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Genetic Variation of the Mitochondrial Cytochrome *b* Sequence in Korean *Rana rugosa* (Amphibia; Ranidae)

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Nucleotide sequences of a 501 base-pair (bp) fragment in the mitochondrial cytochrome *b* (cyt *b*) gene were analyzed for 12 populations of *Rana rugosa* from Korea and Japan using a polymerase chain reaction (PCR) and direct silver sequencing. Two genetically distinct groups (type-A and type-B) were found in Korea. Type-A was found throughout most of South Korea and type-B was restricted to the mid-southeastern regions (Samchok, Yongdok, Chongsong and Pohang). But in the Tonghae population, both types were found. The level of mitochondrial DNA (mtDNA) sequence differences ranged from 0% to 0.8% among six populations of type-A, and 0 to 1.0% among 4 populations of type-B. However, sequence differences between type-A and type-B ranged from 5.4% to 6.6%. Using Kimura's two-parameter distance, the level of genetic sequence divergence between type-A and type-B was 6.7%. The Japanese *R. rugosa* was clustered very far from the Korean *R. rugosa* with 14.7%. In the neighbor-joining and UPGMA tree, all Korean samples were grouped, but subdivided into two types in 99% of the bootstrap iteration.

Rana rugosa $S_{chlegel}$ (wrinkled frog) is widely distributed in Korea, Japan, and northeastern part of China. The karyological and RFLP studies for Korean *R. rugosa* were reported previously (Park, 1990; Lee and Park, 1991; Lee et al., 1992). In the result of RFLP analysis, *R. rugosa* had the largest mtDNA size in vertebrates, and mtDNA size variations among populations were found.

A rapid rate of evolution, lack of recombination and maternal pattern of inheritance have allowed the vertebrate mitochondrial genome a great utility in studies of genetic differentiation within and among conspecific populations and closely related species (Brown et al., 1979; Lansman et al., 1981). With the rise of molecular systematics over the past decade, methods of PCR and direct sequencing now allows the rapid determination of nucleotide sequences of specific regions of the mitochondrial genome (Saiki et al., 1988; Kocher et al., 1989). Since Kocher's work (1989), partial sequence data of the mitochondrial cyt *b* gene have been a popular source of DNA sequences for determining the phylogenetic relationships of various animals (Meyer, 1994) and considered as a good indicator for evaluating intraspecific and/or interspecific variations of the brown frog. Short sequences from the mitochondrial cytochrome *b* gene contain phylogenetic information extending from the intraspecific level to the intergeneric level (Kocher et al., 1989; Tanaka et al., 1994).

In this work, partial nucleotide sequences of the mitochondrial cyt *b* gene were compared among 11 populations of Korean *R. rugosa* and one specimen from Japan to analyze the genetic relationships among populations and intraspecific differentiation.

Materials and Methods

DNA extraction

Tissue samples were obtained from 34 individuals representing eleven populations from Korea and one from Japan (Table 1, Fig. 1). Total DNA was extracted from frozen liver, intestine, stomach and heart (~100 mg) using proteinase K/SDS dissolution and purified by phenol-chloroform extraction.

DNA amplification

PCR was used to amplify the nucleotide fragment (501 bp) from the mitochondrial cyt *b* gene. The primers were obtained from Tanaka et al. (1996) (Table 2). PCR amplification of double-stranded products was performed in 25 or 50 μl volume with 30 cycles (95℃ for 1 min, 50℃ for 1 min, 72℃ for 1 min; Genius Thermal Cycler) using *Thermus aquaticus* DNA polymerase (Saiki et al., 1988).

Direct silver sequencing

DNA PrepMate™ Kit (Bioneer, Korea) was used to

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

Localities	No. of specimens	Collecting date
Sizuichi-Sizuhara, Sakyo, Kyoto, Japan	1	Aug. 1997
Asungjon-Ri, Hyonbuk-Myon, Yangyang-Gun, Kangwon-Do (1)*	3	Sept. 1997
Jokmok-Ri, Buk-Myon, Kapyong-Gun, Kyonggi-Do (2)	3	Sept. 1997
Yangchon-Ri, Yonmu-Up, Nonsan-Shi, Chungchongnam-Do (3)	3	April 1997
Kongjong-Ri, Ansung-Myon, Muju-Gun, Chollabuk-Do (4)	3	Oct. 1997
Jangchon-Ri, Samsan-Myon, Haenam-Gun, Chollanam-Do (5)	3	May 1997
Dongchon-Ri, Samdong-Myon, Namhae-Gun, Kyongsangnam-Do (6)	3	June 1997
Samhwa-Dong, Tonghae-Si, Kangwon-Do (7)	2	Sept. 1998
Bichon-Dong, Tonghae-Si, Kangwon-Do	1	Sept. 1998
Yonghwa-Ri, Kundok-Myon, Samchok-Si (8)	3	Sept. 1998
Saam-Ri, Namjong-Myon, Yongdok-Gun, Kyongsangbuk-Do (9)	3	April 1997
Chuwang Mt., Chongsong-Gun, Kyongsangbuk-Do (10)	3	Aug. 1998
Jungsan-Ri, Pohang-Shi, Kyongsangbuk-Do (11)	3	Aug. 1998

* Locality numbers in Fig. 1.

purify the DNA product. This purified DNA was used as a template in the thermal cycler sequencing reactions with the Top DNA Sequencing Kit (Bioneer, Korea). Sequences was visualized via a Silver Staining Kit (Bioneer, Korea).

Data analysis

A published sequence of *Xenopus laevis* (Roe et al., 1985) was used as an outgroup and sequence data was compared and analyzed by DNASIS software and MEGA softwares (Molecular Evolutionary Genetic Analysis; Kumar et al., 1993). Intra- and inter-populational neighbor-joining method (Saitou and Nei, 1987) and UPGMA clustering method based on Kimura's two-parameter (Kimura, 1980) and Tamura-Nei distance (Tamura and Nei, 1993). The bootstrap resampling technique was used to assess the statistical significance of internal nodes (Felsenstein, 1985). 1,000 replications were performed from the bootstrap analysis.

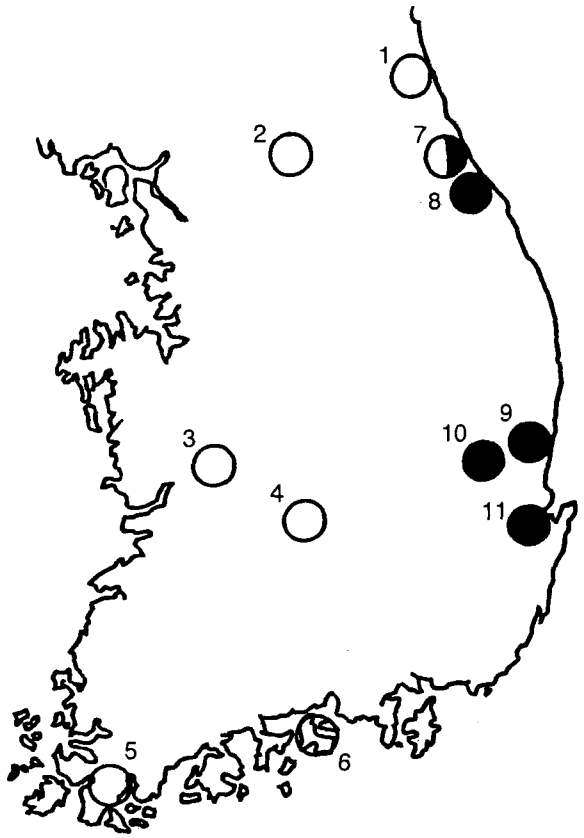


Fig. 1. The sampling localities of 11 populations of Korean *R. rugosa*. Open circle: type-A; solid circle: type-B. Numbers refer to collection localities listed in Table 1. A half-filled circle indicates a mixture of type-A and type-B.

Results

The 501 bp-fragment of the cyt *b* gene was sequenced from 34 samples of *R. rugosa* and aligned with that in *X. laevis* as an outgroup (Fig. 2). Neither insertion nor deletion was detected, but there were 156 variable sites, which account for 31.1% sequence differences, resulting in up to 19.2% amino acid differences. Intrapopulational variation of nucleotide sequence in Korean *R. rugosa* ranged from 0.0% to 0.6%. All Korean samples were separated into two groups (type-A and type-B; Fig. 1). The sequence variation between two types ranged from 5.4% to 6.6% (Table 4, Fig. 2). Intrapopulational variation ranged from 0.0% to 0.8% within type-A, and 0.0% to 1.0% within type-B.

Between type-A and type-B, base substitutions commonly occurred at the third position of the codon (Table 4) and the pattern of substitutions showed that transitions were more frequent. The transition/transversion ratio ranged from 5.2 to 9.0. Especially among populations with the same type, no transition bias was found (Table 4). The type-B individuals were restricted to the mid-southeastern part of the Peninsula. Both types were present at Samhwa-Dong in Tonghae (Fig. 1).

The mean value of the composition of third codon positions consisted of 26.3% of adenine, 19.3% of tymine, 51.1% of cytosine and 3.3% of guanine (Table 3).

Analyses based on Kimura's two-parameter and Tamura-Nei distance did not show any meaningful

Table 2. Sequence of cytochrome *b* oligonucleotides used in PCR amplification

Oligonucleotide	Size	Sequence
L 14850	25 bp	5'-TCTCATCCTGATGAAACTTTGGCTC-3'
H 15410	21 bp	5'-GTCTTTGTAGGAGAAGTATGG-3'

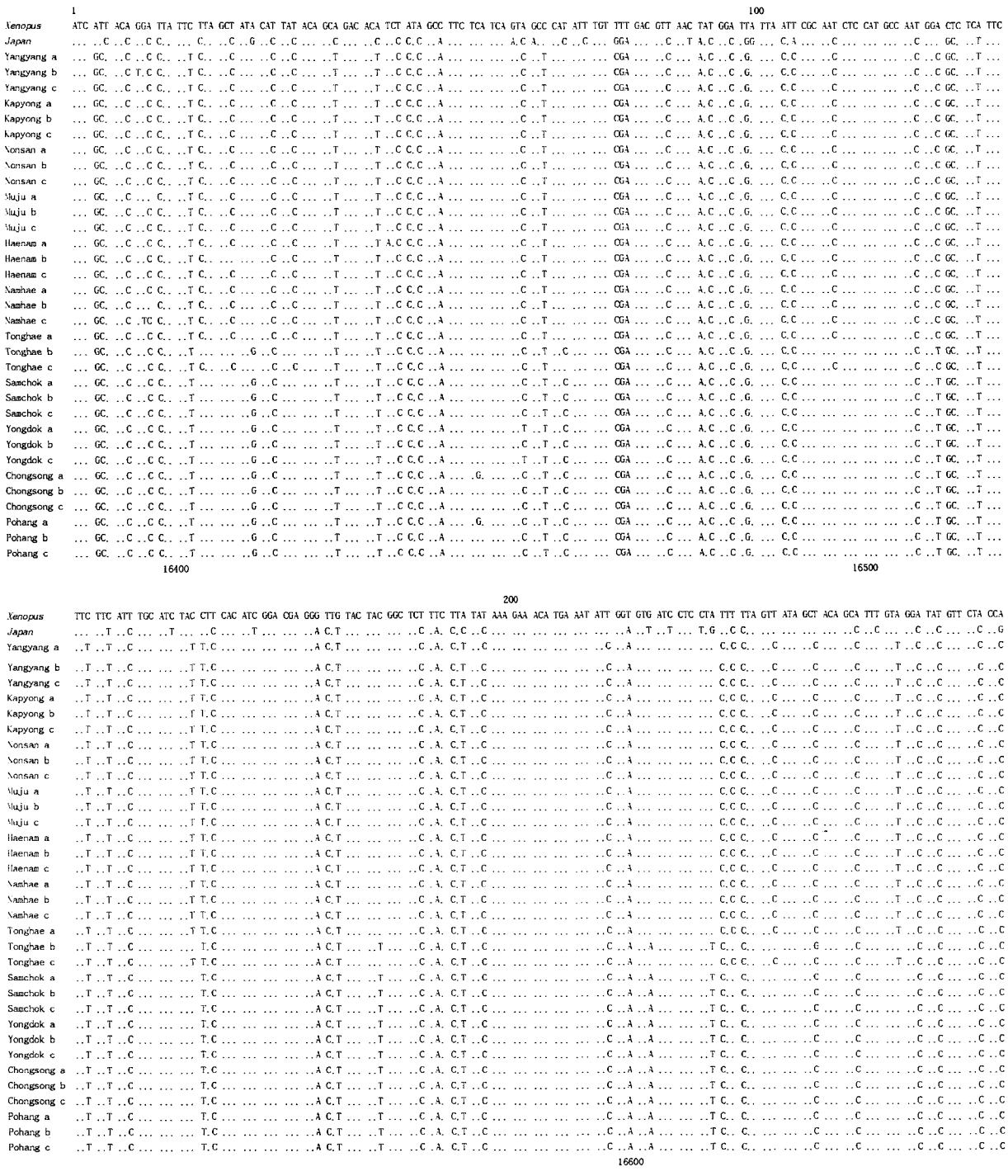


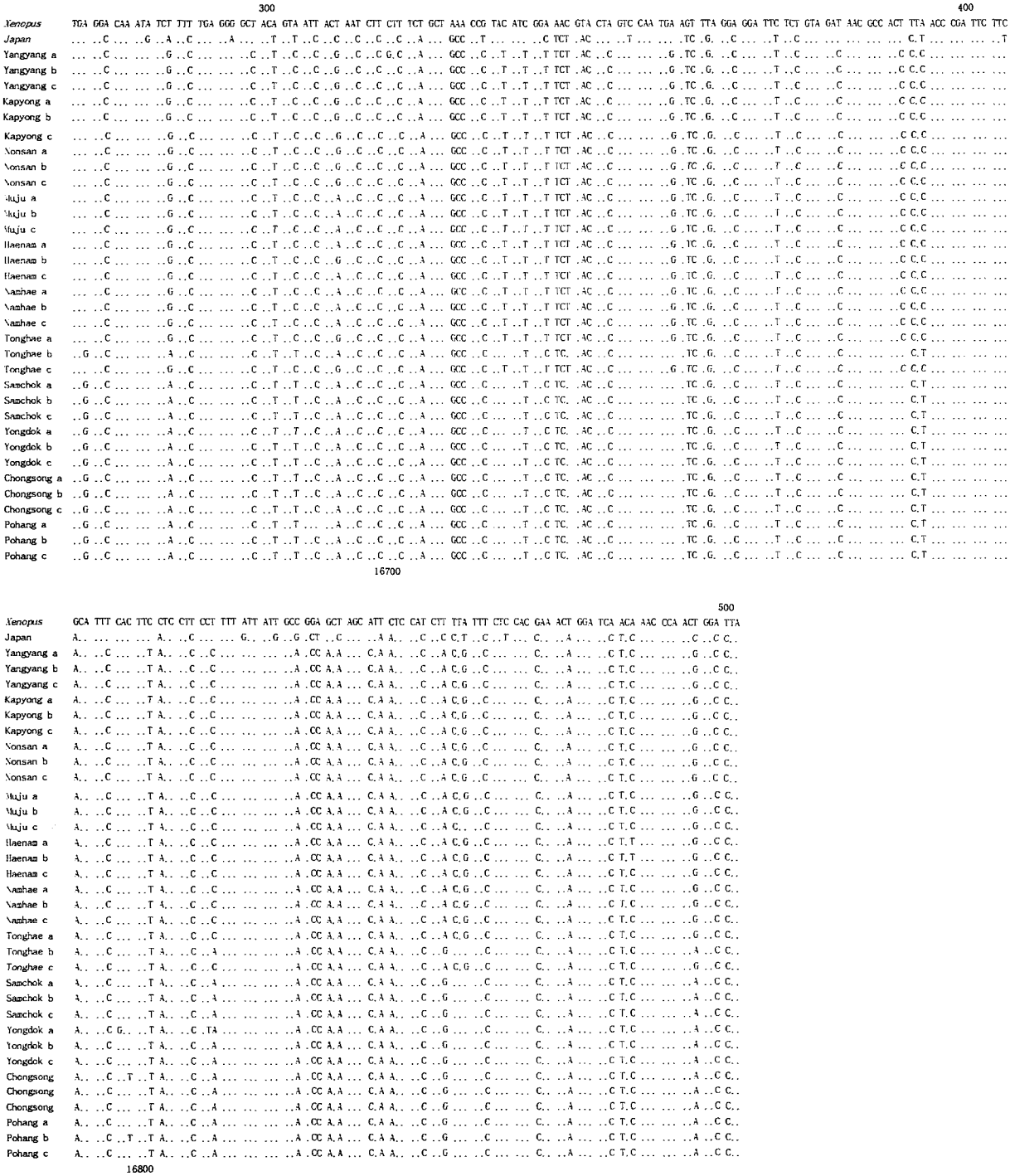
Fig. 2. Sequences of 501-bp of cyt *b* gene from 11 populations of *Rana rugosa*, one Japanese *R. rugosa* and an outgroup of *Xenopus laevis*. Dots indicate sequence identity to *X. laevis*. Nucleotide number given below sequence refer to cyt *b* sequence in *X. laevis* (Roe et al., 1985).

differences. Both the UPGMA tree (Fig. 4) and the neighbor-joining tree (Fig. 5) showed that 11 populations of Korean *R. rugosa* could be clustered into two groups and placed the Japanese *R. rugosa* in the basal position. All Korean samples were grouped in 99% bootstrap support in the neighbor-joining analysis and 100% bootstrap support in UPGMA analysis.

Discussion

Although two mitochondrial haplotypes of Korean *R. rugosa* could not be associated with morphological

Fig. 2. Continued



differences, they can be clearly separated into two groups. The level of sequence variation within populations and among populations of the same type was very low (Table 4). This low intrapopulation variation in the cyt *b* gene is generally observed in the genus *Rana* from Korea, Russia, Japan, and Taiwan (Lee et al., 1997; Tanaka et al., 1994; Tanaka-Ueno et al., 1998a; Tanaka-Ueno et al., 1998b). On the other hand, the sequence divergence of two genetically discrete types ranged from 5.7% to 7.0%, but there were only six amino acid differences (0.036%; Fig. 3). Meyran et al. (1997) stated that most of the intraspecific differences at the nucleotide level were not detectable at the amino acid level. Even in the sequence analysis of closely

Table 3. Frequencies of codon position of cyt *b* sequences in Korean *Rana rugosa*

Nucleotide	Codon position					
	Type-A			Type-B		
	First	Second	Third	First	Second	Third
A	24.03	19.20	25.43	24.00	19.20	27.50
T	25.70	38.33	18.09	26.90	38.35	21.23
C	23.97	25.70	52.55	22.75	25.56	48.87
G	26.30	16.77	3.93	26.35	16.89	2.40

related species, *R. nigromaculata* and *R. plancyi,* few amino acid differences were observed (Lee et al., 1997). This high proportion of silent mutation is expected because this fragment belongs to the coding region of an essential mitochondrial protein, whose structure and function must be maintained (Anderson et al., 1982; Brown et al., 1982).

At the level of nucleotide composition, guanine at the third position of the codon in type-B was lower than type-A (Table 3). This low guanine value is consistent with those of other vertebrates such as rodents, birds, and cranes (Kocher et al., 1989; Carey and King, 1996). A biased base composition is a common feature of animal mtDNA (Brown et al., 1982; Irwin et al., 1991).

In the UPGMA dendrogram based on Kimura's two parameter distance, the result of sequence divergence between type-A and type-B was 6.7%. According to the RFLP analysis of mtDNA of *R. rugosa,* divergency between two groups was 8.2% (Lee et al., 1992). This result supports our present data. The sequence divergence of the cyt *b* gene between *R. nigromaculata* and *R. plancyi* was 8.5% (Lee et al., 1997). In the cyt *b* gene of five Japanese brown frogs, including *R. japonica, R. ornativentris, R. pirica, R. tagoi* and *R. sakuraii,* a mean percentage of sequence divergence was 12.6% (Tanaka et al., 1994). On the other hand, two subspecies of *Taricha torosa* revealed about 7% to 9% sequence divergence (Tan and Wake, 1995). Moreover, in the distance tree obtained from the neighbor-joining method, two clustered groups were strongly supported by 99% bootstrap iterations. Based on the DNA sequence differences, two types of *R. rugosa* were genetically highly diverged as the subspecific level.

In this study, the distribution patterns of two types were clearly divided (Fig. 1). Type-A is widely distributed in South Korea, whereas type-B is restricted to the mid-southeastern parts. Tonghae population revealed the sympatric area which were also consistent with the results of the RFLP analysis. The RFLP analysis reported that these results were caused by discontinuity of gene flow in the boundary of the Taebaek Mountains. But, because the Yangyang population is type-A, a more detailed physiographical investigation may be necessary. Also, to clarify the taxonomic affinity between two types of *R. rugosa,* further investigations are needed by cytogenetical and molecular analysis of nuclear genes.

In comparison to the Korean *R. rugosa*, the Japanese *R. rugosa* was genetically very different, though they were reported as conspecific.

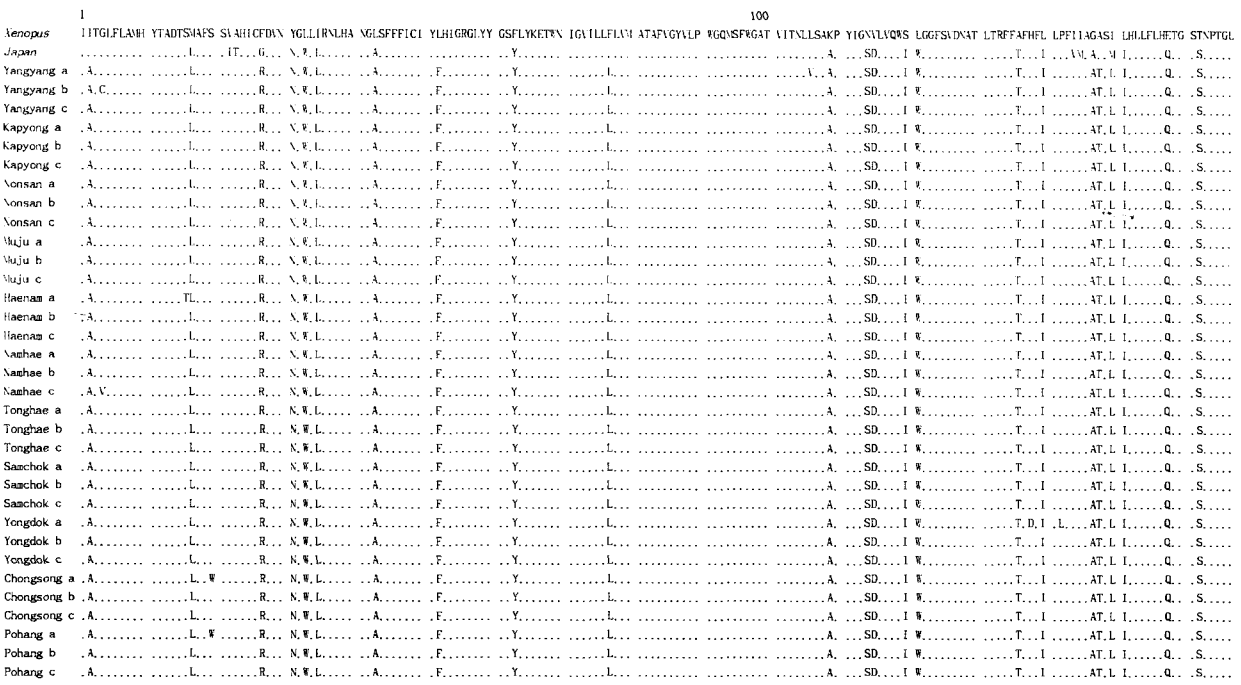


Fig. 3. Amino acid sequences of 167 segments for the cyt *b* gene for 11 populations of *Rana rugosa*, one Japanese *R. rugosa* and an outgroup of *Xenopus laevis.* Dots indicate amino acid sequence identity to *X. laevis.*

Table 4. Matrix of substitution patterns between cyt *b* sequence for 11 populations of *Rana rugosa*, one Japanese *R. rugosa* and an outgroup of *Xenopus laevis*

OTUs		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 *X. laevis*			64:57	63:62	63:62	63:61	63:61	63:60	63:61	62:61	63:62	62:61	63:62	63:61	54:62	53:62	53:63	54:62	55:63	53:63	55:62
R. rugosa																					
2 Japan		87:121		51:15	51:15	51:14	51:14	51:15	51:14	51:14	52:15	53:14	51:15	51:14	47:15	46:15	49:16	48:15	48:16	48:16	48:15
3 Yangyang	a	88:125	54:66		0:2	0:1	0:1	1:2	1:1	2:1	2:2	2:1	1:2	0:1	26:4	25:5	28:5	27:4	27:5	27:5	27:4
4	b	88:125	54:66	0:2		0:1	0:1	1:2	1:1	2:1	2:2	2:1	1:2	0:1	26:4	25:5	28:5	27:4	27:5	27:5	27:4
5 Kapyong	a	88:124	54:65	0:1	0:1		0:0	1:1	1:0	2:0	2:1	2:0	1:1	0:0	26:3	25:4	28:4	27:3	27:4	27:4	27:3
6 Nonsan	a	88:124	54:65	0:1	0:1	0:0		1:1	1:0	2:0	2:1	2:0	1:1	0:0	26:3	25:4	28:4	27:3	27:4	27:4	27:3
7 Muju	a	87:123	55:66	2:3	2:3	2:2	2:2		0:1	1:1	1:2	3:1	0:2	1:1	25:4	24:5	27:5	26:4	26:5	26:5	26:4
8	b	88:124	54:65	1:2	1:2	1:1	1:1	1:1		1:0	1:1	3:0	0:1	1:0	25:3	24:4	27:4	26:3	26:4	26:4	26:3
9	c	87:123	54:65	2:3	2:3	2:2	2:2	2:2	1:1		2:1	4:0	1:1	2:0	24:3	23:4	26:4	25:3	25:4	25:4	25:3
10 Haenam	a	88:125	55:67	2:4	2:4	2:3	2:3	2:3	1:2	2:3		2:1	1:2	2:1	26:4	25:5	28:5	27:4	27:5	27:5	27:4
11	b	87:123	56:67	2:3	2:3	2:2	2:2	4:4	3:3	4:4	2:3		3:1	2:0	26:3	25:4	28:4	27:3	27:4	27:4	27:3
12 Namhae	c	88:125	54:66	1:3	1:3	1:2	1:2	1:2	0:1	1:2	1:3	3:4		1:1	25:4	24:5	27:5	26:4	26:5	26:5	26:4
13 Tonghae	a	88:124	54:65	0:1	0:1	0:0	0:0	2:2	1:1	2:2	2:3	2:2	1:2		26:3	25:4	28:4	27:3	27:4	27:4	27:3
14	b	82:116	50:62	27:30	27:30	27:29	27:29	27:29	26:28	25:27	27:30	27:29	26:29	27:29		0:1	2:1	1:0	1:1	1:1	1:0
15 Samchok	a	82:116	50:62	27:30	27:30	27:29	27:29	27:29	26:28	25:27	27:30	27:29	26:29	27:29	0:0		2:2	1:1	1:2	1:2	1:1
16 Yongdok	a	82:118	51:65	28:33	28:33	28:32	28:32	28:32	27:31	26:30	28:33	28:32	27:32	28:32	1:3	1:3		1:1	3:2	3:2	3:1
17	c	82:116	51:63	28:31	28:31	28:30	28:30	28:30	27:29	26:28	28:31	28:30	27:30	28:30	1:1	1:1	0:2		2:1	2:1	2:0
18 Chongsong	a	83:118	51:64	28:32	28:32	28:31	28:31	28:31	27:30	26:29	28:32	28:31	27:31	28:31	1:2	1:2	2:5	2:3		2:0	0:1
19 Pohang	a	81:116	50:64	28:32	28:32	28:31	28:31	28:31	27:30	26:29	28:32	28:31	27:31	28:31	1:2	1:2	2:5	2:3	2:2		2:1
20	b	83:117	51:63	28:31	28:31	28:30	28:30	28:30	27:29	26:28	28:31	28:30	27:30	28:30	1:1	1:1	2:4	2:2	0:1	2:3	

Ratios of transition: transversion given above the diagonal and ratios of silent: replacement changes given below the diagonal. Individuals which have identical sequences were described as one representative.

Acknowledgements

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References

Anderson S, De Bruijn MHL, Couisen AR, Eperon IC, Sanger F, and Young IG (1982) Complete sequence of bovine mitochondrial DNA. *J Mol Biol* 156: 683-717.

Brown WM, Prager EM, Wang A, and Wilson AC (1982) Mitochondrial DNA sequences of primates: tempo and mode of evolution. *J Mol Evol* 18: 225-239.

Brown WM, George MJ, and Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. *Proc Natl Acad Sci USA* 76: 1976-1971.

Carey K and King DG (1996) Molecular divergence and phylogeny rates and patterns of cytochrome *b* evolution in cranes. *Mol Biol Evol* 13: 21-30.

Felsenstein J (1985) Confidence limits on phylogenetics: an analysis using the bootstrap. *Evolution* 39: 789-791.

Irwin DM, Kocher TD, and Wilson AC (1991) Evolution of the cytochrome *b* gene. *J Mol Biol* 203: 607-617.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16: 111-120.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, and Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc Natl Acard Sci USA* 86: 6196-6200

Kumar S, Tamura K, and Nei M (1993) MEGA, Molecular Evolution Genetics Analysis, Version 1.01 Pennsylvania State University, University Park.

Lansman RA, Shade RO, Shapira JF, and Avise JC (1981) The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. IV. Techniques and potential applications. *J Mol Evol* 17: 214-226.

Lee HY and Park CS (1991) Genetic studies on Korean anurans: on the mitochondrial DNA differentiation in frogs of the genus *Rana*. *Korean J Genet* 13: 1-16.

Table 5. Matrix of Kimura's two-parameter distance (above diagonal) and Tamura-Nei distance (below diagonal) between cyt *b* gene for 11 Korean populations, 1 Japanese *R. rugosa* and an outgroup (*X. laevis*)

OTUs		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 X. laevis			0.2950	0.3063	0.3063	0.3034	0.3034	0.3005	0.3034	0.3002	0.3063	0.3002	0.3063	0.3034	0.2783	0.2783	0.2842	0.2783	0.2842	0.2782	0.2814
R. rugosa																					
2 Japan		0.3010		0.1484	0.1484	0.1461	0.1461	0.1484	0.1461	0.1461	0.1510	0.1513	0.1484	0.1461	0.1381	0.1381	0.1456	0.1407	0.1430	0.1430	0.1407
3 Yangyang	a	0.3170	0.1496		0.0040	0.0020	0.0020	0.0060	0.0040	0.0060	0.0080	0.0060	0.0060	0.0020	0.0633	0.0633	0.0700	0.0655	0.0677	0.0677	0.0655
4	b	0.3168	0.1496	0.0040		0.0020	0.0020	0.0060	0.0040	0.0060	0.0080	0.0060	0.0060	0.0020	0.0633	0.0633	0.0700	0.0655	0.0677	0.0677	0.0655
5 Kapyong	a	0.3139	0.1473	0.0020	0.0020		0.0000	0.0040	0.0020	0.0040	0.0060	0.0040	0.0040	0.0000	0.0612	0.0612	0.0678	0.0634	0.0655	0.0655	0.0634
6 Nonsan	a	0.3139	0.1473	0.0020	0.0020	0.0000		0.0040	0.0020	0.0040	0.0060	0.0040	0.0040	0.0000	0.0612	0.0612	0.0678	0.0634	0.0655	0.0655	0.0634
7 Muju	a	0.3111	0.1496	0.0060	0.0060	0.0040	0.0040		0.0020	0.0040	0.0060	0.0080	0.0040	0.0040	0.0610	0.0610	0.0677	0.0633	0.0654	0.0654	0.0633
8	b	0.3139	0.1473	0.0040	0.0040	0.0020	0.0020	0.0020		0.0020	0.0040	0.0060	0.0020	0.0020	0.0589	0.0589	0.0655	0.0612	0.0633	0.0633	0.0612
9	c	0.3114	0.1473	0.0060	0.0060	0.0040	0.0040	0.0040	0.0020		0.0060	0.0080	0.0040	0.0040	0.0567	0.0567	0.0633	0.0589	0.0610	0.0610	0.0589
10 Haenam	a	0.3170	0.1524	0.0080	0.0080	0.0060	0.0060	0.0060	0.0040	0.0060		0.0060	0.0060	0.0060	0.0633	0.0633	0.0700	0.0655	0.0677	0.0677	0.0655
11	b	0.3102	0.1528	0.0060	0.0060	0.0040	0.0040	0.0080	0.0060	0.0081	0.0060		0.0080	0.0040	0.0612	0.0612	0.0678	0.0634	0.0655	0.0655	0.0634
12 Namhae	c	0.3168	0.1496	0.0060	0.0060	0.0040	0.0040	0.0040	0.0020	0.0040	0.0060	0.0080		0.0040	0.0610	0.0610	0.0677	0.0633	0.0654	0.0654	0.0633
13 Tonghae	a	0.3139	0.1473	0.0020	0.0020	0.0000	0.0000	0.0040	0.0020	0.0040	0.0060	0.0040	0.0040		0.0612	0.0612	0.0678	0.0634	0.0655	0.0655	0.0634
14	b	0.2836	0.1397	0.0634	0.0634	0.0612	0.0612	0.0611	0.0590	0.0568	0.0634	0.0612	0.0611	0.0612		0.0000	0.0060	0.0020	0.0040	0.0040	0.0020
15 Samchok	a	0.2836	0.1397	0.0634	0.0634	0.0612	0.0612	0.0611	0.0590	0.0568	0.0634	0.0612	0.0611	0.0612	0.0000		0.0060	0.0020	0.0040	0.0040	0.0020
16 Yongdok	a	0.2900	0.1476	0.0701	0.0701	0.0679	0.0679	0.0678	0.0657	0.0635	0.0702	0.0679	0.0678	0.0679	0.0060	0.0060		0.0040	0.0101	0.0101	0.0080
17	c	0.2836	0.1425	0.0656	0.0656	0.0635	0.0635	0.0634	0.0613	0.0591	0.0657	0.0635	0.0634	0.0635	0.0020	0.0020	0.0040		0.0060	0.0060	0.0040
18 Chongsong	a	0.2899	0.1448	0.0678	0.0678	0.0656	0.0656	0.0656	0.0634	0.0612	0.0678	0.0656	0.0655	0.0656	0.0040	0.0040	0.0101	0.0060		0.0040	0.0020
19 Pohang	a	0.2831	0.1448	0.0678	0.0678	0.0656	0.0656	0.0656	0.0634	0.0612	0.0678	0.0656	0.0655	0.0656	0.0040	0.0040	0.0101	0.0060	0.0040		0.0060
20	b	0.2870	0.1425	0.0656	0.0656	0.0635	0.0635	0.0634	0.0613	0.0591	0.0657	0.0635	0.0634	0.0635	0.0020	0.0020	0.0081	0.0040	0.0020	0.0060	

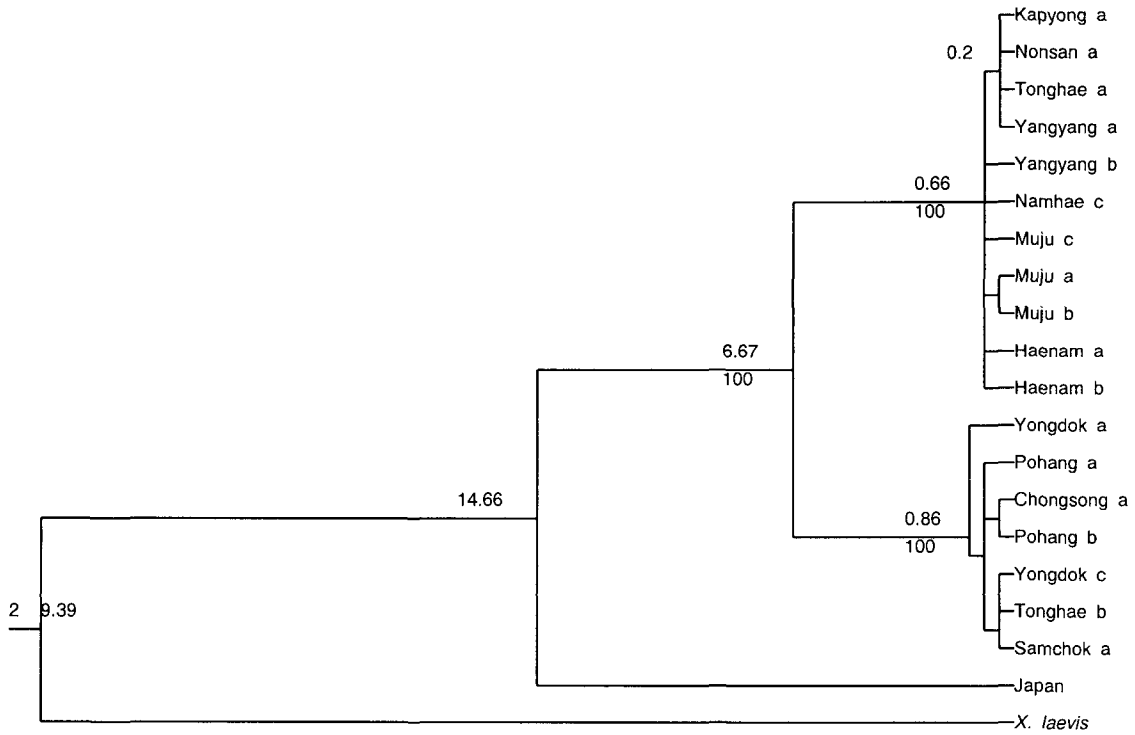

Fig. 4. A UPGMA dendrogram. Numbers indicate percent sequence divergence by Kimura's two-parameter distance. Numbers below the nodes indicate bootstrap values for 1,000 replicates.

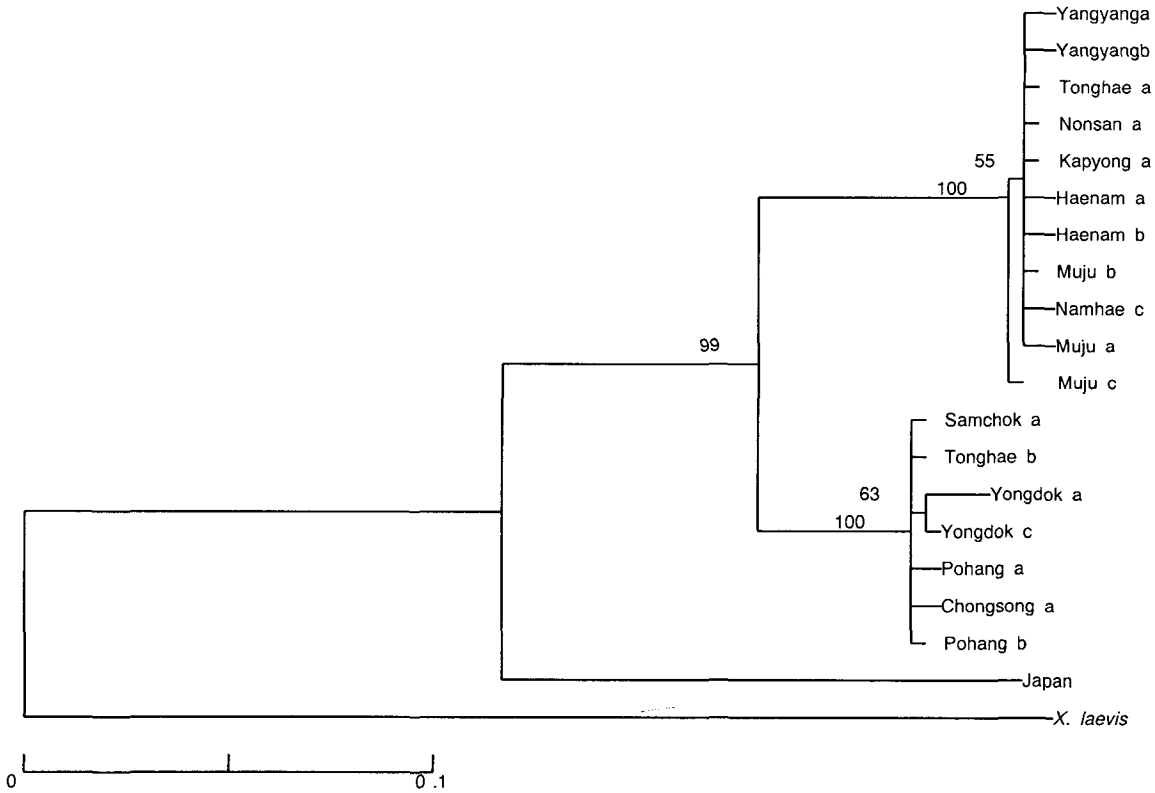

Fig. 5. A neighbor-joining tree based on Kimura's two parameter distance. Numbers above the nodes indicate bootstrap values for 1,000 replicates.

Lee HY, Park CS, Jung EK, and Kim IS (1992) The study on the macromitochondrial DNA using restriction enzymes in Korean anuran, *Rana rugosa*. *Korean J Genet* 14: 303-314.

Lee HY, Oh SJ, Yang DE, Kim YR, and Yang SY (1997) Genetic differentiation between two species in the genus *Rana* of Korea. *Korean J Genet* 19: 317-324.

Meyer A (1994) Shortcomings of the cytochrome *b* gene as a molecular marker. *Trends Ecol Evol* 9: 278-280.

Meyran JC, Monnerot-M, and Taberlet P (1997) Taxonomic status and phylogenetic relationships of some species of the genus *Gammarus* (Crustacea, Amphipoda) deduced from mitochondrial DNA sequences. *Mol Phylogenet Evol* 8: 1-10.

Park CS (1990) Genetic Studies on the Korean Anurans (Amphibia, Anura): Chromosome and Mitochondrial DNA Analysis. Ph. D. Thesis, Inha University, Korea, pp 1-117.

Roe BA, Ma D, Wilson RK, and Wong JFH (1985) The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. *J Biol Chem* 260: 9759-9774.

Saiki RK, Delfand DH, Stooffel S, Scharf SJ, Highchi R, Horn GT, Mullis KB, and Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.

Saitou N and Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4; 406-425.

Tamura K and Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 11: 154-157.

Tan AM and Wake DB (1995) MtDNA phylogeography of the California newt, *Taricha torosa* (Caudata, Salamandridae). *Mol Phylogenet Evol* 4: 383-394.

Tanaka T, Matsui M, and Takenaka O (1994) Estimation of phylogenetic relationships among Japanese brown frogs from mitochondrial cytochrome *b* gene (Amphibia: Anura). *Zool Sci* 11: 753-757.

Tanaka T, Matsui M, and Takenaka O (1996) Phylogenetic relationships of Japanese brown frogs (*Rana*: Ranidae) assessed by mitochondrial cytochrome *b* gene sequences. *Biochem Syst Ecol* 24: 299-307.

Tanaka-Ueno T, Matsui M, Chen SL, Takenaka O, and Ota H (1998a) Phylogenetic relationships of brown frog from Taiwan and Japan assessed by mitochondrial cytochrome *b* gene sequences (*Rana*: Ranidae). *Zool Sci* 15: 283-288.

Tanaka-Ueno T, Matsui M, Sato T, Takenata S, and Takenaka O (1998b) Phylogenetic relationship of brown frogs with 24 chromosomes from Far East Russia and Hokkaido assessed by mitochondrial cytochrome *b* gene sequences (*Rana*: Ranidae). *Zool Sci* 15: 289-294.

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