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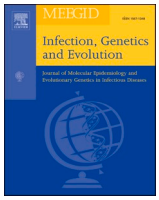

Considerable genetic diversity within *Paragonimus heterotremus* in Luang Prabang, northern Lao People's Democratic Republic

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ABSTRACT

Paragonimiasis, caused by infection with lung flukes of the genus *Paragonimus*, remains a significant public health concern in Southeast Asia. In Lao People's Democratic Republic (Lao PDR), information on the distribution and genetic diversity of *Paragonimus* species is limited. This study investigated *Paragonimus* metacercariae in freshwater (mountain) crabs and analyzed their genetic diversity and phylogenetic relationships. Thirty-six crabs (*Indochinamon* sp.) were received from Xiang Ngeun and Pak Ou in Luang Prabang Province, northern Lao PDR. Partial mitochondrial 16S rRNA sequences obtained from four crabs indicated a moderately close relationship with *Indochinamon ou*. A total of 81 metacercariae identified morphologically as *Paragonimus heterotremus* were found among 13 out of the 32 crabs dissected (40.6 %). Molecular analyses targeting the ribosomal ITS2 region and mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene were conducted on these metacercariae. Phylogenetic analyses revealed that *P. heterotremus* sequences from Lao PDR clustered with those from neighboring countries—China, Myanmar, Vietnam and Thailand—suggesting potential genetic connectivity among eastern Asian populations. Haplotype-network analysis demonstrated significant genetic diversity within *P. heterotremus* populations from Lao PDR, separating into two distinct haplotype groups, one of which was unique to this study. This is the first report that *Indochinamon* sp. crabs serve as key intermediate host for a member of the *P. heterotremus* complex in Luang Prabang Province and highlights the parasite's genetic diversity in this region.

1. Introduction

Paragonimiasis is a zoonotic, food-borne infection caused by lung flukes of the genus *Paragonimus*. It is a major disease due to its potential for significant morbidity and mortality, and long-term health effects (WHO, 2021). Lung flukes commonly cause subacute to chronic inflammatory disease of the lungs. Patients present with chronic cough, chest pain, dyspnea and hemoptysis, symptoms that are also typical of pulmonary tuberculosis and lung cancer. Ectopic paragonimiasis can also occur (Kong et al., 2015; Blair, 2024). Paragonimiasis is a re-emerging disease in modern times (Blair, 2022). In Asia, America, and Africa, there are over 50 known species of *Paragonimus*. In Asia four species complexes exist (based on *Paragonimus heterotremus*, *P. skrjabini*, *P. ohirai* and *P. westermani*) along with two independent species, *P. vietnamensis* and *P. macrorchis* (Nawa et al., 2014; Sanpool et al., 2015). The *Paragonimus heterotremus* complex (*P. heterotremus* and *Paragonimus pseudoheterotremus*) is an important cause of human paragonimiasis in Asia (Blair, 2024; Doanh et al., 2015; Sanpool et al., 2013). Experimental *P. heterotremus* infection in a rat model shows genotoxic potential, most likely due to oxidative stress and an inflammatory environment, and suggests it is a potentially carcinogenic species (Chelomina et al., 2021).

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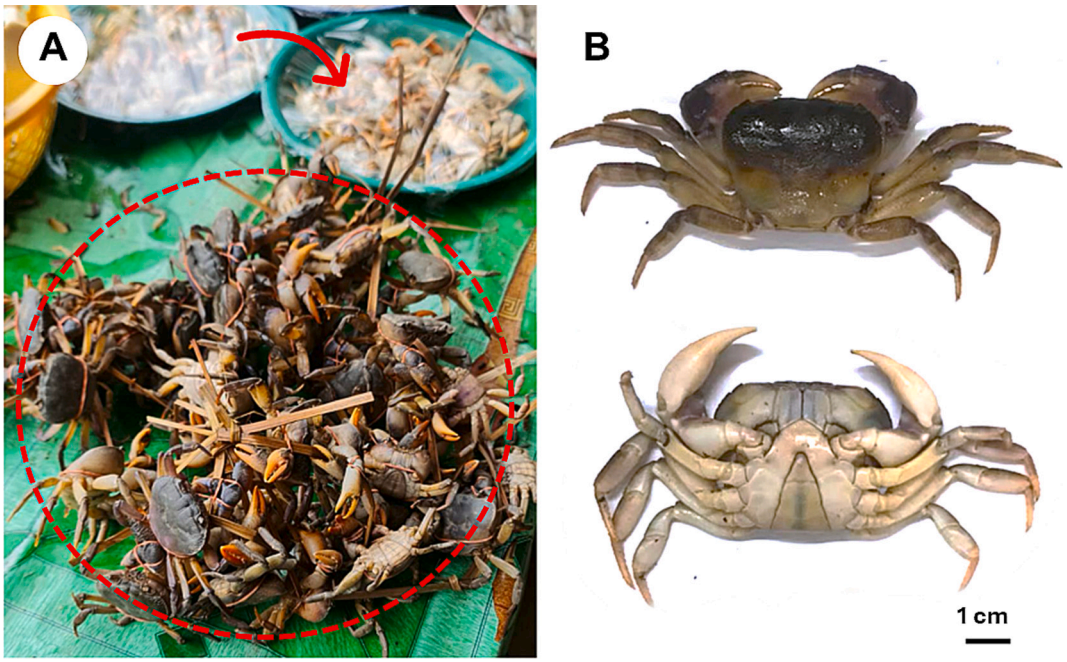

Fig. 1. Freshwater (mountain) crabs, the second intermediate hosts of *Paragonimus* in Lao PDR. A) Freshwater crabs sold in local food market in Luang Prabang Province, Lao PDR; arrow indicates crab meat in a dish and the dashed circle indicates living crabs for sale, B) *Indochinamon* sp. (dorsal and ventral view).

Previously, in Vientiane, central Lao PDR, two species of crabs: *Potamon lipkei* and *Chulathelphusa brandti* were found containing metacercariae of *Paragonimus harinasutai*, *Paragonimus bangkokensis*, *P. heterotremus* and *P. westermani* (Odermatt et al., 2007). Additional species of freshwater crabs have been reported as second intermediate hosts (Odermatt et al., 2007; Yahiro et al., 2008). *Pudaengon arnamicai* collected at Khammouane Province in central Lao PDR was reported as the second intermediate host of *Paragonimus macrorchis* (Sanpool et al., 2015). Sohn et al. (2009) recovered *P. harinasutai* metacercariae in *Indochinamon ou* from Namback District, Luang Prabang Province, Lao PDR.

In this study, we found *P. heterotremus* metacercariae in *Indochinamon* sp. from one out of two localities in Luang Prabang Province. The metacercariae were identified using both morphological characteristics and genetic analysis. Comprehensive phylogenetic analyses were conducted to elucidate the evolutionary relationships among the metacercariae, alongside haplotype network construction to explore genetic diversity and population structure. These findings are discussed with reference to their implications for species differentiation and genetic variation within the population.

2. Materials and methods

2.1. Sample collection and morphological identification

Freshwater (mountain) crabs ($n = 36$) were bought from local food markets that villager collecting from mountain streams at Xiang Ngeun (19°48′23.5"N 102°11′56.9″E) and Pak Ou (20°15′19.8"N 102°21′19.3″E) districts, Luang Prabang Province, northern Lao PDR (Fig. 1A). The crabs were identified morphologically as previously described (Yeo and Ng, 1998, 2007). Muscle tissue was dissected from the legs of four crabs and preserved in 70 % ethyl alcohol for DNA extraction and molecular identification. After collecting the crabs' tissue, the remaining four whole crabs were preserved in 70 % ethyl alcohol and stored at the university as reference vouchers. While the remaining 32 crabs were digested using the pepsin digestion method (Intapan and Maleewong, 2001) and *Paragonimus* metacercariae were collected under a stereomicroscope. These metacercariae were morphologically identified, photographed and measured. The study was approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethics of Animal Experimentation of the National Research Council of Thailand (Reference No. 660201.2.11/799 (122)).

Table 1
PCR conditions for amplifying DNA from freshwater crabs and *Paragonimus heterotremus* metacercariae.

Primer names /genes	PCR programs
Crabs Forward 1471: 5′- CCTGTTTANCAAAAACAT-3′ Reverse 1472: 5′-AGATAGAAACCAACCTGG-3′ (product size 546 base pairs) Mitochondrially encoded 16S RNA (MT-RNR2) (Crandall and Fitzpatrick, 1996)	Initial denaturation at 95 °C for 3 min followed by 30 cycles, 95 °C for 1 min, 42 °C for 1 min, 72 °C for 1.5 min and final incubation at 72 °C for 7 min.
Paragonimus species Forward 3S: 5′- GGTACCGGTGGATCACTCGGCTCGTG-3′ Reverse BD2: 5′-TATGCTTAAATTCAGCGGGT-3′ (product size 432 base pairs) Internal transcribed spacer 2 (ITS2) region (Bowles and McManus, 1993)	Initial denaturation at 94 °C for 5 min followed by 35 cycles, 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30s and final incubation at 72 °C for 7 min.
Forward JB3: 5′- TTTTTTGGGCATCCTGAGGTTTAT-3′ Reverse JB4.5: 5′- TAAAGAAAGAACATAATGAAAATG-3′ (product size 396 base pairs) Mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) (Bowles et al., 1993)	Initial denaturation at 94 °C for 1 min, followed by 10 cycles at 94 °C for 1 min, 40 °C for 1 min, 72 °C for 2 min, 30 cycles of 94 °C for 1 min, 45 °C for 2 min, 72 °C for 2 min, and a postamplification extension for 7 min at 72 °C. similar with Hasegawa et al. (2009) procedure.

2.2. Molecular analyses

Genomic DNA of four individual crabs and 81 *Paragonimus* metacercariae was extracted using the Nucleospin Tissue kit (Macherey-Nagel GmbH & Co., Duren, Germany) according to the manufacturer's instructions. The DNA concentration was measured using a NanoDrop™

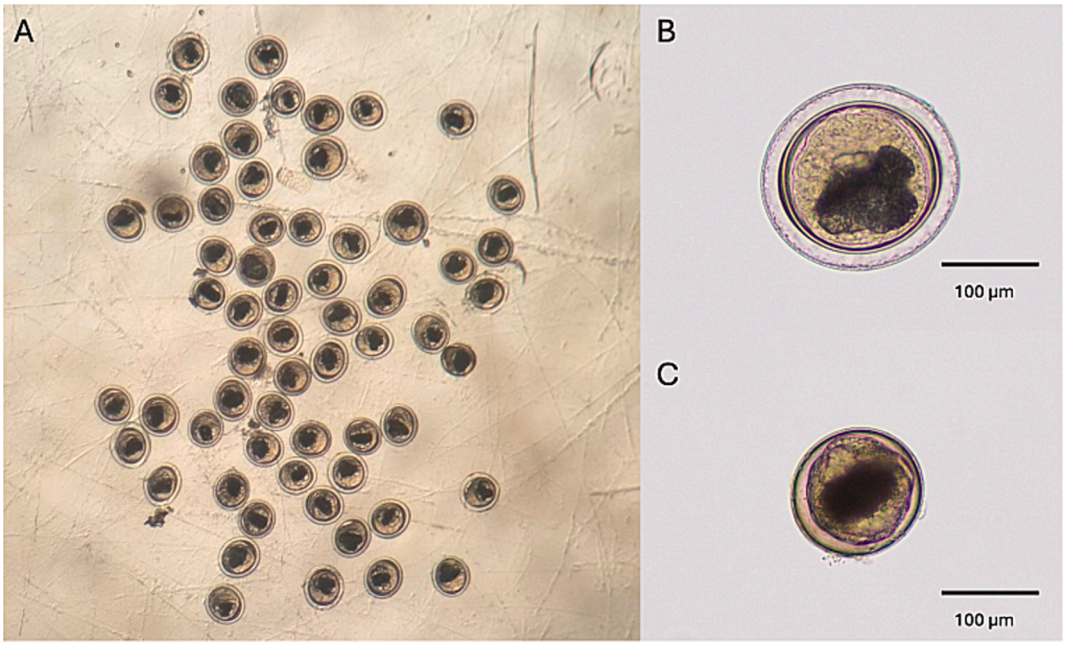

Fig. 2. Two morphological types of *P. heterotremus* metacercariae collected from freshwater crabs in Luang Prabang Province, Lao PDR. Low (A) and high (B and C) magnifications indicate the largest (B) and the smallest (C) sizes, respectively.

One spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and the DNA stored at −20 °C until used. A portion of the mitochondrial 16S rRNA was amplified from each crab sample using primer pair 1471 and 1472 (Crandall and Fitzpatrick, 1996). The PCR products (length 546 bp) were sent to ATGC Co., Ltd. (Thailand) for sequencing in both directions, using PCR primers as the sequencing primers. Sequences were then submitted to the National Center for Biotechnology Information (NCBI) for analysis and comparison using the Basic Local Alignment Search Tool (BLAST), nucleotide BLAST.

Two primer pairs were used to amplify DNA from *Paragonimus* species. For the nuclear ribosomal second internal transcribed spacer (ITS2), primers 3S and BD2 were used (Bowles and McManus, 1993) and for a portion of the mitochondrial *cox1* gene, primers JB3 and JB4.5 were used (Bowles et al., 1993). The PCR amplification conditions for crabs and *Paragonimus* DNA are described in Table 1. PCR was conducted using a GeneAmp® PCR System 9700 (Applied Biosystems, Singapore). The PCR products were sent to ATGC Co., Ltd. (Thailand) for sequencing in both directions, using PCR primers as the sequencing primers. The initial quality check of the sequences was done by comparing the coverage and alignment with previously submitted sequences in NCBI using nucleotide BLAST. Previously submitted sequences of *cox1* and ITS2 from *Paragonimus* species were retrieved from the database and aligned with the new sequences obtained in this study using ClustalW (Thompson et al., 1994).

A maximum likelihood tree was constructed for *Paragonimus* species separately for each gene or region. Four representative sequences of *P. heterotremus* ITS2 from Luang Prabang were used in the ITS2 analysis and aligned with publicly available sequences (alignment length 432 bp when trimmed to the length of the shortest sequence). Of the 81 partial *cox1* gene sequences, 15 were selected for inclusion in the phylogenetic analysis in an alignment of 396 bp. The best-fit substitution model for ITS2 and the *cox1* gene was determined in MEGA11 (Tamura et al., 2021). The Kimura 2-parameter model was the best fit in both cases. An indication of support for each node in the tree was obtained with 1000

Table 2
Morphometric data (µm) of metacercariae of *P. heterotremus* and *P. pseudoheterotremus*.

	P. heterotremus							*P. pseudoheterotremus* (*P. heterotremus* complex) Thailand (Waikagul, 2007)
	India (Singh et al., 2007)	China (Hu, 1998)	Vietnam (Doanh et al., 2015)			Thailand (Doanh et al., 2015)	Laos (This study)	
			Thanh	Yen Bai Group 1	Yen Bai Group 2			
Outer cyst diameter			197–248 × 164–180 (219 × 172)	197–254 × 197–230 (230 × 217)	246–300 × 221–297 (266 × 242)	320–335 × 290–305 (330 × 300)	122.9–178.6 × 127.4–166.2 (157.6 × 145.1)	
Inner cyst diameter	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)	110.5–163.9× 115.0–153.7 (144.6 × 132.1)	180–204 × 168–180 (186 × 176)
The length/ width of the inner cyst diameter			1.2–1.5 (1.3)	1.0–1.2 (1.1)	1.0–1.3 (1.1)			
Thickness 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)		1.6–3.74 (2.5)	
Greatest thickness 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)			

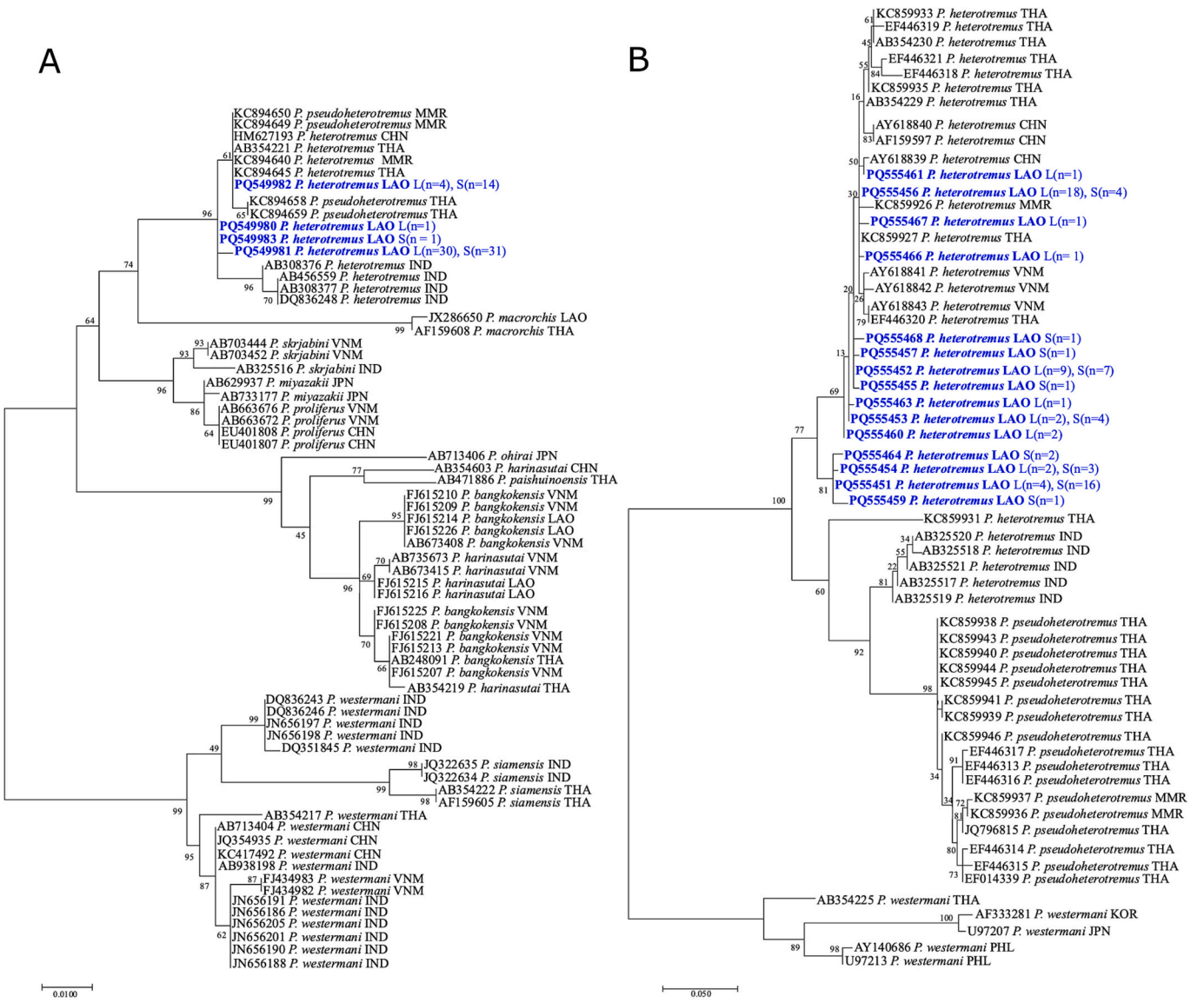

Fig. 3. The maximum likelihood tree reconstructed from ITS2 (A) and *cox1* (B) gene sequences. Bootstrap scores (percentages of 1000 replications) are presented for each node. Samples used to obtain the nucleotide sequences in this study are represented with bold letters. All DNA sequences are shown with GenBank accession no., species name, and country codes (ISO3166-1, alpha-3 code). Small size (S) metacercaria (diameter ≤ 157.66 μm) and large size (L) metacercaria (diameter ≥ 157.66 μm), with (n) representing the number of metacercaria samples with identical nucleotide sequences.

bootstrap replicates.

For the haplotype-network analysis, all *cox1* gene partial nucleotide sequences (396 bp) of *P. heterotremus* obtained in this study were aligned with reference nucleotide sequences from databases using ClustalW in MEGA 11. Haplotype and nucleotide diversity of this alignment were calculated using DnaSP v.6.13.03 (Rozas et al., 2017) and a median-joining network, based on pairwise nucleotide differences between haplotypes, was generated in PopART software version 1.7 (Leigh and Bryant, 2015).

3. Results

3.1. Freshwater crabs as intermediate hosts of Paragonimus

All 36 freshwater crabs were identified by morphology as belonging to the genus *Indochinamon* (Fig. 1B). The 16S rRNA mitochondrial sequences of all four crabs were identical and had 95 % similarity to a sequence from *I. ou* (accession number AB428481) originating from Phongsaly Province, Lao PDR, a province to the immediate north of Luang Prabang. It is unclear whether a similarity of 95 % indicates conspecificity with *I. ou*, so we have referred to the crabs we obtained as *Indochinamon ou*. Levels of similarity with the few other *Indochinamon* 16S sequences in GenBank ranged from 91 % to 93 %. A representative crab sequence has been deposited in the GenBank database with accession number PQ577692.

3.2. P. heterotremus metacercarial morphology

A total of 81 *Paragonimus* metacercariae were found among 13 out of 32 crabs (Supplementary Table 1); the infection rate was 40.6 %. The metacercariae were oval to spherical, had a clear and thick outer cyst wall and varied in size (compare Fig. 2B and C). The overall dimensions of the largest metacercariae were average 178.56 × 165.23 μm (Fig. 2B) and of the smallest metacercariae were 127.43 × 122.99 μm (Fig. 2C). The dimensions of the outer cyst were 122.9–178.6 × 127.4–166.2 μm (average 157.6 × 145.1 μm) ($n = 81$). The inner cyst was 110.5–163.9 × 115.0–153.7 μm (average 144.6 × 132.1 μm). The thickness of the inner cyst wall was 1.6–3.74 μm (average 2.5 μm) (Fig. 2A, Table 2). All

Table 3
Haplotypes of *P. heterotremus* from Luang Prabang, Lao PDR, and *P. heterotremus* and *P. pseudoheterotremus* from other countries.

Group	Haplotype	Country	Accession number
A	1	Thailand	AB354229
	6	Thailand	KC859933, AB354230
	8	Thailand	EF446320
		Vietnam	AY618843
	9	Vietnam	AY618842
	10	Vietnam	AY618841
	11	China	AY618840, AF159597
	12	China	AY618839
	13	Thailand	KC859935
	14	Thailand	KC859931
	15	Thailand	KC859927
		Lao PDR	PQ555456 ($n = 22$)
	16	Myanmar	KC859926
	17	Thailand	EF446321
	18	Thailand	EF446319
	19	Thailand	EF446318
	32	Lao PDR	PQ555452 ($n = 16$)
	33	Lao PDR	PQ555453 ($n = 6$)
	35	Lao PDR	PQ555455 ($n = 1$)
	36	Lao PDR	PQ555457 ($n = 1$)
	38	Lao PDR	PQ555460 ($n = 2$)
	39	Lao PDR	PQ555461 ($n = 1$)
	40	Lao PDR	PQ555463 ($n = 1$)
	42	Lao PDR	PQ555466 ($n = 1$)
	43	Lao PDR	PQ555467 ($n = 1$)
	44	Lao PDR	PQ555468 ($n = 1$)
B	31	Lao PDR	PQ555451 ($n = 20$)
	34	Lao PDR	PQ555454 ($n = 5$)
	37	Lao PDR	PQ555459 ($n = 1$)
	41	Lao PDR	PQ555464 ($n = 2$)
C	20	Thailand	KC859938, KC859940, KC859943- KC859945
	21	Thailand	JQ796815
	22	Thailand	KC859946
	23	Thailand	KC859939, KC859941
	24	Myanmar	KC859937
	25	Myanmar	KC859936
	26	Thailand	EF446317
	27	Thailand	EF446313, EF446316
	28	Thailand	EF446315
	29	Thailand	EF446314
	30	Thailand	EF014339
D	2	India	AB325521
	3	India	AB325520
	4	India	AB325518
	5	India	AB325517
	7	India	AB325519

metacercarial morphology matched closely with that of *P. heterotremus*.

3.3. Molecular phylogenetic analyses

All 81 *P. heterotremus* metacercariae were sequenced for both ITS2 and *cox1* and the data used for phylogenetic analysis and construction of a haplotype network. Sequences from both regions had a high similarity to published *P. heterotremus* data: 99–100 % for ITS2 and 95–100 % for *cox1*. The number of variable sites in our ITS2 and *cox1* sequences were 12 out of 432 (2.78 %) and 46 out of 396 (11.62 %) nucleotide positions, respectively (Supplementary Table 2). A representative nucleotide sequence of each sequence variant has been deposited in GenBank with accession numbers ITS2 ($n = 4$): PQ549980-PQ549983, and *cox1* ($n = 15$ haplotypes): PQ555451-PQ555457, PQ555459-PQ555461, PQ555463-PQ555464, and PQ555466-PQ555468.

The maximum likelihood (ML) tree constructed using ITS2 sequences included 64 sequences retrieved from NCBI GenBank and four *P. heterotremus* sequences obtained from Laung Prabang Province, Lao PDR (this study). All *P. heterotremus* sequences from this study were clustered into one group with high bootstrap support (96 %) containing *P. heterotremus* from China, Myanmar and India and *P. pseudoheterotremus* from Myanmar and Thailand with high bootstrap support (96 %) (Fig. 3A). The phylogenetic tree of partial *cox1* sequences placed sequences from Lao PDR into two groups. The first group also included almost all *P. heterotremus* sequences from Southeast Asian countries, whereas the second group contained only some (28) sequences from Luang Prabang Province (Fig. 3B). The nucleotide sequences from large and small metacercariae (Fig. 2B-2C) were intermingled in both ITS2 and *cox1* trees.

The haplotype network based on all available *cox1* sequences including our 15 sequences (Table 3) and 39 sequences of Asian stain from the GenBank database identified 44 haplotypes, The analyses of the median-joining haplotype network demonstrated that the *P. heterotremus* were genetically separated into four groups (A, B, C, and D; haplotype diversity (Hd) = 0.915 ± 0.002) and nucleotide diversity (π) = 0.0416 (Fig. 4). Group A is the biggest, containing *P. heterotremus* from China, Lao PDR, Myanmar, Thailand and Vietnam (Hap_1, 6, 8–19, 32–33, 35–36, 38–40, 42–44; Hd = 0.838 ± 0.001). The second group (B) consisted only of sequences from Lao PDR obtained during this study (Hap_31, 34, 37 and 41; Hd = 0.439 ± 0.01). The third group (C) included Hap_20–30 (Hd = 0.912 ± 0.003) from Thailand and Myanmar. The final group (D) Hap_2–5 and 7 (Hd = 1 ± 0.016), contained the sequences from India (Fig. 4; Table 3). The geographical distribution of *P. heterotremus* is illustrated in Fig. 5, providing a detailed representation of its spatial occurrence across the studied regions.

4. Discussion

Paragonimus heterotremus is a causative agent of human paragonimiasis in Asia (Blair, 2024). Morphological variation of metacercariae and genetic variation across the geographical range of this lung fluke led to the proposal to recognize a *P. heterotremus* species complex (Doanh et al., 2015). In addition, *P. heterotremus* experimentally raised in rats using metacercariae from crabs (*Potamiscus tannanti*) caught in Yen Bai Province, Vietnam, showed behavioral characteristics of worms, genetic data and host pathology that differed from those reported from this species elsewhere (Voronova et al., 2020). Thus it is likely that *P. heterotremus* infecting humans in different outbreak areas may present different pathologies as well as symptoms and signs, resulting in diverse public health problems. Here, we found metacercarial polymorphism and substantial genetic variation in *P. heterotremus* metacercariae from a freshwater crab species (*Indochinamon* sp.) this is the first molecular data for *P. heterotremus* from Luang Prabang Province, Lao PDR. This study significantly enhances the understanding of *P. heterotremus* distribution and genetic diversity in this area and illustrates the hazard this foodborne parasite poses for human populations.

The identification of *P. heterotremus* metacercariae in freshwater crabs collected from Xiang Ngeun emphasizes the role of these crabs as key intermediate hosts, supporting earlier findings in other regions of Lao PDR (Odermatt et al., 2007; Yahiro et al., 2008; Sohn et al., 2009). The morphological and molecular analyses conducted here, specifically using the ribosomal ITS2 and partial mitochondrial *cox1* sequences, provided robust confirmation of *P. heterotremus* identity and enabled comprehensive phylogenetic and haplotype network analysis. Phylogenetic results indicated that *P. heterotremus* sequences from Lao PDR closely clustered with samples from neighboring countries, including China, Myanmar, and Thailand. In the ITS2 phylogenetic analysis, *Paragonimus* metacercariae from this study was located together with *P. heterotremus* cluster from Myanmar, China, Thailand and to make a group separate from the Indian *P. heterotremus* population. Similarly, in the *cox1* tree, sequences of *P. heterotremus* from Lao PDR were clustered with others from Myanmar, China, Thailand and Vietnam and clearly separate from the Indian *P. heterotremus* population. One outlier from Thailand (KC859331: Hap_14 in Fig. 4) complicated the picture a little. These phylogenetic relationships correlated with previous findings (Doanh et al., 2015; Voronova et al., 2020) and confirmed the genetic diversity of the *P. heterotremus* complex. This clustering of sequences

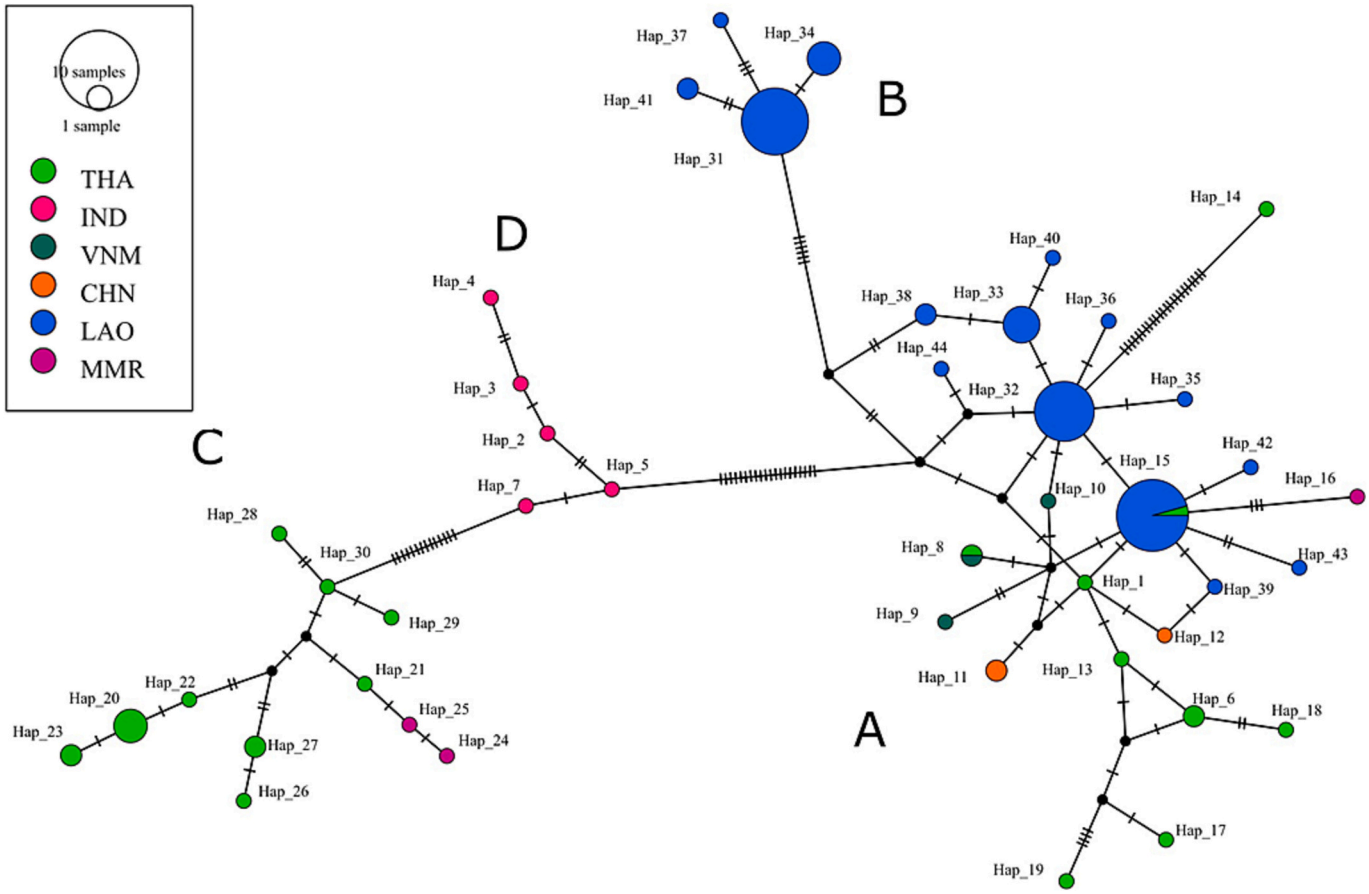

Fig. 4. The median-joining haplotype network of *P. heterotremus* based on *cox1* gene sequences. Note that sequences from *P. pseudoheterotremus* are included in the network. The colour of each circle represents the country of origin, with blue circles indicating sequences from this study. The size of each circle is proportional to the number of individual sequences it represents. Slash marks across lines connecting haplotypes indicate the number of nucleotide changes inferred as occurring between those haplotypes. Small black circles indicate missing haplotypes. See Table 3 for haplotype numbers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from several countries in Group A of Fig. 4 suggests potential genetic connectivity among these Asian populations (Nawa et al., 2014; Sanpool et al., 2015).

The differences in size of metacercariae that we noted were not associated with differences in the ribosomal ITS2 or partial mitochondrial *cox1* sequences: sequences from each type were intermingled in the phylogenetic tree (Fig. 3). The ITS2 sequences exhibited minor variations while *cox1* sequences were much more variable among the metacercariae sampled. As suggested by Blair et al. (1999), the ITS2 sequence is a good marker for species-level identification and the *cox1* sequence is a good marker to distinguish populations within a *Paragonimus* species. The *cox1* sequences, in particular, revealed a clear distinction between *P. heterotremus* and *P. pseudoheterotremus* (Sanpool et al., 2013). The considerable variation in *cox1* sequences found in Luang Prabang Province, Lao PDR indicates that further work is required to demonstrate the full diversity of *P. heterotremus* complex and related forms in Asia.

Interestingly, a subset of the Lao PDR sequences formed a unique cluster, indicating possible regional genetic differentiation, which may reflect localized selection pressures (such as adaptation to a local crab species), host-specific interactions, or restricted gene flow in this region (Tantrawatpan et al., 2021). Whatever the cause, there is a rich genetic diversity in *P. heterotremus* in Lao PDR that could influence transmission patterns and disease dynamics in the region.

These findings highlight the risk of lung fluke disease in this region linked to the consumption of freshwater crabs infected with *P. heterotremus* and point out the importance of understanding the genetic diversity and phylogenetic relationships of the *P. heterotremus* complex to inform targeted control strategies, particularly in endemic areas. As was shown by Voronova et al. (2020) for *P. heterotremus* in Vietnam, experimental completion of the life cycle can greatly increase our understanding of disease risk posed by a local population of this lung fluke. One limitation of our study is that we did not raise and characterize adult worms: that is a task for future researchers.

Enhanced genetic knowledge of *P. heterotremus* populations could aid in designing more effective surveillance systems and in identifying specific genetic markers associated with regional transmission risk. Further research is warranted to explore the ecological and evolutionary drivers of *P. heterotremus* genetic diversity across different geographic and environmental settings, as well as their implications for paragonimiasis control efforts in Asian countries.

5. Conclusions

These findings include the first report of the freshwater crab *Indochinamon* sp. as the intermediate host of *P. heterotremus* in Luang Prabang Province, northern Lao PDR and sheds light on the distribution and genetic diversity of this *Paragonimus* species. Identification of the *P. heterotremus* complex in crabs from this area highlights the key role of crabs in paragonimiasis transmission. Phylogenetic and haplotype analyses confirmed the genetic diversity of the *P. heterotremus* complex, linking Lao PDR populations with those in neighboring countries and revealing unique regional clusters. Understanding this genetic diversity is essential for enhancing surveillance and targeted control efforts,

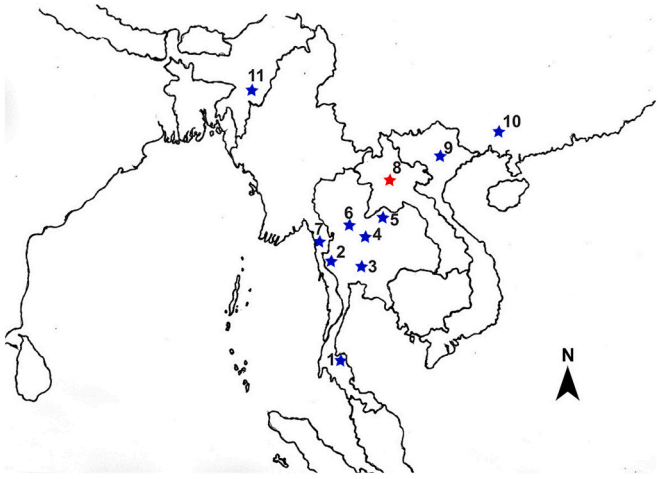

Fig. 5. Geographical distribution of *Paragonimus heterotremus* and *Paragonimus pseudoheterotremus* in Asia. Locations include *P. pseudoheterotremus*: (1) Surat Thani, and (2) Kanchanaburi (Thailand),
Mix *P. heterotremus* and *P. pseudoheterotremus*: (7) Myawaddy (Myanmar), *P. heterotremus*: (3) Saraburi, (4) Phetchabun, (5) Loei, (6) Phitsanulok (Thailand), (8) Luang Prabang (Lao PDR; the study site), (9) Vietnam, (10) Guangxi Zhuang (China), and (11) Manipur (India). Blue stars indicate previously reported locations of *P. heterotremus*, while red stars represent findings from the present study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

especially in endemic areas. Further research on ecological and evolutionary factors influencing diversity of the *P. heterotremus* complex is crucial for improved paragonimiasis control management in eastern Asia.

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CRediT authorship contribution statement

Alongkorn Nonthapa: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Rutchanee Rodpai:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Tongjit Thanchomnang:** Writing – review & editing, Methodology, Investigation, Data curation. **Patcharaporn Boonroumkaew:** Writing – review & editing, Investigation. **Lakkhana Sadaow:** Writing – review & editing, Investigation. **David Blair:** Writing – review & editing, Investigation, Formal analysis. **Pewpan M. Intapan:** Writing – review & editing, Methodology, Investigation. **Wanchai Maleewong:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Virasack Banouvong:** Writing – review & editing, Investigation. **Sakhone Laymanivong:** Writing – review & editing, Investigation. **Oranuch Sanpool:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2025.105718.

Data availability

The authors confirm that the data supporting the finding of this study are available within the article and their supplementary material. The sequences have been deposited in GenBank with accession numbers for ITS2: PQ549980-PQ549983, and for *cox1*: PQ555451-PQ555457, PQ555459-PQ555461, PQ555463-PQ555464, and PQ555466-PQ555468. Meanwhile, the datasets used and analyzed in this study are available at the corresponding author.

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