

Genetic Relationships of Korean Treefrogs (Amphibia; Hylidae) Based on Mitochondrial Cytochrome *b* and 12S rRNA Genes

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The nucleotide sequence of a 447 base pair fragment in the mitochondrial cytochrome *b* gene and the complete sequence of the mitochondrial 12S ribosomal RNA gene, 938 bp, were analyzed to infer inter- and intraspecific genetic relationships of *Hyla japonica* and *H. suweonensis* from Korea and *H. japonica* from Japan. In the mitochondrial cytochrome *b* gene, genetic differentiation among *H. japonica* populations were 9.62% and 15.66% between *H. japonica* and *H. suweonensis*. Based on the Tamura-Nei distance, the level of sequence divergence ranged from 0.45% to 2.75% within Korean *H. japonica*, while 8.31%-8.87% between Korean and Japanese *H. japonica* and 11.51%-12.46% between *H. japonica* and *H. suweonensis*. In the neighbor-joining tree, Korean populations of *H. japonica* were clustered first at 2.22% and followed by Japanese *H. japonica* and *H. suweonensis* at 8.51% and 12.29%, respectively. In mitochondrial 12S rRNA gene, genetic differentiation between *H. japonica* and *H. suweonensis* was 7.17% (68 bp) including 7 gaps. Based on Tamura-Nei distance, the level of sequence divergence ranged 3.53% between Korean and Japanese *H. japonica* and from 4.93% to 5.41% between *H. japonica* and *H. suweonensis*. Phenogram pattern of the 12S rRNA gene sequence corresponded with that of the mitochondrial cytochrome *b* gene.

Kuramoto (1980) reclaimed that there are two species, *Hyla japonica* and *H. suweonensis*, in Korea. *H. japonica* is widely distributed in South Korea, while *H. suweonensis* is restricted to Kyonggi-Do and part of Chung-chung-Do. These sibling species are sympatric and morphologically indistinguishable but reproductively isolated (Kuramoto, 1980; Yang and Park, 1980; Yang, et al., 1997).

Although *H. japonica* and *H. suweonensis* are indistinguishable in morphological analyses, *H. japonica* and *H. suweonensis* differ in chromosomal structure (arm number) and mitochondrial DNA size based on karyological and RFLP analysis. Their chromosomal arm number differences are caused by pericentric inversion in the nucleolus organizer regions (NORs) (Yu and Lee, 1990; Lee and Park, 1991, 1992). Also the genome size differences between two species were found on mitochondrial DNA where *H. japonica* was approximately 1.0 kb larger than *H. suweonensis*. According to RFLP analysis, the level of sequence divergence was

14.2% and the divergence time between two species was 7.1 million years (Lee and Park, 1992). The genetic relationship between two species by isozyme data (Yang, et al., 1997) was rather remote ($S=0.474$, $D=0.755$).

Molecular methods are useful to elucidate specific and generic level relationships. Since the development of conserved primers (Kocher, et al., 1989), mitochondrial DNA genes (2 ribosomal RNAs, 22 transfer RNAs, 13 proteins) have become a routine tool to investigate biological relationships and evolution of various species.

Mitochondrial DNA (mtDNA) has a small genome size (15.0-20.0 Kb) and does not recombine during sexual reproduction. Also mtDNA generally evolves more rapidly than nuclear DNA. Partial or complete sequence of the mitochondrial cytochrome *b* gene has been used to estimate phylogenetic relationships among closely related species. On the other hand, mt rRNA genes have been used to resolve some aspects of higher groups, such as the genus, family, and order (Tanaka, et al., 1994; Ilya and Linda, 1996; Lee, et al., 1997; Sumida, et al., 1998).

In this study, inter- and/or intraspecific relationships of the genus *Hyla* were examined to estimate phylogenetic relationships using a partial sequence of the

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Table 1. Collecting localities, date and numbers of analyzed specimens

Species	Localities	Date	N
<i>Hyla japonica</i>	Kyongju: Kyongju-Shi, Kyongsangnam-Do	July 1996	2
	Pyongtaek: Osong-Myon, Pyongtaek-Gun, Kyonggi-Do	July 1996	2
	Cheju: Cheju-Shi, Cheju-Do	May 1995	2
	Wonju: Wonju-Shi, Kangwon-Do	July 1996	2
	Hadong: Hwagae-Myon, Hadong-Gun, Kyongsangnam-Do	July 1996	3
	Japan: Otomo-Ma, Maebashi city, Gumma-Ken, Japan	July 1994	3
<i>H. suweonensis</i>	Suwon: Suwon-Shi, Kyonggi-Do	May 1995	2
	Pyongtaek: Osong-Myon, Pyungtaek-Gun, Kyonggi-Do	July 1996	1

mt cytochrome *b* gene and a complete sequence of the mt 12S rRNA gene.

Materials and Methods

DNA extraction

We sampled five populations of *H. japonica*, one population of *H. suweonensis* from Korea and one population of *H. japonica* from Japan. Tissue samples were obtained from two specimens per population for the mitochondrial cytochrome *b* gene and one population each of Korean and Japanese *H. japonica* and one population of *H. suweonensis* used for the mitochondrial 12S rRNA study (Table 1). Genomic DNA was extracted from frozen (-70°C) liver and stomach tissue using proteinase K/SDS dissolution and purified by the phenol/chloroform extraction method.

DNA amplification

PCR primer pairs were designed to cover a 470 bp fragment of the cytochrome *b* gene and 1.1 kb fragment of the 12S rRNA gene (Table 2). The cytochrome *b* and 12S rRNA genes were amplified by 30-35 cycles, each cycle consisting of denaturation for 1 min at 95°C, annealing for 1 min at 48°C, and extension for 1 min at 72°C.

Direct silver sequencing

The PCR products were purified by a TragenTM one-step gel extraction kit (Injae, Korea). Purified PCR products were sequenced by a TopTM DNA sequencing kit (Bioneer, Korea) using a silver staining system (Bioneer, Korea).

Table 2. Oligonucleotide sequences for PCR amplification and sequencing of mitochondrial cytochrome *b* and 12S rRNA genes in Korean treefrogs

Name	Oligonucleotide sequences
BP1	5'-TTCCTAGCNATACAYTAYACAGC-3'
HJ4	5'-GGAGTTTAGGCCGGTAGGGTT-3'
H12S1	5'-GCACAGCACTGAAGATGCTGAGA-3'
H12S2	5'-AAACTGGGATTAGATACCCCACTAT-3'
H12S3	5'-GCGTCACGTTGACTCAGTTA-3'
H12S4	5'-ACACACCCGCCGTCACCCCTCT-3'
H12S5	5'-CTAAGATGAACCCTAAAAGTTCT-3'
L12S1	5'-GTAAGCGAAAGGCTTTGGTTAAG-3'
L12S2	5'-TGTGAGGCGTTCTCACAGGGG-3'
L12S3	5'-ATAGTGGGTATCTAATCCAGTTT-3'
L12S4	5'-GCTACTAGGGGCGAAGTGGA-3'

Data analysis

For sequence alignment, CLUSTRAL W software (Thompson, et al., 1994) was used and statistic analysis was performed using MEGA software (Kumar, et al., 1993). *H. suweonensis* was used for the outgroup. The pairwise matrix of sequence divergence was calculated by Tamura-Nei distance (Tamura and Nei, 1993). Genetic relationships among the *Hyla* genus was inferred based on a neighbor-joining method (Saitou and Nei, 1987) with 1000 bootstrap iterations (Felsenstein, 1985).

Results

Mitochondrial cytochrome *b* gene

The 447 bp fragment of the mitochondrial cytochrome *b* gene was obtained from 14 specimens of the genus *Hyla* (Fig. 1). There were 70 bp variable sites and only one amino acid difference within Korean *H. japonica*. The nucleotide differences ranged from 0.45% to 8.05% within Korean and Japanese *H. japonica*, and from 10.74% to 10.96% between Korean *H. japonica* and *H. suweonensis*. Between *H. japonica* from Korea and Japan, nucleotide differences ranged from 7.13% to 7.55%.

Most silent mutations were caused by third codon position substitution; 2.0% at the first codon position, 0.7% at the second position and 43.0% at the third position. Because most amino acid replacements occurred at the first or second position substitution, third position substitution is degenerated into a genetic code. (Kocher, et al., 1989; Friesen, et al., 1993; Cicero and Jhonson, 1995). Also, transitional substitution occurred approximately 3.3 times as frequently as transversion. The 77th amino acid substituted histidine (CAT) for proline (CCA) due to base substitution of the second codon position (Fig. 2).

The average nucleotide compositions were repre-

Table 3. Average percent base composition in each cytochrome *b* gene codon position in Korean treefrogs

Position	A	T	C	G
First	24.2	29.0	20.0	26.8
Second	18.4	40.3	23.9	17.4
Third	31.6	33.5	29.7	5.2
Average	24.7	34.3	24.5	16.5

	1	80
Kyongju	GACACCTCCCTAGCTTTTTTCATCAGTAGCCCATATTTGTCGAGATGTGAATAATGGTTGACTCCTTCGCAATATTCACGC	
PyongtaekC.....T..
Cheju
Wonju a
Wonju b
Hadong
Japan	..T..A.....C.....C.....C.A.....C...TT.A...C...T..	
SuwonG.....C.....C.C.C.....A.....C.C.....T.A.....C.....	
	81	160
Kyongju	AAATGGCGCCTCATTCTTCTTTATCTGCATCTATCTTCACATTGGACGGGGATCTATTACGGATCCTTTTATTTAAAG	
PyongtaekT..C.....T.....
ChejuC.....A.....
Wonju aC.....
Wonju bC.C.....T.....
HadongC.C.....T.....
JapanT..C.....T..C.....C.....A.....T.....	
SuwonC.....C.....T.C.C.T.....A.....G.....	
	161	240
Kyongju	AAACATGAAATATTGGAGTAATCCTTCTTCTTCTAGTTATAGCCACAGCCTTTGTTGGCTATGTTCTTCCATGAGGTCAA	
PyongtaekG.....AT.....
ChejuG.....AT.....
Wonju aG.....AT.....
Wonju bG.....AT.....
HadongG.....AT.....
JapanC.....T.....G.....CT.A.T...C...	
SuwonC.....T.C.....T.....C.C.A.T...C...	
	241	320
Kyongju	ATATCTTTTTGAGGGGCCACAGTCATTACTAATCTTCTTTTCAGCTGCCCATACATCGGAACCGAACTAGTACAGTGAAT	
PyongtaekG.....A.....
Cheju
Wonju a
Wonju b
Hadong
JapanC.....C.....A.....	
SuwonC.....C.....G.A..T...G..A.....	
	281	400
Kyongju	TTGAGGGGGCTTTTCAGTTGACAATGCTACATTAACCCGATTTTTTCACATTCATTTATTTTACCATTATCATTGCAG	
PyongtaekC.....
Cheju
Wonju a
Wonju b
HadongG.....
JapanC.....	
Suwon	C.....A.....C.....C..CC.G.....C.C.....G...T.....	
	351	447
Kyongju	GGGCTTCAATAATTCACCTTCTCTTCTTCCACCAAACAGGATCCTCA	
PyongtaekC.....
Cheju
Wonju a
Wonju b
Hadong
JapanT...T.....G.....	
SuwonC.....T..C.....	

Fig. 1. The 447 nucleotide sequences of the mitochondrial cytochrome *b* gene. Dots indicate identity to the sequence of Kyongju population of *Hyla japonica*. Specimens of this species which have identical sequences were described as one respective.

sented in Table 3. The guanine at the third position of the codon was lower (5.2%) than the other codon. This low guanine value is consistent with those of other

vertebrate mitochondrial genomes in general (Kocher, et al., 1989).

Based on the pairwise matrix of sequence divergence

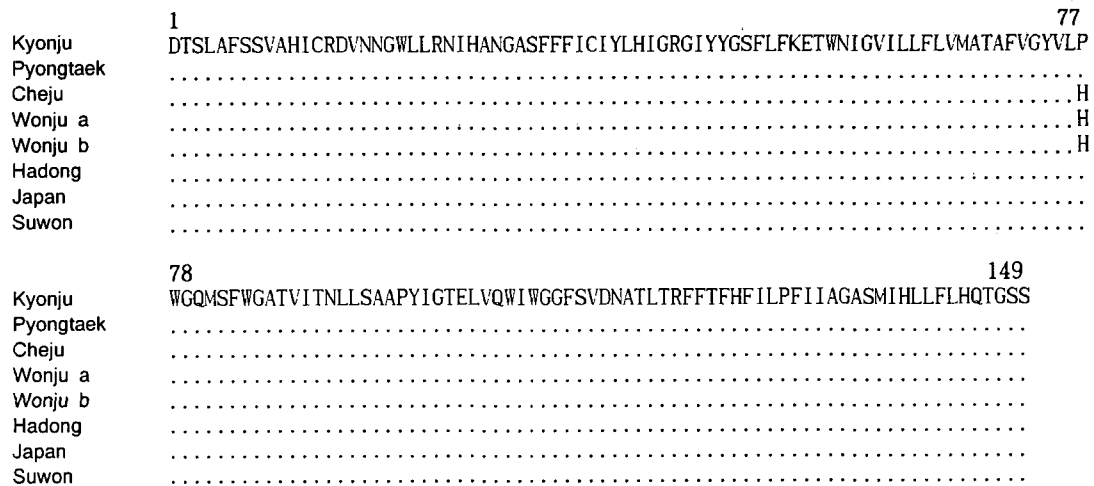


Fig. 2. Amino acid sequence for the cytochrome *b* gene of the genus *Hyla*. Dots indicate identity to the sequence of Kyongju population of *H. japonica*. Specimens of this species which have identical sequences were described as one respective.

calculated by Tamura-Nei distance, intrapopulation sequence divergences of Korean *H. japonica* ranged from 0.45% to 2.75% within 6 populations, but among Korean and Japanese *H. japonica* ranged from 8.31% to 8.87%. On the other hand, the sequence divergences between *H. japonica* and *H. suweonensis* were 11.51%-12.46% (Table 5).

In the neighbor-joining tree, all Korean *H. japonica* clustered first at 99% support of 1000 bootstrap iterations, and followed by Japanese *H. japonica* and *H. suweonensis* (Fig. 4).

Mitochondrial 12S rRNA gene

The 938 bp fragment of the mt 12S rRNA gene was obtained from 3 specimens of the genus *Hyla*. There was a 68 bp variable site including 7 gaps. Using a CLUSTRAL W program (Thompson, et al., 1994), complete mitochondrial 12S rRNA gene sequence of Korean *H. japonica* was 937 bp, 935 bp for Japanese *H. japonica*, and 931 bp for *H. suweonensis* (Fig. 3).

The nucleotide substitutions between Korean and Japanese *H. japonica* were 3.42% and 5.16% between Korean *H. japonica* and *H. suweonensis*, and 4.37% between Japanese *H. japonica* and *H. suweonensis*.

In the nucleotide composition among three specimens, adenine was more abundant than other bases (Table 4).

Table 4. Nucleotide composition of mitochondrial 12S rRNA gene sequences in treefrogs

Species	A	T	C	G
HJK ¹	32.2	24.7	23.7	19.4
HJJ ²	32.2	23.7	24.7	19.4
HSP ³	31.4	24.7	24.1	19.9
Average	31.9	24.4	24.2	19.6

¹Korean *Hyla japonica* from Hadong, ²Japanese *H. japonica*, ³*H. suweonensis* of Pyongtaek.

Based on the pairwise matrix of sequence divergence calculated by Tamura-Nei distance, the sequence divergences were 3.53% between Korean and Japanese *H. japonica* and between *H. japonica* and *H. suweonensis* ranged from 4.93% to 5.41% (Table 6).

In the neighbor-joining tree, *H. japonica* from Korea and Japan clustered first at 3.53% and *H. suweonensis* was separated from *H. japonica* at 5.17% (Fig. 5).

Discussion

The sequence divergence between *H. japonica* and *H.*

Table 5. Pairwise comparisons of the cytochrome *b* gene sequence by Tamura-Nei distance in treefrogs

Species	1	2	3	4	5	6	7	8
<i>Hyla japonica</i> *								
1. Kyongju		0.0183	0.0136	0.0090	0.0136	0.0113	0.0831	0.1236
2. Pyongtaek			0.0275	0.0228	0.0275	0.0253	0.0859	0.1206
3. Cheju				0.0045	0.0090	0.0159	0.0887	0.1151
4. Wonju a					0.0045	0.0113	0.0835	0.1206
5. Wonju b						0.0068	0.0835	0.1206
6. Hadong							0.0859	0.1231
7. Japan								0.1246
<i>H. suweonensis</i> *								
8. Suwon								

* Specimens which have identical distance were described as one respective

Table 6. Pairwise comparisons of the 12S rRNA gene sequence by Tamura-Nei distance in treefrogs

Species	1	2	3
<i>Hyla japonica</i>			
1. Hadong		0.0353	0.0541
2. Japan			0.0493
<i>H. suweonensis</i>			
3. Pyongtaek			

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1
HJK CCAAGGTTTGGTCCTA-ACCTTGTAATCTCTTGTACTTAACTTATACATGCAAGTCTCAGCACCCCTGTGAGAACGCCCTCAATCTTCTTA 92
HJJ .A.....T.A.....T.....C.....T.....
HSP .A.....T.TG.....G...AT.C.....T.....C.....TG.....

93
HJK TTGAAACGAGGAGCTGGTATCAGGCACAATTATTAGCCCAAGACACCTAGCCAGCCACATCCACAAGGAAATTCAGCAGTAATTAACATTG 184
HJJ .....A.....T.....
HSP .....A.....G.....T.....G.....

185
HJK AGTATAAGCGTACGCTTACTCAGTTAAAGTAAAGAGAGCCGGCAAATATGGTGCCAGCCGCCGGGTTACACCACGAGGCTCAAATTGATT 276
HJJ .....G.....
HSP .....T.....-.....

277
HJK ACTTTCGGCGTAAAGCGTGATTAAAGTTACCCATTAAGTATAGTTGATTTTAACTAAGCTGTGACACGCTTGTCTTAAGAACACTCAAAA 368
HJJ .....TT.....T.....
HSP .....C.....T.CCC.....T.G.....

369
HJK ACGAAAGTTACTATATATAAACCAACTTGAATCCACGACAGCTGAGGCACAACTGGGATTAGATACCCCACTATGCTCAGCCGTAAACTTT 460
HJJ .....C.....C.....A.....
HSP .....GC.....C.....A.....

461
HJK AAATTACACCCACATCGCCAGGGAAGTACGAGCAAAGCTTAAACCCAAGGGACTTGACGGTACCCACATCCACTAGAGGAGCCTGTCC 552
HJJ .....T--.....A.....
HSP .....T--.....A.....

553
HJK TATAATCGATAACCCCGCTAACCTCACCATTTTAGCCAATCAGCCTGTATACCTCCGTCGTCAGCTTACCTCGTGAGTGAATCTAGTG 644
HJJ .....G.....C.T.....
HSP .....C.T.G.....

645
HJK AGCTTAATGTCTTTACATCAATACGTCAGGTCAAGGTGAGTAAATAAAATGGGAAGAGATGGGCTACACTCTCTAACTTAGAATATACGAA 736
HJJ .....C.....T.....G.....AG.....GA.....
HSP .....C.....T.....-.....T.....C.C.....

737
HJK AGACTACCTATGAAATCTAGTCAGAAAGCGGATTTAGAAGTAAAGAGACCATAGAGCTCTTCTTAACCCGGCACTGGGGTGTGTACACAC 828
HJJ .....T.....T.....
HSP .....C.....G.....T.....T.....-.....C.....

829
HJK CGCCCGTCACCCTCTTCAAAGCCCAAATAGCAGTATATAACTCAATTTAGCACCACAGAAGAGGCAAGTCGTAACATGGTAAGCGTACCGGA 920
HJJ .....T.....AT.....
HSP .....-.....A.....A.....C.....T.CT.....

921
HJK AGGTGTGCTTGAAACAA 938
HJJ .....
HSP .....

```

Fig. 3. The 938 nucleotide sequences of the mitochondrial 12S rRNA gene. Dots indicate identity to the sequence of Hadong population of *H. japonica*. Quotation marks (-) designates alignment gaps. HJK, Korean *Hyla japonica* from Hadong; HJJ, Japanese *H. japonica*; HSP, Korean *H. suweonensis* from Pyongtaek.

suweonensis was 12.9% by cytochrome *b* gene analysis, while 5.17% by 12S rRNA gene analysis. In the study of interspecific relationships for Korean and Japanese brown frogs, sequence divergence of Japanese brown frogs were 14.24% (Tanaka, et al., 1994) and Korean brown frogs (*Rana amurensis* and *R. dybowskii*) were estimated to be 16.30% using the mitochondrial

cytochrome *b* gene. The sequence divergence between *Rana nigromaculata* and *R. plancyi* which was considered as a subspecies of *R. nigromaculata* was 6.8% (Oh, 1997). In the California newt, *Taricha torosa*, sequence divergence among subspecies was 7.0%-9.0% (Tan and Wake, 1995).

Japanese *H. japonica* which were reported as con-

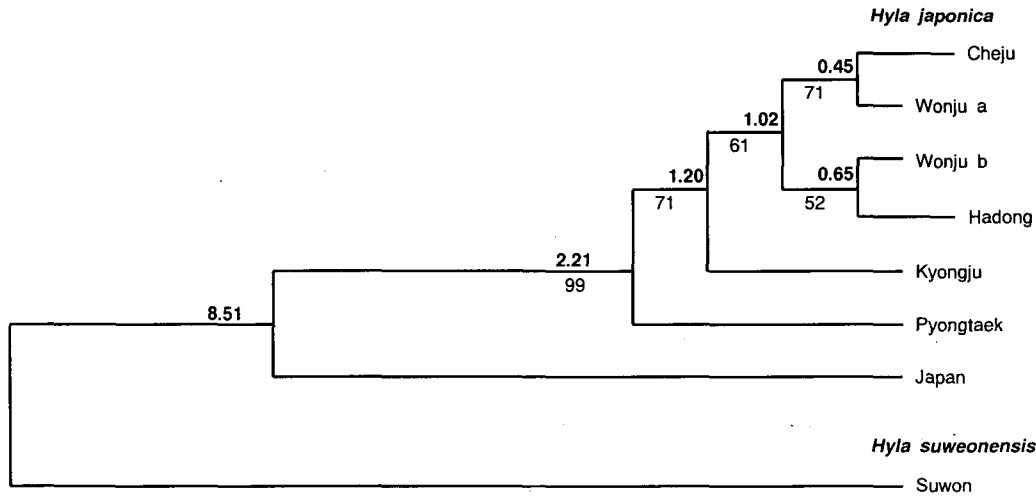


Fig. 4. Neighbor-joining phenogram of mitochondrial cytochrome *b* gene in treefrogs based on Tamura-Nei distance. Nodal values above indicate percentage divergence and below indicate percentage support for branches in 1000 bootstrap replications.

specific was clearly distinct from the Korean population. This value was probably due to geographical differentiation, in contrast to the values of sequence divergence of other conspecifics, for example between *Rana rugosa* (14.7%), *R. dybowskii* (16.39%) and *R. amurensis* (16.44%, unpublished) from Korea, Japan and Russia (Lee, et al., 1999; Kim, 1999).

When 12S rRNA sequence and cytochrome *b* gene sequences were compared, the sequence homology of the mitochondrial 12S rRNA sequence of *H. japonica* and *H. suweonensis* (94.8% -96.5%) showed a rather narrow range compared to cytochrome *b* gene data (87.54%-88.49%). According to the study of evolutionary relationships among Japanese pond frogs (*Rana nigromaculata*, *R. porosa porosa* and *R. p. brevipedata*), percent similarities of nucleotide sequences ranged from 88.5% to 90.3% at the interspecific level and from 95.9% to 96.4% at the subspecific level using the mitochondrial cytochrome *b* gene, whereas those of 12S rRNA genes were 95.95%-96.3% at the interspecific level and 99.5%-99.8% at the subspecific level (Sumida, et al., 1998).

Based on the data presented above, the statuses of *H. japonica* and *H. suweonensis* species were reaffirmed. Our data also confirmed that the mitochondrial

cytochrome *b* gene is more useful in resolving phylogenetic relationships within and between closely related species. 12S rRNA gene sequence analysis is a better method in examining phylogenetic relationships above the specific level rather than within the species.

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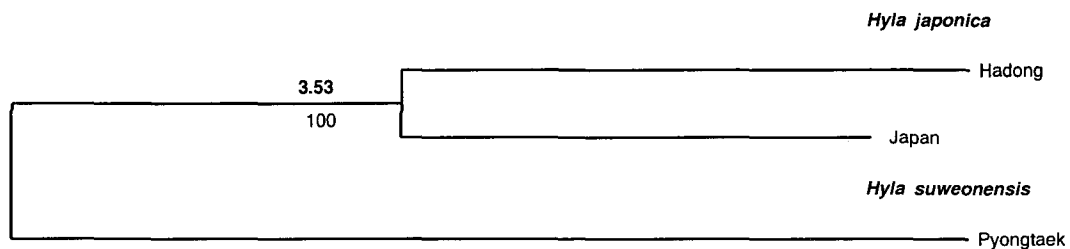


Fig. 5. Neighbor-joining phenogram of mitochondrial 12S rRNA gene in treefrogs based on Tamura-Nei distance. Nodal values above indicate percentage divergence and below indicate percentage support for branches in 1000 bootstrap replications.

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