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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 Holartic 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 Holartic 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 congenic 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 (Ryuzaaki et al., 2006) variations reported within *R. t. tagoi* suggest that it includes cryptic species. Genetically, *R. tagoi* is also diversified as...
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 toptype 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 toptype, 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-
### 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.

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**Notes:**

- **Rana tagoi tagoi**
- **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.**
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 the analyses. The optimum substitution model for each gene was selected using a Bayesian information criterion (AIC). The best model was calculated for each codon position (1st, 2nd, and 3rd positions) of the ND1 using Phylogears. 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).

The robustness of the MP and ML trees were tested using parametric bootstrap analysis (Felsenstein 1985) with 1,000 replicates. We regarded tree topologies with bootstrap value (BS) 70% or greater as significant support (Huelsenbeck and Ronquist, 2001; Shimada et al. 2011). We regarded tree topologies with bootstrap value (BS) 70% or greater as significant support (Huelsenbeck and Ronquist, 2001; Shimada et al. 2011). We regarded tree topologies with bootstrap value (BS) 70% or greater as significant support (Huelsenbeck and Ronquist, 2001; Shimada et al. 2011). We regarded tree topologies with bootstrap value (BS) 70% or greater as significant support (Huelsenbeck and Ronquist, 2001; Shimada et al. 2011).
RESULTS

Sequences and statistics

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

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Fig. 2. Bayesian tree of total 16S rRNA and ND1 mitochondrial genes for three subspecies of R. tagoi, R. sakurai, 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.
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 toptotypic 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 significant, 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 toptotypic 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. t. tagoi* samples from other localities so far studied) from Neba-mura, Nagano (Ruyzaki 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 con-
cluded 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 sub-
clade; 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.

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 allo-
patric 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 cen-
tral 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 symp-
atric with B-2a in almost all ranges of its distribution (local-
ities 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 Sub-
clade A-2 was replaced by Subclade A-6 (southern Chubu)
in western Kanto. Subclades A-4 and A-5 occurred in north-
western 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 sympat-
ic 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, east-
ern (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 Sub-
clade 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 relation-
ships of R. t. tagoi, R. t. yakushimensis, and R. t. okiensis,
such discrepancies surely resulted from insufficient sam-
ping 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.
sakurai), 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. sakurai* 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. sakurai*.

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 haplotype. *Rana sakurai* 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 toptypic 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. sakurai* populations (Ryuzaaki 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. sakurai*. 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 Chuogoku (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. sakurai* (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 post-mating 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. sakurai* 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 sakurai* was divided into two genetic groups (Subclade A-2 and A-3). Of these, Subclade A-2 includes toptypic samples and should be regarded as true *R. sakurai*, in spite of the possibility of past gene introgression from *R. t. tagoi* as discussed above.
Although both subclades of *R. sakurai* 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. sakurai* 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. sakurai*, 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, Hills 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. sakurai* (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 of these frogs. It suggests relatively recent separation among genetic groups and draw definitive taxonomic conclusions. Further studies, including nuclear marker analyses, are necessary to clarify reproductive isolations among genetic groups and draw definitive taxonomic conclusions.

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