

A molecular phylogeographic study based on DNA sequences from individual metacercariae of *Paragonimus mexicanus* from Guatemala and Ecuador

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Abstract

A molecular phylogeographic study of *Paragonimus mexicanus* collected from Guatemala and Ecuador was performed. Genomic DNA was extracted from individual metacercariae, and 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 by the polymerase chain reaction (PCR). Sequences segregated in a phylogenetic tree according to their geographic origins. ITS2 sequences from Ecuador and Guatemala differed at only one site. Pairwise distances among CO1 sequences within a country were always lower than between countries. Nevertheless, genetic distances between countries were less than between geographical forms of *P. westermani* that have been suggested to be distinct species. This result suggests that populations from Guatemala and Ecuador are genetically differentiated perhaps at the level of subspecies.

Introduction

The lung fluke, *Paragonimus mexicanus* Miyazaki & Ishii, 1968, is a medically important trematode occurring throughout Central and South America (Miyazaki & Ishii, 1968a,b). Metacercariae of *P. mexicanus* are unusual because they have no cyst. Human infections with the lung fluke are acquired by eating raw or undercooked freshwater crabs containing live metacercariae.

Paragonimus peruianus Miyazaki, Ibanez & Miranda, 1969 from Peru, and *P. ecuadoriensis* Voelker & Arzube, 1979 from Ecuador are regarded as synonyms of *P. mexicanus* (Vieira *et al.*, 1992). This conclusion has been

based on morphological (the structure of ovaries and the numbers of papillae surrounding the suckers) (Miyazaki *et al.*, 1980; Aji *et al.*, 1984; Tongu *et al.*, 1985; Miyazaki, 1991) and electrophoretic studies (Zillmann & Sachs, 1986). Given the wide geographical range of the species, and the different names that have been applied to it, we considered that a molecular phylogenetic study would improve our understanding of the status of *P. mexicanus*. In this study, we investigate relationships within *P. mexicanus* collected from Guatemala and Ecuador using DNA sequences from two gene regions: partial mitochondrial cytochrome *c* oxidase subunit 1 (CO1) and the second internal transcribed spacer of the nuclear ribosomal gene repeat (ITS2). The molecular phylogeographic relationships between *P. mexicanus* and *Paragonimus* species from Asia using CO1 sequence data were also estimated.

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Materials and methods

Collection and examination of freshwater crabs

Freshwater crabs (*Pseudothelphusa cobanensis* (Guatemala) and *Hypolobocera aequatorialis* (Ecuador)) were collected from Playon and Casilla, Santa Rosa, Guatemala, and from El Canero and Cucaracha River, La Concordia, Esmeraldas, Ecuador. These localities are known to be highly endemic for *P. mexicanus* (Miyazaki *et al.*, 1980; Vieira *et al.*, 1992). Crabs were measured and examined for the presence of metacercariae of *P. mexicanus*. The hepatopancreas of each crab was individually pressed between two glass plates and examined under a dissecting microscope. The remaining part of the body was ground in a small bowl with physiological saline. Crushed tissues of crabs were filtered once through a mesh screen and the filtered sediments examined for metacercariae under the dissecting microscope. The metacercariae collected were fed orally to cats, or were directly used for DNA extraction.

Experimental infection

Three months after oral inoculation of metacercariae into cats, the animals were sacrificed and adult flukes recovered from worm cysts in the lungs. The morphology of adult worms recovered was examined to confirm that they were *P. mexicanus*.

DNA extraction, PCR and sequencing

Genomic DNA was extracted from individual metacercariae, ten from each country. Each metacercaria was incubated for 2 h in an extraction buffer (Invitrogen extraction kit) containing SDS and proteinase K.

Solubilized samples were treated once with an equal volume of phenol equilibrated to pH > 7.8, and once with an equal volume of chloroform. The extracted DNAs were ethanol-precipitated and 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), and *Taq* polymerase (1.75 U/reaction). As primers we used 5'-CGG TGG ATC ACT CGG CTC GT-3' (3S) (forward) and 5'-CCT GGT TAG TTT CTT TTC CTC CGC-3' (A28) (reverse) for the ITS2 region (Bowles *et al.*, 1995) and 5'-TTT TTT GGG CAT CCT GAG GTT TA-3' (FH5) (forward) and 5'-TAA AGA AAG AAC ATA ATG AAA ATG-3' (FH3) (reverse) for the CO1 region (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 ABI PRISM kit. PCR primers were used as sequencing primers. The reactions were prepared according to the manufacturer's instructions and applied to an ABI sequencer 310. DNA from adult worms was also sequenced using the same procedure.

DNA analysis

Alignments were done using the program GENETY-XMAC ver. 9.0 (Software Development Co., Tokyo, Japan). The genetic code was derived from a report by Blair *et al.* (1999a). The partial CO1 nucleotide sequences were translated in DNASIS Ver. 3.2. (Hitachi Software Engineering Co., Japan 1994). The phylogenetic analysis

Table 1. Geographic origins, haplotypes and accession numbers of materials used in this study.

Species	Locality	Gene region	Haplotype	Accession number	Source
<i>P. mexicanus</i>	Ecuador	CO1	E1	AF538934	Present study
<i>P. mexicanus</i>	Ecuador	CO1	E2	AF538935	Present study
<i>P. mexicanus</i>	Ecuador	CO1	E3	AF538936	Present study
<i>P. mexicanus</i>	Ecuador	CO1	E4	AF538937	Present study
<i>P. mexicanus</i>	Guatemala	CO1	G1	AF538938	Present study
<i>P. mexicanus</i>	Guatemala	CO1	G2	AF538939	Present study
<i>P. mexicanus</i>	Guatemala	CO1	G3	AF538940	Present study
<i>P. mexicanus</i>	Guatemala	CO1	G4	AF538941	Present study
<i>P. mexicanus</i>	Guatemala	CO1	G5	AF538942	Present study
<i>P. mexicanus</i>	Guatemala	CO1	G6	AF538943	Present study
<i>P. mexicanus</i>	Guatemala	CO1	G7	AF538944	Present study
<i>P. mexicanus</i>	Ecuador	CO1*	E4	AF159596	Blair <i>et al.</i> , 1999b
<i>P. mexicanus</i>	Guatemala	CO1*	G3	-	Unpublished data
<i>P. mexicanus</i>	Ecuador	ITS2	E	AF538945	Present study
<i>P. mexicanus</i>	Guatemala	ITS2	G	AF538946	Present study
<i>P. miyazakii</i>	Japan	CO1*	-	U97215	Blair <i>et al.</i> , 1999b
<i>P. westermani</i> (2n)	Japan	CO1*	-	U97205	Blair <i>et al.</i> , 1999b
<i>P. westermani</i> (2n)	Malaysia	CO1*	-	U97211	Blair <i>et al.</i> , 1999b
<i>P. westermani</i> (2n)	Philippines	CO1*	-	U97213	Blair <i>et al.</i> , 1999b
<i>P. westermani</i> (2n)	Thailand	CO1*	-	U97212	Blair <i>et al.</i> , 1999b
<i>P. westermani</i> (3n)	Korea	CO1*	-	U97205	Blair <i>et al.</i> , 1997b

*Sequences that came from adult worms.

Table 2. Infection rate of *Paragonimus mexicanus* metacercariae in freshwater crabs, *Pseudothelphusa cobanensis* and *Hypolobocera aequatorialis* collected from Guatemala and Ecuador, respectively.

Country	Locality studied	No. of crabs examined	No. of crabs positive (%)	No. of metacercariae detected (average)		
				Hepatopancreas	Muscle	Total
Guatemala	Playon (Renacimiento)	91	31 (34.1)	115 (3.7)	153 (4.9)	268 (8.6)
	Playon (San Jose)	14	8 (57.1)	14 (1.8)	0 (0)	14 (1.8)
	Casilla	23	7 (30.4)	17 (2.4)	2 (0.3)	19 (2.7)
	Total	128	46 (35.9)	146 (3.2)	155 (3.4)	301 (6.5)
Ecuador	La Concordia (Rio Cucaracha)	52	43 (82.7)	120 (2.8)	12 (0.3)	132 (3.1)
	La Concordia (El Canero)	56	31 (55.4)	52 (1.7)	0 (0)	52 (1.7)
	Total	108	74 (68.5)	172 (2.3)	12 (0.3)	184 (2.5)

was performed using distance and parsimony methods in MEGA (Kumar *et al.*, 2001). Previously reported (Blair *et al.*, 1997, 1999b) partial CO1 nucleotide sequences from other *Paragonimus* species were used (table 1). The numbers of transitions, transversions and amino acid differences in pairwise comparisons among CO1 sequences were calculated in MEGA (Kumar *et al.*, 2001).

Results and Discussion

Metacercarial infection in freshwater crabs

Table 2 shows the infection status of *P. mexicanus* metacercariae in freshwater crabs examined in Guatemala and Ecuador respectively in September 1999. Overall infection rates were 35.9% (Guatemala) and 68.5% (Ecuador). The average number of metacercariae per



Fig. 1. Nucleotide sequences of a region of the ITS2 gene of nuclear ribosomal DNA of *Paragonimus mexicanus* from Ecuador and Guatemala. Alignment gaps are indicated by a hyphen. Sites with a nucleotide identical to that on the top line are indicated by a dot. 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.

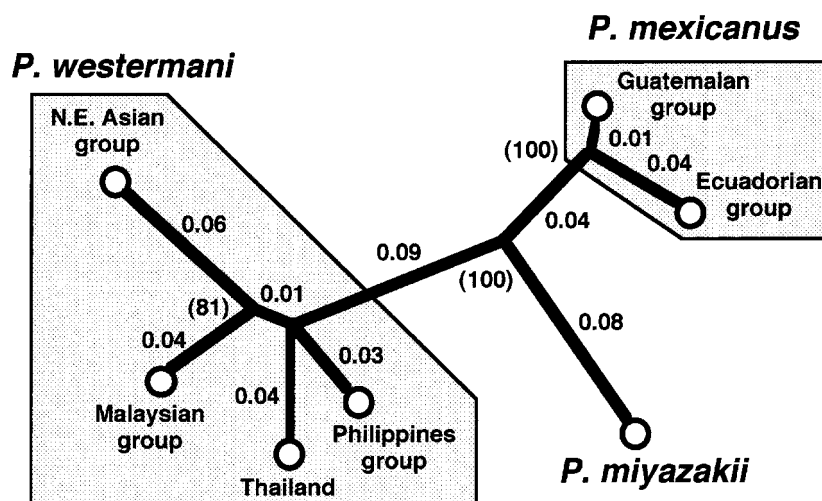


Fig. 3. Unrooted phylogenetic tree of *Paragonimus* species (*P. mexicanus*, *P. westermani* and *P. miyazakii*) based on partial CO1 nucleotide sequence data (383 bp). The tree was constructed with the neighbour joining method using the Kimura 2 parameter model in the MEGA ver. 2.1. All sequence data listed in table 1 were used for the tree analysis. Numbers on each branch show the branch length and numbers in parentheses indicate percentages of 1000 bootstrap replicates. All branches that showed less than 0.01 in distance were omitted. The north-east Asian group includes Japan, Korea and China.

range. This suggests that *P. mexicanus* populations from Guatemala and Ecuador are genetically differentiated at the level of subspecies or below. It will be instructive to obtain data for a population from the type locality of *P. mexicanus* (southern Mexico) and from the southern limit of *P. mexicanus* in Peru.

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