The Korean Journal of Parasitology
Vol. 41, No. 1. 47-55, March 2003

Phylogenetic relationship of ribosomal ITS2 and mitochondrial COI among diploid and triploid *Paragonimus westermani* isolates

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Abstract: We compared patterns of intraspecific polymorphism of two markers with contrasting modes of evolution, nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA), in the lung fluke, diploid and triploid *Paragonimus westermani* from three geographical regions of Korea. The genetic distances between three populations of Korean diploid and triploid *P. westermani* showed no significant difference in the nucleotide sequences of the mitochondrial cytochrome c oxidase subunit I (mtCOI) and ribosomaal second internal transcribed spacer (ITS2) genes. A highly resolved strict-consensus tree was obtained that illustrated phylogenetically useful information of the ITS2 and mtCOI sequences from diploid and triploid *P. westermani*.

Key words: *Paragonimus westermani*, diploid, triploid, ribosomal DNA, mitochondrial DNA, Korea

INTRODUCTION

Approximately 28 species of the genus *Paragonimus* are known: two in Africa, five in the Americas and 21 in Asia (Miyazaki, 1982a). In Korea, three species of lung fluke have been recorded to date, including *P. westermani* (Miyazaki, 1978). *Paragonimus westermani* has long been considered to be the most important causative agent of paragonimiasis in Asia. Eating raw or insufficiently cooked crabs or crayfish, which serve as the second intermediate host, may cause human infection.

Chromosomal studies have proven to be important in the evaluation of competing theories concerning the origin of triploids. Moreover, the discovery of diploid and triploid forms of *P. westermani* in Korea has aroused some debate over the identity and speciation of this parasite. *Paragonimus westermani* is the best known of human pathogens and exists in both diploid and triploid forms. The triploid form of *P. westermani* is presumed to be more pathogenic in humans than the diploid form (Blair et al., 1997). Significantly, the pathology of the conditions caused by the two forms differs; triploid flukes mainly form cysts in the lungs, but the diploid flukes cause lesions in the pleural cavity and in the pleura (Miyazaki, 1982b). Based on C-banding analysis triploidy in *P. westermani* has been suggested to have occurred by hybridization of two closely related species (Hirai and Agatsuma, 1991; Hirai et al., 1992). Moreover, based on both allozyme and cytogenetic studies, it has been proposed that triploids are allotriploids (Hirai, 1987), and triploids from Japan, China and Korea all possess the same alleles at each locus (Agatsuma et al., 1989). Studies using Giemsa

• Received 8 November 2002, accepted after revision 31 December 2002.
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stained chromosomes suggest that triploids arose by autopolyploidy from diploids (Terasaki, 1980). Generally, it is accepted that two mechanisms may give rise to polyploidy: autopolyploidy, in which all chromosome sets are derived from a single ancestor and allopolyploidy, in which all chromosome sets are derived from two or more ancestral species (White, 1977). Whether the triploid species is autoploid or allotriploid is unknown, which makes *P. westermani* taxonomically difficult.

The mitochondrial genome is considered to be clonal and rarely or never undergoes recombination. Moreover, restriction mapping of the mitochondrial genome of *P. westermani* showed that its genome is ca. 21kb in size (Agatsuma et al., 1994; Blair 2000). Slight differences noted in the restriction fragment patterns of diploid and triploid specimens from eastern Asia (Japan and Korea) have been cited as evidence that the triploids are allopolyploids (Agatsuma et al., 1994). The majority of studies undertaken to distinguish species and subspecies have used sequences of nuclear ribosomal second internal transcribed spacer (ITS2) or partial sequences of the mitochondrial cytochrome c oxidase subunit I (mtCOI) gene. Size variations in mtDNA are usually due to differences in noncoding sequences, but is occasionally caused by duplications of coding sequences of other portions of the genome (Zevering et al., 1991). In this paper, we describe phylogenetic relationship among diploid and triploid *P. westermani*, based on the mtCOI and the ITS2 gene sequences from different geographical region.

MATERIALS AND METHODS

Paragonimus westermani metacercariae were recovered from *Cambaroides similis*, which had been collected from Bogil-Island (Wando-gun), Haenam (Haenam-gun), and Youngam (Youngam-gun), Chollanam-do in Korea. Metacercariae were orally fed to dogs and sacrificied at 140-150 days post-infection. Eight fully developed adult flukes from each isolate were used for DNA analysis. Chromosome preparation was obtained by the air-drying technique of Park et al. (2000). Chromosome numbers of the *P. westermani* from the three localities for DNA analysis were investigated: a triploid form from Bogil-Island and Youngam, and a diploid form from Haenam.

DNA was extracted from adult worms. Samples were lyophilized and lysed with lysis buffer containing proteinase K and RNase. DNA was extracted in phenol/chloroform and precipitated by ethanol, as described by Sambrook and Russel (2001). Gene regions were amplified using polymerase chain reaction (PCR). For the mtCOI and ITS2 regions, the primers used were as described by Bowles and McManus (1993) and Bowles et al. (1995). PCR amplification was conducted over 40 cycles using the following conditions: -1 minute at 95°C, 1 minute at 54°C, and 1.5 minutes at 72°C, with a final extension of 7 minutes at 72°C. The PCR products were purified by gel extraction, and ligated into a T cloning vector. Clones were generated by transforming *E. coli* NovaBlue competent cells (provided in the T cloning vector kit), according to the protocol of the supplier. The blue and white color selection used for identifying recombinant plasmid. DNA from the positive recombinants was purified using a QIAprep spin plasmid kit. DNA sequencing was performed using the dideoxy chain termination method and an automated.

DNA sequencer. At least five clones were sequenced per isolate, and additional clones were sequenced, if necessary to resolve ambiguous sites. NCBI (National Center for Biotechnology Information) databases of *P. westermani* collected from various localities were used for sequence similarity analysis. Sequences of *Paragonimus* species obtained from GenBank database were used as outgroups.

Nucleotide sequences were aligned using Clustal X (Thompson et al., 1997) and phylogenetic analysis was performed using the Kimura 2-parameter model (Kimura, 1980). A phylogenetic tree was constructed using the neighbor-joining method (program NEIGHBOR). A majority rule consensus tree was determined from the resulting 100 trees, using the CONSENSE program. Numbers of nucleotide differences and of amino acid differences in pairwise comparison among ITS2 and mtCOI sequences were calculated in MEGA (Kumar et al., 1993).

RESULTS

Nucleotide sequences were obtained for ITS2 of the ribosomal DNA and for a part of the mtCOI gene from different geographical isolates of diploid and triploid *P. westermani*, obtained in Korea. The ITS2 sequences were 363 bases in length and were deposited in the GenBank under accession numbers: AF333276 (Bogil-Island), AF333277 (Youngam) and AF333278 (Haenam). Diploid and triploid sequences were almost identical and differed only at nine of the 363 nucleotides examined. The ITS2 sequences of Korean *P. westermani* were almost identical, and the diploid form (Haenam) and the triploid form (Youngam and Bogil-Island) differed at only two-nucleotide positions. For comparative purposes, the ITS2 sequences of *Paragonimus* species from several countries were obtained from GenBank and they were compared. Identical sequences were obtained in two Chinese, six Japanese, one Korean and two Taiwanese *P. westermani* isolates, regardless of diploid or triploid. Among *Paragonimus*, two branches can be distinguished, one for *P. westermani* and the other for *Paragonimus* species. Two groups were recognized within *P. westermani*: a northeastern Asian group (China, Japan, Korea and Taiwan), which was relatively uniform and included both diploid and triploid forms, and a southeastern Asian group (Malaysia and Philippines), members of which were genetically distinct. Animals of the

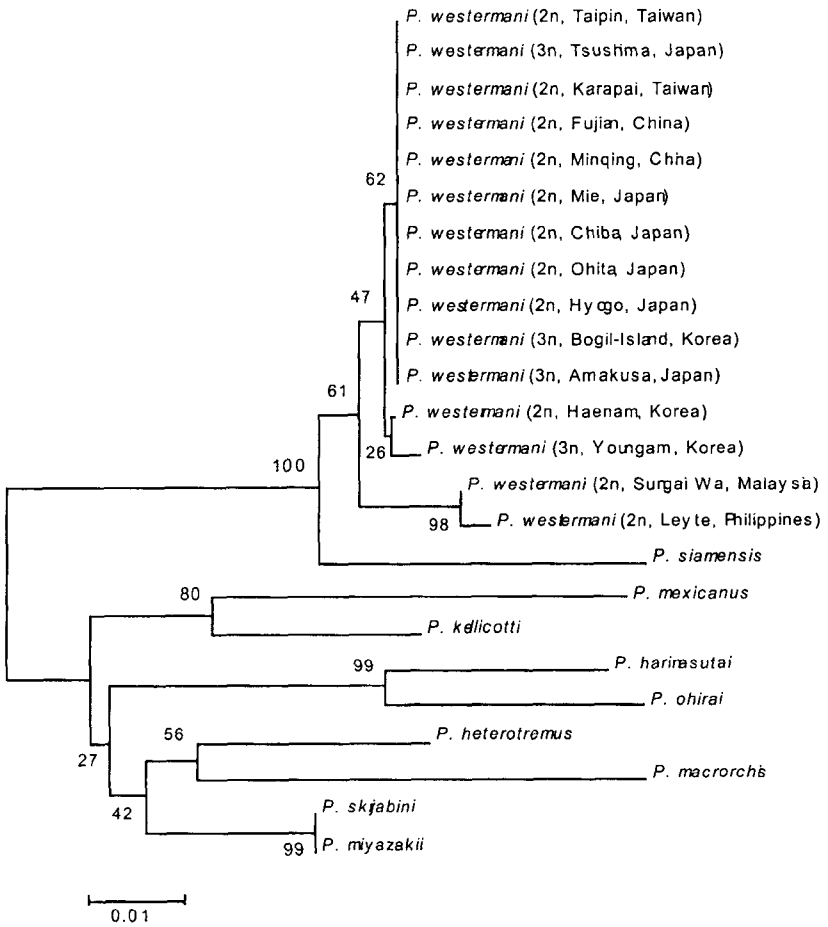

Fig. 1. Tree depicting relationships among *Paragonimus westermani*, inferred from ITS2 data using genus *Paragonimus* as outgroup. A distance matrix was calculated using the Kimura-2-parameter model and the tree was constructed using the minimum evolution. The ITS2 sequences of *P. westermani* and the genus *Paragonimus* used in the analyses were obtained from GenBank: Amakusa, Chiba, Hyogo, Ohita, Mie, Tsushima, Japan (U96907); Minqing, Fugian, China (U96907); Karapai, Taipin, Taiwan (U96908); Leyte, Philippines (U96910); Sungai Wa, Malaysia (U96909); *P. siamensis* (AF159605); *P. heterotremus* (AF159603); *P. macrorchis* (AF159608); *P. skrjabini* (U96913); *P. miyazakii* (U96912); *P. harinasutai* (AF159609); *P. ohirai* (U96911); *P. maxicanus* (AF159607) and *P. kellicotti* (AF159606).

southeastern Asian group (Malaysia and Philippines) were, however, identical to each other in sequence, although they differed from those of other countries (Fig. 1). In the constructed phylogenetic trees, *P. siamensis* is very closely related to *P. westermani*, but the other members including the *Paragonimus* species grouping appear to be alone.

The mtCOI sequences were 387 bases in length. These sequences were also deposited in the GenBank under accession numbers: AF333279 (Bogil-Island), AF333280 (Youngam) and AF333281 (Haenam). Identical mtCOI sequence lengths were found in the three Korean isolates and in those of other countries, regardless of whether they were diploid or triploid. However, the mtCOI sequences of diploid and the triploid Korean *P. westermani* differed at two and seven nucleotide positions, respectively. In contrast to the ITS2 data, only three isolates of *P. westermani* exhibited identical mtCOI nucleotide sequences. They were the diploid form from Hyogo (Japan) and the triploids from Youngam (Korea) and Tsushima (Japan). Intra-specific variations were detected at the very low level of 0-1.29% in the mtCOI sequences of Korean diploid and triploid *P. westermani*. The mtCOI sequences of the Korean diploid and triploid forms were almost identical with those detected in three isolates which exhibited identical ITS2 nucleotide sequences. The mtCOI sequences varied more within *P. westermani* and *Paragonimus* species than the ITS2 sequence. The NJ tree (Fig. 2) shows that three groups were recognized within *P. westermani*, based on their mtCOI sequences: the first group (China,

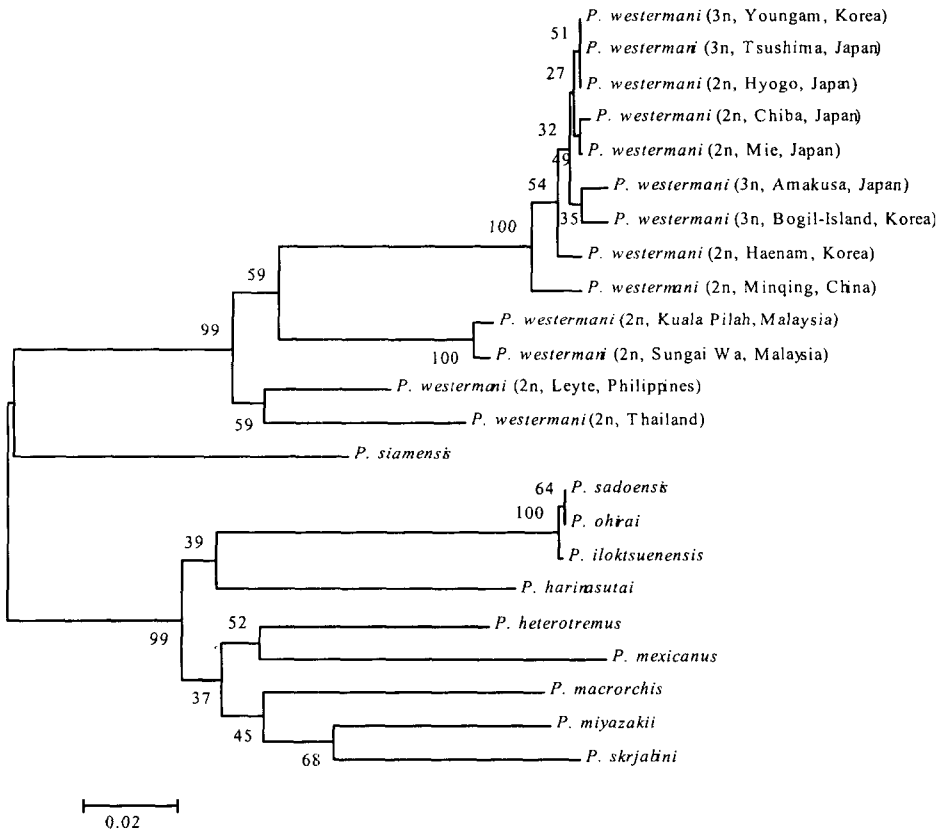

Fig. 2. Phylogenetic relationships among *Paragonimus westermani* inferred from mtCOI data of distance matrix methods using genus *Paragonimus* as outgroups. Minimum evolution analysis by the Kimura-2-parameter distance was used. Numbers on branches indicate the percentage of 100 bootstraps supporting the branching pattern shown. The mtCOI sequence of *P. westermani* and genus *Paragonimus* used in the analyses were obtained from GenBank: Hyogo, Japan (U97205); Amakusa, Japan (U97206); Chiba, Japan (U97207); Mie, Japan (U97208); Minqing, China (U97209); Sungai Wa, Malaysia (U97210); Kuala Pilah, Mlaysia (U97211); Thailand (U97212); Leyte, Philippines (U97213); *P. siamensis* (AF159599); *P. heterotremus* (AF159597); *P. mexicanus* (AF159596); *P. miyazakii* (U97215); *P. skrjabini* (U97216); *P. macrorchus* (AF159598); *P. sadoensis* (AF0008190); *P. ohirai* (AF008189); *P. iloktsuenensis* (AF008188) and *P. harinasutai* (AF159600).

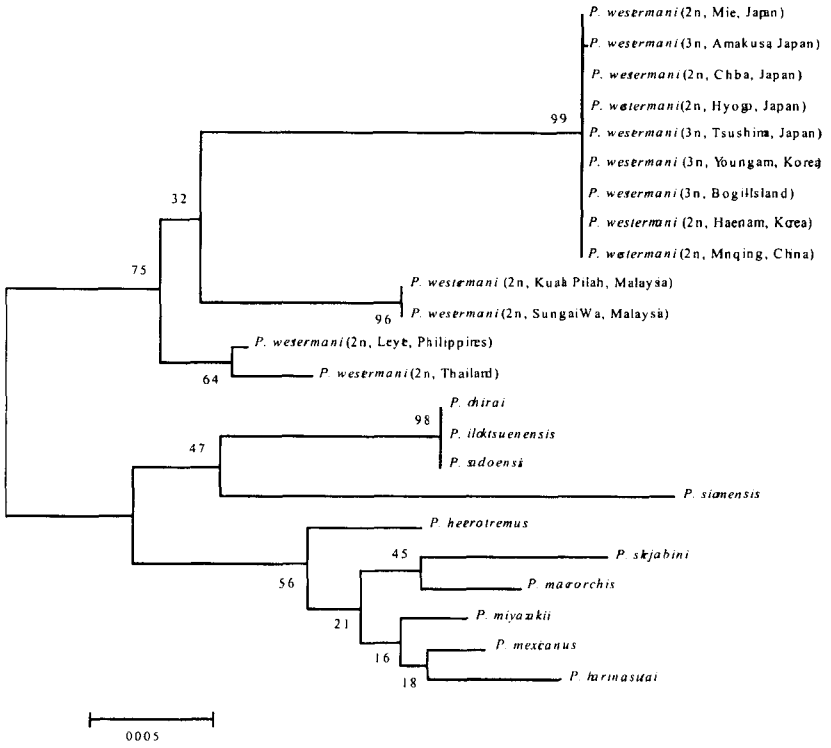

Fig. 3. Phylogenetic tree of *Paragonimus* species based on mtCOI sequences after third codon positions omitted. Neighbor-joining tree based on Kimura-2-parameter distance, using MEGA. Branch lengths are proportional to the scale given in nucleotide substitutions per site. Numbers correspond to bootstrap percent values based on 1,000 replications.

Japan and Korea), which was relatively uniform, differed at 0-13 sites (0-3.36%). The second group (two isolates from Malaysia) differed from the first group at 2-40 sites (0.52-10.34%) and these specimens had almost identical sequences. The third group (Philippines and Thailand) differed from the first and second groups at 27-32 sites (6.98-8.27%) and from each other at 26 sites. In the mtCOI sequences, the third codon position is the most variable in protein coding and the removal of third codon positions from the analysis might increase the consistency index. If third codon positions are omitted, the strict consensus trees collapsed in the first group (Fig. 3). However, the second group and the third group of *Paragonimus* species formed a well-supported clade in trees, even if the third codon positions were omitted. Among the *Paragonimus* species, *Paragonimus siamensis* was the most closely related to *P. westermani* and the others, including the grouping of *Paragonimus* species, appeared to be alone. The genetic distances were relatively very small between diploid and triploid *P. westermani*, but large among the *Paragonimus* species.

DISCUSSION

Studies on phylogeny and/or intraspecific variation in *Paragonimus* species, using ITS2 and mtCOI sequences, have recently been reported (Blair et al., 1997; Iwagami et al., 2000). Homogenization is most likely to occur if the ribosomal genes occur in a single tandem array on one chromosome, as seems to be the case in trematodes (Hirai et al., 1989). Differentiation of the ITS2 gene between populations and species has been reported to be dependent upon many factors, including genetic drift, the relative number and size of repeats, rates of unequal crossover, gene conversion, immigration, and the number of loci (Levinson and Gutman, 1987). Mitochondrial DNA sequences are now widely used for genetic studies on evolutionary population. In particular, lack of recombination and maternal mode of inheritance make them suitable for the study of gene introgression involved in the hybridization event to create a new race such as a triploid. The mtCOI gene exhibits no individual variation and little or no intra-specific variation, which is

consistent with expectations for such gene regions. The results of our analysis of the ITS2 consensus sequence of *P. westermani* are consistent with those of other studies, which also indicate low frequencies and variances of spacer mutants in the genus *Paragonimus*. This similarity between geographically distant populations indicates that divergence is minimal and is limited to regions that are prone to high rates of mutation.

Polyploidy is rare among sexually reproducing animals, presumably because the chromosomal balance for sex determination would be upset. Most polyploids discovered so far are permanently hermaphroditic species, and they are capable of self-fertilization or asexual reproduction, or are parthenogenic. Polyploidy in *P. westermani* has previously been discussed by Hirai and Agatsuma (1991). Miyazaki (1978) reported that diploid *P. westermani* reproduces by bisexual reproduction, while the triploid form reproduces parthenogenetically. A polyploid condition is evident when there is an exact multiplication of the normal chromosomal complement. Polyploidy is the only clearly established mechanism of instantaneous speciation. There are two types of polyploidy. Autopolyploidy arises when more than two haploid chromosome sets of a single species participate in zygote formation. Thus, autopolyploidy is the multiplication of one basic genome. For example, an autopolyploid can be produced by the union of an unreduced (2n) gamete with a normal (n) gamete, or a tetraploid can result from an aberrant mitotic division where the chromosomes duplicate and divide but the nucleus fails to do so, thereby producing a 4n condition. Allopolyploids, on the other hand, have a chromosomal complement that is composed of two or more genomes, each from a different species. The fact that the two species can cross implies at least partial homology between the parent chromosomes. The pattern of association of the chromosomes would depend upon the species involved in the cross. Two species having no cytologically detectable evidence of homology between genomes should show only bivalent forms, while two species with a completely homologous genome would show multivalents or bivalents that are formed at random from the members of the group of four homologues. Triploid forms of *P. westermani* occurring in northeastern countries (China, Japan, Korea and Taiwan), are often sympatric with diploid. Only diploid forms have been found to date in southeastern countries (Malaysia, Philippines and Thailand). However, both diploid and triploid forms of *P. westermani* have been found in Korea (Terasaki, 1980; Hirai et al., 1985). Many studies have been focused on the origins of triploids and their relationships with diploid *P. westermani*, and chromosomal studies have proven to be important for evaluating competing theories with respect to the origins of triploids. Sakaguchi and Tada (1980) and Hirai et al. (1985) analyzed the karyotype of *P. westermani* in Japan and Korea and reported that all 11 sets of three homologous chromosomes from triploids were identical. Terasaki et al. (1995) observed that each chromosome set in diploid and triploid cells from mixoploid lung flukes was identical to that found in diploid and triploid flukes. It is conceivable that mixoploids produce diploids and triploids through parthenogenesis. Accordingly, it cannot be concluded that diploids, triploids, and mixoploids are independent natural groups. On the basis of isoenzyme patterns, Hirai and Agatsuma (1991) proposed that triploidy in *P. westermani* was established by hybridization of two closely related species. They also suggested that triploidy arose only once in *P. westermani*, as this form is invariant where it occurs. In addition, Agatsuma et al. (1992) reported that tetraploids were produced by hybridization between triploid and diploid flukes, based on allozymes. The origin of tetraploids in *P. westermani* has been reported by Terasaki et al. (1989, 1995). They mentioned that the triploid is an autotriploid generated in the ancestral diploid population that was polymorphic for the nucleolar chromosome. Terasaki et al. (1995) reported two tetraploid individuals of *P. westermani* in northeast China, sympatric with diploid and triploid specimens. They considered that tetraploid are autotetraploids, and that these are probably produced by fertilization of diploid and triploid individuals. In the population genetic study between the two types of *P. westermani* using restriction endonuclease analysis, Agatsuma et al. (1994) demonstrated that the triploids may have arisen from hybridization between strains of a

Japanese and a non-Japanese diploid of *P. westermani*. Further clarification of the evolutionary relationships among *P. westermani* lineages may prove to be difficult. In the present study, almost identical ITS2 and mtCOI sequences were obtained from not only three populations of Korean *P. westermani*, but also they were also found to be almost identical with other populations in the northeastern Asian group, included China, Japan and Taiwan, regardless of diploid or triploid. According to both the ITS2 and the mtCOI sequences, *P. westermani* may be partitioned into at least two groups. One group consists of diploids and triploids from China, Japan, Korea and Taiwan, and exhibits relatively little molecular variation. Therefore, we postulate that the triploid form could have arisen somewhere in the northeastern Asian group. The second group (Malaysia, Philippines and Thailand) was less clearly defined, especially through the mtCOI data. Based on the ITS2 and mtCOI data, each isolate in the second group was found to be almost as distant from the others as from any member of the northeast group. Members of the *Paragonimus* species are also different in terms of host specificity and other biological features. *Paragonimus westermani* from China, Japan, Korea and Taiwan utilize Pleuroceridae snails as the first intermediate hosts, while those from Philippines, Malaysia and Thailand utilize Thiaridae snails (Davis et al., 1994). The pathogenicity of *P. westermani* is also known to be quite different. Triploids are much more pathogenic in humans than diploids. Additionally, diploid forms of southeastern isolates are reportedly either non-pathogenic or very weakly pathogenic in humans (Kawashima, 1987). *Paragonimus westermani* develops to adult worm form usually in pairs in lung tissue capsules laid down by the host. Although pairing is usual, cross-fertilization is not essential (Fan et al. 1974). Moreover, in mixed infections of involving two or more species, individuals of different species can develop normally together in the same cyst (Hatsushika, 1981).

From these facts, we propose one possible origin of triploid *P. westermani*. Triploid forms in parthenogenetic *P. westermani* are thought to be derived by autopolyploidy. If triploid *P. westermani* has possibly been established as a hybrid of two or more ancestral species (allopolyploidy), intraspecies variations of *P. westermani* should be evident in mtCOI sequences, however, no intraspecific variability was detected between the isolates obtained from different areas. In particular, when third codon positions from the mtCOI nucleotide sequences were omitted, the strict consensus tree in the northeast Asian group collapsed, indicating that much of the phylogenetic information is contained in the third codon positions. However, Philippines and Thailand isolates yielded phylogenetic trees that were very similar to those constructed from nucleotide sequences with all codon positions, even though the third codon positions were omitted. Terasaki (1980) suggested that the triploid form might be induced by polyploidization of the *P. westermani* (2n) genome, i.e., autotriploid. His suggestion depends mainly on the observation that the three homologous chromosomes are almost homomorphic in triploid individuals. Chromoso-mal behavior similar to that found in parthenogenetic triploid *P. westermani* has been reported in the parthenogenetic triploid *Fasciola* sp. (Terasaki et al., 2000) and in the parthenogenetic triploid *Diphyllobothrium erinacei* (Sasada, 1978).

Using the conventional Giemsa staining technique, Hirai et al. (1985) observed that the triploid was an allotriploid, probably induced by interspecific hybridization between the diploid *P. westermani* and an unknown species. In addition, they suggested that the triploid *P. westermani* was an independent species and synonymous with *P. pulmonalis* (Miyazaki, 1978). On the basis of their mtCOI and ITS2 gene sequences, triploids in our present study could not be distinguished from diploids. There are no supporting data for the proposal that the triploid form should be placed in a separate species, as some workers have suggested (Blair et al., 1999). Evidence from mitochondrial and nuclear genomes suggests that triploid lineages might have arisen more than once (Van Herwerden et al., 1999). Comparative data of chromosome numbers, the ITS2 gene of rDNA and the mtCOI gene sequences suggest that the triploid forms of *P. westermani* was originated from an ancestral species of diploid *P. westermani* by autopolyploidy. Theoretically, natural populations of autopolyploids should exhibit the degree of heterozygosity greater than populations of their

diploid progenitor, because of polysomic inheritance. Additional sequencing data from other conserved protein-encoding genes or microsatellite are required for further confirmation of the proposed hypotheses on the origin of triploidy.

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