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Distinct Species Status of a *Microhyla* from the Yaeyama Group of the Southern Ryukyus, Japan (Amphibia, Anura, Microhylidae)

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Abstract: A Japanese microhylid, *Microhyla okinavensis*, originally described from Okinawajima Island, middle Ryukyus, was long synonymized with *M. ornata* from India. However, molecular phylogenetic studies revealed its distinct species status from *M. ornata*, and more recent phylogenetic study revealed the population from the Yaeyama Group of the southern Ryukyus to be a sister taxon to Chinese *M. mixtura* and not to populations from the remaining group of the Ryukyus, that are sister to another Chinese species, *M. beilunensis*. The Yaeyama and the remaining Ryukyu populations greatly differ phylogenetically, although less clearly morphologically. From these data, we consider the Yaeyama population as a species distinct from *M. okinavensis* from the middle Ryukyus.

Key words: *Microhyla beilunensis*; *Microhyla fanjingshanensis*; *Microhyla mixtura*; *Microhyla okinavensis*; New species; Yaeyama Group

Introduction

A Japanese microhylid, *Microhyla okinavensis* Stejneger, 1901, originally described from Okinawa Shima (=Island, Okinawajima), was later synonymized with *M. ornata* (Duméril and Bibron, 1841) from India (Okada, 1931; Inger, 1947; Nakamura and Uéno, 1963; Maeda and Matsui, 1989). However, Dubois (1987) resurrected the original name without any reason. Then, Matsui et al. (2005) revealed its species status distinct from *M. ornata* through a mitochondrial DNA (mtDNA) phylogenetic study. Meanwhile, the species was long suggested to vary morphologically; the population of the Yaeyama Group of the southern Ryukyus was said to differ from the population of the middle Ryukyus (Inger, 1947), and such conclusion was later strengthened by Kuramoto and Joshy (2006). Distinct relationships of the Yaeyama population from other populations of the Ryukyus were also suggested genetically (Matsui et al., 2005).

A more recent molecular phylogenetic study (Tominaga et al., 2019) revealed that

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the southern Ryukyu population is sister to Chinese *M. mixtura* Liu and Hu, 1966 and not to the middle Ryukyu population *M. okinavensis*, whose sister species is *M. beilunensis* Zhang, Fei, Ye, Wang, Wang, and Jiang, 2018, another Chinese species. Meanwhile, Li et al. (2019) recently described *M. fanjingshanensis* Li, Zhang, Xu, Lv, Jiang, Liu, Wei, and Wang, 2019, which is also phylogenetically related to Japanese and Chinese species shown above. In Li et al. (2019), however, relationships among samples were seemingly different from that reported by Tominaga et al. (2019): two samples of *M. okinavensis* formed a clade with *M. mixtura*, and that clade, *M. beilunensis*, and *M. fanjingshanensis* formed a larger clade with unresolved relationships. Thus, it is necessary to re-examine relationships of two lineages of *M. okinavensis* with Chinese species, including *M. fanjingshanensis*, using larger samples.

Materials and Methods

DNA analyses

DNA sequence data were obtained from GenBank (Accession numbers in Table 1). Phylogenetic (maximum likelihood [ML] and Bayesian inference [BI]) trees were reconstructed from ≤865 base pairs (bp) of partial sequences of mitochondrial 12S (≤345 bp) and 16S rRNA (≤520 bp) genes. We also calculated pairwise comparisons of uncorrected sequence divergences (p-distances) in short fragments (ca. 520 bp) of 16S rRNA by using software MEGA7 (Kumar et al., 2016).

Morphological analyses

In morphological analyses, we used only adult males, because it is more difficult to collect females than males. For specimens of *M. okinavensis* stored in 70% ethanol at the Ryukyu University Museum (Fujukan) (RUMF) and the Faculty of Education, University of the Ryukyus (URE) collections, we took the following 13 body measurements to the nearest 0.1 mm, and when necessary, using a binocular dissecting microscope, following Matsui (1984, 1994): (1) snout–vent length (SVL); (2) head length (HL) from tip of snout to hind border of angle of jaw, not measured parallel with the median line; (3) eye length (EL); (4) head width (HW); (5) internarial distance (IND); (6) interorbital distance (IOD); (7) upper eyelid width (UEW); (8) lower arm and hand length (LAL); (9) hand length (HAL); (10) hindlimb length (HLL); (11) tibia length (TL); (12) tibio-fibula length (TFL); and (13) foot length (FL). Measurement data for the three Chinese species were taken from Li et al. (2019).

We compared SVL by Tukey-Kramer test, while we performed Dunn's multiple comparison tests for ratio values and detection of the presence or absence of differences in the frequency distributions. Significance level of 5% was used in all statistical tests. All statistical analyses were performed by R 3.4.2 (R Core Team, 2017). In the following description, average values are shown with 1 standard deviation (±SD).

For examining overall morphological variation among five taxa, we also conducted multivariate analyses using $\log_{10}$-transformed metric values of all the 13 characters. We conducted canonical discriminant analysis (CANDISC) using a statistical package of SAS (SAS, 2009).

For larvae preserved in 5% formalin, we took the following 14 measurements to the nearest 0.1 mm using a binocular dissecting microscope equipped with a micrometer: (1) total length; (2) head-body length; (3) maximum head-body width; (4) maximum head-body depth; (5) eye-snout distance; (6) eyeball diameter; (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) maximum tail muscle depth. For staging, we followed Shimizu and Ota's (2003) table for *M. okinavensis* from Okinawajima Island.

Acoustic analyses

For the acoustic data, we recorded frog

Table 1. Samples used for mtDNA analysis in this study together with the information on voucher specimens, collection locality, and GenBank accession numbers. URE: University of the Ryukyus, Faculty of Education; KUHE: Graduate School of Human and Environmental Studies, Kyoto University; CIB: Chengdu Institute of Biology.

	Species	Locality	DataBank	Voucher	Reference
1	*M. okinavensis*	Kado, Tatsugo-cho, Kagoshima	LC465622	URE1212	Tominaga et al. 2019
2	*M. okinavensis*	Agina, Setouchi-cho, Kagoshima	LC465623	URE1029	Tominaga et al. 2019
3	*M. okinavensis*	Kamikatetsu, Kikai-cho, Kagoshima	LC465628	URE20130816_1	Tominaga et al. 2019
4	*M. okinavensis*	Ketoku Tokunoshima-cho, Kagoshima	LC465629	URE1053	Tominaga et al. 2019
5	*M. okinavensis*	Tamagusuku, Nanjo-shi, Okinawa	LC465635	URE1448	Tominaga et al. 2019
6	*M. okinavensis*	Hamahigajima, Uruma-shi, Okinawa	LC465636	URE1465	Tominaga et al. 2019
7	*M. okinavensis*	Senbaru, Nishihara-cho, Okinawa	LC465637	URE0279	Tominaga et al. 2019
8	*M. okinavensis*	Nuha, Ogimi-son, Okinawa	LC465641	URE1550	Tominaga et al. 2019
9	*M. okinavensis*	Hirara, Miyakojima-shi, Okinawa	LC465651	URE1396	Tominaga et al. 2019
10	*M. okinavensis*	Hirara-Kugai, Miyakojima-shi, Okinawa	LC465652	URE1441	Tominaga et al. 2019
11	*M. okinavensis*	Gusukube, Miyakojima-shi, Okinawa	LC465653	URE1391	Tominaga et al. 2019
12	*M. okinavensis*	Gusukube, Miyakojima-shi, Okinawa	LC465654	URE1440	Tominaga et al. 2019
13	*M. fanjingshanensis*	Fanjing Mountain, Yinjiang, Guizhou, China	MK087853, MK087856	CIBFJS20180425006	Li et al. 2019
14	*M. fanjingshanensis*	Fanjing Mountain, Yinjiang, Guizhou, China	MK087854, MK087857	CIBFJS20180425007	Li et al. 2019
15	*M. fanjingshanensis*	Fanjing Mountain, Yinjiang, Guizhou, China	MK087855, MK087858	CIBFJS20180425012	Li et al. 2019
16	*M. beilunensis*	Chaiqiao, Beilun, Ningbo, Zhejiang, China	MH234521, MH234535	CIBBL002	Zhang et al. 2018
17	*M. beilunensis*	Chaiqiao, Beilun, Ningbo, Zhejiang, China	MH234522, MH234536	CIBBL003	Zhang et al. 2018
18	*M. beilunensis*	Chaiqiao, Beilun, Ningbo, Zhejiang, China	MH234523, MH234537	CIBBL004	Zhang et al. 2018
19	*M. beilunensis*	Chaiqiao, Beilun, Ningbo, Zhejiang, China	MH234524, MH234538	CIBBL005	Zhang et al. 2018
20	*M. beilunensis*	Chaiqiao, Beilun, Ningbo, Zhejiang, China	MH234525, MH234539	CIBBL006	Zhang et al. 2018
21	*M. kuramotoi*	Midaragawa, Iriomotejima, Taketomi-cho, Okinawa	LC465657	URE0394	Tominaga et al. 2019
22	*M. kuramotoi*	Sakieda, Ishigaki-shi, Okinawa	LC465658	URE0441	Tominaga et al. 2019
23	*M. kuramotoi*	Haemi, Iriomotejima, Taketomi-cho, Okinawa	LC465659	URE0406	Tominaga et al. 2019
24	*M. kuramotoi*	Ibaruma, Ishigaki-shi, Okinawa	LC465660	URE0424	Tominaga et al. 2019
25	*M. kuramotoi*	Haterumajima, Taketomi-cho, Okinawa	LC465662	URE20130814_H1	Tominaga et al. 2019
26	*M. kuramotoi*	Komi, Iriomotejima, Taketomi-cho, Okinawa	LC465665	URE2053	Tominaga et al. 2019
27	*M. kuramotoi*	Bannadake, Ishigaki-shi, Okinawa	LC465666	URE1739	Tominaga et al. 2019
28	*M. kuramotoi*	Midaragawa, Iriomotejima, Taketomi-cho, Okinawa	LC465667	URE2132	Tominaga et al. 2019
29	*M. mixtura*	Sichuan, China	LC465668	CIB2013051806	Tominaga et al. 2019
30	*M. mixtura*	Sichuan, China	LC465669	CIB2013051807	Tominaga et al. 2019
31	*M. mixtura*	Yangxian, Hanzhong, Shaanxi, China	MH234527, MH234532	CIBZMH2017061201	Zhang et al. 2018
32	*M. mixtura*	Yangxian, Hanzhong, Shaanxi, China	MH234526, MH234533	CIBZMH2017061202	Zhang et al. 2018
33	*M. mixtura*	Hua'e mountain, Wanyuan, Sichuan, China	MH234530, MH234541	CIB20170526003	Zhang et al. 2018
34	*M. fissipes*	Ulai, New Taipei City, Taiwan	LC465674	URE2398	Tominaga et al. 2019
35	*M. fissipes*	Huanshan, Anhui, China	LC465676	KUHE32943	Tominaga et al. 2019
36	*M. mukuhelsuri*	Pilok, Thailand	LC465683	KUHE35165	Tominaga et al. 2019
37	*M. mukuhelsuri*	Phrae, Thailand	LC465680	KUHE21982	Tominaga et al. 2019
38	*M. heymonsi*	Guanziling, Taiwan	LC465686	KUHE50505	Tominaga et al. 2019
39	*M. heymonsi*	Pilok, Thailand	LC465688	KUHE K1845	Tominaga et al. 2019

calls in the field using a digital recorder (Olympus LS-11) with an external microphone (Olympus Compact Gun Microphone ME31) at 44.1 kHz/16 bits as uncompressed wave files, and analyzed recordings with Raven Lite 2.0 for Mac OS X on a Macintosh computer. Temporal data were obtained from the oscillogram and frequency information was obtained from the audiospectrograms using Fast Fourier Transformation (1,024-point Hanning window). Definitions of acoustic parameters follow Matsui (1997) and Matsui and Dehling (2012).

Results

MtDNA phylogeny

Of ≤865 bp sequences of two genes, 189 were variable and 101 were parsimony-informative within the ingroup. The best substitution model for maximum likelihood (ML), derived from Kakusan4 (Tanabe, 2011), was the general time reversible model (GTR: Tavaré, 1986) with a G for both partitions. For Bayesian analysis, the GTR model was selected for both partitions, respectively. The likelihood values (lnL) of the ML and Bayesian inference (BI) trees were –2310.98 and –2523.71, respectively.

Phylogenetic analyses employing two different optimality criteria yielded nearly identical topologies, and only the ML tree is presented in Fig. 1. Two lineages of *M. okinavensis* (Yaeyama and other groups) and three Chinese species formed a clade (ML bootstrap values [BS]=91%, Bayesian posterior probabilities [BPP]=1.00) with respect to the clade of *M. fissipes* and *M. mukuhelsuri*. Within the former clade, *M. okinavensis* from the Yaeyama Group and *M. mixtura* formed a group, which was not sufficiently supported as a clade (65%, 0.91), while the remaining *M. okinavensis* lineage and Chinese species formed a weakly-supported clade (71%, 0.96). Thus three clades, (1) *M. mixtura*, (2) *M. okinavensis* from Yaeyama, and (3) the clade of *M. beilunensis*, *M. fanjingshanensis*, and *M. okinavensis* from island groups of Ryukyus other than Yaeyama, were recognized with unresolved relationships. *Microhyla okinavensis* from Amami, Okinawa, and Miyako Groups formed a clade (99%, 1.00) and grouped with *M. fanjingshanensis*, which was not sufficiently supported as a clade (61%, 0.97). Thus, addition of *M. fanjingshanensis* did not alter the relationships reported by Tominaga et al. (2019), and *M. okinavensis* was clearly split phylogenetically into two lineages, one from Amami and Okinawa Groups of the Middle Ryukyus and the Miyako Group of the Southern Ryukyus, and another from the Yaeyama Group of the Southern Ryukyus, as shown by Tominaga (2019).

The uncorrected pairwise sequence divergences at the mitochondrial 16S rRNA gene between the two lineages of *M. okinavensis* were 3.5–4.6%, while divergences between these two lineages and three Chinese species were 2.2–4.3% (Table 2). Thus, *M. okinavensis* from Yaeyama is judged to be distinct from the conspecifics from the other island groups, as are three Chinese species.

Morphological Analyses

Univariate analyses: Unlike genetic separation, the male specimens of *M. okinavensis* from Yaeyama and Okinawa were not well separated from each other in morphometric characters. Results of the Tukey-Kramer test indicated that the samples of *M. okinavensis* from Yaeyama were significantly larger than other samples, including *M. okinavensis* from Okinawa in SVL. However, no significant difference was detected in the ratio characters between Yaeyama and Okinawa samples (Dunn's multiple comparison tests, $P>0.05$), although Yaeyama samples tended to have relatively smaller values in characters related to hindlimb than in Okinawa samples (Table 3). Reflecting this tendency, the tibiotarsal joint reached the posterior edge to the center of the eye in most of the Yaeyama samples (91.7%), but the joint reached the anterior edge of the eye or forward in a larger number (81.2%) of Okinawa samples, when the

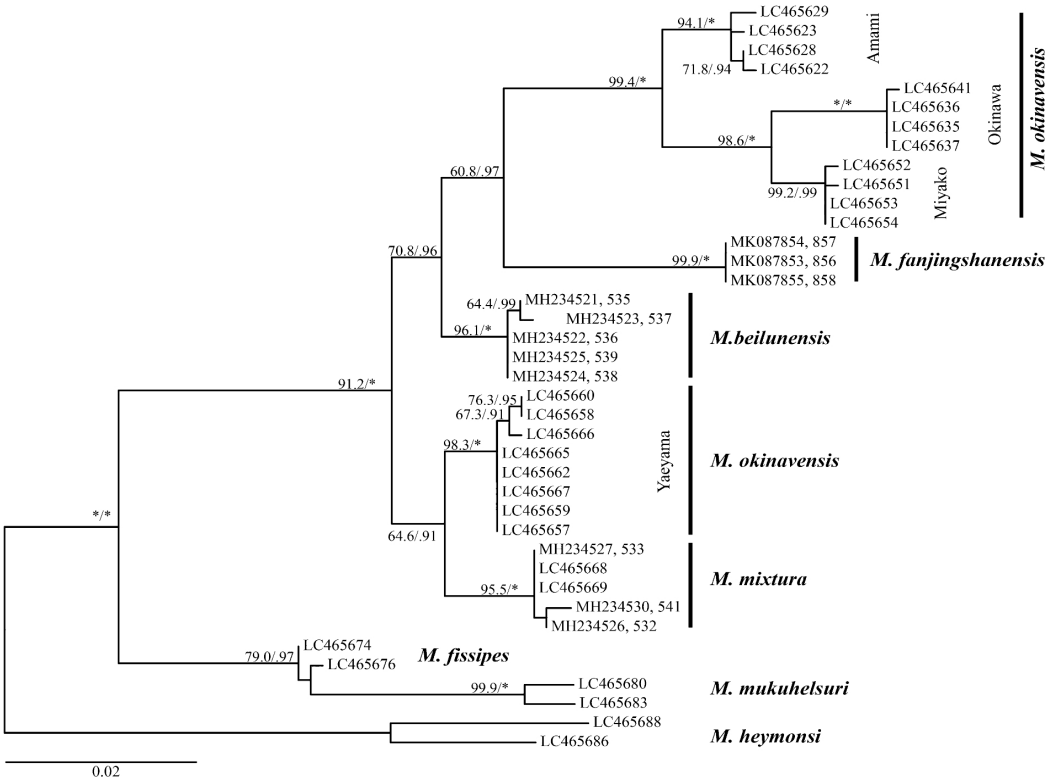

FIG. 1. ML tree from a ≤869 bp sequence of mitochondrial 12S and 16S rRNA genes for samples of *Microhyla*. Numbers above or below branches represent bootstrap supports for ML inference and Bayesian posterior probability (ML-BS/BPP). Asterisks (*) indicate 100% support.

TABLE 2. Uncorrected p-distances (mean±SE %) among samples of *Microhyla* species for short fragments (ca. 520 bp) of 16S rRNA. The analysis involved 33 nucleotide sequences of 16S rRNA.

		1	2	3	4
1	*M. kuramotoi*				
2	*M. okinavensis*	3.89±0.71			
3	*M. mixtura*	1.77±0.52	4.39±0.76		
4	*M. beilunensis*	2.15±0.58	3.60±0.70	2.98±0.70	
5	*M. fanjingshanensis*	4.20±0.86	3.94±0.75	4.25±0.83	2.78±0.72

hindlimb was bent forward along the body (Table 4).

The samples of *M. okinavensis* from Yaeyama significantly differed in ratio (R) to SVL from *M. mixtura* in RHL, RIOD, and RUEW, from *M. fanjingshanensis* in RHL, RHW, RIND, REL, and RTL, and from *M. beilunensis* in RIND, RIOD, RLAL, RHAL, RHLL, RTL, RFL, and RTFL.

Multivariate analyses: CANDISC analysis revealed the ranges of five lineages to be separated from each other except for Yaeyama and Okinawa samples of *M. okinavensis*, that partly overlapped with each other on the first two axes (CAN1–CAN2) (Fig. 2). The eigenvalues of the first (CAN1) and second

Table 3. Measurements in adult males of *Microhyla kuramotoi* sp. nov. and *M. okinavensis*. SVL (mean±SD, in mm) and medians of ratios (R) of other characters to SVL, followed by ranges in parenthesis. See text for character abbreviations.

	M. kuramotoi sp. nov.		*M. okinavensis*	
	n=14		n=16	
SVL	26.0±1.2	(23.8–27.8)	23.7±1.5	(20.8–25.6)
RHL	29.8	(27.9–31.5)	30.6	(27.4–34.6)
RHW	35.2	(33.0–39.1)	36.1	(33.0–41.0)
RIND	8.0	(6.7–9.1)	7.9	(6.4–9.2)
RIOD	9.0	(7.9–11.2)	9.8	(7.2–11.5)
RUEW	7.6	(6.2–8.5)	7.3	(6.5–9.5)
REL	12.8	(11.2–13.8)	12.3	(9.6–13.4)
RLAL	44.4	(40.7–47.5)	43.6	(39.1–47.4)
RHAL	26.9	(25.8–29.8)	27.0	(23.9–29.3)
RHLL	180.8	(165.8–193.4)	184.9	(168.3–198.8)
RTL	57.5	(50.9–60.9)	59.9	(52.8–62.9)
RTFL	84.7	(79.3–90.5)	85.0	(73.2–89.8)
RFL	60.5	(55.3–66.7)	62.0	(56.5–65.7)

Table 4. Variation in the point reached by the tibiotarsal joint when the hindlimb is bent forwards along the body in male *Microhyla kuramotoi* sp. nov. from Yaeyama and *M. okinavensis* from Okinawa. Figures indicate the number of specimens (percentage frequency in parenthesis).

	Post. edge of eye	Center of eye	Ant. edge of eye to nostril	Nostril to tip of snout	Tip of snout or forward
Yaeyama	1 (7.7%)	11 (91.7%)	1 (8.3%)	0	0
Okinawa	0	3 (18.8%)	10 (62.5%)	1 (6.3%)	2 (12.5%)

(CAN2) axes accounted for 11.63 (proportion: 0.60) and 5.76 (proportion: 0.30), respectively. On the first axis, the highest absolute magnitude of the standardized canonical discriminant coefficients was 2.24 of FL, followed by TFL (1.41), SVL (–1.36), TL (–1.36), and HLL (1.00). On the second axis, HL (1.45), HLL (–1.05), and HW (0.96) were high contributors.

Coloration: In the samples of *M. okinavensis* from Yaeyama, brown speckling was evident from the chin and breast to the venter. The speckling is densely distributed to the posterior one-third or more of the abdomen in many specimens. By contrast, in the samples from Okinawa, the speckling was more scattered. It was seen only on the breast in half of the samples, and at most to the posterior one-third of the abdomen (Table 5).

These results of the morphological analyses concur with the genetic separation of Yaeyama and other populations of the Ryukyus, although not strongly. Moreover, results of phylogenetic analyses strongly suggested that each of them is genetically distinct species with relation to the Chinese species. Because the type locality of *M. okinavensis* is Okinawajima Is. of the Central Ryukyus (Stejneger, 1901), the population of the Yaeyama Group of the Southern Ryukyus should be regarded as a distinct species, which we name below.

Table 5. Variation in the distribution of dark spots on the breast and abdomen in male *Microhyla kuramotoi* sp. nov. from Yaeyama and *M. okinavensis* from Okinawa. Figures indicate the number of specimens (percentage frequency in parenthesis).

	On breast	To 1/3 of abdomen	Scattered to 2/3 of abdomen	Dense to 2/3 of abdomen	Dense on 2/3 or more of abdomen
Yaeyama	0	1 (8.3%)	0	2 (16.7%)	9 (75.0%)
Okinawa	8 (50.0%)	4 (25.0%)	4 (25.0%)	0	0

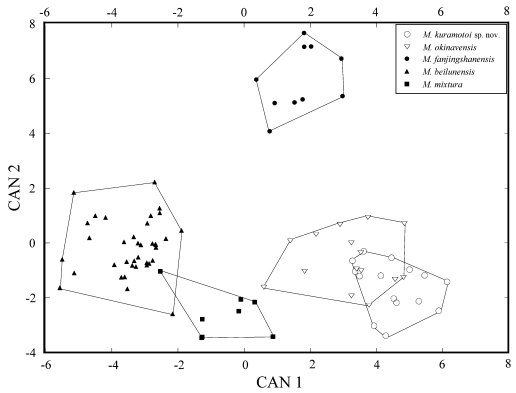

Fig. 2. Canonical components scores of morphological variables for male samples of *Microhyla*. Open circle: *M. kuramotoi* sp. nov., reverse triangle: *M. okinavensis*, closed circle: *M. fanjingshanensis*, closed triangle: *M. beilunensis*, closed square: *M. mixtura*.

Systematics

Microhyla ***kuramotoi*** sp. nov.
[Japanese name: Yaeyama-Himeama-Gaeru]
[English name: Yaeyama Narrow-Mouthed Toad]
Figs. 3 and 4

Microhyla okinavensis Stejneger, 1907, p. 89, (part); Parker, 1934, p. 138, (part); Okada, 1966, p. 42 (part).
Microhyla fissipes Okada, 1930, p. 63 (part); Okada, 1931, p. 71 (part).
Microhyla ornata Gressitt, 1938, p. 164, (part); Inger, 1947, p. 324 (part); Nakamura and Uéno, 1963, p. 66 (part).

Diagnosis

A member of the *Microhyla fissipes* species group of Garg et al. (2019), which is distinguished from other *Microhyla* groups by; small to medium-sized adults; nostrils placed towards the lateral sides of the snout; finger and toe tips rounded; terminal phalanges of toes knobbed or T-shaped; inner metatarsal tubercle present, elongate; outer metatarsal tubercle small, rounded; webbing between toes rudimentary; dorsal skin shagreened to sparsely granular; a narrow mid-dorsal line extending from tip of the snout to the vent.

Etymology

The specific name is dedicated to Dr. Mitsuru Kuramoto, Emeritus Professor of the Fukuoka University of Education, for his great contributions to Asian amphibian biology, including the fauna of the southern Ryukyus.

Holotype

University of the Ryukyus, Ryukyu University Museum (Fujukan) (RUMF-ZH)-01017 (former URE 5226), an adult male collected from Fukai, Ishigaki-shi, Ishigakijima Is., Okinawa Pref., Japan (24°27'30.9" N, 124° 13'35.5" E, 5.4 m asl) by A. Tominaga on 23 June 2019.

Paratypes

Ishigakijima: RUMF-ZH-01018-01031, 12 adult males and two females collected from Nosoko, Ishigaki-shi, by A. Tominaga on 19 September 2011. URE 5227, 5228, a male and a female, data same as the holotype. Iriomotejima: URE 391–392, a male and a

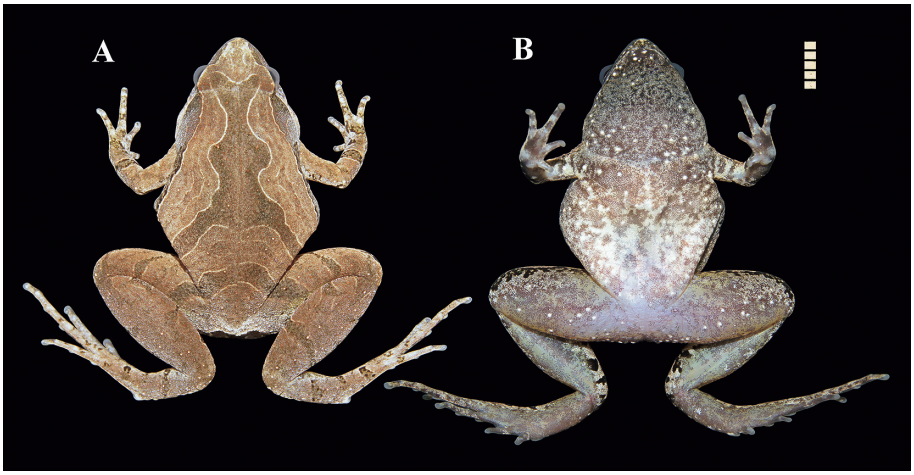

FIG. 3. Dorsal (A) and ventral (B) views of male holotype of *Microhyla kuramotoi* sp. nov. (RUMF-ZH-01017). Scale bar=5 mm.

FIG. 4. Ventral view of hand (A) and foot (B) of male holotype of *Microhyla kuramotoi* sp. nov. (RUMF-ZH-01017). Scale bar=5 mm.

female collected from Aira-gawa, near Komi, Taketomi-cho, Yaeyama-gun, by A. Tominaga on 18 September 2011; URE 406–407, a male and a female collected from Haimi, Taketomi-cho, Yaeyama-gun, by A. Tominaga on 18 September 2011.

FIG. 5. Larval *Microhyla kuramotoi* sp. nov. from Ishigakijima Is., the Yaeyama Group, stage 36 of Shimizu and Ota (2003), total length=20.6 mm. Dorsal (A), lateral (B), and ventral views (C). Scale bar=5 mm.

Description of holotype (measurements in mm)

SVL 28.6; habitus robust (Fig. 3); head triangular, slightly wider (HW, 10.6, 37.1% SVL) than long (HL, 10.5, 36.7%SVL); snout rounded dorsally and in profile, projecting beyond lower jaw; eye as large (EL, 3.3, 11.5%SVL) as snout (3.3, 11.5%SVL);

canthus rostralis rounded; lore sloping, very weakly concave; nostril lateral, below canthus rostralis, closer to tip of snout (1.3, 4.5% SVL) than to eye (1.8, 6.3%SVL); interorbital distance (IOD, 3.0, 10.5%SVL) larger than internarial distance (IND, 1.7, 5.9%SVL), the latter smaller than upper eyelid (UEW, 2.1, 7.3%SVL); pineal spot absent; tympanum hidden; upper jaw edentate; tongue oval, without papillae; slit-like openings to a median subgular vocal sac.

Forelimb short (16.5, 57.7%SVL); fingers free of web, but with slight skin fringes on both sides of second to fourth; finger length formula: I<II<IV<III (Fig. 4A); second finger (measured from inner side, 3.1, 10.8% SVL; outer, 2.0, 7.0%SVL) much longer than first (outer, 1.9, 6.6%SVL), and slightly shorter than fourth (inner, 2.6, 9.1%SVL); tips of three outer fingers not dilated, or forming disks, dorsally without median longitudinal groove; diameter of first finger (0.48, 1.7%SVL) four-fifths that of third finger (0.66, 2.3%SVL), the latter subequaling to width of phalange; a single outer palmar tubercle (1.0, 3.5%SVL) as large as inner (1.0, 3.5%SVL); distinct, rounded subarticular tubercles, formula 1, 1, 2, 2; nuptial pad absent (Fig. 4A).

Hindlimb long (HLL, 50.3, 175.9%SVL) about three times length of forelimb; tibia long (TL, 15.8, 55.2%SVL), heels overlapping when limbs are held at right angles to body; tibiotarsal articulation of adpressed limb reaching to center of eye; foot (FL, 16.1, 56.3%SVL) longer than tibia; tips of toes slightly dilated, wider than those of fingers (diameter of third toe, 0.77, 2.7%SVL), dorsally without median longitudinal groove; third toe (outer, 5.8, 20.2%SVL), longer than fifth (inner, 4.2, 14.7%SVL); webs between toes poorly developed (Fig. 4B), formula (the number of phalanges free of web): I 2–2+ II 2–3+ III 3–4 IV 4—2⅘ V; subarticular tubercles prominent, rounded, formula 1, 1, 2, 3, 2; inner metatarsal tubercle oval, large, length (1.8, 6.3%SVL) two thirds of first toe (2.7, 9.4%SVL); outer metatarsal tubercle elevated, smaller (1.3, 4.5%SVL) than inner.

Skin smooth above with a few low tubercles scattered; eyelid without supraciliary spines; no supratympanic fold discernible; a very low and narrow mid-dorsal skin fold extending from tip of snout to vent; side of body sparsely scattered with tubercles or low ridges; hindlimb dorsally scattered with few tubercles; ventral side of body and limbs smooth. Sides of toes with lateral dermal fringes extending to terminal swelling of toe tips.

Color

Color in life light brown dorsally (Fig. 3A), with a dark, reverse-V shaped mark medially continued from interorbital bar posteriorly to sacral region, followed by one weak and one strong dark bars in front of cloaca; dark marking is edged by narrow light lines; a black lateral stripe from posterior to eye extending above arm to near groin; a cream stripe extending from eye to axilla, dorsally bordering dark lateral stripe; a dark stripe from snout to eye; limbs dorsally with narrow dark brown bars; throat, chest, and anterior two-thirds of abdomen darkly pigmented on cream white (Fig. 3B). In preservative, pattern has not obviously changed, although color has slightly faded.

Variation

Male individuals of the type series are generally similar in size and body proportions (Table 3), although highly variable in coloration. Female paratypes have a larger body size (SVL, 28.8–34.6, 31.0±2.4 mm) than the males (<27.8 mm SVL), but tend to have shorter hindlimb relative to SVL (152–179%, median=167% vs. 166–193%, median= 180% in males). The point reached by the tibiotarsal joint when hindlimb is bent forward along the body is the center of the eye in 92.9% and the anterior border of eye in 7.1% in males, while the point is posterior edge to the center of the eye in 75% and the posterior border of eye in 25% in females. Throats of females are less darkly pigmented than those of males.

There may be some inter-island variations. Matsui and Ota (1984) gave mean SVL values for females from Iriomotejima Is. and Kohamajima Is. The former (29.6 mm) is similar to that of the present study, but the latter (26.3 mm) is much smaller.

Median longitudinal groove on dorsal surface of toe tips is normally absent, and, of 16 males examined, only four had a weak groove on either side of the third, fourth, or fifth toes.

Tadpoles

A total of six tadpoles from St. 34 (head-body length, 5.8 mm) to 36 (8.3 mm) from the type locality of *M. kuramotoi* sp. nov. were closely examined. Head and body flattened above, spheroidal below (Fig. 5); maximum head-body width at level of eye 61–70% (median=65%) of head-body length; maximum head-body depth 76–85% (median=80%) of maximum head-body width; snout broadly rounded, almost truncate in profile; eyes lateral, visible from below, eyeball diameter 9–12% (median=11%) of head-body length; interorbital space very wide, 376–493% (median=423%) of eyeball diameter; eye-snout distance 27–30% (median=28%) of head-body length. Oral disk dorso-terminal, small; lower lip with width 17–21% (median=24%) of maximum head-body width; labial teeth and jaw sheaths entirely absent, but lower labium scattered with small papillae on lateral margin. Spiracle opening median, without free flap, opening 91–96% (median=93%) of distance from tip of snout to end of body; vent median, in form of long tube directed nearly vertically downward, small opening at edge of ventral fin; thick loops of gut visible ventrally. Tail long and lanceolate, abruptly tapering in posterior half and drawn out into a short filament; tail length 157–184% (median=166%) of head-body length, maximum depth 28–35% (median=31%) of length; dorsal fin originating at end of head-body, with a straight margin, sub-parallel with much deeper ventral fin in anterior half of tail; ventral fin deeper than dorsal throughout anterior to tail tip; caudal muscle not strong, maximum tail width 22–31% (median=24%) of maximum head-body width; muscle depth maximum at origin, 35–44% (median=40%) of maximum tail depth, but steadily narrowed posteriorly, with depth at middle of tail shallower than fin depths. Color in life (Fig. 5) light brown on dorsum and laterally, with darker mid-dorsal band, and marking at end of flank; venter grey and belly semi-translucent; tail sparsely dotted with dark brown.

Range

Yaeyama Islands of Southern Ryukyus, Okinawa Pref., Japan: Ishigakijima Is., Taketomijima Is., Kohamajima Is., Iriomotejima Is., and Haterumajima Is. Artificially introduced into Kuroshima Is.

Natural history

Microhyla kuramotoi sp. nov. occurs from lowlands to montane regions, and lives on the ground among leaf litter and grasses. The breeding season extends almost the entire year, but is usually intensive from February to October. Film-like egg mass is laid on the surface of various bodies of still waters including ponds, rice fields, temporary pools, and sometimes slowly flowing small streams. Eggs are dark yellowish brown in the animal hemisphere. Females collected from Iriomotejima Is. and Kohamajima Is., respectively, contained 624–1207 (mean=916.9) and 271–890 (528.9) mature ova of 1.0–1.3 (mean=1.2) mm in diameter (Matsui and Ota, 1984 as *M. ornata*). Larvae form a cohort, swimming slowly in the middle and upper layers of water sucking in plankton.

Calls.

Calls were recorded at Ishigakijima Is. at an air temperature of 26.5°C on 24 June 2019 by A. Tominaga. Calls (36 notes from two males were analyzed) consisted of a series of notes each emitted at an interval (between the beginnings of two successive notes) of 0.48±0.06 (0.37–0.61) s (Fig. 6). Each note

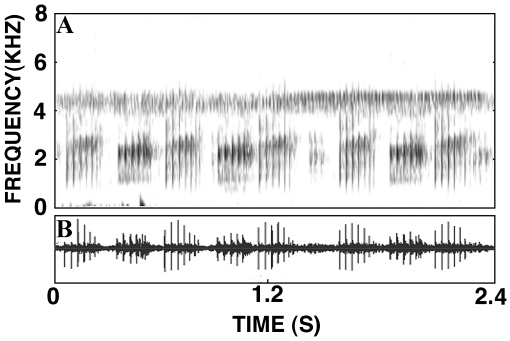

Fig. 6. Advertisement call of *Microhyla kuramotoi* sp. nov. from Ishigakijima Is., the Yaeyama Group, recorded at an air temperature of 26.5°C, showing sonogram (A) and wave form (B).

was composed of 6.2±1.1 (4–8) short pulses and lasted for 0.17±0.11 (0.11–0.21) s. Frequency bands spread over the 0.9–3.7 kHz range, and the dominant frequency was 2.4±0.1 (2.3–2.5) kHz. Frequency and intensity modulations were not marked.

Comparisons

Microhyla okinavensis, long confused with *M. kuramotoi* sp. nov., is placed in the *Microhyla fissipes* group of Garg et al. (2019), who split the group into the *Microhyla okinavensis* subgroup (*M. okinavensis* and *M. mixtura*) and the *Microhyla fissipes* subgroup (*M. chakrapanii* Pillai, 1977, *M. mukhlesuri* Hasan, Islam, Kuramoto, Kurabayashi, and Sumida, 2014, *M. mymensinghensis*, Hasan, Islam, Kuramoto, Kurabayashi, and Sumida, 2014 and *M. fissipes* Boulenger, 1884). Subsequently, *M. beilunensis* Zhang, Fei, Ye, Wang, Wang, and Jiang, 2018 and *M. fanjingshanensis* Li, Zhang, Xu, Lv, Jiang, Liu, Wei, and Wang, 2019 were split from *M. mixtura* (Zhang et al., 2018; Li et al., 2019). Garg et al. (2019) split the two subgroups mainly on the basis of the presence in the *M. okinavensis* subgroup and absence in the *M. fissipes* subgroup of dorso-terminal grooves on toes. However, this is misleading and as shown above (section of variation), development of dorso-terminal grooves on toes is highly variable, and cannot be a diagnostic character in this group.

Within the *Microhyla okinavensis* subgroup, *M. kuramotoi* sp. nov. is most similar to *M. okinavensis*, from which it was separated (e.g., male SVL, 23.8–28.6 mm vs. 20.8–25.6 mm in *M. okinavensis*), but differs from it in having brown speckling from chin and breast to wider area of venter (vs. speckling scattered at most to 2/3 of venter in *M. okinavensis*), and tending to have relatively shorter hindlimb (163%–193%, median=180%) than *M. okinavensis* (168–199%, median=185% in Okinawa population), and when the hindlimb is bent forward along the body, the tibiotarsal joint usually reaches the posterior edge to the center of the eye in *M. kuramotoi* sp. nov., but the joint usually reaches the anterior edge of the eye or forward in *M. okinavensis*.

Microhyla kuramotoi sp. nov. is unique in showing brown speckling from chin and breast to venter in both sexes. The pattern becomes coarse toward the breast and venter. This speckling extends to the lower surface of the thigh. Female *M. okinavensis* of the Okinawajima and Amamioshima populations have an almost immaculate underside, whereas males have a finely mottled pattern in the gular region.

The egg diameter of *M. kuramotoi* sp. nov. (1.20±0.03 [1.0–1.3] mm; Matsui and Ota, 1984 as the southern Ryukyu populations of *M. ornata*) was distinctly larger than that of *M. okinavensis* (0.97±0.04 [0.93–1.04] mm; Shimizu and Ota, 2003 as the Okinawajima populations of *M. ornata*). Larvae of the two species are very similar to each other and have practically no characters to separate them.

Kuramoto (1977) once suggested the presence of call differences between populations of *M. ornata* from Amamioshima Is. (*M. okinavensis*) and Iriomotejima Is. (*M. kuramotois* sp. nov.), in more complex frequency distribution and longer duration of calls in the former than in the latter. However, Kuramoto and Joshy (2006) refuted the previous state-

ment by concluding that advertisement call structures in *M. okinavensis* from Iriomote-jima Is. (*M. kuramotois* sp. nov.), Okinawa-jima Is., and Amamioshima Is. (*M. okinavensis*) were essentially identical.

Microhyla kuramotoi sp. nov. significantly differs from *M. mixtura* by larger SVL (23.8–28.6 mm in males and 28.8–34.6 mm in females vs. 18.8–25.2 mm and 23.8–26.6 mm, respectively, in *M. mixtura*), larger RHL and RUEW, and smaller RIOD, less complex dorsal color pattern, and different point of tibiotarsal articulation of adpressed limb (reaching to center of eye vs. reaching posterior margin of eye in *M. mixtura*). Larval *M. kuramotoi* sp. nov. differs from *M. mixtura* larva by light brown dorsal color and transparent tail fins sparsely dotted with dark brown (vs. dark brown dorsal color and upper and lower fins darkly colored on the margins in *M. mixtura*).

The new species differs from *M. fanjingshanensis* by the lack of a distinct arrow-shaped white stripe on the upper midsection of ventrum (vs. stripe present in *M. fanjingshanensis*), larger SVL (23.8–28.6 mm in males and 28.8–34.6 mm in females vs. 19.0–22.7 mm and 22.5–23.0 mm, respectively, in *M. fanjingshanensis*), and larger REL and smaller RHL, RHW, RIND, and RTL than *M. fanjingshanensis*, different point of tibiotarsal articulation of adpressed limb (reaching to center of eye vs. reaching level between eye and nostril in *M. fanjingshanensis*), and usual absence (vs. presence in *M. fanjingshanensis*) of median longitudinal grooves on tips of toes, except for the first one. In advertisement call, *M. kuramotoi* sp. nov. has each note composed of 4–8 short pulses lasting for 0.11–0.21 s with dominant frequency at 2.3–2.5 kHz (vs. each note with 10–12 pulses, with a duration of 0.31– 0.67 s and the average dominant frequency of 7.7 kHz in *M. fanjingshanensis*).

Microhyla kuramotoi sp. nov. differs from *M. beilunensis* by larger SVL (23.8–28.6 mm in males and 28.8–34.6 mm in females vs. 19.1–23.7 mm and 26.4–28.3 mm, respectively, in *M. beilunensis*), significantly larger RLAL, RHAL, RHLL, RTL, RFL, and RTFL, and smaller RIND and RIOD than *M. beilunensis*, and usual absence (vs. presence in *M. beilunensis*) of median longitudinal grooves on toe tips. In larva, *M. kuramotoi* sp. nov. differs from *M. beilunensis* by having light brown dorsal color and transparent tail fins sparsely dotted with dark brown (vs. dark brown dorsal color and upper and lower fins darkly colored on the margins in *M. beilunensis*).

The new species differs from *M. fissipes* by body size (SVL, 23.8–28.6 mm in males and >28.8 mm in females vs. about 22.0 mm in males and 25.0–26.0 mm in females of *M. fissipes*), possible presence (vs. complete absence in *M. fissipes*) of median longitudinal grooves on toe tips, absence (vs. presence in *M. fissipes*) of X-shaped dorsal marking, and point of tibiotarsal articulation of adpressed limb (reaching to center of eye vs. reaching level near eye in *M. fissipes*).

Microhyla kuramotoi sp. nov. differs from *M. mukhlesuri* by body size (SVL, 23.8–28.6 mm in males and >28.8 mm in females vs. 16.5–21.0 mm in males and 17.3–18.4 mm in female in *M. mukhlesuri*), absence (vs. presence in *M. mukhlesuri*) of X-shaped dorsal marking, and point of tibiotarsal articulation of adpressed limb (reaching to center of eye vs. reaching level to snout in *M. mukhlesuri*).

The new species differs from *M. mymensinghensis* by body size (SVL, 23.8–28.6 mm in males and >28.8 mm in females vs. 14.2–17.6 mm in males, 15.2–21.3 mm in female in *M. mymensinghensis*), and point of tibiotarsal articulation of adpressed limb (reaching to center of eye vs. reaching level between eye and tip of snout in *M. mymensinghensis*).

Microhyla kuramotoi sp. nov. differs from *M. chakrapanii* by possible presence (vs. absence in *M. chakrapanii*) of median longitudinal grooves on toe tips, and absence (vs. presence in *M. chakrapanii*) of minute tubercles dorsally on tibia.

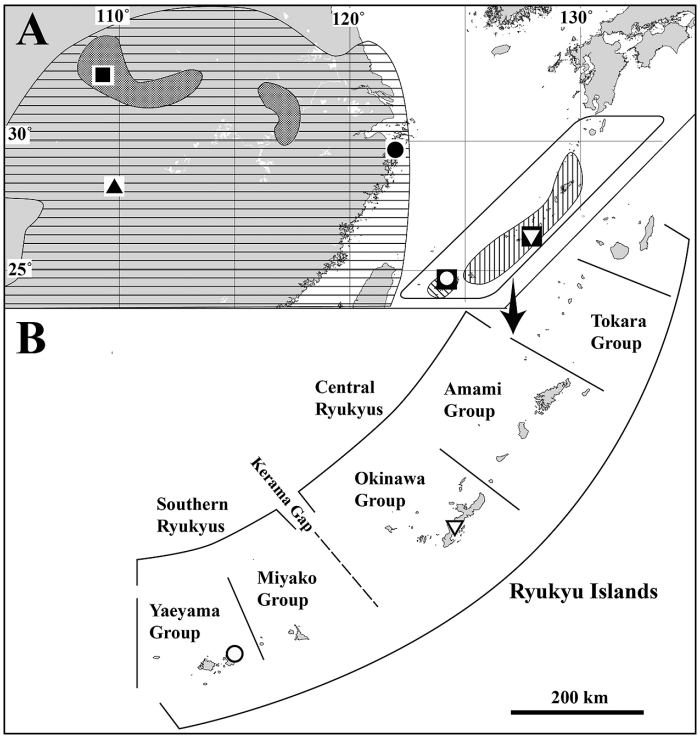

Fig. 7. Map of East Asia (A) and Ryukyu Islands (B), showing distribution of *Microhyla* species. Open circle and obliquely hatched area: *M. kuramotoi* sp. nov., reverse triangle and vertically hatched area: *M. okinavensis*, closed circle: *M. beilunensis*, closed triangle: *M. fanjingshanensis*, closed square and finely dotted area: *M. mixtura*, horizontally hatched area: *M. fissipes*.

Discussion

For a long time, populations of *M. okinavensis* were known to occur in both Southern and Central Ryukyus across the intervening Kerama Gap (Maeda and Matsui, 1999; Matsui et al., 2005), but recent genetic studies suggested that populations from each of these two regions are independent evolutionarily significant units, which might be more appropriately treated as independent species (Fig. 7; Matsui et al., 2005; Matsui et al., 2011; Tominaga et al., 2019).

It was Stejneger (1907) who first studied samples of *M. okinavensis* from Southern Ryukyus (Ishigakijima Is.). Subsequently, Okada (1930, 1931, as *M. fissipes*) and Parker (1934) also studied both Southern and Central Ryukyu specimens, but no authors referred to their differences. In contrast, Inger (1947), as *M. ornata*, found Ishigaki samples (Southern Ryukyus) to have larger body but relatively shorter hindlimb than Okinawa samples (Central Ryukyus). More recently, Kuramoto and Joshy (2006) compared them in relation to *M. ornata* and found morphological differences, some of which we could not confirm.

Matsui et al. (2005) first suggested based on genetic evidence that populations from Southern (now *M. kuramotoi* sp. nov.) and Central Ryukyus (*M. okinavensis*) greatly differ from each other. Later, Tominaga et al. (2019) showed them not to be sister species to each other; *Microhyla kuramotoi* sp. nov. and Chinese *M. mixtura*, and *M. okinavensis* and Chinese *M. beilunensis*, respectively, were sister species. In the present study, we

confirmed a close relationship of *M. kuramotoi* sp. nov. and *M. mixtura*. In contrast, *M. okinavensis* showed a close relationship with newly added *M. fanjingshanensis*, and these two species formed a clade with *M. beilunensis*.

Our present result contrasts sharply with Li et al. (2019), in which *M. okinavensis* formed a clade with *M. mixtura*, and this clade, *M. fanjingshanensis*, and *M. beilunensis* formed a clade with unresolved relationships. However, the result of Li et al. (2019) seems to be distorted by the two sequences of *M. okinavensis* they employed, one from Okinawa (middle Ryukyus) and another from Ishigaki (southern Ryukyus=*M. kuramotoi* sp. nov.). Actually, of the three genes (12S, 16S, and COI) Li et al. (2019) used for their phylogenetic analyses, all genes were examined for only one sample from Okinawa, and only 16S was employed for another sample from Ishigaki.

Tominaga et al. (2019) estimated the ages of divergence between the *M. okinavensis* subgroup and the *M. fissipes* subgroup in the Middle Miocene (ca. 15.8 MYBP), and between the clade of *M. okinavensis* and the clade of *M. kuramotoi* sp. nov. and *M. mixtura* around the Middle to the Late Miocene (ca. 10.7 MYA). Subsequent divergences were thought to have occurred in the Late Miocene between *M. kuramotoi* sp. nov. and *M. mixtura* ca. 5.1 MYBP, after the first divergence in the clade of *M. okinavensis* ca. 7.3 MYBP. *Microhyla kuramotoi* sp. nov. and *M. mixtura* are discretely distributed in the Yaeyama Islands and Inland China, respectively, while *M. okinavensis* is distributed only in the Ryukyu Archipelago north of the Yaeyama Islands with its related lineage, *M. beilunensis*, in a very limited area of coastal China. Much wider intervening areas in the East Asian region are now occupied by *M. fissipes*, which would have forced the species of the *M. okinavensis* subgroup to be relict lineages (Tominaga et al., 2019).

Only two frog species (*M. okinavensis* and *Buergeria japonica*) were long known to occur in both Southern and Central Ryukyus across the intervening Kerama Gap (Nakamura and Uéno, 1963; Maeda and Matsui, 1989; Matsui and Maeda, 2018), but, as shown here, *M. okinavensis* is now restricted to the Central Ryukyus, and the Southern Ryukyu populations are a distinct species, *M. kuramotoi* sp. nov. Similarly, *B. japonica* is also surely to be split into distinct species (Tominaga et al., 2015). However, the pattern of distribution differs between *M. kuramotoi* sp. nov. and *B. japonica*. In the case of *B. japonica*, the Southern Ryukyu population, distinct from the Central Ryukyu population, also occurs on northwestern Taiwan (Tominaga et al., 2015). The reason for this difference is unknown, but the presence of a competitor would have enabled current situations. *B. japonica* and its probable competitor *B.* sp. Southern Taiwan (see Matsui and Tominaga, in press) segregate on Taiwan, the former occurring in the northwest, while the latter occupy the southeastern region of the island. In contrast, *M. fissipes*, a possible competitor for *M. kuramotoi* sp. nov. is dominant, occupying regions throughout Taiwan. This condition is just the same as in the China continent, where *M. mixtura*, and two other relatives of *M. kuramotoi* sp. nov., have relict distributions in the range of *M. fissipes*.

Finally, another biologically interesting problem is the absence of *M. kuramotoi* sp. nov. but presence of *M. okinavensis* on Miyakojima Is. (Tominaga et al., 2019). As discussed by Tominaga et al. (2019), the origin of the non-volant terrestrial fauna of Miyakojima Is. is mysterious. The island is suggested to have been nearly completely submerged in the Middle to the Late Pleistocene (ca. 0.4–1.0 MYBP) (Kizaki, 1985; Iryu et al., 2006), but seems to have never been connected with the Yaeyama Islands during the last glacial maximum (Iryu personal communication with AT on 13 April 2020), in contrast to currently popular hypothesis (Kimura, 2003; Kizaki and Oshiro, 1980). Thus, the idea of dispersal of some amphibian and reptile species from the Yaeyama

Islands (Ota, 1998) also requires reconsideration.

Other than *M. okinavensis*, *Fejervarya sakishimensis* Matsui, Toda and Ota, 2008 (2007) occurs on the island as a native amphibian. The species is replaced by *F. kawamurai* Djong, Matsui, Kuramoto, Nishioka, and Sumida, 2011 in the region north of Miyakojima Is. Genetically, the population of this species from Miyakojima Is. is close to the Yaeyama populations (Toda et al., 1997). Thus the two anuran species native to Miyakojima Is. (Toyama, 1976) are thought to have different origins. The divergence between Okinawa and Miyako subclades of *M. okinavensis* was estimated as 4.6 (3.3–5.9) MYBP, largely overlapping the times of divergence between Yaeyama subclade (*M. kuramotoi* sp. nov.) and *M. mixtura* at 5.1 (3.4–6.9) MYBP (Tominaga et al., 2019). Possibly invasion of ancestral *M. kuramotoi* sp. nov. from Yaeyama to Miyako would have been prevented by already diverging ancestral Miyako subclade of *M. okinavensis*.

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