

# Genetic Relationships of *Rana amurensis* Based on Mitochondrial Cytochrome *b* Gene Sequences

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**Key Words:**

*Rana amurensis*  
Brown frog  
Genetic relationship  
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Inter- and intraspecific genetic relationships between *Rana amurensis* from Korea and Russia and other brown frogs were investigated by nucleotide sequence of a 504 base pair (bp) fragment of the mitochondrial cytochrome *b* gene. Nucleotide sequence similarities among Korean populations of *R. amurensis* ranged from 99.6% to 97.6% and 98.8% within Russian populations. The nucleotide sequence similarity between Korean and Russian *R. amurensis* ranged from 86.9% to 85.5%. Based on Kimura-2-parameter distance, the sequence divergence between *R. amurensis* from Korea and Russia was 16.18% and 18.04% among other related brown frogs. Interspecific sequence divergences among *R. amurensis* and other related brown frogs diverged by 20.3%. Using an estimate of 2-4% mitochondrial DNA sequence divergence per million years, Korean and Russian *R. amurensis* diverged about 8 to 4 million years ago (Mya) and other brown frogs diverged about 9 to 5 Mya from ancestral frogs and distributed from North Asia to Sakhalin in a short time. In the neighbor-joining and UPGMA tree, *R. amurensis* was clustered into two groups with Korean and Russian populations, and the other brown frogs were grouped separately with diverged trichotomous clusters (*R. dybowskii* and *R. pirica*, *R. okinavana* and *R. tsushimensis*, and *R. japonica* and *R. longicrus*).

Recently, the mitochondrial DNA is more frequently used than the nuclear DNA in phylogenetic and population genetic study. The mitochondrial DNA which is maternally inherited consists of small sized circular duplex (15.0-20.0 kb) and has been evolved much more rapidly than single-copy nuclear DNA (Brown et al., 1979; Bermingham et al., 1986). Particularly, the mitochondrial cytochrome *b* gene has been used as a popular source for studying inter- and intraspecific genetic differentiation (Meyer, 1994; Randi, 1996).

Brown frogs are morphologically so similar that it is difficult to prove specific phylogenetic relationships. Many researchers have attempted to clarify molecular evolutionary relationships using genetic variations of the mitochondrial DNA and chromosome analysis. Korean brown frogs have been identified into 2 species, *R. amurensis* (2N=26) and *R. dybowskii* (2N=24). The phylogenetic relationship for *R. dybowskii* has been studied widely using isozyme and the mitochondrial cytochrome *b* gene data (Lee and Kim, 1993; Tanaka et al., 1994, 1996; Matsui et al., 1998; Tanaka-Ueno et al., 1998a, b, c; Kim et al., 1999), but *R. amurensis* has been studied by isozyme (unpublished) and RFLP

analysis (Lee and Kim, 1993).

*R. amurensis* inhabits in western Siberia east of Sakhalin, northern and eastern Mongolia, northeastern China and Korea, but not in Japan. Phylogenetic relationship of *R. amurensis* from Sakhalin and the Maritime Territory was analyzed by the mitochondrial cytochrome *b* gene (Tanaka-Ueno et al., 1998c). First, we intended to compare inter- and intraspecific genetic relationships between Korean and Russian *R. amurensis* (published data) and second, to evaluate phylogenetic relationships between *R. amurensis* and other related brown frogs with 24 chromosomes based on the published cytochrome *b* gene sequence.

## Materials and Methods

The genomic DNA was extracted from frozen liver, heart, or stomach of 14 specimens of Korean *R. amurensis* and

Table 1. Species, localities and collection date of specimens

Species	Localities	Collection date
<i>Rana amurensis</i>	Yangpyong-gun, Kyonggi-do	June 1997
	Kangrung-shi, Kangwon-do	Sep. 1997
	Nonsan-shi, Chungchongman-do	April 1997
	Chongju-shi, Chungchongbuk-do	March 1997
	Wonju-gun, Chollabuk-do	Oct. 1997
	Kyongju-shi, Kyongsangbuk-do	June 1997
	Koje-island, Kyongsangnam-do	Oct. 1997
<i>R. catesbeiana</i>	Puan-gun, Chollabuk-do	May 1998

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	1	89
<i>Xenopus laevis</i>	CAAATCATTACAGGATTATTCTTAGCTATACAITATACAGCAGACACATCTATAGCCCTTCTCATCAGTAGCCCATATTTGCCGTGACGT	
<i>Rana catesbeiana</i>	.....G.C.T.C.....C.....C.....C.....T.....T.CC.T.A.....TA.T.T.C.C.....T..	
<i>R. amurensis</i>		
Yangpyoung	.....GCC.C.C.....C.....C.....T.T.T.T.....A.T.....TA.T.T.C.C.T.A.T..	
Kangrung	.....GCC.C.C.....C.....C.....T.T.T.T.....G.T.....TA.T.T.C.C.T.A.T..	
Nonsan	.....GCC.C.C.....C.....C.....T.T.T.T.....A.T.....TA.T.T.C.C.T.A.T..	
Chungju	.....GCC.C.C.....C.....C.....T.T.T.T.....A.T.....TA.T.T.C.C.TG.A.T..	
Wanju	.....GCC.C.C.....C.....C.....T.T.T.T.....A.T.....TA.T.T.C.C.T.A.T..	
Kyongju	.....GCC.C.C.....C.....C.....C.....T.T.T.T.....G.T.....TA.T.T.C.C.T.A.T..	
Koje	.....GCC.C.C.....C.....C.....C.....T.T.T.T.....G.T.....TA.T.T.C.C.T.A.T..	
Anlva (Russia)	.....TGC.T.C.....C.....C.....G.T.T.T.C.G.A.....TA.C.A.C.C.....A.T..	
Lazo (Russia)	.....TGC.T.C.....C.....C.....G.T.T.T.C.G.A.....TA.C.A.C.C.....A.T..	
<i>R. japonica</i> (Japan)	.....G.GCC.C.C.....TC.G.C.....C.C.....T.T.T.CC.....A.T.....TA.C.....C.C.T..	
<i>R. okinavana</i> (Japan)	.....GCC.C.C.....C.T.C.....C.C.G.T.T.T.CC.....A.T.T.TA.T.....C.C.....C.T..	
<i>R. longicrus</i> (Taiwan)	.....GCC.C.GC.....C.G.C.....C.C.....T.T.T.....A.T.T.CA.C.....C.C.....C.T..	
<i>R. tsushimensis</i> (Japan)	.....GCC.C.CC.....C.G.C.....C.....G.T.....C.CC.....A.T.C.TA.C.....C.C.....T..	
<i>R. pirica</i> (Japan)	.....GCC.C.GC.....TC.....C.....G.T.T.C.....G.T.....CA.C.....C.C.T.A.T..	
<i>R. dybowskii</i> (Korea)	.....TGCC.T.CC.....TC.....C.G.C.C.....T.....T.CC.....G.T.....TA.C.T.C.C.C.A.....	
	90	178
<i>X. laevis</i>	TAACTATGGATGATTAATTGCGCAATCTCCATGCCAATGGAGCCCTCATTCTTCTTCATTGTCATCTACCTTCACATCGGACGAGGGTTGT	
<i>R. catesbeiana</i>	...TA.C.C.C.CC.A.A.....C.....A.....T.T.C.T.T.TT.....C.....C.C.	
<i>R. amurensis</i>		
Yangpyoung	C.TA.....C.TC.....C.T.C.A.C.C.A.....T.T.C.....T.C.T.T.....G.TC.T.	
Kangrung	C.TA.....C.TC.....C.T.C.A.C.C.A.....T.T.C.....T.C.T.T.....G.TC.T.	
Nonsan	C.TA.....C.TC.....C.T.C.A.C.C.A.....T.T.C.....T.C.T.T.....G.TC.T.	
Chungju	C.TA.....C.TC.....C.T.C.A.C.C.A.....G.T.T.C.....T.C.T.T.....G.TC.T.	
Wanju	C.TA.....C.TC.....C.T.C.A.C.C.A.....G.T.T.C.....T.C.T.T.....G.TC.T.	
Kyongju	C.TA.....C.TC.....C.T.C.A.C.C.A.....T.T.C.....T.C.T.T.....G.TC.T.	
Koje	C.TA.....C.TC.....C.T.C.A.C.C.A.....T.T.C.....T.C.T.T.....G.TC.T.	
Anlva (Russia)	...TA.C.....GC.CC.A.....T.C.....C.C.A.....T.....C.T.T.T.....T.T.G.....TC.T.	
Lazo (Russia)	...TA.C.....GC.CC.A.....T.C.....C.C.A.....T.....C.T.T.T.....T.T.G.....TC.T.	
<i>R. japonica</i> (Japan)	C.A.C.C.C.CC.....T.....C.....C.C.....T.....C.....TT.C.....T.G.....CC.T.	
<i>R. okinavana</i> (Japan)	A.A.C.....GC.CC.C.....C.T.C.....C.C.A.....C.....T.....T.....T.....TC.T.	
<i>R. longicrus</i> (Taiwan)	C.A.C.C.C.CC.C.C.T.C.....C.....C.C.A.....C.....T.C.....C.....CC.C.	
<i>R. tsushimensis</i> (Japan)	...A.C.C.C.CC.C.....C.....C.C.A.....T.T.C.T.....G.CC.T.	
<i>R. pirica</i> (Japan)	A.TA.C.C.C.C.TC.C.....C.....C.C.A.....C.....TT.C.....G.G.CC.T.	
<i>R. dybowskii</i> (Korea)	A.A.A.....C.C.TC.....C.T.C.....C.A.....C.....T.C.T.....G.TC.T.	
	179	267
<i>X. laevis</i>	ACTACGGCTCTTTCTTATATAAAGAACATGAAATATGGGTGTGATTCTTCTATTTTTAGTTATAGCTACAGCATTGTAGGATATGTT	
<i>R. catesbeiana</i>	.....C.A.C.T.....C.....A.....A.G.....A.....T.....T.C.....	
<i>R. amurensis</i>		
Yangpyoung	.T.T.....C.A.C.T.C.....G.....C.....A.T.....AT.....C.C.G.....C.....C.C.C.G.C...	
Kangrung	.T.T.....C.A.C.T.C.....G.....C.....A.T.....GT.....C.C.G.....GC.....C.C.C.G.C...	
Nonsan	.T.T.....C.A.C.T.C.....G.....C.....A.T.....AT.....C.C.G.....C.....C.C.C.G.C...	
Chungju	.T.T.....C.A.C.T.C.....G.....C.....A.T.....AT.....C.C.G.....C.....C.C.C.G.C...	
Wanju	.T.T.....C.A.C.T.C.....G.....C.G.A.T.....AT.....C.C.G.....C.....C.C.C.G.C...	
Kyongju	.T.T.....C.A.C.T.C.....G.....C.....A.T.....AT.....C.C.G.....TT.C.....C.C.C.G.C...	
Koje	.T.T.....C.A.C.T.C.....G.....C.....A.T.....AT.....CC.C.G.....C.....C.C.C.G.C.A	
Anlva (Russia)	.T.T.....C.A.C.C.....G.....C.....A.T.C.T.....A.....A.....C.....C.C.....C.C.A	
Lazo (Russia)	.T.T.....C.A.C.C.....G.....C.....T.C.T.....A.....A.....C.....C.C.....C.C.A	
<i>R. japonica</i> (Japan)	.T.....A.A.C.C.C.....G.....C.C.A.A.C.C.G.C.....A.....C.....T.....C.....C	
<i>R. okinavana</i> (Japan)	.T.....C.A.C.C.....C.C.A.C.C.C.C.CC.....A.....C.G.T.C.G.T.C.C	
<i>R. longicrus</i> (Taiwan)	.T.....A.A.C.T.C.....C.C.C.AG.A.GT.G.CC.G.A.....C.....T.....G.C.C.C	
<i>R. tsushimensis</i> (Japan)	.....C.A.C.C.....G.G.C.C.G.T.C.C.C.C.....A.G.C.....C.....G.C.C.C	
<i>R. pirica</i> (Japan)	.T.T.....A.C.T.....G.G.C.....A.A.C.C.G.C.....G.....C.T.....C.....	
<i>R. dybowskii</i> (Korea)	.T.T.....C.A.C.C.....G.G.....C.....G.....C.C.G.....A.....T.....C.....	

Fig. 1. Nucleotide sequence of 504 bp fragment of mitochondrial cytochrome *b* gene from 7 populations of Korean and 2 published Russian *Rana amurensis*, published brown frogs and two outgroups of *Rana catesbeiana* and *Xenopus laevis*. Dots indicate identity to the sequence of *X. laevis*.

Fig. 1. Continued

	268	356
<i>X. laevis</i>	CTACCATGAGGACAAATATCTTTTTGAGGGGCTACAGTAATTACTAATCTTCTTTCTGCTAATCCGTACATCGGAAACGTACTAGTCCA	
<i>R. catesbeiana</i>	.....G. C.....A. C.....C. C.....C.....C. A. CGCC. A.....TTCT. AC. T. T..	
<i>R. amurensis</i>	..C.....T.....A.....G. C. C.....C.....A. CGCC. C.....CTCTAAC. T.....	
Yangpyoung	..C.....T.....A.....G. C. C.....C.....A. CGCC. C.....CTCTAAC. C.....	
Kangrung	..C.....T.....A.....G. C. C.....C.....GG. A. CGCC. C.....CTCTAAC. T.....	
Nonsan	..C.....T.....A.....G. C. C.....C.....A. CGCC. C.....CTCTAAC. T.....	
Chungju	..C.....T.....A.....G. C. C.....C.....A. CGCC. C.....CTCTAAC. T.....	
Wanju	..C.....T.....A.....G. C. C.....C.....A. CGCC. C.....CTCTAAC. T.....	
Kyongju	..C.....T.....A.....G. C. C.....C.....A. CGCC. C.....CTCTAAC. T.....	
Koje	..C.....T.....A.....G. C. C.....C.....A. CGCC. C.....CTCTAAC. T.....	
Anlva (Russia)	..G.....C.....A.....C. C.....C. C.....C. C. G. CGCC. C.....CTCT. AC. C.....	
Lazo (Russia)	..G.....C.....A.....G. C. C.....C. C.....C. C. A. CGCC. C.....CTCT. AC. C.....	
<i>R. japonica</i> (Japan)	T.....C.....G. C. C.....C.....C. C. C. C. A. CGCC. C. T.....CTC. AC. C. T..	
<i>R. okinavana</i> (Japan)	..G. C.....C.....G. A.....T.....T. C. C. C. C.....G. AGCC. C. G.....TC. AC. C.....	
<i>R. longicrus</i> (Taiwan)	..G.....C.....C.....C. C.....T. C.....C. C. A. GCC. C.....CTC. ACT.....	
<i>R. tsushimensis</i> (Japan)	..G.....G. C.....A. C.....C. C.....C. C.....C. A. CGCC. C.....CC. AC. T.....	
<i>R. pirica</i> (Japan)	T.....G.....C. G.....C. C.....C. C.....C. C. A. CGCC. C.....CTTT. ACT. G.....	
<i>R. dybowskii</i> (Korea)	T.....C.....G.....C.....C.....C.....C. C. C. A. GC. C.....CTTT. ACT.....	
	357	445
<i>X. laevis</i>	ATGAATTTGAGGAGGTTTCTCTGTAGATAACGCCACTTTAACCAGATTCTCGCAITTCACITTCCTCCTTCTCTTTATTATTGCGGGAG	
<i>R. catesbeiana</i>	.....G.....T. A.....C.....CC. C.....TA. T.....A.....C. G.....C. A. C..	
<i>R. amurensis</i>	..G. C.....C.....A.....C.....T. CC. C.....T. TA.....A. T. C. A.....A.....CT.	
Yangpyoung	..G. C.....C.....A.....C.....T. CC. C.....T. TA.....GA. T. C. A.....A.....CT.	
Kangrung	..G. C.....C.....A.....C.....T. CC. C.....T. TA.....G.....A. T. C. A.....A.....CT.	
Nonsan	..G. C.....C.....A.....C.....T. CC. C.....T. TA.....G.....GA. T. C. A.....A.....CT.	
Chungju	..G. C.....C.....A.....C.....T. CC. C.....T. TA.....G.....A. T. C. A.....A.....CT.	
Wanju	..G. C.....C.....A.....C.....T. CC. C.....T. TA.....G.....A. T. C. A.....A.....CT.	
Kyongju	..G. C.....C.....A.....C.....T. CC. C.....T. TA.....A. T. C. A.....A.....CT.	
Koje	..G. C.....C.....A.....C.....T. CC. C.....T. TA.....A. T. C. A.....A.....CT.	
Anlva (Russia)	.....C. G. G.....A.....C.....CC. C. T. G.....TA.....A.....C. A.....C. C.....CC.	
Lazo (Russia)	.....C. G. G.....A.....C.....CC. C. T. G.....TA.....A.....C. A.....C. C.....CC.	
<i>R. japonica</i> (Japan)	.....C.....C. T. A.....C.....A. CC. C.....TA. T. C.....A.....C. A.....C.....CTA	
<i>R. okinavana</i> (Japan)	.....C.....C. C.....G.....C.....T. CC. C. T. G. T. TA.....C.....A. T. C. C. C. C.....T. CG.	
<i>R. longicrus</i> (Taiwan)	..G. C.....C.....A.....C. G. A. CC. T. T.....A.....C. T. A. T. C. A. C.....T. CTA	
<i>R. tsushimensis</i> (Japan)	..G. C.....G. C.....G. G. C.....A. CC. G.....T. TA. G. C.....A.....C. C.....G. C. C. T. CTA	
<i>R. pirica</i> (Japan)	.....C. G.....C. G.....C.....T. CC. C.....T. TA.....C.....A.....C. G. C. C. C. A. CTA	
<i>R. dybowskii</i> (Korea)	.....C.....C. C.....G.....C. T. T. CC. C.....G.....TA.....C.....A.....C. G. C. C.....C. A. CTC	
	446	504
<i>X. laevis</i>	CTAGCAATTCCTCCATCTTTTATTCTCCACGAAACTGGATCAACAAACCCCACTGGATTA	
<i>R. catesbeiana</i>	.A. T. GA. ....C. GC. T. C.....TC.....C.....T. C.....A. CC. C	
<i>R. amurensis</i>	T...TC. AA. T. C. CC.....T. A. TC.....A.....T. T.....C. A.....C. T	
Yangpyoung	T...TC. AA. T. C. CC.....T. A. TC.....A.....T. T.....C. A.....C. T	
Kangrung	T...TG. AA. T. C. CC.....T. A. TC.....A.....T. T.....C. A.....C. T	
Nonsan	T...TC. AA. T. C. CC.....T. A. TC.....A.....T. T.....C. A.....C. T	
Chungju	T...TC. AA. T. C. CC.....T. A. TC.....A.....T. T.....C. A.....C. T	
Wanju	T...TC. AA. T. C. CC.....T. A. TC.....A.....T. T.....C. A.....C. T	
Kyongju	T...TC. AA. T. C. CC.....T. A. TC.....A.....T. T.....C. A.....C. T	
Koje	T...TC. AA. T. C. CC.....T. A. TC.....A.....T. T.....C. A.....C. T	
Anlva (Russia)	T...C. AA. T.....C.....T. A. TC.....A.....T. T.....C. G.....C. C	
Lazo (Russia)	T...C. AA. T.....C.....T. A. TC.....A.....T. T.....C. G.....C. C	
<i>R. japonica</i> (Japan)	TA.....AA. T. C. CC.....C. G. TC.....G.....CT. C.....A.....C. T	
<i>R. okinavana</i> (Japan)	.A.....GA.....C.....C.....C. G. TC.....G.....T. C.....C. A.....C. T	
<i>R. longicrus</i> (Taiwan)	TA.....AA.....C.....C.....T.....C.....A. G. CT.....C. A. GC. T	
<i>R. tsushimensis</i> (Japan)	TA.....GA.....C. CC.....C. G. TC.....G.....CT. C.....C. A. TC. C	
<i>R. pirica</i> (Japan)	TA. T. AA. T. C. C. G.....T.....C.....A. C.....T. C. T. C. A.....C. C	
<i>R. dybowskii</i> (Korea)	TA. T. AA. T. C. C. C.....C.....C.....A. C.....T. C.....C. A.....C. C	

one from *R. catesbeiana* for the outgroup taxon (Table 1). DNA extraction, gene amplification and sequencing were used as described previously (Lee et al., 1997; Kim et al., 1999; Lee et al., 1999).

A 560 bp fragment of the mitochondrial cytochrome *b* gene was amplified using polymerase chain reaction (PCR). Primers used for amplification and sequencing were CB1 (5'-CCATCCAACATCTCAGCATGATGAAA-3', Kocher et al., 1989) and 560A (5'-GTCTTTAGGA

GAAATATGG-3', Tanaka et al., 1996).

Statistic data analysis was carried out by MEGA software (Kumar et al., 1993). Genetic distance was performed from the Kimura-2-parameter model (Kimura, 1980) and gene tree was constructed using neighbor-joining (Saitou and Nei, 1987) and UPGMA (Sneath and Sokal, 1973) methods. Using MEGA, UPGMA and neighbor-joining phenogram with 500 replications of bootstrapping test were obtained for the statistical signifi

	1		89
<i>Xenopus laevis</i>	QIIITGLFLAMHYTADTSMFSSVAHICRDVNYGWLIRNLHANGASFFFICIVLHIGRGLYGSFLYKETWNIQVILLFLVMATAFVGYV		
<i>Rana catesbeiana</i>	..V.....L...I.....N...L.....F.....Y.....		
<i>R. amurensis</i>			
Yangpyoung	..A.....L...I.....N...L.....F.....Y.....L.....		
Kangrung	..A.....L...I.....N...L.....F.....Y.....L.G.....		
Nonsan	..A.....L...I.....N...L.....F.....Y.....L.....		
Chungju	..A.....L...I...G...N...L.....V...F.....Y.....L.....		
Wanju	..A.....L...I.....N...L.....V...F.....Y.....S.....L.....		
Kyongju	..A.....L...I.....N...L.....F.....Y.....L.....		
Koje	..A.....S...L...I.....N...L.....F.....Y.....L.....		
Anlva (Russia)	..A.....L...I.....N...L.....F.....Y.....L.....		
Lazo (Russia)	..A.....L...I.....N...L.....F.....Y.....L.....		
<i>R. japonica</i> (Japan)	..A.....L...I.....N...L.....F.....Y.....L.....		
<i>R. okinavana</i> (Japan)	..A.....L...I.....N...L.....F.....Y.....L.....		
<i>R. longicrus</i> (Taiwan)	..A.....L...I.....N...L.....F.....Y.....V.....		
<i>R. tsushimensis</i> (Japan)	..A.....L...I.....N...L.....FF.....Y.....L.....		
<i>R. pirica</i> (Japan)	..A.....L...I.....N...L.....F.....Y.....L.....		
<i>R. dybowskii</i> (Korea)	..A.....L...I...S...N...L.....Y.....		
	90		168
<i>X. laevis</i>	LPWQMFSFWGATVITNLLSAIPYIGNVLVQWIIWGGFSDNATLTRFFAFHLLPFIAGASILHLLFLHETGSTNPTGL		
<i>R. catesbeiana</i>	.....A...SD.....T...I...A...MI...Q...S.....		
<i>R. amurensis</i>			
Yangpyoung	.....A...SN.....T...I...TAV...LI...Q...S.....		
Kangrung	.....A...T...SN.....T...LI...TAV...LI...Q...S.....		
Nonsan	.....A...A...SN.....T...D...I...TAV...VI...Q...S.....		
Chungju	.....A...SN.....T...DLI...TAV...LI...Q...S.....		
Wanju	.....A...SN.....T...D...I...TAV...LI...Q...S.....		
Kyongju	.....A...SN.....T...I...TAV...LI...Q...S.....		
Koje	.....A...SN.....T...I...TAV...LI...Q...S.....		
Anlva (Russia)	.....A...SD.....T...I...AV...LI...Q...S.....		
Lazo (Russia)	.....A...SD.....T...I...AV...LI...Q...S.....		
<i>R. japonica</i> (Japan)	.....A...SD.....T...I...AM...MI...Q...S.....		
<i>R. okinavana</i> (Japan)	.....A...V...HD.....T...I...A...MI...Q...S.....		
<i>R. longicrus</i> (Taiwan)	.....A...SD.....K.....T...I...AM...MI...Q...S.....		
<i>R. tsushimensis</i> (Japan)	.....A...HD.....T...I...V...AM...MI...Q...S.....		
<i>R. pirica</i> (Japan)	.....A...FD.....A.....T...I...AM...MI...Q...S.....		
<i>R. dybowskii</i> (Korea)	.....A...FD.....T...I...AL...MI...Q...S.....		

Fig. 2. Amino acid sequence of 168 fragment of the mitochondrial cytochrome *b* gene from 7 populations of Korean and 2 published Russian *R. amurensis*, published brown frogs and two outgroups of *Rana catesbeiana* and *Xenopus laevis*. Dots indicate identity to the sequence of *X. laevis*.

cance of internal nodes (Felsenstein, 1985).

For phylogenetic relationships among brown frogs, the published sequences of 5 species of Japanese and Taiwanese brown frogs (*R. pirica*, *R. japonica*, *R. okinavana*, *R. longicrus* and *R. tsushimensis* complex), two specimens of *R. amurensis* from Russia and one species of *R. dybowskii* from Korea were used (Tanaka-Ueno et al., 1998 a,c; Kim et al., 1999). Also the published complete sequence of *Xenopus laevis* was used for another outgroup (Roe et al., 1985). The rate of mitochondrial DNA evolution was calculated by 2-4% nucleotide sequence divergence per million year (Brown et al., 1979; Brown, 1985).

**Results**

Genetic relationships among 7 populations of Korean *R. amurensis* were compared with published data of Russian *R. amurensis* and other related brown frogs of the mitochondrial cytochrome *b* gene sequence of 504 bp (Fig. 1).

The mean values of the nucleotide compositions were adenine (24.0%), thymine (31.0%), cytosine (29.0%)

and guanine (16.1%). In the nucleotide composition of each codon, guanine of the third codon position was the least in quantity (5.7%), which is similar to other vertebrates (Table 2) (Kocher et al., 1989). Neither deletion nor addition was found within all species. The 168 amino acid encoded for the 504 nucleotide sequence were analyzed (Fig. 2). Transition was 3.9 times higher than the transversion between Korean and Russian *R. amurensis*.

Interpopulational nucleotide sequence similarity of Korean *R. amurensis* ranged from 99.6% to 97.6% and 86.9% to 85.5% between Korean and Russian *R. amurensis* (Table 3). The 13 amino acid differences (7.7%)

Table 2. Average nucleotide composition of *Rana amurensis*

Position	Nucleotides (%)			
	A	T	C	G
First	24.7	27.1	22.5	25.7
Second	19.6	38.8	24.8	16.7
Third	27.6	27.0	39.6	5.7
Average	24.0	31.0	29.0	16.1

occurred between Korean and Russian *R. amurensis* (Fig. 2). Based on Kimura-2-parameter distance, inter-populational genetic distance of Korean *R. amurensis* ranged from 0.0080 to 0.0242, whereas the distance between Korean and Russian *R. amurensis* ranged from 0.1484 to 0.1667 (Table 3). For the phylogenetic analysis, our data and the published data of *R. amurensis* from Russia and other related brown frogs from Korea, Japan, and Taiwan were combined (Tanaka-Ueno et al., 1998a, c).

In the neighbor-joining and UPGMA tree (Fig. 3), as outgroup taxa, *X. laevis* and *R. catesbeiana* were completely separated from the brown frogs. *R. amurensis* from Korea and Russia were separated into two groups with 84% bootstrap iterations. The other brown frogs were grouped separately with diverged trichotomous clusters (*R. dybowskii* and *R. pirica*, *R. okinavana* and *R. tsushimensis*, and *R. japonica* and *R. longicrus*) and sequence divergences between them was 16.18%. Sequence divergence between *R. amurensis* and other related brown frogs was 20.03% and 18.04% among other related brown frogs.

Although the UPGMA tree was similar to the clustering pattern of the neighbor-joining tree, the nodal values of bootstrap iteration were respectively low. In the neighbor-joining tree, *R. amurensis* group and other brown frog groups were supported by high bootstrap iteration value (98%), but very low iteration (40%) in the UPGMA tree (Fig. 3B).

**Discussion**

The values of interpopulational sequence similarities within Korean *R. amurensis* (97.6-99.6%) and Russian *R. amurensis* (98.8%) are the same as other amphibian species (Tanaka et al., 1994, 1996; Lee et al., 1997, 1998; Matsui et al., 1998; Tanaka-Ueno et al., 1998a, b, c; Lee et al., 1999; Kim et al., 1999). Genetic distance of Korean populations of *R. amurensis* ranged from 0.0080 to 0.0242 and 0.0120 within Russian *R.*

*amurensis* (Table 3). Genetic differentiation from the analyses of allozymes and proteins among populations of *R. amurensis* from Russia, Mongolia, and China ranged from 0.005 to 0.008 (Nishioka et al., 1992).

Although interpopulational sequence similarities within Korean populations and within Russian *R. amurensis* were 97.6% to 99.6%, the similarity value between Korean and Russian *R. amurensis* ranged from 86.9% to 85.5%. The value of the genetic distance between *R. amurensis* from Korea and Russia ranged from 0.1484 to 0.1667. The similarity and genetic distance values of *R. dybowskii* from Korea and Russia were similar to those of *R. amurensis* from Korea and Russia. Matsui et al (1998) suggested that *R. dybowskii* of Russia differs genetically from Korean *R. dybowskii*, which is similar to the Tsushima population.

The sequence divergence of Japanese brown frogs was 14.24% (Tanaka, et al., 1994). In our data, genetic divergence among other related brown frogs was 18.04%. The sequence divergence of the cytochrome *b* gene between *Hyla japonica* and *H. suweonensis* was 12.9% (unpublished) and 4.0-6.8% between *R. nigromaculata* and *R. plancyi* (Lee et al., 1997). On the other hand, two subspecies of *Taricha torosa* showed 7.0% to 9.0% sequence divergence (Tan and Wake, 1995).

The evolutionary dynamics of the cytochrome *b* gene is better characterized with sister species, congeners and confamilial genera than most other molecular systems (Johns and Avise, 1998). The rate of mitochondrial DNA evolution was generally estimated to be 2-4% nucleotide sequence divergence per million year (Brown et al., 1979; Brown, 1985). This calculation was widely applied in studies of genetic relationships using mitochondrial RFLP and the cytochrome *b* gene (Brown et al., 1979; Willson et al., 1985; Irwin et al., 1991; Sumida et al., 1998). Based on this calculation, Korean and Russian *R. amurensis* were diverged about 8 to 4 million years ago (Mya), while other related frogs diverged about 9 to 5 Mya from ancestral frogs

**Table 3.** Matrix of percent similarities of nucleotide sequence (above diagonal) and Kimura-2-parameter distance (below diagonal) among cytochrome *b* gene sequences of brown frogs.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 <i>Xenopus laevis</i>		78.4	74.6	74.4	74.0	73.8	74.2	74.4	74.0	75.6	75.0	74.4	73.2	74.0	73.6	73.8	75.8
2 <i>Rana catesbeiana</i>	.2573		81.9	80.6	81.5	81.2	81.3	81.5	81.2	83.1	83.5	84.3	81.3	80.6	83.7	82.5	83.3
<i>R. amurensis</i>																	
3 Yangpyoung	.3164	.2115		98.4	99.2	99.2	99.0	99.6	98.8	86.5	86.9	82.9	82.7	82.9	82.5	84.3	83.5
4 Kangrung	.3189	.2282	.0161		97.6	98.0	97.6	98.4	98.0	85.5	85.9	82.7	82.1	82.3	81.2	83.9	83.1
5 Nonsan	.3256	.2166	.0080	.0242		98.8	98.6	98.8	98.0	85.9	86.3	82.5	82.1	82.5	82.1	83.9	83.1
6 Chungju	.3281	.2217	.0080	.0201	.0120		99.0	98.8	98.0	85.7	86.1	82.1	81.9	82.1	81.7	83.5	82.7
7 Wanju	.3215	.2188	.0100	.0242	.0140	.0100		98.6	97.8	85.5	85.9	82.1	81.9	81.9	81.5	83.7	83.1
8 Kyongju	.3197	.2172	.0040	.0161	.0120	.0120	.0140		99.2	86.1	86.5	82.9	82.7	82.9	82.1	84.3	83.9
9 Koje	.3256	.2223	.0120	.0201	.0201	.0201	.0222	.0080		85.9	86.3	83.1	82.5	83.1	81.9	84.3	83.1
10 Anlva (Russia)	.3001	.1952	.1540	.1667	.1608	.1635	.1661	.1593	.1620		98.8	83.7	84.5	81.3	82.3	82.5	82.9
11 Lazo (Russia)	.3102	.1899	.1484	.1611	.1552	.1579	.1605	.1537	.1563	.0120		84.1	84.5	81.5	82.9	82.5	83.5
12 <i>R. japonica</i> (Japan)	.3214	.1798	.2008	.2030	.2059	.2109	.2109	.2008	.1973	.1902	.1843		85.3	87.1	86.1	86.5	86.5
13 <i>R. okinavana</i> (Japan)	.3392	.2201	.2023	.2102	.2102	.2124	.2124	.2023	.2045	.1779	.1776	.1667		85.7	86.9	82.1	84.1
14 <i>R. longicrus</i> (Taiwan)	.3273	.2326	.1988	.2067	.2039	.2089	.2118	.1988	.1955	.2218	.2193	.1451	.1610		85.1	85.3	82.5
15 <i>R. tsushimensis</i> (Japan)	.3332	.1869	.2059	.2256	.2109	.2160	.2189	.2116	.2138	.2095	.2005	.1581	.1477	.1704		84.1	83.7
16 <i>R. pirica</i> (Japan)	.3316	.2042	.1806	.1855	.1855	.1905	.1877	.1806	.1801	.2066	.2062	.1524	.2102	.1672	.1828		89.1
17 <i>R. dybowskii</i> (Korea)	.2969	.1936	.1917	.1967	.1967	.2017	.1960	.1861	.1967	.2008	.1920	.1524	.1834	.2059	.1889	.1211	

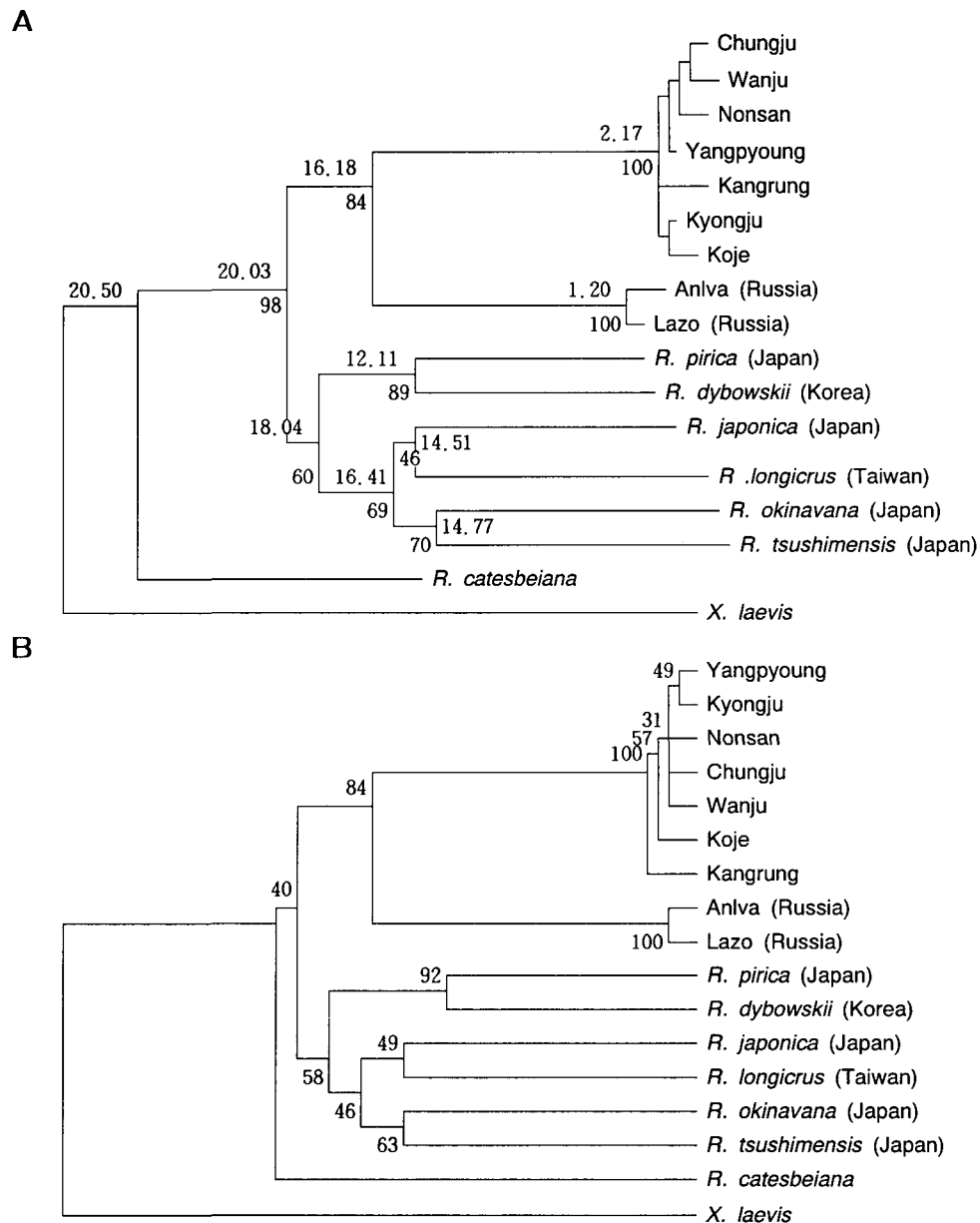


Fig. 3. A neighbor-joining tree (A) and UPGMA tree (B). Numbers above the nodes of A indicated Kimura-2-parameter distance values and numbers below the nodes of (A) and number of (B) indicate bootstrap values for 500 iterations.

and distributed from North Asia to Sakhalin in a short time.

According to the classification of morphological characters (Dubois, 1992), the brown frogs were divided into four groups, i.e. *R. japonica* group (*R. amurensis*, *R. japonica* and *R. longicrus*), *R. temporaria* group (*R. tsushimensis* and *R. okinavana*), *R. chensinensis* group (*R. pirica*, *R. ornativentris* and *R. dybowskii*) and *R. tagoi* group (*R. tagoi* and *R. sakuraii*). However, in our cytochrome *b* gene data, *R. amurensis* does not belong to the *R. japonica* group. Based on both UPGMA and neighbor-joining tree, sequence divergence between

Korean and Russian *R. amurensis* was clustered with 16.18% and other related brown frogs was diverged from them by 20.03%. This result corresponds with the data of Tanaka-Ueno et al. (1998a).

Korean brown frogs are divided into two groups, i.e. chromosome number  $2N=24$  for *R. dybowskii* and  $2N=26$  for *R. amurensis*. According to karyological analysis, 26-chromosome species were translocated into 24-chromosome species (Green, 1983; Lee and Park, 1986). Green and Borkin (1993) proposed that *R. amurensis* having 26-chromosome diverged before the divergence of two groups of European and Asian brown

frogs with 24-chromosomes. The ancestral species of *R. amurensis* is assumed to have been involved in the initial divergence among East Asian brown frogs.

In conclusion, *R. amurensis* from Korea and Russia were diverged first from ancestral frogs about 8 to 4 million years ago. Based on the rate of genetic distance and nucleotide sequence similarity between *R. amurensis* from Korea and Russia ranged from 0.1484 to 0.1667, they are considered as genetically differentiated at the species level. Further investigation should be carried out by morphological, ecological (including vocalization), geographical analyses to clarify their taxonomic status.

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