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Research Article

A New Frog Species of the Genus *Glandirana* from Southeastern Kyushu, Japan (Anura Ranidae)

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Abstract

Glandirana rugosa, long thought to be a single species living in Japan, is separated phylogenetically into four local groups, designated *G. rugosa*-West, -North, -East, and -Central. Based on genetic identity values of the complete mitogenome sequences of the four groups, we propose that *G. rugosa*-West represents *G. rugosa* sensu stricto (s.str.) and *G. rugosa*-East and -Central should have a different species name. *G. rugosa*-North may be separated from *G. rugosa*-West as a new species as well. Recently we have identified a phylogenetically distinct new group of *G. rugosa* in southeastern Kyushu of Japan, designated se-Kyushu. On a phylogenetic tree, se-Kyushu is clustered differently from *G. rugosa*-West, although the two populations inhabit parapatrically in Kyushu. We observed significant differences in eye distance (ED) and distance between tympanum and eye (T-ED) and advertisement calls between se-Kyushu and *G. rugosa*-West. Genetic identity of the mitogenomes between the two populations was much lower than the range of intraspecific genetic identity in the Ranidae frogs. In addition, we observed lower survival rates of embryos and tadpoles of the first reciprocal combination, when the two populations were hybridized. Thus, we concluded that se-Kyushu is a new species in the genus *Glandirana*.

Keywords: *Glandirana nakamurai* sp. nov.; Mitochondrial DNA Phylogeny; Morphometry; New Species; Southeastern Kyushu

Headings

- *Glandirana rugosa* consists of four local groups, designated *G. rugosa*-West, -North, -East, and -Central.
- *G. rugosa*-West should represent *G. rugosa* s.str., since von Siebold probably collected *G. rugosa* frogs in Nagasaki, Japan.
- We identified a phylogenetically distinct new group of *G. rugosa* in southeastern Kyushu of Japan that we named se-Kyushu.
- The habitat of se-Kyushu and *G. rugosa*-West was parapatric in Kyushu.
- On the RAxML phylogenetic tree se-Kyushu was in a cluster different from *G. rugosa*-West.
- We observed significant differences in morphology and advertisement calls between se-Kyushu and *G. rugosa*-West.

- Genetic identity of the mitogenomes between the two populations was much lower than the range of intraspecific genetic identity in the Ranidae frogs.
- Lower survival rates of embryos and tadpoles of the first reciprocal combination were observed, when the two populations were hybridized.
- Thus, se-Kyushu can be described as a new species in the genus *Glandirana*, named *G. nakamurai* sp.nov.

Introduction

During his Japan stay from 1823 to 1829, PH. F. von Siebold spent most of his time in Nagasaki except for five months to make a round trip between Nagasaki and Edo (present day Tokyo) [6]. He collected many specimens of animals and plants in Japan. His collections are detailed in the books of "Fauna Japonica 1 to 4," that he edited. One of his many collections was of the frog, originally described as *Rana rugosa* [27]: the name has since been changed to *Glandirana rugosa* [3]. This species is distributed across Japan, Korea and northeastern China [8,12], but one group of the frogs in China and Korea is described as *G. emeljanovi* [14]. The *G. rugosa* frog is separated phylogenetically into four local groups in Japan, designated *G. rugosa*-West, -North, -East, and -Central within the same species [18]. *G. rugosa*-West inhabits Kyushu, Shikoku, and western Honshu. Recently, in the process of mapping the territory of each group of *G. rugosa*, we have identified a phylogenetically distinct new group of *G. rugosa* in southeastern Kyushu, designated se-Kyushu. Surprisingly se-Kyushu did not phylogenetically cluster with any of the pre-existing groups [19]. In addition, we have observed significant differences in ED and T-ED, advertisement calls and nucleotide sequences of the mitogenomes between se-Kyushu and *G. rugosa*-West, although their territories are parapatric (Figure 1). Moreover, we observed lower survival rate of embryos and tadpoles obtained by crossing between se-Kyushu and *G. rugosa*-West. Thus, we concluded that the se-Kyushu is a new species in the genus *Glandirana*.

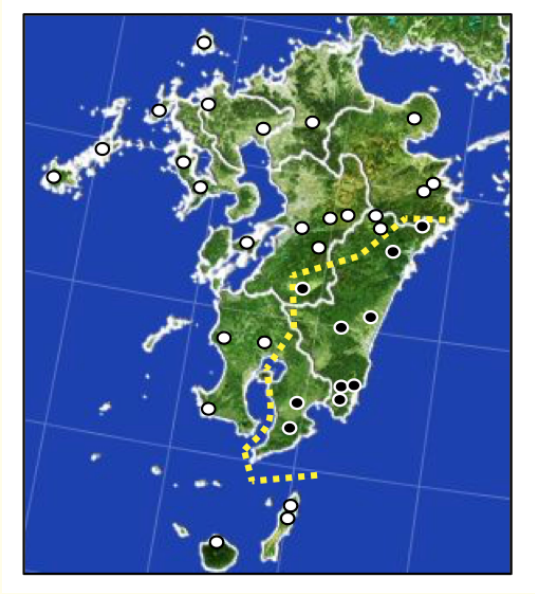

Figure 1: *Distribution of the two populations in Kyushu.*
Black and white circles indicate the sites at which se-Kyushu and G. rugosa-West frogs were captured, respectively. The yellow dotted line indicates a predicted border between the two populations. The figure comes from Oike., et al. [19].

Research Methodology

Ethics statement

Manipulations of embryos, tadpoles, and frogs were approved by the Ethics Committee of Waseda University (Permit Number, 2020-A056). We performed all the animal experiments according to the Fundamental Guidelines for Proper Activities in Academic Research Institutions (Notice No. 71 of the Ministry of Education, Science, and Culture of Japan, 2006) and the Prevention of Cruelty to Animals Act (Notice No. 88 of the Ministry of the Environment of Japan, 2006).

Eggs

Unfertilized eggs were obtained from se-Kyushu and *G. rugosa*-West (Kyushu) frogs that were collected at Higashi-kaminaka, Sadowara-cho, Miyazaki City, Miyazaki Pref. (32°00'43.7"N 131°24'42.7"E), and Hoshino-mura, Yame City, Fukuoka Prefecture (33°16'25.1"N 130°43'32.0"E), respectively. A frog collected in Hoshino-mura (NSMT-H14089) was deposited at the National Museum of Nature and Science in Tsukuba City, Ibaraki Prefecture. The eggs were artificially inseminated with sperm according to Iwade., *et al*. [7]. The thus-fertilized eggs were allowed to develop into juvenile frogs after metamorphosis. Tadpoles were staged according to Shumway [23] and Taylor and Kollros [26].

Morphometry

We captured adult frogs of se-Kyushu, *G. rugosa*-West, and -North and measured 15 morphological traits to the nearest 0.1 mm with a vernier caliper. The number of frog specimens for morphological study are shown in parentheses following the name of each group in table 1. Parameters used are defined elsewhere [19,28] and were: snout-vent length (SVL), head length (HL), head width (HW), snout length (SL), eye diameter (ED), distance between tympanum and eye (T-ED), tympanum diameter (TD), internarial distance (IND), interorbital distance (IOD), forelimb length (FLIMB), hind-limb length (HLIMB), thigh length (THIGH), tibia length (TIBIA), foot length (FOOT) and inner metatarsal tubercle length (IMTL). We also measured lengths of fingers of the forelimbs and hind-limbs. Then, specimens of females and males were soaked in 50% and then 70% ethanol solution and stored.

	Stages		**Stages**		**Stages**		**Stages**	
	St. VI	**St. VI**	**St. XII**	**St. XII**	**St. XX**	**St. XX**	**St. XXV**	**St. XXV**
	G. nakamurai (n = 5)	*G. rugosa* (n = 5)	*G. nakamurai* (n = 6)	*G. rugosa* (n = 6)	*G. nakamurai* (n = 6)	*G. rugosa* (n = 6)	*G. nakamurai* (n = 9)	*G. rugosa* (n = 9)
Body Length (mm)	35.0 ± 2.2	34.6 ± 2.4	45.0 ± 1.9	46.7 ± 2.2	49.8 ± 1.9	48.3 ± 2.9	16.1 ± 0.44	16.0 ± 0.3
Head Length (mm)	11.9 ± 1.3	12.2 ± 0.9	13.5 ± 1.4	13.7 ± 0.8	16.4 ± 1.1	16.2 ± 1.3		

Table 1: *Body and head lengths of G. nakamurai sp. nov. and G. rugosa-West tadpoles. We measured body and head lengths of G. nakamurai sp. nov. and G. rugosa-West tadpoles at different developmental stages. Numbers in parentheses indicate the number of individuals examined.*

Calls

Three males from each group were used to analyze pulse number, pulse interval and durations of calls. Calls of males from se-Kyushu (Kimotsuki, Kagoshima), *Glandirana rugosa*-West (Omura, Nagasaki), and -North (Tokamachi, Niigata) groups were recorded using a

digital voice recorder with a microphone inside (44.1 kHz/16 bits; model ICD-UX560F, SONY, Tokyo) at air and water temperatures of 22.3°C and 19.7°C, on June 10, 2017, 24.5°C and 19.1°C on May 26, 2019, and 20.9°C and 18.5°C on May 24, 2017, respectively. The three parameters were measured using Praat version 6.0.29 [1] and RAVEN LITE 2.0 (Cornell Laboratory of Ornithology), as described by Hasegawa., *et al*. [5].

Molecular phylogeny

We isolated mitochondrial (mt) DNA from liver cells of adult frogs using the mt DNA extractor CT kit (FUJIFILM Wako, Tokyo, Japan) according to the manufacturer's protocol. DNA fragments of non-cording D-loop to the *16S rRNA* gene were amplified from 100 ng of the extracted DNA using two primers (forward 5'-CCTACCCACTTCAGATCCTACAT-3' and reverse 5'-GGCGTGGTAAGACTAGGCG-3'). The fragments amplified by PCR were sub-cloned into the pTAC2 vector (Bio Dynamics, Tokyo, Japan) and sequenced, as described previously [19]. Based on nucleotide sequences of non-cording D-loop to the *16S rRNA* gene in the mt DNA from se-Kyushu (4,445-bp; ACCN LC574079), *G. rugosa* (LC536281-4), *G. emeljanovi* (MH972198), and *Pelophylax nigromaculata* (AB043889), we constructed a RAxML phylogenetic tree using the standard RAxML program (http://github.com/stamatak/standard-RAxML). We used the GTR + G nucleotide substitution model that was obtained by Partition Finder (http://www.robertlanfear.com/partitionfinder/).

A pseudo *tRNA-Ser (AGY)* gene

DNA fragments of a pseudo *tRNA-Ser* (AGY) gene were amplified from 100 ng of the extracted DNA using the primers (forward 5'-TCAACTGGTCTTCAATCGCCT-3' and reverse 5'-AGAAGAAGCGCTTGTTTGGC-3'). The fragments were sub-cloned into the pTAC2 vector (Bio Dynamics, Tokyo, Japan) and sequenced as described previously [19]. Alignment of the pseudo and non-pseudo *tRNA-Ser* (AGY) genes from se-Kyushu, four groups of *G. rugosa*, *G. emeljanovi* (MH972198) and *Pelophylax nigromaculata* (AB043889) was obtained by the multiple alignment program for nucleotide sequences (MAFFT version 7; https://mafft.cbrc.jp/alignment/server/).

Survival rate of embryos and tadpoles

For the crossing experiment, we collected adult male and female frogs from Higashi-Kaminaka, Miyazaki on June 9, 2019 for se-Kyushu, and from Hoshino-mura, Fukuoka for *G. rugosa*-West on June 7, 2019. Fertilized eggs were obtained by mating from three pairs of adult males and females. Then, we allowed them to develop into juvenile frogs just after metamorphosis and determined survival rates of individuals at different developmental stages obtained by crossing between se-Kyushu and *G. rugosa*-West.

Statistics

Data were represented as the mean±SD. Differences ($P < 0.05$) determined using one-way ANOVA were considered statistically significant.

Image acquisition and analysis

Images were scanned and adjusted for brightness and contrast by Adobe Photoshop CS2.

Research Results

Systematics

Glandirana nakamurai sp. nov. (Japanese name: Himuka gaeru. English name: Himuka frog).

Etymology

The specific name was derived from the name of M. Nakamura who found this population.

Holotype

An adult female was collected from Ushiroda, Kimotsuki-cho, Kagoshima Prefecture (31°17'00.3"N 130°57'05.3"E) by M. Nakamura at 14:30 o'clock on June 8, 2019. A specimen (NSMT-H14085) was preserved in 70% ethanol and deposited at the National Museum of Nature and Science.

Paratype

Adult females (NSMT-H14086-8) were collected from Higashi-Kaminaka, Sadowara-cho, Miyazaki Prefecture (32°00'43.7"N 131°24'42.7"E) by M. Nakamura at 10:30 o'clock on June 9, 2019, preserved in 70% ethanol and deposited at the National Museum of Nature and Science.

Description of holotype (measurements in mm)

Snout-vent length (SVL, 46.8), head length (16.6, 34.2% SVL), head width (15.3, 32.7% SVL), snout length (6.0, 12.3% SVL), eye distance (5.8, 11.9% SVL), distance between tympanum and eye (1.4, 2.9% SVL), tympanum diameter (4.2, 8.6% SVL), internarial distance (3.3, 6.8% SVL), interorbital distance (4.0, 8.2% SVL), forelimb length (28.6, 58.8% SVL), hindlimb length (77.7, 159.9% SVL), thigh length (19.9, 40.9% SVL), tibia length (23.4, 48.1% SVL), foot length (35.9, 73.9% SVL), and inner metatarsal tubercle length (5.2, 10.7% SVL). The tibia was about 2.0 times longer than the arm (from the tip of the longest third finger to the elbow; 11.5, 21.6% SVL).

Colors

The dorsal skin of the body, forelimbs and hind-limbs of *G. nakamurai* was brown (Figure 2A and B). The ventral skin color of the body was light yellow (Figure 2B). Some dark brown spots were observed on the skin of the ventral side of the jaw, abdomen, forelimbs, and hind-limbs of adult frogs (Figure 2B). The dorsal skin of the forelimb hands (Figure C) and hind-limb feet (Figure D) of *G. nakamurai* had 12-16 and 6-8 black transect lines, respectively, whereas that of hands and feet possessed 12-15 and 6 such lines [19]. Feet had well-developed webs (Figure 2D): the webbing formula was determined by the criteria of Savage and Heyer [21] and was I1-2II1-2III1-2IV2-1V. Fingers were slender and their tips were slightly depressed. Excision of membrane between the two outer fingers reached the middle subarticular tubercle of the fourth finger when fingers were in contact. A skin fold developed on the outer margin of the fifth finger from near the proximal subarticular tubercle to near the distal articulation. Lengths (mm) of the right forelimb hand and hind-limb foot of *G. nakamurai* female were as follows: right forelimb hand, 1st finger, 6.9; 2nd, 6.2; 3rd, 8.8; 4th, 7.9; and right hind-limb foot, 1st toe, 19.9; 2nd, 28.2; 3rd, 21.5; 4th, 15.3; 5th, 11.7.

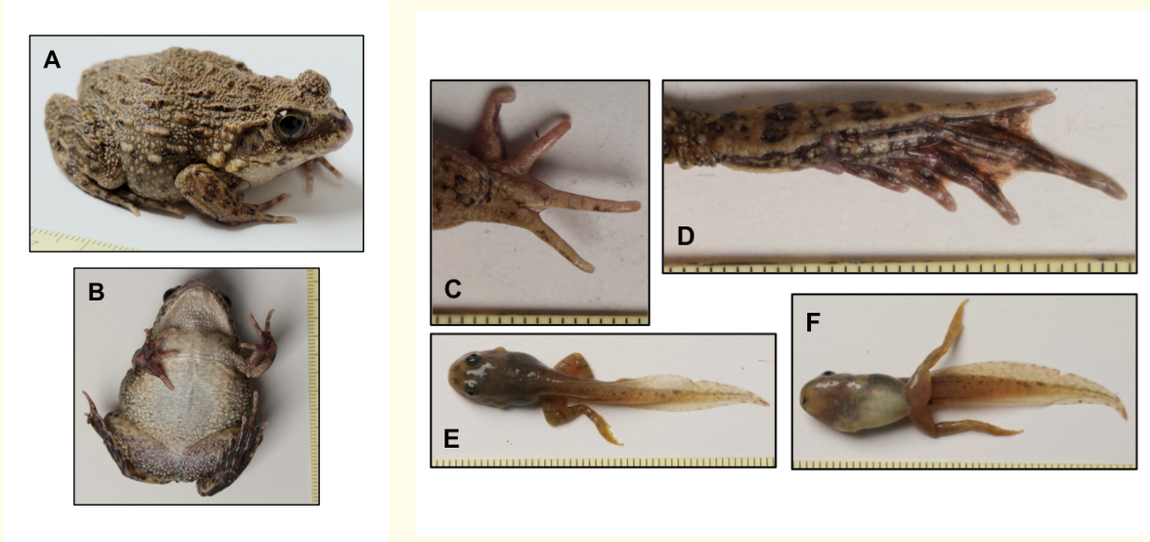

Figure 2: *Specimens of G. nakamurai.*
Dorsolateral (A) and ventral (B) views of an adult female frog. Dorsal views of right hand (C) and foot (D). Dorsal (E) and ventral (F) views of a tadpole at stage XVI. Scale bar is 1 mm.

Tadpoles

The dorsal skin of tadpoles of *G. nakamurai* was light brown as was that of *G. rugosa*-West (Figure 2E and F). Anterior and posterior abdominal skin colors were light brown and creamy, respectively. The tail fin was well-developed at the center (Figure 2E and F). The dorsal and abdominal skin color of tadpoles of *G. nakamurai* was similar to that of *G. rugosa*-West [19]. Next, we measured head and body lengths of tadpoles of *G. nakamurai* and *G. rugosa*-West: the body of a tadpole is considered to consist of a head and a tail. Head and body lengths of *G. nakamurai* vs. *G. rugosa*-West tadpoles at different developmental stages are summarized in table 1. Head lengths of tadpoles at St. VI, XII and XX appeared to be approximately one third of the body lengths (30 to 35%). In addition, the SVLs of juvenile frogs of *G. nakamurai* vs. *G. rugosa*-West at St. XXV just after metamorphosis were similar to each other (Table 1). Thus, there were no significant differences in head and body lengths of tadpoles between the two populations.

Variation

Individuals of *G. nakamurai* were recorded as generally similar to each other in morphology, but the SVL of females was longer than that of males (female (n = 5) vs. male (n = 7); 53.4 ± 2.2 vs. 44.6 ± 1.6 mm, P < 0.01) [19].

Morphological traits

We compared fifteen morphological characteristics of *G. nakamurai* with those of *G. rugosa*-West and -North in table 2. In males (♂) and females (♀), the T-ED of *G. nakamurai* was significantly longer than that of *G. rugosa*-West (*P < 0.05, Table 2) and -North (**P < 0.01). In females (♀) and males (♂), the ED of *G. nakamurai* was significantly smaller than that of *G. rugosa*-West (**P < 0.01 and *P < 0.05, respectively). In females, the ED of *G. nakamurai* was significantly smaller than that of *G. rugosa*-North (*P < 0.05). Evidently there were significant differences in the T-ED and ED between *G. nakamurai* and *G. rugosa*-West and also between *G. nakamurai* and *G. rugosa*-North. In addition, the IND of *G. nakamurai* males was significantly shorter than that of *G. rugosa*-North males (**P < 0.01). Thus, there were significant differences in morphological traits between *G. nakamurai* and *G. rugosa*-West and between *G. nakamurai* and *G. rugosa*-North.

	G. nakamurai	***G. nakamurai***	***G. rugosa***	***G. rugosa***	***G. rugosa***	***G. rugosa***
	(♂)	**(♀)**	**North (♂)**	**North (♀)**	**West (♂)**	**West (♀)**
Characteristics	**n = 7**	**n = 5**	**n = 18**	**n = 12**	**n = 5**	**n = 5**
SVL	44.6 ± 1.6	53.4 ± 2.2	49.4 ± 3.2	55.5 ± 3.1	42.2 ± 1.8	50.0 ± 3.0
	42.5 ~ 47.2	50.7 ~ 56.1	41.7 ~ 54.2	45.3 ~ 59.7	40.3 ~ 44.7	46.0 ~ 53.5
HL	15.4 ± 0.7	16.9 ± 0.9	17.2 ± 1.0	18.0 ± 1.0	15.4 ± 1.4	17.4 ± 0.8
	14.6 ~ 16.8	15.8 ~ 18.1	15.7 ~ 18.9	15.7 ~ 19.5	13.0 ~ 16.9	16.4 ~ 18.7
HW	15.1 ± 0.8	17.6 ± 1.0	16.8 ± 1.0	17.8 ± 1.3	15.5 ± 0.6	17.4 ± 2.0
	13.8 ~ 16.3	16.8 ~ 19.0	14.7 ~ 18.8	15.5 ~ 20.0	14.7 ~ 16.6	15.3 ~ 20.3
SL	4.9 ± 0.5	5.4 ± 0.6	5.5 ± 0.5	5.7 ± 0.5	4.9 ± 0.6	6.4 ± 0.4*
	4.1 ~ 5.6	4.6 ~ 6.1	4.2 ~ 6.3	4.8 ~ 6.5	4.1 ~ 5.4	6.0 ~ 7.0
ED	4.2 ± 0.2	4.6 ± 0.5	4.8 ± 0.6	5.5 ± 0.4*	4.9 ± 0.4*	5.8 ± 0.4**
	4.0 ~ 4.6	4.2 ~ 5.3	4.0 ~ 5.8	4.7 ~ 6.1	4.3 ~ 5.3	5.4 ~ 6.6
T-ED	1.6 ± 0.2	2.0 ± 0.2	1.2 ± 0.2**	1.5 ± 0.2**	1.1 ± 0.2*	1.4 ± 0.2*
	1.2 ~ 1.8	1.7 ~ 2.3	0.8 ~ 1.6	1.1 ~ 2.0	0.9 ~ 1.4	1.1 ~ 1.8
TD	4.1 ± 0.4	4.5 ± 0.2	5.2 ± 0.4**	5.0 ± 0.6	4.2 ± 0.2	4.3 ± 0.2*
	3.3 ~ 4.7	4.3 ~ 4.7	4.2 ~ 5.8	4.2 ~ 6.0	4.0 ~ 4.4	4.0 ~ 4.6
IND	3.5 ± 0.3	3.7 ± 0.1	4.1 ± 0.4**	4.3 ± 0.4	3.4 ± 0.1	3.8 ± 0.3
	2.8 ~ 3.7	3.6 ~ 3.8	3.3 ~ 4.9	3.2 ~ 4.8	3.2 ~ 3.5	3.3 ~ 4.1
IOD	4.0 ± 0.4	5.2 ± 0.6	4.4 ± 0.7	4.9 ± 0.4	4.0 ± 0.6	4.5 ± 0.3
	3.5 ~ 4.6	4.6 ~ 6.0	3.4 ~ 5.9	3.7 ~ 5.5	3.0 ~ 4.7	4.0 ~ 4.9

FLIMB	23.4 ± 2.6	28.7 ± 1.3	25.7 ± 2.4	29.6 ± 2.5	24.7 ± 2.1*	30.8 ± 3.6
	19.3 ~ 27.6	26.9 ~ 30.1	19.6 ~ 27.9	22.4 ~ 34.8	21.3 ~ 27.9	27.0 ~ 30.4
HLIMB	68.4 ± 4.6	76.3 ± 1.7	73.5 ± 3.6	83.7 ± 5.3	66.8 ± 1.8	77.4 ± 2.6
	63.2 ~ 76.7	75.0 ~ 78.6	67.0 ~ 78.5	68.8 ~ 92.3	64.0 ~ 67.4	73.2 ~ 80.1
THIGH	18.9 ± 1.5	21.6 ± 1.4	20.3 ± 0.9	23.2 ± 1.4	19.1 ± 1.0	20.4 ± 2.1
	16.6 ~ 21.3	19.7 ~ 23.0	18.7 ~ 22.2	19.6 ~ 26.0	17.6 ~ 19.4	19.3 ~ 22.8
TIBIA	20.0 ± 1.5	22.3 ± 0.9	22.4 ± 1.0	25.6 ± 1.6*	19.4 ± 0.6	22.5 ± 1.0
	18.3 ~ 22.8	21.3 ~ 23.5	20.5 ~ 24.0	21.5 ~ 28.2	18.9 ~ 20.5	20.8 ~ 23.4
FOOT	31.4 ± 2.2	33.3 ± 1.2	34.2 ± 1.7	38.3 ± 3.1	30.2 ± 1.3	34.1 ± 2.5
	28.8 ~ 34.5	31.8 ~ 34.6	31.0 ~ 37.7	32.7 ~ 43.4	28.3 ~ 31.5	30.3 ~ 36.2
IMTL	3.4 ± 0.4	4.4 ± 0.8	3.9 ± 0.5	4.3 ± 0.9	36 ± 0.7	4.3 ± 0.6
	2.8 ~ 4.0	3.3 ~ 5.0	3.1 ~ 4.9	3.3 ~ 6.8	2.3 ~ 4.0	3.5 ~ 5.2

Table 2: *Measurements of 15 morphological characteristics.*

*Values from Oike., et al. [19] represent mean ± SD. Ranges of values are shown under each characteristic. The letter "n" indicates the number of individuals examined. ♂, male and ♀, female. Asterisks show the probability (G. nakamurai sp. nov. (♂) vs. other groups (♂) and G. nakamurai sp. nov. (♀) vs. other groups (♀), *P < 0.05 and **P < 0.01).*

Calls

Males from three groups of *G. nakamurai*, *G. rugosa*-West, and -North were used to analyze pulse interval, pulse number, and duration of calls. Calls of each group were recorded at air and water temperatures of 22.3°C and 19.7°C, on June 10, 2017, 24.5°C and 19.1°C on May 26, 2019, and 20.9°C and 18.5°C on May 24, 2017, respectively [19]. We observed three types of calls in *G. nakamurai* and *G. rugosa*-West and two types in *G. rugosa*-North. We also determined pulse intervals, pulse numbers and call durations from these groups, as reported previously [19].

Type 1 pulse intervals of males from three groups were as follows: *G. nakamurai* (mean ± SD = 152.90 ± 20.08 ms), *G. rugosa*-West (126.72 ± 12.22 ms), and *G. rugosa*-North (100.34 ± 11.28 ms). Statistically significant differences were observed in pulse intervals between *G. nakamurai* and *G. rugosa*-West (**P < 0.01) and between *G. nakamurai* and *G. rugosa*-North (**P < 0.01) [19]. Pulse numbers of *G. nakamurai*, *G. rugosa*-West, and *G. rugosa*-North were the mean ± SD = 8.21 ± 2.88, 9.05 ± 2.22 and 7.10 ± 2.05, respectively, but there were no statistically significant differences in pulse numbers among these groups of frogs. Call durations of *G. nakamurai*, *G. rugosa*-West, and *G. rugosa*-North were the mean ± SD = 1228.25 ± 2 97.36, 1359.30 ± 3 21.00 and 813.80 ± 266.28 ms, respectively. Call durations of *G. nakamurai* were much longer than those of *G. rugosa*-North (**P < 0.01).

Next, we determined type 2 call durations from *G. nakamurai* and *G. rugosa*-West males. These were 543.25 ± 149.09 and 415.15 ± 107.19 ms, respectively [19]. There was a statistically significant difference in pulse durations between *G. nakamurai* and *G. rugosa*-West (**P < 0.01).

Finally, we observed type 3 calls in *G. nakamurai* (Figure 3, pulse intervals; 20.07 ± 0.87 ms, pulse numbers; 20.05 ± 2.67 and call durations; 407.85 ± 37.94 ms), and *G. rugosa*-West (pulse intervals; 15.42 ± 1.24 ms, pulse numbers; 28.00 ± 3.84 and call durations; 481.60 ±

58.33 ms) [19]. There were statistically significant differences in all three factors of type 3 calls between *G. nakamurai* and *G. rugosa*-West (**P < 0.01).

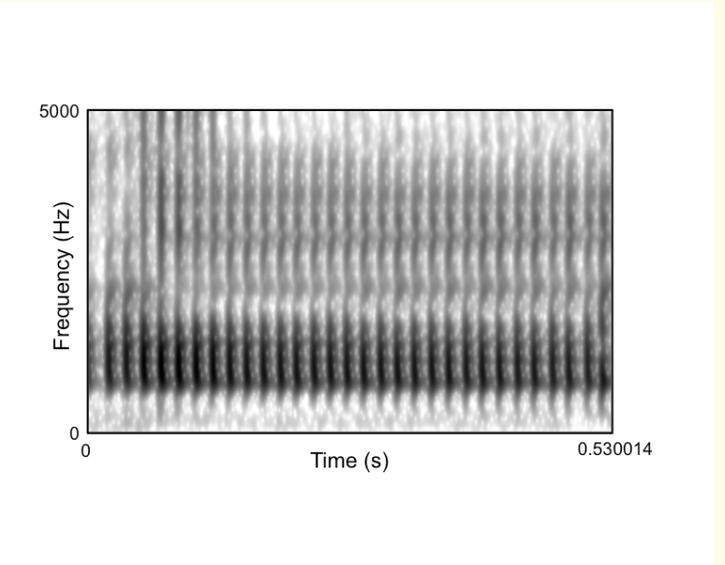

Figure 3: *Spectrogram of type 3 call of G. nakamurai.*
Ordinate, frequency (Hz). Abscissa, time (second). Values indicate the mean ± SD.

Nucleotide sequences of mitochondrial DNA

We constructed a RAxML phylogenetic tree, based on nucleotide sequences (4.5 to 5.3-kb) of non-cording D-loop to the *16S rRNA* gene of the mt DNA from different species. *G. susurra* was not employed, since nucleotide sequence of this region in this species is not currently available for construction of the tree. The RAxML tree recognized two clusters: one cluster contained *G. emeljanovi*, *G. rugosa*-West and -North, and the other included *G. nakamurai*, *G. rugosa*-East and -Central (Figure 4A).

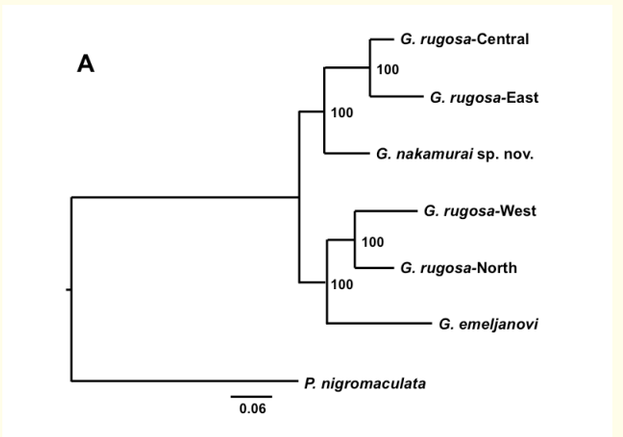

Figure 4: *Mitochondrial DNA analysis.*
Panel A- a RAxML phylogenetic tree. The tree was constructed based on nucleotide sequences of non-cording D-loop to the 16S rRNA gene in the mitogenome of G. rugosa-East, -Central, -West, -North, G. nakamurai, R. emeljanovi, and P. nigromaculata. Numbers at each node indicate the bootstrap values of 1000 replications. Panel B- alignment of nucleotide sequences of the pseudo tRNA-Ser (AGY) genes. Gaps are introduced in the sequences to optimize the alignment by dashes. Asterisks and dots on the bottom of the panel indicate that nucleotides from all seven groups and five groups of G. nakamurai and G. rugosa are identical, respectively.

The previous study showed a pseudo *tRNA-Ser* (AGY) gene in all the mt DNA from the four groups of *G. rugosa* [11]. Nucleotide sequences of the pseudo *tRNA-Ser* (AGY) genes were similar between *G. rugosa*-West and -North and between *G. rugosa*-East and -Central (Figure 4B) [11]. By contrast, *G. emeljanovi* (MH972198) and *P. nigromaculata* (AB043889) do not carry a pseudo *tRNA-Ser* (AGY) gene. Then, we determined the nucleotide sequence of a *tRNA-Ser* (AGY) gene in the mt DNA of *G. nakamurai* to clarify whether *G. nakamurai* carries a pseudo gene as well. We found a pseudo *tRNA-Ser* (AGY) gene in the mt DNA of the species like those in the four groups of *G. rugosa* (Figure 4B). However, the nucleotide sequence of the gene from *G. nakamurai* was not identical to those from *G. rugosa*-West, and -North (Figure 4B). Genetic identity values were as follows: *G. nakamurai* vs the-West, -North, -East and -Central = 84.1, 88.4, 92.8 and 92.8%, respectively. Genetic values indicate that *G. nakamurai* is closer to the latter two groups than the former two, although *G. nakamurai* and the-West inhabit parapatrically in Kyushu.

Survival rates of embryos

We examined survival rates of intraspecific and interspecific hybrid embryos and tadpoles. The rates of embryos and tadpoles obtained from intraspecific mating were over 60% on day 60 after 2-cell division, but those of interspecific hybrids were less than 30% (Table 3). Almost all live tadpoles metamorphosed within one year (Table 3).

Crossing (♀x ♂)	2 Cells	Hatched embryos	Feeding embryos	Day 60	1 year
Gr x Gr	314 (100%)	256 (81.5%)	217 (69.1%)	210 (66.7%)	202 (1) (64.3%)
Gn x Gn	347 (100)	220 (66.2)	216 (62.2)	214 (61.7)	209 (1) (60.8)
Gn x Gr	462 (100)	231 (50.0)	226 (48.9)	148 (32.0)	131 (1) (28.6)
Gr x Gn	694 (100)	298 (42.9)	271 (39.0)	199 (28.7)	181 (0) (26.1)

Table 3: *Survival rates of embryos and tadpoles.*
Interspecific and intraspecific hybrid embryos and tadpoles in water were reared outdoors by feeding them boiled spinach. Numbers on day 60 and 1 year show tadpoles and individuals that have metamorphosed within 1 year after fertilization, respectively. Numbers in parentheses on 1 year indicate tadpoles that have not metamorphosed within 1 year.
Gn, G. nakamurai. Gr, G. rugosa-West.

Discussion

PH. F. von Siebold collected thousands of specimens of animals and plants during his stay in Japan. One of his collections is for the *Glandirana rugosa* frog of which nine specimens are stored in the Naturalis Biodiversity Center (NBC) in Leiden, the Netherlands. Unfortunately, the type locality of *G. rugosa* is stated neither in the original publication [27] nor on the label of the bottle container of the specimens (RMNH 2064) in the NBC. According to Temminck and Schlegel [27], von Siebold kept the frogs alive in tanks at Decima (now known as Dejima) in Nagasaki, Japan, where on hot evenings they showed their presence by lugubrious/lurid cries, that were to be heard often and during the whole night [27]. By this, he learned the frog habits of crying at night and crawling on land, rather than jumping; this habit gave rise to the name of "Tsutsi kaheru" in Japanese. These statements led us to conclude that von Siebold collected live *G. rugosa* frogs in Nagasaki and observed them at Decima. Thus, the type locality of *G. rugosa* is probably "Nagasaki," which agrees with Stejneger [24] and Nakamura [13]. In contrast, Gee and Boring [4] restricted the type locality of *G. rugosa* to Nagasaki. The type locality will be clarified if the place where von Siebold collected the frogs is specified by examining type series of the specimens in the NBC.

We recognized two clusters on the RAxML phylogenetic tree constructed based on nucleotide sequences of non-cording D-loop to the *16S rRNA* gene in the mt DNA from different species: one cluster contains *G. emeljanovi*, *G. rugosa*-West and -North, and the other includes

G. nakamurai, *G. rugosa*-East and -Central (Figure 4A). This tree is compatible with the tree constructed based on the complete mitogenomes of the four groups of *G. rugosa* [11]. The Korean *G. emeljanovi* is a different species from the Japanese *G. rugosa* because of the 84% genetic identity of the mitogenomes between the two species: the range of intraspecific genetic identity values in Ranidae frogs is from 94.0 to 100.0% [2]. The recent study of [11] has shown that genetic identity values of the complete mitogenomes of *G. rugosa*-North, -East, -Central, and *G. emeljanovi* are 88.6, 81.8, 82.4 and 84.8%, respectively, when the *G. rugosa*-West is assigned as 100%. These values are much lower than the range of intraspecific genetic identity in Ranidae frogs. Thus, each cluster should be considered to consist of at least one species in the genus *Glandirana*. In other words, *G. rugosa* s.lat. can be separated into at least two species (see Figure 4A). The West group should represent *G. rugosa* s.str., because it includes *G. rugosa* frogs in Nagasaki [18]. The East and Central groups should have a different species name. The genetic identity of the mitogenomes can be a good criterion to identify a new species in amphibians.

Sekiya., *et al*. [22] described a new species in the genus *Glandirana*. To describe the new species, they determined morphological features, advertisement calls and partial nucleotide sequences of the *12S* and *16S rRNA* genes in the mitogenomes of the yellow morphotype and *G. rugosa*. By crossing experiments between the two groups of the frogs, Ohtani., *et al*. [17] concluded that the yellow morphotype is reproductively isolated from *G. rugosa*-North on Sado Island through all hybrid maleness with scarce fertility. Hence, Sekiya., *et al*. [22] concluded that the yellow morphotype is a new species in the genus *Glandirana*, designated *Glandirana susurra*. According to Oike., *et al*. [18], *G. rugosa* on Sado Island is a member of the North group that arose from the hybridization between the West and East groups [15,16]. Thus, the North group unquestionably emerged later than the West and East. During the round trip between Nagasaki and Edo von Siebold did not visit any places which the *G. rugosa*-North inhabits [6, 29]. This indicates that he did not collect any frogs of the *G. rugosa*-North. Therefore, any frogs in the *G. rugosa*-North can be neither the holotype nor syntype of the *G. rugosa* species. No places where *G. rugosa*-North inhabits can be the type locality of *G. rugosa*, either. Evidently, Sekiya and his colleagues did not employ *G. rugosa* frogs from the type locality to perform crossing experiments. This is unusual. They should have employed the frogs from the type locality for comparison between *G. rugosa* and the yellow morphotype. Much to our surprise, morphological traits and advertisement calls of *G. susurra* are very similar to those of *G. emeljanovi* in northeastern South Korea (the authors' observation). This implies that *G. susurra* could be a subspecies of *G. emeljanovi*.

Recently we have identified a phylogenetically different group from *G. rugosa* in southeastern Kyushu that we named *G. nakamurai*. On the RAxML phylogenetic tree, *G. nakamurai*, *G. susurra*, *G. rugosa*-East and -Central are in one cluster, whereas *G. emeljanovi*, *G. rugosa*-West and -North in the other (Figure 4A and [11, 19]). Needless to mention, *G. rugosa* is a different species from *G. emeljanovi* and *G. susurra*. Interestingly, genetic identity values of nucleotide sequences of the non-cording D-loop to the *16S rRNA* gene in the mitogenomes of *G. rugosa*-West and *G. emeljanovi* are 72.1 and 73.7%, respectively, when the nucleotide sequence of the region from *G. nakamurai* is set as 100%. The value (72.1%) between *G. nakamurai* and *G. rugosa*-West is much lower than those (94.0-100.0%) of interspecific genetic identity in Ranidae frogs. Thus, *G. nakamurai* can be considered to be a different species from *G. rugosa*-West (*G. rugosa* s.str.) at the mitochondrial DNA level. In addition, we observed statistically significant differences in morphology and advertisement calls between the two populations. All *G. susurra*, *G. emeljanovi* and *G. rugosa* are a different species in the genus *Glandirana*. Thus, we conclude that *G. nakamurai* can be described as a new species in the genus *Glandirana*.

Finally, a question arises as to how *G. nakamurai* was established as a new species. Recently, two new species in amphibians have been described in Japan: *Microhyla kuramotoi* [9] and *Buergeria choui* [10]. These two species inhabit small islands in southwestern Japan, indicating that these two species have probably evolved to become a new species under isolated environments. On the other hand, the habitat of *G. nakamurai* and *G. rugosa*-West is parapatric in Kyushu. According to Saito and Tojo [20] and Taira [25], the Japan islands were a part of the Eurasian continent 21 My ago. However, two landmasses, the northern landmass (NL) and the southern landmass (SL), detached from the continent and moved to form the Japanese islands. Then, the SL was reconnected with the Korean peninsula by land due to repetitive arrivals of glacial periods, but most of the NL was under water. Thus, *G. nakamurai* perhaps moved to Japan via the Korean

peninsula earlier than *G. rugosa*-West, because the former inhibits the eastern side of Kyushu, whereas the latter resides on the western side (see Figure 1). Thereafter, *G. nakamurai* remained isolated from *G. rugosa*-West by such geographical barriers as mountain ranges which exist even today. Then, the former probably evolved to become a new species in the genus *Glandirana*. However, there still remains the possibility that *G. nakamurai* inhabited the SL when it was detached from the continent. To clarify this issue, further study is needed.

Conclusion

Based on the results of this study, a phylogenetically distinct group of *Glandirana rugosa* in southeastern Kyushu of Japan can be described as a new species, named *Glandirana nakamurai* sp. nov. in the genus *Glandirana*.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

Author Contributions

MN collected animals and wrote the paper. MN and YN performed animal care and histology, respectively. AO, KT, TS and EI performed molecular analysis. All authors read and approved the final manuscript.

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Data Availability Statements

DNA sequences are deposited in Genbank (Accession Nos. LC574079, LC536281-4). This species is deposited in ZooBank (http://zoobank.org/References/13692B57-A3B9-461D-AA23-BB0CA0D45D7F).

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