PRIMER NOTE

Development and characterization of microsatellite markers for the endangered northern riffleshell mussel *Epioblasma torulosa rangiana* (Bivalvia: Unionidae)

DAVID T. ZANATTA and ROBERT W. MURPHY
Royal Ontario Museum, Department of Natural History, 100 Queen's Park, Toronto, Ontario M5S2C6, Canada

Abstract

Primers for six polymorphic microsatellite loci were developed for the endangered northern riffleshell *Epioblasma torulosa rangiana* from the Sydenham River, Ontario, Canada. These loci along with an additional nine microsatellite loci, developed from other unionoid species, available in the literature, were tested and characterized on individuals from two populations in the Allegheny River; one population in French Creek, PA, USA, and one population from the Sydenham River. Allelic diversity ranged from two to 28 alleles per locus and averaged 13.7 per locus. These primers are being used in a rangewide study on the conservation genetics of remaining extant populations of the northern riffleshell.

Keywords: bivalves, DNA, Epioblasma, freshwater mussel, microsatellites, Unionoida

Received 29 January 2006; revision accepted 17 February 2006

Unionid freshwater mussels are considered to be one of the most endangered groups of animals (Lydeard *et al*. 2004). The northern riffleshell (NRS, *Epioblasma torulosa rangiana*) was listed as endangered in the USA in 1993 and in Canada in 2000. Conservation and recovery efforts will require reintroductions, and population augmentations in several watersheds as this species has been lost from the majority of its range in the Great Lakes and Ohio River drainages (USFWS 1994; Staton *et al*. 1998). To this end, culturing and propagation techniques are being developed for this species in order to restore it to its former range. However, recovery activities for this and many other species will require genetic analysis of remaining populations to help manage recovery efforts.

Mantle tissue (~30 mg) was nondestructively excised following protocols by Berg *et al*. (1995) from 86 specimens of NRS collected from four localities: 23 individuals from the Allegheny River at West Hickory, Forest County, PA, USA; 23 individuals from the Allegheny River at Hunter Station, Forest County, PA, USA; 20 individuals from French Creek, Venango County, PA, USA; and 20 individuals from the Sydenham River near Florence, Chatham-Kent Region, Ontario, Canada.

Correspondence: David T. Zanatta, Fax: 416-586-5553; E-mail: dave.zanatta@utoronto.ca

Several tissue samples from the Sydenham River were sent to BC Research (www.vizonscitec.com) to develop a microsatellite DNA library using a modified version of the magnetic bead capture technique (Kandpal *et al*. 1994). Repeat motifs used for library enrichment were TG_{12} and TC_{12}. A microsatellite-enriched library of 200 clones was received from BC Research. All 200 clones were successfully amplified via polymerase chain reaction (PCR) using M13 universal primers. The 200 amplified clones were sequenced in the forward and reverse directions on an ABI 377 in order to develop primers. Forty-one of the sequenced clones were found to have sufficient flanking regions to have primers developed from them using PRIMER 3 online software (Rozen & Skaletsky 2000).

Total genomic DNA was extracted from ~15 mg of frozen or 70% ethanol preserved mantle tissue samples by standard phenol extraction (Hillis *et al*. 1996) or using the Purgene DNA extraction kit (Gentra Systems, www.gentra.com). Forward PCR primers were ordered with an M13 tail on the 5′ end following the protocol of Schuelke (2000). PCRs were done in a 25-μL solution containing 1.0 μL of genomic DNA, 8 pmol of each the forward primer with M13 tail,

© 2006 The Authors
Journal Compilation © 2006 Blackwell Publishing Ltd

© 2006 The Authors
Journal Compilation © 2006 Blackwell Publishing Ltd

Table 1 Characteristics of 15 polymorphic microsatellite DNA loci from the endangered northern riffleshell (*Epioblasma torulosa rangiana*) developed using DNA from *E. t. rangiana* (this study), *Lampsilis abrupta* (Eackles & King 2002) and *Epioblasma capsaeformis* (Jones *et al*. 2004). The size range of alleles per locus, number of alleles per locus, observed heterozygosity (H_O) and expected heterozygosity (H_E) represent combined data and analysis from populations of *E. t. rangiana* at Allegheny River, Hunter Station, PA; Allegheny River, West Hickory, PA; French Creek, PA; and Sydenham River, ON. Bold *P* values are significant after Bonferroni adjustment for multiple comparisons

Locus	Source	GenBank Accession no.	Primer sequence (5′–3′)	Melting temp. (°C)	Repeat motif	Size range (bp)	No. of Alleles	H_O	H_E	HWE *P* value
Etr90	This study	DQ396403	F: TGTAAAACGACGGCCAGTCCATTCTATAAATTTTTCCACCA	68.7	$(TC)_5AC(TC)_7$	185–238	28	0.89	0.93	$P = 0.011$
$n = 85$			R: CGGACTAGTTTCCCGATCCT	62.5	$(AC)_{14}(ATC)_3$					
Etr114	This study	DQ396404	F: TGTAAAACGACGGCCAGTCCAGTCTTTTCCCAATTGCT	71.2	$(CT)_{15}C(CT)_{34}$	137–181	19	0.78	0.91	$P = 0.158$
$n = 77$			R: GGCAAAACATCATACTCTGCAA	58.9						
Etr124	This study	DQ396405	F: TGTAAAACGACGGCCAGTTGTTTGTACGTTATAGTTGCACGA	70.6	$(CT)_{20}$	99–175	19	0.67	0.77	$P = 0.062$
$n = 84$			R: GGCATTGCACACAAAACAGT	58.4						
Etr140	This study	DQ396406	F: TGTAAAACGACGGCCAGTGCTTTGGAGCTAGTGCCTCT	73.3	$(AC)_{17}$	158–189	15	0.74	0.76	$P = 0.900$
$n = 86$			R: CAGCTCAAAGTAAAGGAATACGC	61.0						
Etr145	This study	DQ396407	F: TGTAAAACGACGGCCAGTAACTCGCTTTTAGTTTATGTGTCA	69.6	$(AG)_{20}$	218–250	12	0.59	0.84	$P < 0.001$
$n = 73$			R: CAAAGAAAGACTTGATGCTTGAAA	57.7						
Etr187	This study	DQ396408	F: TGTAAAACGACGGCCAGTCAGTAGCAACCAGAACTTTTATATCG	71.2	$(GA)_{26}$	168–212	23	0.73	0.92	***P* < 0.001**
$n = 86$			R: AACGTGGGTGTTTCTTCAGG	60.4						
LabC2	Eackles	AF512384	F: TGTAAAACGACGGCCAGTATGGACACCAGAAAGAAAAGG	71.0	$(ATC)_2$ …	157–161	3	0.37	0.41	$P = 0.449$
$n = 80$	& King (2002)		R: GAAGTCACAAGGTCAGGATCTC	62.7	$(ATC)_3$ …					
					$(TCA)_8(GCA)_7$					
LabC24	Eackles	AF512386	F: TGTAAAACGACGGCCAGTTGGACCTATTCTTGCTTGTTG	68.4	$(TGT)_5(TGC)_8$	167–171	2	0.10	0.09	$P = 1.000$
$n = 84$	& King (2002)		R: GTTCTTTCGCCTCCATGTATAG	58.9						
LabD111	Eackles	AF512395	F: TGTAAAACGACGGCCAGTTGCATCAACTCTATTCACAACC	70.9	$(ATCT)_{12}$ …	230–267	9	0.60	0.76	$P = 0.020$
$n = 85$	& King (2002)		R: CAATGATAATGTAAATGTAAGCCTATC	58.5	$(TGTC)_4$					
LabD206	Eackles	AF512395	F: TGTAAAACGACGGCCAGTAAGTGTAGAGGCAGAGAACTGAC	72.7	$(ATCT)_9$	205–253	11	0.68	0.83	***P* = 0.003**
$n = 81$	& King (2002)		R: TCACTGATACAGCATACATAATATAC	58.3						
LabD213	Eackles &	AF512398	F: TGTAAAACGACGGCCAGTATACACAGGGTGCTCTAAATGC	71.9	$(ATCT)_{17}$	130–209	18	0.84	0.91	$P = 0.018$
$n = 83$	King (2002)		R: TTGCCAAAACAACATAGTTCC	56.7						
Ecap4	Jones *et al.*	AY650392	F: TGTAAAACGACGGCCAGTATGCCCCAGTGCTAGACATT	72.2	$(CA)_{10}$	87–133	10	0.42	0.80	***P* < 0.001**
$n = 84$	(2004)		R: AGAACAAAACACCCGTGTCC	60.4						
Ecap6	Jones *et al.*	AY650394	F: TGTAAAACGACGGCCAGTGATTTTGATTTTACGCTCCTGG	70.9	$(GT)_{22}$	243–260	7	0.68	0.79	$P = 0.221$
$n = 85$	(2004)		R: GGTTAGTGTTAGGAGTGACCGG	64.5						
Ecap8	Jones *et al.*	AY650396	F: TGTAAAACGACGGCCAGTTGCAGACATCGTAGCGATATG	72.1	$(CA)_{15}$	149–179	11	0.60	0.74	***P* < 0.001**
$n = 86$	(2004)		R: ATTTCCAGTTGCAAGTCTCATT	57.1						
Ecap9	Jones *et al.*	AY650397	F: TGTAAAACGACGGCCAGTAAAAAGGTGTGGAGAGAGATGG	71.9	$(GT)_{18}$	131–179	19	0.53	0.91	***P* < 0.001**
$n = 81$	(2004)		R: CCACTCTGCAGATATCGTATCG	62.7						

reverse primer and a FAM- or HEX-labelled M13 primer, 0.3 μm dNTP, 1× PCR buffer (2.0 mm Tris-HCl, 10 mm KCl, 0.01 mm EDTA and 0.1 mm DTT), 2.0 μm $MgCl_2$ and 1.5 U *Taq* (Promega or Fisher). Each PCR run (94 °C for 2 min; 35 cycles at 94 °C for 40 s, 56.5 °C for 40 s, 72 °C for 1 min; a final extension step at 72 °C for 1 min and a hold at 4 °C — MJ Research, PTC-200 thermocycler) included a negative control. Double-stranded PCR products were visualized with a 1 KB+ ladder to estimate fragment length on a 1.0% agarose gel stained with ethidium bromide. Non-overlapping amplified microsatellite loci were multiplexed and genotyped with a ROX size standard using Applied Biosystems (ABI) 3100 automated sequencers and scored using ABI's GENESCAN software. GENESCAN was used to determine allele size, and GENPOP (Raymond & Rousset 1995) was used to determine heterozygosity values, perform chi-squared tests for Hardy–Weinberg equilibrium (HWE) and test for linkage disequilibria for each locus.

Of the clones to have primers developed, six were successfully amplified and found to be polymorphic. Amplification used primers from Eackles & King (2002) and Jones *et al*. (2004). Consistently successful and polymorphic PCR products were also recovered from LabC2, LabC24, LabD111, LabD206 and LabD213 developed from *Lampsilis abrupta* (Eackles & King 2002) and Ecap4, Ecap6 and Ecap8, Ecap9 were developed from *Epioblasma capsaeformis* (Jones *et al*. 2004). These loci are characterized in a combined analysis of all four populations in Table 1.

Allelic diversity ranged from two to 28 alleles per locus and averaged 13.7 alleles per locus. The average expected heterozygosity (H_E) per locus ranged from 0.09 to 0.93 and averaged 0.76. Significant deviations from HWE, after Bonferroni correction for multiple comparisons, showing deficiencies of heterozygotes, were observed at six of 15 loci (Table 1). No significant linkage disequilibria, after Bonferroni correction, were encountered when loci were pooled across all populations.

We have described the development and characteristics of six microsatellite primer pairs designed from the DNA of the endangered NRS *E. t. rangiana*. These primers, in conjunction with the primers developed by Eackles & King (2002) and Jones *et al*. (2004), are being used in a rangewide conservation genetic study of remaining extant populations of NRS.

Acknowledgements

This work was supported by grants from the Department of Fisheries and Oceans Canada Species at Risk Program, the Canadian Government's Interdepartmental Recovery Fund, and the National Science and Engineering Research Council (NSERC) of Canada. We wish to thank: J. Metcalfe-Smith, D. McGoldrick, N. Mandrak, R. Villella, T. Smith, R. Evans, R. Hoeh, B. Bowen, A. Lathrop, K. Choffe, and A. Ngo.

References

Berg DJ, Haag WR, Guttman SI, Sickel JB (1995) Mantle biopsy: a technique for nondestructive tissue-sampling of freshwater mussels. *Journal of the North American Benthological Society*, **14**, 577–581.

Eackles MS, King TL (2002) Isolation and characterization of microsatellite loci in *Lampsilis abrupta* (Bivalvia: Unionidae) and cross-species amplification within the genus. *Molecular Ecology Notes*, **2**, 559–562.

Hillis DM, Mable BK, Larson A, Davis SK, Zimmer EA (1996) Nucleic acids IV: sequencing and cloning. In: *Molecular Systematics* (eds Hillis DM, Moritz C, Mable BK), 2nd edn, pp. 321–381. Sinauer Associates, Sunderland, MA.

Jones JW, Culver M, David V *et al*. (2004) Development and characterization of microsatellite loci in the endangered oyster mussel *Epioblasma capsaeformis* (Bivalvia: Unionidae). *Molecular Ecology Notes*, **4**, 649–652.

Kandpal RP, Kandpal G, Weissman SM (1994) Construction of libraries enriched for sequence repeats and jumping clones, and hybridization selection for region specific markers. *Proceedings of the National Academy of Sciences, USA*, **91**, 88–92.

Lydeard C, Cowie RH, Ponder WF *et al*. (2004) The global decline of nonmarine mollusks. *Bioscience*, **54**, 321–330.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Rozen S, Skaletsky H (2000) PRIMER 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, NJ.

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

Staton SK, Metcalfe-Smith JL, West EL (1998) *Status of the Northern Riffleshell, Epioblasma torulosa rangiana, in Canada*, p. 32. Committee on the Status of Endangered Wildlife in Canada (COSEWIC), Ottawa, Canada.

USFWS (1994) *Clubshell* (Pleurobema clava) *and Northern Riffleshell* (Epioblasma torulosa rangiana) *Recovery Plan*. Hadley, Massachusetts, p. 68.

© 2006 The Authors
Journal Compilation © 2006 Blackwell Publishing Ltd