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Comparison of Nucleotide Sequences of 28S rDNA from Two Viviparid Snail Species in Korea: *Cipangopaludina chinensis malleata* and *C. japonica*

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= 국문 요약 =

한국산 논우렁이와 큰논우렁이의 28S rDNA 유전자 염기서열 분석

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한국산 논우렁이(*Cipangopaludina chinensis malleata*)와 큰논우렁이(*C. japonica*)는 형태학적으로 유사하여 그 감별이 용이치 않다. 본 연구는 이 두 종을 대상으로 28S rDNA D1 유전자를 7종의 제한효소로 처리하여 PCR-RFLP기법으로 그 절편을 비교하였다. 절편 상호간에는 차이점을 관찰할 수 없었으나, 두 종으로부터 분석된 28S rDNA D1 유전자의 염기서열에서는 4 부위에서 종간 차이를 보였다.

Key words : Viviparidae, *Cipangopaludina chinensis malleata*, *C. japonica*, 28S rDNA, PCR-RFLP, gene sequence

INTRODUCTION

The Korean rice-field snails belonging to family Viviparidae have regarded as an important fresh-water molluscs, because they are edible as food source in Korea and play a role as the intermediate hosts of some trematode parasites. However, their taxonomic problems have not been clearly solved yet. Kwon *et al.*(1993) recognized two viviparid species in Korea, *Cipangopaludina chinensis malleata* and *C. japonica*. *C. chinensis malleata* distribute nation-widely everywhere in Korea, such as rivers, streams, ponds and rice-fields, but the geographical distribution of *C. japonica* is much restricted only in the southern part of Korea (Park *et al.*, 1997).

In recent years, genetic variations of the organisms have been investigated for phylogenetic analysis using the nuclear genomes. The nuclear ribosomal RNA gene (rDNA) repeat unit has regions evolving at varying rates, and also has been used extensively to study genetic variations and phylogeny at a number of taxonomic levels. Polymerase

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chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques have been developed for molecular genetic identification and examination of the genetic diversity in populations (Bowditch *et al.*, 1993; Liu and Mitton, 1993). Genetic variations of the 28S rRNA gene detected by PCR-RFLP were made as markers of genetic identity (Matsuura *et al.*, 1992; Fujino *et al.*, 1995).

In this study, PCR-RFLP analysis and nucleotide sequences of 28S rDNA between two Korean viviparid species were done to compare their genetic distances. The results obtained in this study were also compared with the nucleotide data from other molluscan species to determine the percentages of homology.

MATERIALS AND METHODS

1. Genomic DNA purification from snails

Snail specimens of *C. chinensis malleata* collected from the Chunchon and Kimhae area and *C. japonica* from the Kimhae area in Korea were used in this study. Adult snail specimens were stored at -70℃ throughout this study. Their shells of snail specimens were removed, then lyophilized and lysed with lysis buffer, proteinase K, and RNAase. The DNAs of the samples were extracted in phenol/chloroform, and precipitated in ethanol by means of a standard method (Sambrook *et al.*, 1989).

2. PCR-RFLP of 28S rDNA D1

PCR was performed using a mixed solution of extracted DNA, primer and reaction mixtures from TAKaRa Ex Taq Kit (TAKARA Shuzo Co., LTD., Japan). Oligonucleotide primers JB9 and JB10 were designed based on evolutionarily conserved regions at the 5′ end of the eukaryotic 28S rDNA. Forward primer JB10 (5′ GATTACCCGCTGAACTTAGCATAT 3′) corresponds to 21-45 positions of the mouse, and reverse primer JB9 (5′ GCTGCATTCACAAA-CACCCCGACTC 3′) corresponds to the positions of 278-302 (Qu *et al.*, 1988). PCR reaction cycles consisted of denaturing at 95℃ for 20 sec, annealing at 55℃ for 30 sec, extending at 72℃ for 30 sec, followed by a final extension of 6 min at the end of 40 cycles. The PCR products were digested by seven restriction endonucleases (*Alu* I, *Msp* I, *Hae* III, *Mbo* I, *Cfo* I, *Rsa* I and *Taq* I) for 1-2hrs at 37℃. The digested products were analyzed with 2% agarose (Metaphor®, FMC BioProducts, Rockland, ME, USA) gel electrophoresis.

3. Sequencing of 28S rDNA

The PCR-products of two viviparid snail species were sequenced by the dideoxy-mediated chain-

Table 1. Pairwise distances between taxa for D1 regions of the 28S rRNA gene*.

Species	1	2	3	4	5	6†
C. chinensis malleata	-	0.016	0.032	0.190	0.108	0.129
C. japonica	4	-	0.045	0.204	0.126	0.142
V. viviparus	8	11	-	0.201	0.116	0.144
Valvata sp.	47	50	50	-	0.181	0.153
C. symbolicum	27	31	29	45	-	0.100
Ampullaria sp.	32	35	36	38	25	-

*Lower diagonal represents absolute distances and upper diagonal represents mean distances.

†Numbers 1 through 6 mean vertically arranged molluscan names from top to bottom.

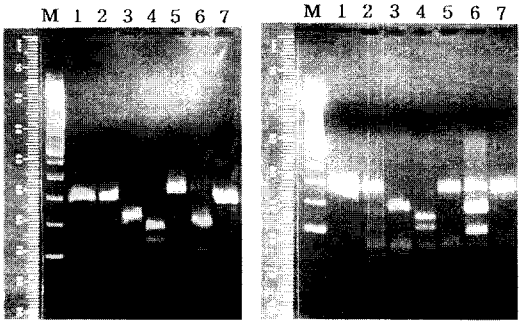

Fig. 1. Agarose gel electrophoretic patterns of PCR-RFLP of 28S rDNA.
Lane M, molecular weight markers(100 bp DNA ladder, GIBCO BRL); lane 1: *Alu* I ; lane 2: *Msp* I; lane 3: *Hae* III; lane 4: *Mbo* I; lane 5: *Cfo* I; lane 6: *Rsa* I; lane 7: *Taq* I. A: *C. chinensis malleata*; B: *C. japonica.*

termination method (Sanger *et al.*, 1977). Sequence reaction mixtures were electrophoresed on 6% polyacrylamide gels and visualized by autoradiography. The nucleotide sequences of two viviparid species were aligned by the Clustal V Multiple Alignment Program (Higgins *et al.*, 1992) with those of *Ampularia* sp. (ASU 78643), *Campanile symbolicum* (CSU79650), *Valvata* sp. (VSU78672) and *Viviparus viviparus* (VVU75863) (data from McArthur *et al.*, 1992). Phylogenetic analyses were performed using both parsimony and distance methods (Swofford, 1993).

RESULTS

RFLP results of PCR-amplified 28S rDNA were analysed to distinguish Korean *Cipangopaludina* species. Seven restriction endonucleases, *Alu* I, *Hae* III, *Mbo* I, *Cfo* I, *Rsa* I and *Taq* I, yielded the same restriction fragment patterns in two viviparid species (Fig. 1A, B). In the D1 region (sequence position number; 1-254), the two species were distinguished each other by the insertions and/or deletions of four inter-specific base pairs (Fig. 2). The results indicate inter-specific differences between two viviparid species. Pairwise distance between taxa for the 5′ 28S rDNA region was 0.016 (Table 1). Fig. 3 shows the most parsimonious tree obtained from a cladistic analysis of sequencing data in Table 1. Bootstrap analysis showed four clades in both distance and parsimonious trees: a clade grouping two experimental viviparid species with *V. viviparus* (88%), a clade grouping with *Campanile symbolicum* (100%), and the other clade with *Valvata* sp. and *Ampullaria* sp. (66%).

DISCUSSION

The technical development of quick and inexpensive methods for the extraction and cleavage of specific sites of DNA has made it possible to solve the traditional questions of population genetics at the DNA level. Obviously, DNA sequencing is the ultimate level at which genetic diversity can be recorded. Untranslated genetic variation can not detected by protein electrophoresis in population genetics. RFLP analysis of rDNA is a good tool for estimating the extent of intra- and inter-specific variations. RFLP analysis was used for studying taxonomic differences between *C. chinensis malleata* and *C. japonica*. No intra-specific variation was detected in RFLP analysis. There are a few hundred copies of rRNA(ribosomal RNA) genes which are composed of tandemly repeated units in the molluscan genomes. Each rRNA gene consists of the transcription units, and the transcription unit is flanked by a nontranscribed spacer. All the repeated copies of rRNA are concertedly evolved (Gerbi, 1986; Long and David, 1980). The nontranscribed spacer region(NTS) is known to evolve more rapidly than the transcribed region (Gerbi, 1986) and the diversity of NTS has been detected at intraspecific level in *Schistosoma* spp. and *Aedes* spp. (Walker *et al.*, 1986; Suzuki *et al.*, 1987; Black *et al.*, 1989). Species-specific restriction sites and inter-specific

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                          1                   20                  40                  60
C. chinensis malleata     CACTAAGCGGAGGAAAAGAAACTAACAAGGATTCCCTCAGTAACGGCGAGTGAAGCGG-A
C. japonica               CACTAAGCGGAGGAAAAGAAACTAACAAGGATTCCCTCAGTGGCGGCGAGTGAAGCGGGA
V. viviparous             CACTAAGCGGAGGAAAAGAAACTAACAAGGATTCCCTCAGTAACGGCGAGTGAAGCGGGA
Campanile symbolicum      TACTAAGCGGAGGAAAAGAAACTAACGAGGATTCCCTCAGTAACGGCGAGTGAAGCGGGA
Valvata sp.               CATTAAGCGGAGGAAAAGAAACTAACAAGGATTCCCTCAGTAACGGCGAGTGAAGCGGGA
Ampullaria sp.            CACTAAGCGGAGGAAAAGAAACTAACCAGGATTCCCTCAGTAACGGCGAGTGAAGCGGGA
                           * ********************** ************** ************** *

                                    80                  100                 120
C. chinensis malleata     CGAGCCCAGCACCGAATCCCCCGGCA--TGCAGCCGGCGGGAAATGTGGTGTGTGGGACG
C. japonica               CGAGCCCAG-ACCGAATCCCC-GGCA--TGCAGCCGG--GGAAATGTGGTGTGTGGGACG
V. viviparous             CAAGCCCAGCACCGAATCCCCCGGCA--TGCAGCCGGTGGGAAATGTGGTGTGCGGGACG
Campanile symbolicum      TGAGCCCAGCACCGAATCCCTCCGTCGCCACGGCGGACGGGACCTGTGGTGTGTGGGGCA
Valvata sp.               AGAGCCCAGCACCGAATCCCCCGGCC--TGTCGTCGGCGGGAACTGTGGTGTATGGGACG
Ampullaria sp.            AGAGCCCAGCACCGAATCCCTCGGCC--TGTCGCCGACGGGAACTGTGGTGTGTGGGACG
                            ******* **********   *        * *   ***  ********  *** *

                                   140                 160                 180
C. chinensis malleata     CCAACTGTCGTCCGCGCTGGAGACCGAAGTCCTCCTGATCGGGGCCTCTCCCACAGCGGG
C. japonica               CCAACTGTCGTCCGCGCTGGAGACCGAAGTCCTCCTGATCGGGGCCTCTCCCACAGCGGG
V. viviparous             CCAACTGTCGTCCGCGTTGGAGACCGAAGTCCTCCTGATCGGGGCCAATCCCACAGCGGG
Campanile symbolicum      CCACCAGTCG-CGGGAACGGCCGCCGAAGTCCTCCTGATCGAGGCTTCTCCCAGAGCGGG
Valvata sp.               CCAACTGCCCACGGCGTTAGTGGCCGAAGTCCTCCTGATCGGGGCCTCTCCCAGAGCGGG
Ampullaria sp.            CCAACTGTCCGCGGTGCCGGCAGCCGAAGTCCCCCTGATCGGGGCCTCTCCCAGAGTGGG
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                                   200                 220                 240
C. chinensis malleata     TGTCAGGCCTTTACTGGTCT--CTGGCCGGGCGGCTGCGAGCGTCTCAGGAGTCGGGGTG
C. japonica               TGTCAGGCCTTTACTGGTCT--CTGCGCGGGCGGCTGCGAGCGTCTCAGGAGTCGGGGTG
V. viviparous             TGTCAGGCCTTTACTGGTCT--CTGGCCGGGCGGCTGCGAGCGTCTCAGGAGTCGGGTTG
Campanile symbolicum      TGTAAGGCCTTTTCGGGCGC--GCCGTCCCGCGGCCGCGAGTGCCTCCGGAGTCGGGTTG
Valvata sp.               TGTCAGGCCTTTACAGGCCA--CCGGCCCGTGGGCTGCGAGCGTCTCCGGAGTCGGGTTG
Ampullaria sp.            TGTGAGGCCTTTGCCGGCTTGCTCGGTCCGCGGGCTGCGAGCGTCTCAGGAGTCGGGTTG
                          *** ******** * **          *     *** ***** * *** ********* **

                                  254
C. chinensis malleata     TTTGTGAATGCAGC
C. japonica               TTTGTGAATGCAGC
V. viviparous             TTTGGGAATGCAGC
Campanile symbolicum      TTTGGGAATGCAGC
Valvata sp.               TTTGGGAATGCAGC
Ampullaria sp.            TTTGGGAATGCAGC
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Fig. 2. Sequence comparison of the D1 region of 28S rDNA from six mollusc species. The nucleotide positions were numbered from the 5'-terminus. Dashes represent gaps in the alignment. Nucleotide sequences of *C. chinensis malleata* and *C. japonica* were compared with those of other molluscs, family Viviparidae (*V. viviparus*), family Valvatidae (*Valvata* sp.), family Campanilldae (*C. symbolicum*) and family Ampullariidae (*Ampullaria* sp.), for the comparison of their homologies. Number of total nucleotide was 257 bp.

variations of fasciolid species were detected as different lengths of the NTS region (Blair and McManus, 1989). In this study, no intra-specific variation in restriction fragment profiles of 28S rDNA was shown between two viviparid species.

A measure called 'nucleotide diversity' has been

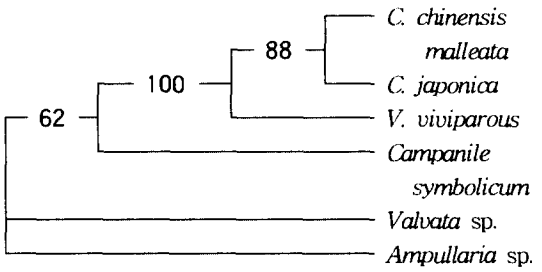

Fig. 3. The most-parsimonious tree obtained using nucleotide sequences of 28S rRNA genes in six molluscan species. Branch lengths and ranges of bootstrap values were obtained using statistical package (Power Macintosh 6100/66, PAUP Ver. 3.1.1.)

proposed to express the degree of polymorphism in a population at the nucleotide level (Nei and Li, 1979; Nei and Tajima, 1981). The more related two species, the more similar their nucleotide sequences. Although the number of specimens was small in the present study, the inter-specific genetic diversity value of pairwise distances between *C. chinensis malleata* and *C. japonica* was 0.016. From these results, four out of 254 nucleotide sequences were different in inter-specific level.

SUMMARY

Two species of family Viviparidae, *Cipangopaludina chinensis malleata* and *C. japonica* are very similar, though there are some distinctions in the shell morphologies. Biomolecular comparison of these closely related two viviparid species was conducted by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis to observe the genetic distance. The PCR products were digested by seven restriction endonucleases (*Alu* I, *Hae* III, *Msp* I, *Mbo* I, *Cfo* I, *Rsa* I and *Taq* I). No intra-specific variation in restriction fragment profiles of 28S rDNA was shown in both species. However, four out of 254 nucleotide sequences were different in inter-specific level. Molecular data obtained from D1 region of the 28S rDNA in this study made sufficient phylogenetic information to determine systematic distinction between two experimental species of family Viviparidae in Korea.

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