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Phylogenetic relationships of snails of the genera *Oncomelania* and *Tricula* inferred from the mitochondrial 12S rRNA gene

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Abstract

The *Schistosoma japonicum* group and *S. sinensium* utilize intermediate snail hosts belonging to the genera *Oncomelania* and *Tricula* (Gastropoda: Pomatiopsidae). In the present study, partial sequences of the mitochondrial 12S rRNA gene from 7 subspecies of *O. hupensis*, two species of *Tricula* (*T. bollingi* and *T. humida*) and *O. minima* were examined to infer a phylogeny for these. Nucleotide differences among subspecies of *O. hupensis* were less than 6.5% and among species from different genera, 10-12%. The phylogenetic tree obtained in this study indicates that *O. hupensis* subspecies fell into four distinct clades ; that is, *O. h. quadrasi* from the Philippines, *O. h. lindoensis* from Indonesia, *O. h. hupensis* from Yunnan, China and the remaining 5 subspecies (*O. h. hupensis* from other parts of China, *O. h. robertsoni* from China, *O. h. formosana* from Taiwan, *O. h. chiui* from Taiwan and *O. h. nosophora* from Japan). The phylogenetic tree also showed that *O. minima* was placed as sister to all of the subspecies of *O. hupensis.* Possible evolutionary relationships among the snail hosts were discussed.

Key Words: *Oncomelania*, *Tricula*, mitochondrial DNA, 12S rRNA gene, phylogenetic tree

INTRODUCTION

Species of *Schistosoma* have been placed in a number of groups based on, amongst other things, egg morphology and/or geographical distribution (Rollinson and Southgate, 1987). For example, African schistosomes of the *S. mansoni* and *S. haematobium* groups develop in pulmonate snails of the family Planorbidae, while the oriental species, namely the *S. japonicum* group (*S. japonicum*, *S. mekongi*, *S. malayensis*) and *S. sinensium*, utilize snails belonging to the family Pomatiopsidae. Pulmonate snails belong to the subclass Pulmonata, while pomatiopsids belong to the subclass Caenogastropoda. These two subclasses diverged a long time ago (Davis, 1980). Generally, specificity for an intermediate host in the genus *Schistosoma* is high (Rollinson and Southgate, 1987). So the phylogeny of these intermediate host snails seems to be very important when the evolution of the schistosome is traced.

Schistosoma japonicum is widely distributed throughout East Asia, China and Japan, and the intermediate host is *Oncomelania hupensis*. Since *O. hupensis* shows geographical variation in morphology, many subspecies have

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been described (Davis *et al.,* 1995). Geographical variation is so great that some researchers prefer to regard the subspecies as independent species (Woodruff *et al.,* 1988; Nihei *et al.,* 1998). In the present study, phylogenetic relationships of the snail hosts of *S. japonicum*, *O. hupensis*, and other related species of *Oncomelania* and *Tricula* were studied using the mitochondrial 12S rRNA gene.

MATERIALS AND METHODS

Snail samples

Sixteen isolates of *O. hupensis*, belonging to 7 subspecies, were examined. We also examined *O. minima*, *T. bollingi* and *T. humida.* The geographical origins and locations of species or subspecies used in this study are shown in Table 1 and Fig. 1.

Preparation of DNA

Genomic DNA from each snail sample was extracted using Easy-DNA Kit (Invitrogen, USA). DNA extracted by this kit contained an inhibitor for PCR, and this inhibitor could not be removed by phenol/chloroform extraction or commercially available spin column etc. So DNA was purified by 0.5% agarose gel electrophoresis with 0.5 X Tris-boric EDTA buffer. After electrophoresis, high molecular weight DNA was cut off with agarose gel and extracted by QIAEX II Gel Extraction Kit (Qiagen, Germany).

Amplification and sequencing of DNA

Purified genomic DNA was used as a template for amplification of DNA fragments by the polymerase chain reaction (PCR). Amplification of a part of the mitochondrial 12 S rRNA gene was carried out using universal primers (Kocher *et al.,* 1989). The sequences of the primers were as follows: L1091; 5'-AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC ACT AT-3' and H1478; 5'-TGA CTG CAG AGG GTG ACG GGC GGT GTG T-3'. PCR was performed using Ampli Taq DNA Polymerase (Perkin Elmer, USA) according to the manufacturer's instructions. After an initial denaturation step (94 ℃ for 3 minutes) there were 30 cycles of denaturation at 94 ℃ for 30 sec, annealing at 50 ℃ for 30 sec and extension at 72 ℃ for 1min. PCR products were purified with QIAquick-spin PCR purification Kit (Qiagen). Purified double-stranded PCR products were directly sequenced with the same primers as those of PCR from both ends using Dye Terminator Cycle Sequencing FS Ready Reaction Kit and a Model 377A DNA sequencer (Perkin Elmer).

Table 1. Geographical origins of subspecies or species of *Oncomelania* and *Tricula* examined.

subspecies or species	Location	Country
Oncomelania hupensis hupensis	Anhui	China
O. h. hupensis	Hunan	China
O. h. hupensis	Hubei	China
O. h. hupensis	Yunnan	China
O. h. robertsoni	Sichuan	China
O. h. formosana	Kaohsiung	Taiwan
O. h. formosana	Shuili	Taiwan
O. h. formosana	Yilan	Taiwan
O. h. chiui	Shimen	Taiwan
O. h. nosophora	Kofu	Japan
O. h. nosophora	Kurume	Japan
O. h. lindoensis	Sulawesi	Indonesia
O. h. quadrasi	Mindoro	Philippines
O. h. quadrasi	Bohor	Philippines
O. h. quadrasi	Asuncion	Philippines
O. h. quadrasi	Digos	Philippines
O. minima	Sado	Japan
Tricula bollingi	Fang	Thailand
T. humida	Sichuan	China

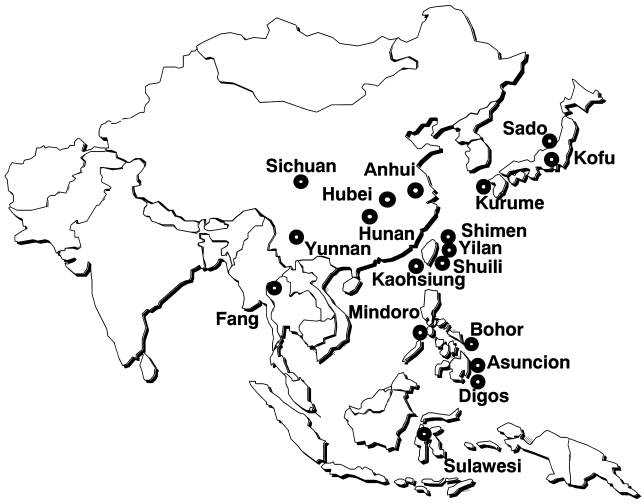

Fig. 1. Geographical locations of species or subspecies of *Oncomelania* and *Tricula* used in this study.

Phylogenetic analyses

DNA sequence data were aligned using the CLUSTAL W computer program. The evolutionary distances were computed by Kimura's two-parameter method (Kimura, 1980), and the phylogenetic tree was constructed by the neighbor-joining method using the neighbor-joining computer program in the PHYLIP 3.5 phylogeny package (Felsenstein, 1993). The tree was evaluated using the bootstrap test based on 1,000 resampling. A sequence from *Littorina littorea* was used for the outgroup (Rumbak *et al.,* 1994).

RESULTS AND DISCUSSION

Using this primer pair, fragments of 365 to 368 bp were amplified in this study. Sequences and partial alignments of 12S rRNA gene are shown in Fig. 2. The maxi-

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OhcShimen          1:TCTTGAAGATAAATAAATTTATACCGGGGCACTACGAATAATCT-TTAGATTTAAAACCCAAAGAGCTTGGCGGTGTTTT
OhfKaohsiung       1:.........................A..................-.....................................
OhfShuili          1:.............................................-.....................................
OhfYilan           1:.............................................-.....................................
OhhAnhui           1:.............................................-.....................................
OhhHubei           1:...........G.................................-.....................................
OhhHunan           1:.............................................-.....................................
OhhYunnan          1:.....................................G..T.-.....................................
OhlSulawesi        1:.........................A...T.............T.-...A.....................................
OhnKoufu           1:.....................................G....-.....................................
OhnKurume          1:.....................................G....-.....................................C
OhqAssuncion       1:..............G..........A.................T.-.C.....................................
OhqBohor           1:..............G..G.......A.................T.-.C.....................................
OhqDigos           1:.........................A.................T.-.C.....................................
OhqMindro          1:..............G..........A.................T.-.C.....................................
OhqSorsogon        1:..............G..........A.................T.-.C.....................................
OhrSichuan         1:.............................................-.....................................
OminimaSado        1:........AG........A.......A................T.A...A.....................................
TbollingiFang      1:.........T.....G-.A.......A...T...........--T.A...A.....................................
ThumidaSichuan     1:........AG..........CA.....A..........A.........T...A.....................................
Llittorea          1:....AG.TG............TA.TTTACCAGAGTACT.CG.A.CA-..A.........T..................CC..

OhcShimen         81:AGACTATTTAGGGGAACTTGTTTCATAATCGATAATCCACGAGATACCTAACCTTCTTTTGTAATCAGTATGTATACCGT
OhfKaohsiung      81:.....................................................................................
OhfShuili         81:.....................C.....................A.....................................
OhfYilan          81:.....................................................T.....................
OhhAnhui          81:.....................................................................G.....
OhhHubei          81:.....................................................................................
OhhHunan          81:.....................................................................................
OhhYunnan         81:.....................................T.G..........T........C.....................
OhlSulawesi       81:.....................................................................................
OhnKoufu          81:.....................................................................................
OhnKurume         81:.....................................................................................
OhqAssuncion      81:.........................G.................A.............C.....................
OhqBohor          81:.........................G.................A.............C.....................
OhqDigos          81:.........................G.................A.............C.....................
OhqMindro         81:.........................G.................A.............C.....................
OhqSorsogon       81:.........................G.................A.............C.....................
OhrSichuan        81:.....................................................G.....
OminimaSado       81:.....G..........C.....................................................G.....
TbollingiFang     81:......C.....................................T.A.....................................
ThumidaSichuan    81:...................C......G.................T.....................................
Llittorea         81:.....T..C........C...C..........C.........ACA....C...C.T.....C..A.....

OhcShimen        161:TGTCGTCAGGTAACTTTTTAAAATAAAAAAGTTA-GCGAAAAAGCCATAAGCTTACACGTCAAATCAAGGTACAGCCTAT
OhfKaohsiung     161:.....................................-..A.................T.....................
OhfShuili        161:.....................................-.....................T.....................
OhfYilan         161:.....................................-.........T..........T.....................T...
OhhAnhui         161:.....................................-.....................T.....................
OhhHubei         161:.....................................-.....................T.....................
OhhHunan         161:.....................................-.....................T.....................
OhhYunnan        161:.....................................-...................C.T.....................
OhlSulawesi      161:.....C...............................-..A.....................................
OhnKoufu         161:.....................................-..........T.........T...................A.....
OhnKurume        161:.....................................-..........T.........T...................A.....
OhqAssuncion     161:.............................G.....-.....................................
OhqBohor         161:.............................G.....-.............G.....................................
OhqDigos         161:.............................G.....-.............G.....................................
OhqMindro        161:.............................G.....-.............G.....................................
OhqSorsogon      161:.............................G.....-.............G.....................................
OhrSichuan       161:.....................................-.....................T.....................
OminimaSado      161:.............................G.......-..A.GG..A.T....AT.C.....................
TbollingiFang    161:..............................T.....AC.A.....ATTG...AT...T.....................
ThumidaSichuan   161:.......................C.T.G......GC.A.....ATTT.T.TT.....................C.C
Llittorea        161:C....C............A......T..CT....GCTA.G...CTAT.T..T..T.......G........G.......
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OhcShimen       241:AAGAAAGAGAGAATGAGTTACAATTAAAATTTATAATAACGGAATAGAAAAAGAAAATTTCTATGAAGGCGGACTTAAAA
OhfKaohsiung    241:.....G......G.....................................................................
OhfShuili       241:.....G.....................................................................
OhfYilan        241:.....G.....................................................................
OhhAnhui        241:.....G.....................................................................
OhhHubei        241:.....G.....................................G.....................................
OhhHunan        241:.....G.....................................................................
OhhYunnan       241:.....G....A.....................................................T.A.........
OhlSulawesi     241:..AG.G.....................................................................
OhnKoufu        241:.....G.....................C.....................................................
OhnKurume       241:.....G.....................C.....................................................
OhqAssuncion    241:GG.GGG.....................C.....................................T...............
OhqBohor        241:GG.GGG.....................C.....................................T...............
OhqDigos        241:...GGG.....................C.....................................T...............
OhqMindro       241:GG.GGG.....................C.....................................T...............
OhqSorsogon     241:GG.GGG.....................C.....................................T...............
OhrSichuan      241:.....G.....................................................................
OminimaSado     241:.T.G.G.........G...........C.......G........A............T...................
TbollingiFang   241:.T...G....A...TG...........C............G..A.........AA.T........A..........
ThumidaSichuan  241:.....G.........G...........C..............A..GT......C..T.T......A..........
Llittorea       241:..A.GG....AG...G.....................T.....TAGTGGTC.A....GCCACT......A......G...

OhcShimen       321:GTAAAAAAATTACTATAGAGACTTTTTGAATCAAGCTCTGAAACGTGC
OhfKaohsiung    321:................A.............................
OhfShuili       321:.....................................................
OhfYilan        321:.....................................................
OhhAnhui        321:.....................................................
OhhHubei        321:.....................................................
OhhHunan        321:....................T.....................................
OhhYunnan       321:............T......A.T.........TG.....................
OhlSulawesi     321:..............CA.............................
OhnKoufu        321:.....................................................
OhnKurume       321:.....................................................
OhqAssuncion    321:............T........T.........C.....................
OhqBohor        321:............T........T.........C.....................
OhqDigos        321:............T........T.........C.....................
OhqMindro       321:............T........T.........C.....................
OhqSorsogon     321:............T........T.........C.....................
OhrSichuan      321:.....................................................
OminimaSado     321:.........CA.T....A.A.T.........TG.....................
TbollingiFang   321:..........A.T....T.A.T.........T.....................
ThumidaSichuan  321:..........A......A.A.T.........T............T..-
Llittorea       321:..--....G...G..ATC.A.TGC.......T.........GG....-
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Fig. 2. Nucleotide sequence alignment of the 12S ribosomal RNA gene in the mitochondrial DNA in the three genera, *Oncomelania, Tricula* and *Littorina.*

Table 2. Pairwise differences in nucleotide sequences of the 12S rRNA gene among subspecies/species of the genus *Oncomelania* and *Tricula.*

species/subspecies	O.h.hup.	O.h.h.Yun	O.h.rob.	O.h.for.	O.h.chi.	O.h.nos.	O.h.lin.	O.h.qua.	*O.minima*	*T.bollingi*
O.h.hupensis (excluding Yunnann)	0.8*									
O.h.hupensis (only Yunnan)**	4.2									
O.h.robertsoni	0.7	4.4	-							
O.h.formosana	1.1	3.7	1.0	1.3*						
O.h.chiui	0.0	4.6	0.8	1.3	-					
O.h.nosophora	1.7	4.8	1.6	2.0	1.8	0.7*				
O.h.lindoensis	3.6	6.4	3.4	3.4	3.4	4.2	0.8*			
O.h.quadrasi	5.1	6.4	5.3	5.0	5.0	6.1	6.2	0.7*		
O.minima	9.4	9.3	9.6	9.3	9.3	9.1	8.3	10.5	-	
T.bollingi	10.0	10.2	10.2	10.4	10.4	9.9	9.8	11.7	8.8	-
T.humida	10.7	12.1	11.0	10.7	10.7	10.5	10.8	12.5	9.0	9.9

* : values for intra-subspecies

**: The Yunnan strain of O.h.h. was listed separately from the other strains, because it differed in a high degree from any other subspecies of O. hupensis.

mum levels of nucleotide variations detected between pairs of species or subspecies for the 12S rRNA gene are shown in Table 2. The values are expressed as pairwise differences in percentage. Nucleotide differences within the subspecies of *O. hupensis* were in general very low. However, in the case of *O. h. hupensis,* the intra-nucleotide difference was very large, because a Yunnan isolate of *O. h. hupensis* differed from all of the other isolates. On the other hand, differences among five of the subspecies, *O. h. hupensis* from China except for Yunnan, *O. h. robertsoni, O. h. formosana, O. h. chiui* and *O. h. nosophora,* were less than 2.0%. Nucleotide differences between these 5 subspecies and *O. h. lindoensis,* or *O. h. quadrasi* or the Yunnan isolate of *O. h. hupensis* were larger, being about 3 to 6%. Very large values of nucleotide differences (about 9 to 12%) were obtained between different species.

As shown in Fig. 3, a phylogenetic tree of the snail hosts was constructed using the neighbor-joining method. *Oncomelania hupensis* subspecies are distributed among 4 groups. That is, four specimens of *O. h. quadrasi* from Philippines form a monophyletic clade. *Oncomelania h. nosophora, O. h. hupensis, O. h. robertsoni, O. h. chiui* and *O. h. formosana* form one group. *Oncomelania h. lindoensis* and *O. h. hupensis* from Yunnan make independent clades. In the meantime, *O. minima, T. bollingi* and *T. humida* also make independent clades, and they are genetically distant from each other as well as from all the subspecies of *O. h. hupensis.*

The sequence of mitochondrial 12S rRNA gene has been utilized to infer the phylogeny of various animals (Kocher *et al.,* 1989). Rumbak *et al.* (1994) examined the phylogenetic relationships among 11 species in the genus *Littorina,* which is a widely distributed marine gastropod, belonging to the same suborder Archaeotaenioglossa as the genus *Oncomelania.* In the case of the genus *Littorina,* nucleotide differences between European species and American species were about 3%. And differences between subgenera in the genus *Littorina* were about 5% or more. In the present study, *O. hupensis* were distributed among 4 groups, and nucleotide differences among these 4 groups were 3 to 6%. Although there is no reason to assume that rates of molecular evolution have been the same in *Littorina* as in the pomatiopsids, comparisons of the percentage differences between the two studies suggest that the subspecies in *O. hupensis* may require re-evaluation as suggested by Woodruff *et al.* (1988). Despite the wide geographic area involved, the genetic differences between the subspecies in Japan, Taiwan and China were very small. All of the examined snails in China were collected from the Yangtze basin except for that of Yunnan Province. In the glacial maxima, only approximately twenty thousand years ago (Wang and Sun, 1994), sea levels were much lower than now, the mouth of the Yangtze was considerably closer to Japan and Taiwan, and the Taiwan channel was dry land. This could explain the high levels of similarity among Japanese, Taiwanese and Yangtse basin samples.

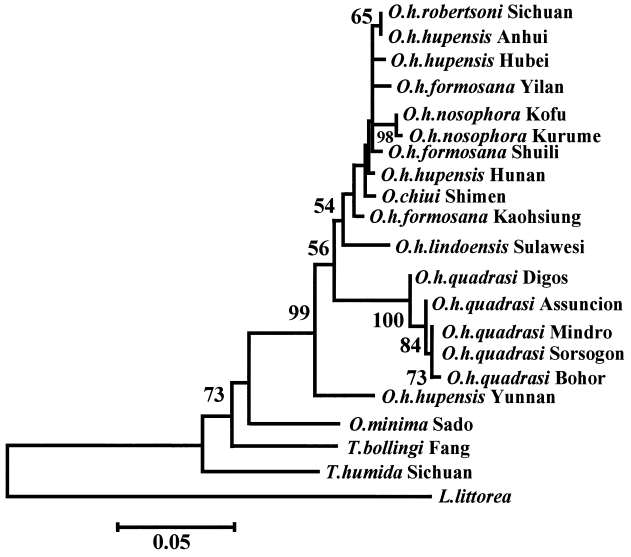

Fig. 3. A phylogenetic tree of the genus *Oncomelania* and *Tricula,* including the snail intermediate hosts of *Schistosoma japonicum,* inferred from 12S rRNA gene in the mitochondrial DNA using the NJ method.

It has been reported that genetic variation among *S. japonicum* populations in Asia, including China, the Philippines, Japan and Indonesia, is very slight (Bowles *et al,* 1993). However, we have shown that genetic variation among their intermediate host snails is quite considerable. Thus, our study supports the idea that *S. japonicum* has been recently introduced to many areas where it now occurs, and has been able to adapt to local strains of *Oncomelania,* as suggested in previous papers (Woodruff, 1988; Davis, 1992; Attwood *et al.,* 2002).

Davis (1980) suggested that *S. sinensium* may constitute a species complex, because of geographical differences in snail host specificity. Our previous studies showed a large difference in egg sizes as well as in nucleotide sequence of CO1 between the two isolates of *S. sinensium* from China and Thailand, supporting Davis's hypothesis (Kawanaka *et al.,* 1998; Agatsuma *et al.,* 2000). The present result of a large distance value between their intermediate hosts may suggest coevolution between the hosts and parasites.

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