Mitochondrial DNA Variations among Three Species of Korean Planorbid Snails: *Gyraulus convexiusculus*, *Hippeutis cantori* and *Segmentina hemisphaerula**

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=국문요약=

한국산 또아리물달팽이과 3종 내의 미토콘드리아 DNA 변이

정평림 · 정영헌 · 정은경 · 김대순

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한국산 담수 또아리물달팽이과(Planorbidae)에 속하는 또아리물달팽이(*Gyraulus convexiusculus*), 수정또아리물달팽이(*Hippeutis cantori*) 및 배꼽또아리물달팽이(*Segmentina hemisphaerula*) 3종에 대한 종간 유전적 변이와 이들 상호간의 분류학적 유연 관계를 생화학적 측면에서 밝히고자 하였다. 즉, 모계유전으로 자손에 유전되고 있는 미토콘드리아 DNA(mitochondrial DNA; mtDNA)의 변이를 보기 위하여 제한효소(restriction enzyme)를 처리하고 잘라진 mtDNA 절편들을 상호 비교하는 restriction fragment length polymorphism(RFLP) 기법을 응용하였다.

본 실험에서 10개의 제한효소 중 *Cla* I, *Dra* I, *Eco* RI, *Hin* dIII, *Kpn* I 및 *Pst* I의 6개 제한효소에서 좋은 결과를 얻어 종간의 공통절편(shared fragments)을 비교하고, 염기분화율(nucleotide divergence rate)을 각각 측정하였다.

미토콘드리아 DNA 크기(genome size)는 또아리물달팽이가 12.08 kb, 수정또아리물달팽이가 14.4 kb, 그리고 배꼽또아리물달팽이가 12.93 kb로 관찰되었다. 염기분화율(p)은 또아리물달팽이/수정또아리물달팽이 군에서 $p = 12.7\%$, 배꼽또아리물달팽이와 상기 2종군 사이의 염기분화율은 $p = 56.6\%$여서 배꼽또아리물달팽이류는 타 2종보다 그 분화율이 매우 높음을 알 수 있었다.

이상의 결과로 보아 분류군 (taxa)의 mtDNA 변이에 의한 RFLP기법이 앞으로 한국산 담수 패류 연구에 널리 응용될 수 있음이 확인되었다.

INTRODUCTION

The Korean freshwater planorbid snails are small species (about 3~8 mm in size) belong-

ing to the pulmonate family Planorbidae, and have been reported so far as only three species, *Gyraulus convexiusculus* (Hutton), *Hippeutis (Helicorbis) cantori* (Benson) and *Segmentina (Polypylis) hemisphaerula* (Benson). These species are especially implicated in the transmission of digenetic trematode infections to the vertebrate hosts including human beings, of

these snails, *H. cantori* was reported as the snail intermediate host of *Fibricola seoulensis,* one of the snail-borne human intestinal trematodes in Korea (Seo *et al.,* 1988). However, the systematic studies of these snails have not been satisfied in Korea.

Mitochondrial DNA (mtDNA) is usually maternally inherited in animals. Urnsprung and Schabtach (1965) showed that in tunicates the paternal mtDNA are lost prior to or concurrent with the entry of the sperm into the oocyte. The rate of evolution of mtDNA may be higher than nuclear DNA (Vawter and Brown, 1986), facilitating the identification of species boundaries and the study of phylogenetic relationships of closely related species. The mtDNA studies have been performed for the population structure, gene flow, biogeography, species hybridization and the systematic studies among taxa.

The phylogeny is estimated mainly by comparing the various characteristic patterns of the taxa of interest. The restriction patterns of mtDNA among taxa are the basic data of restriction fragment length polymorphisms (RFLPs) used to estimate maternal phylogeny. Each pattern of mtDNA is composed of several bands generated by endonuclease digestion of the mtDNA. DNA sequencing has also shown that the differences in the restriction patterns among different mtDNAs are primarily due to single base changes. Individuals or each species that share the same restriction patterns may be considered to be a descendent of the same maternal ancestor. If two individuals or species have different patterns, it may be possible to relate the patterns by the gain or loss of a specific restriction site. The greater the proportion of shared fragments, the closer the two individuals or species are considered to be. The accuracy of the inferred phylogeny depends on the identity of the fragments in size.

Several studies have been done on the genetic variation of the mtDNAs in marine mussel species of *Mytilus* (Skibinski, 1985; Edwards and Skibinski, 1987; Hoeh *et al.,* 1991; Hoffmann *et al.,* 1992; Zouros *et al.,* 1992), on the sequences and size variation of mtDNA in the sea scallop species (La Roche *et al.,* 1990; Gjetvaj *et al.,* 1992) and in tracing the ancestry of introduced land mollusk, *Cepaea nemoralis* (Stine, 1989). By Liu and Mitton (1993), a technique to reveal RFLPs was well developed on a freshwater mollusk, genus *Physa.* However, the mtDNA techniques have not been applied yet on the Korean freshwater mollusks.

This study was carried out to solve the systematic problems of the Korean planorbid snails malacologically and to develop the mtDNA technologies for the freshwater snails.

MATERIALS AND METHODS

1. Collection of snails

The planorbid snails collected for mtDNA studies were *Gyraulus convexiusculus, Hippeutis cantori* and *Segmentina hemisphaerula.* The collected localities, dates, habitats and catalog numbers of three planorbid species are shown in Table 1.

2. Preparation of tissue samples

Only live snail specimens were used in this mtDNA study. The individuals of each species were pooled to get about 1~2 g of tissue specimens. The snail specimens pooled were placed in a watch-glass on a pan filled with crushed ice, and then minced thoroughly with a curved scissors. The minced tissues were

Table 1. Snail specimens of *Gyraulus convexiusculus, Hippeutis cantori* and *Segmentina hemisphaerula* collected from various localities in Korea

Species	Localities collected	Date collected	Habitat	Catalog number*
G. convexiusculus	Onyang-1, Chungnam	July 10, 1994	rice field	IUMC 60
	Songdo, Inchon	July 15, 1994	ditch	IUMC 62
H. cantori	Yangsung, Kyungkido	July 10, 1994	pond	IUMC 59
	Songdo, Inchon	July 15, 1994	ditch	IUMC 63
S. hemisphaerula	Onyang-2, Chungnam	July 10, 1994	rice field	IUMC 61
	Songdo, Inchon	July 20, 1994	ditch	IUMC 64

*The specimens of each species were catalogued as Inha University Medical College (IUMC) voucher specimens.

placed in a glass bar homogenizer with 3 ml of ice-cold 0.25 M sucrose-TEK, and then homogenized about ten times for breaking even very small pieces of snail shells inserted during the preparation process. The tissue specimens ground roughly were moved to the Teflon bar homogenizer (B. Brown Co., German) and homogenized with 30 ml of ice-cold 0.25 M sucrose-TEK (50 mM Tris-HCl, pH 7.5, 200 mM EDTA, 1.5% KCl) including 140 μg/ml of ethidium bromide until well ground. The ethidium is essential for protecting DNA from nucleases. The homogenized tissue specimens were centrifuged at 700×g for 15 min. at 4℃ to get the mitochondria, nuclei and other cell debris in the supernatant.

3. Removal of mucopolysaccharides

Mucopolysaccharides which is inhibiting the action of restriction endonucleases were co-purified with DNA. Using a long-stem pipette, the supernatant with 30 ml of ice-cold 0.25M sucrose-TEK was slowly placed on the layer of 30 ml of ice-cold 1.1 M sucrose-TEK in a test tube, and centrifuged at 13,000×g for 50 min. at 4℃ to collect the mitochondria. The mucopolysaccharides at the interface of sucrose gradients were removed when the supernatant was decanted to get the pellet of

mitochondria on the bottom of the test tube.

4. Isolation of mitochondrial DNA

The pellet of mitochondria was resuspended by a vortex mixer with 4 ml of ice-cold 2% NP-40 in TEK. NP-40 detergent disrupts cell and mitochondrial membranes, but not nuclear membranes. The tissue samples were transferred to the Eppendorf tubes, then placed on the crushed ice for 40 min. and centrifuged in a microcentrifuge at 12,000 r.p.m. for ten min. at 4℃. One part of the supernatant was mixed with one part of phenol in an Eppendorf tube, and incubated at 37℃ for ten min. After putting it on ice for ten min., the tissue specimens were microcentrifuged at 12,000 r.p.m. for ten min. One part of the clear upper phases of the tissue specimens was mixed again with an equal part of a mixture of phenol: chloroform, 1:1, and shaked thoroughly. The specimens were let stand at room temperature for five min. and then microcentrifuged for five min. One part of the upper clear phases was then removed to the new Eppendorf tubes with two parts of cold 100% ethanol and thoroughly shaked. This specimens were stored in a freezer at − 20℃ overnight. The mtDNA samples were precipitated by microcentrifugation at 12,000

r.p.m. for ten min. The supernatant with ethanol was then decanted, and the pellets of mtDNA for drying were stored at 37℃ in an incubator.

5. Digestion of mitochondrial DNA by restriction endonucleases

The DNA pellet was dissolved in 20~40 μl of TE buffer solution (pH 8.0), and stored at −20℃. Six to ten μl of DNA in TE buffer solution were diluted with distilled water up to 14 μl. Two μl of spermidine (0.1 M), two units of enzyme (endonuclease) and two μl of the appropriate digestion buffer were added, and these were mixed by tapping the tube. The digestion solution was incubated overnight (at least 6 hrs.) at appropriate temperatures according to the enzymes, and then four μl of stop buffer was added. Ten restriction endonucleases (*Bam* HI, *Bcl* I, *Bst* EII, *Cla* I, *Dra* I, *Eco* RI, *Hin* dIII, *Kpn* I, *Pst* I and *Xba* I) were employed in this study.

6. Agarose gel electrophoresis

0.5 g of agarose was dissolved in 50 ml of 1 × TEA buffer (1%) by heating the solution to boiling. The agarose solution was let cool to 60℃ and poured into a 12 × 9.5 × 0.5 cm gel tray. A comb was used to make the sample wells. When the agarose became solid, the comb was removed. Then, the gel was submerged in the electrophoresis tank. The samples were loaded into the wells, and electrophoresed in 1× TEA buffer at 60 volts until the bromophenol blue has traveled 6 cm. λ phage DNA digested with *Hin* dIII was used as a standard marker. The gel was immersed in 200 ml of 1× TEA buffer containing 200 μl of ethidium bromide for at least 20 min. for staining, then rinsed the gel two or three times with distilled water.

7. Photography

The gel was placed on a UV-340 nm transilluminator (Vilber Lourmat Co., France) to check the mtDNA bands and the pictures of gel patterns were taken by using a 35 mm camera (Nikon) with orange YA 3 filter (Kenko Co., Ltd, Japan). The gel pictures were exposed to the microfilm for 15~20 sec. at 2.8. Two blue light bulbs (500W/110V) were set up 25 cm far from the gel plates.

8. Calculation of F and *p* values

The number of restriction fragments for each restriction endonuclease and the number of shared restriction fragments between species were calculated. The F value for similarity between species is defined as $F = 2n_{xy}/(n_x + n_y)$, where n_x and n_y are the total number of fragments generated by all of the restriction enzymes used on both the xth and y^{th} species, and n_{xy} is the number of fragments shared by the two species. The fraction of base substitution (nucleotide divergence) were calculated with the following formula (Upholt, 1977):

$$p = 1 - \{[(F^2 + 8F)^{1/2} - F]/2\}^{1/r}$$

Where, p = nucleotide divergence, F = similarity between species, r = recognition site of restriction enzyme.

The distance divergences were clustered by their sequence divergences estimated by UPGMA (Sneath and Sokal, 1973).

RESULTS

Restriction patterns in this study were generated by digesting the mtDNAs of the three Korean planorbids with six endonucleases out of ten endonucleases employed. Data have been collected from a total of 100 snails

Table 2. Mitochondrial DNA fragment numbers of three Korean planorbid snails

Species	Restriction endonucleases										
	Bam HI	*Bcl* I	*Bst* EII	*Cla* I	*Dra* I	*Eco* RI	*Hin* dIII	*Kpn* I	*Pst* I	*Xba* I	Total
G. convexiusculus	0	0	0	1	4	1	3	0	1	0	10
H. contori	0	0	0	2	0	2	2	0	0	0	6
S. hemisphaerula	0	0	0	1	0	4	4	1	1	0	11

Table 3. Shared restriction fragments among three Korean planorbid snails

Species	Restriction endonucleases										
	Bam HI	*Bcl* I	*Bst* EII	*Cla* I	*Dra* I	*Eco* RI	*Hin* dIII	*Kpn* I	*Pst* I	*Xba* I	Total
G. convexiusculus/ *H. cantori*	0	0	0	0	0	0	1	0	0	0	1
G. convexiusculus/ *S. hemisphaerula*	0	0	0	0	0	0	0	0	0	0	0
H. cantori/ *S. hemisphaerula*	0	0	0	0	0	1	0	0	0	0	1

pooled in each experimental species.

The mtDNA genome sizes of three planorbid species were 12.08 kb, 14.48 kb and 12.93 kb in *G. convexiusculus*, *H. cantori* and *S. hemisphaerula*, respectively, based on direct comparisons of linearized mtDNA with the size of co-migrating standard marker, λ phage DNA.

The numbers of mtDNA fragments digested by ten restriction endonucleases are shown in Table 2.

These ten restriction endonucleases produced a total of 10, 6 and 11 restriction fragments from mtDNAs of *G. convexiusculus*, *H. cantori* and *S. hemisphaerula*, respectively. The restriction endonucleases, *Bam* HI, *Bcl* I, *Bst* EII and *Xba* I did not produce any cutting restriction sites; however, six other restriction endonucleases such as *Cla* I, *Dra* I, *Eco* RI, *Hin* dIII, *Kpn* I and *Pst* I generated patterns that differed from their restriction sites

among three species. Especially, *Hin* dIII produced three fragment patterns in *G. convexiusculus*, two fragment patterns in *H. cantori* and four fragment patterns in *S. hemisphaerula*.

Shared restriction fragments among three different Korean planorbid snails are shown in Table 3.

Most restriction endonucleases did not produce shared restriction fragments except *Eco* RI and *Hin* dIII. *G. convexiusculus* shared one restriction fragment for *Hin* dIII at size 6.8 kb with *H. cantori* and did not share any fragment with those of *S. hemisphaerula*. One restriction fragment was also shared for *Eco* RI at size 7.7 kb between *H. cantori* and S. *hemisphaerula*. Figures 1-4 show typical electrophoretic patterns of mtDNA restriction fragments digested with four different restriction endonucleases.
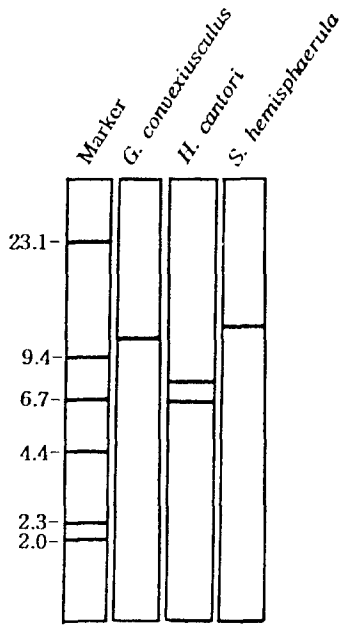
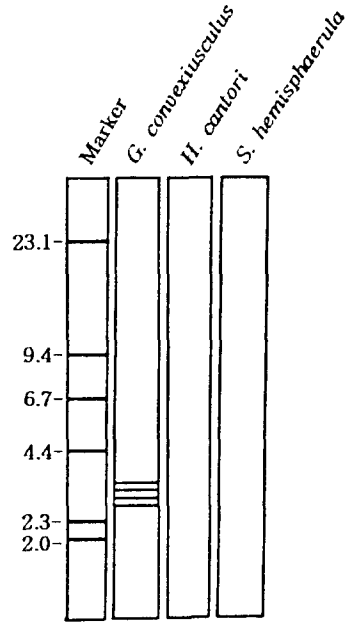The most basic method for assessing the

Fig. 1.

Fig. 2.

Fig. 1-2. Diagramatic representations of digestion patterns for the restriction enzymes *Cla* I (Fig. 1) and *Dra* I (Fig. 2).
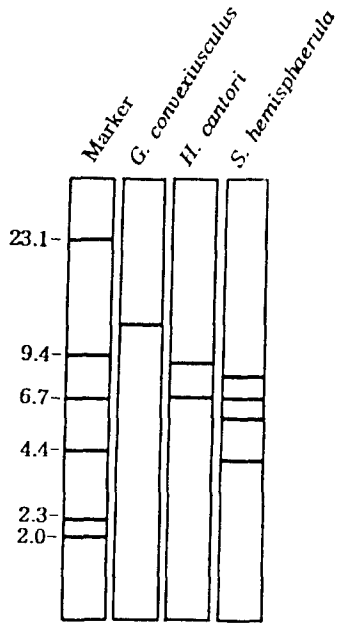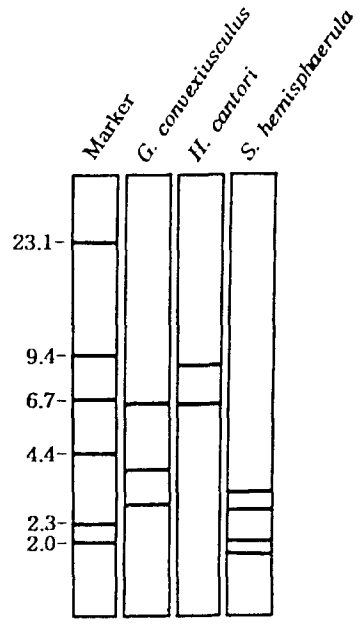

Fig. 3.

Fig. 4.

Fig. 3-4. Diagramatic representation of digestion patterns for the restriction enzymes *Eco* RI (Fig. 3) and *Hin* dIII (Fig. 2).

Table 4. Estimates of genetic divergences of mtDNA among three Korean planorbid snails

	G. convexiusculus	H. cantori	S. hemisphaerula
G. convexiusculus	—	0.125	0.000
H. cantori	0.127	—	0.117
S. hemisphaerula	1.000	0.131	—

The fragment homology values; above diagonal
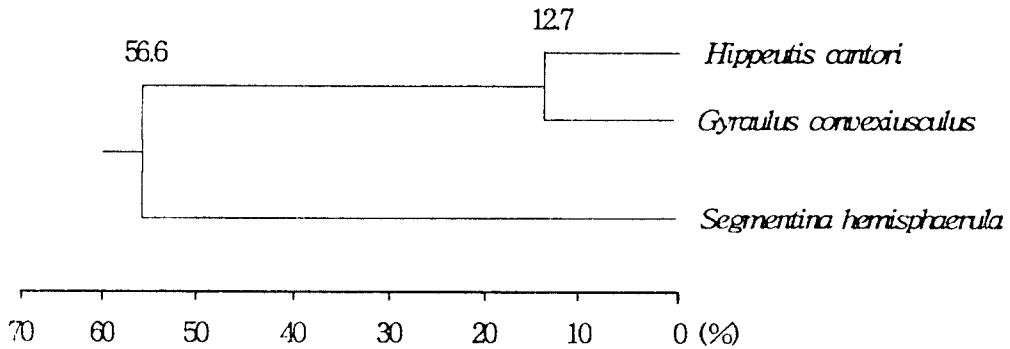The values of nucleotide sequence divergence; below diagonal

Fig. 5. Dendrogram drawn from UPGMA clustering of mtDNA nucleotide divergences among three Korean planorbid snails.

genetic relationships of taxa is to construct networks based on the number of shared fragments among species. The genetic relationships among the species were quantified and summarized by computing the proportion of shared fragments, p value for each pair of species. The fragment homologies (F values) are shown above diagonal, and nucleotide sequence divergences (p values) are shown below diagonal in Table 4.

The fragment homology value between G. convexiusculus and H. cantori was 0.125; but, the value was 0.0 between G. convexiusculus and S. hemisphaerula which shows no similarity at all. The F value between H. cantori and S. hemisphaerula was 0.117. The values of nucleotide sequence divergence (p values) were

estimated to be 0.127 in G. convexiusculus and H. cantori, 1.0 in G. convexiusculus and S. hemisphaerula, and 0.131 in H. cantori and S. hemisphaerula.

A dendrogram was drawn from UPGMA clustering of mtDNA nucleotide sequence divergences among three Korean planorbid snails (Fig. 5). H. cantori and G. convexiusculus were clustered together at 12.7% of divergence rate of nucleotide sequence, while S. hemisphaerula was quite far from the H. cantori and G. convexiusculus clustering at the rate of 56.6%. Mitochondrial DNA sequence divergent time was calculated by using an estimate of 2% sequence divergences per million years (Shields and Wilson, 1987). According to Fig. 5, H. cantori and G. convexiusculus were

diverged about 6.35 million years ago, and *S. hemisphaerula* diverged from these two species about 28.3 million years ago.

DISCUSSION

Mitochondrial DNA from a land snail species, *Cepaea nemoralis*, has been isolated by Stine (1986). He extracted mtDNA from the soft body part of tissues such as foot, genitalia and hepatopancreas excluding mantle, gut, love dart and columella ligaments, since the mantle, gut, and love dart contain mucopolysaccharides, nucleases and calcium, respectively. The columellar ligaments are also collagenous and very difficult to mince and homogenize. The mtDNAs were isolated from the whole animal tissues of the Korean planorbid snails pooled in this study. It was, however, not practical to extract mtDNA directly from soft body parts of the small-sized planorbid snails (3~8 mm in shell length). Therefore, the whole pooled animal body tissues were used for mtDNA extraction.

The most difficult problem encountered in this study was the purification of the mtDNA from the samples. Three approaches were applied to solve this problem: 1) the concentration of EDTA in TEK buffer was increased, 2) ethidium bromide was added during homogenizing, and 3) mucopolysaccharides were removed. These approaches were successful in allowing some but not all enzymes to cleave the DNA completely and reproducibly.

High EDTA concentrations limit the loss mtDNA occurring due to degradation and mitochondrial membrane breakage. Therefore, it may be necessary (particularly when working with small amount of tissue) to determine empirically which EDTA concentration provides the best yields. The concentration of EDTA may be adjusted, depending on levels of DNase activity. EDTA inhibits DNase activities by chelating divalent cations required for their function. A good starting concentration is generally 100mM EDTA. Isolations of mtDNA from mollusks with high levels of DNase activity have been more successful using 200mM EDTA in their grinding buffer, whereas initial studies of teiid lizards and terrestrial mammals worked well with 1mM EDTA (Hillis and Moritz, 1990). Therefore, the starting concentration of EDTA was 200mM in this study.

The ethidium must be added to inhibit nuclease activity while homogenizing the tissue by means of Stine's method (1989). The third series of attempts to improve the isolation and digestion of mtDNA were to remove mucopolysaccharides. The presence of mucopolysaccharides might account for the observed cohesiveness of the mitochondrial pellets. Sucrose gradient steps were employed to remove the mucopolysaccharides physically at the 0.25 M/1.1 M sucrose gradient interface from particles within the homogenate (Stine, 1986, 1989).

The variable results of the mtDNA digestion suggested that the restriction enzymes were operating suboptimally because of inappropriate assay conditions or residual contamination from the isolation procedure. Incompletely purified mtDNA was either not cut or partially cut by restriction enzyme (Stine, 1986). Our extractions of planorbid mtDNAs were not always fully cut by the methods of Stine (1989) and Liu and Mitton (1993). Our works were done mainly by means of Stine's method (1989) with minor modification; that is, the concentration of spermidine was 0.1M instead of 2.5 mM, and the amounts of restriction endonuclease and buffer were in-

creased twice (2 μl). Nevertheless, the mtDNAs were cleaved reliably with *Cla* I, *Dra* I, *Eco* RI, *Hin* dIII, *Kpn* I and *Pst* I; but, not with *Bam* HI, *Bcl* I, *Bst* EII, and *Xba* I.

Widespread variation in the size of mtDNA genomes of invertebrate matazoans and poikilotherm vertebrates has been recently documented. The variation occurs among species, between individuals of the same species, and even among molecules within an individual (Moritz *et al.*, 1987; Rand and Harrison, 1987). The range of genome sizes, previously reported to be from 14.2 kb to 19.5 kb (Fauron and Wolstenholme, 1976), has been extended to 42 kb (Snyder *et al.*, 1987). La Roche *et al.* (1990) reported that the mtDNA size of sea scallop *(Placopecten magellanicus)* is known to be very large (>31 kb). The sizes of mtDNAs in the Korean planorbids ranged from 12.08 kb to 14.48 kb, which means smaller genome sizes as compared with those of the other invertebrates.

Restriction analysis of mtDNA can be expected to be useful in systematics at or below the level of the genus. Several observations support the hypothesis that 70% of the nucleotide sequence does not very. First, based on direct sequencing, approximately 70 % of the bases are identical for mammals: cows (Anderson *et al.*, 1982), mice (Bibb *et al.*, 1981) and humans (Anderson *et al.*, 1981). Second, members of two families of primates were observed to have an average divergence of 25% based on extrapolation from restriction site analysis (Brown *et al.*, 1979).

Aside from structural changes, some coding sequences may be conservative enough to provide characters useful for phylogenetic analysis among taxa. However, because of the small size of many of these sequences, they will have to be characterized with 4-base pair

restriction endonucleases (Kreitman and Aguade, 1986) or sequenced to make a substantial contribution. However, ten restriction endonucleases employed in the present study were the enzymes recognizing 6 base pairs.

We have confirmed that the three species of Korean planorbid snails were very much differentiated on the basis of the F and *p* values among them, and the RFLP studies using mtDNA techniques are able to be applied for the systematic researches on Korean freshwater snails.

SUMMARY

Three species of Korean freshwater planorbids; *Gyraulus convexiusculus*, *Hippeutis cantori* and *Segmentina hemisphaerula*, were employed in this study to make a systematic relationship by means of restriction fragment length polymorphism (RFLPs) of their mitochondrial DNAs (mtDNAs).

Six out of ten restriction endonuclease used; *Cla* I, *Dra* I, *Eco* RI, *Hin* dIII, *Kpn* I and *Pst* I, gave rise to good outcomes making mtDNA differences among three planorbid species. The mtDNA genome sizes of three snail species employed were 12.08 kb, 14.48 kb and 12.93 kb in *G. convexiusculus*, *H. cantori* and *S. hemisphaerula*, respectively. Out of three planorbid snail species, *H. cantori* and *G. convexiusculus* were clustered together at 12,7% of nucleotide divergence rate, however, *S. hemisphaerula* was diverged quite far from *H. cantori/G. convexiusculus* clustering at the rate of 56.6%.

This study shows a possibility that the RFLP studies using mtDNA techniques are able to be applied for the systematics of Korean freshwater snails.

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