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J Carlsson
Virginia Institute of Marine Science

CL Morrison

Kimberly S. Reece
Virginia Institute of Marine Science

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Wild and Aquaculture Populations of the Eastern Oyster Compared Using Microsatellites

Jens Carlsson, Cheryl L. Morrison, and Kimberly S. Reece

From the School of Marine Science, Virginia Institute of Marine Science, College of William and Mary, PO Box 1346, Gloucester Point, VA 23062-1346 (Carlsson, Morrison, and Reece); and the US Geological Survey-BRD, Aquatic Ecology Barnach, 11649 Leetown Road, Kearneysville, WV 25430 (Morrison).

Address correspondence to J. Carlsson at the address above, or e-mail: jc@vims.edu.

Five new microsatellite markers were developed for the eastern oyster (*Crassostrea virginica*), and allelic variability was compared between a wild Chesapeake Bay population (James River) and a hatchery strain (DEBY^{TM}). All loci amplified readily and demonstrated allelic variability with the number of alleles ranging from 16 to 36 in the wild population and from 11 to 19 in the DEBY^{TM} strain. Average observed and expected heterozygosities were estimated at 0.66 and 0.80 in the hatchery sample. The corresponding estimates were 0.91 and 0.75 in the wild sample. Results indicated lower genetic variability in the DEBY^{TM} strain and significant genetic differentiation between the wild population and hatchery strain. These microsatellite loci will prove valuable for future population genetic studies and in tracking of hatchery strains used in restoration.

Historically, the primary commercial oyster species of the eastern United States, *Crassostrea virginica* (Gmelin 1791), supported an extensive fishery in Chesapeake Bay. Overfishing, loss of habitat, pollution and diseases, however, have depleted present populations to a fraction of historical sizes (e.g., Burreson and Andrews 1988; Rothschild et al. 1994). The decline has prompted several restoration strategies including construction of artificial reefs followed by stocking with various oyster strains. Due to high pathogen pressure, the disease-tolerant DEBY^{TM} strain (Burreson 1991; Ragone Calvo et al. 2003) has been used for stocking reefs in the Virginia portion of Chesapeake Bay. Recently developed population genetic statistical methods such as assignment tests (reviewed by Hansen et al. 2001) can determine the population of origin for single or groups of individuals and could potentially be used to examine the breeding success of deployed oysters. These tests, however, require genetic differentiation among potential source populations that often only can be discerned by using highly variable molecular markers, such as microsatellites. Many microsatellite markers may need to be developed and screened in order to identify a suit of loci that are most powerful and efficient for population genetic analyses. Toward this effort, we have developed 5 microsatellite loci and tested them on a wild population and a hatchery strain of *C. virginica.*

Materials and Methods

Primer Development

Microsatellite loci *Cvi8VIMS* and *Cvi12VIMS* were identified from a *C. virginica* genomic library as previously described (Reece et al. 2004). Loci *Cvi4VIMS*, *Cvi5VIMS*, and *Cvi18VIMS* were isolated from *C. virginica* genomic DNA following a modified (McDowell et al. 2002) method of Hamilton et al. (1999). Primers (Table 1) were designed with PRIMER3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). A T3 tail (AATTAACCCTCACTAAAGGG) was added to the 5′ end of the forward primers. DNA was extracted from 2 collections of *C. virginica* (Table 2) by using the Dneasy Tissue Kit (Qiagen Inc., Santa Clara, CA). Polymerase chain reaction (PCR) amplifications (5 μl) contained 5–50 ng template DNA and reagents (Invitrogen, Carlsbad, CA) as follows: 0.5 μl 10× buffer, 1.0–2.0 mM $MgCl_2$, 1 μl of 2% bovine serum albumin, 0.2 mM deoxynucleoside triphosphate mix, 0.025 pmol of forward primer, 0.1 pmol of reverse primer, 0.025 units of *Taq* polymerase, and 0.1 pmol fluorescently labeled T3 primer (source indicated in Table 1). Cycling parameters were 94 °C for 3 min followed by 35 cycles at 94 °C for 1 min, annealing (temperatures indicated in Table 1) for 1 min, 72 °C for 1 min, with a final extension at 72 °C for 7 min. Amplified products where run with a size standard on an ABI 3100 Prism Genetic Analyzer (Applied Biosystems, Forest City, CA) according to the manufacturer's recommendations. Allele scoring was performed with GENEMARKER (SoftGenetics, State College, PA). Optimization of annealing temperatures and $MgCl_2$ concentrations was done by performing gradient PCRs on a set of 8 individuals (24 different temperatures ranging from 45.0 to 65.0 °C) with 1.0, 1.5, and 2.0 mM $MgCl_2$. The annealing

Table 1. PCR amplification parameters, repeat structure, and fluorescent label used for *Crassostrea virginica* microsatellite loci

Locus	Repeat structure	Primer sequence (5′–3′) (F = forward, R = reverse)	$MgCl_2$ (mM)	T_a (°C)	T3 primer fluorescent label
Cvi4VIMS	$(GATT)_9(GATA)_{18}$	F: AAGTCACAATCCACTACAAG R: CTTCCATTCTTTTTTCAAC	1.5	55.0	NED[a]
Cvi5VIMS	$(CT)_{19}$	F: ATAAAAGTCCATTCGTAAGC R: AGATTTGAAGTATTGCTATCG	1.5	47.0	PET[a]
Cvi8VIMS	$(GT)_9$	F: AGAGGTCCATGAGCCACATC R: TTGCAGCATCCTCAGGACTA	1.5	59.0	VIC[a]
Cvi12VIMS	$(CT)_3CCCA(CT)_{24}$	F: CACGCTGGCTTTTTCTGTAA R: CTGCTGATTATGTGCTGTCAGA	1.5	55.0	6-FAM[b]
Cvi18VIMS	$(CT)_{15}$	F: CAAACTGAAACATCCCTAAC R: TTACAAATGGCGAAACG	2.0	47.0	6-FAM[b]

T_a = annealing temperature; Genbank accession numbers: DQ205719–DQ205723.

[a] Source = ABI.

[b] Source = Invitrogen.

temperature and $MgCl_2$ concentration that yielded the clearest amplification and that allowed for unambiguous allele scoring was selected and used for amplifying the remaining individuals.

Sample Comparisons

Oysters were collected from the Horsehead Bar in the lower James River (VA) in 2004, and the sample of hatchery-reared, disease-resistance selected line of DEBY™ oysters were obtained in 2002. The James River has historically produced extensive numbers of seed oysters and is still regarded as one of the most productive oyster habitats in Virginia (cf. MacKenzie 1996). The Horsehead Bar sample should represent a natural condition, uncontaminated by transplantation or stocking. Although numerous oysters have been transplanted from the James River, there are no records of oysters being transplanted to the James River (cf. MacKenzie 1996). The DEBY™ oysters originate from Delaware Bay wild oysters and were brought to the hatchery at Virginia Institute of Marine Science in 1987, where a selective breeding program was initiated that continues to this date (Burreson 1991). The main objective was to create disease-resistant (particularly against Dermo and MSX) brood stock for aquaculture (Burreson 1991; Ragone Calvo et al. 2003).

Samples of 96 wild *C. virginica* from the James River and 96 hatchery-bred DEBY™ strain oysters (F_5 generation) were screened for variation at these 5 microsatellite loci. Approximately 20% of all individuals were rerun to ensure repeatability of allele scoring. Samples failing to amplify the first time were reamplified once. The MICRO-CHECKER 2.2.1 software (van Oosterhout et al. 2004) was used for identifying possible genotyping errors (i.e., stuttering, large allele dropout, and null alleles, 1000 randomizations).

Table 2. Summary statistics for 5 microsatellite loci among eastern oyster collections. Single-locus F_{ST}, number of individuals (*n*), number of alleles (*a*), allele richness per locus (*Rs*), allele size range in base pairs (*as*), expected heterozygosity (H_E), observed heterozygosity (H_O), and probability values of concordance with HWE are given. Values in bold represent significant probability estimates after correction for multiple tests (initial $\alpha = 0.05/5 = 0.01$)

Strain		Locus					
		Cvi4VIMS	*Cvi5VIMS*	*Cvi8VIMS*	*Cvi12VIMS*	*Cvi18VIMS*	Average across loci
	F_{ST}	0.056	0.114	0.035	0.031	0.145	0.076
DEBY™	*n*	90	92	92	84	92	90
	a	16	11	12	19	13	14.2
	Rs	15.7	10.8	11.9	19.0	12.9	14.1
	as	235–347	144–174	145–183	133–185	118–146	
	H_E	0.87	0.74	0.84	0.89	0.67	0.80
	H_O	0.48	0.85	0.60	0.98	0.39	0.66
	HWE	**0.000**	0.069	**0.000**	0.407	**0.000**	
	F_{IS}	0.49	−0.07	0.25	−0.06	0.42	0.21
Wild	*n*	90	92	92	92	92	91.6
	a	36	19	16	32	22	25.0
	Rs	36.3	16.8	15.4	31.5	21.6	24.3
	as	223–365	138–176	141–183	121–191	108–154	
	H_E	0.96	0.91	0.82	0.95	0.93	0.91
	H_O	0.46	0.92	0.65	0.92	0.78	0.75
	F_{IS}	0.53	−0.01	0.21	0.03	0.16	0.19
	HWE	**0.000**	0.150	**0.000**	0.221	0.011	

GENEPOP 3.1b (Raymond and Rousset 1995) was used to identify deviations from Hardy–Weinberg equilibrium, HWE (exact tests, 1000 iterations), observed and expected heterozygosities (indicating an excess or deficiency of heterozygotes), and genotypic disequilibrium (1000 iterations). FSTAT 2.9.3.2 (Goudet 1995) was used to calculate allelic richness and F_{IS} (Weir and Cockerham 1984) per locus and sample. The ARLEQUIN 3.0 software (Excoffier et al. 2005) was used to calculate single-locus F_{ST} and global multilocus F_{ST} values (10 100 permutations) (Weir and Cockerham 1984). Significance levels were adjusted for multiple tests using the sequential Bonferroni correction technique (Rice 1989).

Results and Discussion

Number of alleles varied from 11 at locus *Cvi5VIMS* to 36 at locus *Cvi4VIMS* (Table 2). Significantly fewer alleles were found in the DEBY™ strain compared with the wild sample (Kruskal–Wallis test, $P = 0.027$). Overall allelic richness varied from 10.8 to 36.3 (Table 2), with the wild population showing significantly higher allelic richness than the DEBY™ strain (Kruskal–Wallis test, $P = 0.047$).

The MICRO-CHECKER analysis indicated that the loci *Cvi4VIMS* and *Cvi8VIMS* might be influenced by one or more null alleles in both the wild and hatchery samples and that locus *Cvi18VIMS* in the hatchery sample could be affected by null alleles. This observation indicates that using *Cvi4VIMS* and *Cvi8VIMS* for population genetic analyses that assume HWE may prove to be problematic. Hence, we estimated global multilocus F_{ST} both with and without these 2 loci. With *Cvi18VIMS*, on the other hand, there were indications of null alleles only in the hatchery sample and, therefore, this locus was included in all further analyses.

There were no indications that genotyping errors affected allele scoring (e.g., allele dropouts or stuttering) at any of the markers in any of the samples. Samples that failed to amplify were rare (cf. Table 2) and indicate that null homozygotes were not common. In addition, no sample failed to amplify at more than one locus (data not shown), and this makes it unlikely that poor DNA quality affected our results. Observed heterozygosity ranged from 0.39 at locus *Cvi18VIMS* to 0.98 at locus *Cvi12VIMS*, whereas expected heterozygosity varied from 0.67 at *Cvi18VIMS* to 0.96 at locus *Cvi4VIMS* (Table 2). Inbreeding coefficients (F_{IS}) varied among markers from −0.06 (*Cvi12VIMS*) to 0.49 (*Cvi4VIMS*) in the hatchery strain and from −0.01 (*Cvi5VIMS*) to 0.529 (*Cvi4VIMS*) in the wild sample (cf. Table 2). Average F_{IS} including all markers was 0.21 in the hatchery line and 0.19 in the wild sample. The average F_{IS} when excluding the *Cvi4VIMS* and *Cvi8VIMS* markers was considerably lower and estimated to be 0.10 in the hatchery strain and 0.06 in the wild sample.

Cvi5VIMS and *Cvi4VIMS* showed significant genotypic disequilibrium in the hatchery strain, even after sequential Bonferroni correction for multiple tests (data not shown). Significant departures from HWE were found at *Cvi18VIMS* in the DEBY™ strain and at *Cvi4VIMS* and *Cvi8VIMS* in both samples (Table 2). Corresponding heterozygote deficiency was significant in both the wild and hatchery collection for markers *Cvi4VIMS* ($P < 0.001$) and *Cvi8VIMS* ($P < 0.001$). *Cvi18VIMS* ($P = 0.003$) showed heterozygote deficiency only in the hatchery collection.

Previously, many microsatellites markers developed for *C. virginica* have deviated from HWE usually in the direction of deficiencies of heterozygotes (Reece et al. 2004). General causes for such deviations include substructuring of the population sample, inbreeding, or the presence of null alleles (Zouros and Foltz 1984); however, from results of previous studies on *Crassostrea* spp., null alleles seem to be a likely explanation (McGoldrick et al. 2000; Reece et al. 2004). Of note, such deviations appear to be quite common in microsatellite markers for bivalves (e.g., Eackles and King 2002). High frequency of null alleles may complicate many types of population genetic analyses that rely on HWE, as false homozygotes would be common (e.g., Pemberton et al. 1995; de Sousa et al. 2005). There are available methods to correct allele frequencies for null alleles (cf. Brookfield 1996; Summers and Amos 1997), although using loci with low frequency or absence of null alleles is less complex and preferred.

Significant single-locus F_{ST} estimates between samples were detected at all markers ($P < 0.001$, Table 2), and the global multilocus F_{ST} including all loci was estimated to be 0.076 ($P < 0.001$), and when excluding *Cvi4VIMS* and *Cvi8VIMS* (due to the high likelihood of null alleles), it was estimated at 0.095 ($P < 0.001$). The significant F_{ST} estimates indicate genetic differentiation between the DEBY™ strain and wild sample. Lower allele richness observed in the DEBY™ strain, as compared with the wild sample, may be caused by hatchery selection and inbreeding (cf. Table 2). Continued hatchery amplification of the DEBY™ strain might lead to decreased genetic variability if the number of effective breeders is not maintained. Hence, continued genetic monitoring of the strain is warranted.

There are several studies on aquatic organisms, especially salmonids, which argue that hatchery-bred strains have reduced fitness when exposed to natural environments, as compared with their wild counterparts (see Hansen 2002 and references therein). If reduced microsatellite variability also correlates to reduced variability at genes under selection, the use of genetically depauperate hatchery strains for restoration efforts may be unwise as this reduction in genetic variation can diminish the ability of a population to respond to other stressors. In addition, hatchery strains may be subjected to specific and unique selective forces in the hatchery, which might be very different from those encountered on natural restoration sites. The strong genetic differentiation observed here demonstrates that microsatellite markers can detect population structure in *C. virginica* and will be useful for population genetic studies including assignment tests, pedigree analysis and mapping studies.

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