

Variation of nuclear and mitochondrial DNAs in Korean and Chinese isolates of *Clonorchis sinensis*

Soo-Ung LEE and Sun HUH*

Department of Parasitology, College of Medicine, Hallym University,
Chuncheon 200-702, Korea

Abstract: We compared the DNA sequence difference of isolates of *Clonorchis sinensis* from one Korean (Kimhae) and two Chinese areas (Guangxi and Shenyang). The sequences of nuclear rDNA (18S, internal transcribed spacer 1 and 2: ITS1 and ITS2) and mitochondrial DNA (cytochrome c oxidase subunit 1: *cox1*) were compared. A very few intraspecific nucleotide substitution of the 18S, ITS1, ITS2 and *cox1* was found among three isolates of *C. sinensis* and a few nucleotide insertion and deletion of ITS1 were detected. The 18S, ITS1, ITS2 and *cox1* sequences were highly conserved among three isolates. These findings indicated that the Korean and two Chinese isolates are similar at the DNA sequence level.

Key words: *Clonorchis sinensis*, ribosomal DNA, mitochondrial DNA, China, Korea

Clonorchiasis is endemic in East Asia, and this zoonotic infection ranges from China, Hong Kong, Macao, and Korea, to Laos and Vietnam (Rim, 1986). There is a previous report that isolates of *C. sinensis* of Korea (Kimhae) and of China (Liaoning) showed low level intraspecific variation of DNA. The two populations are very closely related within the range of a genetic identity value of 0.998-1.0 and they have a high homology in the nucleotide sequences of the 18S rDNA, ITS2 (internal transcribed spacer 2) and *cox1* (cytochrome c oxidase subunit 1) (Park and Yong, 2001). However, there had not been sequence data of ITS1 of *C. sinensis*. To understand whether the differ-

ence of the geographical region is related with the differences of the intraspecific variation, we compared the DNA sequences of *C. sinensis* from one Korean and two different Chinese isolates besides of Liaoning. DNA sequences of *C. sinensis* were analyzed for the nuclear rDNA (18S, ITS1 and ITS2) and mitochondrial DNA (*cox1*) to reveal how much the sequence variation is among the geographic isolates.

Metacercariae of *C. sinensis* were collected after the artificial digestion of the muscle of the freshwater fish, *Pseudorasbora parva* in Kimhae, Korea (A), Guangxi (B) and Shenyang (C), China. The adult worms were obtained from the rat (Sprague-Dawley, 4 to 6-week-old male) liver one month post-infection with metacercariae (A and B) or from the rabbit liver five months post-infection (C). The worms collected were then stored at -70°C until assayed. The frozen worms were lyophilized and lysed with a lysis buffer containing 1% SDS, proteinase K (500 µg/ml), and RNase at 37°C for 2-3 hr. The DNA was extracted in phenol/chloroform and precipitated in ethanol as reported by

• Received 23 March 2004, accepted after revision 21 August 2004.

• This study was supported by the grant of Korea Health 21 R & D Project, Ministry of Health and Welfare, the Republic of Korea (HMP96-PJ1-PG2-M-0164).

• Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Numbers See Table 1.

*Corresponding author (e-mail: shuh@hallym.ac.kr)

Table 1. Aligned sequence analysis for *Clonorchis sinensis*

Marker	Isolates ^{a)}	Aligned length ^{b)} (bp)	G+C% ^{c)}	GenBank accession No.
18S	A	1,030	51	AF217093
	B	1,030	51	AF229852
	C	1,031	51	AF217100
ITS1	A	762	54	AF181891
	B	762	54	AF181892
	C	776	54	AF192414
ITS2	A	451	52	AF217094
	B	451	52	AF217097
	C	451	52	AF217099
<i>cox1</i>	A	395	42	AF181889
	B	395	42	AF184619
	C	393	41	AF188122

^{a)} A, isolate from Kimhae, Korea; B, isolate from Guangxi, China; C, isolate from Shenyang, China

^{b)} Aligned length contains no missing data from Clustal W.

^{c)} GC content was calculated from the EMBOSS GEECEE program.

Sambrook and Russell (2001). The PCR primers previously designed for amplification of target DNA region were used (18S, Barker and Blair, 1996; ITS1, Bowles et al., 1993; ITS2, Bowles et al., 1995; *cox1*, Bowles et al., 1992). Each PCR was carried out in a volume of 50 μ l, as follows: 10-100 ng of the extracted genomic DNA as template, 200 μ M of each dNTP, 10 pmoles of each primer and 0.5 unit of Ex Taq enzyme (TAKARA Shuzo Co., Japan). PCR amplification consisted of 40 cycles of 20 second denaturation at 95°C, 30 second annealing at 50°C, 30 second extension at 72°C, followed by a final extension at 72°C for 6 minutes. The amplified PCR products were extracted using QIAEX II Gel extraction Kit (QIAGEN Co., Germany) and ligated into a T cloning vector (pT7Blue Perfectly Blunt Cloning Kit, Novagen Co., USA). Transformation was carried by *E. coli* NovaBlue competent cells provided in T cloning kit. Positive recombinant clones were picked and grown overnight in 2 ml of LB broth (in the presence of 50 μ g/ml ampicillin) at 37°C, and the positive plasmid DNAs were purified using a QIAprep spin plasmid kit (QIAGEN Co.). The recombinant plasmids were selected by blue/white screening using isopropyl- β -thiogalactoside and 5-bromo-4-chloro-3-indolyl- β -D-galactoside. Plasmids DNAs of white colonies were digested with *Bam*HI and *Hind*III restriction enzymes at 37°C, run in 1% agarose gels

and stained with ethidium bromide. DNA sequencing was performed by the dideoxy chain termination method (Sanger et al, 1977) using a Sequenase kit (ABI Prism Dye Terminator Cycle Sequencing Core Kit, Perkin Elmer) and an automated DNA sequencer (Applied Biosystems model 373A, Perkin Elmer) according to manufacture's instructions. Sequencing primers included the universal primers T3 and T7 for both directions. At least two clones were sequenced per isolate with additional clones being sequenced as necessary to resolve ambiguous sites. The aligned sequence was done using the CLUSTAL W program (European Bioinformatics Institute, <http://www.ebi.ac.uk/clustalw/>) among three isolates. Alignment gaps were treated as missing data (Higgins et al., 1992). For sequence analysis, we used BLAST in National Center for Biotechnology Information, NIH, Bethesda, USA. We also calculated the fractional GC content of nucleic acid sequences using an EMBOSS GEECEE program in Sanger Institute, Cambridge, U.K. (http://analysis.molbiol.ox.ac.uk/pise_html/geecee.html).

As results of sequencing, the length for each product of isolates was 1,030-1,031 bp for 18S; 762-776 bp for the ITS1; 451 bp for ITS2 and 393-395 bp for *cox1* (Table 1). A G+C content was 51% (18S), 54% (ITS1), 52% (ITS2), and ranged from 41% to 42% (*cox1*) (Table

Table 2. Analysis of nucleotide differences of the each sequence among *Clonorchis sinensis* isolates

Marker	Isolates ^{a)}	Substitution and indel differences number ^{b)} (bp)		
		A	B	C
18S	A	–	1	1
	B	2	–	1
	C	1	3	–
ITS1	A	–	18	8
	B	1	–	22
	C	1	0	–
ITS2	A	–	0	0
	B	0	–	0
	C	0	0	–
<i>cox1</i>	A	–	0	2
	B	0	–	2
	C	3	3	–

a) A, isolate from Kimhae, Korea; B, isolate from Guangxi, China; C, isolate from Shenyang, China

b) Below the diagonal is the nucleotide substitution number not adjusted for gaps (indel; insertion, deletion), and above the diagonal is the indel number from aligned sequence.

1). Sequences were deposited in GenBank by the accession numbers listed in Table 1. The nucleotide substitution differences among the isolates were 1-3 bp for the 18S, 1 bp for ITS1 and 3 bp for *cox1*. For the ITS2, no variation was detected among and within isolates from Korea and China. The nucleotide gap (insertion, deletion) differences among the isolates were 1 bp for the 18S, 8-22 bp for the ITS1 and 2 bp for *cox1*. For the ITS2, no gap was detected among isolates from Korea and China (data of multiple sequences alignment not shown). The ITS1 sequences showed much higher gap differences at nucleotide composition, 8-22 bp insertion and deletion at the same position among three isolates (Table 2).

According to the above results, it was found that Korean and Chinese isolates of *C. sinensis* are remarkably similar at the DNA sequential level for ribosomal DNA and mitochondrial DNA. A few intraspecific variations in *C. sinensis* were found in the rDNA repeat (18S, ITS1 and ITS2) and *cox1*, and a few base insertion and deletions were detected in the ITS1 sequences. Extensive intraspecific sequence variations

were not found between the Korean and two Chinese isolates. There were other comparable data on the intraspecific variations of ITS2 and *cox1* sequence of parasitic trematodes in human. The nucleotide sequence of ITS 2 and *cox1* of *Opisthorchis viverrini* in northeast Thailand showed intraspecific variation, that has been classified into 5 patterns, but no area-specific pattern was observed. Nucleotide sequences in a region of the ITS2 from different areas were identical (Ando et al., 2001). Those data compared very well to the present results suggested that the geographic differences are not so significant for some DNA markers. The average sequence divergence among the *Echinostoma* species range from 2.2% in the ITS rDNA to about 8% for the *cox1* (Morgan and Blair, 1988). DNA sequence similarities observed between the Korean and Chinese isolates of *C. sinensis* are indicative of a common ancestry. There is negligible intraspecific variation among one Korean and two Chinese isolates of *C. sinensis*.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. Gab-Man Park, Kwandong University College of Medicine, Korea and Yoon Kong, Sungkyunkwan University College of Medicine, Korea for their kind donation of the two Chinese isolates.

REFERENCES

- Ando K, Sithithaworn P, Nuchjungreed C, et al. (2001) Nucleotide sequence of mitochondrial COI and ribosomal ITSII of *Opisthorchis viverrini* in Northeast Thailand. *Southeast Asian J Trop Med Public Health* **32**: 17-22.
- Barker SC, Blair D (1996) Molecular phylogeny of *Schistosoma* species supports traditional groupings within the genus. *J Parasitol* **82**: 292-298.
- Bowles J, Blair D, McManus DP (1992) Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Mol Biochem Parasitol* **54**: 165-173.
- Bowles J, Blair D, McManus DP (1995) A molecular phylogeny of the human schistosomes. *Mol Phylog Evol* **4**: 103-109.
- Bowles J, Hope M, Tiu WU, Liu X, McManus DP (1993)

- Nuclear and mitochondrial genetic markers highly conserved between Chinese and Philippine *Schistosoma japonicum*. *Acta Trop* **55**: 217-229.
- Higgins DG, Bleasby AJ, Fuchs R (1994) CLUSTAL W: Improved software for multiple sequence alignment. *Comput Appl Biosci* **8**: 189-191.
- Morgan JA, Blair D (1998) Relative merits of nuclear ribosomal internal transcribed spacers and mitochondrial COI and ND1 genes for distinguishing among *Echinostoma* species (Trematoda). *Parasitology* **116**: 289-297.
- Park GM, Yong TS (2001) Geographical variation of the liver fluke, *Clonorchis sinensis*, from Korea and China based on the karyotypes, zymodeme and DNA sequences. *Southeast Asian J Trop Med Public Health* **32**(Suppl 2): 12-16.
- Rim HJ (1986) The current pathobiology and chemotherapy of Clonorchiasis. *Korean J Parasitol* **24**(suppl): 5-141.
- Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463-5467.
- Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd ed. Chapter 6, pp 6.1-6.30. Cold Spring Harbor Laboratory Press, New York, USA.