Investigation of cryptic diversity and occurrence of echinostome metacercariae infection in *Anentome helena* (von dem Busch, 1847)

Nithinan Chomchoei[1,2], Chalobol Wongsawad[1,3,4,5], Nattawadee Nantarat[1,3,4,5✉]

[1]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[2]Graduate School, Chiang Mai University, Chiang Mai 50200, Thailand
[3]Applied Parasitology Research Laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[4]Environmental Science Research Center, Faculty of Science, Chiang Mai University, 50200, Thailand
[5]Science and Technology Research Institute, Chiang Mai University, Chiang Mai 50200, Thailand

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ABSTRACT

Objective: To investigate the parasitic infection of *Anentome helena* (*A. helena*) and determine the validity of species boundaries for *A. helena* by combining molecular phylogeny and morphological approaches. **Methods:** A total of 325 individuals of *A. helena* were collected throughout northern Thailand. Shells were measured and compared by *t*-test. Radulae were investigated by using light and scanning electron microscope. Two partial mitochondrial DNA sequences of *COI* and *16S rRNA* from 36 specimens of *A. helena* and related species were used to test the validity of the morphospecies. Phylogenetic trees were constructed using neighbour joining, maximum likelihood and Bayesian inference. Infection of *A. helena* with trematode larva was examined and observed. **Results**: Morphological examination of *A. helena* revealed 2 distinct morphospecies. Genetic divergences supported the separation of the two morphotypes into two distinct groups. Both individual and combined analyses of the two nucleotide fragments revealed two phylogroups that corresponded with shell and radula characteristics. In addition, *A. helena* was found infected with 37-collar spined echinostome metacercariae. The prevalence and intensity of metacercariae was highest in the San Kamphaeng district, Chiang Mai province, with 7.5% and 1.670±0.577, respectively. **Conclusions**: These findings suggest that comprehensive taxonomic revision of this unrecognised species complex is needed. This study represents the preliminary step to reveal new data on the recent distribution of trematode infection in *A. helena*. This information may be useful for developing conservation management of the snail and the practice of targeted regimes to reduce anthelmintic resistance in the future.

1. Introduction

The genus *Anentome* contains carnivorous snails in the Family Nassariidae, one of the largest and most biologically successful families worldwide. This genus consists of 6 species: *Anentome helena(A. helena)*, *Anentome combojiensis*, *Anentome spinosa*, *Anentome jullieni*, *Anentome scalarina* and *Anentome wykoffi*. *A. helena* is widely distributed throughout Southeast Asia, including Cambodia, Indonesia, Malaysia and especially in northern

First author: Nithinan Chomchoei, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand.
✉Corresponding author: Nattawadee Nantarat, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50202, Thailand.
E-mail: n_nantarat@yahoo.com
Tel: +66 53 943346, ext. 2002
Fax: +66 53 892259

Thailand[1]. *A. helena* is commonly known as the "assassin snail" and tend to live on soft, muddy bottoms[2–4]. While *A. helena* is popular in aquariums, its biology remains unclear. Thus, there is increasing apprehension about what would happen if this species was introduced outside its native range[5]. Moreover, *A. helena* in Chiang Mai province was infected with echinostome metacercariae[6]. The parasites from the Family Echinostomatidae can infect the gastrointestinal tract of a variety of animals and humans, which leads to a disease called echinostomiasis. The disease has been generally found in north and northeastern parts of Thailand[7]. Since the disease may be transmitted to humans by ingesting raw or partially cooked snails, there is substantial interest in understanding echinostome metacercariae infection in snail populations in order to control potential echinostomiasis cases in humans[8].

The taxonomic relationships among the members of *Anentome* are ambiguous. Traditional taxonomic classification of gastropods is commonly based on shell morphological characteristics, including shape, size and surface sculpture[1,9]. A review by Brandt[1] examined Thai freshwater snails and synonymised 7 species with *Clea helena* only based on shell morphology and noted that their shell was extremely variable with regard to size, shape and costulation. However, environmental factors can greatly affect *A. helena* shell morphology[10]. Thus, given the flourishing interest in this species across various disciplines, the taxonomy of this snail needs to be robustly explored.

Molecular phylogeny offers a framework to clarify organism classification[11]. During the previous decades, this technique has been used to resolve systematics and evolution issues when morphological and physiological characteristics prove ambiguous[12]. Studies on freshwater snail phylogeny using molecular DNA sequences are potentially useful, and the most common genes utilized in these studies are mitochondrial cytochrome oxidase subunit I (*COI*) and *16S rRNA*[13–16]. Ambiguities in the species emphasize the need for rigorous morphological analyses accompanied by molecular comparisons to clarify the species boundaries of *A. helena* and determine the parasitic infection of this snail. This research will provide useful information that may be valuable for developing public health prevention strategies for echinostomiasis and conservation management of *A. helena* in the future.

2. Materials and methods

2.1. Sampling and identification

A. helena s.l. samples were collected throughout northern Thailand by using the count per minute method (samples collected every 10 minutes per sampling by 5 collectors). Specimens were identified based on shell morphology and compared with original descriptions[1].

2.2. Shell and radula study

One hundred empty shells of each *A. helena* morphotype were investigated. Weight was determined using an electronic scale with 0.01 g precision. Shell height, shell width, aperture height and aperture width were measured with vernier calipers. In order to detect variations in shell morphology between the 2 morphotypes, the mean values of linear measurements (shell or aperture sizes) were compared by *t*-test ($P<0.05$) using IBM SPSS Statistics ver. 22.0[17].

At least 30 radulae of each morphotype were examined. The radulae were extracted from the snail buccal cavity using a stereomicroscope, boiled in 5% sodium hydroxide for 5 min and then washed with distilled water. Dehydration was performed by immersing the radulae in increasing alcohol concentrations (10%, 30%, 50%, 70%, 80% and 95%, respectively). Subsequently, the specimens were mounted on stubs with the help of a sharp-tipped needle on carbon conductive adhesive tapes. The stubs were then coated with gold and observed using a scanning electron microscope (JSM 5910 LV) at the Electron Microscope Research and Service Center, Faculty of Science, Chiang Mai University.

2.3. DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from foot tissues of 15 individuals using 150 μL of Chelex 100 and 3 μL of proteinase K. Samples were incubated at 55 ℃ for 1 h, 95 ℃ for 30 min and then stored at -20 ℃ until use. *COI* and *16S rRNA* mitochondrial genes were amplified using the following primers: LCOI490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') for the *COI* gene[18] and 16sar (5'-CGCCTGTTTATCAAAAACAT-3') and 16sbr (5'-CCGGTCTGAACTCAGATCACGT-3') for the *16S rRNA* gene[19]. PCR was performed in 20 μL reactions with 5 μL 2 Illustra Hot Start Master Mix (GE Healthcare), 1 M each primer, deionized water and approximately 10 ng DNA template. For *COI*, thermal cycling was: 94 ℃ for 2 min, 36 cycles of 94 ℃ for 30 s, 42 ℃ for 2 min and 72 ℃ for 2 min, and a final extension step of 72 ℃ for 5 min. For *16S rRNA*, thermal cycling was performed in the same way as for *COI* except the annealing temperature was changed to 49 ℃ for 30 s. The amplified products were checked with 1% (w/v) agarose gel electrophoresis using 0.5× TBE buffer. Gels were run at 100 V for 45 min and visualised with RedSafe nucleic acid staining solution and UV transillumination. PCR products were purified and sequenced using BigDye® Terminator v3.1 cycle sequencing kit chemistry and 1st BASE DNA Sequencing Services (Applied Biosystems).

2.4. Phylogenetic analysis

The taxonomic sampling scheme used herein included 17

individuals identified as *A. helena* sensu lato, 6 related species[16] and 6 specimens of *Nassodonta dorri*[3]. Chromatograms were visually inspected and edited as necessary with ClustalW[20] as implemented in MEGA 7.0[21]. *COI* was translated to check for amino acid sequence, stop codons and frameshift mutations. All newly generated sequences have been deposited in GenBank (Table 1). Phylogenetic trees were constructed using neighbour joining (NJ), maximum likelihood (ML) and Bayesian inference (BI). The phylogenetic trees were rooted on the genus *Nassodonta* since it has been suggested to represent close relatives of *Anentome*[3,16]. jModeltest 0.1.1[22] was used to evaluate the best evolutionary substitution model under Akaike Information Criterion[23]. NJ analysis was performed using PAUP* v4.0[24] based on appropriate models (*16S:* GTR+I+G; *COI:* GTR+G; concatenated dataset, Kimura 2-parameter (K2P)). One thousand replicates of bootstrap resampling[25] were performed to assign support to branches in the NJ tree. ML was performed using PhyML v.3[26], with randomised stepwise addition parsimony trees (number seed = 13) and with 1 000 bootstrap replicates[25,27]. BI analysis was performed using MrBayes v3.1.2[28], where the tree space was explored using 4 chains for 2 runs of a Markov chain Monte Carlo algorithm. The BI analysis was run for 7 000 000 generations with heating parameter set at 0.095, sampled every 100 generations, burn-in set at 25%, and the last remaining trees were used to determine posterior probabilities of the BI tree. Convergence was observed by verifying that the average standard deviation of the split frequencies (between 2 runs) was below 0.01[29].

2.5. Parasitic infection

Infection of *A. helena* with trematode larva was examined by the crushing method. Metacercariae were covered with a cover slip and observed under a light microscope. The shape, size and cyst wall structure were used for characterisation of the metacercariae. The presence of collar spines was highly indicative of the echinostome-metacercaria type. Different numbers of arranged collar spines have been reported before raging from a minimum of 31 to a maximum of 51. The number of collar spines presented in each echinostome species can be used for identification[6]. So, the numbers of collar spines on the oral collar were counted[6,30]. The metacercaria were compressed, fixed in 5% formalin and stained with haematoxylin. They were dehydrated in ascending grades of alcohols, cleared in xylene and mounted in Permount for permanent slides. Two measures were calculated: prevalence of the parasite species as percentage of hosts infected by that species; intensity of the infection as the mean number of parasite larvae per infected host.

3. Results

3.1. Morphology

A total of 325 *A. helena* were collected throughout Thailand (Figure 1, Table 1). *A. helena* represents one of the most widespread species of *Anentome*. We collected the snail throughout northern Thailand. The *A. helena* shell was conical in shape and had dark brown and yellowish-tan bands around the whorls. The morphological examination of *A. helena* specimens revealed 2 distinct morphotypes. Morphotype A exhibited 2 elongated shell forms, while morphotype B exhibited globate shells (Figure 2A, C). As previously observed, *A. helena* were anatomically cryptic due to large shell structure variation. Average shell dimensions for morphotype A (Figure 2A) were 1.889 cm (range 1.600-2.300 cm) in height and 0.847 cm (range 0.700-1.000 cm) in width; average aperture sizes were 0.659 cm (range 0.590-0.720 cm) in height and 0.341 cm (range 0.300-0.380 cm) in width. For morphotype B (Figure 2C), average shell height was 1.996 cm (range 1.800-2.400 cm) and width was 0.928 cm (range 0.900-1.200 cm); aperture height was 0.753 cm (range 0.660-0.830 cm) and aperture width was 0.350 cm (range 0.30-0.44 cm). The shell and aperture widths were statistically significantly different ($P<0.05$) (Table 2).

Table 1

List of localities and GenBank accession numbers of samples.

No. in map	Locality	Codes	Latitude/Longtitude	Accession numbers	
				COI	16S
1	Chiang Rai (Phan)	CR1-5	19°35'31.7"N 99°43'50.4"E	MH256030 MH256031 MH256032 MH256033	MH256013 MH256014 MH256015 MH256016
2	Phayao (Mae Chai)	PY1-5	19°21'50.6"N 99°48'49.1"E	-	-
3	Chiang Mai (San Kamphaeng)	IN1-3	18°46'05.1"N 99°07'08.9"E	MH256026	MH256009
4	Chiang Mai (Mae Rim)	C3-4,7	18°57'16.5"N 98°56'27.9"E	MH256027 MH256028 MH256029	MH256010 MH256011 MH256012
5	Chiang Mai (Chom Thong)	C10-12	18°24'59.5"N 98° 40'44.2"E	MH256018 MH256019 MH256020	MH256001 MH256002 MH256003
6	Lamphun (Meuang)	LN1-5	18°35'28.0"N 98°58'55.9"E	MH256034	MH256017
7	Lampang (Ngao)	LG1-5	18°43'07.7"N 99°56'49.2"E	MH256021 MH256022 MH256023	MH256004 MH256005 MH256006
8	Phrae (Long)	PE1-5	18°05'43.6"N 99°51'59.8"E	MH256024	MH256007
9	Nan (Wieng sa)	NN1-5	18°34'05.1"N 100° 45'23.6"E	MH256025	MH256008

A. helena radulae were examined using a scanning electron microscope (350 magnification; Figure 2B, D). The radula was a stenoglossan type with a general formula of 1:1:1, each row of radula teeth consisted of 1 central tooth and 1 lateral tooth on each side. Examination of radular morphology also revealed differences

between morphotypes (Figure 2B, D). Radulae of morphotype A (Figure 2B) consisted of approximately 6 cusps (range 5-8 cusps) of the central tooth and 3 cusps on each side of the lateral teeth. The secondary cusp of the lateral tooth was triangular in shape and narrower and shorter than the other cusps. Comparatively, morphotype B had a much more rounded cusp on the central part of the lateral tooth that was similarin shape to the other cusps, but with more convex bases compared to morphotype A. There were approximately 8 cusps (range 6-11 cusps) of the central tooth (Figure 2D). The outermost lateral teeth were similar between morphotypes.

Table 2
Shell measurements of *A. helena* morphotypes.

Morphotypes	Shell		Aperture	
	Height	Width	Height	Width
Morphotype A	1.889 ±0.302	0.847 ±0.018	0.659±0.800	0.341±0.005
Morphotype B	1.996 ±0.240	0.928 ±0.010 *	0.753 ±0.010	0.350 ±0.007*

* Significant difference in width between the two morphotypes at *P*<0.05.
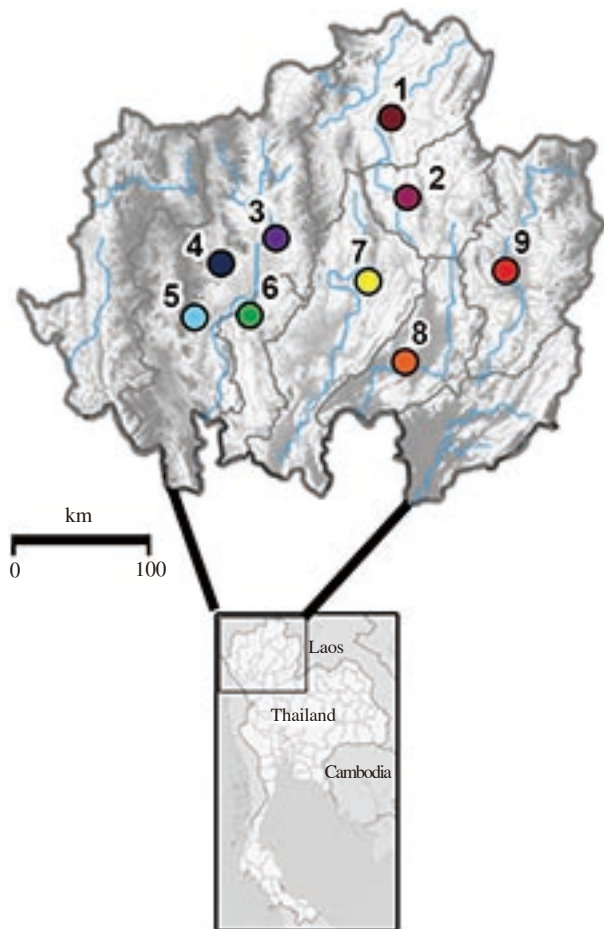

Figure 1. Sampling locations for specimens of *A. helena* in northern part of Thailand.
A. helena morphotype A: No. 5: Chiang Mai, Chom Thong district ; No. 7: Lampang, Ngao district; No. 8: Phrae, Long district; No. 9: Nan, Wieng Sa district. *A. helena* morphotype B: No. 1: Chiang Rai, Phan district ; No. 2: Phayao, Mae Chai district; No. 3 Chiang Mai, San Kamphaeng and No. 4 Mae Rim districts; No. 6. Lamphun, Meuang district.

3.2. Phylogenetic analyses

The *COI* and *16S rRNA*-derived phylogenetic trees shared similar topologies that principally referred to relationships between the 2 morphotypes and division of the outgroup and other related species. All trees showed comparable evolutionary patterns with moderately high statistical support for each clade. The phylogenetic tree (Figure 3) was separated into 2 main clades: clade 1, all *Anentome* spp. and clade 2, outgroup (*Nassodonta* spp.). Clade 1 was also divided into 2 subclades: clade A, all *Anentome* spp. in Thailand and clade B, *Anentome* spp. from other countries; there was 100, 100 and 1.00 bootstrap support, respectively, from the NJ, ML and posterior probability of the BI method. Both morphotypes were placed in clade A and were further separated into clade A1 (morphotype A) and A2 (morphotype B) with moderately high statistical support and 61, 75 and 0.98 bootstrap support (NJ, ML and BI, respectively).
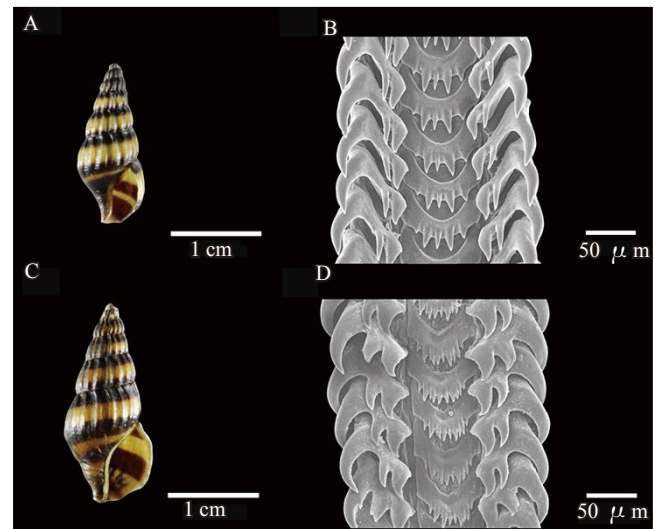

Figure 2. *A. helena* morphotype A and B.
A) Shell of *A. helena* morphotype A; B) Radula of *A. helena* morphotype A;
C) Shell of *A. helena* morphotype B; D) Radula of *A. helena* morphotype B.

3.3 Epidemiology

The prevalence of echinostome metacercariae in *A. helena* was examined from northern Thailand (Figure 4). Metacercariae type was identified by morphology. The 37 collar spines echinostome metacercariae were found and resembled members of *Echinostoma* group[6]. Infected snails were only recorded in San Kamphaeng district, Chiang Mai Province. The highest prevalence (7.5%) was found in *A. helena*. The highest intensity was noted in the same locality with (1.670±0.577) metacercariae per snail.
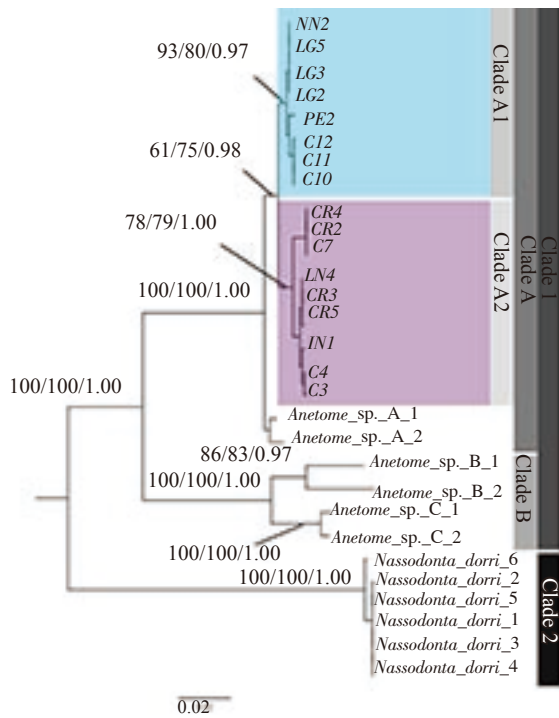
93/80/0.97

61/75/0.98

78/79/1.00

100/100/1.00

100/100/1.00

86/83/0.97
100/100/1.00

100/100/1.00
100/100/1.00

NN2
LG5
LG3
LG2
PE2
C12
C11
C10

CR4
CR2
C7
LN4
CR3
CR5
IN1
C4
C3

Clade A1

Clade A2

Clade A
Clade I

Clade 2

*Anetome*_sp._A_1
*Anetome*_sp._A_2
*Anetome*_sp._B_1
*Anetome*_sp._B_2
*Anetome*_sp._C_1
*Anetome*_sp._C_2
Nassodonta_dorri_6
Nassodonta_dorri_2
Nassodonta_dorri_5
Nassodonta_dorri_1
Nassodonta_dorri_3
Nassodonta_dorri_4

Clade B

0.02

Figure 3. The ML tree obtained from sequence analysis of the concatenated *COI* and *16S rRNA* dataset (1 178 bp).
Numbers above branches are bootstrap percentages of NJ method, ML method and posterior probability of BI method, respectively.
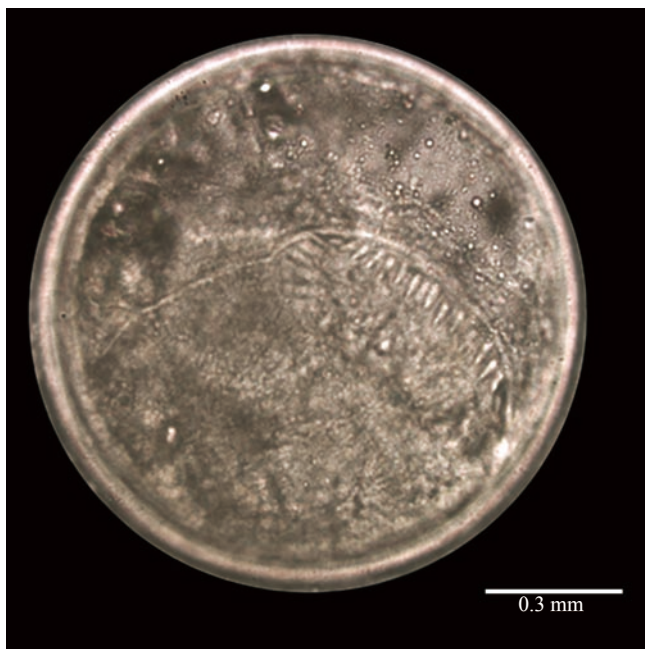

0.3 mm

Figure 4. Echinostome metacercariae infected in *A. helena* from San Kamphaeng district, Chiang Mai Province.

4. Discussion

The molecular relationships among species of the genus *Anentome*

are ambiguous[16]. Furthermore, the taxonomy of *A. helena* is unclear. Originally, this species had the combination name *Melania helena* (von dem Busch, 1847)[31], but subsequent alternative representation led to it being recognised as *Clea* (now *Anentome*) *helena* (von dem Busch, 1847)[1] and also combined with many synonyms. Subsequently, Strong *et al.*[16] suggested that many taxa belonged to the genus *Clea*. Based on the morphology and molecular phylogeny evidence, they suggested that the species should be recognised as *A. helena* because of morphological differences in the shell, operculum and radula from *Clea nigricans*, the type species of *Clea*.

This study initially demonstrated cryptic diversity based on shell morphology in *A. helena* collected from different populations in northern Thailand. Phylogenetic analysis showed moderate support that *A. helena* morphotype A formed a sister group to morphotype B. The morphotypes exhibited different shell and aperture sizes as well as radular structure. Shell characteristics, including shape, size, colour pattern, sculpture and number of whorl have been used to identify the different *Anentome* species[1]. However, morphological classification based on a snail's shell is occasionally misleading for accurate species delimitation[32,33]. In many cases, shell morphological variations may be caused by phenotypic plasticity in response to environmental conditions[32,33]. Our results showed significant differences between the two morphotypes that were supported by both morphological characteristics and phylogeny. The molecular results supported the recognition of two *A. helena* lineages from northern Thailand. *A. helena* morphotype A was recovered from sites in Chiang Mai (Chom Thong district) Lampang (Ngao district), Phrae (Long district) and Nan (Wieng Sa district; No. 9 on map), while *A. helena* morphotype B was recovered from sites in Chiang Rai (Phan district), Phayao (Mae Chai district), Chiang Mai (San Kamphaeng and Mae Rim districts) and Lamphun (Meuang district). This occurrence may be caused by allopatric speciation related to geographic isolation (most likely due to river systems in northern Thailand). Moreover, the current distribution of these populations may reflect the great drainage connectivity across the Chiang Mai and Lumpang river system[34] and/or may be accidental.

While both lineages received high support from analyses of the mitochondrial and concatenated datasets, specimens identified here as species from northern Thailand displayed high K2P average pairwise distances for *COI* and *16S* (*COI*, 0.080; *16S*, 0.036–0.086) when compared with species from eastern Thailand and Malaysia . Thus, the geographic scale of this species complex, the morphological distinctiveness of the lineages and the molecular evidence are provided here. This study has uncovered an unrecognised radiation within *A. helena* from northern Thailand that was formerly recognised as a single species. Thus, comprehensive revision of snail systematics is needed. However, another complicating influence is that the freshwaters in Southeast Asia are affected by human impacts, including habitat loss, agriculture,

pollution and impoundment, and many snail species are believed to already be extinct[35]. Nevertheless, the validity of the currently discovered species remains to be tested.

Our study provided epidemiological data of echinostome metacercariae in *A. helena* from northern Thailand between February 2017 and January 2018. Based on morphological characteristics, the worms showed a prominent head collar with 37 collar spines arranged in a double row. In this case, they were identified as genus *Echinostoma*. In this survey, we found different prevalence in echinostome metacercariae infection. Only the San Kamphaeng district, Chiang Mai Province contained *A. helena* infected with echinostome metacercariae; prevalence and intensity were 7.5% and 1.670±0.577 metacercariae per snail, respectively. The diverse ecosystem in the agricultural area of San Kamphaeng district may provide suitable ecological conditions that allow for the distribution of trematodes in the snails that differ from other localities[6]. The overall prevalence and intensity of metacercarial infection were 0.92% and 1.670±0.577, both of which are considered low infection rates when compared to other snail species (*e.g.*, *Filopaludina* spp. and *Lymnaea* spp). These species were reported to act as the first and second most common intermediate hosts of trematodes in the family Echinostomatidae[6,8,36]. Parasite infection patterns are often related to host dispersal and gathering of parasites, principally during larval stages[37,38]. Moreover, reports have revealed an effect of parasite infection on the population structure, including death, of intermediate host snails[39]. Detection of this infection in this area is important for public health control with regard to monitoring dispersion; echinostomes can be easily transmitted to humans through eating raw or partially cooked snails. Coordinated parasite control strategies across the region are of great importance.

Conflict of interest statement

The authors declare that they have no competing interests.

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