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Source: Zoological Science, 21(6) : 661-669

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.21.661

ZOOLOGICAL SCIENCE **21**: 661–669 (2004)

A New Species of Salamander of the Genus *Hynobius* from Central Honshu, Japan (Amphibia, Urodela)

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ABSTRACT—We describe a small salamander from south Central Honshu, Japan, as a new species, *Hynobius katoi*. The genetic distances between this species and several named species, including sympatric *H. kimurae*, derived from allozyme data from a starch gel electrophoresis, proved to be sufficiently large to differentiate it at a specific rank. Distribution of this species is confined to the montane regions of Shizuoka and Nagano Prefectures, on the Akaishi Mountains of the Chubu District, central Japan. It is regarded as a member of the *naevius* group of *Hynobius*, characterized by small number of large, pigmentless ova. The species differs from the other species of the *naevius* group by the combination of relatively small body size, nearly spotless body, relatively few vomerine teeth forming moderately shallow series, and unique electrophoretic pattern of isozymes.

Key words: Urodela, *Hynobius katoi*, central Japan, electrophoresis, taxonomy

INTRODUCTION

The small salamanders of the genus *Hynobius* are distributed in east Asia (Zhao, 1999) and belong to the family Hynobiidae which is supposed to represent a very primitive lineage within the order Urodela (Duellman and Trueb, 1986). The genus *Hynobius* comprises a large component of the species-level diversity in the herpetofauna of east Asia, and especially, of Japan (Sato, 1943; Matsui, 1996).

In early May of 1978, Mr. Makoto Kato brought us three salamander specimens from Shizuoka, southern part of central Honshu, Japan. Of these, two larger specimens, having yellow dorsal markings, were easily identified as *H. kimurae* Dunn, 1923, whereas the smallest specimen differed from them by lacking dorsal markings, and having much smaller body and shallower vomerine teeth series. Subsequent surveys of additional specimens have proved that the salamander in question could be consistently distinguished from *H. kimurae* on the basis of several characteristics, especially the smaller size and the absence of yellow dorsal marking. However, as is the case for most other Japanese salamanders (Matsui and Miyazaki, 1984; Matsui, 1987), the relatively small salamander from Shizuoka was morphologically similar to other allopatric *Hynobius* species, making its taxonomic status unclear solely on the morphological ground.

In this paper we describe the salamander from Shizuoka as a new species on the basis of both morphological and allozyme variation among its populations and populations of other *Hynobius* species from adjacent regions of central Honshu, from the Kanto to Kinki districts. Isozyme analysis has proven to be a highly useful technique in systematic studies of various lineages in urodele amphibians (e.g., Jackman and Wake, 1994; Hayashi and Matsui, 1988; Chippindale, 2000; Tilley, 2000; Matsui *et al*., 2000, 2001, 2002; Nishikawa *et al*., 2001).

MATERIALS AND METHODS

Morphological comparisons

The unidentified salamanders were collected from several localities of Shizuoka and Nagano Prefectures (Appendix 1). For morphological comparisons with them, specimens belonging to the following three recognized species from surrounding regions of central Japan were used: *H. tokyoensis* Tago, 1931 from Kanagawa, *H. naevius* (Temminck and Schlegel, 1838) from Gifu, Mie, and Osaka, and *H. kimurae* from Shizuoka (syntopic with the unidentified species in some areas). The total number of specimens for each character varied with a maximum of 103. The following eight measurements were taken for those specimens in preserved state: 1) snout-vent length (SVL), from snout to anterior angle of cloaca [=sum of 2) and 3)]; 2) head length (HL), from tip of snout to gular fold; 3) trunk length (TRL), from gular fold to anterior angle of clo-

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aca; 4) tail length (TAL), from anterior angle of cloaca to tip of tail; 5) head width (HW), measured at jaw articulation; 6) maximum tail height (MTAH); 7) length of vomerine teeth series (VTL); and 8) width of vomerine teeth series (VTW). The number of costal grooves, including the axillary groove, was counted. Overlap of finger and toe tips when both limbs were adpressed to the body was recorded by the number of costal folds between the tips with 'plus' indicating overlap and 'minus' separation. Tukey-Kramer tests were used for morphometric comparisons, while Kruskal-Wallis or Dunn's multiple comparison tests were performed for ratio values and detection of the presence or absence of differences in the frequency distributions. A significance level of 95% was used in all statistical tests.

Electrophoresis

Allozymes from a total of 28 specimens [*H.* sp. from Shizuoka (n=13); *H. tokyoensis* from Kanagawa (n=5); *H. kimurae* from Shizuoka (n=5); *H. naevius* from Osaka (n=5)] were examined electrophoretically. In the laboratory, tissue samples of liver were removed from anesthetized salamanders and maintained frozen at –84°C until used for electrophoresis. Voucher specimens were fixed in 10% formalin, later preserved in 70% ethanol for metamorphs and 50% ethanol for larvae, and stored in Graduate School of Human and Environmental Studies, Kyoto University (KUHE) and Mr. Tanabe's private collection (T)(Appendix 2). We analyzed homogenized tissue extracts by standard horizontal starch gel electrophoresis (Murphy *et al*., 1996) at a concentration of 11.5%. We scored the products of 25 loci encoding 18 allozymes as shown in Appendix 3 for all individuals. Genetic interpretations of allozyme data followed Nishikawa *et al*. (2001). Enzyme nomenclature, E. C. numbers, and the notations of loci, electromorphs and genotypes mainly followed Murphy *et al*. (1996). Electromorphs were designated alphabetically with "a" representing the most slowly migrating variant. In order to estimate overall genetic differentiation among samples, coefficients of Nei's (1978) unbiased genetic distance and modified Rogers' distance (Wright, 1978) were computed. We estimated genetic relationships among species from the pairwise matrix of Nei's distance, clustered according to the UPGMA algorithm (Sneath and Sokal, 1973) to facilitate comparison with many similar studies in the literature, and additionally performed a neighbor-joining analysis (Saitou and Nei, 1987) using measures of modified Rogers' distance (Wright, 1978). These analyses were made by use of BIOSYS-1 (Swofford and Selander, 1981). We further performed a maximum-likelihood analysis using CONTML procedure in PHYLIP vers. 3.5 C (Felsenstein, 1993).

DESCRIPTION

Hynobius ***katoi*** Matsui, Kokuryo, Misawa, et Nishikawa sp. nov.
[Japanese name: Akaishi-sansyouo]
Fig. 1

Holotype

KUHE 17946, an adult male from Mt. Takane-San (138°11'E, 34°58'N, alt. 750 m) in Fujieda-shi, Shizuoka Prefecture, collected by Y. Kokuryo and Y. Misawa on 5 May 1994.

Paratypes

A total of 30 specimens, all from Takane-san, Fujieda-shi, Shizuoka Pref. KUHE 14664–14665, two males, alt. 750 m, 6 May 1993, Y. Kokuryo and Y. Misawa; KUHE 16302, one young, alt. 720 m, 24 August 1993, Y. Kokuryo and Y. Misawa; KUHE 16676, one female, alt. 720 m, 17 November, Y. Kokuryo and Y. Misawa; KUHE 17004, one female, alt. 720 m, 10 April 1994, Y. Kokuryo; KUHE 17945, one young, alt. 720 m, 24 April 1994, Y. Kokuryo; KUHE 17947–17953, three young, three males, and one female, alt. 750 m, 3–5 May 1994, Y. Kokuryo and Y. Misawa; KUHE 18349, 18351, 18352, 18391, 18392, two young, two males, and one female, alt. 750 m, 1 April 1995, Y. Kokuryo and Y. Misawa; KUHE 18404–18407, one male and three females, alt. 720 m, 5 May 1995, Y. Kokuryo and Y. Misawa; KUHE 21685–21689, 21709, one young, three males and two females, alt. 750 m, 5 May 1996, Y. Kokuryo and Y. Misawa; KUHE 26098, 26099, one young and one male, alt. 720 m, 3 May 1999, M. Matsui.

Fig. 1. Dorsal views of male holotype (KUHE 17946, SVL=58.4 mm) and young paratype of *Hynobius katoi* (KUHE 17945, SVL=25.7 mm).

Diagnosis

A member of the *naevius* group (Sato, 1943) of *Hynobius*: ova large, pigmentless, very few per clutch; most probably breeding in flowing water under the ground; most similar to *H. naevius* in general body proportion, but with a nearly immaculate dorsum, relatively narrower head, shorter trunk, fewer vomerine teeth, much more shallowly curved

Table 1. Comparisons of snout-vent length (SVL: means±1SD, followed by ranges in parenthesis, in mm) and percentage ratios of each of the other character dimensions to SVL (medians, followed by ranges in parenthesis) in four *Hynobius* species

	H. katoi		*H. naevius*	*H. kimurae*	*H. tokyoensis*
	12 males	10 females	27 males	27 males	28 males
SVL	58.4±3.3	62.7±1.6	57.7±3.9	84.9±5.1	57.4±6.4
	(53.8–64.0)	(60.2–66.1)	(50.8–64.7)	(76.5–93.1)	(45.1–66.9)
HL	24.6	24.3	25.0	23.6	24.6
	(24.3–25.2)	(24.0–24.7)	(23.8–26.4)	(23.0–25.4)	(22.4–27.5)
HW	16.2	15.6	19.1	17.0	17.4
	(15.3–16.7)	(14.8–16.0)	(17.4–204)	(15.5–18.4)	(15.9–19.0)
TRL	75.4	75.7	75.0	76.4	75.4
	(74.8–75.7)	(75.3–76.0)	(73.6–76.2)	(74.6–77.0)	(72.5–77.6)
TAL	73.6	72.9	65.9	88.2	86.6
	(70.7–82.6)	(71.2–73.2)	(60.5–74.2)	(81.2–95.8)	(68.8–97.0)
TAH	9.7	8.9	10.7	11.0	14.0
	(7.8–10.7)	(7.3–10.0)	(8.8–12.5)	(8.1–14.1)	(10.5–16.1)

vomerine teeth series, and large electrophoretic differences; completely different from sympatric *H. kimurae* in having a much smaller, nearly immaculate body, longer head, shorter trunk, fewer vomerine teeth, and much shallower vomerine teeth series, as well as in its electrophoretic allozyme profile.

Description and variation

The following description of *H. katoi* is based on the maximum number of 12 adult males and 10 adult females of the type series. Morphometric data are summarized in Table 1 together with those of the reference species, *H. naevius, H. kimurae*, and *H. tokyoensis*. Although males (mean±SD=58.4±3.2 mm, n=12) tended to be smaller in SVL than females (62.6±1.6 mm, n=10), there was no statistically significant sexual dimorphism in this character (Tukey-Kramer test, $P>0.05$). Head moderately depressed, distinctly longer than broad. Males tended to have relatively longer head (HL) and shorter trunk than in females, when each dimension is converted to percentage ratio to SVL, but there were no statistically significant differences (Kruskal-Wallis test, $P>0.05$). Number of costal grooves including axillary groove 12 or 13 (Table 2), not different between the sexes, the modal number being 13 in both sexes. Limbs short, stout; fore- and hindlimbs almost always separated by at least 1 fold when adpressed; degree of separation greater in females (median=2.5 folds) than in males (median=0.75 fold; Kruskal-Wallis test, $P<0.05$; Table 3). Tail rounded at base, gradually flattening to tip, obtusely pointed at tip; tail only slightly keeled, upper keel very weakly originating at posterior two-fifth, lower one at posterior one-fifth. No development of tail fin even in breeding males. Males tending to have longer and higher tails than females, but without statistically significant support. Fifth toe very short, or rudimentary, or even absent. Vomerine teeth in two small, obliquely arched series, nearly touching at midline, usually forming a shallow “U” (Fig. 2). Combined series wider than long (VTW/

Table 2. Variation in the number of costal grooves, including the axillary groove, in four *Hynobius* species

		Number of costal grooves		
Species	n/Sex	12	13	14
H. katoi	20 males	2	18	
	15 females		15	
H. kimurae	27 males	1	26	
H. naevius	27 males	15	12	
H. tokyoensis	28 males	1	22	5

Table 3. Variation in the number of costal folds between adpressed limbs in four *Hynobius* species

		Overlap of adpressed limbs shown by number of costal folds							
Species	n/Sex	–3	–2.5	–2	–1.5	–1	–0.5	0	0.5
H. katoi	20 males				1	9	9	1	
	15 females	2	8	5					
H. kimurae	27 males			3	6	14	3	1	
H. naevius	27 males		4	14	6	2	1		
H. tokyoensis	28 males				1	5	14	7	1

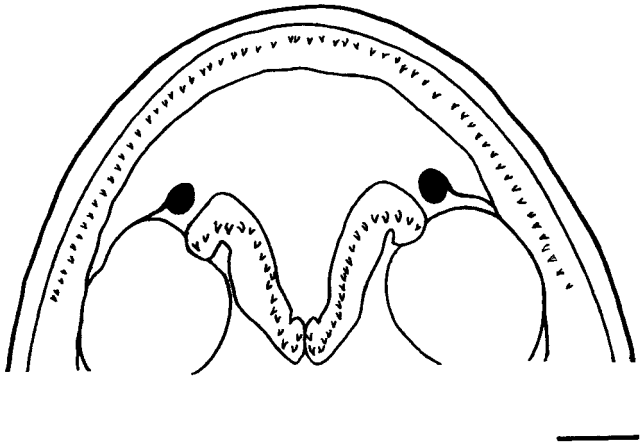

Fig. 2. Open mouth of holotype of *Hynobius katoi* (KUHE 17946) showing shape of vomerine teeth series. Scale bar indicates 1 mm.

VTL=1.18-1.81 in males and 1.18-1.71 in females), not different between sexes; VTL tending to be smaller in males than in females, but without significant difference, either.

Color

In life, dorsum uniformly dark brown, rarely scattered with silvery dots. The silvery dots developing more often in young than in adults. Underside lighter than dorsum, with silvery dots on throat and sometimes on belly. In preservative, dorsal coloration tending to fade, becoming gray brown, or otherwise, without obvious change.

Measurements and counts of the holotype

An adult male with the following measurements (mm; in preservative): HW 10.3, HL 14.2, head depth at posterior angle of jaw 4.2, eyelid length 3.5, anterior rim of orbit to snout 4.4, horizontal orbit diameter 2.9, interorbital distance 3.3, distance separating internal nares 3.0, distance separating external nares 3.1, SVL 58.4, axilla to groin 29.9, TAL 44.6, tail width at base 6.0, tail height at posterior angle of cloaca 4.6, tail height at middle 5.3, MTAH 5.6, axilla to tip of outstretched forelimb 13.9, groin to tip of outstretched hindlimb 16.8, VTW 3.18, VTL 2.13. The numbers of upper jaw teeth 59, lower jaw teeth 67, vomerine teeth 39, costal

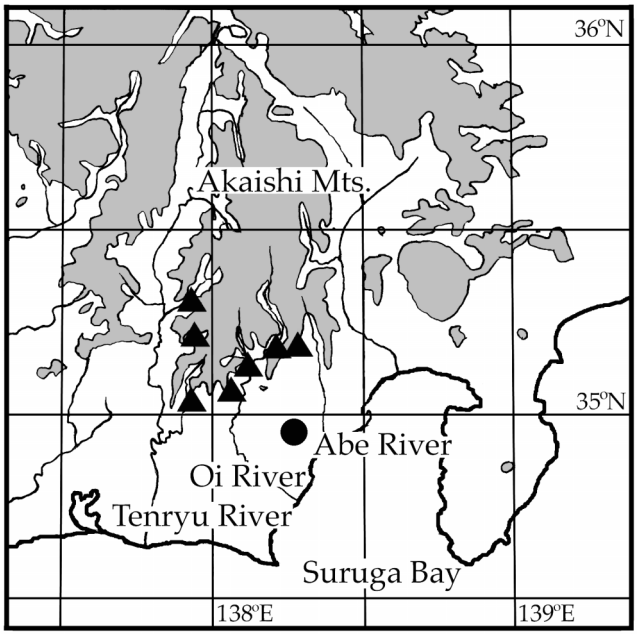

Fig. 3. A map of southern Central Honshu, Japan, showing the type (filled circle) and other known (filled triangle) localities of *Hynobius katoi*. Stippled area>1000 m alt.

Table 4. Allele frequencies of variable loci in four *Hynobius* species

	Species (n)			
Locus	*H. katoi* (13)	*H. tokyoensis* (5)	*H. kimurae* (5)	*H. naevius* (5)
AAT-2	a0.04	c1.00	b1.00	b1.00
	b0.96			
ACH-1	a1.00	a0.10	b1.00	a1.00
		b0.90		
ACH-2	c1.00	d1.00	b1.00	a1.00
EST-1	a1.00	b1.00	a1.00	b0.10
				c0.90
EST-2	d1.00	b1.00	a1.00	b0.40
				c0.60
FUM	b1.00	b1.00	a1.00	b1.00
GDA	a1.00	b1.00	c1.00	c0.40
				d0.60
GPI	a1.00	b1.00	b1.00	b1.00
LDH-1	c1.00	a1.00	b1.00	a1.00
LDH-2	d0.15	b1.00	a1.00	c1.00
	e0.85			
MDH-1	b1.00	a1.00	a1.00	b0.30
				c0.70
MDH-2	a1.00	a1.00	b1.00	a1.00
ME-1	a0.81	c1.00	c1.00	c1.00
	b0.19			
ME-2	a1.00	a0.80	b1.00	a1.00
		b0.20		
PEP-la	c0.08	c1.00	c1.00	a0.10
	d0.92			b0.90
PEP-lg	b1.00	b1.00	b1.00	a1.00
PEP-lgg	a1.00	c1.00	c1.00	b0.04
				c0.60
PGD	b1.00	b1.00	c1.00	a0.10
				b0.90
PGM-C	b1.00	a1.00	d0.20	c1.00
			e0.80	
SDH	c1.00	a1.00	b1.00	b1.00
SOD	a1.00	a1.00	b1.00	a1.00

grooves between axilla and groin including axillary groove 13. Adpressed limbs separated by 0.5 costal folds, fifth digits rudimentary on both hindlimbs.

Etymology

The specific name "*katoi*" is dedicated to Prof. Makoto Kato of Kyoto University who, as an undergraduate student then, first collected the species and has supported our study by offering his collections to us.

Range

Known so far only from south Central Japan, the montane region of the northwestern part of Shizuoka Prefecture (Fujieda-shi, Shizuoka-shi, Misakubo-cho, Honkawane-cho) and southeastern part of Nagano Prefecture (Minamishinano-mura), on the Akaishi Mountains (Fig. 3, Appendix 1)

Table 5. Matrices of Nei's (1978) unbiased genetic distance (above diagonal) and modified Rogers' genetic distance (Wright, 1978, below diagonal) for four *Hynobius* species studied

Species	1	2	3	4
1 *H. katoi*	–	0.795	1.244	0.733
2 *H. tokyoensis*	0.732	–	0.888	0.571
3 *H. kimurae*	0.834	0.761	–	0.958
4 *H. naevius*	0.700	0.643	0.765	–

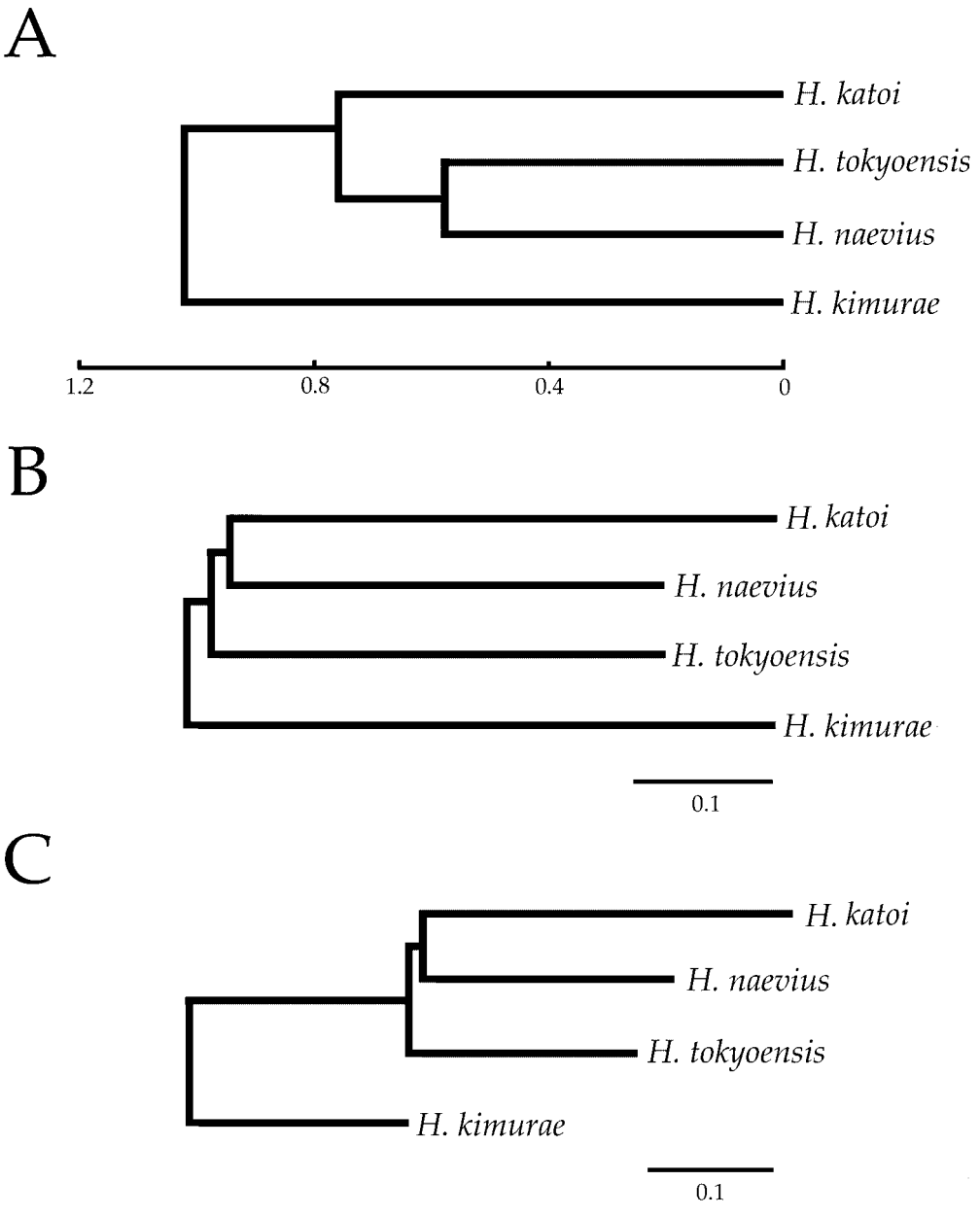

Fig. 4. UPGMA tree (A) constructed from Nei's (1978) distance, NJ tree (B) constructed from modified Rogers' distance (Wright, 1978), and ML tree (C) constructed by CONTML procedure in PHYLIP vers. 3.5 C (Felsenstein, 1993).

and along Oi-gawa and Tenryu-gawa rivers. Known localities, 21 in number, range from 500 m to 1200 m in altitude (median=750 m).

Morphological comparisons

In SVL, *H. katoi* was not significantly different from *H. naevius* from Chubu and Kinki regions and *H. tokyoensis* males from Kanagawa (Tukey-Kramer test, $P>0.05$), but was much smaller than sympatric *H. kimurae* from Shizuoka (Tukey-Kramer test, $P<0.01$).

By comparing percentage ratios of each character dimension relative to SVL, the following significant differences in body shape were detected (Dunn's test, $P<0.05$). In males, *H. katoi* differed from *H. tokyoensis* from Kanagawa in lower tail. *Hynobius naevius* from Chubu and Kinki regions had wider head and longer trunk than *H. katoi*. *Hynobius kimurae* from Shizuoka differed from *H. katoi* in having shorter head and longer trunk. Limited numbers of female samples for the present species prohibited detailed statistical comparisons in this sex.

No statistically significant interspecific differences were observed in the number of costal grooves and the degree of limb overlap. However, in males, *H. katoi* had significantly smaller number of vomerine teeth than *H. kimurae* and *H. naevius* (Tukey-Kramer test, $P<0.01$). The shape of the vomerine teeth series, as expressed by the ratio of width to length, differed among the species with *H. katoi* having vomerine teeth series that are much shallower than *H. naevius* and *H. kimurae* (Dunn's test, $P<0.01$).

Allozymes

Of the 25 loci scored, 21 were polymorphic in at least some species (Table 4). The number of polymorphic loci and the mean number of alleles per locus per sample in *H. katoi* were 12.0 and 1.2, respectively, and were intermediate among the four species (4.0–28.0 and 1.0–1.3, respectively). When compared with *H. kimurae*, *H. naevius*, and *H. tokyoensis*, electromorphs unique to *H. katoi* have been detected at as many as 10 loci (ACH-2, EST-2, GDA, GPI, LDH-1, LDH-2, ME-1, PEP-lgg, PGM-C, and SDH), and at one locus (PEP-la), nearly fixed allelic difference was evident. Nei's (1978) distances among four species (Table 5) were very large, ranging from 0.571 to 1.244, with the highest D value between *H. katoi* and *H. kimurae*. This pattern was nearly identical in the Rogers' distances. The UPGMA tree (Fig. 4A) was slightly different from NJ and ML trees (Fig. 4B, C) in topology. In the UPGMA tree, the first major branching event clearly separated *H. kimurae* from all other species, and the second major branching event separated *H. katoi* from a cluster consisting of *H. tokyoensis* and *H. naevius*. In the NJ and ML trees, *H. katoi* formed a cluster with *H. naevius* and together formed the sister group to *H. tokyoensis*.

Fecundity and natural history

The natural history of *H. katoi* is poorly understood, and egg sacs or larvae are yet to be found in nature despite our extensive field surveys. The numbers of eggs found in ovaries of the two collected females were nine and 13 (mean=11). The mean diameter of ova found in ovaries of the two females were 4.8 and 5.0 (mean=4.9) mm. Both the animal and the vegetal poles are cream in color. Because females with ripe eggs were collected from late April to early May, breeding seems to occur in spring. The smallest young (19.2 mm SVL), collected in late August, is considered to have just metamorphosed. In some larvae, metamorphosis seems to occur in the year of oviposition.

Protection

The new species has been cited in the Red Data Book of Shizuoka Prefecture as an endangered species, *Hynobius* sp.

DISCUSSION

Recent findings of Japanese salamander species have chiefly been supported by the use of biochemical techniques (Matsui, 2000), and isozyme variation appears to be the best descriptor of *H. katoi* as a separate species. The description of *H. katoi* as a new species raises the number of Japanese *Hynobius* species to 16 (Matsui *et al*., 2002).

According to Sato (1943:24, 500), Japanese species of the genus *Hynobius* are split into the lowland, still-water breeding type (lentic breeder) of the *nebulosus* and *lichenatus* groups, and the montane, stream breeding type (lotic breeder) of the *naevius* group. Although this classification has minor problems in a strict sense (Matsui, 1987), it can be generally advocated because each ecological type includes members that are fairly uniform in adult morphology. In contrast to the compressed tails of lentic type salamanders, for example, salamanders of the lotic type have thick tails (Sato, 1943), and the latter is the case in *H. katoi*.

The nine and 13 eggs found in ovaries of the two females of *H. katoi* are much less than the clutch sizes previously reported for Japanese *Hynobius* species (Sengoku *et al*., 1996). Among ten species of lowland, lentic breeders, the mean of the minimum and maximum clutch sizes varies from 50.5 to 110 (median=92.5), whereas in five lotic breeding species it ranges from 26.5 to 40 (median=32). Also in clutch size, therefore, *H. katoi* is much closer to the lotic breeders than to the lentic ones.

Similarly, the non-pigmented and large egg (mean=4.9 mm) of *H. katoi* is again closer to that of the lotic breeders [e.g., mean ovum diameter=4.3–4.5 mm in *H. kimurae* (Misawa and Matsui, 1997); mean=5.1 mm in *H. naevius* (Tominaga, unpublished data)] than to small [e.g., *H. nebulosus* (mean=2.2 mm: Ento and Matsui, 2002); *H. hidamontanus* (mean=2.8 mm: Matsui, 1987)], pigmented eggs of lentic breeders. From these lines of evidence, the newly described *H. katoi*, possessing characteristics common to the lotic breeders, is judged to be a member of the *H. naevius* group of Sato (1943).

As a result of the electrophoretic examination in this work, *H. katoi* was shown to be genetically very distant from the lentic breeding *H. tokyoensis* [Nei's (1978) D=0.795 (Table 5)]. However, at the same time *H. katoi* exhibited similar genetic distances from the lotic breeding *H. naevius* (D=0.733). These large genetic distances seem to indicate that *H. katoi* has a very divergent history from allied species and likely represents a good species. *Hynobius katoi* is largely sympatric with *H. kimurae* throughout its range, but allozyme variation clearly indicated genetic isolation [Nei's D=1.244], and no evidence of introgression or hybridization between these two species was detected.

Although *H. katoi* is assigned to the lotic breeding *H. naevius* group by its adult morphology and ovarian egg characteristics, current knowledge of its ecology is very limited. The distributional range of *H. katoi* is well known as an area with frequent land slipping (Saito and Fujita, 1988), and this seems to imply the presence of a large amount of underground water. Recently two syntopic morphotypes of *H. naevius* were found in northern Kyushu (Tominaga *et al*., 2003). Of these morphotypes, a larger bodied one breeds in small open streams and larvae are easily found, whereas the other, smaller morphotype is thought to breed in underground water and its larvae are yet to be found. This difference in breeding habits is assumed to allow these two types to coexist (Tominaga *et al*., 2003). In the case of *H. katoi*, sympatric *H. kimurae* are much larger in body size and breed in open streams, although the egg sacs are attached to the underside of stones. We consider our failure to detect eggs and larvae of *H. katoi* to be attributed to its possible breeding in small underground streams. The size difference between *H. katoi* and *H. kimurae*, and their occupations of different breeding habitats may enable them to coexist.

Populations of *H. katoi* are distributed in a region surrounding the southern half of the Akaishi Mountains (Fig. 3), and are geographically delimited by the southern edge of the Fossa Magna (Itoigawa-Shizuoka Tectonic Line) and eastern edge of the Median Tectonic Line. Because of the complex topography of their habitat, information on the distribution of *H. katoi* should be much limited yet. Therefore, more detailed surveys are needed for the better understandings of biogeographical history of this salamander. In any case, it seems likely that the range of *H. katoi* is very limited and much smaller than that of most other Japanese *Hynobius* species. The limited distribution of *H. katoi*, coupled with its genetic distinctiveness, clearly argues for the necessity to devise immediate measures for the protection of this component of Japanese endemic biodiversity.

ACKNOWLEDGMENTS

We are particularly grateful to M. Kato for supplying the first specimen of *H. katoi* and valuable information. We thank M. Yamagami, H. Takahashi, H. Ishikawa, and T. Sawahata for information and collection of materials. D. Weisrock kindly corrected verbal errors.

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(Received March 12, 2004 / Accepted March 24, 2004)

Appendix 1 Voucher specimens morphologically examined are stored at Graduate School of Human and Environmental Studies, Kyoto University (KUHE), Dr. Takahashi's private collection (TK), and Mr. Tanabe's private collection (T).

H. katoi: Fujieda-shi, Shizuoka Pref.: KUHE 14664, 14665, 16302, 16676, 17004, 17945–17953, 18349, 18351, 18352, 18391, 18392, 18404–18407, 21685–21689, 21709, 26098, 26099, 29767; Shizuoka-shi, Shizuoka Pref.: KUHE 5404; Misakubo-cho, Shizuoka Pref.: KUHE 21706, 21707, 22885, 22886, 22926–22928, 27154, 27155; Honkawane-cho, Shizuoka Pref.: KUHE 16108, 16109, 29677–29681, 29768–29770; Minamishinano-mura, Nagano Pref.: KUHE 33928, TK unnumbered three specimens.

H. naevius: Fujihashi-mura, Gifu Pref.: KUHE 27396–27399, 27406, 27408, 27531–27533, 27632, 27636, 28762, 28765, 28775, T3045, T3046; Tsuchiyama-cho, Shiga Pref.: KUHE 7504, 28477, 28710, T-2799, T-2804; Suzuka-shi, Mie Pref.: KUHE 6378; Izumi-shi, Osaka Pref.: T-2695, T-, T-2854, T-2989, T-2990.

H. kimurae: Shizuoka-shi, Shizuoka Pref.: KUHE 16942, 16944–16650, 16977, 18114, 18115, 24564, 24565; Misakubo-cho, Shizuoka Pref.: KUHE 17944, 18835–18840, 18844–18848, 21537, 21708.

H. tokyoensis: Yokosuka-shi, Kanagawa Pref.: KUHE 13192–13219.

Appendix 2 Voucher specimens used for electrophoretic analyses are stored at Graduate School of Human and Environmental Studies, Kyoto University (KUHE) and Mr. Tanabe's private collection (T).

H. katoi: Fujieda-shi, Shizuoka Pref.: KUHE 16676, 17004, 17946, 18391, 18392, 18404–18406, 21685–21689.

H. naevius: Chihaya-Akasaka-mura, Osaka Pref.: KUHE unnumbered one specimen; T 2677–2680.

H. kimurae: Shizuoka-shi, Shizuoka Pref.: KUHE 16942–16946.

H. tokyoensis: Yokosuka-shi, Kanagawa Pref.: KUHE 14395, 14397, 14400, 14402, 14405.

Appendix 3 Enzymes, E.C.numbers, locus notations, and buffer systems used in the analyses of allozyme variations in the *Hynobius* species.

Enzyme	E.C. number	Locus	Buffer system*
Aspartate aminotransferase	2.6.1.1	AAT-1	CAPM6
Aspartate aminotransferase	2.6.1.1	AAT-2	CAPM6,TC7
Aconitate hydratase	4.2.1.3	ACH-1	TC8
Aconitate hydratase	4.2.1.3	ACH-2	TC8
Esterase	3.1.1.1	EST-1	TC7
Esterase	3.1.1.1	EST-2	TC7
Fumarate hydratase	4.2.1.1	FUM	TBE8.7
Guanine deamidase	3.5.4.3	GDA	TBE8.7
Glucose-6-phosphate isomerase	5.3.1.9	GPI	CAPM6
Glutamate dehydrogenase	1.4.1.3	GTDH	TC8
Isocitrate dehydrogenase	1.1.1.42	IDH-1	TC7
L-Lactate dehydrogenase	1.1.1.27	LDH-1	CAPM6,TC7
L-Lactate dehydrogenase	1.1.1.27	LDH-2	CAPM6,TC7
Malate dehydrogenase	1.1.1.37	MDH-1	CAPM6,TC8
Malate dehydrogenase	1.1.1.37	MDH-2	CAPM6,TC8
Malic enzyme**	1.1.1.40	ME-1	TC7
Malic enzyme**	1.1.1.40	ME-2	TC7
Peptidase (leucyl-alanine)	3.4.11.–	PEP-la	TBE8.7
Peptidase (leucyl-glycine)	3.4.11.–	PEP-lg	TBE8.7
Peptidase (leucyl-glycyl-glycine)	3.4.11.–	PEP-lgg	TBE8.7
Phosphoglucomutase	5.4.2.2	PGM-A	TC7
Phosphoglucomutase	5.4.2.2	PGM-C	TC7
Phosphogluconate dehydrogenase	1.1.1.44	PGD	TC7
Sorbitol dehydrogenase	1.1.1.14	SDH	CAPM6
Superoxide dismutase	1.15.1.1	SOD	TBE8.7

*Buffer systems-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).
**NADP-dependent malate dehydrogenase.