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# The origin of the triploid in *Paragonimus westermani* on the basis of variable regions in the mitochondrial DNA

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**Abstract**

Triploid, parthenogenetic forms of the lungfluke, *Paragonimus westermani*, occur in Japan, Korea and China. The origin(s) of triploidy has been debated over the years. Sequences of two regions in the mitochondrial DNA, i.e. partial lrRNA (16S), and a portion of the non-coding region, were obtained from natural populations of *P. westermani*. All triploid individuals (Japan, Korea, China) and a single tetraploid individual (China) had identical sequences in the 16S region studied. Some sequence variation was observed among diploids, with those from Taiwan being distinct from the remainder. Both neighbour joining and parsimony trees using the 16S region placed diploid individuals from southwestern Japan close to the triploids and the tetraploid. The fragment amplified from the mitochondrial non-coding region showed dimorphism. One form (type A) consisted of 239 bp comprising two identical tracts of 70 bp separated by a tract of 93 bp. The second form (Type B) consisted of only a single 70 bp tract. All diploid individuals from Taiwan, China and Korea possessed type A, while those from Japan were polymorphic; individuals from Oita and Hyogo had type B, those from Chiba had type A, but both types were found in Mie. On the other hand, all of the triploid individuals and two tetraploid individuals possessed type B. Both the form present in the non-coding region and the 16S sequence suggest an affinity between a south-eastern group of diploid populations in Japan and the triploid form. A possible mechanism responsible for the origin of the triploid is discussed.

## Introduction

Since the first discovery of a triploid form of *P. westermani* in Japan (Terasaki, 1977), various taxonomic, isoenzyme, chromosomal and molecular studies have been done on it (Miyazaki, 1978; Agatsuma & Habe, 1985; Hirai *et al.*, 1985; Blair *et al.*, 1997, 1999). Triploids were subsequently discovered also in Korea, Taiwan and north-east China. In Liaoning Province, north-east China, diploids and triploids co-exist with tetraploid individuals (Terasaki *et al.*, 1989). Metacercariae of all three ploidy

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types can be found in the same individual crustacean host. After his intensive morphological studies, Miyazaki (1991) stated that the triploid is a separate species, and named it *Paragonimus pulmonalis* (Baelz, 1880) Miyazaki, 1978. To investigate the relationship between the two forms, extensive isoenzyme studies were carried out on natural populations from the Philippines, Malaysia, China, Taiwan and Japan in East Asia (Agatsuma, 1987; Agatsuma *et al.*, 1987, 1989; Agatsuma & Su, 1989). All triploid populations were monomorphic at all 16 loci examined, five of which were heterozygotes. Surprisingly, one allele at each of these five loci in Japanese triploids was not present in diploid populations from Japan but was found in diploid populations from Taiwan and China. However, the other allele at each of the five heterozygous loci in the triploid was present in diploid populations from Japan. From these data, Hirai & Agatsuma (1991) hypothesized that the triploid form might have arisen recently on a single occasion by crossing between a diploid individual from Japan and one from another country (perhaps China). Chromosomal studies have also demonstrated that some homologous chromosomes in triploids differ in their C-banding patterns, suggesting that crossing might have been involved in the origin of triploidy through allopolyploidy (Hirai & Agatsuma, 1991). However, other findings have suggested that the triploid could have arisen through autopolyploidy (Tan & Li, 1990; Terasaki *et al.*, 1996; Van Herwerden *et al.*, 1999). One of these studies concerned karyotypes (Terasaki *et al.*, 1996). In another study, Van Herwerden *et al.* (1999) found distinct differences among triploid specimens from Japan, China and Korea, using restriction fragment length polymorphisms (RFLPs) of ribosomal internal transcribed spacers and fingerprint patterns. These findings suggested that triploid lineages may have originated independently on more than one occasion. As this short introduction demonstrates, an exact mechanism responsible for the origin(s) of triploidy, and possible time(s) and place of this event(s), is still in question.

Mitochondrial DNA (mtDNA) has been used as a marker for vagility of organisms and for tracing pedigrees through maternal lineages (Boore, 1999). Previous studies using the whole mtDNA in *P. westermani* demonstrated that, out of 16 restriction enzymes, *Pst* I, *Hae* III and *Rsa* I revealed consistently different cleavage patterns between

Table 1. Number of individuals surveyed in each locality for the two regions of mt DNA (16S and the non-coding region) and PCR-typing for the non-coding region in *Paragonimus westermani.*

| Localities studied and ploidy | Countries | Number of individulas surveyed | | PCR-typing for the non-coding region** | DNA source of materials (reference) | Accession number for 16S |
|---|---|---|---|---|---|---|
| | | 16S | Non-coding region | | | |
| **2n** | | | | | | |
| Chiba | Japan | 1 | 5 | A | Iwagami *et al.* (2000) | Chiba3(AY190050) |
| Mie | Japan | 4 | 5 | A:B = 2:3 | Iwagami *et al.* (2000) | Mie2(AY190051) Mie4(AY190052) |
| Hyogo | Japan | 1 | 10 | B | Iwagami *et al.* (2000)* | Hyogo1(AY190053) |
| Oita | Japan | 1 | 10 | B | Iwagami *et al.* (2000)* | Oita1(AY190054) |
| Haenam-gun | Korea | 1 | 6 | A | Present study | Haenam1(AF540958) |
| Wanbo | China | 1 | 1 | A | Iwagami *et al.* (2000) | Wanbo1(AY190055) |
| PingShan | China | 1 | 1 | A | Iwagami *et al.* (2000) | PingShan1 (AY190056) |
| Xigutai | China | 0 | 5 | A | Present study | |
| Guaofanz | China | 1 | 6 | A | Iwagami *et al.* (2000)* | Guaofanz1(AY190057) |
| Minqing | China | 1 | 1 | A | Iwagami *et al.* (2000) | Minqing1(AY190060) |
| Shaowu | China | 1 | 1 | A | Iwagami *et al.* (2000) | Shaowu1(AY190058) |
| Lishui | China | 1 | 1 | A | Iwagami *et al.* (2000) | Lishui1(AY190059) |
| Karapai | Taiwan | 2 | 1 | A | Iwagami *et al.* (2000) | Karapai1(AY190061) Karapai2(AY190062) |
| Myaoli | Taiwan | 1 | 1 | A | Iwagami *et al.* (2000) | Myaoli1(AY190063) |
| Leyte | Philippines | 1 | 1 | na | Iwagami *et al.* (2000) | Leyte6(AY190064) |
| Kuala Pilah | Malaysia | 0 | 2 | na | Iwagami *et al.* (2000) | |
| Thailand | Thailand | 0 | 1 | na | Iwagami *et al.* (2000) | |
| **3n** | | | | | | |
| Tsushima | Japan | 1 | 3 | B | Present study | Tsushima2-1(AY190065) |
| Amakusa | Japan | 2 | 8 | B | Iwagami *et al.* (2000)* | Amakusa1(AY190066) |
| Yakushima | Japan | 1 | 2 | B | Present study | Yakushima35(AY190067) |
| Bogil-do | Korea | 2 | 4 | B | Iwagami *et al.* (2000) | Bogil-do(AF219379) |
| Beiguhe | China | 0 | 1 | B | Present study | |
| Liujiahe | China | 1 | 4 | B | Present study | Liujiahe20-1(AY190069) |
| **4n** | | | | | | |
| Xigutai | China | 1 | 2 | B | Present study | Xigutai10-8(AY190070) |

*Several new individuals in each locality were added for the non-coding region survey in the present study.
** For A and B in the PCR typing, see text.
na, Not amplified.

Japanese diploids and triploids (Agatsuma *et al.*, 1994). Here we present sequence data from two regions of mtDNA, i.e. lrRNA (16S) and a portion of the long non-coding region, in *P. westermani* in order to examine the origin of the triploid. Both regions were found to be highly variable among natural diploid populations, but not among triploids (or tetraploids). A possible mechanism for the origin of triploidy in *P. westermani* is discussed.

## Materials and methods

Most of the individual specimens of *P. westermani* used in this study are those previously reported (Iwagami *et al.*, 2000). Several new specimens collected in 1989 were used in the present study, as shown in table 1. Recently in Korea, diploid metacercariae were found and collected in Haenam-gun, Chollanam-do. Adult worms were recovered from a dog about 90 days post-infection with the metacercariae. A map of the geographical distributions of three forms, diploid, triploid and tetraploid, of *P. westermani* is shown in fig. 1.

Genomic DNA was extracted from whole worms. Worms were incubated in an extraction buffer (Invitrogen Easy DNA kit) containing SDS and proteinase K. The solubilized samples were treated with phenol and chloroform. A single worm was used in each case. The polymerase chain reaction (PCR) conditions and amplification reactions have been described elsewhere (Iwagami *et al.*, 2000). Purified PCR products were precipitated with ethanol and resuspended in distilled water, and aliquots were sequenced using the PRISM kit (ABI). Polymerase chain reaction primers were used as sequencing primers. The purified reactions were applied to an ABI DNA sequencer. The 16S region was PCR-amplified using primers 5′-ATT TAC ATC AGT GGG CCG TC-3′ (T7-1) (8834-8853)* and 5′-GAT CCA AAA GCA TGT GAA AC-3′ (SP6-1) (9658-9678)*. Sequencing was performed using the same primers as those for PCR and additional two primers, 5′-TAA TGA GTA GTT TGA ATG GC-3′ (T7-2) (9339-9359)* and 5′-ACT CGA CTC CGG CAA CTA GC-3′ (SP6-2) (9134-9154)*. A non-coding region was amplified by PCR using two primers; 5′-GTC ATC CTT TCT GTT TCT CT-3′ (Pw NCR-F) (13669-13689)* and 5′-GCT GTT TTG TAA TTC TGA CA-3′ (Pw NCR-R) (14035-14055)*. For numbers* in parenthesis, see the sequence numbers of triploid *P. westermani* (AF219379). Analyses for multiple sequence alignments were done using the programs CLUSTAL V and GENETYXMAC (ver. 6.0). Phylogenetic analysis was performed using distance (neighbour joining (NJ)) and parsimony methods in MEGA (ver. 2.1) and PAUP (ver. 3.1.1), respectively.

## Results

### *lrRNA (16S)*

The fragment amplified for 16S was 758 bp in size. The alignment for 16S is shown in fig. 2a,b. The sequence from a tetraploid was identical to that from all triploids. No variations in the sequence were present among any triploid or tetraploid individuals, while variations were present among diploid individuals and populations. The Leyte population from the Philippines was observed to have two gaps and the largest differences from the remaining populations in sequence differences, as shown

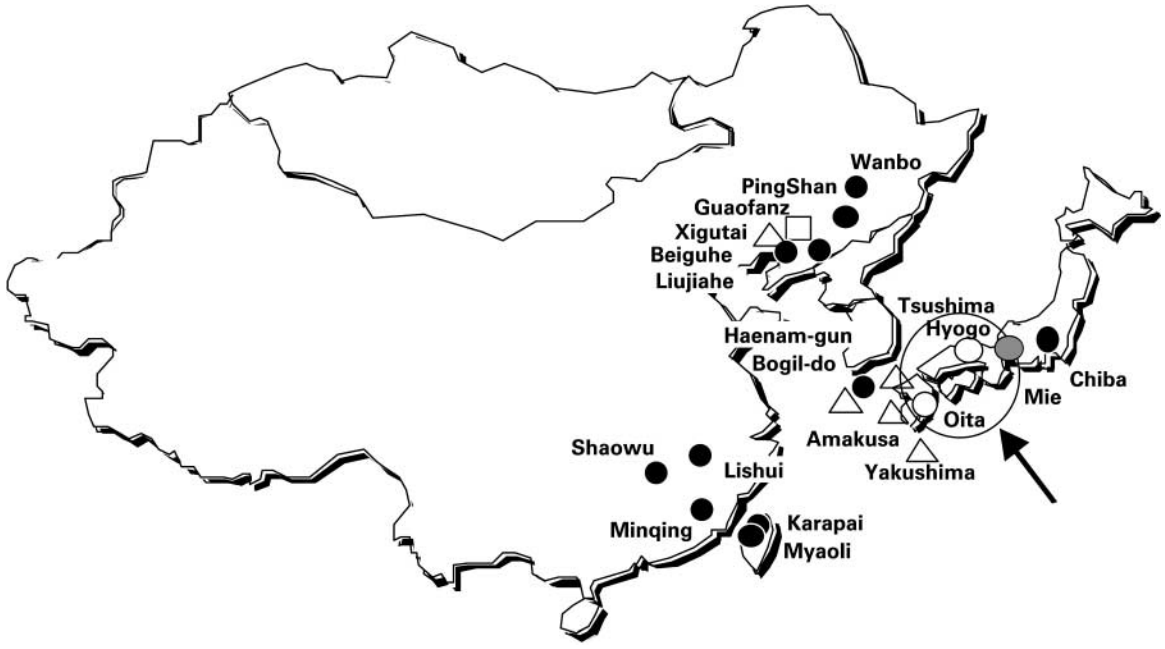


Fig. 1. Geographical distributions of the three forms (diploid, triploid and tetraploid) of *Paragonimus westermani* surveyed in the present study. Circle = diploid; triangle = triploid; square = tetraploid. Black = type A (showing a 93 bp band in the non-coding region), white = type B (a 239 bp band in the non-coding region), grey = occurrence of both types. The arrow indicates the possible area (circle) of the origin of the triploid.

in table 2 and fig. 2. The three Taiwan populations were found to be relatively diverged from the other populations other than the Leyte population. As shown in table 2, the triploid individuals were all found to be closest to diploids from Ohita and Hyogo populations. Geographic locations and numbers of individuals surveyed in each locality are shown in fig. 1 and table 1, respectively.

Based on an outgroup of the Leyte population, phylogenetic trees (fig. 3) were constructed by the NJ and parsimony methods with 1000 cycles of bootstrapping. The tree(s) shows that, among diploids, those from Taiwan are very distinct from the remainder. Both NJ and parsimony trees placed diploid individuals from south-western (SW) Japan close to the triploids and the tetraploid to the exclusion of diploids from all other places. As shown in fig. 3, a NJ tree showed that two large clusters were formed. One cluster includes two diploid populations from Taiwan, and another cluster includes two subclusters in which one subcluster consists of diploid populations derived from China and Korea, and another consists of all diploid populations from Japan plus all populations comprising of triploids and tetraploids. Furthermore, diploid populations from Japan can be divided into two groups, SW (Ohita and Hyogo) and eastern (Chiba and Mie). The triploid population was the most closely related to the SW

a

| | 3 8 | 4 0 | 4 7 | 6 6 | 6 7 | 7 3 | 7 4 | 7 6 | 7 7 | 7 0 | 1 2 8 | 1 4 2 | 1 6 3 | 1 6 6 | 1 9 2 | 2 0 4 | 2 1 0 | 2 3 6 | 2 3 2 | 2 4 4 | 2 5 0 | 2 6 6 | 2 6 2 | 2 7 8 | 2 7 4 | 2 7 5 | 2 8 7 | 2 8 0 | 2 9 7 | 3 0 5 | 3 0 2 | 3 0 3 | 3 1 9 | 3 2 7 | 3 3 1 | 3 3 0 | 3 9 0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chiba3(2n) | T | T | T | C | G | T | C | C | G | G | A | T | C | C | A | A | C | A | T | G | C | G | A | C | A | A | A | C | G | G | C | T | G | T | T | A | G |
| Mie2(2n) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Mie4(2n) | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | T | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Hyogo1(2n) | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | T | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Oita1(2n) | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | T | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Haenam1(2n) | . | . | . | . | . | . | . | T | . | . | . | . | T | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . |
| Wanbo1(2n) | . | . | . | . | . | . | . | T | . | . | . | . | T | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . |
| PingShan1(2n) | . | . | . | . | . | . | . | T | . | . | . | . | T | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . |
| Guaofanz1(2n) | . | . | . | . | . | . | . | T | . | . | . | . | T | T | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . |
| Shaowu1(2n) | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . |
| Lishui1(2n) | . | . | . | . | A | . | . | . | . | . | . | . | T | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . |
| Minqing1(2n) | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . |
| Karapai1(2n) | . | C | . | . | . | . | . | . | . | . | . | . | T | T | . | . | . | C | A | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Karapai2(2n) | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | C | A | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Myaoli1(2n) | . | . | A | . | . | . | . | . | . | . | . | . | T | . | . | . | . | C | A | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Amakusa1(3n) | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | T | G | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Xigutai10(4n) | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | T | G | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Leyte6(2n) | C | C | . | T | A | G | A | T | T | A | G | C | T | T | G | G | . | . | C | . | T | T | G | T | G | G | G | T | A | A | T | C | A | - | . | G | A |

b

| | 3 9 1 | 3 9 4 | 3 9 8 | 4 2 0 | 4 2 3 | 4 2 9 | 4 3 0 | 4 3 4 | 4 4 1 | 4 4 4 | 4 4 5 | 4 5 3 | 4 5 7 | 4 7 1 | 4 7 3 | 4 7 5 | 4 7 7 | 4 9 4 | 4 5 1 | 5 2 6 | 5 2 3 | 5 5 4 | 5 5 1 | 5 6 6 | 6 3 9 | 6 3 2 | 6 5 4 | 6 5 6 | 6 5 8 | 6 6 0 | 6 6 3 | 6 7 5 | 7 7 6 | 7 8 3 | 7 0 0 | 7 1 5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chiba3(2n) | A | G | G | T | C | C | G | C | T | T | C | G | A | G | C | T | T | C | A | G | A | C | C | T | G | G | G | C | C | C | C | T | A | T | G |
| Mie2(2n) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . |
| Mie4(2n) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Hyogo1(2n) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Oita1(2n) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Haenam1(2n) | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Wanbo1(2n) | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| PingShan1(2n) | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Guaofanz1(2n) | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Shaowu1(2n) | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Lishui1(2n) | . | . | . | C | . | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Minqing1(2n) | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Karapai1(2n) | . | . | . | C | T | . | . | . | . | . | . | . | G | . | . | . | . | . | . | A | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Karapai2(2n) | . | . | . | C | T | . | . | . | . | . | . | . | G | . | . | . | . | . | . | A | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Myaoli1(2n) | . | . | . | C | T | . | . | . | . | . | . | . | G | . | . | . | . | . | . | A | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Amakusa1(3n) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Xigutai10(4n) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Leyte6(2n) | G | A | T | . | T | . | T | T | C | C | A | A | . | A | T | C | C | . | G | A | G | T | T | C | T | C | - | T | T | T | T | C | G | C | A |

Fig. 2. Nucleotide sequence alignment in 16S regions of the mitochondrial DNA from natural populations of *Paragonimus westermani* surveyed in the present study. Only variable sites are listed in the alignment, and the numbering scheme does not include the primers. A dot '·' indicates identity with base on top line, and a hyphen '–', gaps.

Table 2. Pairwise differences in nucleotide sequences among 16S sequences of the diploid, triploid and tetraploid in *Paragonimus westermani*.

| Country | Ploidy | Locality | CB3 | ME2 | ME4 | HG1 | OT1 | HN1 | WB1 | PS1 | GF1 | SW1 | LS1 | MQ1 | KP1 | KP2 | ML1 | AK1 | XT | LT6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Japan | 2n | Chiba(CB)3 | | 1/0 | 3/0 | 3/0 | 3/0 | 5/0 | 5/0 | 5/0 | 6/0 | 4/0 | 6/0 | 4/0 | 9/0 | 7/0 | 7/1 | 5/0 | 5/0 | 52/9 |
| Japan | 2n | Mie(ME)2 | 1 | | 4/0 | 4/0 | 4/0 | 6/0 | 6/0 | 6/0 | 7/0 | 5/0 | 7/0 | 5/0 | 10/0 | 8/0 | 8/1 | 6/0 | 6/0 | 51/9 |
| Japan | 2n | Mie(ME)4 | 3 | 4 | | 0/0 | 0/0 | 6/0 | 6/0 | 6/0 | 5/0 | 5/0 | 7/0 | 5/0 | 8/0 | 8/0 | 8/1 | 2/0 | 2/0 | 51/9 |
| Japan | 2n | Hyogo(HG)1 | 3 | 4 | 0 | | 0/0 | 6/0 | 6/0 | 6/0 | 5/0 | 5/0 | 7/0 | 5/0 | 8/0 | 8/0 | 8/1 | 2/0 | 2/0 | 51/9 |
| Japan | 2n | Oita(OT)1 | 3 | 4 | 0 | 0 | | 6/0 | 6/0 | 6/0 | 5/0 | 5/0 | 7/0 | 5/0 | 8/0 | 8/0 | 8/1 | 2/0 | 2/0 | 51/9 |
| Korea | 2n | Haenam(HN)1 | 5 | 6 | 6 | 6 | 6 | | 0/0 | 0/0 | 1/0 | 1/0 | 3/0 | 1/0 | 8/0 | 6/0 | 6/1 | 8/0 | 8/0 | 51/9 |
| China | 2n | Wanbo(WB)1 | 5 | 6 | 6 | 6 | 6 | 0 | | 0/0 | 1/0 | 1/0 | 3/0 | 1/0 | 8/0 | 6/0 | 6/1 | 8/0 | 8/0 | 51/9 |
| China | 2n | PingShan(PS)1 | 5 | 6 | 6 | 6 | 6 | 0 | 0 | | 1/0 | 1/0 | 3/0 | 1/0 | 8/0 | 6/0 | 6/1 | 8/0 | 8/0 | 51/9 |
| China | 2n | Guaofanz(GF)1 | 6 | 7 | 5 | 5 | 5 | 1 | 1 | 1 | | 2/0 | 4/0 | 2/0 | 7/0 | 7/0 | 7/1 | 7/0 | 7/0 | 50/9 |
| China | 2n | Shaowu(SW)1 | 4 | 5 | 5 | 5 | 5 | 1 | 1 | 1 | 2 | | 2/0 | 0/0 | 7/0 | 5/0 | 5/1 | 7/0 | 7/0 | 52/9 |
| China | 2n | Lishui(LS)1 | 6 | 7 | 7 | 7 | 7 | 3 | 3 | 3 | 4 | 2 | | 2/0 | 9/0 | 7/0 | 7/1 | 9/0 | 9/0 | 52/9 |
| China | 2n | Minqing(MQ)1 | 4 | 5 | 5 | 5 | 5 | 1 | 1 | 1 | 2 | 0 | 2 | | 7/0 | 5/0 | 5/1 | 7/0 | 7/0 | 52/9 |
| Taiwan | 2n | Karapai(KP)1 | 9 | 10 | 8 | 8 | 8 | 8 | 8 | 8 | 7 | 7 | 9 | 7 | | 2/0 | 2/1 | 10/0 | 10/0 | 49/9 |
| Taiwan | 2n | Karapai(KP)2 | 7 | 8 | 8 | 8 | 8 | 6 | 6 | 6 | 7 | 5 | 7 | 5 | 2 | | 1/0 | 10/0 | 10/0 | 51/9 |
| Taiwan | 2n | Myaoli(ML)1 | 8 | 9 | 9 | 9 | 9 | 7 | 7 | 7 | 8 | 6 | 8 | 6 | 3 | 1 | | 11/0 | 11/0 | 51/10 |
| Japan | 3n | Amakusa(AK)1 | 5 | 6 | 2 | 2 | 2 | 8 | 8 | 8 | 7 | 7 | 9 | 7 | 10 | 10 | 11 | | 0/0 | 53/9 |
| China | 4n | Xigutai(XT)10 | 5 | 6 | 2 | 2 | 2 | 8 | 8 | 8 | 7 | 7 | 9 | 7 | 10 | 10 | 11 | 0 | | 53/9 |
| Philippines | 2n | Leyte(LT)6 | 61 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 59 | 61 | 61 | 61 | 58 | 60 | 61 | 62 | 62 | |

Values above diagonal are transitions/transversions. Those below are total differences out of 758 bp.

group in Japan. Tree analysis by the parsimony method gave basically the same results as that by the NJ method.

*Portion of the non-coding region*

A portion of the long non-coding region was PCR-amplified for every individual listed in table 1. Polymerase chain reaction products could be assigned to one of two types on the basis of length. Examples of each type were sequenced. One form (type A) consists of a 93 bp region flanked at each end by an identical sequence of 70 bp. The total size of this fragment is therefore 239 bp. The second form (type B) consists of only a single copy of the 70 bp tract. All diploid individuals from Taiwan, China and Korea possessed type A. Diploid individuals from Japan possessed either A or B; those from Oita and Hyogo were all of type B and those from Chiba of type A, but both types were found in Mie. On the other hand, all triploid and tetraploid individuals examined had type B. Diploid worms from the Philippines, Malaysia and Thailand did not show any band, indicating that the primers did not anneal to the region.

## Discussion

In the present study, no intra- or inter-populational variation in the two regions of mt DNA was found in the triploid form derived from Japan, Korea and China, suggesting that the triploid form must have arisen only once.

Previous isoenzyme studies suggested that Liaoning Province (China) could be the place where the triploid originated, since every allele at the five heterozygous loci in the triploid is present in the diploid populations within this area (Agatsuma *et al.*, 1989). However, in the present study, diploid individuals examined from this area were found to have only type A in the non-coding region (as do most diploids from elsewhere). The striking exception is seen in SW Japanese diploid populations (Oita and Hyogo) where individuals have type B only. This finding suggests that a certain individual from a SW Japanese population was involved in the hybridization as the maternal parent (mitochondrial genomes being maternally inherited). The 16S phylogenetic tree obtained in this study also supports this idea. However, this hypothesis still conflicts with a previous finding of the *Pst* I digestion patterns that revealed consistent differences between diploids (two cutting site: Oita, Mie, Chiba) and triploids (one cutting site: Tsushima, Amakusa, Yakushima, Bogil Island) (Agatsuma *et al.*, 1994).

For the origin of the tetraploids, Agatsuma *et al.* (1992) suggested, on the basis of isoenzyme studies, that the tetraploids might have arisen by reproduction between individuals from a triploid and diploid form, whereby a triploid contributes a triploid egg and a diploid, a haploid sperm. Chromosomal studies also supported the reproduction theory mentioned above (Terasaki *et al.*, 1995). If the tetraploids had been of an autoploid origin from diploid individuals, they would have shown type A in the non-coding region, since there is no diploid individual having type B in Xigutai. The present study showed that

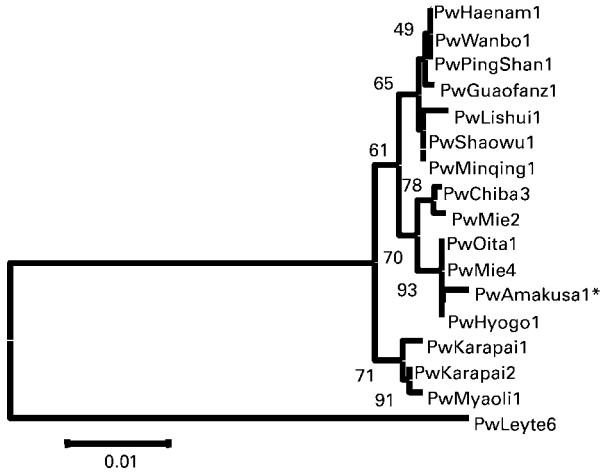


Fig. 3. A phylogenetic tree constructed by the neighbour-joining (NJ) approach in MEGA using 16S sequences in the mitochondrial DNA from natural populations of *Paragonimus westermani* surveyed in the present study. A distance matrix was calculated using the Kimura 2-parameter model. Number under the bar numbers on internodes indicate percentages of 1000 bootstrap replicates. Asterisk (*); identical to the sequence of all of triploids/tetraploids.

the non-coding region in the tetraploids is of type B, supporting the hypothesis.

It has been noted that Korean diploid forms could have an evolutionary linkage with diploid forms from Japan and China and therefore could have contributed to the triploid (Agatsuma, unpublished data). In this study, the Korean diploid was found to have type A in the non-coding region. This means that the Korean diploid could only have played a role as the paternal parent. Clearly, intensive surveys on isoenzymes and RFLP will be needed for the Korean diploid.

The Leyte diploid from the Philippines has been considered to be a separate species based on morphology (Blair *et al.*, 1999), isozyme (Agatsuma *et al.*, 1988) and CO1 sequences (Blair *et al.*, 1997). This idea is supported by extremely large numbers of differences in the 16S gene sequence between the Leyte diploid and all of the remaining individuals. Failure of PCR amplification in the Leyte diploid within the non-coding region also suggested that the amplified region was more different than intra-specific variation.

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