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Genetic Relationships among Six Korean *Rana* Species (Amphibia; Ranidae) Based on the Mitochondrial Cytochrome *b* Gene

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Genetic relationships among six species of the genus *Rana* from Korea were investigated by complete nucleotide sequence analyses of mitochondrial cytochrome *b* gene (1143 bp). Based on Kimura-2-parameter distance, the interspecific sequence differences of cytochrome *b* gene within the genus *Rana* were ranged from 7.83% to 25.00%. The genetic distances were 7.83% between *R. nigromaculata* and *R. plancyi*, 8.47% between two types of *R. rugosa* (type A and B), 16.42% between the brown frogs (*R. amurensis* and *R. dybowskii*), 16.11% between *R. dybowskii* types 1 and 2 and 12.36% between pond frogs (*R. nigromaculata* and *R. plancyi*) and *R. catesbeiana*. In the neighbor-joining and parsimony trees, *R. catesbeiana* was more closely related to pond frogs than brown frogs. *R. dybowskii* types 1 and 2 were considered to be at a distinct and specific level of differentiation (16.11%), while two types of *R. rugosa* were suspected to be at a subspecific level (8.47%).

Mitochondrial DNA (mtDNA) is a small sized circular duplex (15.0-20.0 Kb) and does not recombine during sexual reproduction. The mtDNA has evolved more rapidly than the nuclear DNA. Since the development of versatile primers and PCR (Kocher, et al., 1989), mtDNA genes (2 ribosomal RNAs, 22 transfer RNAs, 13 proteins) have been used extensively to estimate evolutionary phylogenies in a wide range of taxa (Howell, 1989: Kocher et al., 1989: Edwards et al., 1991: Irwin et al., 1991: Kornegay et al., 1993: Ma et al., 1993: Meyer, 1994: Whitmore et al., 1994). In particular, partial or complete sequences of the mitochondrial cytochrome *b* gene have been used to estimate phylogenetic relationships among closely related species. In addition DNA sequences of mitochondrial rRNA genes have been evaluated for appropriate evolutionary rates to resolve some aspects of the higher groups, such as genus, family and order (Ilya and Linda, 1996: Sumida, et al., 1998: Lee, et al., 1999b: Suh, 1999). The short sequence of mitochondrial cytochrome *b* was examined successfully for phylogenetic relationships but longer sequences were need to clarify accurate evolutionary relationships (Kornegray et al., 1993). In many cases, mitochondrial cytochrome *b* gene was often chosen as a phylogenetic indicator because it may be easier to align a protein-coding sequence than to align either mitochondrial rRNA or non-coding sequences (Howell, 1989).

Korean amphibia are classified into 2 order, 6 families, 7 genera and 17 species. Among the 17 species, six species belong to genus *Rana*: *R. amurensis, R. dybowskii, R. nigromaculata, R. plancyi, R. rugosa* and an introduced species, *R. catesbeiana,* inhabiting South Korea. Based on partial sequence analysis of cytochrome *b* gene, two distinct types were found in Korean *R. dybowskii* and *R. rugosa.* Two types of *R. dybowskii* showed specific level (Kim et al., 1999), while two types of *R. rugosa* were of subspecific level (Lee et al., 1999a). Inter- and intraspecific genetic relationships among the Korean amphibia have been investigated based on partial sequences of mitochondrial cytochrome *b* gene (Lee et al., 1997, Oh 1997, Lee et al., 1999a, b, Park et al., 1999).

In the present work, we attempted to elucidate genetic relationships among six species of the genus *Rana* including two distinct types of *R. dybowskii* (types 1 and 2) and *R. rugosa* (types A and B) using complete sequences of cytochrome *b* gene.

Materials and Methods

Ten localities were carefully selected based on published data and preliminary experiments (Table 1). *R. dybowskii* and *R. rugosa* were sampled from two different localities each, because they were separated into two distinct types by mitochondrial cytochrome *b* sequence (Lee et al., 1997, 1999a; Kim et al., 1999).

The methods used for DNA extraction, gene amplifi-

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Table 1. Species, localities and collection date

Species		Localities	Collection date
R. nigromaculata		Nonsan-shi, Chungchongnam-do	June 1996
		Kangrung-shi, Kangwon-do	June 1996
R. placyi		Nonsan-shi, Chungchongnam-do	June 1996
R. catesbeiana		Puan-gun, Chollabuk-do	March 1998
R. amurensis		Nonsan-shi, Chungchongnam-do	March 1997
		Samchuk-shi, Kangwon-do	Feb. 1998
R. dybowskii	type 1	Tonghae-shi, Kangwon-do	March 1997
	type 2	Tonghae-shi, Kangwon-do	March 1997
R. rugosa	type A	Nonsan-shi, Chungchongnam-do	April 1997
	type B	Yongdok-gun, Kyongsangbuk-do	April 1997

cation and sequencing were as described in the previous study (Lee et al., 1997). Primers used were: L14731, 5'-GAAAAACTATCGTTGTTATTCAACTA-3'; L16303, 5'-CCATCCAACATCTCAGCATGATGAAA-3'; L16654, 5'-TGAGGACAAATATCATTCTGAGGGGC-3'; L16797, 5'-TTYATYCTCCCNTTYATTAT-3'; L16937, 5'-TCYTMGG NTTTRTTATTAT-3'; H16915, 5'-GTCTTTGTAGAGAGA AGTATGG-3'; H21 5'-TTATGCTCTATATACATAAG-3'. The positions and orientations of the 8 primers for amplification and sequencing are shown in Fig. 1.

The complete sequences of cytochrome *b* gene for the six species of the genus *Rana* were registered in GenBank under accession numbers AF205087-AF205094. The complete sequences were aligned by DNASIS program. MEGA software (Molecular Evolutionary Genetic Analysis, Version 1.01; Kumar et al., 1993) was utilized for statistic analysis. The published complete mitochondrial cytochrome *b* sequences of *Xenopus laevis* (Roe et al., 1985) was used as an outgroup for the phylogenetic analysis. Phylogenetic trees among genus *Rana* were constructed using UPGMA (Sneath and Sokal, 1973) and neighbor-joining (Saitou and Nei, 1987) method in MEGA package and parsimony method in PAUP 3.1 software (Swofford, 1993). The bootstrap values were obtained with 1000 iterations (Felsenstein, 1985). The evolutionary rate of mtDNA was calculated by 2% nucleotide sequence divergence per million yr (Brown et al., 1979: Brown, 1985).

Results and Discussion

Genetic differences among the six Korean *Rana* species were estimated based on complete sequences (1143 bp) of the mitochondrial cytochrome *b* gene. The published mitochondrial cytochrome *b* gene of *X. laevis* was 3 bp (one amino acid) shorter than those of the *Rana* species due to deletion in the positions 856-858 (amino acid residues 256).

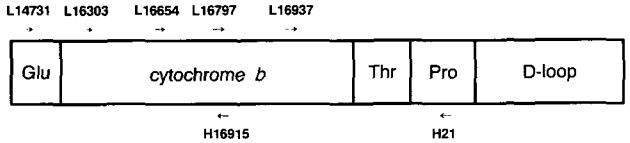

Fig. 1. Positions and orientations of primers used in this study. Names give the DNA strand (L and H) and the position of the 5' end of the oligonucleotide numbered according to the *X. laevis* mt DNA sequence.

The translated product of the 1143 bp nucleotide sequences consisted of 381 amino acid residues. The beginning of the initiation codon was methionine in all species but the termination codon was TAA in *R. nigromaculata, R. plancyi, R. catesbeiana, R. amurensis* and *R. dybowskii* type 1, but TAG in *R. dybowskii* type 2 and *R. rugosa* (types A and B). Transition was 1.6 times higher than transversion. The average nucleotide compositions were adenine (24.9%), thymine (30.1%), Cytosine (30.9%), guanine (14.1%). This pattern was similar to that of the other vertebrates (Kocher et al., 1989).

Based on Kimura-2-parameter distance (Table 2), interspecific genetic distances among mitochondrial cytochrome *b* genes of the *Rana* species ranged from 7.83% to 25.00%. Sequence divergence between *R. nigromaculata* and *R. plancyi* was 7.83% and that between two types of *R. rugosa* (types A and B) was 8.47%. Among the brown frogs, the sequence divergence ranged from 13.07% to 19.77% between *R. amurensis* and *R. dybowskii* (types 1 and 2) but 16.11% between type 1 and 2 of *R. dybowskii*. The genetic distance ranged from 12.29% to 12.46% between *R. catesbeiana* and pond frogs (*R. nigromaculata* and *R. plancyi*) and 12.97% to 19.21% between *R. catesbeiana* and brown frogs. Neighbor-joining tree constructed with Kimura-2-parameter distance showed that *R. nigromaculata* and *R. plancyi* were first clustered with 99% bootstrap value, followed by *R. catesbeiana, R. amurensis, R. dybowskii* types 1 and 2. Second group was clustered with *R. rugosa* types A and B (100% bootstrap value). In the parsimony tree, the clustering pattern was in accordance with the neighbor-joining tree and the bootstrap value was higher than the neighbor-joining tree.

In the neighbor-joining tree, *R. catesbeiana* was more closely related to pond frogs rather than brown frogs. *R. amurensis* was related with *R. dybowskii* type 1, and *R. rugosa* was clustered separately in the neighbor-joining and parsimony trees. *R. dybowskii* types 1 and 2 were considered to be a distinct and specific level of differentiation (16.11%), while two types of *R. rugosa* were suspected to be at subspecific level (8.47%).

Although two distinct types of Korean *R. dybowskii* and *R. rugosa* were morphologically similar to each

Table 2. Pairwise matrix of Kimura-2-parameter distance comparisons for complete sequences of cytochrome *b* gene among the genus *Rana* and *X. laevis*

Species	1	2	3	4	5	6	7	8
1. *R. nigromaculata*								
2. *R. plancyi*	0.0783							
3. *R. catesbeiana*	0.1246	0.1229						
4. *R. amurensis*	0.1423	0.1239	0.1394					
5. *R. dybowskii* type 1	0.1287	0.1428	0.1297	0.1307				
6. *R. dybowskii* type 2	0.1803	0.2023	0.1921	0.1977	0.1611			
7. *R. rugosa* type A	0.1699	0.1699	0.1809	0.1953	0.1718	0.1874		
8. *R. rugosa* type B	0.2161	0.2382	0.2248	0.2500	0.1782	0.2379	0.0847	
9. *X. laevis*	0.3488	0.3603	0.3398	0.3671	0.3488	0.3621	0.3365	0.3416

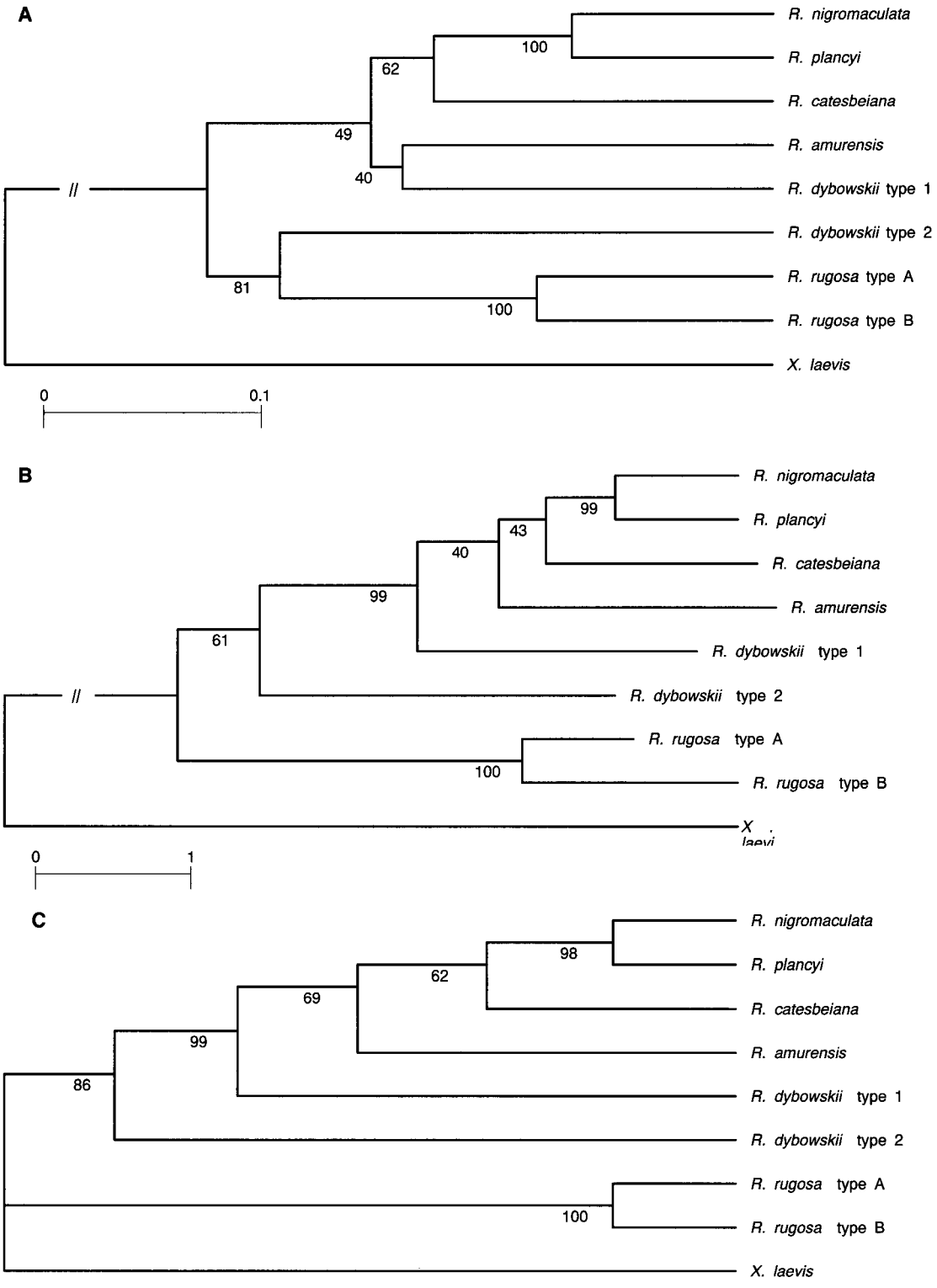

Fig. 2. Phylogenetic relationships among Korean *Rana* species obtained using UPGMA (A), Neighbor-joining (B) and maximum parsimony methods (C). Numbers below each node indicate bootstrapping values obtained from 1000 iteration. Bars indicate genetic distance value.

other, mitochondrial haplotypes separated them into two groups (Kim et al., 1999: Lee et al., 1999a). In the present phenograms constructed by neighbor-joining and parsimony methods, *R. dybowskii* types 1 and 2 were separated into different groups by UPGMA tree with 99% bootstrap value.

According to phenogram of 5 species of genus *Rana* by RFLP analysis, genetic distances were 9.47% between *R. nigromaculata* and *R. plancyi* and 14.20% between *R. amurensis* and *R. dybowskii*. *R. rugosa* and the pond frogs were grouped with 19.80% (Lee and Park, 1991). The results obtained from isozyme analysis showed that the *Rana* species were clustered into two groups. The first group was clustered with *R. nigromaculata* and *R. plancyi* (p=0.476), and the second group *R. amurensis*, *R. dybowskii* (p=0.321) and *R. rugosa* (0.208) (Kim, 1988).

The genetic distances between *R. dybowskii* types 1 and 2 and between *R. rugosa* types A and B were 16.11% and 8.47%, respectively. These rates are similar to the published data of *R. dybowskii* (14.8%) and *R. rugosa* (6.7%) (Kim et al., 1999: Lee et al., 1999a). The sequence divergences were 12.9% between *Hyla japonica* and *H. suweonensis* (Lee et al., 1999b) and 16.3% among Korean brown frogs (Oh, 1997) using partial sequences of mitochondrial cytochrome *b* gene. In the two subspecies of *Taricha torosa*, sequence divergence was from 7.0% to 9.0% (Tan and Wake, 1995). According to the study on evolutionary relationships among Japanese pond frogs (*R. nigromaculata*, *R. porosa porosa* and *R. p. brevipoda*), the percent similarities of nucleotide sequences range from 88.5% to 90.3% at the interspecific level, and 95.9% to 96.4% at the subspecific level on the basis of mitochondrial cytochrome *b* gene (Sumida et al., 1998). Depending upon comparison of sequence divergence, *R. dybowskii* type 1 and type 2 were considered to be at a specific level, but the two types of *R. rugosa* (A and B) were speculated to be at a subspecific level. *R. plancyi* was once considered to be a subspecies of *R. nigromaculata* (Okada, 1928). However, these two species were confirmed to be distinct sympatric species by Shannon (1956) and Yang and Yu (1978). Therefore, further investigations on the two types of *R. rugosa* by cytogenetical, morphological and molecular analyses of other genes are needed.

The genetic divergence of mitochondrial cytochrome *b* gene within sister species, congeners and confamilial genera was phylogenetically informative (Johns and Avise, 1998). Brown et al. (1979) suggested that mitochondrial DNA will be an extremely useful molecule for evolutionary biologists to use in assessing relationships among species and populations that diverged recently within the past 5-10 million yr. The rate of mitochondrial DNA evolution was generally estimated to be in the average of 2% nucleotide divergence per million yr by restriction analysis of primate mitochondrial DNA (Brown et al., 1979: Brown, 1985). Irwin et al. (1991) have analyzed nucleotide sequences of cytochrome *b* genes of more than 20 mammals. Slobodyanyuk et al. (1995) found that the evolution rates determined by Brown et al. (1979) and Irwin et al. (1991) were very similar (2.1% per million yr). Neighbor Joining is a distance matrix method producing an unrooted tree without the assumption of a clock. UPGMA does assume a clock. Assummg that these evolution rates of mitochondrial DNA of genus *Rana*, *R. nigromaculata* and *R. plancyi* diverged about 4 million yr ago and those of *R. amurensis* and *R. dybowskii*, about 6.5 million yr ago. *R. dybowskii* types 1 and 2 diverged about 8 million yr ago and *R. rugosa* types A and B about 4 million yr ago. The pond frogs and *R. catesbeiana* diverged about 6 million yr ago, and *R. rugosa* and *R. dybowskii* type 2 about 9 million yr ago. These evolution time points roughly correspond to that estimated from the mitochondrial RFLP sequence divergence (Lee and Park, 1991).

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