Taxonomic re-evaluation of the Oita salamander *Hynobius dunni*: Description of two new species from Kyushu, Japan

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オオイタサンショウウオ *Hynobius dunni* の分類学的再検討: 日本の九州地方からの 2 新種の記載

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大分県および宮崎県に分布するサンショウウオ属の 2 種を新種として記載した。形態および分子系統解析の結果，*Hynobius dunni* は少なくとも 3 群 (宮崎南部グループ，大分東部グループ，広域分布グループ) に明確に分かれることが判明した。このため，3 群のうち，宮崎南部グループを *Hynobius miyazakiensis* sp. nov.，大分東部グループを *Hynobius amabensis* sp. nov. として，それぞれ記載した。形態比較の結果，*H. miyazakiensis* sp. nov. は，*H. amabensis* sp. nov. と *H. dunni* よりも体側に沿って手足を伸ばした時に重なり合う肋鰭の数が多い傾向にあった。*H. dunni* と *H. amabensis* sp. nov. は形態的に類似しているが，背側の黒色斑点が後者ではほとんどの個体で確認できず，かつこれら 2 種は姉妹群を形成しなかった。本記載により，*H. dunni* は，宮崎県南部には分布しておらず，大分県，熊本県北東部，宮崎県北部の一部に分布域が限られることになる。

In this paper, we described two new species of the genus *Hynobius* from Miyazaki and Oita Prefectures, Japan. Based on morphological and molecular analyses, *Hynobius dunni* could be divided into three groups: southern Miyazaki, eastern Oita, and widely distributed groups. Thus, the southern Miyazaki and eastern Oita groups were described as *Hynobius miyazakiensis* sp. nov. and *Hynobius amabensis* sp. nov., respectively. Morphological comparisons showed that *H. miyazakiensis* sp. nov. has a larger number of costal folds between adpressed limbs than *H. amabensis* sp. nov. and *H. dunni*. In addition, *H. dunni* and *H. amabensis* sp. nov. have the resembled morphology, but distinct black spots on dorsum are usually absent in the latter species. Moreover, these two species are not formed a sister group. Based on the description, *H. dunni* is not distributed in the southern part of Miyazaki Prefecture, and it is limited to the Oita, northeastern part of Kumamoto, and northernmost part of Miyazaki Prefectures.

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INTRODUCTION

Hynobius dunni (Oita salamander) was first described in Shiroyama, Saiki City, Oita Prefecture, Japan (Tago, 1931. It is widely found in Oita, Kumamoto, and Miyazaki Prefectures, Japan (Sugawara *et al*., 2015; Sugawara *et al*., 2018). This species has two genetically distinct groups, the Oita (including the Oita, Kumamoto, and northernmost Miyazaki populations) and Miyazaki (including the populations from the southern part of Miyazaki Prefecture) groups; monophyly of the two groups was supported by maximum likelihood (ML) estimation and Bayesian inference (BI) (Sugawara *et al*., 2018). However, phylogenetic analysis was performed using data obtained from only twelve populations (ten populations from the Oita group and two populations from the Miyazaki group). Thus, re-assessments using genetic data from many populations across the entire distribution range of *H. dunni* are necessary. Sugawara *et al*. (2018) compared the morphology between the Oita and Miyazaki groups, but the comparison is not enough because it only used male individuals, and only eleven individuals were examined from the Miyazaki group. In addition, the number of female individuals examined from the Oita and Miyazaki groups was only two and one, respectively (Sugawara *et al*., 2018). Thus, using more individuals in both sexes is essential to obtain accurate morphological comparisons. Another haplotype group of *H. dunni* was found in eastern Oita Prefecture (Sugawara and Nagano, unpublished), but the morphology of this group has not been investigated. Morphological information of this group is important for comprehensive taxonomic surveys of *H. dunni*.

In the present study, we assessed the species validity of three *H. dunni* groups using morphological, phylogenetic, and evolutionary species concepts. We also clarified the distribution range of the three groups of *H. dunni* in detail.

MATERIALS AND METHODS

Molecular analysis

For molecular analysis, we sampled 60 individuals from 60 localities between February 2013 and March 2022 (Table 1; Fig. 1). A single-tailbud embryo from each paired egg sac or tissue samples from larvae or juveniles were collected and preserved in 99.5% ethanol. DNA extraction and sequence 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 based on the Bayesian Inference (BI), including *Hynobius nihoensis* as the outgroup (Table 1). We estimated the best-fit nucleotide substitution model based on the Bayesian information criterion (Schwarz, 1978) using jModelTest 2 (Darriba *et al*., 2012). We selected the Hasegawa–Kishino–Yano model with gamma distribution. We constructed the Bayesian tree using MrBayes 3.2 (Ronquist *et al*., 2012). 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/). and the first 25% of generations were discarded as burn-in. Monophyly was determined using the posterior probability (PP) based on the criteria described by Huelsenbeck and Rannala (2004) ($PP \geq 0.95$).

Morphological analysis

We sampled 88 *H. dunni* individuals between January 2016 to June 2022. Among them, 29 individuals (24 males and five females) were from two populations (Pops. 2 and 4) of the southern Miyazaki group; 27 individuals (20 males and seven females) were from one population (Pop. 5) of the eastern Oita group, and 32 individuals (19 males and 13 females) were from eight populations (Pops. 16, 19, 20, 24, 35, 36, 40, and 43) of the Oita group (Table 1; Fig. 1). The sampled 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 examined individuals. We measured the following 22 measurements on each specimen by using a vernier caliper: snout–vent length (SVL), trunk length (TRL), axilla–groin distance (AGD), head length (HL), tail

Table 1 Samples using molecular phylogenetic analyses. Population numbers corresponding to the localities shown in Fig. 1. Asterisks after the sampling localities show the type locality of three species (Pops. 4, 5, and 61).

Pop.	Species	Sampling licality	Accession number / Label in Fig. 2
1	*Hynobius miyazakiensis* sp. nov.	Kiyotakecho Kanoko, Miyazaki City, Miyazaki Pref.	LC737911 / MIY01
2	*Hynobius miyazakiensis* sp. nov.	Furujocho, Miyazaki City, Miyazaki Pref.	LC737912 / MIY02
3	*Hynobius miyazakiensis* sp. nov.	Hosoe, Miyazaki City, Miyazaki Pref.	LC737913 / MIY03
4	*Hynobius miyazakiensis* sp. nov.	Tanocho Otsu, Miyazaki City, Miyazaki Pref.*	LC737914 / MIY04
5	*Hynobius amabensis* sp. nov.	Shuki (north), Oita City, Oita Pref.*	LC737915 / AMA01
6	*Hynobius amabensis* sp. nov.	Shuki (south), Oita City, Oita Pref.	LC740565 / AMA02
7	*Hynobius amabensis* sp. nov.	Baba, Oita City, Oita Pref.	LC740566 / AMA03
8	*Hynobius amabensis* sp. nov.	Kisagami (east), Oita City, Oita Pref.	LC740567 / AMA04
9	*Hynobius amabensis* sp. nov.	Sashiu, Usuki City, Oita Pref.	LC740568 / AMA05
10	*Hynobius amabensis* sp. nov.	Sano, Oita City, Oita Pref.	LC737916 / AMA06
11	*Hynobius amabensis* sp. nov.	Takeya, Usuki City, Oita Pref.	LC737917 / AMA07
12	*Hynobius amabensis* sp. nov.	Tai, Usuki City, Oita Pref.	LC740569 / AMA08
13	*Hynobius amabensis* sp. nov.	Fukata, Usuki City, Oita Pref.	LC737918 / AMA09
14	*Hynobius dunni*	Kunimimachi Akane, Kunisaki City, Oita Pref.	LC737919 / DUN01
15	*Hynobius dunni*	Kunisakimachi Nakada, Kunisaki City, Oita Pref.	LC737920 / DUN02
16	*Hynobius dunni*	Kunisakimachi Owara, Kunisaki City, Oita Pref.	LC737921 / DUN03
17	*Hynobius dunni*	Yokogi, Kitsuki City, Oita Pref.	LC737922 / DUN04
18	*Hynobius dunni*	Otakutsukake, Kitsuki City, Oita Pref.	LC737923 / DUN05
19	*Hynobius dunni*	Yamagamachi Odake, Kitsuki City, Oita Pref.	LC737924 / DUN06
20	*Hynobius dunni*	Tashibumanaka, Bungotakada City, Oita Pref.	LC737925 / DUN07
21	*Hynobius dunni*	Jozen, Bungotakada City, Oita Pref.	LC737926 / DUN08
22	*Hynobius dunni*	Eguma, Usa City, Oita Pref.	LC737927 / DUN09
23	*Hynobius dunni*	Kanamaru, Usa City, Oita Pref.	LC737928 / DUN10
24	*Hynobius dunni*	Minamiusa, Usa City, Oita Pref.	LC737929 / DUN11
25	*Hynobius dunni*	Ajimumachi Furukawa, Usa City, Oita Pref.	LC737930 / DUN12
26	*Hynobius dunni*	Ajimumachi Niibaru, Usa City, Oita Pref.	LC737931 / DUN13
27	*Hynobius dunni*	Innaimachi Osoi, Usa City, Oita Pref.	LC737932 / DUN14
28	*Hynobius dunni*	Minamihata, Hiji Town, Oita Pref.	LC737933 / DUN15
29	*Hynobius dunni*	Fujiwara, Hiji Town, Oita Pref.	LC737934 / DUN16
30	*Hynobius dunni*	Yufuincho Kawakami, Yufu City, Oita Pref.	LC737935 / DUN17
31	*Hynobius dunni*	Shonaicho Nishiotsuru, Yufu City, Oita Pref.	LC737936 / DUN18
32	*Hynobius dunni*	Hasamamachi Onigase, Yufu City, Oita Pref.	LC737937 / DUN19
33	*Hynobius dunni*	Beppu, Beppu City, Oita Pref.	LC737938 / DUN20
34	*Hynobius dunni*	Uchinari, Beppu City, Oita Pref.	LC737939 / DUN21
35	*Hynobius dunni*	Dannoharu, Oita City, Oita Pref.	LC737940 / DUN22
36	*Hynobius dunni*	Nakahanda, Oita City, Oita Pref.	LC740570 / DUN23
37	*Hynobius dunni*	Ueno, Oita City, Oita Pref.	LC737941 / DUN24
38	*Hynobius dunni*	Yokoo, Oita City, Oita Pref.	LC737942 / DUN25
39	*Hynobius dunni*	Ichigi, Oita City, Oita Pref.	LC737943 / DUN26
40	*Hynobius dunni*	Kida, Oita City, Oita Pref.	LC740571 / DUN27
41	*Hynobius dunni*	Kisagami (west), Oita City, Oita Pref.	LC737944 / DUN28
42	*Hynobius dunni*	Sugibaru, Oita City, Oita Pref.	LC740572 / DUN29
43	*Hynobius dunni*	Shitsuru, Oita City, Oita Pref.	LC737945 / DUN30
44	*Hynobius dunni*	Kugiono, Usuki City, Oita Pref.	LC740573 / DUN31
45	*Hynobius dunni*	Notsumachi Miyakobaru, Usuki City, Oita Pref.	LC740574 / DUN32
46	*Hynobius dunni*	Fukura, Usuki City, Oita Pref.	LC737946 / DUN33
47	*Hynobius dunni*	Notsumachi Nishihata, Usuki City, Oita Pref.	LC737947 / DUN34
48	*Hynobius dunni*	Hiana, Saiki City, Oita Pref.	LC737948 / DUN35
49	*Hynobius dunni*	Ume Oaza Kiurauchi, Saiki City, Oita Pref.	LC737949 / DUN36
50	*Hynobius dunni*	Asajimachi Torita, Bungoono City, Oita Pref.	LC737950 / DUN37
51	*Hynobius dunni*	Miemachi Hisada, Bungoono City, Oita Pref.	LC737951 / DUN38
52	*Hynobius dunni*	Ogatamachi Oharu, Bungoono City, Oita Pref.	LC737952 / DUN39
53	*Hynobius dunni*	Naoirimachi Oaza Nagayu, Taketa City, Oita Pref.	LC737953 / DUN40
54	*Hynobius dunni*	Tamarai, Taketa City, Oita Pref.	LC737954 / DUN41
55	*Hynobius dunni*	Kariono, Taketa City, Oita Pref.	LC737955 / DUN42
56	*Hynobius dunni*	Kujuno, Taketa City, Oita Pref.	LC737956 / DUN43
57	*Hynobius dunni*	Yamaga, Ubuyama Village, Kumamoto Oita Pref.	LC737957 / DUN44
58	*Hynobius dunni*	Namino Akanita, Aso City, Kumamoto Pref.	LC737958 / DUN45
59	*Hynobius dunni*	Kawara, Takamori Town, Kumamoto Pref.	LC737959 / DUN46
60	*Hynobius dunni*	Gokasho, Takachiho Town, Miyazaki Pref.	LC737960 / DUN47
61	*Hynobius dunni*	Shiroyama, Saiki City, Oita Pref.*	LC633146 / DUN00
	Hynobius nihoensis	Yamashita, Usa City, Oita Prefecture	LC633145 / H. nihoensis

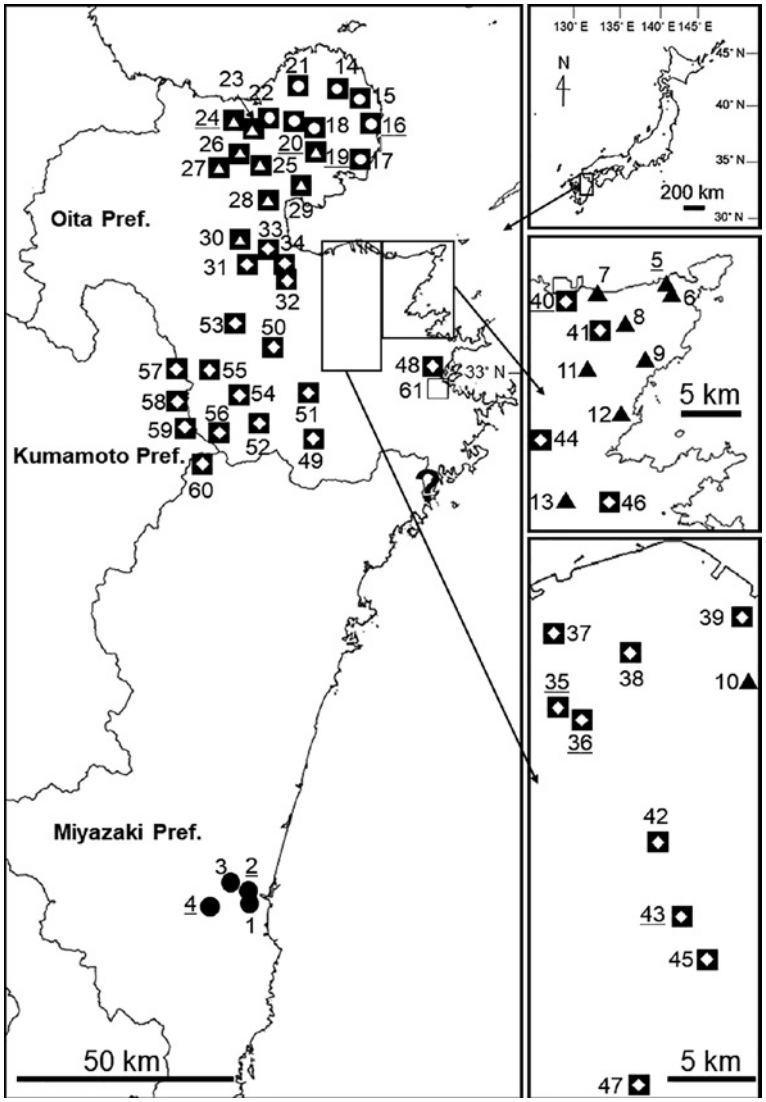

Fig. 1 Sampling localities for three *Hynobius* species in this study. Population numbers correspond to those used in molecular analyses (Table 1 and Fig. 2). The left, middle right, and lower right enlarged areas include the eastern part of Kyushu District, east central part of Oita Prefecture, and central part of Oita City (including around areas), respectively. The closed symbols correspond to the three species: *Hynobius miyazakiensis* sp. nov. (closed circles), *Hynobius amabensis* sp. nov. (closed triangles), and *Hynobius dunni* (closed squares). Closed squares, including the open circle, open triangle, and open diamond, indicate the Kunisaki population, northern Oita population, and southern Oita population of *H. dunni*, respectively (Fig. 2). The open square corresponds to *H. dunni* cited from Sugawara *et al.* (2022). Individuals of the three species were collected from the underlined localities for morphological assessments: Pops. 2 (four males and one female) and 4 (20 males and four females) for *H. miyazakiensis* sp. nov.; Pop. 5 (20 males and seven females) for *H. amabensis* sp. nov.; Pops. 16 (two males), 19 (one male), 20 (one female), 24 (three males), 35 (12 males and seven females), 36 (four females), 40 (one female), and 43 (one male) for *H. dunni*. The question mark on the southeastern part of Oita Prefecture indicates the population of Kamae (only literature record) that cannot identify the species (Sugawara and Nagano, 2019).

length (TAL), median tail width (MTAW), median tail height (MTAH), vomerine 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). Morphological characteristics were also examined: presence of distinct black spots on the dorsum (DBSD), distinct white spots on the venter (DWSV), distinct white spots on the lateral sides of the body (DWSL), distinct yellow lines on the dorsal (DTYLD) and ventral (DTYLV) sides of the tail, 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); we adopted the counting method of CGN by Matsui et al. (2019). In conserving the populations, measured individuals were subsequently returned to their capture sites, except for candidate-type specimens.

Prior to morphological comparisons among the three groups, normality was tested using the Shapiro–Wilk test. When data followed a normal distribution, homoscedasticity was tested using Bartlett's test. When population variances were equal, Tukey–Kramer tests were performed, and when variances were unequal, we performed Games–Howel tests. When data did not follow a normal distribution and population variances were unequal, Steel–Dwass tests were conducted. In evaluating the overall morphological variation among the three groups, we performed canonical discriminant analysis using SVL and standardized values (R = %SVL) for 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 (holotype and paratypes) were fixed in 10% formalin and transferred to 70% ethanol before measurement. The following 44 measurements were checked when we measured the holotype of two new species: 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 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), left snout length (LSL), right snout length (RSL), left upper eyelid width (LUEW), right upper eyelid width (RUEW), left lower jaw length (LLJL), and right lower jaw length (RLJL).

RESULTS

Hynobius dunni was divided into three monophyletic groups based on PP values: southern Miyazaki, eastern Oita, and widely distributed groups (Fig. 2). Although the distribution ranges of the eastern Oita group and widely distributed group of *H. dunni* were closer to each other, the former group formed a monophyletic relationship with the southern Miyazaki group (but not supported statistically) (Fig. 2). The widely distributed group of *H. dunni* was divided into three populations: Kunisaki, northern Oita, and southern Oita populations (Fig. 2).

Morphological measurements and significance levels of each morphological measurement of the three *H. dunni* groups are shown in Table 2 and Table 3, respectively. The mean SVL of the southern Miyazaki group was smaller than 70 mm in both sexes, and the other two groups of SVL were larger than 70 mm in both sexes (Table 2). Between the southern Miyazaki group and the eastern Oita group, five morphological characteristics significantly differed in males, and two characteristics significantly differed in females (Table 3). Between the southern Miyazaki group and the widely distributed group, males showed significantly different measurements for five morphological characteristics, whereas females had no significantly different measurements (Table 3). Males and females of the eastern

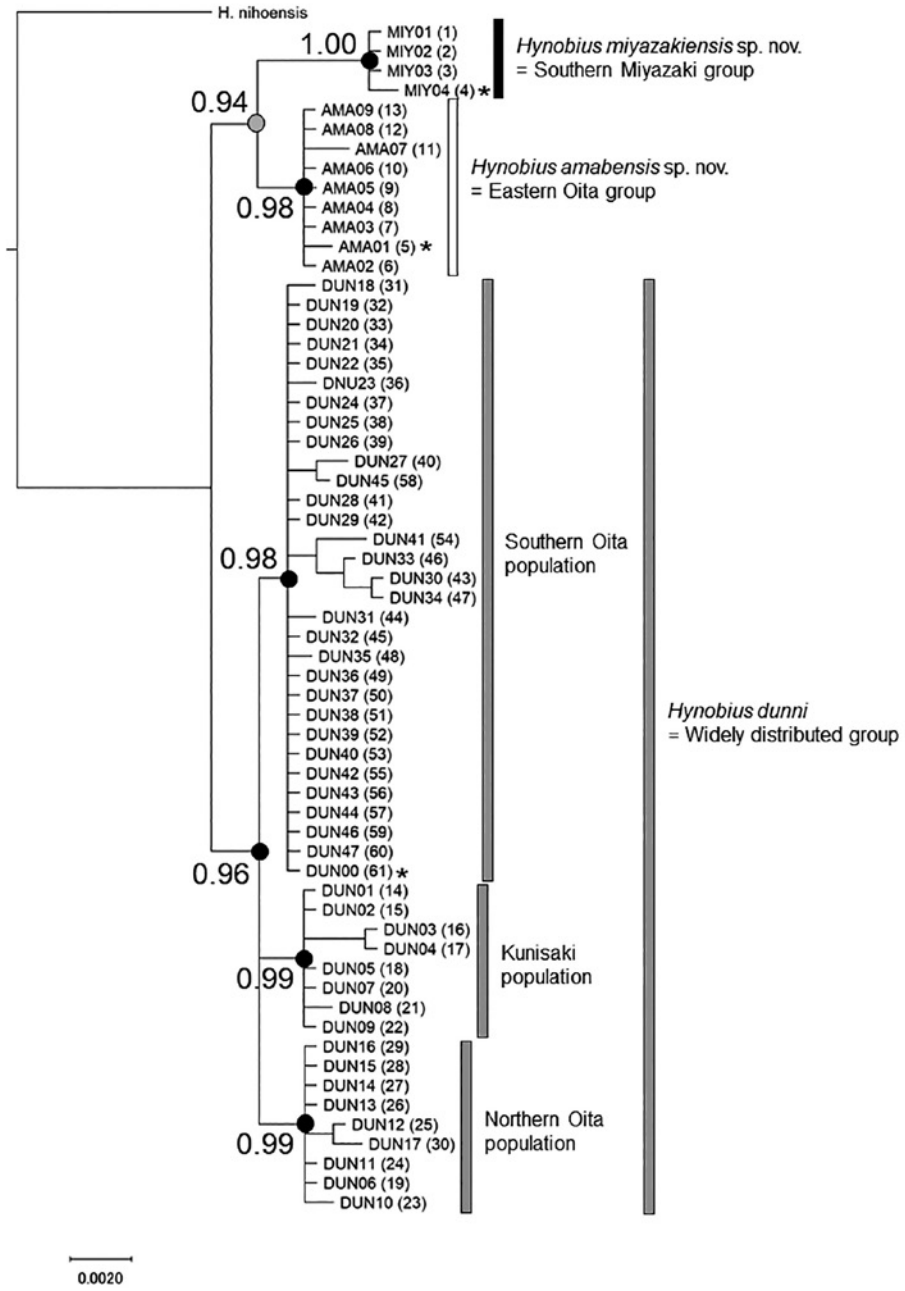

Fig. 2 Bayesian inference tree based on 630-base-pair (bp) cytochrome b sequences rooted with *Hynobius nihoensis*. The scale shows the genetic distance (expected changes per site). Numbers located near nodes are posterior probabilities (PP) (black nodes = 0.95 or more, gray node = less than 0.95). Values enclosed in parentheses after the haplotype names show population localities (Table 1 and Fig. 1). Asterisks after parentheses (Pops. 4, 5, and 61) indicate the type locality of the three species.

Table 2 Morphological measurements (mm) of SVL and characteristic ratios (R = %SVL) of TRL to LJL. The symbol ± and following values indicate the standard deviation, and ranges are shown in parentheses.

	Hynobius miyazakiensis sp. nov.		*Hynobius amabensis* sp. nov.		*Hynobius dunni*	
	Male	Female	Male	Female	Male	Female
Trait	*n* = 24	*n* = 5	*n* = 20	*n* = 7	*n* = 19	*n* = 13
SVL	69.7±2.56	69.4±2.38	72.1±6.93	76.1±3.76	71.4±4.25	71.0±4.30
	(64.8-74.4)	(65.6-71.4)	(60.9-82.9)	(70.0-81.1)	(61.6-78.2)	(61.3-77.6)
RTRL	76.6±0.82	77.5±0.59	76.8±0.85	77.3±0.99	76.4±0.63	76.8±0.89
	(74.7-78.1)	(76.6-78.3)	(75.4-78.8)	(76.0-78.6)	(75.3-77.8)	(75.7-78.0)
RAGD	52.8±1.05	54.0±2.99	53.4±2.24	56.1±1.05	53.4±2.02	53.5±2.81
	(51.4-55.1)	(49.0-56.4)	(50.2-57.7)	(54.5-57.4)	(50.8-58.1)	(48.1-59.7)
RHL	24.5±0.74	23.8±0.87	23.8±0.72	22.9±0.63	24.1±0.74	24.0±1.18
	(23.3-26.3)	(23.2-25.4)	(22.4-24.9)	(22.2-23.7)	(22.6-25.2)	(22.8-26.6)
RTAL	89.6±5.12	76.1±13.87	90.5±12.28	79.8±3.72	92.3±8.98	79.4±7.88
	(79.3-96.2)	(56.0-89.6)	(64.4-109.1)	(74.0-84.2)	(68.5-102.8)	(63.9-92.8)
RMTAW	6.9±0.65	6.3±0.38	6.6±1.01	6.8±0.62	6.4±0.96	6.2±0.90
	(5.4-7.9)	(6.0-6.9)	(4.9-8.7)	(6.3-8.1)	(4.7-8.5)	(4.8-8.6)
RMTAH	14.3±1.80	11.8±0.77	14.1±1.68	12.5±1.04	12.6±1.32	11.1±1.34
	(11.0-17.5)	(10.9-12.7)	(11.3-17.2)	(11.4-13.9)	(9.3-14.8)	(9.1-13.5)
RVTL	5.0±0.36	4.8±0.32	4.7±0.53	4.3±0.34	4.7±0.45	5.0±0.43
	(4.0-5.5)	(4.2-5.0)	(4.0-6.5)	(3.7-4.7)	(4.0-5.6)	(4.5-5.7)
RVTW	5.6±0.25	5.6±0.60	5.9±0.60	5.2±0.34	5.6±0.54	5.8±0.39
	(5.2-6.1)	(4.6-6.1)	(5.2-6.9)	(4.5-5.5)	(4.7-6.8)	(5.3-6.6)
RHW	18.6±0.63	17.9±0.65	18.6±1.10	16.8±0.44	17.8±0.78	17.5±0.93
	(17.2-19.7)	(17.5-19.1)	(16.4-20.5)	(15.9-17.3)	(16.6-19.4)	(15.9-19.4)
RFLL	29.4±1.58	27.1±1.11	27.7±1.32	26.1±0.99	28.2±2.83	26.0±1.49
	(26.8-32.3)	(25.5-28.1)	(25.0-30.5)	(24.9-27.7)	(23.0-36.1)	(23.9-28.9)
RHLL	34.7±1.04	32.5±1.26	34.1±1.32	32.2±1.50	35.1±1.67	33.1±1.86
	(32.5-36.5)	(31.1-34.5)	(31.8-36.9)	(30.3-34.2)	(32.5-39.2)	(28.7-36.1)
R2FL	6.6±0.45	6.2±0.31	6.8±0.55	5.8±0.59	6.6±0.64	5.9±0.72
	(5.7-7.2)	(5.8-6.4)	(5.9-8.1)	(5.0-6.9)	(5.0-7.8)	(4.6-7.1)
R3FL	5.7±0.41	5.6±0.61	5.2±0.50	4.7±1.03	5.6±0.56	5.5±0.64
	(4.9-6.6)	(4.6-6.1)	(4.1-5.8)	(3.0-6.2)	(4.4-6.7)	(3.9-6.4)
R3TL	9.1±0.76	9.2±0.23	9.1±0.61	8.6±0.64	9.4±0.57	9.4±0.57
	(8.1-10.4)	(8.8-9.4)	(8.0-10.1)	(7.5-9.4)	(8.4-10.7)	(7.9-10.2)
R5TL	3.4±0.57	3.7±0.64	3.2±0.73	3.6±0.39	3.4±0.56	3.4±0.48
	(2.3-4.6)	(2.7-4.2)	(1.8-4.8)	(3.0-4.2)	(2.4-4.4)	(2.7-4.5)
RIND	6.3±0.54	5.7±0.46	5.9±0.30	5.6±0.30	6.0±0.43	5.5±0.36
	(4.5-7.0)	(5.0-6.1)	(5.3-6.4)	(5.0-5.9)	(5.2-7.1)	(4.9-6.2)
RIOD	6.8±0.53	6.4±0.56	6.8±0.31	6.2±0.30	6.8±0.39	6.3±0.64
	(5.1-7.6)	(5.6-7.1)	(6.3-7.2)	(5.9-6.7)	(6.1-7.8)	(5.0-7.8)
RUEW	3.5±0.29	3.4±0.19	3.1±0.27	3.3±0.21	3.1±0.36	3.1±0.28
	(2.8-3.9)	(3.1-3.5)	(2.6-3.6)	(2.9-3.6)	(2.5-3.7)	(2.6-3.4)
RSL	7.3±0.39	6.8±0.43	7.0±0.43	6.8±0.45	7.0±0.40	6.4±0.41
	(6.6-8.0)	(6.3-7.4)	(6.2-8.1)	(6.3-7.5)	(6.4-8.0)	(5.7-7.4)
RUEL	4.6±0.24	4.5±0.40	4.6±0.35	4.4±0.16	4.4±0.31	4.2±0.46
	(4.2-5.2)	(4.1-5.2)	(4.0-5.4)	(4.1-4.6)	(3.7-5.1)	(3.6-5.0)
RLJL	14.0±0.72	13.9±0.57	14.3±0.82	13.0±0.60	14.1±0.83	13.5±0.81
	(12.3-15.1)	(13.0-14.5)	(12.5-16.1)	(11.9-13.9)	(12.3-15.6)	(12.5-15.4)

Table 3 Significantly different values among 22 morphological characteristics. Larger values of significant differences are indicated in bold. MIY, AMA, and DUN indicate *Hynobius miyazakiensis* sp. nov., *Hynobius amabensis* sp. nov., and *Hynobius dunni*, respectively.

	Male			Female			MIY	AMA	DUN
	MIY vs. AMA	MIY vs. DUN	AMA vs. DUN	MIY vs. AMA	MIY vs. DUN	AMA vs. DUN	Male vs. Female		
SVL	NS	NS	NS	P < 0.05	NS	P < 0.05	NS	NS	NS
RTRL	NS	NS	NS	NS	NS	NS	P < 0.05	NS	NS
RAGD	NS	NS	NS	NS	NS	NS	NS	P < 0.01	NS
RHL	P < 0.01	NS	NS	NS	NS	NS	NS	P < 0.01	NS
RTAL	NS	NS	NS	NS	NS	NS	NS	P < 0.01	**P < 0.0001**
RMTAW	NS	NS	NS	NS	NS	NS	NS	NS	NS
RMTAH	NS	P < 0.01	P < 0.05	NS	NS	NS	P < 0.01	P < 0.05	P < 0.01
RVTL	NS	NS	NS	NS	NS	P < 0.01	NS	P < 0.05	NS
RVTW	NS	NS	NS	NS	NS	P < 0.05	NS	**P < 0.0001**	NS
RHW	NS	P < 0.01	P < 0.05	P < 0.05	NS	NS	NS	**P < 0.0001**	NS
RFLL	**P < 0.001**	NS	NS	NS	NS	NS	P < 0.01	P < 0.01	P < 0.01
RHLL	NS	NS	NS	NS	NS	NS	**P < 0.001**	P < 0.01	P < 0.01
R2FL	NS	NS	NS	NS	NS	NS	P < 0.05	**P < 0.001**	P < 0.01
R3FL	P < 0.01	NS	P < 0.05	NS	NS	NS	NS	NS	NS
R3TL	NS	NS	NS	NS	NS	P < 0.05	NS	NS	NS
R5TL	NS	NS	NS	NS	NS	NS	NS	NS	NS
RIND	P < 0.01	P < 0.01	NS	NS	NS	NS	**P < 0.0001**	P < 0.01	P < 0.01
RIOD	NS	NS	NS	NS	NS	NS	NS	**P < 0.001**	P < 0.05
RUEW	**P < 0.001**	**P < 0.001**	NS	NS	NS	NS	NS	NS	NS
RSL	NS	P < 0.01	NS	NS	NS	NS	P < 0.01	NS	P < 0.01
RUEL	NS	NS	NS	NS	NS	NS	NS	P < 0.01	NS
RLJL	NS	NS	NS	NS	NS	NS	NS	P < 0.01	NS
P < 0.05	0	0	3	2	0	3	2	2	1
P < 0.01	3	4	0	0	0	1	3	8	6
P < 0.001	2	1	0	0	0	0	1	2	0
P < 0.0001	0	0	0	0	0	0	1	2	1
Total	5	5	3	2	0	4	7	14	8

Oita group and the widely distributed group showed significantly different measurements for three and four morphological characteristics, respectively (Table 3). Canonical discriminant analyses showed that the distribution score areas of the three groups did not overlap (Fig. 3).

The results of morphological observations are shown in Table 4. Of the 24 males of the southern Miyazaki group, 19 had frequently DBSD (79.2%), 22 and 23 had almost always no DWSV (91.7%) and DWSL (95.8%), respectively, 21 had usually no DTYLD (87.5%), 24 never had DTYLV (100%), 21 had usually 12 CGN (87.5%), and 21 usually had ≥ 2.5 CFBALN (87.5%). Of the five females of the southern Miyazaki group, four usually had DBSD (80.0%), DWSV (80.0%), 12 CGN (80.0%), and ≤ 1.0 CFBALN, and five never had DTYLV (100%) and DGM (100%). Of the 20 males of the eastern Oita group, 18, 19, and 19 had almost no DBSD (90.0%), DWSL (95.0%), and DTYLD (95.0%), respectively; 17 usually had no DWSV (85.0%); 14 frequently had 12 CGN (70.0%); 19 almost always had ≤ 2.0 CFBALN (95.0%). Of the seven females of the eastern Oita group, six usually had no DBSD (85.7%), five had frequently less than 0.0 CFBALN (71.43%), five had frequently no DWSL (71.4%) and DTYLD (71.4%), and seven never had DTYLV (100%) and DGM (100%). Of the 19 males of the widely distributed group, 14 had frequently no DWSL (73.7%), 17 had usually no DTYLD (89.5%), 19 never had DTYLD (100%), and 15 had frequently less than 2.5 CFBALN (78.95%). Of the 13 females of the widely distributed group, 11 usually had DBSD (84.6%) and DWSV (84.6%), 10 had frequently no DTYLD (76.9%), 13 never had DTYLV and DGM (100%), and 18 had less than 1.0 CFBALN (92.31%).

Based on the results of molecular and morphological analyses, we considered the southern Miyazaki and

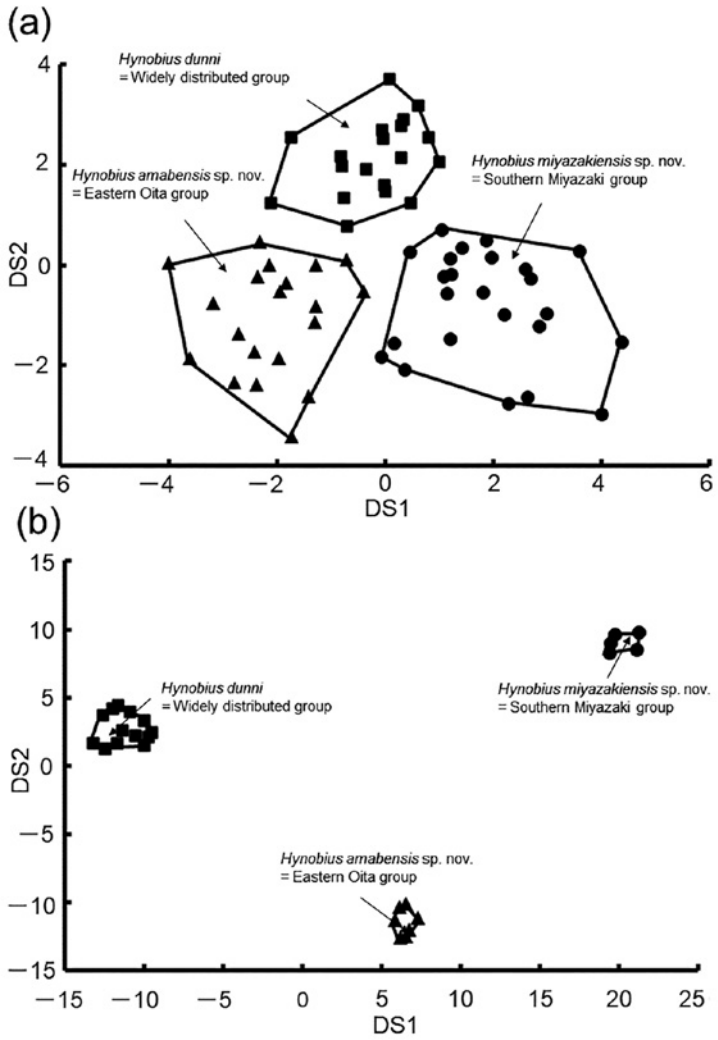

Fig. 3 Two-dimensional plots based on canonical discriminant analyses in both sexes. The x- and y-axes show discriminant score 1 (DS1) and discriminant score 2 (DS2), respectively. The closed symbols correspond to the three species: *Hynobius miyazakiensis* sp. nov. (closed circles), *Hynobius amabensis* sp. nov. (closed triangles), and *Hynobius dunni* (closed squares). The contribution ratios of DS1 and DS2 in (a) males and (b) females are as follows: DS1 = 57.79% (male), 73.73% (female); DS2 = 42.21% (male), 26.27% (female).

eastern Oita groups as new species in accordance with three species concepts.

SYSTEMATIC DESCRIPTION

Hynobius miyazakiensis Sugawara, Nagano et Sueyoshi sp. nov.

(Figs. 4, 5)

ZooBank LSID: urn:lsid:zoobank.org:act:1676F31B-6AC3-4A51-AAD3-39CC7D87DF8F

Hynobius dunni Iwasaki (1998: 1–10); Sueyoshi (2001: 1–8); Sueyoshi (2002: 1–6); Sueyoshi and Kushima (2004: 5–12)

Holotype: An adult male was collected by Masahiro Nagano in Tanocho Otsu, Miyazaki City, Miyazaki Prefecture, Kyushu, Japan (31° 51' N, 131° 16' E; elevation = 180 m above sea level [a.s.l.]; in all cases, datum = WGS84) on 14 February 2022. This specimen (specimen number = A-000055) was deposited in the Miyazaki Prefectural Museum of Nature and History: 2-4-4, Jingu,

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

		Hynobius miyazakiensis sp. nov.		*Hynobius amabensis* sp. nov.		*Hynobius dunni*	
		Male	Female	Male	Female	Male	Female
Character	Condition	*n* = 24	*n* = 5	*n* = 20	*n* = 7	*n* = 19	*n* = 13
DBSD	Absent	5 (20.8%)	1 (20.0%)	18 (90.0%)	6 (85.7%)	7 (36.8%)	2 (15.4%)
	Present	19 (79.2%)	4 (80.0%)	2 (10.0%)	1 (14.3%)	12 (63.2%)	11 (84.6%)
DWSV	Absent	22 (91.7%)	1 (20.0%)	17 (85.0%)	4 (57.1%)	12 (63.2%)	2 (15.4%)
	Present	2 (8.3%)	4 (80.0%)	3 (15.0%)	3 (42.9%)	7 (36.8%)	11 (84.6%)
DWSL	Absent	23 (95.8%)	3 (60.0%)	19 (95.0%)	5 (71.4%)	14 (73.7%)	6 (46.2%)
	Present	1 (4.2%)	2 (40.0%)	1 (5.0%)	2 (28.6%)	5 (26.3%)	7 (53.8%)
DTYLD	Absent	21 (87.5%)	3 (60.0%)	19 (95.0%)	5 (71.4%)	17 (89.5%)	10 (76.9%)
	Present	3 (12.5%)	2 (40.0%)	1 (5.0%)	2 (28.6%)	2 (10.5%)	3 (23.1%)
DTYLV	Absent	24 (100%)	5 (100%)	20 (100%)	7 (100%)	19 (100%)	13 (100%)
	Present	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
DGM	Absent	9 (37.5%)	5 (100%)	9 (45.0%)	7 (100%)	10 (52.6%)	13 (100%)
	Present	15 (62.5%)	0 (0%)	11 (55.0%)	0 (0%)	9 (47.4%)	0 (0%)
CGN	11	1 (4.2%)	0 (0%)	1 (5.0%)	0 (0%)	0 (0%)	0 (0%)
	12	21 (87.5%)	4 (80.0%)	14 (70.0%)	4 (57.1%)	12 (63.2%)	7 (53.8%)
	13	2 (8.3%)	1 (20.0%)	5 (25.0%)	3 (42.9%)	7 (36.8%)	6 (46.2%)
CFBALN	4.0	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (5.26%)	0 (0%)
	3.5	1 (4.2%)	0 (0%)	0 (0%)	0 (0%)	1 (5.26%)	0 (0%)
	3.0	3 (12.5%)	0 (0%)	0 (0%)	0 (0%)	1 (5.26%)	0 (0%)
	2.5	17 (70.8%)	0 (0%)	1 (5.0%)	0 (0%)	1 (5.26%)	0 (0%)
	2.0	2 (8.3%)	0 (0%)	5 (25.0%)	0 (0%)	6 (31.58%)	0 (0%)
	1.5	1 (4.2%)	1 (20.0%)	6 (30.0%)	0 (0%)	6 (31.58%)	0 (0%)
	1.0	0 (0%)	2 (40.0%)	7 (35.0%)	0 (0%)	3 (15.79%)	1 (7.69%)
	0.5	0 (0%)	1 (20.0%)	1 (5.0%)	0 (0%)	0 (0%)	3 (23.08%)
	0.0	0 (0%)	1 (20.0%)	0 (0%)	2 (28.57%)	0 (0%)	4 (30.77%)
	-0.5	0 (0%)	0 (0%)	0 (0%)	2 (28.57%)	0 (0%)	2 (15.38%)
	-1.0	0 (0%)	0 (0%)	0 (0%)	3 (42.86%)	0 (0%)	3 (23.08%)

Miyazaki City, Miyazaki Prefecture, 880-0053, Japan. Further details are available only by contacting the corresponding author or the Miyazaki Prefectural Museum of Nature and History to prevent the overcollection of this species.

Paratype: An adult female from the same locality as that of the holotype was collected by Toyofumi Sueyoshi on 17 February 2022. This specimen (specimen number = A-000056) was deposited in the Miyazaki Prefectural Museum. An adult male was collected by Masahiro Nagano in Furujocho, Miyazaki City, Miyazaki Prefecture, Japan (31° 53' N, 131° 23' E, elevation = 15 m a.s.l.) on 14 February 2022. This specimen (specimen number = YCM-RA593) was deposited in the Yokosuka City Museum: 95 Fukadadai, Yokosuka City, Kanagawa Prefecture, 238-0016, Japan. To prevent the overcollection of this species, further details are available only by contacting the corresponding author or the Yokosuka City Museum.

Diagnosis: This species (mean SVL of 69.7 mm in males and 69.4 mm in females) is larger in the Japanese lentic *Hynobius*: distinct black spots on dorsum frequently present in males and usually present in females; distinct white spots on the venter almost always absent in males and usually present in females; distinct white spots on the lateral side of the body almost always absent in males; distinct yellow line on the dorsal edge of the tail usually absent in males; distinct yellow stripe on the ventral edge of the tail always absent in both sexes; distinct gular mottling never present in females; fifth toe of hindlimb always present; U-shaped (rarely V-shaped) vomerine teeth series; usually 12 (rarely 11 or 13) costal

grooves in both sexes; the number of costal folds between adpressed limbs usually ranges from 2.5 to 3.5 in males and 0.5 to 1.5 in females; coil-shaped egg sacs.

Comparisons: The new species statistically differs from *H. dunni* in the following measurements: RMTAH, RHW, RIND, RUEW, and RSL in males. These measurements are significantly longer in *H. miyazakiensis* sp. nov. than in *H. dunni*. A distinct characteristic that separates them is the CFBALN ratio in males, which is usually ≥ 2.5 in *H. miyazakiensis* sp. nov. (21/24 = 87.5%), and frequently ≤ 2.0 (15/19 = 78.9%) in *H. dunni*. Other *Hynobius* species are not distributed sympatrically with this new species.

Description of holotype: This specimen is large with following characteristics: HL larger than HW; VTW larger than VTL; TAL 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; five toes on each hindlimbs; U-shaped vomerine teeth series; smooth and shiny skin; DBSD present (but unclear after preservation); DWSV and DWSL absent; DTYLD and DTYLV absent; DGM absent. The holotype measurements are as follows (mm): SVL = 68.3, TRL = 52.5, AGD = 34.9, HL = 15.2, TAL = 56.9, MTAW = 3.5, MTAH = 9.5, BTAW = 8.6, BTAH = 7.7, VTL = 3.5, VTW = 3.8, HW = 12.0, MXHW = 12.2, LFLL = 21.9, RFLL = 20.6, LHLL = 22.3, RHLL = 21.6, L1FL = 1.0, L2FL = 4.4, L3FL = 4.4, L4FL = 3.0, R1FL = 1.5, R2FL = 4.0, R3FL = 3.6, R4FL = 2.1, L1TL = 2.0,

Fig. 4 *Hynobius miyazakiensis* sp. nov. holotype (A-000055, adult male): (a) dorsal view, (b) ventral view, and (c) lateral view.

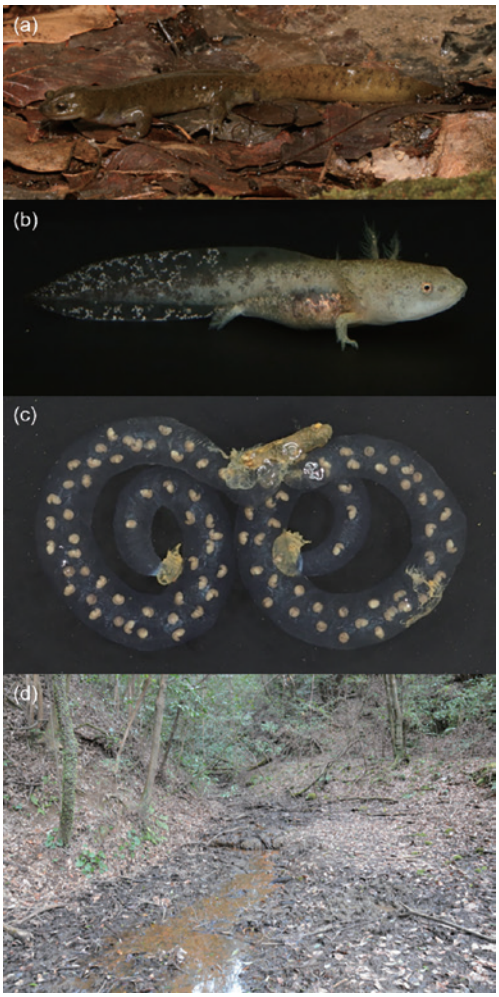

Fig. 5 *Hynobius miyazakiensis* sp. nov.: (a) adult, (b) larva, (c) egg sacs, and (d) type locality.

L2TL = 4.1, L3TL = 5.9, L4TL = 5.1, L5TL = 3.0, R1TL = 1.4, R2TL = 4.2, R3TL = 5.9, R4TL = 5.7, R5TL = 2.9, IND = 4.2, IOD = 4.6, LUEW = 2.1, RUEW = 2.5, LSL = 5.4, RSL = 5.2, LUEL = 2.9, RUEL = 2.8, LLJL = 8.9, RLJL = 8.7, CGN = 12.

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 RMTAH, RFLL, RHLL, R2FL, RIND, and RSL and relatively shorter RTRL than females. Skin markings are listed in Table 4. The dorsum is yellowish brown or dark brown. DBSD is rarely absent in males (5/24 = 20.8%) and females (1/5 = 20.0%). The venter is lighter than the dorsum, with DWSV rarely present in males (2/24 = 8.3%) and rarely absent in females (1/5 = 20.0%). DTYLD is rarely present in males (3/24 = 12.5%) and occasionally present in females (2/5 = 40%). DGM is occasionally absent in males (9/24 = 37.5%). CGN is hardly ever 11 (1/24 = 4.2%) or 13 (2/24 = 8.3%) in males and rarely 13 (1/5 = 20.0%) in females. CFBALN is rarely ≤ 2.0 in males (3/24 = 12.5%) and occasionally ≤ 0.5 in females (2/5 = 40.0%). The dorsal coloration tended to fade to dark gray when preserved in 70% ethanol.

Etymology: The specific epithet "*miyazakiensis*" refers to Miyazaki City where the new species occurred. The suggested common name in Japanese is Miyazaki-sanshouo.

Distribution: This new species is endemic to Miyazaki

Fig. 6 *Hynobius amabensis* sp. nov. holotype (A-000051, adult male): (a) dorsal view, (b) ventral view, and (c) lateral view.

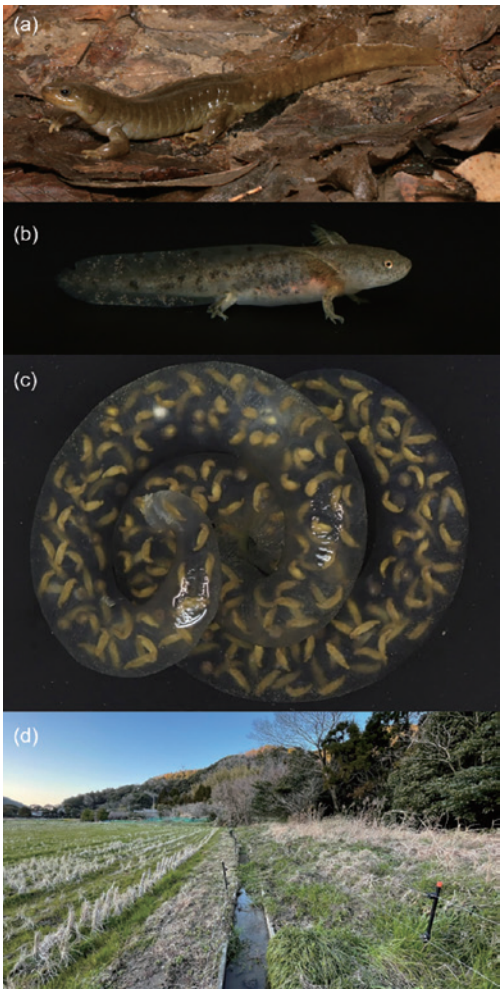

Fig. 7 *Hynobius amabensis* sp. nov.: (a) adult, (b) larva, (c) egg sacs, and (d) type locality.

Prefecture and found only in Miyazaki City (including the former Miyazaki City and Sadowara, Kiyotake, and Tano Towns). Populations from the former Sadowara Town (northernmost populations) may be extinct, and sustainable fragmented populations may be less than five populations based on our recent surveys.

Natural History: The main dominant vegetation type of habitat is a mixed forest consisting of chinquapin (*Castanopsis*), live oak (*Quercus*), and Japanese cedar (*Cryptomeria japonica*). Larvae have black spots on the lateral sides of the body and tail (Fig. 5-b). The fingers and toes of claws are absent. One pair of balancers are present during early larval developmental stages. Egg sacs are coil-shaped (Fig. 5-c) and attached to fallen branches or leaves in puddles, ponds, or swamps at forest edges from December to March. The number of eggs in a sac ranges from 24 to 108, and the clutch size ranges from 50 to 201 (Sueyoshi, 2001; Sueyoshi, 2002). This species forms the mating ball during breeding (Sueyoshi and Kushima, 2004).

Hynobius amabensis Sugawara et Nagano sp. nov.
(Figs. 6, 7)

ZooBank LSID: urn:lsid:zoobank.org:act:19CED49D-B4AD-4713-AF63-086D7BE2A7C7

Holotype: An adult male was collected by Masahiro Nagano in Shuki, Oita City, Oita Prefecture, Kyushu, Japan (33° 14' N, 131° 50' E; elevation = 10 m above sea level [a.s.l.]; in all cases, datum = WGS84) on 23 February 2022. This specimen (specimen number = A-000051) is stored in the Miyazaki Prefectural Museum of Nature and History: 2-4-4, Jingu, Miyazaki City, Miyazaki Prefecture, 880-0053, Japan. Further details are available only by contacting the corresponding author or the Miyazaki Prefectural Museum of Nature and History to prevent the overcollection of this species.

Paratype: An adult female (specimen number = A-000052) and male (specimen number = YCM-RA594) from almost the same locality of the holotype was collected by Masahiro Nagano on 24 January 2022. The female and male specimens are stored in the Miyazaki Prefectural Museum of Nature and History and the Yokosuka City Museum, respectively. Further details of these specimens are available only by contacting the corresponding author or the stored museum of each specimen.

Diagnosis: This species (mean SVL of 72.1 mm in males and 76.1 mm in females) is one of the largest species in lentic *Hynobius* species; distinct black spots on dorsum almost always absent in males and usually absent in females; distinct white spots on the venter usually absent in males; distinct white spots on lateral sides of the body almost always absent in males and frequently absent in females; distinct yellow stripe on the dorsal edge of the tail almost always absent in males and frequently absent in females; distinct yellow line on the ventral side of the tail always absent in both sexes; distinct gular mottling always absent in females; fifth toe of hindlimb always present; V or U-shaped vomerine teeth series; frequently 12 costal grooves (rarely 11 or 13) in males; number of costal folds between adpressed limbs almost always 1.0 to 2.5 in males and frequently −0.5 to −1.0 in females; head of male sometimes triangular; coil-shaped egg sacs.

Comparisons: The new species statistically differs from *H. dunni* in the following measurements: RMTAH, RHW, and R3FL in males; SVL, RVTL, RVTW, and R3TL in females. These measurements are significantly longer in *H. amabensis* sp. nov. than in *H. dunni*, except for R3FL in males and RVTL, RVTW, and R3TL in females. DBSD are almost always absent in males (18/20 = 90.0%) and usually absent in females (6/7 = 85.7%) of *H. amabensis* sp. nov. but often present in males (12/19 = 63.2%) and usually present in females (11/13 = 84.6%) of *H. dunni*. This new species also statistically differs from *H. miyazakiensis* sp. nov. in the following measurements: RHL, RFLL, R3FL, RIND, and RUEW in males; SVL and RHW in females. These measurements are significantly shorter in *H. amabensis* sp. nov. than in *H. miyazakiensis* sp. nov., except for SVL in females. DBSD is almost always absent in males (18/20 = 90.0%) and usually absent in females (6/7 = 85.7%) of *H. amabensis* sp. nov. but frequently present in males (19/24 = 79.2%) and usually present in females (4/5 = 80%) of *H. miyazakiensis* sp. nov. CFBALN is almost always ≤ 2.0 in males (19/20 = 95.0%) and always ≤ 0.0 in females (7/7 = 100%) of *H. amabensis* sp. nov. but usually ≥ 2.5 in males (21/24 = 87.5%) and usually ≥ 0.5 in females (6/7 = 80%) of *H. miyazakiensis* sp. nov.

Description of holotype: This specimen is large with following characteristics: HL larger than HW; VTW

larger than VTL; TAL shorter than SVL; body almost cylindrical; rounded snout; gular fold present; tail gradually compressed toward the tip; largely expanded cloaca; webbing between digits absent; four fingers on each forelimb; V-shaped vomerine teeth; skin smooth and matte; absent DBSD, DWSV, DWSL, DTYLD, and DTYLV; present DGM. The holotype measurements are as follows (mm): SVL = 72.2, TRL = 54.9, AGD = 36.4, HL = 17.3, TAL = 67.6, MTAW = 4.5, MTAH = 10.8, BTAW = 10.7, BTAH = 9.4, VTL = 3.8, VTW = 4.0, HW = 13.4, LFLL = 22.4, RFLL = 21.4, LHLL = 24.9, RHLL = 23.4, L1FL = 1.9, L2FL = 4.8, L3FL = 4.1, L4FL = 2.2, R1FL = 1.2, R2FL = 4.1, R3FL = 3.7, R4FL = 1.9, L1TL = 1.8, L2TL = 4.3, L3TL = 6.4, L4TL = 5.2, L5TL = 2.5, R1TL = 2.0, R2TL = 4.2, R3TL = 5.9, R4TL = 4.9, R5TL = 2.6, IND = 4.3, IOD = 4.6, LUEW = 2.2, RUEW = 2.1, LSL = 5.9, RSL = 5.6, LUEL = 2.9, RUEL = 3.1, LLJL = 10.0, RLJL = 9.8, and CGN = 12.

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, RVTW, RHW, RFLL, RHLL, R2FL, RIND, RIOD, RUEL, and RLJL and relatively shorter RAGD than females. Skin markings are listed in Table 4. The dorsum is uniformly greenish brown or dark brown. The venter is lighter than the dorsum. DBSD is rarely present in males (2/20 = 10.0%) and females (1/7 = 14.3%). DWSV is rarely present in males (3/20 = 15.0%) and sometimes present in females (3/7 = 42.9%). DWSL is rarely present in males (1/20 = 5.0%) and hardly present in females (2/7 = 28.6%). DTYLD is rarely present in males (1/20 = 5.0%) and rarely present in females (2/7 = 28.6%). DGM is sometimes absent in males (9/20 = 45.0%). CGN is hardly ever 11 (1/20 = 5.0%) or rarely 13 (5/20 = 25.0%) in males and sometimes 13 in females (3/7 = 42.9%). CFBALN is almost always < 1.0 in males (1/20 = 5.0%) and rarely more than −0.5 (2/7 = 42.86%) in females. When preserved in 70% ethanol, the dorsal coloration tends to fade to dark gray.

Etymology: The specific epithet "*amabensis*" refers to the Amabe county that located in the southeastern part of Oita Prefecture where the new species occurs. Suggested common name in Japanese: Amabe-sanshouo.

Distribution: This new species is endemic to Oita Prefecture and found in Oita (only including the former Oita City and Saganoseki Town) and Usuki (only including the former Usuki City) Cities.

Natural History: The main dominant vegetation type of the habitat is a mixed forest consisting of Japanese cedar (*Cryptomeria japonica*), live oak (*Quercus*), and chinquapin (*Castanopsis*). The larval morphology of *H. amabensis* sp. nov. is similar to that of *H. miyazakiensis* sp. nov. and *H. dunni* (Fig. 7-b). Egg sacs are coil-shaped (Fig. 7-c) and attached to fallen branches or leaves in puddles, ponds, swamps, or brooks at forest edges from January to March. No ecological studies of this species have been conducted. Therefore, such studies should be performed for the conservation of this species.

Remarks: This new species may be formed a monophyletic group with *H. miyazakiensis* sp. nov., although the PP value was not supported the monophyly based on the criteria of Huelsenbeck and Rannala (2004) (PP ≥ 0.95) (Fig. 2).

DISCUSSION

Hynobius dunni has been regarded as a single species, but it was divided into three groups based on the phylogenetic and evolutionary species concepts using the criteria of Huelsenbeck and Rannala (2004) (Fig. 2). The morphological difference between *H. miyazakiensis* sp. nov. and *H. amabensis* sp. nov. was based on the presence of DBSD (79.2% vs. 10.0% in males; 80.0% vs. 14.3% in females) and CFBALN (usually ≥ 2.5 vs. almost always ≤ 2.0 in males; usually ≥ 0.5 vs. always ≤ 0.0 in females) in both sexes (Table 4) and males of RFLL ($P < 0.001$) and RUEW ($P < 0.001$, Table 3). The morphological difference between *H. miyazakiensis* sp. nov. and *H. dunni* was relatively unclear compared with the differences between *H. miyazakiensis* sp. nov. and *H. amabensis* sp. nov., although males of CFBALN tended to differ with one another (usually ≥ 2.5 vs. frequently ≤ 2.0 in males; usually ≥ 0.5 vs. often ≤ 0.0 in females). In addition, males of RUEW ($P < 0.001$) were significantly different with one another (Tables 3 and 4). The difference between *H. amabensis* sp. nov. and *H. dunni* was relatively unclear compared with the differences between *H. miyazakiensis* sp. nov. and *H. dunni*, although

the presence of DBSD in females was relatively clear (absent = 85.7% in *H. amabensis* sp. nov. vs. present = 84.6% in *H. dunni*). However, *H. amabensis* sp. nov. and *H. dunni* were not monophyletic based on phylogenetic analyses (Fig. 2), although their morphological characteristics were similar to each other (Table 4). Thus, three groups of *H. dunni* should be divided into three species based on the three species concepts.

The distribution area of *H. dunni* is restricted to Oita Prefecture and the surrounding area (Table 1; Fig. 1). The distribution record of *H. dunni* was summarized by Sugawara and Nagano (2019), who found the literature record of *H. dunni* in the former Kamae Town (Saiki City at present), Oita Prefecture (Fig. 1). Specimens from this area have not been collected, although the picture of the individual from this area is present (Sugawara and Nagano, 2019). Therefore, *H. dunni* of this area may be extinct at present based on our field surveys. However, Kamae's population may have survived elsewhere. In addition, the Kamae's population may not belong to *H. dunni* but *H. amabensis* sp. nov. Thus, the final decision of the distribution area of *H. dunni* and *H. amabensis* sp. nov. should be determined after discovering some new populations of *H. dunni* from the former Kamae Town. The boundary of *H. dunni* and *H. amabensis* sp. nov. belong to the eastern part of former Oita and Usuki Cities (Fig. 1). Given the geographical continuity of distribution ranges, introgression may occur between *H. dunni* and *H. amabensis* sp. nov. as is the case with *Pelophylax nigromaculatus* and *Pelophylax porosus* (Komaki et al., 2012). In the future, studies about population genetics or conservation genetics must be performed on *H. dunni* and *H. amabensis* sp. nov. using microsatellite or single nucleotide polymorphism (SNP) markers.

The distribution area of *H. miyazakiensis* sp. nov. is limited to Miyazaki City (Table 1; Fig. 1). Geographically, this species is clearly isolated from *H. amabensis* sp. nov. and *H. dunni*. Thus, the current introgression to *H. miyazakiensis* sp. nov. from *H. dunni* and *H. amabensis* sp. nov. is less likely to occur. According to Sugawara *et al*. (2015) who used microsatellite markers, the southern Oita population of *H. dunni* was clearly separated from the northern Oita population of *H. dunni* and *H. miyazakiensis* sp. nov., but the genetic separation between the northern Oita population of *H. dunni* and *H. miyazakiensis* sp. nov. was unclear. However, analysis was performed only using three microsatellite markers, which lacked credibility. Moreover, no evidence has shown that *H. miyazakiensis* sp. nov. and the northern population of *H. dunni* are phylogenetically nested each other. Thus, *H. miyazakiensis* sp. nov. should be separated from *H. dunni* based on the morphological, phylogenetic, and evolutionary species concepts. The population of the northern limit (former Sadowara Town) and several populations from Miyazaki City were already extinct by the residential development or reformation into well-drained paddy field, and sustainable populations of this species may be fewer than five or six populations based on our field surveys. Thus, management plans of this new species must be established as soon as possible.

Based on our field surveys and some papers, the distribution area of true *H. dunni* are as follows: Kunisaki (including the former Kunimi, Kunisaki, Musashi, and Aki Towns), Bungotakada (only including the former Bungotakada City and Matama Town), Kitsuki (including the former Kitsuki City, Yamaga Town, and Ota Village), Usa (including the former Usa City and Ajimu and Innai Towns), Beppu, Yufu (including the former Hasama, Shonai, and Yufuin Towns), Oita (including the former Oita City and Notsuharu Town), Usuki (including the former Usuki City and Notsu Town), Tsukumi, Saiki (only including the former Saiki City and Yayoi and Ume Towns), Bungo-Ono (including the former Inukai, Mie, Ono, Asaji, and Ogata Towns, and Chitose and Kiyokawa Villages), and Taketa (only including the former Taketa City and Naoiri and Kuju Towns) Cities, and Hiji Town, Oita Prefecture, Aso City (only including the former Namino Village), Takamori Town, and Ubuyama Village, Kumamoto Prefecture, and Takachiho Town, Miyazaki Prefecture (Sato and Horie, 2000; Sugawara and Nagano, 2019). Whereas the distribution range was elucidated sufficiently, on the morphological characters of this species are necessary to reassess yet. The type locality of *H. dunni* is Shiroyama (Saiki City), but individuals from this area are not included in this study because this population is designated as a natural monument. Although topotype was not described in this study, the redescription of *H. dunni* using individuals from the type locality should be performed for further morphological

assessment of true *H. dunni*.

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