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A Comparison of Mating Calls Recorded around the Type Localities of *Rana tagoi* and *R. neba* (Amphibia: Anura: Ranidae)

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Abstract: Two species of frogs endemic to Japan, *Rana neba* and *R. tagoi*, have previously been separated based on differences in the number of chromosomes and the dominant frequencies of their advertisement calls. However, through a comparison of advertisement calls around the type localities of both species, we found that interspecific differences are instead apparent in the fundamental frequencies, not in the dominant frequencies. Additionally, the calls of *R. neba* are more easily distinguished by their unique first notes, which can be differentiated by the strong frequency modulation, longer and fewer pulses, and lower pulse rate. Compared with *R. neba*, the calls of *R. tagoi* are composed of notes with similar acoustic structure, although the last note is longer in duration, contains more pulses, and has longer inter-pulse intervals than in the other notes in the call. Based on these results, we present a new acoustic diagnosis for *R. neba* and *R. tagoi*.

Key words: Acoustic characters; Advertisement call; *Rana neba*; *Rana tagoi*; Type locality

Introduction

The number of described amphibian species has increased rapidly over the last century (Glaw and Köhler, 1996; Hanken, 1999), and the speed has not decreased in recent decades (Frost, 2020). Most of recently described amphibians are so-called "cryptic species", which morphologically resemble to known species, but are instead a distinct species. These cryptic species have been identified through the use of genetic and karyological tools, as well as ethological characters. For example, anurans use distinct advertisement calls, often resulting in reproductive isolation, and many researchers have focused on the difference in acoustic characters in the calls of sympatric allies (e.g., Gerhardt, 1994; Forester, 1973; Littlejohn and Watson, 1974).

In Japanese anurans, recent studies suggest that variation in calling might elucidate unknown diversity in the *Rana tagoi* complex, especially in *Rana neba* Ryuzaki, Hasegawa, and Kuramoto, 2014 and *R. tagoi* Okada,

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1928 (Ryuzaki et al., 2014a, b). These recently separated species are known to share most morphological characters with each other, and no diagnostic keys applicable for fixed specimens have been reported. Nevertheless, *R. neba* is unique by having 2n=28 chromosomes (Ryuzaki et al., 2014a), while *R. tagoi* has 2n=26. Additionally, it is noted that the advertisement calls of *R. neba* are higher in dominant frequency (ca. 1.3 and 1.7 kHz) than the calls of *R. tagoi* (0.5 to 0.7 kHz) (Ryuzaki et al., 2014a). Although there is no reliable information on whether these two species are truly sympatric or not, interbreeding is possible because both species inhabit Central Japan and their breeding seasons largely overlap (Matsui and Maeda, 2018). Thus, variation in advertisement calls between these species might result in reproductive isolation.

While the dominant frequency of advertisement calls has previously been used to separate *R. neba* and *R. tagoi*, further study of their calls is needed for two reasons. First, although Ryuzaki et al. (2014a) showed only a narrow range of dominant frequencies for both species, a much wider range (1–2 kHz) was reported in the advertisement calls of *R. tagoi* (Ryuzaki et al., 2014b). If it is the case, the variation in ranges of the dominant frequencies of the species largely overlap and this character might not be useful as a diagnostic character. Second, Eto et al. (2016) preliminarily suggested that frequency modulation in the first note of *R. neba* advertisement calls might be unique in that species. However, such modulations were not mentioned in previous studies (Ryuzaki et al., 2014a) and no studies have compared this call characteristic with adequate sampling. Considering these two points, a careful re-evaluation of the advertisement calls of these species is needed. We collected advertisement calls around the type localities of *R. tagoi* (the former Kamitakara Village, Gifu Prefecture, Japan: Okada, 1928; Shibata, 1988) and *R. neba* (Neba Village, Nagano Prefecture, Japan: Ryuzaki et al., 2014a) and compared them based on 12 acoustic parameters. Through the results from the comparison, we made a diagnostic table to distinguish calls of *R. tagoi* and *R. neba*.

Materials and Methods

Sample collection

Advertisement calls of *R. tagoi* and *R. neba* were recorded during their mating seasons (middle to late May) with an IC recorder (SONY PCM-D100, LPCM 44.1 kHz/16 bit) with a built-in microphone affixed at the top of 1 m metal stick. Recordings of 45 *R. tagoi* calls were collected from six sites in the former Kamitakara Village (now Takayama City), Gifu Prefecture, Japan, while recordings of 26 *R. neba* calls were collected from five sites around Mt. Chausu, Neba Village, Nagano Prefecture, Japan (Table 1, Fig. 1). Calls were recorded separately for each individual male frog, and only a single typical call from each male was used for the analysis. During the fieldwork, frogs often stop calling because of our disturbance. In such cases, we waited for several minutes until the calling became stable. After the recording, we measured the air and water temperatures using a thermometer (EXTECH 39240).

Acoustic analyses

We picked a typical call from each individual and obtained a spectrogram using WavePad v.10.38 (NCH Software). We also obtained an oscillogram using RAVEN Pro 1.6.1 (Center for Conservation Bioacoustics, 2019). Calls of *R. tagoi* and *R. neba* were compared based on 12 acoustic parameters. The first eight properties obtained from the oscillogram included (1) call length, (2) note number per call, (3) average inter-note length, (4) average note length, (5) pulse number per note, (6) average pulse length, (7) average inter-pulse length, and (8) pulse rate (pulse repetition rate; the ratio of the number of pulses and the note length). The last four acoustic properties obtained from the spectrogram included (9) dominant frequency, (10)

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Table 1. Sampling localities, altitudes, dates, number of records, temperatures (air and water). Numbers correspond with Fig. 1.

No.	Site	Altitude (m)	Date	N	Air temp. (°C)	Water temp. (°C)
R. tagoi						
1	Kashiate, Okuhida-Onsenkyo, Takayama city, Gifu Pref.	783	2019/5/27	13	—	—
2	Kanakido, Kamitakara, Takayama city, Gifu Pref.	876	2019/5/26	1	21.9	12.7
3	Arahara, Kamitakara, Takayama city, Gifu Pref.	895	2019/5/26	3	26.9	13.6
4	Nezumochi "A", Kamitakara, Takayama city, Gifu Pref.	689	2019/5/27	20	18.6	17.6
5	Nezumochi "B", Kamitakara, Takayama city, Gifu Pref.	976	2019/5/27	2	26.1	8.6
6	Kurabashira, Kamitakara, Takayama city, Gifu Pref.	836	2019/5/27	6	27.7	12.6
R. neba						
7	Takahashi, Neba village, Nagano Pref.	1030	2014/5/20	2	13	11.1
8	Nokigawa, Urugi village, Nagano Pref.	1145	2019/5/17	18	14.5	10.4
9	Utsubo, Hiraya village, Nagano Pref.	1065	2014/5/20	2	11.7	10.7
10	Houchi, Shimokurogawa, Toyone village, Aichi Pref.	962	2015/5/17	1	—	—
11	Men'noki, Inabu, Toyota city, Aichi Pref.	1025	2012/5/17	2	17.7	11.8
			2019/5/17	1	14.5	10.4

fundamental frequency, and the presence or absence of (11) frequency modulation and (12) harmonics. We refer to parameters (1)–(3) and (4)–(12) as "call–level parameters" and "note–level parameters", respectively. A value of call-level parameter was obtained from every call, and a value of note-level parameter from every note. In some recordings, the fundamental frequency was difficult to define, especially in the second or later note. It would be largely because of the background noise such as water sounds, or diffusion by the surrounding environment, since males of *R. neba* and *R. tagoi* call inside cavities along springs and subterranean streams (Matsui and Maeda, 2018). Thus, we measured this character only from the first note recorded with a good quality.

For the term "frequency modulation" pertaining to the *R. tagoi* complex, we found two different definitions in previous studies. Daito and Kawakami (1992) reported that all calls of *R. tagoi* in their sampling localities (Fukuoka, Ehime, Hiroshima, Hyogo, Kyoto, and Niigata Prefectures) exhibited "mountain-shaped frequency modulations", but as far as we observed in the spectrograms

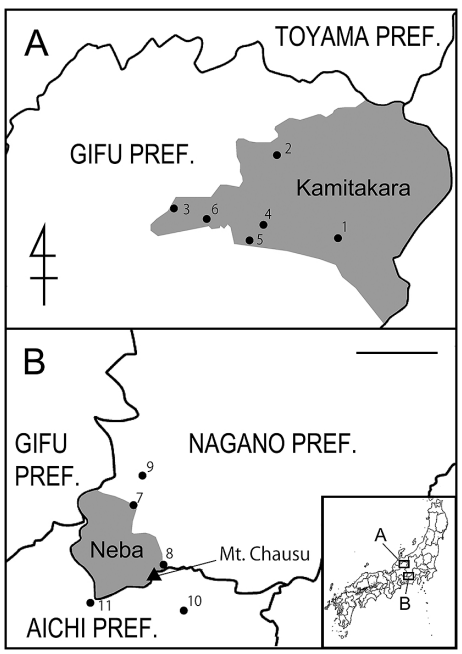


Fig. 1. Research sites of our recording survey; *Rana tagoi* (A): 1–6, Kamitakara, Takayama City, Gifu Prefecture; *R. neba* (B): 7. Neba Village, 8. Urugi Village, 9. Hiraya Village, Nagano Prefecture, 10. Toyone Village, 11. Toyota City, Aichi Prefecture. Scale bar = 10 km. See Table 1 for details.

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shown in their paper, such a modulation pattern was not observed. Instead, because central pulses of each note tend to exhibit a larger number of harmonics reaching a higher range of frequencies compared with peripheral pulses, the shape of the spectrogram of each note looked mountain-shaped. We suspect that their definition of "frequency modulation" might indicate changes of the highest harmonic recognized, probably caused by the change of sound energy over time. In other studies, "frequency modulation" indicates a temporal change of frequency in each harmonic within a pulse or corresponding harmonics of subsequent pulses (e.g., Maeda and Matsui, 1989; Matsuo et al., 2011; Sueyoshi et al., 2013). This is a commonly accepted definition of "frequency modulation" in bioacoustics including studies on other anurans (e.g., Köhler et al., 2017) and we also follow it in this study. Other terminology basically follows Köhler et al. (2017).

In some records, we detected a weak irregular sound produced before and/or after the main call. Such sounds are usually composed of an extremely long pulse or intermittent short pulses separated with apparently longer intervals than ordinary notes (Fig. 2B, D). We refer to these call elements as extra "pre-calls" (Daito and Kawakami, 1992) and "post-calls", and excluded them from the acoustic analyses.

Statistical Analyses

We compared the call-level parameters of *R. neba* and *R. tagoi* calls using nonparametric Mann–Whitney *U*-tests. Temperatures of breeding sites were also compared using *U*-tests. On the other hand, the note-level parameters apparently vary among notes (see RESULTS). To analyze such inter-note variation, we chose three notes from each call; the first, middle, and last notes. The first and the last notes was taken from the first and the last part of the call, ignoring the pre- and the post-calls. For the middle note, we chose a central note of the call [If we express the note number of a call as "2x" (even number) or "2x-1" (odd number) (x=1, 2, 3…), the x-th note was chosen as a middle note, while we did not take any middle note for the two-note call]. We compared six categories of notes (the first, the middle, and the last notes from each of two species) using Kruskal-Wallis tests with Dunn's multiple comparisons. All analyses were conducted using the software R version 3.0.3. (R Core Team, 2014). The significance level was set at 0.05.

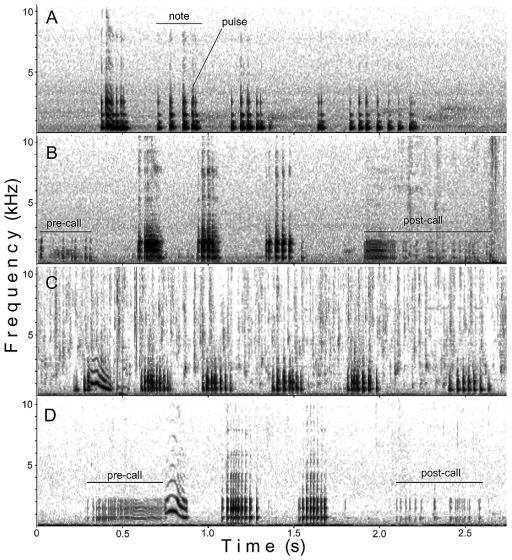


FIG. 2. Spectrograms of advertisement calls of *Rana tagoi* (A, B) and *R. neba* (C, D), recorded in and around the type locality. B and D additionally illustrate the pre-call and a post-call elements. Window type=Hann, window size=512 samples, frequency grid spacing=86.1 Hz.

RESULTS

Temperatures in the breeding sites

The air temperature in the type locality of *R. tagoi* (average=24.2°C) was significantly higher than in the type locality of *R. neba* (average=14.3°C; W=25, p=0.012). Unlike air temperature, no significant difference was detected in the water temperature between the type localities of *R. tagoi* (average=13.0°C) and *R. neba* (average=10.9°C; W=20, p=0.143).

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Table 2. Acoustic status (mean±SD, followed by range in parenthesis) of call-level parameters (call length, note number, and inter-note length).

	N	Call length (S)	Note number (notes/call)	Inter-note length (S)
R. tagoi				
1	13	2.33±1.29 (1.05–5.80)	5.08±1.49 (3–8)	0.27±0.07 (0.16–0.44)
2	1	1.28	2	0.44
3	3	1.36 (0.67–2.00)	3.00 (2–4)	0.44 (0.37–0.48)
4	20	1.43±0.67 (0.17–2.90)	3.50±0.74 (3–5)	0.30±0.08 (0.14–0.53)
5	2	2.16±0.05 (2.13–2.19)	4.00 (4–4)	0.39±0.02 (0.37–0.41)
6	6	1.47±0.35 (1.10–1.90)	3.17±0.37 (3–4)	0.33±0.07 (0.21–0.41)
total	45	1.72±0.93 (0.17–5.80)	3.87±1.28 (2–8)	0.31±0.09 (0.14–0.53)
R. neba				
7	2	2.28 (2.18–2.38)	6.00 (6–6)	0.26 (0.25–0.27)
8	18	2.06±0.56 (0.78–3.07)	5.00±0.94 (3–7)	0.28±0.05 (0.19–0.42)
9	2	1.47 (1.42–1.52)	5.00 (5–5)	0.26 (0.25–0.27)
10	1	1.42	4	0.15
11 (2012)	2	1.78 (1.33–2.24)	4.5 (4–5)	0.30 (0.28–0.33)
11 (2019)	1	0.96	3	0.26
total	26	1.94±0.56 (0.78–3.07)	4.92±0.96 (3–7)	0.28±0.05 (0.15–0.42)

Acoustic parameters

Interspecific calls from each type locality exhibited similar acoustic structures, and no apparent geographic variations among calls of the same species were detected. Thus, we combined data for *R. tagoi* (six sites) and *R. neba* (five sites). Acoustic parameters obtained from each species are shown in Table 2 (call-level parameters) and Table 3 (note-level parameters). Here, we show the results of statistic comparisons, listing up all tendencies regarded as significant in each analysis. The acoustic diagnosis between the topotypic populations of *R. tagoi* and *R. neba* are summarized in Table 4.

(1) Call length (Fig. 3A): *Rana neba* had significantly greater call length than *R. tagoi* ($W=413$, $p=0.040$).

(2) Note number per call (Fig. 3B): *Rana neba* had a significantly larger note number than *R. tagoi* ($W=269.5$, $p<0.001$).

(3) Average inter-note length (Fig. 3C): No significant difference was detected between *R. neba* and *R. tagoi* ($W=722$, $p=0.104$).

(4) Note length (Fig. 3E): A significant difference was detected ($\chi^2=66$, $df=5$, $p<0.001$). In the multiple comparisons, the last note of *R. tagoi* was significantly longer than the first and middle note of both species. The last note of *R. neba* was significantly longer than its first note.

(5) Pulse number per note (Fig. 3F): A significant difference was detected ($\chi^2=82$, $df=5$, $p<0.001$). In the multiple comparison, the first note of *R. neba* had significantly fewer pulses than in others. The last note of *R. tagoi* had significantly more pulses than in its first and middle notes.

(6) Average pulse length (Fig. 3G): A significant difference was detected ($\chi^2=96$, $df=5$, $p<0.001$). In the multiple comparison, the first note of *R. neba* had significantly larger values than in other notes of both species. The first note of *R. tagoi* also exhibited significantly larger values than some other notes (the last note of *R. tagoi*, the middle and the last notes of *R. neba*).

(7) Average inter-pulse length (Fig. 3H): In this parameter, we excluded the first notes of *R. neba*, because this note often contains only a single pulse, and no inter-pulse length could be defined in many records. A significant

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TABLE 3. Summary of acoustic status (mean±SD, followed by range in parenthesis) of note-level parameters (note length, pulse number, pulse length, inter-pulse length, pulse rate, dominant frequency, fundamental frequency, and absence/presence of modulation and harmonics) of each species. *: 38 finely-recorded calls were examined; ** 22 calls were examined.

Note position	N	Note length (S)	Pulse number (pulses/note)	Pulse length (S)	Interpulse length (S)	Pulse rate (pulse/S)	Dominant frequency (kHz)	Fundamental frequency (kHz)	Modulation			Harmonics	
									Absent	Obscure	Obvious	Absent	Present
R. tagoi													
First note	45	0.15±0.04 (0.06–0.33)	5.22±2.73 (1–13)	0.029±0.023 (0.006–0.117)	0.013±0.006 (0.002–0.029)	36.09±17.64 (14.99–88.90)	1.06±0.33 (0.51–1.78)	*0.72±0.17 (0.45–1.04)	40	4	1	0	45
Middle note	40	0.18±0.11 (0.08–0.76)	5.13±1.96 (2–11)	0.016±0.009 (0.006–0.049)	0.027±0.015 (0.005–0.071)	30.25±9.56 (13.16–62.96)	1.06±0.43 (0.43–2.06)	—	39	1	0	0	40
Last note	45	0.37±0.18 (0.10–0.79)	8.20±3.68 (2–21)	0.013±0.007 (0.005–0.044)	0.038±0.014 (0.010–0.079)	23.49±6.02 (11.33–40.56)	1.01±0.38 (0.46–1.86)	—	43	2	0	0	45
R. neba													
First note	26	0.13±0.04 (0.05–0.22)	1.65±0.96 (1–4)	0.090±0.040 (0.030–0.170)	—	17.87±5.31 (9.82–25.57)	1.22±0.30 (0.45–1.75)	**1.34±0.23 (1.01–1.88)	0	1	25	2	24
Middle note	26	0.17±0.04 (0.08–0.23)	6.77±2.52 (3–13)	0.014±0.008 (0.004–0.041)	0.016±0.006 (0.005–0.028)	40.03±10.85 (21.63–62.99)	1.16±0.38 (0.44–1.73)	—	26	0	0	0	26
Last note	26	0.20±0.08 (0.09–0.37)	6.35±1.80 (3–11)	0.010±0.004 (0.004–0.021)	0.026±0.012 (0.010–0.060)	35.33±12.36 (16.39–64.98)	1.05±0.39 (0.43–1.68)	—	26	0	0	0	26

TABLE 4. Acoustic diagnosis between the topotypic population of *Rana tagoi* and *R. neba*, suggested in this study.

Character		*R. tagoi*	*R. neba*
First note	Impression in human ear	Usually sounds similar with other notes	Sounds differently from other notes
	Modulation	Usually absent	Present
	Pulse number	Many (usually ≥3, mode=5)	Few (usually ≤2, mode=1)
	Pulse length	Short (usually <0.06 s, average=0.029 s)	Long (>0.06 s in 2/3 of examined records, average=0.090 s)
	Distances between harmonics in a spectrogram	Short (<1.0 kHz)	Long (≥1.0 kHz)
	Fundamental frequency	Low (usually <1.0 kHz, average=0.72 kHz)	High (usually >1.0 kHz, average=1.34 kHz)
Last note	Impression in human ear	Sometimes sounds slower than other notes	Sounds similar with other notes
	Note length	Long (>0.4s in the half of examined records, average=0.37s)	Short (usually <0.4s, average=0.20s)
	Pulse number	Many (≥8 in the half of examined records, mode=9)	Few (usually <8, mode=5)
	Inter-pulse length	Long (>0.04s in the half of examined records, average=0.038s)	Short (Usually <0.04s, average=0.026s)
Note number		Few (<4 in the half of examined samples, mode=3)	Many (Usually ≥4, mode=5).

difference was detected ($\chi^2=82$, df=4, $p<0.001$). In the multiple comparisons, the last note of *R. tagoi* exhibited larger values than first and middle notes and the middle note of *R. neba*. The last note of *R. neba* exhibited larger values than in the first note of *R. tagoi*. The middle note of *R. tagoi* exhibited larger values than its first note.

(8) Pulse rate (Fig. 3I): A significant difference was detected ($\chi^2=55$, df=5, $p<0.001$). In the multiple comparison, the first note of *R. neba* had a significantly smaller value than in others except for the last note of *R. tagoi*, which also exhibited a smaller value than some of others (the first note of *R. tagoi* and the middle and last note of *R. neba*).

(9) Dominant frequency (Fig. 3J): No significant differences were detected ($\chi^2=7.8$, df=5, $p=0.200$).

(10) Fundamental frequency in the first note (Fig. 3D): The first note of *R. neba* had a significantly higher fundamental frequency than in *R. tagoi* ($p<0.001$).

(11) Frequency modulation: Almost all calls of *R. neba* (25 out of 26 samples; 96.2%) exhibited an obvious frequency modulation in the first note (Figs. 2C, 2D, 4C; Table 3), while only one record had obscure modulation (Fig. 4D). The frequency modulation typically showed a mountain-shape with a flat top followed by gradual descent of frequency (Fig. 2C, 2D), which is sometimes obscure (Fig. 4D). In some cases, there are several unmodulated short pulses before a modulated long pulse (Fig. 2C), but such pulses were seldom observed after it. In *R. tagoi*, most records (40 out of 45 samples; 88.9%) lack any frequency modulations (Fig. 2B). An obscure (weak) modulation was observed in four records (8.9%), but it occupies only a part (around the beginning) of the note (Fig. 4B). An obvious frequency modulation (Fig. 4A) was recorded from only one record (2.2%).

(12) Harmonics: Harmonics were observed in almost all notes of both species, and only two records (7.7%) of *R. neba* lacks harmonics in the first note. The distance between

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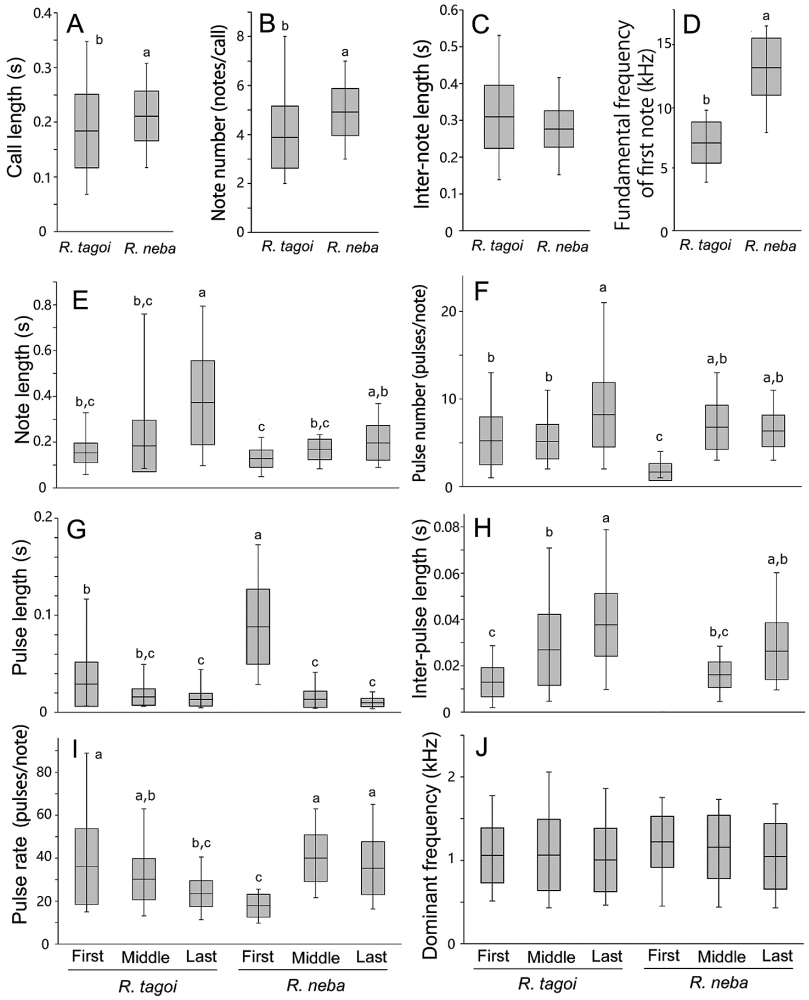


FIG. 3. Acoustic characteristics of *Rana tagoi* and *R. neba* calls. The horizontal bar, the gray box, and the vertical bar indicate average, SD, and range, respectively. A: call length, B: note number per call, C: inter-note length, D: fundamental frequency of the first note, E: note length, F: pulse number per note, G: average pulse length, H: average inter-pulse length, I. pulse rate, and J: dominant frequency. In E–J, the first, the middle, and the last notes were shown separately. The different letters on the bar indicate the significant difference in the multiple comparisons.

each harmonic in the spectrogram seems to be wider in *R. neba* than in *R. tagoi* reflecting a higher fundamental frequency. In the spectrogram of *R. tagoi*, the distance is apparently narrower than 1.0 kHz even at maximum. In contrast, in *R. neba*, the maximum distance between each harmonic is approximately equal to, or wider than 1.0 kHz (Fig. 3D).

DISCUSSION

Our results show that the advertisement calls of *R. neba* and *R. tagoi* are distinguishable, however, the distinguishing characters are different from those previously proposed (Ryuzaki et al., 2014a). The advertisement calls of frogs and toads are species-specific traits that can act as isolating mechanisms for

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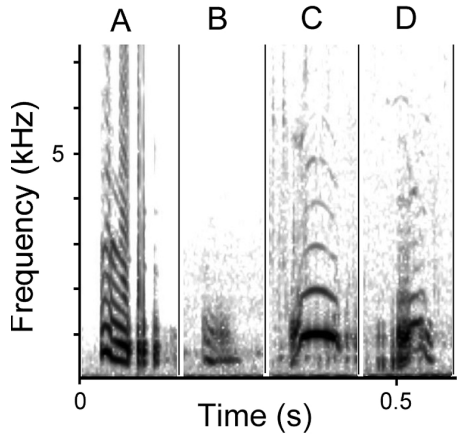


FIG. 4. Exceptional variations of the first note of advertisement calls: A, *Rana tagoi* with an obvious frequency modulation; B, *R. tagoi* with an obscure frequency modulation; C, *R. neba* with a frequency modulation lacking obvious descent of frequency; D, *R. neba* with an obscure frequency modulation. Window type=Hann, window size=512 samples, frequency grid spacing=86.1 Hz.

allied species. Thus, many anuran taxonomists focus on advertisement calls when describing species (Köhler et al., 2017). However, to utilize this character for taxonomy, we must pay attention to the factors affecting acoustic parameters. First, body size of the male is known to affect the pitch of its calling (Köhler et al., 2017). Inconveniently, in the *R. tagoi* complex, it is usually difficult to measure the body size of calling males in wild, because they call from hidden cavities near the stream or spring, with several males often occupying a single cavity (Daito and Nakamizo, 1993; our unpublished data). Because we detected no variation in body size between the type localities of *R. tagoi* and *R. neba*, or between present and previous studies (Ryuzaki et al., 2014a; our unpublished data), body size is unlikely to affect our results. Second, environmental temperatures are also reported to affect some acoustic parameters, such as call length, pulse rate, and dominant frequency (Köhler et al., 2017). In *R. tagoi*, Daito and Kawakami (1992) reported that environmental temperature exhibited a positive correlation with the dominant frequency of advertisement calls. A similar effect from temperature might affect the results of our study. In our dataset, air temperatures during recording were significantly higher in *R. tagoi* compared to *R. neba*, but no differences were detected in water temperatures. Males of both species call in the water running underground and are likely more affected by water temperature than air temperature (Daito and Kawakami, 1992). Thus, the differences observed in air temperature may not have a large effect on our results. Yet, it should be noted that our results might be affected by such environmental factors irrespective of their intrinsic differences. However, given that the acoustic differences between the two localities are large and not clinal, it is unlikely that environmental factors explain such differences. Thus, we propose that differences we observed in the calls between the type localities accurately reflects the interspecific variation in calls between *R. tagoi* and *R. neba*.

The acoustic diagnosis between *R. tagoi* and *R. neba* proposed in this study (Table 4) is not concordant with those previously reported. Although Ryuzaki et al. (2014a) identified the dominant frequency of advertisement calls as one of the major diagnostic characters of *R. tagoi* (0.5 to 0.7 kHz) and *R. neba* (1.3 and 1.7 kHz), our result cast doubt on their conclusion. In our survey of type localities of each species, both of them exhibit great variation in dominant frequencies and no significant differences were detected (Fig. 3). Thus, the calls of *R. neba* and *R. tagoi* cannot be distinguished following the acoustic diagnosis reported in Ryuzaki et al. (2014a).

Unlike dominant frequencies, there was a significant difference in the fundamental frequency of the first note between *R. tagoi* and *R. neba* calls (Figs. 3D, 4). Unfortunately, this character could not be applied to all cases, as there were several recordings in which estimating a reliable fundamental frequency value was difficult (15.6% in *R. tagoi* and 15.4% in *R. neba*). If, however, calls are recorded in a high quality (i.e., loud

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sound with low background noise), we propose that fundamental frequency is a good diagnostic character for these species.

In addition to fundamental frequency of the call, frequency modulation in the first note of call could also be used as a reliable diagnostic character (Fig. 4; Eto et al., 2016). Obvious frequency modulation was absent in many recordings of *R. tagoi* collected in our study, whereas almost all samples of *R. neba* exhibited apparent frequency modulation. As previously stated by Ryuzaki et al. (2014b), *R. tagoi* also infrequently (11.1% in this study) emit calls with frequency modulation (Fig. 4A, B). Such calls of *R. tagoi* with modulation also could be differentiated from the calls of *R. neba* due to their lower fundamental frequency, which appears as closer distances between each harmonic in the spectrogram, as well as weaker modulation (Figs. 2A, 2C, 4A, and 4B). Additionally, the first note of *R. neba* is also characterized by longer and fewer pulses and lower pulse rate than in other notes (Fig. 3F, 3G, and 3I). Because of these unique acoustic characters, *R. neba*'s first note sounds notably different from the following notes by the human ear, while *R. tagoi*'s first note usually sounds similar to the following notes.

In contrast to *R. neba*, the calls of *R. tagoi* are composed of notes with similar acoustic structures. Yet, its last note can be characterized by its larger values in note length, pulse number, and inter-pulse length, and smaller values in pulse rate compared with the other notes. These four tendencies found in the last note were also reported in the description of advertisement calls of "Kamitakara group of *R. tagoi*" in Ryuzaki et al. (2014b). To the human ear, the last note of *R. tagoi* often sounds slower than the other notes. Daito and Kawakami (1992) recognized "fast" and "slow" advertisement calls of *R. tagoi* (their "call" corresponds to "note" in our terminology) and mentioned that "fast calls" were followed by only a single "slow call" in many localities (Fukuoka, Ehime, Kyoto, and Niigata). It is difficult to compare their result with ours precisely, but at least a part of their "slow call" might correspond with the unique last note of *R. tagoi* that we report here.

Among the variations in advertisement call of the *R. tagoi* complex reported so far (Daito and Kawakami, 1992; Daito and Nakamizo, 1993; Doi, 2017; Kimura et al., 2019; Kuramoto, 1979; Maeda and Matsui, 1989; Matsui and Maeda, 2018; Matsuo et al., 2011; Ryuzaki et al., 2014a, b; Sueyoshi et al., 2013; Sugahara and Matsui, 1995), calls of *R. neba* are unique in containing two apparently different parts in one call. In anurans, such clear differentiation of the parts in an advertisement call sometimes reflects differences in social function. For example, the calls of *Engystomops pustulosus* contains introductory and secondary notes, and the repeating of the latter notes enhances the territorial and mate attracting function of the call (Rand and Ryan, 1981). Furthermore, it has been observed that two types of notes are used for mate attraction and territoriality, respectively, in several frog species, e.g, *Dendropsophus ebraccatus* (Wells and Schwartz, 1984), *Eleutherodactylus coqui* (Narins and Capranica, 1978), and *Geocrinia* spp. (Littlejohn and Watson, 1974; Littlejohn and Harrison, 1985). Considering these studies, there might also be a separation in the function of the first note and the following notes in *R. neba*. A call-playback experiment using male and female frogs of this species would be helpful in resolving this issue.

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