

Landscape genetics: the correlation of spatial and genetic distances of *Oncomelania hupensis*, the intermediate host snail of *Schistosoma japonicum* in mainland China

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Abstract. Two internal transcribed spacer (ITS1 and ITS2) sequences of the non-coding region of the rDNA and a 16S RNA gene fragment of the mtDNA of *Oncomelania hupensis*, the intermediate host snail of *Schistosoma japonicum* in mainland China, have been investigated with a view to illustrate the influence of the environment on genetic differentiation. Thirteen populations of *O. hupensis* snails, representing four types of ecological settings, were collected to compare genetic and spatial distances. The length of the ITS1-5.8S-ITS2 gene sequence showed a range from 752 to 796 bp with a guanine-cytosine (GC) content of 49.1-50.3%, while that of the 16S sequence ranged from 505 to 508 bp with a GC content of 33.5-35.1%. The 5.8S fragment was shown to be highly conserved and it was therefore removed in the subsequent analysis. In contrast, the ITS flanking sequences and the 16S fragment were found useful for further study as their degree of polymorphism amounted to 13.2% and 8.6%, respectively. The genetic relationship was investigated using tools based on maximum parsimony, minimum evolution and neighbour-joining algorithms. Four branches of *O. hupensis* were found to be clearly represented on the 16S phylogenetic tree, namely (i) the mountainous region population (from Sichuan and Yunnan provinces); (ii) the Karst region population (from Guangxi autonomous region); (iii) the population representing the region of swamps and lakes along the Yangtze River basin which stretches through the Anhui, Hunan, Hubei, Jiangxi, Jiangsu and Zhejiang provinces; and (iv) the littoral, hilly region population (from Fujian province). These four population branches were found to be correlated to environmental features based on the concept of landscape ecology attributing genetic differentiation to differences in ecological features. However, only three main branches could be found on the ITS1-ITS2 phylogenetic tree. The swamps and lakes population (from the Yangtze River basin) and the littoral, hilly population (from Fujian province) were clustered on the third branch in spite of these two populations not being spatially related, i.e. no firm genetic demarcation between their snail populations was found. Thus, it seems that the Fujian population does not constitute a separate branch but belongs to the third branch. This fact, together with the strong genetic evidence that the subspecies *O. hupensis guangxiensis* represents a discrete branch, support the hypothesis that genetic differentiation of *O. hupensis* in mainland China is ultimately structured by landscape ecology.

Keywords: *Oncomelania hupensis*, ITS1-5.8S-ITS2, 16S, genetic variation, subspecies, snail population, landscape genetics, China.

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Introduction

Oncomelania hupensis is the sole intermediate snail host of *Schistosoma japonicum*, which therefore completely depends on this snail species for its transmission (Wang et al., 2009).

The epidemiology and control of schistosomiasis continue to be a strong research focus due to the great importance of this disease in mainland China (Davis et al., 1995; Chitsulo et al., 2000; Zhou et al., 2005, Chen et al., 2007; Wang et al., 2008, 2009). This includes the development and application of molecular systematic techniques for the classification and population genetics of the snail (Hope et al., 1994; Spolsky et al., 1996; Xu and Zheng, 2003). As publicized in the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/>), *Oncomelania* is a recognized taxonomic group comprised of seven subspecies in the world, three of which are distributed in mainland China, namely *O. hupensis hupensis*, *O. hupensis robertsoni* and *O. hupensis tangi* (<http://www.ncbi.nlm.nih.gov/Taxonomy/>). However, there are uncertainties regarding *O. hupensis tangi* (Davis et al., 1997), while an additional recognized subspecies, *O. hupensis guangxiensis*, is now considered a discrete subspecies based on geographic distribution and amplified fragment length polymorphism (AFLP) (Zhou et al., 2007a,b).

From the spatial point of view, the areas where *Oncomelania* is mainly located in mainland China fall into four different ecological settings, i.e. the region of swamps and lakes in the Yangtze River basin (part of the Anhui, Hubei, Hunan, Jiangsu, Jiangxi and Zhejiang provinces), the mountainous region of the Sichuan and Yunnan provinces, the hilly, littoral part of the Fujian province, and the Karst landscape of Guangxi autonomous region (Chen et al., 2007; Zhou et al., 2007a). As the ecology varies considerably between these areas, it seems likely that features of the landscape have influenced and channeled the genetic variations of *O. hupensis*, into the different subspecies we see today, a situation not only demanding an understanding of ecology, but also a detailed knowledge of population genetics.

Landscape genetics is a relatively new discipline focusing on how the environment models populations and subspecies of organisms at the DNA level.

This approach combines the tools of molecular genetics with the principles of landscape ecology to spatially map allele frequencies and correlate genetic patterns with the limits set by the environment. The new concept promises to facilitate our comprehension of how geographical and environmental features structure the genetic variation at the population and speciation levels (Manel et al., 2003; Manel and Segelbacher, 2009). In spite of considerable amount of research on the population genetics of *O. hupensis*, the knowledge of the molecular genetics of this snail is still relatively limited. For example, only few sets of gene sequences have been deposited in GenBank (<http://www.ncbi.nlm.nih.gov/>), most of them focused on the mitochondrial sequences of the cytochrome oxidase subunit 1 (COI) (Wilke et al., 2000, 2006; Shi et al., 2002).

This study aimed to amalgamate spatial statistics, geographical information system (GIS) and informative molecular markers with ecological biogeography and molecular ecology to better understand population biology and the evolution of *O. hupensis*. It is based on the supposition that the four types of environment mentioned above have channeled the genetics of this snail through environmental features resulting in four different ecotypes. To investigate this from the population genetics perspective, we have sequenced part of the *O. hupensis* mitochondrial 16S gene, as well as the nuclear 5.8S rRNA gene with its two internal transcribed spacer (ITS) fragments (ITS1 and ITS2). The latter, flanking sequences emanate from a non-coding rDNA region and are characterized by a relatively fast evolutionary rate (Stothard et al., 1996), which facilitated the exploration of the genetic variation and phylogeny of this snail species.

Materials and methods

Snail sampling

Snails of the *O. hupensis* species were collected from 13 sites in 10 provinces in mainland China, representing four different landscape ecology populations, making up a total of 30 different isolates,

which incorporated the three subspecies publicized in GenBank. We also included an *O. hupensis quadrasi* isolate (coded as Quadrasi) from the Philippines in the study as an out-group for the planned phylogeny analysis. The geographical origins and locations of all isolates investigated are shown in Table 1 and Figure 1.

DNA preparation

After removal of the gut and digestive glands from the soft parts of the snails, the muscle tissues from each single isolate were digested for 3 hours at 56°C in water bathing with proteinase K (Amresco Inc. Solon, OH, USA) followed by the standard DNA

Table 1. Information regarding the collected samples of *Oncomelania hupensis*.

Population	Site, province, country	Site code	Date	Coordinates
Swamps and lakes region (n _{ITS1-ITS2} = 18, n _{16S} = 15)	Tongling, Anhui, China	AHTL	5/12/2007	29.94904 N; 118.45947 E
	Yueyang, Hunan, China	HNYX	3/28/2007	29.08614 N; 113.05955 E
	Hanshou, Hunan, China	HNHS	4/14/2007	28.92238 N; 112.15158 E
	Yangxin, Hubei, China	HBYX	3/29/2007	29.82804 N; 115.23305 E
	Yugan, Jiangxi, China	JXYG	4/14/2007	28.79958 N; 116.34376 E
	Yangzhou, Jiangsu, China	JSYZ	4/14/2007	32.55622 N; 119.45517 E
	Jinhua, Zhejiang, China	ZJJH	3/23/2007	28.85230 N; 119.46883 E
Mountainous region (n _{ITS1-ITS2} = 4, n _{16S} = 4)	Weishan, Yunnan, China	YNWS	7/04/2007	25.16897 N; 100.14422 E
	Meishan, Sichuan, China	SCMS	4/27/2007	30.18314 N; 103.73853 E
Littoral hilly region (n _{ITS1-ITS2} = 3, n _{16S} = 2)	Fuqing, Fujian, China	FJFQ	3/22/2007	25.40488 N; 119.64710 E
	Changle, Fujian, China	FJCL	8/26/2007	25.70914 N; 119.58699 E
Karst landscape region (n _{ITS1-ITS2} = 5, n _{16S} = 3)	Yizhou, Guangxi, China	GXYZ	9/04/2007	24.21169 N; 108.50488 E
	Jingxi, Guangxi, China	GXJX	9/26/2007	23.36546 N; 106.29970 E

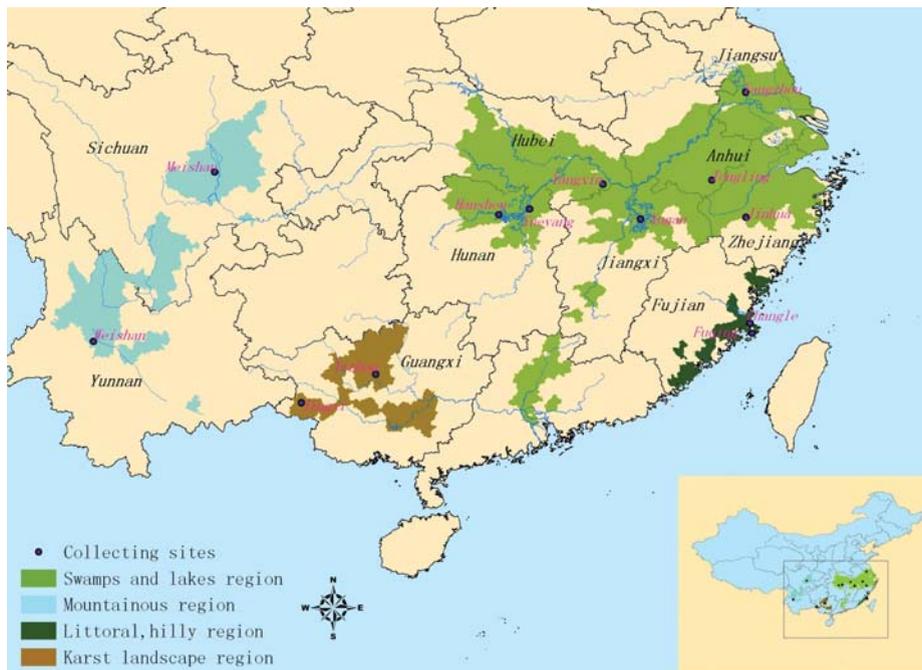


Fig. 1. Distribution of snail landscape ecology populations and collecting sites in schistosome-endemic areas of China.

extraction procedure (Parayre et al., 2007) using Easy-DNA Kit (QIAGEN GmbH, Hilden, Germany).

DNA amplification and sequencing

The 5.8S rRNA gene, flanked by the two ITS fragments, was extracted, together with the 16S gene sequence, from the 30 snail isolates collected from the four ecological populations. At least four specimens were sequenced from each population. The gene sequences were amplified with a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA), using the polymerase chain reaction (PCR) as follows: (i) an initial denaturation at 94°C for 3 min; (ii) 35 cycles of 94°C for 30 sec, annealing at 52°C (ITS1-5.8S-ITS2) or at 56°C (16S) for 30 sec and 72°C for 45 sec; and (iii) final extension at 72°C for 10 min. The primers applied were 16Sar (5'-CGC-CTGTTTATCAAAAACAT-3') and 16Sbr (5'-CCG-GTCTGAACTCAGATCACGT-3') to amplify a 515 bp region of the 16S gene (Attwood et al., 2004), while P1158SF (5'-GAGGATGGATTGGGTTCT-CAAGTGT-3') and P1258SR (5'-CTGCGTTA-GAAAGGCATGTGGGTTT-3') were applied for the 5.8S rRNA gene together with the two ITS fragments using a design based on an alignment of the U93222 and U93220 sequences of *O. hupensis formosana*, deposited in GenBank by Lo CT (1997). The resulting PCR products were purified by electrophoresis in 1% agarose and the bands cut out of the gel with a QIAQuick PCR purification kit (QIAGEN GmbH, Hilden, Germany). The sequences were identified by an ABI 377 automated sequencer (Shanghai Sangon Co. Ltd, Shanghai, China).

Genetic differentiation analysis

The DNA sequences were assembled and edited using Chromas version 2.3 and BioEdit 7.0.1 (Hall, 1999). The *O. hupensis quadrasi* sequences were used as the out-group. All sequence datasets were aligned using Clustal X v1.83 (Thompson et al., 1997) and manually checked for accuracy. The

analysis of the nucleotide differentiation and the calculation of the genetic distances between the different populations were carried out according to substitution models of the Kimura 2-parameter in MEGA version 4 (Tamura et al., 2007) for closely related isolates. The phylogenetic relationships were analyzed using the methods of maximum parsimony (MP), minimum evolution (ME) and neighbour-joining (NJ) algorithms according to Saitou et al. (1989). Statistical support for the inferred nodes was obtained by bootstrapping in MEGA version 4.0 (Tamura et al., 2007). All bootstrap values were based on the performance of 1000 replicates for the NJ, MP and ME trees.

Spatial correlation analysis

All coordinates from the sites where the *O. hupensis* snails were collected were recorded and labeled using Arc View GIS software (ESRI Corp, Redlands, CA, USA) to visualize the geographic distribution map of the *O. hupensis* collected sites. The geographical distances between collected sites were measured using the mapped data followed by correlation analysis, comparing the spatial and genetic distances for each *O. hupensis* isolate based on Microsoft Excel (2003). The *F*-test, the regression coefficient (*R*) and the decision coefficient (*R*²) between isolates were calculated using SPSS11 (Statistical Products & Service Solutions) package for Windows (SPSS Inc., Chicago, USA).

Results

Genetic distance based on the ITS1-5.8S-ITS2 sequence

The various ITS1-5.8S-ITS2 sequences from the 30 individual isolates, representing the four ecological populations, ranged from 752 to 796 bp and showed guanine-cytosine (GC) contents from 49.1% to 50.3%, respectively (Table 2). The alignment of the ITS1-5.8S-ITS2 sequences generated 826 characters with 13.21% (109) polymorphic

sites, of which 78.0% (85) were informative with respect to parsimony. The remaining 22.0% (24) were singletons and 18 haplotypes were found among them. The 5.8S rRNA gene was located between site 384 and site 521 in the alignment sequence. Only three polymorphic sites were found: a guanine/adenine (G/A) mutation at site 424 (in one isolate from Yangzhou city), a thymine/cytosine (T/C) mutation at site 502 (in five isolates from Guangxi) and a G/A mutation at site 514 (in three isolates from Zhejiang). Most of the polymorphic sites were found in the ITS1 and ITS2 fragments where they formed two "hot spots" at the 160-378 region and the 587-729 region, respectively.

The intra-population genetic distances across the ITS1-5.8S-ITS2 sequence ranged from 0.000-0.011 (in the Karst population from Guangxi) to 0.000-0.020 (in the population from the swamps and lakes region). The divergence between the populations was calculated from the inter-population sequences divergence matrix. The genetic distances ranged from 0.0098 between Fujian and the swamps and lakes region to 0.0536 between high-altitude regions (Yunnan and the Karst region of Guangxi) with an average genetic distance of 0.0236. The population from the mountainous region (Yunnan and Sichuan) was found to be the most divergent one. It differed from all the other populations by a genetic distance ranging from 0.0329 to 0.0536 (Fig. 2).

Genetic distance based on the 16S sequence fragment

The length of the 16S rRNA mitochondrial gene from 24 individual isolates (the other six isolates failed the DNA sequencing attempts) from four landscape ecological populations ranged from 505 to 508 bp with the corresponding GC content varying from 33.5% to 35.1% (Table 2). At the 16S locus, with 511 characters generated from the alignment sequences, 14 haplotypes were found. However, only 8.6% (50) of the sites were polymorphic and 54.0% (27) of these were informative with respect to parsimony, while the others were single-

tons. All the polymorphic sites were scattered in the sequences without any marked regions of concentration.

Intra-population variation was detected in all populations with the greatest seen in the mountainous population from the Yunnan and Sichuan provinces, which showed a maximum genetic distance of 0.0392 between individual isolates. The least genetic distances were seen in the population from the swamps and lakes region (0.000-0.006). When the divergence between the study populations was calculated, the least distance (0.004) was seen between the populations from Fujian and that of the swamps and lakes region (Anhui, Hunan, Jiangxi and Zhejiang). The largest distance (0.0478) was observed between an isolate from Sichuan and an isolate from Guangxi (Fig. 3).

Phylogenetic analysis

Because the ITS1 and ITS2 sequences, in contrast to the 5.8S gene fragment, were found to include a large number of polymorphic sites, the latter sequence was removed. The two ITS fragments were subsequently combined and subjected to phylogenetic analysis with the aim of finding the phylogenetic relationships between the populations from the four geographical areas. The *O. hupensis quadrasi* was sequenced as an outgroup for the phylogeny analysis.

When the datasets were combined, three substitution models (the maximum composite likelihood, the

Table 2. Statistics related to each locus used in the phylogenetic analyses.

Index	ITS1-5.8S-ITS2	16S
Length (bp)*	752-796	505-508
GC content (%)	49.1-50.3	34.2
Haplotypes (no.)	18	14
Sites (bp)**	825	511
Polym. sites (bp)***	109 (13.2%)	50 (9.8%)
PS (bp)****	85 (78%)	27 (54%)

*Length of amplicon discounting primer sequences; **total number of sites excluding those with alignment gaps; ***number of polymorphic sites; ****number of polymorphic sites with parsimony informative sites within parentheses.

Jukes-Cantor and the Kimura-2-parameter) were applied to generate the phylogenetic tree according to Tamura et al. (2007). The MP, ME and NJ algorithms resulted in the same phylogenetic topology trees with the level of support being greater for all nodes on the NJ tree (Fig. 2). In comparison to all other populations, three main distinct clades in the phylogenetic trees stood out, i.e. (i) the population from the mountainous regions (Yunnan and

Sichuan); (ii) the population from the Guangxi Karst region; and (iii) the population from the swamps and lakes regions (which included the six provinces mentioned before), including the Fujian population. In this third clade, the Zhejiang isolates were clearly clustered together with Fujian isolates forming a mixed small branch on the phylogenetic tree.

For the 16S datasets, the main topology of the phylogenetic trees were equal to those generated

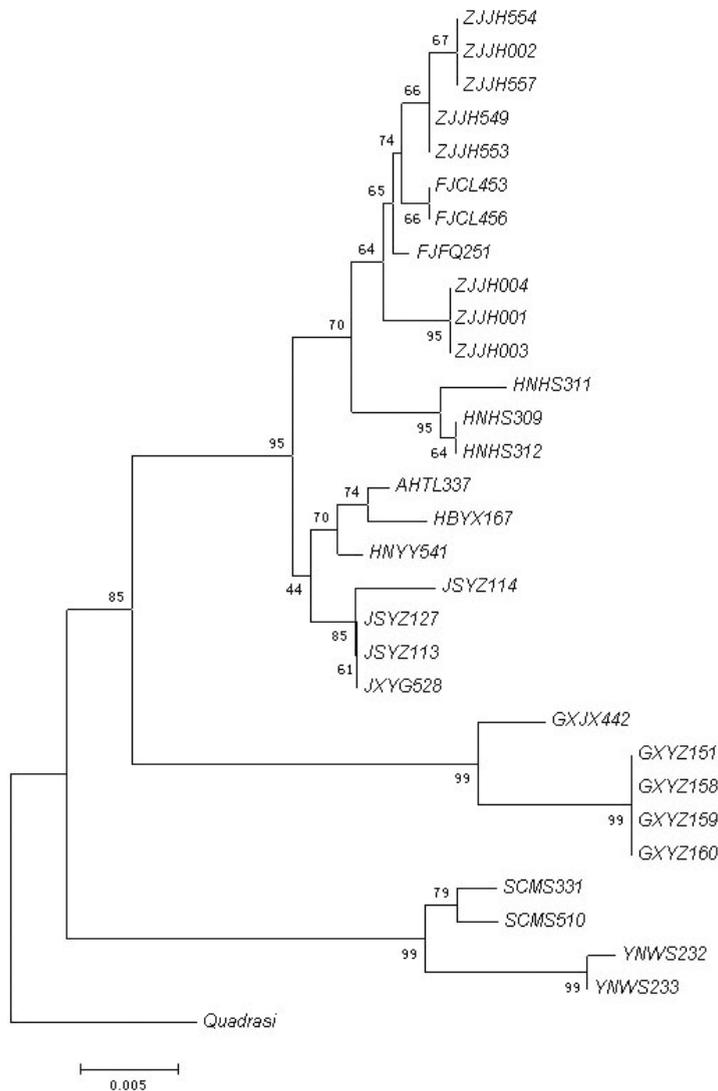


Fig. 2. Phylogeny of the *O. hupensis* populations based on the combined ITS1 and ITS2 sequences. The tree was generated by neighbour-joining (NJ) analysis, and bootstrap proportions were shown at nodes. Site code was followed by the sample serial number.

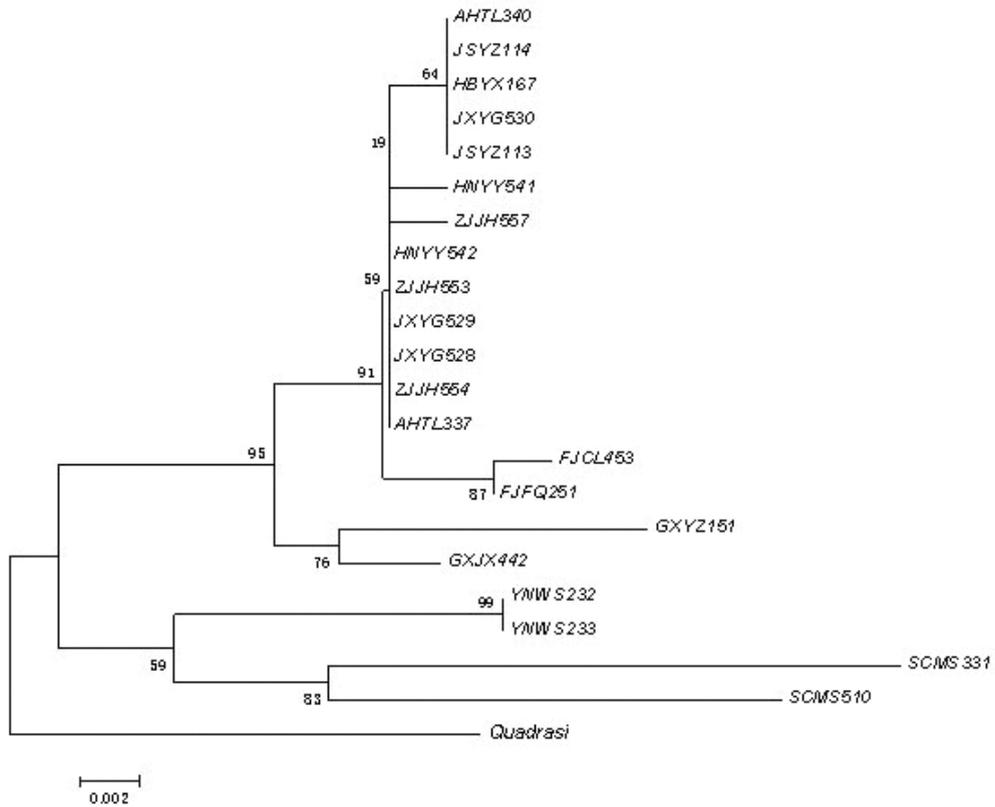


Fig. 3. Phylogeny of the *O. hupensis* populations based on 16S sequences. The tree was generated by minimal evolution (ME) analysis, and bootstrap proportions were shown at nodes. Site code was followed by the sample serial number.

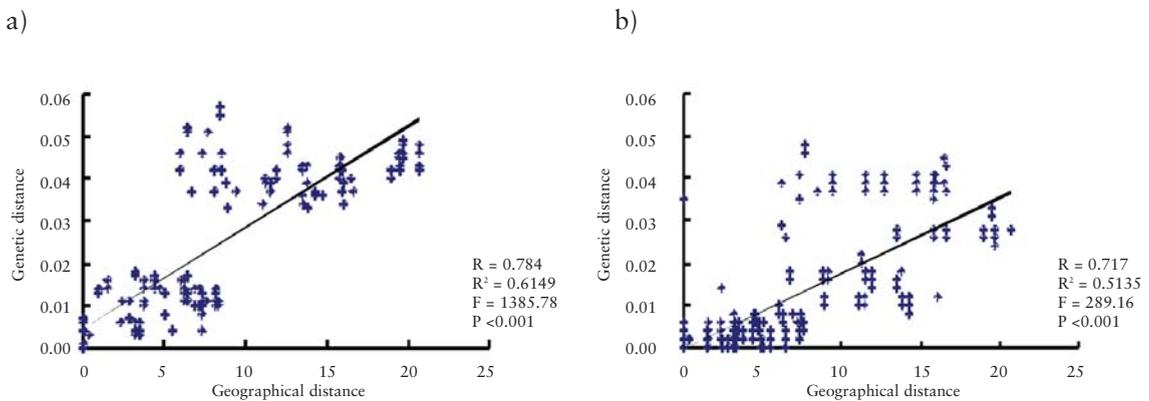


Fig. 4. Correlograms between genetic distance and geographical distance of ITS1-ITS2 and 16S of *O. hupensis*: a) ITS1-ITS2, b) 16S.

from the combined ITS1 and ITS2 datasets regardless which method (NJ or ME) was chosen from the Jukes-Cantor, maximum composite likelihood or Kimura-2-parameter models (Fig. 3). Three main clades were observed but the Fujian branch in the third clade was clearly separated from the closed population from the swamps and lakes regions.

Spatial correlation analysis

The geographical isolation of the four different landscape populations from where the 30 *O. hupensis* isolates were collected is clearly seen on the map (Fig. 1). The geographical distances between these snail populations showed significant correlations with the genetic distances based on the ITS1-ITS2 and 16S molecular markers ($P < 0.001$) (Fig. 4), confirming a correlation between genetic differentiation and geographical distance at the population level ($R_{\text{ITS1-ITS2}} = 0.78$, $R_{16S} = 0.72$) (Figs. 4a and 4b). Moreover, the decision coefficient R^2 ranged from 51.4% to 61.5% ($R^2_{\text{ITS1-ITS2}} = 0.61$, $R^2_{16S} = 0.51$) which indicates the degree of genetic differentiation driven by the geographical difference in the equation.

Discussion

Since *O. hupensis* is the only intermediate host that can transmit *S. japonicum* from one final host snail to another, it is clearly of practical importance to establish a robust and ecologically meaningful classification and phylogeny. Efforts to explain the heterogeneities in transmission intensity across mainland China as due to subspecies classification remain controversial (Zhou et al., 2006). However, the investigation presented here, which is based on an integrated approach combining landscape ecology with population genetics, makes it possible to understand genetic differentiation as a combined effect of various characteristics where landscape features influences the gene flow in *O. hupensis*. The following three main questions require further discussion.

First, is the discovered genetic differentiation driven by the ecology? The study of molecular identities has become a fundamental approach for species differentiation and population genetics (Ma et al., 2006). They provide a unique perspective on landscape genetics and emphasize computational and biochemical analyses to better understand evolutionary and functional relationships. An important finding in this respect regards the correlation between genetic differentiation and geographical distance at the population level, which clearly illustrates that genetic differentiation can be attributed to differences in ecological features. This is further supported by the finding that the contribution to genetic differentiation in the snail populations by geographical difference was 51% to 61% of all causes in the correlative equation (R^2). The notion of genetic differentiation as a continuous process issuing from a common origin and driven by ecological variation is strengthened by two molecular markers (ITS1-ITS2 and 16S) producing similar results. However, the phylogeny trees show three branches according to the ITS1-ITS2 marker and four branches according to the 16S marker, namely (i) the mountainous region population (Sichuan and Yunnan); (ii) the Karst region population of Guangxi; (iii) the swamps and lakes region population; and (iv) the littoral, hilly region of Fujian. The first and second branches were identical according to both markers, while a difference was noted regarding the third branch, i.e. both markers agreed with the existence of a third branch though the 16S data-sets indicated that the third branch should be divided into two (a third and a fourth branch). The two latter branches were clearly observed with the 16S data-sets, which differentiated between the two landscape ecological populations from the littoral hilly regions and the swamps and lakes region of the Yangtze River basin, which is supported by previous AFLP results (Zhou et al., 2007b,c) An interesting discovery is that ITS1-ITS2 data-sets integrated the Fujian isolates with the Zhejiang isolates of the swamps and lakes region. Whether or not this is an example of convergent evolution is discussed in the

last paragraph of this paper.

The second question is whether the Guangxi population is monophyletic. Based on differences in morphological characters, geographical distribution, ecological environment and parasite susceptibility, *O. hupensis guangxiensis* was considered a monophyletic subspecies (Liu et al., 1981). They put forward the hypothesis that five subspecies exist in mainland China, namely, *O. hupensis hupensis*, *O. hupensis fausti*, *O. hupensis tangi*, *O. hupensis guangxiensis* and *O. hupensis robertsoni* (Liu et al., 1981). However, the later, systematic analysis by Davis et al. (1997), based on morphological data, isoenzyme differences and DNA sequence variation, overturned this analysis with respect to *O. hupensis fausti*, which is now accepted as a smooth-shelled variant of *O. hupensis hupensis* influenced by ecosystem variations and other environmental factors (Yang et al., 2007, 2008; Zhou et al., 2008). In accordance with Liu et al. (1981), Davis et al. (1997) considered *O. hupensis guangxiensis* to be sufficiently distinct from *O. hupensis hupensis* to be regarded as a separate subspecies. This has led to the 3-subspecies classification that includes *O. hupensis tangi* and *O. hupensis guangxiensis* as two additional, but not sufficiently different, uncertain taxa. This classification has been widely accepted in China even though an in-depth study of *O. hupensis guangxiensis* is lacking (Qian et al., 1994; Iwanaga, 1995; Yang et al., 2008). However, molecular genetic evidence, together with detailed ecological, morphological and geobiological data, support the 4-subspecies classification according to which the distinction of *O. hupensis guangxiensis* from the Guangxi province is accepted even if full molecular evidence is lacking. The Guangxi samples were clustered with those from Fujian (*O. hupensis tangi*) in the same clade in AFLP analysis (Zhou et al., 2007b,c). However, in the study presented here, the clade of *O. hupensis guangxiensis* population from Guangxi was distinctly separated from the other populations and formed a separate monophyletic clade based on phylogenetic analysis of two molecular sequences.

Finally, the third question is whether there is convergent evolution between *O. hupensis tangi* and other snails. Although there is no evidence that gene introgression has taken place between *O. hupensis tangi* (or Fujian isolates) and other snails, it was considered a discrete subspecies or uncertain taxa in the classification system (Liu et al., 1981; Davis et al., 1997; Zhou et al., 2007a,b). The phylogenetic analysis based on the combined ITS1 and ITS2 datasets showed that *O. hupensis tangi* had clustered with *O. hupensis hupensis* (Zhejiang) with an indistinct geo-boundary between the two landscape ecological populations in spite of the close geographical distance presented. In contrast, the clades in the 16S genetic cluster, *O. hupensis tangi* were sufficiently different from *O. hupensis hupensis* to support its independence.

ITS1 and ITS2 have a fast evolutionary rate because of their location at a non-coding rDNA region and are therefore widely used for species identification and analysis of genetic relationships (Stothard et al., 1996). The mitochondrial 16S rRNA gene is relatively conserved and thus exhibits less informative sites with regard to parsimony. It was therefore not surprising that our study found the *O. hupensis tangi* subspecies distributed southeast littoral region of Fujian province to be close to *O. hupensis hupensis* in the Zhejiang province, which shares a similar environment. Consequently, there maybe a convergent evolution or gene change on the ITS1 and ITS2 sequences between *O. hupensis tangi* and the Zhejiang isolates of *O. hupensis hupensis*, which resulted in a shorter genetic distance. This could have been caused by the same type of stress from similar environmental features even if the areas are separated by the Cangshan Mountain (Zhou et al., 2007a), which effectively blocks any exchange.

We concluded that based on the molecular phylogenetic trees investigated, and in accordance with classification according to landscape ecology, this study supports the existence of four main branches of *O. hupensis* largely driven by ecological variations. The hypothesis of landscape ecology as the ultimate arbiter is supported by the unclear genetic

boundary according to the ITS1-ITS2 datasets between the population from the swamps and lakes region along the Yangtze River basin and that of the littoral hilly region (Fujian). This is further strengthened of *O. hupensis guangxiensis* now being accepted as a discrete *O. hupensis* subspecies. However, the convergent evolution between *O. hupensis tangi* and Zhejiang isolates of *O. hupensis hupensis* needs to be investigated further in the future.

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