Morphological and Molecular Identification of Lymnaeid snail and Trematodes Cercariae in Different Water Bodies in Perak, Malaysia

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Abstract

Lymnaeid snails are vital in transmitting trematode cercariae as an intermediate host that can infect buffalo and other ruminants and humans, causing significant economic losses. The study aimed to conduct morphological and molecular identification of snails and cercariae collected from the selected buffalo farms under palm oil integration in Perak, Malaysia. A cross-sectional study was conducted, and 35 water were investigated for the presence or absence of snails. A total of 836 lymnaeid snails were collected from three marshes wetlands. To identify the snail family and species, morphological identification was performed on each snail's shell, and to identify trematode cercariae types; the crushing method was used to observe the cercarial stage inside each snail's body. In addition, the Internal Transcribed Spacer 2 (ITS2) region and Cytochrome c oxidase subunit 1 (*cox*1) was used as the target gene to identify the snail species and cercarial types up to the species level. The result showed that the collected snails belong to the family Lymnaeidae and *Radix rubiginosa* species. The infection rate by cercarial emergence in snails was 8.73%. Five morphological cercarial types were observed which include: echinostome, xiphidiocercariae, gymnocephalous, brevifurcate-apharyngeate distome, and vivax. Using molecular methods, the identified cercariae belong to the three families, including Echinostomatidae, Plagiorchiidae, and Fasciolidae. This is the first report on *R. rubiginosa* and different types of trematodes cercariae in buffalo farms under palm oil integration in Perak. Our finding confirmed that *R. rubiginosa* could serve as an intermediate host for a range of parasitic trematodes in Perak.

Introduction

Freshwater snails play an important role as intermediate hosts of several trematodes species in the superfamilies such as Paramphistomoidea, Fascioloidea, Clinostomoidea, Diplostomoidea, Echinostomatoidea, Pronocephaloidea and Schistosomatoidea (Bawm et al. 2022; Martin & Cabrera, 2018; Islam et al. 2013). These trematodes parasites in many tropical and sub-tropical countries cause public health threats and socioeconomic problems (Tookhy et al. 2022; Prasopdee et al. 2015). Family Lymnaeidae receives much interest among freshwater snails since they are involved in the life cycle of several trematodes with substantial biomedical and veterinary implications (Saijuntha et al. 2021; Mohammed et al. 2016; Bargues et al. 2011).

The cercarial stage is a free-living stage that lives inside the snail's intermediate host and emerges when it is entirely mature to hunt for a suitable second intermediate host or some species that encyst on water plants (Poulin & Cribb 2002). On the plants, the cercariae encyst to metacercariae are ingested by appropriate ruminant hosts and later become adults in the rumen, which causes significant economic losses (Huson et al. 2017; Chaudhry et al. 2017). Because data on cercarial infection in snails is essential for epidemiological modelling and integrated management, studies on trematode larval stages in snail hosts have received more attention in recent years (Prastowo et al. 2022; Nguyen et al. 2021; Wiroonpan et al. 2021).

Trematode cercariae can be divided into different types based on their morphological features, such as Pleurolophocercous cercariae, Furcocercous cercariae, Monostome cercariae, Echinostome cercariae, Xiphidiocercariae, Gymnocephalous cercariae, and Amphistome cercariae (Lockyer et al. 2004; Ito 1978). Additionally, distinct types of cercariae morphologically could be assigned to families, superfamilies, or genera, such as amphistome cercariae, which have been identified as the family Paramphistomatidae and Paramphistomum genera (Frandsen & Christensen 1984), pleurolophocercous cercariae as Opisthorchiidae family and Opisthorchis genera, Heterophyidae family and Heterophyes genera (Chontananarth & Wongsawad 2010), and echinostome cercariae as Echinostomatidae family and Echinostome genera (Chontananarth & Wongsawad 2013).

Many studies have previously reported the incidence of trematode infections in various snail species in different countries and reported various types of cercariae with different ranges (0.94–84%) in Thailand (Prastowo et al. 2022; Tapdara et al. 2022; Dunghungzin & Chontananarth 2020), Vietnam (Nguyen et al. 2021; Doanh et al. 2020), Myanmar (Bawm et al. 2022), Nepal (Devkota et al. 2011), Philippines (Martin & Cabrera 2018), Germany (Soldánov et al. 2010), Bangladesh (Islam et al. 2013), Iran (Rivaz et al. 2014), Sudan (Mohamfmed et al. 2016), Nigeria (Luka & Mbaya 2015), Central Europe (Faltýnková et al. 2008), France (Abrous et al. 1999), Egypt (Ibrahim & Ahmed 2019) and Ethiopia (Mereta et al. 2019). Although snail infection rates are frequently low, one infected snail may release many parasite larvae (Piratae 2015). Currently, the world is experiencing issues with the fast-growing snail population, especially near dams and lakes where parasitic diseases transmitted by snails could spread widely (Sokolow et al. 2017).

The conventional method used to identify snails and trematode cercariae by morphological characteristics is underestimated, and the method is fragrantly complex due to morphological similarity (Chontananarth et al. 2017; Anucherngchai et al. 2016; Correa et al. 2011). Identification of cercarial specimens at the family and occasionally genus levels using only morphological characteristics is possible (Frandsen & Christensen 1984). However, it is challenging to distinguish minor changes in cercarial morphology between several trematode genera and between various species within each trematode genus. It is necessary to conduct a correct cercarial identification on the genus and species level (Frandsen & Christensen, 1984). Additionally, within lymnaeid snails, intraspecific variation in shell shape is particularly well-marked and depends on environmental factors that complicate the identification (Correa et al. 2011; Pfenninger et al. 2006).

Therefore, molecular methods provide better resolution on snail and trematode identification of larval stage and understanding of the life cycle (Locke et al. 2011; Caron et al. 2011; Kaset et al. 2010). Notably, the ITS2 and *cox*1 could be used for the identification of snails and different stages such as cercariae, metacercariae, and adult stages in intermediate and/or definitive hosts and used in phylogenetic relationship analysis (Nguyen et al. 2021; Wiroonpan et al. 2021; Japa et al. 2021; Chontananarth et al. 2017; Dunghungzin & Chontananarth 2020; Anucherngchai et al. 2016; Sripalwit et al. 2015).

Previous to this study, no morphological and molecular sequence data analysis from freshwater snails and trematode cercariae had been done in Perak and other parts of Malaysia. Therefore, this study aims to use both morphological and molecular identification of snails and cercariae collected from the

water bodies around the selected buffalo farms under palm oil integration in Perak.

Materials And Methods

The study areas and sampling strategy

The study was conducted in Perak, Malaysia. Perak is a state in the northwest of Peninsular Malaysia. It is between 4° 41' 38.2200" N latitude and 101° 7' 3.2772" E longitude (Fig. 1). Its geographical area is 20.976 km². The annual rainfall in Perak is around 2169 mm. The average annual temperature is 26.5°C. The annual average humidity is 86.5% (Malaysian metrological department). In Perak, there are many rainy seasons, especially October, the wettest month, and July, the driest month (Malaysian metrological department).

Snail Collection And Identification

In the study site, semi-quantitative snail sampling was carried out. A total of 35 sites which include five types of water bodies (ditches = 6 sites; ponds = 9 sites; irrigation canals 6 sites; river = 2 sites and wetlands = 12 sites), were surveyed in two trips during March and July 2022 for present and absent of snails (Figs. 2 and 3). Snail sampling was conducted at each site for 20 minutes between 8 to 11 am. Snail collection was always performed by the same person using a scoop net with wire mesh measuring 1.5 mm on an iron frame (40x30 cm) and was mounted on a 1.5 m long iron handle (Mereta et al. 2019; Opisa et al. 2011). The snails were placed individually in a 45 ml capacity container with a perforated cover to provide them with good aeration and prevent the captive snail from escaping (Ramitha & Vasandakumar 2015). Each container was filled with water from their habitats, and the snails were transported to the Parasitology Laboratory, Faculty of Veterinary Medicine, University Putra Malaysia, for further processing.

Snail Dissection And Morphometric

All snails were classified according to shell morphology and identified according to Burch (1984) and Samadi et al. (2000). The flesh of each snail was carefully removed from the shell, and a sterile surgical blade was used to dissect the foot tissue. Shell was examined grossly and then under a stereomicroscope. The parameter such as shell shape, whorls, aperture, spire, inner lip, parietal wall, and columella was examined. In addition, the height and width of the body, spire length, aperture length, and width are measured and recorded using digital callipers (Vernier Caliper) as described previously (Al-Asadi 2011). For further molecular studies, foot and soft body tissue were separated separately in microfuge tubes and kept at − 20°C. The measurements were made using digital callipers.

Morphological Identification Of Cercariae

To investigate the presence of cercariae in the snails, each snail was inspected for the presence or absence of cercariae under a stereomicroscope using a crushing method (Tapdara et al. 2022; Wiroonpan et al. 2021). Later, the motility and morphology of live cercariae were recorded under the microscope and photographed for classification based on gross characteristics, resting position, swimming behaviour, and further cercarial development (Schell et al. 1985; Frandsen & Christensen 1984). Cercariae were collected and preserved in 70% ethanol in a 1.5 ml sterile tube for DNA extraction.

Dna Extraction Of Snail And Cercariae

Out of 836 snails, about 2% (19) of snail samples were randomly selected and used for DNA extraction. In addition, fresh specimens of cercariae were preserved in microcentrifuge tubes with absolute ethanol (99%). The representative of each group (approximately 10 cercariae) was randomly selected and used for DNA extraction. The DNA extraction was performed by DNeasy Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's instructions. The extraction DNA was stored at − 80°C until used for PCR.

Polymerase Chain Reaction (Pcr)

PCR was set up to amplify partial fragments of the ITS2 and *cox*1 of snail and cercariae DNA. PCR reaction of 25 µl was carried out for both snail and cercariae containing 4 µl of DNA template, 8.4 µl DH20, GoTaq® G2 Hot Start Green Master Mix (Promega, USA), and 1 µl of forward and reverse primers. Details of sequences of three types of primers used in this study and PCR cycling profiles are given in Table 1. In addition, the amplicons were examined by 1.4% agarose gel electrophoresis in Tris-acetate-EDTA (TAE) and stained with 5 µl RedSafe Nucleic Acid Staining Solution (Iron Biotech, Korea).

Table 1
Summary of primers used in PCR amplification

Gene	Primer sequence 5' >3	PCR condition	Reference
ITS2	F: news TGTGTCGATGAAGAACGCAG R: Rixo2 TTCTATGCTTAAATTCAGGGG	94°C for 2 min, 30 cycles of 98°C for 30 s, 50°C for 30 s, 72°C for 30 s, 72°C for 7 min	Correa et al. (2011)
ITS2	F: ITS3 TCC TCC GCT TAT TGA TAT GC R: ITS3 TCC TCC GCT TAT TGA TAT GC	94°C for 4 min 30 cycles of 98°C for 10 s, 50°C for 15 s, 68°C for 1 min, 68°C for 5 min	Barber et al. (2000)
*Cox*1	F: LCO1490 TAAACTTCAGGGTGACCAAAAAATCA R: HCO2198 TAAACTTCAGGGTGACCAAAAAATCA	95°C for 5 min 35 cycles of 95°C for 1 min, 40°C for 1 min, 72°C for 90 s, 72°C for 7 min	Folmer et al. (1994)

Sequence Analysis

The purification and bidirectional DNA sequence analysis were done by 1st BASE Company (Malaysia) using the same primers pairs as for the PCR product. The obtained sequences of snail and cercariae were edited using BioEdit version 7.2 (North Carolina State University. Raleigh, NC, USA). In addition, the ITS2 sequences of snail and cercariae, which were discovered in the present study submitted to the GenBank database (National Center for Biotechnology Information, Bethesda, Maryland, USA), and the accession number was provided. All the sequences obtained in the present study are available in the GenBank database under the accession numbers OP646430 - OP646448, OP647125–126, OP647206–216, and OP647263–270, respectively.

Results

Morphological identification of the snails

In the present study, 35 sites have been investigated in the study area. Except for three marshes wetlands, the remaining sites were found to be negative for the presence of snails. Table 2 shows five types of water bodies, the number of sites, and several snails collected from each site in Perak. Out of 836 snails examined, all were morphologically identified as the family Lymnaeidae and *R. rubiginosa* species.

Table 2 Five different types of water bodies, number of sites, and number of snails collected from each site in Perak

Site type	The number of sites investigated	No. of snails collected
Ditches	6	0
Ponds	9	0
Irrigation canal	6	0
Rivers	2	0
Wetlands	12	876
Total	35	876

Shell morphology of *Radix rubiginosa*

The shell was medium, elongated, cylindrical, and coiled to the right (dextral). The number of whorls was five. The basal whorl was markedly swollen, the aperture was longer than the spire, and the inner lip was extended over the parietal wall. Columella was almost twisted. Partially translucent with evident transverse striae (Fig. 4). The measurement of shell length, aperture length, aperture width, and spire length are described in Table 3. The morphological specification led to the identification of *R. rubiginosa*.

Table 3 Shell morphological parameters, minimum, maximum and average size of R. rubiginosa in this study

Shell character	Minimum (mm)	Maximum (mm)	Mean ± SE (mm)
Shell height	10.5	19.5	15
Shell width	5.6	9.9	7.7
Aperture height	7	11.4	9.2
Aperture width	4.1	7.5	5.8
Spire height	2.1	6.3	4.25

Morphological identification of cercariae and infection rate

In the present study, five morphological types of cercariae (types A-E) were identified as follows: echinostome cercariae (type A), xiphidiocercariae (type B), gymnocephalous cercariae (type C), brevifurcate-apharyngeate distome cercariae (type D), and vivax cercariae (type E) (Fig 5). Table 4 details the infection rates of each type of cercariae in *R. rubiginosa*. The overall infection rate of cercariae in snails was 8.73% (73/836). The results show that echinostome cercariae had the highest infection rate at 69.86% (51/73), followed by xiphidiocercariae at 21.9% (16/73), gymnocephalous cercariae at 4.1% (3/73), brevifurcate-apharyngeate distome cercariae 2.73% (2/73), and vivax cercariae had the lowest infection rate 1.36% (1/73).

Table 4 Prevalence of cercarial infections in marshes wetlands in R. rubiginosa in buffalo farms in the present study

No. of collected Snail	No. of examined snail	No. of snail infected	No. of the infected snail with cercariae species					Prevalence % (no. of infected/no. of examined	Mixed infection
			Gymnocephalous	Echinostome cercariae	Xiphidiocercariae	Brevifurcate-apharyngeate distome cercariae	Vivax cercariae		
876	836	73	4.1% (3/73)	69.86% (51/73)	21.91% (16/73)	2.73% (2/73)	1.36% (1/73)	8.73% (73/836)	0.97% (8/836)

Morphological description of cercarial morphotypes

Echinostome cercariae

The body was elongated and pear-shaped. The oral sucker (os) lacks a stylet and circular shape and is present at the sub-terminal section of the body, which is surrounded by a spiny collar (sc). The ventral sucker (vs) was more significant than the oral sucker and was spherical, located approximately two-thirds of the body length measured from the front. The primary collecting tubes (mct) were located on both sides of the body from the pharynx to the ventral sucker and contained large, blackish-bordered granules. The tail (ta) was slender, almost the same as the body, and was unforked. Eyespot was absent (Fig. 5-a).

Xiphidiocercariae

The body of the cercariae was small, flat, and oblate-shaped. The oral sucker was small, circular, and sub-terminal, while the ventral sucker was present in the middle of the body but smaller than the oral sucker. A stylet was found within the centre of the oral sucker. The tail was unforked and shorter than the body length. The eyespot was absent (Fig. 5-b).

Gymnocephalous cercariae

The ventral sucker was located at the mid-ventral surface of the body, and the size was the same as an oral sucker. Not stylet and the spiny collar was absent, eyespot was absent, and the tail was unforked and was longer than the body (Fig. 5-c).

Brevifurcate-apharyngeate distome cercariae

The oral sucker and ventral sucker were present. The pharynx was absent. The tail was elongated, longer than the body, and bifurcated with longitudinal muscular fibers. (Fig. 5-d and e).

Longifurcate-pharyngeate monostome cercariae (Vivax cercariae)

The body was small and oval. The oral sucker was present, but the ventral sucker was vestigial, smaller than the oral sucker, and existed at around two-thirds of the body length. Body finfold was not present, but sometimes furcal finfold is present. Caudal bodies (cb) in the tailstem were not present. The tail was longer and broader than the body, spined, blunt spined, and bifurcated, with longitudinal muscle fibers and furcae length shorter than the tail length and sharp (Fig. 5-f).

Molecular identification of snail

BioEdit software was used to review the sequences and ensure the sequence quality. The ITS2 sequence length ranged from 534-548 bp (Fig. 6), and fragments were trimmed to 454 bp for homology analysis with published sequences in the GenBank using NCBI BLAST (National Center for

Biotechnology Information). The *cox*1 sequence length ranged from 672 – 680 bp (Fig. 7) and was trimmed to 656 bp. The BLAST result showed that all the ITS2 sequences had 98.41 – 99.34% similarities with *R. rubiginosa* in Thailand (KX056267) and Vietnam (LC659106, KF042385). *cox*1 sequence had similarities of 96 – 96.65% with *R. rubiginosa* in Indonesia (KY574609, KX056255), Thailand (KM067685), and Vietnam (LC658537, LC658538).

Molecular identification of cercariae

Of 24 samples of five morphological types of cercariae, 21 were successfully amplified and sequenced for ITS2 and *cox*1. 21 novel sequences of the ITS2 region (401 – 588 base pairs) (Fig. 8) were identified. All the cercarial sequences were compared with NCBI BLAST results. The cercarial sequences in the present study had (92.47-99.61%) similarities with one of the trematode species in GenBank. The results show that the sequence of echinostome (type A) cercariae was most likely to be *Echinoparyphium mordvilkowi* and *E. bolschewense* and was aligned with *E. mordvilkowi* from Lithuania (KJ542640) and *E. bolschewense* from Russia (MZ517175). Xiphidiocercariae (Type B) is likely to be *Plagiorchis maculosus* and was aligned with *P. maculosus* from the Czech Republic (KJ533391). Gymnocephalous cercariae (type C) looks to be *Fasciola gigantica* and has more similarities with *F. gigantica* in buffalo from Vietnam (MT429177) and in *Bos indicus* from India (KT199360). In addition, Cox1 also ran to reconfirm the types of cercariae, but the sequence result in the BLAST showed similarities of 96.6% for *R. rubiginosa*.

Discussion

The present study is the first morphological and molecular identification of freshwater snails and cercariae in water bodies in Malaysia. In the present study, out of five types of water bodies (ditches, ponds, irrigation canals, rivers, and wetlands) investigated, only marshes wetlands were found to be positive for snails. We detected only one species of snail, *R. rubiginosa*. *Radix rubiginosa* has also been reported from different water bodies in Singapore, Thailand, Israel, Cambodia, Laos, Vietnam, Philippines, and France (Stelbrink et al. 2019; Ng et al. 2016; Aksenova et al. 2016; Roll et al. 2009; Brandt, 1974). Our morphological finding on *R. rubiginosa* is almost the same as that of Dung et al. (2013), Monzon et al. (1994), and Appleton & Miranda (2015), which carried on *R. rubiginosa* in Vietnam, Thailand, and South Africa. Generally, freshwater snail shells typically provide crucial taxonomic data that can be utilized to demonstrate species differences and reveal evolutionary links among various taxa (Opeyemi & Alexander 2018).

Radix rubiginosa may grow in several habitats, including rivers, lakes, rivers, rice fields, ponds, wetlands, and canals in its native location because it is tolerant to a broader range of temperatures, conductivity, and pH (Chitramvong et al. 1981; Brandt 1974). It is well-documented that physical geography, physiochemical characteristics, bottom oil deposits, and macrophyte abundance all influence the diversity of freshwater snails (Omudu & Iyough 2005). Environmental changes without genetic change may result in distinct non-genetic changes in snail shell morphology. Rundle et al. (2004) indicated that calcium contributes to shell formation, and calcium levels in the environment act as limiting factors and selective pressure on the shell morphology. Most times, calcium and water pH correlate favourably, and snail shells are eroded and damaged easily in an environment with low calcium and low pH (Glass & Darby 2009). In our study, we found the presence of Lymnaea snails in marshes wetlands, and the pH values were neutral to alkaline and ranged from 7–8.3. There are different types of wetlands, including swamps, marshes, and bogs. The area where we collected the Lymnaea snails is the marshes wetlands. According to the United States Environmental Protection Agency (EPA), Marshes are frequently or continually inundated wetlands with water, characterized by emergent soft-stemmed vegetation adapted to saturated soil conditions. Standley et al. (2013) reported the presence of Lymnaea/Galba in shallow water bodies with alkaline pH, similar to the present study. According to Olsen et al. (2015), environmental variables such as wetlands, streams, and pastures were positively associated with *F. hepatica* in cattle. Marshes wetlands are known to have humid circumstances favourable for intermediate host snail survival and growth, host snail infection development, and transfer of free-living fluke stages (Kuerpick et al. 2013; Boray & Love 2007). In our study, the snails were collected from the buffalo farms under palm oil integration, and except for three marshes wetlands, all types of water bodies were negative for the presence of snails. This absence of snails in the water bodies could be due to palm oil farm, as in the forest area, there is a lack of enough sunlight, which limit the growth of food algae which is essential for the snail to breed and survive (Kuerpick et al. 2013; Pullan et al. 1972).

In addition, this study's infection rate by cercarial infection in *R. rubiginosa* was 8.73% (73/836). Mixed infections of two or more cercarial types in *R. rubiginosa* were also detected in 0.97% (8/836) of snails. Our findings are in line with the studies by Tapdara et al. (2022) in Thailand, Veeravechsukij et al. (2018) in Thailand, and Rafiq et al. (2022) in Pakistan who reported the infection rate of cercariae in freshwater snails as 5.57%, 10.46%, and 8.19% respectively. The infection rate in the present study was higher than Prastowo et al. (2022) in Indonesia, Japa et al. (2021) in Thailand, Mereta et al. (2019) in Ethiopia, and Luka & Mbaya (2015) in Nigeria, and it lower than Ibrahim & Ahmed (2019) in Egypt, Martin & Cabrera (2018) in Philippines, Okeke & Ubachukwu (2017) in Nigeria, and Maye et al. (2016) in Nepal. The difference in infection rates in different countries could be due to several factors, such as climatic conditions, study sites, free grazing animals, movement of wild animals, and sample size.

Based on morphological identification, five types of cercariae were recorded: echinostome, xiphidiocercariae, gymnocephalous, brevifurcate-apharyngeate distome, and vivax cercariae. Echinostome cercariae and brevifurcate-apharyngeate distome cercariae were reported in *R. rubiginosa* by Bawm et al. (2022) in Myanmar, Japa et al. (2021) in Thailand, and Imani-Baran et al. (2013) in Iran which are consistent with our study. The presence of brevifurcate-apharyngeate distome and vivax cercariae indicates Schistosoma infection in the area (Owojori et al. 2006; Frandsen & Christensen, 1984). Brandt (1974) indicated that known to harbour the larval stage of *Schistosoma incognitum*. This blood fluke causes cercarial dermatitis in humans. It is also the first intermediate host of *F. hepatica*, *F. gigantica*, *Orientobilharzia barinasutai* and several avian species of blood fluke, which cause cercarial dermatitis in Thailand. In an Indonesian study, lymnaeid snails harboured gymnocephalous cercariae (Duan et al. 2021), which is consistent with the present study. Likewise, other previous studies reported different types of cercariae in freshwater snails, such as echinostome cercariae, xiphidiocercariae, brevifurcate-apharyngeate distome, vivax cercariae, strigea cercariae, parapleurolophocercous cercariae, gymnocephalous cercariae, monostome cercariae,

megarulous cercariae, and virgulate cercariae from Myanmar (Thu et al. 2016), Vietnam (Nguyen et al. 2021), Thailand (Wiroonpan et al. 2021; Dunghungzin & Chontananarth, 2020; Chontananarth et al. 2017), and Denmark (Duan et al. 2021). The variation of cercariae types in different countries could be due to the types of snails in freshwater, types of water bodies, and climatic conditions.

According to the molecular investigation, cercariae investigated, the cercarial types are classified into three families of digenean trematodes, including Echinostomatidae (*E. mordvilkowi* and *E. bolschewense* species), Plagiorchiidae (*P. maculosus*), and Fasciolidae (*F. gigantica*). *Echinoparyphium* is an important taxon in the Echinostomatidae family and has medical, veterinary, and wildlife importance (Fried 2001). *E. mordvilkowi and E. bolschewense* belong to the genus *Echinostoma*; intestinal parasites infect vertebrate host species, including humans (Fu et al. 2019). According to Toledo & Esteban (2015), human echinostomiasis is endemic in Southeast Asia and the Far East, including China, India, Indonesia, Korea, Malaysia, Nepal, Philippines, and Thailand. In addition, in the present study, we detected *Plagiorchis* spp. from *R. rubiginosa*. *Plagiorchis* spp. has a three-host life cycle, freshwater snail act as the first intermediate host, arthropods as the second intermediate, and vertebrates, including humans, as definitive hosts (Gordy et al. 2016; Guk et al. 2007). There are reports of the zoonotic importance of *Plagiorchis* spp. from Thailand, Indonesia, Korea, Philippines, and Japan (Guk et al. 2007).

In the present study, we detected *F. gigantica* from *R. rubiginosa*. Fascioliasis is a foodborne trematodiases and is among the Neglected Tropical Diseases (NTDs) given priority by the World Health Organization (WHO 2020; WHO 2013). The parasite causes zoonotic diseases that infect mammals, especially herbivorous ruminants, and humans also become infected by ingestion of infective metacercariae through food and drinking water (Mas-Coma et al. 2018). Naresh et al. (2006) reported A case of human fascioliasis in a man in Malaysia.

It is possible to classify the snails and cercariae by both morphological and molecular methods, but molecular methods are more sensitive and identify the snail and trematode cercariae at the specie level (Mereta et al. 2019; Bin Dajem 2012). Several gene regions, including ITS1, ITS2, 16S, 18S, and *cox*1, have been employed in the taxonomy and phylogenetic analysis of Lymnaeidae snails and cercariae (Bawm et al. 2022; Prastowo et al. 2022; Schniebs et al. 2022; Japa et al. 2021; Nguyen et al. 2021; Correa et al. 2010; Bargues & Mas-Coma 2005). In the present study, ITS2 was more sensitive than *cox*1 for identifying Lymnaeid snails and cercariae. Bargues & Mas-Coma (2005) and Choi et al. (2015) indicated that the nuclear rDNA ITS2 had been the most useful molecular marker in lymnaeid snails and cercariae studies.

Conclusion

Our study confirmed the presence of *R. rubiginosa* in buffalo farms under palm oil integration in Perak by morphological and molecular methods for the first time. In addition, three trematode families (Echinostomatidae, Plagiorchiidae, and Fasciolidae) in *R. rubiginosa* were confirmed by molecular method. It is found that marshes wetlands appear as a suitable environment for *R. rubiginosa*, which plays a vital role as an intermediate host for various trematodes. *R. rubiginosa* snail was found to harbour five types of cercariae, indicating the importance of freshwater snails in transmitting trematodes.

Declarations

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Authors contribution Conceptualization: Nazir Ahmad Tookhy, Nur Mahiza Md Isa, Rozaihan Mansor, Yasmin Abd Rahman, and Nur Indah Ahmad. Methodology: Nazir Ahmad Tookhy, Dung Thi Bui, Lokman Hakim Idris, Norhadila Zulkifli. First draft of the manuscript: Nazir Ahmad Tookhy. Supervision: Nur Mahiza Md Isa. Editing: Noor Hazfalinda Hamzah. Approval of the final version of the manuscript: All authors.

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Data availability The sequences generated for the ITS2 and *COX*1 gene in the present study are available in the GenBank database under the accession numbers OP646430 - OP646448, OP647125 - 126, OP647206 – 216, and OP647263 – 270, respectively.

Ethical approval All experimental procedures involving animals were approved by the Institute Animal Care and Use Committee (IACUC) in the Faculty of Veterinary Medicine, University Putra Malaysia (UPM/IACUC/AUP-R043/2022).

Consent to participate Not applicable.

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Conflict of interest The authors declare that they have no conflict of interest.

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Figures

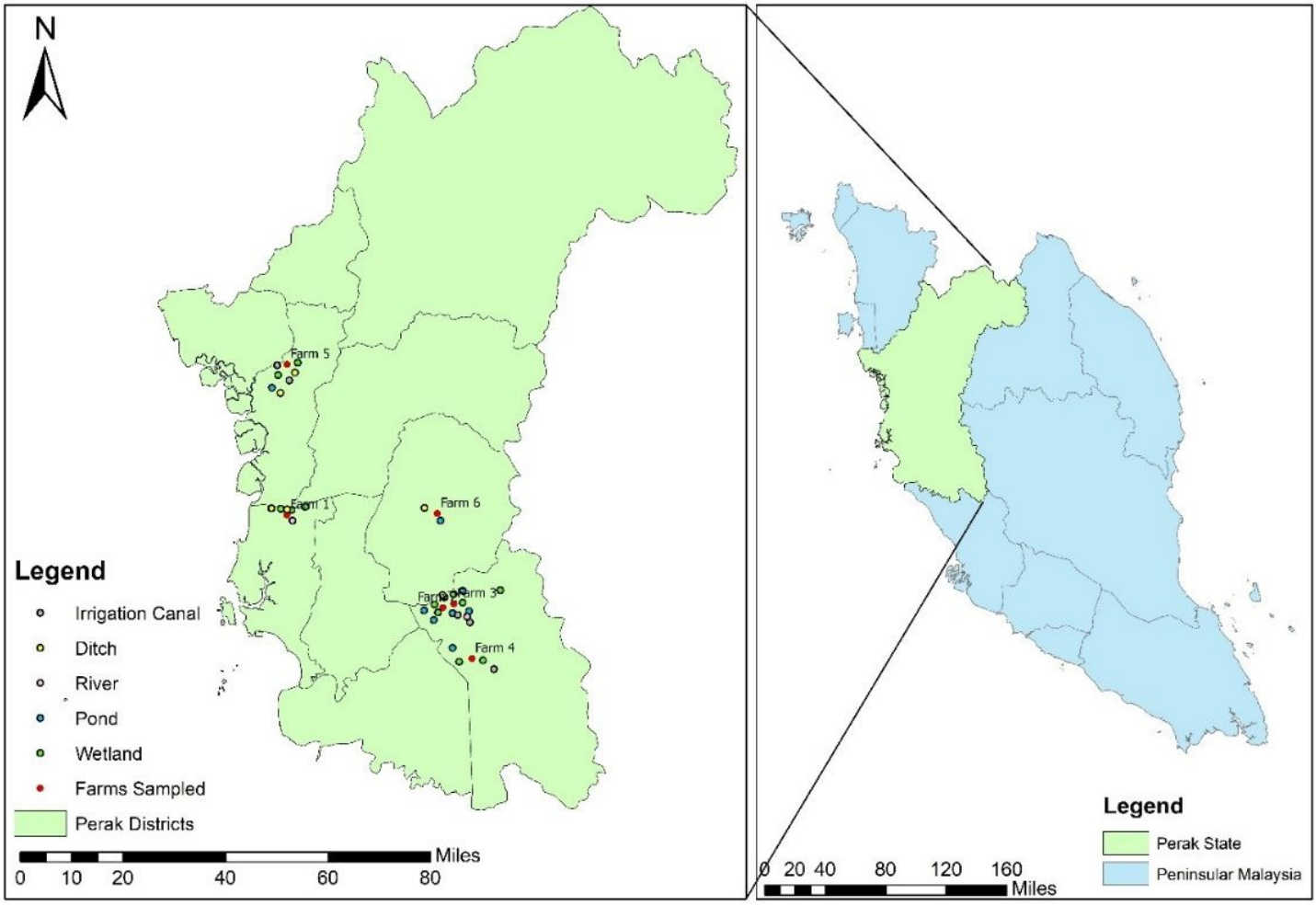

Figure 1

Sampling sites (water resource) around buffalo farms in Perak

Figure 2

Different water bodies investigated during the survey in Perak: (a) irrigation canal, (b) ditches, (c) river, (d) marshes wetlands, (e) Pond

Figure 3

The presence of R. rubiginosa snails in marshes wetlands in this study

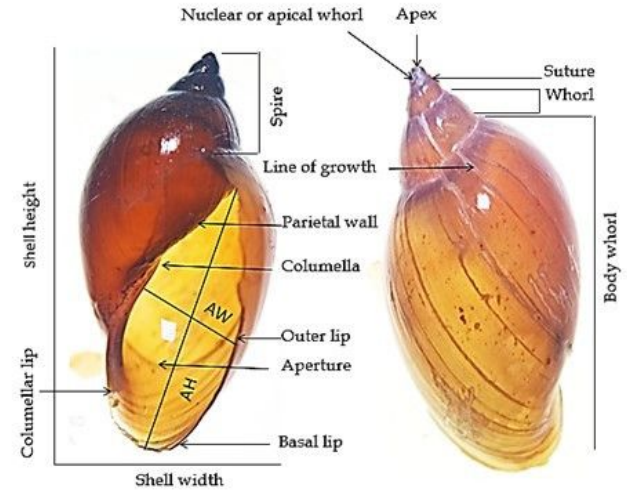

Figure 4

Shell morphology of R. rubiginosa in the present study (the body parts named according to Burch (1984)

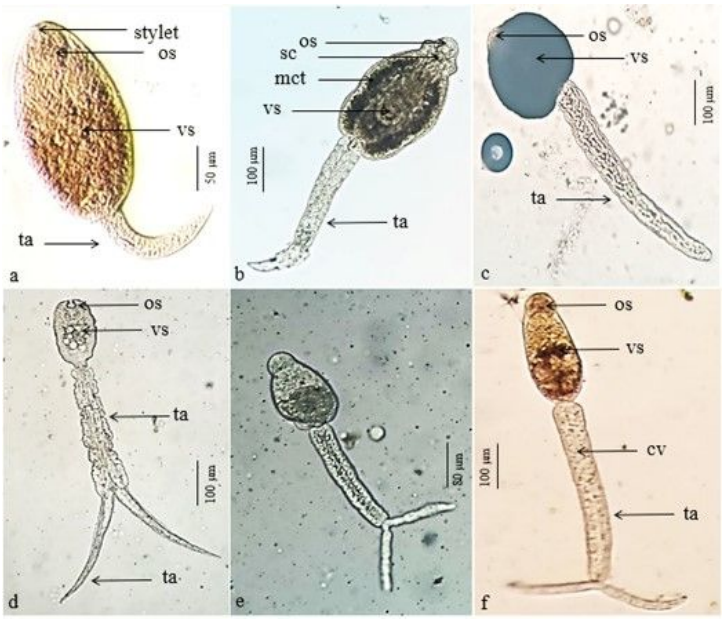

Figure 5

Five types of cercaria observed in *R. rubiginosa* snails collected in this study: (a) Xiphidiocercariae, (b) Echinostome cercariae, (c) Gymnocephalous cercariae (d, e) Brevifurcate-apharyngeate distome cercariae, and (f) Longifurcate-pharyngeate monostome cercariae (Vivax cercariae).

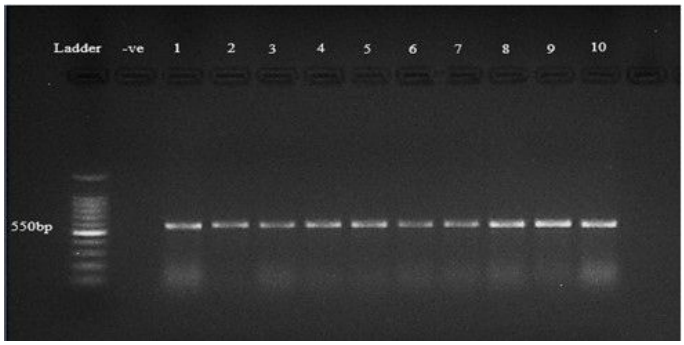

Figure 6

Agarose gel electrophoresis results showing the PCR products containing ITS2 from R. rubiginosa. Lane -ve shows negative control, and lane 1 – 10 shows 550-based pair amplified PCR product of R. rubiginosa.

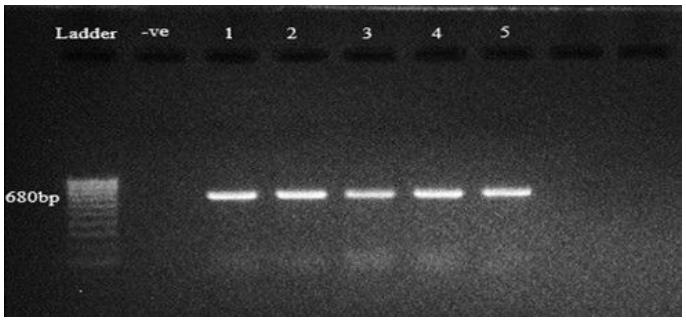

Figure 7

Agarose gel electrophoresis results showing the PCR products containing Cox1 from R. rubiginosa. Lane -ve shows the negative control and lane 1-5 show the 680-based pair amplified PCR product of R. rubiginosa.

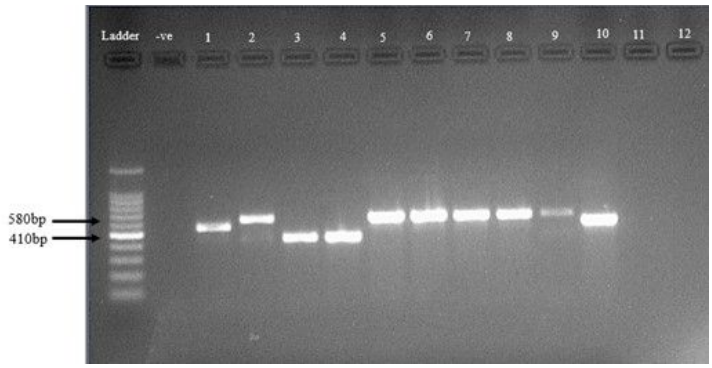

Figure 8

Agarose gel electrophoresis results showing PCR products containing ITS2 of different types of cercaria isolated from R. rubiginosa. -ve lane showing negative control. Lanes 1-10 show the 580-based pair and 410-based pair amplified PCR products of different types of cercaria: Xiphidiocercaria (1.2.3.4), Echinostome cercaria (5.6.7.8), Gymnocephalous cercaria (9.10), while land 11 and 12 (Furcocercous cercariae) showing no amplification.