# Notes on a discrepancy in Mitochondrial DNA and Allozyme Differentiation in a Pond Frog Rana nigromaculata

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# Notes on a discrepancy in Mitochondrial DNA and Allozyme Differentiation in a Pond Frog *Rana nigromaculata*

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**ABSTRACT**—Analyses of complete 1143-base pair sequence of the mitochondrial cytochrome b gene demonstrated a sister relationship between Japanese *R. nigromaculata* and Korean *R. plancyi chosenica*, but not with Korean *R. nigromaculata*, while the allozyme data strongly supported the monophyly of the Korean and Japanese populations of *R. nigromaculata*. We surmise this discordance to be the result of the inheritance of introduced mtDNA and the dilution of introduced nuclear DNA in mixed lineages after past hybridization and genome introgression between the two species, although the direction of introgression is unknown.

**Key words:** Korea, Japan, *Rana nigromaculata*, mitochondrial cytochrome b gene, allozymes

## INTRODUCTION

In an analysis of partial mtDNA cyt b gene sequences for a pond frog *Rana nigromaculata* (Kim *et al*., 1999), we found the level of sequence divergence (Tamura-Nei distance=6.6–8.0%) was markedly greater than that obtained from allozyme analyses (Nei's D<0.1: Yang *et al*., 1999) between Korean and Japanese populations. On the other hand, Lee *et al.* (2000) found that the level of sequence divergence in entire mtDNA cyt b (Kimura-2-parameter distance, *p*=7.8%) was equivalent to the allozymic divergence (Nei's D>0.53: Yang *et al*., 1999) between *R. nigromaculata* and its relative *R. plancyi chosenica*, that are sympatric in South Korea.

This means that despite the close allozyme affinity, the Korean population of *R. nigromaculata* is as distant from the conspecific Japanese population as is heterospecific *R. p. chosenica*. These results prompted us to re-examine the relationships of these three genetic groups using mt cyt b sequences. We directly compared Japanese *R. nigromaculata* and Korean *R. p. chosenica* by adding several new samples of *R. nigromaculata* from Japan and extended our analyses of mtDNA to the complete cyt b sequence to detect more variation in the sequence. Based on the results of this study and hitherto accumulated information, we discuss the evolutionary history of Korean and Japanese pond frogs.

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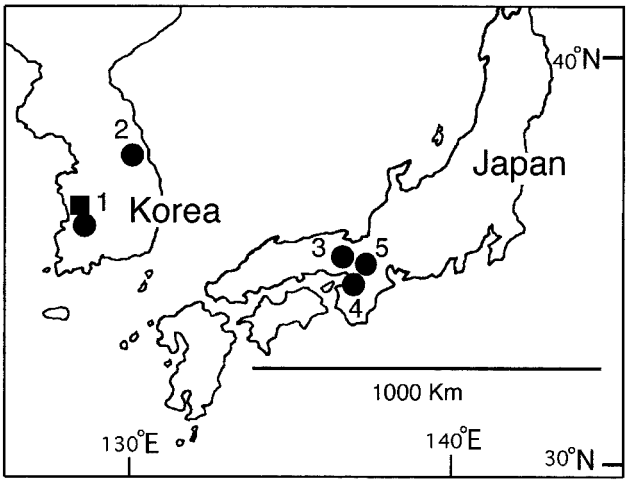


**Fig. 1.** Sampling localities of *Rana nigromaculata* (Solid circles) and *R. plancyi chosenica* (Solid square) in Korea and Japan. Numbers refer to collection localities in Table 1.

**Table 1.** Sampling localities, number of specimens, haplotypes, and accession numbers of cyt b gene.

| Species and locality | N of Specimen | *Haplotype | Accession No |
|---|---|---|---|
| ***Rana nigromaculata* (Korea)** | | | |
| 1. Yeonmu, Nonsan, Chungcheongnam-do | 1 | RN-Korea | AF205087 |
| 2. Jukwang, Koseong, Kangweon-do | 1 | RN-Korea | AF205087 |
| ***R. nigromaculata* (Japan)** | | | |
| 3. Sizuhara, Sakyo, Kyoto | 2 | RN-Kyoto | AY315755 |
| 4. Ogi, Izumisano, Osaka | 1 | RN-Osaka | AY315756 |
| 5. Katada, Otsu, Shiga | 1 | RN-Otsu | AY315757 |
| ***R. plancyi chosenica* (Korea)** | | | |
| 1. Yeonmu, Nonsan, Chungcheongnam-do | 1 | RP-Korea | AF205089 |

* Same abbreviations are used in other tables and figures.

## MATERIALS AND METHODS

All of the samples of *Rana nigromaculata* and *R. plancyi chosenica* two pond frog species, except some from the Japanese *R. nigromaculata* populations, have been used for partial mtDNA sequence (Kim *et al*., 1999; Lee *et al*., 2000) analyses. The two pond frog species are sympatric at Nonsan (pop. 1: Fig. 1). Methods of sample preparation and extraction of DNA are same as previously reported (Kim *et al*., 1999).

The following primers designated to match many groups of amphibians (Kocher, 1989; Tanaka *et al*., 1996; Sumida *et al*., 1998; Kim *et al*., 1999; Lee *et al*., 2000) were used for PCR amplification: L14731, 5'-GAAAAACTATCGTTGTTATTCAACTA-3'; L16303, 5'-CCATCCAACAT CTCAGCATGATGAAA-3'; L16654, 5'-TGAGGACAAATATCATTCTGAGGGGC-3'; L16797, 5'-TTYATYCTCCCNTTYATTAT-3'; L16937, 5'-TCYTMGGNTTTRTTATT AT-3'; H16915, 5'-GTCTTTGTAGAGAGAAGTATGG-3'; and H21, 5'-TTATGCTCTAT ATACATAAG-3'. The PCR products purified using a Tragene$^{TM}$ one-step gel extraction kit (Injae, Korea) were sequenced with a Top$^{TM}$ DNA sequencing kit (Bioneer, Daejon, Korea) using a silver staining system (Bioneer).

Nucleotide sequences (see Table 1 for GenBank accession number) were aligned using the program GENETYX-WIN (Ver. 5.0; Software Development Co., Tokyo, Japan). The alignments revealed no deletions or insertions. The genetic relationships among haplotypes were estimated based on the pairwise matrix of sequence divergence (*p*) calculated using Kimura's two-parameter method (Kimura, 1980). To infer phylogenetic relationships among haplotypes, the neighbor-joining (NJ: Saitou and Nei, 1987), maximum-likelihood (ML: Felsenstein, 1981), and maximum parsimony (MP) methods were applied using the programs included in the PHYLIP package (Felsenstein, 1993) and PAUP 4.0b (Swofford, 1998). Bootstrap values were computed from 1,000 repetitions to obtain approximate confidence levels for all trees. The reported nucleotide sequences of the Hiroshima population of Japanese *R. nigromaculata* (Sumida *et al*., 2001), *R. catesbeiana* (Lee *et al*., 2000), and *Xenopus laevis* (Roe *et al*., 1985) were used as references or outgroups to construct gene trees.

## RESULTS

We determined the complete 1143-bp sequences of six individuals from two Korean and three Japanese populations of *R. nigromaculata* and one *R. p. chosenica*. The two Korean *R. nigromaculata* (pops. 1 and 2) had the identical haplotype (RN-Korea: Table 1). In contrast, all three Japanese populations of *R. nigromaculata* had different haplotypes (RN-Kyoto, RN-Otsu, and RN-Osaka; Table 1).

The genetic differences among the three haplotypes of Japanese *R. nigromaculata* ranged from 1 to 4 bp, whereas there were much greater differences (74–75 bp) between the Korean and Japanese *R. nigromaculata*. The haplotypes of *R. nigromaculata* and *R. p. chosenica* differed at 55-84 bp. In addition, the degrees of nucleotide sequence divergence (*p*) among the haplotypes were much lower within Korean ($p$=0%) or Japanese *R. nigromaculata* ($p$=0.09–0.35%) than between the Korean and Japanese *R. nigromaculata* haplotypes ($p$=6.79–6.89%) or between the *R. nigromaculata* and *R. p. chosenica* haplotypes ($p$=4.99–7.74%: Table 2).

In all the NJ, MP, and ML trees, the ingroup pond frogs were clearly separated from the outgroup *X. laevis* and *R. catesbeiana*, with 100% bootstrap support, although they were divided in a curious order, Korean *R. nigromaculata* first split from the remainings encompassing *R. p. chosenica* and the Japanese *R. nigromaculata* (Fig. 2). The latter cluster had very high bootstrap support of 96–100%.

**Table 2.** Percentage divergences [above diagonal: Kimura's two-parameter distance (Kimura, 1980)] and number of different nucleotides (below diagonal) among haplotypes in Korean and Japanese *R. nigromaculata* (RN) and *R. plancyi chosenica* (RP). * The numbers of transition (Ts) and transversion (Tv) sites. ** The percentage of different nucleotide.

| Haplotypes | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1. RN-Korea | – | 6.80 | 6.89 | 6.79 | 7.74 |
| | | (52/22*) | (54/21) | (53/21) | (66/18) |
| 2. RN-Kyoto | 74(6.5%**) | – | 0.35 | 0.09 | 5.00 |
| | | | (3 /1) | (0 /1) | (35/20) |
| 3. RN-Osaka | 75(6.6%) | 4(0.3%) | – | 0.26 | 4.99 |
| | | | | (3 /0) | (36/19) |
| 4. RN-Otsu | 74(6.5%) | 1(0.1%) | 3(0.3%) | – | 4.99 |
| | | | | | (36/19) |
| 5. RP-Korea | 84(7.3%) | 55(4.8%) | 55(4.8%) | 55(4.8%) | – |

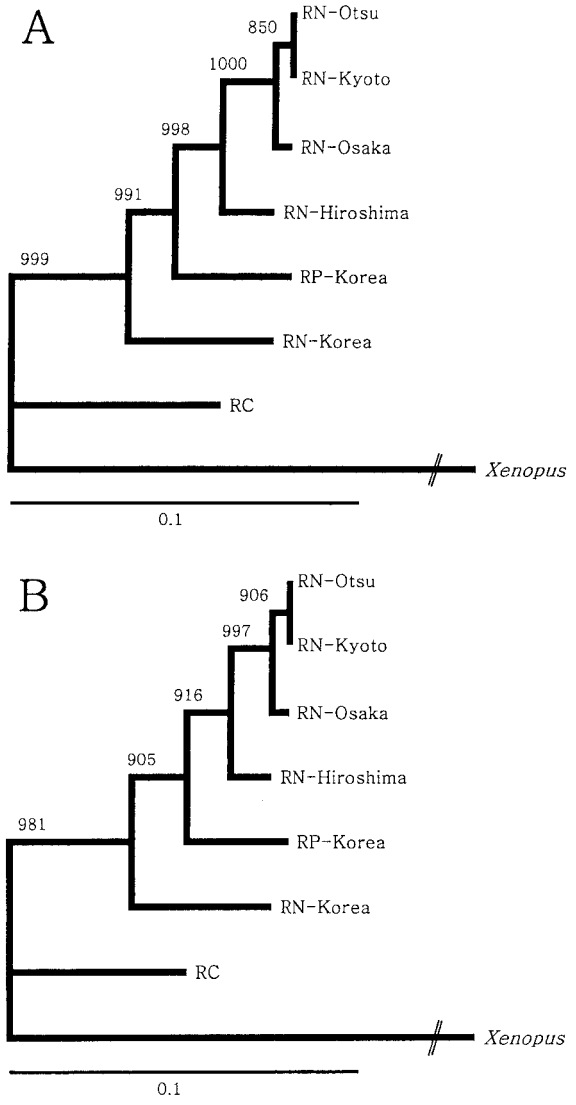


**Fig. 2.** NJ (A) and ML (B) trees based on Kimura's two-parameter distance (Kimura, 1980) from the mitochondrial cytochrome b gene sequences. Numbers on nodes are bootstrap values based on 1,000 replications and a scale bar represents branch length in terms of percentage divergences.

## DISCUSSION

We found interpopulation variation in the complete mt cyt b sequences of *R. nigromaculata* within Japan, and our Japanese samples also differed slightly from the published data for another Japanese population (Hiroshima: Sumida *et al.*, 2001). Moreover, the degree of divergence was very high between populations from Korea and Japan, which are isolated by a sea barrier. These results indicate the necessity of caution in using a 'species-specific complete sequence' of a gene for comparisons across different taxa. On the other hand, the genetic divergences between Korean and Japanese populations of *R. nigromaculata* did not much differ between the partial ($p$=7.4% in 243 bp: Kim *et al.*, 1999) and complete ($p$=6.8–6.9%) sequences of mtDNA cyt b genes. The resultant gene trees were also similar using the complete (Fig. 2) and partial (Kim *et al.*, 1999) sequences. Therefore, for practical purposes, even partial sequences, if properly chosen, can be informative to a considerable degree.

However, on comparing *R. p. chosenica* with Japanese *R. nigromaculata* directly, we found the degree of divergence between them ($p$=5.0%) to be even smaller than that between Korean and Japanese *R. nigromaculata* (see above), in striking contrast to the results of the allozyme analyses, in which Nei's D ranged from 0.00 to 0.02 between Korean and Japanese *R. nigromaculata*, which was much smaller than the values between Japanese *R. nigromaculata* and *R. p. chosenica* (0.56 to 0.57: Kim *et al.*, unpublished data). Moreover, unexpected phylogenetic relationships were suggested on gene trees. Thus, results of our analyses of the longer mt cyt b sequences and addition of new samples were well in concordance with those reported previously (Kim *et al.*, 1999; Yang *et al.*, 1999; Lee *et al.*, 2000), and we failed to solve the problem of the apparent discordance between mitochondrial DNA and allozyme differentiation, which was the starting point of this study.

Whether this discordance is exceptionally extreme should be evaluated by comparisons with data for other frog groups. However, because no comparable data are currently available, our discussion is limited to our own data. Unlike nuclear DNA expressed as allozymes, mt genes are inherited maternally and clonally, and recombination is rare. Further, the evolutionary rate of mt genes is generally much higher than that of nuclear genes, perhaps five to tenfold faster than that of typical single copy nuclear DNA (*e.g.*, Brown *et al.*, 1979; Tan and Wake, 1995). Even considering this faster evolutionary rate of the mt cyt b gene, the discordance between the mtDNA and allozyme divergence in *R. nigromaculata* seems great.

Because allozyme analyses showed no evidence of current hybridization among samples used in this study (Kim *et al.*, unpublished data), the above discordance could be explained as the result of the inheritance of introduced mtDNA and the dilution of introduced nuclear DNA in mixed lineages after past hybridization and genome introgression between *R. nigromaculata* and *R. p. chosenica*.

Kawamura and Nishioka (1975) reported that all male F1 hybrids between these two species were sterile, while half of the F1 females were probably fertile. Thus, it is likely that the maternal contribution to the nuclear genome in the female hybrid lineage decreases in each generation, whereas the mtDNA was inherited maternally during repeated backcrossing. Sumida (1997) experimentally found no paternal mtDNA in the reciprocal hybrids and backcross offspring between *R. nigromaculata* and another Japanese pond frog *R. porosa brevipoda*, although the proportions of the original maternal nuclear genes as estimated by allozyme loci constantly decreased.

Thus, the problem of discordance between mtDNA and allozyme could be partially explained with reference to

results of these laboratory experiments. However, if the past hybridization is the case in reality, very limited samples we report here, especially lacking *R. p. chosenica* from localities allopatric with *R. nigromaculata*, prevent further discussion on the direction of genome introgression, and date and place of the events. Further analyses of cyt b sequences of many additional samples from different localities are clearly necessary.

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