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Estimation of Phylogenetic Relationships among Japanese Brown Frogs from Mitochondrial Cytochrome b Gene (Amphibia: Anura)

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ABSTRACT—We investigated phylogenetic relationships among five species of Japanese brown frogs by the analysis of nucleotide sequences in the cytochrome b gene of mitochondrial DNA (mtDNA). The sequence of the 251-base pairs, which cover approximately 22% of the cytochrome b gene, was determined by PCR-Direct sequencing method. Phylogenetic relationships were analyzed by UPGMA, neighbor-joining, maximum-likelihood, and maximum parsimony analyses. The sequences only slightly varied within one population of *Rana japonica*. Intraspecific variation in sequences varied among species, and *R. tagoi* showed more pronounced variation than did *R. ornativentris*. *Rana japonica* and *R. tagoi* share 2n=26 chromosomes with each other, but the former was closer to *R. pirica* and *R. ornativentris*, both with 2n=24, than to the latter. Phylogenetic relationships estimated from the nucleotide sequence of the cytochrome b gene generally conformed to the idea hitherto proposed chiefly on the bases of morphological and ecological evidences.

INTRODUCTION

Recently, DNA sequences have often been used to infer phylogenetic relationships among various animal groups [11]. Moreover, PCR (Polymerase Chain Reaction: [19]) enables us easily to obtain many DNA fragments for determination of the nucleotide sequences.

In evolutionary studies, mitochondrial DNA (mtDNA) is more often used than nucleic DNA. This is because mutation occurs at much higher rates in mtDNA than in nucleic DNA. Such a rapid rate of change in mtDNA is effective in investigating evolutionary relationships among population of a species and/or among closely related species [4, 13]. In addition, mtDNA is simple, because of its maternal inheritance [25], and is suitably used as a molecular clock by which we can determine the time elapsed since each divergence point in the phylogenetic tree [25]. Nucleotide sequences in cytochrome b gene of mtDNA have recently been successfully employed to estimate phylogenetic relationships of various animals representing a wide range of divergence time [2, 3, 8, 12].

The genus *Rana* contains approximately 300 species [7], and 19 species of this genus occur in Japan. Among them, brown frogs of the *Rana temporaria* group, consist a rather large group, including eight species: *R. japonica, R. tsushimensis, R. okinavana, R. tagoi, R. sakuraii, R. pirica, R. ornativentris*, and *R. dybowskii* [16]. Although these frogs can be grouped into two types by the number of diploid chromosomes (2n=24 or 26), they all are quite similar in morphology, and it is hard to infer their phylogenetic relationships [17].

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We investigated a phylogenetic relationship of Japanese brown frogs from nucleotide sequences in the cytochrome b gene in mtDNA by the following approaches: 1) Analysis of polymorphism within one population of *R. japonica*; 2) Analysis of the geographic distribution of polymorphisms in *R. tagoi* and *R. ornativentris*; 3) Estimation of phylogenetic relationships among five species of brown frogs using several methods that have different assumptions.

MATERIALS AND METHODS

DNA sources

We examined a total of 36 frogs as shown in Appendix. *R. tagoi* from Kyoto contains two sympatric populations that differ in the body size (Large type and Small type: [26]). *Rana catesbeiana* was used as an outgroup taxon from morphological studies made by Dubois [5].

Liver, muscle, heart and egg were immediately removed from sacrificed individuals under deep anesthesia with acetone chloroform and stored at −80°C. Frozen tissue samples (10 mg-100 mg) were homogenized at 4°C using a homogenizer in 1.5 ml of a solution containing 0.25 M sucrose, 0.01 M Tris-HCl , and 1 mM EDTA, pH 7.4–7.6. The homogenate was centrifuged at 600×g for 2 min at 4°C. The aqueous phase was transferred to a new tube and centrifuged at 5500×g for 20 min at 4°C. The pellet was suspended in STE (10 mM Tris/Cl pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0). Mitochondrial fraction was lysed by the addition of 1% sodium dodecyl sulfate. Proteins were digested with proteinase K (0.1 mg/ml) for 3 hr at 52°C. The solution was treated with phenol and chloroform/isoamyl alcohol and DNA was precipitated with ethanol. DNA precipitates were dried and dissolved in 1ml of TE (10 mM Tris/HCl, 1mM EDTA, pH 8.0) and 50 μl was subjected to PCR amplification.

Amplification and sequencing of mitochondrial cytochrome b gene

Mitochondrial sequences containing cytochrome b gene were

amplified by PCR. Primers for amplification and sequencing were designed according to the method of Kocher et al. [15] on the basis of conserved areas of nucleotide sequences of humans [1] and *Xenopus laevis* [21]. Primers were synthesized using a ABI/381A DNA synthesizer. Sequences of primers were L14850 (5′-TCTCCGCATGATGAAACTTCGGCTC-3′) and H15168 (5′-AAGTTTGTAATTACTGTGGCCCCTC-3′). The numbering system followed that of the human sequence [1]. DNA segment was purified by TaKaRa/EASYTRAP™ Ver.2 Kit after electrophoresis in a 4% NuSieve GTG (FMC BioProducts) agarose gel. Sanger dideoxy reaction [23] was carried out using Pharmacia/Cycle Sequencing Kit and two primers, L14850 and H15150 (5′-TCAGAATGATATTTGGCCTC-3), respectively.

Data Analysis

Genetic relationships among taxa were estimated based on the pairwise matrix of distance calculated by Kimura's 2-parameter model [14]. When a taxon included several haplotypes, we considered the one which appeared most frequently as a representative haplotype for the taxon. UPGMA algorithm [24] and a neighbor-joining method [22], using the program included with PHYLIP [6], were applied to the data set. In the latter analysis, we located a root at the midpoint of the longest path. Using the branch-and bound algorithm in version 3.5C of PHYLIP [6], we bootstrapped the data set 1,000 times to obtain a consensus tree.

In addition to above phenetic analyses, a continuous maximum-likelihood (CONTML) analysis was made using the program included with version 3.5C of PHYLIP [6].

The sequence data were also subjected to a cladistic parsimony analysis using version 3.0 of PAUP [28], and the branch-and-bound algorithm to find the shortest trees. We again bootstrapped the data set 1,000 times, using the branch-and-bound algorithm, to obtain approximate confidence on the tree.

In the analyses of neighbor-joining, maximum-likelihood, and maximum parsimony, a transition-to-transversion ratio was assumed to be 2.0.

RESULTS

Intraspecific differences

We could determine the nucleotide sequence of 251 bp in the Japanese brown frogs' cytochrome b gene. The nucleotide sequence within one population of *R. japonica* from Tateyama, Chiba showed three different haplotypes (type 1–3). The type 1, 2, and 3 appeared in seven (58.3%), three (25%), and two samples (16.7%), respectively. These three types differed from each other by 2 bp, and therefore, similarities between them were invariably 99.2%.

The nucleotide sequences of two samples from a Large type population of *R. tagoi* from Kyoto showed a difference in 2 bp. The similarity between them was 99.2%, which value was identical to those obtained among different haplotypes within one population of *R. japonica*. Differences in nucleotide sequences among six populations of *R. tagoi* collected from five different localities of Tohoku to Kyusyu were 8–17 bp. Similarities among these populations ranged from 93.2–96.8%, which were smaller than the similarity found within a population. The pattern of variation among populations was very complex, and a simple geographic cline was not observed. For instance, the sample of the Large type population from Kyoto was quite dissimilar in the sequence to that of the sympatric Small type population. On the other hand, this Small type was similar in sequences to specimens from Aomori or Kochi, both are fairly remote from Kyoto geographically.

The two samples of *R. ornativentris* from Toyama differed in 2 bp, with the similarity value of 99.2%, which was equal again to the similarity found within a population of *R. japonica* or *R. tagoi*. Differences in nucleotide sequences among five populations of *R. ornativentris* from Tohoku to Kyusyu regions were 2–10 bp, and similarities ranged from 99.2 to 96.0%. These values were larger than those found among populations of *R. tagoi*. The five populations could be roughly divided into northeastern (Aomori and Toyama) and southwestern (Hyogo, Kochi and Oita) groups by the degree of similarities in sequences.

Interspecific differences

Within the five brown frogs' cytochrome b sequences,

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R. japonica          1 : AGATCGCCACCGGACTATTGCTGGCCATACACTACACAGCTGATACTTCCCTAGCATTTTCATCTATCGCCCATATCTGCCGCGATGTCAACAACGGCTG
R. ornativentris     1 : ·A··········CT·A··T·····T·····C··T·····C·····A·····A·····T·····
R. pirica            1 : ·A·····G·····T··A·····T·····G·····T·····G·····C·····C·····T··A·····A··T·····
R. tagoi             1 : ·A·····C·····C·····T·····G··C·····C·····C·····C·····A··T··T·····
R. sakuraii          1 : ·A·····T··C·····C·····G··C·····C·····C··G·····T·····C·····A··T··T·····
R. catesbeiana       1 : ·A····T···T··CT····C··A·····T·····C··C·····T·····C·····T··C·····T·····T··T·····

R. japonica        101 : ACTCCTTCGTAATCTCCACGCCAACGGCGCCTCATTTTTCTTCATCTGCATCTATTTCCACATTGGGCGAGGCCTTTATTACGGCTCATACCTCTACAAA
R. ornativentris   101 : ···T·····C··C·····A·····C·····T·····T·····A·····T··T··C·····T···
R. pirica          101 : ···T··C··C··C·····A·····C·····C·····G·····T·····T·····T··T···
R. tagoi           101 : ·····C··C··C··T·····A·····C·····C·····A·····T·····T·····T··T···
R. sakuraii        101 : ·····C··C··C··T·····A·····C·····C··A·····T·····T·····T··T···
R. catesbeiana     101 : ·····A··A·····T·····A··A·····C··T··T·····T··T·····C··C·····C··C·····C·····T··T···

R. japonica        201 : GAGACATGAAACATCGGAGTAATCCTCCTGTTCTTAGTAATAGCCACAGCT
R. ornativentris   201 : ·····T·····A··TC·····
R. pirica          201 : ··A··G··G·····T·····G·····T··C···
R. tagoi           201 : ··A·····G··T·····T··T·····G·····
R. sakuraii        201 : ··A·····G··T··T··T··T·····G·····
R. catesbeiana     201 : ··A·····T·····T·····T··A·····T·····T·····
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FIG. 1. Aligned sequences of a 251-bp segment of the cytochrome b gene from five brown frogs and *R. catesbeiana* as an outgroup. Dots indicate identity to the sequence of *R. japonica*.

TABLE 1. Pairwise comparisons of cytochrome b sequences among five Japanese brown frog species and one outgroup taxon *Rana catesbeiana*. The percentage sequence differences are shown in the above diagonal, and the distance obtained by Kimura's 2-parameter model [14] are shown below.

	1	2	3	4	5	6
1. *R japonica*	—	88.8	86.9	88.0	86.9	83.7
2. *R. ornativentris*	0.1158	—	88.4	88.0	85.3	83.7
3. *R. pirica*	0.1397	0.1149	—	86.5	85.3	83.3
4. *R. t. tagoi*	0.1303	0.1247	0.1440	—	97.2	85.3
5. *R. sakuraii*	0.1444	0.1573	0.1583	0.0283	—	85.3
6. *R. catesbeiana*	0.1851	0.1796	0.1857	0.1640	0.1640	—

251 bp, (Table 1), all of 57 substitutions occurred at first and third positions of codons. On the other hand, there were no replacements at second positions. The number of nucleotide substitution at first and third position of codons were 2, 55, respectively. Both of them at first position of codon were transitions (i. e. the interchange of pyrimidines, C⇔T ,or purines, A⇔G). Transition occurred approximately five times as frequently as transversion (i. e. a change from a purine to a pyrimidine or vice versa) at third position of codon. Although most of nucleotide replacement were silent mutations, amino acid replacement occurred only in the sequence of *R. japonica*. The amino acid residue changed from phenylalanin to Leucine.

Figure 2A shows the tree obtained from the pairwise matrix of genetic distance (Table 1) with UPGMA method.

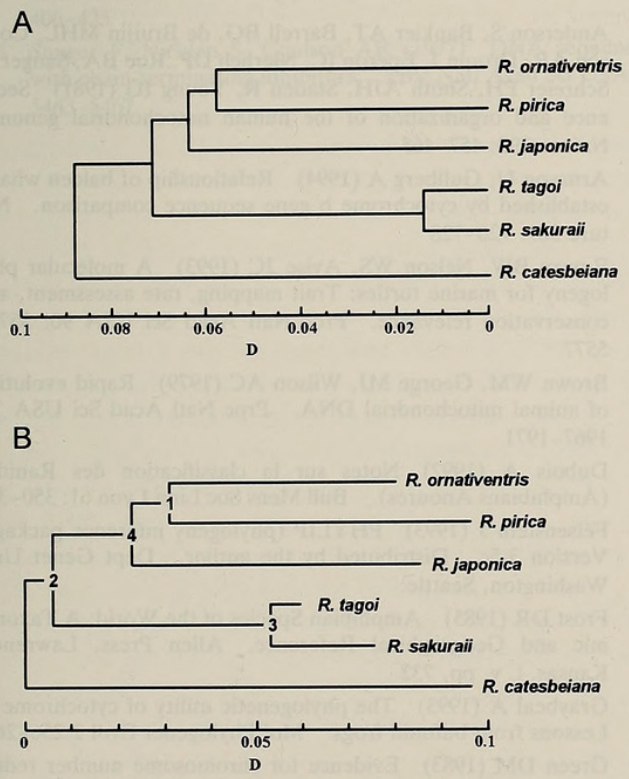

FIG. 2. A UPGMA phenogram (A) and a neighbor-joining tree (B) constructed from a distance matrix obtained from nucleotide sequences by the Kimura "2-parameter" model. The latter tree was rooted at the midpoint of the longest path. Numbers on the nodes in B correspond to those shown in Table 2.

In this tree, the outgroup species, *R. catesbeiana*, is clearly separated from the ingroup five species of brown frogs. In the ingroup, *R. tagoi* and *R. sakuraii* constitute a cluster and split from another cluster of all the remaining species. In the latter cluster, *R. pirica* and *R. ornativentris* formed a subcluster, and split from another subcluster of *R. japonica*. The tree constructed by the neighbor-joining method (Fig. 2B) showed a topology identical to the UPGMA tree. The ingroup relationships of brown frogs were supported in 805/1,000 bootstrap iterations. Within the ingroup, the sister relationship of *R. tagoi* and *R. sakuraii* was nearly completely supported (998/1,000 bootstrap iterations), while the sister relationship of *R. ornativentris* and *R. pirica* was supported in only 512/1,000 iterations. The relation of the latter two species with *R. japonica* was more strongly supported (770/1,000 iterations).

The maximum-likelihood analysis, using *R. catesbeiana* as an outgroup, produced a result identical to that obtained by the two analyses discribed above, although the tree contained a collapsing branch in which the 95% confidence interval includes zero (Table 2).

In the parsimony analysis, only one shortest tree, with a minimum of 94 steps and a consistency index of 0.707 (excluding uninformative characters) was produced. The topology of this tree slightly differed from that found in the above analyses. The sister relationship of *R. pirica* and *R.*

TABLE 2. Branch lengths and their approximate confidence intervals obtained in the maximum likelihood analysis for Japanese brown frogs, with an outgroup taxon *R. catesbeiana*. Nodes are those shown in Fig. 2B

Branch between nodes		Branch length	Approximate confidence interval
R. catesbeiana	2	0.11775	0.07052, 0.16629
2	3	0.04541	0.01373, 0.07739
3	*R. tagoi*	0.00398	0.00000, 0.01354
3	*R. sakuraii*	0.02441	0.00428, 0.04553
2	4	0.02908	0.00123, 0.05717
4	*R. japonica*	0.06004	0.02492, 0.09540
4	1	0.02592	0.00210, 0.04993
1	*R ornativentris*	0.04886	0.01681, 0.08121
1	*R. pirica*	0.06706	0.03043, 0.10369

ornativentris was less strongly supported (338/1,000 bootstrap iterations) than that of *R. japonica* and *R. ornativentris* (569/1,000 iterations).

DISCUSSION

The four nucleotide replacements found in a population of *R. japonica* from Tateyama, Chiba were transitions, all of which being silent substitutions in the third position of codons. It is well-known that transitions occur more frequently than transversions. The variation in sequences within a population of *R. japonica* from Tateyama, Chiba, was very low, and suggested that only a few samples may represent features of the sequence specific to a population or a taxon. This assumption was supported by low haplotype variations within population exhibited by *R. tagoi* from Kyoto (a population of the large type) and *R. ornativentris* from Oyama, Toyama.

There were great differences in nucleotide sequences between the large and the small type of *R. tagoi* from Kyoto, and the degree of difference (similarity=94.6%) roughly equaled to those found among populations that are geographically remote (e. g., 94.4% between populations from Aomori and Miyazaki). This genetic divergence was concordant with differentiations found in morphology and ecology of the two types [26, 27]. Previous studies on isozymes and blood proteins in several populations of *R. tagoi* from west Japan suggested that genetic differentiation has proceeded well within this species [20]. Differentiation of nucleotide sequences also seemed to have progressed well in this species. In order to clarify further genetic differentiation in this species, we must investigate larger number of populations in detail. On the other hand, differences in nucleotide sequences among populations of *R. ornativentris* were lower than in *R. tagoi*. This may indicate that the genetic divergence occurred more recently in this species than in *R. tagoi*.

Like most other ranid frogs, *R. tagoi, R. sakuraii*, and *R. japonica*, have diploid chromosomes of 2n=26 [9]. However, in none of the trees constructed by varying methods, *R. japonica* constituted a cluster with the remaining two species. Instead, *R. japonica* was clustered with the subcluster of *R. pirica* and *R. ornativentris* both with 2n=24 chromosomes. Green and Borkin [10] reported similarly remote phylogenetic relationships of *R. tagoi* and *R. japonica* through the analysis of isozymes. These results suggest an early divergence of *R. tagoi*, and probably of *R. sakuraii*, from the other brown frogs, and this is in agreement with a high degree of specialization of these two species in reproductive strategies, i. e., breeding in underground, small streams (*R. tagoi*) or under the stones of montane streams (*R. sakuraii*) unlike others that breed in open, still water [18].

Unlike the above three species, *R. pirica* and *R. ornativentris* have 2n=24 chromosomes, and have been regarded as closely related with each other from isozyme, acoustic, and morphological evidences [17]. These two species formed a group in UPGMA, neighbor-joining, and maximum-likelihood trees, but their sister relationship was not always strongly supported as shown by a low value of bootstrap iterations (512/1,000) in the consensus tree obtained by the neighbor-joining method. Further, in the parsimony analysis, *R. ornativentris* formed a group not with *R. pirica*, but with *R. japonica*, although the topology of the tree was not strongly supported (569/1,000 bootstrap iterations). This finding suggests equally remote relationships of *R. pirica* and *R. japonica* to *R. ornativentris*, that have never been pointed out before. Further studies of these species from various approaches will reveal the validity of the present findings.

This study suggested that differences in nucleotide sequences would increase in the order of within a population, among populations, and among species. Generally, nucleotide sequences of cytochrome b gene are regarded as good indicators for evaluating intraspecific and/or interspecific variation of brown frogs. Determinations and comparisons of nucleotide sequences of longer areas would clarify divergence times in Japanese brown frogs, in addition to intraspecific and/or interspecific phylogenies.

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APPENDIX
SPECIMENS EXAMINED

A total of 36 frogs are stored at the Graduate School of Human and Environmental Studies, Kyoto University and in T. Sugahara private collection.

Rana japonica (n=12): Tateyama-shi, Chiba (n=12).
Rana tagoi (n=7): Towadako-machi, Aomori (n=1); Hayakawa-cho, Yamanashi (n=1); Kyoto-shi, Kyoto (Kyoto-L=Large type of Sugahara [26]) (n=2); Kyoto-shi, Kyoto (Kyoto-S=Small type of Sugahara [26]) (n=1); Tosayama-mura, Kochi (n=1); Gokase-cho, Miyazaki (n=1).
Rana sakuraii (n=4): Okutama-machi, Tokyo (n=2); Kiyokawa-mura, Kanagawa (n=1); Miyama-cho, Kyoto (n=1).
Rana pirica (n=3): Obihiro-shi, Hokkaido (n=1); Sapporo-shi, Hokkaido (n=2).
Rana ornativentris (n=6): Towadako-machi, Aomori (n=1); Oyama-machi, Toyama (n=2); Sasayama-cho, Hyogo (n=1); Tosayama-mura, Kochi (n=1); Bungotakada-shi, Oita (n=1).
Rana catesbeiana (n=4): Inuyama-shi, Aichi (n=2); Bungotakada-shi, Oita (n=2).