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Sequences of Internal Transcribed Spacers and Two Mitochondrial Genes: Effective Genetic Markers for *Metorchis orientalis*

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Abstract: The present study examined sequence variations in the Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA (rDNA) and two mitochondrial DNA (mtDNA) regions, namely cytochromec oxidase subunit 1 (cox1), NADH dehydrogenase subunits 1 (nad1), among *Metorchis orientalis* metacercaria isolates from Guangxi in China. The sequences of ITS, pcox1 and pnad1 were amplified from 6 individual *M. orientalis* metacercariae and sequenced. The relevant sequences of other 7 trematode species belonging to 6 genera in 4 families were downloaded from GenBank and their phylogenetic relationships were re-constructed using the combined pcox1 and pnad1 mt DNA sequences with *Trichinella spiralis* as outgroup. The results showed that sequences of ITS rDNA, pcox1 and pnad1 of *M. orientalis* were 1131, 654 and 650 bp, respectively and they were quite conserved among the *M. orientalis* isolates. However, they were quite different from that of other species, phylogenetic analysis of the combined pcox1 and pnad1 mt DNA sequences were able to distinguish *M. orientalis* from different species of the *Opisthorchiidae* and trematodes in other families. Therefore, the ITS, cox1 and nad1 mt DNA sequences provide effective genetic markers for the specific identification of trematodes of the *Opisthorchiidae* family and have implications for studying their population biology, genetic structure, as well as molecular epidemiology.

Key words: *Metorchis orientalis*, metacercaria, Opisthorchiidae, trematodes, Internal Transcribed Spacers (ITS), mitochondrial DNA (mtDNA), cytochromec oxidase subunit 1 (cox1), NADH dehydrogenase subunits 1 (nad1), phylogenetic analysis, genetic marker

INTRODUCTION

Trematodes in the family Opisthorchiidae are divided into Opisthorchiina and Metoriinae which can infect mollusks and vertebrates. Some of the trematodes in Opisthorchiidae such as *Clonorchis sinensis*, *Opisthorchis felineus* and *Metorchis orientalis* are of zoonotic importance, infecting both humans and animals and causing death and health problem as well as significant economic losses (Lin *et al*., 2001; Shekhovtsov *et al*., 2009; Sohn, 2009). *M. orientalis* is recognized as one of the causative agents of trematode diseases in domestic animals and humans (Sohn *et al*., 1992; Cheng *et al*., 2005; Zhu *et al*., 2006). About 26 species of Metorchis have been reported worldwide of which 8 species parasitize in mammals and 18 species in birds. The final hosts of *M. orientalis* were always considered to be ducks or other poultry. However, Lin and Cheng (1986) firstly reported that cats, dogs were naturally infected by this trematode. It also can infect guinea pigs, rats, mice and domestic cats experimentally. Moreover, infection of humans with *M. orientalis* has been detected (Lin *et al*., 2001). The fish *Psetidorasbora parvaas* is the second intermediate host of this parasite in which the metacercaria stage develops (Cheng *et al*., 2005).

Currently, the identification and classification of *M. orientalis* is based on morphological characters, especially body length and width. However, it is difficult to accurately discriminate between *M. orientalis* and

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other *Opisthorchiidae* trematodes, especially at the metacercaria stage because of their morphological similarities (Yossepowitch *et al*., 2004; Schuster *et al*., 1999; Kang *et al*., 2008; Skov *et al*., 2008; Sherrard-Smith *et al*., 2009; Traub *et al*., 2009; Cai *et al*., 2010). The objectives of the present study were to determine the sequences of the Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA (rDNA) and two mitochondrial DNA (mtDNA) regions namely cytochromec oxidase subunit 1 (cox1), NADH dehydrogenase subunits 1 (nad1) from *M. orientalis* metacercaria isolates from Guangxi in China and then to study the phylogenetic relationships among Opisthorchiidae trematodes using combined cox1 and nad1 sequences.

MATERIALS AND METHODS

Parasites and isolation of genomic DNA: *M. orientalis* isolates were collected from Guangxi Zhuang Nationality Autonomous Region, China. Sample codes, host and GenBank™ accession number are shown in Table 1. The metacercariae were stored in 70% molecular grade ethanol and stored at -20°C before extraction of genomic DNA. Total genomic DNA was extracted from individual metacercariae by SDS/proteinase K treatment, column-purified (Wizard® SV Genomic DNA Purification System, Promega) and eluted into 30 μL H_2O according to the manufacturer's recommendations (Zhao *et al*., 2009a, b, 2010; Ai *et al*., 2010).

Enzymatic amplification and sequencing: The rDNA region comprising ITS-1, 5.8S and ITS-2 plus primer flanking sequences were amplified by Polymerase Chain Reaction (PCR) from trematode DNA using primers BD1 and BD2 (Luton *et al*., 1992). A portion of the cox1 gene (pcox1) was amplified with primers JB3 and JB4.5 (Bowles *et al*., 1992), part of the nad1 gene (pnad1) with primers nad1-F and nad1-R (Li *et al*., 2008a, b) (Table 2). PCR reactions (25 μL) were performed in 2 mM of $MgCl_2$ (2.5 mM for ITS and pnad1, 3 mM for pcox1), 2.5 μM of each primer, 2.5 μL 10x r*Taq* buffer, 0.2 mM of each dNTPs, 1.25 U of r*Taq* DNA polymerase (TAKARA) and 2 μL of DNA sample in a thermocycler (Biometra) under the following conditions: after an initial denaturation at 94°C for 5 min, then 94°C for 30 sec (denaturation); 50°C (for ITS and pnad1) or 55°C (for pcox1) for 30 sec (annealing); 72°C for 30 sec (extension) for 35 cycles, followed by a final extension at 72°C for 5 min.

These optimized cycling conditions for the specific and efficient amplification of individual ITS and mtDNA fragments were obtained after varying annealing temperatures.

Samples without genomic DNA (no-DNA controls) were included in each amplification run and in no case were amplicons detected in the no-DNA. Each amplicon (5 μL) was examined by agarose gel electrophoresis to validate amplification efficiency. Positive amplicons were selected, purified and sequenced using an ABI 377 automated DNA sequencer (using BigDye Terminator Chemistry) employing the same

Table 1: Metacercaria samples of *Metorchis orientalis* from Guangxi, China used in the present study as well as their GenBank™ accession numbers for sequences of the Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA (rDNA), a portion of mitochondrial DNA (mtDNA) cytochromec oxidase subunit 1 (pcox1) and NADH dehydrogenase subunits 1 (pnad1)

Sample codes	Location	Intermediate host	Stage	Identification by morphology	GenBank™ accession number		
					ITS	pcox1	Pnad1
Momgx1	Guangxi, China	*Psetidorasbora parvaas*	Metacercaria	*M. orientalis*	HM347223	HM347229	HM347235
Momgx2	Guangxi, China	*P. parvaas*	Metacercaria	*M. orientalis*	HM347224	HM347230	HM347236
Momgx3	Guangxi, China	*P. parvaas*	Metacercaria	*M. orientalis*	HM347225	HM347231	HM347237
Momgx4	Guangxi, China	*P. parvaas*	Metacercaria	*M. orientalis*	HM347226	HM347232	HM347238
Momgx5	Guangxi, China	*P. parvaas*	Metacercaria	*M. orientalis*	HM347227	HM347233	HM347239
Momgx6	Guangxi, China	*P. parvaas*	Metacercaria	*M. orientalis*	HM347228	HM347234	HM347240
Cs1	Unknown	Unknown	Adult	*Clonorchis sinensis*		NC_012147	NC_012147
Cs2	Unknown	Unknown	Adult	*C. sinensis*		FJ381664	FJ381664
Of1	Unknown	Unknown	Unknown	*Opisthorchis felineus*		NC_011127	NC_011127
Of2	Unknown	Unknown	Unknown	*O. felineus*		EU921260	EU921260
Pw1	South Korea	Unknown	Unknown	*Paragonimus westermani*		AF540958	AF540958
Pw2	Unknown	Unknown	Unknown	*P.westermani*		AF219379	AF219379
Fh1	Australia	Unknown	Unknown	*Fasciola hepatica*		NC_002546	NC_002546
Fh2	Australia	Unknown	Unknown	*F. hepatica*		AF216697	AF216697
Sj1	Unknown	Unknown	Unknown	*Schistosoma japonicum*		NC_002544	NC_002544
Sj2	Unknown	Unknown	Unknown	*S. japonicum*		AF215860	AF215860
Sm1	Puerto Rico	Unknown	Unknown	*Schistosoma mekongi*		AF216698	AF216698
Sm2	Laos	Unknown	Unknown	*S. mekongi*		AF217449	AF217449
Tr1	Unknown	Unknown	Unknown	*Trichobilharzia regenti*		DQ859919	DQ859919
Tr2	Unknown	Unknown	Unknown	*T. regenti*		NC_009680	NC_009680
Mb	Unknown	Unknown	Unknown	*Metorchis bilis*		FJ423739	
Mx	Unknown	Unknown	Unknown	*Metorchis xanthosomus*		FJ423740	
Ts	Unknown	Unknown	Unknown	*Trichinella spiralis*		NC_002681	NC_002681

Table 2: Sequences of primers used to amplify the Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA (rDNA), a portion of cytochromec oxidase subunit 1 (pcox1) and NADH dehydrogenase subunits 1 (pnad1) of metacercaria samples of *Metorchis orientalis* from Guangxi, China

Name of primer	Sequence (5'-3')	References
For ITS		
BD1	GTCGTAACAAGGTTTCCGTA	Luton *et al.* (1992)
BD2	TATGCTTAAATTCAGCGGGT	Luton *et al.* (1992)
For pcox1		
JB3	TTTTTTGG GCATCCTGAGGTTTAT	Bowles *et al.* (1992)
JB4.5	TAAAGAA A GAACAT AATGAAA ATG	Bowles *et al.* (1992)
For pnad1		
nad1-F	TTCTTATGAGATTGCTTTT	Li *et al.* (2008a)
nad1-R	TATCATAACGAAAACGAGG-	Li *et al.* (2008b)

primers (individually) as used in the PCR. The ITS, pcox1 and pnad1 sequences are available from DDBJ, EMBL and GenBank™ under the accession numbers shown in Table 1.

Sequences analysis and reconstruction of phylogenetic relationships: The pcox1 and pnad1 sequences were separately aligned using the computer program Clustal ×1.83 (Thompson *et al.*, 1997). Sequence Differences (D) were calculated by pair-wise comparison using the formula D = 1-(M/L) in which M is the number of alignment positions at which the two sequences have a base in common and L is the total number of alignment positions over which the two sequences are compared (Chilton *et al.*, 1995). To study the phylogenetic relationships between *M. orientalis* and other 7 trematode species belonging to 6 genera in 4 families, the combined pcox1 and pnad1 sequences of *M. orientalis* as well as that of *Clonorchis sinensis*, *Opisthorchis felineus*, *Fasciola hepatica*, *Schistosoma japonicum*, *Schistosoma mansoni*, *Trichobilharzia regenti* and *Paragonimus westermani* obtained from GenBank (Table 1) were used for phylogenetic analyses with *Trichinella spiralis* (NC_002681) as the outgroup (GenBank™ accession number can be shown in Table 1).

Three methods namely Neighbor Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP) were used for phylogenetic re-constructions. NJ and MP analysis were carried out using PAUP 4.0 Beta 10 programme (Swofford, 2002) and ML analyses were performed using PUZZLE 4.1 (Strimmer and von Haeseler, 1996) under the default setting. The consensus tree was obtained after bootstrap analysis of 1000 replications and values above 50% were reported. Phylograms were drawn using the Tree View program version 1.65 (Page, 1996).

RESULTS AND DISCUSSION

Genomic DNA was prepared from 6 individual metacercariae from Guangxi in China (Table 1). Amplicons of ITS, pcox1 and pnad1 (~1300, 720, 720 bp, respectively) were amplified individually and subjected to agarose gel electrophoresis. For each DNA region, no size variation was detected on agarose gel among any of the amplicons

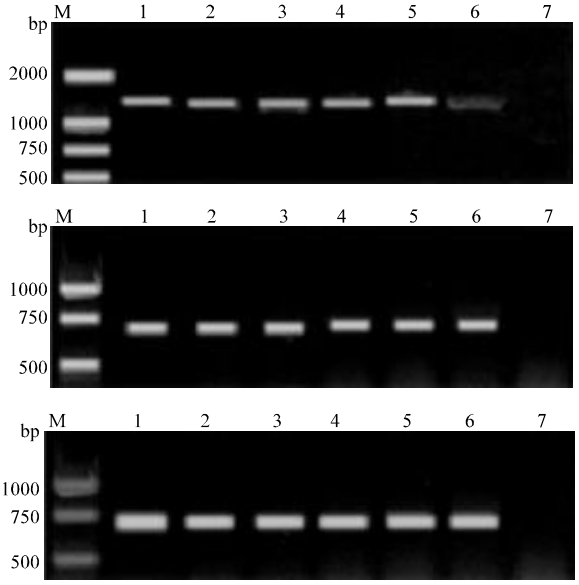

Fig. 1: Representative PCR products of metacercaria samples of *Metorchis orientalis* for the Internal Transcribed Spacers (ITS) of rDNA (upper), a portion of cytochrome c oxidase subunit 1 (pcox1, middle) and NADH dehydrogenase subunits 1 (pnad1, bottom) from Guangxi in China. Lanes 1-7 represent samples Momgx1, Momgx2, Momgx3, Momgx4, Momgx5, Momgx6 and negative control, respectively. M represents a DNA size marker (ordinate values in bp)

examined (Fig. 1). To examine sequence variations in the ITS and two mt DNA regions among isolates, the amplicons were subjected to direct sequencing. The sequences of ITS, pcox1 and pnad1 were 1131, 654 and 650 bp in length, respectively. The A+T contents of the sequences were 46.42-46.51% (ITS), 59.39-59.57% (pcox1) and 62.54-63.11% (pnad1), respectively. Sequence variations among *M. orientalis* isolates were 0.0-0.3% for ITS, 0.0-0.4% for pcox1 and 0.0-1.2% for pnad1. Sequence difference in the ITS and 5.8S between *M. orientalis* and *Metorchis bilis* (EU038154) were 4.0-4.2%, between *M. orientalis* and *Opisthorchis felineus*

Table 3: Pairwise comparison of sequence differences (%) in the partial mitochondrial cytochrome *c* oxidase subunit 1 gene (pcox1, above the diagonal), NADH dehydrogenase subunits 1 genes (pnad1, below the diagonal) among *Metorchis orientalis* isolates from Guangxi in China

Sample codes	Mom gx1	Mom gx2	Mom gx3	Mom gx4	Mom gx5	Mom gx6	Cs1	Cs2	Of1	Of2	Pw1	Pw2	Fh1	Fh2	Sj1	Sj2	Sm1	Sm2	Tr1	Tr2
Momgx1	-	0.4	0.2	0.2	0.4	0.2	23.5	23.5	25.5	25.5	31.3	31.3	36.5	36.5	37.5	37.5	34.7	34.7	35.4	35.6
Momgx2	0.4	-	0.2	0.2	0.4	0.2	23.5	23.5	25.5	25.5	30.1	30.1	36.6	36.6	36.1	36.1	34.5	34.5	36.6	36.5
Momgx3	0.4	0.0	-	0.0	0.2	0.0	23.3	23.3	25.3	25.3	31.1	31.1	36.6	36.6	37.4	37.4	34.5	34.5	35.3	35.8
Momgx4	0.9	0.4	0.4	-	0.2	0.0	22.5	22.5	23.4	23.4	35.1	35.1	32.6	32.6	35.3	35.3	37.2	37.2	38/0	38.0
Momgx5	0.0	0.4	0.4	0.9	-	0.2	23.5	23.5	22.7	22.7	35.2	35.2	33.2	33.2	35.4	35.4	36.2	36.2	37.5	37.3
Momgx6	0.4	0.0	0.0	0.4	0.4	-	0.1	31.5	31.6	32.1	32.8	35.3	35.4	33.9	33.9	35.7	32.2	32.2	35.4	35.6
Cs1	24.1	24.6	24.6	25.0	24.1	24.6	-	0.1	32.2	32.5	33.5	33.5	36.4	36.7	38.4	38.6	38.2	38.3	36.4	36.4
Cs2	24.6	24.6	24.5	24.5	24.5	24.2	0.2	-	29.0	29.0	28.4	28.4	29.2	35.3	34.0	34.3	33.7	33.9	35.6	35.6
Of1	24.2	24.2	24.6	26.3	26.3	26.5	26.8	26.8	-	0.2	39.5	39.5	39.7	38.6	38.4	39.2	39.9	39.9	39.8	39.7
Of2	24.4	24.4	24.5	24.6	24.6	24.6	26.4	26.4	0.3	-	31.2	31.2	35.8	35.8	36.4	38.1	36.5	36.5	37.4	38.0
Pw1	38.9	39.0	39.0	38.7	38.7	38.7	38.1	38.2	38.0	38.1	-	0.4	33.7	34.6	34.6	35.5	32.2	32.3	35.6	35.6
Pw2	38.8	38.9	38.8	38.8	38.9	38.8	38.3	38.3	39.2	39.1	0.2	-	22.3	30.9	32.5	38.3	38.3	38.1	39.2	39.2
Fh1	39.9	39.5	39.5	39.2	39.5	39.5	38.4	38.9	38.6	38.9	35.0	35.0	-	0.0	24.7	24.7	22.3	22.3	32.1	32.1
Fh2	39.9	39.5	39.5	39.2	39.5	39.5	38.4	38.9	38.6	38.9	35.0	35.0	0.0	-	24.7	24.7	22.3	22.3	33.0	33.0
Sj1	40.2	40.2	40.5	40.5	40.3	40.3	42.5	42.5	42.4	42.8	41.0	41.2	41.9	41.5	-	0.0	38.0	38.0	38.4	38.5
Sj2	40.2	40.3	40.3	40.5	40.3	40.3	42.5	42.5	42.4	42.8	41.0	41.2	41.9	41.5	0.1	-	38.0	38.0	37.9	37.6
Sm1	41.1	41.4	41.4	41.1	41.4	41.2	41.1	41.5	41.3	41.3	42.8	42.8	42.9	43.0	18.9	18.9	-	0.0	39.1	38.4
Sm2	41.1	41.4	41.4	41.1	41.4	41.2	41.1	41.5	41.3	41.3	42.8	42.8	42.9	43.0	18.9	18.9	0.0	-	39.2	38.4
Tr1	41.3	41.2	42.0	41.3	41.3	41.6	42.4	42.5	42.8	42.2	42.5	42.6	42.0	42.0	42.3	42.3	42.6	42.6	-	0.1
Tr2	41.3	41.2	42.1	41.3	41.3	41.5	42.4	42.5	42.8	42.2	42.5	42.6	42.0	42.0	42.3	42.3	42.6	42.6	0.1	-
Mb	7.0	7.0	7.2	7.2	7.2	7.0	24.2	24.5	24.5	24.6	38.7	38.8	39.5	39.2	41.0	41.2	42.0	42.2	43.5	43.5

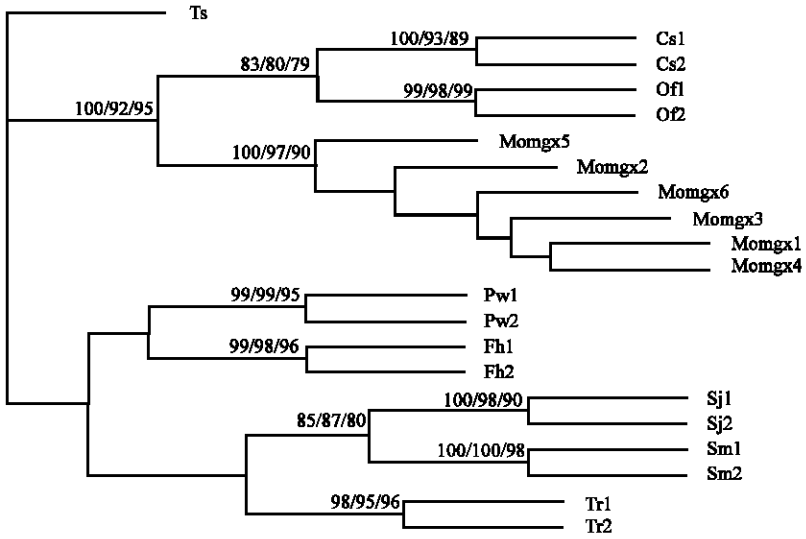

Fig. 2: Phylogenetic relationship of *Metorchis orientalis* with other trematodes inferred by Neighbor-Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses using the combined pcox1 and pnad1 sequences, with *Trichinella spiralis* as outgroup. Bootstrap values (in percentage) above 50% from 1,000 pseudo–replicates are shown for the NJ (the first value), MP (the second value) and ML analyses (the third value). weak = node resolved by method but very weak (<50%). Scale bar indicates an evolutionary distance of 10 substitutions per site in the sequence

(EU038137) were 6.0-6.1% and between *M. orientalis* and *Clonorchis sinensis* (AF181892) were 10.0-10.1%. Then, pcox1 and pnad1 mtDNA sequences were assessed whether they could provide a suitable marker for examining relationships between *M. orientalis* and other trematodes. In order to examine sequence differences in the pcox1 and pnad1 mtDNA among other trematodes, sequences of *M. orientalis* isolates in China were aligned into a consensus sequence. The genetic difference between *Clonorchis* and *Opisthorchis* in the combined pcox1 and pnad1 sequences was 22.7-23.5% for pcox1 and 24.1-26.5% for pnad1 and was 32.2-37.5% for pcox1 and 40.2-43.0% for pnad1 between Schistosoma and Trichobilharzia, respectively (Table 3).

The combined sequences of pcox1 and pnad1 mtDNA were aligned over a consensus length of 1304 bp. Topologies of the combined pcox1 and pnad1sequences inferred by different methods (NJ, MP and ML) with different building strategies and/or different distance models were similar (Fig. 2). The phylogenetic tree was

consisted of two large clades: the first one contained all examined trematodes of the family Opisthorchiidae and the other one includes all other examined trematodes. Within the first clade, all the trematodes belonging to the family Opisthorchiidae were divided into two groups, *C. sinensis* and *O. felineus*. For the Opisthorchiidae cluster, *M. orientalis* isolates were grouped together and the isolates of *C. sinensis* and *O. felineus* were clustered together with high bootstrap value (>50%), respectively (Fig. 2). Within the second clade, *Fasciola* (Fasciolidae) trematodes and *Paragonimus* (Paragonimidae) flukes were clustered together, *Schistosoma* samples were clustered together, respectively. This clustering is in agreement with the results of traditional classifications.

CONCLUSION

The results of the present study were the first charactrization of *M. orientalis* metacercariae in China by a genetic approach using ITS rDNA, pcox1and pnad1 mtDNA as genetic markers. The combined pcox1 and pnad1 sequences are useful for re-construction of phylogenetic relationships between *M. orientalis* and other trematodes. The ITS, cox1 and nad1 mt DNA sequences provide effective genetic markers for the specific identification of trematodes of the Opisthorchiidae family and have implications for studying their population biology, genetic structure as well as molecular epidemiology.

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