

# Distinct Specific Status of the Korean Brown Frog, *Rana amurensis coreana* (Amphibia: Ranidae)

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We used morphological and genetic data to assess the taxonomic status of *Rana amurensis coreana* and *R. a. amurensis*. Morphological comparisons revealed these two subspecies to be different from each other in size of body, nature of tubercles on dorsal stripe, degree of development in toe webbing, and condition of lateral spots of trunk. They were also different in sequence of mitochondrial 16S rRNA gene, with genetic distance as large as those observed among different species of brown frogs. Therefore, Korean populations previously considered a subspecies of *R. amurensis* should be regarded as a distinct species (= *R. coreana*).

**Key words:** mitochondrial 16S rRNA, morphology, taxonomy, phylogeny, China, Russia

## INTRODUCTION

*Rana* is the largest genus of ranids, with approximately 240 species (Frost, 2004). In Korea, six species of *Rana* have been recorded: three brown frogs (*R. amurensis coreana*, *R. dybowskii*, *R. huanrenensis*), two pond frogs (*R. nigromaculata*, *R. plancyi chosonica*) and one wrinkle frog (*R. rugosa*) (Kang and Yoon, 1975; Zhao and Adler, 1993; Sengoku *et al.*, 1996; Maeda and Matsui 1999, Yang *et al.*, 2000; Yang *et al.*, 2001). Among these, species of brown frogs are very difficult to identify because they are morphologically similar (Nakamura and Uéno, 1963). *Rana amurensis coreana* is known only from South Korea, and was originally described as a subspecies of European *R. temporaria* (Okada, 1928). Later, it was moved on morphological grounds to a subspecies of *R. amurensis*, a species occurring Russian Far East and northeastern China (Shannon, 1956). Since Shannon's review, this taxonomic arrangement has been widely accepted (Dixon, 1956; Yang and Yu, 1978).

Recently, mitochondrial DNA (mtDNA) sequences have been widely used to estimate phylogenetic relationships of various organisms (*e.g.*, Brown *et al.*, 1982; Smith and Patton, 1991; Moritz *et al.*, 1992; Tan and Wake, 1995; Tanaka-Uedo, 1998). Mitochondrial DNA has a smaller genome size (15.0–20.0kb) and evolves more rapidly than the nuclear DNA (perhaps 5–10 times faster than a typical single-copy nuclear DNA) and is maternally inherited. For

solving phylogenetic problems at the level of species, genus or family, 16S rRNA gene has been used among mtDNA genes. Preliminary analyses of ranid frogs, including *R. a. coreana* (Song *et al.*, 2003) indicate that 16S may be applied to solve relationships of closely related brown frogs.

The aim of this study is to clarify phylogenetic and taxonomic relationships of *R. a. coreana* with the nominotypical subspecies from outside of Korea using concordant morphological and genetic sequence data of 16S rRNA.

## MATERIALS AND METHODS

### Morphological analysis

We studied a total of 36 specimens of *Rana amurensis* (*R. a. amurensis*: n=16; *R. a. coreana*: n=20) and 50 specimens of three brown frog species (*R. tsushimensis*: n=14; *R. dybowskii*: n=16; *R. huanrenensis*: n=20) for comparisons. These brown frogs were collected from different areas in Korea, China, and Japan (Table 1). All specimens were anesthetized with chloroform, dissected for tissue samples, and then fixed in 10% formalin.

These specimens and tissues are kept in 70% ethanol, in the Ecological Laboratory of Kyonggi University (KUEL).

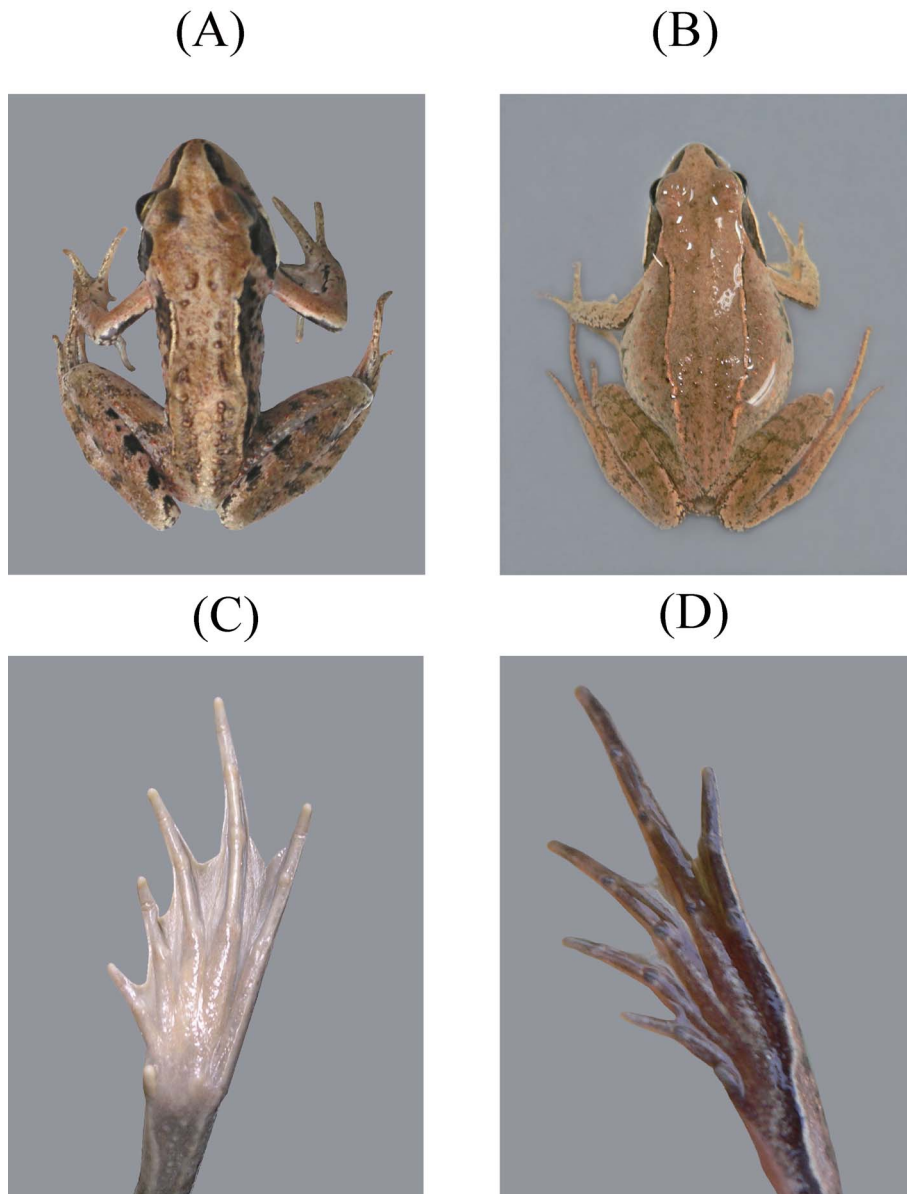
Examinations of qualitative characteristics were done using a stereoscopic microscope (SZ-ST, Olympus Co.) and a CCD color camera (No. IK-642K, Toshiba Co.). We analyzed following morphological characteristics: (1) shape of toe and finger tips; (2) marking on lower jaw; (3) line of upper lip; (4) size of tympanum; (5) degree of development in toe webbing; (6) degree of development of vomerine teeth series; (7) pattern of dorsolateral fold; (8) shape of snout; (9) length of hindlimb; (10) tubercles on dorsum. Terminology followed Kang and Yoon (1975), and Maeda and Matsui (1999).

For morphometric analysis, the method modified from Matsui (1984) was used, and measurements were made with digital calipers to 0.1 mm for the following 15 characters: (1) snout-vent length (SVL); (2) head length (HL); (3) nostril-eyelid length (N-EL); (4)

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**Table 1.** Collection localities and sample size of brown frogs and outgroup species used in this study

Species	Locality	Sample size	
		Male	Female
<i>R. a. amurensis</i>	Tonghe, Heilongjiang Province, China	3	3
	Pilevskoskovo, Sakhalin, Russia	3	3
	Aniva, Sakhalin, Russia	3	1
<i>R. a. coreana</i>	Mt. Gwangkyo, Paldal-Gu, Suwon, South Korea	4	2
	Daecheon, Boryeong, Chungcheongnam-do, South Korea	4	5
	Mokgeri, Uhmjeong-Myeon, Chungju, South Korea	3	2
<i>R. huanrenensis</i>	Mt. Seorak, Inje-Gun, Gangwan-do, South Korea	15	5
<i>R. dybowskii</i>	Mt. Gwangkyo, Paldal-Gu, Suwon, South Korea	3	6
	Mupo, Daehongdan-Gun, Ryanggang-do, North Korea	4	3
<i>R. tsushimensis</i>	Mt. Tatera, Izuhara, Nagasaki Prefecture, Japan	8	6
<i>R. chensinensis</i>	Keiher, Daxinganling, Heilongjiang Province, China	–	1
<i>R. catesbeiana</i>	Gangwha bridge, Gimpo, Gyeonggi-do, Korea	1	–

**Fig. 1.** Dorsal surface of *R. a. amurensis* (A) and *R. a. coreana*, and Plantar surface of left foot of *R. a. amurensis* (C) and *R. a. coreana* (D).

snout length (SL); (5) eye length (EL); (6) tympanum-eye length (TEL); (7) tympanum diameter (TD); (8) head width (HW); (9) internarial distance (IND); (10) interorbital distance (IOD); (11) radioulna length (RUL); (12) tibia length (TL); (13) foot length (FL); (14) thigh length (THIGH); (15) inner metatarsal tubercle length (IMTL).

Statistic analyses were performed by SPSS 11.5 software package (Statistical Package of the Social Science). For morphometric comparisons, we applied t-test(ANOVA and Tukey HSD) for absolute size, and Mann-Whitney's U tests for ratios. We also performed Canonical Discriminant Analyses (CANDISC) to examine overall size and shape differences.

### Genetic analysis

In order to examine phylogenetic relationships among Korean brown frogs, we obtained partial sequences of 16S rRNA (ca. 420-bp) from seven species of *Rana*. Additionally, four specimens (Graduate School of Human and Environmental Studies, Kyoto University (=KUHE) 11639, 11640, 33644, and 33647) of *R. a. amurensis* from Russia were sequenced. We selected *R. catesbeiana* as an outgroup (Table 1).

Total DNA was extracted from frozen (-70C) tissue samples of the hindlimb muscle, using an extraction buffer (150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% sodium dodecyl sulfate), proteinase K, and phenol. Amplification were done by the polymerase chain reaction (PCR), using the primers 16S1F (5'-GAG-GTCCAGCCTGCCAG-3') and 16S1R (5'-CCCTGATACCAACATCGAG-3') (Song *et al.*, 2003). The amplified fragments were sequenced in an automated DNA sequencer (ABI PRISM 3100) using the PCR primers and following the manufacturer's instructions.

Sequences were aligned using CLUSTAL W 1.4. The nucleotide sequences of 16S were combined into a single data set of 414-bp. We used Maximum Likelihood (ML) to estimate phylogeny in PAUP\* 4.0b(Swofford, 1998). Heuristic searches were performed using 100 replicates of a stepwise addition of taxa, and Bootstrapping (Felsenstein, 1985) was employed to assess relative nodal support (100 replicates). Pairwise comparisons of corrected sequence divergences [Kimura-2 parameter (K2p) distances (Kimura, 1980)] were also made with PAUP. In these analyses, ratio of transition: transversion bias was equally weighted.

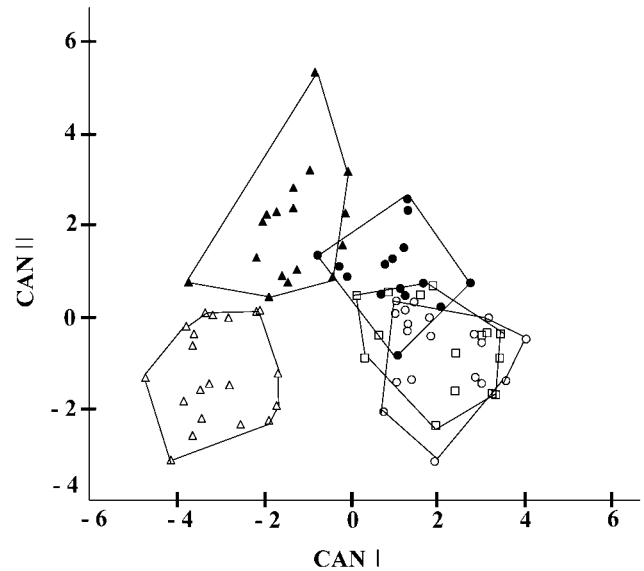
## RESULTS

### Morphological analysis

Our examination of color variation in *R. amurensis* revealed patterns fixed color difference between populations. *Rana a. amurensis* had two dorsal stripes having tubercles each with a black spot, whereas *R. a. coreana* had

two dorsal stripes with black spots but not with tubercles. *Rana a. amurensis* has many tubercles on dorsum and ventrum, whereas *R. a. coreana* has smooth skin dorsally and ventrally (Fig. 1). Similarly, sides of body are rough in *R. a. amurensis*, but are smooth in *R. a. coreana*. *Rana a. amurensis* has dark brown or red spots on sides of body, that are absent in *R. a. coreana*. Further, small tubercles on ventral side of the thigh are more developed in *R. a. amurensis* than in *R. a. coreana*. Toe webbing of *R. a. amurensis* (I 1<sup>1/4</sup>-3<sup>3/4</sup> II 3<sup>3/4</sup>-2<sup>1/4</sup> III 3<sup>3/4</sup> IV 1<sup>3/4</sup>-1<sup>1/4</sup> V) is much more developed than in *R. a. coreana* (I 2<sup>3/4</sup>-2<sup>3/4</sup> II 2<sup>1/4</sup>-1<sup>3/4</sup> III 1<sup>3/4</sup>-1<sup>3/4</sup> IV 2<sup>3/4</sup>-1<sup>3/4</sup> V).

Body size in male *R. a. coreana* (mean=38.4±3.9mm SD) was significantly smaller than *R. a. amurensis* (53.3±5.7mm SD; t-test, p<0.05) but female *R. a. coreana* (44.4±3.6mm SD) were statistically indistinguishable (p>0.05) from females of *R. a. amurensis* (45.0±8.7mm SD). This may probably be due to small sample size of females.



**Fig. 2.** Plot of first against second canonical variates for brown frogs compared. *R. a. coreana* (open triangles); *R. a. amurensis* (closed triangles); *R. tsushimensis* (open squares); *R. dybowskii* (closed circles); *R. huanrenensis* (open circles).

**Table 2.** Pairwise Kimura's (1980) distance among brown frogs (1–8) and an outgroup (9). 1: *R. dybowskii* (S. Korea); 2: *R. dybowskii* (N. Korea); 3: *R. huanrenensis* (S. Korea); 4: *R. chensinensis* (China); 5: *R. tsushimensis* (Japan); 6: *R. a. coreana* (S. Korea); 7: *R. a. amurensis* (China); 8: *R. a. amurensis* (Russia); 9: *R. catesbeiana*.

	1	2	3	4	5	6	7	8
2	0.0000	–						
3	0.0380	0.0380	–					
4	0.0516	0.0516	0.0487	–				
5	0.0647	0.0646	0.0672	0.0698	–			
6	0.0835	0.0835	0.0838	0.0867	0.0842	–		
7	0.0948	0.0948	0.0950	0.0896	0.0786	0.0458	–	
8	0.0948	0.0948	0.0950	0.0896	0.0786	0.0458	0.0000	–
9	0.1917	0.1917	0.1901	0.1969	0.1740	0.1645	0.1670	0.1670

When compared with other species, *R. a. coreana* was similar with *R. tsushimensis* (35.9±3.8mm) but was smaller than *R. huanrenensis* (48.1±3.3mm) and *R. dybowskii* (59.5±9.6mm) (t-test,  $p < 0.05$ ).

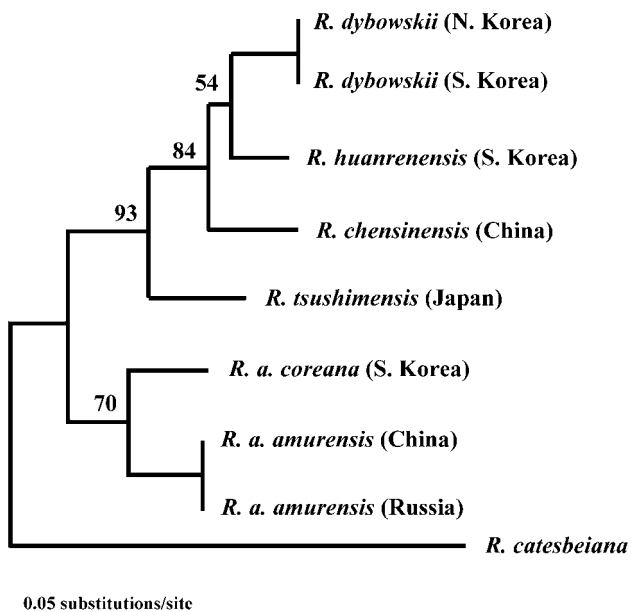
The post-hoc analysis by the Tukey HSD test showed that the mean foot length of *R. a. coreana* was relatively long compared with that of the other species, and ANOVA revealed a significant difference in the relative foot length between all brown frogs ( $F = 7.916$ ,  $P < 0.05$ ). The ratio of foot length to tibia length (=100FL/TL) was significantly larger in *R. a. coreana* (119.6±4.8%) than in *R. a. amurensis* (114.7±5.7%) and other species compared (*R. tsushimensis*, 101.3±5.8%; *R. dybowskii*, 103.3±7.9%; *R. huanrenensis*, 104.8±5.9%) ( $U = 87.0$ ,  $p = 0.20$ ).

As a result of CANDISC, nine characters (N-EL, EL, T-EL, TD, HW, IND, RUL, TL, FL and IMTL) revealed to be useful in differentiating each brown frog taxon. The each eigenvalue and the percent variance accounted for 4.606 and 56.2% (I), 1.539 and 18.8% (II), 1.294 and 15.8 (III), and 0.756 and 9.2 (IV). In the first two axes, *R. dybowskii*, *R. huanrenensis* and *R. tsushimensis* largely overlapped, but each of *R. a. amurensis* and *R. a. coreana* did not overlap with other species (Fig. 2).

### Genetic analysis

Of the 414 nucleotide positions aligned, 93 were variable. The gene sequence was completely identical between Chinese and Russian *R. a. amurensis*. By contrast, *R. a. coreana* was very remote from these populations of *R. a. amurensis* with large K2p distance of 0.0458, which value was similar to those observed (0.0380–0.0698) among different species of other brown frogs (*R. chensinensis*, *R. huanrenensis*, *R. dybowskii*, and *R. tsushimensis*) (Table 2).

In the maximum-likelihood tree (-lnL=1241.04706), two



**Fig. 3.** A ML tree for brown frogs compared. Numbers on nodes indicate bootstrap proportions.

main clades were recognized within brown frogs (Fig. 3). One main clade, supported by 70% iterations, contained *R.*

*a. amurensis* and *R. a. coreana*, and another, supported by 93% iterations, included *R. dybowskii*, *R. tsushimensis*, *R. huanrenensis*, and *R. chensinensis*. In the latter main clade, however, relationships among taxa included were not resolved except that *R. tsushimensis* was sister to the remaining three taxa.

### DISCUSSION

Recognition of more than one species of brown frogs in Korea dates back to Stejneger (1907), who identified a Korean brown frog in the collection of Philadelphia Academy of Sciences from Chemulpo as *R. japonica*, and also recorded *R. amurensis* from the same locality. Thereafter, Okada (1928) recognized three subspecies of *R. temporaria* from Korea, and described one of them as a new taxon *R. temporaria coreana*.

Later, Shannon (1956) changed the name of this taxon to *R. amurensis coreana*. His identification was made on the bases of (1) the lack of vocal sacs in male Korean frog, which is possessed by male *R. temporaria*, and (2) morphologically close similarity of Korean frog with Russian *R. amurensis*. However, he (Shannon, 1956) did not give morphological criteria adequate to differentiate these two subspecies. Since then, no detailed taxonomic study was made to reassess relationships of the two subspecies of *R. amurensis*. Thus, Korean authors have treated this frog variously as a distinct subspecies or without discriminating subspecies in biological works from many aspects (Kang and Yoon, 1975; Yang *et al.*, 2001).

As clearly shown by our genetic results, the two "subspecies" of *R. amurensis* are monophyletic and at the same time they are genetically divergent to the same degree as observed among other full species of Brown frogs. *Rana a. amurensis* has a very wide range of distribution, but is known to be extensively uniform genetically (Tanaka-Ueno *et al.*, 1998). Thus, presence of a subspecies only in South Korea is biogeographically unlikely.

Our morphological analyses also resulted in clear distinction of the two "subspecies" of *R. amurensis*. As shown in the result of CANDISC, *R. a. coreana* differed morphologically from *R. a. amurensis* with the magnitude larger than that observed between two good species, *R. dybowskii* and *R. huanrenensis*.

We conclude from both genetic and morphological evidence that the Korean brown frog should be elevated to the status of full species. The correct specific epithet for this species is *Rana coreana* (Korean name, Hankook-sangaeguri).

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**Appendix 1.** Nucleotide sequence alignment of the mitochondrial 16S rRNA genes (414bp) in brown frogs and an outgroup, *R. catesbeiana*.

	1	60
<i>R. catesbeiana</i>	GCCGCGGTACCCTAACCGTG–CGAAGGTAGCATAATCACTTGTCTTTAAATAGGGACTC	
<i>R. dybowskii</i> (N.Korea)	..... – .....	G .....
<i>R. dybowskii</i> (S.Korea)	..... – .....	G .....
<i>R. huanrenensis</i> (S.Korea)	..... – A .....	C .....
<i>R. chensinensis</i> (China)	..... G .....	– .....
<i>R. tsushimensis</i> (Japan)	..... T .....	G .....
<i>R. a. coreana</i> (S.Korea)	..... – .....	T .....
<i>R. a. amurensis</i> (China)	..... – .....	C .....
<i>R. a. amurensis</i> (Russia)	..... – .....	C .....
	61	120
<i>R. catesbeiana</i>	GTATCAACGGCATCACGAGGGCTATACTGTCTCCTTTCTCCAATCAGTGAAACTGATCTT	
<i>R. dybowskii</i> (N.Korea)	..... CT .....	T .. T .....
<i>R. dybowskii</i> (S.Korea)	..... CT .....	T .. T .....
<i>R. huanrenensis</i> (S.Korea)	..... CT .....	T .. T .....
<i>R. chensinensis</i> (China)	..... CT .....	T .. T .....
<i>R. tsushimensis</i> (Japan)	..... CT .....	T .. T .. A .....
<i>R. a. coreana</i> (S.Korea)	..... CT .. T .....	C .. T .....
<i>R. a. amurensis</i> (China)	..... CT .. T .....	T .....
<i>R. a. amurensis</i> (Russia)	..... CT .. T .....	T .....
	121	180
<i>R. catesbeiana</i>	CCCGTGAAGAAGCGGGAATTATTATATAAGACGAGAAGACCCCATGGAGCTTTAAACTC A	
<i>R. dybowskii</i> (N.Korea)	..... G .. AA .....	.....
<i>R. dybowskii</i> (S.Korea)	..... G .. AA .....	.....

Appendix 1. Continued

<i>R.huanrenensis</i> (S.Korea)	.....G...G.A.....	
<i>R.chensinensis</i> (China)	.....G...AAG.....	
<i>R.tsushimensis</i> (Japan)	.....C..C.AA.....	
<i>R.a.coreana</i> (S.Korea)	.....G.....C.....	
<i>R.a.amurensis</i> (China)	.....G..C.AA.....	
<i>R.a.amurensis</i> (Russia)	.....G..C.AA.....	
	181	240
<i>R.catesbeiana</i>	ATATATATTTCTACGCACCTTATATCACTATTAGCCTAA-GAAAACATATGTATTAGTTTTA	
<i>R.dybowskii</i> (N.Korea)	CC..GC.CC...GT..C.CCC-..AT.CT.A.A.AC..-CGG.C..GCA.GC.....	
<i>R.dybowskii</i> (S.Korea)	CC..GC.CC...GT..C.CCC-..AT.CT.A.A.AC..-CGG.C..GCA.GC.....	
<i>R.huanrenensis</i> (S.Korea)	CC..GC.CC...GT..T.C.--.AC.CT.A.A.AC..-C.G.C..GCA.G.....	
<i>R.chensinensis</i> (China)	C...GC.CC...GT..T.C.--.CAC.CT.A.A.AC..-T.GGT..GCA.G.....	
<i>R.tsushimensis</i> (Japan)	C...GC.CC...GT..C.C----AC.ACA..A.AC..GT.GTC..GCA.....	
<i>R.a.coreana</i> (S.Korea)	CC...C.CC...T.TTACC.-TATC.C-A...AC..-..G.C..G.A.G.....	
<i>R.a.amurensis</i> (China)	C.G..C.CC...T.TT.CT.-TATC..TA..A.AC..-...C..G.AC.....	
<i>R.a.amurensis</i> (Russia)	C.G..C.CC...T.TT.CT.-TATC..TA..A.AC..-...C..G.AC.....	
	241	300
<i>R.catesbeiana</i>	GGTTGGGGGGACCGCGGAGAAAAAATTAACCTCCACGACAAATAGGCCAACGCCTTTATC	
<i>R.dybowskii</i> (N.Korea)	.....T...A...T.C..C.....AG.....G...T..TA..C.....	
<i>R.dybowskii</i> (S.Korea)	.....T...A...T.C..C.....AG.....G...T..TA..C.....	
<i>R.huanrenensis</i> (S.Korea)	.....T...A...T.T..C.....AG.....G...T..TA..C.....	
<i>R.chensinensis</i> (China)	.....T...A...T.T..C.....TAG.....G...T..TA..C.....	
<i>R.tsushimensis</i> (Japan)	.....A...T.C.....A.....CG...T...A..G...C..	
<i>R.a.coreana</i> (S.Korea)	.....A...C.C..C.....TA.....G...T...A..C.....	
<i>R.a.amurensis</i> (China)	.....A...T.C..C.....TA.....G...T...A..C.....	
<i>R.a.amurensis</i> (Russia)	.....A...T.C..C.....TA.....G...T...A..C.....	
	301	360
<i>R.catesbeiana</i>	TATGAACCACAATTCTAAGAATCAATAAACTGATGTTTAAATGATCCAATTTTTTGATCA A	
<i>R.dybowskii</i> (N.Korea)	C.C..GA.....C.....T.T..T...A...C...T...C..G..A..C-.....	
<i>R.dybowskii</i> (S.Korea)	C.C..GA.....C.....T.T..T...A...C...T...C..G..A..C-.....	
<i>R.huanrenensis</i> (S.Korea)	C.C..GA.....C.....T.C.....A...C...C-.C..G..A..C-.....C..	
<i>R.chensinensis</i> (China)	C.C..GA.....C.....T.T.....A...C...C-.C..G..A..C-.....	
<i>R.tsushimensis</i> (Japan)	C.C..GA.....C.....T.C.....A...C...C-.C..G..A..C-.....	
<i>R.a.coreana</i> (S.Korea)	C.C..GA.....C.....T.C.....A...C...T-.C..G..A..C-.....	
<i>R.a.amurensis</i> (China)	C.C..GA.....C.....T...G..A...C...T-.C..G..A..C-.....	
<i>R.a.amurensis</i> (Russia)	C.C..GA.....C.....T...G..A...C...T-.C..G..A..C-.....	
	361	
<i>R.catesbeiana</i>	CGAACCAAGTTACCTGGGGATAACAGCGCAATCTACTTCAAGAGCTCCTATCG	
<i>R.dybowskii</i> (N.Korea)	T.....C.A.....	
<i>R.dybowskii</i> (S.Korea)	T.....C.A.....	
<i>R.huanrenensis</i> (S.Korea)	T.....C.A.....	
<i>R.chensinensis</i> (China)	T...T.....C.A.....	
<i>R.tsushimensis</i> (Japan)	T.....C.A.....	
<i>R.a.coreana</i> (S.Korea)	T.....A.....	
<i>R.a.amurensis</i> (China)	T.....A.....	
<i>R.a.amurensis</i> (Russia)	T.....A.....	