Taxonomic revision of the Yamaguchi salamander *Hynobius bakan*: Description of two new species from Chugoku and Kyushu, Japan

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ヤマグチサンショウウオ *Hynobius bakan* の分類学的再検討：中国地方および九州地方からの 2 新種の記載

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We describe two new species of the genus *Hynobius* from Yamaguchi and Oita Prefectures, Japan. *Hynobius bakan* could be clearly divided into at least three groups based on morphological and molecular analyses; thus, the Yamaguchi group and the Usa-Bungotakada population of the Oita group were described as *Hynobius nagatoensis* sp. nov. and *Hynobius nihoensis* sp. nov., respectively. Morphological comparisons showed that *H. nagatoensis* sp. nov. has a significantly longer tail length than *H. bakan* in males, and *H. nihoensis* sp. nov. has a significantly narrower head width than *H. bakan* in both sexes. The distribution area of *H. bakan* was largely revised and is limited to the southernmost part of Yamaguchi Prefecture.

本論文において，日本の山口県および大分県から得られたサンショウウオ属の 2 種を新種として記載する。形態および分子系統解析の結果，ヤマグチサンショウウオは少なくとも 3 群（山口グループ，大分グループの宇部集団，大分グループの宇佐・豊後高田集団）に明確に分かれることが判明した。したがって，3 群のうち，山口グループを *Hynobius nagatoensis* sp. nov.，大分グループの宇佐・豊後高田集団を *H. nihoensis* sp. nov. として，それぞれ記載した。形態比較の結果，前者は雄において *H. bakan* よりも尾長が有意に長く，後者は両性共に頭幅が *H. bakan* よりも有意に狭かった。本記載により，*H. bakan* の分布域は大きく変更となり，本種は山口県南部に分布域が限られることになる。

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INTRODUCTION

The Yamaguchi salamander (*Hynobius bakan*) was first described in Warisaka, Kurumaji, Ube City, Yamaguchi Prefecture, and is distributed in Yamaguchi and Oita Prefectures across the Seto Inland Sea (Suonada) (Matsui *et al*., 2019). Mitochondrial DNA analyses revealed that two genetically distinct groups comprise the species, the Yamaguchi group (excluding Ube populations) and the Oita (including Ube populations) group; monophyly of the two groups was not supported by maximum likelihood estimation (ML) and Bayesian inference (BI) (56/0.63) (Matsui *et al*., 2019). However, the phylogenetic analysis was performed using data from only seven populations, warranting reassessment using data from many populations across the entire distribution of *H. bakan*. Additionally, morphological uniformity was not previously compared between the Oita and Yamaguchi groups of *H. bakan* (Matsui *et al*., 2019). The Oita group contains two distinct populations, the Ube (Yamaguchi Prefecture) population and the Usa-Bungotakada (Oita Prefecture) population, which are isolated by the Seto Inland Sea (Matsui *et al*., 2019). Geographically isolated populations can exhibit morphological differences (e.g., Rodríguez *et al*., 2020). Thus, morphological comparisons among the three *H. bakan* groups (the Yamaguchi group, the Ube population of the Oita group, and the Usa-Bungotakada population of the Oita group) are necessary.

In the present study, we evaluated the species validity of three *H. bakan* groups using morphological, phylogenetic, and evolutionary species concepts, as described in Sugawara *et al*. (2018). To resolve the taxonomic uncertainty surrounding *H. bakan*, we performed statistical analyses to compare morphological characteristics among the three groups. Additionally, we used DNA sequence data collected from specimens across the entire distribution range of the species to reconstruct the phylogeny of *H. bakan*. Finally, we describe the distribution ranges of the three groups of *H. bakan* in detail.

MATERIALS AND METHODS

Molecular analysis

For the phylogenetic analysis, we sampled 26 individuals from 26 localities, suggested by closed symbols in Fig. 1, between February 2007 and April 2021 (Table 1; Fig. 1). We collected a single tailbud embryo from each paired egg sac or tissue samples from larvae and preserved them in 99.5% ethanol. DNA extraction and sequencing analyses were conducted following the methods of Sugawara *et al*. (2018). We deposited the acquired sequences into the DNA Data Bank of Japan (Table 1). We aligned DNA sequences using MEGA X (Kumar *et al*., 2018) and performed phylogenetic analysis using BI and maximum likelihood (ML) estimation, including several *Hynobius* species and *Salamandrella keyserlingii* as the outgroup (Table 1). We estimated the best-fit nucleotide substitution model based on the Bayesian information criterion (Schwarz, 1978) and corrected Akaike's information criterion (AICc) (Sugiura, 1978) using jModelTest 2 (Darriba *et al*., 2012). We selected the Hasegawa-Kishino-Yano model (gamma distribution) for BI and the same model (gamma distribution with invariant sites) for ML. We constructed Bayesian and maximum likelihood trees using MrBayes 3.2 (Ronquist *et al*., 2012) and MEGA X (Kumar *et al*., 2018), respectively. For Bayesian analyses, we performed two independent MCMC runs for 2,000,000 generations, with a sampling frequency of 100. We examined the stationarity of the sampled tree likelihood scores using Tracer version 1.7 (http://tree.bio.ed.ac.uk/software/tracer/); the first 25% of generations were discarded as burn-in. Monophyly was assessed using posterior probability (PP) and bootstrap (BS) values based on the criteria described by Huelsenbeck and Rannala (2004) (PP $\geq$ 0.95) and Hillis and Bull (1993) (BP $\geq$ 70).

Morphological analysis

We sampled 126 *H. bakan* individuals between January 2017 and April 2021. Among them, 37 individuals (27 males and 10 females) were from eight Yamaguchi group populations (Pops. 1, 2, 6, 8, 9, 12, 15, 16), 19 individuals (14 males and 5 females) were from

Table 1 List of specimens in molecular analyses. Population numbers correspond to the localities where individuals were collected (Fig. 1). The number of adults is the individuals of both sexes used in morphological analyses. Asterisks after the sampling localities show the type locality of three species.

Pop.	Species	Sampling licality	Accession number / Label in Fig. 3
1	*Hynobius nagatoensis* sp. nov.	Shuhocho Kama, Mine City, Yamaguchi Pref.*	LC633121 / NAGA01
2	*Hynobius nagatoensis* sp. nov.	Shuhocho Akiyoshi, Mine City, Yamaguchi Pref.	LC633122 / NAGA02
3	*Hynobius nagatoensis* sp. nov.	Mitocho Aka, Mine City, Yamaguchi Pref.	LC633123 / NAGA03
4	*Hynobius nagatoensis* sp. nov.	Mitocho Naganobori, Mine City, Yamaguchi Pref.	LC633124 / NAGA04
5	*Hynobius nagatoensis* sp. nov.	Nishikibe, Ube City, Yamaguchi Pref.	LC633125 / NAGA05
6	*Hynobius nagatoensis* sp. nov.	Omi Island, Senzaki, Nagato City, Yamaguchi Pref.	LC633126 / NAGA06
7	*Hynobius nagatoensis* sp. nov.	Hekikami, Nagato City, Yamaguchi Pref.	LC633127 / NAGA07
8	*Hynobius nagatoensis* sp. nov.	Hohokucho Tsunoshima, Shimonoseki City, Yamaguchi Pref.	LC633128 / NAGA08
9	*Hynobius nagatoensis* sp. nov.	Toyotacho Azakami, Shimonoseki City, Yamaguchi Pref.	LC633129 / NAGA09
10	*Hynobius nagatoensis* sp. nov.	Toyouracho Uka, Shimonoseki City, Yamaguchi Pref.	LC633130 / NAGA10
11	*Hynobius nagatoensis* sp. nov.	Kikugawacho Oaza Nanami, Shimonoseki City, Yamaguchi Pref.	LC633131 / NAGA11
12	*Hynobius nagatoensis* sp. nov.	Ouchi, Shimonoseki City, Yamaguchi Pref.	LC633132 / NAGA12
13	*Hynobius nagatoensis* sp. nov.	Ozukicho, Shimonoseki City, Yamaguchi Pref.	LC633133 / NAGA13
14	*Hynobius nagatoensis* sp. nov.	Toyouracho Kuroi, Shimonoseki City, Yamaguchi Pref.	LC633134 / NAGA14
15	*Hynobius nagatoensis* sp. nov.	Fujigatani, Shimonoseki City, Yamaguchi Pref.	LC633135 / NAGA15
16	*Hynobius nagatoensis* sp. nov.	Kitaku, Moji Ward, Fukuoka Pref.	LC633136 / NAGA16
17	*Hynobius nagatoensis* sp. nov.	Jyumonji, Mitocho Mana, Mine City, Yamaguchi Pref.	LC436411 / H48
18	*Hynobius nagatoensis* sp. nov.	Ominecho, Mine City, Yamaguchi Pref.	LC436412 / H49
19	*Hynobius nagatoensis* sp. nov.	Oba, Hohokucho Tasuki, Shimonoseki City, Yamaguchi Pref.	LC436410 / H47
20	*Hynobius nagatoensis* sp. nov.	Yamadacho, Shimonoseki City, Yamaguchi Pref.	LC436413 / H50
21	*Hynobius bakan*	Kori, Sanyo-Onoda City, Yamaguchi Pref.	LC633137 / BAKA01
22	*Hynobius bakan*	Warisaka, Kurumaji, Ube City, Yamaguchi Pref.*	LC436416 / H53
23	*Hynobius bakan*	Nishikiwa, Ube City, Oita Pref.	LC633138 / BAKA02
24	*Hynobius bakan*	Norisada, Ube City, Yamaguchi Pref.	LC633139 / BAKA03
25	*Hynobius nihoensis* sp. nov.	Shinei-nyuzubaru, Bungotakada City, Oita Pref.	LC633140 / NIHO01
26	*Hynobius nihoensis* sp. nov.	Haraida, Bungotakada City, Oita Pref.	LC633141 / NIHO02
27	*Hynobius nihoensis* sp. nov.	Sakai, Bungotakada City, Oita Pref.	LC633142 / NIHO03
28	*Hynobius nihoensis* sp. nov.	Yama, Usa City, Oita Pref.	LC633143 / NIHO04
29	*Hynobius nihoensis* sp. nov.	Waki, Usa City, Oita Pref.	LC633144 / NIHO05
30	*Hynobius nihoensis* sp. nov.	Yamashita, Usa City, Oita Pref.*	LC633145 / NIHO06
31	*Hynobius nihoensis* sp. nov.	Shinei, Bungotakada City, Oita Pref.	LC436414 / H51
32	*Hynobius nihoensis* sp. nov.	Usa City, Oita Pref.	LC436415 / H52
33	*Hynobius* sp.	Onoda, Sanyo-Onoda City, Yamaguchi Pref.	LC422612 / SP01
	Hynobius dunni		LC633146 / H. dunni
	Hynobius nebulosus		LC436409 / H. nebulosus
	Hynobius tsuensis		LC436448 / H. tsuensis
	Hynobius iwami		LC436417 / H. iwami
	Hynobius okiensis		LC436446 / H. okiensis
	Salamandrella keyserlingii		NC_026032 / S. keyserlingii

three Ube populations (Pops. 21, 22, 23) of the Oita group, and 70 individuals (57 males and 13 females) were from five Usa-Bungotakada populations (Pops. 25, 27, 28, 29, 30) of the Oita group (Table 1; Fig. 1). The adults assessed in the morphological analyses are listed in Table 1. The collected individuals were anesthetized using ethyl 3-aminobenzoate methanesulfonate salt (Sigma-Aldrich®, St. Louis, MO, USA) diluted 1,000 times with water (Bennett 1991). We photographed the dorsal, ventral, and lateral sides of all individuals on a black background and collected tissue samples (preserved in 99.5% ethanol) from the tail tips of all individuals. We used a vernier caliper to measure the following 22 measurements (refer to Nishikawa *et al*., 2007) on each specimen: snout–vent length (SVL), trunk length (TRL), axilla–groin distance (AGD), head length (HL), tail length (TAL), median tail width (MTAW), median tail height (MTAH), vomerine

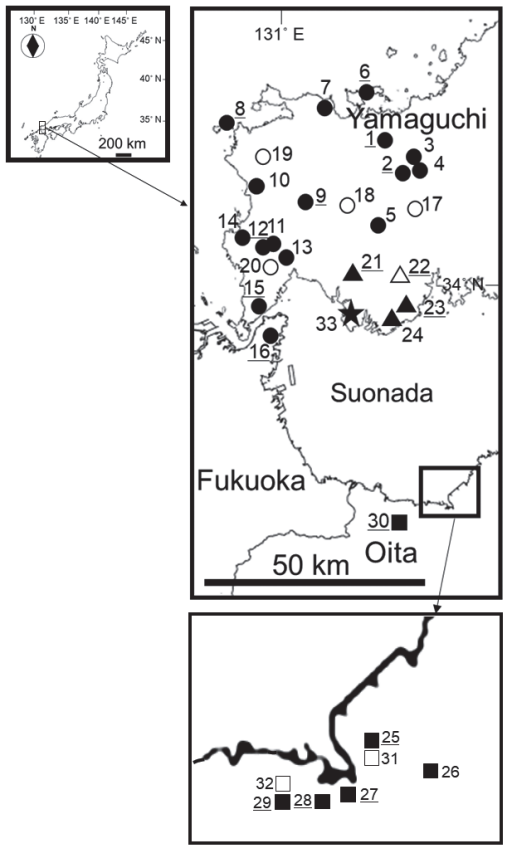

Fig. 1 Localities for three *Hynobius* species populations sampled in their distribution areas. Population numbers match those used for molecular analyses (see Table 1 and Fig. 3). The enlarged areas above and below include the westernmost part of Chugoku and the northeastern part of Kyushu and the northeastern part of Oita Prefecture, respectively. The closed symbols correspond to the three species and one unidentified species sampled in this study: *H. nagatoensis* sp. nov. (closed circles), *H. bakan* (closed triangles), *H. nihoensis* sp. nov. (closed squares), and *H.* sp. (closed star). The open symbols correspond to the three species cited from Matsui *et al.* (2019): *H. nagatoensis* sp. nov. (open circles), *H. bakan* (open triangles), and *H. nihoensis* sp. nov. (open squares). For the morphological assessments, individuals of the three species were collected from the underlined localities: Pops. 1 (type locality of *H. nagatoensis* sp. nov.: 17 males and 7 female), 2 (2 males and 2 females), 6 (1 male), 8 (2 males), 9 (1 male), 12 (2 males and 1 female), 15 (1 male), and 16 (1 male) for *H. nagatoensis* sp. nov.; Pops. 21 (9 males and 2 females), 22 (4 males and 3 females), and 23 (1 male) for *H. bakan*; Pops. 25 (12 males and 1 female), 27 (5 males), 28 (6 males and 1 female), 29 (28 males and 8 females), and 30 (type locality of *H. nihoensis* sp. nov.; 6 males and 3 females).

teeth length (VTL), vomerine teeth width (VTW), head width (HW), forelimb length (FLL), hindlimb length (HLL), second finger length (2FL), third finger length (3FL), third toe length (3TL), fifth toe length (5TL), internarial distance (IND), interorbital distance (IOD), upper eyelid length (UEL), snout length (SL), upper eyelid width (UEW), lower jaw length (LJL). We also recorded the presence of distinct black spots on the dorsum (DBSD), distinct white spots on the venter (DWSV), distinct and bright yellow lines on the dorsal (DBYLD) and ventral (DBYLV) sides of the tail (Fig. 2), and distinct gular mottling (DGM) on the ventral side of the head. The number of costal folds between the adpressed limbs (CFBALN) and the number of costal grooves (CGN) were counted; we used the counting method of Matsui *et al.* (2019) for CGN. To conserve the populations, measured individuals were subsequently returned to their capture sites, excluding the candidate type specimens.

Prior to performing morphological comparisons among the three groups, we tested for normality using the Shapiro–Wilk test. When data followed a normal distribution, we tested for homoscedasticity using Bartlett's test. When population variances were equal, we performed Tukey–Kramer tests, and when variances were unequal, we performed Games–Howel

Fig. 2 Representative images of (a) a distinct and bright yellow line (*H. nagatoensis* sp. nov.) and (b) an indistinct and dull yellow line (*H. nihoensis* sp. nov.) on the dorsal side of the tail. In this study, we did not recognize the latter as a distinct yellow line.

tests. When data did not follow a normal distribution and population variances were unequal, we performed Steel–Dwass tests. To evaluate the overall morphological variation among the three groups, we conducted a canonical discriminant analysis using SVL and standardized values (R = %SVL) for the 21 measurements: RTRL, RAGD, RHL, RTAL, RMTAW, RMTAH, RVTL, RVTW, RHW, RFLL, RHLL, R2FL, R3FL, R3TL, R5TL, RIND, RIOD, RUEW, RSL, RUEL, RLJL. All statistical analyses were performed using R with $\alpha = 0.05$ (Ihaka and Gentleman, 1996).

Type specimens measurements

The collected type specimens (holotype and paratypes) were fixed in 10% formalin and transferred to 70% ethanol before measurement. We took 43 measurements from the holotype (also topotype), namely, SVL, TRL, AGD, HL, TAL, MTAW, MTAH, basal tail width (BTAW), basal tail height (BTAH), VTL, VTW, HW, left forelimb length (LFLL), left hindlimb length (LHLL), right forelimb length (RFLL), right hindlimb length (RHLL), left first finger length (L1FL), left second finger length (L2FL), left third finger length (L3FL), left fourth finger length (L4FL), right first finger length (R1FL), right second finger length (R2FL), right third finger length (R3FL), right fourth finger length (R4FL), left first toe length (L1TL), left second toe length (L2TL), left third toe length (L3TL), left fourth toe length (L4TL), left fifth toe length (L5TL), right first toe length (R1TL), right

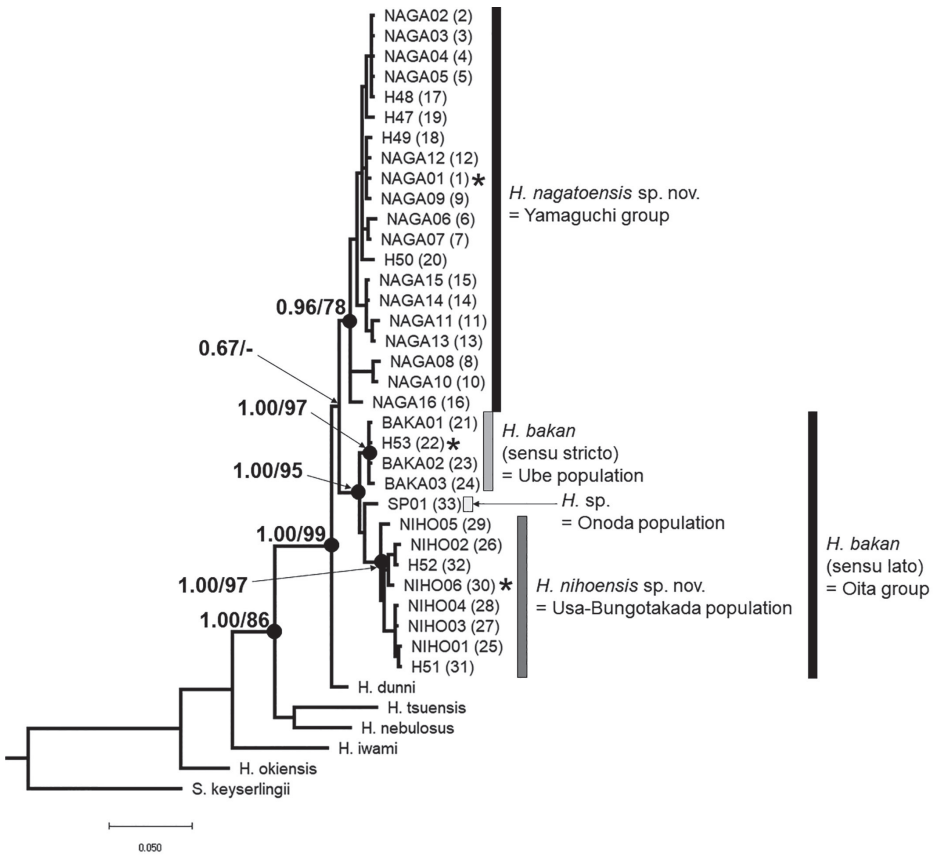

Fig. 3 Bayesian inference phylogenetic tree based on 630-base-pair (bp) cytochrome b sequences rooted with *Salamandrella keyserlingii*. The scale shows the genetic distance (expected changes per site). Numbers located near the branches are posterior probabilities (PP) for Bayesian inference and bootstrap (BS) values for the maximum likelihood estimation. Numbers appearing in parentheses after the haplotype names correspond to population localities, as indicated in Table 1 and Fig. 1. Asterisks after parentheses (Pops. 1, 22, and 30) indicate the type locality of the three species.

second toe length (R2TL), right third toe length (R3TL), right fourth toe length (R4TL), right fifth toe length (R5TL), IND, IOD, left upper eyelid length (LUEL), right upper eyelid length (RUEL), SL, left upper eyelid width (LUEW), right upper eyelid width (RUEW), LJL.

RESULTS

The monophyly of *H. bakan* and *H. dunni* was completely supported by PP and almost completely supported the BS value (Fig. 3). The monophyly of *H. bakan* was not supported by PP nor BS (Fig. 3). Monophyly of the Oita group of *H. bakan* was strongly supported (Fig. 3), and monophyly of the Yamaguchi group of *H. bakan* was supported by PP and BS

Table 2 Measurement (mm) of SVL and characteristic ratios (R = %SVL) of TRL to LJL. Ranges are shown in parentheses. See Materials and Methods section for morphological trait definitions.

	H. nagatoensis sp. nov.			*H. bakan*			*H. nihoensis* sp. nov.		
		Male	Female		Male	Female		Male	Female
Trait	Holotype	*n* = 27	*n* = 10	Topotype	*n* = 14	*n* = 5	Holotype	*n* = 57	*n* = 13
SVL	59.8	56.5±4.73	59.8±5.30	53.9	51.7±3.40	53.9±2.88	56.8	57.7±7.23	59.2±2.16
		(48.9-65.3)	(48.1-66.1)		(44.0-56.5)	(50.2-56.9)		(41.6-70.3)	(56.0-62.9)
RTRL	79.1	77.1±1.53	77.9±1.51	77.0	76.0±0.67	77.2±1.31	78.5	78.1±1.23	79.5±1.05
		(74.4-82.5)	(75.5-80.2)		(74.7-77.5)	(76.0-78.9)		(74.7-80.7)	(77.3-81.5)
RAGD	53.7	51.7±1.88	54.8±1.94	51.8	51.4±1.30	54.2±2.06	56.3	54.6±1.93	57.7±1.47
		(47.6-55.5)	(52.0-57.8)		(48.9-53.8)	(51.5-56.4)		(50.8-58.9)	(55.9-60.1)
RHL	22.4	24.2±1.10	23.2±0.96	23.6	24.2±0.81	23.6±1.36	21.0	22.6±1.09	20.9±0.86
		(21.6-26.3)	(21.5-24.9)		(22.7-26.1)	(22.2-25.5)		(20.5-25.1)	(19.8-23.3)
RTAL	96.7	84.2±6.45	75.4±4.19	76.6	69.5±6.96	71.6±7.02	75.5	74.8±8.02	64.9±4.95
		(74.0-97.1)	(68.1-82.5)		(55.8-76.6)	(63.9-80.8)		(52.3-90.0)	(54.9-72.3)
RMTAW	6.2	6.0±0.86	5.5±0.83	5.4	5.8±1.03	5.3±0.81	5.1	5.3±0.89	5.0±0.73
		(4.6-8.0)	(4.3-7.1)		(4.1-7.5)	(4.3-6.5)		(3.6-7.2)	(3.8-6.2)
RMTAH	15.2	13.2±1.49	11.1±0.79	10.9	10.7±1.35	8.9±2.52	11.3	11.9±1.51	9.3±0.98
		(11.2-17.5)	(9.7-12.4)		(8.5-12.9)	(4.9-11.8)		(9.0-18.8)	(6.5-10.7)
RVTL	5.5	6.0±0.54	5.6±0.44	5.9	6.0±0.48	5.9±0.55	4.8	5.3±0.59	5.0±0.37
		(5.0-7.5)	(4.7-6.3)		(5.2-6.7)	(5.1-6.4)		(4.4-6.8)	(4.4-5.5)
RVTW	5.4	5.7±0.42	5.3±0.39	5.4	5.7±0.45	5.5±0.72	5.1	5.4±0.56	5.2±0.48
		(4.9-6.5)	(4.9-6.1)		(4.8-6.6)	(4.8-6.4)		(4.4-6.8)	(4.3-5.9)
RHW	18.4	18.4±0.97	17.3±0.52	17.3	17.3±0.39	16.7±0.49	15.0	16.1±0.77	15.2±0.58
		(16.4-20.5)	(16.6-18.3)		(16.9-18.0)	(15.9-17.3)		(14.5-17.6)	(14.3-16.3)
RFLL	23.1	23.6±1.23	22.4±1.42	21.9	24.6±1.24	23.0±1.48	21.0	21.9±2.08	19.8±2.33
		(21.0-26.3)	(20.3-24.6)		(21.9-26.9)	(21.9-25.6)		(18.1-27.0)	(17.4-25.1)
RHLL	32.1	29.7±1.50	28.6±1.52	30.6	30.9±0.88	29.9±1.19	26.4	27.8±1.45	26.1±1.30
		(27.0-32.1)	(26.8-31.3)		(29.3-32.4)	(28.5-31.5)		(24.6-30.3)	(24.7-30.1)
R2FL	4.2	4.8±0.48	4.9±0.59	5.4	5.1±0.48	4.8±0.28	4.6	4.6±0.59	4.3±0.34
		(4.0-5.9)	(4.2-5.9)		(4.3-5.8)	(4.5-5.2)		(3.0-6.2)	(3.8-4.8)
R3FL	4.2	4.4±0.41	4.6±0.59	4.6	4.0±0.34	4.3±0.20	4.2	3.8±0.62	4.1±0.38
		(3.6-5.3)	(3.9-5.7)		(3.4-4.6)	(4.2-4.7)		(2.4-5.4)	(3.4-4.8)
R3TL	7.4	7.9±0.66	7.4±0.43	8.3	7.7±0.47	7.9±0.83	7.2	7.2±0.67	6.8±0.68
		(6.9-9.8)	(6.7-8.0)		(6.8-8.3)	(7.2-9.3)		(5.0-8.5)	(6.0-8.5)
R5TL	1.7	2.2±0.93	2.6±0.75	2.8	1.9±0.37	2.1±0.70	2.5	2.0±0.47	2.3±0.38
		(0.2-3.7)	(1.6-3.9)		(1.4-2.8)	(1.2-2.9)		(0.6-3.2)	(1.5-3.1)
RIND	4.5	4.6±0.48	4.2±0.75	3.9	4.9±0.54	4.9±0.29	4.8	4.2±0.40	3.9±0.23
		(3.8-5.4)	(3.1-5.8)		(3.7-5.8)	(4.6-5.2)		(3.1-4.8)	(3.6-4.3)
RIOD	6.7	6.4±0.57	5.8±0.49	6.7	6.3±0.37	5.8±0.39	6.0	5.5±0.49	5.3±0.39
		(5.5-7.6)	(5.2-7.0)		(5.7-6.8)	(5.2-6.2)		(4.6-7.1)	(4.6-5.9)
RUEW	3.2	3.4±0.46	3.2±0.36	2.8	3.2±0.24	3.0±0.44	2.5	3.2±0.40	3.2±0.27
		(2.5-4.2)	(2.5-3.9)		(2.7-3.5)	(2.5-3.6)		(2.4-4.1)	(2.5-3.6)
RSL	7.5	6.6±0.46	6.2±0.63	6.7	6.2±0.35	5.8±0.73	6.5	5.8±0.46	5.3±0.49
		(5.6-7.8)	(5.6-7.3)		(5.6-6.8)	(4.7-6.4)		(4.7-6.6)	(4.6-6.6)
RUEL	4.5	4.1±0.45	4.1±0.58	4.6	4.7±0.26	4.5±1.03	3.5	4.1±0.33	4.0±0.25
		(3.3-5.0)	(3.5-5.2)		(3.4-5.1)	(3.7-6.2)		(3.4-5.2)	(3.4-4.3)
RLJL	13.5	14.2±1.06	13.4±1.16	13.5	14.0±0.50	13.5±0.76	11.3	12.8±0.86	12.4±0.96
		(11.7-16.0)	(12.0-15.0)		(13.2-14.9)	(12.4-14.4)		(11.2-14.5)	(11.2-14.3)

Table 3 Significantly different values among 22 morphological characteristics between three species of both sexes and between sexes for each species. Larger values of significant differences were bold. See Materials and Methods section for definitions of morphological traits. NAGA, *Hynobius nagatoensis* sp. nov.; BAKA, *H. bakan*; NIHO, *H. nihoensis* sp. nov..

	Male			Female			NAGA	BAKA	NIHO
	NAGA vs. BAKA	NAGA vs. NIHO	BAKA vs. NIHO	NAGA vs. BAKA	NAGA vs. NIHO	BAKA vs. NIHO	Male vs. Female		
SVL	P < 0.01	NS	**P < 0.001**	P < 0.05	NS	P < 0.05	NS	NS	NS
RTRL	P < 0.05	P < 0.01	**P < 0.0001**	NS	P < 0.05	P < 0.01	NS	NS	**P < 0.001**
RAGD	NS	**P < 0.0001**	**P < 0.0001**	NS	P < 0.01	P < 0.01	**P < 0.0001**	P < 0.01	**P < 0.0001**
RHL	NS	**P < 0.0001**	**P < 0.0001**	NS	**P < 0.001**	P < 0.01	P < 0.05	NS	**P < 0.0001**
RTAL	**P < 0.0001**	**P < 0.0001**	NS	NS	**P < 0.001**	P < 0.05	**P < 0.001**	NS	**P < 0.0001**
RMTAW	NS	P < 0.05	NS	NS	NS	NS	NS	NS	NS
RMTAH	**P < 0.0001**	**P < 0.001**	P < 0.05	NS	**P < 0.001**	NS	**P < 0.0001**	NS	**P < 0.0001**
RVTL	NS	**P < 0.0001**	**P < 0.001**	NS	P < 0.01	P < 0.01	NS	NS	P < 0.05
RVTW	NS	NS	NS	NS	NS	NS	P < 0.05	NS	NS
RHW	**P < 0.001**	**P < 0.0001**	**P < 0.0001**	NS	**P < 0.0001**	**P < 0.0001**	P < 0.01	P < 0.05	**P < 0.001**
RFLL	NS	**P < 0.0001**	**P < 0.0001**	NS	P < 0.05	P < 0.05	P < 0.05	NS	P < 0.01
RHLL	P < 0.05	**P < 0.0001**	**P < 0.0001**	NS	P < 0.01	P < 0.01	NS	NS	**P < 0.001**
R2FL	NS	NS	P < 0.05	NS	P < 0.05	NS	NS	NS	P < 0.05
R3FL	P < 0.01	**P < 0.0001**	NS	NS	NS	NS	NS	P < 0.01	NS
R3TL	NS	**P < 0.0001**	P < 0.05	NS	NS	P < 0.01	P < 0.05	NS	NS
R5TL	NS	NS	NS	NS	NS	NS	NS	NS	NS
RIND	NS	P < 0.01	**P < 0.0001**	NS	NS	P < 0.01	NS	NS	P < 0.01
RIOD	NS	**P < 0.0001**	**P < 0.0001**	NS	P < 0.05	NS	P < 0.01	P < 0.05	NS
RUEW	NS	NS	NS	NS	NS	NS	NS	NS	NS
RSL	P < 0.05	**P < 0.0001**	P < 0.05	NS	P < 0.01	NS	NS	NS	**P < 0.001**
RUEL	**P < 0.0001**	NS	**P < 0.0001**	NS	NS	NS	NS	NS	NS
RLJL	NS	**P < 0.0001**	**P < 0.0001**	NS	NS	NS	P < 0.05	NS	NS
P < 0.05	3	1	4	1	4	3	5	2	2
P < 0.01	2	2	0	0	4	7	2	2	2
P < 0.001	1	1	2	0	3	0	1	0	4
P < 0.0001	3	12	10	0	1	1	2	0	4
Total	9	16	16	1	12	11	10	4	12

Table 4 Skin marking characteristics among the three *Hynobius* species. The values show the number of individuals exhibiting that characteristic with percentages in parentheses. See Materials and Methods section for morphological characteristic definitions.

		H. nagatoensis sp. nov.		*H. bakan*		*H. nihoensis* sp. nov.	
		Male	Female	Male	Female	Male	Female
Character	Condition	*n* = 27	*n* = 10	*n* = 14	*n* = 5	*n* = 57	*n* = 13
DBSD	Absent	9 (33.3%)	3 (30.0%)	13 (92.9%)	3 (60.0%)	57 (100%)	12 (92.3%)
	Present	18 (66.7%)	7 (70.0%)	1 (7.1%)	2 (40.0%)	0 (0%)	1 (7.7%)
DWSV	Absent	2 (7.4%)	0 (0%)	0 (0%)	0 (0%)	36 (63.2%)	0 (0%)
	Present	25 (92.6%)	10 (100%)	14 (100%)	5 (100%)	21 (36.8%)	13 (100%)
DBYLD	Absent	3 (11.1%)	1 (10.0%)	4 (28.6%)	0 (0%)	39 (68.4%)	2 (15.4%)
	Present	24 (88.9%)	9 (90.0%)	10 (71.4%)	5 (100%)	18 (35.6%)	11 (84.6%)
DBYLV	Absent	0 (0%)	0 (0%)	0 (0%)	0 (0%)	22 (38.6%)	0 (0%)
	Present	27 (100%)	10 (100%)	14 (100%)	5 (100%)	35 (61.4%)	13 (100%)
DGM	Absent	9 (33.3%)	10 (100%)	10 (71.4%)	5 (100%)	35 (61.4%)	13 (100%)
	Present	18 (66.7%)	0 (0%)	4 (28.6%)	0 (0%)	22 (38.6%)	0 (0%)
CGN	12	3 (11.1%)	0 (0%)	3 (21.4%)	2 (40.0%)	2 (3.5%)	0 (0%)
	13	22 (81.5%)	9 (90.0%)	11 (78.6%)	2 (40.0%)	52 (91.2%)	10 (76.9%)
	14	2 (7.4%)	1 (10.0%)	0 (0%)	1 (20.0%)	3 (5.3%)	3 (23.1%)
CFBALN	1.5	2 (7.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	1	3 (11.1%)	0 (0%)	2 (14.3%)	0 (0%)	0 (0%)	0 (0%)
	0.5	5 (18.5%)	0 (0%)	2 (14.3%)	0 (0%)	0 (0%)	0 (0%)
	0	8 (29.6%)	2 (20.0%)	5 (35.7%)	2 (40.0%)	0 (0%)	0 (0%)
	-0.5	5 (18.5%)	2 (20.0%)	1 (7.1%)	1 (20.0%)	0 (0%)	0 (0%)
	-1	4 (14.8%)	1 (10.0%)	4 (28.6%)	1 (20.0%)	4 (7.0%)	0 (0%)
	-1.5	0 (0%)	3 (30.0%)	0 (0%)	1 (20.0%)	12 (21.0%)	0 (0%)
	-2	0 (0%)	2 (20.0%)	0 (0%)	0 (0%)	21 (36.8%)	1 (7.7%)
	-2.5	0 (0%)	0 (0%)	0 (0%)	0 (0%)	14 (24.6%)	2 (15.4%)
	-3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.8%)	7 (53.8%)
	-3.5	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (3.5%)	1 (7.7%)
	-4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (3.5%)	1 (7.7%)
	-4.5	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.8%)	1 (7.7%)

(Fig. 3). Additionally, *H. bakan* of the Oita group was genetically divided into three genetic clades (the Ube population, Onoda population, and Usa-Bungotakada population; Fig. 3).

Morphological measurements of the three *H. bakan* groups are shown in Table 2. Yamaguchi group males exhibited the longest average RTAL, which was more than 14% and 9% longer than those of the Oita group Ube population and Usa-Bungotakada population, respectively (Table 2). In both sexes, the average RHW was shortest in the Usa-Bungotakada population of the Oita group, and the value was less 2% and 1% shorter than that of the Yamaguchi group and the Ube population of Oita group, respectively (Table 2). Furthermore, the RHW range in females did not overlap among the Yamaguchi group and the Usa-Bungotakada population of the Oita group (Table 2). All significantly different measurements among the three groups are listed in Table 3. Between the Yamaguchi group and the Ube population of the Oita group, nine morphological characteristics significantly differed for males, and one significantly differed for females (Table 3). Between the Yamaguchi group and the Usa-Bungotakada population of the Oita group, males and females showed significantly different measurements for 16 and 12 morphological characteristics, respectively (Table 3). Males and females of the Ube and Usa-Bungotakada populations of the Oita group had significantly different measurements for 16 and 11 morphological characteristics, respectively (Table 3). Canonical discriminant analyses showed that the three groups were different, and the distribution score areas did not overlap (Fig. 4).

The morphological observation results are shown in Table 4. Males of the Yamaguchi group always had DBYLV and CFBALN ≥ − 1.0 (27/27 = 100%), almost always had DWSV (25/27 = 92.6%), usually had DBYLD (24/27 = 88.9%) and 13 CGN (22/27 = 81.5%). Females of the Yamaguchi group always had DWSV, DBYLV, and CFBALN ≤ 0 (10/10 = 100%), almost always had DBYLD and 13 CGN (9/10 = 90%), and never had DGM (10/10 = 100%). Males of the Ube population of the Oita group always had DWSV, DBYLV, and CFBALN ≥ − 1.0 (14/14 = 100%), and almost always had no DBSD (13/14 = 92.9%). Females of the Ube population of the Oita group always had DWSV, DBYLD, DBYLV, and CFBALN ≤ 0 (5/5 = 100%), and never had DGM (5/5 = 100%). Males of the Usa-Bungotakada population of the Oita group almost always had 13 CGN (52/57 = 91.2%) and CFBALN ≤ − 1.5 (53/57 = 93%), and never had DBSD (57/57 = 100%). Females of the Usa-Bungotakada population of the Oita group always had DWSV and DBYLV (13/13 = 100%), almost always had CFBALN ≤ − 2.5 (12/13 = 92.3%), usually had DBYLD (11/13 = 84.6%), almost always had no DBSD (12/13 = 92.3%), and never had DGM (13/13 = 100%)

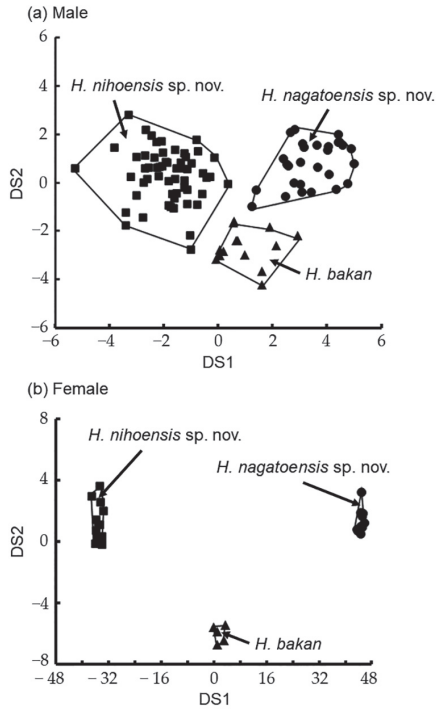

Fig. 4 Two-dimensional plots based on canonical discriminant analyses in both sexes. The x- and y-axes show discriminant score 1 (DS1) and discriminant score 2 (DS2), respectively. The contribution ratio of DS1 and DS2 in (a) males and (b) females are as follows: DS1 = 81.19% (male), 99.40% (female); DS2 = 18.81% (male), 0.60% (female).

Fig. 5 *Hynobius nagatoensis* sp. nov. holotype (YPYM-H-101, adult male): (a) dorsal view, (b) ventral view, and (c) lateral view.

Based on the results of molecular and morphological analyses, the *Hynobius bakan* Yamaguchi group and Usa-Bungotakada population of the Oita group are distinct species. We describe the new species in the following sections.

SYSTEMATIC DESCRIPTION

Hynobius nagatoensis Sugawara, Tahara, Matsukoji et Nagano sp. nov.

ZooBank LSID: urn:lsid:zoobank.org:act:1A9416D1-42BD-4574-894A-E30CB0A36A59

(Figs. 5, 7–8)

Hynobius nebulosus Abe (2001: 25–28); Kawano and

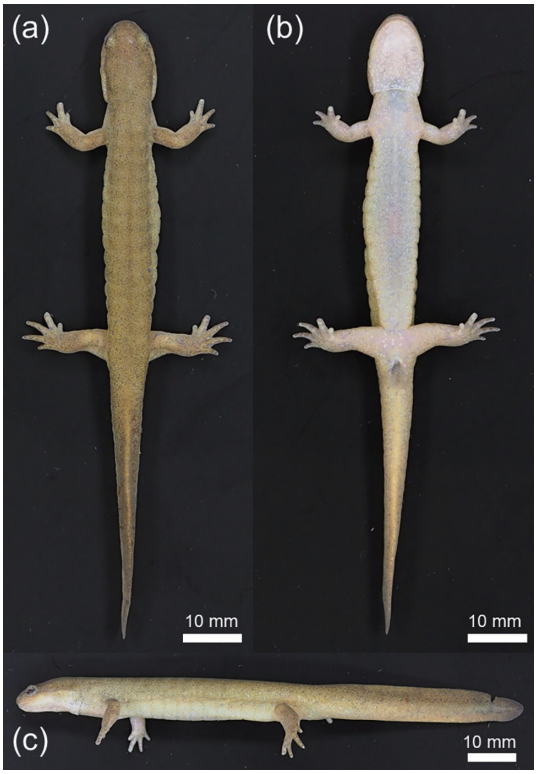

Fig. 6 *Hynobius nihoensis* sp. nov. holotype (YPYM-H-103, adult male): (a) dorsal view, (b) ventral view, and (c) lateral view.

Tokunaga (2008: 69–84)

Hynobius bakan Matsui *et al.* (2019: 60–62), in part.

Holotype: An adult male was collected by Kazuhiro Tahara on private land in Shuhocho Kama, Mine City, Yamaguchi Prefecture, Chugoku, Japan (34°16' N, 131°15' E; elevation = 120 m above sea level [a.s.l.]; in all cases, datum = WGS84) on 16 January 2018. We obtained permission from the landowner to collect specimens. This specimen (specimen number = YPYM-H-101) is stored in the Yamaguchi Prefectural Museum: 8-2, Kasugacho, Yamaguchi City, Yamaguchi Prefecture, 753-0073, Japan. To avoid the overcollection of this species, further information is available only by contacting the corresponding author or the Yamaguchi Prefectural Museum.

Paratype: An adult female from the same locality of the holotype was collected by Yoshihiro Tahara on 16 January 2018. This specimen (specimen number = YPYM-H-102) is stored in the Yamaguchi Prefectural Museum. An adult male was collected by Yoshihiro Tahara in Fujigatanicho, Shimonoseki City, Yamaguchi Prefecture, Chugoku, Japan (33°59' N, 130°57' E, elevation = 140 m a.s.l.) on 27 March 2021. This specimen (specimen number = YCM-RA591) is stored in the Yokosuka City Museum: 95 Fukadadai, Yokosuka City, Kanagawa Prefecture, 238-0016, Japan. To avoid the overcollection of this species, further information is available only by contacting the corresponding author or the Yokosuka City Museum.

Diagnosis: A comparatively small species (mean SVL of 56.5 mm in males and 59.8 mm in females) in the Japanese lentic salamander *Hynobius*. Distinct white spots on venter almost always present in males and always present in females; distinct and bright yellow line on dorsal edge of the tail usually present in males and almost always present in females; distinct yellow stripe on ventral edge of the tail always present in both sexes; distinct gular mottling never present in females; fifth toe of hindlimb always present; V-shaped vomerine teeth series; 13 (rarely 12 or 14) costal grooves; number of costal folds between adpressed limbs always −1.0 to 1.5 in males and −2.0 to 0 in females; ratio of TAL/SVL almost always more than 76% in males.

Comparisons: The new species statistically differs from *H. bakan* in the following measurements: SVL, RTRL, RTAL, RMTAH, RHW, RHLL, R3FL, RSL, and RUEL in males and SVL in females; these measurements are significantly longer in *H. nagatoensis* sp. nov. than in *H. bakan*, excepting RHLL and RUEL in males. One of the most distinct characteristic separating them is the TAL/SVL ratio in males, which is almost always more than 76% in *H. nagatoensis* sp. nov. (24/27 = 89.9%), and usually 76% or less (12/14 = 85.7%) in *H. bakan*.

Description of holotype: A moderately large individual: HL larger than HW; TAL slightly shorter than SVL; body almost cylindrical; rounded snout; gular fold present; tail gradually compressed toward the tip; clearly expanded cloaca; webbing between digits absent; four fingers on each forelimb, order of length Ⅲ = Ⅱ < Ⅰ = Ⅳ on left and Ⅱ < Ⅲ < Ⅳ < Ⅰ on right; five toes on each hindlimb, order of length Ⅲ < Ⅳ < Ⅱ < Ⅰ = Ⅴ on both sides; V-shaped vomerine

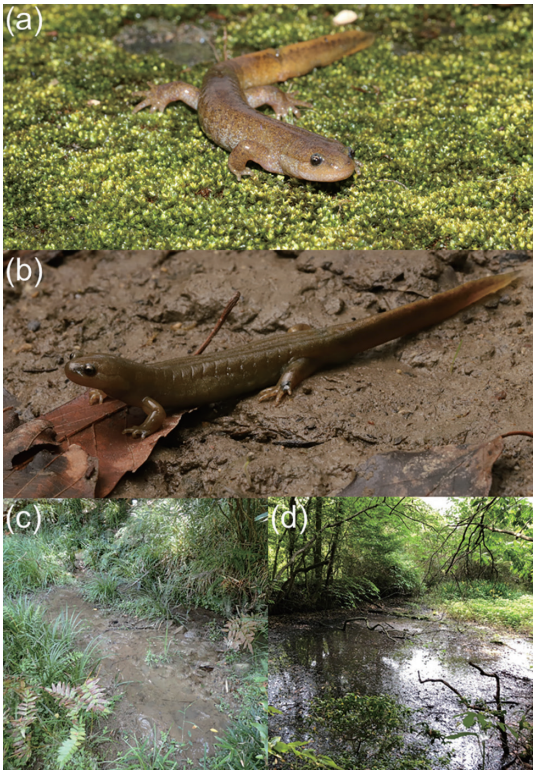

Fig. 7 Live individual and habitat at the type locality of *Hynobius nagatoensis* sp. nov. (a, c) and *H. nihoensis* sp. nov. (b, d). These individuals are not type specimens, but they accurately reflect the diagnosis of each species.

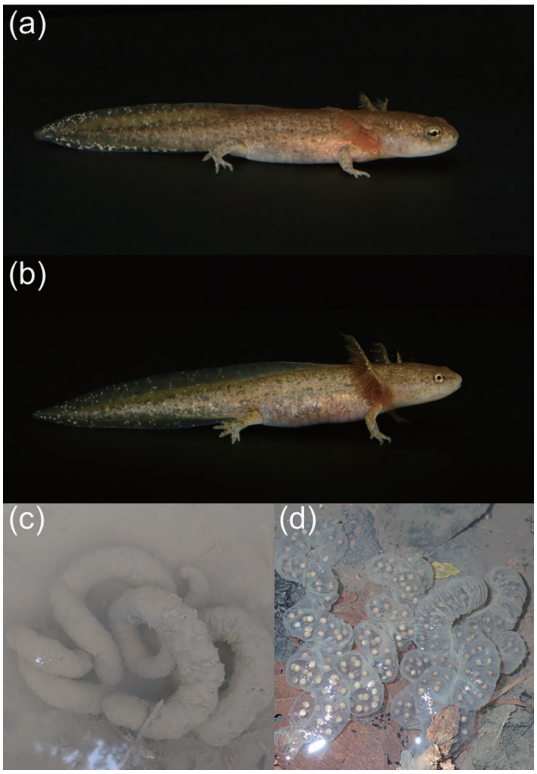

Fig. 8 Larva and egg sacs of *Hynobius nagatoensis* sp. nov. (a, c) and *Hynobius nihoensis* sp. nov. (b, d).

teeth; skin smooth and shiny; DBSD present; DWSV densely present; DBYLD and DBYLV present; DGM present. The holotype measurements are as follows (mm): SVL = 59.8, TRL = 47.3, AGD = 32.1, HL = 13.4, TAL = 57.8, MTAW = 3.7, MTAH = 9.1, BTAW = 8.2, BTAH = 7.5, VTL = 3.3, VTW = 3.2, HW = 11.0, MXHW = 11.2, LFLL = 13.8, RFLL = 13.9, LHLL = 19.2, RHLL = 19.4, L1FL = 1.2, L2FL = 2.5, L3FL = 2.5, L4FL = 1.2, R1FL = 1.1, R2FL = 2.8, R3FL = 2.6, R4FL = 1.4, L1TL = 1.1, L2TL = 3.1, L3TL = 4.4, L4TL = 3.9, L5TL = 1.0, R1TL = 1.0, R2TL = 2.9, R3TL = 4.7, R4TL = 3.8, R5TL = 0.7, IND = 2.7, IOD = 4.0, LUEW = 1.9, RUEW = 1.7, SL = 4.5, LUEL = 2.7, RUEL = 2.8, LJL = 8.1, CGN = 13.

Variation: Morphometric measurements and observations are presented in Tables 2 and 4, respectively. Significant different measurements between sexes are listed in Table 3. Males have relatively longer RHL, RTAL, RMTAH, RVTW, RHW, RFLL, RHLL, R3TL, RIOD, and RLJL and relatively shorter RAGD than females. Skin markings are listed in Table 4. DBSD are frequently present in males (18/27 = 66.7%) and females (7/10 = 70%). DWSV are rarely absent in males (2/27 = 7.4%). DTBYLD is rarely absent (3/27 = 11.1% in males; 1/10 = 10% in females). DGM is often present in males (18/27 = 66.7%). CGN is rarely 12 (3/27 = 11.1%) or 14 (2/27 = 7.4%) in males and 14 (1/10 = 10%) in females. Dorsal coloration can be found between yellowish-brown to blackish-brown, and ventral coloration is usually bluish-white, reddish-white, or whitish-indigo. The iris coloration can be found between dark brown to light brown. When preserved in 70% ethanol, the dorsal coloration tended to fade to dark gray.

Etymology: The specific epithet "*nagatoensis*" refers to the old name of Yamaguchi Prefecture (Nagato), where the new species occurred. Suggested common name in Japanese: Nagato-sanshouo.

Distribution: It is known from Shimonoseki (including the former Shimonoseki City, and Hohoku, Toyota, Toyoura, and Kikugawa Towns), Nagato (including the former Nagato City, and Yuya and Heki Towns), Mine (including the former Mine City, and Shuho and Mito Towns), and Ube (including the former Kusunoki Town) Cities in Yamaguchi Prefecture, and Moji Ward, Kitakyushu City, Fukuoka Prefecture. DNA samples from the former Yuya Town was not included in this study, however, this species is distributed in the former Yuya Town based on the previous field surveys (Y. Tahara, personal observation). A population of the western part of Ube City (former Ube City) has the haplotype of *H. nagatoensis* sp. nov. based on the DNA data of one larva (Sugawara and Nagano, unpublished), but it is necessary to confirm that this population is native or nonnative.

Natural History: The dominant vegetation type in the type locality is a mixed forest consisting of chinquapin (*Castanopsis*), live oak (*Quercus*), and Japanese cedar (*Cryptomeria japonica*) (Fig. 7-c). Larvae have distinct black dots on the lateral sides of the tail (Fig. 8-a). Claws on the tips of the fingers and toes are absent. One pair of balancers are present during early larval developmental stages. Egg sacs are coil-shaped and attached to fallen branches or leaves in puddles, ponds, or swamps at forest edges from December to April (Fig. 8-c).

Remarks: The new species forms a monophyletic group with *H. bakan* and *H. dunni* based on Matsui *et al.* (2019) and our analysis (Fig. 3).

Hynobius nihoensis Sugawara, Nagano et Nakazono sp. nov.

ZooBank LSID: urn:lsid:zoobank.org:act:C14D5C4D-5AC8-4DF9-9AB3-B26D453D045A

(Figs. 6–8)

Hynobius nebulosus Sato and Horie (2000: 1), in part; Sugawara *et al.* (2017: 54–56).

Hynobius bakan Matsui *et al.* (2019: 60–62), in part.

Holotype: An adult male was collected by Aoi Nagano in Yamashita, Usa City, Oita Prefecture, Kyushu, Japan (33° 31' N, 131° 18' E; elevation = 40 m a.s.l.); in all cases, datum = WGS84) on 23 March 2019. This specimen (specimen number = YPYM-H-103) is stored in the Yamaguchi Prefectural Museum: 8-2, Kasugacho, Yamaguchi City, Yamaguchi Prefecture, 753-0073, Japan. To avoid the overcollection of this species, further information is available only by contacting the corresponding author or the Yamaguchi Prefectural Museum.

Paratype: An adult female from the same locality of the holotype was collected by Masahiro Nagano on 2 February 2020. This specimen (specimen number = YPYM-H-104) is stored in the Yamaguchi Prefectural Museum. An adult male was collected by Hirotaka Sugawara in Shinei-nyuzubaru, Bungotakada City, Oita Prefecture, Kyushu, Japan (33° 34' N, 131° 26' E; elevation = 10 m a.s.l.) on 22 March 2017. This specimen (specimen number = YCM-RA592) is stored in the Yokosuka City Museum: 95 Fukadadai, Yokosuka City, Kanagawa Prefecture, 238-0016, Japan. To avoid the overcollection of this species, further information is available only by contacting the corresponding author or the Yokosuka City Museum.

Diagnosis: A comparatively small species (mean SVL of 57.7 mm in males and 59.2 mm in females) within the Japanese lentic salamander species complex of *Hynobius*. Distinct black spots on dorsum absent in adults (rarely present in females); distinct white spots on venter always present in females; distinct and bright yellow stripe on dorsal edge of the tail usually present in females; distinct yellow line on ventral side of the tail always present in females; distinct gular mottling always absent in females; yellowish-brown to blackish-brown dorsum; fifth toe of hindlimb always present; V-shaped vomerine teeth series; 13 costal grooves (rarely 12 or 14); number of costal folds between adpressed limbs almost always −4.5 to −1.5 in males and −4.5 to −2.5 in females; ratio of HW/SVL less than 17% (rarely more than 17%) in males and always less than 16.5% in females; longer coil-shaped egg sacs.

Comparisons: The new species statistically differs from *H. bakan* in the following measurements: SVL, RTRL, RAGD, RHL, RMTAH, RVTL, RHW, RFLL, RHLL, R2FL, R3TL, RIND, RIOD, RSL, RUEL, and RLJL in males; SVL, RTRL, RAGD, RHL, RTAL, RVTL, RHW, RFLL, RHLL, R3TL, and RIND in

females; these measurements are significantly shorter in *H. nihoensis* sp. nov. than in *H. bakan*, excepting SVL, RTRL, RAGD, and RMTAH in males and SVL, RTRL, and RAGD in females. One of the most distinct characteristic separating them is the number of costal folds between adpressed limbs, which is always − 1.0 to 1.0 in males (14/14 = 100%) and usually − 1.0 to 0.0 (4/5 = 80%) in females of *H. bakan*, and almost always − 4.5 to − 1.5 in males (53/57 = 93.0%) and − 4.5 to − 2.0 in females (13/13 = 100%) of *H. nihoensis* sp. nov. Additionally, the ratio of HW/SVL is almost always 17% or more in males (13/14 = 92.9%) and usually 16.5% or more in females (4/5 = 80%) of *H. bakan*, whereas this measurement is usually less than 17% in males (47/57 = 82.5%) and always less than 16.5% in females (13/13 = 100%) of *H. nihoensis* sp. nov. The new species statistically differs from *H. nagatoensis* sp. nov. in the following length measurements: RTRL, RAGD, RHL, RTAL, RMTAW, RMTAH, RVTL, RHW, RFLL, RHLL, R3FL, R3TL, RIND, RIOD, RSL, and RLJL in males and RTRL, RAGD, RHL, RTAL, RMTAH, RVTL, RHW, RFLL, RHLL, R2FL, RIOD, and RSL in females; the lengths of these measurements, excepting RTRL and RAGD in both sexes, are significantly shorter in *H. nihoensis* sp. nov. than in *H. nagatoensis* sp. nov. The most distinct characteristic separating them is the number of costal folds between the adpressed limbs, which is almost always − 4.5 to − 1.5 in males (53/57 = 93.0%) and − 4.5 to − 2.5 (12/13 = 92.3%) in females of *H. nihoensis* sp. nov., and always − 1.0 to 1.5 in males (27/27 = 100%) and − 2.0 to 0 in females (10/10 = 100%) of *H. nagatoensis* sp. nov.

Description of holotype: A moderately large individual with HL larger than HW; TAL shorter than SVL; body almost cylindrical; rounded snout; gular fold present; tail gradually compressed toward the tip; slightly expanded cloaca; webbing between digits absent; four fingers on each forelimb, order of length Ⅱ < Ⅲ < Ⅳ < Ⅰ ; five toes on each hindlimb, order of length Ⅲ < Ⅳ < Ⅱ < Ⅴ < Ⅰ ; V-shaped vomerine teeth; skin smooth and matte; DBSD absent; DWSV absent; DBYLD and DBYLV absent; DGM absent. The holotype measurements are as follows (mm): SVL = 56.8, TRL = 44.6, AGD = 32.0, HL = 11.9, TAL = 42.9, MTAW = 2.9, MTAH = 6.4, BTAW = 5.8, BTAH = 6.1, VTL = 2.7, VTW = 2.9, HW = 8.5, MXHW = 8.6, LFLL = 11.9, LHLL = 15.0, RFLL = 12.4, RHLL = 15.2, L1FL = 1.0, L2FL = 2.6, L3FL = 2.4, L4FL = 1.3, R1FL = 1.0, R2FL = 2.9, R3FL = 2.7, R4FL = 1.2, L1TL = 1.2, L2TL = 2.7, L3TL = 4.1, L4TL = 3.7, L5TL = 1.4, R1TL = 0.9, R2TL = 2.5, R3TL = 4.1, R4TL = 3.5, R5TL = 1.5, IND = 2.7, IOD = 3.4, LUEW = 1.4, RUEW = 1.1, SL = 3.7, LUEL = 2.0, RUEL = 2.1, LJL = 6.4, and CGN = 13.

Fig. 9 *Hynobius bakan* topotype (KPM-NFA 955, adult male): (a) dorsal view, (b) ventral view, and (c) lateral view.

Variation: Morphometric measurements and observations are presented in Tables 2 and 4, respectively. Significant different measurements between sexes are listed in Table 3. Males have relatively longer RHL, RTAL, RMTAH, RVTL, RHW, RFLL, RHLL, R2FL, RIND, and RSL and relatively shorter RTRL and RAGD than females. Skin markings are listed in Table 4. DBSD are rarely present in females (1/13 = 7.7%). DWSV are often absent in males (36/57 = 63.2%). DBYLD is frequently

absent in males (39/57 = 68.4%) and rarely absent in females (2/13 = 15.4%). DBYLV is frequently present in males (35/57 = 61.4%). DGM is frequently absent in males (35/57 = 61.4%). CGN is rarely 12 (2/57 = 3.5%) or 14 (3/57 = 5.3%) in males and 14 in females (3/13 = 23.1%). CFBALN is rarely ≥ − 1.0 in males (4/57 = 7.0%) and rarely ≥ − 2.0 (1/13 = 7.7%) in females. Dorsal coloration can be found between yellowish-brown to blackish-brown, and ventral coloration is usually bluish-white, reddish-white, or whitish-Indigo. The iris coloration can be found between dark brown to light brown. When preserved in 70% ethanol, the dorsal coloration tends to fade to dark gray.

Etymology: The specific name is derived from "Niho." In ancient times, Oita Prefecture was divided into two areas (Buzen and Bungo), and where the two areas met was called Niho. The boundary was located at the border of the current Usa (Buzen area) and Bungotakada (Bungo area) Cities, Oita Prefecture, where the new species occurs. Suggested common name in Japanese: Niho-sanshouo.

Distribution: This new species is endemic to Oita Prefecture and found in Bungotakada (only former Bungotakada City) and Usa (only former Usa City) Cities. This species may be present in Nakatsu City, although DNA data is absent.

Natural History: The dominant vegetation type in the type locality is a mixed forest consisting of chinquapin (*Castanopsis*), live oak (*Quercus*), and Japanese cedar (*Cryptomeria japonica*) (Fig. 7-d). The larval morphology of *H. nihoensis* sp. nov. is similar to that of *H. nagatoensis* sp. nov. (Fig. 8-b). Egg sacs are long and coil-shaped and attached to fallen branches, leaves, or stones in puddles, ponds, swamps, or brooks at forest edges from January to March (Fig. 8-d).

Remarks: The new species forms a monophyletic group with *H. bakan*.

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(Fig. 9)

Holotype: An adult male (specimen number = KUHE OU 0391) was collected by Hiroshi Okawa in Warisaka, Kurumaji, Ube City, Yamaguchi Prefecture, Chugoku, Japan (34° 50' 36" N, 131° 18' 12" E, alt. 70 m) on 5 March 2010 (Matsui *et al*., 2019) . This specimen is stored in the Graduate School of Human and Environmental Studies, Kyoto University: Yoshidahonmachi, Sakyo Ward, Kyoto City, Kyoto Prefecture, 606-8501, Japan. Furthermore, there may be errors in the GPS data (Matsui *et al*., 2019) because of the point maps on the sea.

Diagnosis: A comparatively small species (mean SVL of 51.7 mm in males and 53.9 mm in females) within the Japanese lentic salamander species complex of *Hynobius*. Distinct black spots on dorsum almost always absent in males; distinct white spots on venter always present in both sexes; distinct and bright yellow stripe on dorsal edge of the tail always present in females; distinct yellow line on ventral side of the tail always present in both sexes; distinct gular mottling always absent in females; fifth toe of hindlimb always present; V-shaped vomerine teeth series; 13 costal grooves (rarely 12 or 14); costal folds between adpressed limbs always − 1.0 to 1.0 in males and always − 1.5 to 0 in females; coil-shaped (not long) egg sacs.

Description of topotype: An adult male was collected by Yoshihiro Tahara around Warisaka, Kurumaji, Ube City, Yamaguchi Prefecture, Chugoku, Japan (34° 03' N, 131° 17' E; elevation = 40 m a.s.l.); in all cases, datum = WGS84) on 9 March 2019. The specimen (specimen number = KPM-NFA 955) is stored in the Kanagawa Prefectural Museum of Natural History: 499 Iryuda, Odawara City, Kanagawa Prefecture, 250-0031, Japan. To avoid the overcollection of this species, further information is available only by contacting the Kanagawa Prefectural Museum. A moderately large individual with HL larger than HW; TAL shorter than SVL; body almost cylindrical; rounded snout; gular fold present; tail gradually compressed toward the tip; slightly expanded cloaca; webbing between digits absent; four fingers on each forelimb, order of length Ⅱ < Ⅲ < Ⅳ < Ⅰ; five toes on each hindlimb, order of length Ⅲ < Ⅳ < Ⅱ < Ⅴ < Ⅰ; V-shaped vomerine teeth; skin smooth and matte; DBSD absent; DWSV present; DTBYLD and DTBYLV present; DGM absent. The topotype measurements are as follows (mm): SVL = 53.9, TRL = 41.5, AGD = 27.9, HL = 12.7, TAL = 41.3, MTAW = 2.9, MTAH = 5.9, BTAW = 5.8, BTAH = 6.0, VTL

= 3.2, VTW = 2.9, HW = 9.3, MXHW = 9.6, LFLL = 11.8, LHLL = 16.5, RFLL = 11.7, RHLL = 16.5, L1FL = 1.0, L2FL = 2.9, L3FL = 2.7, L4FL = 1.5, R1FL = 0.9, R2FL = 2.8, R3FL = 2.5, R4FL = 1.2, L1TL = 1.2, L2TL = 2.9, L3TL = 4.5, L4TL = 3.8, L5TL = 1.5, R1TL = 1.3, R2TL = 2.9, R3TL = 4.6, R4TL = 3.6, R5TL = 1.8, IND = 2.1, IOD = 3.6, LUEW = 1.5, RUEW = 1.2, SL = 3.6, LUEL = 2.5, RUEL = 2.6, LJL = 7.3, and CGN = 13.

Variation: Morphometric measurements and observations are presented in Tables 2 and 4, respectively. Significant different measurements between sexes are listed in Table 3. Males have relatively longer RHW and RIOD and relatively shorter RAGD and R3FL than females. Skin markings are listed in Table 4. DBSD are rarely present in males (1/14 = 7.1%) and sometimes present in females (2/5 = 40%). DBYLD is rarely absent (4/14 = 28.6%) in males. DGM is rarely present (4/14 = 28.6%) in males. CGN is rarely 12 (3/14 = 21.4%) in males and sometimes 12 (2/5 = 40.0%) or rarely 14 (1/5 = 20.0%) in females. Dorsal coloration can be found between yellowish-brown to blackish-brown, and ventral coloration is usually bluish-white, reddish-white, or whitish-indigo. The iris coloration can be found between dark brown to light brown. When preserved in 70% ethanol, the dorsal coloration tended to fade to dark gray.

Distribution: This species is endemic to Ube and Sanyo-Onoda Cities, Yamaguchi Prefecture. A population of the northern part of Usa City (Oita Prefecture) has the haplotype of *H. bakan* based on the DNA data of one larva (Sugawara *et al*., unpublished), but it is necessary to confirm that this population is native or nonnative.

DISCUSSION

Hynobius nagatoensis sp. nov. and *H. bakan* were previously regarded as the same species, but the monophyly was rejected based on the criteria of Huelsenbeck and Rannala (2004) and Hillis and Bull (1993) (Matsui *et al*., 2019) (Fig. 3). In addition to mitochondrial data, phylogenetic analyses using allozyme data also did not support a monophyletic relationship (Matsui *et al*., 2006). Thus, there is no evidence that these two species comprise a monophyletic group as closest relatives. Moreover, the two are morphologically distinguished (Tables 2–4; Fig. 4). Based on the three species concepts, *H. nagatoensis* sp. nov. is a distinct species. Monophyly of *H. bakan* and *H. nihoensis* sp. nov. was supported (Matsui *et al*., 2019) (Fig. 3), but the morphological differences between *H. bakan* and *H. nihoensis* sp. nov. are more distinct than those between *H. bakan* and *H. nagatoensis* sp. nov. (Tables 2–4). Based on the morphological species concept, all three are different species. Unlike the result reported by Matsui *et al*. (2019), *H. bakan* had another distinct clade (Onoda population = Pop. 33) based on our molecular analyses (Fig. 3). However, we collected only one larva from this population; we were unable to collect adult individuals. It is possible that, in 2021, this population is already extinct; thus, we were unable to perform morphological analyses that included this clade. There is no morphological evidence that this group is *H. bakan*; hence, this population should be named *Hynobius* sp. at present. Further field surveys are needed to discover new populations belonging to this clade. Furthermore, additional morphological surveys are required to decide the taxonomic status of the Onoda population of the Oita group.

The distribution area of *H. bakan* is largely changed due to this description of two new species; *H. bakan* is only found in Ube and Sanyo-Onoda Cities, Yamaguchi Prefecture (Table1; Fig. 1). In Usa City of Oita Prefecture, one individual larva with the haplotype of *H. bakan* was discovered (Sugawara and Nagano, unpublished). In addition, one larva from a population in the western part of Ube City (former Ube City), Yamaguchi Prefecture had the haplotype of *H. nagatoensis* sp. nov. (Sugawara *et al*., unpublished). Currently, additional individuals have not been discovered in these populations, so it is possible that the collected individual had been introduced from other populations. The genetic data on these larvae were removed from the present study, but further studies are warranted to investigate the inhabitation of *H. bakan* in Oita Prefecture and *H. nagatoensis* sp. nov. in the former Ube City based on morphological

and molecular perspectives. The specific name of Yamaguchi salamander, "*bakan,*" refers to the old name of Shimonoseki City in Yamaguchi Prefecture; however, this species is not distributed in that area. Based on our field surveys, *H. bakan* and *H. nihoensis* sp. nov. habitats are limited and fragmented; these small populations are unsustainable due to their small sizes as well as their locations, which are often near development areas. Therefore, *H. bakan* and *H. nihoensis* sp. nov. viabilities may be affected by catastrophes or human activities, such as deforestation and development. The conservation status of these species must be reassessed in light of these new species descriptions, and management plans for its conservation are imminently needed to save *H. bakan* and *H. nihoensis* sp. nov. from extinction.

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