7-2004

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Journal of Heredity 2004:95(4):346–352
doi:10.1093/jhered/esh058

Microsatellite Marker Development and Analysis in the Eastern Oyster (*Crassostrea virginica*): Confirmation of Null Alleles and Non-Mendelian Segregation Ratios

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Eighteen microsatellite markers were developed for the *Crassostrea virginica* nuclear genome, including di-, tri-, and tetranucleotide microsatellite repeat regions that included perfect, imperfect, and compound repeat sequences. A reference panel with DNA from the parents and four progeny of 10 full-sib families was used for a preliminary confirmation of polymorphism at these loci and indications of null alleles. Null alleles were discovered at three loci; in two instances, primer redesign enabled their amplification. Two to five representative alleles from each locus were sequenced to ensure that the targeted loci were amplifying. The sequence analysis revealed not only variation in the number of simple sequence repeat units, but also polymorphisms in the microsatellite flanking regions. A total of 3626 bp of combined microsatellite flanking region from the 18 loci was examined, revealing indels as well as nucleotide site substitutions. Overall, 16 indels and 146 substitutions were found with an average of 4.5% polymorphism across all loci. Eight markers were tested on the parents and 39–61 progeny from each of four families for examination of allelic inheritance patterns and genotypic ratios. Twenty-six tests of segregation ratios revealed eight significant departures from expected Mendelian ratios, three of which remained significant after correction for multiple tests. Deviations were observed in both the directions of heterozygote excess and deficiency.

Eastern oyster (*Crassostrea virginica* [Gmelin 1791]) populations have been heavily impacted by the protistan parasites *Haplosporidium nelsoni* and *Perkinsus marinus* (Burreson and Ragone Calvo 1996). These parasites threaten both the establishment of aquaculture with *C. virginica* and the restoration of natural populations. A potential solution to the disease problem in *C. virginica* is development of genetically improved disease-resistant strains that can grow to market size despite challenge. Selective breeding may be accelerated by identifying genetic markers associated with traits such as disease resistance or growth rate. Such markers would allow marker-assisted selection for disease-resistant lines and identification of quantitative trait loci (QTL) via a genetic linkage map. In addition, these markers would provide a means of genetically tracking larval transport and monitoring genetic exchange among oyster populations in the natural environment.

Microsatellite and allozyme loci, expressed sequence tags (ESTs), randomly amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), and short interspersed nuclear elements (SINEs) have all been used as molecular markers for constructing genetic linkage maps in aquaculture species (Agresti et al. 2000; Kocher et al. 1998; Liu et al. 1999a,b,c; McGoldrick and Hedgecock 1997; McGoldrick et al. 2000; Moore et al. 1999; Young et al. 1998). Microsatellite markers—tandem arrays of short nucleotide repeats—have proven particularly useful for creating saturated linkage maps because they are often polymorphic and relatively easy to detect (Naciri et al. 1995; Sakamoto et al. 1999). In addition, they have been presumed to be dispersed fairly evenly throughout the genome, and selectively neutral, although some more recent studies bring these assertions into question (Li et al. 2002).

We have developed 18 microsatellite markers for *C. virginica* that were tested for polymorphisms on the parents and progeny of 10 single-pair matings. Eight of these loci were also examined for Mendelian inheritance in four of the

families by screening the parents and 39–61 adult progeny from each family. We looked at inheritance patterns for an indication of null (nonamplifying) alleles and tested for deviations from expected Mendelian ratios, phenomena reported in many other studies of bivalve molluscs, including *Ostrea* and *Crassostrea* oyster species, using a variety of molecular markers (Beaumont et al. 1983; Bierne et al. 1998; Gaffney and Scott 1984; Hu and Foltz 1996; Launey and Hedgecock 2001; Launey et al. 2002; McGoldrick and Hedgecock 1997; McGoldrick et al. 2000; Zouros and Foltz 1984).

Materials and Methods

A small-insert *C. virginica* genomic library was constructed by J. Pierce at the University of the Sciences at Philadelphia (Gaffney et al. 2003). Random clones from this library were sequenced at Dupont's Genome Sciences DNA Sequencing Facilities (Newark, DE), resulting in approximately 700,000 bp of single-pass sequence data. A total of 743 clone sequences were screened for microsatellite repeats by eye. Sequences containing simple sequence repeats were imported into MacVector 6.5.3 (Oxford Molecular, Princeton, NJ), and locus-specific polymerase chain reaction (PCR) primers were designed using the software's primer pair design function and purchased from either Invitrogen (Carlsbad, CA) or Sigma-Genosys (The Woodlands, TX).

Crassostrea virginica families (PG families) were produced by single-pair matings in 1996 at the Haskin Shellfish Research Laboratory at Rutgers University. DNA was extracted from ethanol-preserved tissue of parents and 50–65 adult progeny from 10 of these families, using a FastDNA kit (BIO 101, Vista, CA) on a FastPrep FP120 instrument (BIO 101, Vista, CA) following the manufacturer's protocol. DNA-containing supernatant was purified further by extraction with 500 µl of 1:1 phenol-chloroform, and precipitated with 1/10 volume 3 M sodium acetate and two volumes of ethanol. DNA was pelleted by centrifugation at 13,000 rpm for 20 min, washed with 70% ethanol, vacuum dried, and resuspended in 50 µl 0.1× TE (1 mM Tris-HCl pH 8.0, 0.1 mM EDTA).

The PCR amplifications were run in either a PTC-100 DNA thermal cycler (MJ Research Inc., Watertown, MA) or a TGradient thermal cycler (Biometra, Göttingen, Germany). Initial optimization reactions were carried out in a 25 µl volume containing approximately 25 ng of template DNA from reference animals, 2% bovine serum albumin (BSA) at 1 mg/ml, $MgCl_2$ at 1.5 mM or 2.0 mM, dNTPs at 200 µM, Tris-HCl pH 8.3 at 20 mM, KCl at 50 mM, primers at 1.0 µM, and 0.025 U of *Taq* polymerase. Initial denaturation for 1 min at 95°C was followed first by 40 cycles of denaturation at 95°C for 30 s, annealing at 46°C–53°C for 1 min, and extension at 72°C for 2 min, then by a final extension at 72°C for 10 min. Products were electrophoresed on a 2.0% agarose gel (in 1× Tris-boric acid-EDTA [TBE; 0.09 M Tris, 0.09 M boric acid, 20 mM EDTA at pH 8.0]) containing 0.5 µg/ml ethidium bromide and visualized by ultraviolet (UV) illumination to ensure that amplification was successful.

A reference panel that included DNA from parents and four progeny from the 10 PG families was initially screened for microsatellite polymorphisms and the presence of null alleles. Samples were amplified in 5 µl volumes using the reaction conditions above with optimized annealing temperatures and $MgCl_2$ concentrations (Table 1) and with reduced primer concentrations ranging from 50 to 200 nM. One primer was IR labeled (LI COR, Inc., Lincoln, NE) to allow for detection by the laser on an automated DNA sequencer (model 4200L, LI COR Inc., Lincoln, NE). Following amplification, 4 µl of formamide stop dye (95% deionized formamide, 0.08% bromophenol blue, 20 mM EDTA pH 8.0) was added to each reaction. Products (1 µl) were electrophoresed on a 7% polyacrylamide gel. ddGTP-labeled plasmid carrying an amplified fragment from the major histocompatibility complex of blue marlin (*Makaira nigricans*) was run as a size standard. Alleles were scored using RFLPScan (Scanalytics, Fairfax, VA).

Inheritance ratios were analyzed at eight microsatellite loci (*Cvi*1g3, *Cvi*2g14, *Cvi*2i4, *Cvi*2i23, *Cvi*2j10, *Cvi*2j24, *Cvi*2k14, and *Cvi*2m14) using DNA from parents and 39–61 progeny from each of four PG families. Amplification and scoring were as described above. Goodness-of-fit *G* tests with Bonferroni corrections (Rice 1989) were used to compare genotypic ratios in progeny to Mendelian expectations.

Two to five representative size-polymorphic alleles were subcloned and sequenced to determine whether size polymorphisms reflected microsatellite repeat number variation. Prior to cloning, PCR amplification products were electrophoresed as above on 2% agarose gels to ensure that products of the expected size had amplified. They were then cloned either directly using a TA Cloning kit (Invitrogen, Carlsbad, CA) or following excision from an agarose gel and purification using a Concert Gel Purification kit (Life Technologies, Gaithersburg, MD). Plasmids were extracted using a Qiagen Midi Plasmid Prep kit (Qiagen Inc., Santa Clara, CA) and digested with *Eco*RI to check for inserts, which were then sequenced by bidirectional cycle sequencing with a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, Cleveland, OH) and M13 IR-labeled forward and reverse primers (LI COR, Inc., Lincoln, NE). Sequencing reaction products were electrophoresed on a 7.0% polyacrylamide gel and detected on a LI COR 4200 automated sequencer.

Results

From the 743 genomic library clones examined, 49 primer pairs were designed to amplify microsatellite-containing regions of the genome. Eighteen markers were optimized and used to amplify microsatellite loci across a panel of four progeny and both parents from 10 reference families. Table 1 lists the repeat unit found in the genomic clone and the primer sequences that were used to PCR amplify the microsatellite loci. In those cases where the primers were redesigned to amplify a null allele, the redesigned primers are given. The optimal annealing temperatures and $MgCl_2$

Table 1. Microsatellite markers

Locus	Repeat sequence in genomic library clone	Primers (5′ to 3′)	T_A	$MgCl_2$ conc. (mM)	Observed allele size range (no. of alleles)
*Cvi*1a13	$(TTG)TTA(TTG)_6C(TTG)$	CGTATTAGAGTGTTCAAAGACTATC CTTCAGACCCTGGCATCC	51	2.0	218–227 (3)
*Cvi*1g3	$(TAA)_2TA(TAA)$	CATAAAGTTAATGCTTC ATAGCGAGTTGAGGAACC	51	2.0	215–239 (3)
*Cvi*1g4	$(ACCC)_4$	TCATAAACAATCAGTGACACAG GCAAAGTAAGGGGTAAGATG	50	2.0	298–318 (5)
*Cvi*1g8	$(CT)_{16}$	GCTACACACGAAAAATGGG TCAAATGAAGAGCACCTCC	46	2.0	249–289 (14)
*Cvi*1i24b	$(CA)_{10}$	TGACACCTCCCCACACTGTTG TCCTTAGAAAACCCATAGCAATGC	51.5	1.5	96–128 (8)
*Cvi*1j14	$(TTGA)_4$	TGTGTGGGTAACTGTCCATC CGAGATACTTTGTGCCAAG	48.5	1.5	168–172 (2)
*Cvi*2g14	$(TC)_4TT(TC)_{20}$	GTCCTAACTACATTTATCACAC TTCTTCTTACAAAACAGACTG	52	1.5	199–217 (6)
*Cvi*2i4	$(GATT)_{24}$	AATAATACAAAATCCAGTAGC CCAATCAAATCTCACTAAAG	46	2.0	327–403 (11)
*Cvi*2i20	$(CTGT)_3CCGT(CTGT)_4$ CCGT(CTGT)	CAGTATCAAGGTTTGGCTG CCCCAGTGGTTTTGGAGG	50	2.0	311–327 (3)
*Cvi*2i23	$(GTTT)_7$	TAACACAAAGCCAACATCGCC AAGTAAAAGACGGTCAAAGGGTCC	51.5	1.5	372–472 (10)
*Cvi*2j10	$(AT)_4AG(AT)_2$	CCCACACAGTTGCCACACAAAC CCACAATAGATTTCCATCCCTTCC	49	2.0	166–178 (4)
*Cvi*2j24	$(CAAT)_9$	CGTAGCCAGAAGGGGGGTTTC GCAGTGAGACACGATAAAAGAAGCC	53	1.5	379–399 (4)
*Cvi*2k14	$(CAG)_4$	CCAAAGAAGGTGGAGTATGCC GGATGATGAAATGGATGACGG	50	1.5	205–211 (3)
*Cvi*2m4	$(TA)_4$	ATGTTCAGTGGTGTCAGG AATCAGTGTGTAGGTCCG	51	2.0	245–253 (4)
*Cvi*2m6	$(CTC)_3$	TTCAATAAAAAACATAACAC GGGAGGGGGAAAAGGTGG	49	2.0	370–379 (3)
*Cvi*2m10	$(GA)_6GG(GA)_4$; $(GATA)_3G(GATA)_4AATA(GATA)_6$	CGAATCTGTGTTGATGAAAGG CCATGTCTCTCATCGTCAGTGCC	50	2.0	360–533 (8)
*Cvi*2m14	$(TG)_5$	TCAAGCTTGCATGTGC GAAGTTCCAATGAGAGGTAG	50	1.5	259–269 (3)
*Cvi*2n8	$(TATT)_3$	TCAGAAGTGAAAAAAGTGATTG CACAGAAATAGGAACTCAAAGC	48.5	1.5	255–303 (4)

Repeat sequences, primer sequences, and optimal annealing temperatures (T_A in °C), and $MgCl_2$ concentrations that were identified for 18 *C. virginica* microsatellite loci. The allele size and number of alleles that were observed at these loci in 10 *C. virginica* families are also given.

concentrations that were determined for amplification in our laboratory are also listed. The allele size ranges and number of alleles observed among the 10 PG families studied are given for each locus. Representative sequences for all 18 of the microsatellite regions were deposited into GenBank under the accession numbers AY644651–AY644668.

About 67% of the primers that were initially designed appeared to successfully amplify a single targeted locus when PCR products were analyzed by agarose-gel electrophoresis. However, when these PCR products were subsequently analyzed on polyacrylamide gels, approximately 50% were not scorable due to excessive "stutter," apparent amplification of multiple loci (more than two bands), and/or failure to amplify DNA from a large number of individuals, despite attempts at reoptimization. That is, only 37% of the primer pairs (18) were ultimately successfully employed.

Eight of the 18 functional PCR primer pairs were used for analysis of Mendelian ratios. One of the progeny in family PG1 and five individuals in family PG6 possessed alleles that were not seen in the parents. These individuals were most likely hatchery contaminants and were removed from the analyses.

Segregation of alleles at locus *Cvi*2m14 conformed to predicted inheritance patterns in two of the four families (families PG6 and PG10) (Table 2). However, segregation patterns in families PG1 and PG2 departed from Mendelian ratios and were consistent with the presence of null alleles. In family PG1, the dam appeared to be a heterozygote (A/B) and the sire a homozygote (B/B). In family PG2, the dam appeared to be a homozygote (B/B) and the sire a heterozygote (A/B) (Table 2). If these were the "true" genotypes of the parent, genotypes A/B and B/B in a 1:1

Table 2. Segregation analysis of microsatellite alleles in four *C. virginica* families

Locus	Dam	Sire	N[a]	Genotypes of progeny	Expected ratio	Observed ratio
Family PG1						
*Cvi*1g3	A/B	A/C[b]	51	A/C:A/A:B/C:A/B	1:1:1:1	13:18:5:15*[c]
*Cvi*2g14	A/B	C/D[b]	42	A/C:A/D:B/C:B/D	1:1:1:1	7:18:5:12
*Cvi*2i4	A/B	A/C	46	A/A:A/C:B/A:B/C	1:1:1:1	5:13:10:18*[c]
*Cvi*2i23	A/B	C/D	45	A/C:A/D:B/C:B/D	1:1:1:1	4:17:9:15*[c]
*Cvi*2j10	A/A	A/A	47	A/A	1	47
*Cvi*2j24	A/B	C/D	47	A/C:A/D:B/C:B/D	1:1:1:1	16:7:16:8
*Cvi*2k14	A/A	A/B	50	A/A:A/B	1:1	13:37**[c]
*Cvi*2m14	A/B	B/X[d]	50	A/B:A/X:B/B + B/X	1:1:2	9:6:35*[c]
Family PG2						
*Cvi*1g3	A/A	A/A	58	A/A	1	58
*Cvi*2g14	C/D[b]	A/E	61	C/A:C/E:D/A:D/E	1:1:1:1	12:14:20:15
*Cvi*2i4	C/D	E/F	55	C/E:C/F:D/E:D/F	1:1:1:1	7:19:19:10
*Cvi*2i23	A/C	E/F	55	A/E:A/F:C/E:C/F	1:1:1:1	18:9:9:19
*Cvi*2j10	A/A	A/A	53	A/A	1	53
*Cvi*2j24	C/D	A/C	57	C/A:C/C:D/A:D/C	1:1:1:1	15:12:11:19
*Cvi*2k14	A/A	A/B	61	A/A:A/B	1:1	32:29
*Cvi*2m14	B/X[d]	A/B	59	B/A:B/B + X/B:X/A	1:2:1	23:24:12
Family PG6						
*Cvi*1g3	A/B	A/A	56	A/A:B/A	1:1	35:21
*Cvi*2g14	B/B	A/B	51	B/A:B/B	1:1	28:23
*Cvi*2i4	G/H	G/H	53	G/G:G/H:H/H	1:2:1	24:3:26**[c]
*Cvi*2i23	C/G	A/H	55	C/A:C/H:G/A:G/H	1:1:1:1	17:12:15:11
*Cvi*2j10	A/B	A/A	51	A/A:B/A	1:1	19:32
*Cvi*2j24	C/C	A/D	54	C/A:C/D	1:1	32:22
*Cvi*2k14	A/B	B/B	56	A/B:B/B	1:1	21:35
*Cvi*2m14	A/B	B/C	57	A/B:A/C:B/B:B/C	1:1:1:1	30:24:3:0**[c]
Family PG10						
*Cvi*1g3	A/A	A/A	53	A/A	1	53
*Cvi*2g14	A/B	B/B	48	A/B:B/B	1:1	21:27
*Cvi*2i4	I/J	J/K	47	I/J:I/K:J/J:J/K	1:1:1:1	10:11:18:8
*Cvi*2i23	G/H	G/I	45	G/G:G/I:H/G:H/I	1:1:1:1	11:11:11:12
*Cvi*2j10	A/A	A/B	39	A/A:A/B	1:1	13:26*[c]
*Cvi*2j24	C/D	A/D	50	C/A:C/D:D/A:D/D	1:1:1:1	16:17:9:8
*Cvi*2k14	A/B	A/B	53	A/A:A/B:B/B	1:2:1	18:27:8
*Cvi*2m14	C/C	A/C	55	C/A:C/C	1:1	33:22

[a] N = number of progeny scored at each locus.

[b] Null alleles that were revealed after redesign of PCR primers.

[c] *Indicates nominal significant deviation from expected Mendelian ratio ($P < .05$); ** indicates significance after sequential Bonferroni correction.

[d] X indicates an inferred null allele.

ratio would be expected in each family. Progeny in both families, however, exhibited genotypes A/A, A/B, and B/B. The A/A genotype would not be expected in either cross, suggesting that a null allele occurred in the sire of PG1 and the dam of PG2 (Table 2). The inferred null alleles could not be amplified despite redesign of primers. Null alleles initially apparent at loci *Cvi*2g14 and *Cvi*1g3, on the other hand, were successfully amplified with redesigned primers, which are given in Table 1.

Eight of 28 segregations available for analysis (29%) exhibited distorted segregation ratios. Three of these were significant after Bonferroni correction for multiple tests. Locus *Cvi*2k14 in family PG1 had an excess of heterozygotes (A/B), while *Cvi*2i4 in PG6 was heterozygote deficient for the genotype G/H. Locus *Cvi*2m14 in family PG6 was deficient in one heterozygote (B/C) and the homozygote (B/B) genotype (Table 2).

Sequence analysis of two to five alleles at each of these 18 microsatellite loci confirmed that single targeted loci were amplified with each primer pair, and that the variation in allele size was due to varying numbers of microsatellite repeat sequences and/or flanking region indels. Substantial flanking region polymorphism was found at most loci in this study, even though only a few alleles from the families were sequenced and compared (Table 3). The flanking region sequence length ranged from 50 bp at *Cvi*1i24 to 327 bp at *Cvi*2m6 in the genomic library clones. Indels were observed in the flanking region of half the loci. A significant flanking region deletion of 20 bp was found in an allele of the *Cvi*1g3 locus, which resulted in a null allele. This allele was

Table 3. Flanking region polymorphisms identified at each locus by sequencing alleles of varying sizes

Locus (no. of alleles sequenced)	Flanking region length sequenced (bp)	Flanking region polymorphisms		
		No. of indels (length)	No. of substitutions	% polymorphism
*Cvi*1a13 (3)	200	0	1	0.1%
*Cvi*1g3 (4)	191	1 (20 bp)	6	3.7%
*Cvi*1g4 (3)	257	2 (1 of 2–5 bp; 1 of 1 bp)	22	9.3%
*Cvi*1g8 (3)	188	0	46	24.5%
*Cvi*1i24 (3)	50	0	1	2.0%
*Cvi*1j14 (4)	129	1 (13–16 bp)	4	3.9%
*Cvi*2g14 (4)	120	1 (1 bp)	6	5.8%
*Cvi*2i4 (3)	267	4 (3 of 2 bp; 1 of 1 bp)	6	3.7%
*Cvi*2i20 (2)	250	0	0	—
Cvi 2i23 (5)	327	1 (1 bp)	6	2.1%
*Cvi*2j10 (2)	118	0	2	1.7%
*Cvi*2j24 (3)	309	2 (2 of 1 bp)	12	4.5%
*Cvi*2k14 (2)	160	1 (3 bp)	0	0.6%
*Cvi*2m4 (3)	199	0	0	—
*Cvi*2m6 (2)	327	1 (1 bp)	16	5.2%
*Cvi*2m10 (4)	138	0	0	—
*Cvi*2m14 (3)	114	0	1	0.9%
*Cvi*2n8 (3)	282	3 (2 of 1 bp; 1 of 3 bp)	17	7.1%
Overall (56)	3626	17	146	4.5%

successfully amplified after redesign of one of the PCR primers to bind outside of the indel. No flanking region polymorphisms were observed among the alleles analyzed at *Cvi*2i20, *Cvi*2m4, or *Cvi*2m10. The proportion of polymorphic nucleotide positions ranged from 0.1–24.5% at variable loci, with overall 4.5% polymorphism observed at these loci using the conservative calculation that each deletion, regardless of length, was weighted equally with substitutions.

Discussion

A high attrition rate of potential microsatellite markers, almost 73% overall, was experienced from the primer design step following identification of a simple sequence repeat, through agarose gel analysis, to polyacrylamide gel optimization and final analysis. The relatively low success rate of primers may be due at least in part to high intraspecific polymorphism in the *C. virginica* genome, as was seen in the analysis of flanking region sequences in this study. In a similar vein, Cameron et al. (1999) attributed variation in amplification efficiency of sea urchin microsatellite loci to a high level of genomic polymorphism. Null alleles for single-copy, PCR-based DNA markers are often the result of polymorphisms in the microsatellite flanking regions of DNA to which PCR primers are designed to bind (Callen et al. 1993; Jones et al. 1998; McGoldrick et al. 2000). The resulting failure to amplify the target sequence can often be overcome by primer redesign. Polymorphisms at primer binding sites, such as those detected by sequence comparisons among a few alleles at each locus in this study, may have thus prevented amplification in some individuals and families. We detected null alleles with 3 of the 18 initially designed primer pairs. Null alleles at loci *Cvi*2g14 and *Cvi*1g3 were successfully amplified via primer redesign. An apparent null allele problem at a third locus, *Cvi*2m14, however, was not successfully resolved.

McGoldrick et al. (2000) proposed that in family analyses, null alleles can be treated as recessive alleles for mapping purposes. For a cross such as AX × AB, the genotypes AX and AA may be combined into one genotypic class (AA) and the expected genotypic ratio changed to 2 AA:1 AB:1 BB. While such an approach may reduce the utility for mapping of markers exhibiting null alleles, it would also eliminate the need to redesign primers or to discard those loci where nulls could not be resolved. In population studies, the presence of null alleles may complicate the interpretation of deviations from Hardy-Weinberg equilibrium, and multiple nulls could confuse analyses of population structure.

Many marine invertebrates, and particularly bivalve molluscs, frequently exhibit non-Mendelian segregation ratios of alleles, which can confound the creation of a linkage map (Brown et al. 2000; Buroker et al. 1975; Hare et al. 1996; Kocher et al. 1998; Launey and Hedgecock 2001; Naciri et al. 1995). Heterozygote deficiency has been well documented with respect to allozyme loci and single-copy nuclear DNA in several wild populations of marine species (Brown 1991; Foltz 1986a; Hare et al. 1996; Hedgecock et al. 1996; Karl and Avise 1993; Singh and Green 1984; Zouros et al. 1980). Explanations for heterozygote deficiencies for allozymes include selection (Beaumont et al. 1983; Singh and Green 1984; Zouros et al. 1980), epistasis (Hedgecock et al. 1996), null alleles (Foltz 1986a), and scoring errors (Buroker et al. 1975; Fairbairn and Roff 1980).

Unlike allozyme loci, which tend to show genotypic ratios distorted in the direction of heterozygote deficiency

(Foltz 1986b; Hare et al. 1996; Hedgecock et al. 1996; Singh and Green 1984), microsatellite loci reveal distortion in both directions: heterozygote deficiency and heterozygote excess (Bierne et al. 1998, 2000; Huang et al. 2000; Launey and Hedgecock 2001; McGoldrick et al. 2000). Departures from Mendelian ratios appear to result from two primary causes: null alleles and zygotic viability selection. McGoldrick et al. (2000) documented the frequent occurrence of null microsatellite alleles in the Pacific oyster. Naciri et al. (1995) suggested that high genetic load with resulting strong zygotic selection during the larval stage was the cause of segregation distortion in the flat oyster (*Ostrea edulis*), a phenomenon recently demonstrated experimentally in *Crassostrea gigas* families by Launey and Hedgecock (2001). By genotyping progeny at 6 h after fertilization and then 2–3 months later, they confirmed that segregation distortion was minimal at the early zygote stage and increased during development, supporting the theory that some of the microsatellite alleles were selected against because of their linkage to highly deleterious fitness gene alleles.

In our study, the Mendelian segregation ratios in adult progeny from single-pair matings also deviated from expected in the direction of both heterozygote excess and deficiency. Locus *Cvi*2k14 revealed significant heterozygote excess in family PG1, but did not show significant distortion in any other family. *Cvi*2i4 revealed significant heterozygote deficiencies in family PG6. *Cvi*2m14 revealed deficiencies in both a heterozygote and a homozygote genotype in family PG6. The incidence of segregation distortion that we observed was slightly lower than that reported in other bivalve species. Launey and Hedgecock (2001) reported that 36% of analyses in *C. gigas* exhibited non-Mendelian segregation ratios before Bonferroni correction, with 21% significant after correction. Likewise, McGoldrick et al. (2000) reported that 37% of analyses deviated from expected ratios in *C. gigas* once null alleles were taken into account, and Naciri et al. (1995) reported that approximately 20% of segregations were distorted in *O. edulis*. With these *C. virginica* microsatellite markers we found approximately 29% of segregations distorted before Bonferroni correction, but only 11% significant after correction.

These microsatellite markers should prove to be a useful addition to the collection of other molecular markers that are now available for *C. virginica*, including allozymes [see Gaffney (1996) for a review], mitochondrial markers (Reeb and Avise 1990; Wakefield and Gaffney 1996), single-copy nuclear markers (Hare et al. 1996; Karl and Avise 1992), AFLPs (Yu and Guo 2003), and other microsatellites (Brown et al. 2000). Currently several of the markers in this study are being used for mapping, to characterize *C. virginica* populations in the Chesapeake Bay and to genetically track the survival and spread of hatchery stocks used in restoration efforts.

Acknowledgments

The authors thank Karen Hudson, Elizabeth Walker, and the VIMS/ABC hatchery personnel for technical support and Dr. Cheryl Morrison and Nancy Stokes for helpful suggestions.

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Received August 10, 2003
Accepted March 18, 2004

Corresponding Editor: Brian Bowen