UNIVERSITI PUTRA MALAYSIA

MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF LYMNAEID SNAILS FROM SELECTED BUFFALO FARMS UNDER OIL PALM INTEGRATION IN PERAK, MALAYSIA.

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Morphological and Molecular Identification of Lymnaeid snails from selected buffalo farms under oil palm integration in Perak, Malaysia.

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CERTIFICATION

It is hereby certified that we have read this project paper entitled “Morphological and Molecular Identification of Lymnaeid snails from selected buffalo farms under oil palm integration in Perak, Malaysia”, by Arvinderan A/L Joganathan, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Final Year Project.

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DEDICATION

This thesis is dedicated to my:

Supervisor

Dr. Nur Mahiza Md Isa

Co-supervisors

Dr. Mohd Akmal Mohd Noor

My beloved family

My friends

Sorga Vaasal

My batchmates and juniors

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 –Projek Tahun Akhir.

Pengenalpastian Morfologi dan Molekul siput Lymnaeid dari ladang kerbau terpilih di bawah integrasi kelapa sawit di Perak, Malaysia.

Oleh

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2022

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Keluarga siput air tawar Lymnaeidae mempunyai kepentingan utama dalam perubatan veterinar dan manusia di seluruh dunia kerana menjadi perumah perantaraan trematoda digenean seperti *Paramphistomum sp* dan *Fasciola sp*. Pengenalpastian tepat perumah perantaraan untuk trematod ini adalah kunci untuk memahami epidemiologi penyakit dan kawalan yang berkesan. Walau bagaimanapun, di sebalik peranan penting mereka dalam kitaran hayat parasit, penyelidikan tentang pengecaman dan pengedaran siput air tawar di Malaysia adalah terhad dengan

maklumat yang boleh dipercayai hanya terdapat di negara jiran. Kajian ini bertujuan untuk mengenal pasti Lymnaeids di ladang kerbau di bawah integrasi kelapa sawit di Perak melalui pendekatan morfologi dan molekul. Sebanyak 106 ekor siput air tawar telah dikumpulkan di kawasan berair di dua ladang kerbau dan tanah lembap dan paya bersebelahan di Perak. Pencirian morfologi bagi sampel siput dilakukan melalui analisis tujuh parameter morfometrik cangkerangnya seperti ketinggian cangkerang (SH), lebar cangkerang (SW), tinggi spire (SpH), lebar apertur (AW), tinggi apertur (AH), bilangan whorls (NoW) dan orientasi cangkerang (OS), berdasarkan kunci dan sastera lain untuk pengenalpastian. Analisis molekul pada lima sampel terpilih menggunakan tisu kaki untuk pengekstrakan DNA dan spacer transkripsi dalaman kedua (ITS2) DNA ribosom nuklear sebagai penanda molekul mendedahkan bahawa Lymnaeid yang dikumpul adalah daripada spesies *Radix rubiginosa*, spesies perumah perantaraan biasa untuk kedua-dua *Paramphistomum sp*. dan *Fasciola sp.*

Kata kunci : Lymnaeidae; siput; pengenalpastian molekul; pengenalpastian morfologi; Perak

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 – Final Year Project.

Morphological and Molecular Identification of Lymnaeid snails from selected buffalo farms under oil palm integration in Perak, Malaysia.

by

Arvinderan A/L Joganathan

2022

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The Lymnaeidae family of freshwater snails are of key interest in veterinary and human medicine worldwide due to being intermediate hosts of digenean trematodes such as *Paramphistomum sp* and *Fasciola sp*. Accurate identification of the intermediate host for these trematodes is key to understanding disease epidemiology and effective control. However, despite their essential role in the parasite's life cycle, the research about freshwater snails identification and distribution in Malaysia is scarce with reliable information only available in neighboring countries. This study aimed to identify Lymnaeids in buffalo farms

under oil palm integration in Perak through morphological and molecular approaches. A total of 106 freshwater snails were collected at waterlogged areas in two buffalo farms and the adjacent wetlands and marshes in Perak. Morphological characterization of the snail samples was done through analysis of seven morphometric parameters of their shells such as shell height (SH), shell width (SW), spire height (SpH), aperture width (AW), aperture height (AH), number of whorls (NoW) and orientation of shell (OS), in reference to a key and other literature for identification. Molecular analysis on the five selected samples using foot tissue for DNA extraction and the second internal transcribed spacer (ITS2) of the nuclear ribosomal DNA as molecular markers revealed that Lymnaeids collected were of the species *Radix rubiginosa*, a common intermediate host species for both *Paramphistomum sp* and *Fasciola sp*.

Keywords: Lymnaeidae; snails; molecular identification; morphological identification; Perak

INTRODUCTION

Freshwater snails of the Lymnaediae family are of significant medical and veterinary importance as vectors of digenean parasites. The intimate relationship between digenean families, moluscan families and vertebrate hosts is primitive in nature and Crib et al. (2001) stated that Digenea were possibly first parasites of mollusc prior to acquiring vertebrate hosts. A large portion of concern regarding this relationship is on the adverse health effects on the vertebrate host (ruminants and mammals in the case of zoonotic transmission) but the vertebrate hosts are not the only party detrimentally affected by digenean parasitic infections, the snail intermediate hosts also suffer physiological effects such as stunting, gigantism and parasitic castration (Sorensen and Minchella, 2001).

Perhaps the most important role of lymnaeid snails is as intermediate hosts of *Fasciola sp* and *Paramphistomum sp*, agents of fascioliasis and paramphistomosis respectively, infecting ruminants causing reduced meat, weight and milk production, decrease in fertility and even death in some cases (Hambal, 2020). There is a positive correlation between snail populations, their distribution and the occurrences of these parasitic infections within ruminant populations reared as livestock (Khan and Maqbool, 2012) implying that for the purpose of understanding disease epidemiology and concentrating control efforts, it is essential to correctly identify such intermediary snail hosts, especially when resources are scarce and the host range of the parasites is broad (Kane et al., 2008).

Several studies on the morphology and molecular characteristics of Lymnaeid snails have been conducted in neighboring Southeast Asian countries. However, presently there is only a single study on morphological identification of Lymnaeids (Saad, 2017) and a complete absence on molecular identification of Lymnaeid snails in Malaysia. In light of this, the objective of this study was to identify and characterize a single or multiple Lymnaeid snail species collected from buffalo farms under oil palm integration in Temoh, Perak by both morphological and molecular techniques.

1.1 Justification

Although there has been extensive research regarding the parasitic trematode agent itself, there seems to be a lack of emphasis and studies in Malaysia on the intermediate hosts, lymnaeid snails, which are pivotal in the life cycle of the parasite. Understanding the interactions between specific lymaeid species and trematodes can only be done through properly identifying these snails and their epidemiological role in the transmission of the parasite. Therefore, rapid and accurate identification would be warranted and hopefully the findings of this study will aid farmers, vets and researchers in identifying the snails, subsequently allowing proper control measures to be taken in order to reduce occurrences of trematodiasis

1.2 Objective

The overall objective is to perform morphological and molecular identification of Lymnaeid snail species collected from the selected buffalo farms under oil palm integration in Perak, Malaysia

1.3 Hypothesis

The null hypothesis is that only a single species or no species of lymnaeid snail would be identified in water resources around buffalo farms in Perak, Malaysia while the alternative hypothesis is that different species of lymnaeid snail would be identified in water resources around buffalo farms in Perak, Malaysia

LITERATURE REVIEW

2.1 Lymnaeidae

Lymnaeidae Rafinesque, 1815 , commonly known as pond snails, are one of the most diverse and functionally significant families of pulmonate freshwater snails, with a distribution that is almost universal (Aksenova et al., 2018). Prior to the 21st century, there was no agreement on the exact number of genera and species existing within this family until the revolution of molecular zoological systematics came about. The Integrated Taxonomic Information System recognizes 11 genera: *Acella*, *Bulimnea, Erinna, Fisherola, Galba, Idaholanx, Lanx, Lymnaea, Pseudosuccinea, Radix and Stagnicola but* Vinarski (2013) argues that a total of 25 genera exists under the family Lymnaeidae : *Corvusiana, Ladislavella, Omphiscola, Aenigmomphiscola, Hinkleyia, Walterigalba, Walhiana, Sphaerogalba, Myxas, Pacifimyxas, Cerasina, Pectinidens, Limnobulla, Orientogalba, Austropeplea, Bullastra* and *Lantzia*. Presently, there are less than 100 species of Lymnaeids, comprising of 40% of all gastropod species (Corea et al., 2010)

Snails within this family have similar features that are shared across a wide array of species, be it characteristics of their shells or soft tissue anatomy. Shell wise, the majority of Lymnaeidae have dextral shells, that is when facing the aperture of the shell with the spire away from the holder, the aperture is on the right side. Lymnaeidae and other Posobronch families tend to have the typical trait of well defined indented sutures at the margin of whorls that elevate into the structure known as the spire (Russel-Hunter, 1983). Furthermore, another key feature would be the columella, the inner lip of the shell formed by reflection of the aperture over the largest whorl, the body whorl. Key feature of Lymnaeids regarding their soft tissue anatomy on the other hand include presence of the eyes at the base of their tentacles which differ from their terrestrial relatives that posses eyes at the tip of the tentacles, tentacles that are wide at the base and triangular in shape, lacking an operculum seen in their prosobranch counterparts and a head divided into two lateral lobes (Pechenik, 1985).

2.2 Distribution, Natural Habitat and Life cycle of Lymnaeids

Lymnaeids being one of the most globally distributed gastropod family can be found at all corners of the globe inhabiting rivers, lakes, swamps, streams, underground caves, springs, ditches and drainage as well as seasonal waters, with fossilized remains found even in the frigid conditions of Antarctica (Ashworth, 2003). The greatest diversity of these freshwater snails exists in the Palearctic (Euro-Siberia; the Mediterranean Basin; the Sahara and Arabian Deserts; and Western, Central and East Asia) and Nearctic (North America) but many species can be found in the Afrotropical, Australasian and Oriental regions (Strong, 2008). In their endemic regions, freshwater snail spatial

distribution is mostly influenced by environmental factors that affect the amount of water in an area, such as proximity to water bodies, texture of soil, temperature, frequency of rainfall, vegetation, and the potential for flooding (Sangwan et al., 2016). Some evidence even shows that regions where rice is grown, freshwater snails tend to be more prevalent (Sangwan et al., 2016). A study by Saad (2017) in cattle farms in Terengganu revealed that lymnaeid snails can be found in waterlogged areas of clear water with an abundance of *Ipomoea* sp. (kangkung), high calcium content and pH approximately neutral which coincides with the natural habitat requirements stated in Rajamanickam et al. (1996).

Diversity of Lymnaeidae and their distribution in Malaysia is poorly studied and current information is purely based on preliminary assessments of general diversity patterns across the region even though Malaysia is a notable biodiversity hotspot (Aksenova et al., 2018). There is a lack of information regarding this family of snails in Malaysia, their distribution and currently available literature tends to revolve around specific species and terrestrial gastropods rather than the Lymnaeidae family as a whole. Furthermore, a significant amount of studies regarding snails and gastropods in Malaysia focus on usage of snails as feed in fish and efficacy of molluscicides in the agricultural sector.

Majority of freshwater snails are herbivores and detritivores, with some species known to occasionally feed on carrion as well as small invertebrates (Cuker, 1983). Lymnaeids have a preference for ingesting algae, diatoms from rock and macrophytes, with a large portion of their diet being herbivorous however ingesting animal tissue has been known

to allow rapid growth in many species (Bovbjerg, 1968) such as the omnivorous lymnaeid *Pseudosuccinea columella* (Kesler et al., 1986).

Lymnaeid snails are oviparous hermaphrodites with a preference of cross fertilization as do all pulmonate freshwater snails. These snails are also annual and semelparous in nature in which throughout their entire lifespan prior to death, they would only reproduce once. The genus *Lymnea* can be rather interesting as differences in climate can oftentimes affect their lifecycle as seen in temperate regions where most species are annual, reproducing in the spring and die, with a complete replacement of a generation while *Lymnea* in the tropics can experience up to three reproductive intervals and sometimes have a life expectancy of five years (Calow, 1978). The pace of their life cycle can also be greatly affected by the environmental temperature with warmer temperatures encouraging faster growth rates. In temperatures of 20℃ - 35℃, depending on the choices of feed available, lymnaeids can attain sexual maturity between 25 to 93 days (Aziz and Raut, 1996).

2.3 Role of Lymnaeids in Transmission of Trematodes

The family Lymnaeidiae is of particular interest in modern medicine, both of human and veterinary importance as they are vectors of zoonotic parasitic trematodes, leading to implications in human health, livestock, and economic losses (Kaplan, 2001). Lymnaeids participate in the life cycles of at least 71 trematode species belonging to 13 different families whose members use birds and both domestic and sylvatic mammals as definitive hosts, including humans (Brown, 1978). In many cases, the same

lymnaeid species is even used by more than one digenean species simultaneously (Moukrim et al., 1993). Examples of trematodes that are of interest in human medicine include several species of Echinostomatidae (Graczyk and Fried, 1998) and Schistosomatidae while trematodes of veterinary importance are such as *Fasciola hepatica, Fasciola gigantica* and *Paramphistomum sp* which infect ruminants. Paramphistomosis in Malaysia is of particular interest, with its increasing prevalence (Saad, 2019; Naim et al., 2021) due to the highly suitable tropical climate of Southeast Asia (Gupta et al., 1978), leading to significant production loss in both buffalo and cattle.

Figure 1 shows the life cycle of *Paramphistomum sp* and the involvement of lymnaeid snails as intermediate hosts. The life cycle begins when the adult flukes shed their eggs within the stomach of the infected host, with the egg being through feces into the environment. Under appropriate conditions, the eggs hatch releasing the miracidia which then search for the intermediate host: freshwater snail within a period of 24 hours (de Waal, 2010). The miracidium invades the molluscan host's soft tissue and within the snail, undergoes three stages of development (sporocyst, redia and cercaria). The cercaria is then released from the snail and encysts on vegetation, forming metacercaria that remain viable up to three months (Kahl et al., 2021). Evidently, the metacercaria is ingested by the definitive host along with the vegetation and excystation occurs when it reaches the small intestine. The juvenile fluke then hatches and migrates to the rumen where it attaches to ruminal pillars and develops into the adult stage.

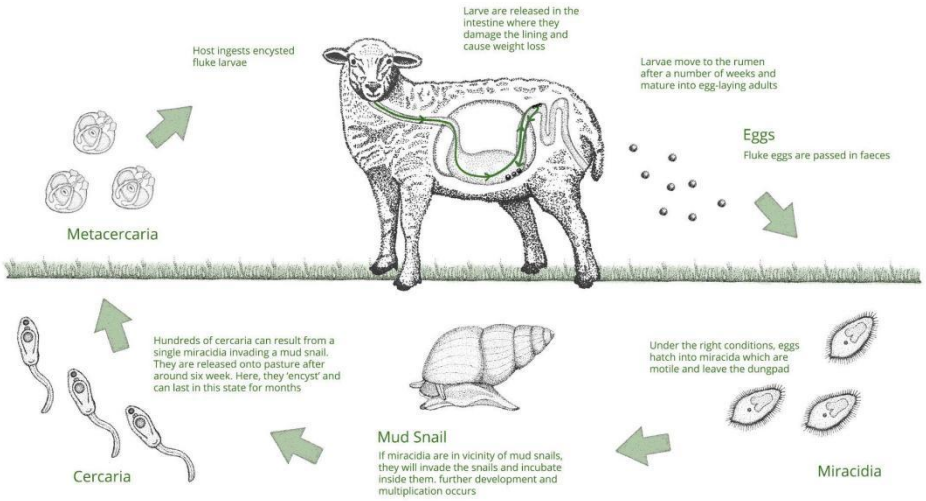

Figure 1 : Life cycle of *Paramphistomum sp.* The Lymnaeid snails serve as an intermediate host where the miracidia invade their soft tissue and undergo further development.

2.4 Identification of Lymnaeids

2.4.1 Morphological Identification

In gastropod taxonomy, it is pivotal to accurately identify one species from another, with some species having involvement in transmission of parasites of zoonotic potential. Due to the necessity of identification, morphological classification was established using an array of criteria. Criteria for identification may vary according to taxa but generally include morphological characteristics such as shell shape, colour and size, characteristics of the whorls, sutures and aperture, sculpture of the shell and radula.. Most studies employ methods of identification measuring the relative dimensions of the shell and utilizing morphometric parameters (Madsen and Hung, 2014). These characteristics and parameters can then be contrasted and compared to a regional key

as reference, identifying the taxonomy of the snail based on the corresponding observations. In Southeast Asia, useful keys for identifying freshwater snails are such as Burch (1980) and Upatham et al. (1983).

Issues and difficulties tend to arise when identifying specimens at the species level within the Lymnaeidae family. This is due in large measure to the uniformity of interspecific morphological and anatomical features seen in many species (Oviedo et al., 1995). One method that has been proven effective in distinguishing closely related species within the family is by identifying characteristics of the reproductive tract (Jackiewicz, 1988). However, numerous reports have still shown that the reproductive tract among different species exhibits extreme homogeneity (Pointier et al., 2006).

Historically, a significantly large portion of taxonomic studies on Lymnaeid snails were solely based on conchology, exclusively using morphometric parameters and characteristics of their shells as a basis of diagnosing genera, subgenera, species and even intraspecific varieties. This led to researchers accepting a wide array of varieties within a few species and in some cases, even augmenting these varieties into the ranks of a full species even though molecular analysis of their DNA sequence would say otherwise (Aksenova, 2018).

2.4.2 Molecular identification

Molecular identification of snails arose as a more reliable classification system at a higher resolution, allowing accurate identification at the species level. A comprehensive

molecular identification system for the Lymnaeidae family began with Hubendick (1951), Kruglov and Starobogatov (1993) and Jackiewicz (1998). However, when initially established, there were instances where the systems would not correspond to each other and sometimes would even be contradictory. Since then, a number of established molecular markers have been utilized to identify Lymnaeids due to their ease to amplify and sequence, as well as having a wide array of sequences publicly available to compare with Lawton et al. (2015). Markers most used in molecular studies are such as the nuclear ribosomal gene 16s and 18s, the internal transcribed spacer regions ITS-1 and ITS-2 (Bargues et al., 2001) and the international barcoding gene cytochrome oxidase 1 (cox1). Currently, there is no consensus on which marker to use and choice of marker can vary depending on intended efficacy as well as personal preference, however most studies concluded that the ITS-2 and cox1 markers are generally effective at differentiating closely related species (Pfenninger et al., 2006; Schniebs et al., 2011; Kane et al., 2008).

MATERIALS AND METHOD

3.1 Sampling Location

Sampling location was at buffalo farms and surrounding marshes and wetlands in Temoh, Perak. Exact locations comprised of two farms and three wetlands (**Figure 2**)

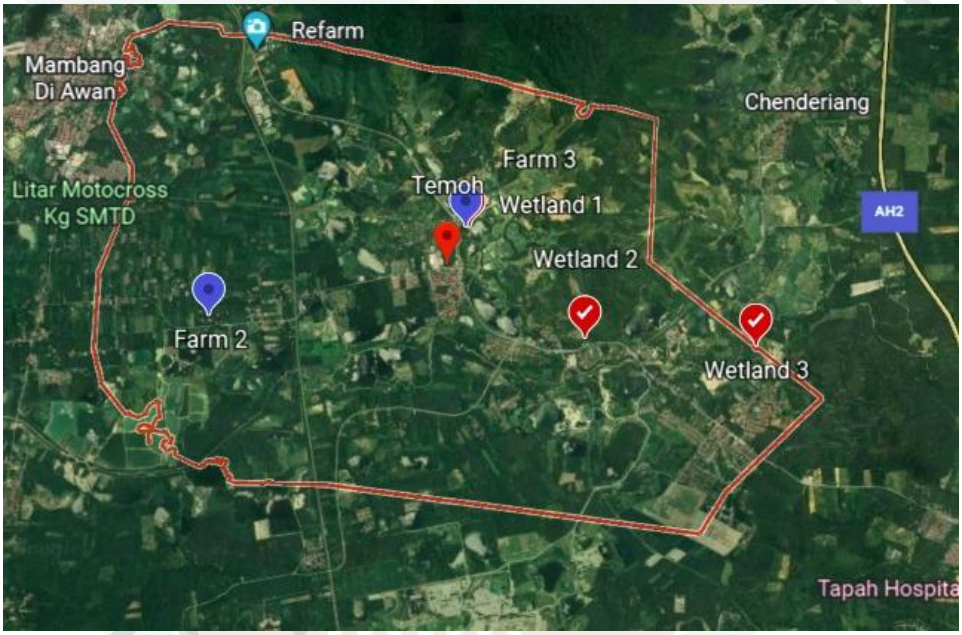

Figure 2: Sampling location at buffalo farms and surrounding wetlands in Temoh, Perak.

GPS Coordinates for sampling sites consisting of both farms and adjacent wetlands :

Farm 2: 4°14’53°N 101°11’49°E

Farm 3: 4°14’53°N 101°11’48°E

Wetland 1 (adjacent to Farm 3): 4°14’53°N 101°11’49°E

Wetland 2: 4°14’05°N 101°12’41°E

Wetland 3: 4°14’02°N 101°13’54°E

The sampling locations are characterized by shallow waterlogged areas with adequate vegetation and foliage (**Figure 3**).

Figure 3 : Shallow waterlogged areas with lymnaeid snails present

3.2 Sampling Size and Method

Initial sampling size consisted of 100 snails and sampling technique was convenience sampling by randomly collecting noticeable snails within water-logged marshes and wetlands surrounding a buffalo farm in Temoh, Batang Padang District of Perak. Sample collection is done 3-4 hours during the morning hours 6:00 a.m. - 10:00 a.m. At each water resource, an initial observation is done to grossly identify the density of snail populations within the area. If there is a high positive of snail population, only a single person should remain within the water resource to collect the samples. Aside from manually collecting the snails using a pair of gloves, sieve trays or bowls can be used to filter out snails from mud and sediment in waterlogged areas.

3.3 Sample Storage

Snails are collected and initially stored into plastic test tubes filled with water from the water source they were found (**Figure 4**). They are then transferred into large glass jars together with the water and kept at room temperature until morphological examination is performed.

Figure 4 : Storage of snail samples in plastic test tubes

3.4 Morphological Examination based on Morphometric Parameters

Snails were euthanized by immersion in 90-100% ethanol and removal of the body tissue using a pair of forceps was done. Each snail shell was then examined, measured, and recorded under a Nikon ECLIPSE E200 Stereomicroscope at 20X magnification for the following seven parameters and characteristics (**Figure 5**): shell height, shell width, spire height, aperture height, aperture width, orientation of shell and number of whorls.

Based on the recorded parameters, morphological identification was performed using the key of Burch (1980) as reference. Pictures were then taken and edited on Adobe Illustrator CS6 and Procreate for illustrative purposes and presentation.

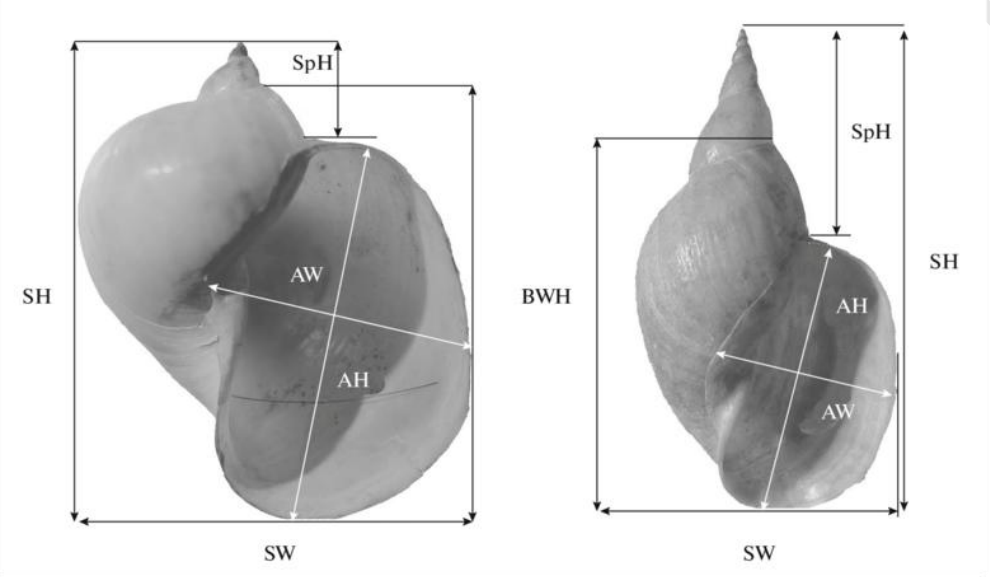

Figure 5: Standardized measurements of Lymnaeid shells (Vinarksi, 2016): shell height (SH), shell width (SW), spire height (SpH), aperture height (AH), aperture width (AW).

3.5 Molecular Examination

3.5.1 DNA Extraction

Once morphological identification had been performed, five snail samples were chosen at random out of the 106 collected samples for DNA extraction using the QIAGEN DNeasy® Blood & Tissue Kit Quick-Start Protocol. A piece of foot tissue from each snail sample was cut to approximately 20 - 25 mg and was then further cut into smaller pieces followed by placing each cut sample into a

1.5 ml microcentrifuge tube. 180 µl of Buffer ATL was added into each tube followed by 20 µl of proteinase K. Solutions and cut tissue were mixed by vortexing and then incubated at 56℃ until completely lysed. Once completely lysed, the samples were vortexed again directly for 15 s. 200 µl of Buffer AL is added and mixed thoroughly by vortexing followed by incubation at 56°C for 10 min.

Subsequently, 200 µl of ethanol (96-100%) is added and again, mixed by vortexing. The mixture was pipeted ino a DNeasy spin column placed in a 2 ml collection tube and centrifuged at 6000 x g (8000 rpm) for 1 min. The flow through and collection tube is discarded and the spin column is placed in a new 2 ml collection tube. 500 µl of Buffer AW1 is added and then centrifuged at 6000 x g (8000 rpm) for 1 min. Similarly, to the previous step, the flow through and collection tube is discarded and the spin column is placed in a new 2 ml collection tube. Next, 500 µl of Buffer AW2 is added and centrifuged at 20,000 x g (14,000 rpm) for 3 min. Flow through and the collection tube is discarded. The spin column is transferred to a new 1.5 ml of 2 ml microcentrifuge tube. The DNA is eluted by adding 200 µl Buffer AE to the center of the spin column membrane followed by incubation for 1 min at room temperature. Microcentrifuge tube and its contents are centrifuged for 1 min at 6000 x g (8000 rpm).

3.5.2 PCR Amplification

A total volume of 25 µL per each 1.5 ml microcentrifuge tube was used for amplification consisting of 10 µl master mix, 10.4 µl deionized distilled water, 2 µl DNA template, 1 µl NEWS (Lawton et al., 2015) forward primer (5'-TGTGTCGATGAAGAACGCAG-3') and 1 µl reverse primer (5'-TTCTATGCTTAAATTCAGGGG-3'). The master mix contains Taq DNA Polymerase, PCR buffer and dNTPs. The rDNA ITS-2 primer sequence used in amplification was previously described in Corea et al. (2011). A negative control was included containing deionized distilled water as a substitute for the master mix while no positive control was included in the amplification process. Once all five samples and negative control were prepared with aforementioned reagents, the samples were loaded into a thermo-cycler and the amplification was run under the following thermo cycling profile: 2 mins at 94℃ (initial denaturation) followed by 30 cycles comprising of 30 s at 94℃ (denaturation), 30s at 50℃ (annealing), 30s at 72℃ (elongation),and a final elongation at 72℃ for 7 min.

3.5.3 Gel Electrophoresis and UV Illumination

A 0.75% agarose gel was prepared with 0.75 g of Hydrogel powder, 50 ml of Tris-acetate-EDTA(TAE) buffer and stained with 3 µl of RedSafe™ Nucleic Acid Staining Solution. Aliquots 10 µl of each PCR amplified products were placed in the wells of the agarose gel with the negative control in the second well followed by the samples in the subsequent wells 3,4,5,6 and 7. A 5 µl of DNA ladder and 1 µl of dye were filled into the first well. The agarose gel was then run in the

electrophoresis machine for 50 min at 80 V and 400 mA . Once the electrophoresis was complete, visualization of the agarose gel was performed under short UV wave illumination in an Ultraviolet (UV) transilluminator.

3.5.4 DNA sequencing and Bioinformatics Analysis of gene sequence

Samples and primers were sent to Apical Scientific Sdn Bhd (formerly known as First Base Laboratories Sdn Bhd) for sequencing using the Sanger sequencing method.

Sequences received were assessed for similarity with other available sequences in the GenBank database by utilization of BLAST® Standard Nucleotide BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify and confirm Lymnaeid species and its degree of homologous to available sequences.

RESULTS

4.1 Morphological Analysis of Snails

Throughout the process of morphological analysis, only a single morphological type of varying size was distinguished using the key of Burch (1980) as reference. All 106 samples are dextral (coiled to the right), a feature specific to the family Lymnaeidae. As observed in **Figure 6,** the shell appears cylindrical and is elongated with well-rounded whorls and indented sutures. The shells also possess a twisted columella, creating a fold at the inner margin of the aperture which led to assumption of the genus *Radix*.

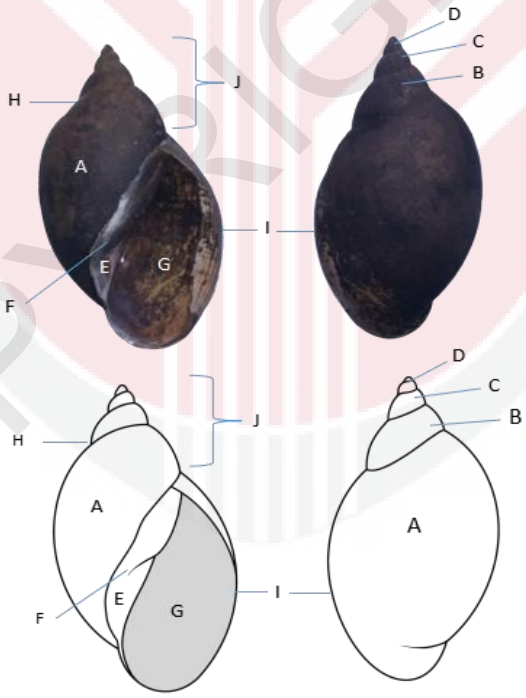

Figure 6 : Gross morphology of snail shells collected. Body whorl (A), penultimate whorl (B), apical whorl (C), apex (D), columella (E), columella fold (F), aperture (G), suture (H), outer lip (I), spire (J).

Under examination of the stereomicroscope as seen in **Figure 7**, the shell appears thin, partially translucent with clear transverse striae. Similarly to the gross examination , the twisted columella can also be appreciated at the inner margin of the aperture. These characteristics, along with referencing the key of Burch led to the identification of *Radix rubginosa (Lymnaea sp.).*

Figure 7: Stereomicroscopic examination of snail shell morphology, 20X magnification. Black arrows depict the clear transverse striae along the shell. The white arrow depicts the twisted columella fold.

Table 1 depicts the range and average value of each morphometric parameter measurement starting with the shell height ranging from 10.29-21.54 mm while the shell width ranges from 5.72-11.7 mm. The spire is relatively high, measuring between 2.5-7.08 mm, approximating one thirds of the shell length. The aperture is relatively large but does not extend outwards while the margin of the outer lip maintains a generally ovoid margin. The aperture height ranges from 7.44-14.46 mm while the width ranges between 4.26-8.83 mm.

Table 1 : Minimum, maximum and average values of morphometric parameters of the snail shells.

Morphometric Parameter	Min (mm)	Max (mm)	Average (mm)
Shell Height	10.29	21.54	15.88
Shell Width	5.72	11.7	8.63
Spire Height	2.5	7.08	5.09
Aperture Width	7.44	14.46	10.79
Aperture Height	4.26	8.83	6.69

4.2 Molecular Analysis

Figure 8 shows the PCR amplification of genomic DNA extracted from snails resulted in fragments of approximately 550 bps from all five samples, suggestive of snails under the Lymnaeidiae family.

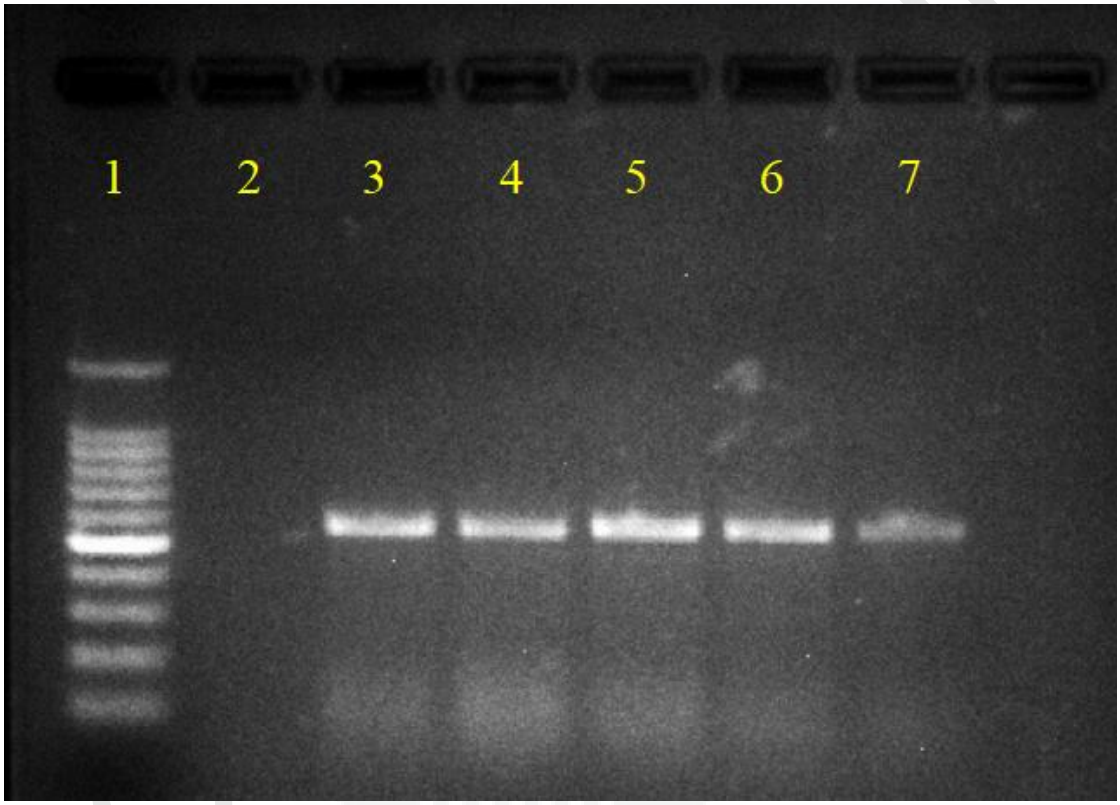

Figure 8: Agarose gel electrophoresis of PCR amplification products of ribosomal DNA of lymnaeid snail from buffalo farms in Temoh, Perak. Lane 1: 100 bps DNA marker, Lane 2 : negative control, Lane 3-7 : snail samples

4.2.1 Bioinformatics analysis of gene sequence

Table 2 shows the GenBank data search using the BLAST® Standard Nucleotide BLASTn application using all five samples sent for sequencing exhibiting the same genomic sequence displayed 99% homology with the 5.8S rRNA,ITS2 and 28S rRNA region of *Radix rubiginosa* from two isolates in Vietnam (LC659106.1, KF042385.1). Our results also shows that *Radix natalensis* (HQ283270.1) from Réunion Island exhibits a 99% homogeneity to the sample at the 5.8S ribosomal RNA and ITS2 region.

Table 2 : Reference isolates from GenBank that are identical with sample isolates from experiment.

Description of Isolates	Percentage of homologous (Percent identity)	Accession Number
Radix rubiginosa L1320 genes for 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence (Vietnam)	99%	LC659106.1
Radix natalensis 5.8S ribosomal RNA gene and internal transcribed spacer 2, partial sequence (Réunion Island, Indian Ocean)	99%	HQ283270.1
Radix rubiginosa 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (Vietnam)	99%	KF042385.1
Radix rubiginosa isolate 1 internal transcribed spacer 2, partial sequence (Australia)	99%	EU556316.1
Radix rubiginosa L1320 genes for 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence	99%	LC659106.1
Radix rubiginosa voucher Mlym-63 internal transcribed spacer 2, partial sequence (Thailand)	98%	KX056267.1
Radix alticola L1413 genes for 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence (Bangladesh)	93%	LC659114.1

DISCUSSION

Morphological analysis led to the conclusion that all 106 snails were *Radix rubiginosa* based on characteristics and morphometric parameters of their shells. The findings of this study coincide with the ranges of morphometric parameters and shell characteristics of *Radix rubginosa* in previous studies (Dung et al., 2013 and Nadasan, 2011). Identifying the family and genus which were Lymnaeidae and *Radix* were relatively direct and uncomplicated due to the presence of distinct features of the shell that are specific to these groups, as stated in the key of Burch (1980). However, there was difficulty at identifying at the species level due to the similarities of the shell characteristics of snails within the genus *Radix* (phenotypic plasticity) and that shell shape could vary depending on the environmental conditions (Pfenninger et al., 2006).Morphological techniques alone does not always result in accurate identification, especially in the hands of a non--expert malachologist (Corea et al., 2011). In fact, Pfenninger, 2006 argues that using shell morphological characteristics as a means of identification in the *Radix* genus would eventually result in completely inaccurate diagnosis of specific species. This prompts us to incorporate molecular techniques.

With that being said, molecular identification of gene sequence from the snail specimens in this study did raise certain conflicts, particularly in the genetic identity of the species. Using the ITS2 genetic marker, both *Radix rubiginosa* (LC659106.1, KF042385.1, EU556316.1 and LC659106.1) and *Radix natalensis* (HQ283270.1) had a 99% homogeneity with the specimens tested thus raising the issue which species would be more accurately corresponding to the identity of the snail specimens. ITS2 could be the source of this conflict whereby using this specific molecular sequence in

molecular analysis results in low levels of variation between closely related species (Lawton et al., 2015) which could be seen in this case with *Radix rubiginosa* and *Radix natalensis.* Similar situations of low degree of differentiation between species within the genus *Radix* have been recorded in previous studies (Schniebs et al., 2011).

An alternative molecular marker that would be recommended to clarify this confusion would be the Cox1 marker. Compared to ITS2, Cox1 has a greater phylogenetic signal and is better able to distinguish between species that are closely related (Pfenninger et al., 2006). Studies using Cox1 also showed more similar results in *Radix* identification concurrent with studies that incorporate other mitochondrial markers such as 16s (Correa et al., 2010) and cyt-b (Schniebs et al., 2011) compared to studies using ITS2. Using Cox1 as the molecular marker could possibly have resulted in a difference in percentage of homology between the two closely related species thus allowing a more distinct identification of the species. Based on the bioinformatics analysis using BLAST® Standard Nucleotide BLASTn, *Radix rubiginosa* was chosen as the molecular identity of all five snail samples due to the samples having the highest number of matches with the genetic sequence from the database. Even with the minor conflict encountered in this study regarding the genetic identity of the snail samples, molecular identification is still superior and significantly more objective compared to morphological idenfitication (Pfenninger et al., 2006).

This study resulted in the identification of only a single Lymnaeid species which led to the speculation of a number of possible reasons. One of it being the environmental conditions the snails were collected from, particularly the salinity of the water bodies

within the area. A study by Saad, (2017) recorded the identification of a number of Lymnaeid species and the corresponding salinity, specifically calcium concentration, in different types of water bodies and drainages. The study showed that *Radix rubiginosa* could only be found in water bodies with a calcium concentration of 33.06 mg/L while other species could be found in a broad range of water bodies with differing calcium concentrations. This however does not address a significant correlation as no statistical analysis were performed and does not address any potential confounding factors such as water temperature and pH. The correlation between salinity and density of freshwater snail populations had been previosly studied in Lymnaeid snails in palm oil plantations in Perak (Rajamanickam, 1996). In that study, it mentioned that there is a positive correlation between snail population density and the high calcium concentration in the soil of palm oil plantations. In this study however, the calcium concentrations of the water bodies from the sampling sites were not measured, therefore a proper correlation could not be made.

The species *Radix rubiginosa* and the genus *Radix* is an important factor in trematode prevalence, especially in tropical and subtropical regions. *Radix rubginosa* has been known to be intermediate host to larvae of the superfamilies *Fasciolidae*, *Paramphistomoidea*, *Echinostomatoidea*, *Schistosomatoidea* and *Diplostomoidea*, many of which are involved in zoonotic transmissions between humans and animals (Martin et al., 2018). The study of the diseases caused by these parasites are possibly impaired if the species of snail intermediate host involved in their transmission is not accurate identified.

CONCLUSION

As a conclusion, snails collected from buffalo farms under palm oil integration in Temoh, Perak were identified as lymnaieds , specifically *Radix rubiginosa* of the genus *Radix* and were proven through both morphological and molecular techniques. Incorporation of both morphological and subsequent molecular techniques in this study was pivotal in accurately identifying the lymnaeid species.

RECOMMENDATION

This is the first study done in Malaysia on the morphological and molecular classification of lymnaeid snails. This study alone could not provide sufficient data and information regarding the distribution of lymnaeid snails, as only a single species from a single genus was identified. Similar studies should be carried out employing a larger sample size and expanding the range of sampling sites to obtain a more diverse array of snail species. This would provide more reliable data on species specific distribution and help correlate to occurrences of

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APPENDICES

APPENDIX A : List of materials and equipment for sampling,storage and morphological identification

Sampling and storage

List	Amount
Materials	
Gloves	A few pairs
Plastic Test Tubes	1 plastic
Sieve tray/bowl	1
Glass container	4

Morphological identification

List	Amount
Materials	
Tray	1
Glass petri dish	2
Paper towels	1 box
Equipment	
Electronic Digital Caliper	1
Forceps	1
Stereo Microscope Nikon ECLIPSE E200	1

APPENDIX B : List of materials and equipment for DNA extraction

List	Amount
Materials	
Water bath	56℃
Deionised distilled water	1 bottle
Ethanol	70%
Proteinase K	10 µl
Buffer ATL	180 µl
Buffer AL	200 µl
Snail foot tissue sample	20 - 25 mg
Ethanol	95-100%
Buffer AW1	500 µl
Buffer AW2	500 µl
Buffer AE	200 µl
Equipment	
PCR tubes	1 plastic
Microcentrifuge tubes	1 jar
Pipet tips (10,100,1000 µl)	3 boxes
2 ml collection tubes	1 plastic
DNeasy mini spin column	1 plastic
Forceps	1
Centrifuge machine	1
Incubator	1
Vortexer	1

APPENDIX C : List of materials and equipment for gel electrophoresis and UV illumination

List	Amount
Materials	
Hydrogel powder	0.75 g
Tris-acetate-EDTA (TAE) Buffer	50 ml
RedSafe™ Nucleic Acid Staining Solution	3 µl
DNA ladder	5 µl
Dye	1 µl
Deionised distilled water (Negative control)	10 µl
Amplified PCR products (Samples)	10 µl
Equipments	
Microwavable flask	1
Well comb	1
Casting tray	1
Gel box (electrophoresis unit)	1
UV Transilluminator	1
Vortexer	1

APPENDIX D : Measurement of morphometric parameters of 106 snail samples from buffalo farm in Temoh, Perak

SH : Shell Height, SW : Shell Width, SpH: Spire Height, AH : Aperture Height, AW : Aperture Width, NoW : Number of Whorls, OS : Orientation of Shell

Sample ID	Morphometric parameters						
	SH (mm)	SW (mm)	SpH (mm)	AH (mm)	AW (mm)	NoW (mm)	OS
1	17.47	9.68	5.57	11.9	8.83	3 1/4	Dextral
2	21.54	11.7	7.08	14.46	8.12	3 1/4	Dextral
3	19.03	10.51	6.2	12.83	8.44	3 1/4	Dextral
4	17.93	9.51	6.47	11.46	7.4	3 1/4	Dextral
5	19.33	9.78	6.83	12.5	7.67	3 1/4	Dextral
6	17.07	9.49	5.58	11.49	7.7	3 1/4	Dextral
7	17.24	9.26	5.81	11.43	7.81	3 1/4	Dextral
8	16.47	9.77	4.73	11.74	7.76	3 1/4	Dextral
9	17.09	9.68	6.27	10.82	7.34	3 1/4	Dextral
10	17.2	9.34	5.12	12.08	7.38	3 1/4	Dextral
11	17.42	9.76	5.67	11.75	8.09	3 1/4	Dextral
12	18.44	10.12	6.44	12	8.2	3 1/4	Dextral
13	19.31	10.35	5.84	13.47	7.94	3 1/4	Dextral
14	17.74	9.48	5.93	11.81	7.38	3 1/4	Dextral
15	19.43	9.81	6.57	12.86	7.97	3 1/4	Dextral
16	18.26	9.74	6.66	11.6	7.91	3 1/4	Dextral
17	16.9	9.45	5.09	11.81	7.66	3 1/4	Dextral
18	16.58	9.16	5.69	10.89	6.83	3 1/4	Dextral
19	18.68	9.58	6.07	12.61	7.89	3 1/4	Dextral
20	17.02	9.5	6.03	10.99	6.87	3 1/4	Dextral
21	19.36	10.45	6.49	12.87	7.13	3 1/4	Dextral
22	18.94	9.77	5.9	13.04	7.33	3 1/4	Dextral
23	17.34	9.2	5.49	11.85	7.73	3 1/4	Dextral
24	17.98	9	6.7	11.28	7.63	3 1/4	Dextral

25	17.82	9.8	5.35	12.47	7.65	3 1/4	Dextral
26	17.23	9.83	5.62	11.61	7.86	3 1/4	Dextral
27	17.72	9.09	5.87	11.85	7.24	3 1/4	Dextral
28	17.32	8.97	6.47	10.85	7.2	3 1/4	Dextral
29	17.09	8.99	5.56	11.53	7.38	3 1/4	Dextral
30	17.81	9.05	6.69	11.12	7.23	3 1/4	Dextral
31	17.08	8.69	5.64	11.44	7.26	3 1/4	Dextral
32	15.93	9.26	5	10.93	6.78	3 1/4	Dextral
33	18.29	9.7	5.8	12.49	8.13	3 1/4	Dextral
34	17.23	9.76	6.41	10.82	7.54	3 1/4	Dextral
35	16.87	9.87	5.49	11.38	7.1	3 1/4	Dextral
36	17.05	9.43	5.41	11.64	7.21	3 1/4	Dextral
37	18.15	9.84	6.24	11.91	8.02	3 1/4	Dextral
38	16.89	9.3	5.17	11.72	7.28	3 1/4	Dextral
39	16.76	9.71	5.6	11.16	7.1	3 1/4	Dextral
40	17.11	9.73	6.07	11.04	7.78	3 1/4	Dextral
41	17.78	9.8	6.02	11.76	7.65	3 1/4	Dextral
42	16.11	9.17	4.77	11.34	7.31	3 1/4	Dextral
43	16.22	9.29	4.89	11.33	7.33	3 1/4	Dextral
44	16.58	8.85	5.66	10.92	7.04	3 1/4	Dextral
45	15.83	9.23	6.03	9.8	6.76	3 1/4	Dextral
46	17.19	9.76	5.75	11.44	7.27	3 1/4	Dextral
47	16.82	9.29	5.82	11	7.14	3 1/4	Dextral
48	15.38	9.03	4.95	10.43	6.71	3 1/4	Dextral
49	17.42	9.27	6.02	11.4	7	3 1/4	Dextral
50	14.98	8.02	5.23	9.75	5.88	3 1/4	Dextral
51	19.08	10.08	6.54	12.54	7.29	3 1/4	Dextral
52	18.53	10.11	6.09	12.44	7.1	3 1/4	Dextral
53	16.3	9.02	5.3	11	6.71	3 1/4	Dextral
54	17.04	9.47	5.5	11.54	7.75	3 1/4	Dextral
55	16.15	8.75	5.61	10.54	6.41	3 1/4	Dextral

56	16.42	8.99	4.88	11.54	6.79	3 1/4	Dextral
57	15.72	8.86	5.1	10.62	6.4	3 1/4	Dextral
58	15.81	8.84	5.07	10.74	6.55	3 1/4	Dextral
59	17.2	9.66	5.67	11.53	7.19	3 1/4	Dextral
60	15.41	8.52	5.4	10.01	6.16	3 1/4	Dextral
61	15.49	9.23	5.79	9.7	6.76	3 1/4	Dextral
62	16.3	8.97	5.14	11.16	6.87	3 1/4	Dextral
63	15.01	8.47	4.95	10.06	6.26	3 1/4	Dextral
64	13.66	7.82	3.71	9.95	6.01	3 1/4	Dextral
65	13.76	7.25	3.58	10.18	5.43	3 1/4	Dextral
66	14.34	7.65	4.23	10.11	5.53	3 1/4	Dextral
67	12.99	7.1	3.71	9.28	6.1	3 1/4	Dextral
68	13.32	7.37	3.8	9.52	5.16	3 1/4	Dextral
69	12.91	6.74	2.88	10.03	5.1	3 1/4	Dextral
70	12.61	7.16	3.05	9.56	5.55	3 1/4	Dextral
71	14.38	6.83	4.2	10.18	5.84	3 1/4	Dextral
72	13.36	7.49	3.91	9.45	6.09	3 1/4	Dextral
73	12.91	6.85	3.42	9.49	5.65	3 1/4	Dextral
74	13.95	7.46	3.89	10.06	5.2	3 1/4	Dextral
75	12.73	7.15	3.32	9.41	5.28	3 1/4	Dextral
76	12.85	6.71	3.57	9.28	5.32	3 1/4	Dextral
77	13.09	6.85	4.1	8.99	5.45	3 1/4	Dextral
78	11.82	6.21	3.5	8.32	4.87	3 1/4	Dextral
79	11.66	5.93	3.67	7.99	4.26	3 1/4	Dextral
80	16.46	9.66	4.72	11.74	7.09	3 1/4	Dextral
81	17.18	9.03	6.25	10.93	6.96	3 1/4	Dextral
82	16.49	9.11	5.15	11.34	6.1	3 1/4	Dextral
83	15.67	8.38	5.05	10.62	6.56	3 1/4	Dextral
84	14.75	8.12	4.26	10.49	6.41	3 1/4	Dextral
85	14.22	7.89	4.12	10.1	5.76	3 1/4	Dextral
86	15.67	8.92	4.71	10.96	6.47	3 1/4	Dextral

87	15.29	7.65	4.99	10.3	6.1	3 1/4	Dextral
88	15.8	8.55	5.24	10.56	5.89	3 1/4	Dextral
89	14.07	7.69	4.68	9.39	6.4	3 1/4	Dextral
90	14.43	7.7	4.15	10.28	6.26	3 1/4	Dextral
91	16.53	7.03	5.91	10.62	8.83	3 1/4	Dextral
92	14.89	8.53	4.41	10.48	7	3 1/4	Dextral
93	15.01	8.72	4.38	10.63	6.23	3 1/4	Dextral
94	14.64	7.73	4.54	10.1	6.15	3 1/4	Dextral
95	14.86	7.38	5.1	9.76	5.2	3 1/4	Dextral
96	14.3	7.99	4.07	10.23	6.52	3 1/4	Dextral
97	13.16	7.46	3.99	9.17	5.17	3 1/4	Dextral
98	14.1	7.43	3.85	10.25	5.69	3 1/4	Dextral
99	13.22	7.41	3.6	9.62	5.9	3 1/4	Dextral
100	15.41	8.18	4.56	10.85	6.6	3 1/4	Dextral
101	14.64	7.57	4.19	10.45	6.07	3 1/4	Dextral
102	12.78	6.88	3.49	9.29	5.37	3 1/4	Dextral
103	11.56	5.72	4.08	7.48	4.71	3 1/4	Dextral
104	11.48	6.1	4.04	7.44	4.55	3 1/4	Dextral
105	11.57	6.38	3.52	8.05	4.95	3 1/4	Dextral
106	10.29	6.09	2.5	7.79	4.35	3 1/4	Dextral

APPENDIX E : DNA sequence of Lymnaeid snail isolates obtained from buffalo farm in Temoh, Perak

R 11
CCGCGGATATGTGAATTGCAGAACACATTGAACATCGATATCTTGAACGCATATGG
CGGCCTCGGGTCAATCCCGGGGCCACGCCCGTCTGAGGGTCGGCTAGTGTCAAA
AACAATCGTGTCGCTTTGCTCGTGCGACGCGCTCTGGTCCGTCGCGGCCATAAAA
TCCAGCGTTCACCGCCGCATCGCTTTGCTCGGCGATGTCGTGTGTGTGATGTGTC
TGGTGGCCCCGTGGTCTTAAGCACAAGCCGCGCCGTTGTCCGTGTTCGTCTCGG
GACGTCCGCGACGCCGCCTTGCTCTCGGCGGCGGCCAAATTTTTTTTTTCCATCT
GCGTCACCGCTAAGCGGGACCCGGCTCGCTCTCGCTAACGGGCCCGCTTATACG
AAGCTCAAGGGTGATTGCGGAGGGGGGAAAAAAACGCCGACGCTCGCTTGACAA
ATCGGCGCCCGTACGAATTTGAAATGAAGAAAAACGCTGTTTTTTCTCTCATTCATA
TCTCCGACCTCAGATCGGACGAGATTACCCCCTGAATTAAGCCATAGAAAA

R 12
CCCGGGNATATGTGAATTGCAGAACACATTTGAACATCGATATCTTGAACGCATAT
GGCGGCCTCGGGTCAATCCCGGGGCCACGCCCGTCTGAGGGTCGGCTAGTGTC
AAAAACAATCGTGTCGCTTTGCTCGTGCGACGCGCTCTGGTCCGTCGCGGCCATA
AAATCCAGCGTTCACCGCCGCATCGCTTTGCTCGGCGATGTCGTGTGTGTGATGT
GTCTGGTGGCCCCGTGGTCTTAAGCACAAGCCGCGCCGTTGTCCGTGTTCGTCTC
GGGACGTCCGCGACGCCGCCTTGCTCTCGGCGGCGGCCAAATTTTTTTTTTCCAT
CTGCGTCACCGCTAAGCGGGACCCGGCTCGCTCTCGCTAACGGGCCCGCTTATA
CGAAGCTCAAGGGTGATTGCGGAGGGGGGAAAAAAACGCCGACGCTCGCTTGAC
AAATCGGCGCCCGTACGAATTTGAAATGAAGAAAAACGCTGTTTTTTCTCTCATTC
ATATCTCCGACCTCAGATCGGACGAGATTACCCCCTGAATTAANGCATAGAAAA

R 13
CCGGGGATATGTGAATTGCAGAACACATTTGAACATCGATATCTTGAACGCATATG
GCGGCCTCGGGTCAATCCCGGGGCCACGCCCGTCTGAGGGTCGGCTAGTGTCAA
AAACAATCGTGTCGCTTTGCTCGTGCGACGCGCTCTGGTCCGTCGCGGCCATAAA
ATCCAGCGTTCACCGCCGCATCGCTTTGCTCGGCGATGTCGTGTGTGTGATGTGT
CTGGTGGCCCCGTGGTCTTAAGCACAAGCCGCGCCGTTGTCCGTGTTCGTCTCG
GGACGTCCGCGACGCCGCCTTGCTCTCGGCGGCGGCCAAATTTTTTTTTTCCATC
TGCGTCACCGCTAAGCGGGACCCGGCTCGCTCTCGCTAACGGGCCCGCTTATAC
GAAGCTCAAGGGTGATTGCGGAGGGGGGAAAAAAACGCCGACGCTCGCTTGACA
AATCGGCGCCCGTACGAATTTGAAATGAAGAAAAACGCTGTTTTTTCTCTCATTCAT
ATCTCCGACCTCAGATCGGACGAGATTACCCCCTGAATTAAGCCATAGAAAA

R 14

CCCGCGGATATGTGAATTGCAGAACACATTGAACATCGATATCTTGAACGCATATG
GCGGCCTCGGGTCAATCCCGGGGCCACGCCCGTCTGAGGGTCGGCTAGTGTCAA
AAACAATCGTGTCGCTTTGCTCGTGCGACGCGCTCTGGTCCGTCGCGGCCATAAA
ATCCAGCGTTCACCGCCGCATCGCTTTGCTCGGCGATGTCGTGTGTGTGATGTGT
CTGGTGGCCCCGTGGTCTTAAGCACAAGCCGCGCCGTTGTCCGTGTTCGTCTCG
GGACGTCCGCGACGCCGCCTTGCTCTCGGCGGCGGCCAAATTTTTTTTTTCCATC
TGCGTCACCGCTAAGCGGGACCCGGCTCGCTCTCGCTAACGGGCCCGCTTATAC
GAAGCTCAAGGGTGATTGCGGAGGGGGGAAAAAAACGCCGACGCTCGCTTGACA
AATCGGCGCCCGTACGAATTTGAAATGAAGAAAAACGCTGTTTTTTCTCTCATTCAT
ATCTCCGACCTCAGATCGGACGAGATTACCCCCTGAATTAAGCCATAGAAAAA

R 15

CCCGGGNATATGTGAATTGCAGAACACATTGAACATCGATATCTTGAACGCATATG
GCGGCCTCGGGTCAATCCCGGGGCCACGCCCGTCTGAGGGTCGGCTAGTGTCAA
AAACAATCGTGTCGCTTTGCTCGTGCGACGCGCTCTGGTCCGTCGCGGCCATAAA
ATCCAGCGTTCACCGCCGCATCGCTTTGCTCGGCGATGTCGTGTGTGTGATGTGT
CTGGTGGCCCCGTGGTCTTAAGCACAAGCCGCGCCGTTGTCCGTGTTCGTCTCG
GGACGTCCGCGACGCCGCCTTGCTCTCGGCGGCGGCCAAATTTTTTTTTTCCATC
TGCGTCACCGCTAAGCGGGACCCGGCTCGCTCTCGCTAACGGGCCCGCTTATAC
GAAGCTCAAGGGTGATTGCGGAGGGGGGAAAAAAACGCCGACGCTCGCTTGACA
AATCGGCGCCCGTACGAATTTGAAATGAAGAAAAACGCTGTTTTTTCTCTCATTCAT
ATCTCCGACCTCAGATCGGACGAGATTACCCCCTGAATTAAGCCATAGAAAAA