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Molecular identifications of land snails (Gastropoda) and determination of its associated parasites

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Abstract

Freshwater snail is known to be an intermediate host for parasites. However, little is known on terrestrial snails whether they can become host for any parasites as well. The identification of the land snails also need confirmation as there are disagreements on the classification result of land snail species. Therefore, this study was conducted to determine the prevalence of snail parasites in livestock and poultry farms from Penang and Kedah, Malaysia. Identification of the snail species was conducted based on the morphological features and mitochondrial cytochrome c oxidase subunit I (COI) gene analysis. Out of 105 individual snails collected, four species were found in this study with the highest prevalence of 47.62% (50/105) snails identified as *Physella acuta* followed by *Macrochlamys* sp. with 31.4% (33/105). While 14.3% (15/105) were identified as *Physella cubensis* and the lowest prevalence was identified as *Filopaludina* sp. with 6.7% (7/105). Among these four species, only *Physella acuta* and *Macrochlamys* sp. were found to be infected with helminth parasites namely trematode and nematode. *Macrochlamys* sp. recorded the highest prevalence of parasitic infection with the prevalence of 87.9%. Besides, there are 134 of endoparasite and 36 of ectoparasite found in this study namely, *Barchylaima fuscatum* (helminth), 3 unknown nematode, and 36 *Riccardoella limacum* or slug mite which was found on the surface and in the mantle cavity of the snails.

1. INTRODUCTION

Phylum Mollusca constitutes of a total of eight classes and Gastropoda is one them. Gastropoda includes land, freshwater and marine snails and slugs Snails is a member of the molluscan in the class Gastropoda. Its range in size from less than 1 mm to almost 1 m in length (Aktipis *et al.,* 2008). Gastropods can be further divided between pulmonates, which breath with a lung and evolved from terrestrial snails, and caenogastropoda, which respire with a gill and evolved from marine gastropods (Pyron and Brown, 2015). Therefore, snails can be found in many parts of the world such as marine, brackish, freshwater and terrestrial areas (Pyron and Brown, 2015). Snails possess a single shell with varies in shape, shell coloration, banding pattern and ornamentation considerably among major groups, which can be used for taxonomic purposes to classify genera (Jordaens *et al.,* 2008; Pyron and Brown, 2015).

Morphological taxonomy has been used in species identification for over a century. However, similarities in physical characteristics among species has become an issue in identification of species based on morphological characteristics. Previous studies have raised this issue due to the discrepancies within the method and the disagreements on the classification result of snail species (Bin Dajem, 2012; Friedheim, 2016). Besides, there are also issues regarding the phenotypic plasticity or convergence of shell shapes, the limited descriptions of cryptic species and the difficulty in applying species-diagnostic characters to juvenile snails (Wade *et al.,* 2001; Pyron and Brown, 2015; Palasio *et al.,* 2017). Recently, various molecular marker techniques have been developed for biological species identification in which DNA barcoding has been routinely facilitated the identification of species (Friedheim, 2016; Palasio *et al.,* 2017). This technique is based on the polymorphism of a short region (approximately 600 bp long) of the mitochondrial cytochrome coxidase I (COI) gene which is relatively quick and easy to be conducted (Hebert *et al.,* 2003; Palasio *et al.,* 2017).

Most of the aquatic gastropods species, are known to serve as intermediate hosts for different parasites that cause diseases such as fascioliasis, schistosomiasis and paragonimiasis. Different snails species have different ability to maintain the parasite life cycle during its infections. Recent studies show that land snails may also harbour parasites as they serve as reservoir hosts. Previously, a study by Rashed (2008) reported a new parasite metacercaria of the *Brachylaima* from the kidney of infected snail, *Monarcha obstucta* in Egypt. However, no

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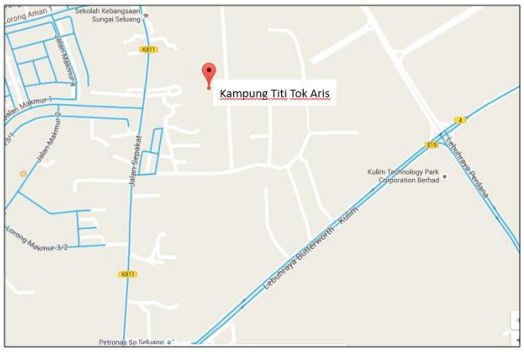
report regarding parasitism on land snails from Malaysia. There are many studies about snail as intermediate host for certain parasite up to today but only few are recently published. This study is urgently needed for local checklist documentation on this matter. Therefore, this study was conducted to confirm the identification of land snails and also to determine the snails parasites found from Penang and Kedah, Malaysia.

2. MATERIALS AND METHODS

2.1. Snails sampling

Snails were sampled from Kampung Valdor (5º14'07.0"N,100º29'20.9"E), Sungai Bakap, Penang, Kampung Titi Tok Aris (5º26'39.5"N,100º33'16.9"E), and Sungai Kob, Karangan, Kedah (5º42'35.25"N,100º64'24.12"E) (Figure 1). The snails were collected randomly in one quadrat per site (1 m x 1 m) using wire-mesh scoop , forceps or by hand. Three replicates had been conducted on each sampling site. Then, the snails were placed in separated labelled plastic aquarium. The mouths of the aquarium were covered with fine meshed nets to prevent the escape of snails and to provide good aeration. For each species, 30 adult-sized snails in habitats were collected. All the snails were brought to laboratory in Universiti Sains Malaysia for further analysis.

a)



b)

c)

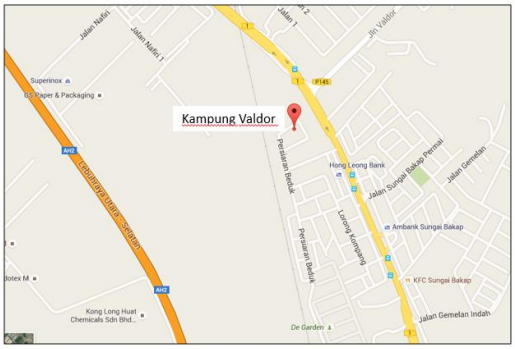


Figure 1: Google maps for sampling location in **a)** Kampung Titi Tok Aris, Kedah **b)** Sungai Kob, Kedah **c)** Kampung Valdor, Penang.

2.2. Identification of samples

2.2.1. Morphological examination

Alive snails were observed for their characteristics and were identified to family, genera, species level where possible The snails were observed under a dissecting microscope in which the shell characteristics such as shell height, shell width, colours, characters, surface characters, body whorls, and body length and the presence or absence of an operculum were measured using digital callipers. The shell of snail gave distinguishable characteristic for identification and was observed under dissecting microscope. The identification was based on various key identification from combination of multiple literature materials authored by Kathryn et al. (2004), Somsak and Burch (2004), Perez et al. (2004), Thompson (2004), and Dan and Judy (2006). The snails were then dissected to remove its soft body parts from the shell whereby the foot tissue was preserved in 75% alcohol for molecular analysis.

2.2.2. Molecular analysis

Polymerase Chain Reaction (PCR) was used in this study to identify and confirm the taxonomy of the snail species by using COI gene. Total genomic DNA was extracted from 25 mg of snail foot tissue using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. The concentration and purity of the DNA extracted were measured using a Nanodrop spectrophotometer (ACTGene, UK) at an absorbance of 260 and 280 nm wavelengths. The DNA samples were stored at –20°C. A fragment of the COI gene (~650 bp) was amplified with the primer pair LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198(5'TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). A final volume of 25 µl containing 14 µl of sterile distilled water, 3.5 µl green buffer, 3 µl $MgCl_2$, 1 µl dNTP, 1 µl primers, 1 µl DNA template and 0.5 µl Taq polymerase was prepared for the

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DNA amplification reaction. The polymerase chain reaction (PCR) was performed on a Bio-Rad MyCycler Personal Thermal Cycler (Bio-Rad) under the following parameters: pre-denaturation at 92°C for 2 min, 35 cycles of denaturation at 92°C for 40 second, annealing at 50°C for 1 min, extension at 68°C for 90 second, followed by a final extension at 68°C for 7 min. PCR products were visualized on 0.8% agarose gel electrophoresis with Lucigen 1 kb DNA ladder as a marker. The PCR products were sent for DNA purification and sequencing at Genomics BioScience and Technology Co., Ltd.. The sequences were edited using BioEdit Sequence Alignment Editor and then aligned using BLAST Sequence Analysis Tool which was performed by NCBI Database (GenBank). BLAST Analysis Tool was used to finding the similarity and comparison between sequences through the public sequence database.

2.2.3. Identification of Parasites

All collected snails were cleaned up with tap water. The body was pulled from apex and foot using forceps in the petri dish. For external parasite examination, the diagnosis was done on alive snails as parasites are easily seen on alive host. The shell of the individual snail was removed by crushing it on a petri dish. Later, the whole body of the snail was firmly squashed by pressing it with a heavy glass plate on the petri dish with a little water to allow parasites escaped to the distilled water. The specimen was observed for any parasite presents under a dissecting microscope. The parasite found was transferred into a bijou bottle containing 70% alcohol by using a small tip dropper or dissecting pin. Each bottle was labelled with snail species, date, and the location. Then the parasite was preserved on slides and staining procedure using Safranine was conducted for further identification. DPX Mountant was then dropped using wooden stick onto the parasite. Cover slip was placed carefully on the DPX to avoid formation of air bubbles. The slide was left for air dry. All possible parasites that were seen under a dissecting microscope was counted and recorded. Identification of parasites was based on Frandsen and Christensen (1984) and Awharitma et al. (2003).

2.3 Data analysis

The one-way analysis of (ANOVA) was used to investigate the variance between components with different variable. It was used to determine the significant different of the snail species and the type of parasite. Pearson correlation was used to find the relationship for both variable.

3. RESULT AND DISCUSSION

3.1 Characteristics of snails

Four snails' species were found in this study (Table 1) with the greatest parasite prevalence were identified in *Physella acuta* (47.6%) snails followed by *Macrochlamys* sp. with 31.4% and *Physella cubensis* with 14.3%. The shells of *Physella acuta* were dull black and patchy pattern, elongated cylindrical, left side of whorls coiled and the opercula was absent. Meanwhile *Macrochlamys* sp shells was transparent and yellowish brown, discoidal, right-side whorls coiled and absent of the opercula.

Macrochlamys sp. has the longest body length and the largest shell width with mean size 15.1±0.34 mm and 14.6±0.27 mm respectively. The shells of *Filopaludina* sp. (Figure 2) were dark brown and black bands shell, globose, straight columella with the plait, transverse or growth lines, right side of whorls coiled and present of the operculum. This species has the highest shell height and the largest body whorls with mean size 14.2±1.50 mm and 11.7±1.21 mm respectively (Table 2).

Macrochlamys sp. is a terrestrial pulmonate snail which belongs to the family Ariophantidae (Schilthuizen & Rutjes, 2001). It is a diverse group of terrestrial snails from the family of Helicarionidae that was found in Africa, Southeast Asia and Australia (Hyman et al., 2007). Shell of the snail that belong to this family have many variety of shape. However, the similarity between the shells may cause confusion in identifying the taxonomy of snail species.

Table 1: The relative density (%) of snails based on the collection sites.

Snail species	Mean ± SE (mm)				No. of whorls
	Shell height	Shell width	Body whorls	Body length	
Macrochlamys sp.	8.23±0.17	14.59±0.27	7.56±0.15	15.09±0.34	4
Physella acuta	7.29±0.11	3.92±0.06	5.96±0.09	3.67±0.09	4
Physella cubensis	7.14±0.24	5.87±0.20	3.54±0.13	3.86±0.11	4
Filopaludina sp.	14.17±1.50	10.28 0.72	11.74±1.21	6.79±0.58	3

Table 2: Mean ± SE (mm) of the shell measurements for the identified snails.

Snail species	Location	
	Kedah	Penang
Macrochlamys sp.	-	100
Physella acuta	69.5	-
Physella cubensis	20.8	-
Filopaludina sp.	9.7	-

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Figure 2: *Filopaludina* sp.

Thirty-three (33) snail size of this species from the sample has about 6-8 mm shell height and about 12- 16 mm shell width, were within the size range. Meanwhile, *Physella acuta* and *Filopaludina* sp. are the freshwater snails. *Physella acuta* is a pulmonate snail and belongs to the family of Physidae (Wethington & Wise, 2009). This snail species is the most successful physid which is found worldwide in a variety of freshwater habitats. From seven snails that had been collected during this study, the size of the *Filopaludina* sp. measured and recorded were about 15-22 mm height and 10-13 mm width. According to Taylor (2003), about 80 species of Physidae that have been classified according to the penial complex. Meanwhile, *Filopaludina* sp. is a caenogastropoda snail belongs to the family Viviparidae which is a subclass of Prosobranchia and has been recorded in Europe, Asia and Eastern North America (Thompson, 2004). This family can usually be found at the rivers, lakes, ponds, swamps, and canals (Strong et al., 2008).

Both genetic and morphological method used may give precise taxonomy of snail and accurate classification of gastropod (Bin Dajem, 2012). The gene regions that usually used in determined snail taxonomy was COI which commonly used by many researcher include Wethington and Lydeard (2007); Hyman *et al.*, (2007), Aktipis *et al.,* (2008) and Wethington and Wise (2009). Thus, in this study the result has assured that the three snails were *Macrochlamys* sp., *Filopaludina* sp. and *Physella acuta*. The results obtained from PCR shows that *Physella cubensis* had the similar sequence as *Physella acuta.* Based on the previous study by Paraense and Pointier (2003), it was proved that *Physella cubensis* was synonymy under name of *Physella acuta.*

From the BLAST result (Table 3), snails that was found at Sungai Kob was *Filopaludina* sp. had disclosed sequence of *Bellamya* sp. which was 87 % identity from 37 % quary cover (quary length). The BLAST report indicates the alignment sequence of *Filopaludina* sp. from 304 to 496 amino acid sequence had match with 15 to 207 sequence provide by database. While snails at Kampung Valdor, *Macrochlamys* sp. had higher sequence percentage of query cover, with 98 % identity. The others species from Kampung Titi Tok Aris was *Physella acuta* with identities 100 % match to the database sequence. From BLAST result, sequence of *Physella cubensis* had confirm was *Physella acuta* which identity 93 % query. However, the result was compare to the morphological characteristic, the species was different. The result was referring to the accession that was provide from the public sequence database. Analysis of homolog using BLAST revealed that *P. acuta* is correctly identified with 100% similarity (NCBI:txid109671) and *Macrochlamys* sp. revealed 99% similarity to *Macrochlamys* sp. (EF015438.1) by Hyman and Jermiin (2007). The analysis of homolog for *Filopaludina* sp. Revealed 87% similarity to the sequence of Grabner et al. (2014) which is *Bellamya* sp. (KF412773.1).

Table 3: The confirmation of snail species based on BLAST

Snail species	Query cover (%)	E-value	Identities (%)	Accession
Filopaludina sp.	37	3e-51	87	KF4122773.1
Macrochlamys sp.	98	0.0	99	EF015438.1
Physella acuta	93	0.0	100	KT280442.1
Physella cubensis	92	0.0	99	KT280442.1

3.2 Parasites determination

Out of 105 snail samples collected, 33.4% were found to be infected with parasites (Table 4). Only *Physella acuta* and *Macrochlamys* sp. snails were recorded positive for parasites such as trematode and nematode. *Macrochlamys* sp. recorded the highest number of parasitic infection with 87.9%. However, no parasites were found in *Filopaludina* sp. and *Physella cubensis*. Interestingly, in this study, the snails were also infested with ectoparasite, *Riccardoella limacum* or slug mite which was found on the surface and in the mantle cavity of the snails (Table 5). Meanwhile, five *Physella acuta* was reported with an unknown trematode. The infection rate of *Macrochlamys* sp. snails was 87.9% and they had been infected with trematode *Brachylaima fuscatum* together with mites, *Riccardoella limacum.* One-way ANOVA was tested to find the significant difference between snail species and the type of parasite. The result indicates there was a significant difference in snail species and the trematode ($P<0.05$). Pearson correlation shows *Macrochlamys* sp. had relationship with the trematode, nematode and mite occurrences.

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Table 4: Number of parasites recovered in snails from Kedah and Penang, Malaysia

Snail Species	Number of samples	Endoparasite		Ectoparasite
		Trematode	Nematode	Mites
Macrochlamys sp.	33	126	3	36
Physella acuta	50	5	-	-
Physella cubensis	15	-	-	-
Filopaludina sp.	7	-	-	-
Total	105	131	3	36

Table 5: Percentage of snail infection based on type of parasite found in each species of snail

Snail species	No. of snail	No. of snail infected	Type of parasite	Percentage of infection (%)
Macrochlamys sp.	33	29	*Brachylaima fuscatum*	87.9
		2	Unknown nematode	6.1
		6	*Riccardoella limacum*	18.2
Physella acuta	55	5	Unknown trematode	10
Physella cubensis	15	0	-	0
Filopaludina sp.	7	0	-	0

Land and freshwater snails are intermediate hosts in the life cycle of various parasites. Most parasites require a specific snail species as their intermediate host. For example, the life cycles of *Brachylaima fuscatum* require *Macrochlamys* sp. as their intermediate hosts. A total of 126 trematodes were found as *B. fuscatum* on *Macrochlamys* sp. *Brachylaima fuscatum* is a digenetic trematode of the family Brachylaimidae with worldwide distribution (Sirgel et al., 2012). However, this parasite was rarely found in human and domestic animals (Heneberg et al., 2016). Other than *Macrochlamys* sp. these parasites also can affect other terrestrial snails namely, *Helicella* sp., *Oxychilus* sp., and *Agrolimax* sp. (Awharitma et al., 2003). *Riccardoella limacum* (Schrank) also known as slug mite is an ectoparasite which belongs to the family of Ereynetidae. This parasitic mite was found on the body surface of the snail as well as inside the mantle cavity and moved rapidly over the snail. A total of 36 individual of mites was identified as *Riccardoella limacum* infesting *Macrochlamys* sp. Although it has not been recorded that this species can be a host for this mite, it is possible because *Macrochlamys* sp. is one of the pulmonates snails. According to a study by Faltýnková (2005), *Physella acuta* had served as an intermediate host for *Echinostoma* sp., *Echinoparyphium aconiatum* and *Opisthioglyphe ranae* with low infection for *P.acuta*. Unfortunately, identification based on morphological characteristics of five trematodes found in *P.acuta* was not successful due to their small size and number. Apart from that, *Filopaludina* sp. found in this study was free from any parasitic infection. Only detritus worms were present from the dissection of *Filopaludina* sp.

4. CONCLUSION

A total of four species of snails namely, *Macrochlamys* sp., *Physella acuta*, *Physella cubensis* and *Filopaludina* sp. were successfully identified based on the morphological shell characteristics and mitochondrial cytochrome c oxidase subunit I (COI) gene analysis. Besides, based on morphological and molecular technique, the snail species that was found at Kampung Valdor and Kampung Titi Tok Aris had confirmed as *Macrochlamys* sp. and *Physella acuta*. According to the result given, the sequence of PCR product was successful compare from the GenBank of database provided. Both morphology and molecular techniques are important in order to identify the taxonomy of snail. There is a particularly similarity in the morphology that was extremely confuse during shell examination. Thus, molecular technique is reliable and comparable for the species identification. Out of four species of snails, trematode, *Brachylaima fuscatum* with mites, *Riccardoella limacum* was recorded from *Macrochlamys* sp.

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