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Genetic Comparison of *Oncomelania hupensis quadrasi* (Möllendorf, 1895) (Gastropoda: Pomatiopsidae), the Intermediate Host of *Schistosoma japonicum* in the Philippines, Based on 16S Ribosomal RNA Sequence

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ABSTRACT

Schistosomiasis japonica is a water-borne trematode infection transmitted by different subspecies of *Oncomelania hupensis*. As parasites may either co-evolve or locally adapt with their hosts, snail diversity, as revealed by morphometric and genetic studies, may reflect parasite diversity and elucidate snail susceptibility and transmission patterns. This study aimed to compare isolates of *O. h. quadrasi* based on a 342-bp fragment of

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the 16S ribosomal RNA gene. *O. h. quadrasi* isolates were collected from nine provinces known to have *S. japonicum* in the Philippines, namely Cagayan Valley, Bohol, Negros Occidental, Leyte, Davao, Davao del Sur, Mindoro Oriental, Northern Samar, and Sorsogon. *O. h. hupensis* and *O. h. nosophora* isolates were also collected from China and Japan, respectively. The 16S ribosomal RNA gene of each specimen was amplified and sequenced. Phylogenetic and network analyses based on the 221 16S rRNA gene sequences revealed that *O. h. quadrasi* clustered as a distinct clade from the two other subspecies. Of the four identified haplotypes for *O. h. quadrasi*, two haplotypes were from Negros Oriental (Ohq2 and Ohq3), and one haplotype was from Bohol (Ohq4). The isolates from the remaining seven provinces shared a common haplotype (Ohq1). The current study was able to show the relationship among *O. hupensis* subspecies and demonstrate the limited ability of mitochondrial 16S ribosomal molecular marker in differentiating *O. h. quadrasi* geographic strains in the Philippines.

Keywords: Oncomelania hupensis quadrasi, *Schistosoma japonicum*, *Schistosomiasis japonica*, Snail Intermediate Host, Haplotype, 16S Ribosomal RNA Gene

INTRODUCTION

Oncomelania hupensis (Gastropoda: Pomatiopsidae) is an amphibious freshwater snail that acts as the intermediate host of *Schistosoma japonicum*, a parasitic worm of great public health concern. Nine geographical subspecies found throughout Asia have been recognized for the *O. hupensis* species complex (WHO 1993). The four subspecies in China are *O. h. hupensis* (Yangtze Basin)*, O. h. robertsoni* (Sichuan and Yunnan)*, O. h. tangi* (Fujian), and *O. h. guangxiensis* (Guangxi). In other Asian countries, the subspecies present are *O. h. nosophora* in Japan, *O. h. lindoensis* in Indonesia, *O. h. chiui* and *O. h. formosana* in Taiwan, and *O. h. quadrasi* in the Philippines (Davis et al. 1995a). The subspecies mainly differ in shell dimensions, electromorphic and antigenic patterns, reproductive potential, growth rate, and compatibility with various geographic strains of *S. japonicum* (Davis et al. 1995b; Zhao et al. 2010).

Oncomelania hupensis quadrasi in the Philippines was first recognized as the intermediate host of *S. japonicum* by Tubangui, based on observations made in Palo, Leyte (Blas 1988; Leonardo et al. 2016). An adult *O. h. quadrasi* shell is

colored light brown to black, usually measures 3 to 5 mm, and has 5 to 7 whorls (Figure 1). These snails are dioecious and thrive in many different kinds of wet shaded environments. These habitats include flood plains, swampy areas, and grass lands. Although considered transient, rice fields and other man-made habitats, such as irrigation canals and burrow pits, also support these snails. They prefer areas with dense vegetation where flow of water is sluggish, and rivers that are not very deep or too wide. *O. h. quadrasi* colonies have been known to occur over most of Mindanao and Samar, eastern Leyte, Bohol, in small areas in southeastern Luzon, and eastern Mindoro (Pesigan et al. 1958). *O. h. quadrasi* colonies have been recently discovered in Cagayan Valley in northern Luzon and in Negros Occidental (Leonardo et al. 2015).

Figure 1. *Oncomelania hupensis quadrasi* (Mollendorf 1895) collected from the province of Leyte in eastern Philippines. Juvenile (right) snails measure a maximum of 3 mm while adult snails reach more than 3 mm in size.

Genetic studies on the intermediate host are important in determining the relationship of the intermediate host with the parasite, as the presence or absence of co-evolution may significantly affect transmission patterns. Co-evolution implies the restriction of the parasite to certain snail hosts, limiting transmission to areas where snails are compatible with the parasite (Davis et al. 1999), whereas the absence of co-evolution, as evidenced by recent studies, may result in host-switching or acquisition, making it possible for the parasite to spread in other areas (Attwood et al. 2015). Previous studies have reported the different compatibilities of snail populations from different geographic locations with strains of *S. japonicum,* and variation in susceptibility to sodium pentachlorophenate (NaPCP), a molluscicidal agent (Cross et al. 1984; Hong et al. 1995; Rachford 1997). Determination of genetic variants within geographical isolates of *O. h. quadrasi* is necessary for the evaluation of their potential in transmitting *S. japonicum*.

Past research revealed only minor variation among geographic isolates of *O. h. quadrasi* in the Philippines (Viyanant et al. 1987; Woodruff et al. 1988); however,

these studies only involved techniques that tested phenotypic characters, which are subject to environmental influences. Some enzyme-coding genes are only expressed under certain environmental conditions (Davis et al. 1995a). Hope and McManus (1994) used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to directly examine the internal transcribed spacer (ITS) gene, and found minor variation among Philippine snails. A recent paper of Saijuntha et al. (2014) studied 12S ribosomal RNA gene sequences of *O. h. quadrasi* from three provinces with *S. japonicum*, and they were able to show population substructuring associated with geographical origin.

To further investigate the phylogeography of *O. h. quadrasi* in the Philippines, this study examined genetic variation among snail populations collected from 12 localities in seven islands based on the 16S rRNA gene. Snail samples from two newly identified endemic foci for *S. japonicum* in Cagayan Valley and Negros Occidental were included in the analysis. The 16S rRNA gene was selected since previous studies have demonstrated its utility to assess genetic variation in populations in the aquatic snail *Potamopyrgus antipodorum* (Stadler et al. 2005), the slug *Arion subfucus* (Pinceel et al. 2005), the giant African snail *Achatina fulica* (Fontanilla et al. 2014), and the pomatiopsid snail *Neotricula aperta* (Attwood et al. 2008).

MATERIALS AND METHODS

Snail Collection

Geographic isolates of *O. h. quadrasi* were collected from nine provinces across seven islands of the Philippines: Cagayan and Sorsogon on Luzon island; Mindoro Oriental on Mindoro island; Negros Occidental on Negros island; Bohol on Bohol island; Leyte on Leyte island; Samar on Samar island, and Davao and Davao del Sur on Mindanao island (Table 1). Thirty individual snails from each locality were examined. Snails collected were identified as *O. h. quadrasi* by comparison with previously identified voucher specimens archived in the Department of Parasitology, College of Public Health, University of the Philippines Manila. Samples of *O. h. hupensis* from China and *O. h. nosophora* from Japan were also included in the study. Snails were washed with 0.9% NaCl and placed in a vial with 70% ethanol before being transported to the laboratory. The head-foot muscle was dissected under the microscope, and this was used in the DNA extraction.

Table 1. Source organisms, localities, and sample sizes of 16S sequences analysed

Sample Code	Locality	Province	Island	Country	Subspecies	No.
MCag	Magrafil	Cagayan Valley	Luzon	Philippines	*O. h. quadrasi*	27
TCag	Tapel	Cagayan Valley	Luzon	Philippines	*O. h. quadrasi*	3
TSor	Tulay	Sorsogon	Luzon	Philippines	*O. h. quadrasi*	30
BDMin	Batong Dalig	Mindoro Oriental	Mindoro	Philippines	*O. h. quadrasi*	30
BNeg	Bonbon	Negros Occidental	Negros	Philippines	*O. h. quadrasi*	22
HNeg	Hinag-ongan	Negros Occidental	Negros	Philippines	*O. h. quadrasi*	6
MBoh	Magsaysay	Bohol	Bohol	Philippines	*O. h. quadrasi*	30
PLey	Palo	Leyte	Leyte	Philippines	*O. h. quadrasi*	13
DLey	Dagami	Leyte	Leyte	Philippines	*O. h. quadrasi*	11
Sam	Catarman	Northern Samar	Samar	Philippines	*O. h. quadrasi*	18
Dav	Compostela Valley	Davao	Mindanao	Philippines	*O. h. quadrasi*	29
Das		Davao del Sur	Mindanao	Philippines	*O. h. quadrasi*	2
WAnh	Wuwei	Anhui	Mainland China	China	*O. h. hupensis*	4
TAnh	Tongling	Anhui	Mainland China	China	*O. h. hupensis*	4
HAnh	Hexian	Anhui	Mainland China	China	*O. h. hupensis*	4
XAnh	Xiuzhou	Anhui	Mainland China	China	*O. h. hupensis*	4
Lanh	Lingjiang	Anhui	Mainland China	China	*O. h. hupensis*	4
DAnh	Dongzhi	Anhui	Mainland China	China	*O. h. hupensis*	4
SAnh	Shitai	Anhui	Mainland China	China	*O. h. hupensis*	4
JHun	Jiangshan	Hunan	Mainland China	China	*O. h. hupensis*	4
CZhe	Chizhou	Zhejiang	Mainland China	China	*O. h. hupensis*	4
NYam	Nirasaki	Yamanashi	Honshu	Japan	*O. h. nosophora*	5
KYam	Kofu	Yamanashi	Honshu	Japan	*O. h. nosophora*	2

DNA extraction, PCR amplification, and DNA sequencing

DNA extraction was carried out using the Qiagen® Genomic DNA Extraction Kit™ (USA) following the manufacturer's protocol. DNA was quantified using Thermo Scientific®NanoDrop™ 2000c (USA). PCR amplification of the 16S rRNA gene was performed in a total volume of 50 µL containing 20mM buffer (Tris-HCl, $MgCl_2$, and KCl), 10 pmol each of forward and reverse primers, 2.5 mM dNTP, 1.25 units Roche® Taq DNA polymerase, and 8.0 µL DNA template. The forward and reverse primers used were 16S1ionco5 5'–TGACCGTGCGAAGGTAGCAT–3', which was designed for this study, and 16SSCP2i 5'– CCTAGTCCAACATCGAGGTC–3', which was obtained from Fontanilla et al. (2010). This primer pair amplifies a 342-bp fragment of the 16S rRNA gene that corresponds to its secondary structure's domain IV (Lydeard et al. 2000), which is also the same fragment used in other previous studies (Stadler et al. 2005; Pinceel et al. 2005; Attwood et al. 2008; Fontanilla et al. 2014). Initial denaturation temperature was 94°C for 3 minutes succeeded by 43 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, and extension at 65°C for 1 minute, which was followed by one final extension step at 72°C for 5 minutes. PCR products were purified from agarose using Qiagen® QIAquick Gel Extraction Kit™ (USA) following the manufacturer's protocol. Purified PCR products were sent to 1st BASE Pte. Ltd. in Malaysia for DNA sequencing.

DNA Sequence Analysis

Sequences were aligned using BioEdit Version 7.0.9.0 (Hall 2008). Aligned sequences were inspected manually and distinct haplotypes were identified using the Data Analysis in Molecular Biology and Evolution (DAMBE) Version 5.2.79 software (Xia and Xie 2001). Consensus sequences were submitted to NCBI's GenBank (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov/) for the assignment of accession numbers (Table 2).

For the phylogenetic analyses, three methods of tree construction, two requiring a model of DNA substitution and one method that did not, were employed. For the model-based methods, the best model with optimized parameters was determined using the Bayesian inference criterion (BIC) in jModel Test Version 0.1.1 (Posada 2008). Once the optimal model was identified, the maximum likelihood (ML) tree (Cavalli-Sfroza and Edwards 1967; Felsenstein 1981) and neighbor joining tree (Saitou and Nei 1987) were constructed using PHYML Version 2.4.4 (Guindon and Gascuel 2003) and PAUP Version 4.0b10 package (Swofford 2002), respectively.

For the non-model based method, the maximum parsimony (MP) tree (Eck and Dayhoff 1966; Fitch 1977) was constructed using MEGA6 (Tamura et al. 2013). All three methods rooted the trees on *O. minima*, and bootstrap resampling (Felsenstein 1985) with 1000 replicates was also undertaken. An ML tree that includes the bootstrap supports for the clades based on the ML, NJ, and MP analyses was consequently generated.

Table 2. Genbank accessions of 12 haplotypes of the 16S rRNA gene of various *Oncomelania* spp.

Sequence ID	Country	Isolate	GenBank Accession	Subspecies
Ohq1	Philippines: Palo, Leyte	L6	KY432666	*O. h. quadasi*
Ohq2	Philippines: Calatrava, Negros Occidental	N28	KY432668	*O. h. quadrasi*
Ohq3	Philippines: Calatrava, Negros Occidental	N15	KY432667	*O. h. quadrasi*
Ohq4	Philippines: Magsaysay, Bohol	B1	KY432669	*O. h. quadrasi*
Ohh1	China: Wuwei, Anhui	1AHwuwei	KY432670	*O. h. hupensis*
Ohh2	China: Lingjiang, Anhui	1AHlingjiang	KY432671	*O. h. hupensis*
Ohh3	China: Hexian, Anhui	1AHhexian	KY432673	*O. h. hupensis*
Ohh4	China: Xiuzhou, Anhui	1AHxiuzhou	KY432674	*O. h. hupensis*
Ohh5	China: Shitai, Anhui	1AHshitai	KY432675	*O. h. hupensis*
Ohh6	China: Chizhou, Zhejiang	1ZJchizhou	KY432676	*O. h. hupensis*
Ohh7	China: Jiangshan Hunan	1HNjiangshan	KY432672	*O. h. hupensis*
Ohn1	Japan: Kofu, Yamanashi	1Kofu	KY432677	*O. h. nosophora*

A median joining network of the distinct haplotypes was also constructed using the Network Version 4.502 program (Bandelt et al. 1999), in order to elucidate the relationships of the different *O. hupensis* subspecies, and in particular, the Philippine *O. hupensis quadrasi* haplotypes. DNAsp5 was utilized to compute for the haplotype number (Slatkin and Hudson, 1991; Librado and Rozas, 2009).

RESULTS

Analysis of *O. hupensis* subspecies

A total of 221 *16S* rRNA sequences were obtained for *O. h. quadrasi*, 36 for *O. h. hupensis*, and seven for *O. h. nosophora* (Table 1). Alignment revealed 20 polymorphic sites consisting of 19 transitions and one insertion/deletion (Table 3). The variable

sites yielded 12 haplotypes: four haplotypes of *O. h. quadrasi,* seven haplotypes of *O. h. hupensis*, and a single haplotype of *O. h. nosophora* (Tables 2, 3, and 5). The HKY85+G model was identified to be the best model for sequence evolution with optimized parameters using the Bayesian inference criterion (BIC). Median-joining network analysis supports the ML tree findings as four similarly distributed clusters were identified. The *O. h. quadrasi* haplotypes clustered in one group and were assigned to be closely related to an *O. h. robertsoni* haplotype (Figure 2). The Haplotype network analysis suggests that Philippine haplotypes share one, or possibly two putative haplotype ancestors (mv5 and mv6; Figure 3), which split into two lineages. The first lineage includes the common haplotype (Ohq1) found in majority of the sampled areas, which in turn gave rise to the Bohol haplotype (Ohq4). The other lineage gave rise to the two haplotypes in Negros Occidental, the Hinabo (or Hinab-ongan) haplotype (Ohq2) and the Bonbon haplotype (Ohq3).

Table 3. Variable positions of the 16S rRNA gene sequences of *O. h. quadrasi* (Ohq), *O. h. hupensis* (Ohh), and *O. h. nosophora* (Ohn) haplotypes. Nucleotide position number is based on *O. h. hupensis* isolate SCMS complete mitochondrial genome sequence [GenBank: NC_012899]

Haplotype	Nucleotide Position																			
						1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	9	9	9	9	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	3	5	5	9	0	0	0	1	2	2	2	2	3	4	4	4	5	9	9
	1	5	1	5	2	0	2	6	9	1	2	4	8	5	3	5	6	8	7	8
Ohq1	G	A	A	T	G	C	C	G	G	C	A	T	A	G	A	A	-	A	T	A
Ohq2	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	T	.	.	.
Ohq3	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	T	.	C	.
Ohq4	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	-	.	.	.
Ohh1	A	.	.	C	.	T	T	.	A	.	.	.	G	A	.	G	-	G	.	G
Ohh2	A	.	.	C	.	T	T	.	A	.	.	.	G	A	.	G	-	.	.	G
Ohh3	A	.	.	C	.	T	T	.	A	.	.	.	G	A	G	.	-	.	.	G
Ohh4	A	.	.	C	.	T	T	A	A	.	.	.	G	A	.	G	-	.	.	G
Ohh5	A	.	.	C	.	T	T	.	.	.	.	.	G	A	.	G	-	.	.	G
Ohh6	A	.	.	C	.	T	T	.	A	.	G	C	G	A	.	G	-	.	.	G
Ohh7	A	G	.	C	.	T	T	.	A	.	.	.	G	A	.	G	-	.	.	G
Ohn1	A	.	G	C	A	T	T	.	A	T	.	.	G	A	.	G	-	G	.	G

There were no common or shared haplotypes among the three subspecies. The ML tree also showed that *O. h. quadrasi* formed a highly supported (92% ML, 95% MP, and 90% NJ bootstraps) cluster that was distinct from the other subspecies (Figure 3). The phylogenetic tree contradicts current subspecies classification based on

geographic origin as *O. h. hupensis* subspecies clustered with *O. h. formosana* from Taiwan and *O. h. nosophora* from Japan. *O. h. robertsoni* and *O. h. quadrasi* were observed to be monophyletic.

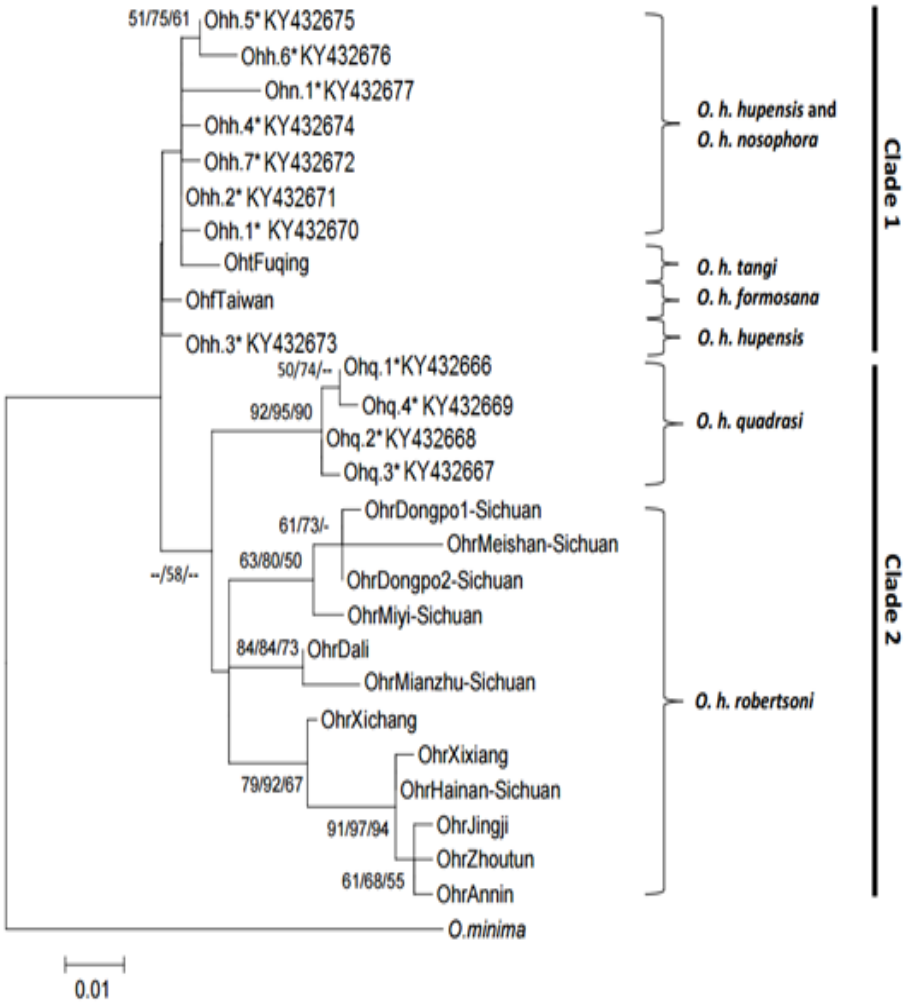

Figure 2. Maximum likelihood tree of *O. hupensis* based on 342 nucleotides of the 16S rRNA gene and using the HKY85+G model of DNA substitution. Haplotypes with (*) represent samples from this study while the rest were obtained from GenBank. The tree was rooted on *O. minima*. Values on nodes represent bootstrap support percentage (out of 1000 bootstrap samples) for ML/MP/NJ; values less than 50% are not shown. Bootstraps for NJ were likewise based on the HKY85+Γ model. Bootstraps for MP were based on six equally parsimonious trees. Scale bar represents one nucleotide substitution for every 100 nucleotides.

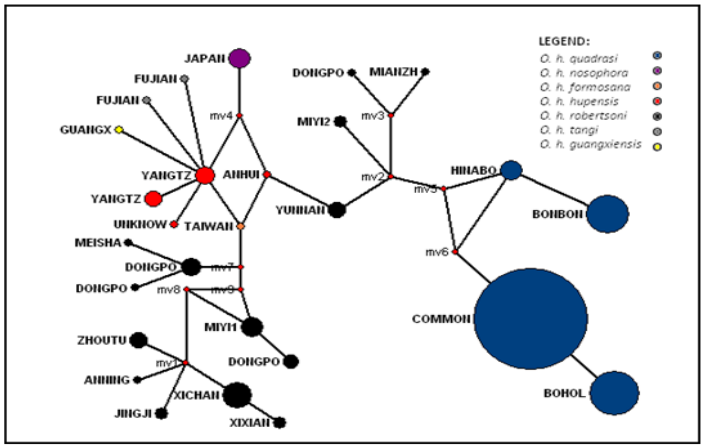

Figure 3. Median-joining haplotype network of three subspecies of *O. hupensis* based on 16S rRNA gene sequences. The size of the circles represents the frequency of each haplotype (found inside parenthesis) among the isolates. Red dots represent putative haplotypes. Ohq = O. *h. quadrasi*; Ohh = *O. h. hupensis*; Ohn = *O. h. nosophora*.

Genetic Diversity of *O. h. quadrasi*

Genetic distance ranged from 0.30 to 7.69% among subspecies haplotypes, and 0.30 to 0.95% among *O. h. quadrasi* haplotypes (Table 4). Of the four haplotypes of *O. h. quadrasi*, two haplotypes were from Bonbon and Hinab-ongan in Negros Occidental (Ohq2 and Ohq3), and a single haplotype was from Magsaysay, Bohol (Ohq4). The isolates from the remaining seven provinces formed a single shared haplotype (Ohq1) (Table 5). The haplotype and nucleotide diversities for the Philippine population were 0.429 ± 0.037 and 0.00230 ± 0.00024, respectively (Table 4). There was no substantial genetic subdivision formed among the nine provinces, suggesting that high rates of gene flow occur among these regions.

Table 4. Molecular variations of the *O. h. quadrasi* from the Philippines grouped according to island of origin.
n = number of samples; h = number of haplotype;
μ = number of non-overlapping haplotype; hd = haplotype diversity;
Π = nucleotide diversity

Population	n	h	μ	hd ± SD	π ± SD
Luzon	60	1	0	0.000 ± 0.000	NA
Mindoro	30	1	0	0.000 ± 0.000	NA
Leyte	24	1	0	0.000 ± 0.000	NA
Samar	18	1	0	0.000 ± 0.000	NA
Negros	28	2	2	0.349 ± 0.090	0.00125 ± 0.00001
Bohol	30	1	1	0.000 ± 0.000	NA
Mindanao	31	1	0	0.000 ± 0.000	NA
All populations	226	4	4	0.429 ± 0.037	0.00230 ± 0.00024

DISCUSSION

The study was able to show that *O. h. quadrasi* is genetically different from the other subspecies of *O. hupensis* found in other Asian countries based on the 16S rRNA sequences. However, only limited genetic substructuring existed among the geographical isolates of *O. h. quadrasi* in the nine provinces of the Philippines. Two major clades were observed in the ML tree. *O. h. hupensis, O. h. tangi, O. h. formosana,* and *O. h. nosophora* formed clade 1. *O. h. robertsoni* isolates grouped with *O. h. quadrasi* in clade 2. The four haplotypes of *O. h. quadrasi* clustered together to form a distinct subclade. These results agree with those of a recent comparative phylogenetic study of *Oncomelania hupensis* and *Schistosoma japonicum* using mitochondrial genes, in which *O. h. tangi* and *O. h. formosana* also clustered with *O. h. hupensis*, suggesting that they are not valid subspecies but rather are *O. h. hupensis* variants (Attwood et al. 2015). The same study likewise found *O. h. robertsoni* to be not monophyletic (Attwood et al. 2015).

The phylogenetic tree in this study closely resembles the trees previously constructed using the mitochondrial 12S ribosomal gene (Okamoto et al. 2003), and combined mitochondrial *COI* and 16S genes (Wilke et al. 2006). In the 12S tree, *O. h. hupensis* also clustered with *O. h. nosophora* and *O. h. formosana*, while *O. h. quadrasi* also formed a monophyletic clade. Similarly, they identified a Bohol haplotype and a shared haplotype from Mindoro and Sorsogon. The previous study also identified two haplotypes from different sampled areas in Davao, Digos, and Asuncion, while the current study using the 16S genetic marker identified the Davao representative from Compostela Valley to be identical with the common haplotype from the remaining provinces. It is therefore possible that increased sampling effort from other areas in Davao could lead to the discovery of other 16S rRNA gene haplotypes; this may also be the case for the other provinces.

Cagayan Valley and Negros Occidental have only been recently identified as endemic for schistosomiasis in 2002 and 2005, respectively (Leonardo et al. 2015). The current hypothesis for these two areas is that *O. h. quadrasi* had always been present in these places, and recent migration of schistosome-infected human and/or animal hosts resulted in sustained transmission. However, results of the network analysis suggest the possibility of Negros Occidental snail isolates evolving independently from other geographic isolates. Therefore, the same apparent barriers to dispersal that led to the snails from Negros Occidental being genetically distinct from the other populations could lead to similar isolation and divergence of the schistosomes present there. This may likely have been the result of allopatric

speciation, in which new strains arise in non-overlapping geographic locations. This suggests that Negros Occidental had always been endemic for schistosomiasis but clinical cases were unreported or misdiagnosed. For Cagayan Valley, the founder effect seems more likely as the local snails were discovered to be identical with the isolates from other schistosomiasis-positive provinces, which also raises the possible endemicity of the *O. h. quadrasi* haplotypes in other Luzon provinces. Performing a molecular clock analysis could be applied to confirm either hypothesis. This type of analysis has been successfully accomplished for *O. h. robertsoni* (Hauswald et al. 2011) and Japanese pomatiopsids (Kameda and Kato 2001).

The presence of fewer *O. h. quadrasi* haplotypes compared to the Chinese populations may be explained by isolation by distance, or either founder effect or population bottlenecking. Genetic differentiation among individuals increases as geographical distance increases, which is brought about by isolated populations being prevented from mating with other populations (Wright 1943). This could explain why China, which is much larger than the Philippines, has more snail haplotypes. Founder effect occurs when populations originate from only a few individuals (Freeman and Herron 2004), such as in *Achatina fulica* populations (Fontanilla et al. 2014). This may be the case if *O. h. quadrasi* were introduced by migratory birds from Chinese *O. hupensis* ancestors. Population bottlenecking, the other possibility, may have been the result of early schistosomiasis control efforts, wherein the use of molluscicides successfully reduced snail populations. However, when the use of molluscicides was halted due to adverse environmental effects, the surviving snail populations repopulated the affected areas, resulting in reduced genetic variation among progenies. The use of molluscicides could also have acted as a selection pressure that caused the appearance of new strains, which is a possible explanation of the emergence of the Bohol haplotype from the shared haplotype. The founder effect seems to be more likely since Negros Occidental and Cagayan Valley were recently discovered to be schistosomiasis endemic areas and were previously not subjected to control efforts. However, *O. h. quadrasi* populations in these two areas may have been unintentionally reduced together with crop pests as they co-inhabit rice fields (Freeman and Herron 2004).

With most isolates under a common haplotype, effects of existing snail control measures (e.g., clearing of vegetation and exposure of habitats to direct sunlight) on these haplotypes would unlikely vary and are expected to have the same impact as in previous instances. However, more detailed studies on tolerance ranges, habitat preferences, and molluscicide susceptibility, particularly on other haplotypes, are needed to ascertain this assumption.

Table 5. Distribution of 16S rRNA gene haplotypes at various localities sampled for *O. h. quadrasi, O. h. hupensis* and *O. h. nosophora*.

Locality	n	Number of samples in each haplotype of *O. h. quadrasi*				Number of samples in each haplotype of *O. h. hupensis*							Number of samples in each haplotype of *O. h. nosophora*
		Ohq1	Ohq2	Ohq3	Ohq4	Ohh1	Ohh2	Ohh3	Ohh4	Ohh5	Ohh6	Ohh7	Ohn1
MCag	27	27	-	-	-	-	-	-	-	-	-	-	-
TCag	3	3	-	-	-	-	-	-	-	-	-	-	-
TSor	30	30	-	-	-	-	-	-	-	-	-	-	-
BDMin	30	30	-	-	-	-	-	-	-	-	-	-	-
BNeg	22	-	22	-	-	-	-	-	-	-	-	-	-
HNeg	6	-	-	6	-	-	-	-	-	-	-	-	-
MBoh	30	-	-	-	30	-	-	-	-	-	-	-	-
PLey	13	13	-	-	-	-	-	-	-	-	-	-	-
DLey	11	11	-	-	-	-	-	-	-	-	-	-	-
Sam	18	18	-	-	-	-	-	-	-	-	-	-	-
Dav	29	29	-	-	-	-	-	-	-	-	-	-	-
Das	2	2	-	-	-	-	-	-	-	-	-	-	-
WAnh	4	-	-	-	-	4	-	-	-	-	-	-	-
TAnh	4	-	-	-	-	2	2	-	-	-	-	-	-
HAnh	4	-	-	-	-	-	-	4	-	-	-	-	-
XAnh	4	-	-	-	-	-	-	-	4	-	-	-	-
LAnh	4	-	-	-	-	-	4	-	-	-	-	-	-
DAnh	4	-	-	-	-	-	-	-	-	2	2	-	-
SAnh	4	-	-	-	-	-	-	-	-	4	-	-	-
JHun	4	-	-	-	-	-	2	-	-	-	-	2	-
CZhe	4	-	-	-	-	-	-	-	-	-	4	-	-
Kofu	7	-	-	-	-	-	-	-	-	-	-	-	7
		163	22	6	30	6	8	4	4	6	6	2	7
TOTAL:	264	221				36							7

The use of additional markers as concatenated sequences is recommended to further characterize the different haplotypes present in the Philippines. The lack of genetic substructuring among the *O. h. quadrasi* isolates based on the 16S gene in this study is in contrast to what Saijuntha et al. (2014) observed. Combining the markers may provide greater phylogenetic signal and more variable positions. It should be noted that the evolutionary histories of taxa are reflected by the evolutionary histories of their genomes rather than by single genes, which may have different evolutionary histories that interact with each other and with the environment (Morrison 2006).

CONCLUSIONS

The current study was able to demonstrate *O. h. quadrasi*'s genetic diversity using the 16S rRNA gene. *O. h. quadrasi* is a distinct subspecies with four haplotypes, two from Negros Occidental (Ohq2 and Ohq3), a single haplotype in Bohol (Ohq4), and one from the remaining provinces in the study (Ohq1). There might be a need for the re-evaluation of the *O. hupensis* subspecies classification as Chinese isolates spread into the two clades.

COMPETING INTERESTS

The authors do not have any competing interests.

AUTHORS' CONTRIBUTIONS

JC, IF, MK, NH, YC, and LL conceptualized the project. JC, PT, IT, and LL performed snail collection in the Philippines, while TA provided samples from China and Japan. JC, IF, ERDC, PT, IT, RJF, and TA performed PCR, sequence alignment, and data analysis. JC, IF, LL, IT, and RJF wrote the manuscript with the suggestions of all the other authors.

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