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ORIGINAL PAPER

Discovery of *Paragonimus westermani* in Vietnam and its molecular phylogenetic status in *P. westermani* complex

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Abstract *Paragonimus westermani* is the most well-known species among the genus *Paragonimus*. It is widely distributed in Asia with considerable genetic diversity to form *P. westermani* species complex. While *P. westermani* distributed in Japan, Korea, China, and Taiwan are genetically homogeneous to form the East Asia group, those found in other geographic areas are heterogeneous and would be divided into several groups. Recent discoveries of *P. westermani* in India and Sri Lanka highlighted new insights on molecular phylogenetic relationship of geographic isolates of this species complex. Since Vietnam is located at the east end of Southeast Asia, the intermediate position between South and East Asia, it is of interest to see whether *P. westermani* is distributed in this country. Here, we report that *P. westermani* metacercariae were found in mountainous crabs, *Potamiscus* sp., collected in Quangtri province in the central Vietnam. Adult worms were successfully obtained by experimental infection in cats. Molecular phylogenetic analyses revealed that *P. westermani* of Vietnamese isolates have high similarities with those of East Asia group.

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Introduction

Among about 50 species of the genus *Paragonimus* (Blair et al. 1999), *Paragonimus westermani* was first described as early as in 1878 and is the most well-known species because of its wide geographical distribution and of its medical importance. By molecular phylogenetic analyses, *P. westermani* was considered as a species complex consisting of at least two groups: the East Asia group endemic in Japan, Korea, China, and Taiwan and the Southeast Asia group distributed in Malaysia, Thailand, and the Philippines (Blair et al. 1997; Iwagami et al. 2000; Park et al. 2003). However, recent discoveries of *P. westermani* in south Thailand (Binchai et al. 2007; Sugiyama et al. 2007), India (Tandon et al. 2007), and Sri Lanka (Iwagami et al. 2008) provided evidences that *P. westermani* complex was constructed with more than two groups (Blair et al. 2007). In spite of broad geographical distribution of *P. westermani* in Asia, genetic information on geographical isolates from Indochina peninsula is limited to those from Thailand and Malaysia. During our recent surveys on

paragonimosis and *Paragonimus* in Vietnam, we found metacercariae of *P. westermani* in mountainous crabs, *Potamiscus* sp., in Quangtri province in the central Vietnam. We also successfully obtained adult worms by experimental infection in cats. Adult worms were, together with metacercariae, used for molecular study. The phylogenetic analyses of the nuclear ribosomal second internal transcribed spacer region (ITS2), and the partial mitochondrial cytochrome oxidase subunit 1 gene (CO1) sequences showed that *P. westermani* of Vietnamese isolate was genetically close to the East Asia group.

Materials and methods

Materials

Mountainous crabs, *Potamiscus* sp., collected in Dackrong district of Quangtri province in the central Vietnam were examined for the presence of *Paragonimus* metacercariae by the methods of Habe et al. (1993). In brief, gills and liver of each crab were separately pressed between two glass plates and examined under a stereomicroscope. Muscles of the body and legs were chopped into small pieces and ground with a wooden rod on the stainless sieve immersed in tap water. The homogenates were kept still for a few minutes and then the supernatant was discarded. The sediment was washed several times by repeating sedimentation/decantation and finally examined under a stereomicroscope for detecting *Paragonimus* metacercariae. After morphological observation such as the shape, size and cyst wall structure, and the position of metacercaria in the cyst, some freshly isolated metacercariae were fixed separately in 70% ethanol and stored at −20°C until use for molecular analyses. Some other freshly isolated metacercariae were used for experimental infection.

Three cats, two dogs, and ten rats, all of which were examined for negative with *Paragonimus* or any other parasite eggs in stool, were used for experimental infection. Except for one cat, which was infected by intraperitoneal injection with 50 metacercariae, all animals were fed orally with ten to 50 live *P. westermani* metacercariae per animal (Table 1). Presence of *Paragonimus* eggs in feces was monitored 1 month post-infection (PI) and onward. Infected animals were autopsied on the designated days for collecting adult worms.

Molecular analyses

For molecular analyses, we amplified and sequenced the ITS2 and CO1 of each three metacercariae and adults of *P. westermani* collected from Quangtri province by the methods described in our previous paper (Doanh et al. 2007b). In brief, genomic DNA from individual metacercaria was extracted by proteinase K digestion and phenol/chloroform/isoamyl alcohol extraction procedures, followed by ethanol precipitation for purifying extracted DNA. The ITS2 was amplified using the primer pair of 3S (Bowles and McManus 1993) and BD2 (Bowles et al. 1993). For partial CO1 gene amplification, we used Pw-F8049 (5′-TTT TTT GGG CAT CCT GAG GTT TA-3′ = FH3 in Iwagami et al. 2008) as a forward primer and Pw-R8447 (5′-CAG AGA CAA GAC GTA ATG AAA ATG-3′) as a reverse primer, instead of generally used primer pair of JB3 and JB4.5 (Bowles et al. 1993). The new reverse primer for CO1 was designed based on the mitochondrial genome sequences of *P. westermani* (AF219379; Agatsuma and Iwagami, unpublished). PCR products were purified using Qiaquick PCR Purification Kit (Qiagen). These products were primed using Big-Dye Terminaitor Cycle Sequencing Kit v3.1 (ABI) and both strands directly sequenced (Model 310 or 3100, Applied Biosystems).

Phylogenetic analyses

Six each of ITS2 and CO1 sequences of the samples were determined. These sequences were deposited in the Gen Bank/EMBL/DDBJ nucleotide sequence database with

Table 1 The result of experimental infections in animals with *P. westermani* metacercariae collected from Quangtri

Host no.	Route of infection	Dose of metacercariae	Autopsy (days PI)	No. of worms recovered (%)	No. of worms in	
					Lungs	Pleural cavity
Cat no. 1	Oral	50	45	18 (36.0%)	18	–
Cat no. 2	Oral	50	160	22 (44.0%)	2	20
Cat no. 3	IP	30	170	19 (54.3%)	12	7
Dog no. 1	Oral	50	160	–		
Dog no. 2	Oral	50	200	–		
Rat no. 1–5	Oral	10	60	–		
Rat no. 6–10	I.P	10	60	–		

IP intraperitoneal injection

accession no. FJ434982–FJ434993. For the alignment and phylogenetic tree analyses, all ITS2 and CO1 sequences of *P. westermani* available from the DNA database were downloaded from the GenBank. Two phylogenetic trees were reconstructed based on two sequence data sets, ITS2 and CO1. For CO1 dataset, we downloaded 48 sequences, but omitted three sequences (AF096228, AF219379 and AY140677), because of lacking or ambiguity of the localities of collection. In addition, some completely identical sequences from the same locality were treated as single operational taxonomic unit (OTU). Therefore, final CO1 data set with Vietnamese samples includes 387 bp of 43 OTUs. For ITS2 data set, we downloaded 21 sequences for analyses. Because only partial ITS2 sequences of 287 bp were recorded for two Sri Lanka isolates (AY240942 and AY240943; Iwagami et al. 2008), phylogenetic tree analyses with ITS2 dataset was performed using the overlapping region of the ITS2 sequences between our data and Sri Lanka isolates. The finally aligned length with Vietnamese samples was 282 bp from 24 OTUs. In addition, CO1 and ITS2 sequences of *Paragonimus heterotremus*, *Paragonimus vietnamensis*, and *Paragonimus proliferus* (Doanh et al. 2007b, 2008) were used as out group. All sequences were aligned using Clustal-X v1.83 (Thompson et al. 1997) with default options.

For tree reconstruction, we used neighbor-joining (Saitou and Nei 1987) method with the Kimura-2-parameter model (Kimura 1980). The genetic distances of Kimura-2-parameter model were calculated in consideration with all substitutions and with missing and/or gaps as unambiguous changes. In addition, the statistical confidence of branching patterns was evaluated by the bootstrap test (Felsenstein 1985) and is expressed as the proportion of 1,000 replication at each node. These phylogenetic analyses were carried out with the software package PAUP* ver 4.0 (Sinauer Associates, Sunderland, MA, USA).

Moreover, we analyzed the genetic diversities of *P. westermani* for CO1 gene data set. Forty-three OTUs were divided into four subdivisions based on the collecting localities: East Asia (N=20 from Japan, Korea, China, and Taiwan), South Asia (N=5 from India and Sri Lanka), Southeast Asia (N=12 from Malaysia, Thailand, and the Philippines), and Vietnam (N=6). For each subdivision, we calculated nucleotide diversity (Pi), haplotype diversity (Hd), and average number of nucleotide differences (k) using DnaSP 4.5 (Rozas et al. 2003).

Results

Prevalence of *P. westermani* metacercariae in crabs

Fifty two crabs, *Potamiscus* sp., collected in Dackrong district of Quangtri province in the central Vietnam were examined for the presence of *Paragonimus* metacercariae. Of these, 49 (94.2%) were infected with the intensity of infection range of nine to 586 (78 in average) metacercariae/crab. Metacercariae (Fig. 1) were uniform in size (407± 20 μm in diameter based on 25 samples) and appearance and were morphologically identified as *P. westermani*.

Experimental infection in mammalian hosts

Three cats, two dogs, and ten rats were infected with *P. westermani* metacercariae collected from Quangtri province. As shown in Table 1, mature and immature adult worms were obtained from three cats, but not from dogs or rats. Cat no. 1 infected orally with 50 metacercariae was autopsied 45 days PI, and 18 (36.0%) worms were recovered from the cysts in the lungs. All of them were immature. Cat no. 2 fed with 50 metacercariae was monitored for longer time by fecal egg examinations, but was negative until 160 days PI. When the cat was killed at this time, 22 (44.0%) worms were recovered, 20 immature worms from the pleural cavity, and two from one cyst in the lung. One worm from the lung cyst had eggs in the uterus but did not yet lay eggs, and the other was immature. Cat no. 3, which was infected by intraperitoneal inoculation with 30 metacercariae, became fecal egg positive at 170 days PI. The cat was immediately autopsied, and 19 (54.3%) worms were recovered, seven from the pleural cavity and 12 from the cysts in the lungs. Among all these worms, only two were fully mature adult (Fig. 2) producing eggs.

Molecular analysis

Six each of ITS2 and CO1 sequences were successfully obtained from three each of metacercariae and adults of *P. westermani* Vietnamese isolates. ITS2 sequences were

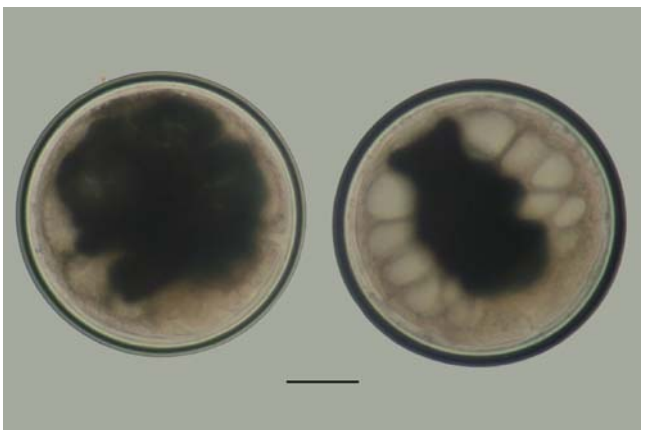

Fig. 1 Metacercariae of *P. westermani* from a crab (*Potamiscus* sp.) collected from Quangtri province. *Scale bar* 100 μm

Fig. 2 Fully adult of *P. westermani* recovered 170 days PI from cat no. 3. *Scale bar* 1 mm

completely and CO1 sequences were almost completely identical with each other to form a distinct clade among *P. westermani* complex (Figs. 3 and 4). Including our present data, 22 characters (7.8%) are parsimony informative sites within 46 variable sites of ITS2 data set (282 sites) among all *P. westermani* species complex, whereas 94 characters (24.3%) are parsimony informative sites within 114 variable sites of CO1 data set (387 bp). In the phylogenetic tree analyses, using *P. heterotremus*, *P. proliferus*, and *P. vietnamensis* as the out group, all *P. westermani* samples from different geographic locations were, including our Vietnamese samples, clustered in the same clade in both ITS2 (Fig. 3) and CO1 (Fig. 4) trees with the bootstrap value of 100%.

In *P. westermani* complex, ITS2 sequences of *P. westermani* Vietnamese isolates showed high similarities (98.7% on average) to those of East Asia group (Japan, Korea, and Taiwan) in which their sequence similarities were 100–99.3%. Similarly, in the CO1 tree, Vietnamese isolates showed high similarities (94.8% on average) with East Asian isolates. Furthermore, East Asian isolates of CO1 gene showed very low genetic diversities (Pi=0.010

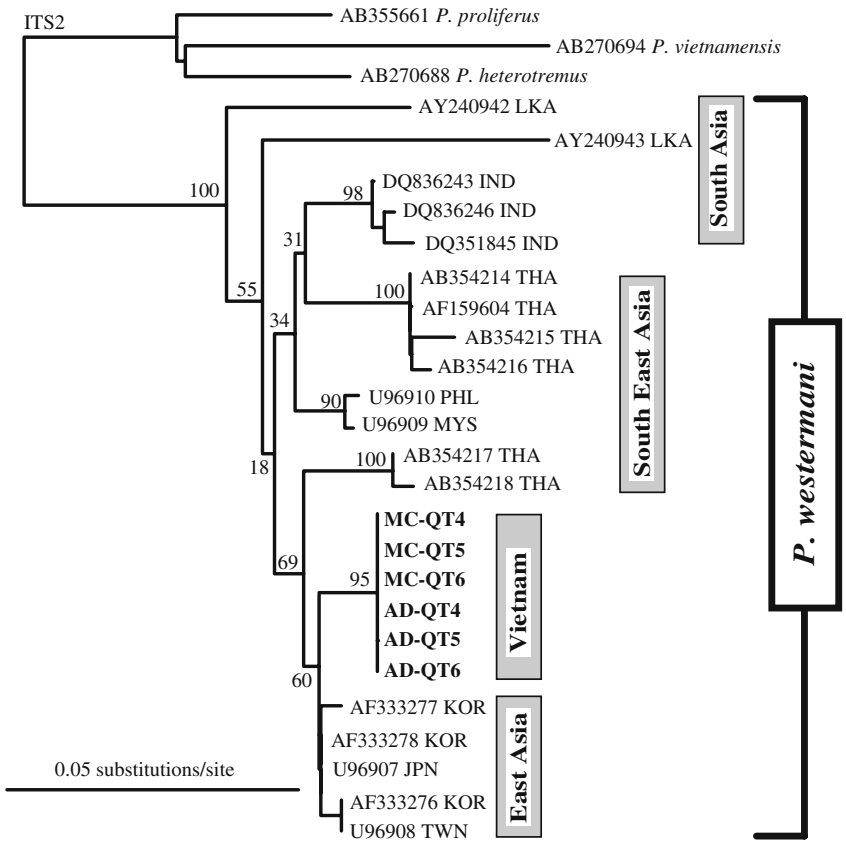

Fig. 3 The neighbor-joining tree reconstructed from ITS2 gene sequences (282 bp). Bootstrap scores (percentages of 1,000 replications) are presented for each node. Samples of *P. westermani* from DNA database are shown with accession no. and country code (ISO 3166-1 alpha-3 codes). The *boxes* indicated the collecting subregions of Asia

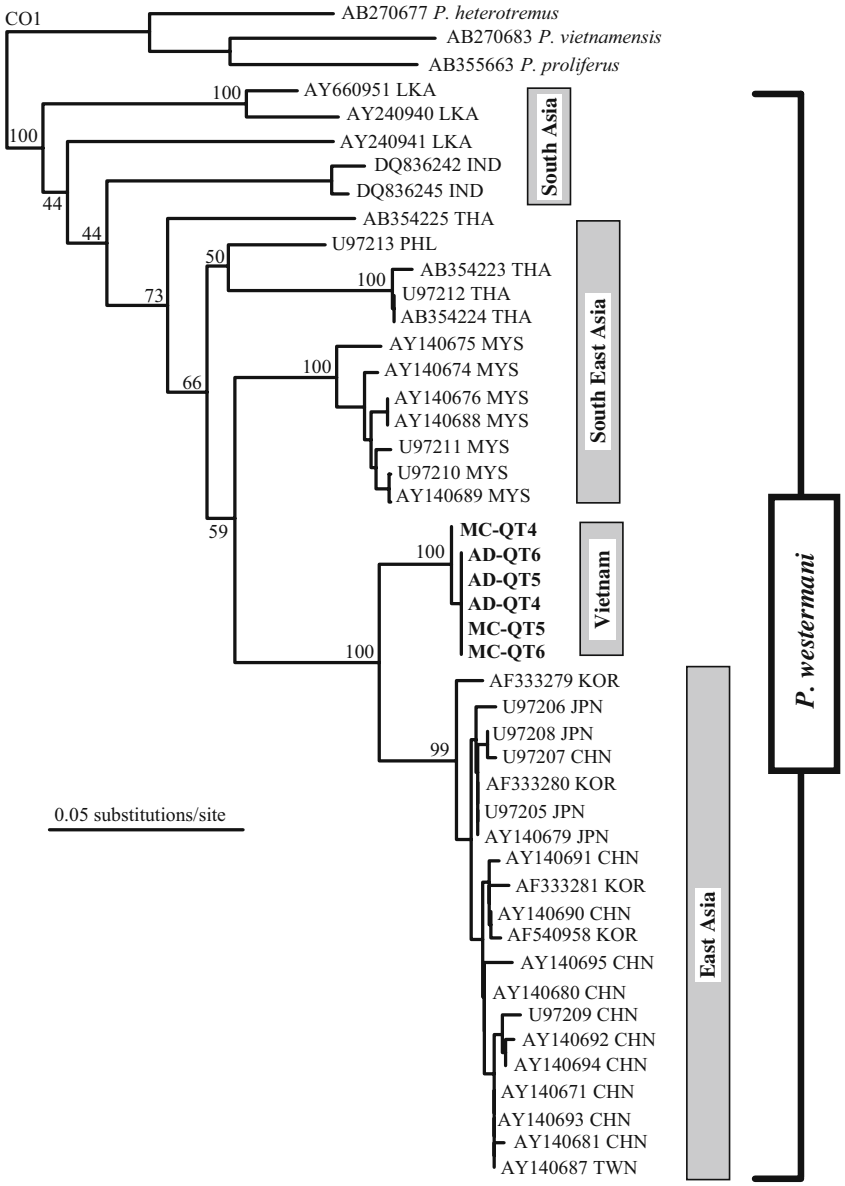

Fig. 4 The neighbor-joining tree based on CO1 gene sequences (387 bp). Bootstrap scores expressed as percentages of 1,000 replications are given at each node. Sequences of *P. westermani*, obtained from DNA database, are indicated with accession no. country code (ISO 3166-1 alpha-3 codes). The *shaded boxes* indicate the collecting subregions of Asia: one Southeast Asian sample (from Philippines; AY140677; *arrow*) is clustered into East Asia group (see text)

and k=3.889), even when Vietnamese isolates were included in East Asia group, the genetic diversity (Pi= 0.024 and k=9.335) of this group remained much lower than that of Southeast Asia (Pi=0.057 and k=22.000) and South Asia (Pi=0.110 and k=42.110) group (Table 2).

Discussion

Paragonimosis is a serious public health issue in northern Vietnam, and *P. heterotremus* was identified as the important pathogen for human paragonimosis in this area

Table 2 Genetic diversities of *P. westermani* within the subdivisions of Asia

Subdivision	Samples	*H*	Hd	Pi	*k*
All	43	32	0.979	0.082	30.735
South Asia	5	5	1.000	0.110	42.110
Southeast Asia	12	9	0.955	0.057	22.000
East Asia	20	16	0.968	0.010	3.889
Vietnam	6	2	0.333	0.001	0.333
East Asia + Vietnam	26	18	0.951	0.024	9.335

h Number of haplotype, *Hd* haplotype diversity, *Pi* nucleotide diversity, *k* average number of nucleotide differences

(Doanh et al. 2005; Le et al. 2006). During epidemiological surveys in Vietnam, in addition to *P. heterotremus*, we have found two more *Paragonimus* species, *P. vietnamensis* (Doanh et al. 2007a) and *P. proliferus* (Doanh et al. 2008) in northern mountainous area of this country. The present results added *P. westermani* as the fourth *Paragonimus* spp. in Vietnam and Quangtri province as the new endemic area in this country. In northern Vietnam, *Potamiscus* crabs often harbor two or more species of *Paragonimus* metacercariae with the extreme predominance with *P. heterotremus* (Doanh et al. 2007b). Related to this, geographic distribution of *Paragonimus* spp. is supposed to be dependent primarily on the snail hosts (Wilke et al. 2000; Blair et al. 2007). To elucidate the reason why only *P. westermani* was found in Quangtri province in this research, more extensive survey for snail, crustacean, and mammalian hosts in this and other areas of Vietnam is necessary.

Concerning the pathogenicity, the present results showed that *P. westermani* Vietnamese isolates were infective to cats, but not to dogs or rats (Table 1). Even in cats, only few mature worms were obtained after a long (170 days) incubation period. Similar to our results, Habe et al. (1996) reported that Felidae are the most important final hosts for Malaysian *P. westermani*, while Canidae are unsuitable hosts, and Malaysian *P. westermani* require long incubation period (four or more months) to reach mature adults in cats (Habe 1987). In contrast, cats and dogs are known as the excellent experimental hosts to obtain adult worms of *P. westermani* in Japan, Korea, and China with the short (2.5 months) maturation period (Habe 1978; Shibahara 1983; Shibahara et al. 1989). In the rodent hosts, we could not obtain any mature or immature worms from rats 60 days after infection with *P. westermani* metacercariae. In contrast, *P. westermani* in the Philippines mature readily in rats (Miyazaki and Habe 1979), and rats are usually the paratenic hosts of *P. westermani* in Japan, China, and Malaysia (Habe 1983; Habe et al. 1996; Shibahara et al. 1989). Thus, Vietnamese *P. westermani* showed unique mammalian host specificity. Further study is required for the identification of the natural final and paratenic hosts for *P. westermani* in Vietnam.

In the present study, the results of the molecular phylogenetic analyses of ITS2 and CO1 sequences revealed that *P. westermani* Vietnamese isolates have high similarities with those of East Asian isolates to form distinct group among *P. westermani* species complex. The genetic homogeneity of East Asia group has already been pointed out by several authors (Blair et al. 1997; Iwagami et al. 2000; Park et al. 2003). Previously, *P. westermani* distributed in Malaysia, Thailand, and the Philippines were, because of their genetic differences from East Asia group, considered to form another group among *P. westermani* species complex (Blair et al. 1997; Iwagami et al. 2000; Park et al. 2003). However, recent discoveries of *P. westermani*-like isolate in Thailand (Binchai et al. 2007; Sugiyama et al. 2007) and *P. westermani* in India (Tandon et al. 2007) and Sri Lanka (Iwagami et al. 2008) provided evidences that the grouping of *P. westermani* species complex was more complicated than it was thought to be (Blair et al. 2007). In particular, recent work of Binchai et al. (2007) demonstrated that, by ITS2 sequence analyses, *P. westermani* Thailand isolates could be divided into two different groups (AF159604, AB354214, AB354215, and AB354216) and (AB345217 and AB345218) based on the sequence similarities between them. In our tree analyses, the latter group (so-called *P. westermani*-like; by Binchai et al. 2007) was clustered with East Asia type with moderate support value (69%). Nevertheless, the CO1 sequence (AB354223) of this isolate in Thailand was far away from the East Asia group. Moreover, the results of molecular phylogenetic analyses showed that *P. westermani* of South and Southeast Asia groups have larger genetic diversities than those from East Asia group (Table 2; Figs. 3 and 4). These probably indicate a kind of bottleneck event or recent rapid expansion in East Asian region. Since only few ITS2 and CO1 sequences were available, this hypothesis must be justified by multiple sampling from more diverse localities in Asia.

Blair et al. (2001) proposed an evolutionary scenario, whereby populations of *P. westermani* arose first in Southeast Asia and utilized thiarid snails. Later, by range expansion and the addition of pleurocerid snail hosts, they were able to establish populations in East Asia. More recently, Blair et al. (2007) brushed up this scenario to put origin of *P. westermani* from the Indian subcontinent. Our present results seemed to support these hypotheses.

In conclusion, *P. westermani* is highly endemic in Quangtri province, the central Vietnam. The susceptibility of Vietnamese isolate of *P. westermani* in experimental animals is similar to that of Malaysian isolate; however, its genetic feature is rather similar to those distributed in East Asia. Although *P. westermani* East Asian isolates are pathogenic to humans (Blair et al. 2007), the proven human case has never been reported in Quangtri province until now. More extensive and careful survey, including sputum egg examination and sero-diagnosis for humans, is necessary in this area. Whatsoever is the pathogenicity of this *P. westermani* Vietnamese isolate to humans, the discovery of these isolates having molecular similarities with East Asia type might provide a kind of missing ring in the evolution of this species.

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