Genetic Structure Inferred from Mitochondrial 12S Ribosomal RNA Sequence of *Oncomelania quadrasi*, the Intermediate Snail Host of *Schistosoma japonicum* in the Philippines

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Abstract. Species and subspecies of the *Oncomelania hupensis* species complex are recognized as intermediate hosts of *Schistosoma japonicum*. Of these species and subspecies, *O. quadrasi* is distributed throughout the Philippines. This study used 12S ribosomal RNA sequences to explore the genetic structure of *O. quadrasi* populations in the Philippines. Three subspecies, *O. h. hupensis*, *O. h. formosana*, and *O. h. chiuui* of this group were also examined. The phylogenetic tree and haplotypes network showed that *O. quadrasi* separated from the subspecies. Ten *O. quadrasi* haplotypes (Oq1–Oq10) clustered in relation to their geographic origin. Genetic differentiation (F_{ST}) and estimated gene flow (Nm) among populations showed significant differences, ranging from 0.556–1.000 to 0.00–0.74, respectively. Genetic differences among groups ($F_{CT} = 0.466$), populations within a group ($F_{SC} = 0.727$), and populations ($F_{ST} = 0.854$) were observed. These results indicate that the *O. quadrasi* populations in the Philippines have a substructure associated with their geographic origin.

INTRODUCTION

Snails of the *Oncomelania hupensis* group are recognized as the intermediate hosts of *Schistosoma japonicum*, a major causative agent of schistosomiasis in countries in Asia. There are many species and subspecies in the *O. hupensis* complex that act as the intermediate hosts of *S. japonicum*, i.e., *O. hupensis hupensis*, *O. h. tangi* and *O. h. robertsoni* in China, *O. h. nosophora* in Japan, *O. h. lindoensis* in the Indonesian islands, *O. h. formosana*, *O. h. chiuui* in Taiwan, and *O. quadrasi* in the Philippines.^{1,2} During the 1970s, *S. japonicum* was still endemic to many areas in the Philippines. Currently 560,000 persons are estimated to be infected and more than 6.7 million are at risk of infection in 28 of the 79 provinces in the country, particularly among rice farmers and fishermen.^{1,2} Moreover, not only are humans infected, but there are many animals that act as final reservoir hosts of *S. japonicum* in the Philippines, including dogs, cats, pigs, rats, goats, cattle, and water buffalos.^{3,4}

The genetic diversity of *O. hupensis* complex, especially *O. h. hupensis*, *O. h. tangi*, and *O. h. robertsoni* from mainland China, has been intensively investigated.^{5,6} Evidence of close co-evolutionary relationships between *S. japonicum* and its intermediate snail host *O. hupensis* has been demonstrated. This finding has led to an increased interest in the phylogenetics and population genetic structure of the snail.^{5,7} Because there are co-evolutionary relationships and close genetic interactions between the snails and their parasites, a snail population may reflect population genetic parameters of the parasite and vice versa.^{8,9} Genetically diverse snail populations appear to be more susceptible to infection with *S. japonicum* than homogeneous populations.¹⁰

In spite of a considerable amount of research on the genetic diversity within populations of *O. quadrasi*, only a limited assessment has been made on the significance of this diversity in a population structure or phylogenetic context. For instance, there are some reports of genetic diversity of *O. quadrasi* based on several genetic markers, including allozymes, nuclear DNA, and mitochondrial DNA sequences.^{11–13} These markers could be potentially used to demonstrate genetic diversity within *O. quadrasi* and its differentiation from other species and subspecies within the *O. hupensis* complex. However, knowledge of the genetic structure of *O. quadrasi* populations in the Philippines is virtually unknown.

The taxonomic status of variously designated subspecies of *O. hupensis* is controversial because some authors favor subspecies-specific characterization while others consider certain previously designated subspecies, including *O. quadrasi*, to be full species based on morphologic (e.g., shell sculpture, operculum) and molecular characteristics and geographic distributions.^{14,15} Recent studies strongly support the specific status of *O. quadrasi*.^{13,14} For a better understanding of the population genetic structure of *O. quadrasi* in the Philippines, this study investigated 12S ribosomal RNA sequence variation from six localities in three provinces on three islands. Three subspecies, *O. h. hupensis* from China, and *O. h. formosana* and *O. h. chiuui* from Taiwan, were also included in the analysis to determine the levels of interspecific genetic variation and phylogenetic relationships.

MATERIALS AND METHODS

Sample collection. Snails were collected from the field from different countries: *O. quadrasi* from the Philippines, *O. h. hupensis* from China, *O. h. chiuui*, and *O. h. formosana* from Taiwan (Table 1). Collections from the Philippines were from three provinces: Cagayan, Oriental Mindoro, and Leyte (Table 1), which are located on the islands of Luzon, Mindoro, and Leyte, respectively. Snails were washed in sterile 0.3%

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TABLE 1
Localities of *Oncomelania quadrasi* and *O. hupensis* ssp., the Philippines

Sample code	Locality	Province*	Country	Subspecies	No.
AN	Aling Nene	Oriental Mindoro	Philippines	<i>O. quadrasi</i>	10
MB	Malabo	Oriental Mindoro	Philippines	<i>O. quadrasi</i>	10
DG	Dagami	Leyte	Philippines	<i>O. quadrasi</i>	10
MG	Magrafil	Cagayan	Philippines	<i>O. quadrasi</i>	10
SM	Sta. Maria	Cagayan	Philippines	<i>O. quadrasi</i>	10
TP	Tapel	Cagayan	Philippines	<i>O. quadrasi</i>	9
AH	Guichi	Anhui	China	<i>O. h. hupensis</i>	5
SC	Tianquan	Sichuan	China	<i>O. h. hupensis</i>	5
YN	Weishan	Yunnan	China	<i>O. h. hupensis</i>	5
KS	Yue-Mei	Kaohsiung	Taiwan	<i>O. h. formosana</i>	5
TA	Shimen	Taipei	Taiwan	<i>O. h. chiui</i>	5

*Oriental Mindoro, Leyte, and Cagayan are located on the islands of Mindoro, Leyte, and Luzon, respectively.

NaCl and cracked to break their shells before being placed in individual vials containing 80% ethanol to transport to the laboratory for genetic investigation. Each snail was washed several times in sterile 0.3% NaCl, and the head-foot muscle dissected under a microscope and dried using a vacuum dryer. The tissues were then used for DNA extraction.

DNA extraction, PCR amplification, and DNA sequencing.

Total genomic DNA of each individual snail was extracted by using the E.Z.N.A.[®] Mollusc DNA Kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's instructions. The 12S ribosomal RNA gene was amplified by using primers and polymerase chain reaction (PCR) conditions as reported by Okamoto and others.¹² All PCR products were gel-purified using GENECLAN[®] II Kit (Q-BIO Gene, Carlsbad, CA). The purified PCR products were cycle-sequenced by using ABI BigDye v3.1 chemistry and run on an ABI Prism 377 automated sequencer (Applied Biosystems, Foster City, CA).

Data analyses. Sequences were assembled and edited by eye using the software program ABI sequence scanner v1.0. Multiple sequence alignment was performed using a BioEdit

version 5.0.6. Genetic differentiation (F_{ST}), gene flow estimation (Nm), and other parameters of DNA sequence polymorphism, including number of segregation site (S), haplotype diversity (Hd), nucleotide diversity (π), genetic diversity (θ_s), and the mismatch distribution under an expected population growth-decline model,¹⁶ were computed and generated by using DnaSP v5 software.¹⁷ Genetic structure, fixation indices, and evolutionary neutrality were calculated by using Arlequin 3.5.¹⁸ Minimum spanning haplotype network was constructed by using the Network 4.6.1.0 program based on median-joining algorithm.¹⁹ A phylogenetic tree was constructed based on an unweighted pair group method with arithmetic mean analysis by using Phylip program version 3.6.²⁰ The relative support for clades was determined by using 1,000 bootstrap replicates.

RESULTS

Interspecific analyses of *O. hupensis* ssp. and *O. quadrasi*.

The 357 bp partial sequence of the mitochondrial 12S ribosomal

TABLE 2

Variable positions of 12S ribosomal RNA sequences of *Oncomelania quadrasi* (Oq), *O. h. hupensis* (Ohh), *O. h. chiui* (Ohc) and *O. h. formosana* (Ohf) haplotypes, the Philippines*

Haplotype		Nucleotide sequence																																							
		3	4	5	6	6	6	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3		
		3	4	8	1	3	4	8	2	3	6	0	2	3	4	2	3	0	1	6	5	0	2	7	4	7	8	0	1	2	4	5	8	9	9	0	2	6	8	0	6
Oq1		A	A	A	T	T	C	T	G	T	A	A	T	T	C	T	T	A	T	G	G	T	C	C	T	G	G	G	G	G	A	G	C	G	T	G	A	T	T	G	
Oq2		.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Oq3		.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Oq4		.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Oq5		.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Oq6		.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Oq7		.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Oq8	G	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.		
Oq9	G	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Oq10	G	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	A	A	.	.	.	.	.	.	.	.	.	.	.	.	
Ohh1	.	G	.	C	.	T	.	A	.	.	G	.	C	T	.	C	.	G	.	A	A	.	T	.	.	A	A	A	A	.	.	.	T	.	A	.	.	.	C	.	
Ohh2	.	G	.	C	.	T	.	A	.	.	G	.	C	T	.	C	.	.	.	A	A	.	T	.	.	A	A	A	A	.	.	.	T	.	A	.	.	.	C	.	
Ohh3	.	G	.	C	.	T	.	A	.	G	G	C	C	T	.	C	.	.	.	A	A	.	T	.	C	A	A	A	A	.	.	.	T	.	A	.	G	.	C	.	
Ohh4	.	G	.	C	.	T	.	A	.	G	G	C	C	T	.	C	.	C	.	A	A	.	T	.	C	A	A	A	A	.	.	.	T	.	A	.	G	.	C	.	
Ohh5	.	T	.	C	.	T	.	A	.	G	.	C	C	T	.	C	.	.	.	A	A	.	T	.	.	A	A	A	A	.	.	.	T	.	A	.	.	.	C	.	
Ohh6	.	G	.	C	C	T	.	A	.	G	G	C	C	T	.	C	.	.	.	A	A	.	T	.	A	A	A	A	A	.	.	.	T	.	A	.	G	C	C	.	
Ohh7	.	G	G	.	.	T	.	A	.	.	T	G	.	T	C	C	.	.	.	A	A	C	T	.	.	A	A	A	A	.	.	.	A	T	.	A	.	.	A	.	
Ohh8	.	G	G	.	.	T	.	A	.	.	T	A	.	T	C	C	.	.	.	A	A	C	T	.	.	A	A	A	A	.	.	.	A	T	.	A	.	.	A	.	
Ohc1	.	G	.	C	.	T	.	A	.	.	G	.	C	T	.	C	.	.	.	A	A	.	.	.	.	A	A	A	A	A	.	.	.	T	.	A	.	.	C	.	
Ohf1	.	G	.	C	.	T	C	A	.	.	.	.	C	T	.	C	.	.	.	A	A	.	T	.	.	A	A	A	A	.	.	.	T	.	A	.	.	.	C	.	
Ohf2	.	G	.	C	.	T	C	A	.	.	.	.	C	T	.	C	.	.	.	A	A	.	T	.	.	A	A	A	A	.	.	G	.	T	.	A	.	.	.	C	.

*, = identity.

RNA gene was compared within and between populations of *O. quadrasi*, *O. h. hupensis*, *O. h. formosana*, and *O. h. chiui*. In total, 40 variable positions consisting of 34 transitions, 2 transversions, 1 insertion/deletion, and 3 multiple mutational changes were observed (Table 2). Of these positions, 1–16 (0.28–4.48%) variable positions were observed within each taxon, which were classified into 21 haplotypes: 10 haplotypes of *O. quadrasi* (Oq1–Oq10), 8 haplotypes of *O. h. hupensis* (Ohh1–Ohh8), 2 haplotypes of *O. h. formosana* (Ohf1–Ohf2), and 1 haplotype of *O. h. chiui* (Ohc1) (Table 2 and Figure 1). The nucleotides sequences of all haplotypes detected in this study were submitted to GenBank under the following accession numbers: KC763451–60 are haplotypes of *O. quadrasi*, KC763461–68 are *O. h. hupensis*, KC763470–71 are *O. h. formosana*, and KC763469 is *O. h. chiui*. A minimum spanning haplotype network showed that the network of 10 haplotypes of *O. quadrasi* was clearly separated from the other haplotypes at 13 mutational points (Figure 1). The haplotypes of subspecies *O. h. hupensis*, *O. h. formosana*, and *O. h. chiui* were grouped in the same network except the two haplotypes of *O. h. hupensis* from Yunnan, which were distinctly separated from the others at 10 mutational points (Figure 1). No common haplotype was shared among/between populations of the four subspecies. The phylogenetic tree also

showed that *O. quadrasi* formed a distinct cluster from the other subspecies, whereas *O. h. hupensis*, *O. h. chiui*, *O. h. formosana*, *O. h. nosophara*, and *O. h. lindoensis* were clustered as a monophyletic group (Figure 2).

Intraspecific analyses of *O. quadrasi*. Of the 10 haplotypes of *O. quadrasi*, 1–2 unique haplotypes existed in each population. Two haplotypes were shared between two populations: Oq3 and Oq10 shared between the populations MG with TP, and AN with MB, respectively (Table 2 and Figure 1). The haplotype, nucleotide, and genetic diversity were 0.859 ± 0.019 , 0.218 ± 0.012 , and 0.215 ± 0.087 , respectively. In the neutrality test, the observed values of both Tajima's D (0.034, $P = 0.721$) or Fu's F_s (–1.279, $P = 0.100$) suggest that natural selection may have occurred (Table 3). Significant genetic differentiation (F_{ST}) and estimated gene flow (Nm) between populations ranged from 0.556 to 1.000 and from 0.00 to 0.74, respectively (Table 4). Substantial genetic subdivision and low gene flow existed among the six populations ($F_{ST} = 0.854$; $P < 0.001$ and $Nm = 0.06$, $P < 0.001$), respectively (Table 4). The network of *O. quadrasi* haplotypes shows a star-like pattern and is significantly separated into three groups according to three provinces/islands by $F_{CT} = 0.466$ ($P < 0.05$). A significant $F_{SC} = 0.727$ ($P < 0.001$) was also observed among populations within groups (Table 5). The mismatch distribution of 12S

FIGURE 1. Minimum spanning haplotype network of the three subspecies of *Oncomelania hupensis* generated in based on 12S ribosomal RNA sequence. A network of *O. quadrasi* corresponds to their geographic localities in three provinces of the Philippines. The area of the circles represents the proportion of sample number (indicate in parentheses) found in each haplotype. Oq = *O. quadrasi*; Ohh = *O. h. hupensis*; Ohf = *O. h. formosana*; and Ohc = *O. h. chiui*.

FIGURE 2. Phylogenetic tree generated from 12S ribosomal RNA sequences of *Oncomelania* snails. The star represents the haplotype samples examined in this study; other haplotype samples were obtained from Okamoto and others.¹² *Tricula hortensis* was obtained from GenBank under accession no. NC_013833 and used as an outgroup.

TABLE 3
Summary statistics of molecular variation and neutrality analysis in six populations of *Oncomelania quadrasi*, the Philippines*

Population	n	S	H	Uh	Hd	$\pi \pm SD$	$\theta_s \pm SD$	Tajima's D	Fu's <i>F_s</i>
AN	10	2	2	1	0.533 ± 0.095	0.107 ± 0.019	0.107 ± 0.054	1.641 (0.970)	2.338 (0.854)
MB	10	0	1	0	0.000 ± 0.000	NA	NA	NA	NA
DG	10	0	1	1	0.000 ± 0.000	NA	NA	NA	NA
MG	10	2	3	2	0.378 ± 0.181	0.056 ± 0.028	0.071 ± 0.054	-0.691 (0.218)	-0.594 (0.105)
SM	10	1	2	2	0.200 ± 0.154	0.020 ± 0.015	0.035 ± 0.035	-1.112 (0.179)	-0.339 (0.164)
TP	9	2	3	2	0.722 ± 0.097	0.106 ± 0.016	0.074 ± 0.056	1.494 (0.958)	0.453 (0.527)
All populations	59	10	10	8	0.859 ± 0.019	0.218 ± 0.012	0.215 ± 0.087	0.034 (0.721)	-1.279 (0.100)

*n = number of samples; S = number of segregating sites; H = number of haplotypes; Uh = unique haplotype; Hd = haplotype diversity; π = nucleotide diversity; θ_s = genetic diversity estimated from segregating sites; NA = not analyzed. *P* values are indicated in parentheses.

TABLE 4

Genetic differentiation (F_{ST}) (lower triangle) and estimated gene flow (Nm) (upper triangle) among *Oncomelania quadrasi* populations, the Philippines*

Population	AN	MB	DG	MG	SM	TP
AN	—	0.20†	0.11	0.16	0.07	0.17
MB	0.556†	—	0.00	0.02	0.01	0.04
DG	0.704	1.000	—	0.03	0.01	0.06
MG	0.614	0.916	0.879	—	0.09	0.74
SM	0.838	0.980	0.976	0.843	—	0.13
TP	0.588	0.868	0.820	0.258	0.789	—

* Among all populations, $F_{ST} = 0.854$ and $Nm = 0.06$ (significant at $P < 0.001$). Comparison values were significant at $P < 0.001$.

† $P < 0.01$.

ribosomal RNA sequences was unimodal and almost fitted the expected distribution (Figure 3).

DISCUSSION

We found that substructuring occurred between the six populations of *O. quadrasi* examined according to their geographic origin in the Philippines, similar to that described for the subspecies *O. h. hupensis* and *O. h. robertsoni* from mainland China.^{5,6} The genetic differences found for *O. quadrasi* populations from different localities in the Philippines islands may be caused by lack or low level of gene flow, especially between the three provinces located on separated islands. Furthermore, significant genetic differentiation was detected between populations within each province, e.g., three populations in Cagayan and two populations in Oriental Mindoro provinces, suggesting that the level of gene flow between these populations is currently low.

The mismatch distribution of the 12S ribosomal RNA haplotypes of *O. quadrasi* is markedly unimodal, which is observed when the genealogy of the sample resembles a star network, which in turn is observed during demographic expansions.¹⁶ A high level of haplotype and nucleotide diversity of *O. quadrasi* populations was observed in this study, in agreement with the results of previous studies of *O. hupensis* ssp. in mainland China.^{5,6} Because of the short generation times and large effective population sizes, invertebrates, including snails, usually show a high level of genetic diversity.²¹ Moreover, in the case of *O. quadrasi*, the observed high genetic variability is also potentially related to the low mobility of the species, leading to population subdivision and potential increase global genetic diversity. Unique haplotypes for each population were found, except for MB, for which only one haplotype was observed, which was shared with an adjacent AN population from Oriental Mindoro province. The results in this study strongly indicate that the *O. quadrasi* populations

TABLE 5

Analysis of molecular variance of six populations of *Oncomelania quadrasi* classified into three groups defined by island, i.e., groups of Luzon, Mindoro, and Leyte islands, the Philippines*

Source of variation	df	Sum of squares	Variance components	% Variation	Fixation indices
Among groups	2	40.057	0.75	46.60	$F_{CT} = 0.466†$
Among populations within group	3	19.012	0.62	38.83	$F_{SC} = 0.727‡$
Within population	53	12.422	0.23	14.57	

* Level of significance is based on 1,000 permutations. df = degrees of freedom.

† $P < 0.05$.

‡ $P < 0.001$.

FIGURE 3. Mismatch distribution of the 59 partial 12S ribosomal RNA sequences (357 basepairs) of six *Oncomelania quadrasi* populations from the Philippines. The mismatch distribution is the distribution of the number of pairwise differences among sequences. The expected distribution under a model of expansion is given as a continuous line, and the observed distribution is given as a dashed line. Exp = expected; Obs = observed.

in the Philippines have a substructure based on their geographical origin. However, a larger sample size from a wider range of geographic areas is needed to confirm that all unique haplotypes are specific to each particular geographic area.

Genetic differences between some *S. japonicum* isolates in the Philippines have also been noted; the isolate from Mindoro is moderately genetically differentiated from the Leyte and Luzon populations.²² We have now determined that the genetic structure of *O. quadrasi* populations from Leyte, Oriental Mindoro, and Cagayan also show significant genetic differences from each other, which was in concordance with findings of a previous report.¹³ This finding may be caused by reciprocal evolution between the parasite and its snail hosts, as indicated in several host-parasite systems, e.g., *O. viverrini* and its *Bithynia* snail hosts,^{23,24} and *S. mansoni* and its *Biomphalaria* snail hosts.⁹ Moreover, the three provinces, i.e., Cagayan, Oriental Mindoro, and Leyte, are located on separated islands so that gene flow is likely to be highly restricted, eventually resulting in high genetic differentiation between the different geographic areas. This conclusion is suggested by the significant F_{CT} differences. Such a situation has also been observed between populations of *O. h. hupensis* in mainland China, where the population genetic structure is related to geographic area and is correlated with differing shell characteristics.⁶

Another objective of our study was to establish whether *O. quadrasi* was genetically distinct from three subspecies of *O. hupensis* examined. A significant difference between *O. quadrasi* and the three subspecies was found by phylogenetic analysis of 12S ribosomal RNA. Our phylogenetic tree shows that *O. quadrasi* forms a distinct clade when compared with the *O. hupensis* ssp. Moreover, the genetic relationships of *O. h. hupensis* from Yunnan province are distinct from the other subspecies, which is in concordance with findings of previous studies.^{6,12} Although previous morphologic and biogeographic studies of *O. hupensis* ssp. across mainland China indicate that *O. h. robertsoni* and *O. h. hupensis* have the same shell growth allometry, the shell length is on average different.²⁵ *Oncomelania h. robertsoni* has a patchy distribution in Sichuan and Yunnan provinces above the Three Gorges, whereas *O. h. hupensis* occurs in the Yangtze River drainage below the Three Gorges.^{26,27} Both snails are probably different

at a specific level. In addition, *S. japonicum* from Yunnan also showed significant genetic differences from the other geographic isolates.²⁸ However, subspecies validity and the assignment of *O. hupensis* in mainland China and *O. quadrasi* in the Philippines remain controversial in these areas and need to be further clarified by intensively analyzing snail morphology and using more powerful molecular markers.

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