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Genetic Studies on Korean Anurans: Length and Restriction Site Variation in the Mitochondrial DNA of Tree Frogs, *Hyla japonica* and *H. suweonensis*

***Hei Yung Lee and Chang Shin Park**

Department of Biology, Inha University, Incheon 402-751, Korea

The genetic variation in mitochondrial DNA (mtDNA) was analysed within and between two species of tree frogs, *Hyla japonica* and *H. suweonensis* from South Korea. Purified mtDNAs were digested with each of 11 restriction enzymes which cleave at six base recognition sequences. The genome size of *H. japonica* revealed two types (20.0 ± 0.3 and 19.6 ± 0.3 kb) and this difference is explained by either addition or deletion of about 0.4 kb fragment. On the other hand, the genome size of *H. suweonensis* was about 19.0 ± 0.4 kb only. For the analysis, level of fragment homology (F) and nucleotide sequence divergence (*p*) were estimated from comparisons of digestion profiles. Among four populations of *H. japonica*, substantial mean sequence divergence was 0.017 (range 0.001-0.026); between identical types, 0.001 (small type) and 0.004 (Large type) respectively; between different ones, 0.024 (range 0.023-0.026). The level of sequence divergence between two species was 0.142 (range 0.131-0.146). This result suggested that two species were distinctly differentiated species. The divergence time between two species was estimated 7.1 million years.

KEY WORDS: ***Hyla*, Mitochondrial DNA, Nucleotide sequence divergence**

The analysis of genetic variation in mitochondrial DNA (mtDNA) with restriction endonucleases has proved to be a useful complement to other molecular and morphological methods for study of natural populations. MtDNA study has been performed in invertebrates (Hale and Singh, 1986; Latorre *et al.*, 1986; Rand and Harrison, 1989) and vertebrates (Avise and Lansman, 1983; Brown, 1983; Carr *et al.*, 1987; Lee *et al.*, 1988, 1989; Riddle and Honeycutt, 1990). An application of mtDNA is the measurement of divergence within and between species and reconstruction of phylogenetic relationships. In general, phylogeny is a genetic trace of a gene pool in each taxon over evolutionary time (Zink and Avise, 1990). So the phylogenetic study on a common set of organisms should be carried out by several molecular methods which expose genetic variation directly.

The restriction-enzyme fragment analysis of mtDNA was carried out with two sympatric species of *Hyla japonica* and *H. suweonensis*. *H. japonica* is widely distributed in South Korea but *H. suweonensis* has restricted area as well as sympatric population with *H. japonica* (Yang and Park, 1988). In South Korea tree frog was reported to be one species, *H. arborea japonica*, until Kuramoto (1980) demonstrated that *H. a. japonica* renamed into *H. japonica* comparing with European *H. arborea* and the existence of a sympatric species, *H. suweonensis*, from analysis of mating call differences.

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Yang *et al.* (1981) confirmed these results with sonogram, morphometrics and electrophoresis. They also reported that these two species were morphologically indistinguishable sibling species. Although these species are morphologically similar, allozyme and chromosome data suggest that they have distinct genetic differences (Yang and Park, 1988; Yu and Lee, 1990).

In this study we examined mtDNA differentiation between two species of *Hyla*, and compared divergence times estimated from independent calibration of mtDNA and previous allozymes.

Materials and Methods

Samples of *H. japonica* were obtained from 4 localities and *H. suweonensis* from 1 locality where two species distributed sympatrically. These collection sites were listed in Table 1. Alive individuals from each locality were transported to the laboratory and took out the fresh tissues (liver and heart) which were pooled because of a small amount of samples. Preparation and purification of mtDNA were conducted according to Zimmerman *et al.* (1988).

Eleven restriction endonucleases with hexanucleotide recognition sites were used. Enzyme digestions were conducted overnight under conditions recommended by the enzyme suppliers (Promega). The mtDNA fragments were electrophoresed in 0.8% agarose gel and detected by ethidium-bromide staining according to the published procedure (Lee and Park, 1991b). A *Hind* III digest of Lambda DNA was used as size standards of each gel.

All analyses were based on mtDNA fragment variation. Sequence divergence (p value) that was calculated from shared fragments (F value) between mtDNAs compared was estimated according to Upholt's (1977) fragment method. The distance divergency was clustered by UPGMA (Sneath and Sokal, 1973) using the average of p values.

Results

The 11 restriction endonucleases produced an average of 40 scorable fragments among 4 populations of *H. japonica* and 29 in 1 population of *H. suweonensis*. The numbers of fragment for all restriction endonuclease digestions were listed in Table 2. The fragments smaller than 0.5 kb were not detected. We assumed that fragments with the same mobilities have identical nucleotide sequences.

MtDNA genome size

Fragment patterns of the mtDNA present in *H. japonica* revealed two different types of its genome size, with large type (designated by 'L') being 20.0 $\pm$ 0.3 kb and small type ('S') being 19.6 $\pm$ 0.3 kb. Type S is found in central populations (abbreviations of localities, SW and KC) whereas type L occurs in the southern populations (CL and MD) of south Korea. These size variation within species was observed in the largest fragment of digests which were treated with *Bam* HI, *Bgl* I, *Bgl* II, *Eco* RI, *Pvu* II and *Xba* I enzymes. Four examples of them were presented in Figs. 1 and 2. Only *Pst* I, however, showed different fragment pattern in the smallest one of mtDNA (Fig. 2). In the other enzymes, it hardly be found the

Table 1. Localities and numbers of specimens used for mitochondrial DNA analysis

Species Localities (abbreviations)	Dates of collection	Number of specimens
Hyla japonica		
Suhdun-dong, Suwon-si, Kyonggi-do (SW)	1989. 5	10
Kangchon, Chunsong-gun, Kangwon-do (KC)	1989. 7	12
Chili-mt., Hadong-gun, Kyongsangnam-do (CL)	1989. 6	8
Mudung-mt., Dong-gu, Kwangju-si (MD)	1989. 6	8
H. suweonensis		
Suhdun-dong, Suwon-si, Kyonggi-do (SW)	1989. 5	6

Table 2. Comparative analysis and estimate number of mtDNA fragments between and within 2 species of the genus *Hyla*.

Species	Localities	Restriction enzymes											
		Ava I	*Bam* HI	*Bcl* I	*Bgl* I	*Bgl* II	*Bst* EII	*Cla* I	*Eco* RI	*Pst* I	*Pvu* II	*Xba* I	Total
H. japonica													
	SW	4	3	5	2	3	5	2	5	4	4	3	40
	KC	4	3	5	2	3	5	2	4	4	4	3	39
	CL	4	3	7	2	3	3	2	4	4	4	3	39
	MD	4	3	7	2	5	3	2	4	4	4	4	42
	SW/KC	4	3	5	2	3	5	2	4	4	4	3	39
	SW/CL	4	2	4	1	2	2	1	3	2	3	2	26
	SW/MD	4	2	4	1	2	2	1	3	2	3	2	26
	KC/CL	4	2	4	1	2	2	1	3	2	3	2	26
	KC/MD	4	2	4	1	2	2	1	3	2	3	2	26
	CL/MD	4	3	7	2	3	3	2	4	4	4	2	38
H. suweonensis													
	SW	5	3	4	2	3	0	2	2	2	4	2	29
H. japonica/ H. suweonensis													
	SW, KC, CL/SW	0	0	0	0	0	X	1	1	0	1	0	3
	MD/SW	0	0	0	0	1	X	1	1	0	1	0	4

The letter of x indicates no data for restriction enzyme.

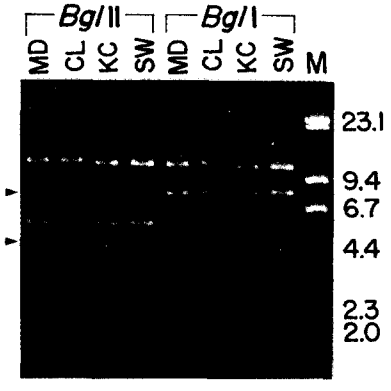

Fig. 1. Electrophoretic patterns of *Hyla japonica* mtDNA. The two different types of genome size are distinguished in the largest fragment. Arrow heads indicate fragments showing interindividual variation from that of MD population. M: marker.

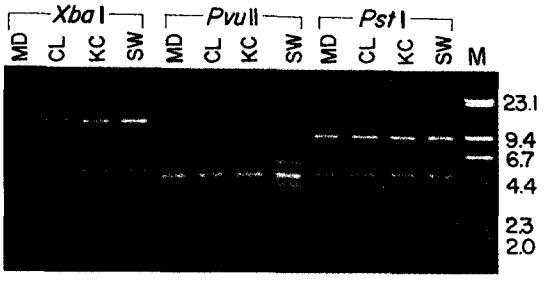

Fig. 2. Electrophoretic patterns of *Hyla japonica* mtDNA. Two types in which differed by either addition or deletion of about 0.4 kb fragment are apparent in comparing the small (19.6 kb) and large genome (20.0 kb); (*Pvu* II) one fragment becomes larger if there is no cleavage site for the restriction enzyme within the fragment; (*Xba* I) one additional fragment is present if the fragment includes a cleavage site. M: marker.

distinctive fragments which express the difference of genome size. The difference between two types in the genome size was also found in the electrophoretic pattern of the crude samples which were induced by spoid pumping (Fig. 3A). Because the crude samples produced by spoid pumping contain not only supercoiled and nicked circular but linear types which were cut randomly, we could easily compare the linear types without restriction enzyme digestion. The above proce-

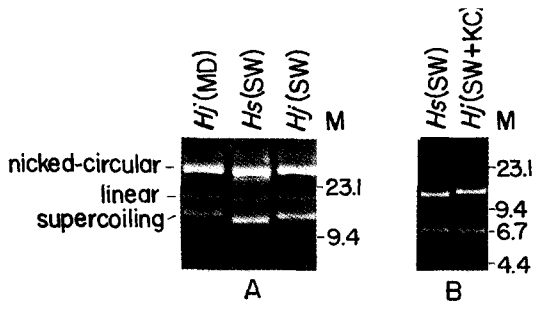

Fig. 3. Electrophoretic patterns of undigested (A) and *Cla* I-digested (B) mtDNA from two species (*Hj*: *H. japonica*, *Hs*: *H. suweonensis*). M: marker.

dures, therefore, could be valuable data for the comparisons of the genome size within and between species together with the results of restriction enzyme digestion.

The mtDNA of *H. suweonensis* analyzed only in one population has no evidence for genome size difference within the population and the estimated size was approximately 19.0 ± 0.4 kb. A comparison of the genome sizes between mtDNA of two species evidently appeared in both the restriction enzyme digests and the electrophoretic pattern of crude samples as those of *H. japonica* (Figs. 3A, B).

Variation within and between species

Of the 4 populations of *H. japonica* examined, intrapopulation variation of mtDNA fragments was identified from three populations: *Bst* EII and *Eco* RI digests from SW population, *Bst* EII from KC, and *Bgl* II from MD. For *H. suweonensis*, two genotypes of mtDNA were observed within the sympatric population (SW) and these differed only in one *Bgl* II enzyme. The differences within 3 populations of *H. japonica* and between the two genotypes of *H. suweonensis* are attributed to the presence or absence of recognition sites by base substitution. No intrapopulation variation was observed in digests with the other enzymes. These variant restriction fragments are mainly analyzed in sum of the total fragments for each restriction enzyme and in the comparisons with mtDNA fragments of other populations (e.g., Fig. 1; *Bgl* II).

Overestimates than the genome size in sum of the total fragments, because mtDNA samples examined were pooled from each population, have a difficulty indistinguishable heteroplasmy that contain mtDNAs of more than two types within a individual. However, we included them into intrapopulation variation as adopted in the comparisons within and between two species.

Two kinds of mtDNA variation were detected among 4 populations of *H. japonica*: 1) variation in the number of fragments, attributable to base substitutions, 2) variation in the length of a fragment without alterations in the number of fragments. The first was observed in the *Xba* I digests from MD population, differing by the one polymorphic restriction site (Fig. 2). For comparisons within species, the most common genotype examined was chosen as the reference genotype. Therefore, a change in fragment pattern *Xba* I was due to the gain of a restriction site relative to the reference patterns. The second variation was mainly found in the largest fragment of digests with the remaining other enzymes and it may be due to the addition or deletion of small fragment, about 0.4 kb.

In the comparison between two species, only one fragment was shared from digests produced with *Cla* I, *Eco* RI and *Puu* II enzymes (Table 2).

MtDNA sequence divergence

The fraction of shared restriction fragments (F) and the nucleotide sequence divergence (*p*) ere reported for both intra- and interspecific comparisons in Table 3. The mean nucleotide sequence divergences between the identical types in mtDNA genome size from *H. japonica* were 0.10% (S

Table 3. Intra- and interspecific mtDNA differentiation in the genus *Hyla*.

Species		Localities				
		SW	KC	CL	MD	SW
H. japonica	SW	–	.987	.658	.634	.094
	KC	.001	–	.667	.642	.095
	CL	.024	.023	–	.938	.094
	MD	.026	.025	.004	–	.119
H. suweonensis	SW	.146	.145	.146	.131	–

Results are based on restriction profiles of 11 enzymes. Data above the diagonal are total proportions of shared restriction fragments (F), those below the diagonal are nucleotide sequence divergence (*p*).

type) and 0.40% (L type), whereas the value between the different types was more higher, about 2.45%. These results indicate that the types might be grouped into two distinct assemblages.

The *p* values were used to generate the phenogram (UPGMA) shown in Fig. 4. The mtDNA types in *H. japonica* could be grouped into two distinct phylogenetic assemblages. The average *p* value between mtDNA fragment patterns of the two species was 14.20%. This level of *p* value between the two species of tree frog is typical of well differentiated congeneric species.

Discussion

MtDNA variation within species

There are two distinct mtDNA types within *H. japonica* species that differ in nucleotide sequence divergences (2.45%) estimated by Upholts' formura (1977). The differences between two types were observed in the genome size by addition or deletion of large or small mtDNA fragment rather than restriction site by base substitution. Although nucleotide base substitutions are dominantly responsible for intraspecific variation among mtDNA genotypes, addition or deletion have also been reported (Cann and Wilson, 1983; Bermingham *et al.*, 1986). The results for comparisons between the central (SW and KC) and the southern populations (CL and MD) of *H. japonica* suggest that an addition or deletion of about 0.4 kb fragment have occured in pathway leading from common mtDNA type. Most addition or deletion are only a few base pairs in length (Cann and Wilson, 1983), but others have been several hundred base pairs long (Bermingham *et al.*, 1986; Lee and Park, 1991b). Large additions appear to be a recurrent feature of amphibian (and possibly reptilian and fish) mtDNA; for example, intraspecific variation of the genome size has been observed in frogs *R. amurensis* and *R. rugosa* of the genus *Rana* resulted from the difference by about 0.5 kb fragment (Lee and Park, 1991b). Also, Bermingham *et al.* (1986), and Becker *et al.* (1988), noted that size polymorphisms tended to be more prevalent in lower vertebrates than in mammals.

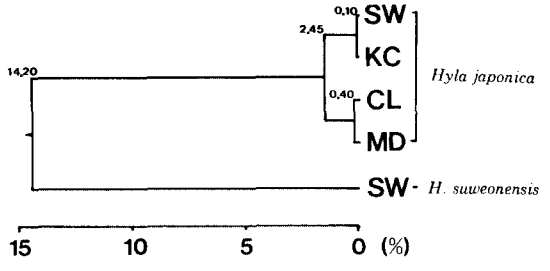

Fig. 4. Genetic phenogram of the genus *Hyla* based on the analysis of mtDNA sequence divergence within and between two species.

In general, length variants are useful in revealing population subdivision as this study where restriction site variation is limited. The estimated sequence divergence between mtDNAs from the two types, 2.45%, is about ten times greater than that (average of 0.25%) between the populations of identical types, and is also greater than that within species of other lower vertebrates such as fishes (Avise *et al.*, 1986; Becker *et al.*, 1988) and frogs (Lee and Park, 1991a, b). If we assume that divergence occurs at a rate of approximately 2% per million years (Brown, 1985), these two types have diverged over 1 million years ago.

Of other studies addressing intraspecific variation in *H. japonica*, allozyme data (Yang and Park, 1988) showed that genetic variation of *H. japonica* populations was about two fold more variable than that of *H. suweonensis* populations. *H. suweonensis* have not only restricted populations but also low intraspecific allozyme variation. Because *H. suweonensis* mtDNA was carried only in one population, comparison among populations could not be analysed. But it could suggested that the low genetic variation within species, based on the similar patterns between allozyme and mtDNA in other studies, can be caused by bottle necking after speciation (Becker *et al.*, 1988).

MtDNA differentiation between species

Hyla japonica was distributed in all areas, while *H. suweonensis* had restricted sites and was sympatric species with *H. japonica* (Yang and Yu, 1978). These sibling species are morphologically indistinguishable but reproductively isolated. Yang and Park (1988) reported that genetic relation between two species was rather remote (S = 0.520, D = 0.634), and interspecific similarity was usually similar to that of other amphibia.

Interspecific mtDNA sequence divergence was

calculated to be 14.20%. The estimate of percent sequence divergence between *H. japonica* and *H. suweonensis* is higher than the level of interspecific differences observed in several frog and toad species. Spolsky and Uzzell (1984) identified mtDNA types separated by 8.1% between congeneric species of the frog genus *Rana*. Szymura *et al.* (1985) give a value of 9.4% between the frog species *Bombina bombina* and *B. variegata*. Lee and Park (unpublished) also found a level of divergence between the toad species *Bufo bufo* and *B. stejnegeri* was 7.31%. Whereas much higher variation than present result has been reported in mtDNA analyses among Korean *Rana* species and the mean sequence divergence was 19.2% (range 9.2-21.9) (Lee and Park, 1991b). Although mtDNA sequence divergence has been estimated for several species of vertebrates, these values may not be directly comparable because of differences in the numbers and types of restriction enzymes used in the various studies and in the evolutionary rates from different groups. Furthermore, there are also variable methods to measure the sequence divergence. Our results were based on mtDNA fragment variation but most studies were estimated by restriction site maps. Analyses of sites and fragments, however, yield nearly congruent (Zink and Kittmann, 1991). Because the results of Lee and Park (1991b) containing present one were assayed with identical suites of restriction enzymes, data can be compared directly to each other. The estimation of mtDNA sequence divergence between the two species of tree frogs is typical of well differentiatied congeneric species. These interspecific difference also support by that two species differentiated by pericentric inversion (Lee and Yu, 1988; Yu and Lee, 1990) play an important role in the process of speciation. With above other survey we suggest that both the size and site variation are responsible for interspecific mtDNA difference.

Using an estimate of a 2% mtDNA sequence divergence per million years as intraspectific comparison, we could conclude that the two species diverged about 7 million years ago. This age is more later than the date of 3.2 million years ago, which has been estimated from allozyme genetic distances (Yang and Park, 1988). The difference between the two estimates may be related to independent calibration for their divergent times.

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한국산 무미류에 대한 유전학적 연구 : 청개구리속 2종(***Hyla japonica, H. suweonensis***)에 대한 mtDNA의 크기 및 제한효소 인식위치의 변이

이혜영 · 박창신(인하대학교 이과대학 생물학과)

한국산 청개구리속 2종의 종내 및 종간 mitochondrial DNA 변이를 분석하였으며 총 11개의 제한효소를 사용하였다. *H. japonica*의 종내 mtDNA 크기에서는 2가지 형(20.0 ± 0.3, 19.0 ± 0.3 kb)이 관찰되었으며 이는 약 0.4 kb 절편의 부가 또는 소실로 설명될 수 있다. 그러나 *H. suweonensis*의 mtDNA 크기는 19.0 ± 0.4 kb로 종간 차이만 나타났다. 절편양상에서 공통절편수의 비율(F값)과 염기분화정도(p값)를 산출하였다. *H. japonica*의 4개 집단간 평균 염기분화정도는 1.7%였고 mtDNA 크기의 동일형 간에는 각각 0.1%(small형)와 0.4%(large형)로 변이가 낮았으나 다른형 간에서는 2.4%로 다소 높은 변이를 보였다. 또한 2종간 염기분화정도의 값 14.2%는 오래전에 분화된 종간차이임을 제시하며 그 분화연대는 약 7백만년전으로 추정된다.