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A new frog species of the genus *Rugosa* from Sado Island, Japan (Anura, Ranidae)

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Abstract

We describe a new frog species in the genus *Rugosa* from Sado Island, Japan. This new species, *Rugosa susurra*, is genetically close to *R. rugosa* Temminck and Schlegel, but is morphologically distinguishable and postzygotically isolated from the latter species. The skin over the entire body is much smoother, and the abdomen and ventral surface of hindlimb are deep yellow whereas these are pale yellow or grayish yellow in *R. rugosa*. In addition, duration of the advertisement call of *Rugosa susurra* is definitely longer than that of *R. rugosa*. *Rugosa susurra* is the first endemic amphibian species to Sado Island in Japan.

Key words: *Rugosa susurra* sp. nov., advertisement call, postzygotic isolation, smooth skin

Introduction

The wrinkled frog, *Rugosa rugosa* is a species of a convoluted taxonomic history. Fei *et al.* (1991 “1990”) proposed a new genus name of *Rugosa* to the three frog species of *Rana rugosa* Temminck and Schlegel, *R. emeljanovi* Nikolsky and *R. tientaiensis* Chang, and the name of *Glandirana* to the species *Rana minima* Ting and Ts’ai. Dubois (1992) relegated *Rugosa* and *Glandirana* as subgenera of *Rana* and set them into the different sections (*Rugosa* into the section *Pelophylax* and *Glandirana* into the section *Hylarana*). However, the two sections were considered polyphyletic by Che *et al.* (2007). More recently, Frost *et al.* (2006) combined the two genera, *Rugosa* and *Glandirana*, into one genus *Glandirana* based on the molecular phylogenetic data. However, *Glandirana* (sensu Frost *et al.* 2006) was sometimes considered as non-monophyletic (Wiens *et al.* 2009). In addition, several morphological characters, particularly toe webbing, of *Rana minima* were largely different from the remaining three species (Frost *et al.* 2006). Thus, we consider that it is premature to recognize *Glandirana* (sensu Frost *et al.* 2006) as a good genus for the four species. Also, we consider the Dubois’s classification invalid, because the two subgenera *Rana* (*Glandirana*) and *Rana* (*Rugosa*) were placed into polyphyletic sections as mentioned above. In contrast, monophyletic relationship of the three species *Rana rugosa*, *R. emeljanovi* and *R. tientaiensis* seems stable although the name of *R. rugosa* was not used in several studies (Frost *et al.* 2006; Che *et al.* 2007; Wiens *et al.* 2009). Therefore, in this study, we follow Fei’s classification.

The wrinkled frog, *Rugosa rugosa* was thought to be distributed in the northeastern parts of Asia, including Japan archipelago (Maeda & Matsui 1999). However, the Chinese populations of *R. rugosa* was given a distinct species name, *Rugosa emeljanovi* (Fei *et al.* 1991 “1990”). Recently, the populations in Korean peninsula were also treated as *R. emeljanovi* (Kuzumin *et al.* 2004). Following these treatments, we use the species name *Rugosa rugosa* for only the Japanese populations in this study. The type locality of the species was restricted to Nagasaki of Kyusyu district in the Western part of Japan (Stejneger 1907). The unique biological characteristic of *R. rugosa* is the diversity in sex determining mechanisms. A total of five geographic races are currently recognized on the basis

of molecular phylogenetic and cytogenetic studies (Miura 2007). In three (XY, ZW and Neo-ZW) of the five races, chromosomes 7 out of 13 haploid complements are the heteromorphic sex chromosomes, while they are homomorphic in the remaining two races (West- and East-Japan). The type locality of the species is on the area of West-Japan race (Fig.1). Very interestingly, two (ZW and Neo-ZW) of the five races have a female heterogametic sex determining mechanism whereas the remaining three have a male heterogametic one (Nishioka *et al.* 1994; summary in Fig. 1). Since co-existence of the two different kinds of sex determining mechanisms within a single species is very rare, this frog is reputed to be an excellent and valuable research material for the study on evolutionary mechanisms of sex determination (Graves 2008). In contrast to the high diversity of sex chromosomes and sex determining mechanisms, the species is poor in variation of the external morphology (Maeda & Matsui 1999), and thus evoked little taxonomical interest. The dorsum of this species is largely muddy brown and bears many short ridges, and the abdomen is pale yellow or grayish yellow. Recently, we have found a unique morphotype of this species on Sado Island, Japan, whose abdomen and ventral surface of hindlimb are deep yellow and the skin is much smoother (Sekiya *et al.* 2010). Also, *R. rugosa* (ZW race) whose color of abdomen is pale yellow inhabits the southern hilly area (Kosado Hills) and the north part of the same island, while most of the unique morphotype occupies flat lowland (Kuninaka Plain) in the central region of the island (Fig.1). The two forms are genetically differentiated and are postzygotically isolated from each other (Ohtani *et al.* 2012). Almost all of the reciprocal hybrids produced from artificial crossings become males with low abundance of sperm in the testes, and showing an extremely low fertility when backcrossed to the females of both types (Ohtani *et al.* 2012). We describe this unique morphotype occurring in Sado Island as a new species of the genus *Rugosa*.

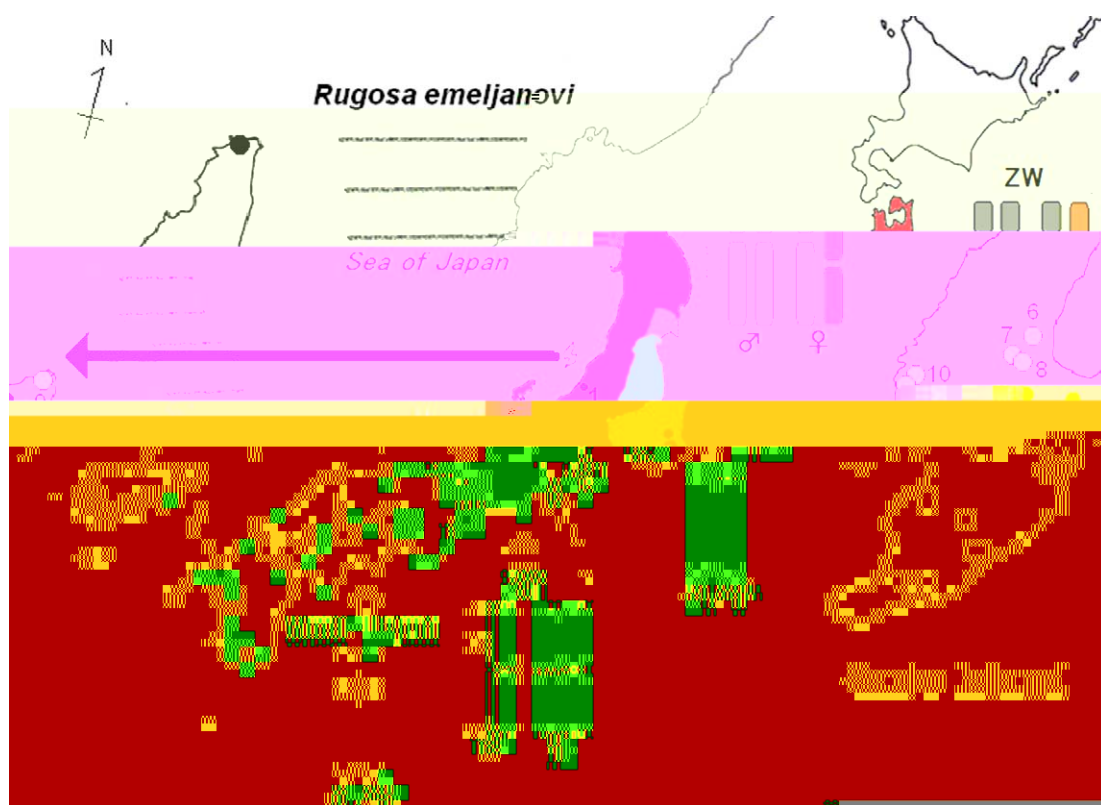


FIGURE 1. Distribution map of the five geographic races of *Rugosa rugosa* and the localities of specimens (closed circle) used in this study including those (yellow circle) of *R. susurra* in Sado Island. Circles without number are the localities of *R. rugosa* on Sado Island referred from Sekiya *et al.* (2010). Chromosomes 7 are given for the groups except Neo-ZW. Names of localities are as follows: 1, Nagaoka; 2, Hitachi-Oomiya; 3, Motosu; 4, Kinomoto; 5, Higashi-Hiroshima; 6, Umetsu; 7, Nagae; 8, Akitsu; 9, Suitsu; 10, Tassyia; 11, Ogawa; 12, Nakaoku; 13, Niibo-Iuchi; 14, Akadama; 15, Hamochi-Ohashi.

Materials and methods

Morphology. Adult specimens of the new species and *Rugosa rugosa* were collected in the period from June 2008 to July 2011. The collected specimens were fixed with 10% formalin and were finally stored in 80% ethanol after being rinsed with water. Tadpoles of the new species were fixed in 10% formalin. A set of 13 characters of preserved adult specimens and head-body length of preserved tadpole specimens were measured to the nearest 0.05mm with dial calipers by mainly following the study of Ueda *et al.* (1998). Ten of the earlier mentioned characters are snout-vent length (SVL), head width (HW), head length (HL, in parallel with the line connecting angle of jaw), eye distance (ED), tympanum diameter (TD), distance between tympanum and eye (T-ED), interorbital distance (IOD), snout length (SL, in parallel with the line connecting angle of jaw), tibia length (TIBIA) and internarial distance (IND). Upper eyelid width (UEW) and distance between nostril and eye (N-ED) were measured following the method of Maeda and Matsui (1999). Arm length (AL) was defined as a distance from the proximal end of inner metacarpal tubercle to elbow in the present study. Webbing formula is based on that of Myers and Duellman (1982). Furthermore, we examined the presence of skin granules on the ventral side of jaw in all specimens. For tadpole specimens, dental formula and developmental stages were examined according to Gosner's staging table (1960). Size of ova was measured to the nearest 0.05mm under a binocular dissecting microscope with dial calipers. All statistical analyses were conducted using R 2.11.1 software of R development core team (2010); males and females were separated for each analysis, while the data of specimens less than five in total number were excluded from analysis. Also, the data of granule distribution on the ventral surface of jaw from plural populations of each species were put together and used for statistical analysis. Specimens used in this study were deposited in Kanagawa Prefectural Museum of Natural History, Japan (KPM) and Institute for Amphibian Biology, Graduate School of Science, Hiroshima University, Japan (IABHU). Specimens of *R. rugosa* for morphological comparison were shown in Appendix

Call. Advertisement call was recorded in the field from males of the new species belonging to one population on 19 June 2011. In order to compare the calls of new species and *Rugosa rugosa*, we injected pituitary suspension of *R. rugosa* or *Pelophylax porosus brevipoda* Ito into the abdomen of three males each of the new species and *R. rugosa* both collected from Sado Island, and recorded their calls at laboratory following the method of Hasegawa *et al.* (1999). For recording, we used a microphone (NTG2, Rode) and a digital recorder (44.1kHz, 16bit, ICR-PS004M, Sanyo). Call duration and number of pulse in a single call were measured using Praat ver 5.1.44 (Paul 2001) and Raven lite version 1.0 (Cornel lab of ornithology). Mean value of each parameter for three males was calculated.

Molecular phylogeny. A mitochondrial gene tree was constructed based on the sequences of 12SrRNA (357bp) and 16SrRNA (510bp) genes from *Rugosa rugosa* (6 populations), the new species, *R. emeljanovi* (Korean and Chinese populations), *R. tientaiensis* Chang and *Glandirana minima* Ting and Ts'ai from Fujian Province, China obtained from NCBI database (*R. rugosa*, AB430340-430345 and AB430352; the new species, AB430349; *R. emeljanovi*, Korean population AB430346; Chinese population, AF205561 and AF315155; *R. tientaiensis*, DQ359954 and DQ359985; *G. minima*, DQ359968 and DQ359998). DNA sequences of the new species, *R. rugosa* and *R. emeljanovi* (Korean population) were determined in our previous study (Sekiya *et al.* 2010). The sequence of *Pelophylax nigromaculatus* Hallowell was used as an outgroup (DQ283137). Phylogenetic analysis was conducted by the methods of neighbor-joining, maximum parsimony and maximum likelihood with MEGA ver.5 software (Tamura *et al.* 2011). DNA distances among the sequences were estimated by Kimura's two-parameter methods with the same software.

Systematics

Rugosa susurra sp. nov.

(Japanese name: Sado gaeru)

(Figs. 2–4)

Rana rugosa (yellow type): Sekiya *et al.* 2010, p. 71, figs. 2A–B.

Diagnosis. Genetically close to *Rugosa rugosa* than other species of the genus *Rugosa*. A medium sized species of *Rugosa*, with males 33.0–44.2 mm, females 38.1–49.6 mm in SVL; web rather well developed; dorsum khaki in color with many dermal ridges of varying size, dorso-lateral fold absent, supratympanic fold strong; venter

posteriorly deep yellow; anterior ventral less granulated than posterior ventral, especially around jaw granule almost absent. This new species is differentiated from *R. rugosa* by its yellow ventral coloration and jaw almost without granules and no vocal sac in male.

Holotype: KPM-NFA000078, an adult male from Akitsu, Sado city, Sado Island, Niigata Prefecture, Japan (38°4'N, 138°24'E, 11m a.s.l), Collected on 14 July 2009 by M. Ogata.

Paratypes: Two adult females and an adult male (KPM-NFA000075-77) from the same locality and collected on the same day by M. Ogata and Y. Ogata.

Referred specimens: Is. Sado, Akitsu (10 males, 7 females, collected on 22 June 2008 by M. Ogata IABHU F2462-F2471, F2475-F2481, 3 males collected on 30 May 2011 by M. Ogata IABHU F2472-F2474, one female collected on 11 July 2011 by M. Ogata IABHU F2482) and Suitsu (38°4'N, 138°33'E, 33m a.s.l, 10 males, 3 females, collected on 22 June 2008 by M. Ogata, IABHU F2483-F2495), Nakaoku (38°3'N 138°21'E, 187m a.s.l, 9 tadpoles, collected on 1 July 2010 by K. Sekiya, IABHU F2513-F2521).

Description of holotype (measurements in millimeters). Snout-vent length 37.6; head about as long (14.0) as broad (13.8); snout (5.5) longer than eye (5.0), tip of snout rounded; distance between nostril and eye (3.4) about as same as upper eyelid width (3.3) and internarial distance (3.5), but narrower than interorbital width (4.4), snout dorsally concave and slightly pointed; lore concave; supratympanic fold strong; tympanum conspicuous, diameter (3.8), separated from eye by about one-fifth of tympanum diameter (0.7).

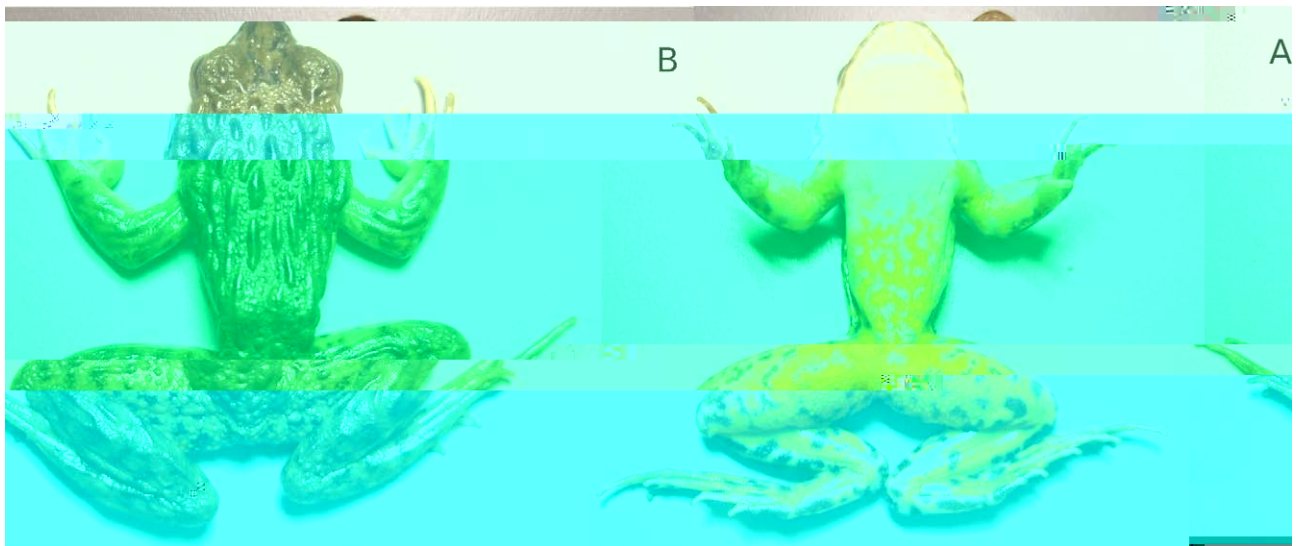


FIGURE 2. Holotype of *Rugosa susurra* (KPM-NFA000078) in life. Dorsal side (A) and ventral side (B).

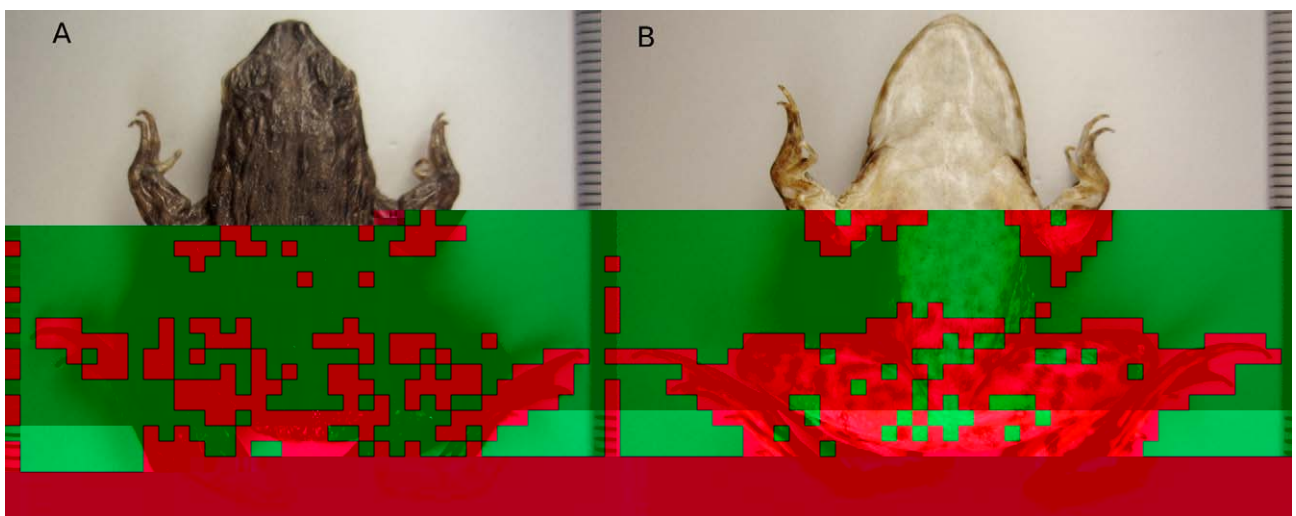


FIGURE 3. Holotype of *Rugosa susurra* (KPM-NFA000078) in alcohol. Dorsal side (A) and ventral side (B). Scale bars represent 1mm.

1	15.7 (15.4–17.5)	17.3–18.8 (16)	(13.5–15.8)	31.9 (39.8–44.6)	41.1 (39.8–44.6)
3–15.0)	10.5 (10.0–11.6)	11.0–11.8 (9)	(8.1–12.0)	9.8 (8.0–10.6)	9.2 (8.0–10.6)
5–10.3)	40.2 (38.2–41.8)	43.0–44.9 (4)	(35.8–39.2)	42 (8.0–11.4)	9.5 (8.0–11.4)
3	10.2 (8.4–11.7)	10.5–10.4 (8)	(7.4–9.8)	37.6±2.72 (33.5–42.6)	37.6±2.72 (33.5–42.6)
7–9.4)	9.0 (7.9–9.7)	9.1–10.4 (9)	(7.5–10.8)	17.9 (14.0–19.5)	17.9 (14.0–19.5)
5–9.6)	ratio to SVL (range); See the text for abbreviations of the other characters and <i>R. rugosa</i> (six populations).				
e): Other characters: median of morphological characters in males					
<i>Rugosa rugosa</i> aoka					
tsu	Akadama (ZW)	Nagaw	Hitachi-Oomiya (East-Japan)	Fotoşu, (Neo-ZW)	Kimomoto (Neo-ZW)
10	n=8	n=16	n=15	n=8	n=8
3±2.09	41.5±2.79 (37.1–45.3)	41.5–47.2 (36)	39.5±1.21 (37.2–41.5)	41.7±1.53 (39.5–44.0)	41.7±1.53 (39.5–44.0)
3–44.2)	19.8 (18.4–21.5)	17.7–19.0 (16)	18.4 (16.8–19.4)	18.0 (15.5–21.2)	18.0 (15.5–21.2)
0	53.6 (48.4–55.3)	54.6–57.1 (50)	48.9 (46.4–51.3)	49.9 (45.7–52.8)	49.9 (45.7–52.8)
.6–19.8)	14.0 (13.3–16.2)	15.3–16.4 (12)	12.3 (10.5–14.0)	13.1 (12.6–14.9)	13.1 (12.6–14.9)
7	11.4 (9.4–12.6)	12.7–12.9 (10)	11.2 (9.3–12.3)	11.0 (9.6–12.0)	11.0 (9.6–12.0)
9–52.0)	3.4 (2.4–4.2)	3.1–3.3 (2)	3.5 (2.2–3.9)	2.9 (1.8–3.8)	2.9 (1.8–3.8)
3	9.4 (8.1–10.9)	9.3–9.6 (7)	9.8 (8.6–10.8)	9.2 (8.8–11.1)	9.2 (8.8–11.1)
2–16.4)	42.0 (39.3–43.7)	40.5–43.4 (3)	37.4 (35.3–39.3)	40.8 (38.5–42.2)	40.8 (38.5–42.2)
3–11.5)			14.8	16.3 (14.9–17.5)	16.3 (14.9–17.5)
3–3.5)				9.7 (8.9–11.4)	9.7 (8.9–11.4)
5–11.3)				8.6 (8.0–10.7)	8.6 (8.0–10.7)
6				8.6 (8.0–10.7)	8.6 (8.0–10.7)
.4–38.3)				ers.	ers.

roshima
(n)

Rugosa in the following characters (Chang 1933; Nikolsky 1918; Fei *et al.* 1991“1990”; 2005): dorsal surface with slightly large dermal ridges and minute tubercles (*R. tientaiensis* has oval or elongated narrow warts on dorsal surface, Chang 1933) and smooth ventral skin without granule especially around jaw (*R. emeljanovi* and *R. tientaiensis* have rough skin both on dorsum and ventral side, Chang 1933; Fei *et al.* 1991“1990”; 2005). Ventral surface is deep yellow (dirty-yellow in *R. emeljanovi*, Nikolsky 1918). Since the genus *Glandirana* was considered as related with *Rugosa* (Che *et al.* 2007), we compared *R. susurra* and *G. minima* to clarify their morphological difference. *R. susurra* differs from *G. minima* in the following characters: webbing on toes is well developed (half-webbed on toes in *G. minima*, Fei *et al.* 1991“1990”), and ventral surface is deep yellow (gray in *G. minima* Ting & Ts'ai 1979).

TABLE 2. Distribution of granules on the ventral side of jaw in *Rugosa susurra* and *R. rugosa*.

Species	Locality (Geographic race)	Number of frogs with granules		Number of frogs without granules	
		Male	Female	Male	Female
<i>Rugosa susurra</i>	Akitsu	1	1	11	8
	Suitsu	0	0	10	3
<i>Rugosa rugosa</i>	Akadama (ZW)	8	6	0	0
	Nagaoka (ZW)	6	5	0	0
	Hitachi-Oomiya (East-Japan)	11	8	4	0
	Motosu (XY)	14	2	0	0
	Kinomoto (Neo-ZW)	8	3	0	0
	Higashi-Hiroshima (West-Japan)	9	6	0	0

Eggs. A female (IABHU F2482 collected from Akitsu on 11 July 2011) contained a total of 710 matured ova in a pair of ovaries. The diameter of twenty ova ranged from 1.2 to 1.5mm (mean±2SE=1.38±0.04). The animal pole is dark brown while the vegetable pole is cream in color.

Tadpole. Developmental stages of the nine tadpoles (IABHU F2513-F2521 collected from Nakaoku) varied from St.29 to St.42. In preserved specimens, dorsum almost grayish brown, abdomen white (Fig. 5). Tail and tail fin fine and grayish brown network. A lot of small white spots were distributed on entire body including tail fin. Head and body oval shaped, body slightly rounded below. Dermal ridges strongly on the dorsum of tadpoles at St.41 and St.42. Head-body length of two tadpoles at St.29 was 16.5mm and 18.0mm and that of tadpoles at St. 31 and St. 32 was 19.1 mm and 19.9 mm. Head-body length of four tadpoles at St.41 and St.42 varied from 22.6 mm to 27.0 mm whereas that of tadpole at St. 39 is 25.0 mm. Tail fin slightly develop around the center of tail.

Oral slightly below. Dental formula of six tadpoles (St.29-St.32 and St.39-St.42) was 1:1+1/1+1:2 having relatively large interrupted portion on upper dentition, while that of the remaining three tadpoles was 1:1+1/3 (St.41) or 1/1+1:2 (St.29).

Range. *Rugosa susurra* is mainly distributed in the Kuninaka Plain of Sado Island including the type locality (Fig. 1), not in any areas outside Sado Island, Japan. Five localities are known other than the three as mentioned above, Umetsu (38°6'N, 138°24'E, 75m a.s.l.), Nagae (38°5'N, 138°23'E, 54m a.s.l.), Tassya (38°4'N, 138°15'E, 77m a.s.l.), Ogawa (38°3'N, 138°15'E, 29m a.s.l.), Niibo-Iuchi (38°1'N, 138°27'E, 102m a.s.l.). The elevations of the eight localities range from 11m to 187m (in a.s.l.). Five of the eight localities are on the Kuninaka Plain (Fig.1, No.6–No.8 and No.12–No.13)

Calls. A total of five calls from one male of *Rugosa susurra* were recorded in Akitsu at air and water temperatures of 18.5°C and 21.5°C, respectively. Call duration ranged from 1112 to 1646 ms (mean±SD = 1341.7±226.9 ms), whereas number of pulse in a single call ranged from 67 to 93 (mean±SD = 78.8±11.6) (Fig. 6). When the male frog was calling, we could not observe any apparent expansion of vocal sac as *R. rugosa* is doing. Recording at laboratory was conducted at air temperatures of 24.6–26.8°C for *R. susurra* collected from Akitsu on 30 May 2011 (IABHU F2472-2474) and *R. rugosa* from Akadama on Sado Island on 30 May 2011 (ZW race, IABHU F2504-2506). A total of 16 calls (*R. susurra*) and 19 calls (*R. rugosa*) were recorded. Call duration of *R. susurra* is much longer than that of *R. rugosa* (*R. susurra*, mean±SD = 1333.5±382.4ms; *R. rugosa*, mean±SD = 111.7±9.1ms, t-test p<0.05). Also, number of pulse in a single call is completely different between them (*R.*

susurra, mean±SD =63.0±6.4; *R. rugosa*, 6.8±0.7, $p<0.005$, t-test). In the three male specimens of *R. susurra*, any vocal sac and vocal opening could not be identified inside the mouth by dissection. Therefore, *R. susurra* has no vocal sac.



FIGURE 5. Tadpole of *Rugosa susurra* (IABHU F2521). Dorsal (A), ventral (B) and lateral (C) views. Developmental stage is 41 (Gosner 1961). Scale bars represent 1mm.

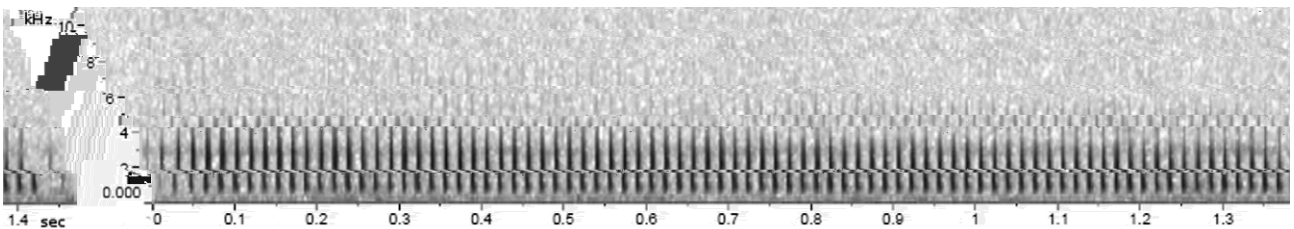


FIGURE 6. Spectrogram of an advertisement call of *Rugosa susurra*. Air temperature and water temperature are 18.5°C and 21.5°C, respectively

Natural history. Adult frogs of *Rugosa susurra* are always observed near the water, such as in rims of rice field, small streams and ponds. Egg masses laid are attached to short withered branches or water grass (Fig. 7). Breeding seems to occur from middle May to early August, because egg masses are observed in ponds during the season. In winter, both adult frogs and tadpoles are observed in the mud under water. Thus, the tadpoles of *R. susurra* can hibernate successfully like those of *R. rugosa* (Maeda & Matsui 1999), although we have no information whether all tadpoles that are produced in the season hibernate or not.



FIGURE 7. Egg masses of *Rugosa susurra* from Tassya on Sado Island.

Karyotype. $2n = 26$ (males and females) with no heteromorphic pair of sex chromosomes, and the chromosome 7 is very similar in morphology and late replicating banding pattern to that of East-Japan (Kanto) race of *R. rugosa*, suggesting their genetically close relationship (cf. Fig. 3 in Sekiya *et al.* 2010).

Molecular phylogeny. The bootstrap consensus tree of mitochondrial genes was constructed by maximum likelihood method (Fig. 8). *Rugosa rugosa* comprises two main lineages: one contains East-Japan (Kanto), XY and Neo-ZW races, while the other contains West-Japan and ZW races. *R. susurra* was always clustered with the lineage comprising East-Japan (Kanto), XY and Neo-ZW races of *R. rugosa* in the three topologies with high bootstrap probability. Topologies of the other two methods were almost the same as that of Fig. 6, although two sequences of *R. emeljanovi* were clustered with the lineage (East-Japan +XY+Neo-ZW + *R. susurra*) in maximum parsimony methods with low bootstrap probability (<50%). DNA distances between *R. susurra* and *R. rugosa* are 0.041 to 0.062 whereas those of *R. susurra* and the remaining three species are 0.068–0.075 (*R. emeljanovi*), 0.071 (*R. tientaiensis*) and 0.096 (*Glandirana minima*).

Etymology. The specific epithet is derived from "susurrus" in Latin meaning "whispering". The advertisement call of *Rugosa susurra* is much quieter than those of other anuran species in the same locality (*Hyla japonica* Günther, *Rhacophorus arboreus* Okada and Kawano), and sounds like whispering among them.

FIGURE 8. Maximum-likelihood tree based on the 867bp sequences of the mitochondrial 12S rRNA and 16S rRNA genes of genus *Rugosa*. Numeral at each node denotes the bootstrap support (% in 1000 replicates). *R. susurra* indicates a specimen

hybridization between the primary West-Japan and East-Japan (Kanto) races and subsequently expanded its distribution to the northwestern mainland of Japan and finally reached Sado Island (Sekiya *et al.* 2010). Although there is no information about the time when *R. rugosa* (ZW race) invaded Sado Island, the small value of DNA distance (0.001) between the two populations of the ZW race (Niigata vs Sado) indicate that the invasion of the ZW race was much more recent than speciation of *R. susurra*. Thus, it is evident that *R. susurra* has been isolated from *R. rugosa* for a long time on the island. And such a long time isolation would have made several differences in morphology and call (advertisement call duration, color of abdomen, and smooth skin), and evolved a postzygotic isolation between *R. susurra* and *R. rugosa* (ZW race). In sharp contrast, there are no postzygotic isolations among the five races of *R. rugosa* itself on the basis of artificial crossings: reciprocal hybrids among the races are all

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