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CITATION:

ISSUE DATE:
1994-10

URL:
http://hdl.handle.net/2433/65057

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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].

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 Rana 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 supernatant 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 the conserved areas of nucleotide sequences of humans [1] and Xenopus laevis [21]. Primers were synthesized using an ABI/381A DNA synthesizer. Sequences of primers were L14850 (5'-TCTCCGCA-TGATGAAACTTCGGCTC-3') and H15168 (5'-AAAGTTGTAA-TTACTGTGGCCCCTC-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'-TCAGAATGATATTT-GGCCTC-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 joining method [22], using the program included with PHYLIP [6], the one which appeared most frequently as a representative model [14]. When a taxon included several haplotypes, we considered the one which appeared most frequently as a representative haplotype for the taxon.

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, the similarities between each pair of populations were 99.0%–99.7%, and these values were larger than those among populations within a single species. The similarity value between the Dewa type population of R. japonica and the specious Small type population was 99.0%, which was equal again to the similarity found within a population of R. japonica. The differences 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.
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.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>R. japonica</em></td>
<td>0</td>
<td>88.8</td>
<td>86.9</td>
<td>88.0</td>
<td>86.9</td>
<td>83.7</td>
</tr>
<tr>
<td>2. <em>R. ornativentris</em></td>
<td>0.1158</td>
<td>0.1149</td>
<td>0.1397</td>
<td>0.1303</td>
<td>0.1444</td>
<td>0.1851</td>
</tr>
<tr>
<td>3. <em>R. pirica</em></td>
<td>0.1397</td>
<td>0.1149</td>
<td>0.1140</td>
<td>0.1440</td>
<td>0.1583</td>
<td>0.0283</td>
</tr>
<tr>
<td>4. <em>R. t. tagoi</em></td>
<td>0.1303</td>
<td>0.1247</td>
<td>0.1440</td>
<td>0.1440</td>
<td>0.1583</td>
<td>0.1583</td>
</tr>
<tr>
<td>5. <em>R. sakuraii</em></td>
<td>0.1444</td>
<td>0.1573</td>
<td>0.1583</td>
<td>0.0283</td>
<td>0.0283</td>
<td>0.0283</td>
</tr>
<tr>
<td>6. <em>R. catesbeiana</em></td>
<td>0.1851</td>
<td>0.1796</td>
<td>0.1857</td>
<td>0.1640</td>
<td>0.1640</td>
<td>0.1640</td>
</tr>
</tbody>
</table>

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

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.

**ACKNOWLEDGMENTS**

We received help in the acquisition of tissue samples from T. Tanabe, Y. Misawa, T. Sugahara and K. Kasugai. T. Hikida helped in the statistic analyses. Part of the study was supported by the National Geographic Society (No. 4505–91) to M. Matsui.

**REFERENCES**

Frog Phylogeny from Cytochrome b Gene

24-chromosome species group and the significance of chromosome number change. Zool J Linn Soc 109: 1–25
24 Sneath PHA, Sokal RR (1973) Numerical Taxonomy. W H Freeman, San Francisco

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); Kiyokawamura, 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).