

GEOGRAPHICAL VARIATION OF THE LIVER FLUKE, *CLONORCHIS SINENSIS*, FROM KOREA AND CHINA BASED ON THE KARYOTYPES, ZYMODEME AND DNA SEQUENCES

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Abstract. Genetic characterization was carried out in order to reveal the geographical variations of the oriental liver fluke, *Clonorchis sinensis* (Trematoda: Opisthorchiidae), collected in Korea and China. The chromosome number was $2n=56$ in both Korean (Kimhae) and Chinese (Liaoning) flukes, and chromosomes were divided into two groups based on their sizes; consisting of 8 pairs of large and 20 pairs of small chromosomes. However, the karyotypes showed some differences between Korean and Chinese flukes. Isozyme analysis showed that two loci from each enzyme of aconitase and esterase (α -Na and β -Na); only one locus each from six enzymes, glucose-6-phosphate dehydrogenase (G6PD), α -glycerophosphate dehydrogenase (GPD), 3-hydroxybutyrate dehydrogenase (HBDH), malate dehydrogenase (MDH), phosphoglucose isomerase (PGI) and phosphoglucomutase (PGM). Most of loci in two populations of *C. sinensis* showed homozygous monomorphic banding patterns and one of them, GPD was specific as genetic markers between two different populations. Two populations were very closely clustered within the range of genetic identity value of 0.998-1.0. We also compared patterns of intraspecific polymorphism of two markers with contrasted modes of evolution, nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) of the liver fluke from Kimhae, Guangxi and Liaoning. They showed a high homology. In conclusion, three populations of *C. sinensis* from Korea and China showed high homogeneity in the nucleotide sequences of the 18S rDNA, ITS2 and mtCOI gene.

INTRODUCTION

Clonorchis sinensis Looss 1907, "the Chinese or the oriental liver fluke", is an important human parasite and is widely distributed in southern Korea, China, Taiwan, and northern Vietnam, and clonorchiasis is one of the most important endemic diseases in Korea. Although reports of this infection are infrequent in western countries, infection can be acquired by eating frozen, dried, or pickled freshwater fish imported from endemic areas. Several Korean workers conducted studies of *C. sinensis* on its biology, epidemiology, pathology, clinical symptoms as well as treatment (Chu *et al.*, 1982; Cho *et al.*, 1983).

Enzyme electrophoresis and restriction site analyses are applicable to most studies of intra-specific variation. Most enzymes are under the control of nuclear genes which undergo a straight-forward Mendelian pattern of inheritance. This indicates that enzyme variants are of particular value in genetic work including inter-specific hybrid studies. These methods remain the useful tools choice, although DNA sequencing may be needed to resolve particularly difficult problems (Andrews and Chilton, 1999). A few have been reported on intra- or inter-specific hybridization experiments using enzyme variants (isozyme) in the parasitic helminth (Wright and Southgate, 1976; Wright and Ross, 1980).

Cytotaxonomic studies of the parasites are

important not only for the parasites of systematic analyses, but also for the basic information of the parasitic agents. From this point of view chromosomal studies on trematode parasites have been noticeably useful in systematic studies (Park *et al.*, 1998); but, until recently there have been relatively a few studies on the chromosomes of trematode parasites. Diploid chromosome numbers of the trematodes ranged from 16-22 (Short *et al.*, 1989; Terasaki *et al.*, 1995). Until now, no studies on the chromosome of *C. sinensis* from Korea and China have been attempted.

During the past decade, molecular genetic characterization is being used increasingly to distinguish among morphologically similar parasites. In fact, two of the most popular markers used in molecular evolution, mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA), have quite different properties, which could translate into different consequences of mutation, migration and selection on patterns of geographical variation and molecular divergence. Until now, no studies on the sequence variation of the 18S, ITS2 and mtCOI gene of *C. sinensis* from Korea and China populations have been attempted.

The purpose of this study was to analyze the chromosome numbers, chromosomes and rDNA and mtDNA gene for intra-species variation of the liver fluke, *C. sinensis*, collected from Korea and China.

MATERIALS AND METHODS

The adult flukes used in this study were obtained from rabbits experimentally infected with *Clonorchis* metacercariae which were removed from the host fishes, *Pseudorasbora parva*, collected from Kimhae in Kyungsangnam-do, Korea and from Liaoning Province and Guangxi Province, China. The rabbits were sacrificed about 5 months after infection.

The adult parasites for isozyme were homogenized and centrifuged at 12,000 rpm individually and the supernatants were applied for starch gel electrophoresis. The eight enzymes in two different kinds of buffer systems (TC-I and TC-II) examined in this study were as follows: aconitase-I, II (ACO I, II), esterase (EST α -Na, β -Na), glucose-6-phosphate dehydrogenase (G6PD), α -glycerophosphate dehydrogenase (GPD), 3-hydroxybutyrate dehydrogenase (HBDH), malate dehydrogenase (MDH), phosphoglucose isomerase (PGI), and phosphoglucomutase (PGM) (Table 1). For the discussion of relative phylogenetic relationships among two populations of *Clonorchis* species, the normalized identity of gene and the standard genetic distance were used (Nei, 1972).

The chromosomes in the gonadal tissues of the fluke were studied by the simple cell cultivation method of Park's (1994). Morphological features of the chromosomes used to compare karyotypes were the relative length and total lengths of the mitotic metaphase chromosomes. The prepared slides were observed under an Olympus BX50 microscope with a 100X oil immersion objective and a 10X ocular. The nomenclature of the centromeric position for the classification of chromosomes was followed as proposed by Levan *et al* (1964).

Fully developed adult flukes were used for DNA / analysis. DNA extraction and purification of rDNA and mtDNA were as described previously (Agatsuma *et al*, 1994). Gene regions were amplified using the polymerase chain reaction (PCR). For the 18S rDNA, the primers used were as described by Barker and Blair (1996), ITS2 gene and mtCOI gene by Bowles *et al* (1995). All sequences were determined directly from the PCR products. Cycle sequencing reactions were run on an ABI 373A Automated Sequencer. Nucleotide sequences were aligned using Clustal X program.

RESULTS

Isozyme patterns

Eleven loci were presumed from eight enzymes (Fig 1). Five out of 11 loci were polymorphic ((EST (α -Na), GPD, HBDH, MDH, PGI)), and the number of alleles per polymorphic locus varied from two to three. A single monomorphic band of ACO-I, II, EST β -Na, G6PDH, MDH-I, II, PGM were observed in all individuals employed. Monomorphic double bands of MDH-I showed strong and fast band. MDH-II was slow and the activity was poorer than MDH-I. α -glycerophosphate dehydrogenase (GPD) was composed of three bands. This locus was population-specific. The slowest band was demonstrated only in Chinese population. The fast and intermediate bands were observed in Korean population only. In other words, the enzyme GPD in Korean population showed typical monomeric banding patterns, whereas those in Chinese population were monomorphic without any variation. This result indicates that monomorphic patterns of GPD in Chinese population are supported by genetic stability in the populations. Three bands of EST(α -Na) occurred. The intermediate band was common in all individuals of two

Table 1
Eight enzymes assayed in two different buffer systems in this study.

Enzymes (EC Number)	No. loci scored	Abbreviation	Buffer system
Aconitase (4.2.1.3)	2	ACO	TC-I ^a
Esterase (3.1.1.1.)	2	EST	TC-II ^b
Glucose-6-phosphate dehydrogenase (1.1.1.49)	1	G6PDH	TC-II
α -Glycerophosphate dehydrogenase (1.1.1.8)	1	GPD	TC-II
3-Hydroxybutyrate dehydrogenase (3.1.1.31)	1	HBDH	TC-II
Malate dehydrogenase (1.1.1.37)	2	MDH	TC-II
Phosphoglucose isomerase (5.3.1.9)	1	PGI	TC-II
Phosphoglucomutase (2.7.5.1)	1	PGM	TC-II

^a TC-I: Tris/HCl-0.2 M, pH 8.0; ^b TC-II: Tris/HCl-0.5 M, pH 8.0.

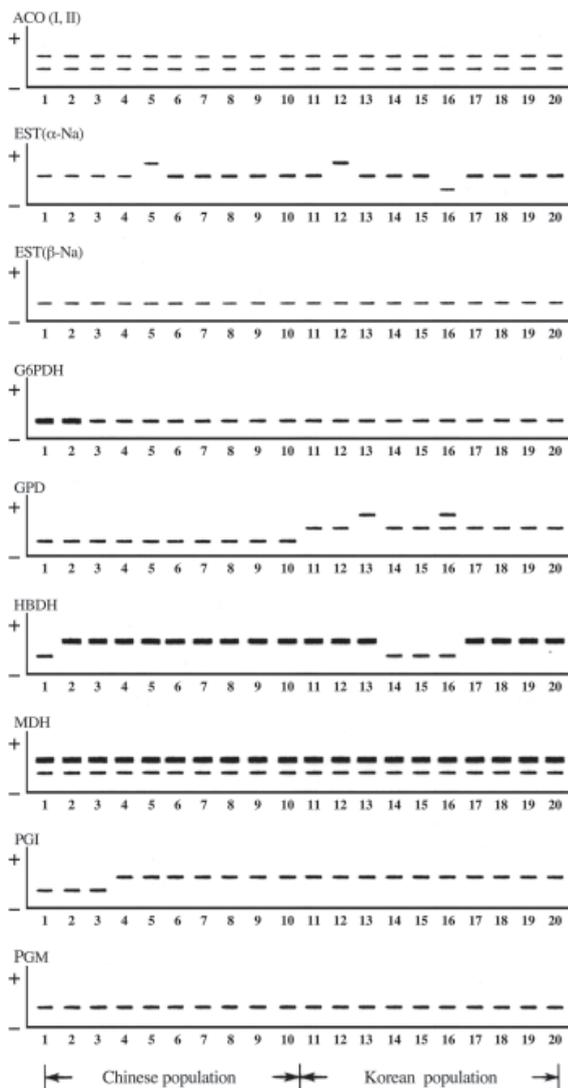


Fig 1- Zymograms of eight enzymes of liver fluke, *Clonorchis sinensis* from Kimhae, Korea and from Liaoning, China.

populations. The slow moving band occurred in Korean population. Average heterozygosity frequency of each Korean and Chinese population was 0.146 and 0.078, respectively (Table 2). Accordingly, genetic variance of Korean population was a little higher than Chinese populations, and the average heterozygosity frequency of the two populations was 0.112. The two populations of *C. sinensis* in Korea and China are so similar in their phenotypic distributions, in allele frequencies and isozyme band patterns, as to be indistinguishable.

Table 2
Allele frequencies for two populations of *Clonorchis sinensis* from Kimhae in Korea and Liaoning in China.

Locus	Allozyme	Population	
		China	Korea
ACO-I	a	1.0	1.0
	H ^a	0	0
	ACO-II	a	1.0
EST(α-Na)	H	0	0
	a	0.1	0.1
	b	0.9	0.8
EST(β-Na)	c		0.1
	H	0.28	0.34
	a	1.0	1.0
GPD	H	0	0
	a	1.0	
G6PDH	b		0.85
	c		0.15
	H	0	0.26
	a	1.0	1.0
HBDH	H	0	0
	a	0.9	0.4
	b	0.1	0.6
MDH-I	H	0.18	0.48
	a	1.0	1.0
	H	0	0
MDH-II	a	1.0	1.0
	H	0	0
PGI=(GPI)	a	0.7	1.0
	b	0.3	
	H	0.42	0
PGM	a	1.0	1.0
	H	0	0
Heterozygosity frequency		0.078	0.146
No. of mean allele		14/11 (1.27)	15/11 (1.36)

^a Heterozygosity per locus.

Chromosomes

Mitotic chromosomes were observed in 118 cells from 12 individuals in Korea and in 121 cells from 18 individuals in China. All of the metaphase figures of mitosis in *C. sinensis*, 56(2n) chromosomes were recognized (Figs 2-5). According to the size and shape of the chromosomes, the karyotypes were established on plates for 28 pairs. Although there were size variations in the chromosomes, the complements were tentatively ordered into pairs according to size. They

could be divided into two groups by size, 8 pairs of large and 20 pairs of small chromosomes. The karyotypes of Korean population consisted of three metacentric pairs, one meta-/submetacentric pair, 16 submetacentric pairs and eight subtelocentric pairs of chromosomes (Fig 3). The karyotypes of Chinese population consisted of two metacentric pairs of chromosomes, two meta-/submetacentric pairs, 16 submetacentric pairs and eight subtelocentric pairs of chromosomes (Fig 5).

Intra-species variation of DNA

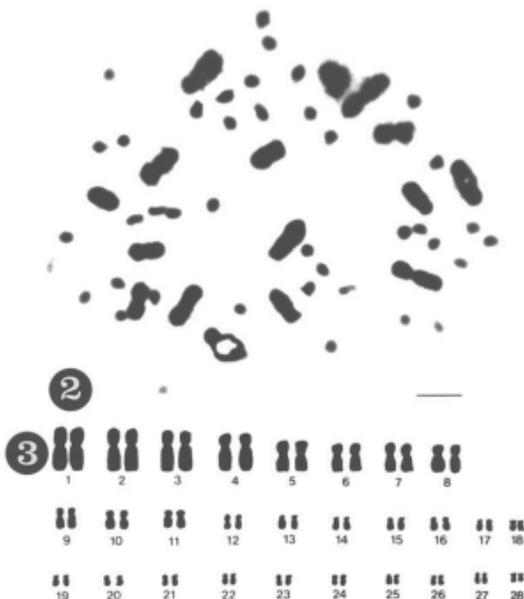
We compared patterns of intra-species polymorphism of three markers with contrasted modes of evolution, nuclear ribosomal DNA and mitochondrial DNA from Korea (Pusan) and from China (Guangxi, Liaoning). They showed a very high homology.

DISCUSSION

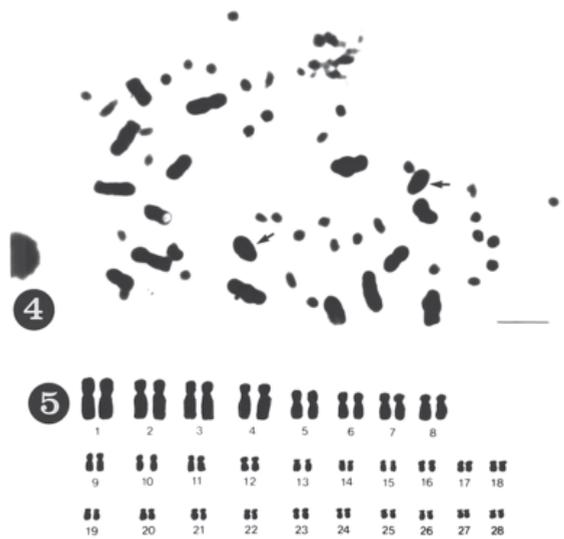
Electrophoresis has been widely used in parasite systematics in recent years (Mueller and Fried, 1999). Genotypic information from this method is useful especially in systematics between lower taxa. Most geographically separated populations share the same alleles at monomorphic and polymorphic loci, and at similar frequencies in the latter (Ferguson, 1980). Genetic identity values of sibling species by isozyme electrophoresis patterns is 0.626 (0.539-0.777 at invertebrate) and 0.567 (0.300-0.833 at invertebrate)

in distinct species reported by Ferguson (1980). In this study, genetic identity of the two populations was 0.998-1.0. Consequently, in this point of view, *C. sinensis* collected from Kimhae in Korea and Liaoning in China showed little variation and suggested identical species.

The morphology of chromosomes among individuals of the same species is sufficiently constant that karyotypes are recognized as a definite species character (White, 1973). The study of chromosome numbers has been used as a valuable complement to biochemical methods for identifying species, hybrids and more rarely populations. It has also been of particular importance in the application of polyploidy. In fact, relationships within groups of species cannot be considered complete, in an evolutionary sense, without good cytotaxonomic data to reinforce conclusions drawn from morphological criteria. Barsiene (1993) reviewed chromosome numbers and karyological data for 230 species of trematodes. Chromosome numbers of trematodes ranged from $2n=16$ for *Schistosoma mansoni* (Short *et al.*, 1989), $2n=20$ for *Fasciola* spp (Vi and Ye, 1990), *Neodiplostomum seoulense* (Park *et al.*, 1998), *Pegosomum asperum* and *P. saginatum* (Aleksandrova and Podgornova, 1978) to $4n=44$ for *Paragonimus westermani* (Terasaki *et al.*, 1995). In this study, the chromosome number of *C. sinensis* from Korea and China was $2n=56$. Prior to this study no *C. sinensis* have been investigated cytologically. The karyotypes of *C. sinensis* from



Figs 2-3-Mitotic chromosomes of *Clonorchis sinensis* from Korea. Fig 2. Diploid chromosomes in metaphase I. Scale bar=10 μ m. Fig 3. Camera lucida drawings of Fig 2.



Figs 4-5-Mitotic chromosomes of *Clonorchis sinensis* from China. Fig 4. Diploid chromosomes in metaphase I. Scale bar=10 μ m. Fig 5. Camera lucida drawings of Fig 4.

Korea and China were different. Differences in chromosomal morphology could be due to inversions, additions, deletions, etc which might still represent within species variability. Nonetheless, this speculation enhances the authors conclusion that more study is warranted for these groups of flukes. This chromosomal data will provide basic information on the liver flukes, which can then be used for genome study.

The usefulness of molecular genetic techniques based on nuclear and mitochondrial DNA was emphasized whilst addressing problems of the identification, characterization, and phylogeny of parasites (McManus and Bowles, 1996). The mtCOI gene exhibits no intra-individual variation and little or no intra-species variation, which is in line with expectation for such gene regions, as they are expected to undergo homogenization by convergent evolution (Hoffmann *et al.*, 1992). In this study, nuclear and mitochondrial DNA sequences of *C. sinensis* showed a high homology. This similarity between geographically distant populations indicates that divergence is minimized and limited to regions that are prone to high rates of mutation.

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