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Differences in Radular Morphology in Relation to Microhabitat of Assassin Snails *Anentome Helena* from Northern Thailand (Mollusca: Gastropoda)

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ABSTRAC

Anentome helena is a widespread freshwater snail, especially in Thailand. It is a successful predator in the ecosystem and an intermediate host of trematodes. The radula is a specific character of gastropods for feeding and often uses to limited species. However, it's neither all morphological features of radulae and radular teeth are functionally adaptive or optimized for a specific function, nor all morphology should be viewed in an evolutionary context. This study aims to compare the radular morphology of assassin snail *A. helena* from different microhabitats in Northern Thailand and performs DNA fingerprints analysis using the high annealing temperature-random amplified polymorphic DNA marker (HAT-RAPD) of *A. helena* and related species. A total of 140 adult individuals of *A. helena* were collected from 14 different localities throughout Northern Thailand. There were 4 different types of microhabitats of *A. helena* including sand, mud, cement and plant root. The results showed that there were two morphotypes in the *A. helena* complex. The radula was investigated by using a light microscope and scanning electron microscope (SEM). The radula of all *A. helena* was stenoglossan but different in number and size of cusps on a central tooth and lateral teeth. Moreover, the radular teeth of *A. helena* from sand differed from those other microhabitats with more blunt-cusps teeth. HAT-RAPD analysis with 8 primers could be identified species-specific banding patterns for each freshwater snail species. In addition, it was separated two morphotypes of *A. helena* into two groups and showed the result that it was not related to their microhabitats. This finding has demonstrated morphological adaptation of *A. helena* radula in number and size of cusps which seems to be related to preferred substrata. This study will help to understand the ecology that can affect to the radular morphology of *A. helena*. In addition, HAT-RAPD profiling was a useful tool to determine the genetic relationship between freshwater snail species and variation that might occur on their radular morphology.

INTRODUCTION

Anentome helena (von dem Busch, 1847) belongs to phylum Mollusca, class Gastropoda, and family Nassariidae. It is a freshwater snail that commonly known as the "assassin snail". The snail is a non-selective predator and scavenger and very popular in the aquarium for its ability to hunt down pest snail species [1], [2], [3]. Assassin snail is commonly found in the broad lower reaches of coastal rivers, as well as lakes and ponds. It widely distributed throughout Southeast Asia, including Cambodia, Indonesia, Laos, Malaysia, Vietnam and Thailand [1], [4], [5]. Moreover, *A. helena* serves as an intermediate host of trematode especially the Echinostomatidae that cause of disease in avian and mammals including humans [6].

In heterogeneous environments, natural selection is more probable to effect in the maintenance of genetic polymorphism if altered genotypes are able to choose the habitats in which they are the greatest fit [7]. In some snails such as *Cepaea nemoralis*, habitat choice is supposed to be important in maintaining the polymorphism; since banded shells absorb more solar energy than do unbanded shells and the snails adapt their exposure to the sun behaviourally [8]. There are also reports on microhabitat selection by freshwater snails *Biomphalaria pfeifferi* and *Lymnaea natalensis* may contribute to additional disease transmission and relate to adaptation of some morphological characteristics [9]. Classification of gastropods is largely based solely on shell morphology [3], [10] but often show geographic variation and phenotypic plasticity [11], [12]. Radula is the characteristic feeding organ of gastropods which consists of a long ribbon of tissue with repeated rows of teeth along its length [13], [14]. The radula

has been frequently investigated because it's a tool in supraspecific systematic to diagnose the species and recognized as an important morphological criterion for the taxonomic allocation. It shows general similarities at family and generic levels with consistent differences at the species level [15], [16]. Moreover, the differences of radula types are based on feeding pattern of gastropods. The special food sources and all different shapes of radula teeth has its special performance and are appropriate to any kind of food. For examples, the carnivore gastropods *Conus textile* need less teeth and herbivores need more teeth than carnivores [13]. Scanning Electron Microscope (SEM) is the most powerful tool in radular study. However, the morphological characters are used to separate species of gastropod are difficult to score and identification [17].

Nowadays, molecular taxonomic methods have successfully identified freshwater snail species in various groups. The molecular techniques are proved useful in cases where morphological features failed. High annealing temperature-randomly amplified polymorphic DNA (HAT-RAPD) marker is a useful procedure to differentiate between closely related and morphologically indistinct species because high annealing temperature gives greater polymorphisms, reproducibility, and resolution [18].

In this study, we compare the radular morphology of *A. helena* from different microhabitats in Northern Thailand and combine analysis of morphological and molecular marker to determine the validity of species boundaries for *A. helena* in Northern Thailand.

METHODOLOGY

Sampling and identification

140 adults of *Anentome helena* were sampling from 14 localities in Northern Thailand during February 2017 - June 2018 (Table 1, Figure 1) by using hand held scoops. Sampling was done in a 100m ×2m belt transect for approximately 30 minutes. Specimens were identified based on shell morphology [4].

Study of shell morphology

120 shells of A. helena from 4 difference microhabitats (n=30) were investigated and the remained 20 samples were used for radular study. Shell height (SH), shell width (SW) were measured for each individual to the nearest 0.01 mm. precision by using Vernier calipers.

Study of radular morphology

Radular were extracted from the radular sac under stereo microscope, boiled with 10% Sodium hydroxide about 5 mins, washed in distilled water. Dehydration was done by immersing the radula in increasing concentration of alcohol (10%, 20%, 30%, 50%, 70%, 85% and 95%, respectively). Then, the specimens were mounted on SEM stubs using carbon conductive adhesive tapes. The SEM stubs were then coated with gold and observed on LV-Scanning Electron Microscope: JSM 5910 LV at Electron Microscope Research and Service Center, Faculty of science, Chiang Mai University.

Total genomic DNA extraction

Genomic DNA of freshwater snails was extracted from foot tissue using 150 µm of Chelex® 100 [19] and 3 µm of Proteinase K. Then, incubated at 55 °C for 1 hours and 95 °C for 30 minutes. All the DNA samples were stored at -20 °C until further use.

Table 1. List of localities of *Anentome helena* samples.

No.	Localities	GPS coordinates
1	Chiang Rai (Phan)	19°35'31.7"N 99°43'50.4"E
2	Phayao (Mae Chai)	19°21'50.6"N 99°48'49.1"E
3	Chiang Mai (Doi Saket)	18°52'37.0"N 99°08'18.8"E
4	Chiang Mai (San Kamphaeng)	18°46'05.1"N 99°07'08.9"E
5	Chiang Mai (San Sai)	18°53'20.1"N 99°00'03.8"E
6	Chiang Mai (Meuang)	18°48'16.8"N 98°57'14.5"E
7	Chiang Mai (Mae Rim)	18°57'16.5"N 98°56'27.9"E
8	Chiang Mai (Hang Dong)	18°41'19.6"N 98°57'20.7"E
9	Chiang Mai (San Pa Tong)	18°36'52.3"N 98°53'59.4"E
10	Chiang Mai (Chom Thong)	18°24'59.5"N 98°40'44.2"E
11	Lamphun (Meuang)	18°35'28.0"N 98°58'55.9"E
12	Lampang (Ngao)	18°43'07.7"N 99°56'49.2"E
13	Phrae (Long)	18°05'43.6"N 99°51'59.8"E
14	Nan (Wiengsa)	18°34'05.1"N 100°45'23.6"E

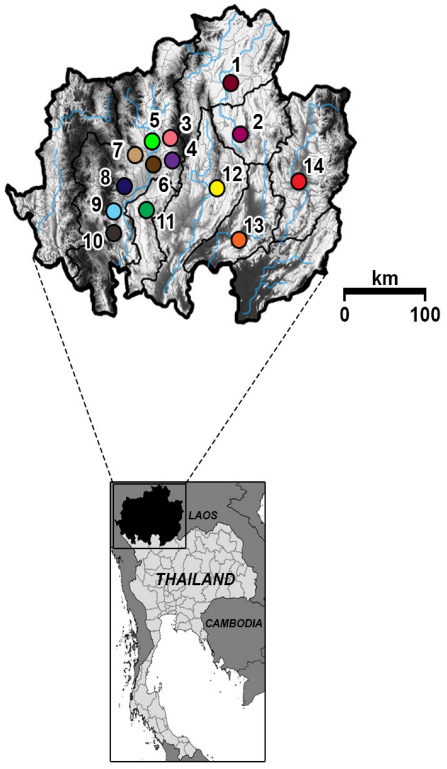

Figure 1. Sampling locations for specimens of *Anentome helena* in Northern Thailand.

HAT-RAPD analysis

Eight commercially available arbitrary 10-mer primers (Operon Biotechnology, Huntsville, Alabama, USA) including OPA02, OPA03, OPA07, OPA10, OPN02, OPN05, OPN06 and OPN07 were performed to use individually in HAT-RAPD PCR.

The reaction was carried out in a final volume of 20 µl mixture containing; 2 µl of 1x PCR buffer, 0.7 µl of $MgCl_2$, 0.4 µl of dNTP, 1 µl of each primer, 1 µl of Vivantis Taq DNA polymerase, 13.9 µl of deionized H_2O and 1 µl of DNA template. PCR protocols were indicated as follows: 1 cycle of 94°C for 2 min, 36 cycle of 94°C for 30 sec, 42°C for 2:45 min, 72°C for 45 sec, and 1 cycle of final extension at 72°C for 7 min. HAT-RAPD PCR products were separated on 1.0% TBE agarose gel electrophoresis, stained with RedSafe nucleic acid staining solution and electrophoresed in 0.5X TBE buffer, run at 100 V for 45 minute and the bands were visualized under UV light.

Data analysis

Photographs of agarose gels showed different banding patterns were digitalized and analysed. HAT-RAPD fragments were scored as 1 for the presence of a band, and 0 for absent. Ambiguous bands that could not be clearly distinguished were not scored. A dendrogram was constructed based on the genetic distance matrix by applying an unweighted pair group method with arithmetic averages (UPGMA) analysis [20] by using Phylip software V.3.697 [21] and phylogenetic tree were performed by using FigTree software V.1.4.3 [22].

RESULTS AND DISCUSSIONS

In the genus *Anentome*, *A. helena* is one of the most widespread species in Northern Thailand. A total number of 140 individuals of *A. helena* were collected from 14 localities throughout Northern Thailand. There were 4 different microhabitat types of *A. helena* including sand (localities No. 10 and 13 on map), mud (localities No. 1, 5, 8, 9 and 14 on map), cement (localities No. 2, 3, 6 and 7 on map) and plant root (localities No. 4, 11 and 12 on map).

The shell of *A. helena* was conical in shape. The sculpture consists of axial ribs with dark brown and yellowish bands around the whorls. There was a siphonal canal through the aperture which the siphon protrudes, and the operculum is light brown. The morphological examination of the specimens of *A. helena* showed the presence of two distinct morphotypes; mostly in apex, spire whorl and aperture, consisted of elongate (morphotype A) from sand in locality No. 13 (Figure 2B) and plant root in locality No. 12 (Figure 2D) and globate shells (morphotype B) from cement in locality No. 7 (Figure 2F) and mud in locality No. 1 (Figure 2H). Average shell dimensions from each microhabitat types showed in Table 2.

The radular of *A. helena* were examined using light microscope and scanning electron microscope (SEM). The radula morphology of all *A. helena* was stenoglossan type with 1:1:1 general formula. There were three teeth per row consisted of 1 central tooth and 1 lateral tooth on each side. Moreover, the radular teeth of *A. helena* from 4 microhabitat types were differed in shape, number and size of cusps on central tooth and lateral teeth.

Sand

The central tooth of the radular from sand was spade-like shape, consisting of 5 main cusps on posterior margin with central and lateral cusps smaller. The lateral teeth were tricuspid with more blunt-cusps teeth. The central cusp much smaller, narrower, and closer to the inner cusp and the outer cusp was the largest cusp with C shape (Figure 2A).

Table 2. Shell measurements of *Anentome helena* from each microhabitat types.

Microhabitats	Shell	
	height	width
Sand (n=30)	1.05-1.90 cm (1.56±0.24)	0.55-0.88 cm (0.73±0.08)
Plant root (n=30)	1.05-2.05 cm (1.55±0.04)	0.51-0.99 cm (0.71±0.15)
Cement (n=30)	1.40-2.24 cm (1.84±0.06)	0.66-1.05 cm (0.85±0.10)
Mud (n=30)	1.44-2.44 cm (1.86±0.03)	0.66-1.10 cm (0.09±0.12)

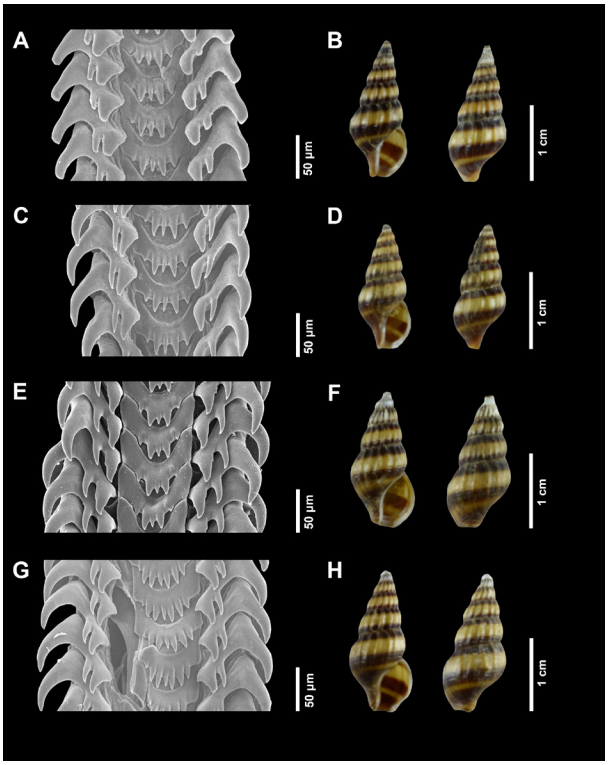

Figure 2. Radular and shell morphology of *Anentome helena* from different microhabitats (A, B) sand; (C, D) plant root; (E, F) cement and (G, H) mud.

Mud

The central tooth of the radular from mud was spade-like shape consisted of 5 main cusps on posterior margin with central and lateral cusps smaller. The lateral teeth were tricuspid. The central cusp much smaller, narrower, and closer to the inner cusp and the outer cusp was the largest cusp with C shape (Figure 2C).

Cement

The central tooth of the radular from cement consisted of 5 main cusps on posterior margin with central cusp smaller. The lateral teeth were tricuspid. The central cusp much narrower and closer to the inner cusp, a small denticle present on one side of the inner cusp and the outer cusp was the largest cusp with C shape (Figure 2E).

Plant root

The central tooth of the radular from plant root consisted of 7 main cusps on posterior margin with central cusp smaller, a small denticle present on each side of the lateral cusps. The lateral teeth were tricuspid with central cusp much narrower and closer to the inner cusp and the outer cusp was the largest cusp with C shape (Figure 2G).

HAT-RAPD analyses

All freshwater snails used in this study were genetically profiled using HAT-RAPD PCR with 8 random arbitrary primers; OPA02, OPA03, OPA07, OPA10, OPN02, OPN05, OPN06 and OPN07. The results showed that the DNA profiles of freshwater snails are shared bands among closely species (Figure 3).

UPGMA dendrogram was constructed which showed that separated into 2 main clades: clade A, all operculate freshwater snails and clade B, outgroup (*Physa acuta*). Clade A was divided into 2 subclades: clade 1, All *Anentome helena* from each microhabitat types and clade 2, *Filopaludina martensi martensi*, *F. doliaris*, *F. sumatrensis polygramma*, and *Pomacea canaliculata*. Clade 1 was also divided into 2 subclades: clade 1A, *A. helena* from cement and mud and clade 1B, *A. helena* from sand and plant root (Figure 4).

The morphological characteristics of shell and radula showed that there was difference between microhabitat types. However, the dependence on shell and radula characters as taxonomic characteristics may possible causes by geographical variations and phenotypic plasticity [17], [23]. Moreover, the influence of the environment on the organisms and the response of the latter to its environment are equally important. The various environmental factors often mutually affect one another, and their combined effect influences the species [24], [25]. In addition, the substratum of the freshwater organisms which consists of the sediments composes of the mineral component and varies with regard to chemical, mineral and granulometric qualities, all of which influence the chemical composition of the water and affect the freshwater snail species [25]. So, the correlation difference between radular morphology on each microhabitat types suggests that the environmental factor is a major factor in the adaptive radiation of *Anentome helena*.

HAT-RAPD analysis has revealed considerable DNA differences among 6 species of freshwater snails in Northern Thailand. In addition, HAT-RAPD analysis of *Anentome helena* has revealed remarkable variation between each microhabitat types. For the analysis of phylogenetic relationships, we were found that *Anentome helena* from cement (Mae Rim, Chiang Mai locality No. 7 on map; Figure 1) and mud (Phan, Chiang Rai locality No. 1 on map; Figure 1) were separated from *A. helena* from sand (Long, Phrae locality No. 13 on map; Figure 1) and plant root (Ngao, Lampang locality No. 12 on map; Figure 1). However, the result of phylogenetic relationships supported the differences between the 2 morphotypes and showed that it was not related to their microhabitat. This situation supports the allopatric speciation of this snail [26], [27]. The occurrence may also cause by the distribution of *A. helena* in different areas which differ in their topographic and environment conditions.

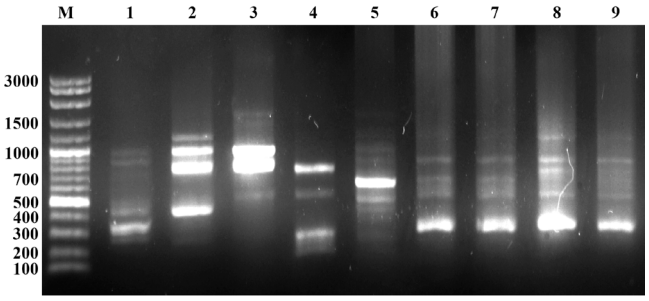

Figure 3. HAT-RAPD DNA profiles of freshwater snail species in Northern Thailand generated with the OPA02 primer. Lane M, 100 bp ladder; Lane 1, *Filopaludina martensi martensi*; Lane 2, *F. doliaris*; Lane 3, *F. sumatrensis polygramma*; Lane 4, *Pomacea canaliculata*; Lane 5, *Physa acuta*; Lane 6, *Anentome helena* (cement); Lane 7, *A. helena* (mud); Lane 8, *A. helena* (sand) and Lane 9, *A. helena* (plant root).

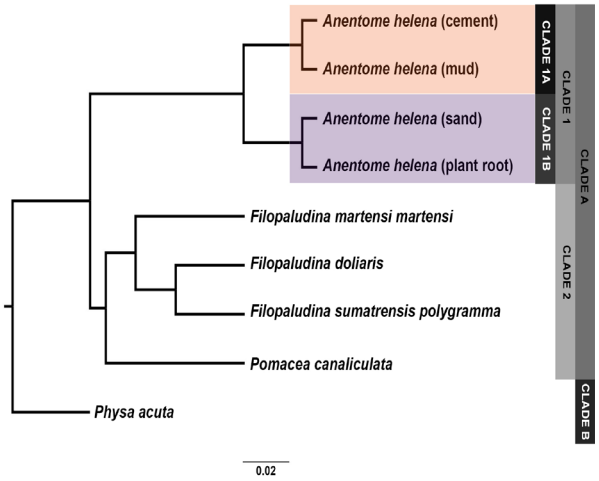

Figure 4. UPGMA dendrogram showed the relationships between freshwater snail species in Northern Thailand and demonstrated calculated based on polymorphism of HAT-RAPD markers.

CONCLUSIONS

Based on the morphological and molecular analyses of *Anentome helena* in Northern Thailand, we found differences between two morphotypes that may cause by the allopatric speciation. Moreover, the influence of the environment factors is equally important that affect the adaptive radiation of *Anentome helena* radula in number and size of cusps which seems to be related to preferred substrata. This study will help to understand the ecology that can affect to the radular morphology of *A. helena*. In addition, HAT-RAPD profiling was a useful tool to determine the genetic relationship between freshwater snail species and variation that might occur on their radular morphology.

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