Journal of Helminthology (2015) **89,** 182–188 doi:10.1017/S0022149X13000734

Metacercarial polymorphism and genetic variation of *Paragonimus heterotremus* (Digenea: Paragonimidae), and a re-appraisal of the taxonomic status of *Paragonimus pseudoheterotremus*

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(Received 7 July 2013; Accepted 1 October 2013; First Published Online 14 November 2013)

Abstract

Paragonimus heterotremus, which is an important pathogen for human paragonimiasis in Asia, is recognized as having the smallest metacercariae (maximum diameter $<300\,\mu m$) of any previously reported *Paragonimus* species. Recently, *P. pseudoheterotremus* has been described from Thailand as a new species having metacercariae (about $200\,\mu m$) slightly smaller than those of Thai *P. heterotremus*. In fact, the small size of *P. pseudoheterotremus* metacercariae is compatible with those of *P. heterotremus* from India and China. In this study in Vietnam, we found variably sized small metacercariae which are expected to consist of both *P. heterotremus* and *P. pseudoheterotremus*. Contrary to expectation, the adult flukes obtained by separate infection of experimental cats with different sized metacercariae were all identified as *P. heterotremus*, using both morphological and molecular characteristics. The molecular analyses of an extensive collection of *P. heterotremus/P. pseudoheterotremus* isolates from Asian countries also indicated that genetic distances between different populations of *P. heterotremus* are even larger than that between *P. pseudoheterotremus* and *P. heterotremus*. The haplotype network showed that all *P. heterotremus* and *P. pseudoheterotremus* isolates formed a *P. heterotremus* complex consisting of three groups with strong geographical origins. In addition, the Indian *P. heterotremus* group is the root of the other *P. heterotremus* and *P. pseudoheterotremus* populations. Based on the observed metacercarial polymorphisms and genetic variation in *P. heterotremus*, *P. pseudoheterotremus* should be considered a geographically isolated population of the *P. heterotremus* complex.

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Introduction

Lung flukes of the genus *Paragonimus* parasitize the lungs of animals and/or humans, causing a typical food-borne zoonosis called paragonimiasis. More than 50 nominal species of the genus have been described morphologically (Blair *et al.*, 1999). Recent molecular data, however, placed some species as synonyms or subspecies of others, and closely related species in a single clade form a species complex, such as *P. westermani*, *P. skrjabini* and *P. bangkokensis/P. harinasutai* complexes (Blair *et al.*, 1999, 2005; Doanh *et al.*, 2008, 2012). On the other hand, new *Paragonimus* species, such as *P. vietnamensis* from Vietnam (Doanh *et al.*, 2007a) and *P. pseudoheterotremus* from Thailand (Waikagul, 2007), have been described recently. Of these, *P. pseudoheterotremus* is morphologically and genetically similar to *P. heterotremus*. Both of them have small metacercariae (<300 μm in the length/width of cyst walls). In a comparison of Thai samples, *P. pseudoheterotremus* has metacercariae slightly smaller than, and is genetically distant from *P. heterotremus* inferred from the mitochondrial cytochrome oxidase subunit 1 (CO1) gene, but almost completely identical in the nuclear ribosomal second internal transcribed spacer region (ITS2) (Waikagul, 2007; Thaenkham & Waikagul, 2008). During our surveys for *Paragonimus* spp. in Vietnam, we found variably sized small metacercariae which appeared to be of *P. heterotremus* and *P. pseudoheterotremus*. However, the adults obtained separately by experimental infection with different sizes of metacercariae were morphologically homogeneous, and all of them were identified as *P. heterotremus* with the strong support of molecular data. The aim of this study is, therefore, to clarify the taxonomic status and phylogenetic relationship of *P. heterotremus* and *P. pseudoheterotremus* based on morphological and molecular analyses of all available *P. heterotremus* populations from India, Thailand, Vietnam and China, and *P. pseudoheterotremus* from Thailand. Their phylogenetic relationship and taxonomic status are discussed in detail.

Materials and methods

Collection of metacercariae from mountain crabs

Paragonimus metacercariae were collected from mountain crabs, *Indochinamon tannanti*, caught from two distant locations (about 400 km apart; Luc Yen district, Yen Bai province and Cam Thuy district, Thanh Hoa province, Vietnam), by the method described previously (Doanh *et al.*, 2007b). *Paragonimus* metacercariae of small size (maximum diameter <300 μm), which were supposed to be of *P. heterotremus* and *P. pseudoheterotremus*, were tentatively separated further into two groups by size. Since metacercariae from Thanh Hoa province were more uniform and the diameter of the inner cyst was smaller than 200 μm, these samples were placed in one group. Conversely, the metacercariae from Yen Bai province were also small but exhibited some variation and were divided into two groups by the inner cyst wall diameter of smaller and larger than 200 μm. The morphometric measurements (length/width of cyst walls, length/width ratio, thickness of the inner cyst wall) of metacercariae from both provinces were statistically compared by paired *t*-test.

Experimental infection

Four cats, all of which were free of *Paragonimus* eggs before infection, were orally administered with small metacercariae which were supposed to be *P. heterotremus* and *P. pseudoheterotremus*. Cat 1 and cat 2 were infected with metacercariae from Thanh Hoa province; while cats 3 and 4 were administered with metacercariae of <200 and >200 μm from Yen Bai province, respectively. This experiment was approved by the Ethical Committee for Animal Experiments, Institute of Ecology and Biological Resources, Vietnam Academy of Sciences and Technology, and all procedures of the experiment followed the National Guidelines in Vietnam. The presence of *Paragonimus* eggs in faeces of the infected cats was examined 1 month post-infection (PI) and thereafter. The cats were sacrificed 70 days after being infected to collect adult flukes. A small piece of about 2 mm^2 of the posterior end of each fluke was cut, separately fixed in 100% ethanol and stored at −20°C for DNA extraction. For morphological identification the remainder of each fluke was flattened between two glass slides and fixed in 70% ethanol for about 2 weeks, and these were then stained with carmine, cleared in xylene, mounted individually on to a glass slide with Canada balsam, and covered with a cover slip for permanent preparation.

Phylogenetic analyses

Four samples (two metacercariae and two adults) from Thanh Hoa province and six samples from Yen Bai province, including two metacercariae and one adult fluke from each size group of metacercariae (<200 and >200 μm) were subjected to molecular phylogenetic analyses (table 1).

The ITS2 and CO1 gene were amplified by polymerase chain reaction (PCR) (Doanh *et al.*, 2007b) using primer pairs 3S and A28 (Bowles *et al.*, 1995) and JB3 and JB4.5 (Bowles *et al.*, 1993), respectively. The PCR products were purified using Qiaquick PCR purification Kit (Qiagen Inc., Tokyo, Japan). Both strands were directly sequenced

Table 1. Samples of metacercariae and adults of *Paragonimus heterotremus* examined for molecular analyses, with the inner cyst wall diameter of metacercariae being <200 μm (VNM1–7) or >200 μm (VNH8–10).

Sample codes	Location	Stage
VNM1	Thanh Hoa	Metacercaria
VNM2	Thanh Hoa	Metacercaria
VNM3	Thanh Hoa	Adult
VNM4	Thanh Hoa	Adult
VNM5	Yen Bai	Metacercaria
VNM6	Yen Bai	Metacercaria
VNM7	Yen Bai	Adult
VNM8	Yen Bai	Metacercaria
VNM9	Yen Bai	Metacercaria
VNM10	Yen Bai	Adult

https://doi.org/10.1017/S0022149X13000734 Published online by Cambridge University Press

by Genetic Analyzer 3130 (Applied Biosystems Japan Ltd, Tokyo, Japan) using the PCR primers as sequencing primers and a Big-Dye terminator cycle sequencing kit v3.1 (Applied Biosystems). For the phylogenetic analyses, we downloaded from GenBank all ITS2 and *CO1* sequences registered as *P. heterotremus/P. pseudoheterotremus* from India, China, Thailand and Vietnam,. Those of *P. vietnamensis* were used as an outgroup. The ITS2 and CO1 sequence data sets for phylogenetic analyses were prepared and aligned using Clustal-W (Thompson *et al.*, 1994) with default options. Evolutionary analyses were performed using the MEGA5 software package (Tamura *et al.*, 2011). The best model of nucleotide substitution for each sequence dataset was tested, and maximum likelihood trees were reconstructed using the Kimura 2-parameter model for the ITS2 tree and Hasegawa–Kishino–Yano + invariant site substitution model for the CO1 tree. The statistical confidence of branching patterns was evaluated by the bootstrap test with 1000 replications.

For inferring intraspecific phylogenies, the haplotype network of *P. heterotremus* and *P. pseudoheterotremus* was generated based on CO1 sequences using SplitsTree 4 (Huson & Bryant, 2006).

Results

Morphological variation of P. heterotremus *metacercariae*

Metacercariae collected from Thanh Hoa province were oval or spherical in shape with the outer cyst wall 197–248 × 164–180 μm (mean 219 × 172; $n = 50$), the inner cyst wall 180–234 × 148–156 μm (mean: 200 × 155), and the length/width ratio ranging between 1.2 and 1.5 (mean: 1.3); the thickness of the inner cyst wall was variable with or without bipolar thickening, measuring 1.2–4.0 μm (mean 3.0) at the side and 1.2–18.0 μm (mean 8.1) at the poles (fig. 1a, table 2). In contrast, metacercariae collected from Yen Bai province were rounder and more variable in size (fig. 1b), the outer cyst wall measuring 197–300 × 197–297 μm (mean: 256 × 235; $n = 50$) and the inner cyst wall 156–271 × 156–243 μm (mean: 205 × 182). The length/width ratio ranged between 1.0 and 1.2 (mean: 1.1); the thickness of the inner cyst wall was also variable with or without bipolar thickening, measuring 2.0–5.0 μm (mean 3.3) at the side and 2.0–17.0 μm (mean 7.8) at the poles. The width and length/width ratio, but not the thicknesses of the inner cyst wall, of metacercariae from Thanh Hoa and from Yen Bai provinces were significantly different ($P < 0.05$).

Metacercariae from Yen Bai province were divided into two groups based on the diameters of the inner cyst wall, with measurements of group 1 being <200 μm (156–197 × 156–189; mean 184 × 171); and group 2 >200 μm (205–271 × 200–243; mean 218 × 205) (table 2). Both groups of metacercariae from Yen Bai and the group from Thanh Hoa were administered separately to four cats, all of which showed eggs in the faeces between 50 and 55 days post-infection. Adult flukes obtained from the cats were all identical with each other and shared the morphological characteristics of *P. heterotremus* (data not shown).

Molecular phylogenetic analyses

We successfully obtained a total of ten ITS2 and ten CO1 sequences, which are deposited in GenBank with accession numbers AB827360–AB827379. The results of molecular analyses showed that all metacercariae and adults from Thanh Hoa and Yen Bai provinces were completely and almost completely identical with each other in ITS2 and CO1 sequences, respectively. They were also identical with *P. heterotremus* from China, Thailand and from other locations in Vietnam, forming a single cluster (fig. 2). However, *P. heterotremus* specimens from India differed markedly (1.5% and 9.0% differences in ITS2 and CO1, respectively) from other populations, forming a separate group in both trees. In contrast, ITS2 sequences of *P. pseudoheterotremus* were almost completely identical (99.7% similarity) with those of *P. heterotremus* from China, Thailand and Vietnam and were clustered in this group; however, the CO1 sequences of *P. pseudoheterotremus* were considerably different (10.4%) from the Chinese, Thai and Vietnamese *P. heterotremus* populations, and relatively closer (5.7% difference) to the Indian *P. heterotremus* population.

The haplotype network (fig. 3) revealed that all *P. heterotremus/P. pseudoheterotremus* isolates were genetically divided into three groups, namely *P. heterotremus* from India, *P. heterotremus* from China, Thailand and

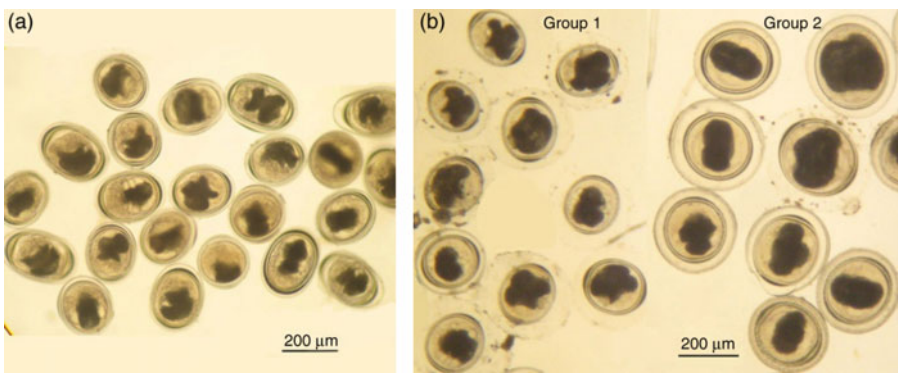

Fig. 1. Metacercariae of *Paragonimus heterotremus* from crab hosts in the provinces of Thanh Hoa (a) and Yen Bai (b), Vietnam.

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Table 2. Morphometric data (μm) of *P. heterotremus* and *P. pseudoheterotremus* metacercariae from different geographical regions in India, China, Vietnam and Thailand.

	P. heterotremus							
			Vietnam					
				Yen Bai				
	India (Singh *et al.*, 2007)	China (Hu, 1998)	Thanh Hoa	Group 1	Group 2	Thailand	*P. pseudoheterotremus* from Thailand (Waikagul, 2007)
Outer cyst wall			197–248 × 164–180 (219 × 172)	197–254 × 197–230 (230 × 217)	246–300 × 221–297 (266 × 242)	320–335 × 290–305 (330 × 300)	
Inner cyst wall	163–215 × 133–188 (196 × 162)	163–210 × 135–189 (180 × 164)	180–234 × 148–156 (200 × 155)	156–197 × 156–189 (184 × 171)	205–271 × 200–243 (218 × 205)	285–310 × 250–270 (300 × 260)	180–204 × 168–180 (186 × 176)
The length/width of the inner cyst wall			1.2–1.5 (1.3)	1.0–1.2 (1.1)	1.0–1.3 (1.1)		
Thickness at the side of inner cyst wall	4.2–10.4 (6.3)		1.2–4.0 (3.0)	2.0–4.0 (3.2)	2.0–5.0 (3.3)		
Thickness at the poles of inner cyst wall	10.4–27.1 (18.2)		1.2–18.0 (8.1)	2.0–12.0 (7.7)	2.0–17.0 (7.8)		

Numbers in parentheses indicate mean values.

Vietnam and *P. pseudoheterotremus* from Thailand. The genetic distance between the Indian *P. heterotremus* population and other populations of this species from Vietnam, China and Thailand was 7.7%, which was larger than the distance (4.9%) between *P. heterotremus* from India and *P. pseudoheterotremus* from Thailand. On the contrary, there was a larger genetic distance of 13.0% between *P. pseudoheterotremus* from Thailand and *P. heterotremus* from China, Thailand and Vietnam.

Discussion

The traditional taxonomic classification of *Paragonimus* species has been based on morphological characteristics of adults and metacercariae (Blair *et al.*, 1999). However, morphology-based classification often produces difficulties and complexity in identification, particularly in species that exhibit only minor morphological differences. Fortunately, the application of DNA sequence analysis to taxonomy has helped to clarify the phylogenetic relationship between species and their taxonomic status (Blair *et al.*, 1999, 2005; Doanh *et al.*, 2008, 2012). However, the use of molecular differences for taxonomy has brought about a trend to describe new species based on differences of only a few nucleotides in their ribosomal spacer DNA sequences (Kunz, 2002).

In the genus *Paragonimus*, a new *Paragonimus* species, named *P. pseudoheterotremus*, has been described recently from Thailand, although it is very similar to *P. heterotremus* in both morphology and ITS2 sequences (Waikagul, 2007; Thaenkham & Waikagul, 2008). Since both of these species have been reported to cause human paragonimiasis (Pariyanonda *et al.*, 1990; Blair *et al.*, 1999; Le *et al.*, 2006; Devi *et al.*, 2007; Singh *et al.*, 2007; Yahiro *et al.*, 2008; Intapan *et al.*, 2012), clarification of the taxonomic status of these two species is very important. According to the original description, the prominent morphological differences between *P. pseudoheterotremus* and *P. heterotremus* were the sizes of metacercariae and the body spines of adults: metacercariae of *P. pseudoheterotremus* were slightly smaller than those of *P. heterotremus*; in contrast, the body spines of adults of the former species were slightly bigger than those of the latter species (Waikagul, 2007). However, the sizes of body spines and metacercariae have limitations for distinguishing *Paragonimus* species because of the change of body spines during maturation in the definitive host and their variation across the body surface (Blair *et al.*, 1999). Also, metacercarial polymorphisms of *Paragonimus* species have been reported, as can be seen in *P. westermani* metacercariae (Sugiyama *et al.*, 2007; Devi *et al.*, 2013). The size of *P. pseudoheterotremus* metacercariae was reported to be smaller than those of *P. heterotremus* from Thailand only (Waikagul, 2007). In fact, the size of *P. pseudoheterotremus* metacercariae was compatible with those of *P. heterotremus* from China (Hu, 1998), India (Singh *et al.*, 2007) and Thanh Hoa province of Vietnam in this study (table 1). Not only the size but also the thickness of the cyst walls of metacercariae may be used to distinguish species (Chung *et al.*, 1975; Blair *et al.*, 1999). The bipolar thickening of the inner cyst wall has been reported in *P. heterotremus* and *P. pseudoheterotremus* metacercariae

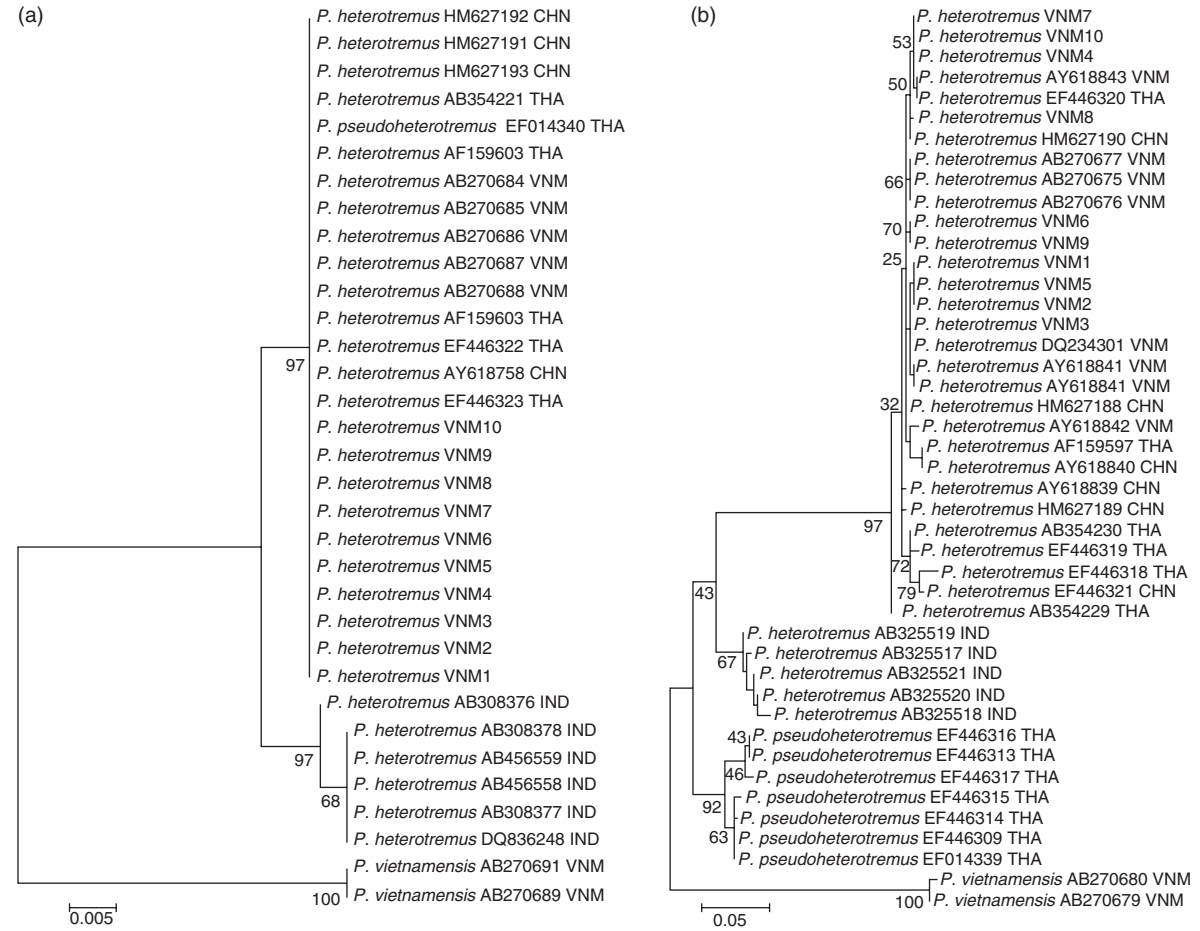

Fig. 2. Maximum likelihood trees reconstructed from (a) ITS2 and (b) CO1 sequences; bootstrap scores expressed as percentages of 1000 replications are given at each node, new sequences given as VNM1–10, and the remainder with the accession numbers from GenBank.

(Chung *et al.*, 1964; Singh *et al.*, 2007; Waikagul, 2007). However, this characteristic was not always observed in all *P. heterotremus* (fig. 1) or *P. pseudoheterotremus* metacercariae (Waikagul, 2007). The thickness of the inner cyst wall of *P. heterotremus* from Vietnam seems to be much less than in specimens from India, but a similar thickness (1.2–17.0 μm; mean at poles 12 μm) to *P. tuanshanensis* from China (Chung *et al.*, 1964), which was considered a synonym of *P. heterotremus* (Blair *et al.*, 1999). Thus, the shape, size and thickness of the inner cyst wall of *P. heterotremus* metacercariae seem to represent different geographical populations, indicating the metacercarial polymorphism of *P. heterotremus*. The measurements and characteristics of *P. pseudoheterotremus* metacercariae are within the range of *P. heterotremus*. Moreover differences in the coefficients of *P. heterotremus* and *P. pseudoheterotremus* metacercariae were higher than those among subspecies (Waikagul, 2007), but differences in adult worms did not support the separation of these species. It is, therefore, difficult to find sufficiently clear morphological differences between *P. heterotremus* and *P. pseudoheterotremus* to separate them as distinct species.

Genetically, when comparing *P. pseudoheterotremus* with *P. heterotremus* from within Thailand, Thaenkham & Waikagul (2008) observed only one base difference of 0.3% in the ITS2 sequences and a considerable difference of 10.6% in the CO1 sequences. As highlighted by Blair *et al.* (1999), the ITS2 sequence is a good marker for inter-species and the CO1 sequence is a good marker for intra-species for identification of *Paragonimus* species, and the extensive study of Blair *et al.* (2005) revealed a large variation of ITS2 sequences in *P. skrjabini* (0–6 variable sites in 363 nucleotides) and *P. westermani* (1–11 variable sites in 363 nucleotides). Thus, the difference of only one base in ITS2 sequence of *P. pseudoheterotremus* and *P. heterotremus* is not sufficient for separating *P. pseudoheterotremus* as a distinct species. Moreover, the molecular analyses in this study, based on an extensive collection of *P. pseudoheterotremus* and *P. heterotremus* populations from different countries, revealed that the differences between geographical populations (India and other countries) of *P. heterotremus* were even larger than those between *P. heterotremus* and *P. pseudoheterotremus* within Thailand. In the phylogenetic trees, *P. pseudoheterotremus* was clustered with *P. heterotremus* from China, Thailand and Vietnam to make a group

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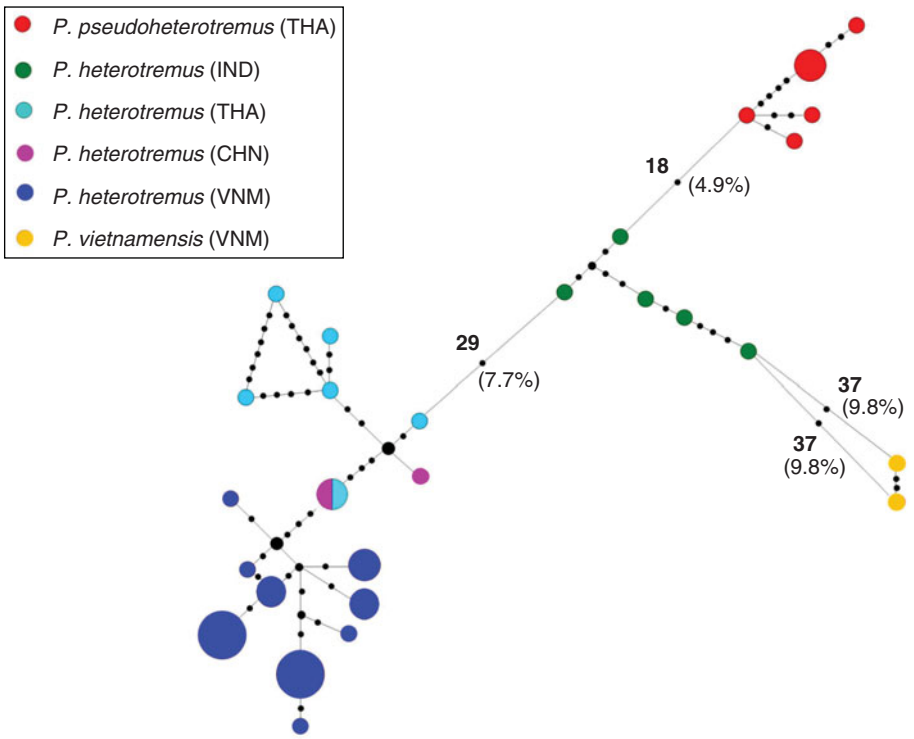

Fig. 3. The median joining network of *P. heterotremus* species complex determined by CO1 sequences.

separated from the Indian *P. heterotremus* population in the ITS2 tree. However, in the CO1 tree, *P. pseudoheterotremus* formed a separate group closer to the Indian *P. heterotremus* population. In the CO1 tree and the haplotype network, three groups (*P. heterotremus* from India; *P. heterotremus* from China, Thailand and Vietnam; and *P. pseudoheterotremus* from Thailand) were clearly differentiated. Although their CO1 sequence variations were markedly different, the genetic distances were either less than, or similar to, those observed in the *P. skrjabini* (9.5–11.5%) or *P. westermani* (29.5%) complexes (Blair *et al.*, 2005; Doanh *et al.*, 2009, 2013). Therefore, these three groups of *P. heterotremus* and *P. pseudoheterotremus* should be considered as members of a species complex, *P. heterotremus*, with the results of the haplotype network suggesting the Indian *P. heterotremus* group be considered as the root of the cluster.

In conclusion, using an expansive collection of different geographical populations, the morphological and molecular analyses performed in this study clearly showed that *P. heterotremus* exhibits metacercarial polymorphism and genetic variation as reported in other *Paragonimus* species complexes. In addition, the findings suggested that *P. pseudoheterotremus* couldn't be considered a distinct species; instead, it should be considered a geographical variation in the *P. heterotremus* complex.

Financial support

This research is funded by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.12-2012.52.

Conflict of interest

None.

Ethical standards

This work was approved by the Ethical Committee for Animal Experiments, Institute of Ecology and Biological Resources, Vietnam Academy of Sciences and Technology, and all procedures followed the National Guidelines in Vietnam.

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