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Phylogenetic Relationships of Brown Frogs with 24 Chromosomes from Far East Russia and Hokkaido Assessed by Mitochondrial Cytochrome *b* Gene Sequences (*Rana*: Ranidae)

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ABSTRACT—Comparisons of nucleotide sequences of mitochondrial cytochrome *b* gene revealed that the brown frog from Sakhalin has genetically little differentiated from *Rana pirica* from Hokkaido. Although they show some differences in adult morphology, they are considered to be conspecific. These frogs have sequences substantially different from *R. dybowskii* from the Maritime territory of Russia, and the current taxonomic idea to specifically separate them is genetically supported. On the other hand, *R. dybowskii* from the Maritime territory is genetically well differentiated from a conspecific population from Tsushima.

INTRODUCTION

The Eurasian brown frogs are very difficult to classify (Matsui, 1991). Especially, members with 24 chromosomes are morphologically quite similar with each other and have a highly complicate taxonomic history. The Ezo brown frog from Hokkaido, Japan, now recognized as a distinct species *Rana pirica*, was once regarded as a population of the European common frog, *R. temporaria* (Boulenger, 1886; Stejneger, 1907), and was not accorded a distinct taxonomic status even as a subspecies for a long time (Okada and Kawano, 1923; Okada, 1930). Later, Nakamura and Uéno (1963) considered the population from Hokkaido as taxonomically identical with the population from the Maritime territory, and treated both of them as a subspecies of *R. temporaria*, *R. t. dybowskii*. On the other hand, Kawamura (1962) considered the brown frog from Hokkaido as a species different from *R. temporaria* on the bases of their differential chromosome number (Witschi *et al*., 1958). He instead identified the brown frog from Hokkaido as *R. chensinensis* which was originally described from Tsingling, Shaanxi, China, by David (1875). Nakamura and Uéno (1972) partially followed this idea and used the name of *R. chensinensis dybowskii* for the Hokkaido population. On the contrary, Okada (1966) insisted to regard the Hokkaido population as a subspecies of the European common frog and used the name, *R. temporaria chensinensis*.

On Sakhalin Island, there occurs a brown frog other than *R. amurensis*. This frog was first reported in 1870, and has long been considered as conspecific with *R. temporaria* (Stejneger, 1907; Okada, 1930, 1931). Similarly the occurrence of *R. temporaria*, together with *R. amurensis*, has long been known on the Maritime territory of Russia (Stejneger, 1907; Nikolsky, 1918; Terent'ev and Chernov, 1949). Günther (1876) described *R. dybowskii* from Abrek Bay not far from Vladivostok in the Maritime territory. This form, however, was usually synonymized with *R. temporaria* (Boulenger, 1886; Stejneger, 1907). Nikolsky (1918), without actually examining type specimens, suspected that Günther's (1876) *R. dybowskii* is not even a brown frog but is conspecific with a water frog, *R. nigromaculat*a. At the same time, he (Nikolsky, 1918) described *R. semiplicata* from Poltawka, which is very near the type locality of *R. dybowskii*. These taxonomically complicate brown frogs from the Maritime and Sakhalin as a whole were once identified as *R. semiplicata* (Bannikov *et al*., 1977), but, most subsequent authors referred them as *R. chensinensis* (e.g., Orlova *et al*., 1977; Green and Borkin, 1993). The name of *R. dybowskii* began to be used quite recently (Kuzmin, 1995).

Matsui (1991), while describing the Ezo brown frog from Hokkaido as a distinct species *R. pirica*, showed that *R. pirica* is genetically fairly diverged from *R. dybowskii* from Tsushima and the Maritime territory of Russia on the bases of isozyme

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analyses. However, the brown frog from Sakhalin, long regarded as conspecific with the Hokkaido population (Okada, 1930), has never been analyzed electrophoretically. On the other hand, Matsui *et al.* (1994), in the morphometric comparisons of adult external characters among brown frogs from Sakhalin, *R. pirica* from Hokkaido, and *R. dybowskii* from Tsushima and Maritime, demonstrated that the frogs from Sakhalin and Hokkaido are morphologically somewhat divergent. In this way, systematic relationships between frogs from Sakhalin and Hokkaido, both long treated as *R. temporaria* (see above), are still poorly understood.

In the present study, we determined base pair sequences of the mitochondrial cytochrome *b* gene in the brown frog from Sakhalin, *R. pirica* from Hokkaido, and geographically adjacent *R. dybowskii* from Maritime territory, and compared the data among these samples, as well as with published data for other brown frogs having 2n = 24 chromosomes (*R. chensinensis* from Tsingling, China, *R. dybowskii* from Tsushima, and *R. ornativentris* from Japan main islands: Tanaka-Ueno *et al.*, unpublished). Our purpose is to infer phylogenetic relationships among species and populations of brown frogs with 2n = 24 chromosomes from this region, and to determine taxonomic allocation of the brown frog from Sakhalin accordingly.

MATERIALS AND METHODS

We extracted DNA from small amounts (less than 50mg) of frozen liver and muscle of four specimens of *Rana* sp. from Sakhalin, four of *R. pirica* from Hokkaido, and two of *R. dybowskii* from Lazo, Maritime territory, Russia (Fig. 1; see Appendix). Unpublished sequence data (Tanaka-Ueno *et al.*, unpublished) for *R. chensinensis* (topotypes) and published data for *R. dybowskii* from Tsushima, *R. ornativentris* (all 2n = 24), *R. japonica*, and *R. amurensis* from Sakhalin (all 2n = 26) (Tanaka-Ueno *et al.*, 1998) were utilized for comparisons. Data for *Xenopus laevis* (Dunon-Bluteau *et al.*, 1985) and *R. catesbeiana* (Tanaka *et al.*, 1996) were also used as those for the outgroup.

The methods, same as described previously (Tanaka *et al.*, 1994, 1996), were used to extract, amplify, and sequence DNA. We amplified part of the mitochondrial cytochrome *b* gene using the polymerase chain reaction (PCR) and sequenced approximately 600 bp region of this gene. We used two primers, L14850 (5'-TCTCATCCTGATGAAACTTTGGCTC-3') and H15502 (5'-GGATTCGCTGGTGTGAAATTGTCTGGG-3'). The numbering system for sequence followed that of the human sequence (Anderson *et al.*, 1981).

In order to infer relationships among taxa, three different methods were employed. We applied neighbor-joining method (Saitou and Nei, 1987) using the PHYLIP package program (Felsenstein, 1993). For the resultant network, we designated a root at the midpoint of the longest path. We also performed Maximum parsimony analyses using the branch-and-bound search algorithm in PAUP (Swofford, 1993) with the sequences added in a random order for 1,000 bootstrap repetitions.

RESULTS

Genetic differentiation among taxa was estimated using nucleotide sequence data of 587 bp that were constantly obtained for all samples (Fig. 2).

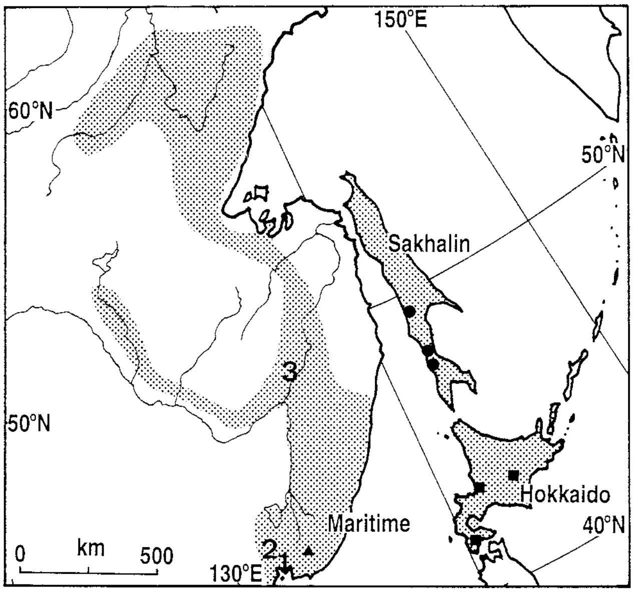


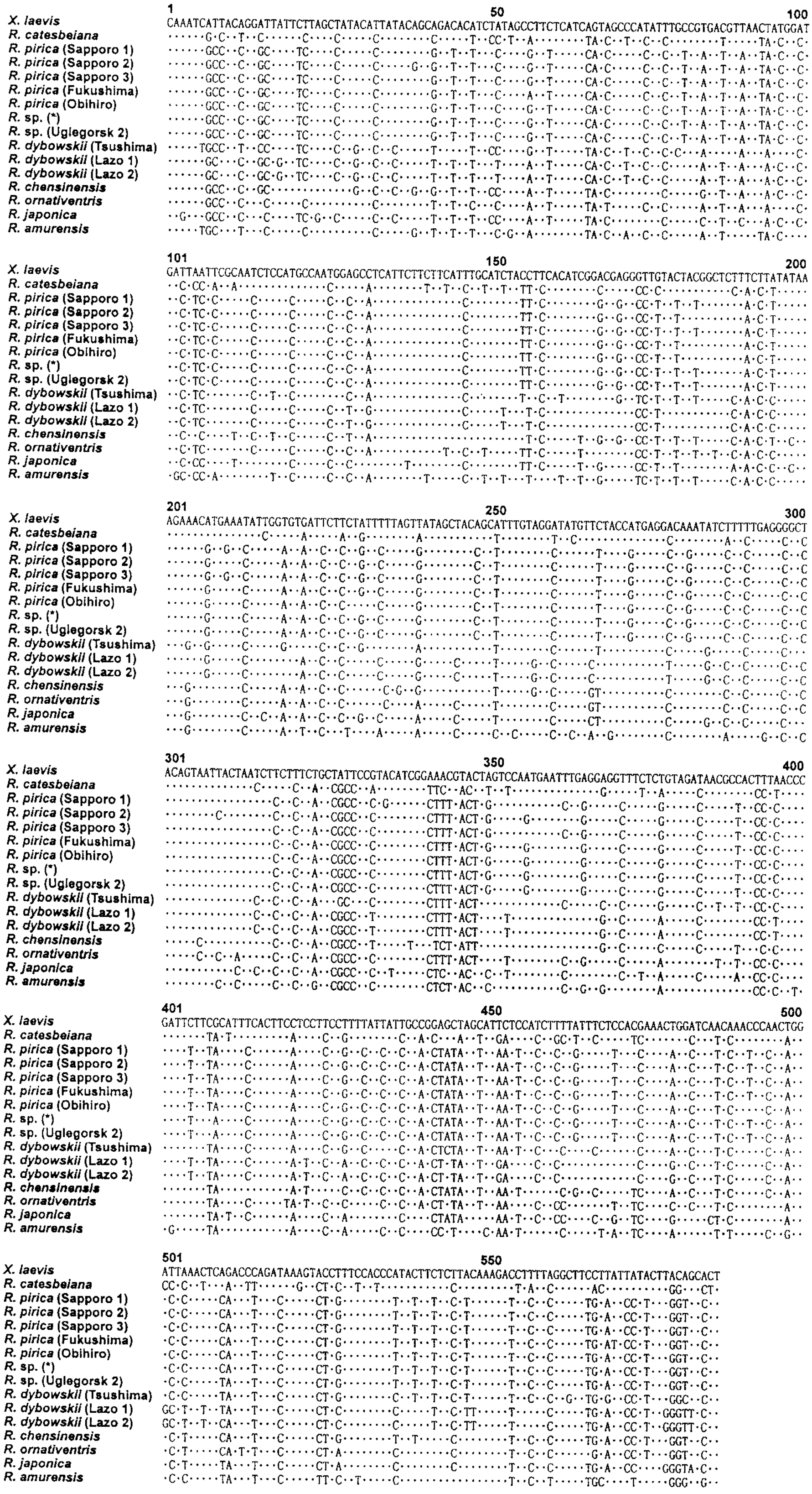
Fig. 1. Map of Far Eastern Russia and northern Japan showing localities where samples of *R.* sp. (dark circle), *R. pirica* (dark square), and *R. dybowskii* (dark triangle) used in the present study were collected, and reported their distributional range (stippled: after Maeda and Matsui, 1993 and Kuzmin, 1995). Taxonomically important localities also included: 1 = Vladivostok, 2 = Plotawka, 3 = Khabarovsk.

The brown frog from Sakhalin exhibited very small intrapopulation variation (sequence similarity = 100–99.8%). Intrapopulation variation in *R. pirica* was also small with sequence similarities of 99.8–98.8%. The similarity between the brown frog from Sakhalin and *R. pirica* ranged 99.3–99.0%, which is within the range of between-individual variation in *R. pirica*. Two individuals of *R. dybowskii* from Lazo had nearly similar sequences (similarity = 99.8%).

Phylogenetic analyses resulted in the formation of a single group by brown frogs. The outgroup taxa, *X. laevis* and *R. catesbeiana*, were separated from the remaining frogs, and the cluster of ingroup was supported by high bootstrap iterations, 93.0% in neighbor-joining, and 95.5% in maximum-parsimony trees.

In the tree obtained by the neighbor-joining method (Fig. 3A), sister relationship of *R. pirica* with *R.* sp. from Sakhalin was strongly supported (95.3% bootstrap iterations). This result obviously derives from high genetic similarities of the two populations as mentioned above. Individuals of *R. pirica* showed nearly polytomous relationships and their sister relationship was supported in less than 50% iterations. Similarly, exclusively close affinity of the two samples of *R.* sp. from Sakhalin was supported in only 54.3% iterations.

The cluster including *R.* sp. from Sakhalin and *R. pirica* showed a sister relationship with *R. dybowskii* from Tsushima, but the iterations supporting their association was as low as 51.8%. The cluster including these three species next formed a clade with *R. dybowskii* from Lazo, but the iterations were even lower (50.1%). These Far Eastern brown frogs exhibited a sister relationship with Chinese *R. chensinensis* (iterations



*R. sp. (*)= Uglegorsk 1/Slepikovskogo/Tomari

Fig. 2. Aligned sequences of a 587 bp segment of the cytochrome *b* gene. Dots indicate identity to the sequence of *Xenopus laevis.*

= 66.2%), and these were then outgrouped by *R. ornativentris* with high iterations of 79.0%. All these brown frogs with 2n = 24 chromosomes formed a single group with high iterations (99.7%) and split from *R. japonica* and then from *R. amurensis*, both of which have 2n = 26 chromosomes.

In the maximum-parsimony analyses (Fig. 3C), sister relationship of *R. pirica* and *R.* sp. from Sakhalin was even more strongly supported (iterations = 100%) than in the neighbor-joining tree. Unlike in the latter tree, the clade of *R. pirica* and *R.* sp. from Sakhalin showed a polytomous relationship with *R. dybowskii* from Lazo, *R. dybowskii* from Tsushima, and *R. chensinensis* in the parsimony tree. Their association was supported only by low iterations (54.8%), and they were outgrouped by *R. ornativentris* (iterations = 56.1%), *R. japonica* (93.2%), and *R. amurensis* (95.5%) in order. Results of the maximum-parsimony analyses were same with those of the neighbor-joining analyses in that species with 2n = 24 chromosomes form a monophyletic group with high iterations (93.2%), being separated from species with 26 chromosomes.

In this manner, *R.* sp. from Sakhalin is shown to be genetically nearly identical with *R. pirica* from Hokkaido, and is remote from *R. dybowskii* from Lazo, which in turn is clearly differentiated from the conspecific population from Tsushima.

DISCUSSION

Brown frogs occurring in Hokkaido, Sakhalin, and Maritime territory of Russia have a complicate taxonomic history as stated in introduction (for details, see Nishioka *et al.*, 1992). However, the brown frogs generally are genetically well differentiated locally notwithstanding their conservativeness in morphological variation (e.g., Tanaka *et al.*, 1994; Sumida, 1996). Therefore, the conspecificity of the brown frogs from geographically remote Far East and western China appears unlikely, especially when such account merely depends on similarities of only a few external characters between populations involved. On the bases of morphological and biogeographic evidence, Matsui (1991) described the brown frog from Hokkaido as *R. pirica* a species distinct from *R. chensinensis*, and subsequently further demonstrated their morphological difference by examining topotypes of the latter (Matsui *et al.*, 1993). Independent taxonomic status of these two species were also confirmed by a comparison of nucleotide sequences of mitochondrial cytochrome *b* in topotypic *R. chensinensis* and *R. pirica* (Tanaka-Ueno *et al.*, unpublished).

Both the two analyses performed here resulted in a grouping of *R.* sp. from Sakhalin and *R. pirica* as one exclusive unit (Bootstrap iterations: neighbor-joing = 95.3%, maximum-par-

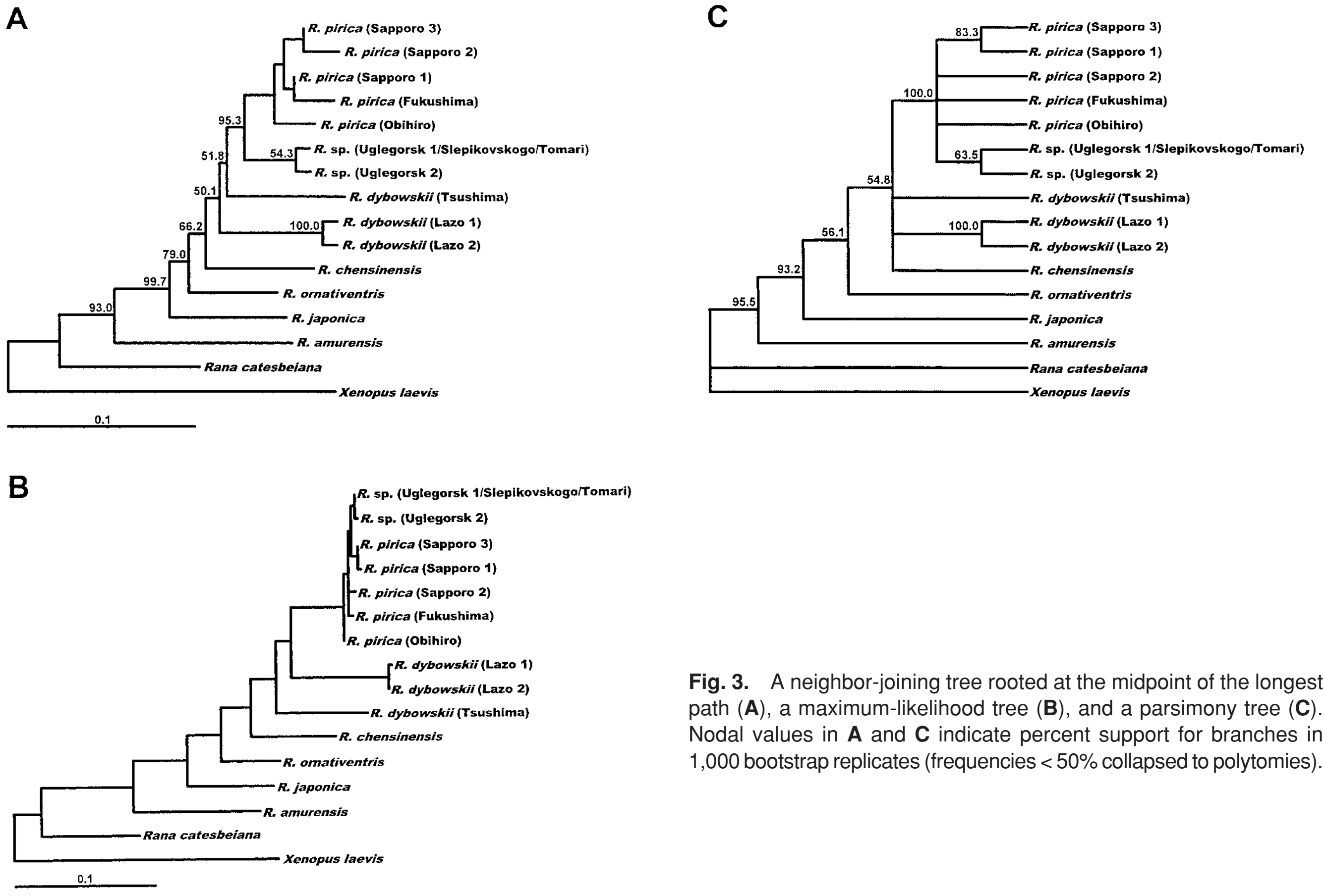


Fig. 3. A neighbor-joining tree rooted at the midpoint of the longest path (**A**), a maximum-likelihood tree (**B**), and a parsimony tree (**C**). Nodal values in **A** and **C** indicate percent support for branches in 1,000 bootstrap replicates (frequencies < 50% collapsed to polytomies).

simony = 100.0%). From these results, it seems obvious that the population from Sakhalin belongs to *R. pirica*. However, the Sakhalin and Hokkaido populations are reported to show some morphometric differences in adults (Matsui *et al*., 1994), and in order to examine the possibility of their taxonomic splitting at the subspecific rank, further studies using specimens from wider areas of the two regions are strongly desired.

Close genetic affinity between *R. pirica* populations from Sakhalin and Hokkaido suggests the presence of gene flow between them until very recently. In fact, the Soya Strait separating Hokkaido and Sakhalin is considered to have been formed less than 12,000 y. B.P. (Ohshima, 1990). It is likely that the common ancestor, widely distributed in these regions during their land bridge connection, was separated by the formation of the strait. Morphological differentiation reported by Matsui *et al*. (1994) may have occurred after this geographic isolation.

Green and Borkin (1993), in examining phylogenetic relationships of the brown frogs with 2n=24 chromosomes from isozyme variation, found that "*R. chensinensis chensinensis*" from Hokkaido and Kunashiri (a Japanese island northeast of Hokkaido) differed in only one genetic locus. Also, they reported that the genetic distance between the brown frogs from Kunashiri and Khabarovsk in continental Russia is slightly larger than intrapopulation distances in *R. arvalis* and *R. amurensis*, and is slightly smaller than the interspecific distance between two brown frogs from Caucasus. There should have been a land bridge between Sakhalin and the continent until the last glacial period (Ohshima, 1990). Further, *R. amurensis*, coinhabiting *R. pirica* on Sakhalin, has a wide range of distribution on the continent and its invasion into the Sakhalin Island is estimated to be a rather recent event (Tanaka-Ueno *et al*., 1998). From these considerations, it would be safe to suspect that a brown frog having a close phylogenetic affinity with *R. pirica* occurs in the continental regions adjacent to Sakhalin, such as Khabarovsk.

Continental *R. dybowskii* used in the present study was collected from Lazo which is separated from Khabarovsk by a chain of mountains. The population was genetically close to *R. pirica*, but was still clearly different from the latter. Nishioka *et al*. (1992), in a study of variation in isozymes, reported that *R. chensinensis* from Siberia (= Maritime territory) and *R. dybowskii* from Tsushima form a cluster, splitting from *R. chensinensis* from Hokkaido (= *R. pirica*). If their *R. chensinensis* from Siberia is regarded as *R. dybowskii*, their results are not discordant with ours. Further, Green and Borkin (1993) also reported that *R. chensinensis semiplicata* from the Maritime territory was equally different from *R. c. chensinensis* from Kunashiri and Khabarovsk and *R. dybowskii* from Korea.

On the other hand, a fossil identified as *R. pirica* has been found from Aomori, northernmost Honshu of Japan (Hasegawa *et al*., 1988). The Tsugaru Strait separating Honshu from Hokkaido is considered to have been formed more than 60,000 y. B.P. (Ohshima, 1990). Results of the present study indicate that continental *R. dybowskii* is closer to *R. pirica* than to *R. ornativentris* from Japan, south to Hokkaido. This result suggests that the common ancestor of *R. dybowskii* and *R. pirica* had a wider distribution range than those species do at present in the Far East, including Russia, Sakhalin, Hokkaido, and Honshu. The result obtained by Green and Borkin (1993) does not contradict to this view. Part of this ancestral form, leading to *R. pirica*, should have invaded from the continent through Sakhalin into Japan using northern land bridges (now under the straits of Mamiya, Soya, and Tsugaru), at least before the formation of the Tsugaru Strait. After the formation of that strait, however, the population in Honshu should have become extinct, possibly through competitions with the *R. ornativentris* lineage which should have had a similar ecological niche.

The ancestral form of *R. amurensis* is suspected to have been involved in the initial divergence among East Asian brown frogs (Green and Borkin, 1993; Tanaka-Ueno *et al*., 1998). The present result also confirmed remote relationships of *R. amurensis* from all other species, and particularly from sympatric *R.* sp. from Sakhalin. This finding again seems to support the idea that the date of invasion into Sakhalin differs between *R. pirica* and *R. amurensis* (Tanaka-Ueno *et al*., 1998).

From the present survey, the topotypic *R. chensinensis* from western China was proved to be genetically considerably different from *R. dybowskii*, and previous taxonomic treatments to synonymize Russian brown frogs with *R. chensinensis* (e.g., Green and Borkin, 1993) was judged to be erroneous. Green and Borkin (1993) stated that *R. c. semiplicata* from the Maritime territory is taxonomically different from Korean *R. dybowskii*, and suggested that their "*semiplicata*" might be a synonym of "*dybowskii*". Our results also strongly suggest a differentiation at the specific rank between *R. dybowskii* from the Maritime and *R. dybowskii* from Tsushima. This coincides with the results from previous isozyme analyses [Matsui, 1991; Nishioka *et al*., 1992 (population from the Maritime treated as *R. chensinensis* in the latter)]. Future DNA studies on the Korean population will clarify phylogenetic relationships of brown frogs currently lumped under the name of *R. dybowskii*.

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APPENDIX

Material Examined: Sources of tissue samples used are as follows. A total of 10 frogs are stored at the Graduate School of Human and Environmental Studies, Kyoto University (KUHE). *Rana pirica* (n = 4): Obihiro-shi, Hokkaido (n = 1: KUHE10203); Fukushima-cho, Hokkaido (n = 1: KUHE14494); Sapporo-shi, Hokkaido (n = 2: KUHE14539, 1 uncatalogued specimen). *Rana* sp. (n = 4): Slepikovskovo, Sakhalin (n = 1: KUHE11656); Uglegorsk, Sakhalin (n = 2: KUHE11617,11668); Tomari, Sakhalin (n = 1: KUHE11679). *Rana dybowskii* (n = 2): Lazo, Ussuriysk, Maritime territory, Russia (n = 2: KUHE11728, 11731).

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