher's exact test indicated that Pop. 2 (Kanto) was most distinct, differing from the other 20 populations in the frequency of the four color types ( $P < 0.05$ ). Populations 1, 3 (Kanto), and 4 (Chubu) followed it, each differing from the other 19 populations. Thus, the eastern populations proved to be unique in color pattern. However, Pop. 5 (Chubu) was exceptional, with a significant difference from only seven other populations. By contrast, least unique were Pops. 10 (Chubu), 14 (Kinki), and 24 (Chugoku), each of which differed from only four other populations. Among the western populations, Pop. 18 (Kinki) was most unique, differing from the other 14 populations, and Pop. 17 (Kinki) followed it, differing from 13 populations. Population 19 (Kinki) differed from only 11 other populations, but did not differ from all eastern populations, strongly differing from other western populations.

### DISCUSSION

Populations of *H. kimurae* from eastern Japan were

reported to have larger body than those from western Japan (Matsui, 1981). However, the present results indicated a more complicated pattern of SVL variation, in which populations from the easternmost and westernmost regions tended to be larger than the geographically intermediate populations. This kind of size variation among populations has been attributed both to the effects of environmental factors, reflected in differences in the length of the active season determined by differences in latitudes and altitude of the habitat, on life-history characters (including differential growth rate, age at sexual maturity, and longevity) (e.g., Caetano and Castanet, 1993), and to genetic factors (e.g., Carr, 1996).

Populations 2 (Tokyo) and 16 (Kyoto) of *H. kimurae* were from localities similar in latitude and altitude, but differed greatly in SVL. This difference has been proved to derive from different size at metamorphosis, which in turn is affected by the different life histories of the populations (Misawa and Matsui, 1997, 1999). This suggests that life-history traits such as growth rate, age at first reproduction, and longevity affect variation in body size in the remaining populations of *H. kimurae*.

Additionally, effects of coexisting species might affect the body size of *H. kimurae*. Tominaga et al. (2005b) considered that differences in body size allowed the coexistence of *Hynobius* species in Chugoku District. In eastern Chugoku, *H. kimurae* is larger than *H. naevius*, which in turn is larger than *H. nebulosus* (Temminck and Schlegel, 1838), and in western Chugoku, where the smallest *H. nebulosus* is absent, the size difference between *H. kimurae* and *H. naevius* increases by the further miniaturization of the latter species. Thus, the larger body size in the westernmost populations might represent an example of character displacement (Matsui, 1994). In some of the eastern *H. kimurae* populations with large body size, the situation seems similar, because *H. kimurae* and smaller-sized *H. katoi* Matsui, Kokuryo, Misawa, and Nishikawa, 2004 occur sympatrically (Matsui et al., 2004), although they are segregated during larval life, unlike in Chugoku, where the larger larvae of *H. kimurae* are known to feed on the smaller *H. naevius* larvae (Okada et al., 1997). Interestingly, in some of intermediate regions of Chubu and southern Kinki, *H. kimurae* is not markedly large, but it is still larger than sympatric *H. yatsui* (Tominaga and Matsui, 2008).

Resemblance of these remote populations was not remarkable in other morphological traits. Analyses of allozyme variations in *H. kimurae* showed that geographically distant easternmost and westernmost populations greatly differ genetically (Matsui et al., 2000), and their similarly large body size is surely more affected by ecological factors than by genetic ones.

CANDISC, based on scores from MGPCA, indicated a separation of the eastern (Pops. 1–5) from the central-western (Pops. 6–24) populations. This result was strongly concordant with the genetic separation of *H. kimurae* populations (Matsui et al., 2000), except for the discordance in the placement of Pop. 6 (in this study, Toyota). This population fell into the central-western group morphologically, but into the eastern group genetically. Separation of the eastern and central-western groups was also evident in some meristic characters (UJTN and LJTN), but the details differed



slightly. For example, Pops. 1–4 in the eastern group differed from many populations in the western group in having high frequencies of individuals with few dorsal yellow spots. By contrast, Pop. 5, although also a member of the eastern group, did not differ in body color from many populations of the western group. Patterns of morphological and genetic variation in *H. kimurae* thus generally coincide, but the boundary of the two groups is not strictly concordant among characters compared. It is thus necessary to clarify in greater detail the boundary between the eastern and central-western groups, in order to elucidate the evolutionary history and taxonomic relationships of *H. kimurae* populations.

A significantly positive relationship was found between morphometric (Mahalanobis distance from CANDISC based on scores from MGPCA) and genetic distance (Matsui et al., 2000) when the two groups were combined. This tendency conforms well to that reported in the *Plethodon glutinosus* complex (Carr, 1996) and suggests that genetic factors greatly affect morphometric variation among populations of *H. kimurae*. Like morphometric characters, patterns of variation in UJTN, LJTN, and color pattern tended to differ between the eastern and central-western populations, and seem to suggest the effects of genetic factors on morphological variation.

In the simple regression analysis of relationships between environmental factors and morphological variation, 19 morphological characters showed correlations, among which one character was correlated with three, and seven characters with two, environmental parameters, and the remaining 11 characters were correlated with one parameter each. Among the five parameters studied, longitude had the largest number of correlations (with eight characters) and latitude had the smallest (with two characters). Tominaga et al. (2005b) conducted a similar study, also using simple regressions, in a lotic breeder (*H. naevius* sensu lato) and found many correlations in small-sized Group B (now *H. yatsui* [Tominaga and Matsui, 2008]). Among the significant correlations in that study, those of VTN with latitude; RLJL, RSL, and RMTAW with longitude; and R2FL, R3FL, and R3TL with precipitation are common to *H. kimurae* (analysis of all populations). However, the relationships of these morphological characters with environmental parameters are reversed in the two species for all characters except RLJL and RSL.

From this comparison, it appears that some characters have actually been affected by environmental factors among these salamanders, though the direction they act varies. However, such a simple association should be made with caution, because the results of simple and partial regressions strongly differed in *H. kimurae*. Nishikawa et al. (2007) studied a lotic-breeding species complex of *H. boulengeri* (Thompson, 1912) and *H. stejnegeri* Dunn, 1923 using partial rank correlation. Their results strongly differ from those obtained in our partial regression analyses, and only a positive correlation of SVL with longitude (in the case of all populations of *H. kimurae*) is common to the two studies. This difference may stem from the different methods employed, and also from the fact that the salamanders studied by Nishikawa et al. (2007) surely included more than one species. Apart from these problems, the significant correlations commonly observed by partial regression analyses in

both the total and western populations of *H. kimurae* (positive correlation of RUEL and RSL with longitude, of RTRL with latitude and altitude, and of RAGD with latitude, and negative correlation of RMTAW with longitude, and of RHL with latitude and altitude) might indicate causal relationships between morphological variation and environmental factors. The fact that none of these relationships was detected by simple regression analysis indicates that the method should be carefully applied in this kind of study. Thus, the general relationships between environmental factors and morphological variation in Japanese lotic-breeding salamanders remain unclear, and further studies using a larger number of cases are required.

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#### Appendix 1. Measurements taken.

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1) SVL, snout-vent length from tip of snout to anterior tip of cloaca; 2) HL, head length from tip of snout to gular fold; 3) HW, head width at the level of anterior tip of parotoid gland; 4) MXHW, maximum head width; 5) LJL, lower jaw length from tip of lower jaw to articulation of upper and lower jaws; 6) SL, snout length from tip of snout to anterior tip of upper eyelid; 7) IND, minimum internarial distance; 8) IOD, minimum interorbital distance; 9) UEW, maximum upper eyelid width; 10) UEL, upper eyelid length, distance between anterior and posterior angles; 11) OL, orbit length; 12) AGD, axilla-groin distance; 13) TRL, trunk length from gular fold to anterior tip of cloaca; 14) TAL, tail length from anterior tip of cloaca to tip of tail; 15) BTAW, basal tail width at the level of anterior tip of cloaca; 16) MTAW, tail width at middle level of tail; 17) MXTAH, maximum tail height; 18) MTAH, tail height at middle level of tail; 19) FLL, forelimb length from base of forelimb to tip of the longest finger; 20) HLL, hindlimb length from base of hindlimb to tip of the longest toe; 21) 2FL, second finger length from base to tip; 22) 3FL, third finger length from base to tip; 23) 3TL, third toe length from base to tip; 24) 5TL, fifth toe length from base to tip; 25) VTW, maximum width of vomerine tooth series; 26) VTL, maximum length of vomerine tooth series.

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