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On the Brown Frogs from the Ryukyu Archipelago, Japan, with Descriptions of Two New Species (Amphibia, Anura)

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Abstract: A recent taxonomic study revealed that the name *Rana okinavana* Boettger, 1895 is a subjective senior synonym of *R. psaltes* Kuramoto, 1985 from the Yaeyama Island Group of the Southern Ryukyus and Taiwan. This led the brown frog of the genus *Rana* from the Okinawa and the Amami Island Groups of the Central Ryukyus, long been referred to as *R. okinavana* in various fields of zoology, to be unnamed. Moreover, molecular phylogenetic analyses revealed that the brown frogs from the Okinawa and the Amami Groups are so divergent genetically as to be recognized as two distinct species. Because there are no available names for these brown frogs, I describe the populations from the Okinawa and the Amami Groups as two new species, *R. ulma* and *R. kobai*, respectively.

Key words: Biogeography; MtDNA phylogeny; *Rana okinavana*; Ryukyu Archipelago; Taxonomy

INTRODUCTION

Although most brown frogs of the subgenus *Rana* Linnaeus, 1758 (Dubois, 1992) belong to the Palearctic fauna, some have extended their ranges into the Oriental region. Among them are the brown frogs from the Amami and Okinawa Island Groups in the middle of the Ryukyu Archipelago (Central Ryukyus), Japan (Fig. 1). These frogs have been referred to as *R. okinavana* Boettger, 1895 without any doubt over 60 years since Inger's taxonomic assignment in 1947 (e.g., Nakamura and Uéno, 1963; Maeda and Matsui, 1999). However, Matsui (2007) examined the type specimens of *R. okinavana* stored in Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt a. M. and clarified that the species is actually a member of the subgenus *Nidirana* Dubois, 1992 (Dubois, 1992; Chou, 1999) or the genus *Babina* Thompson, 1912 (Frost et al., 2006), and is identical with the frog so far called *R. psaltes* Kuramoto, 1985 described from the Yaeyama Island Group of the Southern Ryukyus.

Thus, the brown frogs of the subgenus *Rana* from the Central Ryukyus, long referred to as *R. okinavana*, are assuredly undescribed, for which no names are available. Because quite a few biological works have been conducted on these frog populations under this name (e.g., Kuramoto, 1972, 1974, 1979; Kawamura et al., 1981; Borkin, 1979; Nishioka et al., 1992; Tanaka et al., 1996; Tanaka-Ueno et al., 1998), paucity of their proper names is strongly hin-

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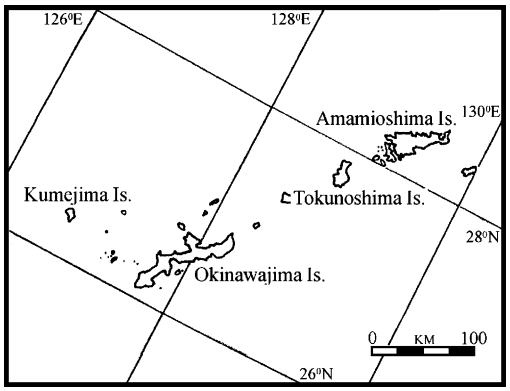

FIG. 1. Map of the middle part of the Ryukyu Archipelago (Central Ryukyus), Japan, showing four islands where the brown frogs occur. Kakeromajima Island, located close to the western coast of Amamioshima Island, is not specified.

dering current studies (e.g., Mori and Toda, 2011). Furthermore, preliminary analyses of mitochondrial (mt) cytochrome b (cyt b) gene strongly suggested presence of a high degree of genetic divergence between brown frog populations from the Okinawa and the Amami Groups (Tanaka et al., 1996).

In order to confirm such divergence, I obtained larger samples from all those Central Ryukyu islands where the brown frogs were known to occur (Maenosono and Toda, 2007), and studied them phylogenetically using longer sequences including sequences of additional mtDNA genes. I also conducted univariate and multivariate analyses using morphometric data for these samples. As a result, I found two genetically and morphologically distinct lineages that warrant specific recognition. I, therefore, describe these lineages as two new species.

MATERIALS AND METHODS

Molecular phylogenetic analyses

I obtained DNA sequence data from muscle or liver tissue samples preserved in 99% ethanol. I reconstructed phylogenetic trees from approximately 2400 base pairs (bp) of partial sequences of mitochondrial 12S and 16S rRNA, and intervening $tRNA_{val}$ genes from 24 specimens, including six specimens from Okinawajima Is. and two from Kumejima Is. of the Okinawa Group, six from Amamioshima Is., one from Kakeromajima Is. and five from Tokunoshima Is. of the Amami Group, and four of the outgroup taxa (one specimen each of *R. tsushimensis* Stejneger, 1907 from Tsushima Is.; *R. sauteri* Boulenger, 1909 from Alishan, Taiwan; *R. temporaria* Linnaeus, 1758 from Frahi Lazhe, Czech; and *Lithobates sylvaticus* [LeConte, 1825] from Quebec, Canada).

Methods for DNA extraction, and amplification and sequencing of the mtDNA fragments are the same as those reported by Matsui et al. (2010). Briefly, I amplified and sequenced total DNA by PCR with the primers shown in Shimada et al. (2011). The PCR cycling, precipitation, and sequencing procedures were identical to those described by Matsui et al. (2010). The resultant sequences were deposited in GenBank (Accession numbers AB639591–639592, 685766–685787).

The alignment matrices with 2435 mtDNA nucleotide sites (945 sites for 12S rRNA; 80 sites for $tRNA_{val}$; and 1410 sites for 16S rRNA) were subjected to estimate phylogenetic relationships using maximum likelihood (ML) and Bayesian inference (BI). Pairwise comparisons of uncorrected sequence divergences (p-distance) for 16S rRNA were also calculated. Details for these procedures are given in Matsui et al. (2010).

Morphological analyses

For adult specimens stored in 70% ethanol, I took the following 16 body measurements to the nearest 0.1 mm with dial calipers following Matsui (1984): (1) snout-vent length (SVL); (2) head length (HL), (3) snout length (SL); (4) eye length (EL); (5) tympanum diameter (TD); (6) head width (HW); (7) internarial distance (IND); (8) interorbital distance (IOD); (9) upper eyelid width (UEW); (10) lower arm and hand length (LAL); (11) forelimb length (FLL); (12) hindlimb length (HLL); (13) tibia length (TL); (14) foot length (FL); (15) inner

metatarsal tubercle length (IMTL); and (16) first toe length (1TOEL). The system of description of toe-webbing states followed that used by Savage (1975).

In the univariate comparisons, SVL was compared by Tukey-Kramer test, while the ratios (R) of the remaining characters to SVL were compared by Dunn's multiple comparisons test. For examining overall morphological variation among populations, I also conducted multivariate analyses. Only males were used for these analyses because the number of females was limited. Using $\log_e$-transformed metric values, I first conducted Principal Component Analysis (PCA). Then, in order to examine variation only in proportions among samples, I employed Canonical Discriminant Analysis (CDA) with the values obtained in the Multiple-Group Principal Component Analysis (MGPCA: Thorpe, 1988), which excludes a contribution of size variation (Thorpe, 1988; Overton et al., 1997). I used a statistical package (SAS, 1990) and ran analyses through the facilities of the Data Processing Center of Kyoto University.

For preserved larvae, the following 13 measurements were taken to the nearest 0.01 mm using a binocular dissecting microscope equipped with a micrometer: (1) total length (TOTL); (2) head-body length (HBL); (3) maximum head-body width (HBW); (4) maximum head-body depth (HBD); (5) eyeball diameter; (6) internarial distance; (7) interorbital distance; (8) snout-spiracle opening distance; (9) oral disk width; (10) tail length; (11) maximum tail depth; (12) maximum tail width, and (13) muscle depth at middle of tail. I followed Gosner's (1960) table for staging.

Calls recorded in the field were analyzed using the computer programs SoundEdit Vers. 2 and SoundEdit Pro (MacroMind-Paracomp, Inc.) on a Macintosh computer. Methods of analyses are as described elsewhere (Matsui, 1997).

Preserved specimens of *Rana* (sensu stricto) examined for morphological comparisons are stored at Graduate School of Human and Environmental Studies, Kyoto University (KUHE), National Museum of Nature and Science, Tokyo (NSMT), and Osaka Museum of Natural History (OMNH).

RESULTS

Molecular phylogenetic analyses

I obtained 2435 bp of concatenated fragments of mtDNA genes for 24 samples, including out-groups. For the ML analysis, the best substitution models were J2 model with a Gamma (G) shape parameter of 0.155, HKY85+G of 0.100, and the general time reverse (GTR) model+G of 0.188 for 12S rRNA, $tRNA_{val}$, and 16S rRNA, respectively. For the BI analysis, GTR+G of 0.199, HKY85+G of 0.027, and GTR+G of 0.235, respectively, were selected as the best models. The likelihood values (–lnL) of the ML and BI trees were 6890.125 and 6947.565, respectively.

Phylogenetic analyses employing two different optimality criteria yielded identical relationships. As shown in the ML tree in Fig. 2, samples of brown frogs from the Central Ryukyus formed a monophyletic group with respect to outgroup taxa (99 and 100% in ML bootstrapping nodal supports and Bayesian posterior probabilities, respectively). Two fully supported clades, the clade of the Okinawa Group samples (Okinawajima Is. and Kumejima Is.) and the clade of the Amami Group samples (from Amamioshima Is., Kakeromajima Is., and Tokunoshima Is.) exhibited sister group relationships. Each of these two clades was further split into two reciprocally sister subclades, the Amamioshima subclade (99 and 100%, respectively) and Tokunoshima subclade (99 and 100%, respectively) in the Amami clade, and Okinawajima subclade (99 and 100%, respectively) and Kumejima subclade (99 and 100%, respectively) in the Okinawa clade. A sample from Kakeromajima Is. was nested in the Amamioshima subclade.

The genetic distances (uncorrected p-distance in 16S rRNA) between samples within each of the four islands (Kakeromajima Is. included in the Amamioshima Is.) were very small, with the means of 0.1–0.2%. The distances between

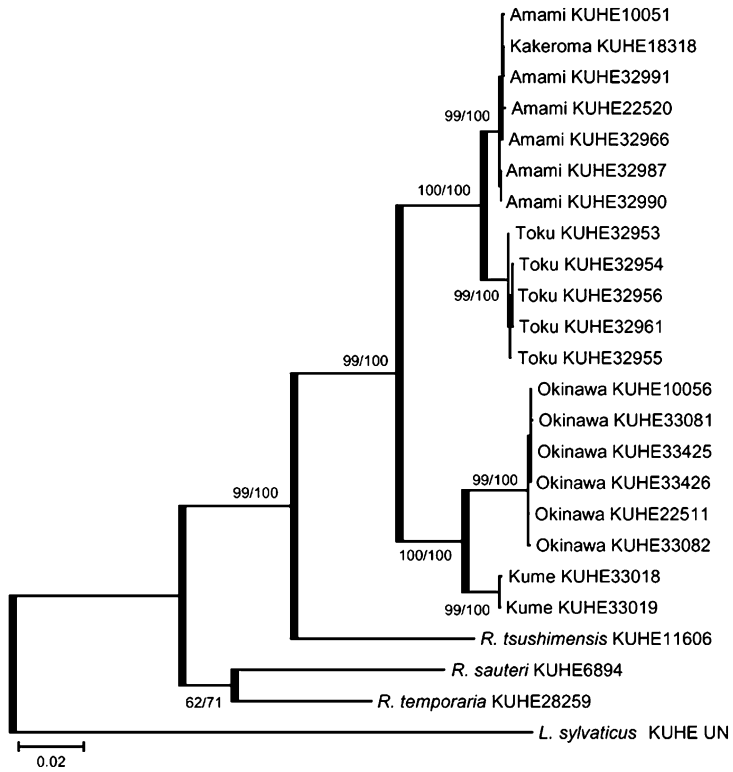

FIG. 2. ML tree from a 2435 bp sequence of mitochondrial 12S rRNA, $tRNA^{val}$ and 16S rRNA genes for samples of brown frogs from the Central Ryukyus and related species. Numbers above or below branches represent bootstrap supports for ML inferences and Bayesian posterior probabilities (ML-BS/BPP).

the two subclades were moderate in the Amami clade (1.3–1.5 [$\bar{x}$=1.4]%), but were relatively large in the Okinawa clade (2.9–3.1 [$\bar{x}$=3.0]%). The differences between these two clades were much greater, being 4.7–5.3 ($\bar{x}$=5.0)%.

Morphological analyses

Univariate analysis for two clades: When samples from each island were grouped into the two genetic clades (see above), significant difference was detected in SVL between sexes in both the Okinawa and Amami clades, where females had larger value than males (Tukey-Kramer test, $P<0.05$). I, therefore, separated sexes in comparing morphometric values between the two genetic clades. As a result, significant sexual difference was found in RIOD, REL, RLAL, and RFL in the Amami clade, and in RTD, RTL, and R1TOEL in the Okinawa clade. Except for RTL and R1TOEL in the Okinawa clade, males had larger values than females ($P<0.05$). Between the two clades, specimens of the Amami clade were significantly larger than those of the Okinawa clade in male RIOD, REL, RLAL, RFL, RTL, RFL, RHLL, and R1TOEL (U test, $P<0.05$).

MGPCA for two lineages: A PCA using a total 16 characters resulted in the first eigenvector to indicate positive values with nearly similar magnitudes in all the characters examined. Because the first eigenvector highly correlated to SVL ($r=0.916$, $df=51$, $P<0.0001$), I employed MGPCA, which is thought to exclude an effect of the eigenvector reflecting size (SVL) variation. Using the size-independent 15 character values obtained from the MGPCA, I subsequently conducted CDA. The eigenvalues of the first three axes in CDA (CAN1–

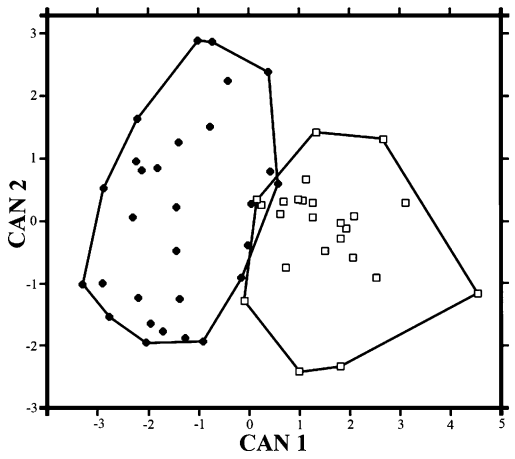

FIG. 3. Plot of first against second canonical variables for male specimens of the Okinawa clade (open squares) and the Amami clade (closed circles) of the brown frogs from the Central Ryukyus.

CAN3) accounted for 63.8%, 17.6%, and 6.0% of total variation, respectively (Wilks' Lambda=0.1891, P<0.0001). Males of the Okinawa and Amami clades were nearly completely separated by the first axis (Fig. 3).

Qualitative analyses: In males, the two genetic clades much differed in the toe webbing, which was much more developed in the samples from Amamioshima Is. than those from Okinawajima Is.: phalanges free of web in the Amamioshima samples were often $2^1/_3$ and up to $2^1/_2$ (vs. often $2^3/_4$ and up to 3 in the Okinawajima samples) on the inner (preaxial) side of the third toe, often one (vs. $1^1/_2$) on the outer (postaxial) side of the third, usually $2^3/_4$ (vs. three or more) on the inner side of the fourth, and usually 2 (vs. more than $2^1/_2$) on the outer side of the fourth.

SYSTEMATICS

The populations of brown frogs from the Central Ryukyus examined here are genetically clearly split into two clades that substantially differ from each other by large genetic distances (uncorrected p-distance in 16S rRNA of 4.7–5.3%), equivalent to those usually observed among good species of frogs (Fouquet et al., 2007). Furthermore, adults of these two clades were also separated morphologically in congruent with genetic separation. Each of these clades, although occurring allopatrically, should therefore be recognized as a distinct species from the lineage-based species concept (Wiley, 1978; Frost and Hillis, 1990; De Queiroz, 1998). Thus, I describe the two unnamed clades as follows:

Rana ulma n. sp.

(Japanese name: Ryukyu-Aka-gaeru)
Figs. 4A, B

Rana macropus (non Boulenger): Okada, 1930, p. 89 (part); Okada, 1931, p. 97 (part); Okada, 1966, p. 64 (part).
Rana sauteri: Okada, 1934, p. 20 (part); Koba, 1957, p. 198.
Rana okinavana: Inger, 1947, p. 311 (part); Shibata and Matsui, 1985, p. 3 (part).
Rana (*Rana*) *okinavana*: Nakamura and Uéno, 1963, p. 43 (part); Maeda and Matsui, 1989, p. 53 (part).
Rana sp.: Matsui, 2007, p. 202.

Etymology

The specific name is derived from Uruma, a dialect of the Ryukyus, meaning a space of a piece of coral, or the coral island, Okinawajima, where the new species inhabits.

Holotype

KUHE 28141, an adult male from Taiho, Ogimi-son, Okinawajima Is., Okinawa Prefecture, Japan (26°39'N, 128°08'E, 150 m asl), collected on 10 December 2000 by Mamoru Toda.

Paratypes

KUHE 28142–28161 (18 males and two females), paratopotypes with the same collection data as the holotype; OMNH Am 15573–15576 (two males, one female, and one juvenile) and OMNH Am 15616–15618 (three males) from Yona, Kunigami-son, Okinawajima Is., Okinawa Prefecture, Japan (26°45'N, 128°13'E), collected on 5 January 1973 and 31 December 1992, respectively, by Mitsuru Kuramoto.

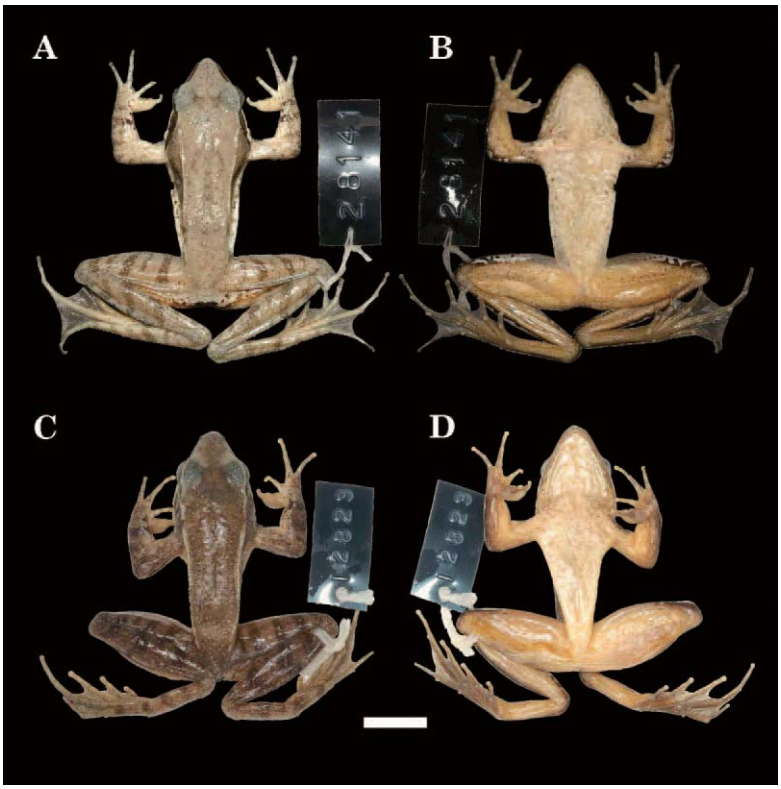

FIG. 4. Male holotypes of (A, B) *Rana ulma* (KUHE 28141), and (C, D) *R. kobai* (KUHE 12823); dorsal (A, C) and ventral views (B, D). Scale bar=10 mm.

Referred specimens

KUHE 12704–12706 and 12713 (one male and three females) from Kumejima-cho, Kumejima Is., Okinawa Prefecture, Japan (26°20'N, 126°48'E, <300 m asl), collected on 27 December 1991 by Masanao Toyama and Mamoru Toda; KUHE unnumbered six tadpoles from Mt. Nishimedake, Kunigami-son, Okinawajima Is., Okinawa Prefecture, Japan (26°48'N, 128°48'E, <420 m asl), collected on 19 April 2008 by Atsushi Tominaga.

Diagnosis

A small-sized species (SVL 33–39 mm in males and 42–51 mm in females) of the genus *Rana* (sensu stricto); females larger than males; snout low and fairly pointed; tympanum relatively large, more than three-fifths of eye length; dorsolateral fold rather thin; hindlimb long, tibiotarsal articulation of adpressed limb reaching far anterior to snout tip; knee seldom touching axilla when hindlimb bent and adpressed to body; tips of digits swollen, but lacking grooves; outer side of fourth toe with two and half or more phalanges free of broad web in males; a dark mask covering tympanum; a white stripe on upper half of upper lip; dark spots on ventral side clear; skin covered with small tubercles with asperities on posterior half of upper eyelid, dorsum, sides, and tarsus; males without vocal sacs; eggs not laid in a large globular mass; larva of normal type, without modification on ventrum.

Description of holotype (measurements in mm)

Snout-vent length 38.0; body slender; head slightly elongate, longer (13.9) than wide

(12.2); snout triangular, tip slightly blunt in dorsal outline; projecting beyond lower jaw, slightly rounded in lateral profile; canthus rostralis distinct; lores very weakly oblique, concave; nostril below canthus, nearer to tip of snout (2.3) than to anterior margin of upper eyelid (3.0); internarial distance (3.1) subequal to distance from nare to eye; eye moderate, length (5.2) less than twice eye-nostril distance and smaller than snout length (5.7); interorbital flat, narrower (2.75) than width of upper eyelid (3.0) and internarial distance; tympanum distinct, nearly circular, about three-fifths of eye length (2.95); vomerine teeth in two, small, oval, and obliquely raised series (each of three and five teeth), center on line connecting posterior margins of choanae, narrowly separated from each other, but widely separated from choanae; tongue moderately notched, without papilla; no vocal sac or vocal opening.

Forelimb (24.2) rather stout; fingers slender, unwebbed; first finger only slightly longer than second, fourth longer than second; finger tips rounded forming small disk without grooves; no fringes of skin along fingers; three large palmar tubercles, and distinct supernumerary tubercles; subarticular tubercles prominent, oval; distinct creamy nuptial pads on dorsal, medial, and ventral surfaces of first finger extending from its base to subarticular tubercle, covered with minute asperities medially reaching nearly to finger tip (Fig. 5B).

Hindlimb long (71.2), about three times length of forelimb; tibia (22.7) longer than foot (21.2); heels well overlapping when limbs held at right angles to body; tibiotarsal articulation of adpressed limb reaching far anterior to snout tip; toe tips similar to finger tips; third toe shorter than fifth; toes moderately webbed, formula I 1–2 II $1^{1}/_{3}$–$2^{1}/_{2}$ III 2–3 IV 3–$1^{1}/_{2}$ V (Fig. 5A); excision of membrane between two outer toes reaching midway of middle and proximal subarticular tubercles of fourth when toes in contact; webs not crenulate; subarticular tubercles prominent, oval; inner metatarsal tubercle distinct, oblong (1.5), one-third length of first toe (4.4); outer metatarsal tubercle small, but distinct, round and raised; sides of tarsus raised, but not forming distinct tarsal fold.

Dorsal skin scattered with small tubercles; supratympanic fold from posterior margin of eye above and behind tympanum to above arm insertion; thin dorsolateral fold from

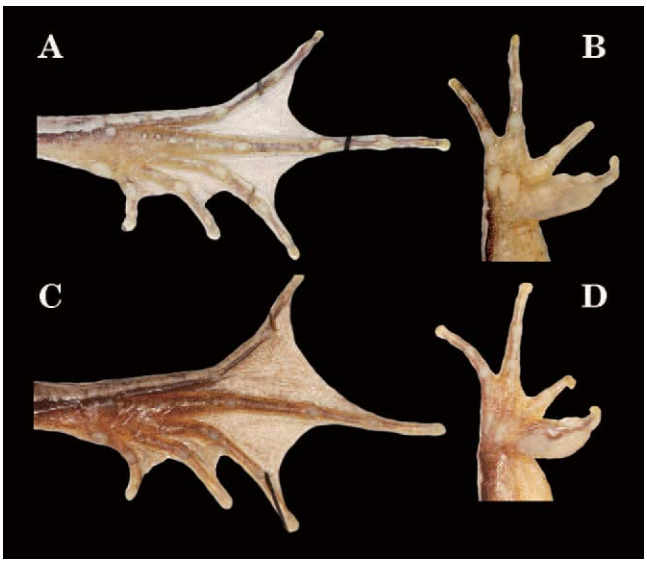

FIG. 5. Ventral views of right feet (A, C) and hands (B, D) of male holotypes of (A, B) *Rana ulma* and (C, D) *R. kobai*.

supratympanic fold to groin; sides scattered with small tubercles; throat and chest smooth; abdomen smooth, coarsely granular posteriorly; small tubercles on posterior half of upper eyelid, dorsum, sides, and tarsus equipped with white asperities.

Color in alcohol (after formalin fixation)

Light gray brown dorsally on head and body; a narrow dark gray interorbital bar and a weak dark gray chevron marking in scapular region; no vertebral line; lores with dark gray marking below canthus; no gray marking on whitish labial; a distinct dark brown marking from behind eye, covering tympanum and reaching above arm insertion (Fig. 6A); dorsal surfaces of limbs marked with alternating, dark crossbars; rear of thigh dusted irregularly; throat and abdomen whitish scattered with small dark gray markings; foot ventrally dark grayish.

Variation

Morphometric data are summarized in Table 1. Females are larger (42.3–50.9, $\bar{x}\pm$ SD= 47.3 mm in SVL) than males (32.7–39.4, 36.4±1.7 mm in SVL). Males have relatively larger tympanum than females in the Okinawajima population (RTD: 7.3–9.2, median= 8.5%SVL in males vs. 6.8–8.3, median= 7.3%SVL in females). Snout is usually longer than eye, but in a few individuals it is equal to or shorter than the latter. Interorbital space is usually narrower than upper eyelid, but the former equals to or wider than the latter in some individuals. Similarly, internarial distance is usually larger, but is equal to or smaller than upper eyelid width in a few individuals. Development of toe webbing is variable, but males tend to have more developed toe webbing than females in outer sides of third and fourth toes (Table 2). White asperities on the back are much more developed in males than in females. Except for ground body color metachrosis, specimens are fairly constant in coloration, except for the absence or presence and the extent of dark dots on the back along dorsolateral fold.

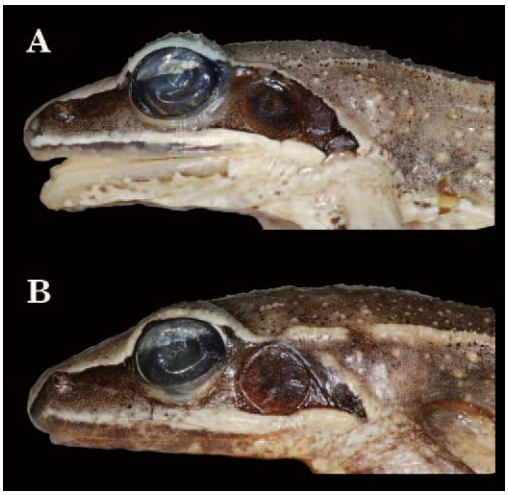

FIG. 6. Lateral views of heads of male holotypes: (A) *Rana ulma*; (B) *R. kobai*.

Populations from Okinawajima Is. and Kumejima Is. differ substantially in mtDNA sequences (see above), although small number of specimens prohibited morphological comparisons.

Eggs and larvae

Eggs are usually laid in small, loosely tied masses each containing about ten eggs, but are sometimes scattered. The clutch size is approximately 320. The diameter of ova artificially squeezed from females ranged from 2.2–2.6 ($\bar{x}\pm$2SE=2.44±0.14) mm. The animal pole is dark brown and the vegetal pole is grayish white in color.

A total of six tadpoles from stages 33 (TOTL=26.4 mm, HBL=9.9 mm) to 39 (33.0 mm, 10.8 mm), collected from Mt. Nishimedake, Okinawajima Is., were closely examined. Head and body slightly flattened above, spheroidal below; HBW maximum posterior to level of spiracle 64–73% (median=66%) of HBL; HBD 54–79% (median=57%) of HBW; snout rounded; eyes dorsolateral, not visible from below, eyeball 11–15% (median= 13%) of HBL; interorbital moderate, 138–167% (median=156%) of eyeball diameter; nostril open, dorsolateral, rim raised, closer to tip of snout than to eye; internarial 72–100

TABLE 1. Morphological variation in the brown frogs from the Central Ryukyus. SVL ($\bar{x}\pm1SD$) mm and medians of ratios (R) of other characters to SVL followed by ranges. See text for character abbreviations.

	Males				Females			
	Amamioshima	Tokunoshima	Okinawajima	Kumejima	Amamioshima	Tokunoshima	Okinawajima	Kumejima
(n)	22	8	24	1	5	4	4	3
SVL	36.4±1.7	33.8±1.2	36.4±1.7	36.8	42.6±3.6	40.2±4.1	47.2±2.1	47.5±4.6
	33.7–40.6	31.7–36.1	32.7–39.4	—	38.9–46.4	34.5–44.3	44.9–49.9	42.3–50.9
RHL	37.3	37.0	37.3	38.0	36.0	36.8	37.7	36.4
	35.5–38.7	36.6–38.2	36.2–39.6	—	35.0–37.0	35.6–38.6	35.9–38.9	34.4–37.6
RHW	33.2	32.5	32.4	34.8	32.3	33.5	31.9	32.6
	30.8–35.0	31.3–34.2	29.1–34.4	—	30.7–34.2	32.2–33.9	31.6–33.5	31.0–33.7
RIND	9.2	8.6	9.1	9.5	8.9	8.5	8.4	8.3
	8.1–9.9	7.9–10.2	7.7–9.7	—	8.2–9.3	8.1–8.7	8.0–8.6	7.7–9.0
RIOD	8.4	8.3	7.9	9.0	7.8	7.6	8.1	8.3
	6.8–9.3	7.4–9.1	6.9–8.6	—	6.9–9.7	7.5–7.9	7.7–8.5	8.0–8.7
RUEW	8.8	8.2	8.3	9.2	8.7	8.2	8.4	8.3
	7.7–9.9	7.6–9.6	7.5–9.2	—	7.1–9.3	7.8–8.6	8.2–8.9	7.5–9.0
RSL	14.8	15.7	15.0	15.8	14.8	15.0	15.1	14.9
	13.6–16.3	14.9–16.4	14.1–16.3	—	13.8–15.4	13.9–15.3	14.5–16.0	13.9–15.2
REL	15.7	15.2	14.2	15.2	13.9	14.2	14.1	14.2
	13.8–16.6	14.1–16.4	13.2–15.9	—	13.4–14.3	13.9–15.4	13.1–14.5	14.1–14.4
RTD	7.9	8.3	8.5	7.9	7.8	7.9	7.3	7.7
	6.8–9.3	7.6–8.9	7.3–9.2	—	6.9–8.4	7.4–8.7	6.8–8.3	7.3–7.8
RLAL	49.6	49.7	47.8	49.5	47.8	46.6	47.6	46.2
	46.1–52.4	48.8–55.0	45.3–49.6	—	44.7–48.0	45.8–49.2	46.3–49.0	45.9–47.4
RFLL	68.0	70.0	65.8	68.2	63.1	62.9	65.1	63.1
	62.2–71.4	68.6–72.2	59.3–67.4	—	62.5–63.6	62.0–66.1	63.1–66.5	62.2–63.3
RTL	62.3	64.6	59.7	62.2	63.1	63.9	63.9	61.7
	58.3–66.6	61.1–65.8	57.4–63.7	—	58.1–64.5	60.6–66.4	61.7–66.1	60.0–63.2
RFL	59.2	60.1	57.8	61.1	58.0	58.1	59.5	56.6
	57.4–62.9	58.6–65.5	55.4–60.5	—	55.5–62.8	56.5–60.7	55.7–64.1	55.3–57.7
RHLL	196.4	199.3	188.7	194.6	194.9	192.8	192.0	188.7
	187.9–208.4	191.4–212.6	181.2–200.5	—	186.6–198.7	187.5–204.3	190.8–205.2	187.4–189.6
RIMTL	4.0	3.9	4.3	4.6	4.3	4.1	3.8	3.8
	3.6–4.8	3.3–4.7	3.4–5.7	—	4.0–4.6	3.2–4.7	3.4–4.3	3.5–4.5
R1TOEL	12.7	12.9	11.9	13.3	12.2	12.6	12.8	13.0
	10.6–14.4	12.0–14.0	10.0–12.7	—	11.1–14.3	11.6–13.1	12.2–14.0	12.8–13.4

(median=93)% of interorbital. Oral disk anteroventral, emarginate, width 29–32% (median=31%) of HBW; marginal papillae on upper labium with wide gap, short papillae in one row at corners, submarginal papillae present; lower labium with a continuous row of papillae, submarginal papillae present near corners; denticles 4(2–4)/4(1); beaks with black outer margins; outer surface smooth; margin finely serrate; upper beak weekly convex medially; neither beak divided. Spiracle sinistral, opening 47–61% (median=54%) of HBL; tube pointing upward and backward, free of body wall slightly. Anal tube dextral, attached to ventral fin; thick loops of gut visible ventrally. Tail long and lanceolate, both margins weakly convex, tapering gradually to slightly rounded tip; tail length 162–221% (median=185%) of HBL, maximum depth 27–33% (median=31%) of length; dorsal fin origin at posterior end of body, deeper than ventral fin except near tail tip; ventral fin

TABLE 2. Development of toe webbing in the brown frogs from the Central Ryukyus as shown by the number of phalanges free of broad web.

	1st outer						2nd inner					2nd outer					3rd inner				
	1-	1	$1^1/_3$	$1^1/_2$	$1^3/_4$	2	$1^1/_3$	$1^1/_2$	$1^3/_4$	2	$2^1/_3$	1-	1	$1^1/_3$	$1^1/_2$	2	2	$2^1/_3$	$2^1/_2$	$2^3/_4$	3
Males																					
Okinawajima		6	**7**	5	1					**18**	1		**11**	7	1		1	4	4	**9**	1
Amamioshima	3	**15**	2	1			3	2	3	**13**		1	**18**	1		1	5	**10**	6		
Tokunoshima	1	**7**							**7**	1		2	**6**				1	**4**	2	1	
Females																					
Okinawajima			1	1						**2**			1		1					2	
Amamioshima		1		**1**		1				**3**			**2**		1					**2**	1
Tokunoshima		1	**2**	1						**3**	1		2		2				2	2	

	3rd outer					4th inner					4th outer						5th inner				
	1	$1^1/_3$	$1^1/_2$	$1^3/_4$	2	$2^1/_2$	$2^3/_4$	3	$3^1/_3$	$3^1/_2$	$1^3/_4$	2	$2^1/_3$	$2^1/_2$	$2^3/_4$	3	1-	1	$1^1/_3$	$1^1/_2$	$1^3/_4$
Males																					
Okinawajima			8	**7**	4			**15**	3	1				8	**5**	6		4	2	**12**	1
Amamioshima	**15**		4	1	1	7	**4**	9	1		1	**19**		1			3	**17**		1	
Tokunoshima	1	1	**4**		2	2		**6**				1	3	**4**				**8**			
Females																					
Okinawajima				1	1			1	1							**2**			1		1
Amamioshima			**2**	1				**3**						**3**				1	**2**		
Tokunoshima			2	2				**4**				1		**2**	1			1	1	2	

origin continuous to vent; caudal muscle moderately strong, maximum tail width 26–32% (median=28%) of HBW; muscle depth at middle of tail 35–41% (median=37%) of tail depth, steadily narrowed posteriorly, shallower than either fin in distal half of tail. Indistinct supranaso-orbital, infranaso-orbital, oral, mental, and pregular lateral line pores discernible. In formalin head-body light brown dorsally and laterally, scattered with small black spots dorsally; caudal muscle dark with light mottling; fins scattered with small dark spots. SVL of juveniles at metamorphosis approximately 12 mm.

Call

Mating calls, recorded by Masataka Matsui on 16 December 1997 at Mt. Yonaha-dake, Kunigami-son, Okinawajima Is. (water temperature=16.5C), were like those of chickens and showed very complicate structure (Fig. 7). Moreover, males aggregated formed a chorus and called synchronously, making the later analyses very difficult. Each call was emitted either singly or consisted of 3–7 ($\bar{x}\pm SD=4.5\pm1.6$, n=6) very short notes, each with many fine pulses. The note duration varied from 3–22 (9.2±4.2, n=30) msec, and the inter-note duration from 46–114 (76.3±14.8, n=21) msec. The call duration in multinoted calls varied from 155 msec with three notes to 436 msec with seven notes (276.6±106.8, n=6), and in the call groups, inter-call duration varied from 183–776 (313.4±255.5, n=5) msec. Frequency band spread over 1850 to 4500 Hz range, with the dominant from 2250 to 3400 (2895.8±291.9, n=12) Hz. Each note showed weak frequency modulation, and frequency modulation was also seen in a multinoted call, with the dominant frequency of the initial notes (1850–2100 [1960.0±96.2, n=5] HZ) rising towards the middle (2350–3050 [2620.0±295.0, n=5] Hz) and falling at the end (2800–3200 [2950.0±169.6, n=5] Hz) of the call. Second frequency band was found between 7901 and 9815 Hz, with the dominant

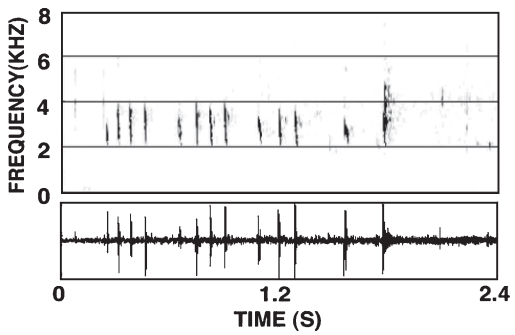

FIG. 7. Sonagram (top) and wave form (bottom) of advertisement call of *Rana ulma*.

from 8580 to 9321 (8945.5±261.2, n=12) Hz. In a multi-noted call, frequency modulation of successive notes was also seen (e.g., from 9012 through 9259 to 8580 Hz).

Comparisons

Rana ulma closely resembles *R. kobai*, another new species described below, but differs from it in having relatively smaller IOD, EL, LAL, FL, TL, FL, HLL, and 1TOEL, and less developed toe web (outer side of fourth toe with two and half or more phalanges free of broad web vs. usually two phalanges free in *R. kobai*) in males (Fig. 5A, C). In addition, it tends to have a thinner dorsolateral fold than *R. kobai* (Fig. 6), and has clearer dark spots on ventral side than does *R. kobai*. Further, in *R. ulma*, knee seldom touches axilla when hindlimb is bent and adpressed to body, but it touches or overlaps axilla in *R. kobai*.

Among members of brown frogs from East Asia, *R. ulma* and *R. kobai* superficially most resemble *R. tsushimensis* from Tsushima Is. and *R. sauteri* (including *R. multidenticulata* Chou and Lin, 1997) from Taiwan, but differ from them in some morphological and ecological characteristics. *Rana ulma* has less rounded snout and relatively larger tympanum (more than three-fifths eye length) than *R. tsushimensis* (half the eye length). In males, *R. ulma* has a relatively long hindlimb with the tibiotarsal joint reaching the point beyond the tip of snout, whereas in *R. tsushimensis* it reaches at most to the snout tip when the hindlimb is bent forward along the body. *Rana ulma* has more pointed snout and flatter head than *R. sauteri* in which the snout tip is rounded and head is high. Most conspicuous difference is the absence in *R. ulma* of horizontal groove on digital tip, which is present in *R. sauteri*.

Eggs of *R. ulma* are laid in many small masses containing small eggs, but *R. tsushimensis* lays a large globular egg mass. Larvae of *R. ulma* and *R. kobai*, as well as of *R. tsushimensis*, are normal type, without modification on spherical ventrum, but larval *R. sauteri* possesses a weak sucker-like structure ventrally (Chou and Lin, 1997). The dental formula is also completely different in *R. sauteri* with much more rows of denticles on both lips (e.g., 7[4–7]/8[1]) than in *R. ulma* (4[2–4]/4[1]).

Range

So far known only from Okinawajima Is. and Kumejima Is. of the Okinawa Island Group, Central Ryukyus, Japan (Maeda and Matsui, 1999).

Natural History

Rana ulma occurs from lowland to montane regions, and inhabits in forests near montane trails and around small streams. It breeds usually in a short period in December (Mori et al., 2009) in shallows of headwaters of small montane streams, but sometimes also in small pools near montane trails. Eggs are usually laid in small, loosely tied masses containing about ten eggs. The water temperatures at the time of breeding are between 11 and 14.5C (Utsunomiya and Utsunomiya, 1983). This species is eaten by a pitviper, *Ovophis okinavensis* (Boulenger, 1892) (Masunaga et al., 2008), and breeding adults are its major prey (Mori and Toda, 2011). Also, the frog is reportedly predated by a dicroglossid frog *Limnonectes namiyei* (Stejneger, 1901) (Mori, 2009). In most non-breeding season, adult *R. ulma* seem to spend their lives scattered in montane forests.

Conservation

The population of Kumejima Is. is designated as a locally protected population by the Okinawa Prefecture (Anonymous, 1996).

***Rana kobai* n. sp.**

(Japanese name: Amami-Aka-gaeru)

Figs. 4C, D

Rana macropus (non Boulenger): Okada, 1930, p. 86 (part); Okada, 1931, p. 95 (part); Okada, 1966, p. 62 (part).

Rana sauteri: Okada, 1934, p. 20 (part); Koba, 1955, p. 151; Koba, 1956, p. 154; Okada, 1966, p. 131.

Rana okinavana: Inger, 1947, p. 332 (part); Koba, 1962, p. 97: Shibata and Matsui, 1985, p. 3 (part).

Rana (*Rana*) *okinavana*: Nakamura and Uéno, 1963, p. 43 (part); Maeda and Matsui, 1989, p. 53 (part).

Etymology

The species name is dedicated to the late Dr. Kazuo Koba, Professor Emeritus of Kumamoto University, who was the distinguished pioneer of herpetological survey in the Amami Island Group of the Central Ryukyus, Japan.

Holotype

KUHE 12823, an adult male from Kinsakubaru, Amami-shi (former Sumiyo-son), Amamioshima Is., Kagoshima Prefecture, Japan (28°21'N, 129°27'E, 300 m asl), collected on 12 February 1992 by Mamoru Toda.

Paratypes

KUHE 12815–12822, 12824–12834, 12836–12839 (22 males and one female), paratopotypes with the same collection data as the holotype; NSMT H-01732, H-01733 from Mt. Yuwandake, Uken-son, Oshima-gun, Amamioshima Is., Kagoshima Prefecture, Japan (28°17'N, 129°19'E, 500 m asl), collected on 17 November 1962 by Yoshinori Imaizumi; NSMT H-04097 from Mt. Yuwandake, Uken-son, Oshima-gun, Amamioshima Is., Kagoshima Prefecture, Japan (28°17'N, 129°19'E, 100 m asl), collected on 23 November 1981 by Mamoru Owada.

Referred specimens

KUHE 12841, 12842, 12848–12857 (eight males and four females) from Mikyo, Amagi-cho, Oshima-gun, Tokunoshima Is., Kagoshima Prefecture, Japan (27°45'N, 128°52'E), collected between 9 and 11 February 1992 by Mamoru Toda; KUHE unnumbered: two tadpoles and eight just metamorphosed juveniles from Otana, Yamato-son, Amamioshima Is., Kagoshima Prefecture, Japan (28°19'N, 129°21'E), collected on 22 April 2005 by Atsushi Tominaga.

Diagnosis

A small-sized species (SVL 32–41 mm in males and 35–46 mm in females) of the genus *Rana* (sensu stricto); females larger than males; snout low and fairly pointed; tympanum relatively large, more than three-fifths of eye length; dorsolateral fold rather thick; hindlimb long, tibiotarsal articulation of adpressed limb reaching far anterior to snout tip; knee usually touching or overlapping axilla when hindlimb bent and adpressed to body; tips of digits swollen, but lacking grooves; outer side of fourth toe usually with two phalanges free of broad web; dark mask covering tympanum; a white stripe on upper half of upper lip; dark spots on ventral side not clear; skin covered with small tubercles with asperities on posterior half of upper eyelid, dorsum, sides, and tarsus; males without vocal sacs; eggs not laid in a large globular mass; larva of normal type, without modification on ventrum.

Description of holotype (measurements in mm)

Snout-vent length 38.1; body moderately slender; head slightly elongate, longer (14.4) than wide (12.1); snout triangular, tip slightly blunt in dorsal outline; projecting beyond lower jaw, slightly rounded in lateral profile; canthus rostralis distinct; lores very weakly oblique, concave; nostril below canthus, nearer

to tip of snout (2.7) than to anterior margin of upper eyelid (3.2); internarial distance (3.1) subequal to distance from nare to eye; eye moderate, length (5.5) less than twice eye-nostril distance and smaller than snout length (6.0); interorbital flat, subequal (3.2) to width of upper eyelid (3.2) and internarial distance; tympanum distinct, nearly circular, about three-fifths of eye length (3.3); vomerine teeth in two, oval, oblique raised series (each of 5 teeth), center on line connecting centers of choanae, equidistantly separated from each other and from choanae; tongue moderately notched, without papilla; no vocal sac or vocal opening.

Forelimb (25.2) rather stout; fingers slender, unwebbed; first finger only slightly longer than second, fourth longer than second; finger tips rounded forming small disk without grooves; no fringes of skin along fingers; three large palmar tubercles, and indistinct supernumerary tubercles; subarticular tubercles prominent, oval; distinct grayish nuptial pads on dorsal, medial, and ventral surfaces of the first finger extending from its base to subarticular tubercle, covered with minute asperities medially reaching nearly to finger tip (Fig. 5D).

Hindlimb long (72.2), less than three times length of forelimb; tibia (22.2) subequal to foot (22.3); heels well overlapping when limbs held at right angles to body; tibiotarsal articulation of adpressed limb reaching far anterior to snout tip; toe tips similar to finger tips; third toe slightly shorter than fifth; toes moderately webbed, formula I 1–2 II 1–2$^{1}/_{2}$ III 1–3 IV 2–1 V (Fig. 5C); excision of membrane between two outer toes reaching near middle subarticular tubercle of fourth when toes in contact; webs not crenulate; subarticular tubercles prominent, oval; inner metatarsal tubercle distinctly raised, oblong (1.4), less than one-third length of first toe (5.1); outer metatarsal tubercle small; sides of tarsus raised, but not forming distinct tarsal fold.

Dorsal skin scattered with small tubercles; supratympanic fold from posterior margin of eye above and behind tympanum to above arm insertion; distinct dorsolateral fold from supratympanic fold to groin; sides scattered with small tubercles; throat, chest and abdomen smooth; upper lip, dorsolateral fold, outer metatarsals, and small tubercles on part of upper eyelid, dorsum, sides, and tarsus equipped with asperities.

Color in alcohol (after formalin fixation)

Light brown dorsally on head and body; narrow dark gray interorbital bar and very weak dark gray chevron marking in scapular region; dorsolateral fold creamy; no vertebral line; lores with dark brown marking below canthus; no gray marking on whitish labial; a distinct dark brown marking from behind eye, covering tympanum and reaching above arm insertion (Fig. 6B); dorsal surfaces of limbs marked with alternating, dark crossbars; front and rear of upper arm, front of tibia, distal thirds of thigh, and around vent marked with dark brown; rear of thigh dusted with brown; throat and abdomen whitish dotted with brown especially on chin; foot ventrally light grayish.

Variation

Females (34.5–46.4, $\bar{x}\pm SD=41.5\pm4.0$ mm in SVL) are larger than males (31.7–40.6, 35.9 ± 2.2 mm). Tympanum is relatively larger in males (RTD: 6.8–9.3, median=7.9%SVL) than females (6.9–8.4, median=7.8%SVL) in the population from Amamioshima Is., and in the Tokunoshima Is. population, foot is relatively larger in males (RFL: 58.6–65.5, median=60.1%SVL) than females (56.5–60.7, median=58.1%SVL). In the Amamioshima population, snout is longer than eye in females, but more than half of males have the snout equal to or shorter than eye. Variation in the width of upper eyelid with respect to interorbital and internarial distances tends to be similar to the cases found in *R. ulma*. In the Amamioshima population, males tend to have more developed toe webbing than females in outer sides of first, third, and fourth, and inner sides of third and fifth toes. As in *R. ulma*, asperities on the back are less developed in females than in males, and some specimens have dark dots on the back along dorsolateral fold.

Eggs and larvae

Eggs are usually laid scattered (Utsunomiya and Utsunomiya, 1983). The clutch size is about 510. Two tadpoles of St. 35 (TOTL= 28.8 mm, HBL=10.6 mm) and 36 (30.3 mm, 10.4 mm) from Yamato-son, Amamioshima Is. were closely examined. HBW 69–73% (median= 71%) of HBL; HBD 62–64% (median=63%) of HBW; eyeball 11–12% (median=12%) of HBL; interorbital 170–177% (median=174%) of eyeball diameter; internarial 67–91 (median= 79)% of interorbital. Oral disk width 15–26% (median=20%) of HBW; denticles 4(2–4)/ 4(1); Spiracle opening 48–53% (median= 51%) of HBL; Tail length 172–192% (median= 182%) of HBL, maximum depth 31–33% (median=32%) of length; maximum tail width 26–29% (median=28%) of HBW; muscle depth at middle of tail 35–41% (median= 38%) of tail depth. Otherwise the characteristics are similar to those of larval *R. ulma*. Color in life light brown on dorsum and laterally, with darker markings at interorbital, and on middle and end of body; venter grey scattered with silver; tail scattered with black and silver spots (Fig. 8).

SVL of eight juveniles at the time of metamorphosis 9.6–10.6 ($\bar{x}\pm SD=10.1\pm0.3$) mm.

FIG. 8. Dorsal (A), lateral (B), and ventral (C) views of larval *Rana kobai* from Amamioshima Is. (Stage 36, total length=30.3 mm).

Karyotype

Diploid chromosome number is 26, with five large and eight small pairs. Chromosomes forming pairs 3, 6, 8, 9, 11, and 13 are submetacentric, and the remaining seven pairs are metacentric. Secondary constrictions are recognized on the shorter arms of pair 9 (data from Kuramoto, 1972).

Call

Calls are similar to those of *R. ulma*. The call duration varies from 500 to 1000 msec with five to eight notes. Each note shows clear harmonics, each with conspicuous frequency modulation, and the fundamental frequency is about 1800 Hz (Maeda and Matsui, 1989).

Comparisons

Rana kobai differs from *R. ulma* as noted above. It differs from *R. tsushimensis* and *R. sauteri* in the same way as *R. ulma* does.

Range

Known only from Amamioshima Is., Kakeromajima Is., and Tokunoshima Is. of the Amami Island Group, Central Ryukyus, Japan (Maeda and Matsui, 1999).

Natural History

Breeding is done in the winter months, from late November to early January, but sometimes as late as in April. Eggs are laid in small clumps in headwaters of small montane streams, small springs near montane trails, small ponds, and marshy places. The water temperatures at the time of breeding are between 8.5 and 16C (Kuramoto et al., 1971; Utsunomiya and Utsunomiya, 1983). Metamorphosis takes place usually by the end of March, but sometimes after May.

DISCUSSION

There is a complicate nomenclatorial history regarding *R. ulma* and *R. kobai* (details in Shibata and Matsui [1985] as *R. okinavana*). Okada (1930, 1931) applied the name *R. macropus* Boulenger, 1886 to the brown frogs

from the Central Ryukyus, but this name was actually a junior synonym of *Ixalus japonicus* Hallowell, 1860 (now *Buergeria*). Inger (1947: 332) placed *R. macropus* sensu Okada to the synonymy of *R. okinavana* Boettger, 1895, which he studied only on samples from Ishigakijima Is. of the Southern Ryukyus but included the Amami and the Okinawa Groups as its range of distribution. All the subsequent Japanese authors, except for Okada (1966) followed this view and used *R. okinavana* for the brown frogs from the Central Ryukyus (e.g., Koba, 1962; Nakamura and Uéno, 1963; Utsunomiya, 1979; Maeda and Matsui, 1989, 1999). Nakamura and Uéno (1963) included the Southern Ryukyus in the range of *R. okinavana* probably following Inger (1947), but no brown frogs have actually been recorded there. Instead, *R. psaltes*, which is not a brown frog but is now placed in *Nidirana* (Chuaynkern et al., 2010) or *Babina* (Frost et al., 2006), was found there, and the species was eventually revealed to be true *R. okinavana* (Matsui, 2007).

Tanaka et al. (1996) first found the presence of a high degree of genetic divergence in cyt b gene between the brown frogs from Okinawajima Is. and Amamioshima Is. More recently, Iwanari et al. (2010) reported a genetic distance of 0.155 in 20 loci of 14 allozymes and complete substitution in LDH-A locus between the two populations. They also detected sequence divergence of 5.2% in 12S rRNA and 3.1% in 16S rRNA genes of mtDNA. Moreover, offspring they artificially produced between females from Okinawajima Is. and males from Amamioshima Is. developed normally, but mostly became males that showed abnormal spermatogenesis. From these results, Iwanari et al. (2010) considered the two populations to have diverged in a degree to be recognized at least as different subspecies. Although information of reciprocal cross is still unavailable, different species status of brown frog populations from Okinawajima Is. (*R. ulma*) and Amamioshima Is. (*R. kobai*) is most likely because production of at most sterile males has been reported between *R. kobai* (as *R. okinavana*) and six different species of browns from Japan and Taiwan (Kuramoto, 1974).

In general appearance, *R. ulma* and *R. kobai* are most similar to *R. tsushimensis* from Tsushima Is. located between mainland Japan and Korea, sharing the 2N=26 chromosome number, slender body, and long limbs, although they are well differentiated morphologically and ecologically. Monophyly of the group of *R. ulma* and *R. kobai* (reported as *R. okinavana*) and *R. tsushimensis* has been reported by Tanaka et al. (1996) on the basis of the cyt b gene sequences. To the south of the Central Ryukyus, two species of brown frogs, *R. longicrus* and *R. sauteri*, occur in Taiwan, of which one of the cryptic lineages of the latter is also reportedly close to *R. tsushimensis* and then to the Central Ryukyu species (as represented by "*R. okinavana* from Okinawajima island" [i.e., *R. ulma*]) (Tanaka-Ueno et al., 1998). However, there are no brown frogs in the Southern Ryukyus located between Taiwan and the Central Ryukyus. This suggests that the common ancestor of *R. ulma* and *R. kobai* reached the Central Ryukyus from the north, not from the southwest (i.e. Taiwan: Tanaka et al., 1996) unlike many other amphibians occurring in this region (Ota, 1998; Matsui et al., 2010). Similarly, *Cynops ensicauda* (Hallowell, 1861) and *Rhacophorus viridis* (Hallowell, 1861), also distributed in the Central Ryukyus, are thought to be descendants from the north, with their phylogenetically closest relatives on mainland Japan (*Cynops pyrrhogaster* [Boie, 1826] [Hayashi and Matsui, 1988] and *Rhacophorus schlegelii* [Günther, 1858] [Maeda and Matsui, 1989], respectively).

It is also noteworthy that genetic differentiation between *R. ulma* from the Okinawa group and *R. kobai* from the Amami group is so great as to merit different taxonomic status. This conforms to several other amphibians from the two island groups, that have diverged to the specific (*Babina holsti* [Boulenger, 1892] vs. *B. subaspera* [Barbour, 1908][Matsui and Utsunomiya, 1983]; *Odorrana narina* [Stej-

neger, 1901] vs. *O. amamiensis* [Matsui, 1994]; *O. ishikawae* [Stejneger, 1901] vs. *O. splendida* Kuramoto, Satou, Oumi, Kurabayashi, and Sumida, 2011) or subspecific level (*Cynops ensicauda popei* [Inger, 1947] vs. *C. e. ensicauda* [Hayashi and Matsui, 1988; Tominaga et al., 2010]; *Rhacophorus viridis viridis* vs. *Rh. v. amamiensis* Inger, 1947 [Matsui, unpublished data]). Extensive genetic differentiations between the two island groups would be the results of bottleneck effects experienced by founder populations of these taxa.

Finally, mean uncorrected p-distance in 16S rRNA of 3.0% obtained between samples from Okinawajima Is. and Kumejima Is. in *R. ulma* corresponds to that reported between cryptic species of frogs (Fouquet et al., 2007), and taxonomic relationships between the two populations remain to be studied.

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