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. chiuui* 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 sub-

class 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. bolingi* 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 reac-

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

tion (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°C for 3 minutes) there were 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C 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).

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-

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

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

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OhcShimen      1:TCTTGAAGATAAAATAAATTTATACCGGGGCACTACGAATAATCT-TTAGATTTAAACCCAAAGAGCTTGGCGGTGTTTT
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.....T..A.....
Llittorea      1:....AG.TG.....TA.TTTACCAGAGTACT.CG.A.CA-..A.....T.....CC..

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OhcShimen      81:AGACTATTTAGGGGAACCTTGTTTCATAATCGATAATCCACGAGATACCTAACCTTCTTTTGTAAATCAGTATGTATACCGT
OhfKaohsiung   81:.....A.....
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.....

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OhcShimen      161:TGTCGTCAGGTAACTTTTAAATAAAAAAGTTA-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.....
OhqAssuncion   161:.....G.....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: AAGAAAGAGAGAATGAGTTACAATTAAATTTATAATAACGGAATAGAAAAAGAAAATTTCTATGAAGGCGGACTTAAAA
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:A.....
OhfYilan	321:A.....
OhhAnhui	321:A.....
OhhHubei	321:A.....
OhhHunan	321:T.....
OhhYunnan	321:T.....A.T.....TG.....
OhlSulawesi	321:CA.....
OhnKoufu	321:A.....
OhnKurume	321:A.....
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:A.....
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....-

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.	<i>O.minima</i>	<i>T.bollingi</i>
<i>O.h.hupensis</i> (excluding Yunnann)	0.8*									
<i>O.h.hupensis</i> (only Yunnan)**	4.2									
<i>O.h.robertsoni</i>	0.7	4.4	-							
<i>O.h.formosana</i>	1.1	3.7	1.0	1.3*						
<i>O.h.chiui</i>	0.0	4.6	0.8	1.3	-					
<i>O.h.nosophora</i>	1.7	4.8	1.6	2.0	1.8	0.7*				
<i>O.h.lindoensis</i>	3.6	6.4	3.4	3.4	3.4	4.2	0.8*			
<i>O.h.quadrasi</i>	5.1	6.4	5.3	5.0	5.0	6.1	6.2	0.7*		
<i>O.minima</i>	9.4	9.3	9.6	9.3	9.3	9.1	8.3	10.5	-	
<i>T.bollingi</i>	10.0	10.2	10.2	10.4	10.4	9.9	9.8	11.7	8.8	-
<i>T.humida</i>	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 (Ko-

cher *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 Yangtze basin samples.

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.

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.

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