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Genetic diversity and population structure of the helminths intermediate host *Anentome helena* (von dem Busch, 1847) in northern Thailand

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ABSTRACT

Objective: To investigate the genetic diversity and population structure of the helminth intermediate host, *Anentome (A.) helena*, populations from northern Thailand and reveal the effect of parasitic infection on snail host populations.

Methods: *A. helena* was collected throughout river drainages from northern Thailand and investigated for larval trematode infection. Seventy-three *A. helena* specimens were used to examine and reconstruct the molecular phylogenetics (neighbour-joining, maximum likelihood, and Bayesian inference methods), genetic diversity, and population structure based on the concatenated datasets of COI and *16S* rRNA (1 178 bp).

Results: The infection of zoogonid cercariae, heterophyid metacercariae, and echinostome metacercariae in the snail was found from 2 localities, including Mae Sa and Mae Pu Kha populations, with a total prevalence of 6.7% and 16.3%, respectively. The phylogenetic tree and haplotype network revealed three main clades and three haplogroups for each region (COI and *16S* rRNA). High levels of genetic diversity in terms of haplotype diversity were found in parasitic infected populations.

Conclusions: This is the first time that *Echinostoma mekongi* has been discovered from *A. helena* in northern Thailand. The results also showed that parasitic infection can affect the population genetic structure of the snail intermediate host, *A. helena*. Moreover, habitat and historical demographic changes could be other important factors that can affect the degree of population genetic structure in *A. helena*. Understanding the population structure of intermediate host species can inform parasite transmission dynamics and is useful for the creation of effective public health management strategies.

KEYWORDS: Cercariae; Metacercariae; Phylogeny; Parasite; Host; Freshwater snail

1. Introduction

Freshwater snails receive extensive attention as they are the intermediate hosts of some trematodes that cause diseases in animals and humans and can adversely affect both agriculture and the economy[1]. Many freshwater snails are medically important, including *Biomphalaria* spp., *Radix* spp., *Filopaludina* spp., and *Anentome* spp.[2-5]. Most of the diseases caused by snail-borne trematodes are prevalent in the tropic and subtropic regions of the world[6]. Hence, the parasites distribution is greatly dependent on the intermediate snail host's distribution[7]. The nature and dynamics of host-parasite relationships has attracted much scientific attention[8]. The case study in *Biomphalaria* spp. showed that *Schistosoma mansoni* caused of high levels of genetic diversity in *Biomphalaria choanomphala* populations[8-10] and reduces snail

Significance

This study aims to investigate the genetic diversity and population structure of the helminth's intermediate snail host, *Anentome helena*. The findings showed a deeper understanding of larval trematodes infection in *Anentome helena* populations with high genetic diversity, and population structure. These efforts could be used to determine the direction, transmission and development of the parasite management system and may be useful for public health implications.

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fitness[11]. However, there is no report about the effect of parasites in *Anentome* in Thailand. *Anentome (A.) helena* is one of the most common freshwater snails in Thailand, especially northern part[12]. It has been reported to be infected with some larval trematodes that are important parasitic trematodes of mammals and humans, including Heterophyidae, *Echinostoma revolutum*, and *Echinostoma mekongi*. These parasites can lead to diseases like heterophyiasis and echinostomiasis[5,7,13,14].

A deeper understanding of the population structure, genetic diversity of freshwater snail hosts is crucial because the host plays a major role as a vector in the transmission of parasites[8,15,16]. Likewise, the mitochondrial DNA has been shown to be a valuable tool to study the genetic diversity of freshwater snails, especially, mitochondrial cytochrome oxidase subunit I (COI) and *16S* ribosomal RNA (*16S* rRNA)[8,17–21]. So, this study aims to investigate the population structure and genetic diversity of *A. helena* from northern Thailand to better understand factors that may contribute to parasite transmission.

2. Materials and methods

2.1. Ethics

All animal experiments were done according to the guidelines approved by the Institute of Animals for Scientific Purpose Development (IAD), National Research Council of Thailand (permit number U1-07724-2561, issued to Nithinan Chomchoei on 22 October 2023 and U1-03304-2559, issued to Nattawadee Nantarat on 19 October 2023). This study complied with all the relevant national regulations and institutional policies for the humane care and use of animals.

2.2. Taxon sampling and specimen identification

A. helena samples were collected throughout northern Thailand (Figure 1, Table 1, and Supplementary Table 1)[22,23]. All specimens were identified based on shell morphology and compared with original description and Brandt[12].

2.3. Parasitic infections

The parasitic infections in *A. helena* were examined by the crushing method under an Olympus SZ40 stereomicroscope[24]. Larval trematodes were covered with a cover slip, observed, and photographed with a microscope camera (Optikam Pro 3LT-4083.11LT with Olympus CX31). The specimens were fixed with 4% formalin, stained with haematoxylin, dehydrated using an ethanol gradient, cleared with xylene, and mounted in Permount. The cercariae and metacercariae were then classified according to morphological characteristics described by Frandsen & Christensen[25], Kanev *et al*.[26] and Schell[27], and using the molecular method. The prevalence of the parasite species was recorded.

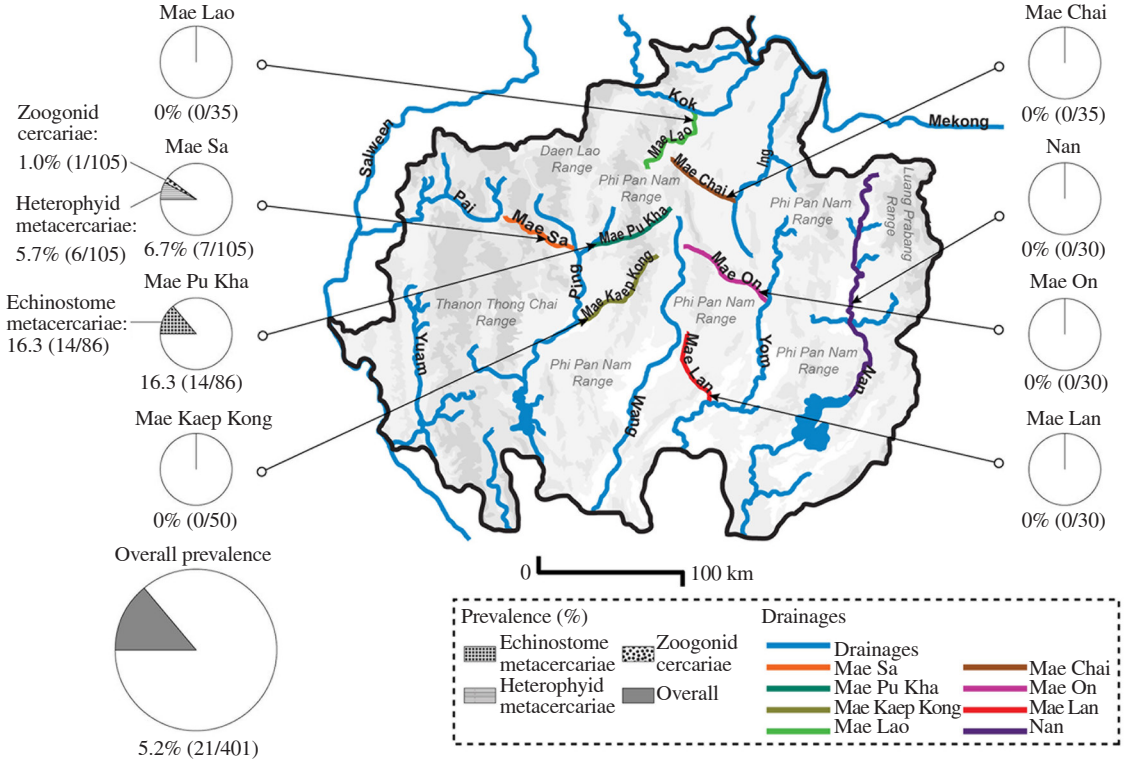

Figure 1. Map of sampling localities of *Anentome helena* populations in northern Thailand. The map shows the sampling localities with different shades of colors based on river drainages. The pie charts show the prevalence of larval trematode infections in *Anentome helena* of each population.

2.4. Molecular analyses for larval trematodes

2 Individuals of each types of cercariae or metacercariae were used to extract genomic DNA using Chelex® method[28]. PCR was performed in reaction volumes of 20 μL containing 0.3 μL of *Taq* DNA polymerase, 0.7 μL of 50 mM magnesium chloride, 1 μL of each primer, 2 μL of 10X ViBuffer A, 0.4 μL of deoxynucleoside triphosphates (dNTPs) and 1 μL of the DNA template and deionized water up to 20 μL. Thermal cycling conditions for COI (primer: JB3 and JB4.5[28]) were as follows: 95 ℃ for 3 min, followed by 40 cycles of 95 ℃ for 1 min, 50 ℃ for 1 min, 72 ℃ for 1 min and a final extension step of 72 ℃ for 7 min. The amplified products were checked with 1% (w/v) agarose gel electrophoresis. PCR products were purified and sequenced using BigDye® Terminator v3.1 cycle sequencing kit chemistry and 1st BASE DNA Sequencing Services (Applied Biosystems, Selangor, Malaysia).

The sequences were aligned and compareed using BLASTn algorithm to verify the correct group of organisms and compare the closely related species from the obtained sequences[29]. All new sequences were deposited in GenBank (MZ822059- MZ822064).

2.5. Molecular analyses for A. helena

Genomic DNA of the foot tissues of *A. helena* were extracted using Chelex® method[28]. The mitochondria *16S* rRNA (*16S*ar and *16S*br[30]) and COI genes (LCOI490 and LCOI490[31]) were amplified. PCR was performed in reaction volumes of 20 μL containing 10 μL of 2× Illustra Hot Start Master Mix (GE Healthcare), 1 μL of each primer (10 mM), approximately 1 ng of DNA template and deionized water up to 20 μL. Thermal cycling conditions were as follows: 94 ℃ for 2 min, followed by 36 cycles of 94 ℃ for 30 sec, 45 ℃ for 1 min, 72 ℃ for 1 min and a final extension step of 72 ℃ for 5 min for *16S* rRNA, and 94 ℃ for 2 min, followed by 36 cycles of 94 ℃ for 30 sec, 40 ℃-42 ℃ for 2 min, 72 ℃ for 2 min and a final extension step of 72 ℃ for 5 min for COI. The amplified products were checked with 1% (w/v) agarose gel electrophoresis. PCR products were purified and sequenced using BigDye® Terminator v3.1 cycle sequencing kit chemistry and 1st BASE DNA Sequencing Services (Applied Biosystems, Selangor, Malaysia).

DNA sequences were edited and aligned with ClustalW[32] as implemented in MEGA v 7[33]. The alignment was refined manually. Phylogenetic analyses of the *A. helena* populations were re-constructed by using neighbour-joining (NJ) with MEGA v 7[33]; 1 000 bootstrap replicates[34], maximum likelihood (ML)[35] with PhyML v 3[36]; 1 000 bootstrap replicates, and Bayesian inference (BI) with MrBayes v 3.1.2[37]; 4 chains for 10 000 000 generations with the heating parameter set at 0.05, tree sampling every 100 generations and burn-in set at 25%. HKY+G were used for all datasets. Tree topologies were drawn with FigTree v 1.4.3[38].

2.6. Haplotype network analyses

COI and *16S* rRNA haplotype networks of the *A. helena* populations from northern Thailand were generated and visualized with the median joining method as implemented in PopART v1.7[39]. The river drainage systems where the detected haplotypes of *A. helena* were used as traits and employed in building the haplotype networks.

2.7. Population genetics analyses

Population genetics analyses were designed to examine the genetic diversity and population

Table 1. List of localities of *Anentome helena* and overview of larval trematode infections in *Anentome helena* from northern Thailand.

No.	Localities	Populations	Habitat types	Overall			Echinostome metacercaria			Heterophyid metacercaria			Zoogonid cercariae		
				No. of snails examined	No. of snails infected	Prevalence (%)	No. of snails infected	Prevalence (%)		No. of snails infected	Prevalence (%)		No. of snails infected	Prevalence (%)	
1	Mae Sa, Mae Rim, Chiang Mai	Mae Sa	Stream	105	7	6.7	0	-		6	5.7		1	1.0	
2	Mae Pu Kha, San Kamphaeng, Chiang Mai	Mae Pu Kha	Temporary pond	86	14	16.3	14	16.3		0	-		0	-	
3	Rim Ping, Meuang, Lamphun	Mae Kaep Kong	Stream	50	0	-	0	-		0	-		0	-	
4	San Klang, Phan, Chiang Rai	Mae Lao	Irrigation canal	35	0	-	0	-		0	-		0	-	
5	Si Thoi, Mae Chai, Phayao	Mae Chai	Irrigation canal	35	0	-	0	-		0	-		0	-	
6	Ban Pong, Ngao, Lampang	Mae On	Stream	30	0	-	0	-		0	-		0	-	
7	Ban Pin, Long, Phrae	Mae Lan	Stream	30	0	-	0	-		0	-		0	-	
8	Klang Wiang, Wiang Sa, Nan	Nan	River	30	0	-	0	-		0	-		0	-	
Total				401	21	5.2	14	3.5		6	1.5		1	0.2	

structure of *A. helena* populations from northern Thailand. To estimate the genetic diversity of *A. helena* populations, the number of segregating sites (*s*), the number of haplotypes (*H*), haplotype diversity (*h*), and nucleotide diversity (π) were calculated with DNASP v6[40]. A one-sample *t* test was applied with IBM SPSS Statistics v20[41] software to analyse the differentiation of the haplotype diversity and nucleotide diversity between *A. helena* populations. For measuring the genetic differentiation among *A. helena* populations, F_{ST} values were calculated with a significance test of *F*-statistics using 10000 random permutations and performed in Arlequin v3.5.2.2[42].

3. Results

3.1. Snail sampling and larval trematode infections

A total of 401 *A. helena* samples were collected throughout northern Thailand. The larval trematode infections in *A. helena* were examined from all 8 populations in northern Thailand (Figure 2A). The infected populations were recorded in only 2 populations from the Mae Sa (% total prevalence=6.7%) and Mae Pu Kha (% total prevalence=16.3%) drainages, with an overall prevalence of 5.2% (21/401) (Table 1 and Figure 1). According to type of the parasite, there were three types of larval trematodes infecting *A. helena*, including (1) zoogonid cercariae (Figure 2B; from Mae Sa, % prevalence=1.0%), (2) heterophyid metacercariae (Figure 2C-2D; from Mae Sa; % prevalence=5.7%), and (3) echinostome metacercariae with 37 collar spines (Figure 2E-2F; from Mae Pu Kha; % prevalence=16.3%).

The sequence comparison (COI) from BLASTn revealed zoogonid cercariae similarities with trematodes in the Zoogonidae family: *Zoogonus lasius* at 88.64%-90.15%; heterophyid metacercariae similarities with trematodes in the Heterophyidae family: *Pygidiopsis macrostomum* at 84.57%-85.06%; and echinostome metacercariae similarities with trematodes in the Echinostomatidae family: *Echinostoma mekongi* at 96.61%-97.31%.

3.2. Molecular phylogenetic and haplotype network analyses of A. helena

The COI (657 bp), *16S* rRNA (521 bp), and concatenated dataset of both DNA regions (1178 bp) were used to re-construct the phylogenetic tree. The phylogenetic relationships of *A. helena* samples are shown in Figure 3A with high statistical supports. *A. helena* was monophyletic, forming a moderately high supported clade which was divided into three well-supported main clades (Clade A-C).

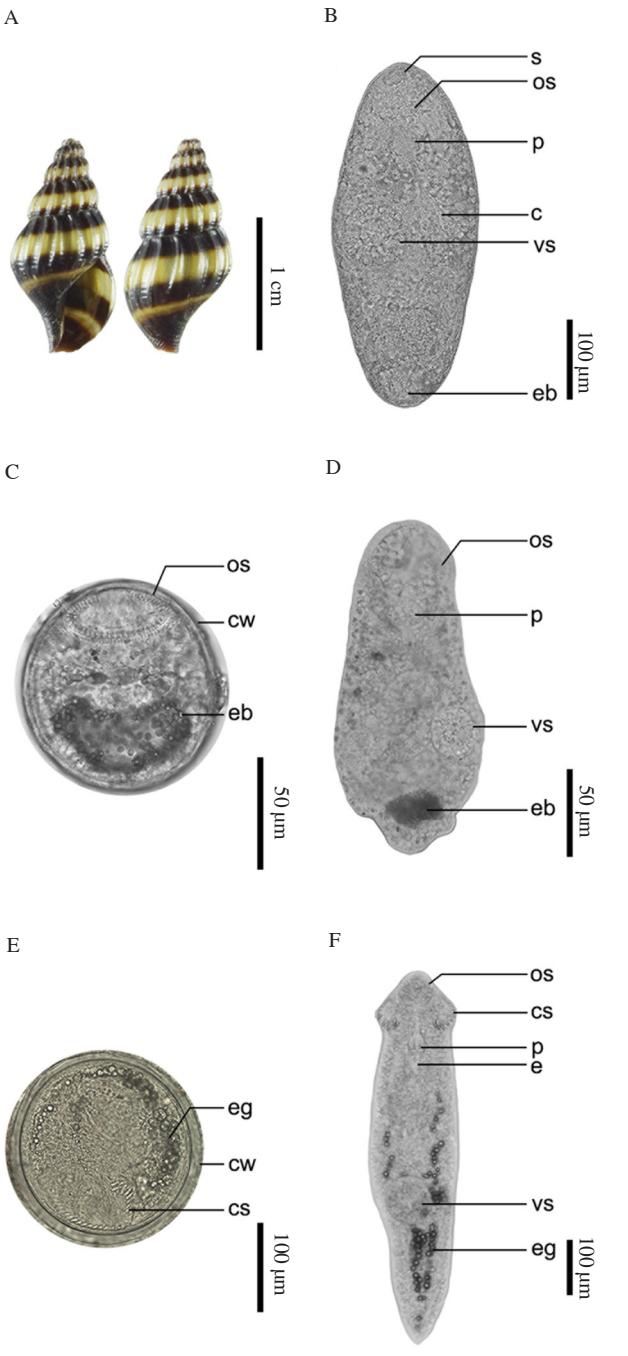

Figure 2. (A) Shell photo of *Anentome helena* specimen voucher CMUZ A172040, and light microscopic photographs of trematode cercariae and metacercariae infecting *Anentome helena* in northern Thailand: (B) zoogonid cercariae; (C) heterophyid metacercarial cyst; (D) excysted heterophyid metacercaria; (E) echinostome metacercarial cyst; (F) excysted echinostome metacercaria. (Abbreviations: c, caecum; cs, collar spine; cw, cyst wall; e, esophagus; eb, excretory bladder; eg, excretory granule; os, oral sucker; p, pharynx; s, stylet and vs, ventral sucker.)

A median joining haplotype network constructed from COI and *16S* rRNA sequences also demonstrated that three haplogroups of each region could be classified (haplogroups A, B, and C for the COI

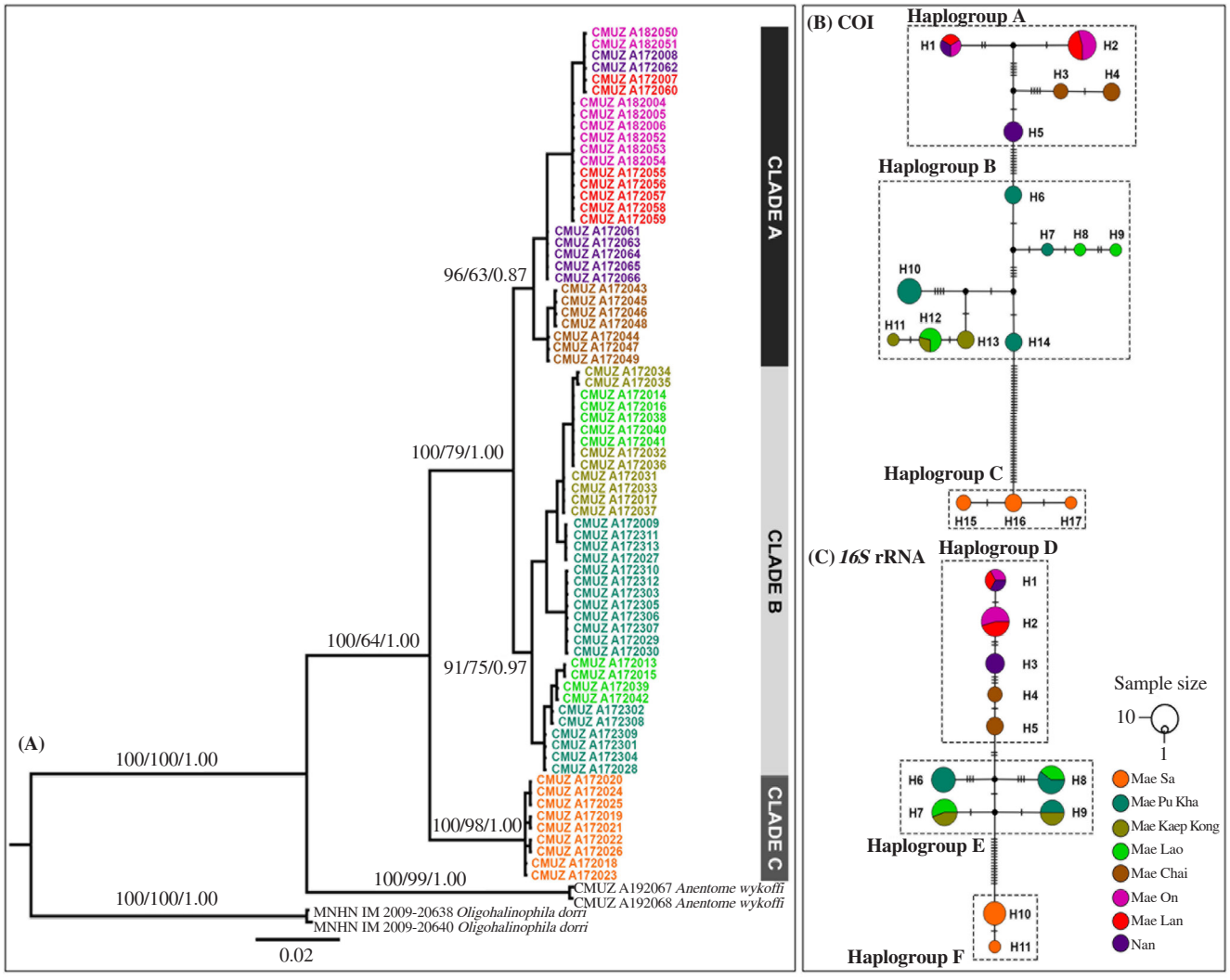

Figure 3. (A) The BI phylogenetic tree of the *Anentome helena* in northern Thailand and related species obtained from DNA sequences analysis of the concatenated datasets of COI and *16S* rRNA (1 178 bp). Numbers at the branches are NJ bootstrap (BP)/ML bootstrap (BP)/BI posterior probability (PP) values, respectively. Scale bar refers to a phylogenetic distance of nucleotide substitutions per site. Median joining haplotype networks for *Anentome helena* based on mitochondrial COI (B), and *16S* rRNA (C). 8 Populations based on river drainages were represented in different shades of colors. Hatch marks along the branches indicate the numbers of mutational steps and black dots represent hypothetical haplotypes (H = Haplotype).

network and haplogroups D, E, and F for the *16S* rRNA network) (Figure 3B and 3C). Four populations from the Mae Chai, Mae On, and Mae Lan and Nan drainages contained the haplotypes from haplogroups A and D, while three populations from the Mae Pu Kha, Mae Kaep Kong, and Mae Lao drainages contained the haplotypes from haplogroups B and E. However, we found that haplogroups C and F are only from the Mae Sa drainage population. In the COI network, there were 17 haplotypes (5, 9, and 3 haplotypes from haplogroups A, B, and C, respectively), while the *16S* rRNA network included 11 haplotypes (5, 4, and 2 haplotypes from haplogroups D, E, and F, respectively). Shared haplotypes were found in haplotypes (H=haplotype) H1, H2, and H12 (from haplogroups A and B; COI network) and H1, H2, H7, H8, and H9 (from haplogroups D and E; *16S* network) (Figure 3B and 3C).

3.3. Genetic diversity of *A. helena* populations

The COI gene and *16S* rRNA fragments from all 73 samples were amplified and sequenced. The number of segregating sites (s), the number of haplotypes (H), haplotype diversity (h), and nucleotide diversity (π) of all 8 populations are reported in Table 2.

The total number of segregating sites from COI and *16S* rRNA were 62 and 27, respectively, while the total number of haplotypes from COI and *16S* rRNA were 17 and 11, respectively. High haplotype diversity and low nucleotide diversity were found in both COI (h = 0.935 ± 0.011; π = 0.027 ± 0.003) and *16S* rRNA (h = 0.906 ± 0.010; π = 0.015 ± 0.001). The haplotype diversity of *A. helena* populations in this study ranged from 0.429 ± 0.169 to 0.732 ± 0.069 (COI) and 0.389 ± 0.164 to 0.680 ± 0.056 (*16S*), while nucleotide diversity ranged from 0.001 ± 0.000 to 0.007 ± 0.001 (COI) and 0.001 ± 0.000 to 0.007 ± 0.001 (*16S*).

Table 2. Diversity indices of COI and *16S* rRNA sequences in the *Anentome helena* populations from 8 different localities in northern Thailand (grey color indicate the parasitic infected populations).

Populations	Number of sequences	COI				*16S* rRNA			
		s	*H*	*h* ± SD	π ± SD	*s*	*H*	*h* ± SD	π ± SD
Mae Sa	9	2	3	0.722±0.097*	0.001±0.000*	1	2	0.389±0.164*	0.001±0.000*
Mae Pu Kha	18	11	4	0.732±0.069*	0.007±0.001*	8	3	0.680±0.056*	0.007±0.001*
Mae Kaep Kong	8	2	3	0.714±0.123*	0.002±0.000*	2	2	0.571±0.094*	0.002±0.000*
Mae Lao	9	9	3	0.667±0.132*	0.007±0.001*	5	2	0.556±0.090*	0.005±0.001*
Mae Chai	7	1	2	0.571±0.119*	0.001±0.000*	1	2	0.571±0.119*	0.001±0.000*
Mae On	8	3	2	0.429±0.169*	0.002±0.001*	1	2	0.429±0.169*	0.001±0.000*
Mae Lan	7	3	2	0.476±0.171*	0.002±0.001*	1	2	0.476±0.171*	0.001±0.000*
Nan	7	8	2	0.476±0.171*	0.006±0.002*	3	2	0.476±0.171*	0.003±0.001*
Total	73	62	17	0.935±0.011	0.027±0.003	27	11	0.906±0.010	0.015±0.001

*Significantly different at $P<0.05$ (one-sample *t*-test); *s* = the number of segregating sites; *H* = the number of haplotypes; h ± SD = haplotype diversity ± standard deviation; π ± SD = nucleotide diversity ± standard deviation. The table retains values to three decimal places, and if the value equals 0, it will be displayed as 0.000.

3.4. Population genetic structure of A. helena populations

Population pairwise F_{ST} values among 8 populations in northern Thailand based on COI and *16S* rRNA sequences revealed that the pairwise F_{ST} ranged between 0.000 and 0.983 (COI) and 0.000 and 0.977 (*16S* rRNA) (Supplementary Tables 2 and 3). For COI, the populations from the Mae Sa and Mae Chai drainages showed the greatest pairwise differentiation (F_{ST}=0.983, $P<0.05$), and the populations from the Mae On and Mae Lan drainages showed the least differentiation (F_{ST}=0.000, $P>0.05$). For *16S* rRNA, the populations from the Mae Sa and Mae On drainages showed the greatest pairwise differentiation (F_{ST}=0.977, $P<0.05$), and the populations from the Mae On and Mae Lan drainages showed the least differentiation (F_{ST}=0.000, $P>0.05$). The pairwise population showing genetic distances lower than 0.1 was the populations from Mae On versus Mae Lan drainages at 0.000 (COI and *16S* rRNA).

4. Discussion

A. helena is a freshwater snail species of medical and veterinary importance as it transmits mammal, bird, and human parasites worldwide. To the best of our knowledge, this is the first study of the genetic diversity and population structure of *A. helena* in Thailand. The infected populations of *A. helena* were found from Mae Sa and Mae Pu Kha populations. One type of cercariae (zoogonid cercariae) and two types of metacercariae (heterophyid metacercariae and echinostome metacercaraie) were found. The overall prevalence of *A. helena* infected with trematode larvae found in this study (5.2%) was higher than that observed in previous studies[13,43,44]. The distribution of *A. helena* infected with trematode larvae may involve many factors, including the abundance and type of parasites, water levels, season, water temperature, pH, salinity, habitat complexity, and the number of snails hosts *etc*. All of them can affect the prevalence of larval trematode infections[5,45–47]. In this case, we suggested that both the Mae Sa and Mae Pu Kha populations contain diverse hosts and agricultural environments that may be maintained poor hygienic-sanitary and environmental conditions, which had a significant influence on the transmission of parasites[48].

The Heterophyidae and Echinostomatidae families can lead to heterophyiasis and echinostomiasis in human, respectively[49]. However, the trematode in the Zoogonidae family has been reported as the intestinal trematodes of teleosts and occasionally elasmobranchs[50–52]. In this case, the nassariids have been reported to be the dominant intermediate hosts for the parasites in Zoogonidae family that congruence with our study[53]. The 37-collar spines parasite, *Echinostoma mekongi* was normally found from the small intestine of riparian people along the Mekong River in Cambodia[54]. This is the first report of *Echinostoma mekongi* infection in *A. helena* from northern Thailand. This may be because the echinostomes have a wide range of second intermediate hosts, and are generally less specific[55]. According to heterophyid metacercariae, it could infect *A. helena* depending on the predatory behaviour of the snail and the potential of parasite transmission[56].

The phylogenetic tree of *A. helena* populations based on concatenated datasets revealed three main clades of each region, which are consistent with the haplotype network construction. Shared haplotypes were found from either Mae On, Mae Lan, and Nan populations (haplogroup A and D) or Mae Pu Kha, Mae Lao, and Mae Kap Kong populations (haplogroup B and E), while the shared haplotypes from the Mae Sa population (haplogroup C and F) were not found. This occurrence may be caused by the result of the dispersal of these snails by humans or flooding[57]. They may transfer these snails via the ornamental pet industry or the transportation of aquatic plants for aquariums, which are known to be the habitats for *A. helena*[58,59].

A. helena populations showed high haplotype diversity but low nucleotide diversity. High haplotype diversity in concurrence with low nucleotide diversity has been linked to population growth after a period of low effective population[60]. However, the genetic diversity of the *16S* rRNA was lower than COI[19–21]. In terms of the great evolution rate, COI is superior to the *16S* rRNA region[61]. Moreover, the results showed that the infected populations (Mae Sa and Mae Pu Kha populations) had a higher genetic diversity compared to the uninfected populations ($P<0.05$). High genetic diversity may be consistent with what would be expected if parasitism were strongly

influencing population structure[62,63]. Additionally, as presented by Standley *et al*[8], high levels of snail genetic diversity were consistent with an effect of parasitic infection on the population structure of intermediate host snails. Thus, we hypothesised that parasitic infections may be one of the factors that can increase the genetic diversity of the snail intermediate hosts. As such, it has been predicted that the natural population structure of *A. helena* would produce a tightly coupled system in which parasites and snails co-adapt, leading to localised compatibility and comparable population structures but also causing local high variety[8]. In addition, there are reports that the parasitic infection with high levels of genetic diversity in snail host also reduces snail fitness, which involves a higher mortality rate for the snails population[11]. Moreover, the highest genetic diversity of this study was found in the Mae Pu Kha populations, which is a temporary pond. In the permanent ponds, selfing was found to be a major factor shaping the genetic diversity of snail populations, while in the temporary ponds, selfing and migration both have an effect, as flooding facilitates snails' quick recolonisation of dried ponds in aestivation or refuges[64-66].

In this study, the population genetic structure of the pairwise FST values showed a significant genetic differentiation between the populations, which was consistent with the phylogenetic analysis and haplotype network construction. The Mae Sa population differed from other populations with a high value of F_{ST} (COI F_{ST} = 0.918-0.983 and *16S* rRNA F_{ST} = 0.857-0.977). This suggested that the Mae Sa population revealed a high level of genetic differentiation from the other populations. The patchily distributed habitats and different origin of those drainages, which can act as natural barriers blocking the migration and gene flow of freshwater snail populations[21], result in isolation-by-distance between populations and encourages inbreeding, and this is likely the most significant factor shaping the genetic structure of freshwater snails[15]. In contrast, two populations from the Mae On and Mae Lan drainages revealed the lowest of the pairwise FST values (COI = 0.000 and *16S* rRNA = 0.000), which is likely due to the close geographical distance between these populations. Therefore, the populations from the same river systems tended to be more closely related to the other[67].

Our analysis revealed that high genetic diversity and high population structuring of *A. helena* are reliable influencing factors for parasitism. These findings contribute to filling knowledge gaps regarding parasite transmission and are crucial for understanding disease epidemiology. In addition to genetic factors, environmental and demographic stability may also play a role in shaping the observed population structure. We noted that this study only represented the genetic diversity and population structure of the intermediate hosts, *A. helena* populations collected from northern Thailand. Parasite transmission is likely influenced by larger-scale factors, such as human migration and national treatment campaigns, which operate at national or regional levels. From a public health perspective, incorporating these broader scales into control strategies is essential for designing effective and sustainable interventions.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Authors' contributions

N.C. collected, and examined the specimens, performed the molecular analysis. Both C.W. and P.B. authors examined the parasitic parts. N.N. designed the study, collected, and examined the specimens. All authors participated in data analysis, prepared the manuscript, read, and approved the final version.

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