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Highly Complex Mitochondrial Dna Genealogy in an Endemic Japanese Subterranean Breeding Brown Frog *Rana Tagoi* (Amphibia, Anura, Ranidae)

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Highly Complex Mitochondrial DNA Genealogy in an Endemic Japanese Subterranean Breeding Brown Frog *Rana tagoi* (Amphibia, Anura, Ranidae)

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The endemic Japanese frog *Rana tagoi* is unique among Holarctic brown frogs in that it breeds in small subterranean streams. Using mitochondrial 16S ribosomal RNA and NADH dehydrogenase subunit 1 genes, we investigated genealogical relationships among geographic samples of this species together with its relative *R. sakuraii*, which is also a unique stream breeder. These two species together form a monophyletic group, within which both are reciprocally paraphyletic. *Rana tagoi* is divided into two major clades (Clade A and B) that are composed of 14 genetic groups. *Rana sakuraii* is included in Clade A and split into two genetic groups, one of which forms a clade (Subclade A-2) with sympatric *R. tagoi*. This species-level paraphyly appears to be caused by incomplete taxonomy, in addition to introgressive hybridization and/or incomplete lineage sorting. *Rana tagoi* strongly differs from other Japanese anurans in its geographic pattern of genetic differentiation, most probably in relation to its unique reproductive habits. Taxonomically, *R. tagoi* surely includes many cryptic species.

Key words: *Rana tagoi*, Japan, mtDNA, paraphyly, cryptic species, subterranean breeding, genetic divergence

INTRODUCTION

The genus *Rana* historically represented a very large group of frogs that occurred almost worldwide (Boulenger, 1920; Frost, 1985; Dubois, 1992), but is now restricted to smaller number of Holarctic brown frogs (Frost et al., 2006) that are generally similar in adult morphology and ecology. Most congeners breed in still (lentic) waters, such as ponds and rice paddies (e.g., *R. temporaria* Linnaeus from Europe: Nöllert and Nöllert, 1992), and only a few (e.g., *R. graeca* Boulenger from Europe and *R. sauteri* Boulenger from Taiwan) in flowing (lotic) waters of open streams (Nöllert and Nöllert, 1992; Tanaka-Ueno et al., 1998). Compared with such species, Japanese *R. tagoi* Okada (type locality: restricted by Shibata [1988] to Kamitakara-mura, currently included in Takayama-shi, Gifu Prefecture) is unique in that it breeds in small underground streams (Maeda and Matsui, 1999). This subterranean breeding habit is highly specialized and is not known in any other congeneric species.

Rana tagoi is endemic to the main (Honshu, Shikoku, and Kyushu) and some adjacent, smaller (Yakushima, Oki, and Goto) islands of Japan. Eggs laid in subterranean streams are few in number and large in size, and once hatched tadpoles can metamorphose without feeding (Maeda and Matsui, 1999). Such traits appear to be an adaptation to this unique breeding environment. Another brown frog, *R. sakuraii* Matsui and Matsui (type locality: Okutama-machi, Nishitama-gun, Tokyo Prefecture) occurs only on Honshu Island and breeds in wider open streams in mountain regions. Other than the difference in breeding environment, this species is generally similar to *R. tagoi* in morphology and ecology, and is thought to be a close relative of *R. tagoi*, having originated from a *R. tagoi*-like subterranean breeding ancestor (Maeda and Matsui, 1999).

Steep mountains that provide many streams and rivers occupy the larger part of the main islands of Japan. Reflecting this environmental trait, there are various amphibian species that are adapted to lotic environments (e.g., *Bufo torrenticola* Matsui; *Buergeria buergeri* [Temminck and Schlegel]). Recent extensive surveys have revealed high cryptic diversity in some lotic breeding salamanders of the genera *Hynobius* Tschudi and *Onychodactylus* Tschudi (Nishikawa et al., 2007; Yoshikawa et al., 2008). A similar situation is expected in the case of lotic breeding *R. tagoi*, as the species is unique among Japanese frogs in that it contains three distinct subspecies (*R. t. tagoi* from main islands of Japan, *R. t. okiensis* Daito from Oki Islands, and *R. t. yakushimensis* Nakatani and Okada from Yakushima Island). In addition, morphological, breeding ecological (Sugahara, 1990; Sugahara and Matsui, 1992, 1993, 1994, 1995, 1996, 1997), and karyological (Ryuzaki et al., 2006) variations reported within *R. t. tagoi* suggest that it includes cryptic species. Genetically, *R. tagoi* is also diversified as

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shown by the analyses of allozymes (Nishioka et al., 1987) and mitochondrial DNA (mtDNA; Tanaka et al., 1994). In contrast, variations within *R. sakuraii* have been poorly studied.

These previous studies suggest the presence of phylogenetic and/or taxonomic problems in *R. tagoi*, while such information is lacking for *R. sakuraii*. To date, few studies (e.g., Ryuzaki et al., 2006) have compared a large number of samples from the entire distributional range of the two species, leaving the comprehensive patterns of intra- or inter-specific variations unresolved. In this study, we conducted a phylogenetic analysis using two mitochondrial genes, relatively conservative 16S ribosomal RNA (16S rRNA) and rapidly evolving NADH dehydrogenase subunit 1 (ND1; Mueller, 2006), to reveal patterns of genetic differentiation and genealogical relationships in terms of mtDNA among samples of *R. tagoi* and *R. sakuraii*.

MATERIALS AND METHODS

We collected 183 specimens of *R. t. tagoi*, including the topotypic population, from 145 localities covering its entire distributional range in Honshu, Shikoku, and Kyushu. The large and small types of *R. t. tagoi* from Kinki (Sugahara, 1990) were distinguished according to the diagnosis of Sugahara and Matsui (1994). We also collected two specimens of *R. t. yakushimensis* from Yakushima Island and three specimens of *R. t. okiensis* from the Oki islands. Furthermore, we collected 19 specimens of *R. sakuraii*, including the topotype, from 16 localities in Honshu. Detailed sampling localities are shown in Fig. 1 and Table 1.

As outgroups, we used *R. tsushimensis* from Tsushima Islands, Japan, and *Lithobates sylvaticus* from Quebec, Canada. The latter species is morphologically and ecologically similar to members of the genus *Rana*, but has been placed recently in another ranid genus, *Lithobates* (Frost et al., 2006).

Total DNA was extracted from frozen or ethanol-preserved tissues by standard phenol-chloroform extraction procedures (Hillis et al., 1996). Fragments containing the entire 16S rRNA and ND1 sequences, approximately 2.9 kb long, were amplified by polymerase chain reaction (PCR). The PCR cycle included an initial heating at 94°C for 4 min; 33 cycles of 94°C (30 s), 50°C (30 s), and 72°C (2 min 30 s); and a final extension at 72°C for 7 min. The amplified PCR products were purified by polyethylene glycol (PEG) precipitation procedures. The cycle sequence reactions were carried out with ABI PRISM Big Dye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems) and sequencing was performed on an ABI 3130 automated sequencer. We used the primers listed in Table 2 to amplify and sequence the fragments, and all samples were sequenced in both directions. The obtained sequences were depos-

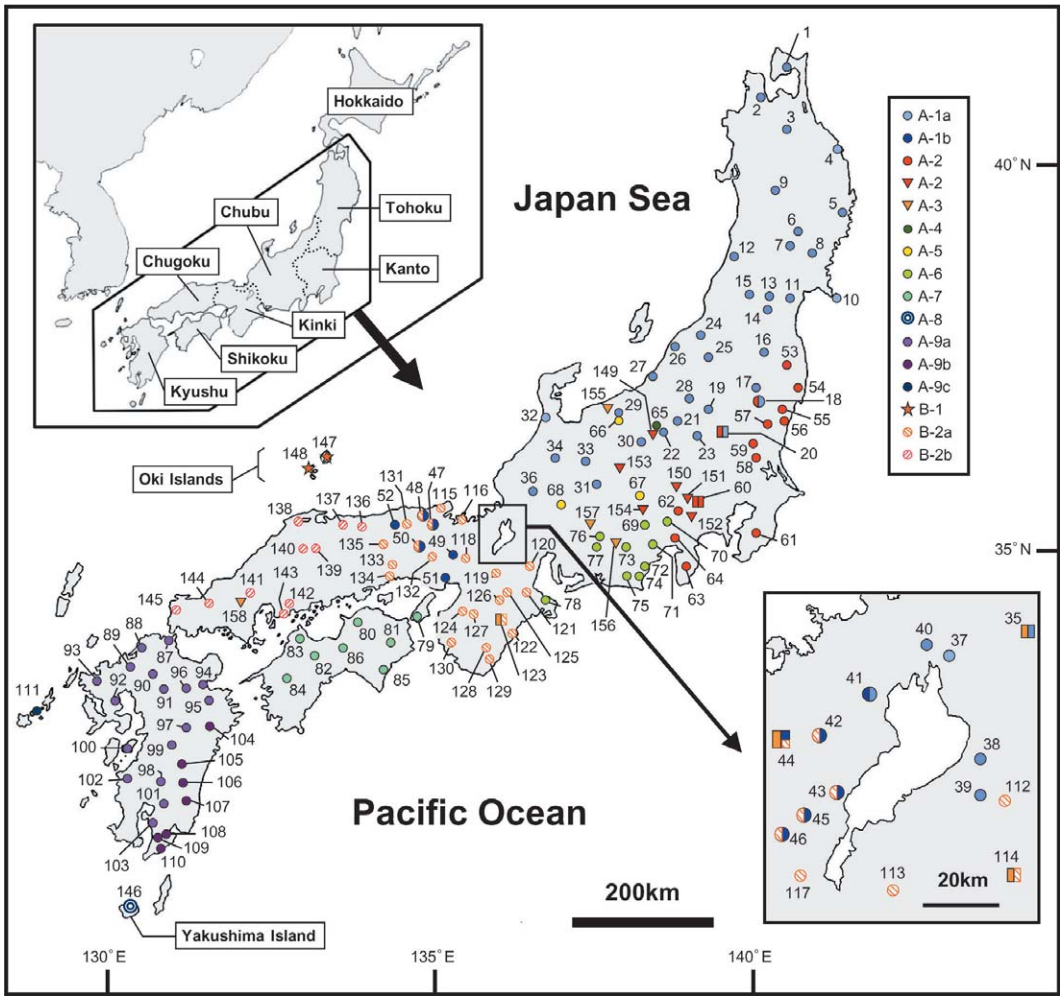

Fig. 1. Map of Japan showing sampling localities of *Rana t. tagoi* (circles), *R. t. yakushimensis* (double circle), *R. t. okiensis* (stars), and *R. sakuraii* (triangles). Squares indicate localities with sympatry of *R. t. tagoi* and *R. sakuraii*. For names of localities and genetic groups, see Table 1.

Table 1. Samples used for mtDNA analysis in this study with the information of voucher and collection locality. KUHE: Graduate School of Human and Environmental Studies, Kyoto University; TMP: Temporary numbered; UN: Unnumbered.

Sample no	Locality	genetic group	Voucher (KUHE)	GenBank 16S rRNA	GenBank ND1
Rana tagoi tagoi					
1	Mutsu-shi, Aomori Pref.	A-1a	44827	AB639413	AB639593
2	Goshogawara-shi, Aomori Pref.	A-1a	36949	AB639413	AB639594
3	Towada-shi, Akita Pref	A-1a	13932	AB639413	AB639603
4	Noda-mura, Iwate Pref.	A-1a	37028	AB639413	AB639595
5	Kamaishi-shi, Iwate Pref.	A-1a	27750	AB639411	AB639596
6	Oshu-shi, Iwate Pref.	A-1a	32889	AB639413	AB639597
7	Ichinoseki-shi, Iwate Pref.	A-1a	35268	AB639412	AB639603
8	Fujisawa-cho, Iwate Pref.	A-1a	36699	AB639413	AB639598
9	Senboku-shi, Akita Pref.	A-1a	27351	AB639413	AB639603
10	Ishinomaki-shi, Miyagi Pref.	A-1a	41545	AB639414	AB639603
11	Sendai-shi, Miyagi Pref.	A-1a	37121	AB639415	AB639599
12	Sakata-shi, Yamagata Pref.	A-1a	37544	AB639416	AB639600
13	Yamagata-shi, Yamagata Pref.	A-1a	37543	AB639417	AB639601
14	Kaminoyama-shi, Yamagata Pref.	A-1a	29360	AB639420	AB639602
15	Nishikawa-machi, Yamagata Pref.	A-1a	37548	AB639418	AB639603
16	Nihonmatsu-shi, Fukushima Pref.	A-1a	29595	AB639419	AB639604
17	Shirakawa-shi, Fukushima Pref.	A-1a	21629	AB639420	AB639605
18a	Daigo-machi, Ibaraki Pref.	A-1a	42344	AB639420	AB639605
18b		A-2	43886	AB639421	AB639646
19	Nikko-shi, Tochigi Pref.	A-1a	36719	AB639426	AB639609
20a	Kanuma-shi, Tochigi Pref.	A-1a	40166	AB639422	AB639609
21	Minakami-machi, Gunma Pref.	A-1a	27539	AB639429	AB639612
22	Nakanojo-machi, Gunma Pref.	A-1a	27930	AB639424	AB639606
23	Shibukawa-shi, Gunma Pref.	A-1a	29485	AB639425	AB639607
24	Agano-shi, Niigata Pref.	A-1a	29600	AB639426	AB639608
25	Aga-machi, Niigata Pref.	A-1a	UN	AB639426	AB639609
26	Yahiko-mura, Niigata Pref.	A-1a	27765	AB639427	AB639610
27	Kashiwazaki-shi, Niigata Pref.	A-1a	36892	AB639428	AB639611
28	Uonuma-shi, Niigata Pref.	A-1a	36896	AB639429	AB639612
29	Otari-mura, Nagano Pref.	A-1a	43367	AB639430	AB639613
30	Ueda-shi, Nagano Pref.	A-1a	18752	AB639431	AB639614
31	Kiso-machi, Nagano Pref.	A-1a	43382	AB639432	AB639615
32	Hodatsushimizu-cho, Ishikawa Pref.	A-1a	41053	AB639433	AB639616
33	Takayama-shi, Gifu Pref.	A-1a	27613, 43018	AB639434	AB639617
34	Shirakawa-mura, Gifu Pref.	A-1a	26104	AB639435	AB639618
35a	Ibigawa-cho, Gifu Pref.	A-1a	27388	AB639436	AB639619
36	Ikeda-cho, Fukui Pref.	A-1a	40441	AB639438	AB639624
37	Nagahama-shi, Shiga Pref.	A-1a	41470, 41471	AB639439	AB639621
38	Maibara-shi, Shiga Pref.	A-1a	37610, 37614	AB639440	AB639622
39a	Taga-cho, Shiga Pref.	A-1a	41287	AB639440	AB639622
39b			41551	AB639441	AB639623
40	Nagahama-shi, Shiga Pref.	A-1a	40385	AB639442	AB639624
41a	Takashima-shi, Shiga Pref.	A-1a	TMP_T3395	AB639443	AB639625
41b			40437	AB639444	AB639625
41c			TMP_T3402	AB639445	AB639625
41d		A-1b	TMP_T3392	AB639446	AB639626
42a	Takashima-shi, Shiga Pref.	A-1b	25993	AB639447	AB639627
42b		B-2a	43609	AB639448	AB639711
42c		B-2a	25996	AB639453	AB639711
43a	Otsu-shi, Shiga Pref.	A-1b	41414, 43428	AB639449	AB639628
43b		B-2a	41090	AB639450	AB639629
43c			43148	AB639451	AB639713
44a	Nantan-shi, Kyoto Pref.	A-1b	41408	AB639452	AB639630
44b		B-2a	41406	AB639453	AB639711
44c			41426	AB639547	AB639713
45a	Kyoto-shi, Kyoto Pref.	A-1b	43324	AB639457	AB639635
45b		B-2a	41730	AB639458	AB639633
45c			38698	AB639459	AB639634
46a	Kyoto-shi, Kyoto Pref.	A-1b	42034, 44828	AB639460	AB639635
46b		B-2a	44835	AB639462	AB639711
46c			42396	AB639463	AB639711
46d			41439	AB639461	AB639713
46e			42319	AB639464	AB639712
47a	Toyooka-shi, Hyogo Pref.	A-1b	25664	AB639465	AB639636
47b		B-2a	25662	AB639564	AB639729
48a	Toyooka-shi, Hyogo Pref.	A-1b	42711	AB639466	AB639637
48b		B-2a	42714	AB639467	AB639729
49a	Sasayama-shi, Hyogo Pref.	A-1b	10285	AB639468	AB639638
49b			10307	AB639469	AB639639
50a	Asago-shi, Hyogo Pref.	A-1b	10319	AB639470	AB639642
50b		B-2a	36586	AB639471	AB639640
51	Kobe-shi, Hyogo Pref.	A-1b	22647	AB639472	AB639641
52	Wakasa-cho, Tottori Pref.	A-1b	34743	AB639473	AB639642
53	Nihonmatsu-shi, Fukushima Pref.	A-2	36330	AB639474	AB639643
54	Hirono-machi, Fukushima Pref.	A-2	44829	AB639475	AB639644
55	Kitaibaraki-shi, Ibaraki Pref.	A-2	27544	AB639476	AB639645
56	Hitachi-shi, Ibaraki Pref.	A-2	27550	AB639477	AB639646
57	Hitachiomiya-shi, Ibaraki Pref.	A-2	43711	AB639478	AB639647
58a	Tsukuba-shi, Ibaraki Pref.	A-2	42747	AB639479	AB639648
58b			42751	AB639480	AB639649
59	Mashiko-machi, Tochigi Pref.	A-2	25968	AB639481	AB639650
60a	Akiruno-shi, Tokyo Pref.	A-2	42452	AB639483	AB639651
61	Ichihara-shi, Chiba Pref.	A-2	28409	AB639482	AB639652
62	Otsuki-shi, Yamanashi Pref.	A-2	28064	AB639483	AB639653
63a	Izu-shi, Shizuoka Pref.	A-2	36715	AB639484	AB639654
63b			43468	AB639485	AB639655
64	Fuji-shi, Shizuoka Pref.	A-2	43473	AB639486	AB639656
65	Nakanojo-machi, Gunma Pref.	A-4	22930, 22936	AB639487	AB639657
66	Nagano-shi, Nagano Pref.	A-5	18005	AB639488	AB639658
67	Hokuto-shi, Yamanashi Pref.	A-5	43483	AB639489	AB639659
68a	Gujo-shi, Gifu Pref.	A-5	14228	AB639490	AB639660
68b			44832	AB639491	AB639661
69	Hayakawa-cho, Yamanashi Pref.	A-6	14208	AB639492	AB639662
70	Fujikawaguchiko-machi, Yamanashi Pref.	A-6	43480	AB639493	AB639663
71a	Shizuoka-shi, Shizuoka Pref.	A-6	42977	AB639494	AB639664
71b			24561	AB639495	AB639665
72	Shizuoka-shi, Shizuoka Pref.	A-6	29933	AB639496	AB639666
73	Kawanehon-cho, Shizuoka Pref.	A-6	42270	AB639497	AB639667
74	Fujieda-shi, Shizuoka Pref.	A-6	17955	AB639498	AB639668
75	Kakegawa-shi, Shizuoka Pref.	A-6	39980	AB639499	AB639669
76	Neba-mura, Nagano Pref.	A-6	27335	AB639500	AB639670
77	Shitara-cho, Aichi Pref.	A-6	27251	AB639501	AB639671
78a	Ise-shi, Mie Pref.	A-6	42829	AB639502	AB639672
78b			42830	AB639503	AB639672
79	Minamiawaji-shi, Hyogo Pref.	A-7	43885	AB639504	AB639673
80	Manno-cho, Kagawa Pref.	A-7	TMP_T2882	AB639505	AB639674
81	Kamiyama-cho, Tokushima Pref.	A-7	TMP_T2876	AB639506	AB639675
82	Saijo-shi, Ehime Pref.	A-7	27679	AB639507	AB639676
83	Imabari-shi, Ehime Pref.	A-7	27506	AB639508	AB639677
84	Seiyo-shi, Ehime Pref.	A-7	TMP_T2241	AB639509	AB639678
85	Toyo-cho, Kochi Pref.	A-7	29464	AB639510	AB639679
86	Kochi-shi, Kochi Pref.	A-7	36184	AB639511	AB639680
87	Kitakyushu-shi, Fukuoka Pref.	A-9a	28614	AB639512	AB639681
88	Koga-shi, Fukuoka Pref.	A-9a	26841	AB639513	AB639682
89	Fukuoka-shi, Fukuoka Pref.	A-9a	26238	AB639514	AB639683
90	Yame-shi, Fukuoka Pref.	A-9a	26643	AB639515	AB639684
91	Asakura-shi, Fukuoka Pref.	A-9a	27137	AB639516	AB639685
92	Isahaya-shi, Nagasaki Pref.	A-9a	9660	AB639517	AB639686
93	Sasebo-shi, Nagasaki Pref.	A-9a	27140	AB639518	AB639687
94	Beppu-shi, Oita Pref.	A-9a	43637	AB639519	AB639688
95	Bungo-ohno-shi, Oita Pref.	A-9a	27146	AB639520	AB639694
96	Kokonoe-machi, Oita Pref.	A-9a	26148	AB639521	AB639689
97	Gokase-cho, Miyazaki Pref.	A-9a	44834	AB639522	AB639690
98	Ebino-shi, Miyazaki Pref.	A-9a	41284	AB639523	AB639694
99	Yatsushiro-shi, Kumamoto Pref.	A-9a	27562	AB639524	AB639691
100	Amakusa-shi, Kumamoto Pref.	A-9a	30342	AB639525	AB639692
101	Soo-shi, Kagoshima Pref.	A-9a	42191	AB639526	AB639693
102	Izumi-shi, Kagoshima Pref.	A-9a	27564	AB639527	AB639694
103	Kanoya-shi, Kagoshima Pref.	A-9a	27295, 43404	AB639530	AB639697
104	Nobeoka-shi, Miyazaki Pref.	A-9b	27121	AB639528	AB639695
105	Nishimera-son, Miyazaki Pref.	A-9b	26088	AB639529	AB639696
106	Aya-cho, Miyazaki Pref.	A-9b	42194	AB639531	AB639698
107	Miyakonojo-shi, Miyazaki Pref.	A-9b	30907	AB639532	AB639699

Continued

Table 1. Continued

Sample no	Locality	genetic group	Voucher (KUHE)	GenBank 16S rRNA	GenBank ND1
108	Kimotsuki-cho, Kagoshima Pref.	A-9b	43397	AB639533	AB639700
109a	Kanoya-shi, Kagoshima Pref.	A-9b	43401	AB639534	AB639701
109b			43403	AB639535	AB639702
110a	Kinko-cho, Kagoshima Pref.	A-9b	27678	AB639536	AB639703
110b			41250	AB639537	AB639704
111	Goto-shi, Nagasaki Pref.	A-9c	31539	AB639538	AB639705
112a	Taga-cho, Shiga Pref.	B-2a	43508	AB639539	AB639706
112b			43509	AB639540	AB639707
113	Konan-shi, Shiga Pref.	B-2a	18763	AB639541	AB639708
114a	Koka-shi, Shiga Pref.	B-2a	28466	AB639542	AB639709
115	Kyotango-shi, Kyoto Pref.	B-2a	24566	AB639544	AB639729
116	Maizuru-shi, Kyoto Pref.	B-2a	TMP_T3345	AB639545	AB639711
117a	Kyoto-shi, Kyoto Pref.	B-2a	27168	AB639546	AB639712
117b			41431	AB639547	AB639714
118	Kameoka-shi, Kyoto Pref.	B-2a	41553	AB639548	AB639713
119	Joyo-shi, Kyoto Pref.	B-2a	41554	AB639549	AB639714
120	Komono-cho, Mie Pref.	B-2a	26744	AB639550	AB639715
121	Matsuzaka-shi, Mie Pref.	B-2a	41484	AB639551	AB639716
122	Owase-shi, Mie Pref.	B-2a	26990	AB639552	AB639717
123a	Odai-cho, Mie Pref.	B-2a	40190	AB639553	AB639718
124	Izumi-shi, Osaka Pref.	B-2a	TMP_T3425	AB639556	AB639721
125	Soni-mura, Nara Pref.	B-2a	24435	AB639557	AB639722
126	Sakurai-shi, Nara Pref.	B-2a	18893	AB639558	AB639723
127	Kudoyama-cho, Wakayama Pref.	B-2a	24546	AB639559	AB639724
128	Hongu-cho, Wakayama Pref.	B-2a	26784	AB639560	AB639725
129	Shingu-shi, Wakayama Pref.	B-2a	24540	AB639560	AB639726
130	Gobo-shi, Wakayama Pref.	B-2a	41229	AB639561	AB639727
131	Kami-cho, Hyogo Pref.	B-2a	43603	AB639562	AB639728
132	Taka-cho, Hyogo Pref.	B-2a	10330	AB639564	AB639729
133	Sayo-cho, Hyogo Pref.	B-2a	41021	AB639563	AB639729
134	Kamigori-cho, Hyogo Pref.	B-2a	41022	AB639563	AB639729
135	Mimasaka-shi, Okayama Pref.	B-2a	27659	AB639564	AB639730
136	Misasa-cho, Tottori Pref.	B-2b	24574	AB639565	AB639731
137	Daisen-cho, Tottori Pref.	B-2b	36824	AB639566	AB639732
138	Unnan-shi, Shimane Pref.	B-2b	18877	AB639567	AB639734
139a	Shobara-shi, Hiroshima Pref.	B-2b	36037	AB639568	AB639733
139b			36040	AB639569	AB639734
140	Shobara-shi, Hiroshima Pref.	B-2b	24553	AB639570	AB639735
141	Hatsukaichi-shi, Hiroshima Pref.	B-2b	UN	AB639571	AB639736
142	Higashihiroshima-shi, Hiroshima Pref.	B-2b	30262	AB639572	AB639737
143	Higashihiroshima-shi, Hiroshima Pref.	B-2b	30220	AB639573	AB639738
144	Hagi-shi, Yamaguchi Pref.	B-2b	42848	AB639574	AB639739
145	Shimonoseki-shi, Yamaguchi Pref.	B-2b	34516	AB639575	AB639740
R. t. yakushimensis					
146a	Yakushima-cho, Kagoshima Pref.	A-8	10182	AB639578	AB639741
146b			43326	AB639577	AB639741
R. t. okiensis					
147a	Okinoshima-cho, Shimane Pref.	B-1	10818	AB639576	AB639742
147b			22341	AB639579	AB639742
148	Nishinoshima-cho, Shimane Pref.	B-1	43647	AB639580	AB639742
R. sakuraii					
20b	Kanuma-shi, Tochigi Pref.	A-2	43635	AB639423	AB639744
35b	Ibigawa-cho, Gifu Pref.	A-3	36297	AB639437	AB639620
44d	Nantan-shi, Kyoto Pref.	A-3	UN	AB639454	AB639631
44e			41412	AB639455	AB639632
44f			41413	AB639456	AB639632
60b	Akiruno-shi, Tokyo Pref.	A-2	42450	AB639583	AB639744
114b	Koka-shi, Shiga Pref.	A-3	TMP_T2666	AB639543	AB639710
123b	Odai-cho, Mie Pref.	A-3	27647	AB639554	AB639719
123c			40309	AB639555	AB639720
149	Naganohara-machi, Gunma Pref.	A-2	27937	AB639581	AB639744
150	Chichibu-shi, Saitama Pref.	A-2	43736	AB639582	AB639743
151	Okutama-machi, Tokyo Pref.	A-2	UN	AB639583	AB639744
152	Kiyokawa-mura, Kanagawa Pref.	A-2	14276	AB639584	AB639745
153	Matsumoto-shi, Nagano Pref.	A-2	22887	AB639585	AB639746
154	Fujikawa-cho, Yamanashi Pref.	A-2	43481	AB639586	AB639747
155	Itoigawa-shi, Niigata Pref.	A-3	31300	AB639587	AB639748
156	Hamamatsu-shi, Shizuoka Pref.	A-3	UN	AB639588	AB639749
157	Nakatsugawa-shi, Gifu Pref.	A-3	18201	AB639589	AB639749
158	Iwakuni-shi, Yamaguchi Pref.	A-3	43893	AB639590	AB639750
R. tsushimensis					
	Tsushima-shi, Nagasaki Pref.		11606	AB639592	AB639752
Lithobates sylvaticus					
	Quebec, Canada		UN	AB639591	AB639751

ited in GenBank (Table 1).

Sequences obtained were aligned using Clustal W (Thompson et al., 1994), and gaps and ambiguous areas were excluded from alignments using Gblocks 0.91b (Castresana, 2000) with default settings. We then constructed phylogenetic trees from the combined alignments using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). The MP analysis was performed using PAUP*4.0b10 (Swofford, 2002). We used a heuristic search with the tree bisection and reconnection (TBR) branch-swapping algorithm and 100 random additions replicates, and the number of saved trees was restricted to 5,000. Transitions and transversions were equally weighted. The ML and BI analyses were respectively performed using TREEFINDER ver. Oct. 2008 (Jobb, 2008) with Phylogears 1.5.2010.03.24 (Tanabe, 2008) and MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Different substitution models were applied for each gene partition in both of the analyses. The optimum substitution model for each gene was selected by using Kakusan4 (Tanabe, 2010), based on the Akaike information criterion (AIC). The best model was calculated for each codon position (1st, 2nd, and 3rd positions) of the ND1 genes. In the BI analysis, two independent runs of four Markov chains were conducted for 7,000,000 generations (sampling frequency: one tree per 100 generations). We used TRACER v. 1.4 (Rambaut and Drummond, 2007) to determine the burn-in size and when the log likelihood of sampled trees reached stationary distribution, and the first 7,001 trees were discarded (burn-in = 700,000).

Table 2. Primers used to amplify mtDNA in this study.

Target	Name	Sequence	Reference
16S	L1507	TACACACCGCCCGTCACCCTCTT	Shimada et al. (2011)
	H1923	AAGTAGCTCGCTTAGTTTCGG	Shimada et al. (2011)
	L1879	CGTACCTTTTGCATCATGGTC	Shimada et al. (2011)
	H2315	TTCTTGTTACTAGTTCTAGCAT	Shimada et al. (2011)
	L2188	AAAGTGGGCCTAAAAGCAGCCA	Matsui et al. (2006)
	Wilkinson_6	CCCTCGTGATGCCGTTGATAC	Wilkinson et al. (2002)
	16L1	CTGACCGTGCAAAGGTAGCGTAATCACT	Hedges (1994)
	16H1	CTCCGGTCTGAACTCAGATCACGTAGG	Hedges (1994)
ND1	L3032	CGACCTCGATGTTGGATCAGG	Shimada et al. (2011)
	ND1_Htago	GRGCRTATTTGGAGTTTGARGCTCA	this study
	ND1_Ltago	GACCTAAACCTCAGYATYCTATTTAT	this study
	tMet_H	AGGAAGTACAAAGGGTTTTGATC	Shimada et al. (2011)

The robustness of the MP and ML trees were tested using nonparametric bootstrap analysis (Felsenstein 1985) with 1,000 replicates. We regarded tree topologies with bootstrap value (BS) 70% or greater as sufficiently supported (Huelsenbeck and Hillis, 1993). For the BI, we regarded Bayesian posterior probability (BPP) 0.95 or greater as significant support (Huelsenbeck and Ronquist, 2001;

Leaché and Reeder 2002). Uncorrected p-distances for each gene were also calculated using PAUP* ver. 4.0b10.

RESULTS

Sequences and statistics

We obtained complete 16S rRNA (1,625 bp long) and ND1 (973 bp) sequences from 207 individuals and two outgroup taxa. After excluding gaps and ambiguous areas, a combined 2,521 nucleotide sites, of which 624 were variable

Table 3. Alignment statistics for total 16S rRNA and ND1. The number of base pairs (bp), variable sites (vs), number of parsimony informative sites (pi), and transition-transversion ratio (ti/tv) are given for ingroups only.

	bp	vs	pi	ti/tv
16SrRNA	1554	310	206	6.65
ND1	967	314	250	9.38
Combined	2521	624	456	8.04

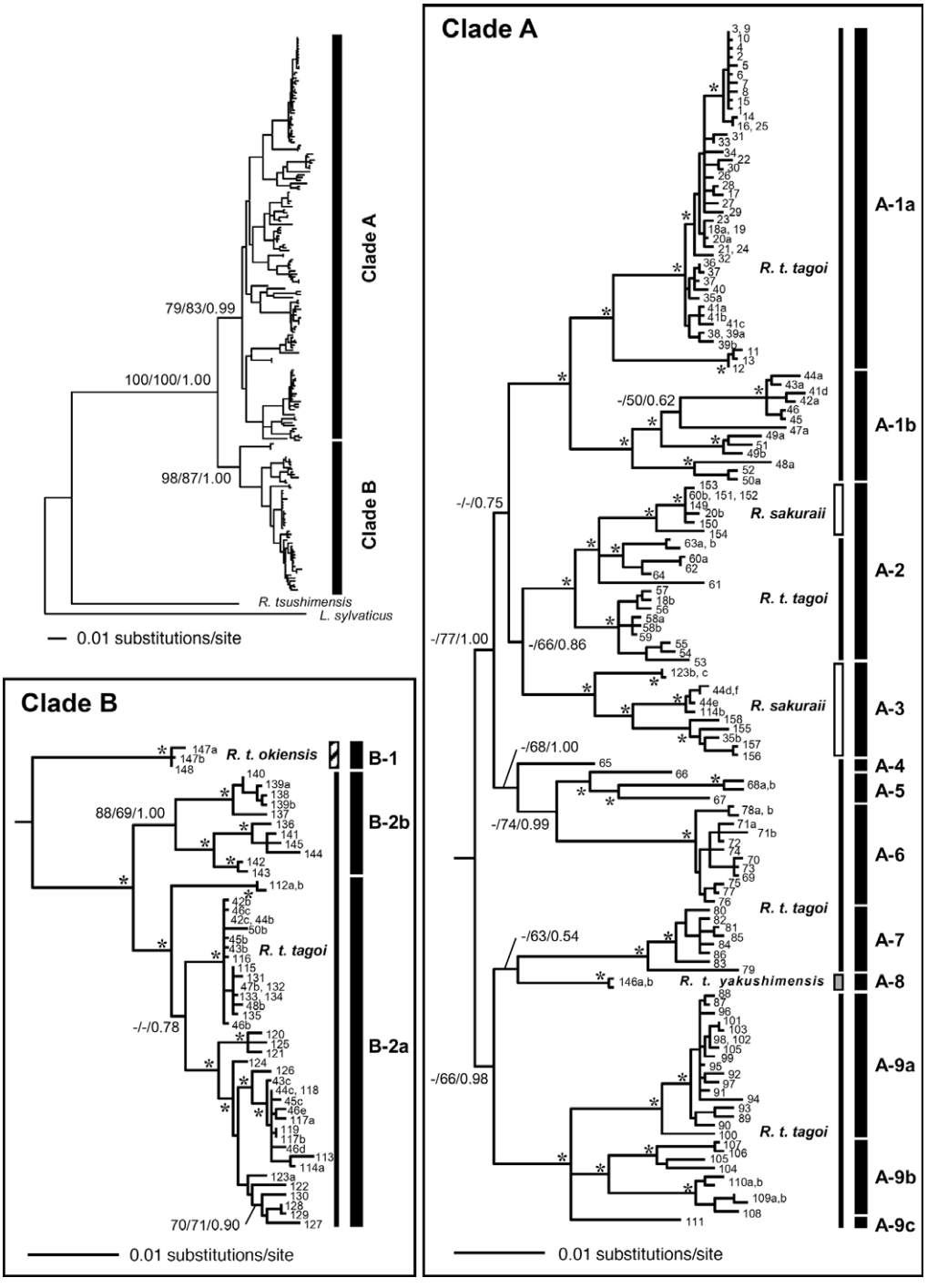

Fig. 2. Bayesian tree of total 16S rRNA and ND1 mitochondrial genes for three subspecies of *R. tagoi*, *R. sakuraii*, and outgroup taxa. Nodal values indicate bootstrap supports for MP and ML, and Bayesian posterior probability (MP-BS/ML-BS/BPP). Asterisks indicate nodes with MP-BS and ML-BS = 70% and BPP = 0.95. For locality numbers, see Table 1 and Fig. 1.

and 456 parsimoniously informative (Table 3), were used for phylogenetic analysis. We detected 190 haplotypes within the ingroup, of which 168 were in *R. t. tagoi*, two in *R. t. yakushimensis*, three in *R. t. okiensis*, and 17 in *R. sakuraii*.

The MP analysis produced 5,000 equally most parsimonious trees (L = 2007, CI = 0.519, RI = 0.901). For the ML analysis, the best substitution model of 16S rRNA estimated by Kakusan 4 was J2 model with a Gamma (G) shape parameter. In ND1, Hasegawa-Kishino-Yano-1985 (HKY85) model + G, HKY85 + G, and J2 + G were selected for the 1st, 2nd, and 3rd codon positions, respectively. For the BI analysis, the general time reverse (GTR) model + G was selected for 16S rRNA. In ND1, HKY85 + G, HKY85 + G, and GTR + G were selected for the 1st, 2nd, and 3rd codon positions, respectively. The likelihood values (–lnL) of the ML and BI trees were 14439.77 and 15102.97, respectively.

Table 4. Mean uncorrected p-distances (%) among genetic groups of three subspecies of *R. tagoi* and *R. sakuraii* for 16S rRNA (above diagonal) and ND1 (bellow diagonal). Darkly shaded areas indicate distances among groups with sympatric distribution and lightly shaded areas indicate distances among groups with parapatric distribution.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. A-1a	–	1.3	1.6	1.9	1.6	1.4	1.5	1.9	1.9	1.7	1.8	2.0	1.7	3.0	3.3	3.0
2. A-1b	4.1	–	1.8	2.1	1.7	1.6	1.6	1.9	2.1	1.6	2.0	2.3	1.8	2.8	3.2	2.9
3. A-2 (*R. t. tagoi*)	3.9	4.3	–	1.1	1.7	1.3	1.6	1.9	1.9	1.7	1.9	2.2	1.8	2.9	3.1	2.8
4. A-2 (*R. sakuraii*)	4.2	4.2	2.1	–	1.9	1.4	1.8	2.0	2.1	1.9	2.1	2.4	2.1	3.0	3.1	2.8
5. A-3	4.7	5.0	3.7	3.6	–	1.4	1.5	1.9	2.1	1.8	1.9	2.2	1.8	2.7	3.0	2.6
6. A-4	3.9	4.3	3.0	3.3	4.1	–	1.3	1.7	1.8	1.5	1.7	2.0	1.6	2.7	2.8	2.6
7. A-5	4.4	4.9	3.5	3.8	4.4	3.0	–	1.5	2.1	1.7	2.0	2.3	1.9	2.9	3.1	2.8
8. A-6	5.0	4.7	4.3	4.3	5.4	3.4	4.1	–	2.3	2.0	2.3	2.5	2.1	2.9	3.3	2.8
9. A-7	4.7	5.2	3.8	4.4	5.1	4.1	4.7	5.3	–	1.8	2.0	2.4	1.9	3.0	3.1	2.7
10. A-8	4.0	4.4	3.1	3.4	3.9	2.8	3.6	4.0	3.1	–	1.8	2.1	1.6	2.7	2.8	2.7
11. A-9a	5.2	5.3	4.2	4.4	5.4	4.4	4.8	5.4	4.4	3.4	–	1.7	1.3	3.2	3.2	2.9
12. A-9b	4.7	5.1	3.8	4.1	4.8	3.7	4.2	4.9	4.1	2.8	3.0	–	1.4	3.3	3.5	3.1
13. A-9c	5.0	5.2	4.1	3.9	5.0	4.0	4.7	5.3	4.1	3.3	3.2	2.9	–	2.9	3.1	2.8
14. B-1	6.1	6.4	5.6	6.0	6.5	5.3	5.9	6.1	5.9	5.4	6.3	6.2	6.4	–	2.0	1.8
15. B-2a	6.9	6.7	5.9	6.3	6.5	5.9	6.3	6.7	6.3	5.8	6.7	6.6	6.6	4.1	–	1.3
16. B-2b	7.0	6.9	5.7	6.1	7.0	5.9	6.5	6.6	6.3	6.0	6.4	6.4	6.4	4.4	2.9	–

Phylogenetic relationships

The ML and BI analyses yielded essentially identical topologies. The MP tree was also similar to these, although support values tended to be lower. The BI tree is shown in Fig. 2. *Rana tagoi* and *R. sakuraii* formed a fully supported monophyletic group, but both were paraphyletic with respect to each other. The ingroup was divided into two major clades, Clade A (MP-BS = 79%, ML-BS = 83%, BPP = 0.99) and Clade B (98%, 87%, 1.00, respectively), with uncorrected p-distances of 2.1% to 3.9% in 16S rRNA and 4.9% to 8.5% in ND1 between them. Each clade contained several subclades, some of which were further divided into two or three groups. Sequence divergences as measured by the mean uncorrected p-distances among these subclades and groups are shown in Table 4.

Clade A, which contained a subset of *R. t. tagoi*, *R. t. yakushimensis*, and *R. sakuraii* samples, was divided into nine subclades (Subclade A-1 to A-9). Subclade A-1 (94%, 98%, 1.00) contained *R. t. tagoi* samples from Tohoku, northern Chubu, and northern Kinki regions. This subclade was divided into two groups, Group A-1a (97%, 99%, 1.00) and A-1b (96%, 99%, 1.00), with sequence divergences of 0.9% to 1.9% in 16S rRNA and 3.3% to 4.9% in ND1 between them.

Group A-1a contained *R. t. tagoi* from Tohoku, northern Chubu, and northeastern Kinki (localities 1 to 41), including topotypic samples (locality 33) and a part of the *R. t. tagoi* large type (Sugahara, 1990) (locality 41). Except for samples from localities 11 to 13, which were divergent from the others, genetic variation within Group A-1a was small, despite its wide range of distribution. Group A-1b consisted of all samples of the *R. t. tagoi* small type from northern Kinki (localities 41 to 52). Within this group, genetic variation among haplotypes was signifcant, and four divergent subgroups were recognized.

Subclade A-2 (96%, 99%, 1.00) contained *R. t. tagoi* from Kanto region (localities 18 and 53 to 64) and was divided into two divergent groups. Interestingly, *R. sakuraii* from eastern Honshu (localities 20, 60, and 149 to 154), including topotypic samples (locality 151), was completely embedded in one of these groups. Within Subclade A-2, *R. sakuraii* was not much divergent from *R. t. tagoi* (0.8% to 1.3% in 16S; 1.3% to 3.0% in ND1).

Subclade A-3 (99%, 99%, 1.00) contained *R. sakuraii* from western Honshu (localities 35, 44, 114, 123, and 155 to 158), and was divided into three groups. Subclades A-2 and A-3 tended to form a clade, but their monophyly was not supported (< 50%, 66%, 0.86).

Subclade A-4 contained only one sample of *R. t. tagoi* from Nakanojo-machi (former Kuni-mura), Gunma (locality 65), while Subclade A-5 (78%, 75%, 1.00) contained divergent haplotypes of *R. t. tagoi* from central Chubu (localities 66 to 68). Subclade A-6 (all 100%, or 1.00) contained *R. t. tagoi* from southern Chubu (localities 69 to 77) and Shima Peninsula (locality 78), where variation among haplotypes was small. This subclade included *R. t. tagoi* with 2n = 28 chromosomes (vs. 2n = 26 chromosomes in *R. tagoi* samples from other localities so far studied) from Neba-mura, Nagano (Ryuzaki et al., 2006; locality 76). Subclades A-4 to A-6 tended to form a clade, but their monophyly was not unambiguously supported (< 50%, 68%, 1.00). Subclades A-1 to A-6 also tended to form a clade, but the MP support of this node was low (< 50%, 77%, 1.00).

Subclade A-7 (99%, 99%, 1.00) contained *R. t. tagoi* from Shikoku (localities 80 to 86) and Awaji Island (locality 79), with small genetic variations within the group. Subclade A-8 (all 100%, or 1.00) contained *R. t. yakushimensis* from Yakushima Island (locality 146), and was close to Subclade A-7, although their monophyly was not supported (< 50%, 63%, 0.54).

Subclade A-9 (90%, 99%, 1.00) contained *R. t. tagoi* from Kyushu and tended to form a clade with A-7 and A-8 but their monophyly was not supported (< 50%, 66%, 0.98). Subclade A-9 was divided into three groups, Groups A-9a (99%, 100%, 1.00), A-9b (93%, 94%, 1.00), and A-9c (only

one sample) with divergences between them being 1.3% to 1.7% in 16S, and 2.9% to 3.2% in ND1. Group A-9a contained samples from northwestern Kyushu (localities 87 to 103), and genetic variation within the group was small. Group A-9b consisted of samples from southern Pacific side of the island (localities 104 to 110) and was divided into two subgroups. Group A-9c contained one sample from Narujima Island (locality 111).

Clade B contained *R. t. okiensis* and a part of *R. t. tagoi* samples and was divided into two subclades. One of them, Subclade B-1 (all 100% or 1.00) contained *R. t. okiensis* from Oki islands (localities 147 and 148), while another, Subclade B-2 (99%, 95%, 1.00), consisted of *R. t. tagoi* from western Honshu. Two groups, with divergences of 0.8% to 1.6% in 16S rRNA and 2.1% to 4.0% in ND1, were recognized within this subclade; Group B-2a (99%, 95%, 1.00) and Group B-2b (88%, 69%, 1.00). Group B-2a contained samples from Kinki (localities 42 to 48, 50, and 112 to 135) and was divided into three subgroups. A large portion of the *R. t. tagoi* large type (Sugahara, 1990) samples (localities 42 to 48 and 50) was included in this group. Group B-2b contained samples from Chugoku (localities 136 to 145) and was divided into two subgroups.

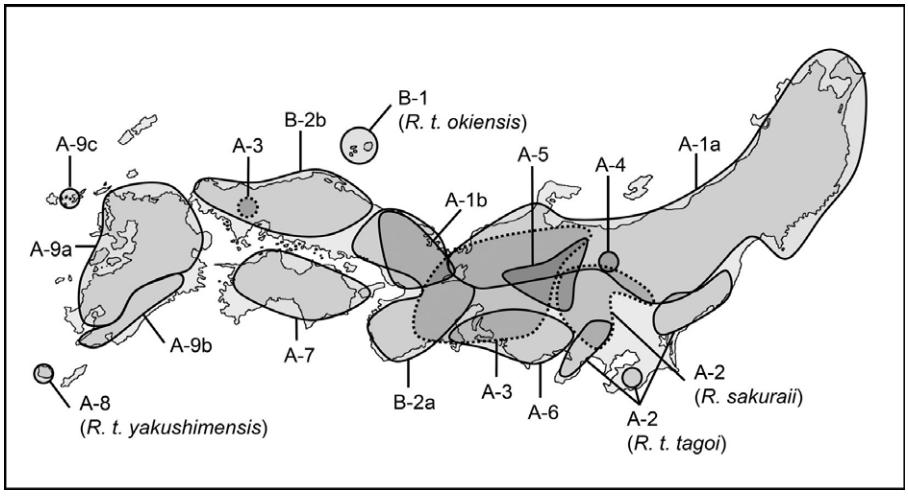

Fig. 3. Distributional range of each genetic group of *Rana tagoi* (solid line) *and R. sakuraii* (dotted line). For names of genetic groups, see Table 1 and Fig. 2.

Geographic distribution of genetic groups

Genetic groups recognized in two major clades of *R. tagoi* (sensu lato) and *R. sakuraii* (totally 15 subclades/groups) showed a complex pattern of geographic distribution, with sympatric or parapatric occurrence in some (Figs. 1, 3 and Table 4). Only *R. t. yakushimensis* (A-8), *R. t. okiensis* (B-1), *R. t. tagoi* from Awaji Island and Shikoku (A-7), and *Rana t. tagoi* from Kyushu (A-9a, b, and c) were allopatric from the other genetic groups, although A-9a and A-9b were parapatric within Kyushu.

Rana t. tagoi Group A-1a was widely distributed throughout northeastern Honshu to the northern part of central Honshu. It was transposed by *R. t. tagoi* Groups A-1b and B-2a in northeastern Kinki, the westernmost area of its distributional range. Group A-1a and A-1b were parapatric, with the exception of one sympatric site (locality 41). Group A-1b was distributed in northern part of Kinki, and was sympatric with B-2a in almost all ranges of its distribution (localities 42 to 48 and 50).

Group A-1a was transposed by *R. t. tagoi* in Subclade A-2 in northern Kanto. They were mostly parapatric, but were sympatric in one site (locality 18). *Rana t. tagoi* in Subclade A-2 was replaced by Subclade A-6 (southern Chubu) in western Kanto. Subclades A-4 and A-5 occurred in northwestern Kanto to central Chubu, between Group A-1a in the Sea of Japan side and Subclade A-6 in the Pacific side. Subclade A-4 was sympatric with A-1a, and A-5 also seemed to overlap with A-1a. Subclade A-6 widely occurred covering southern Chubu, and was replaced by Group B-2a in the Shima Peninsula (locality 78).

Group B-2a of *R. t. tagoi* from Kinki, which was sympatric with the *R. t. tagoi* small type (A-1b) as shown above, was transposed in the west by B-2b, which widely occurred in Chugoku, western Honshu.

Rana sakuraii was divided into two genetic groups, eastern (A-2) and western (A-3) subclades. In western Kanto, *R. sakuraii* was sympatric with *R. t. tagoi* and together formed Subclade A-2. Also, in the northern part of its distribution, *R. sakuraii* in Subclade A-2 was sympatric with *R. t. tagoi* A-1a (locality 20) and parapatric with A-4 (localities 160 and 67), and furthermore, seemed to overlap with A-5 in central Chubu. Subclade A-2 was transposed by *R. sakuraii* Subclade A-3 in the most western range of its distribution. Subclade A-3 largely overlapped with *R. t. tagoi* genetic groups in western Honshu (e.g., A-5, A-6, and B-2b), and sympatric with A-1a (locality 35), A-1b (locality 44), and B-2a (localities 44 and 114).

DISCUSSION

Phylogenetic relationships and genetic differentiation

Using allozymes and proteins, Nishioka et al. (1987) constructed a phenogram in which *R. t. yakushimensis* (A-8 in this study) was shown to be divergent from *R. t. tagoi* from western Japan. Within the latter, populations from Kinki (B-2a), Chugoku (B-2b), and Shikoku (A-7) formed one group, and those from Kyushu (A-9a) and *R. t. okiensis* (B-1) formed another. These results are completely discordant with results obtained by us or by Tanaka et al. (1996) from the mitochondrial cyt *b* gene. Our results showed common features with those reported by Tanaka et al. (1994, 1996: i.e., paraphyly of *R. tagoi*; large differentiation between large [B-2a] and small [A-1b] types of *R. t. tagoi* from Kyoto). Although there are superficial differences between Tanaka et al. (1994, 1996) and the present study, in the relationships of *R. t. tagoi*, *R. t. yakushimensis*, and *R. t. okiensis*, such discrepancies surely resulted from insufficient sampling in the Tanaka et al. (1994, 1996) study (e.g., Tanaka et al. [1996] used seven samples from five localities of *R. t. tagoi*, one sample of *R. t. yakushimensis*, three samples of *R. t. okiensis*, and six samples from three localities of *R.*

sakuraii), and results obtained from mtDNA analyses are considered essentially similar.

Discordance between trees based on nuclear (i.e., allozymes) and mitochondrial markers is generally explained by the paralogy of genes, introgressive hybridization, and incomplete lineage sorting with ancestral polymorphism (Ballard and Whitlock, 2004). However, these factors are difficult to differentiate without additional studies, in which nuclear marker analyses are made on the samples used in the present mtDNA analysis. In contrast to mitochondrial genes, allozymes are of limited value in estimating phylogenies, as historical relationships among alleles remain unclear (Avise, 2000). Thus, phylogenetic trees based on mitochondrial genes should be more valid than the allozymic ones, although the possibility of mitochondrial gene introgression, which leads to a strongly biased gene tree, is not precluded.

The geographic pattern of genetic differentiations obtained for *R. tagoi* is quite unique among Japanese anurans in that samples from western Honshu (Clade B) first diverge from the others (Clade A). In wide-ranging Japanese anurans (e.g., *Bufo japonicus*: Matsui, 1984; Igawa et al., 2006; *R. japonica*: Sumida and Ogata, 1998; *R. rugosa*: Sekiya et al., 2010; *Buergeria buergeri*: Nishizawa et al., 2011), populations from western Honshu and those from Shikoku and Kyushu tend to form a clade, unlike in *R. tagoi*, in which populations from eastern to central Honshu, Shikoku, and Kyushu form a clade (Clade A). This unique distribution suggests that geographical and environmental factors that separated Clades A and B of *R. tagoi* differ from those that affected the distribution of other Japanese anurans. Our results do not contradict Matsui and Matsui's (1990) hypothesis that the probable common ancestor of *R. tagoi* and *R. sakuraii* would have a habit of subterranean breeding, which is quite unique among Japanese anurans. The availability of subterranean environments, which was not so critical in other anurans, may have been a major factor that caused population fragmentation and subsequent genetic divergence in the ancestor of *R. tagoi* and *R. sakuraii*.

The current wider distribution of Clade A compared to Clade B indicates the Clade A ancestor was dominant throughout Honshu, including Kinki and Chugoku, in the past, whereas Clade B now predominates. Later, ancestral Clade B appears to have arisen somewhere in western Honshu and expanded its range towards east while affecting Clade A by exclusion through competition, and/or causing gene introgression to lose its original haplotypes. *Rana sakuraii* and the small type of *R. t. tagoi* are sympatric with, and specifically distinct from Clade B in Kinki and Chugoku. It is possible that these two groups have already sufficiently differentiated ecologically to avoid competition or introgressive hybridization with Clade B for coexistence in these regions.

Taxonomic relationships

Of the many genetic groups recognized, Group A-1a should be considered true *R. t. tagoi* as it included the topotypic population from Kamitakara of the current Takayama-shi (locality 33), Gifu (Okada, 1928; Shibata, 1988). The small type of *R. t. tagoi*, one of the two types of *R. t. tagoi* from Kinki (Sugahara, 1990), represented Group A-1b and was sympatric with the large type (parts of A-1a and B-2a). The small type differs from the large type in morphological, acoustic, and breeding ecological traits (Sugahara, 1990; Sugahara and Matsui, 1992, 1993, 1994, 1995, 1996, 1997). Thus, *R. t. tagoi* small type (A-1b) should be regarded as a distinct species. In contrast, *R. t. tagoi* morphologically identified as the large type was placed in two genetic groups (A-1a and B-2a), both with samples from the regions other than Kinki, and its taxonomic status is still unclear.

Subclade A-4 from one locality in Chubu has a unique breeding ecology and morphology different from sympatric Group A-1a (Misawa, private communication; Eto et al, 2012) and would be a distinct species. *Rana t. tagoi* from Neba-mura, Nagano, in Subclade A6 could also be another distinct species as it has 2n = 28 chromosomes in contrast to 2n = 26 in other *R. tagoi* and *R. sakuraii* populations (Ryuzaki et al., 2006). In our resultant tree, however, samples from Neba-mura (locality 76) were very close to and formed Subclade A6 with *R. t. tagoi* from southern Chubu and Shima Peninsula. It is thus necessary to investigate the chromosome number of the other populations in A-6 to determine taxonomic status of the Neba-mura population.

Details of morphological and ecological variations among other genetic groups of *R. t. tagoi* are generally poorly studied. Most of them are generally too similar to distinguish morphologically, but there are some exceptions. For example, representatives of Group A-1a and *R. t. tagoi* in Subclade A-2, at their range of sympatry in northern Kanto, are morphologically differentiated although slightly (Eto et al., unpublished data). Thus *R. t. tagoi* seems to include more cryptic taxa than previously suggested.

Rana t. yakushimensis formed Subclade A-8 by itself, and was split from the other *R. tagoi* subspecies and *R. sakuraii*. This result suggests its specific, rather than subspecific status, although it is morphologically very similar to *R. t. tagoi* (Maeda and Matsui, 1999). Supporting this idea, Nishioka et al. (1987) reported that *R. t. yakushimensis* was slightly isolated from *R. t. tagoi* from Chugoku (B-2b) by a low degree of hybrid inviability.

Another subspecies, *R. t. okiensis* also formed a distinct subclade (B-1) and split from other genetic groups. This subspecies is morphologically distinct from the other subspecies of *R. tagoi* and *R. sakuraii* (Maeda and Matsui, 1999), and there is little doubt to treat it as a distinct taxon. Conlon et al. (2010) suggested *R. t. okiensis* and *R. t. tagoi* to be heterospecific from antimicrobial peptide structure, and Nishioka et al. (1987) and Daito et al. (1998b) reported postmating isolation of the two subspecies. These previous studies and present result strongly suggest that *R. t. okiensis* should be treated as a species distinct from *R. t. tagoi*.

The phylogenetic relationships obtained by our group, in which *R. tagoi* and *R. sakuraii* are revealed to be paraphyletic, are in disagreement with current taxonomy. This result may be partly due to imperfect taxonomy (i.e., insufficient detection of cryptic species), in addition to the evolutionary processes as mentioned above. *Rana sakuraii* was divided into two genetic groups (Subclade A-2 and A-3). Of these, Subclade A-2 includes topotypic samples and should be regarded as true *R. sakuraii*, in spite of the possibility of past gene introgression from *R. t. tagoi* as discussed above.

Although both subclades of *R. sakuraii* are sympatric with some genetic groups of *R. t. tagoi* in Honshu (Table 4), the two species are known to be reproductively isolated by differences in the season, site, and behavior of breeding (Maeda and Matsui, 1999). Moreover, *R. sakuraii* in A-2 is completely isolated from *R. t. tagoi* from Kinki (large type from Kyoto: B-2a) and *R. t. okiensis* (B-1) by postmating isolating mechanisms (Daito et al., 1998a; Daito, 1999). Because no obvious morphological and ecological differences have been detected between the two genetic groups of *R. sakuraii*, it seems safe at present to retain it as a single species.

It is now popular to regard uncorrected p-distances in 16S rRNA of 3–5% to be thresholds between intra- and inter-specific divergence levels in anurans (Vences et al., 2005; Fouquet et al., 2007). However, Hillis and Wilcox (2005) reported interspecific sequence divergences of 16S rRNA among American ranid frogs to be 1.2–18.7% (uncorrected p-distances calculated from GenBank data). Thus, sequence divergence alone is not an absolute indicator to draw taxonomic conclusions, though it can be considered useful in detecting candidate species. As to ND1, Vredenburg et al. (2007) separated *R. sierrae* and *R. muscosa*, with 4.6% sequence divergence in ND1 and ND2, as distinct species.

In the light of these reports, divergences among genetic groups of *R. tagoi* and *R. sakuraii* (1.3–3.5% in 16S rRNA and 2.9–7.0% in ND1) are generally not very large. Of the cryptic lineages discussed above, A-1b (small type) could be regarded as heterospecific with B-2a (large type: divergences of 3.2% in 16S rRNA and 6.7% in ND1), although its divergence from true *R. t. tagoi* (A-1a) is not large enough to indicate specific separation (1.3% and 4.1%). Of other unique groups observed, Subclade A6, including a population with extra number of chromosomes, differed from the other groups by divergences of 1.5–3.3% (16S rRNA) and 3.4–6.7% (ND1). Likewise, divergences were 1.8–3.3% and 4.1–6.4% between *R. t. okiensis* and the other groups, and 1.5–2.8% and 2.8–6.9% between *R. t. yakushimensis* and the other groups. These values partly exceed proposed thresholds or reported values for specific separation (Fouquet et al., 2007; Vredenburg et al., 2007). Other combinations produced even smaller divergences (1.4% and 3.9% between Subclade A-4 and Group A-1a; 1.7% and 4.0% between Group A-1a and *R. t. tagoi* in Subclade A-2; and 1.1% and 2.1% between *R. sakuraii* and *R. t. tagoi* in A-2), in spite of their sympatric occurrences, and posed questions about the universality of threshold values in DNA barcoding.

In frogs, sister species sometimes exhibit very small sequence divergences in spite of their distinct morphological and/or ecological differences (e.g., Matsui et al., 2006), and similar situations appear to apply to unique genetic groups recognized in *R. tagoi* and *R. sakuraii*. Small sequence divergences, like morphological and ecological similarities, suggest relatively recent separation among genetic groups of these frogs.

This study provided a large amount of new information about the complex genetic diversity and consequential taxonomic problems with respect to *R. tagoi* and *R. sakuraii*. However, mtDNA along is not a conclusive indicator of reproductive isolation, due to its maternal mode of inheritance. Further studies, including nuclear marker analyses, are necessary to clarify reproductive isolations among genetic groups and draw definitive taxonomic conclusions.

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