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Identities of two *Paragonimus* species from Sri Lanka inferred from molecular sequences

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Abstract

Metacercariae of *Paragonimus* spp. were obtained from field-collected freshwater crabs in Sri Lanka. Genomic DNA was extracted from single metacercariae. Two gene regions (partial mitochondrial cytochrome c oxidase subunit 1 (CO1) and the second internal transcribed spacer of the nuclear ribosomal gene repeat (ITS2)) were amplified using the polymerase chain reaction. Two differing sequences were obtained for each of these gene regions. Phylogenetic analyses placed the type 1 sequences as sister to a clade containing *P. westermani* and *P. siamensis* whereas the type 2 sequences were close to published sequences of *P. siamensis* from Thailand. The possible taxonomic status of these two types are discussed. This is the first report of molecular data about *Paragonimus* from Sri Lanka.

Introduction

Lung flukes (genus *Paragonimus* (Trematoda: Paragonimidae)) are important zoonotic agents distributed all over the world except Europe and Australia. Approximately 40 species have been named, although some of these are regarded as synonyms (Blair *et al.*, 1999c). To date, four *Paragonimus* species have been reported in Sri Lanka; *P. westermani* (see Dissanaike & Paramananthan, 1962: adults in a rusty spotted cat, *Felis rubiginosa* and leopards, *Panthera pardus*); *P. compactus* (see Dissanaike & Paramananthan, 1962: adults in a civet cat, *Viverricula indica mayori* and a fishing cat, *Felis viverrina*); *P. macrorchis* (see Kannangara, 1969: metacercariae in freshwater crabs, *Parathelphusa rugosa* and *P. enodis*) and *P. siamensis* (see Kannangara & Karunaratne, 1969: metacercariae in *Parathelphusa ceylonensis* and *P. rugosa* and adult worms in a grey mongoose, *Herpestes lanka*).

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The four species were identified based on the morphological features of adult worms, such as the branching of ovaries, the arrangement of cuticular spines, and the morphology of metacercariae. The validity of *P. compactus*, however, was not certain (Dissanaike & Paramananthan, 1962). No cases of human paragonimiasis have been reported from Sri Lanka so far. This is probably due to the custom of Sri Lankan people who do not normally eat freshwater crabs (Dissanaike & Paramananthan, 1962). In the present paper, a molecular phylogenetic study of Sri Lankan *Paragonimus* species has been performed using DNA extracted from single metacercariae obtained from freshwater crabs in an effort to identify the species present. It should be noted that material is increasingly difficult to collect and that ethical and logistical considerations make it difficult to raise adult worms in experimental animals. Consequently, there is a real advantage to being able to identify species using molecular sequences from metacercariae. The disadvantage, of course, is that there is no adult specimen from which to obtain morphological information.

Materials and methods

Collection and dissection of the freshwater crabs

Freshwater crabs (*Parathelphusa ceylonensis* and *P. rugosa*) were collected from Naula (Matale District, Central Province), Minneriya (Polonnaruwa District, Northern Central Province), Kottukachchiya (Puttalam District, Western Central Province), Hedeniya, Gampola and Barigama (Kandy District, Central Province) in August 2000. The crabs were measured and examined for the presence of metacercariae of *Paragonimus* species. The hepatopancreas of each crab was individually pressed between glass plates and examined under a dissecting microscope. The remaining part of the body was ground in a small bowl with physiological saline. The crushed tissues of crabs were filtered once through a mesh screen and the filtered sediments examined for metacercariae under the dissecting microscope. Metacercariae were preserved in ethanol when found and taken back to Japan for DNA extraction.

DNA extraction, PCR and sequencing

Genomic DNA was extracted individually from metacercariae. Each specimen was incubated in an extraction buffer (Easy DNA Kit) containing SDS and proteinase K either for 2 h or until the metacercariae were solubilized. The solubilized samples were treated once with an equal volume of phenol equilibrated to pH > 7.8, and treated once with an equal volume of chloroform. The extracted DNAs were ethanol-precipitated. The DNA obtained from metacercariae was resuspended in 10 μl of distilled water. 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 1 min, 72°C for 2 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), Taq polymerase (1.75 U per reaction, Roche Expand TM High Fidelity PCR System) and aliquot of DNA template. For the ITS2 region, we used the primers 3S (5′-CGG TGG ATC ACT CGG CTC GT-3′) (forward)and A28 (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′) (reverse) (Bowles *et al.*, 1995). For the COI region, we used FH5 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) (forward) and FH3 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′) (reverse) (Bowles *et al.*, 1993). The PCR products were purified using Gene Clean kit (BIO 101) and resuspended in 20 μl of distilled water. These 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 310.

DNA analysis

Alignment analyses were done using the program GENETYXMAC ver. 9.0 (Software Development Co., Tokyo, Japan). Genetic code was derived from a report by Blair *et al.* (1999a). The partial CO1 nucleotide sequences were translated with DNASIS ver. 3.2. (Hitachi software Engineering Co., Japan 1994). Phylogenetic analysis was performed using distance and parsimony methods in MEGA ver. 2 (Kumar *et al.*, 2001). For the distance method, the neighbour joining (NJ) approach was applied using Kimura's two-parameter model. For the parsimony method (MP), the heuristic search was used. The topologies of the inferred trees were assessed by 1000 bootstrap resamplings. The nucleotide sequence data of several species of *Paragonimus* were from previous reports (Blair *et al.*, 1997, 1999c; Iwagami *et al.*, 2000) as listed in table 1. The partial CO1 sequence of *F. hepatica* was used as an out-group. Numbers of transitions, transversions and amino acid differences in pairwise comparisons

Table 1. Species used, their geographical origin and nucleotide sequence data.

Species	Origin	ITS2	CO1
Paragonimus sp. type 1	Sri Lanka	AY240942 (present study)	AY240940 (present study)
Paragonimus sp. type 2	Sri Lanka	AY240943 (present study)	AY240941 (present study)
P. siamensis	Thailand	AF159605	AF159599
P. westermani (2n)	Chiba, Japan	–	U97207
	Kuala Pilah, Malaysia	(U96909)*	U97211
	Leyte, Philippines	U96910	U97213
	Thailand	AF159604	U97212
P. westermani (3n)	Korea	–	U97205
P. macrorchis	Thailand	AF159608	AF159598
P. harinasutai	Thailand	AF159609	AF159600
P. heterotremus	Thailand	AF159603	AF159597
P. skrjabini	Sichuan, China	U96913	U97216
P. miyazakii	Rokuroshi, Japan	Iwagami *et al.* (2000)	Iwagami *et al.* (2000)
P. ohirai	Kinosaki, Japan	(U96911)**	Iwagami *et al.* (2000)
P. mexicanus	Ecuador	AF159607	AF159596
P. kellicotti	USA	AF159606	–
Fasciola hepatica	Australia	–	M93388

* ITS2 sequence identical to that of *P. westermani* from Sungai Wa, Malaysia.
** ITS2 sequence identical to that of *P. ohirai* from Tanegashima, Japan.
2n, diploid, 3n triploid.

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Table 2. Infection rates of *Paragomimus* metacercariae in the freshwater crabs collected from Sri Lanka.

Locations studied	No. of crabs examined	No. of crabs positive (%)	No. of metacercariae detected (average per crab)			DNA typing
			Hepatopancreas	Muscle	Total	
Naula	25	0 (0.0)	0	0	0	
Minneriya	38	4 (10.5)	4 (1.0)	0	4 (1.0)	type 1, type 2
Kottukachchiya	133	5 (3.8)	8 (1.6)	0	8 (1.6)	type 2
Hedeniya	11	7 (63.6)	51 (7.3)	0	51 (7.3)	type 1
Barigama	6	0 (0.0)	0	0	0	
Gampola	14	0 (0.0)	0	0	0	
Total	227	16 (7.0)	63 (3.9)	0	63 (3.9)	

among *Paragonimus* sequences were calculated in MEGA ver. 1.01 (Kumar *et al.*, 1993).

Results and Discussion

Infection rates in crabs

Table 2 shows the infection rates of *Paragonimus* metacercariae in freshwater crabs examined in Sri Lanka in August 2000. The infection rates of crabs from Hedeniya, Minneriya and Kottukachchiya were 63.6%, 10.5% and 3.8% respectively. Metacercariae were found only in the hepatopancreas of the crabs. The mean number of metacercariae per infected crab was 3.9.

Morphology of metacercariae

Two types of metacercariae were found in the freshwater crabs in the present study. One type was semi-oval and the other, oval. Both of the metacercariae had two cyst walls, an outer and inner. The inner cyst of the semi-oval one measured $377 \times 328\,\mu$m in diameter. This is remarkably different from that of the spherical *P. westermani*. In this study, no spherical forms were observed. On the other hand, the inner cyst of the oval one measured 459 by $295\,\mu$m in diameter. This metacercaria was similar to *P. siamensis* in appearance, although the diameter of the inner cyst was smaller than that described previously (Miyazaki & Wykoff, 1965; Kawashima *et al.*, 1989). The semi-oval

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Paragonimus sp. SL type1     1 TAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATTTCCC
Paragonimus sp. SL type2     1 .....................................C..................C....
P.siamensis Thailand         1 .....................................C..................C....
P.westermani Thailand        1 .....C...............................................C....
P.macrorchis Thailand        1 ...........A............T...........C..................

Paragonimus sp. SL type1    61 CAACCTGGTCCTGTGCCAGTGGGGTGCCAGATCTATGGCGTTTCCCTAACATGCTCGTGC
Paragonimus sp. SL type2    61 ...T.A....TC.....T.....................................A....G..
P.siamensis Thailand        61 ...T.A....TC.....T........T.............................A....G..
P.westermani Thailand       61 ...T......T......T.....................................A....C..
P.macrorchis Thailand       61 ........C.TC...T.T..........G.....G..............A.AATC..GA.

Paragonimus sp. SL type1   121 GCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAGTG
Paragonimus sp. SL type2   121 .....................................G.....................A
P.siamensis Thailand       121 ..........................................................A
P.westermani Thailand      121 ..........................................................A
P.macrorchis Thailand      121 .T....GT....T..............T..................G.............

Paragonimus sp. SL type1   181 AATGATTTATGTGCGCGTTCCGCTGTTCTGTCCTCATCTGTGGTTTATGTTGCGCGTGGT
Paragonimus sp. SL type2   181 G....C..................T...C.....T.........................
P.siamensis Thailand       181 G....A..................T...C.....T.........................
P.westermani Thailand      181 ....................T......C.....T............C.............
P.macrorchis Thailand      181 ..AA.....................C.....A......T.....G.......AT.....

Paragonimus sp. SL type1   241 CTGCGCTCGATGTTGACCTACGTATGTGCCATTTGGTTCATTCTCCT
Paragonimus sp. SL type2   241 ...T.T....C.........TC.....  ..A.........T....
P.siamensis Thailand       241 ...T.T....C.........TC.....  ..A..............
P.westermani Thailand      241 .....T......C.....................G....C......T..
P.macrorchis Thailand      241 -----T......C.......T..T.....G.GC..C..........
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Fig. 1. Nucleotide sequences of a region of the ITS2 gene of nuclear ribosomal DNA of *Paragonimus* sp. from Sri Lanka. SL represents Sri Lanka. Alignment gaps indicated by a hyphen. Sites with a nucleotide identical to that on the top line indicated by a dot.

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Table 3. Pairwise differences (%) and transitions/transversions among ITS2 sequences of *Paragonimus* species.

Species	Countries	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 *Paragonimus* sp.	Sri Lanka type 1	–	18/5	16/6	11/4	11/6	15/2	28/11	29/8	24/9	21/9	22/8	30/9	26/8	27/6
2 *Paragonimus* sp.	Sri Lanka type 2	8.0	–	5/1	15/3	12/5	17/3	35/10	35/9	28/8	27/7	28/7	32/10	30/11	28/7
3 *P. siamensis*	Thailand	7.7	2.1	–	13/4	10/6	15/4	33/11	33/10	28/9	25/8	26/8	30/11	30/12	28/8
4 *P. westermani*	Kuala Pilah, Malaysia	5.2	6.3	5.9	–	3/4	6/2	30/8	29/8	25/6	23/6	24/6	29/9	23/10	25/6
5 *P. westermani*	Leyte, Philippines	5.9	5.9	5.6	2.4	–	5/4	29/9	28/10	23/9	21/8	22/8	28/11	22/11	23/8
6 *P. westermani*	Thailand	5.9	7.0	6.6	2.8	3.1	–	31/9	30/8	27/7	25/6	26/6	30/9	24/8	26/6
7 *P. macrorchis*	Thailand	13.6	15.7	15.5	13.4	13.4	14.1	–	25/9	18/6	16/7	17/7	26/10	25/8	23/5
8 *P. harinasutai*	Thailand	12.9	15.3	15.0	12.9	13.2	13.2	12.0	–	22/7	19/6	20/6	14/3	26/8	24/4
9 *P. heterotremus*	Thailand	11.6	12.6	13.0	10.9	11.2	11.9	8.5	10.2	–	10/5	11/5	23/8	19/7	16/3
10 *P. skrjabini*	Sichuan, China	10.2	11.9	11.6	10.2	10.2	10.9	8.2	8.8	5.3	–	1/0	20/7	22/8	19/4
11 *P. miyazakii*	Rokuroshi, Japan	10.5	12.3	11.9	10.5	10.5	11.2	8.5	9.1	5.7	0.4	–	21/7	23/8	20/4
12 *P. ohirai*	Kinosaki, Japan	13.6	14.6	14.3	13.2	13.6	13.6	12.7	5.9	10.9	9.5	9.8	–	26/9	23/5
13 *P. mexicanus*	Ecuador	11.9	14.4	14.7	11.6	11.6	11.2	11.7	11.9	9.2	10.6	11.0	12.3	–	14/4
14 *P. kellicotti*	USA	11.7	12.4	12.8	11.0	11.0	11.3	10.0	9.9	6.8	8.2	8.6	9.9	6.4	–

Values above the diagonal are transitions/transversions. Those below are pairwise differences (%).

Table 4. Pairwise differences (%) and transitions/transversions among partial CO1 sequences of *Paragonimus* species.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 *Paragonimus* sp. Sri Lanka type 1	–	32/18	30/22	50/14	52/14	38/15	39/17	38/17	33/28	41/22	38/31	33/29	38/27	37/18	37/25	49/84
2 *Paragonimus* sp. Sri Lanka type 2	13.1	–	29/8	49/12	49/12	39/15	36/15	43/13	31/30	33/28	38/27	37/31	37/29	36/28	37/23	51/84
3 *P. siamensis* Thailand	13.6	9.7	–	47/14	46/14	43/19	37/17	39/17	29/32	33/32	37/27	32/31	35/31	32/30	35/25	46/84
4 *P. westermani* Chiba, Japan	16.7	15.9	15.9	–	2/0	31/5	35/3	39/5	48/26	49/26	48/23	51/29	51/29	51/26	62/21	67/82
5 *P. westermani* Bogil Island, Korea	17.2	15.9	15.7	0.5	–	33/5	35/3	39/5	49/26	49/26	47/23	52/29	52/29	52/26	61/21	69/82
6 *P. westermani* Kuala Pilah, Malaysia	13.8	14.1	16.2	9.4	9.9	–	23/6	26/6	39/25	41/25	39/28	38/28	43/26	39/25	50/24	58/85
7 *P. westermani* Leyte, Philippines	14.4	13.3	14.1	9.9	10.0	7.6	–	23/2	38/27	36/25	35/24	36/28	40/28	36/25	36/22	53/83
8 *P. westermani* Thailand	14.4	14.6	14.6	11.5	11.5	8.4	6.5	–	44.27	43/23	40/24	43/30	43/28	44/25	43/22	56/83
9 *P. macrorchis* Thailand	15.9	15.9	15.9	19.3	19.6	16.7	17.0	18.5	–	30/28	30/15	22/17	28/15	29/24	30/19	46/80
10 *P. harinasutai* Thailand	16.4	15.9	17.0	19.6	19.6	17.2	15.9	17.2	15.1	–	21/25	29/23	35/19	37/10	31/23	45/86
11 *P. heterotremus* Thailand	18.0	17.0	16.7	18.5	18.3	17.5	15.4	16.7	11.7	12.0	–	33/14	34/14	34/23	27/14	49/77
12 *P. skrjabini* Sichuan, China	16.2	17.8	16.4	20.9	21.1	17.2	16.7	19.1	10.2	12.3	12.3	–	26/4	30/17	35/18	44/83
13 *P. miyazakii* Rokuroshi, Japan	17.0	17.2	17.2	20.9	21.1	18.0	17.8	18.5	11.2	12.5	12.5	7.8	–	35/15	39/16	49/83
14 *P. ohirai* Kinosaki, Japan	14.4	16.7	16.2	20.1	20.4	16.7	15.9	18.0	13.8	14.9	14.9	12.3	13.1	–	34/21	50/86
15 *P. mexicanus* Ecuador	16.2	15.7	15.7	21.7	21.4	19.3	15.1	17.0	12.8	10.7	10.7	13.8	14.4	14.4	–	43/79
16 *Fasciola hepatica* Australia	34.7	35.2	33.9	38.9	39.4	37.3	35.5	36.3	32.9	32.9	32.9	33.2	34.5	35.5	31.9	–

Values above the diagonal are transitions/transversions. Those below are pairwise differences (%).

metacercaria was tentatively named type 1, and the oval one, type 2.

DNA analysis

ITS2 region

The ITS2 region could be amplified from eight of eleven metacercariae; one from Kottukachchiya, two from Minneriya and five from Hedeniya. The alignment of ITS2 nucleotide sequences was 287 bp in length (excluding portions of the flanking 5.8S and 28S genes). Two types of sequences were found; type 1 sequences from Hedeniya and type 2 from Kottukachchiya, and both types from Minneriya (fig. 1). Pairwise differences and transition/transversion ratios between the two types were 8.0% and 3.6 respectively (table 3). These differences are not the lowest of the pairwise differences among *Paragonimus* species shown in table 3. The type 1 sequence appears closest to members of the *P. westermani* group (pairwise differences = 5.2–5.9%, Ts/Tv ratios = 1.8–2.8). On the other hand, type 2 was closest to *P. siamensis* from Thailand (pairwise differences = 2.1%, Ts/Tv ratios = 5).

CO1 region

Metacercariae from which the ITS2 had been successfully amplified were used in attempts to amplify the COI region. Successful amplification was obtained from one metacercaria from Kottukachchiya, two from Minneriya and three from Hedeniya. The alignment of the partial CO1 nucleotide sequences was 383 bp in length. Two types, type 1 and type 2 were also found in the CO1 region; type 1 from Hedeniya, type 2 from Kottukachchiya, and both types from Minneriya, following results for the ITS2 region (fig. 2). There were no sequence differences in each type. Pairwise differences and transition/transversion ratios among them were 13.1% and 1.8 respectively (table 4). The number of amino acid substitutions among them was four. Again, these are not the lowest values among species and strains used in the analyses (table 4). The smallest value, 9.7%, of pairwise

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Paragonimus  sp. SL type1     1 TCTTACCGGGGGTTCGGAATTGTGAGTCATATCTGTATGACCTTAACTAACAATGATTCGT
Paragonimus  sp. SL type2     1 .T..G..A..T..T.............C.............G..C..T........TC
P.siamensis  Thailand         1 .T..G..T..T..T..G........A.....T........T..G..G..T........TC
P.westermani Thailand         1 .T..G........T..T..C........C.....C.....T..G.............C.
P.macrorchis Thailand         1 .T..G..A.....T..TG.......A.....T........G........T..C..C..T.

Paragonimus  sp. SL type1    61 TGTTTGGGTATTATGGGTTGGTGTTTGCTATGGGGGCTATTGTGTGTTTGGGAAGTGTTG
Paragonimus  sp. SL type2    61 ............................G.........................G..C....
P.siamensis  Thailand        61 .......T...........A........G.....A..........................
P.westermani Thailand        61 ............C.....A........G........C..C.....C.....C.......
P.macrorchis Thailand        61 .......T........T..A..T.....C.............T........G.....G.

Paragonimus  sp. SL type1   121 TGTGGGCTCACCACATGTTTATGGTTGGTCTGGATGTTAAGACTGCTGTGTTTTTTAGTT
Paragonimus  sp. SL type2   121 ....A..A..T..T.............T..............................
P.siamensis  Thailand        121 .......G..T..T.............T..............................
P.westermani Thailand        121 .......G.....................T.....................C........C.
P.macrorchis Thailand        121 .T.....G..T..T.................T.................T........C.

Paragonimus  sp. SL type1   181 CCGTTACGGGGGTGATTGGTATTCCTACGGGGATTAAGGTTTTTTCTTGGTTGTTTATGT
Paragonimus  sp. SL type2   181 .T..C..C..C..T.....A..A........T.............C...C.........
P.siamensis  Thailand        181 .T..C..C.....T.....G..A........T.................A..A..C....
P.westermani Thailand        181 .T..C..T...........G..A.................C..C...........C....
P.macrorchis Thailand        181 .T..............................T............................C

Paragonimus  sp. SL type1   241 TAGGTGGTGCCCGTTTGCGGTTTTGGGATCCTGTTTTGTGATGGATACTTGGATTCATTT
Paragonimus  sp. SL type2   241 .......AA.T.........C.....................AG.T.....T.......
P.siamensis  Thailand        241 .......G..T........TC.............C.....G.....T.....T.......
P.westermani Thailand        241 .G.....AT.T.....A..A.....A.............G.....C.....G.......
P.macrorchis Thailand        241 ....G..AA.T..C..A.............C..AA.T.....A..TT.G.....T....

Paragonimus  sp. SL type1   301 TTTTGTTTACGATAGGTGGTGTGACGGGTATTATTCTCTCCTCTTCCATATTGGATAGCT
Paragonimus  sp. SL type2   301 ..........C.............T......G..T.G..T.....G...C.......T.
P.siamensis  Thailand        301 .C........T.............C......G..T.G........A...........T.
P.westermani Thailand        301 ..C....C..C.....G........T..C..C...T.G..T........CC.......TC
P.macrorchis Thailand        301 ..C.T.....T........G........G......T.G..T.....T..T..A.....T.

Paragonimus  sp. SL type1   361 TGTTGCATGACACATGATTTGTT
Paragonimus  sp. SL type2   361 .......C..T..T..G......
P.siamensis  Thailand        361 ..........T..G..G.....G
P.westermani Thailand        361 ............G..G......
P.macrorchis Thailand        361 ..........T..T..G......
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Fig. 2. Nucleotide sequences of a region of the CO1 gene of mitochondrial DNA of *Paragonimus* sp. from Sri Lanka. SL represents Sri Lanka. Sites with a nucleotide identical to that on the top line indicated by a dot.

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differences was obtained comparing type 2 and *P. siamensis* with a relatively high value of Ts/Tv ratio (3.6). On the other hand, type 1 showed high pairwise differences (13.6–18.0%) with Ts/Tv ratios 1.1–3.6, when compared with any species from the genus *Paragonimus* (table 4).

Tree analysis

As shown in figs 3 and 4, trees constructed using either gene region lead us to the same conclusions. As has been found in earlier studies (Blair *et al.*, 1999b), *P. westermani* and the related species *P. siamensis* form a clade to the exclusion of all other members of the genus. In the present study, trees show that both type 1 and type 2 sequences also fall within this clade. Type 1 sequences are placed as sister to *P. siamensis* and all *P. westermani* samples. Nevertheless, this basal position in the clade does not lead to greatly reduced support for the clade: bootstrap values are high. Type 2 sequences are close to those reported for *P. siamensis* from Thailand. It is not hard to accept that the type 2 sequences belong to *P. siamensis*, or to a sibling of the Thai species. The metacercarial form also supports this. However, the identity of the worms from which the type 1 sequences were obtained is less clear. Biological and other molecular evidence suggests that *P. westermani* populations in north-east Asia (China, Japan, Taiwan, Korea), utilizing pleurocerid snails and those from southern Asia (Philippines, Thailand, Malaysia), utilizing thiarid snails, represent distinct but cryptic species. *Paragonimus westermani* has also been reported from India and Sri Lanka, but hitherto no molecular data has been available for material from these areas. The present data of DNA sequences and metacercarial morphology suggests that the type 1 sequence might belong to another cryptic member of the *P. westermani* group. If the type 1 sequence represents another form of *P. westermani*, then some interesting biogeographic and host-specificity questions arise. Basal members of the *P. westermani*

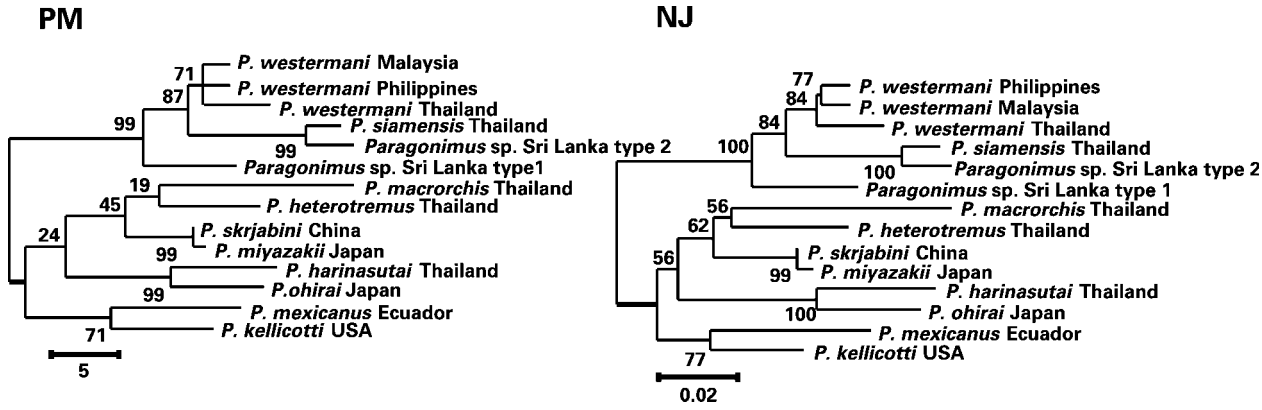

Fig. 3. Phylogenetic trees of *Paragonimus* species based on ITS2 nucleotide data, constructed with the parsimony (PM) and neighbour joining (NJ) methods using the Kimura 2 parameter model. Numbers on internodes indicate percentages of 1000 bootstrap replicates.

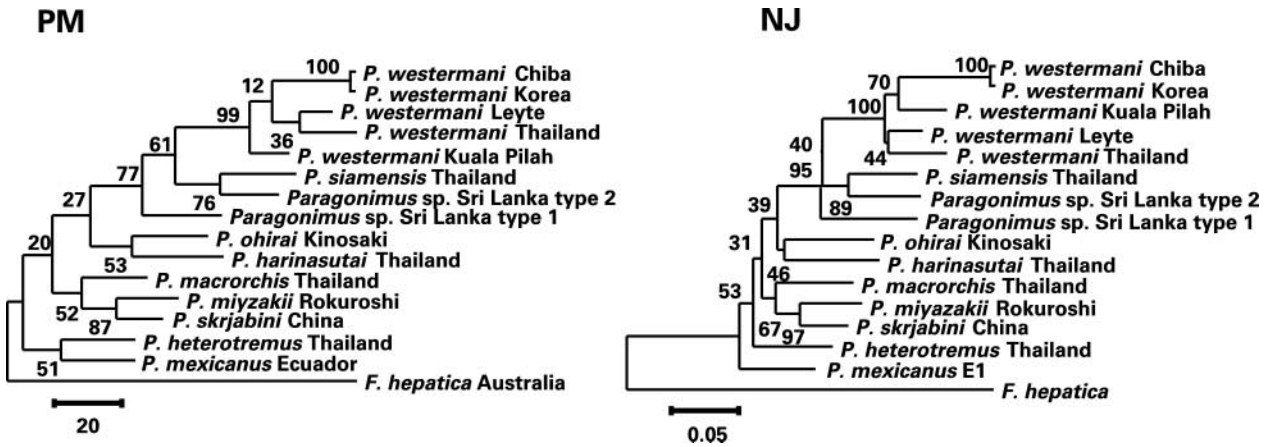

Fig. 4. Phylogenetic trees of *Paragonimus* species based on partial CO1 nucleotide data, constructed with the parsimony (PM) and neighbour joining (NJ) method using the Kimura 2 parameter model. Numbers on internodes indicate percentages of 1000 bootstrap replicates.

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clade occur in southern Asia, and more derived clades in China, Japan and Korea. This suggests a range expansion towards the north by this group, probably in association with the addition of pleurocerid snail hosts (Blair *et al.*, 2001). The range expansion and host-addition seems to have prompted speciation events, albeit that the sibling species are indistinguishable as adults. *Paragonimus siamensis* (or perhaps sibling cryptic species in Sri Lanka and Thailand) also belongs to this clade, and can be distinguished morphologically from *P. westermani*. In both these countries, *P. siamensis* and *P. westermani* are broadly sympatric. It is not clear where, or by what mode of speciation, these two have separated. Molluscan host switching or host addition could have played a role, but here we can only speculate. The molluscan host for *P. siamensis* in Thailand is said to be *Filopaludina* (*Siamopaludina*) *martensi martensi* (Frauenfeld) (see Yaemput *et al.*, 1994), a viviparid snail phylogenetically far removed from known hosts of *P. westermani*.

The key to understanding the evolution of the clade containing *P. westermani* and *P. siamensis* lies in obtaining relevant material from India and molecular sequences from this. Such material might also help resolve nomenclatural problems noted by several authors (see Blair *et al.*, 1999c). The type locality for *P. westermani* is probably India (Blair *et al.*, 1999c). However, it is far from clear that what might be called *P. westermani* in India belongs to the same species as *P. westermani* from north-eastern or southern Asia. The Indian species *P. compactus* might also repay further investigation. This species has been tentatively identified in Sri Lanka and might in fact be close to *P. siamensis* in morphology.

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