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A New Brown Frog from the Goto Islands, Japan with Taxonomic Revision on the Subspecific Relationships of *Rana tagoi* (Amphibia: Anura: Ranidae)

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Abstract: Recent molecular analyses cast doubt on the subspecific relationships of Japanese brown frogs *Rana tagoi tagoi*, *R. t. okiensis*, and *R. t. yakushimensis*. Many samples of *R. tagoi* from three main islands, i.e., Honshu, Shikoku, and Kyushu tend to form a genetic unit with rich variation. On the other hand, *R. t. okiensis*, *R. t. yakushimensis*, and some insular samples are clearly isolated from *R. t. tagoi* and each other. Thus, we propose to treat each subspecies of *R. tagoi* as full species and describe part of the Goto Islands populations as *Rana matsuoi* sp. nov. The new species and *R. tagoi* show a complicated distributional pattern within the Goto Islands but the former is distinguishable from the latter by their smaller male body size, relatively longer limbs, more developed toe webs, nuptial pad formation in breeding male, and advertisement call with repetition of short notes.

Key words: Goto Islands; Oki Islands; *Rana matsuoi* new species; Taxonomy; Yakushima Island

Introduction

The Japanese brown frog, *Rana tagoi* Okada, 1928, is a common species in mountain areas of this country except for Hokkaido and most of the Ryukyus (Matsui and Maeda, 2018). Although some studies recognized several cryptic species within it (Matsui and Matsui, 1990; Ryuzaki et al., 2014; Eto et al., 2022), *R. tagoi* is still considered as a widespread species occurring on most of the main islands of Japan, i.e., Honshu, Shikoku, and Kyushu Islands (Matsui and Maeda, 2018; Eto et al., 2022). Three subspecies are recognized in *R. tagoi*: *R. t. tagoi* from main islands (holotype: Sp. no. 2962 stored at the Museum of Zoological Institute, Faculty of Science, Tokyo Imperial University [Okada, 1928]; probably lost already [Matsui and Maeda, 2018]), *R. t. yakushimensis* Nakatani and Okada, 1966 from

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Yakushima Island (type not designated [Nakatani and Okada, 1966]), and *R. t. okiensis* Daito, 1969 from the Oki Islands (type not designated [Daito, 1969]), although each of their original descriptions have problems (for detailed discussion, see Shibata, 1988). Shibata (1988) carefully checked their original descriptions and related articles, and recommended that all three names should be treated as available following ICZN (https://www.iczn.org). He considered information on their type localities to be incorrect or insufficient and redefined them as follows: alt. 3000 feet of Kamitakaramura, Gifu Pref., central Honshu for *R. t. tagoi*; the River Anbo, Yakushima Isl., Kagoshima Pref. for *R. t. yakushimensis*; and the Oki Islands (probably Dogo Isl.), Shimane Pref. for *R. t. okiensis* (Shibata, 1988). His redefinition is generally accepted (e.g., Eto et al., 2012; Matsui and Maeda, 2018). On the other hand, several molecular studies revealed that their genetic relationships were highly complicated (e.g., Nishioka et al., 1987, Eto et al., 2012; Eto and Matsui, 2014) and their evolutionary history is still uncertain, but it is clear that the taxonomic status of subspecies of *R. tagoi* needs reconsideration. For example, *R. t. tagoi* and *R. t. okiensis* would be heterospecific because an artificial cross breeding test demonstrated that they were reproductively isolated (Daito et al., 1998).

Other than the known insular subspecies, *R. t. tagoi* populations in the Goto Islands (Fig. 1) were reported to have unique characteristics. The Goto Islands (Isls.) are located ca. 20–60 km west of Kyushu Island and form a northeast-southwest-trending chain consisting of five major and hundreds of small islands (Fig. 1). Matsuo et al. (2011) and Sueyoshi et al. (2013) noted that the populations in two major islands of the Goto Isls., Nakadori and Fukue, had call characters and breeding phenology different from the populations in the main islands of Japan. Here we reanalyzed molecular biological information related to subspecies of *R. tagoi* including the Goto Isls. populations, as well as morphology and calls, and review their taxonomic status.

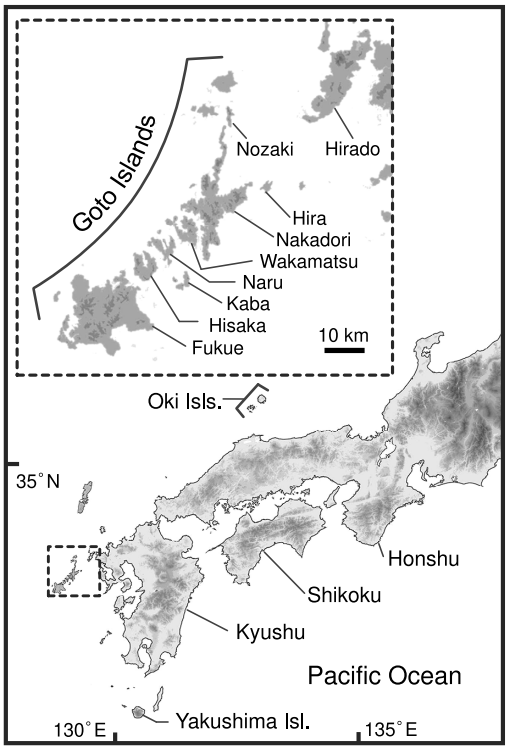

Fig. 1. Map showing western part of Japan, the main sampling area of this study.

Materials and Methods

Sampling

We examined adult specimens of *Rana tagoi* subspecies, including from their suggested type localities (Shibata, 1988): *R. t. tagoi* from Honshu, Shikoku, Kyushu and peripheral islands; *R. t. okiensis* from the Oki Islands, near the western part of Honshu; and *R. t. yakushimensis* from Yakushima Island, near southern part of Kyushu (Fig. 1). Samples from seven islands of the Goto Isls. and the nearby island, Hirado (Fig. 1) were included. These specimens are stored in the Graduate School of Human and Environmental Studies, Kyoto University (KUHE), Kitakyushu Museum of Natural History and Human History (KMNH), the Institute for Amphibian Biology, Hiroshima University (IABHU), and National Museum of Nature and Science,

Tokyo (NSMT). In this study we excluded samples labeled as *R. t. tagoi* from the central- to eastern-part of Honshu Island except for their topotypes, largely because some populations in this area show a geographic mosaic of traits which diagnose *R. t. tagoi* and its relative *R. neba* (e.g., advertisement call and chromosomes: Eto et al., 2016) making species identification difficult. We defer judgement on the taxonomic status of these populations and await future research. See Appendix for voucher numbers.

Molecular analyses

We reanalyzed sequence data of one mitochondrial (mt: NADH dehydrogenase subunit 1 [ND1]) and five nuclear (nc: sodium-calcium exchanger 1 [NCX1=SLC8A1], nuclear factor I/A [NFIA], pro-opiomelanocortin [POMC], sodium-calcium exchanger 3 [SLC8A3], and tyrosinase [TYR]) genes of related taxa deposited in GenBank (accession nos. AB639593–AB639742, AB968646–AB969281). In addition, we determined partial sequences of ND1 of some samples from the Goto Islands and Hirado by using laboratory protocols reported in previous works (e.g., Eto et al., 2012). Newly obtained mitochondrial sequences (GenBank accession nos. LC769391–LC769411) were used for DNA barcoding by comparing reference sequences of each mt-lineage previously reported.

Based on the previous study (Eto and Matsui, 2014), the basic phylogenetic approach using sequence data of nuclear genes would not work well in the *Rana tagoi* species complex, probably because of the incomplete lineage sorting. Instead, we used a population genetic approach to evaluate genetic relationships among populations. We used PHASE ver. 2.1 (Stephens et al., 2001) to determine haplotypes for heterozygous nuclear genes. Then, principal co-ordinate analysis (PCoA) was conducted based on pairwise Nei's genetic distance (Nei, 1972). These downstream analyses, as well as calculation of other statistics, were performed by GenAlEx 6.5 (Peakall and Smouse, 2012). Deviation from Hardy-Weinberg equilibrium (HWE) of each locus by population was tested by exact tests using R 4.1.2 (R Core Team, 2021). Mitochondrial lineages were used for candidate genetic populations in these analyses.

Morphological analysis

For adult specimens preserved in 70% ethanol, we took the following 22 body measurements to the nearest 0.1 mm with dial calipers (Fig. 2); (1) snout-vent length (SVL); (2) head length (HL), from tip of snout to posterior end of jaw joint; (3) head width (HW) at the angle of jaw; (4) snout length (SL); (5) snout-nostril length (S-NL); (6) nostril-eyelid length (N-EL); (7) eye length (EL), length of upper eyelid; (8) tympanum-eye length (T-EL); (9) maximum tympanum diameter (TD); (10) internarial distance (IND); (11) intercanthal distance (ICD); (12) interorbital distance (IOD), shortest distance between upper eyelids; (13) upper eyelid width (UEW); (14) lower arm and hand length (LAL), from elbow to tip of third finger; (15) hand length (HAL), distance from proximal edge of inner palmer tubercle to tip of longest (third) finger; (16) hindlimb length (HLL); (17) tibia length (TL); (18) foot length (FL); (19) inner metatarsal tubercle length (IMTL); and (20, 21, 22) first, third, and fifth toe length (1, 3, and 5TOEL). For comparisons, candidate measurements of the holotype and referred specimens of *R. tagoi* in Okada (1931; but see Shibata [1988] about its reliability) were also used. Because the holotype is identified as a female (Okada, 1928), we chose the measurements of three females numbered ZM2962 (number same as holotype in the original description: probably from Gifu Pref. [see Shibata, 1988]), ZM2954 (Gifu Pref,?), and ZM1912 (Ehime Pref.?) in Okada (1931). In addition, toe-webbing was evaluated following Savage & Heyer (1967; 1997). We also evaluated the pigmentation of ventral side of thigh and tibia in three categories: (1) absent or very little pigmentation; (2) pigmentation forming fine dots; and (3) pigmentation forming blackish blotches or mottling, following Eto et al. (2022).

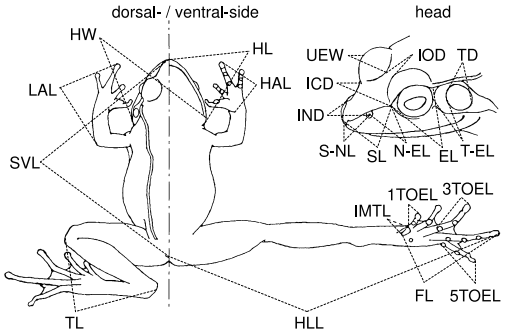

FIG. 2. Character legend for adult morphology. See text for definition of each measurement.

We compared SVL of samples using the Tukey-Kramer test and Student's *t*-test. We used Dunn's multiple comparison test and the Kruskal-Wallis test for ratio values and detection of the presence or absence of differences in frequency distributions. A significance level of 5% was used in all statistical tests. To examine overall morphological variation, we conducted principal component analysis (PCA) using $\log_{10}$ transformed values of each measurement. We used all 22 characters for males, whereas the following 15 characters commonly measured by us and Okada (1931) were used in female analysis: SVL, HL, HW, SL, EL, S-NL, N-EL, T-EL, TD, IND, IOD, UEW, HLL, TL, and FL. All statistical analyses were performed using R 4.1.2 (R Core Team, 2021).

We also examined eggs (in fresh) and larval specimens (preserved in 5% formalin). For larvae, we took the following eight measurements to the nearest 0.01 mm with dial calipers under a binocular microscope: (1) total length (TTL), between tip of snout and posterior end of tail; (2) head-body length (HBL), snout tip to the posterior end of the body; (3) head-body width (HBW), maximum width of head and body; (4) maximum head-body depth (HBD); (5) IOD; (6) eye-snout distance (ESD), distance between the tip of snout and anterior corner of eye; (7) maximum oral-disc width (ODW), and (8) maximum tail height (TAH). We determined developmental stages of larva following staging tables of Gosner (1960). For eggs, we measured the diameter of 10 eggs for each egg mass taken from ovary/oviduct of mature females.

Bioacoustic analysis

We recorded frog calls in the field using digital recorders (Olympus LS-11) at 44.1 kHz/24 bit as uncompressed wave files and analyzed them with Raven Pro 1.6 for Windows (http://www.birds.cornell.edu/raven). Water or air temperatures of calling site were measured. We conducted recording at the following sites: Arikawa, Nakadori Isl. of the Goto Isls. (25 Feb. 2012); Tanoura, Hisaka Isl. of the Goto Isls. (24 Feb. 2012); along the River Anbo, Yakushima Isl. (5 Feb. 2013); Mt. Daimanji, Dogo Isl. of the Oki Islands. (9 Feb. 2010).

For each call, temporal data were obtained from the oscillograms and frequency information was obtained from the audiospectrograms using Fast Fourier Transformation (256 and 512 point Hanning window). We evaluated and compared the following characters: (1) call length (CL), duration of a single call unit; (2) note length (NL), duration of a single note; (3) number of notes (NN); (4) dominant frequency (DF), measured for each note; and (5) number of pulses (PN) of each note for the calls recorded in good conditions (i.e., with little background noise), although we categorized a series of pulses with a pulse repetition rate of 200/s or larger as one continuous pulse unit. Following Shimada et al. (2021), we chose at most three notes from each call to evaluate the above characters except for CL: the first, the middle, and the last note. For the calls containing only one-note, we consider it as the first note. For two-notes call, we considered each of two as the first and the last.

Following Eto et al. (2022), we categorized each call into several call types. First, we categorized each call into four groups following NN: (I) single-note call, (II) two-note call, (III) three-to-five-note call, and (IV) call with six or more notes. Second, we evaluated each call by the variation of NL of the first and the last notes as follows: (A) shorter first note with longer last note (ratio of the first/last NL is 2/3

or less); (B) longer first with shorter last (3/2 or more); and (C) stable NL among first to last (more than 2/3, less than 3/2). Considering these two factors, we categorized each call as I, II-A, III-C etc.

Results

DNA barcoding using mitochondrial gene

We analyzed a total of 187 sequences of ND1 (619 bp), including 19 sequences newly obtained, from *Rana tagoi* subspecies and their allies. Sequences obtained from eight islands around the Goto Isls. (Fig. 1) belonged to mt-lineages A-9a and A-9c in Eto et al. (2012). Minimum uncorrected p-distances were 0.0–0.3% between each of the sequences and sequences in the previous study. Such a distance is smaller than the minimum sequence divergence among lineages reported previously (1.3% in ND1: Eto et al., 2012). By combining the results of previous studies (Eto et al., 2012: Eto and Matsui, 2014), our analysis revealed a complicated distributional pattern of mt-lineages in the Goto Islands and the adjacent area. Mt-lineage A-9a was widely distributed in Kyushu and Hirado islands. On the other hand, mt-lineage A-9c occurred in Nozaki, Nakadori, Wakamatsu, Naru, and Fukue islands of the Goto Isls. However, A-9a also occurred in Hira, Kaba, and Hisaka of the Goto Isls., and the distributional ranges of A-9a and A-9c were interrupted by each other in this area (Fig. 1). We could not recognize co-occurrence of two mt-lineages in any islands. Sequence divergences of ND1 among haplotypes within A-9a was 0.1–1.4%, and those within A-9c was 0.2–3.8%. No genetic variation was observed among the Goto samples of A-9a (uncorrected p-distance=0.0).

Population genetic analysis using nuclear genes

Basic statistics of each of five nc-genes were summarized in Table 1. No deviation from HWE were detected by exact tests in each locus ($P>0.05$). Insular populations tended to have a high proportion of unique haplotypes. For example, all three haplotypes observed in POMC and all four in TYR of *R. t. yakushimensis* were unique to this subspecies. Similarly, all two haplotypes observed in NFIA of *R. t. okiensis* and all six haplotypes observed in TYR of the samples belong to mt-lineage A-9c were unique to each group. In contrast, no such complete substitution of haplotype was observed in any locus of the other groups, i.e., A-1a, A-7, A-9a, A-9b, B-2a, and B-2b.

Two-dimensional plots based on 1st, 2nd, and 3rd axes of PCoA are shown in Fig. 3. Percentage of variation explained by these three axes were 45.4, 21.9, and 16.7%, respectively. Mt-lineages A-8 (=*R. t. yakushimensis*), B-1 (=*R. t. okiensis*), and A-9c (a part of the Goto Isls. population) were clearly separated from the others (A-1a, A-7, A-9a including another part of the Goto population, A-9b, B-2a, and B-2b) in the axis 1. Then, the former three lineages were separated in axis 2, whereas the latter six lineages tended to form a cluster in the axes 1 and 2. This cluster was finally separated into two in the axis 3 (Fig. 3). These results and presence of unique haplotypes suggest that A-8, B-1, and A-9c were genetically isolated from each other and the remaining cluster, which also might be subdivided. Because the topotypes of *R. tagoi* are included in A-1a in this study, we considered this cluster as *R. t. tagoi*, and the other three as mutually independent operational taxonomic units (OTUs) for the following analyses.

Morphological analyses

Measurement values are summarized in Table 2. Multivariate analyses were conducted using 15 characters for females including the candidate holotype of *R. tagoi*, and 22 characters for males. Sample size of each OTU, i.e., *R. t. tagoi*, *R. t. okiensis*, *R. t. yakushimensis*, and mt-lineage A-9c were 19, 6, 6, and 15 in females, and 43, 7, 5, and 18 in males. Contributions of the 1st, 2nd, and 3rd principal components (PC) were 50.7, 12.6, and 8.4% in females, and 63.6, 9.2, and 5.1% in males. Plots of each sex based on the 1st and 2nd PCs are shown in Fig. 4. Each OTU did not tend to

Table 1. Number of allele (=haplotype: Na), observed (Ho) and expected (He) heterozygosities of five nuclear genes among mitochondrial lineages of *Rana*.

	Taxon	*Rana tagoi tagoi*						*R. t. yakushimensis*	*R. t. okiensis*	*Rana* sp. Goto
	MtDNA	A-1a	A-7	A-9a	A-9b	B-2a	B-2b	A-8	B-1	A-9c
	n	18	7	7	3	12	8	3	3	6
POMC	Na	6	1	3	1	7	4	3	5	5
	Ho	0.50	—	0.29	—	0.33	0.38	0.67	0.67	0.33
	He	0.71	—	0.26	—	0.82	0.33	0.50	0.78	0.61
NCX1	Na	8	3	5	3	3	6	4	1	7
	Ho	0.61	0.43	0.57	0.33	0.08	0.50	1.00	—	0.67
	He	0.70	0.50	0.62	0.50	0.60	0.78	0.72	—	0.81
SLC8A5	Na	5	2	2	4	1	6	1	4	1
	Ho	0.28	0.29	0.29	1.00	—	1.00	—	1.00	0.00
	He	0.30	0.41	0.24	0.72	—	0.83	—	0.72	0.00
TYR	Na	12	7	5	9	3	13	4	5	6
	Ho	0.33	0.57	0.43	0.67	0.25	1.00	1.00	1.00	0.67
	He	0.80	0.79	0.47	0.78	0.59	0.91	0.67	0.78	0.78
NF	Na	5	3	2	1	2	2	3	2	5
	Ho	0.22	0.29	0.29	—	0.25	0.13	0.67	0.33	0.50
	He	0.60	0.26	0.24	—	0.22	0.12	0.50	0.28	0.69

form mutually unique clusters in females except for *R. t. okiensis*, which was separated from the others in the 1st PC. Distributions of samples labeled as *R. t. tagoi*, including its candidate holotype, and *R. t. yakushimensis* were indistinguishable in both 1st and 2nd PCs. Samples belonging to A-9c tended to form a cluster but its separation from the others was unclear. In contrast, A-9c was separated from the others in males, whereas subspecies of *R. tagoi* including *R. t. okiensis* were mutually indistinguishable (Fig. 4).

The adult SVL and ratio values of the other measurements to SVL was compared among OTUs. Males of A-9c were significantly smaller than three subspecies of *R. tagoi*, although no statistical difference was detected among subspecies ($P>0.05$). In contrast, females of *R. t. okiensis* were significantly larger than the others, but no statistical difference was detected among the other three OTUs. Among ratio values of 21 characters in males, significant differences were detected by Kruskal-Wallis tests in 16 characters excepting HL, SL, ICD, IOD, and IMTL. Dunn's multiple comparison tests detected significant difference in three characters between *R. t. tagoi* and *R. t. okiensis* (SL, LAL, and HAL) and three characters between *R. t. tagoi* and *R. t. yakushimensis* (EL, LAL, and HAL), whereas no difference was detected between *R. t. okiensis* and *R. t. yakushimensis* in any characters. On the other hand, significant difference was detected in 12 characters between mt-lineage A-9c and *R. t. tagoi* (HW, EL, N-EL, S-NL, LAL, HAL, HLL, TL, FL, and 1, 3, 5TOEL) and five between A-9c and *R. t. okiensis* (SL, S-NL, IND, HLL, and TL). In contrast, only relative HLL showed significant difference between A-9c and *R. t. yakushimensis*.

These results are possibly affected by bias of the sample sizes, but it would be safe to say that four OTUs are mutually differentiated morphometrically at least in either sex, and

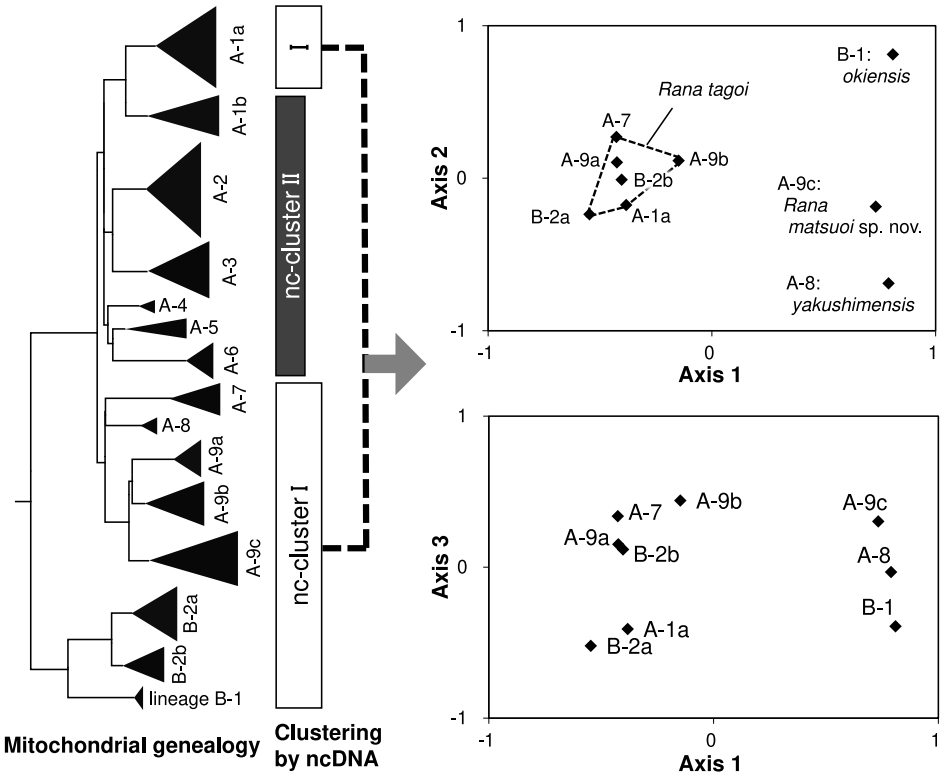

FIG. 3. Known genetic units related to the *Rana tagoi* species complex (left) and two-dimensional plots obtained from PCoA (right). Mitochondrial genealogy and clustering based on ncDNA were cited from those in Eto and Matsui (2014) with modification.

that males from A-9c have small body size and relatively long hindlimbs compared to subspecies of *R. tagoi*.

Comparisons of qualitative traits of adult

We examined the following number of adult males for comparisons of toe-webbing: n=56 for *R. t. tagoi*, n=7 for *R. t. okiensis*; n=8 for *R. t. yakushimensis*, and n=22 for mt-lineage A-9c. Webs between 1st and 2nd toes, and between 4th and 5th toes were compared. Generally, *R. t. tagoi* tended to have less developed webs than all the others (Table 3). For example, in the outer side of the 4th toe, the margin of webbing reached the 3rd phalange in more than half the males of *R. t. tagoi*, whereas the margin was between the 2nd and 3rd phalanges in a majority of males of *R. t. okiensis*, *R. t. yakushimensis*, and A-9c. *Rana t. okiensis* had the most developed webbing among them, and the margin of webbing in the inner side of the 5th toe was near the distal end of the 1st phalange, reaching the disc. The margin of webbing was located near the articulation of the 1st and 2nd phalanges in the inner side of the 5th toe in most males of *R. t. tagoi*, *R. t. yakushimensis*, and A-9c. No clear difference was recognized in the toe webs between of *R. t. yakushimensis* and A-9c (Table 3).

When focused on the pigmentation of ventral side of the thigh, a majority of males of mt-lineage A-9c had blackish blotches or mottling (grade 3, n=5) or fine dots (grade 2, n=14) on their skin, and only a few males lacked such markings (grade 1, n=2). In contrast, a majority of *R. t. tagoi* lacked apparent

TABLE 2. Comparisons of SVL and the other measurements of four *Rana* species. Means followed by ranges in parenthesis. *: measurements in Eto et al. (2022). **: measurements in Okada (1931).

	Rana matsuoi sp. nov. (mt-lineage A-9c)		*Rana tagoi*					*Rana yakushimensis*		*Rana okiensis*	
			topotypes*		candidate measurements of type and related specimens**						
	male	female	male	female	ZM2962 female holotype	ZM2954 female	ZM811 female	male	female	male	female
SVL	38.0 (36.1–40.3)	43.2 (38.9–46.7)	43.0 (38.5–45.5)	41.8 (40.6–43.0)	41	39	44	42.1 (37.4–44.9)	46.5 (42.1–50.6)	43.6 (40.8–45.6)	56.4 (54.3–58.6)
N	*32*	*18*	*12*	*7*				*12*	*13*	*15*	*6*
HL	15.5 (12.9–14.0)	15.8 (14.3–16.6)	15.5 (14.7–16.3)	15.4 (14.4–16.1)	15	15.5	15.5	15.5 (14.3–16.6)	16.9 (15.5–18.2)	15.7 (13.9–16.4)	21.1 (19.9–22.4)
HW	13.0 (12.1–14.3)	15.2 (14.4–16.6)	16.6 (14.8–18.5)	15.5 (14.8–16.1)	14.8	15	14.5	15.5 (15.0–16.1)	16.7 (15.7–18)	15.6 (14–17.3)	21.5 (21–22)
SL	4.8 (4.4–5.2)	5.6 (5.2–6.0)	5.6 (5.2–6.2)	5.6 (5.1–5.9)	6	6	6	5.6 (5.4–5.8)	6.0 (5.5–6.5)	5.9 (5.3–6.4)	7.6 (6.8–8.3)
S-NL	2.3 (2.0–2.5)	2.6 (2.5–2.8)	2.9 (2.8–3.1)	3.0 (2.9–3.2)	3.5	3.5	3.8	2.8 (2.6–3.0)	2.7 (2.4–2.9)	3.1 (2.7–3.4)	3.8 (3.7–4.0)
N-EL	2.2 (1.8–2.5)	2.7 (2.2–3.0)	2.2 (1.8–2.5)	2.3 (2.2–2.4)	2.5	2.5	3	2.4 (2.3–2.6)	2.9 (2.4–3.5)	2.4 (2.1–2.6)	3.5 (3.1–3.8)
EL	6.0 (5.6–6.4)	6.6 (5.9–7.1)	6.1 (5.1–6.9)	5.6 (5.2–6.6)	5	4.5	4.5	6.9 (6.4–7.3)	6.9 (6.3–7.4)	6.7 (5.9–7.4)	8.7 (8.2–9.1)
T-EL	0.7 (0.5–1.1)	1.0 (0.6–1.4)	1.2 (1.0–1.4)	1.1 (1.0–1.2)	1.5	1.3	1.5	0.9 (0.5–1.3)	1.4 (0.8–1.7)	1.1 (0.8–1.4)	1.7 (1.5–1.9)
TD	1.8 (1.5–2.4)	2.6 (2.1–3.0)	2.1 (1.3–2.8)	2.3 (1.8–2.6)	2.5	2.5	2.2	2.3 (1.8–2.8)	2.6 (2.3–3.4)	1.6 (1.1–1.9)	2.7 (2.6–2.8)
IND	3.8 (2.6–4.2)	4.3 (4.0–4.8)	4.6 (4.0–5.1)	4.4 (4.2–4.6)	4	4	4.5	4.6 (4.3–5.1)	4.7 (4.3–5.0)	4.8 (4.2–5.4)	5.9 (5.8–6.0)
ICD	5.6 (4.3–6.4)	6.2 (2.9–6.8)	6.77.3–6.5	6.26.2–6.2				6.2 (5.8–6.5)	6.9 (6.5–8.0)	6.6 (6.1–7.1)	8.2 (7.9–8.6)
IOD	2.6 (2.1–3.1)	3.3 (2.7–4.4)	3.1 (2.5–3.7)	3.4 (2.8–3.8)	3.5	3	3.5	2.8 (2.7–3.1)	3.4 (2.6–4.0)	2.7 (2.3–3)	3.9 (3.8–4.0)
UEW	3.6 (3.1–4.2)	3.9 (2.6–4.6)	3.9 (3.5–4.4)	3.8 (3.4–4.1)	3.6	3.5	3.8	4.3 (4.0–4.6)	4.6 (3.7–5.1)	4.4 (4–4.7)	5.4 (4.9–5.9)
LAL	18.1 (17.4–19.2)	19.9 (18.9–21.4)	19.0 (18.0–20.0)	18.0 (16.6–18.9)				20.6 (20.0–22.2)	21.8 (20.4–23.1)	20.7 (19.4–22.6)	26.0 (24.6–27.4)
HAL	10.0 (9.2–11.0)	11.4 (10.6–12.7)	10.5 (9.7–11.4)	9.8 (9.7–10.0)				11.4 (10.7–12.4)	12.7 (12.0–13.6)	11.6 (10.9–12.6)	15.2 (14.5–15.8)
HLL	72.4 (66.7–76.3)	82.0 (76.7–86.1)	73.3 (67.1–78.7)	72.2 (68.5–74.2)	69	69	69	75.5 (72.5–79.3)	82.0 (75.2–88.7)	75.8 (69.8–80.5)	95.3 (91.1–99.5)
TL	22.7 (20.9–24.1)	26.2 (24.6–27.8)	23.5 (21.3–25.2)	22.6 (21.9–23.2)	21	21	23	23.8 (23.0–24.8)	26.2 (24.0–28.5)	23.5 (21.9–24.7)	30.1 (29.0–31.2)
FL	22.1 (20.4–23.3)	24.2 (22.5–26.0)	21.9 (19.5–24.4)	21.1 (19.6–22.4)	20.5	21	21.5	23.7 (23.2–24.3)	24.3 (22.4–26.3)	23.7 (21.4–26.7)	29.0 (27.1–30.9)
IMTL	1.9 (1.7–2.3)	2.1 (1.7–2.6)	2.2 (2.0–2.5)	2.1 (2.0–2.4)				2.0 (1.7–2.3)	2.3 (2.0–2.5)	2.1 (1.9–2.4)	2.4 (2.3–2.5)
1TL	4.5 (3.7–5.0)	4.9 (3.7–5.6)	4.1 (3.7–4.9)	3.4 (3.2–3.7)				4.8 (4.2–5.6)	5.1 (4.4–5.9)	5.1 (4.7–5.9)	6.6 (6.0–7.1)
3TL	7.8 (7.1–8.5)	8.6 (7.4–9.5)	7.9 (7.1–8.8)	7.4 (6.5–8.3)				8.2 (8.1–8.6)	8.8 (8.1–9.8)	8.6 (7.9–9.7)	10.8 (9.8–11.8)
5TL	8.3 (7.4–9.4)	9.0 (8.0–9.9)	8.1 (7.5–9.1)	7.4 (6.6–8.0)				8.6 (8.2–9.1)	9.0 (8.2–10.1)	8.8 (8.1–10.1)	10.6 (10.0–11.2)
N	*18*	*15*	*10*	*7*				*5*	*6*	*7*	*6*

TABLE 3. Development of toe webbing between toes I–II and between toes IV–V of four *Rana* species (number of individuals).

No. of phalange(s) free from web	*Rana matsuoi* n. sp. (n=22)				*R. tagoi* (n=56)				*R. yakusimensis* (n=8)				*R. okiensis* (n=7)			
	I-out	II-in	IV-out	V-in	I-out	II-in	IV-out	V-in	I-out	II-in	IV-out	V-in	I-out	II-in	IV-out	V-in
(reach disc)																3
1	21			22	41			48	7			7	7			4
1 1/4								1				1				
1 1/2	1				6			4								
1 3/4					7			2	1							
2		22	1		2	55		1		7				7	3	
2 1/4			7			1	2			1	4				2	
2 1/2			5				4				3				2	
2 3/4			5				13									
3			4				37				1					

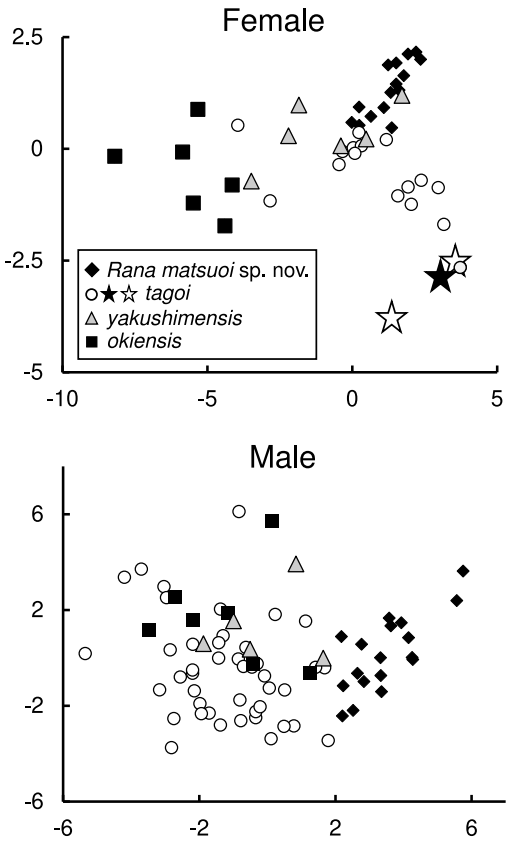

FIG. 4. Plots of first against second PC based on PCA for female and male specimens. Filed star indicates candidate holotype of *R. tagoi*, and open stars indicate specimens used in Okada (1931).

pigmentation (grade 1, n=34) but some males had markings (n=14 and 8 for grades 2 and 3, respectively). Both of *R. t. okiensis* (n=1, 4, 2 for grades 1, 2, 3, respectively) and *R. t. yakushimensis* (n=2 and 6 for grades 2 and 3, respectively) had pigmentation in most cases.

These results and acoustic traits described in the next section suggest the heterogeneity between mt-lineage A-9c in the Goto Islands and *R. tagoi* subspecies. In addition, present analyses using the samples obtained from the candidate type localities of three subspecies, as well as the previous studies (Nishioka et al., 1987; Tanaka et al., 1994; Daito et al., 1998; Eto and Matsui, 2014), also suggest that *R. t. tagoi*, *R. t. okiensis*, and *R. t. yakushimensis* are mutually heterospecific. Here we propose each subspecies of *R. tagoi* as a full species, and describe mt-lineage A-9c from the Goto as a new species below.

SYSTEMATICS

Rana ***matsuoi*** sp. nov.
(Suggested English name: Goto Tago's brown frog)
(Suggested Japanese name: Goto-tago-gaeru)
(Figs. 5, 6)

Synonymy:
Rana t. tagoi: Matsuo et al., 2011, p. 11 (part); Sueyoshi et al., 2013, p. 17 (part)

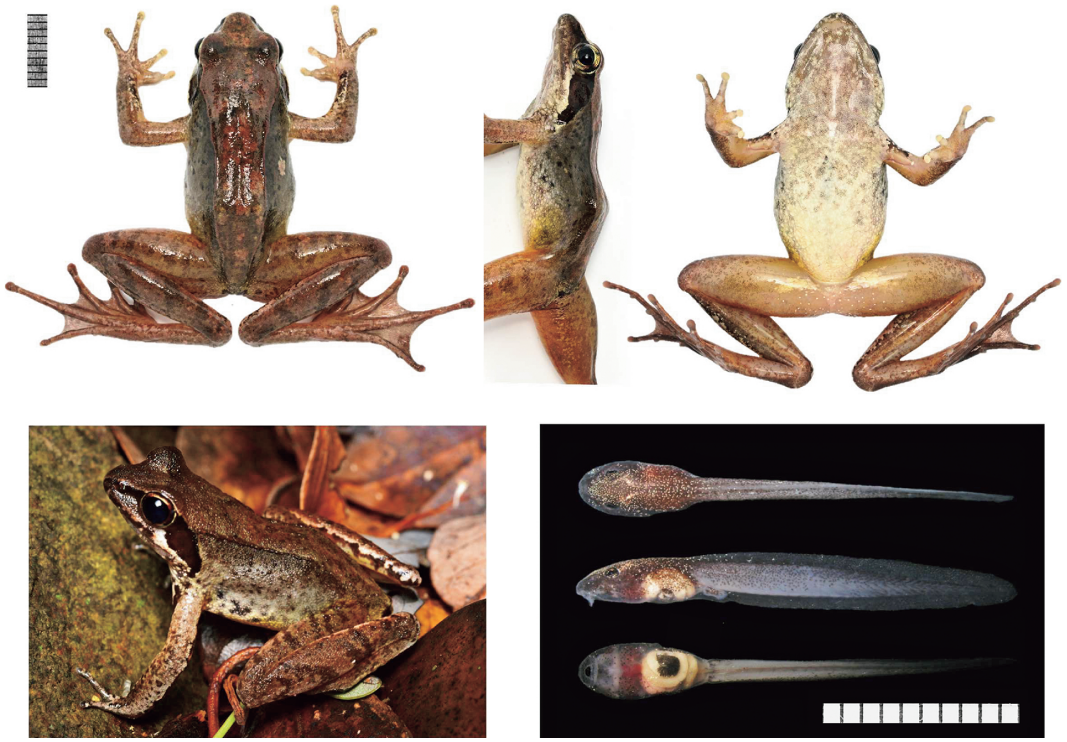

FIG. 5. Dorsal, lateral, and ventral view of holotype of *Rana matsuoi* sp. nov. (top, scale=10 mm), a male paratype KMNH 2023419VR-KE2056 in life (bottom left, not scaled), and tadpoles (bottom right, scale=10 mm).

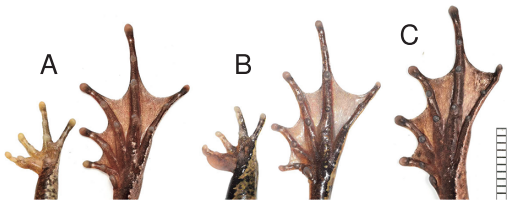

FIG. 6. Fore- and hindlimbs of holotype (A), a male paratype caught in breeding season (KUHE 45131, B), and hindlimb of a female paratype (KMNH 2023419VR-2059, C) of *Rana matsuoi* sp. nov. Scale=10 mm.

Rana t. tagoi (lineage A-9c): Eto et al., 2012, p. 666; Eto & Matsui 2014, p. 234

Holotype

KMNH 20230419VR-KE2058, adult male from Arikawa, Nakadori Island, Nagasaki Pref., Japan (32.9679° N, 129.1306° E, 100 m above sea level), collected on 15 June 2022 by K. Eto.

Paratypes

All from Nakadori Island. KUHE 45130–45135 and 45163–45167, eight males and three females collected on 24 October 2011 by Takanori Matsuo and K. Eto; NSMT-H 18521 (former KUHE 45366), one male collected on 25 February 2012 by K. Eto; KMNH 20230419VR-KE1861–1863, 1885–1887, three males and three females collected on 10–11 March 2022 by Ryo Ugawa, Yutaro Ariyoshi and K. Eto; KMNH 20230419VR-KE2056, 2057, 2059, two males and one female collected on 15 June 2022 by K. Eto.

Referred specimens

All from the Goto Islands, Nagasaki Pref., Japan. Nozaki Isl.: KUHE UN130113-1a, b,

larvae and froglets collected by Akihiro Yamane. Wakamatsu Isl.: KMNH 20230419 VR-KE2064, one female collected by K. Eto; Naru Isl.: KUHE 35138, one female collected by Tomohiko Shimada; KMNH 20230419VR-KE2075, one female collected by K. Eto; Fukue Isl.: KUHE 44316 and 44317, male and female collected by Hiroyuki Fujita; KUHE 45126, 45128, 45129, 45137–45146, and 45149–45158, 15 males and eight females collected by Takanori Matsuo and K. Eto; KUHE 45355 and 45356, two males collected by Tatsuhiko Nishimura and K. Eto; KMNH 2021X01VR-KE1271–1273, KMNH 2023419 VR-KE2090, one female collected by K. Eto; and NSMT-H 13734, one male collected by Natsuhiko Yoshikawa.

Etymology

The specific epithet is dedicated to Mr. Takanori Matsuo, who is a pioneer of herpetofaunal study in the insular regions of Nagasaki Prefecture including the Goto Islands.

Diagnosis

A medium- to small-sized form of the genus (adult males 36.1–40.3 mm, females 38.9–46.7 mm in SVL after fixation); reddish-, dark-, or pale-brown dorsally; a dark mask covering tympanum; a dark shoulder chevron of inverted V shape present but indistinct and interrupted; dorsal and lateral skin nearly smooth with minute granules; supratympanic and dorsolateral fold present; brown mottling present in throat, chest, and ventrolateral area of trunk; usually dark dots present in flunks and ventral side of leg; heels overlapping when limbs are held at right angles to body; tibiotarsal articulation of adpressed limb beyond nostril; tip of digit blunt, slightly dilated but not grooved; toe web developed moderately; males with internal vocal sacs; larvae small, poorly-pigmented. The new species is similar to the other members of the *Rana tagoi* species complex but can be distinguished from them by the combination of the following characters: small adult body size; relatively long limbs; moderately developed toe webs; and advertisement call with high dominant frequency and repetition of short notes.

Description of holotype (measurements in mm)

An adult male SVL 39.3 (Fig. 5); habitus moderately stout; head as long as broad (HL 14.5, 36.9%SVL; HW 13.6, 34.4%SVL); snout rounded; eye large, eyelid length slightly greater than snout length (EL 5.9, 15.0%SVL; SL 4.7, 12.1%SVL); snout projecting beyond lower jaw, rounded in lateral profile; canthus rostralis rounded; lore slightly oblique, concave; nostril lateral, below canthus, nearer to tip of snout than to eye (S-NL 2.1, 5.4%SVL; N-EL 2.2, 5.6%SVL); internarial distance (IND 4.1, 10.5%SVL) wider than interorbital distance (IOD 2.8, 7.2%SVL) and upper eyelid (UEW 3.5, 8.9%SVL) but narrower than intercanthal distance (ICD 5.9, 10.0% SVL); pupil elliptical; tympanum distinct, diameter (TD 2.4, 6.1%SVL) smaller than half of eye, and separated from posterior corner of eye by more than half of tympanum diameter (T-EL 0.9, 2.4%SVL); vomerine teeth in oval, oblique series (right with three and left with two teeth), beginning from a line connecting the centers of choanae and extending posteromedially, groups more widely separated from each other than from choanae, and anterior margin of series outlined by dark pigmentation; tongue notched posteriorly, without papilla; vocal sac bipartite, consisting of two laterally situated parts, with a pinhole-like openings near each jaw commissure.

Forelimb moderately long (LAL 18.0, 45.9%SVL: Figs. 5, 6); hand longer (HAL 9.9, 25.3%SVL) than half of lower arm; fingers unwebbed; third finger longest (5.4, 13.8% SVL); finger length formula: II<I<IV<III; finger tips blunt, slightly dilated but not grooved; subarticular tubercles distinct; no fringes of skin along fingers; nuptial pads diminished but present on medial surface of first finger, forming a continuous callus covering 1st to 3rd phalanges.

Hindlimb long (HLL 73.8, 187.6%SVL: Figs. 5, 6); tibia long (TL 23.2, 59.1%SVL), heels overlapping when limbs are held at right

angles to body; tibiotarsal articulation of adpressed limb reaching snout; foot (FL 22.0, 55.8%SVL) shorter than tibia; toe length formula I<II<III<V<IV; fifth toe (5TOEL 8.2, 20.8%SVL) slightly longer than the third (3TOEL 7.8, 19.9%SVL); toe tips similar to those of fingers; webbing developed moderately, formula: I 1–2 II 1–2 3/4 III 1 1/4–3 IV 2 3/4–1 V; toes without lateral fringes; subarticular tubercles distinct; inner metatarsal tubercle oval, length (IMTL 2.1, 5.3%SVL) shorter than first toe (1TOEL 4.5, 11.4%SVL); outer metatarsal tubercle rounded and small.

Dorsal skin nearly smooth and both sides of body relatively granular (Fig. 5); chevron marking in scapular region indistinct and formed by sparse black blotches; a distinct supratympanic fold from posterior margin of eye to axilla; a dorsolateral fold from supratympanic fold to groin; a junction of supratympanic and dorsolateral folds swelling outside; throat and chest smooth; abdomen weakly rugose.

Color in life brown dorsally with sparse light mottling; sides of body grayish brown with dark blotches, and one large ivory blotch on right side; dorsolateral fold brown with darker outline; head with dark interorbital bar; lore with distinct black bar below canthus; labium with heavy, dusty dark pigmentations; distinct dark brown marking below supratympanic fold, from behind eye, covering tympanum, and toward above arm insertion; iris gold with reddish portions anteriorly and posteriorly, and with blackish portions superiorly and inferiorly; dorsal surface of hind limbs marked with alternating, dark brown crossbars, three to five on thigh and three on tibia; ventrum cream with pale brown mottling on throat and chest; pigmentation relatively sparse in abdomen and absent on lower part; palm sparsely pigmented; ventral side of arm unpigmented in radial side but heavily pigmented in ulnar side; ventral side of thigh nearly unpigmented on proximal side but with dark dots on distal side; ventral side of tibia and tarsus with dark dots, forming sparse mottling in some parts; under side of foot dark brown. In preservative, coloration faded and became grayish but no obvious change in pattern observed.

Variation

Summaries of morphometric data and toe web development are shown in Tables 2 and 3. Adult females (38.9–46.7 mm SVL) are larger than males (36.1–40.3 mm). Breeding males have a nuptial pad covering 1st to 3rd phalanges continuously, although the pad is more or less diminished in non-breeding seasons (Fig. 6). Skin on both siden of the body becomes loose in breeding males, but is taut in females and non-breeding males. When the hindlimb is bent forward along the body, tibiotarsal articulation reaches beyond nostril to beyond snout in both males and females. Development of toe web varies moderately in males (from I $1^1/_2$–2 II 1–$2^1/_2$ III $1^1/_2$–3 IV 3–1 V to I 1–2 II 1–$2^1/_4$ III 1–3 IV 2–1 V) and females (from I 1–2 II 1–$2^3/_4$ III 2–3 IV 3–1 V to I 1–2 II 1–$2^1/_4$ III 1–2 IV $2^1/_4$–1 V). Dorsal ground color varies from orange to dark brown. In some individuals chevron marking is indistinct and hard to recognize. Ventral ground color varies from pale yellow, cream, to pale orange. Development of dark pigmentation in ventral side also varies among individuals, and breeding males tend to develop more dense mottling in throat and chest, although the pigmentation is sparse along median line of throat. A majority of males have fine dots on the ventral side of legs. Ground color of ventral side of legs varies from light yellow to pale pink.

Eggs and larvae

A female (KUHE 44317) had 42 eggs in left oviduct (i.e., estimated clutch size was ca. 80) with a mean diameter of 2.62 mm in fresh (SD=0.11). Another female (KUHE 45146) had 70 eggs in both ovaries with a mean diameter of 2.81 mm when fresh (SD=0.05). Eggs yolky, dark brown in animal hemisphere and white to cream in vegetal hemisphere. Three larvae from the type locality (stage 34–36 following Gosner [1960]) monotonous gray dorsally without obvious marking; ventrum largely unpigmented, and yolky intestine visi-

ble through the skin; intestine cream with patchy dark parts, probably derived from yolk and gut contents; head-body oval, depressed; eyes dorsolateral, not visible from below; tail long; dental formula 1:1+1/1+1:2 with inverse U shaped upper beak and V shaped lower beak, although the upper beak was not observed in one larva (Fig. 5). These specimens had the following measurements (mm) after preservation: TTL 19.4–20.6; HBL 5.7–6.1; HBW 2.5–3.2; HBD 2.3–2.7; IOD 0.8–1.0; ESD 1.4–1.7; ODW 1.0–1.6; TAH 2.7–3.0. Four larvae (St. 30–32 at first) caught in late November 2012 at Nozaki Island, reared without feeding under a water temperature of ca. 10°C completed metamorphosis on 16 January 2013 with SVL of 7.3–8.2 mm. Thus, the larvae would be able to complete metamorphosis only by consuming their large yolk, although they might feed in the wild.

Call characteristics

The advertisement call was recorded at the type locality on 25 February 2012 (Fig. 7; Table 4). Water temperature was 8.7°C near the calling site. Of 14 calls measured, one was a single-note call (call type I), four were two-note calls without NL variation (call type II-C: Fig. 7), five were three- to five-note call without NL variation (III-C: Fig. 7), two were six- and seven-note calls without NL variation (IV-C), and two were six- and 12-note calls with shorter first notes (IV-A). Mean (±SD) call duration (s) of call types I, II-C, III-C, and IV-A/C were 0.08, 0.28±0.15, 0.55±0.10, and 1.45±0.63, respectively (Table 4). Note length (NL) was generally short, varying from 0.03 to 0.17 s in the first note. Each note had two to six pulses, although some notes had a continuous pulse as defined above. Dominant frequency varied from 750 to 1,500 Hz, and the first note seemed lower than the middle (Table 4). Each note contained harmonics. Frequency modulation was not clear probably because of recording quality, but weak modulation was recognized in some calls. These characteristics seem to be essentially same as those previously reported (Matsuo et al., 2011; Sueyoshi et al., 2013) with minor differences.

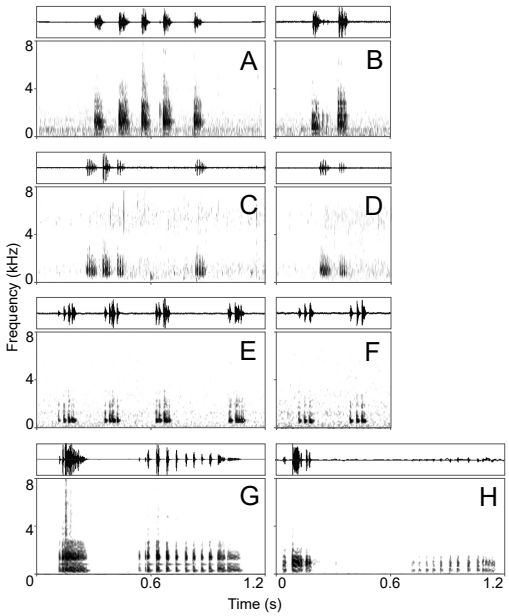

FIG. 7. Wave form and sonagram of advertisement calls. (A) five-note call of *Rana matsuoi* sp. nov. recorded in the type locality; (B) two-note call of *Rana matsuoi* sp. nov. recorded under the same condition with A; (C) three-note call of *R. yakushimensis*, with following single-note call; (D) two-note call of *R. yakushimensis* recorded under the same condition with C; (E) four-note call of *R. okiensis*; (F) two-note call of *R. okiensis* recorded under the same condition with E; (G) two-note call of *R. tagoi* topotype; and (H) two-note call of *R. tagoi* from Hisaka Island. Air temperature at recording was 14.4°C for G, and the other information is listed in Table 4.

Comparisons

By its blunt digit tips without grooves, subterranean breeding habit, and small, potentially endotrophic larva, *Rana matsuoi* sp. nov. can be distinguished from all the other congeners except for taxa belonging to the *R. tagoi* species complex (Eto and Matsui, 2014).

Rana matsuoi sp. nov. is distinct by its smaller body size of adult males (ca. 36–40 mm) compared to members of the *R. tagoi* species complex except for *R. kyoto* (29–

Table 4. Bioacoustic characteristics of advertisement call of *Rana* species. Asterisks indicate that the note contains continuous pulse(s). Data of *R. tagoi* topotypes cited from Eto et al. (2022).

call type	n	Note Number	Call Length (mean±SD)	1st/last NL (mean±SD)	note position	Note Length (s) (mean±SD)	Pulse Number (mean±SD)	Dominant Frequency (Hz) (mean±SD)
Rana matsuoi sp. nov. (Water temperature: 8.7°C)								
I	1	1	0.08	—	—	0.08	1.0*	1312.5
II-C	4	2	0.28±0.15	1.06±0.15	first	0.12±0.14	2.3±1.0	1031.3±324.8
					last	0.12±0.14	2.0±0.8	1031.3±397.7
III-C	5	3–5	0.55±0.10	1.00±0.20	first	0.06±0.02	2.0±1.0	1062.5±216.5
					middle	0.05±0.01	3.0±1.0	1125.0±265.2
					last	0.07±0.03	2.0±1.0	1406.3±132.6
IV-A, C	4	6–12	1.45±0.63	0.59±0.23	first	0.04±0.01	1.0*±0.0	1218.8±496.1
					middle	0.06±0.01	2.3±1.0	1546.9±740.2
					last	0.08±0.03	4.0±1.4	1125.0±216.5
R. tagoi topotypes (Temperature: 8.6–17.6°C)								
II-A	3	2	1.12±0.22	0.40±0.08	first	0.24±0.11	4.5*±2.1	1205.9±258.4
					last	0.59±0.20	10.7±0.6	1033.6±455.7
III-A, C	16	3–5	1.54±0.64	0.40±0.23	first	0.15±0.68	4.3*±4.0	936.0±276.0
					middle	0.22±0.14	5.6*±3.2	1010.7±474.4
					last	0.46±0.18	11.9±4.7	970.6±417.7
IV-A	2	7–8	3.80±0.71	0.32±0.05	first	0.16±0.01	4.0*±2.8	990.5±548.1
					middle	0.26±0.10	7.0±0.0	990.5±548.1
					last	0.51±0.05	10.5±0.7	947.5±243.6
R. tagoi mt-lineage A-9a (Water temperature: 10.1°C)								
I	2	1	0.15±0.02	—	—	0.15±0.02	3.0*±1.4	947.5±243.6
II-A	9	2	1.14±0.17	0.29±0.10	first	0.14±0.02	2.5*±1.6	1093.9±58.1
					last	0.50±0.1	11.4±4.5	872.1±192.3
R. yakushimensis (Water temperature: 13.2°C)								
I	20	1	0.06±0.01	—	—	0.06±0.01	6.5*±1.1	1016.37±59.9
II-B, II-C	8	2	0.14±0.01	1.28±0.36	first	0.06±0.00	6.3*±1.6	1044.36±55.2
					last	0.05±0.01	3.7*±0.8	990.53±112.8
III-B, III-C	10	3	0.22±0.01	1.50±0.19	first	0.06±0.01	6.9±0.9	1033.59±57.4
					middle	0.05±0.01	4.4*±1.0	1016.37±79.2
					last	0.04±0.00	3.3*±0.9	973.3±70.9
R. okiensis (Water temperature: 5.4°C/Air: 7.0°C)								
I	31	1	0.10±0.02	—	—	0.10±0.02	5.06±1.06	870.97±263.2
II-B, II-C	10	2	0.39±0.06	1.15±0.4	first	0.10±0.04	5.1±2.56	731.25±224.5
					last	0.08±0.01	4.3±0.67	787.5±193.7
III-C	9	3–4	0.77±0.21	1.08±0.08	first	0.09±0.01	4.2±1.2	666.67±98.8
					middle	0.08±0.01	3.9±0.3	750±0.0
					last	0.08±0.01	3.9±0.6	750±0.0
IV-A	1	6	1.64	0.58	first	0.097	5	562.5
					middle	0.11	7	1312.5
					last	0.15	8	937.5

44 mm), i.e., *R. tagoi* (38–58 mm), *R. okiensis* (38–46 mm), *R. yakushimensis* (37–48 mm), *R. sakuraii* (38–56 mm), and *R. neba* (37–48 mm) (Matsui and Maeda, 2018; Eto et al., 2022; present study). Compared to *R. tagoi*, males of the new species have a relatively narrower head (ratio [r] of HW mean±SD=34.1±1.0%, vs 37.1±1.5% in *R. tagoi*), shorter snout (rSL 12.6±0.5%, vs 12.9±1.0%), larger eye (15.5± 0.6%, vs 14.0±1.0%), longer forelimb and hands (rLAL 47.2±2.0%, vs 44.9±1.8%), longer hindlimb and toe (rHLL 188.3±6.7%, vs. 172.4±7.2%). Compared to *R. okiensis*, the new species had shorter snout (rSL 12.6±0.5%, vs 13.5±0.6% in *R. okiensis*), narrower internarial distance (rIND 9.9±1.0%, vs 11.1±0.6%), and longer hindlimb (rHLL 188.3±6.7%, vs 173.4±3.4%) and tibia (rTL 58.9±2.3%, vs 53.8±0.3%). Morphometric traits of *R. matsuoi* sp. nov. and those of *R. yakushimensis* are resembling except for smaller SVL and longer hindlimb (rHLL 188.3±6.7%, vs 175.7±6.4% in *R. yakushimensis*). Hindlimb of male of the new species is also apparently longer than that of *R. kyoto* (rHLL 170.4±7.7%, calculated from Appendix of Eto et al., 2022).

In adult toe webs, *Rana matsuoi* sp. nov. shows more developed webs than *R. tagoi* (see Table 3). Webs of the new species (in most males, the margin of outermost web reaches the 1st phalanges of 5th toe) is more developed than those of *R. kyoto* (in most males, the margin of outermost web does not reach the 1st phalanges of 5th toe), but less developed than those of *R. okiensis* and *R. sakuraii* (at least a part of males have web which reaches to the tip disk of 5th toe). By the frequent presence of dark dots on ventral surface of legs, adult males of the new species are also different from those of *R. tagoi*, *R. neba* (topotypes of both species usually lacking such markings). The nuptial pad of the 1st finger without any division by smooth skin distinguishes breeding males of *R. matsuoi* sp. nov. from *R. tagoi*, *R. neba*, and *R. kyoto* (nuptial pad of 1st finger into two parts separated by smooth skin). Also, in mature males, the new species can be distinguished from *R. sakuraii* by the presence of a vocal sac (vocal sac absent in *R. sakuraii*).

Mating calls of *Rana matsuoi* sp. nov. are characterized by high dominant frequency (DF ca. 0.8–1.5 kHz in average), weak or no frequency modulation, and large number (NN 1–12) of short notes (NL 0.03–0.17 s in the first note). Note length does not drastically change depending on the note position (NL ratio of first/last notes is less than 3/2 and more than 2/3 in many calls), although a long last note is present in some six- to 12-note calls (Fig. 7; Table 4). Compared to the new species, calls of *R. tagoi* generally show a lower note number (2–8 in topotypes, 1–2 in the Goto Islands) with the last note apparently longer than the first (NL ratio of first/last notes is 2/3 or less) (Shimada et al., 2021; Fig. 7; Table 4). Calls of *R. yakushimensis* show less NN (1–5: this study; Daito and Nakamizo, 1980) with longer first note (NL ratio of first/last notes is 3/2 or more in many calls) and slightly lower DF (Fig. 7; Table 4). Frequency modulation is observed in some calls of *R. yakushimensis* (Daito and Nakamizo, 1980; Matsui and Maeda, 2018; present study) (vs. obvious modulation is absent in the new species). As noted by Daito and Nakamizo (1980), three-note calls would be most common in this species, although one or two single-note call(s) sometime follow the three-note call (Fig. 7). Calls of *R. okiensis* show similar NL and NL ratio of the first/last notes with the new species but show fewer NN and apparently lower DF than those of the new species (Fig. 7; Table 4). Calls of *R. neba* show obvious frequency modulation within the first note (Shimada et al., 2021) (vs. obvious modulation is absent in the new species). Calls of *R. kyoto* are distinguishable from those of the new species by lower DF (ca. 0.7–0.9 kHz), fewer NN (1–4), and longer first note than the last (generally NL ratio of first/last notes is 3/2 or more) (Eto et al., 2022). Calls of *R. sakuraii* (DF: 0.9/3.1 kHz; NN: 4; obvious modulation absent: Matsui and Maeda, 2018) are not fully examined.

Range

Known from following islands of the Goto Islands, Nagasaki Pref., western Japan: Nozaki, Nakadori, Wakamatsu, Naru, and Fukue (Fig. 1).

Natural history

Inhabiting montane forests of the Goto Islands. *Rana matsuoi* sp. nov. is a subterranean breeding species, with the males calling inside crevasses of rock beds or under rocks near small streams in autumn to winter (October to next February: Matsuo et al., 2011; Sueyoshi et al., 2013; present study). Multiple males often call in a same crevasse or under the same rock. Such a space is partially filled with water, and males sit inside soaking their bodies in the water. Females probably lay eggs inside such a space, and hatched tadpoles stay inside or flow out around the oviposition site. Sometimes swarms of tadpoles are observed under rock(s) or water bottom, and a swarm observed at the type locality consisted of more than 120 tadpoles. Larvae probably can metamorphose even without feeding like those of a close relative *R. tagoi* but that has not been fully tested. At least they can develop without food after St. 30 until metamorphosis as written above. The frogs are often found in forest floor habitats in non-breeding seasons. Other biological information including food habits and maturation age are unknown. A close relative, *R. tagoi*, occurs in Hira, Kaba, and Hisaka of the Goto Isls., although it breeds later than the new species, in late February to mid-March in this region (Sueyoshi et al., 2013; present study).

Discussion

Rana matsuoi sp. nov. is endemic to the Goto Islands, where its relative *R. tagoi* also occurs. Their distributions are isolated but the geographic pattern is not simple: from eastern- to western-islands in this area, *R. tagoi* occurs in Kyushu (main island), Hidado, and Hira; then *R. matsuoi* occurs in Nakadori, Nozaki, Wakamatsu, and Naru; and then *R. tagoi* reappears in Hisaka and Kaba; and *R. matsuoi* reappears in the westernmost island, Fukue (see RESULTS and Fig. 1). The oldest rock of the Goto Isls. date back to ca. 22.6 Ma, although most of the islands consist of rocks derived from younger volcanic material (Kiyokawa et al., 2022). Because the depth of sea around the Goto Isls. and between the Goto and Kyushu is generally shallow (mostly less than 100 m depth: Katsura and Nagano, 1976), these areas would have been connected repeatedly by a land bridge when the sea level dropped in glacial periods during the Pleistocene. Therefore, these two species are suspected to have been in contact in recent glacial periods. Nevertheless, they are suggested to be genetically isolated (Eto and Matsui, 2014; present study), and the presence of some unknown mechanism is expected to prevent gene flow between them. Differentiation of breeding habits, e.g., breeding seasons and advertisement calls as described above, might prevent their interbreeding even if their distributional ranges overlapped in glacial periods.

As repeatedly mentioned in previous studies (e.g., Eto and Matsui, 2014; Eto et al., 2022), mitochondrial phylogeny cannot be definitive in the estimation of evolutionary history of the *Rana tagoi* species complex largely because of their shallow divergence and past introgression. However, apparently larger sequence divergences of mtDNA among samples of *Rana matsuoi* sp. nov. than those of *R. tagoi* around Kyushu suggest that the former has lived in the Goto Isls. for a longer time than the latter. Divergence time estimation based on mtDNA supports this idea (Eto and Matsui, 2014), although such an analysis may not be definitive as noted above. A previous study estimated that the ancestor of *R. matsuoi* sp. nov. (mt-lineage A-9c) split from the common ancestor of A-9a, b, c around 2.0–1.7 Ma, and was subdivided around 1.5 Ma within the Goto Isls. In contrast, subdivision of local lineages within *R. tagoi* A-9a started around 0.6–0.5 Ma (Eto and Matsui, 2014), and their dispersal from Kyushu to the Goto should have occurred more recently. Very small haplotype diver-

gence among *R. tagoi* A-9a from Hira, Hisaka, and Kaba Islands observed in the present study also suggests recent dispersal of this lineage among the Goto Isls. If that is the case, *R. matsuoi* sp. nov. or its ancestor would have dispersed to the ancient Goto area at first, and then *R. tagoi* would have migrated later. A similar phylogeographic scenario is possible in other Japanese ranids including the *Odorrana narina* species group, but co-existence of two relatives in the same islands is observed in that case (Matsui, 1994). Similar to *R. matsuoi* sp. nov., the other insular relatives *R. okiensis* on the Oki Islands and *R. yakushimensis* on Yakushima Island occur solely on each island. Based on submarine topography, these islands are also estimated to have connected with the main islands of Japan in glacial periods (e.g., Tozaki et al., 1978; Kimura, 1996), and secondary contact between each species and *R. tagoi* is also assumed when considering their estimated divergence time (Eto and Matsui, 2014). Future study is requested to determine the factors that allow or prevent co-existence of closely related amphibians on small islands.

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Appendix

Specimens examined for analyses. Vouchers are stored in the Graduate School of Human and Environmental Studies, Kyoto University (KUHE), Kitakyushu Museum of Natural History and Human History (KMNH), the Institute for Amphibian Biology, Hiroshima University (IABHU), and National Museum of Nature and Science, Tokyo (NSMT).

Rana matsuioi sp. nov.—All from Nagasaki Prefecture. Nozaki Island: KUHE 20130116_1; Nakadori Island: KUHE 45130–45135, 45163–45167, 20111023; KMNH 2023419VR-KE1861–1863, KE1885–1887, KE2056–2059; NSMT-H 18521 (former KUHE 45366); Wakamatsu Island KMNH 2023419VR-KE2064; Naru Island: KMNH 2023419VR-KE2075; Fukue Island: KUHE 45126, 45128,

45129, 45137–45146, 45149–45158, 45355, 45356, 45366, KMNH 2023419VR-KE2090; NSMT-H 13734.

Rana tagoi—Honshu Island: KUHE 41217, 41218, 41279, 41288–41293, 41295–41299, 41301, 41303, 41403, 41406, 41418, 41550, 41694, 41985, 42029, 42043, 42046–42049, 42237, 42238, 42308, 42309, 42344, 42319, 42381, 42396, 42473, 42719, 43018, 43211, 44159, 44238, 44602, 44783, 44785, 44786, 44867–44869, 44974, 46243, 48058, 48272, 49345, 49386, 49793, 49910, IABHU F2560–2568; Shikoku Island: KUHE 45690; Kyushu Island: KUHE 44344; Hirado Island: KMNH 2023419VR-KE1901, 1902; Hira Island: KMNH 2023419VR-KE1867, KE1868, KE1872, KE1873; Kaba Island: KUHE 45362, 45364, KMNH 2023419VR-KE1850–1852; Hisaka Island: KUHE 45360, KMNH 2023419VR-KE2085.

Rana okiensis—Dogo Island, Shimane Pref.: KUHE 43639–43646, 43650–43653, 43663–43665.; Nishinoshima Island, Shimane Pref.: KUHE 43647–43649, 43666.

Rana yakushimensis—Yakushima Island, Kagoshima Pref.: KUHE 32006, 32007, 45175–45182, 45352–45354, 46922–46932, 56659, 56668.