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Source: Malacologia, 53(1):147-153. 2010.
Published By: Institute of Malacology
DOI: 10.4002/040.053.0107
URL: http://www.bioone.org/doi/full/10.4002/040.053.0107

MALACOLOGIA, 2010, 53(1): 147–153

IDENTIFICATION OF POLYMORPHIC MICROSATELLITES FOR THE INTERMEDIATE HOST *ONCOMELANIA HUPENSIS* OF *SCHISTOSOMA JAPONICUM* IN CHINA

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INTRODUCTION

The snail *Oncomelania hupensis* in the family Pomatiopsidae is the unique intermediate host of *Schistosoma japonicum* in some Far East countries. In China, this snail host is widely distributed, which determines largely the epidemic area of schistosomiasis. Despite the wide geographical distribution, the habitats of *O. hupensis* can be in general grouped into three ecological categories: (1) lake/marshland representing the shore area of lakes and the Yangtze River, (2) flood-plain and water channel representing the area in the flood-plain of the River and all water channel systems in the plain, and (3) hilly and mountainous regions, with the former in some areas in the middle and lower reaches of the Yangtze River, and the latter mostly in Sichuan, Yunnan provinces now, but also including Fujian, Guangdong provinces and Guangxi autonomous region (Zhou et al., 2005).

Snails distributed in these different habitats vary in their morphotypes. In general, two morphotypes, that is ribbed-shelled and smooth-shelled, have been reported in China (Liu et al., 1981; Davis et al., 1995). Snails distributed in areas of the middle and lower reaches of the Yangtze River, genetically defined as *O. hupensis hupensis,* are polymorphic with ribbed and smooth shells that always have a varix (Davis et al., 1995, 1999). Davis et al. (2006) recently demonstrated polymorphism in shell sculpture from heavily-ribbed shells to slightly-ribbed to smooth-shelled conditions in the same populations in canals in Hubei province. The ribbed shelled morph lives on the flood plains while smooth-shelled snail live in hilly areas or in canals removed from Yangtze River flooding (Davis et al., 1995, 1999, 2006). *Oncomelania hupensis robertsoni*, living in Yunnan and Sichuan provinces, is genetically quite distinct from *O. hupensis hupensis* with unique population genetic attributes (Wilke et al., 2006). *Oncomelania hupensis robertsoni* has a smooth shell, is uniformly smaller than *O. hupensis hupensis*, and never has a varix.

Oncomelania hupensis in Fujian province and in southwest China, Guangxi autonomous region, have smooth-shelled snails with pronounced varix (Liu et al., 1984; Davis et al., 1995). In Fujian province, genetic data support the classification *O. hupensis tangi*, while the classification *O. hupensis guangxiensis* in Guangxi autonomous region is still under consideration(Liu et al., 1981; Davis et al., 1995; Li et al., 2009). The subspecies of *O. hupensis* are significantly different from each other genetically. Overall, *O. hupensis* exhibits a high degree of genetic diversity throughout China. Several studies, using mitochondrial or nuclear genes or even allozymes have detected genetic difference in *O. hupensis* in China, and genetic diversity in *O. hupensis hupensis* and also in *O. hupensis robertsoni* (e.g., Davis et al., 1995, 1999; Wilke et al., 2000, 2006; Li et al., 2009; Zhao et al., 2010). In consideration of the wide geographical distribution, it is thus hypothesized, as also suggested by Davis et al. (1998, 2006), Wilke et al. (2006), and Zhao et al. (2010) that the intermediate host may have considerable genetic diversity at population level, thus within subspecies, which should be examined by using more powerful molecular markers such as microsatellite markers. The understanding of genetic diversity of the intermediate hosts may shed light on the genetic complexity of their parasite, *Schistosoma japonicum*.

Microsatellites or simple sequence repeats (SSRs) have been used widely and efficiently as molecular markers in studying population genetics and parentage and kinship analyses because of their high level of polymorphism and codominant Mendelian inheritance (O'Connell & Wright, 1997). In this study, we employed SSRs methods to screen polymorphic molecular markers from nuclear genome of *O. hupensis*, in order to provide effective microsatellite markers for investigating the population genetics of the intermediate hosts in China.

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MATERIAL AND METHODS

Oncomelania hupensis were obtained with forceps from a wide range of schistosomiasis endemic areas in mainland China in six localities of five provinces, representing principally three different ecological categories (Table 1). The diagnosis of subspecies of *O. hupensis* followed that of Davis et al. (1995). Snails obtained were then brought back to laboratory, where they were washed and captured for several days, and those confirmed without parasite infection under a microscope were preserved in 95% ethanol. The head-foot muscle of about 200 snails from each locality were dissected out and fixed in 70% ethanol before being used for the extraction of the total genomic DNA using a standard proteinase K/SDS extraction method (Sambrook et al., 2001). The DNA concentration was determined by measuring the optical density using a BioPhotometer (Eppendorf, Germany). All samples were then diluted to a concentration of 50 ng/μl.

In order to obtain well-behaved polymorphic microsatellite sequences suitable for studying population genetics, it is necessary to mix the extracted total genomic DNA from samples of six localities (Table 1) for the construction of genomic library. Microsatellite-enriched genomic library for the repeat motif $(CA)_n$ and $(CT)_n$ was constructed essentially following the fast isolation by AFLP of sequences containing repeats (FIASCO) protocol (review in Zane et al., 2002). Enriched fragments were ligated into pMD18-T vector (TaKaRa) and propagated in the DH5α strain of *Escherichia coli*. After polymerase chain reaction (PCR) confirmation, 632 positive amplicons about 300–700 bp long were sequenced with ABI PRISM 3700.

The cloned sequences were analysed for the repeat regions using software Tandem Repeats Finder (Benson, 1999), and 174 putative microsatellite sequences were examined using primers designed with the software Primer3 (Rozen & Skaletsky, 2000). The polymorphism of these microsatellite sequences was further examined with *Oncomelania hupensis* collected from the above mentioned 6 localities (Table 1), and further analyzed with thirty snails collected from Jiangling County of Hubei Province, China in the present study. In general, the PCR amplification for detecting the polymorphism was carried out in a mixture of 25 μl volume on a PTC-100 thermocycler (Biorad, USA), containing 1 × PCR buffer (Tiangen), 50 ng genomic DNA, 0.25 μm for each primer, 150 μmol/L dNTPs, 1.5 mm $MgCl_2$ and 0.25 U Taq DNA polymerase (Tiangen), with the following programme: an initial denaturation at 94°C for 5 min; 35 cycles including denaturation at 94°C for 40 s, annealing at the proper temperature (Table 1) for 40 s and elongation at 72°C for 40 s, and a final elongation at 72°C for 5 min. PCR products were visualized on 8% polyacrilamide gel stained with ethidium bromide, as reported by Tong et al. (2002). The pBR322 DNA/MspI molecular weight marker (Tiangen) was used as standard to identify alleles.

DNA sizing and microsatellite analyses were performed with the Quantity-One software (Biorad). The analyses of polymorphism, including number of alleles (N_a), observed heterozygosity

TABLE 1. Samples of *Oncomelania hupensis* ssp. used for the construction of microsatellite-sequence-enriched library.

Subspecies	Shell sculpture	Locality (County/City, Province)	Habitat	Date	Geographical location
O. h. hupensis	Ribbed	Jiangling, Hubei	Irrigation channel	2008.09.27	30°00’31”N 112°34’43”E
O. h. hupensis	Ribbed	Jiujiang, Jiangxi	Swamp	2008.10.11	29°3’26”N 115°5’43”E
O. h. hupensis	Smooth, varix	Yushan, Jiangxi	Hilly area	2008.09.07	28°4’51”N 118°1’21”E
O. h. hupensis	Smooth, varix	Nanlin, Anhui	Hilly area	2006.05.10	30°53’18”N 118°2’27”E
O. h. robertsoni	Smooth	Xichang, Sichuan	Mountainous region	2006.06.16	27°4’04”N 102°2’10”E
O. h. robertsoni	Smooth	Dali, Yunnan	Mountainous region	2008.07.30	25°2’59”N 100°1’54”E

TABLE 2. Characterization of 41 novel polymorphic microsatellites in *Oncomelania hupensis* ssp.*

Locus	Repeat motif	Primer sequence (5'−3')	Ta (°C)	N	Size (bp)	H_O	H_E	*P* value	GenBank Accession #
Oh10	(GAA)12	F: 5'-AAATGCCCCTTGTGCCTGGAAT-3' R: 5'-TAGGACTGGTTTAGGGTTAGGGTTC-3'	58	3	136	0.5000	0.6576	0.542	GQ892883
Oh12	(ATG)22	F: 5'-CCCCACCACAACCACAAATC-3' R: 5'-GGTCGCTCAGGGTCAGAATA-3'	56	7	204	0.5667	0.6763	0.7832	GQ892884
Oh13	(TG)53	F: 5'-TGCATAAATTCCGTAGTTTT-3' R: 5'-CCCTCTTCTTCTGCCTCCTT-3'	56	4	264	0.5667	0.6808	0.0301	GQ892885
Oh14	(CA)37	F: 5'-CGTCACAAAGGAGCAACTGG-3' R: 5'-TTTACTGCTCTGCATAGCAAAGGAT-3'	56	3	274	0.5000	0.6565	0.4202	GQ892886
Oh20	(ATC)26	F: 5'-GCAGAACCCGCCAACCATTT-3' R: 5'-CAGTAGCGAGTGCTCCAACCT-3'	56	5	223	0.6333	0.7379	0.256	GQ892887
Oh30	(CT)21	F: 5'-TGTGGAAGTGGGTGGTGAGG-3' R: 5'-TGGTAAACACCGCTGTTTTTGT-3'	56	6	206	0.4203	0.5782	0.2603	GQ892888
Oh35	(AG)29	F: 5'-ATCCCATCCTATGACTATTTCCAAG-3' R: 5'-CAAACTGTAAAAGAGAGGGGTGTGT-3'	58	3	209	0.4000	0.5391	0.5721	GQ892889
Oh36	(TC)21	F:5'-AGGGTTGCTGAGGACAGAGAAAGAG-3' R:5'-CCAACTCACAACTCAGAATCCCCAT-3'	56	5	204	0.4667	0.5492	0.6475	GQ892890
Oh47	(AC)38	F: 5'-CACACGTGGTGTAGACCTACTTCTC-3' R: 5'-AACCGTTTGGGATGTAATAAATGTA-3'	58	6	218	0.4668	0.5320	0.1662	GQ892892
Oh57	(CT)59	F: 5'-GCAGCACCTCCAAACGGATTATG-3' R: 5'-GTGTGTATGTTTGTTCGGGGTTGTA-3'	56	6	318	0.6000	0.7576	0.3441	GQ892893
Oh63	(CA)32(CTT)6	F: 5'-CCGCTCCTCATCTTATCTTCTT-3' R: 5'-AAGATAGTAACCGAGAACAGTAGGG-3'	56	5	319	0.3145	0.5236	0.262	GQ892894
Oh68	(CA)36	F: 5'-AGCAGAACATTCTCAAACAAAGCG-3' R: 5'-TCGCCTCAGCTAACAGACCATCC-3'	58	4	174	0.6667	0.7571	0.4231	GQ892895
Oh69	(TG)23(CA)19	F: 5'-GCAAATACACCCAGTAACTGTCTTG-3' R: 5'-GGGCACCCTAGACAGTCCTCTGAT-3'	58	8	337	0.5340	0.5096	0.0459	GQ892896
Oh73	(TCA)24	F: 5'-ACCCTATATTTTCACTGAAGCCTCT-3' R: 5'-CATGATACGACGTCCTTTCTACAAT-3'	56	4	195	0.5333	0.6870	0.0706	GQ892897
Oh80	(CA)64	F: 5'-CCTGTAGTATGTAAGAACAATGGGT-3' R: 5'-GCGCAGTTTGGCGTGTCAGT-3'	56	3	293	0.4613	0.5433	0.047	GQ892898

(continues)

(continued)

Locus	Repeat motif	Primer sequence (5'–3')	Ta (°C)	N	Size (bp)	H_O	H_E	*P* value	GenBank Accession #
Oh94	(TG)57	F: 5'-TAGCTCTCTGATGAAACCAAATCAG-3' R: 5'-TAACGGGGGCGTAAAACAAGTC-3'	56	8	240	0.6100	0.5977	0.5021	GQ892899
Oh95	(TG)16(GA)16	F: 5'-ATAACGCCTTCCAGAAAATCAATAG-3' R: 5'-AGGGCCTTATACGGAATAAACTTGT-3'	56	6	335	0.6730	0.5301	0.0607	GQ892900
Oh100	(CA)44	F: 5'-TATATTTGAAAGCTCGGTTTGCCAT-3' R: 5'-TCCATCTGCGTACGTGTGTCTGC-3'	56	8	233	0.3446	0.4890	0.0441	GQ892901
Oh105	(ATC)62	F: 5'-GTGTCGGAAGTAGAGTAACTGTCGT-3' R: 5'-CTCCACCCTGTTTGCCTTTGC-3'	56	3	296	0.4031	0.5545	0.4463	GQ892902
Oh111	(GA)41	F: 5'-ATTGGTTGCGGCTCCAGTAT-3' R: 5'-CTTCCCTCCCCTTTCCAGAC-3'	58	4	234	0.7231	0.6043	0.0321	GQ892903
Oh125	(CT)23	F: 5'-TGGAGGCTAGGGTGAAGAAG-3' R: 5'-GCAGACACGGAGGAGAACGA-3'	56	7	186	0.5129	0.4897	0.471	GQ892904
Oh132	(GA)43(GAA)6(ATC)17	F: 5'-GTCCAAAAGTGAGGAGAGAGGGT-3' R: 5'-CTTGATGGGAATATAAGTGGCTGT-3'	56	10	276	0.8333	0.8689	0.9617	GQ892905
Oh136	(TG)32	F: 5'-TGTTTATATTACACGCCCACCCTCT-3' R: 5'-CAGGTTCCTCCAAGTATTCATTTTC-3'	56	4	187	0.5543	0.3491	0.1965	GQ892906
Oh150	(GAA)12	F: 5'-AGGCGACACCAATTTTACATGAGG-3' R: 5'-TGTGAAATAAAGGCTTGTGGG-3'	56	6	356	0.4620	0.2135	0.56	GQ892907
Oh151	(TG)24(AG)28	F: 5'-TCACGACCAGTAATGGGG-3' R: 5'-GCAATCCTTCTTGCCTCA-3'	56	7	162	0.6803	0.5458	0.1192	GQ892908
Oh157	(CT)28	F: 5'-CTATGTTCGGATGGCAAGATTG-3' R: 5'-GACTGCGTGTAGCTTGTAAGTGC-3'	55	8	358	0.3215	0.4531	0.0651	GQ892909
Oh158	(TG)47	F: 5'-TCGTAAACAGCAAAGAGAAGAAAAG-3' R: 5'-AGTACACGCATACGCATCTACATAG-3'	56	6	251	0.4436	0.4601	0.2139	GQ892910
Oh161	(TG)16	F: 5'-CGTCCGGGCGATGGAATA-3' R: 5'-GCCAGTGAGGTTCGGTTT-3'	56	6	223	0.5792	0.7550	0.2395	GQ892911
Oh164	(TG)13	F: 5'-ACAGTCATCAGAATGTCGTTTTTAC-3' R: 5'-CAAGTAGGCATGTCAATACAAGTTA-3'	56	7	147	0.4367	0.5280	0.0941	GQ892912
Oh188	(TC)51(CA)7	F: 5'-ATAGACGAATACGCCTACATAAAAC-3' R: 5'-CGCAAAAAAGTGGGTAGACAAGAAT-3'	58	8	308	0.5870	0.5714	0.0431	GQ892913

(continues)

(continued)

Locus	Repeat motif	Primer sequence (5'−3')	Ta (°C)	N	Size (bp)	H_O	H_E	*P* value	GenBank Accession #
Oh199	(CT)12(CA)10(CT)43(CA)11	F: 5'-CTCCAACATCACGCTCCCCTACG-3' R: 5'-AAGAAAGGATTATGTGGGCATGGAT-3'	56	5	256	0.7130	0.5437	0.0453	GQ892914
Oh211	(CA)27	F: 5'-GCCACGTATTCTCCCTCTCTCCC-3' R: 5'-GGGTTCGTTCCTTTTCAGTTGCG-3'	56	6	166	0.2252	0.4301	0.771	GQ892915
Oh212	(ATC)20	F: 5'-GTTCGATCTCTTCACCACCACTAC-3' R: 5'-ACAAACGAACAACAGTAGCAATGGT-3'	56	4	215	0.5392	0.6311	0.1241	GQ892916
Oh217	(TG)46	F: 5'-TAGTGTGCAGCATAATGAGTGCA-3' R: 5'-TTATTGAGTCTCTTGACACACCTGC-3'	58	5	232	0.3000	0.5371	0.0644	GQ892917
Oh225	(CA)45	F: 5'-CTTTTTGCTCTCTCACTCGTTCTG-3' R: 5'-ATACACCTTCAAGCGTTGGTATTG-3'	58	4	210	0.4350	0.5321	0.0419	GQ892918
Oh235	(CA)39	F: 5'-TCAGATAGCCGTAACGGCAGAAAT-3' R: 5'-GCCATCAGAAACACCAAAGAGCAC-3'	58	3	272	0.6980	0.6190	0.0331	GQ892919
Oh410	(AC)31(CT)23(CA)14	F: 5'-TCAGCTTTTTGTGATGTGGTTTTT-3' R: 5'-ATGGGTTAGTGGGCATTTTTAGATT-3'	60	9	270	0.8000	0.8475	0.0701	GQ892920
Oh440	(TG)14(AG)23(GT)31	F: 5'-GGGTTAGTGGGCATTTTTAGATTTT-3' R: 5'-TTTCAGCTTTTTGTGATGTGGTTTT-3'	58	10	270	0.4667	0.496	0.0002	GQ892921
Oh448	(TC)33	F: 5'-TTCGCAGTGCCTCTACATTTCC-3' R: 5'-ATCCTCAAGCTCATTTTCTGTAACC-3'	58	6	223	0.5373	0.5689	0.9617	GQ892922
Oh500	(GTT)16(ATG)9	F: 5'-AGCTTCCATCGCCTGCTTCAC-3' R: 5'-GCAACGCTACATCACTACCCTTACT-3'	60	4	180	0.4243	0.4560	0.0587	GQ892923
Oh573-1	(TG)27	F: 5'-TGGAAGAAAAAAGAAGTGCTTATGC-3' R: 5'-GAAGTGGAAGTGAAGATGGAGGAG-3'	58	4	182	0.5937	0.6977	0.0643	GQ892924

*T_a, annealing temperature; N, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; *P* value, exact test for the HWE after Bonferroni correction.

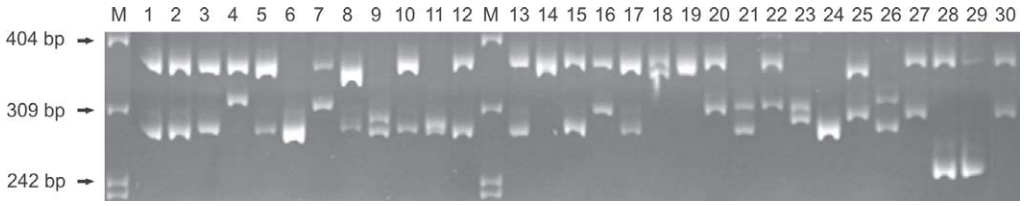

FIG. 1. Amplification pattern of microsatellite locus Oh 132. Lanes 1–30: 30 individuals of *O. hupensis hupensis* collected from Jiangling county of Hubei province, China as listed in Table 1; M: pBR322/ Msp I marker.

(H_O), expected heterozygosity (H_E), and pairwise tests for linkage disequilibrium (LD) and exact tests for the Hardy-Weinberg equilibrium (HWE) were performed by using Popgene version 1.31 (http://www.ualberta.ca/~fyeh/) and Arlequin version 3.1 (Excoffier et al., 2005; Excoffier & Heckel, 2006). The probability of occurrence of null alleles was tested using MicroChecker 2.2.3 (http://www.microchecker.hull.ac.uk/) (Van Oosterhout et al., 2004).

RESULTS AND DISCUSSION

A total of 76, out of 174 pairs of primers were proved to have specific amplification products, but only 41 primers (Table 2) deposited in GenBank (Accession # GQ892883–GQ892890, GQ892892–GQ892924) were polymorphic in the population of *O. hupensis* individuals collected from Jiangling county, Hubei province (Fig. 1). It was also proved that these 41 primers were polymorphic in samples obtained from Anhui, Jiangxi, Sichuan and Yunnan Province (data not shown).

For the samples obtained from Jiangling county, Hubei province, the number of alleles per locus ranged from three to ten with an average of 5.5, and the observed heterozygosity, *H*o and expected heterozygosity, *H*e, ranged from 0.2252 to 0.8333 (average 0.5105) and 0.2135 to 0.8475 (average 0.5704), respectively (Table 2). These loci were shown to be polymorphic. When the *H*o and *H*e value scale of alleles was higher, polymorphism of locus was more obvious, for example Oh132. All pairwise tests for linkage disequilibrium among loci and exact tests for the HWE were non-significant after applying sequential Bonferroni correction ($P < 0.05$). Using the software MicroChecker showed that there were not null alleles at all loci.

In conclusion, novel polymorphic microsatellite sequences have been successfully identified in the intermediate host, *Oncomelania hupensis*, of *Schistosoma japonicum* in the present study. It has been further proved that these microsatellite markers can be employed effectively to examine the population variations at least in the so far confirmed two subspecies, *O. hupensis hupensis* and *O. hupensis robertsoni* (Davis et al., 1995, 1998). However, it may be also of great interest if these microsatellite markers can be used to detect other sub-species of *Oncomelania hupensis*, such as *O. hupensis tangi* and *O. hupensis guangxiensi* at population level.

ACKNOWLEDGEMENTS

This study was funded by a grant (Project no. 30770353) from the National Natural Science Foundation of China, and by a grant (project no. KSCX2-YW-N-055) from the Knowledge Innovation Programme of the Chinese Academy of Sciences. Many colleagues and friends working in the front line of schistosomiasis control in China should be acknowledged for their assistance in sample collection, whose names are just too many to list here.

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Revised ms. accepted 10 April 2010