Molecular phylogeographic studies on *Paragonimus westermani* in Asia

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Abstract

The lung fluke, *Paragonimus westermani* (Kerbert, 1878), is widely distributed in Asia, and exhibits much variation in its biological properties. Previous phylogenetic studies using DNA sequences have demonstrated that samples from north-east Asia form a tight group distinct from samples from south Asia (Philippines, Thailand, Malaysia). Among countries from the latter region, considerable molecular diversity was observed. This was investigated further using additional DNA sequences (partial mitochondrial cytochrome *c* oxidase subunit 1 (COI) and the second internal transcribed spacer of the nuclear ribosomal gene repeat (ITS2)) from additional samples of *P. westermani*. Phylogenies inferred from these again found three or four groups within *P. westermani*, depending on the method of analysis. Populations of *P. westermani* from north-east Asia use snail hosts of the family Pleuroceridae and differ in other biological properties from populations in south Asia (that use snail hosts of the family Thiaridae). It is considered that the populations we sampled can be divided into two species, one in north-east Asia and the other in south Asia.

Introduction

Paragonimus westermani (Kerbert 1878), widely distributed in Asia, is one of the most medically important lung flukes. Numerous studies (ecological, morphological, immunological, cytological and molecular – reviewed in Blair *et al*., 1999) have detected much variability, both within regions and across the range of the species. For example, chromosome studies have found diploid, triploid and tetraploid individuals in north-east Asia (Japan, China, Korea, Taiwan). All individuals elsewhere appear to be diploid only. The results of isozyme and molecular (DNA sequence) analyses have shown that the Philippines and peninsular Malaysian populations are genetically rather distant from one another, and both are also very different from those from north-east Asian countries

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(Agatsuma *et al*., 1993; Blair *et al*., 1997). There are also differences in host specificity and other biological features. *Paragonimus westermani* from the Philippines and peninsular Malaysia utilize thiarid snails while those from Japan, Korea, China and Taiwan utilize pleurocercid snails (Davis *et al*., 1994). *Paragonimus westermani* in the north-east group are usually found in dogs, cats or foxes, which are relatively poor hosts in Malaysia (Habe, 1987). In the Philippines, the species is naturally parasitic in rats, which are also excellent experimental hosts (Miyazaki & Habe, 1979). Given these differences, it is tempting to suggest that *P. westermani* should be split into at least two species, one occurring in north-east Asia, and the other in south Asia. To gather more evidence that might support this, we obtained additional DNA sequences from the second internal transcribed spacer (ITS2) of the nuclear ribosomal gene repeat and the mitochondrial cytochrome *c* oxidase subunit 1 gene (CO1) (partial). Specimens sequenced came from seven countries, Japan, Korea, China, Taiwan, Thailand, Malaysia and The Philippines. The results were interpreted in the light of previous findings.

Materials and methods

Sources of material used are listed in table 1. Adult worms were kept at –80°C until used. Genomic DNA was extracted from whole worms. Worms were incubated in extraction buffer (Invitrogen extraction kit) containing SDS and proteinase K either overnight or until the tissues were solubilized. The solubilized samples were treated with an equal volume of phenol equilibrated to pH >7.8 either two or three times, and treated once with an equal volume of chloroform. The extracted DNAs were ethanol-precipitated. A single worm was used in each case. ITS2 and CO1 regions were amplified using the polymerase chain reaction (PCR). The PCR conditions were as follows: 94°C for 1 min, 50°C for 2 min, 72°C for 3 min, for 30 cycles. Amplification reactions were performed in a final volume of 50 μl containing primers (3.2 pmol), deoxynucleoside triphosphates (dNTPs, 0.2 mM), and *Taq* polymerase (1.75 U/reaction). As primers we used 5′-CGG TGG ATC ACT CGG CTC GT-3′ (3S) as a forward primer and 5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′ (A28) as a reverse primer for the ITS2 region (Bowles *et al*., 1995) and 5′-TTT TTT GGG CAT CCT GAG GTT TA-3′ (FH5) as a forward primer and 5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′ (FH3) as a reverse primer for the CO1 region (Bowles *et al*., 1993). The PCR products were treated with chloroform and purified using either high performance liquid chromatography (HPLC) or MicroSpin Columns (Pharmacia Biotech). The purified DNA was precipitated with ethanol and resuspended in 20 μl of distilled water, and aliquots were sequenced using the PRISM kit (ABI). PCR primers were used as sequencing primers. The reactions were purified according to the manufacturer's instructions (ABI) and applied to an ABI sequencer (373A or 377). Alignment analyses were done using the program GENETYXMAC ver. 6.0 (Software Development Co., Tokyo, Japan). Codon usage was derived from a report of Garey & Wolstenholme (1989), except that the codon ATA was translated to isoleucine rather than methionine (Bowles *et al*., 1992) and that AAA was translated to asparagine rather than lysine (Ohama *et al*., 1990) in DNASIS ver. 3.2. (Hitachi software Engineering Co., Japan 1994). Phylogenetic analysis was performed using distance and parsimony methods in PHYLIP (ver. 3.572; Felsenstein, 1989) and MEGA (ver. 1.01; Kumar *et al*., 1993).

Table 1. Material used and geographical origin.

Species	Origin and numbers of isolates used	
Paragonimus westermani (2n)	Chiba, Japan	5
	Mie, Japan	5
	Hyogo, Japan	5
	Oita, Japan	5
	Ping Shan, Heilongjiang, China	5
	Wanbo, Jilin, China	5
	Lishui, Zhejiang, China	9
	ZhaoWu, Fujian, China	5
	Guaofanz, Liaoning, China	5
	Xi Kou, Hubei, China	5
	Minqing, Fujian, China	5
	Zong An, Fujian, China	5
	Myaoli, Taiwan	5
	Karapai, Taiwan	4
	Taipin, Taiwan	2
	Uru Langat, Malaysia	5
	Kuala Pilah, Malaysia	4
	Sungai Wa, Malaysia	1
	Leyte, Philippines	8
	Sorsogon, Philippines	7
	Thailand	1
P. westermani (3n)	Amakusa, Japan	10
	Bogil Island, Korea	4
P. ohirai	Kinosaki, Japan	1
P. miyazakii	Rokuroshi, Japan	1

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Results

ITS2 gene

The alignment of ITS2 sequence was 454 bp in length including a part of the 5.8S gene and a part of the 28S gene (fig. 1). The end point of the 5.8S rRNA gene was determined by comparative alignment of the sequences of the *Schistosoma* species published by Bowles *et al*. (1993). A length of 287 bp was estimated for the whole ITS2 gene, as was that of 119 bp for a part of the 5.8S gene and that

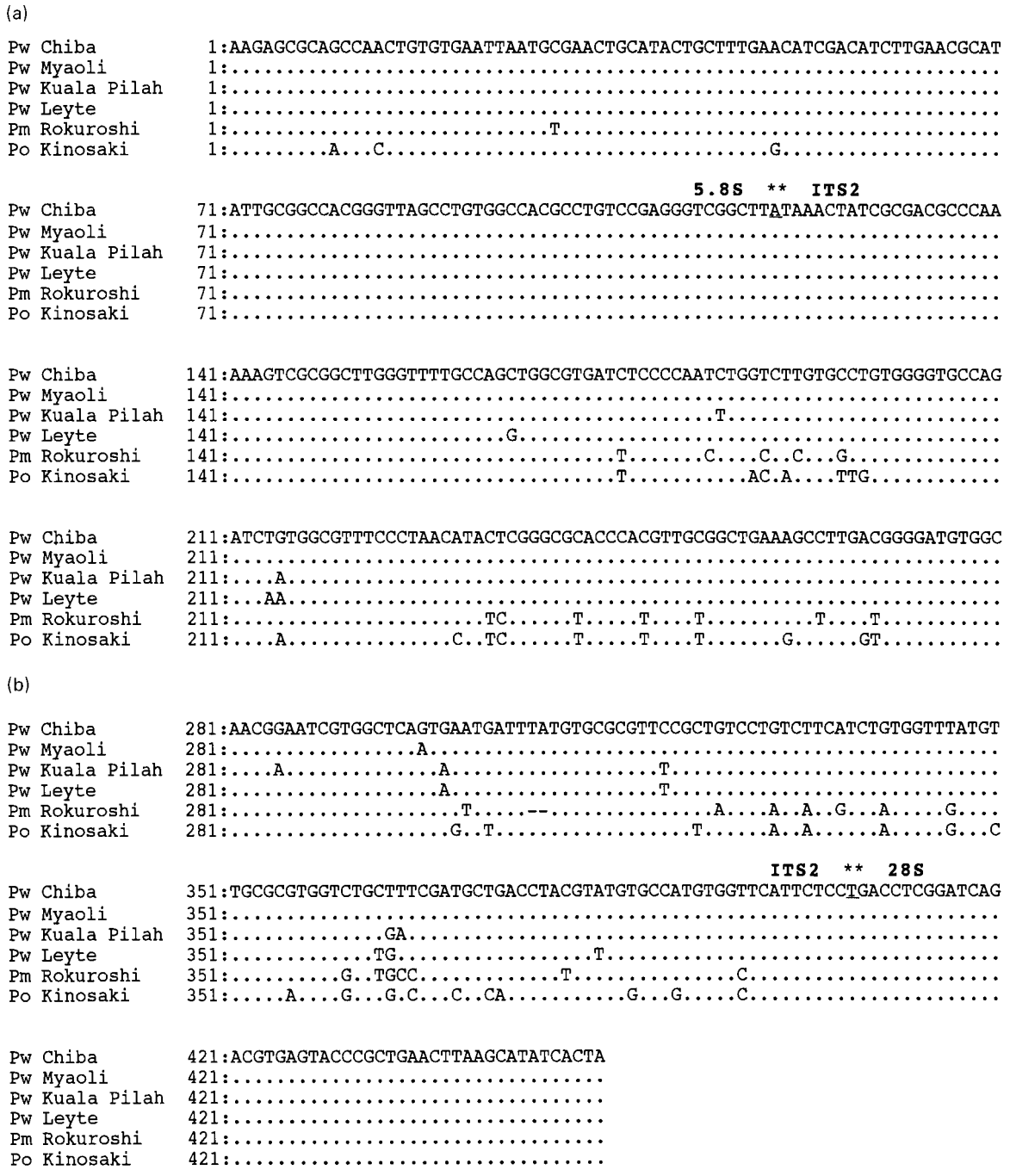

Fig. 1. Nucleotide sequences of a region of the ITS2 gene of nuclear ribosomal DNA of *Paragonimus* species. Alignment gaps indicated by a hyphen. Sites with a nucleotide identical to that on the top line indicated by a dot. All isolates from Japan, China and Korea had sequences identical to that from Chiba, Japan. The presumed beginning and end of the actual spacer region are marked by asterisks. The 5′ end of the sequence is of 5.8S origin, whereas a small portion of 28S sequence is shown at the 3′ end.

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Table 2. Pairwise differences among ITS2 sequences.

Species	Origin	2	3	4	5	6	7
Paragonimus westermani	1 Chiba, Japan	1	7	8	10	27	38
	2 Myaoli, Taiwan		8	9	11	28	39
	3 Kuala Pilah, Malaysia			7	9	31	41
	4 Leyte, Philippines				10	31	42
	5 Thailand					34	43
P. miyazakii	6 Rokuroshi, Japan						32
P. ohirai	7 Kinosaki, Japan						

All isolates from Japan, China and Korea had sequences identical to that from Chiba, Japan.

of 48 bp for the 28S gene. Average nucleotide frequencies of A, C, G and T were 15.5%, 25.5%, 29% and 30%, respectively. Thus the G-C content is 54.5%. Little variation was observed among the ITS2 sequences and only a few gaps needed to be inserted for unambiguous alignment (fig. 1). Pairwise differences among ITS2 sequences were shown in table 2. All isolates of *P. westermani* from Japan, Korea and China had identical ITS2 sequences. Three Taiwan isolates from Myaoli, Taipin and Karapai were identical to one another but differed from Japanese isolates at one site. Specimens from Kuala Pilah, Sungai Wa and Ulu Langat (Malaysian) were identical to one another but differed from those of other countries. Specimens from Leyte and Sorsogon (Philippine) were also identical to each other in sequence but differed from those of other countries. The peninsular Malaysian isolates differed from Philippine ones at seven sites.

Mitochondrial CO1 gene

The alignment of partial CO1 nucleotide sequences was 383 bp in length (fig. 2). There were no insertions or deletions. In the *P. westermani* from Japan, Korea and China, average nucleotide frequencies of A, C, G and T were 14%, 19%, 30% and 37%, respectively. Thus the G-C content is 49%. Pairwise differences among CO1 sequences were shown in tables 3 and 4. In contrast to the ITS2 data, many variations were observed in the CO1 data, even though isolates were from the same localities. As shown in table 2 and 3, three diploid individuals from Oita, Hyogo and Mie, and two triploid individuals from Amakusa and Bogil islands were all identical in the CO1 sequence. On the other hand, three diploid individuals from Guaofanz, Zong An and Myaoli were identical to each other, but differed from the above five isolates at three sites. The Japanese *P. westermani* differed from the Chinese *P. westermani* at four to seven sites. Sequences from peninsular Malaysia, the Philippines and Thailand differed from those from Japan and China at many sites (tables 2 and 3). Nucleotide substitutions were mainly transitions and most of them occurred at the third codon. Most nucleotide substitutions were synonymous: no two sequences differed by more than four amino acid residues (tables 2 and 3). Trees inferred from the partial CO1 sequences are shown in fig. 3. For trees 3a and 3b, a distance matrix was constructed using the Kimura 2-parameter model and trees inferred using the UPGMA method (fig. 3a) or the neighbour joining method (fig. 3b). The parsimony tree in fig. 3c was found by a heuristic search.

Discussion

ITS2 sequences were conserved relative to CO1 sequences. In trematodes, intraspecific variation in ITS2 sequences is minimal or non-existent (Morgan & Blair, 1995; Despres *et al*., 1992; Hashimoto *et al*., 1997). According to the CO1 sequences, the *P. westermani* samples sequenced fall into three or four groups (depending on the method of analysis). The first group, the north-east group, consists of isolates from Japan, Korea, China and Taiwan; the second, those from Malaysia; and the third, those from the Philippines (fig. 3a). In parsimony analyses (fig. 3c) the single worm from Thailand is sister to all other *P. westermani*. In trees inferred using NJ or UPGMA, this worm is sister to the Philippine samples. In the UPGMA tree, all south Asian samples form a monophyletic group to the exclusion of the north-east Asian samples. A similar grouping was found using isozyme studies (Agatsuma *et al*., 1993). However, in the present study, the other two tree-building methods placed the Malaysian samples in a sister group to the north-east Asian samples, and the Philippine samples as sister to both of these. Miyazaki & Chiu (1980) suggested that the diploid forms from Taiwan were morphologically similar to the populations from the Philippines. However, our CO1 sequences from Taiwan samples were clearly placed within the north-east group and very distant from the Philippine sequences. Philippine worms exhibit some morphological peculiarities (Miyazaki, 1978): one or two lobes of one or both testes are often separate from the rest and directly connected to the vas deferens. In addition, the metacercaria is much smaller than that from other countries (Miyazaki, 1981). Among the synonyms for *P. westermani* from south Asia are *P. macacae* Sandosham, 1953 (re-examined by Miyazaki, 1956) and *P. filipinus* Miyazaki, 1978. If the south Asian populations utilizing thiarid snails are to be recognized as a single separate species, *Paragonimus macacae* is the oldest available name. However, we are reluctant to propose formally that the name *P. macacae* Miyazaki, 1978 be re-introduced for the south Asian forms. There are two reasons for this. Firstly, our molecular data are equivocal about placing all south Asian populations in a single clad. Only the UPGMA analysis does this. The other tree building methods suggest that a taxon containing all south Asian forms is paraphyletic (i.e. it does not include all

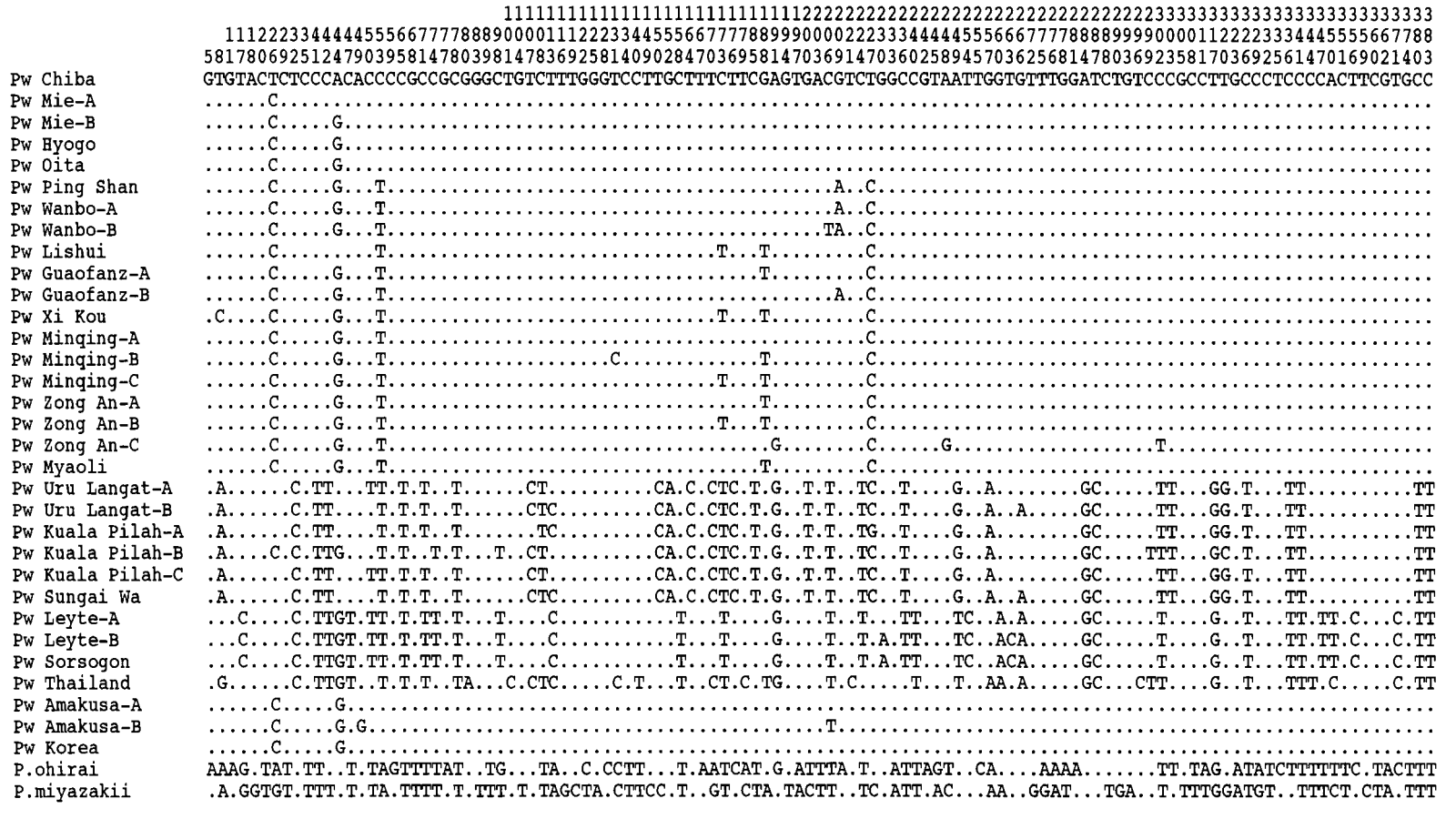

Fig. 2. Nucleotide sequences of a region of the CO1 gene of mitochondrial DNA of *Paragonimus* species. Only variable sites were listed. Sites with a nucleotide identical to that on the top line indicated by a dot.

Table 3. Pairwise differences among CO1 sequences.

Species	Origin	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	28	19	20	21	22	23	24	25	26
P.w. (2n)	1 Chiba(3), Japan	–	1/0	2/0	2/0	5/0	6/0	5/0	6/0	4/1	4/1	5/0	6/1	4/0	5/1	5/1	4/1	5/1	7/0	4/1	33/4	35/3	39/5	2/0	4/0	2/0	61/21
	2 Mie-A(2), Japan	0	–	1/0	1/0	1/0	4/0	4/0	5/0	3/1	3/1	4/0	5/1	3/0	4/1	4/1	3/1	4/1	6/0	3/1	34/4	36/3	40/5	1/0	2/0	1/0	61/21
	3 Mie-B(2), Japan	0	0	–	0/0	0/0	3/0	3/0	4/0	4/1	2/1	3/0	4/1	2/0	3/1	3/1	2/1	3/1	5/0	2/1	35/4	35/3	39/5	0/0	2/0	0/0	60/21
	4 Hyogo(3), Japan	0	0	0	–	0/0	3/0	3/0	4/0	4/1	2/1	3/0	4/1	2/0	3/1	3/1	2/1	3/1	5/0	2/1	35/4	35/3	39/5	0/0	2/0	0/0	60/21
	5 Oita(2), Japan	0	0	0	0	–	3/0	3/0	3/0	4/1	2/1	3/0	4/1	2/0	3/1	3/1	2/1	3/1	5/0	2/1	35/4	35/3	39/5	0/0	2/0	0/0	61/21
	6 Ping Shan(3), China	0	0	0	0	0	–	0/0	1/0	3/1	1/1	0/0	3/1	1/0	2/1	2/1	1/1	2/1	4/0	1/1	34/4	36/3	40/4	3/0	5/0	3/0	61/21
	7 Wanbo-A(1), China	0	0	0	0	0	0	–	1/0	3/1	1/1	0/0	3/1	1/0	2/1	2/1	1/1	2/1	4/0	1/1	34/4	36/3	40/4	3/0	5/0	3/0	60/21
	8 Wanbo-B(4), China	0	0	0	0	0	0	0	–	4/1	2/1	1/0	4/1	2/0	3/1	3/1	2/1	3/1	5/0	2/1	33/3	35/3	39/5	4/0	4/0	4/0	60/20
	9 Lishui(3), China	0	0	0	0	0	0	0	0	–	2/0	3/1	2/0	2/1	3/0	1/0	2/0	1/0	5/1	2/0	31/5	35/4	39/4	4/1	6/1	4/1	60/20
	10 Guaofanz-A(4), China	0	0	0	0	0	0	0	0	0	–	1/1	2/0	0/1	1/0	1/0	0/0	1/0	3/1	0/0	33/5	35/4	39/4	2/1	4/1	2/1	61/21
	11 Guaofanz-B(1), China	0	0	0	0	0	0	0	0	0	0	–	3/1	1/0	2/1	2/1	1/1	2/1	4/0	1/1	34/4	36/3	40/4	3/0	5/0	3/0	58/20
	12 Xi Kou(1), China	0	0	0	0	0	0	0	0	0	0	0	–	2/1	3/0	1/0	2/0	1/0	5/1	2/0	32/5	35/4	38/4	4/1	6/1	4/1	60/21
	13 Minqing-A(1), China	0	0	0	0	0	0	0	0	0	0	0	0	–	1/1	1/1	0/1	1/1	3/0	0/1	33/4	35/3	39/5	2/0	4/0	2/0	61/20
	14 Minqing-B(1), China	0	0	0	0	0	0	0	0	0	0	0	0	0	–	2/0	1/0	2/0	4/1	1/0	34/5	36/4	38/4	3/1	5/1	3/1	59/20
	15 Minqing-C(3), China	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	1/0	0/0	4/1	1/0	32/5	34/4	38/4	3/1	5/1	3/1	60/20
	16 Zong An-A(1), China	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	1/0	3/1	0/0	33/5	35/4	39/4	2/1	4/1	2/1	59/20
	17 Zong An-B(1), China	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	4/1	1/0	32/5	34/4	38/4	3/1	5/1	3/1	59/21
	18 Zong An-C(1), China	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	3/1	32/4	34/3	38/5	5/0	7/0	5/0	60/20
	19 Myaoli(4), Taiwan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	33/5	35/4	39/4	2/1	4/1	2/1	52/23
	20 Uru Langat-A(3), Malaysia	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	–	25/5	28/5	35/4	35/4	35/4	43/25
	21 Leyte-A(3), Philippines	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	–	23/2	35/3	35/3	35/3	40/28
	22 Thailand(1)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	–	39/5	39/5	39/5	43/28
P.w. (3n)	23 Amakusa-A(4), Japan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	–	2/0	0/0	52/29
	24 Amakusa-B(5), Japan	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	1	–	2/0	52/29
	25 Bogil Island (4), Korea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	–	52/29
P.m.	26 Rokuroshi(1), Japan	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	2	3	2	–

Values above diagonal are transitions/transversions. Those below are amino acid differences. Numbers in parentheses are numbers of adult worms examined. A, B and C in the locality names show different haplotypes. 2n and 3n show diploid and triploid respectively. *P.w.*, *Paragonimus westermani*; *P.m.*, *P. miyazakii*.

Table 4. Pairwise differences among CO1 sequences.

Species	Origin	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
P.w. (2n)	1 Chiba(3), Japan	–	4/1	33/4	34/4	31/5	37/3	33/4	34/4	35/3	37/3	37/3	39/5	2/0	4/0	2/0	51/26	51/29
	2 Guaofanz-A(4), China	0	–	33/5	34/5	32/6	33/4	33/5	34/5	35/4	37/4	37/4	39/4	2/1	4/1	2/1	43/27	50/30
	3 Uru Langat-A(3), Malaysia	1	1	–	3/0	3/1	7/1	0/0	3/0	25/5	37/5	27/5	28/5	35/4	35/4	35/4	30/24	43/25
	4 Uru Langat-B(2), Malaysia	1	1	0	–	2/1	8/1	3/0	0/0	24/5	26/5	26/5	25/5	36/4	36/4	36/4	42/24	45/25
	5 Kuala Pilah-A(2), Malaysia	1	1	0	0	–	8/1	3/1	2/1	23/6	25/6	25/6	26/6	33/5	33/5	33/5	39/25	43/26
	6 Kuala Pilah-B(3), Malaysia	1	1	0	0	0	–	7/1	8/1	27/4	29/5	29/4	30/4	35/3	35/3	35/3	44/23	45/26
	7 Kuala Pilah-C(4), Malaysia	1	1	0	0	0	0	–	3/0	25/5	27/5	27/5	28/5	35/4	35/4	35/4	40/24	43/25
	8 Sungai Wa(1), Malaysia	1	1	0	0	0	0	0	–	24/5	26/5	26/5	25/5	36/4	36/4	36/4	42/24	45/25
	9 Leyte-A(3), Philippines	1	1	1	1	1	1	1	1	–	1/0	1/0	23/2	35/3	35/3	35/3	36/25	40/28
	10 Leyte-B(2), Philippines	1	1	1	1	1	1	1	1	0	–	0/0	25/2	37/3	37/3	37/3	38/25	42/28
	11 Sorsogon(3), Philippines	1	1	1	1	1	1	1	1	0	0	–	25/2	37/3	37/3	37/3	38/25	42/28
	12 Thailand(1)	1	1	1	1	1	1	1	1	0	0	0	–	39/5	39/5	39/5	44/25	43/28
P.w. (3n)	13 Amakusa-A(4), Japan	0	0	1	1	1	1	1	1	1	1	1	1	–	2/0	0/0	52/26	52/29
	14 Amakusa-B(5), Japan	1	1	2	2	2	2	2	2	2	2	2	2	1	–	2/0	52/26	52/29
	15 Bogil Island (4), Korea	0	0	1	1	1	1	1	1	1	1	1	1	0	1	–	52/26	52/29
P. ohirai	16 Kinosaki(1), Japan	1	1	2	2	2	2	2	2	2	2	2	2	1	2	1	–	35/15
P.m.	17 Rokuroshi(1), Japan	2	2	3	3	3	3	3	3	3	3	3	3	2	3	2	1	–

For abbreviations, see table 3.

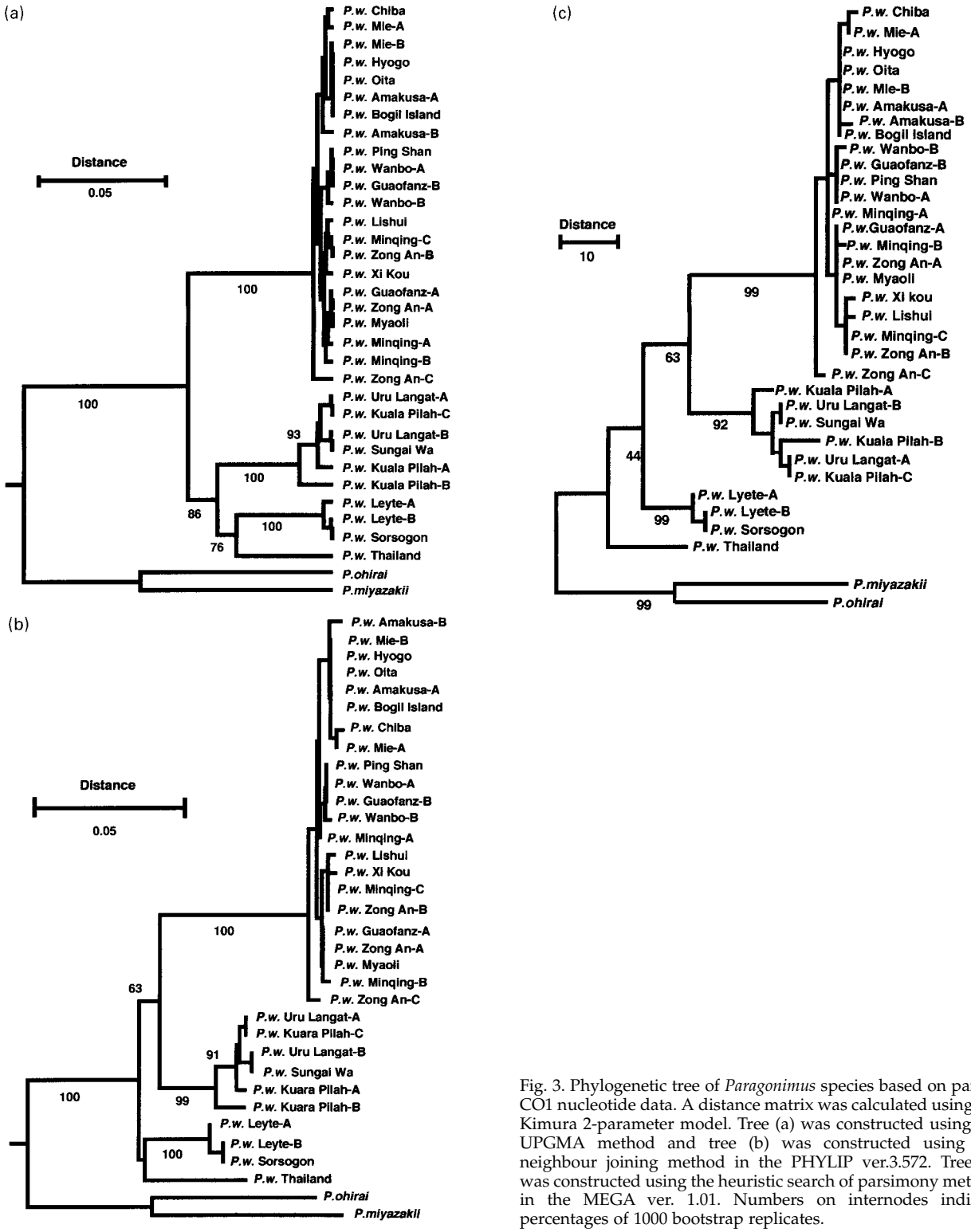

Fig. 3. Phylogenetic tree of *Paragonimus* species based on partial CO1 nucleotide data. A distance matrix was calculated using the Kimura 2-parameter model. Tree (a) was constructed using the UPGMA method and tree (b) was constructed using the neighbour joining method in the PHYLIP ver.3.572. Tree (c) was constructed using the heuristic search of parsimony method in the MEGA ver. 1.01. Numbers on internodes indicate percentages of 1000 bootstrap replicates.

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descendants from its most-recent common ancestor). Indeed, it might be more appropriate, given the genetic distances between them, to place the Philippine forms in a species separate from the Malaysian worms (in which case the name *P. filipinus* Miyazaki could be revived). The second reason concerns the type specimen and locality of *P. westermani* itself. The species was described from worms found in the lungs of a Bengal tiger that died in the Amsterdam Zoo in 1877 (see Blair *et al*., 1999). Records of the Amsterdam Zoo make no mention of the origin of the tiger, but it is commonly assumed to have come from India. Neither detailed morphological nor molecular data are available for *P. westermani* from India. Future work might demonstrate that the Indian form is conspecific with, for example, the Malaysian form. If *P. westermani* sensu lato is split into several species, the name *P. westermani* could be used for Malaysian populations, but not for populations from, for example, north-east Asia. In the interests of stability, we prefer to suggest no nomenclatural changes until populations from India can be characterized.

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