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Genetic Differentiation among the Mitochondrial *ND2* Gene and *tRNA*^{Trp} Gene Sequences of Genus *Rana* (Anura) in Korea

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The genetic variations among six species of *Rana* from Korea (*R. nigromaculata, R. plancyi, R. dybowskii, R.* sp, *R. rugosa* type A, B and *R. amurensis*) were investigated using 499 bases of mitochondrial DNA sequences for *ND2* (NADH dehydrogenase subunit 2) gene and *tRNA*^{Trp} gene. Partial sequences of *ND2* gene (427 bp) and full sequences of *tRNA*^{Trp} gene (73 bp) were identified. The level of sequence divergences ranged from 0.2 to 5.2% within species and 4.9-28.0% among 6 species of the genus *Rana*. The *tRNA*^{Trp} gene of the genus *Rana* was composed of 77 nucleotides which showed a two dimensional "cloverleaf" structure. The secondary structure of *tRNA*^{Trp} was not found compensatory changes which could potentially confound phylogenetic inference. In the neighbor-joining tree, brown frogs were clustered first with the level of sequence divergence of 13.20% between *R. amurensis* and *R. dybowskii,* and 9% between *R. dybowskii* and R. sp. supported by 99% bootstrap iterations, respectively. *R. nigromaculata* and *R. plancyi* were clustered into another group with 5.1% divergence supported by 100% bootstrap iteration. *R. rugosa* A and B types were grouped by 4.9% divergence and clustered into the last group with other two groups with 100% bootstrap iterations.

The mammalian mitochondrial genome containes 2 ribosomal RNAs, 22 tRNAs, 13 proteins and sequences for the displacement loop (D-loop) region and L-strand replication origin.

Inter- and intraspecific genetic relationships among Korean amphibia were investigated by RFLP and partial sequence of the mitochondrial cytochrome *b* gene (Park, 1990; Jung, 1992; Lee et al., 1997; Lee et al., 1999a, b). Also, the systematic relationship between Korean amphibia was carried out by rRNA genetic analysis in the mitochondria (Suh, 1999).

In this study, this is the first attempt that phylogenetic relationships among the Korean genus *Rana* are inferred by comparing the nucleotide sequences of mitochondrial *ND2* and *tRNA*^{Trp} genes.

Materials and Methods

Two specimens of each species and type were used for this study (Table 1). The total DNA was extracted from frozen (-70℃) liver, intestine and stomach using proteinase K/SDS dissolution and purified by the phenol/chloroform extraction method. PCR primer pairs were designed from *R. catesbeiana* (Fujii et al., 1988) to cover a 499 bp fragment of partial mitochondrial *ND2* gene and full sequences of the *tRNA*^{Trp} gene. PCR amplification was performed in 50 or 100 μl volume with 30-35 cycles (95℃ for 1 min, 52℃ for 1 min, 72℃ for 1 min; Genus Thermal Cycler) using *Taq* (*Thermus aquatics*) DNA polymerase (Saiki et al., 1988). The amplified DNA fragments were purified with a High Pure PCR Product Purification Kit (Boehringer Mannheim Corp., USA).

The amplified PCR product was sequenced directly using a Top Polymerase Sequencing Kit (Bioneer, Korea) using a Silver Staining Kit (Bioneer, Korea). The DNA sequences were aligned with the CLUSTAL-W software (Thompson et al., 1994), and statistical analysis was employed for MEGA software Kumar et al., 1993). Phylogenetic trees of the genus Rana were constructed based on neighbor-joining (Saitou and Nei, 1987) and maximum parsimony (MP) methods. The published data of *ND2* gene and *tRNA*^{Trp} gene sequence of *R. catesbeiana* (Fujii et al., 1988) was used for a reference group of aligment and construct phenogram. The bootstrap values were obtained with 1000 iterations (Felsenstein, 1985). MP analysis of the aligned sequences was conducted using the branch-and-bound

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Table 1. Localities, collection date and number of specimens used

Species	Locality	Collecting Date	No. of specimens
Rana nigromaculata	Yon-gi-gun, Chungchongnam-do	6. 26. 1996	2
R. plancyi	Yon-gi-gun, Chungchongnam-do	6. 26. 1996	2
R. dybowskii	Kapyong-gun, Kyounggi-do	4. 23. 1997	2
R. dybowskii sp.	Chongson-gun, Kangwon-do	4. 27. 1997	2
R. amurensis	Kyongji-si Kyongsangbuk-do	6. 20. 1997	2
R. rugosa (A)	Kapyong-gun, Kyounggi-do	9. 27. 1997	2
R. rugosa (B)	Yongdok-gun, Kyongsangbuk-do	4. 25. 1997	2

search procedure of PAUP (Swofford, 1993), in which all the base substitutions received equal weighting.

Results

Genetic relationships among six species of Korean genus *Rana* were estimated using partial sequence of mitochondrial *ND2* and *tRNA*^{Trp} genes of 499 bp (Fig. 1).

The mean value of the base composition consisted of adenine (23.6%), thymine (34.3%), cytosine (23.0%) and guanine (18.5%) for the *ND2* region. *tRNA*^{Trp} base composition consisted of adenine (33.8%), thymine (24.8%), cytosine (23.0%) and guanine (18.5%). The guanine composition was lower than the other bases. This phenomenon is similar to the pattern of other vertebrate mitochondrial genomes in general (Kocher et

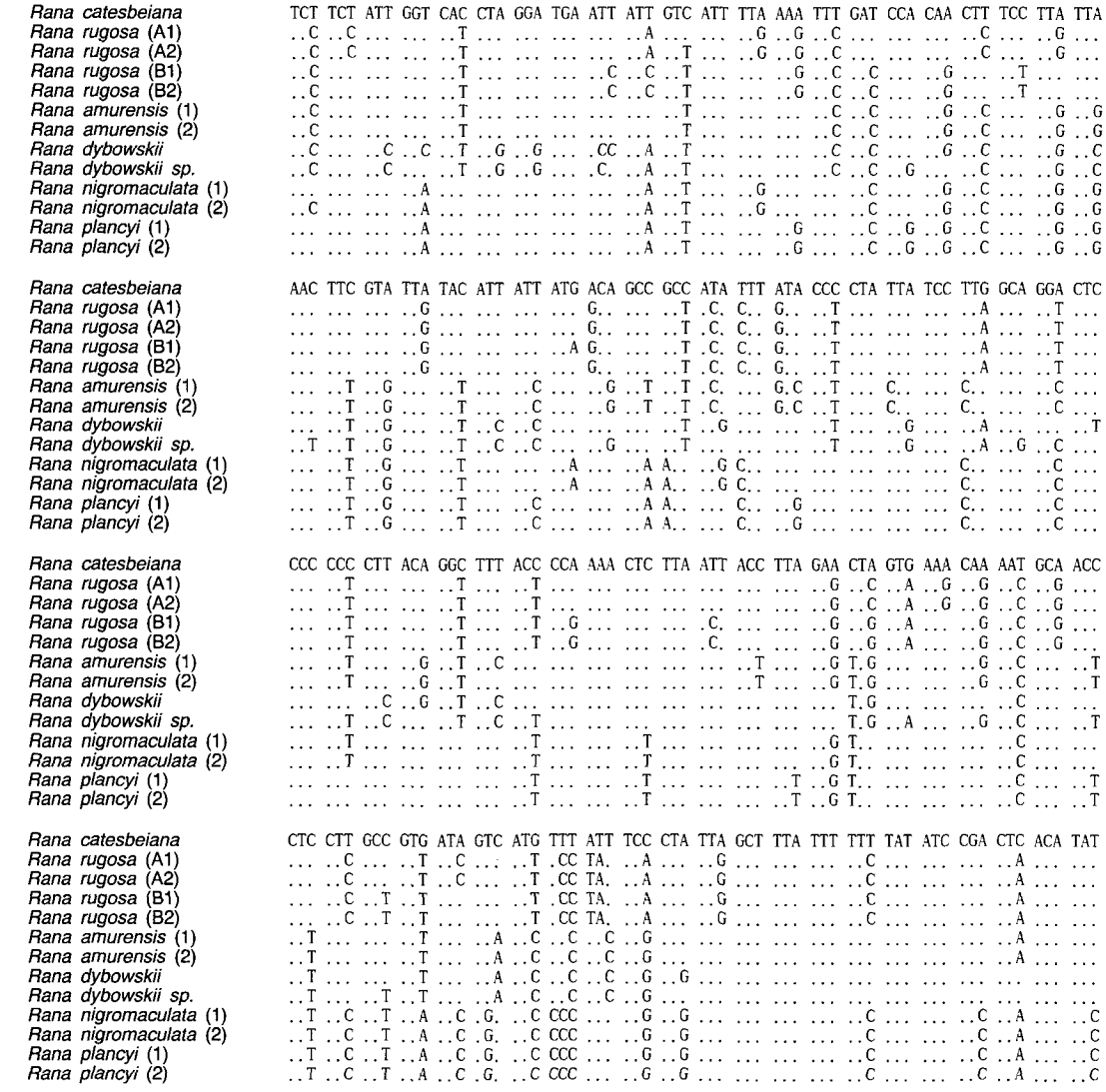

Fig. 1. Nucleotide sequences of 499 bp of the genus *Rana* mitochondrial *ND2* and *tRNA*^{Trp} genes. Dots indicate nucleotide sequence identity of *R. catesbeiana*.

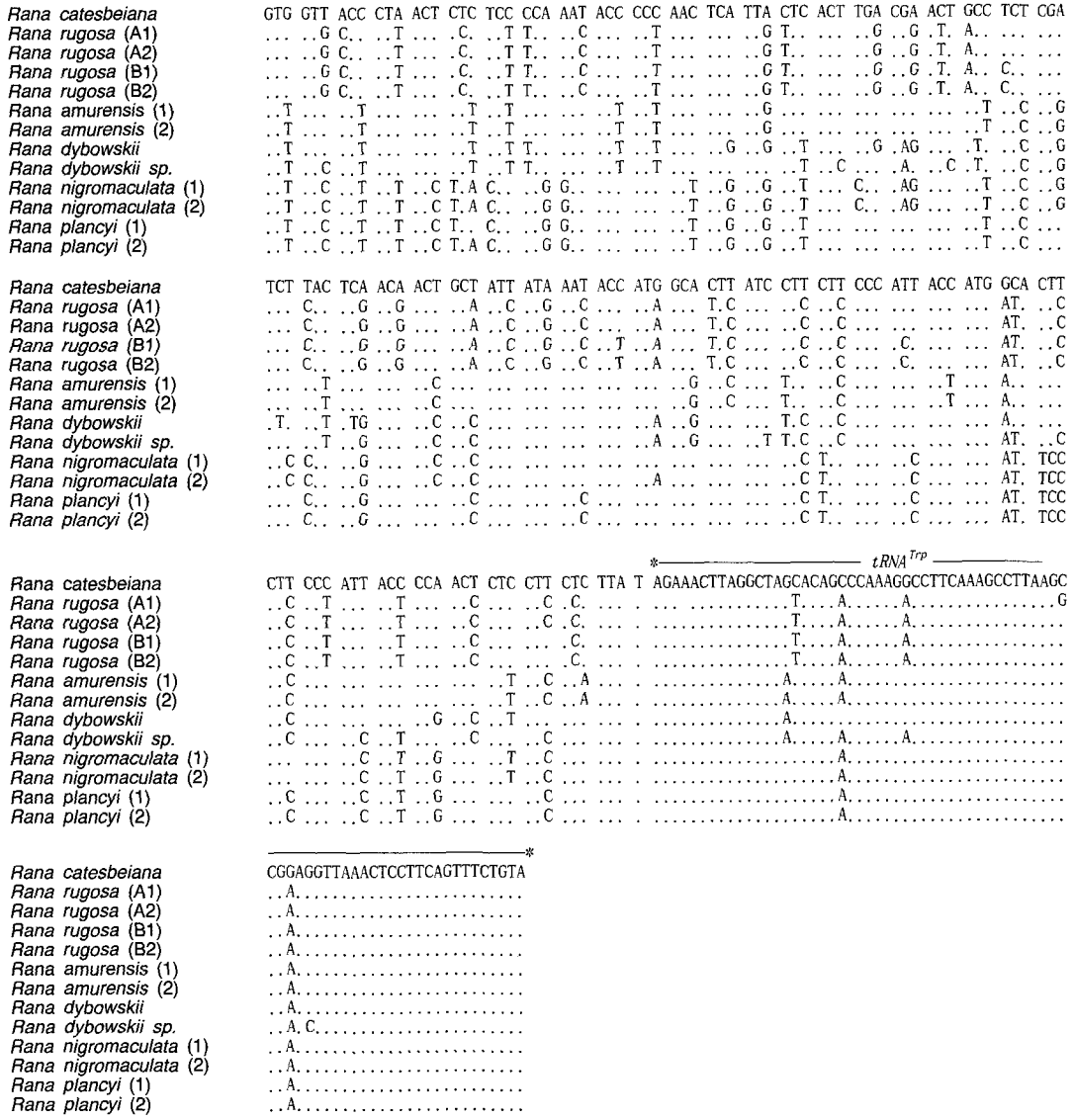

Fig. 1. *Continued*

et al., 1989).

The intrapopulational sequence divergences ranged from 0.0 to 0.5% within each species with zero to two nucleotide substitutions (Table 2). The interpopulational sequence divergences of *R. rugosa* (type A and B) ranged from 4.2 to 5.2% with 20 to 21 substitutions and 9.0% sequence divergence with 36 substitutions between *R. dybowskii* and *R. sp.* (Table 2). The genetic sequence divergences between *R. nigromaculata* and *R. plancyi* ranged from 4.7 to 5.4% with 19 to 22 substitutions, and the sequence divergences between *R. dybowskii* and *R. amurensis,* ranged from 12.9 to 13.5% with 49 to 52 substitutions. The genetic distance between *R. catesbeiana* and other six species ranged from 17.1 to 23.1% with 63 to 81 substitutions.

One hundred forty three amino acid sequences were deduced from the 427 bp segments of the *ND2* gene (Fig. 1). Although many nucleotide substitutions occurred at the third codon position and were silent mutations, five amino acid replacements occurred between *R. catesbeiana* and *R. amurensis*, and 13-20 replacements occurred between *R. catesbeiana* and the other species (Fig. 1).

The *tRNA*^{Trp} gene was conserved and composed of 73 nucleotide sequences with no length variation among the species. The nucleotide sequence of the mitochondrial *tRNA*^{Trp} gene was shown in Fig. 1 and 3. To compare with *R. nigromaculata tRNA*^{Trp}, Korean *Rana species tRNA*^{Trp} showed nucleotide variation at seven sites (15, 16, 21, 28, 45, 47 and 49 position) (Fig. 1).

Both the neighbor-joining tree and the maximum

Table 2. A number of nucleotide substitutions (transition : transversion) in parenthesis (above diagonal) and numbers of nucleotide substitutions per site estimated by the Kimura-2-parameter method (below diagonal) among haplotypes of the mitochondrial *ND2* gene in genus *Rana*

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *R. catesbeiana*		(61:14)	(62:14)	(69:11)	(69:11)	(55: 9)	(54: 9)	(67: 7)	(72: 8)	(66:13)	(68:13)	(62:13)	(62:14)
2. *R. Rugosa* (A1)	0.2093		(1: 0)	(18: 3)	(18: 3)	(72:13)	(71:13)	(80:13)	(75:12)	(83:13)	(81:13)	(79:13)	(79:14)
3. *R. Rugosa* (A2)	0.2127	0.0024		(17: 3)	(17: 3)	(79:13)	(80:13)	(79:13)	(74:12)	(82:13)	(80:13)	(78:13)	(87:14)
4. *R. Rugosa* (B1)	0.2285	0.0516	0.0490		(1: 0)	(79:10)	(76:10)	(82:12)	(81:11)	(88:14)	(86:14)	(84:14)	(84:15)
5. *R. Rugosa* (B2)	0.2249	0.0490	0.0464	0.0024		(79:10)	(78:10)	(82:12)	(81:11)	(88:14)	(86:14)	(84:14)	(84:15)
6. *R. amurensis*(1)	0.1746	0.2456	0.2419	0.2628	0.2589		(1: 0)	(43: 6)	(46: 5)	(68:12)	(68:12)	(66:12)	(65:13)
7. *R. amurensis* (2)	0.1713	0.2419	0.2382	0.2589	0.2551	0.0024		(44: 6)	(47: 5)	(67:12)	(67:12)	(65:12)	(64:13)
8. *R. dybowskii* (1, 2)	0.2093	0.2763	0.2723	0.2810	0.2770	0.1288	0.1318		(35: 1)	(71:12)	(69:12)	(66:12)	(65:13)
9. *R. dybowskii* sp (1, 2)	0.2266	0.2500	0.2462	0.2698	0.2659	0.1321	0.1351	0.0895		(75:11)	(73:11)	(72:11)	(71:12)
10. *R. nigromaculata* (1)	0.2238	0.2883	0.2842	0.3123	0.3166	0.2279	0.2243	0.2388	0.2469		(2: 0)	(18: 2)	(18: 1)
11. *R. nigromaculata* (2)	0.2310	0.2802	0.2763	0.3039	0.3081	0.2279	0.2243	0.2315	0.2394	0.0047		(20: 2)	(20: 1)
12. *R. plancyi* (1)	0.2097	0.2723	0.2684	0.2956	0.2916	0.2208	0.2172	0.2575	0.2357	0.0491	0.0543		(0: 1)
13. *R. plancyi* (2)	0.2127	0.2755	0.2716	0.2989	0.2948	0.2202	0.2167	0.2569	0.2352	0.0466	0.0518	0.0024	

parsimony tree showed almost the same pattern that six species of the genus *Rana* were clustered into three groups. Because intraspecific nucleotide sequence similarities in six species were 99.5-100%, in the neighbor-joining tree, each species are clustered within them. In the first cluster, the genetic divergence between *R. amurensis* and *R. dybowskii* was 13.2% supported by a 99% bootstrap iteration. In the second cluster, a 5.1% divergence between *R. nigromaculata* and *R. plancyi* was supported by 100% bootstrap iterations. In the third cluster, *R. rugosa* A and B types diverged by 4.9% with 100% bootstrap iterations and grouped last with the other two groups.

Discussion

Genetic differentiation among the genus *Rana* was estimated using nucleotide sequence data of 499 bp of *ND2* and *tRNA*^{Trp} genes where each species specific nucleotide sequence difference was shown (Fig. 1).

Base composition of nucleotide sequences is known to vary among taxa and this may obscure phylogenetic information. Most base change at the third codon positions are silent, and this position demonstrates the highest level of compositional bias (Muto and Osawa, 1987) as reflected by a higher standard deviation at this codon position. As for other mitochondrial genomes guanine residues (G) are under represented on the L strand in the opossum (Gadelta et al., 1989). This observation is most apparent at the second codon position, where the average G content is 6.2% and average G content of the first position and the thymine content of the second position are high.

All the sequenced genes appear functional; *tRNA*^{Trp} sequences form stable secondary structure and protein-coding gene for *ND2* have no premature stop codon. The result also indicates that organization of these genes in the mitochondrial genomes of the Korean genus *Rana* were the same as that found in *R. catesbeiana, Xenopus laevis* and in several mammals studied so far (Anderson et al., 1981, 1982; Roe et al., 1985).

Based on Anderson et al. (1981), stop codons for *ND2* appear to be formed by polyadenylation during processing of the primary transcript, 6 species have

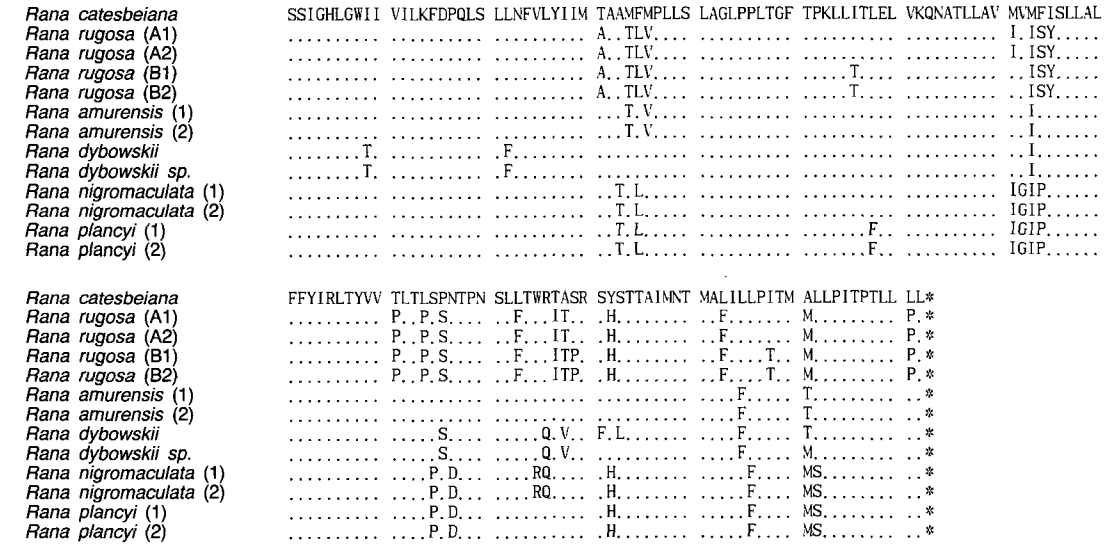

Fig. 2. Amino acid sequences of 143 segments of the *ND2* gene in six species of the genus *Rana*. Dots indicate amino acid sequence identity of *R. catesbeiana* and asterics indicate stop codons.

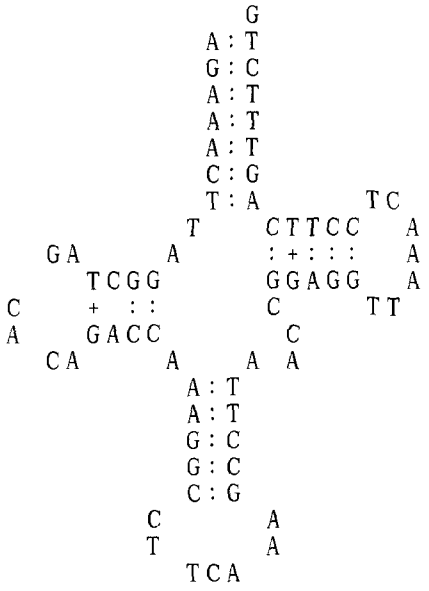

Fig. 3. Secondary structure of genus *Rana* $tRNA^{Trp}$: The clover leaf structures of genus *Rana* $tRNA^{Trp}$ gene are shown. The symbol (:) indicates Watson-Crick base pairs and (+) indicates G: T pairs.

incomplete termination codons, which are presumably formed by polyadenylation of the transcription that termination codon inferred to use TAA. For all the taxa, extension of the *ND2* mRNA including part of the adjacent $tRNA^{Trp}$ from the primary transcript would generate a stop codon. However, this mechanism would preclude production of complete *ND2* mRNA and $tRNA^{Trp}$ molecules from the same primary transcript.

Base composition of nucleotide sequences is known to vary among taxa, and this may obscure phylogenetic information. Most base change at the third codon positions are silent, and this position demonstrates the highest level of compositional bias (Muto and Osawa, 1987) as reflected by the higher standard deviation at this codon position. As for other mitochondrial genomes (Gadeleta et al., 1989) guanine residues (G) are underrepresented on the L strand in the opossum, an observation most apparent at the third codon positions, where the average G content is 12.7% and average G content is 11.6%. Furthermore, the adenine content of first positions and the thymine content of second positions are high.

The nucleotide sequence of the mitochondrial $tRNA^{Trp}$ gene of the Korean *Rana* species showed no length variation and only a few base substitutions. This result suggests extreme conservation of the tRNA gene among anurans (Macey et al., 1997). $tRNA^{Trp}$ gene has many characteristic features that are common in mitochondrial tRNAs of the animals examined so far (Anderson et al., 1981; Roe et al., 1985).

According to Oh (1997), the sequence divergences of the cytochrome *b* gene ranged from 6.3 to 6.9% between *R. nigromaculata* and *R. plancyi* and 15% between *R. dybowskii* and *R. amurensis*. Based on

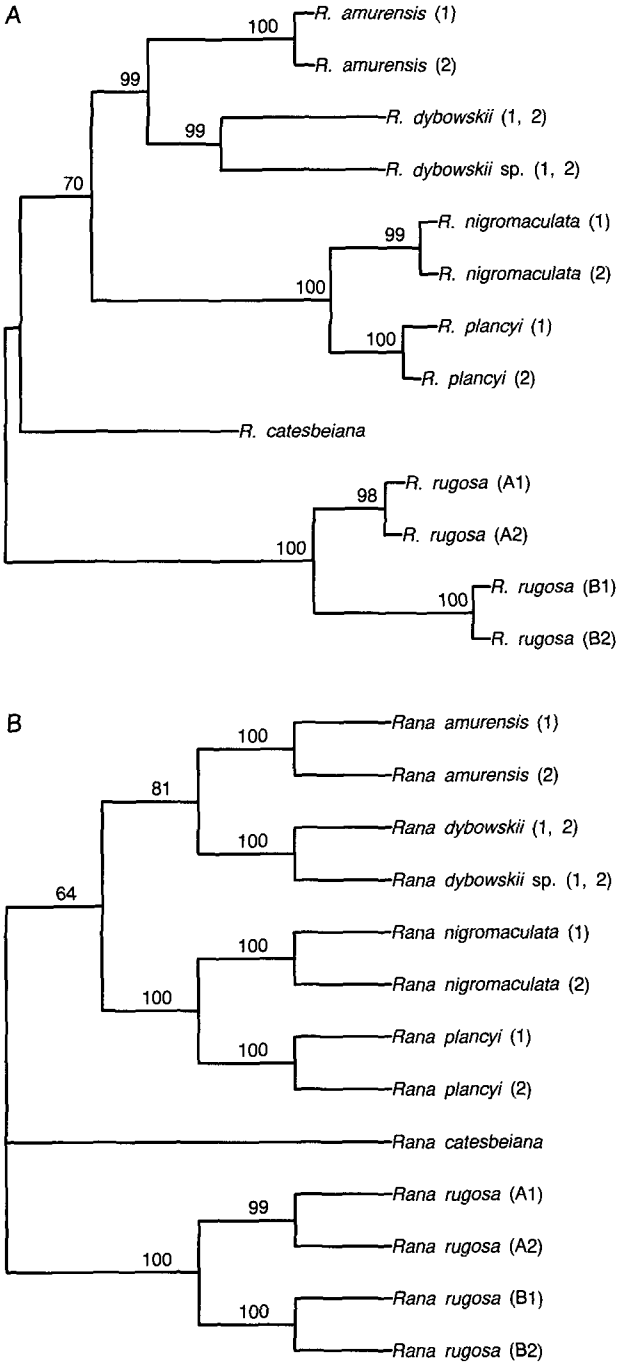

Fig. 4. A neighbor-joining tree rooted at the midpoint of the longest path (A), and a maximum parsimony tree (B). Nodal values in (A) and (B) indicate percent support for branches in 1,000 bootstrap replicates.

sequence divergence of the 12S rRNA gene (Suh, 1999), the genetic divergences between *R. nigromaculata* and *R. plancyi* were 4.2% and, 6.3 to 7.3% between *R. dybowskii* and *R. amurensis*. The sequence divergence value of present study was intermediate between partial sequences of the cytochrome *b* gene and 12S rRNA. In the phenogram of 12S rRNA, the clustering

pattern was similar to present data. Also for phylogenetic relationships using full sequences of the cytochrome *b* gene (*personal communication*), the UPGMA tree were rearly similar to *ND2* and *tRNA*^{Trp} phenogram. Various functional regions of mtDNA evolve at different rates. The nonsynonymus sites, D-loop central domains, tRNA and rRNA genes change much more slowly than synonymus sites and the two peripheral ETAS and CSB domains of the D-loop region. In protein-coding genes, the synonymus rate can be considered roughly uniform in all mitochondrial genes (Graziano et al., 1999). Combining variable data set is useful in reconstructing phylogeny among closely related species (Zink and Blackwell, 1996; Montgelard et al., 1997; Todd et al., 1997; Bloomer and Crowe, 1998).

Phylogenetic relationships were very well resolved among the Korean *Rana* species in this study. Based on the data presented above, interspecific sequence divergences were clustered into three groups. In the Kimura 2-parameter distance, genetic divergence between *R. dybowskii* and *R. sp.* was 9% and following by 13.2% divergence between *R. amurensis* and *R. dybowskii*. Genetic distance between *R. nigromaculata* and *R. plancyi* was 5.1% with another group followed by brown frogs. In the third group, *R. rugosa* A and B types diverged by 4.9% clustering last.

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