We thank the BDFFP for the logistical support, the IBAMA for the permission to transport biological material from Brazil, and Lee Weigt and the Museum Support Center, Smithsonian Institution, for making the molecular work possible.

References

Berry F, Kress WJ (1991) *Heliconia: An Identification Guide*. Smithsonian Institution Press, Washington, DC.

Bruna EM (2003) Are plants in rain forest fragments recruitment limited? Tests with an Amazonian herb. *Ecology*, **84**, 932–947.

DeWoody JA, Schupp J, Kenefic L, Busch J, Murfitt L, Keim P (2004) Universal method for producing ROX-labeled size standards suitable for automated genotyping. *BioTechniques*, **37**, 348–352.

Jones KC, Levine KF, Banks JD (2002) Characterization of 11 polymorphic tetranucleotide microsatellites for forensic applications in California elk (*Cervus elaphus canadensis*). *Molecular Ecology Notes*, **2**, 425–427.

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program Cervus accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*, **16**, 1099–1006.

Kress WJ (1985) Pollination and reproductive biology of *Heliconia*. In: *The Botany and Natural History of Panama* (eds D'Arcy WG, Correa MD), pp. 267–271. Missouri Botanical Garden, St. Louis, Missouri.

Laurance WF, Lovejoy TE, Vasconcelos HL *et al*. (2002) Ecosystem decay of Amazonian forest fragments: a 22-year investigation. *Conservation Biology*, **16**, 605–618.

Rousset F (2007) GenePop'007: a complete re-implementation of the GenePop software for Windows and Linux. *Molecular Ecology Resources*, **8**, 103–106.

doi: 10.1111/j.1755-0998.2009.02627.x

Development and characterization of 30 polymorphic microsatellite markers for the Atlantic surfclam, *Spisula solidissima* (Dillwyn, 1817)

YAN WANG*†, AIMIN WANG* and XIMING GUO†

**Ocean College, Hainan University, Key Laboratory of Tropic Biological Resources, MOE, Hainan Key Laboratory of Tropical Hydrobiological Technology, 58 Renmin Road, Haikou, Hainan 570228, China, †Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349, USA*

Abstract

Thirty polymorphic microsatellite markers were developed for the Atlantic surfclam, *Spisula solidissima*, from an enriched library and characterized in 24 clams from a wild population. The number of alleles ranged from 3 to 16 per locus. The expected and observed heterozygosities ranged from 0.1942 to 0.9238 and 0.0833 to 0.875 respectively. Six loci showed significant ($P<0.05$ after Bonferroni correction) deviation from Hardy–Weinberg equilibrium, probably because of the presence of null alleles. Three primer pairs amplified duplicated loci with two involving tandem mini-satellite repeats. Most of the microsatellite markers developed here should be useful for genetic studies in this species.

Keywords: Atlantic surfclam, fishery, microsatellites, *Spisula solidissima*

Received 16 December 2008; revision accepted 9 February 2009

The Atlantic surfclam, *Spisula solidissima* (Dillwyn, 1817), is a marine bivalve widely distributed along the Atlantic coast of North America. Its geographic distribution stretches from the southern Gulf of St. Lawrence to Cape Hatteras, North Carolina. It is mostly found on mud and sandy bottoms from the intertidal zone to 70 m in depth. It occurs at high densities in certain areas and plays an important role in benthic ecology. The Atlantic surfclam is a popular model bivalve used in studies on fertilization and early development. It is also an important fishery resource and supports an annual landing of about 150 000 metric tons. Studies on the population structure of the Atlantic surfclam have been limited partly because of a lack of genetic markers. Here we report the development and characterization of the first set of microsatellite makers for this species.

Microsatellite markers were developed from an enriched genomic library constructed following the method of Tong *et al*. (2006). Genomic DNA was extracted from ethanol-preserved adductor muscles from 12 individuals with the

Correspondence: Ximing Guo, Fax: +01 856 785 1544;
E-mail: xguo@hsrl.rutgers.edu or aimwang@163.com

Cell/Tissue Genomic DNA extraction kit (TianGen) and digested with *Mbo*I (Takara). Fragments of 400–1000bp were collected by TianGen Gel DNA Extraction Kit. Double-stranded *Mbo*I adapters, Linker A (5′-GATCGTCGACGG-TACCGAATTCT-3′) and Linker B (3′-CAGCTGCCAT-GGCTTAAGAACTG-5′) (250 pmol, excess) were ligated to DNA fragments with T4 DNA ligase (Takara) at 16 °C overnight. Excess adapters were removed by washing on an Ultrafree column (Pall). DNA fragments were amplified 10 cycles with linker B and purified. The products were denatured and hybridized to biotin-labelled $(CA)_{12}$, $(GA)_{12}$, $(ACA)_8$, $(AGA)_8$, $(GACA)_6$ and $(GATA)_6$ oligo-nucleotides (mixed in advance at the ratio of 3:1:1:1:2:2) in 0.5X SSC at 68 °C for 1 h. DNA fragments bound to these probes were captured with Promega Streptavidin MagneSphere® Paramagnetic Particles and eluted by DNase-free water after being washed four times in 0.1× SSC at room temperature, and then amplified for 10 cycles with Linker B.

The enriched fragments were ligated to pGEM-T-easy vector (Promega) at 4 °C overnight, and then transformed into Promega high-efficiency competent *Escherichia coli* cells (Dh5α), which were cultured on Luria–Bertani solid medium containing 100 mg/mL ampicillin. One-hundred and ninety-nine white positive clones were picked and sequenced with the T7 primer on ABI 3730xl DNA analysers at the Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Sequences were screened for microsatellites containing at least six di-, five tri-, five tetra-, four penta-, three hexa- and three octa-nucleotide repeats using the software MISA (http://pgrc.ipk-gatersleben.de/misa/). One-hundred and twenty-six (excluding five duplicates) microsatellite-containing sequences were identified and consisted of 28.1% di-, 7.8% tri-, 22.7% tetra-, 6.3% penta-, 1.6% hexa-, 4.7% octa-nucleotide and 35.2% compound microsatellite repeats. Fifty-seven good sequences with sufficient flanking regions were selected for primer design with Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). An M13 (-21) universal leading sequence (18bp) 5′-TGTAAAACGACGGCCAGT was added to the 5′ end of each forward primer listed in Table 1 (Schuelke 2000). PCR solution (10 μL) contained 10–50 ng of template DNA and the following reagents: 1× Promega Colorless GoTaq® Flexi Reaction Buffer (pH 8.5, content proprietary), 1.5–2.5 mM $MgCl_2$, 0.6 mg/mL bovine serum albumin, 0.2 mM each dNTP, 0.2 pmol M13 (-21) tailed forward primer, 0.8 pmol reverse primer, 0.8 pmol M13(-21) primer labelled with fluorescent dyes (FAM, VIC, NED or PET; Applied Biosystems) and 0.3 units of *Taq* polymerase. PCR was conducted on either a GeneAmp 9700 thermocycler (Perkin Elmer) or a PTC-200 DNA engine (MJ Research Inc.). Cycling parameters were 94 °C for 5 min followed by 34 cycles of 94 °C for 30 s, annealing (temperatures indicated in Table 1) for 45 s, 72 °C for 45 s, followed by 10 cycles of 94 °C for 30 s, 53 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. Amplified products were detected and sized on an ABI 3130xl Prism Genetic Sequencer (Applied Biosystems) using GS-600LIZ as size standard. Allele scoring was performed with GeneMapper v4.0. Thirty primer pairs were successfully amplified and genotyped in 24 individuals from a wild population collected offshore at Rockaway Beach, Long Island, New York.

Observed (H_O) and expected (H_E) heterozygosities were calculated with ARLEQUIN 3.0 (Excoffier *et al.* 2005). Hardy–Weinberg equilibrium (HWE) for each locus and linkage disequilibrium (LD) for all pairs were tested with GENEPOP (Fisher's exact test, 1000 iterations, http://genepop.curtin.edu.au/). All tests were corrected for multiple comparisons by sequential Bonferroni correction (Rice 1989). The 30 microsatellite markers showed variable levels of polymorphism, with the number of alleles ranging from 3 to 16 (mean 6.80 ± 3.78), H_O ranging from 0.0833 to 0.8750 (mean 0.4389 ± 0.2449) and H_E ranging from 0.1942 to 0.9238 (mean 0.6440 ± 0.2304) (Table 1). No LD was detected among all loci at $P < 0.05$ (with Bonferroni correction). Six loci (RUSS05, RUSS18, RUSS23, RUSS26, RUSS48 and RUSS59) showed significant ($P < 0.05$ after Bonferroni correction) deviation from HWE. These six and four other loci (RUSS10, RUSS47, RUSS51 and RUSS53) showed signs of null alleles as indicated by MICRO-CHECKER (1000 randomizations) (van Oosterhout *et al.* 2004), and these 10 loci had the lowest *P*-values when tested for HWE, suggesting that the presence of null alleles is the main cause for the deviation from HWE at these loci.

Three primer pairs RUSS33, RUSS47 and RUSS57 amplified more than two fragments per individual, suggesting that they belong to duplicated loci. RUSS33 amplified three to six fragments per individual with well-defined patterns. It can be explained by the amplification of a tandem mini-satellite repeat in a model of Fprimer-25bp-Fprimer-75bp-Fprimer-(18bp)n-Rprimer. RUSS47 (and RUSS45, not presented here) had clusters of fragments that differed by 8bp, with a cluster-to-cluster distance of 145bp: Fprimer-[(8bp)n]N-Rprimer-145bp-Rprimer-145-Rprimer. RUSS57 amplified one monomorphic locus at 152 bp and another polymorphic locus.

This is the first time that microsatellite markers have been developed for *S. solidissima*. Most of the markers developed here are highly polymorphic and in HWE. They should be useful in genetic studies on this important model and fishery species.

Acknowledgement

This study was supported by a Key Project for the International Science and Technology Cooperation (2004DFA07200), the National Natural Science Foundation of China (40676075), the National High Technology Research and Development Program of China (863 Program, 2006AA10A409) and the Natural Science Foundation of Hainan (80616, 80412). Yan Wang's visit to HSRL was supported by Rutgers University.

Table 1 Primer sequences and characteristics of 30 *Spisula solidissima* microsatellite markers. The number of individuals analysed (*n*), number of alleles (*a*), allele size range in base pairs (a_s), observed (H_O) and expected (H_E) heterozygosities are presented for each locus. T_a is the annealing temperature and P_{HWE} is the probability of Hardy–Weinberg equilibrium

Locus	GenBank Accession no.	Repeat motif§	Primer sequences (5′–3′)	T_a (°C)	*n*	*a*	a_s	H_E	H_O	P_{HWE}
RUSS02	EU446421	$(CCCG)_N$	F: caacagaaaccaaatgttgagc R: tcagattcctgtaaggacatgg	50	21	3	142–150	0.3298	0.1250	0.0239
RUSS03	EU446422	$(ACA)_n$	F: tcgtaacgcgtgaaatcaatag R: agtctacgcatctcgtcttgg	55	24	3	127–136	0.3697	0.4583	0.6350
RUSS05†	EU446424	$(GT)_N$	F: aaatatgggtcatcgggagtc R: catgccggacacagacatac	50	19	9	131–185	0.8635	0.2917	0.0000*
RUSS07	EU446425	$(TCGG)_N$	F: tcaatgaaacctgtggaaagc R: aaccattcggtctacctaccg	50	24	5	213–245	0.5576	0.5833	0.6573
RUSS08	EU446426	$(CCGT)_N$	F: gcaccctatgatgctatgacg R: ccaataaagcacaacggaatg	55	24	3	183–187	0.1968	0.1667	1.0000
RUSS10†	EU446428	$(CAGA)_N$	F: cactgaaatccaatcacaatgg R: aagtggggagacatattgttgtg	55	23	7	108–138	0.6676	0.3333	0.0107
RUSS11	EU446429	$(GTCT)_n$	F: tttgctgcgctgtgctatac R: ggactggggaaacacgataac	55	24	6	240–280	0.7243	0.8333	0.0424
RUSS13	EU446430	$(GACA)_N$	F: ccagtttttgcatacggacag R: ccaaggtgtgcttaaactaacaag	57	24	5	121–137	0.6871	0.6667	0.8678
RUSS14	EU446431	$(CAA)_N$	F: agtgcaagcaagtcaaactcc R: aatagccgccgttataccttc	50	24	7	220–250	0.6809	0.8333	0.3629
RUSS15	EU446432	$(CT)_N$	F: aacaccgagcctacttctgg R: ccgtaggctatttgcttgatg	58	22	5	187–207	0.6286	0.6667	0.3800
RUSS18†	EU446435	$(GTCC)_N$	F: caagacggttatgaaggttgc R: gacctttaagtgtgaccttgacc	58	20	10	263–323	0.9034	0.2083	0.0000*
RUSS20	EU446436	$(GACA)_N$	F: cggtggttaagggaaaaagtg R: gacaaggaaagaatccgttgg	50	20	3	302–314	0.4832	0.0833	1.0000
RUSS21	EU446437	$(AAACAC)_N$	F: atcatccctcttttccatcc R: ttaaagcaatatgccgtcagg	55	24	3	179–191	0.6489	0.4583	0.2027
RUSS23†	EU446439	$(CAGA)_N(AT)_n$	F: aacagtgtatgggtgctggtg R: tcagctgggaacattgtgatag	50	22	7	254–314	0.8289	0.2500	0.0000*
RUSS24	EU446440	$(TG)_N$	F: gttttggccctgtttttcag R: agcaagcaatttggagaagc	50	22	5	236–246	0.6614	0.5833	0.8519
RUSS26†	EU446441	$(AAC)_n$	F: gccaggaatggttcaaattc R: gacaatcaaagcatttaaagttgag	53	19	8	130–289	0.8750	0.3750	0.0000*
RUSS29	EU446443	$(GTCT)_N$	F: agccctttgattgttactgaac R: gatgtttgatgaggacatacagg	55	24	3	249–257	0.2314	0.1250	0.1965
RUSS33‡	EU446445	$(GTT)_n$	F: tgggattgttgtagtggttgg R: ctttaacagcacccggttttc	55	23	4	226–265	0.3076	0.1250	0.0520
RUSS34	EU446446	$(CA)_N$	F: tcaggttaatatcaccgcatc R: cgacacgtctgatgaattgac	55	20	8	185–205	0.8289	0.8750	0.0120
RUSS35	EU446447	$(TGT)_N$	F: aagtgtaatgttgcccgtcag R: gacaccctcgaaaagtaacacc	55	24	6	222–252	0.4858	0.3333	0.0125
RUSS40	EU446451	$(AACAG)_N(CA)_N$	F: caacagttttgaatttttaaatgg R: ccccaatatgcagttctttg	55	23	10	112–229	0.7340	0.5000	0.1518
RUSS44	EU446452	$(GTT)_N$	F: cgataacatggtaaggaattttgg R: tcaagcagaaagtggattgttg	53	23	6	189–219	0.4903	0.5000	1.0000
RUSS46	EU446453	$(GT)_N$	F: cgacaattttgtttgatgacg R: taagcaattaaaaatagccaagc	60	24	10	190–220	0.7952	0.6667	0.0729
RUSS47†‡	EU446454	$(GGTCTGTT)_N$	F: caactaaagagtgcgggacag R: cgcactactaggcgttatgttg	55	21	4	275–299	0.7899	0.2500	0.0152
RUSS48†	EU446455	$(GATA)_N$	F: tgcgcacctgttgataatacc R: tcgacgggtagatatgacgac	55	24	16	104–186	0.9238	0.5417	0.0006*
RUSS51†	EU446456	$(CGCA)_n$	F: ccacgcacaagtgactaacc R: cgttatgcgacgatatttctg	53	21	11	334–394	0.8954	0.4583	0.0104
RUSS53†	EU446457	$(GAAGAC)_N(GAA)_N$	F: tcgatgctctaaaggaagacg R: tgatcacattgtgttgttggtg	55	23	15	143–203	0.9202	0.6250	0.0063

Table 1 *Continued*

Locus	GenBank Accession no.	Repeat motif§	Primer sequences (5′–3′)	T_a (°C)	n	a	a_s	H_E	H_O	P_{HWE}
RUSS54	EU446458	$(ACA)_N$	F: cctaattacagtaacgtgcacagg R: ttgtcggtgatgtagttgtgg	55	24	3	316–322	0.1942	0.2083	1.0000
RUSS57‡	EU446460	$(GAA)_N$	F: gttctatcatttgagacactgtgc R: aggaggccaaactgtgagac	57	24	15	190–247	0.9220	0.8750	0.1138
RUSS59†	EU446461	$(ACA)_N$	F: gttggctgcttaaaacaggtg R: tgaactacagtcagtgagtccatc	55	20	4	134–155	0.6959	0.1667	0.0003*

§N, pure; n, interrupted.
*Significant deviation from HWE ($P < 0.05$) after Bonferroni correction.
†Loci showing evidence for null alleles.
‡Loci amplified more than two bands per individual.

References

Excoffier L, Laval G, Schneider S (2005) ARLEQUIN ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47–50.

van Oosterhout C, Hutchinson WF, Wills DPM *et al.* (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology*, **18**, 233–234.

Tong G, Kuang Y, Yin J, Liang L, Sun X (2006) Isolation of microsatellite DNA and analysis on genetic diversity of endangered fish, *Hucho taimen* (Pallas). *Molecular Ecology Notes*, **6**, 1099–1101.

doi: 10.1111/j.1755-0998.2009.02660.x

Eleven new microsatellite loci for the tiger rattlesnake (*Crotalus tigris*)

A. MUNGUIA-VEGA, K. PELZ-SERRANO, M. GOODE and M. CULVER
Conservation Genetics Laboratory, Arizona Cooperative Fish & Wildlife Research Unit (USGS), School of Natural Resources, The University of Arizona, Tucson, AZ 85721, USA

Abstract

Eleven microsatellite loci were isolated from an enriched genomic library from the tiger rattlesnake *Crotalus tigris*. Average observed heterozygosities in two populations were 0.456 and 0.427, respectively, and mean number of alleles were 7.54 (range 2–14) and 4.72 (range 2–13) respectively. No evidence of linkage disequilibrium was found across pairs of loci. The markers will be used in a long-term study examining the potential effects of urbanization on population dynamics and connectivity of this species in the mountain ranges surrounding Tucson, Arizona.

Keywords: microsatellites, rattlesnake, urban development, Viperidae

Received 17 January 2009; revision accepted 9 February 2009

The tiger rattlesnake *Crotalus tigris* (Viperidae) is a medium-sized, ground-dwelling snake found primarily on rocky slopes or in washes within rocky mountains and foothills. The species ranges from central Arizona south to southern Sonora, Mexico, including Isla Tiburon in the Gulf of California (Grismer 2002). We developed 11 novel microsatellite loci for *C. tigris*, which along with six markers previously described for the species (Goldberg *et al.* 2003), will be used to infer patterns of fine-scale genetic structure,

Correspondence: Adrian Munguia-Vega, Fax: (520) 621 8801;
E-mail: airdrian@email.arizona.edu