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Morphological and Allozymic Variation in *Hynobius boulengeri* and *H. stejnegeri* (Amphibia: Urodela: Hynobiidae)

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We studied morphological and allozymic variation in populations of Japanese salamanders, *Hynobius boulengeri* and *H. stejnegeri*. Adult *H. boulengeri* showed sexual dimorphism, and juveniles differed greatly from adults in many morphological characters. From the results of multivariate analyses of morphological characters, the populations were divided into four groups: (I) *H. boulengeri* from Honshu, (II) *H. boulengeri* from Shikoku, (III) *H. boulengeri* from the Sobo-Katamuki Mountains of Kyushu and *H. stejnegeri*, and (IV) *H. boulengeri* from the Amakusa Islands and the Osumi Peninsula. Phenotypic relationships among the four groups were identical to relationships clarified by allozymic analyses, except for group IV, which was included in group III in the allozyme tree. Some morphometric characters were significantly correlated with environmental variables. We consider *H. stejnegeri* to be a valid species based on its unique color pattern, morphometric characters, and allelic composition, even though it was nested within group III of *H. boulengeri* by both morphological and allozymic analyses. We propose that group I from Honshu and group II from Shikoku should be treated as *H. boulengeri* sensu stricto and *H. hirosei*, respectively. Resolving the taxonomic status of the remaining populations of groups III and IV from Kyushu requires further study.

Key words: geographic variation, interspecific relationships, intrapopulation variation, Japan, morphological similarity

INTRODUCTION

The hynobiid genus *Hynobius* consists of about 30 species mainly from eastern Asia (AmphibiaWeb, 2006). Most species of *Hynobius* are small salamanders and are generally difficult to identify because of their morphological similarities (Matsui and Miyazaki, 1984; Matsui *et al*., 2002). More than half of the species of this genus occur in the Japanese islands, but their identification is often difficult without information on sample locality (Sato, 1943; Matsui *et al*., 2004). In contrast to morphology, these species are well differentiated genetically (Matsui, 1987; Matsui *et al*., 2001, 2004). Indeed, recent genetic studies have suggested the presence of some unnamed cryptic species (Nishikawa *et al*., 2001; Tominaga *et al*., 2005a, 2006; Matsui *et al*., 2006). However, few morphological assessments of these cryptic species have been made.

According to Sato (1943), Japanese species of *Hynobius* are split into the lowland, still-water breeding type (lentic breeders), belonging to the *nebulosus-lichenatus* group, and the montane, stream breeding type (lotic breeders), belonging to the *naevius* group. *Hynobius boulengeri* (Thompson, 1912) is a lotic breeder and is considered a member of the *naevius* group of Sato (1943). This species is morphologically distinct among congeners, with a large body size and monotonous slate body color, and was originally described from Honshu Island as a species of the distinct genus *Pachypalaminus* (Thompson, 1912). Its generic status has been debated for decades (Dunn, 1923; Sato, 1934b; Nakamura and Uéno, 1963; Nishio *et al*., 1987), but intraspecific variation and even the distribution of this species have been addressed only recently (Nishikawa *et al*., 2001, 2003, 2005).

Currently, *H. boulengeri* is known to occur in discrete regions of Honshu, Shikoku, and Kyushu (Sobo-Katamuki Mountains, Amakusa Islands, and Osumi Peninsula; Fig. 1). Sato (1934b) synonymized *H. hirosei* Lantz, 1931 from Shikoku Island with this species, while noting that it could be treated as a distinct subspecies. Nishikawa *et al*. (2001, 2005) studied *H. boulengeri*, as well as another distinct species, *H. stejnegeri*, genetically and recognized three well-differentiated groups (Honshu, Shikoku, and Kyushu). Surprisingly, *H. stejnegeri* was nested within the Kyushu group. This finding was unexpected, because *H. stejnegeri* is well known for its blotched dorsum (Dunn, 1923; Sato,

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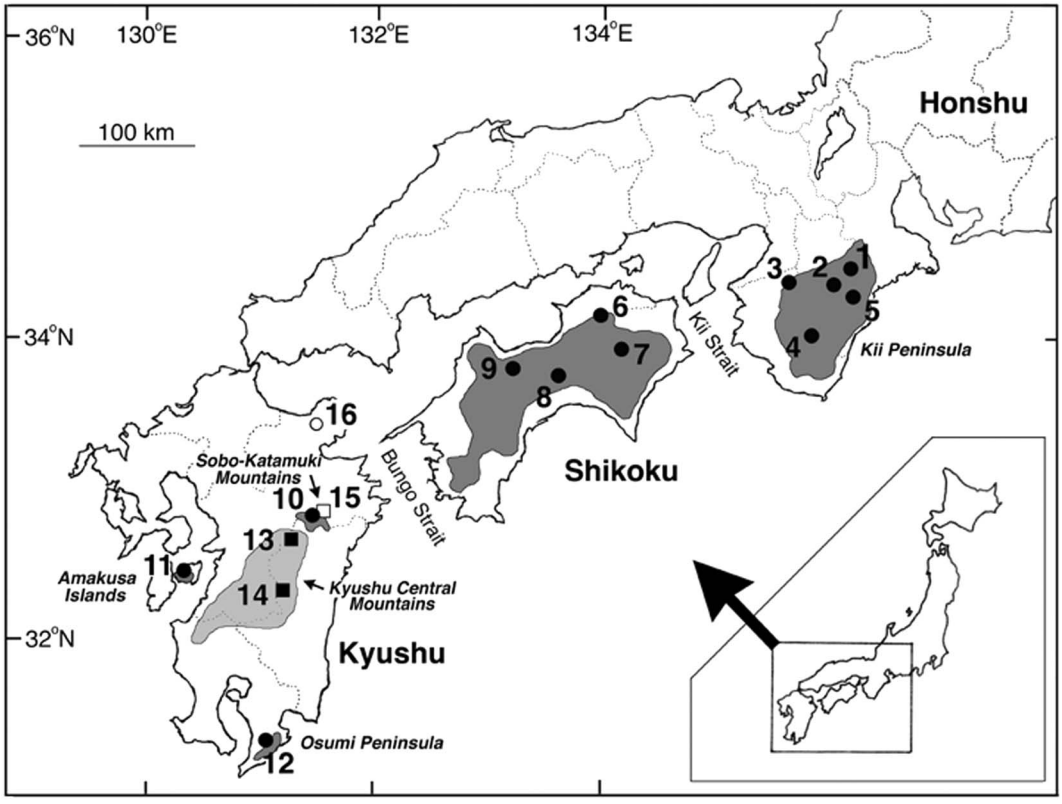

Fig. 1. Western Japan, showing the distributional ranges of *H. boulengeri* (darkly shaded) and *H. stejnegeri* (lightly shaded). Numbers indicate populations (Pops) of hynobiid salamanders used in this study. Localities of *H. boulengeri* (Pops 1–12), *H. stejnegeri* (Pops 13 and 14), *H. naevius* (Pop 15), and *H. dunni* (Pop 16) are indicated by filled circles, filled squares, an open square, and an open circle, respectively.

1943), which contrasts with the monotonous body color of *H. boulengeri*. In fact, the dorsal markings of *H. stejnegeri* have caused it to be confused with *H. naevius* (Stejneger, 1907). As is the case in many other *Hynobius* species, morphological variation in *H. boulengeri* and *H. stejnegeri* has not been studied extensively, making taxonomic assessment of these species difficult.

In this paper, we examined intra- and inter-population variation in the external morphology of *H. boulengeri* and *H. stejnegeri* to delimit morphological entities. We estimated phylogenetic relationships among populations through a study of allozymes, and we correlated morphological and genetic variation in these two species. Our study supplements previous allozyme studies (Nishikawa *et al*., 2001, 2005). We also examined the relationships between environmental factors and patterns of variation in morphological characters. Using our current findings, we propose a classification of *H. boulengeri* and *H. stejnegeri*.

MATERIALS AND METHODS

Morphological analysis

We examined a total of 258 specimens from 14 populations (Pops 1–14) of *H. boulengeri* and *H. stejnegeri* collected mainly in the breeding seasons from 1954 to 2003 (Fig. 1, Table 1). We collected many more adult males than adult females, because males stay in streams much longer than females at the breeding site (Nishikawa *et al*., unpublished data). We could not examine sufficient numbers of females and juveniles to assess their geographic variation. For this reason, we combined samples collected from nearby locations into one population (see Appendix 1).

Salamanders captured in the field were fully anesthetized and tissues were removed for genetic study. They were then fixed in 10% formalin before final preservation in 70% ethanol. Sex and maturity were observed directly by gonad dissection. All specimens were stored at the Graduate School of Human and Environmental Studies, Kyoto University (KUHE). We also examined preserved specimens in private collections of Ms. M. Sakamoto (SK), Mr. S. Sato (ST), and Mr. S. Tanabe (T), and in collections of the National Science Museum of Tokyo (NSMT) and the Osaka Museum of Natural History (OMNH; Appendix 1).

To examine morphological variation, we chose a total of 31 (26 metric and five meristic) characters that covered overall sizes and proportions of the salamanders (see Fig. 2 and Appendix 2). All measurements were taken to the nearest 0.1 mm with a dial caliper. We used a stereoscopic binocular microscope to measure characters with small absolute values (<10 mm) and to count the number of teeth. We also recorded the number of individuals that had a regenerated tail.

Intrapopulation variation

Amphibian metamorphs generally exhibit allometric changes in some morphological characters (*e.g*., Matsui, 1984), but we could not study allometry to detect ontogenetic variation because of the lack of sufficient numbers and sizes of juveniles. We examined ontogenetic variation in character ratios (R, % ratio of each character to snout-vent length, SVL) and meristic characters among adults and juveniles in a Kyushu population of *H. boulengeri* (Pop 10). Most of the juveniles used were recently metamorphosed. We studied the length/width ratio of the vomerine tooth series (VTL/VTW), because morphology of the series has been regarded as a key character for diagnosing species of *Hynobius* (Sato, 1934a, c; Ebitani, 1952). Statistical significance of differences was analyzed by the Kruskal-Wallis test with Dunn's multiple comparisons test

Table 1. Population number (Pop), species, and sample size of salamanders used for morphological and allozyme analyses. Environmental variables and sympatric salamander species (Hn: *Hynobius naevius*; Oj: *Onychodactylus japonicus*) are provided for the populations studied.

		Sample size					Environmental variable					
		Morphology			Allozyme		Latitude	Longitude	Altitude	Mean annual temperature	Mean annual precipitation	Sympatric
Pop	Species	Adult male	Adult female	Juvenile	Metamorph	Larva	(°N)	(°E)	(m)	(°C)	(mm)	species
Ingroup												
1	*H. boulengeri*	29	–	–	–	–	34° 22’	136° 05’	1270	5.6	1486	Hn
2	〃	18	–	–	–	–	34° 11’	135° 56’	1250	5.7	1524	Hn, Oj
3	〃	13	–	–	17	27	34° 14’	135° 36’	780	11.0	1837	Hn
4	〃	13	–	–	10	–	33° 53’	135° 39’	1050	8.7	2077	Hn, Oj
5	〃	23	–	–	10	–	34° 11’	136° 06’	1380	6.5	3253	Hn, Oj
6	〃	8	–	–	8	–	34° 07’	134° 02’	790	11.2	1339	Hn
7	〃	8	–	–	7	10	33° 52’	134° 10’	1380	6.6	2837	Hn, Oj
8	〃	27	15	–	25	–	33° 40’	133° 31’	910	10.1	2784	Hn, Oj
9	〃	21	–	–	1	10	33° 46’	133° 13’	1290	7.5	2992	Hn, Oj
10	〃	25	6	9	16	11	32° 45’	131° 25’	830	11.3	2872	Hn
11	〃	9	–	–	9	–	32° 26’	130° 20’	420	14.0	2065	
12	〃	8	–	–	13	2	31° 08’	130° 53’	610	13.3	2590	
13	*H. stejnegeri*	19	–	–	15	–	32° 43’	131° 11’	1110	9.2	2622	Hn
14	〃	7	–	–	11	–	32° 18’	131° 06’	1600	6.4	2856	Hn
Outgroup												
15	*H. naevius*	–	–	–	9	–	32° 48’	131° 30’	740	–	–	–
16	*H. dunni*	–	–	–	9	–	33° 32’	131° 27’	100	–	–	–

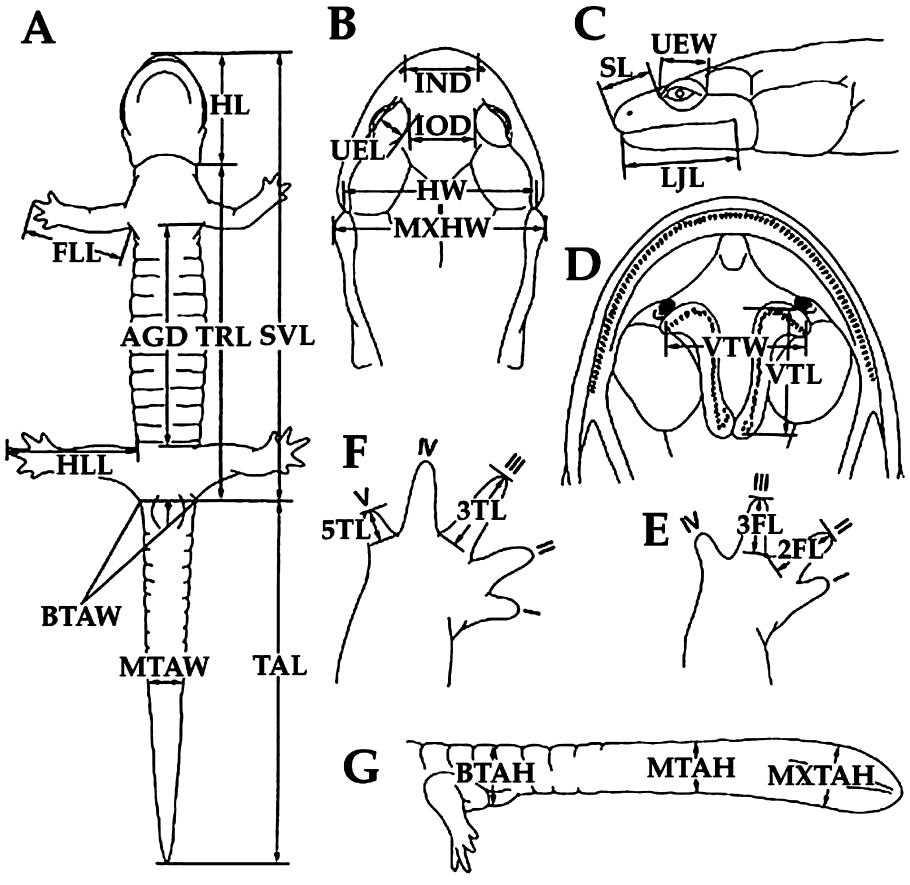

Fig. 2. Character dimensions. See the character definitions in Appendix 2. **(A)** Ventral view of a specimen; **(B)** dorsal view of the head; **(C)** lateral view of the head; **(D)** palatal view of the upper jaw showing vomerine tooth series; **(E)** dorsal view of the left hand; **(F)** dorsal view of the left foot; **(G)** lateral view of the tail. Roman numerals indicate digit numbers.

(Zar, 1984).

We assessed sexual difference in morphometric characters by analysis of covariance (ANCOVA) with Tukey-like tests (Zar, 1984), using SVL (see Fig. 2 and Appendix 2) as an independent variable, in a Shikoku population of *H. boulengeri* (Pop 8), which contained both sexes in sufficient numbers for comparison. In this analysis, all metric values (x) were $\log_e$ transformed, and their allometric relationship to SVL was expressed by the standard formula (Huxley, 1932): $\log_e x = \alpha \log_e SVL + \log_e b$ (α: allometric constant; b: initial growth index). Sexual differences in SVL were examined with Student's *t*-test, whereas meristic characters and character ratios (R) were examined with a Mann-Whitney *U*-test. We compared sexual dimorphism in allometry and ratios to assess the validity of ratio values for the analysis of morphological variation.

Geographic variation

Among populations examined, we compared SVL by one-way analysis of variance (ANOVA) with the Tukey-Kramer test, and 26 character ratios against SVL and five meristic characters using the Kruskal-Wallis test with Dunn's multiple comparisons test. We omitted tail length (TAL; see Appendix 2 for a list of all character abbreviations used) from the analyses because some individuals had regenerated tails, although we could use other tail characters (BTAW, MTAW, BTAH, MXTAH, and MTAH) that could be measured in an intact condition. Frequencies of tail regeneration were compared between populations by Fisher's exact probability test.

We conducted multivariate analyses for examining overall morphological variation among populations. When we found a high correlation between some pairs of characters, we omitted one of them so as to exclude overweighting effects of these characters on the analyses. Using $\log_e$-transformed metric values, we first conducted principal components analysis (PCA). Then, to examine variation only in proportions among samples, we employed canonical discriminant analysis (CDA) with the values obtained in the multiple-group principal component analysis (MGPCA: Thorpe, 1988), which excludes the contribution of size variation from multivariate analysis (Thorpe, 1988; Overton *et al.*, 1997). We constructed a phenogram by the unweighted pair-group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973) using Mahalanobis's (1936) distance (Morph D) obtained from the analysis above, to examine overall morphological similarity among populations under the assumption of a constant rate of differentiation among all branches.

Homogeneity of variances was assessed by Bartlett's test before applying parametric tests (ANCOVA, ANOVA, and Student's *t*-test). The significance level was set at $P<0.05$. All statistical analyses were performed with SAS (1990).

Allozyme analysis

To combine published allozyme data from different sources (Nishikawa *et al*., 2001, 2005) we conducted new electrophoresis analyses and confirmed the homogeneity of zymograms obtained in previous studies. The experimental conditions, techniques, and interpretation of the zymograms are basically the same as described by Nishikawa *et al*. (2005); for all buffers and enzymes used, see Appendix 3. We combined allozyme data from adjacent samples to facilitate comparison with populations (Pop) defined in the morphological analyses (for combinations of localities, see Appendix 1). As a result, we compared ten *H. boulengeri* (Pops 3–12, data not available for Pops 1–2) and two *H. stejnegeri* (Pops 13–14) populations (Table 1, Appendix 1). We also used one *H. naevius* (Pop 15) and one *H. dunni* (Pop 16) population in the comparisons (see below).

We calculated the mean number of alleles per locus (*A*), the percentage of polymorphic loci (*P*), and the direct-count heterozygosity (*H*). The degree of genetic differentiation across populations was calculated using Nei's (1978) unbiased genetic distance (Gen D).

To infer phylogenetic relationships among populations, we constructed a UPGMA tree in BIOSYS-1 (Swofford and Selander, 1981) using Gen D. We also constructed a continuous maximum likelihood (CONTML) tree using PHYLIP vers. 3.5C (Felsenstein, 1993). Wiens (2000) recently reported that some distance-based methods, including UPGMA and maximum likelihood (CONTML) tests, outperformed other methods currently utilized in phylogenetic studies using allozyme data. Several modifications of coding or weighting of frequency data for parsimony analysis have been proposed (Mabee and Humphries, 1993; Murphy, 1993; Mardulyn and Pasteels, 1994), but most of these parsimony methods have been claimed to produce erroneous results (Wiens, 2000). We constructed a maximum parsimony (MP) tree using the method designated by the MANOB criterion (Swofford and Berlocher, 1987). This criterion utilizes step matrices to weight changes between populations based on Manhattan distance (Sneath and Sokal, 1973), making the best use of raw data on allozyme frequency (Swofford *et al*., 1996; Berlocher and Swofford, 1997); it has often been employed in phylogenetic studies using allozymes (Anderson, 2000; Morris *et al*., 2001; Brown and Guttman, 2002). The MP tree was constructed by PAUP* vers. 4.0 (Swofford, 2002) with global rearrangement and random input order options. We used *H. dunni* (Pop 16), a lentic breeder of the *nebulosus-lichenatus* group, as an outgroup to root CONTML and MP trees.

We treated the trees obtained in the above analyses to represent phylogenies. Support for each node in these trees was assessed using 1,000 bootstrapped pseudoreplicates (Felsenstein, 1985). Although Hillis and Bull (1993) noted that a clade supported by ≥95% bootstrap probability was reliable, we considered bootstrap support ≥70% as moderate to strong, and about 50% as weak but meaningful, as reported in several recent phylogenetic studies using allozyme data (*e.g.*, Chippindale *et al*., 2000; Brown and Guttman, 2002).

Correlation of morphological characters with environmental variables

To investigate effects of genetic and environmental factors on overall morphological variation, we used the Mantel test (Mantel, 1967) and the partial Mantel test (Smouse *et al.*, 1986) to analyze autocorrelation between the matrix of Morph D and each of the two matrices, Gen D and Geo D. For Geo D, we measured the straight-line geographic map distance in km between each pair of populations. In the Mantel and partial Mantel tests, probabilities were read directly from the distributions of 10,000 randomized matrices. This analysis was conducted using ARLEQUIN ver 2.000 (Schneider *et al*., 2000).

We assessed correlations between 32 morphological characters (SVL, 26 ratios and five meristic characters) and environmental variables (latitude, longitude, altitude, mean annual temperature, mean annual precipitation, and the number of sympatric salamander species) by Spearman's rank correlation test. Meteorological values were means for the last 20 years 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 available sites encircling actual collection sites from the source above, and averaged the data from them (see method in Matsui, 1984). Data for the number of sympatric species were taken from the literature (*e.g.*, Environmental Agency of Japan, 1981), personal communications, and our own field observations.

When we found significant correlations among environmental variables, we employed a partial rank correlation test to assess correlations between morphological characters and each of these environmental variables by controlling the others.

RESULTS

Morphological variation

Measurement data for 31 characters in 12 populations of *H. boulengeri* and two populations of *H. stejnegeri* are shown in Table 2.

Table 2. Means ± SD of SVL (in mm), medians of ratios of a metric character (% SVL) and meristic characters. Ranges are shown in parentheses. For character abbreviations, refer to Appendix 2 (M: Adult male; F: Adult female; J: Juvenile).

Species		*H. boulengeri*									
Region		Honshu					Shikoku				
Pop		1	2	3	4	5	6	7	8		9
Age/Sex		M	M	M	M	M	M	M	M	F	M
Character	(N)	(29)	(18)	(13)	(13)	(23)	(8)	(8)	(27)	(15)	(21)
Metric character											
SVL		89.8±5.0	98.3±5.4	89.9±4.0	92.7±5.0	93.9±5.3	84.2±4.0	90.4±9.2	83.0±5.4	91.5±3.8	84.8±7.1
		(83.6–101.4)	(84.8–107.3)	(84.7–97.1)	(79.7–97.9)	(83.8–104.5)	(77.9–89.0)	(76.2–102.3)	(73.5–91.9)	(83.0–98.5)	(73.3–103.3)
Ratio (%)											
RHL		24.2	26.0	25.4	23.8	25.1	26.6	25.1	26.8	25.0	25.8
		(22.0–27.1)	(24.0–27.9)	(23.0–26.5)	(22.1–25.5)	(22.0–29.0)	(25.7–27.2)	(23.8–26.5)	(24.4–28.6)	(23.9–26.0)	(24.4–28.0)
RHW		18.9	18.1	20.2	18.7	18.3	17.5	17.5	18.1	16.8	17.7
		(18.1–21.3)	(17.3–20.3)	(19.4–21.1)	(18.0–21.0)	(16.4–19.2)	(16.5–18.4)	(16.1–18.8)	(16.8–21.1)	(15.6–17.5)	(16.2–19.2)
RMXHW		19.9	19.2	20.9	20.3	19.2	18.3	18.2	19.0	17.2	18.3
		(18.2–21.9)	(17.8–21.3)	(20.0–21.9)	(19.1–22.1)	(17.4–20.8)	(17.2–19.0)	(16.8–19.8)	(17.5–21.1)	(16.2–18.1)	(16.8–20.2)
RLJL		15.4	15.1	15.4	15.3	15.3	15.1	14.9	15.6	14.6	15.3
		(13.9–16.4)	(14.0–16.1)	(14.1–17.3)	(14.6–16.4)	(14.1–17.6)	(14.5–15.6)	(14.0–16.1)	(14.5–17.6)	(13.6–15.2)	(14.0–16.5)
RSL		7.3	7.1	7.6	7.4	7.3	7.3	7.2	7.8	7.1	7.5
		(6.8–8.1)	(6.7–7.7)	(6.8–8.1)	(7.0–8.0)	(6.6–8.0)	(6.9–7.9)	(6.7–7.4)	(6.8–9.0)	(6.5–7.7)	(6.8–8.1)
RIND		7.0	6.7	7.0	7.0	6.9	6.1	6.3	6.6	6.1	6.5
		(6.0–7.8)	(5.8–7.2)	(6.5–7.3)	(6.3–7.4)	(6.2–7.4)	(5.9–6.3)	(5.9–7.4)	(6.0–7.5)	(5.3–6.7)	(5.8–7.2)
RIOD		6.3	6.5	6.9	6.3	6.2	5.8	5.4	5.7	5.4	5.7
		(5.8–6.8)	(5.9–7.2)	(6.1–7.5)	(6.1–7.0)	(5.7–7.1)	(5.4–6.0)	(4.9–5.9)	(5.1–6.5)	(4.9–5.6)	(5.1–6.5)
RUEW		3.5	3.1	3.5	3.2	3.1	3.7	3.7	4.1	3.9	4.0
		(3.0–4.1)	(2.8–3.6)	(3.0–3.8)	(2.8–3.6)	(2.8–3.7)	(3.3–4.2)	(3.4–4.7)	(3.6–4.6)	(3.6–4.2)	(3.4–4.6)
RUEL		5.4	5.1	5.7	5.3	5.2	5.3	5.9	5.8	5.2	5.8
		(4.9–6.0)	(4.8–5.5)	(5.2–6.0)	(4.9–5.9)	(4.7–5.8)	(5.1–5.8)	(5.1–6.2)	(5.3–6.8)	(4.9–5.7)	(5.1–6.3)
RAGD		51.5	52.1	50.7	51.6	51.4	50.6	52.4	51.2	54.0	51.4
		(49.2–55.6)	(49.1–54.1)	(47.8–54.0)	(49.0–53.9)	(45.6–53.8)	(48.2–52.8)	(50.5–55.0)	(46.9–53.9)	(50.7–55.2)	(48.8–53.4)
RTRL		75.8	74.0	74.6	76.2	75.0	73.5	74.9	73.2	75.0	74.2
		(72.9–78.0)	(72.1–76.0)	(73.5–77.0)	(74.5–77.9)	(71.0–78.0)	(72.8–74.3)	(73.5–76.3)	(71.4–75.6)	(74.0–76.1)	(72.0–75.6)
RTAL*		78.4	80.9	75.1	76.4	82.4	84.1	83.8	83.9	81.2	85.6
		(68.3–87.5)	(73.3–87.4)	(68.1–81.3)	(68.6–83.7)	(68.3–90.4)	(68.8–88.7)	(76.3–92.1)	(64.0–95.8)	(74.7–87.1)	(69.8–100.5)
RBTAW		12.2	11.1	13.3	12.5	11.4	10.1	10.9	11.1	9.6	11.0
		(9.1–14.4)	(9.7–12.2)	(12.4–14.6)	(11.3–14.0)	(9.7–14.2)	(9.9–11.1)	(9.5–11.8)	(9.1–12.5)	(8.7–10.7)	(9.9–12.0)
RMTAW		10.0	8.1	10.7	9.5	8.0	6.5	7.6	7.8	5.9	7.1
		(8.1–13.9)	(6.5–9.7)	(9.0–12.4)	(8.9–11.8)	(5.7–9.7)	(6.1–8.0)	(5.0–9.1)	(5.7–9.9)	(4.1–6.9)	(6.0–9.9)
RBTAH		10.2	9.4	11.3	10.5	10.5	10.8	9.3	10.7	10.4	10.0
		(8.3–11.9)	(7.1–10.2)	(10.1–12.9)	(8.9–12.4)	(7.7–13.8)	(10.0–11.1)	(8.7–10.7)	(8.1–12.5)	(8.4–11.2)	(8.0–11.0)
RMXTAH		15.0	12.6	16.3	12.8	13.8	13.5	10.6	12.5	10.7	10.6
		(10.8–19.9)	(10.0–14.9)	(12.4–21.4)	(9.6–14.9)	(10.7–16.3)	(11.5–14.6)	(9.7–12.2)	(9.6–15.8)	(8.6–12.7)	(7.3–13.9)
RMTAH		10.3	9.9	13.3	10.6	11.4	12.0	9.9	11.3	10.0	9.7
		(9.0–12.3)	(8.5–10.7)	(10.4–15.6)	(9.3–11.9)	(9.0–13.1)	(10.7–13.2)	(8.6–11.0)	(9.0–14.8)	(7.8–12.1)	(7.4–12.4)
RFLL		23.7	23.6	23.0	23.5	24.3	23.8	23.7	23.7	22.2	24.6
		(20.9–25.1)	(22.6–25.2)	(21.1–24.7)	(21.9–24.8)	(22.7–26.0)	(22.0–24.7)	(21.2–25.2)	(21.7–26.5)	(20.3–23.3)	(22.4–26.5)
RHLL		27.8	28.1	28.4	27.8	28.8	29.4	28.9	29.7	27.4	29.9
		(25.2–29.7)	(26.7–30.0)	(26.0–30.0)	(26.3–30.4)	(27.2–32.1)	(28.2–30.3)	(26.3–31.7)	(26.2–31.8)	(25.2–30.0)	(27.6–32.7)
R2FL		3.8	3.9	3.8	4.1	4.3	5.1	4.6	4.4	4.0	4.8
		(3.2–4.6)	(3.5–4.5)	(3.3–4.5)	(3.2–5.1)	(3.8–5.1)	(4.8–5.3)	(3.6–5.2)	(3.6–5.4)	(2.7–4.7)	(3.9–5.3)
R3FL		3.4	3.8	3.6	3.7	4.0	4.7	4.4	4.4	4.3	4.6
		(3.0–4.0)	(2.9–4.3)	(3.2–3.8)	(3.3–4.3)	(2.7–4.6)	(4.6–5.5)	(3.6–5.0)	(2.7–5.1)	(3.2–4.6)	(3.4–5.3)
R3TL		5.9	6.3	5.7	6.1	6.3	7.1	6.7	7.3	6.4	7.1
		(5.1–6.5)	(4.9–6.8)	(4.2–6.1)	(5.4–6.5)	(5.7–7.1)	(6.4–7.3)	(6.1–8.1)	(5.1–8.1)	(5.7–7.2)	(5.7–8.0)
R5TL		2.1	2.7	2.3	2.3	2.7	3.1	3.0	3.1	3.0	2.9
		(1.4–2.9)	(2.0–3.2)	(1.9–2.9)	(2.0–3.1)	(1.8–3.2)	(2.9–3.8)	(2.5–3.3)	(0.1–4.2)	(2.5–4.8)	(0.1–3.8)
RVTW		6.9	6.4	6.7	6.7	6.5	6.2	6.4	6.5	5.9	6.2
		(5.3–7.5)	(4.6–7.5)	(6.0–7.4)	(6.0–7.5)	(6.0–8.1)	(5.6–6.9)	(5.8–6.7)	(5.6–7.5)	(5.1–7.1)	(5.0–7.7)
RVTL		6.6	6.3	7.1	7.0	6.8	4.0	4.0	4.4	4.1	4.1
		(5.8–7.6)	(5.4–7.8)	(6.4–8.0)	(6.3–7.7)	(5.6–8.3)	(3.8–4.7)	(3.7–4.4)	(3.7–5.2)	(3.5–5.0)	(3.3–4.7)
VTL/VTW		96.7	101.8	105.5	103.5	103.2	64.1	66.1	69.1	68.5	66.7
		(86.4–111.3)	(85.9–118.3)	(90.5–123.2)	(87.5–112.5)	(80.0–112.5)	(57.9–83.7)	(56.9–68.5)	(58.3–78.3)	(62.5–82.7)	(49.1–79.1)
Meristic character											
UJTN		80	84.5	86	83	81	71	74	73	75	76
		(60–89)	(78–96)	(71–92)	(71–89)	(64–92)	(65–79)	(70–83)	(56–80)	(62–84)	(66–95)
LJTN		79	85	76	80	76	72.5	74	69	71	71
		(64–92)	(76–95)	(69–89)	(74–85)	(65–99)	(63–79)	(66–84)	(60–81)	(62–84)	(58–85)
VTN		69	71	69	64	72	36.5	43	42	43	40
		(50–91)	(55–86)	(51–94)	(52–81)	(52–94)	(34–47)	(37–46)	(35–55)	(35–63)	(32–55)
CGN		13	13	13	13	13	13.5	13	13	14	13
		(13–14)	(12–13)	(12–13)	(12–13)	(12–13)	(13–14)	(13–14)	(12–13)	(12–14)	(12–14)
LON		-2.0	-1.5	-1.5	-1.5	-1.5	-1.0	-1.5	-1.0	-2.5	-0.5
		(-3.0– -1.0)	(-2.5– -0.5)	(-2.5– -1.0)	(-3.0– -1.0)	(-2.0–0.5)	(-2.0– -0.5)	(-2.5– -1.0)	(-2.0–1.5)	(-4.0– -1.0)	(-2.0–1.0)

*excluding individuals with regenerated tail, see text.

					H. stejnegeri	
Sobo–Katamuki			Amakusa	Osumi		
10			**11**	**12**	**13**	**14**
M	F	J	M	M	M	M
(25)	(6)	(9)	(9)	(8)	(19)	(7)
83.0±5.5	83.6±9.6	44.7±8.9	72.1±5.1	68.5±3.2	80.4±4.1	81.5±7.8
(73.6–92.4)	(70.8–95.8)	(35.4–62.3)	(64.8–79.0)	(64.4–73.6)	(72.2–87.7)	(69.0–93.2)
24.5	23.9	26.2	23.8	23.6	23.5	23.5
(22.8–27.2)	(22.7–26.3)	(24.5–28.2)	(22.1–25.2)	(21.3–25.0)	(22.0–25.8)	(23.0–24.6)
17.5	17.4	20.6	18.1	17.0	18.1	17.8
(15.6–20.0)	(15.6–18.2)	(16.2–22.9)	(17.0–20.5)	(15.2–18.6)	(16.8–20.0)	(17.3–19.4)
18.2	17.9	22.0	18.9	17.9	18.6	18.2
(16.3–20.9)	(15.9–18.6)	(16.9–23.4)	(17.8–21.1)	(15.6–19.1)	(17.1–20.6)	(17.5–19.9)
15.3	14.9	16.2	14.9	13.5	15.3	15.6
(13.6–17.0)	(14.1–16.7)	(12.9–18.8)	(13.8–16.0)	(13.3–14.3)	(14.1–16.2)	(14.6–16.2)
7.6	7.5	8.2	6.9	6.3	7.3	7.5
(6.9–8.3)	(6.8–8.8)	(7.0–9.6)	(6.3–7.3)	(5.4–6.8)	(6.9–7.8)	(6.8–7.8)
6.4	6.0	7.5	5.7	5.4	6.1	6.1
(5.8–7.1)	(5.8–7.2)	(6.5–9.3)	(4.9–6.1)	(4.8–5.7)	(5.6–7.1)	(6.0–6.7)
5.8	5.5	6.8	5.5	5.6	5.5	5.4
(4.9–6.5)	(4.9–6.8)	(5.8–7.7)	(4.7–5.9)	(5.0–5.7)	(4.9–6.0)	(4.9–6.0)
3.6	3.5	3.8	3.4	3.4	3.6	3.6
(3.1–4.3)	(3.2–3.8)	(3.5–4.8)	(3.2–3.6)	(3.2–3.6)	(3.1–4.4)	(3.3–4.0)
5.7	5.5	6.9	5.9	5.5	5.7	5.6
(4.9–6.1)	(5.2–6.2)	(5.8–7.9)	(5.4–6.3)	(5.3–6.2)	(5.4–6.0)	(5.2–6.2)
52.1	54.8	48.6	51.5	53.0	50.4	53.0
(49.9–57.1)	(49.7–61.7)	(48.5–55.5)	(50.2–53.0)	(49.0–55.7)	(48.4–53.1)	(50.4–53.7)
75.5	76.1	73.8	76.2	76.4	76.5	76.5
(72.8–77.2)	(73.7–77.3)	(71.8–75.4)	(74.8–77.9)	(75.0–78.7)	(74.2–78.0)	(75.4–77.0)
89.3	82.1	80.3	66.1	72.5	84.4	86.9
(63.3–100.6)	(65.3–90.9)	(75.1–92.6)	(61.5–66.5)	(69.4–75.1)	(76.5–92.2)	(79.4–97.3)
11.0	9.0	11.7	12.3	11.0	10.6	11.3
(8.5–12.7)	(8.0–11.9)	(9.1–14.1)	(10.9–13.2)	(9.5–15.8)	(8.9–12.4)	(9.3–11.7)
7.3	5.6	7.1	10.4	8.1	7.9	8.6
(5.1–8.6)	(4.7–6.1)	(4.8–9.3)	(9.2–11.5)	(6.7–14.0)	(5.8–10.5)	(6.8–9.7)
9.2	7.6	11.0	10.7	9.8	9.6	9.3
(7.3–11.6)	(6.7–9.0)	(9.3–12.1)	(9.6–12.1)	(8.7–14.1)	(7.8–11.3)	(8.7–10.4)
10.6	8.4	11.6	11.3	10.2	10.5	11.4
(7.4–14.6)	(7.5–9.9)	(9.5–13.3)	(8.9–12.5)	(9.3–15.7)	(9.0–14.0)	(10.4–14.1)
10.2	8.1	11.2	10.9	10.0	9.7	10.5
(7.3–12.1)	(7.5–9.0)	(9.3–13.0)	(8.9–12.1)	(9.2–15.7)	(8.5–12.6)	(9.6–12.3)
25.1	24.1	25.7	21.0	22.5	24.0	23.2
(23.6–27.6)	(22.1–28.5)	(22.1–28.7)	(19.9–22.2)	(20.3–23.5)	(21.1–26.4)	(21.8–25.4)
30.2	29.0	30.1	27.6	28.8	29.3	28.8
(28.7–33.2)	(27.0–34.4)	(27.9–33.1)	(25.8–29.0)	(25.1–30.5)	(27.4–32.6)	(27.0–30.9)
4.6	4.7	5.4	4.1	4.1	4.3	4.2
(4.0–5.7)	(4.0–5.2)	(4.0–5.6)	(3.4–4.5)	(3.4–4.8)	(3.4–5.3)	(3.9–4.5)
4.9	4.8	5.6	3.9	4.1	4.3	4.4
(4.3–5.7)	(4.7–5.4)	(3.6–6.1)	(3.1–4.2)	(3.1–4.3)	(3.3–5.1)	(3.7–4.8)
6.8	7.4	7.7	6.5	5.8	7.4	6.8
(6.1–8.2)	(6.6–8.5)	(6.5–8.7)	(6.1–7.1)	(5.2–6.8)	(6.2–8.2)	(6.4–7.5)
3.4	3.5	3.4	2.5	2.4	3.1	2.8
(2.6–4.3)	(3.3–4.8)	(2.6–4.0)	(1.8–2.7)	(1.6–2.7)	(2.5–3.8)	(2.6–3.0)
6.4	6.3	7.1	5.7	5.6	5.6	5.9
(5.1–7.5)	(6.0–7.3)	(5.8–8.8)	(5.2–6.4)	(5.3–6.1)	(4.7–6.3)	(5.2–6.7)
5.0	4.9	4.5	5.5	5.1	5.1	5.1
(4.6–5.9)	(4.8–5.9)	(3.5–5.2)	(4.8–6.3)	(4.9–6.0)	(4.4–5.7)	(4.8–5.6)
80.8	81.1	63.9	95.6	92.3	93.5	85.7
(66.1–100.0)	(74.1–91.5)	(39.4–70.4)	(75.6–112.8)	(84.2–107.7)	(74.0–105.1)	(77.4–95.7)
84	85.5	72	82	70	76	78
(74–97)	(82–98)	(46–81)	(77–92)	(63–75)	(68–91)	(68–88)
80	85.5	70	80	68	79	75
(65–92)	(75–93)	(52–87)	(72–85)	(62–74)	(63–90)	(68–86)
52	55.5	34	59	53	48	51
(44–63)	(46–61)	(29–46)	(51–65)	(42–57)	(39–58)	(41–58)
14	13.5	14	13	13	13	13
(13–14)	(13–14)	(13–14)	(13)	(13–14)	(13–14)	(13–14)
-1.0	-1.8	-1.0	-2.5	-2.0	-1.0	-2.0
(-1.5–0.5)	(-2.5–0.0)	(-1.5–0.0)	(-3– -1.5)	(-3.0– -1.0)	(-2.0–0.5)	(-2.5–0.0)

Ontogenetic variation

Results of the Kruskal-Wallis test in Pop 10 indicated significant heterogeneity between adults and juveniles in 21 characters. According to Dunn's multiple comparisons test, juveniles had significantly larger values than adults of both sexes in nine characters of the head (RHL, RHW, RMXHW, RIND, RIOD, and RUEL) and tail (RBTAW, RMTAW, and RBTAH), and smaller values in seven characters of the trunk (RAGD and RTRL) and teeth (RVTL, VTL/VTW, UJTN, LJTN, and VTN). In the remaining five characters, juveniles had significantly larger values than males in RSL, RUEW, and RVTW, and larger values than females in RMXTAH and RMTAH.

Sexual variation

In Pop 8, the sexes differed in SVL, and females were significantly larger than males (t=5.41, df=40, P<0.001). Analysis of covariance revealed significant regressions on SVL in both sexes for HL, HW, MXHW, LJL, SL, IOD, UEL, AGD, TRL, BTAW, and FLL; only in males for IND, UEW, HLL, VTW, and VTL; and only in females for TAL, MTAW, and BTAH.

All 11 characters significantly regressed to SVL differed between sexes in the y-intercept ($\log_e$ b), but not in the slope (α). Regression lines showed smaller y-intercept values in females than in males in all characters except AGD and TRL (Table 3).

Sexual differences in character ratios in Pop 8 are summarized in Table 3. Sexes differed significantly (P<0.05) in all ratios examined, except RTAL, RBTAH, R3FL, R5TL, RVTL, and VTL/VTW. These sexually dimorphic ratios were often larger in males than in females, and this relationship was reversed only for RAGD and RTRL. The sexes differed in only two meristic characters; females had larger CGN (U=91.50, P<0.001) and smaller LON (U=19.50, P<0.001) than males.

Geographic variation

Among the 14 populations examined, the mean SVL of males varied greatly, ranging from 68.5 to 98.3 mm ($F_{13,214}$=28.94, P<0.001). Population 2 from Honshu had the largest mean SVL, which was significantly larger than that in all other populations except Pops 4 and 5 from Honshu and Pop 7 from Shikoku. In contrast, Pop 12 from Osumi had a significantly smaller SVL than all others except Pop 11 from Amakusa. Populations from Honshu (Pops 1–5) tended to have a larger SVL, whereas those from Amakusa and Osumi (Pops 11–12) were significantly smaller than the remaining populations (Pops 6–10, 13–14).

For ratios, RIOD showed the largest number of significant differences (in 42 population pairs), followed by VTL/VTW, RVTL, and RUEL. For meristic characters, interpopulation differences were largest in VTN (31 pairs), followed by UJTN and LON. We found individuals with regenerated tails in nine populations (Pops 1, 3, 5, 8, 10–14). The frequency of regeneration was highest (66.7%) in Pop 11 (Amakusa), followed by Pop 12 (Osumi: 50.0%). The former percentage was significantly higher than those for Pops 1–9 and 13, and the latter was higher than for Pops 1, 2, and 4–9 (Fisher's exact probability test, P<0.05).

In character ratios and meristic characters, populations from Honshu (Pops 1–5) tended to form a group, although a total of 11 characters differed significantly among them.

Table 3. Sexual differences in allometry and ratios in a Shikoku population (M: Adult male; F: Adult female). Growth type abbreviations are: I=isomorphy (α=1), B=bradymorphy (α<1), T=tachymorphy (α>1). For character abbreviations, refer to Appendix 2 (*:P<0.05; **:P<0.01; ***:P<0.001; ns:P≧0.05; –:one or both of the sexes showed no significant regression).

		Allometry								Ratio	
			Slope				Y-intercept				
Character	Sex	n	α	Comparison with α=1	Growt type	Sexual difference	$\log_e$ b	Sexual difference	Character	*U*	Sexual Difference
HL	M	27	0.716	$F_{1,25}$=5.74*	B	ns	–0.029	M>F*	RHL	38.00	M>F***
	F	15	0.740	$F_{1,13}$=3.64ns	I		–0.092				
HW	M	27	0.648	$F_{1,25}$=7.37*	B	ns	–0.038	M>F**	RHW	13.00	M>F***
	F	15	0.623	$F_{1,13}$=2.73ns	I		–0.061				
MXHW	M	27	0.698	$F_{1,25}$=4.65*	B	ns	–0.141	M>F***	RMXHW	19.00	M>F***
	F	15	0.753	$F_{1,13}$=0.98ns	I		–0.279				
LJL	M	27	0.876	$F_{1,25}$=0.69ns	I	ns	–0.567	M>F**	RLJL	40.00	M>F***
	F	15	0.988	$F_{1,13}$=0.01ns	I		–0.811				
SL	M	27	0.715	$F_{1,25}$=2.32ns	I	ns	–0.564	M>F**	RSL	55.00	M>F**
	F	15	1.161	$F_{1,13}$=0.22ns	I		–1.467				
IND	M	27	0.559	$F_{1,25}$=6.29*	B	–	–0.331	–	RIND	61.50	M>F**
	F	15	0.397	$F_{1,13}$=3.14ns	I		–0.035				
IOD	M	27	0.895	$F_{1,25}$=0.32ns	I	ns	–1.038	M>F**	RIOD	58.50	M>F**
	F	15	0.948	$F_{1,13}$=0.03ns	I		–1.171				
UEW	M	27	0.476	$F_{1,25}$=10.50**	B	–	–0.379	–	RUEW	65.50	M>F**
	F	15	0.667	$F_{1,13}$=1.04ns	I		–0.765				
UEL	M	27	0.512	$F_{1,25}$=7.89**	B	ns	–0.293	M>F***	RUEL	13.00	M>F***
	F	15	0.677	$F_{1,13}$=2.20ns	I		–0.648				
AGD	M	27	1.140	$F_{1,25}$=2.97ns	I	ns	–0.561	F>M**	RAGD	34.00	F>M***
	F	15	0.998	$F_{1,13}$=0.01ns	I		–0.268				
TRL	M	27	1.104	$F_{1,25}$=5.94*	T	ns	–0.335	F>M*	RTRL	38.00	F>M***
	F	15	1.086	$F_{1,13}$=3.63ns	I		–0.294				
TAL	M	27	0.490	$F_{1,25}$=1.78ns	I	–	0.887	–	RTAL	138.00	ns
	F	14	1.465	$F_{1,12}$=3.28ns	I		–1.004				
BTAW	M	27	0.549	$F_{1,25}$=4.63*	B	ns	–0.088	M>F**	RBTAW	34.00	M>F***
	F	15	1.202	$F_{1,13}$=0.26ns	I		–1.410				
MTAW	M	27	0.306	$F_{1,25}$=2.57ns	I	–	0.211	–	RMTAW	31.00	M>F***
	F	15	2.541	$F_{1,13}$=3.59ns	I		–4.272				
BTAH	M	27	0.419	$F_{1,25}$=2.44ns	I	–	0.135	–	RBTAH	161.00	ns
	F	15	1.715	$F_{1,13}$=1.66ns	I		–2.398				
MXTAH	M	27	0.305	$F_{1,25}$=3.56ns	I	–	0.432	–	RMXTAH	53.00	M>F**
	F	15	1.113	$F_{1,13}$=0.03ns	I		–1.204				
MTAH	M	27	0.405	$F_{1,25}$=2.54ns	I	–	0.197	–	RMTAH	85.00	M>F**
	F	15	0.748	$F_{1,13}$=0.10ns	I		–0.516				
FLL	M	27	0.627	$F_{1,25}$=7.59*	B	ns	0.093	M>F**	RFLL	25.00	M>F***
	F	15	0.705	$F_{1,13}$=1.64ns	I		–0.081				
HLL	M	27	0.806	$F_{1,25}$=2.11ns	I	–	–0.156	–	RHLL	50.00	M>F**
	F	15	0.395	$F_{1,13}$=4.52ns	I		0.627				
2FL	M	27	0.583	$F_{1,25}$=1.81ns	I	–	–0.556	–	R2FL	106.00	M>F*
	F	15	–0.513	$F_{1,13}$=3.22ns	I		1.563				
3FL	M	27	0.525	$F_{1,25}$=1.45ns	I	–	–0.463	–	R3FL	168.00	ns
	F	15	–0.124	$F_{1,13}$=3.07ns	I		0.819				
3TL	M	27	0.261	$F_{1,25}$=7.01*	B	–	0.264	–	R3TL	77.00	M>F**
	F	15	0.125	$F_{1,13}$=4.90*	B		0.523				
5TL	M	27	0.136	$F_{1,25}$=3.81ns	I	–	0.146	–	R5TL	165.00	ns
	F	15	1.330	$F_{1,13}$=0.08ns	I		–2.141				
VTW	M	27	0.726	$F_{1,25}$=1.85ns	I	–	–0.661	–	RVTW	103.50	M>F*
	F	15	0.538	$F_{1,13}$=0.58ns	I		–0.316				
VTL	M	27	0.674	$F_{1,25}$=1.33ns	I	–	–0.724	–	RVTL	128.00	ns
	F	15	0.971	$F_{1,13}$=0.01ns	I		–1.325				

Populations from Shikoku (Pops 6–9) were grouped more clearly, with a significant difference in only two characters among them. In contrast, we found greater variation in Kyushu populations. Populations of Amakusa (Pop 11) and Osumi (Pop 12) differed only in UJTN, but they differed greatly from the remaining Kyushu populations (Pops 10 and 13–14) in up to 12 characters. Populations of *H. stejnegeri* (Pops 13–14) did not differ from each other, but they differed from the Sobo-Katamuki population (Pop 10) in two characters.

Multivariate analysis

We found a high correlation between HW and MXHW (r=0.990, df=226, P<0.0001), and TRL and AGD (r=0.963, df=226, P<0.0001). For each of these character sets, we omitted the variable with the highest coefficient of variation in conducting the multivariate analysis. We also omitted TAL as noted above.

A PCA using a total of 23 characters resulted in the first eigenvector indicating positive values of similar magnitudes for all characters examined. Because the first eigenvector was highly correlated with SVL (r=0.954, df=226, P<0.001), we employed an MGPCA, which was thought to exclude an eigenvector effect reflecting size (SVL) variation. Using the 23 size-independent character values obtained from the MGPCA, we subsequently conducted CDA. The eigenvalues of the first three axes in CDA (CAN1–CAN3) accounted for 62.4%, 17.7%, and 6.8% of the total variation, respectively (Wilks's lambda=0.0007, P<0.0001).

Two-dimensional plots of the first two canonical variables (CAN1 and CAN2) completely discriminated the following three groups: (I) *H. boulengeri* from Honshu (Pops 1–5), (II) *H. boulengeri* from Shikoku and Sobo-Katamuki Mountains, and *H. stejnegeri* (Pops 6–10 and 13–14), and (III) *H. boulengeri* from Amakusa and Osumi (Pops 11–12; Fig. 3A). Plots of the third against the first variables (CAN1 and CAN3) resulted in clearer separation of Shikoku populations (Pops 6–9) from the Sobo-Katamuki population and the *H. stejnegeri* populations (Pops 10, 13–14) than in plots of the first two canonical variables (Fig. 3B). However, the population from Osumi (Pop 12) largely overlapped with two populations from Honshu (Pops 2 and 5) in the third and first axes.

In contrast, group II recognized in CDA was split in the UPGMA phenogram based on Morph D (Fig. 4), and four clear groups emerged: (I) *H. boulengeri* from Honshu (Pops 1–5), (II) *H. boulengeri* from Shikoku (Pops 6–9), (III) *H. boulengeri* from Sobo-Katamuki and *H. stejnegeri* (Pops 10, 13–14), and (IV) *H. boulengeri* from Amakusa and Osumi (Pops 11–12). Hereafter, we treat populations of *H. boulengeri* and *H. stejnegeri* as consisting of these four morphological groups (I–IV).

As seen in Fig. 4, these four groups showed trichotomous relationships, and groups II and III were closer to each other than to groups I and IV. It is noteworthy that Amakusa and Osumi populations in group IV were distant from each other with the longest branch length among all branches observed.

Salamanders of the Honshu group had a large body and were characterized by a wider head and tail, a higher tail, and more teeth in jaws and vomer than those of the remaining groups. The vomerine tooth series was deeper than in

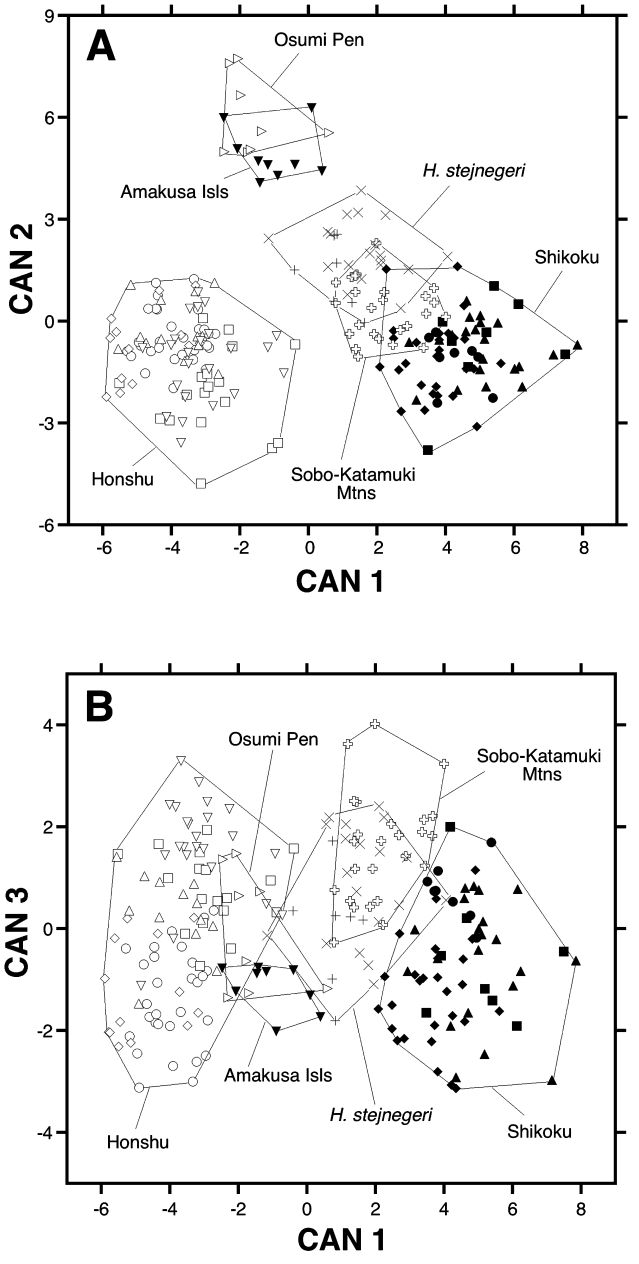

Fig. 3. Two-dimensional plots of **(A)** the first two canonical variables and **(B)** the first and third variables of morphological characters for the samples. Each of 14 populations is represented by a different symbol.

the Shikoku (Pops 6–9) and Sobo-Katamuki (Pop 10) groups. Specimens from Shikoku had a moderate body size, and longer head, eyelid, fingers, and toes than in Honshu, a smaller number of jaw and vomerine teeth than in Sobo-Katamuki, and a shorter trunk than in *H. stejnegeri* (Pops 13–14). Also, the vomerine tooth series in Shikoku samples was shallower than in Honshu, *H. stejnegeri*, and Amakusa (Pop 11). Samples from Sobo-Katamuki had intermediate morphology between the Honshu and Shikoku samples, and had longer tail, fore- and hindlimbs, fingers, and toes than most of the remaining populations. *Hynobius stejnegeri* had a moderate body size. It had longer trunk, fingers, and toes

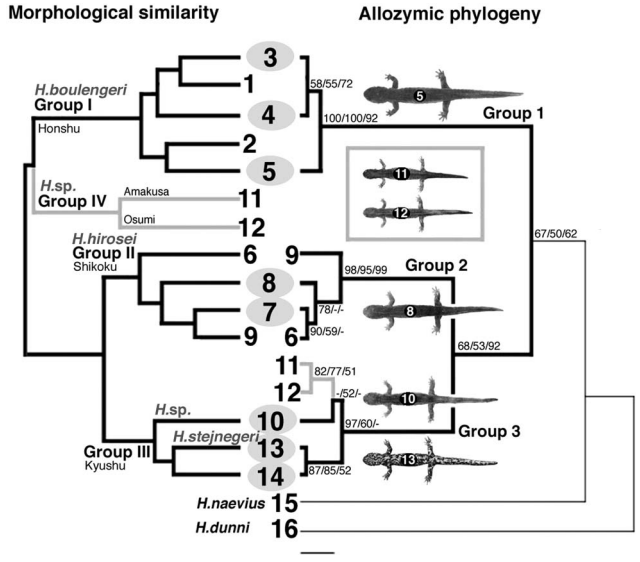

Fig. 4. Two UPGMA trees constructed by Morph D (left) and Gen D (right). Numbers at the nodes show bootstrap support (%) in the three trees obtained from allozyme analyses (UPGMA/CONTML/MP). The number on each salamander indicates its population number. A bar indicates 0.1 Gen D.

than Honshu specimens, a longer trunk and deeper vomerine tooth series than Shikoku specimens, and narrower vomerine tooth series and shorter forelimbs than Sobo-Katamuki specimens. This species had a distinctively blotched body. Amakusa and Osumi samples (Pops 11–12) tended to show smaller values in as many as 22 characters than the remaining samples. These samples were characterized by a small body size, small head characters, short limbs, long trunk, and deep vomerine tooth series.

Allozyme analyses

Of the 22 loci resolved, two (*mAat-A* and *Pep-lgg)* were monomorphic across all populations. Values of *A*, *P*, and *H* varied greatly, ranging from 1.1 in Pops 11 and 16 to 2.3 in Pop 8, from 9.1% in Pop 11 to 54.5% in Pop 8, and from 1.0% in Pop 16 to 10.3% in Pop 14, respectively.

In the parsimony analysis based on the MANOB criterion, a single parsimonious tree was obtained (score=53.9). Only about 30 bootstrap pseudoreplicates were analyzed in the CONTML program of PHYLIP, because subsampling resulted in populations with identically transformed gene frequencies. We repeated this procedure until accumulating 100 pseudoreplicates for assessing nodes in the CONTML tree. Topologies of UPGMA, CONTML, and MP trees were consistent except for the order of branching at deeply nested samples (only the UPGMA tree is shown in Fig. 4). These three trees consistently indicated that all populations of *H. boulengeri* and *H. stejnegeri* are monophyletic, although bootstrap support was not high (bootstrap values of 67, 50, 62% for UPGMA, CONTML, and MP, respectively). The populations were first split into *H. boulengeri* from Honshu (Pops 3–5, corresponding to morphological group I), and the others were further split into *H. boulengeri* from Shikoku (Pops 6–9, group II) and the remaining populations from Kyushu (Pops 10–14, groups III and IV). Thus, three main groups, *i.e.*, (1) Honshu, (2) Shikoku, and (3) Kyushu, could be recognized genetically.

Monophyly of group 1 was strongly supported (100, 100, and 92% in the UPGMA, CONTML, and MP trees, respectively), whereas that of groups 2 and 3 was moderately to weakly supported (68, 53, and 92%, respectively; Fig. 4). Group 2 was found to be monophyletic (98, 95, and 99%, respectively), but monophyly of group 3 was ambiguous (97, 60, and ≤50%, respectively). Within-group genetic divergence was minimal in group 1, compared to other two groups (Fig. 4). In group 2 (Pops 6–9), Pop 9 tended to diverge from the remaining populations as seen in the UPGMA tree (Fig. 4), but this relationship was not always sufficiently supported. Group 3 (Pops 10–14) showed a trichotomy of subgroups, *i.e.*, *H. boulengeri* from Sobo-Katamuki (Pop 10), *H. boulengeri* from Amakusa and Osumi (Pops 11–12), and *H. stejnegeri* (Pops 13–14). Monophyly was moderately to weakly supported in *H. boulengeri* from Amakusa and Osumi (82, 77, and 51%, respectively) and in *H. stejnegeri* (87, 85, and 52%, respectively).

Environmental variation and number of sympatric species

Among sampling sites, latitude, longitude, and altitude ranged from 31°08' to 34°22'N, 130°20' to 136°06'E, and 420 to 1,600 m asl, respectively. Mean annual temperature and annual precipitation ranged from 5.6 to 13.3°C and from 1,339 to 3,253 mm, respectively. For the five environmental variables, we found significant correlations only between latitude and longitude (r=0.926, P<0.001), longitude and annual mean temperature (r=–0.635, P=0.015), and altitude and annual mean temperature (r=–0.871, P<0.001).

Altitudes for groups I to III were similar (mean=1,093–1,146 m) and much higher than for group IV (mean=515 m). Mean annual temperature for group I (7.5°C) was slightly lower than for groups II and III (8.9 and 9.0°C), but both of these were much lower than for group IV (13.7°C). Mean annual precipitation was lowest for group I (2,035 mm) and highest for group III (2,783 mm). Groups II and IV had intermediate precipitation (2,428 and 2,328 mm). Thus, group IV (Amakusa and Osumi) was characterized by having lower altitude and warmer temperature than the remaining groups.

Small salamanders known to be sympatric with *H. boulengeri* and *H. stejnegeri* include *Onychodactylus japonicus* and *H. naevius* (personal observations); the former sympatric species is absent from Kyushu. The number of sympatric species varied from zero in Amakusa and Osumi to two in six populations from Honshu and Shikoku.

Table 4. Results of Mantel and partial Mantel tests examining relationships among Morph D, Gen D, and Geo D.

Comparison	r	P
Morph D × Gen D	0.656	<0.001
Morph D × Geo D	0.462	0.003
Gen D × Geo D	0.464	0.003
(Morph D × Geo D) – Gen D*	0.235	0.054
(Morph D × Gen D) – Geo D*	0.562	<0.001

*"(A × B) – C" tested the partial correlation between A and B in which the effect of C was controlled.

Table 5. Correlation coefficients of morphological characters with environmental variables. Only characters significantly correlated with at least one variable are shown. For character abbreviations, refer to Appendix 2 (*:$P<0.05$; **:$P<0.01$).

Character	Latitude	Longitude	Altitude	Temperature	Precipitation	Sympatric species
SVL	–0.145	0.632*	–0.247	0.170	–0.059	0.775**
RHL	0.333	0.079	–0.195	0.253	–0.130	0.555*
RIND	0.077	0.393	–0.401	–0.093	–0.064	0.603*
RIOD	0.441	0.243	–0.716**	–0.379	–0.446	0.230
RTRL	–0.343	–0.044	0.187	–0.238	0.147	–0.555*
RTAL	–0.177	–0.107	0.656*	0.364	0.525	0.258
RMXTAH	0.563*	–0.094	–0.535	–0.229	–0.420	0.225
RHLL	–0.135	0.135	0.617*	0.633*	0.547*	0.249
R2FL	–0.117	0.050	0.734**	0.703*	0.468	0.191
R3FL	–0.174	–0.029	0.731**	0.612*	0.495	0.029
R5TL	–0.104	–0.021	0.576*	0.473	0.371	0.114

Correlation of morphological characters with environmental variables

As described in the results of multivariate analyses, we omitted RMXHW and RAGD from this analysis because MXHW and AGD were highly correlated with HW and TRL, respectively. The Mantel test gave significant autocorrelations between the matrix of Morph D and each matrix of Gen D and Geo D (Table 4). Because we also found a significant correlation between matrices of Gen D and Geo D, as expected from the isolation-by-distance model (Wright, 1943), we conducted a partial Mantel test to control for the effect of the correlation between Gen D and Geo D on the correlation test between Morph D and each of these two distances. The results indicated that Morph D was correlated with Gen D, but not with Geo D (Table 4).

We next compared each morphological character with five environmental variables and the number of sympatric species. We found significant correlations among several of these variables, as mentioned above. Therefore, in conducting partial correlation tests to assess correlation of morphological characters with these variables, we employed only one variable and controlled another in each of these comparisons of variables. The number of sympatric species was not correlated with any of the environmental variables in the partial correlation test.

Of the morphological characters examined, only eight correlated significantly with any of the five environmental variables (Table 5): six characters (RIOD, RTAL, RHLL, RFL2, RFL3, and RTL5) correlated with altitude, three (RHLL, RFL2, and RFL3) with temperature, and RMXTAH, SVL, and RHLL with latitude, longitude, and precipitation, respectively. Of these, RIOD was negatively correlated with altitude, but all other correlations were positive. Regarding the number of sympatric species, four characters (SVL, RHL, RIND, and RTRL) exhibited correlations, of which only RTRL was negative (Table 5). Thus, we found a total of 16 correlations between morphological characters and environmental variables or number of sympatric species. Among these correlations, seven (43.8%) were exhibited by characters related to limbs (RHLL, R2FL, R3FL, and R5TL).

DISCUSSION

Intrapopulation variation in morphology

Juveniles showed larger values relative to SVL than adults in nine characters, six of which were related to the head. This pattern of juveniles with relatively large head organs is a general trend in various vertebrates (Huxley, 1932), including salamanders (Lynch, 1981), and it suggests that organs essential for life, such as the eyes, jaw, and brain case, in which width was approximated by IOD, form earlier in the course of morphogenesis. Although we could not analyze the allometric pattern of these characters, most of them are thought to be negatively correlated with SVL.

The shape of the vomerine tooth series, which has been regarded as diagnostic in *Hynobius* species (Sato, 1934a, b, c), did not differ sexually in adults. However, length of the series relative to width was shorter in juveniles than in adults. Formation of vomerine tooth series is known to proceed with longitudinal growth of the vomer in a lotic breeder, *H. kimurae* (Misawa, unpublished Ph. D. dissertation), and similar morphological change is expected in *H. boulengeri*. Ontogenetic morphological changes of vomerine tooth series must be considered in examining geographic variation and identifying species of *Hynobius* using this character.

The sexual differences in allometric patterns of characters against SVL largely corresponded to sexual dimorphism in character ratios. From these results, ratio values can be safely employed in analyzing morphological variation, although statistical comparison of ratio values should be made with caution (Atchley *et al.*, 1976; James and McCulloch, 1990).

In a population examined for sexual dimorphism (Pop 8), females had larger SVL than did males. This tendency was also found in Pop 10, and agreed with patterns reported for amphibians in general (Shine, 1979; Duellman and Trueb, 1986) and for *Hynobius* in particular (*e.g.*, Sato, 1933; Misawa and Matsui, 1999; Ento and Matsui, 2002; Tominaga *et al*., 2005b). The larger body size of females would be dictated by egg-carrying capacity.

Compared to females, males showed relatively large values in all body proportions except RAGD and RTRL. Males of *H. boulengeri* and *H. stejnegeri* may combat conspecific males for fertilizing eggs, because during the breeding season the number of males is often smaller than the number of egg-sac pairs which they attend (our personal observations), and some males showed head wounds, which may indicate combat with superior conspecific males. Relatively large heads and long limbs and tails may increase male reproductive success in *H. boulengeri* and *H.*

stejnegeri, as has been reported for lentic breeding *Hynobius* species (Park and Park, 2000; Sato, 1992; Tanaka, 1989; Usuda, 1997) and other urodeles (Houck and Arnold, 2003).

Congruence and incongruence between morphological similarity and allozymic phylogeny

Except for the Amakusa and Osumi populations, phenetic relationships estimated from overall morphology largely agreed with phylogenetic relationships from allozyme data among populations. Partial Mantel tests also showed that Morph D was significantly correlated with Gen D but not with Geo D. These results indicate that morphometry of external characters can be used to estimate phylogeny of closely related species or within a species, among which we often cannot find suitable non-metric characters to utilize in phylogenetic analyses.

Of course, these results do not mean that there are causal relationships between genetic and morphological variation, because the genetic data used here were obtained from multi-locus allozymes, which are regarded as neutral in organic evolution (Avise, 1994; Murphy *et al*., 1996).

The correlations between morphological and genetic variation may have been caused by factors common to both sources of variation. Because genetic differentiation among populations can be ascribed to geohistorical isolation of habitats by the formation of straits or other geographical barriers (Nishikawa *et al*., 2001, 2005), morphological evolution may have been driven by similar geohistorical events.

In contrast, morphological differentiation would also have been induced without accompanying phylogenetic divergence through such geohistorical events. We also found incongruence between morphological and genetic relationships in the position of the Amakusa and Osumi populations. These populations are genetically close to geographically adjacent populations from Kyushu, but their smaller bodies are unique, and they are remarkably divergent morphologically from the latter populations in the UPGMA phenogram, which assumes a constant rate of differentiation among all lineages. This means that the unique morphology of these two populations evolved more rapidly than that of the remaining lineages. A similar case is recognized in the evolution of the unique coloration in *H. stejnegeri*, in which body color is assumed to have evolved rapidly through its cryptic effect against predators (Nishikawa *et al.*, 2005). Congruence between morphological similarity and allozymic phylogeny may be violated by such selection, which occurred in each local environment.

Environmental factors that affect morphological variation

Body size (SVL) and certain allometric proportions were significantly correlated with environmental variables. Because poikilothermic animals such as amphibians are more seriously constrained by the local environment than homeotherms, clinal variation in body size and proportions in relation to varying environments has been reported (*e.g.*, Ruibal, 1957; Nevo, 1973; Lynch, 1981; Matsui, 1984). We found a positive correlation between SVL and longitude when all populations were combined, but the variation in SVL cannot be explained by longitude alone, because SVL differed significantly between the Osumi specimens and one *H. stejnegeri* population (Pop 14), both of which occur at similar longitudes. Further, Tominaga *et al*. (2005b) found a negative correlation between SVL and longitude in *H. naevius* (Type B in their study), which occurs sympatrically with *H. boulengeri* and *H. stejnegeri* (Table 1). This reverse clinal trend in these sympatric species suggests that the presence of sympatric species, in addition to abiotic environmental factors, affects size variation.

We found that some of the limb characters tended to become relatively large with increasing altitude or temperature. However, such trends were not reported for *H. naevius* (Tominaga *et al*., 2005b), and morphoclines seem to differ among species, probably because of different properties of morphogenesis in relation to environmental changes among species. We found two characters of the head (RHL and RIND) to be positively correlated with the number of sympatric species. As shown by Adams and Rohlf (2000), sympatric distribution may cause food segregation and subsequent morphological differentiation in head characters among species.

The Amakusa and Osumi populations were from the southern- and westernmost localities among the populations examined. Their localities were also the lowest in altitude, and hence the warmest. The two populations were further characterized by a lack of sympatric salamander species. Thus, the distinct morphology in these populations could be explained partly by these abiotic and biotic factors. However, only 11 characters (34.4% of the 32 characters examined) correlated with these factors, although the two populations differed from the other populations in as many as 22 characters (68.8%). Thus, the unique morphology in Amakusa and Osumi cannot be explained simply by clinal effects of environmental variables.

The mean size at metamorphosis was smaller in Amakusa (SVL=26 mm: Nishikawa *et al*., 2003) and Osumi (31 mm: Nishikawa *et al*., unpublished) than in *H. boulengeri* from Kyushu (39 mm: Nishikawa *et al*., unpublished). Small size at metamorphosis induces small size at maturity and subsequent adult body size (Misawa and Matsui, 1999). Frequencies of regenerated tails in Amakusa and Osumi were the highest among the populations examined, although the frequencies were significantly higher than those of only a portion of the remaining populations. A high frequency of regenerated tails implies frequent encounters with predators. There are several possible reasons for this result: (1) enemies are relatively abundant; (2) salamanders are old and have had more chances to be attacked; (3) salamanders are relatively slow; and (4) suitable hiding places are scarce.

Although no ecological data are available at present to assess these four possibilities, at least the age of salamanders seems to be precluded, because our preliminary data indicate that salamanders from Amakusa and Osumi were not markedly older than those of the remaining populations. Similarly, individuals from the two populations did not seem particularly slow in their activity compared to those from the other populations. Habitats of salamanders in Amakusa and Osumi are similar to those in other localities in terms of the presence of dead leaves, rotten wood, and stones and rocks where salamanders hide (Nishikawa,

personal observation). Thus, the high frequency of tail regeneration in Amakusa and Osumi suggests a high relative abundance of predators. If this is the case, salamanders may mature and begin reproductive activity early (Kusano, 1982), resulting in small asymptotic size. However, our preliminary results of age estimation do not show obvious differences in age at maturity among Kyushu populations.

Small body size in Amakusa and Osumi might be expected to be accompanied by heterochrony (Gould, 1977), in which characteristics of juveniles are retained in the adult stage. However, adults of these two populations did not exhibit juvenile characteristics as estimated from a population of *H. boulengeri* (Pop. 10 from Sobo-Katamuki). Therefore, rapid evolution in many morphological characters without a significant amount of genetic differentiation in these two populations should be ascribed to reasons other than heterochrony. More detailed morphological analyses using juveniles and subadults would help clarify this issue.

Body size and sympatry

Body size of *H. boulengeri* and *H. stejnegeri* tended to increase with the number of sympatric species. From central Kyushu southwards, except for the Amakusa and Osumi regions, the small type of *H. naevius* (Type B in Tominaga *et al*., 2005b) is distributed sympatrically with *H. boulengeri* and *H. stejnegeri*. The SVL of male *H. naevius* (51–72 mm in Type B from southern Kyushu: Tominaga *et al*., 2005b) was smaller than in a sympatric population of *H. boulengeri* from Sobo-Katamuki (74–92 mm in Pop 10) and *H. stejnegeri* from the Kyushu Central Mountains (72–88 mm in Pop 13; 69–93 mm in Pop 14). In contrast, the size of *H. naevius* largely overlapped with that of allopatric populations of *H. boulengeri* from Amakusa (65–79 mm) and Osumi (64–74 mm). From the results of this study, the ancestor of *H. boulengeri* and *H. stejnegeri* is estimated to have had a large body size, and miniaturization in Amakusa and Osumi, at the periphery of the distributional range of these two species, seems to have occurred as a secondary event in their evolution. Although we have no reliable data at present, this miniaturization seems to have caused body-size overlap with the small type of *H. naevius*, which would have prevented segregation of habitat (Maiorana, 1978) or food (Lynch, 1985). For further discussion, phylogenetic relationships of the *naevius* group (sensu Sato, 1943) in Kyushu (*H. boulengeri*, *H. stejnegeri*, and *H. naevius*) should be clarified, and much more information obtained on the distribution, life history, and ecology of species in the group.

Taxonomic considerations

Sato (1934a, b, c, 1943) utilized skull morphology in determining closely related species of *Hynobius*, although he failed to adopt quantitative methods and conduct statistical comparisons. Sato (1934b) noted that *H. boulengeri* from Shikoku, *i.e*., *H. hirosei*, might be treated as a distinct subspecies because it differed from the nominate Honshu population in skull morphology, especially in having shorter vomerine tooth series. In our study, we found head characters that contributed substantially to the discrimination of the four groups (I–IV) among *H. boulengeri* and *H. stejnegeri*. Because external characters of the head reflect skull morphology, it is worth reanalyzing skull morphology of these species, as well as other species of *Hynobius*, in a systematic study of this genus.

From analyses of allozyme variation, Nishikawa *et al*. (2001, 2005) suggested that the populations of *H. boulengeri* from Honshu, Shikoku, and Kyushu were genetically differentiated from each other and should be recognized as separate species. Our study not only confirmed these results but also revealed that these three groups are well differentiated in external morphology. We therefore propose to restrict *H. boulengeri* (Thompson, 1912) to group I from Honshu, and to revive the name *H. hirosei* Lantz, 1931 for group II from Shikoku.

Taxonomic relationships of the remaining populations from Kyushu (groups III and IV) are more complicated. As reported by Nishikawa *et al*. (2005) and reconfirmed in our study, *H. stejnegeri* (part of group III) was genetically close to *H. boulengeri* from Kyushu (part of groups III and IV). Our study clarified that they are also close to each other in morphometric characters. However, the current recognition of *H. stejnegeri* as a distinct species should be retained, because it has unique characteristics in color pattern, proportions of some body parts (*e.g*., trunk length and width of vomerine tooth series), and some allelic compositions (*e.g.*, *Ldh-B* and *sSod-A*; see Nishikawa *et al*., 2003, 2005). The remaining population in group III, *i.e.*, the Sobo-Katamuki population, can be distinguished from group IV (Amakusa and Osumi) by certain morphological (*e.g.*, body size, tail length, and forelimb length) and genetic features (*mAat-A*, *Pgm-C*, and *sSod-A*). The Amakusa and Osumi populations formed a group in both the morphological and the allozyme analyses, although they differed slightly from each other morphologically as shown by the UPGMA tree. Taxonomic assessment of the Sobo-Katamuki, and Amakusa and Osumi populations is now underway.

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REFERENCES

Adams DC, Rohlf FJ (2000) Ecological character displacement in *Plethodon*: biomechanical differences found from a geometric morphometric study. Proc Natl Acad Sci USA 97: 4106–4111

AmphibiaWeb (2006) AmphibiaWeb: Information on amphibian biology and conservation. Available online at http://amphibiaweb.org/.

Anderson FE (2000) Phylogenetic relationships among loliginid squids (Cephalopoda: Myopsida) based on analyses of multiple data sets. Zool J Linn Soc 130: 603–633

Atchley WR, Gaskins CT, Anderson D (1976) Statistical properties of ratios. I. Empirical results. Syst Zool 25: 137–148

Avise JC (1994) Molecular Markers, Natural History and Evolution. Chapman and Hall, New York

Berlocher SH, Swofford DL (1997) Searching for phylogenetic trees under the frequency parsimony criterion: an approximation using generalized parsimony. Syst Biol 46: 211–215

Boyer SH, Fainer DC, Watson EJ (1963) Lactate dehydrogenase variation from human blood: evidence for molecular subunit. Science 141: 642–643

Brown RM, Guttman SI (2002) Phylogenetic systematics of the *Rana signata* complex of Philippine and Bornean stream frogs: reconsideration of Huxley's modification of Wallace's line at the Oriental-Australian faunal zone interface. Zool J Linn Soc 76: 393–461

Chippindale PT, Hillis DM, Wiens JJ, Price AH (2000) Phylogenetic relationships and systematic revision of central Texas hemidactyliine plethodontid salamanders. Herpetol Monogr 14: 1–80

Clayton JW, Tretiak DN (1972) Amine-citrate buffers for pH control in starch gel electrophoresis. J Fish Res Board Can 29: 1169–1172

Duellman WE, Trueb L (1986) Biology of Amphibians. Johns Hopkins University Press, Baltimore, Maryland

Dunn ER (1923) The salamanders of the family Hynobiidae. Proc Amer Acad Arts Sci 58: 445–523

Ebitani Y (1952) On morphological relation between *Hynobius nebulosus* and *H. tokyoensis*. J Sci Hiroshima Univ Ser B Div 1 13: 1–12

Ento K, Matsui M (2002) Estimation of age structure by skeletochronology of a population of *Hynobius nebulosus* in a breeding season (Amphibia, Urodela). Zool Sci 19: 241–247

Environmental Agency of Japan (1981) Final Report of Reptiles and Amphibians Survey of the Second National Survey on the Natural Environment in Japan, 1978. Nature Conservation Society of Japan, Tokyo

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791

Felsenstein J (1993) PHYLIP (phylogeny inference package) Version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle, Washington

Gould SJ (1977) Ontogeny and Phylogeny. Harvard University Press, Massachusetts

Hillis DM, Bull JJ (1993) An empirical test of the bootstrap as a method for assessing confidence in phylogenetic analysis. Syst Biol 42: 182–192

Houck LD, Arnold SJ (2003) Courtship and mating behavior. In "Reproductive Biology and Phylogeny of Urodela" Ed by DM Sever, Science Publishers, CITY, New Hampshire, pp 383–424

Huxley J (1932) Problems of Relative Growth. Methuen, London

James FC, McCulloch CE (1990) Multivariate analysis in ecology and systematics: panacea or Pandora's box? Ann Rev Ecol Syst 21: 129–166

Kusano T (1982) Post metamorphic growth, survival, and age at first reproduction of the salamander, *Hynobius nebulosus tokyoensis* Tago in relation to a consideration on the optimal timing of first reproduction. Res Pop Ecol 24: 329–344

Lantz LA (1931) Description of two new salamanders of the genus *Hynobius*. Ann Mag Nat Hist Ser 10 7: 177–181

Lynch JF (1981) Patterns of ontogenetic and geographic variation in the black salamander, *Aneides flavipunctatus* (Caudata: Plethodontidae). Smithsonian Contr Zool (324): 1–53

Lynch JF (1985) The feeding ecology of *Aneides flavipunctatus* and sympatric plethodontid salamanders in Northwestern California. J Herpetol 19: 328–352

Mabee PM, Humphries J (1993) Coding polymorphic data: examples from allozymes and ontogeny. Syst Biol 42: 166–181

Mahalanobis PC (1936) On the generalized distance in statistics. Proc Natl Inst Sci India 2: 49–55

Maiorana VC (1978) Difference in diets as an epiphenomenon: space regulates salamanders. Can J Zool 56: 1017–1025

Mantel N (1967) The detection of disease clustering and a generalized regression approach. Cancer Res 27: 209–220

Mardulyn P, Pasteels JM (1994) Coding allozyme data using step matrices: defining new original states for the ancestral taxa. Syst Biol 43: 567–572

Matsui M (1984) Morphometric variation analyses and revision of the Japanese toads (genus *Bufo*, Bufonidae). Contr Biol Lab Kyoto Univ 26: 209–428

Matsui M (1987) Isozyme variation in salamanders of the *nebulosus-lichenatus* complex of the genus *Hynobius* from eastern Honshu, Japan, with a description of a new species. Jpn J Herpetol 12: 50–64

Matsui M, Miyazaki K (1984) *Hynobius takedai* (Amphibia, Urodela), a new species of salamander from Japan. Zool Sci 1: 665–671

Matsui M, Nishikawa K, Tanabe S, Misawa Y (2001) Systematic status of *Hynobius tokyoensis* from Aichi Prefecture, Japan (Amphibia; Urodela). Comp Biochem Physiol B 130: 181–189

Matsui M, Nishikawa K, Misawa Y, Kakegawa M, Sugahara T (2002) Taxonomic relationships of an endangered Japanese salamander *Hynobius hidamontanus* Matsui, 1987 with *H. tenuis* Nambu, 1991 (Amphibia: Caudata). Cur Herpetol 21: 25–34

Matsui M, Kokuryo Y, Misawa Y, Nishikawa K (2004) A new species of salamander of the genus *Hynobius* from central Honshu, Japan (Amphibia, Urodela). Zool Sci 21: 661–669

Matsui M, Nishikawa K, Utsunomiya T, Tanabe S (2006) Geographic allozyme variation in the Japanese clouded salamander, *Hynobius nebulosus* (Amphibia: Urodela). Biol J Linn Soc 89: 311–330

Misawa Y (1989) The method of counting costal grooves. In "Current Herpetology in East Asia" Ed by M Matsui, T Hikida, RC Goris, Herpetol Soc Japan, Kyoto, pp 129–134

Misawa Y, Matsui M (1999) Age determination by skeletochronology of the Japanese salamander *Hynobius kimurae* (Amphibia, Urodela). Zool Sci 16: 845–851

Morris MR, de Queiroz K, Morizot DC (2001) Phylogenetic relationships among populations of northern swordtails (*Xiphophorus*) as inferred from allozyme data. Copeia 2001: 65–81

Murphy RW (1993) The phylogenetic analysis of allozyme data: invalidity of coding alleles by presence/absence and recommended procedures. Biochem Syst Ecol 21: 25–38

Murphy RW, Sites JW Jr, Buth DG, Haufler CH (1996) Proteins: isozyme electrophoresis. In "Molecular Systematics" 2nd ed Ed by DM Hillis, C Moritz, BK Mable, Sinauer Associates, Sunderland, Massachusetts, pp 51–120

Nakamura K, Uéno S (1963) Japanese Reptiles and Amphibians in Color. Hoikusha, Osaka

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583–590

Nevo E (1973) Adaptive variation in size of cricket frogs. Ecology 54: 1271–1281

Nishikawa K, Matsui M, Tanabe S, Sato S (2001) Geographic enzyme variation in a Japanese salamander, *Hynobius boulengeri* Thompson (Amphibia: Caudata). Herpetologica 57: 281–294

Nishikawa K, Matsui M, Tanabe S, Sakamoto M (2003) Occurrence of a lotic breeding *Hynobius* salamander (Amphibia, Urodela) on Kamishima of the Amakusa Islands, Japan. Cur Herpetol 22: 1–8

Nishikawa K, Matsui M, Tanabe S (2005) Biochemical phylogenetics and historical biogeography of *Hynobius boulengeri* and *H. stejnegeri* (Amphibia: Caudata) from the Kyushu region, Japan. Herpetologica 61: 54–62

Nishio K, Matsui M, Tasumi M (1987) The lacrimal bone in salamanders of the genera *Hynobius* and *Pachypalaminus*: A reexamination of its taxonomic significance. Monit Zool Ital (NS) 21: 307–331

Overton JL, Macintosh DJ, Thorpe RS (1997) Multivariate analysis of the mud crab *Scylla serrata* (Brachyura: Portunidae) from four locations in Southeast Asia. Mar Biol 128: 55–62

Park D, Park SR (2000) Multiple insemination and reproductive biology of *Hynobius leechii*. J Herpetol 34: 594–598
Ruibal R (1957) An altitudinal and latitudinal cline in *Rana pipiens*. Copeia 1957: 212–221
SAS (1990) SAS/STAT User's Guide. SAS Institute, Cary
Sato I (1933) On abnormalities of the individual dimension in *Hynobius kimurai* Dunn. Zool Mag Tokyo 45: 327–336
Sato I (1934a) Taxonomical study of the salamanders of the *nebulosus* group, with special reference to the morphology of the skull. Zool Mag Tokyo 46: 214–224
Sato I (1934b) Studies on salamanders from Shikoku. I. On the salamanders found at Ishizuchiyama and its vicinity. Zool Mag Tokyo 46: 464–472
Sato I (1934c) On the species of *Hynobius* of the *naevius* group with special reference to the morphology of the skull. Bot Zool 2: 1843–1852
Sato I (1943) A Monograph of the Tailed Batrachians of Japan. Nippon Shuppan-sha, Osaka
Sato T (1992) Reproductive behavior in the Japanese salamander *Hynobius retardatus*. Jpn J Herpetol 14: 184–190
Schneider S, Roessli D, Excoffier L (2000) ARLEQUIN Ver 2.000. Distributed by the authors, Department of Anthropology and Ecology, University of Geneva, Geneva
Shaw CR, Prasad R (1970) Starch gel electrophoresis of enzymes—a compilation of recipes. Biochem Genet 4: 297–330
Shine R (1979) Sexual selection and sexual dimorphism in the Amphibia. Copeia 1979: 297–306
Smouse PE, Long JC, Sokal RR (1986) Multiple regression and correlation extensions of the Mantel test of matrix correspondence. Syst Zool 35: 627–632
Sneath PHA, Sokal RR (1973) Numerical Taxonomy. WH Freeman, New York
Stejneger L (1907) Herpetology of Japan and adjacent territory. Bull US Natl Mus 58: 1–577
Swofford DL (2002) PAUP*: Phylogenetic Analysis using Parsimony and Other Methods, Version 4.0. Sinauer Associates, Sunderland, Massachusetts
Swofford DL, Berlocher SH (1987) Inferring evolutionary trees from gene frequency data under the principle of maximum parsimony. Syst Zool 36: 292–325
Swofford DL, Selander RB (1981) BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. J Hered 72: 281–283
Swofford DL, Olsen GJ, Waddell PJ, Hillis DM (1996) Phylogenetic inference. In "Molecular Systematics" 2nd ed Ed by DM Hillis, C Moritz, BK Mable, Sinauer Associates, Sunderland, Massachusetts, pp 407–514
Tanaka K (1989) Mating strategy of male *Hynobius nebulosus* (Amphibia: Hynobiidae). In "Current Herpetology in East Asia" Ed by M Matsui, T Hikida, RC Goris, Herpetol Soc Japan, Kyoto, pp 437–448
Thompson JC (1912) Description of a new genus and species of salamander from Japan. Proc Calif Acad Sci Ser 4 3: 183–186
Thorpe RS (1988) Multiple group principal component analysis and population differentiation. J Zool Lond 216: 37–40
Tominaga A, Matsui M, Nishikawa K, Tanabe S (2006) Phylogenetic relationships of *Hynobius naevius* (Amphibia: Caudata) as revealed by mitochondrial 12S and 16S rRNA genes. Mol Phylogenet Evol 38: 677–684
Tominaga A, Matsui M, Nishikawa K, Tanabe S, Sato S (2005a) Genetic differentiations of *Hynobius naevius* (Amphibia, Hynobiidae) as revealed by allozyme analysis. Biochem Syst Ecol 33: 921–937
Tominaga A, Matsui M, Nishikawa K, Tanabe S, Sato S (2005b) Morphological discrimination of two genetic groups of a Japanese salamander, *Hynobius naevius* (Amphibia, Caudata). Zool Sci 22: 1229–1244
Usuda H (1997) Individual relationship of male aggressive behavior during the reproductive season of *Hynobius nigrescens*. Jpn J Herpetol 17: 53–61
Wiens JJ (2000) Reconstructing phylogenies from allozyme data: comparing method performance with congruence. Biol J Linn Soc 70: 613–632
Wright S (1943) Isolation by distance. Genetics 28: 114–138
Zar JH (1984) Biostatistical Analysis. 2nd ed, Prentice Hall, Englewood Cliffs

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Appendix 1. Specimens used in morphological and allozymic analyses. See text for abbreviations of private and institutional collections.

Morphological analysis

Hynobius boulengeri

Pop 1: Higashiyoshino-mura, Nara Prefecture and Matsusaka-shi (formerly Iitaka-cho), Mie Prefecture (KUHE 21692–21697, 24928–24938, 27080, 27081, 27086–27195); Pop 2: Tenkawa-mura, Nara Prefecture (KUHE 22894–22904, 22906, 22908–22912, 27074); Pop 3: Kudoyama-cho, Wakayama Prefecture (KUHE 24550, 24766–24775, 24966, 28541); Pop 4: Totsukawa-mura, Nara Prefecture (KUHE 24544, 24835–24841, 27066–27070); Pop 5: Kamikitayama-mura, Nara Prefecture (KUHE 1528, 5609, 7095, 8312, 8876, 8877, 16044, 22741–22744, 22772, 22775, 25647–25650, 25654–25658, T 2694); Pop 6: Mannou-cho (formerly Kotonami-cho), Kagawa Prefecture (KUHE 9677–9684); Pop 7: Miyoshi-shi (Higashiiyayama-son), Naka-cho (formerly Kisawa-son), and Kamiyama-cho, Tokushima Prefecture (KUHE 8333, 8334, T 2863–2865, 2867, NSMT 1154, 3889); Pop 8: Kochi-shi (formerly Tosayama-mura), Kochi Prefecture (KUHE 9525–9531, 9533, 9534, 9536, 9538, 9540, 18016–18019, 18021, 18024, 18028, 18031, 18033, T 1561, 1567, 1570, 1982, 1983, 2119); Pop 9: Kumakogen-cho (formerly Omogo-mura) and Saijyo-shi, Ehime Prefecture and Ino-cho (formerly Hongawa-mura), Kochi Prefecture (KUHE 24136, 24467, T 2119, 2162–2164, 2173–2178, 2180–2183, 3002–3006); Pop 10: Takachiho-cho, Miyazaki Prefecture and Saiki-shi (formerly Ume-cho), Oita Prefecture (KUHE 18920, 21619, 22813, 22889, 24878, 24967, 25096, 26142, 27125, 27183, 27184, 28748–28756, 32394–32396, 2 ST unnumbered); Pop 11: Amakusa-shi (formerly Sumoto-machi), Kumamoto Prefecture (KUHE 30332–30337, 30339, SK 4, T 3117); Pop 12: Kinko-cho (formerly Tashiro-cho), Kimotsuki-cho (formerly Koyama-cho), and Minamiosumi-cho (formerly Sata-cho), Kagoshima Prefecture (KUHE 18923, 22892, 24961, 24962, 28539, NSM 3664, 4534, OMNH 108).

H. stejnegeri

Pop 13: Gokase-cho, Miyazaki Prefecture (KUHE 12983, 14955, 14956, 22815, 22817–22819, 26065–26068, 27156–27560, 1 ST unnumbered, T 2537, 2808); Pop 14: Nishimera-son, Miyazaki Prefecture (KUHE 26073–26078, 26083).

Appendix 1. Continued

Allozyme analysis

H. boulengeri

Pop 3: Kudoyama-cho, Wakayama Prefecture (KUHE 24050–24076, 24766–24781, 24966); Pop 4: Totsukawa-mura, Nara Prefecture (KUHE 24835–24844); Pop 5: Kamikitayama-mura, Nara Prefecture (KUHE 25647–25656); Pop 6: Mannou-cho (formerly Kotonami-cho), Kagawa Prefecture (KUHE 9677–9684); Pop 7: Miyoshi-shi (Higashiiyayama-son), Tokushima Prefecture (KUHE 18627, 11 KUHE unnumbered, T 1965, 2598–2600, 2919); Pop 8: Kochi-shi (formerly Tosayama-mura), Kochi Prefecture (KUHE 9513–9521, 18015–18022, 18028–18031, 24196–24200); Pop 9: Saijyo-shi, Ehime Prefecture (KUHE 24467, 10 KUHE unnumbered); Pop 10: Takachiho-cho, Miyazaki Prefecture and Saiki-shi (formerly Ume-cho) and Ogata-cho, Oita Prefecture (KUHE 18921, 18922, 21619, 21850, 22813, 22889, 24798, 24799, 24878, 24967, 25096, 25097, 27183–27185, 27334, 11 KUHE unnumbered); Pop 11: Amakusa-shi (formerly Sumoto-machi), Kumamoto Prefecture (KUHE 30332–30339, T 3117); Pop 12: Kinko-cho (formerly Tashiro-cho) and Kimotsuki-cho (formerly Koyama-cho), Kagoshima Prefecture (KUHE 18923, 22892, 24093, 24095, 24795, 24797, 24834, 24961–24963, 27331–27333, 2 KUHE unnumbered).

H. stejnegeri

Pop 13: Gokase-cho, Miyazaki Prefecture (KUHE 12983, 14955, 14956, 22815, 22816, 25405, 26064–26068, 27559, 27560, 1 ST unnumbered, 1 T unnumbered); Pop 14: Nishimera-son, Miyazaki Prefecture (KUHE 26070–26080).

H. naevius

Pop 15: Saiki-shi (formerly Ume-cho), Oita Prefecture (KUHE 22890, 22891, 24968–24972, 25098, 25099).

H. dunni

Pop 16: Bungotakada-shi, Oita Prefecture (KUHE 14351, 14352, 14356, 14358, 14366, 14367, 14370, 14372, 14373).

Appendix 2. Character definitions.

Metric characters (see also Fig. 2)

SVL (snout-vent length): tip of snout to anterior tip of vent; HL (head length): tip of snout to wrinkle of throat; HW (head width): measured at angle anterior to parotid grand; MXHW (maximum head width): measured at widest point; LJL (lower jaw length): tip of lower jaw to the angle of jaw; SL (snout length): tip of snout to anterior tip of upper eyelid; IND (internarial distance): minimum distance between the external nares; IOD (interorbital distance): minimum distance between the upper eyelids; UEW (upper eyelid width): greatest width of the upper eyelid; UEL (upper eyelid length): greatest length of upper eyelid; AGD (axilla-groin distance): minimum distance between axilla and groin; TRL (trunk length): wrinkle of throat to anterior tip of vent; TAL (tail length): anterior tip of vent to tail tip; BTAW (basal tail width): tail width measured at root of tail; MTAW (medial tail width): tail width measured at middle; BTAH (basal tail height): tail height measured at base of tail; MXTAH (maximum tail height): tail height measured at highest point; MTAH (medial tail height): tail height measured at middle; FLL (forelimb length): distance from axilla to tip of the longest finger; HLL (hindlimb length): distance from groin to tip of the longest toe; 2FL (second finger length): distance from junction of second and third fingers to tip of the second finger; 3FL (third finger length): distance from junction of second and third fingers to tip of the third finger; 3TL (third toe length): distance from junction of third and fourth toes to tip of the third toe; 5TL (fifth toe length): distance from junction of fourth and fifth toes to tip of the fifth toe; VTW (vomerine tooth series width): the greatest width of vomerine tooth series; VTL (vomerine tooth series length): the greatest length of vomerine tooth series.

Meristic characters

UJTN (number of upper jaw teeth); LJTN (number of lower jaw teeth); VTN (number of vomerine teeth); CGN (number of costal grooves following Misawa, 1989); LON (number of costal folds between adpressed limbs; plus and minus values indicate overlap and separation, respectively).

Appendix 3. Buffers and enzymes used.

Buffers

CAPM6: citrate-aminopropylmorpholine, 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).

Enzyme codes, enzymes, loci, and buffer systems

E.C. 4.2.1.3 aconitate hydratase (*mAcoh-A*, *sAcoh-A*; TC8), E.C. 1.1.1.1 alcohol dehydrogenase (*Adh-A*; TBE8.7), E.C. 2.6.1.1 aspartate aminotransferase (*mAat-A*, *sAat-A*; CAPM6, TC7), E.C. 4.2.1.1 fumarate hydratase (*Fumh-A*; TBE8.7), E.C. 5.3.1.9 glucose-6-phosphate isomerase (*Gpi-A*; CAPM6), E.C. 1.4.1.3 glutamate dehydrogenase (*Gtdh-A*; TC8), E.C. 1.1.1.42 isocitrate dehydrogenase (*mIdh-A*, *sIdh-A*; TC7), E.C. 1.1.1.27 L-lactate dehydrogenase (*Ldh-A*, *Ldh-B*; CAPM6, TC7), E.C. 1.1.1.37 malate dehydrogenase (*mMdh-A*, *sMdh-A*; CAPM6, TC8), E.C. 1.1.1.40 malic enzyme (NADP-dependent malate dehydrogenase) (*mMdhp-A*, *sMdhp-A*; TC7), E.C. 3.4.11.- peptidase (leucyl-alanine) (*Pep-la*; TBE8.7), E.C. 3.4.11.- peptidase (leucyl-glycyl-glycine) (*Pep-lgg*; TBE8.7), E.C. 1.1.1.44 phosphogluconate dehydrogenase (*Pgdh-A*; TC7), E.C. 5.4.2.2 phosphoglucomutase (*Pgm-A*, *Pgm-C*; TC7), and E.C. 1.15.1.1 superoxide dismutase (*sSod-A*; TBE8.7).