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PRIMER NOTE

Development and characterization of microsatellite loci in the endangered oyster mussel *Epioblasma capsaeformis* (Bivalvia: Unionidae)

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Abstract

Primers for 10 polymorphic microsatellite loci were developed and characterized for the endangered oyster mussel *Epioblasma capsaeformis* from the Clinch River, Tennessee. Microsatellite loci also were tested in four other populations or species. Amplification was successful for most loci in these closely related endangered species or populations; therefore, a high level of flanking sequence similarity was inferred for this group of species and populations. Allelic diversity ranged from nine to 20 alleles/locus, and averaged 13.6/locus. This study demonstrated the feasibility of using polymerase chain reaction (PCR) primers to amplify microsatellite loci across freshwater mussel species to conduct population genetics studies.

Keywords: DNA, *Epioblasma*, freshwater mussel, microsatellites

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North America contains the greatest diversity of freshwater mussels in the world, including nearly 300 species. However, the mollusk superfamily Unionoida is the most imperiled group of animals in the United States, with 213 species (72%) listed as endangered, threatened, or of special concern (Williams *et al*. 1993). Most of the endangerment is caused by habitat loss or destruction affecting the natural structure and function of free-flowing rivers. Without immediate efforts to recover imperiled species in U.S. watersheds, the extinction of additional species is likely. To address the threat of species losses, biologists have developed techniques to propagate and culture endangered freshwater mussels for release of juveniles into rivers to augment or restore populations. However, recovery activities of many species will require genetic analysis of source and recipient populations to help manage species recovery.

Samples of mantle tissue were collected from the following species and locations: (i) *Epioblasma capsaeformis* in the Clinch River, Hancock Co., TN, and Duck River, Maury, Co., TN; (ii) *Epioblasma florentina walkeri* in the upper Clinch River, Tazewell Co., VA, and Big South Fork Cumberland River, Scott County, TN; and (iii) *Epioblasma torulosa rangiana* from the Allegheny River, Venango County, PA. A small piece of mantle tissue (20–30 mg) was collected nonlethally from six to 20 live mussels from each population. Tissues were preserved in 95% ethanol and stored at –20 °C prior to DNA extraction. Total genomic DNA was isolated from ~20 mg of fresh mantle tissue using the Purgene DNA extraction kit (Gentra Systems, Inc., Minneapolis, MN, USA). DNA concentration was determined by flourescence assay

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(Hoefer TKO 1000 Flourometer, Hoefer Scientific Instruments, San Fransisco, CA), and its quality was visually inspected in a 0.8% agarose gel.

Microsatellite loci were isolated using a modified nonradioactive capture-hybridization method (Refseth *et al*. 1997; Sarno *et al*. 2000). Polymerase chain reaction (PCR) amplification conditions followed those of Eackles & King (2002) and consisted of 100 ng of genomic DNA, 1× PCR buffer (Perkin Elmer), 2 mM $MgCl_2$, 250 μM dNTPs, 0.5 μM each primer, and 1.0 U Ampli*Taq* DNA polymerase [Perkin-Elmer Applied Biosystems (ABI)] in a total volume of 20 μL. PCR thermal cycling conditions were as follows: 94 °C for 2 min; followed by 35 cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 1 min; followed by a final extension at 72 °C for 1 min; and a hold at 4 °C (Eackles & King 2002). Microsatellite loci initially were examined for polymorphism using a 7% polyacrylamide silver stained gel. Exact allele size was determined by labelling primers of selected loci with fluorescent dye, followed by separation of dye-labelled PCR products on an Applied Biosystems (ABI) 3100 automated sequencer using a ROX 400 (Perkin Elmer) internal size standard. The GENOTYPER (ABI) software determined allele size, and POPGENE32 was used to determine heterozygosity values, perform Chi-square tests for Hardy–Weinberg equilibrium and test for linkage disequilibria for each locus.

Of 24 primer pairs tested, only 10 primer pairs amplified microsatellite DNA loci. The name of each locus, primer sequences, primer melting temperature, repeat motif of each locus, base pair size range of alleles/locus, number of alleles/locus, observed heterozygosity (H_O), and expected heterozygosity (H_E) for a combined analysis of all five populations are reported in Table 1. Allelic diversity ranged from nine to 20 alleles/locus, and averaged 13.6 alleles/locus, while average expected heterozygosity (H_E) per locus ranged from 0.78 to 0.92, and averaged 0.86. Cross amplification also was conducted separately for each population (Table 2).

Significant deviations from HWE ($\alpha = 0.05$), primarily showing deficiencies of heterozygotes, were observed at *Ecap1–9* (Table 1), and at various loci in all five populations (Table 2). Linkage disequilibria (LD) ($\alpha = 0.05$) were observed at 13 pairs of alleles in *E. capsaeformis* (CR); zero pairs in *E. capsaeformis* (DR); 1 in *E. f. walkeri* (BSF); 1 in *E. f. walkeri* (IC); and zero in *E. t. rangiana* (AR). Deviations from HWE and LD could be the result of null alleles, recent population bottlenecks or significant levels of close inbreeding, perhaps due to hermaphroditic reproduction, which is known to occur in populations of unionids (van der Schalie 1970).

We have described the development and characteristics of 10 microsatellite primer pairs designed from DNA of the endangered oyster mussel *Epioblasma capsaeformis*, collected

Table 1 Characteristics of 10 microsatellite DNA loci developed using DNA from the endangered oyster mussel (*Epioblasma capsaeformis*). The size range of alleles/locus, number of alleles/locus, observed heterozygosity (H_O), and expected heterozygosity (H_E) represent combined data and analysis from the following species and populations: 1) *E. capsaeformis*, Clinch River, TN; 2) *E. capsaeformis*, Duck River, TN; 3) *E. florentina walkeri*, Indian Creek, upper Clinch River, VA; 4) *E. florentina walkeri*, Big South Fork Cumberland River, TN; and 5) *E. torulosa rangiana*, Allegheny River, PA

Locus	Primer Sequence (5′–3′)	Melting Temp. °C	Repeat Motif	Size Range (bp)	No. of Alleles	H_O	H_E	HWE *P*-value	GenBank Accession Number
*Ecap*1	F: TGCATCATATGAAATGTGTTCG	59.4	$(AG)_{17}(GT)_{17}$	146–190	20	0.54	0.89	$P < 0.001$	AY650389
	R: TCAGCATATTTCAAAGCAAACA	58.5							
*Ecap*2	F: ATCCTCAGGTTGGTGGTCAG	60.0	$(GT)_{14}$	107–129	9	0.33	0.80	$P < 0.001$	AY650390
	R: TTTGAAAACCTTGTGATTGGC	60.0							
*Ecap*3	F: GGATGATGGGGAAAATAGATG	59.7	$(GT)_{15}$	236–286	7	0.46	0.90	$P < 0.001$	AY650391
	R: TGCAACATTACCTGCCTTCCA	60.3							
*Ecap*4	F: ATGCCCCAGTGCTAGACATT	60.1	$(CA)_{10}$	98–120	10	0.45	0.83	$P < 0.001$	AY650392
	R: AGAACAAAACACCCGTGTCC	59.9							
*Ecap*5	F: TTTGAACACATTCGCCTCAG	59.8	$(GT)_{29}$	176–224	20	0.55	0.92	$P < 0.001$	AY650393
	R: GAATTTGCCTCATCAGCCAC	60.6							
*Ecap*6	F: GATTTTGATTTTACGCTCCTGG	60.0	$(GT)_{22}$	186–240	13	0.31	0.78	$P < 0.001$	AY650394
	R: GGTTAGTGTTAGGAGTGACCGG	59.9							
*Ecap*7	F: ACGAAAAATGTTGTCATCCATT	58.4	$(CA)_{25}$	106–130	12	0.59	0.87	$P < 0.05$	AY650395
	R: GCCTAGACGACAAGCAAACC	59.9							
*Ecap*8	F: TGCAGACATCGTAGCGATATG	59.9	$(CA)_{15}$	127–159	11	0.35	0.88	$P < 0.001$	AY650396
	R: ATTTCCAGTTGCAAGTCTCATT	57.9							
*Ecap*9	F: AAAAAGGTGTGGAGAGAGATGG	59.6	$(GT)_{18}$	130–162	12	0.55	0.84	$P < 0.001$	AY650397
	R: CCACTCTGCAGATATCGTATCG	59.8							
*Ecap*10	F: ACACTGCAGACATCGTAGCG	60.1	$(AC)_{20}$	115–143	12	0.72	0.87	$P = 0.200$	AY650398
	R: TCACATACTTTGGGGACTTTCA	59.5							

Table 2 Cross amplification of the 10 microsatellite loci in five populations or species belonging to the genus *Epioblasma*. The river of occurrence of each population and number of individuals typed is shown in parenthesis. However, the actual number of individuals type varied slightly (± 1–2 individuals) for some of the examined loci. *** means no amplification product was obtained. Heterozygosity values in **bold** are not in Hardy–Weinberg equilibrium ($P < 0.05$). None of the products have been sequenced

	Epioblasma capsaeformis (Clinch) 20				*Epioblasma capsaeformis* (Duck) 12				*Epioblasma florentina walkeri* (upper Clinch) (8)				*Epioblasma florentina walkeri* (Cumberland) 14				*Epioblasma torulosa rangiana* (Allegheny) (6) H_E			
Locus	Size range	No. of alleles	H_O	H_E	Size range	No. of alleles	H_O	H_E	Size range	No. of alleles	H_O	H_E	Size range	No. of alleles	H_O	H_E	Size range	No of alleles	H_O	H_E
*Ecap*1	146–190	17	0.89	0.94	156–160	3	**0.42**	**0.52**	174–176	2	**0.00**	**0.40**	154–166	6	**0.69**	**0.82**	150–160	6	**0.17**	**0.89**
*Ecap*2	107–129	5	**0.20**	**0.68**	115–129	6	0.58	0.64	121–129	3	**0.50**	**0.59**	115–119	2	0.14	0.14	111–127	5	0.50	0.79
*Ecap*3	264–286	9	**0.50**	**0.89**	278–280	2	0.25	0.52	***			0	242–264	5	0.64	0.81	236–260	5	0.50	0.89
*Ecap*4	100–120	8	**0.42**	**0.74**	102–110	5	**0.50**	**0.70**	102–112	4	0.50	0.65	98–110	4	0.42	0.42	102–112	4	0.50	0.65
*Ecap*5	176–220	13	**0.73**	**0.90**	186–212	4	**0.25**	**0.64**	192–222	4	0.40	0.78	192–202	4	0.56	0.52	188–224	8	0.67	0.92
*Ecap*6	216–240	7	**0.47**	**0.76**	230–236	3	0.25	0.54	232–234	2	**0.00**	**0.36**	234	1	**0.00**	**0.00**	226–238	4	0.83	0.76
*Ecap*7	106–130	11	**0.75**	**0.86**	114–130	6	0.50	0.67	110–122	2	0.25	0.54	114–128	6	0.50	0.58	***	0		
*Ecap*8	127–155	7	0.47	0.77	131–159	5	**0.40**	**0.73**	133–141	2	0.33	0.30	137	1	**0.00**	**0.00**	131–149	2	0.40	0.53
*Ecap*9	134–162	10	0.89	0.88	136–148	5	0.80	0.81	136–138	2	0.25	0.23	136–138	2	**0.08**	**0.23**	130–150	5	0.40	0.84
*Ecap*10	115–143	10	1.00	0.92	123–131	2	0.29	0.26	123–131	4	1.00	0.87	133–137	3	0.83	0.71	119–125	2	0.33	0.33

from the Clinch River, TN. These primer pairs have been used in a taxonomic study of species belonging to the genus *Epioblasma*, and represent only the second set of microsatellite primers to be published for freshwater mussels (Jones 2004). The first set of microsatellite primers was developed by Eackles & King (2002) for the endangered pink mucket *Lampsilis abrupta*. These authors developed primer pairs to amplify 15 loci in *L. abrupta*. We recommend that both sets of microsatellite primers be further screened using DNA from additional species in the freshwater mussel subfamily Lampsilinae to determine the applicability of these DNA loci for intraspecific population genetic studies.

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