RESEARCH ARTICLE

Detection of bloodworm larvae (Diptera: Chironomidae) in the golden apple snail *Pomacea canaliculata* (Lamarck, 1819) (Gastropoda: Ampullariidae) in Metro Manila

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ABSTRACT

Background: *Pomacea canaliculata* (Lamarck, 1819) is an invasive freshwater snail in the Philippines that damages crops but is consumed as food. It is known to harbor parasites, some of which are pathogenic to humans.

Objectives: The objective of this study is to examine *P. canaliculata* individuals present in Metro Manila for parasite infection and identify the parasites and other organisms associated with the snail using molecular identification.

Methodology: *P. canaliculata* were collected from rivers and marketplaces in Metro Manila. Individuals were crushed and digested in Ash's digestive fluid and observed under a microscope. Collected parasites were subjected to DNA barcoding of the COI gene for putative identification.

Results: A total of 462 snails were gathered from 15 sites, eight of which were market areas that sourced the snails from outside Metro Manila. No known parasites were found. Two snails were found to contain insect larvae in the mantle (0.43% infection). The closest BLAST matches for the two insect larvae were the chironomid fly *Nilodorum tainanus* (91.0% identity) from a snail in Sucat, Muntinlupa, and another chironomid *Parachironomus* sp. (92.8% identity) found in a snail originally from Cavite and brought to Calumpang, Marikina.

Conclusion: This study is the first report of the presence of chironomids in Philippine *P. canaliculata*. This could have an impact on the allergenic status of these mollusks if consumed while containing these chironomids. The absence of infection of other medically important parasites is possibly due to the patchy distribution of the snails and few interactions with the definitive hosts of known parasites.

Keywords: *Accidental Infection, Phoresy, Non-biting Midge, Freshwater Snail*

Introduction

The golden apple snail *Pomacea canaliculata* (Lamarck, 1819), locally known as *kuhol*, is a common species of freshwater snail that occurs in the Philippines. It was introduced in the 1980's as an alternative food source; however, the species is highly invasive and has infiltrated rice fields and displaced native populations of the freshwater snail species *Pila conica* (=*luzonica*) (Gray, 1828) [1]. On top of its ecological and economic importance, *P. canaliculata* is also a known intermediate host of the nematode *Angiostrongylus cantonensis* (Chen, 1935) [2]. Although this nematode is a natural lungworm parasite of rats, ingestion by humans through raw or uncooked intermediate or paratenic hosts causes eosinophilic meningoencephalitis or cerebral angiostrongyliasis [3,4]. Trematodes such as amphistome, diastome, and echinostome metacercariae were also found to parasitize *P. canaliculata* with an infection rate of 30.6% [5]. Infectious microorganisms are also known to be present in *P. canaliculata* such as *Mycobacterium ulcerans* that causes ulcerated lesions [6]. In the Philippines, *P. canaliculata* has been reported to harbor *A. cantonensis* in rice farming villages, although eosinophilic meningitis has not been reported locally [7].

P. canaliculata is also known to occur in urban areas. This freshwater snail is able to survive in environments with very

low dissolved oxygen levels and shows high tolerance to polluted water [8]. In addition, *P. canaliculata* can also estivate in the soil for many months during periods of dry weather [8]. The snail is primarily macrophytophagous, but true opportunistic, invasive behavior has also been reported by feeding on other freshwater snails, bryozoans, cyanobacteria, green algae, diatoms, and insects [9]. The competition for limited space in urban areas often results in increased species associations in a habitat. Occurrence of definitive hosts of disease-causing parasites together with *P. canaliculata* may result in infection of the snails as is the case with *A. cantonensis* and various trematode species.

Since *P. canaliculata* is a potential food source for humans and other urban mammals, this study aimed to evaluate the presence of parasites and other associated organisms in *P. canaliculata* occurring in Metro Manila and those that are sold in its major public markets.

Methodology

Sampling of P. canaliculata in Metro Manila

The sampling sites selected for the study fall into two categories: urban habitats (e.g. riverbanks, fisheries, commercial areas, and parks) and public markets. Lakes, rivers, and their tributaries were identified in each city in Metro Manila. Purposive sampling was done as the accessibility of these sites was taken into consideration since some bodies of water were highly polluted with garbage and some did not have road access. Markets that sold *P. canaliculata* as food were also identified. Based on these criteria, a total of 24 sites were examined for the presence of *P. canaliculata*; of these, 16 were urban habitats and eight were markets (Table 1).

The urban sites sampled varied in condition but were mostly polluted with little to no vegetation, except for Marikina Riverbanks and Laguna de Bay Lakeshore, Taguig where the vegetation was dominated by water lilies. The sites were inspected for *P. canaliculata* by looking for their distinct pink egg clusters in the walls/substrate near the banks or in the surrounding vegetation. The residents were also interviewed if they were familiar with the snail species, showing them empty shells as reference. When eggs were detected, the nearby substrate was manually searched for adult individuals. Around 30-40 specimens were opportunistically collected in each site, except in Catmon, Malabon (CAM, Table 1) where only seven live specimens were found. During transport to the lab, all samples were stored in plastic containers with water obtained from the sites or the market and lightly covered for proper aeration. Live *P. canaliculata* were obtained from seven (7) of the 16 urban habitats. Market samples were bought by weight equivalent to 30-40 specimens. A total of 462 individuals were collected and processed.

Parasite Examination

The snail samples were processed immediately in the lab after sampling. They were weighed, cut into pieces, and digested overnight in petri plates using Ash's digestive fluid [10]. The digested tissues in petri plates were then observed under a light dissecting microscope to manually detect parasites following the protocol of Constantino-Santos *et al.* [11] as well as other organisms that may be encountered. Visual examination of the morphology and isolation for third-stage larval nematodes followed those of Ash [10]. Arthropod larvae that were observed were immediately isolated and the entire specimens were used for DNA barcoding.

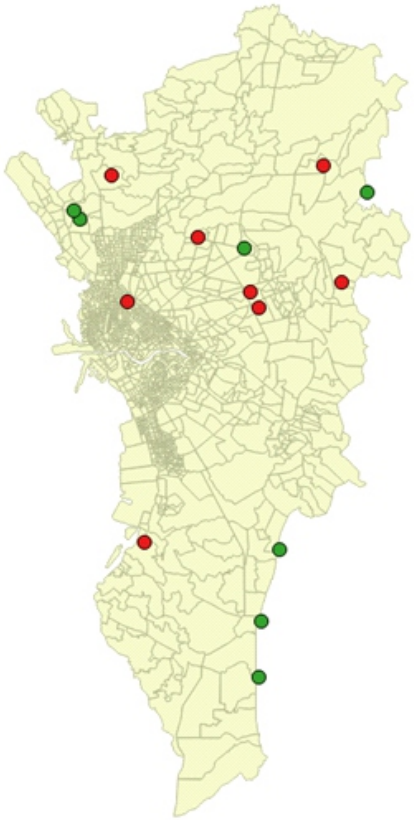

Figure 1. *Sampling sites that had populations/samples of P. canaliculata (n=15). Urban habitats (Green) and marketplaces (Red) were sampled for live P. canaliculata that were subjected to parasite detection.*

Table 1. *Sampling sites for P. canaliculata in Metro Manila. A total of 24 sites were visited in the study. Of the 16 urban habitats, only seven sites were positive for P. canaliculata populations.*

City	District	Area Type	*P. canaliculata*	Code
Caloocan	EDSA	Market[1]	30	EDC
Las Piñas	Pamplona	Field	-	-
Makati	Pio del Pilar	Commercial area	-	
Malabon	Catmon	Lake, commercial area	7	CAM
	Niugan	Canal	32	NIM
Manila	Ermita	Park area	-	-
	Malate	Park area	-	-
	Santa Cruz	Market[2]	30	SCM
Marikina	Calumpang (a)	River	-	-
	Calumpang (b)	Market[3]	30	CLM
Muntinlupa	Sucat	River	31	SUM
	Alabang	River	39	ALM
Paranaque	San Dionisio	Market[4]	30	SDP
Pasig	Francisco Legaspi (a)	Park area	-	-
	Francisco Legaspi (b)	River	-	-
Quezon City	Batasan Hills	River	41	BHQ
	Commonwealth	Market[5]	30	CWQ
	Cubao	Market[4]	30	CUQ
	Diliman (a)	Lagoon area	-	-
	Diliman (a)	Lake, park area	32	DIQ
	EDSA	Market[6]	30	EDQ
Valenzuela	Coloong	Lake	-	-
	Karuhatan	Market[6]	30	KAV
Taguig	Laguna de Bay Lakeshore	Lake	40	LBT

[1]snails originally from Malabon,[2]snails originally from Nueva Ecija,[3]snails originally from Cavite,[4]snails originally from Pampanga,[5]snails originally from Bicol
[6]snails originally from Bulacan

DNA Barcoding

Genomic DNA was extracted from tissue samples of the arthropod larvae using Invitrogen™ Purelink® Genomic DNA Mini Kit (Invitrogen, USA). The DNA extracted from the parasites were stored at -20°C prior to use.

Amplification of the COI gene fragment was done using the Folmer primers HCO (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO (5'-GGTCAACAAATCATAAAGATATTGG-3'), which are two of the most widely used for the COI barcodes in many animal groups [12, 13]. These primers have also been used in the laboratory to amplify the same COI barcodes in other species of the Diptera [14]. A 50 µL PCR mastermix consisting of: 10 µL of My Taq™ PCR buffer (Bioline, UK), 2.5 µL of dimethyl sulfoxide (DMSO), 2.5 µL of 10 µM for each primer, 0.25 µL of U My Taq™ DNA polymerase (Bioline, UK), 5-20 ng/µL of DNA extract, and nuclease-free water was made for the two samples processed for DNA barcoding. Amplification was done in 94°C for three minutes; 43 cycles at 94°C for 30 seconds, 45°C for 30 seconds, and 65°C for one minute; and 72°C for five minutes. The products from the PCR were visualized in 1% agarose gels with ethidium bromide. The PCR products were then extracted from the gel using a Qiagen™ Gel Extraction Kit (Qiagen™, USA). The purified PCR products were sent to 1st BASE Laboratories (Selangor, Malaysia) for capillary sequencing.

The DNA sequences were assembled in STADEN package version 1.5.3 [15]. The sequences were used for a query search on the Basic Local Alignment Search Tool in Genbank to determine possible identities [16].

Results and Discussion

No Nematode Infection in Metro Manila P. canaliculata

Considering the absence of parasite infection of *P. canaliculata* in Metro Manila, it is highly likely that the definitive hosts of nematode and trematode parasites are not present or have minimal interactions with the snails in the sampling sites. It is noted that both *A. cantonensis* and *Ancylostoma caninum* (Ercolani, 1859), nematodes known to parasitize urban snails such as *Achatina fulica* as intermediate or accidental hosts, have terrestrial definitive mammalian hosts like rats and dogs, respectively [11]. *A. fulica* is a terrestrial snail and has a better opportunity to interact with the parasites' definitive hosts or with their habitats. This may not be the case for an aquatic species such as *P. canaliculata*.

P. canaliculata has been noted to harbor a relatively low infection load in the Philippines as well as in other regions. A study in rice farming villages in Nueva Ecija investigated the parasite load of aquatic snails in direct association with *Rattus* sp., the definitive hosts of *A. cantonensis* [7]. While there was a recorded prevalence of 31% infection of the nematode species in the rats examined, only 2% of the 200 *P. canaliculata* sampled in their study were positive for *P. canaliculata*. A similar infection rate of *A. cantonensis* was also observed in a multi-country study of Laos, Cambodia, and Vietnam with a prevalence rate of 2.5% in all 1291 snails sampled [17]. In the same study, no infection was recorded in Laos. In a meta-analysis of 38 studies from 2005-2015 in China, a 7.6% infection rate was observed in a total of 41,299 *P. canaliculata* individuals [18,19].

It is important to note the patchiness of *P. canaliculata* distribution. These snails are not distributed evenly across the geographical range of Metro Manila because species abundance is rendered variable by resource availability and habitat suitability [20]. Excluding the samples from market sites, which sourced the snails from outside Metro Manila, the patchy distribution of *P. canaliculata* depends on the number of freshwater sites in Metro Manila with the resources that they require. While the definitive hosts and snails in Metro Manila may share the same habitat, their association with each other is not always direct. Although *Rattus* species have been recorded to consume snails in rice fields, they do so non-exclusively and feed on other invertebrates that are present [21]. The common rat species, *Rattus tanezumi* Temminck, 1844 and *R. norvegicus* (Berkenhout, 1769) can be found nesting near aquatic areas such as rice fields but they are usually found above the water level and prefer fully terrestrial nests [7,22,23]. *Pomacea* snails, on the other hand, are aquatic operculate snails that generally do not stray far from the water, only leaving it to deposit their eggs above the waterline or to feed on aquatic or semi-aquatic vegetation. The physiologies and ecological requirements of rats and snails also have little overlap, leading to the difficulty in the assessment of the complex transmission of *A. cantonensis* [7,17].

Parasite distribution may differ from that of their host. The hosts represent areas or patches of livable habitat in an environment that could otherwise be potentially unsuitable. Parasites are not distributed uniformly among these areas or patches, resulting in some having more parasites than the average number while others having fewer [24]. In parasitic ecology, parasite aggregation leads to many or most of the host individuals having no or few parasites while some hosts are infected with many [20]. Since only a limited number of individuals were sampled for each of the 15 sites through opportunistic sampling, it is possible that uncollected or overlooked individuals may be infected.

There is also the possibility that the method of isolating samples from snail specimens in the study might not have been optimal. DNA barcoding can accurately identify the species of nematode specimens upon isolation [7,11]. However, the main hurdle is in the actual isolation of the nematodes themselves. Several techniques in the isolation of *A. cantonensis* in snails are being used such as microanatomy, direct isolation from the lung tissues of the host snail [25], and DNA detection in environmental samples (e-DNA) [26]. Enzymatic digestion of snail tissues, the method used in this study, is a standard, and several studies that have isolated *A. cantonensis*, including those in Philippine snails and slugs, have used this method as well (10, 11, 27, 28). However, the reliance of this method to proper collection, storage, and the skill of the observer may be reduced or eliminated entirely in future studies. The use of several methodologies, in particular e-DNA, is recommended to more thoroughly sample snails in urban habitats. Only seven of the urban sites were positive for P. *canaliculata*, but the method of detection here was reliant on direct observation for their presence. The use of e-DNA may be able to detect *A. cantonensis* in the bodies of water even in the absence of physical specimens of *P. canaliculata*.

While a low infection rate is consistent in China and Southeast Asian regions, eosinophilic meningitis caused by

the consumption of this snail species is still observed [29]. Albeit nematode infection was not detected in the specimens collected in Metro Manila, the presence of the known vector *P. canaliculata* in urban habitats where they can be harvested by locals and those that are sold in markets implies that continuous surveillance needs to be implemented.

Association of Chironomid Larvae with P. canaliculata

Two snails (0.43% of the total sampled), one from CLM (Calumpang Market, Marikina, originally from Cavite), and another from SUM (Sucat, Muntinlupa), were observed to contain arthropod larvae. The larvae were found in the mantle of the snail, which they could have easily accessed through its siphon. It is likely that this is a form of phoresy wherein a species attaches itself to a host for dispersal [30]. These larvae were initially identified as non-biting midges (Diptera: Chironomidae) based on their morphology and blood-red coloration. Larval Chironomidae are difficult to identify to species level as most descriptions rely on the adult forms. The closest BLAST matches for these individuals were *Nilodorum tainanus* (91.0% identity), from SUM, and *Parachironomus* sp. (92.8% identity), from CLM. Both are members of family Chironomidae (Insecta: Diptera). However, it is important to note that these are only the closest matches for the two sequences. Due to the low percent identity of the query sequences against available GenBank sequences (91.0% for *Nilodorum tainanus* and *Parachironomus* sp. for 92.8%), only the samples' familial designation could be confirmed but not their taxonomic designation. Both of the BLAST matches belong to the subfamily Chironominae, of which only four genera are recorded in the Philippines, none of which are the same as those in the Genbank database [31]. For family Chironomidae, studies using COI as a DNA barcode report that the gene region is not problematic in species-level chironomid identification [32]. However, the Philippine insect fauna is poorly represented in DNA databases, which is the primary reason for the failure to provide molecular identification at the genus level for the specimens.

No literature yet has reported the presence of chironomid larvae in *P. canaliculata* in the Philippines. Chironomids have been detected in *Pomacea* sp. in its native distribution in South America, albeit in low densities and frequencies [33]. Chironomid larvae were previously detected in the lymnaeid snail *Chilina dombeyana* (Bruigière, 1789) in what the authors suggest as a case of phoresy [34]. Non-biting midges can co-occur with populations of *P. canaliculata* in freshwater habitats, but the frequency of snail infection in this study cannot correlate into such an interaction.

Larvae of chironomid flies proliferate in aquatic environments especially if eutrophication has occurred in the area [35]. Many of the species in Chironomidae have been labeled as pestiferous. They degrade plants such as water hyacinths, horseradishes, and lotuses, and pose an agricultural threat as they also harm rice fields [36]. Chironomids are also considered as bioindicators of water and sediment quality and can pose public health risks due to their association with bacteria such as *Vibrio cholerae*, *Aeromonas*, and *Salmonella* and the high tolerance of larvae to mercury, lead, and cadmium [36-38]. Chironomid larvae have also been reported to cause respiratory allergies and nephrotic syndrome due to the handling of the larvae itself and of fish foods, which also contain the larvae [35,39].

The association of chironomids to pollution and certain diseases pose health risks to humans, especially those that consume *P. canaliculata*. Chironomids are not known to be inherently parasitic to snails; the observation in this study could be a case of an accidental association, although more work has to be done to determine the cause of the interaction. While no medically important parasite was detected in *P. canaliculata*, consistent and frequent monitoring of helminth parasites of this edible snail species is still advised as it remains a staple food in many regions of the Philippines.

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