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PRIMER NOTE

Characterization of 79 microsatellite DNA markers in the Pacific oyster *Crassostrea gigas*

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Abstract

We characterized 79 microsatellite DNA markers, which were obtained from genomic libraries enriched for CA, GA, ATG and TAGA motif repeats, in the Pacific oyster *Crassostrea gigas*. For eight F_1 grandparents or great-grandparents of mapping families, the average heterozygosity, 0.705, and average number of alleles per locus, 5.7, did not vary among motif-repeat or motif-complexity categories. Non-amplifying polymerase chain reaction null alleles, which were confirmed by segregation in the mapping families, were detected at 41 (51.9%) of the 79 loci. Cross-species amplifications from *C. angulata*, *C. sikamea*, *C. ariakensis* and *C. virginica* showed a precipitous decline with distance from the focal species *C. gigas*.

Keywords: *Crassostrea gigas*, cross-specific polymerase chain reaction, library, microsatellite, oyster, polymerase chain reaction null allele

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The Pacific oyster *Crassostrea gigas*, introduced from Japan to all continents except Antarctica, has the largest production and second largest value of farmed aquatic animals worldwide (FAO 2001). Although cultured oysters are at an early stage of domestication and quite commonly propagated from wild-caught brood stock, the few large-scale selection and cross-breeding programmes that have been initiated (e.g. Langdon *et al*. 2003) would benefit from a linkage map. Thirty-five dinucleotide repeat microsatellite loci have been reported for *C. gigas* (Magoulas *et al*. 1998; Huvet *et al*. 2000; McGoldrick *et al*. 2000). In this study, we characterized 79 new microsatellite loci (GenBank accession nos AF468524–AF468602) that are being mapped in *C. gigas* (Hubert *et al*. 2002), using three families derived by crosses among F_2 and F_3 hybrids from six different inbred lines (see Launey & Hedgecock 2001).

Four microsatellite-enriched libraries were constructed by Genetic Identification Services (Chatsworth, CA, USA) using DNA from four oysters that originated from a naturalized population of *C. gigas* in Dabob Bay, Washington. Fragments of genomic DNA, 300–700 bp in length and enriched for a microsatellite motif (CA, GA, ATG or TAGA repeat), were ligated into the *HindIII* cut site of *p*UC19 plasmid and transformed into *Escherichia coli* strain DH5α. Purified clone DNA was prepared using QIAprep 96 Turbo Miniprep Kit Protocol (QIAGEN, Valencia, CA, USA). Inserts longer than 300 bp were selected for sequencing (Davis Sequencing, Davis, CA, USA).

After removing vector sequences, cloned sequences were aligned with each other and with those published previously to check for duplication, using SEQUENCHER (Gene Codes, Ann Arbor, MI, USA). Alignments required low stringency (minimum match 60% and minimum overlap 50 bp) owing to variation in repeat lengths, which can prevent alignment, and were each verified to have substantial overlap on both sides of the repeat region. Polymerase chain reaction (PCR) primers were designed from unique flanking sequences using PRIMER3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) with annealing temperatures of 57–62 °C and expected amplicon lengths of 200–500 bp (optimum 250 bp). Inferred amino acid sequences were matched to entries in the GenBank protein database by BLASTX (http://www.ncbi.nlm.nih.gov/blast/blast_FAQs.html).

Optimal PCR conditions were determined with reactions containing 2 μL of template DNA, 125 μM of dNTP, 400 μM of tetramethylrhodamine-6-dATP (NEL 470; NEN, Boston, MA, USA), 1.0 μM of each primer, 0.325 U of *Taq* (Promega) and one of three levels of Mg^{2+} (0.75, 1.50 and

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2.25 mM) in a final volume of 10 μL. The PCR conditions comprised an initial denaturing period of 92 °C for 2 min followed by 30 cycles of 30 s denaturing at 92 °C, 30 s annealing over a gradient of 12 temperatures from 50 to 62 °C, 45 s at 72 °C and a final 5-min extension at 72 °C in a PTC-225 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). Products were separated on 8% acrylamide gels and imaged with an FMBIO II scanner (Hitachi). Optimal Mg^{2+} and annealing temperatures were determined for subsequent PCRs.

Motifs were classified into di-, tri- or tetranucleotide repeats and into categories of motif complexity (simple or compound vs. pure or interrupted, following Chambers & MacAvoy 2000; with compound and complex motifs combined). We typed eight F_1 grandparents or great-grandparents of three mapping families {$[(7 \times 6) \times (5 \times 2)]$, $[(2 \times 5) \times (7 \times 9)]$, $[(7 \times 9) \times (2 \times 5)]$; Launey & Hedgecock 2001}. Differences in the observed proportion of heterozygous individuals per locus, H_O (arcsine square-root transformed), the number of alleles per locus, n_a, and the number of null alleles among classes of microsatellite motifs were tested by ANOVA. For comparison, we obtained DNA from five or six individuals of three other Asian *Crassostrea* species (*C. angulata*, *C. sikamea* and *C. ariakensis*) and the American species *C. virginica*.

DNA was purified from 1121 microsatellite-containing clones. We sequenced 403 of these clones, 75 of which clustered into 28 alignments of two or more sequences (AF468524, 468529, 496560–496632 and 520585–520589); we designed 490 sets of PCR primers and obtained optimal PCR conditions for 190 potential markers. Ultimately, 92 markers were successfully genotyped on the parents and grandparents of the mapping crosses, of which 79 proved informative and were successfully genotyped for the linkage map (Table 1). Based on the starting number of clones sequenced, 47% yielded optimal PCR protocols, 23% were typed on parents and grandparents and 20% were mapped.

Only 10 clones (AF468542, 468560, 468570, 468574, 468581, 468585, 468586, 468596, 468600 and 468601) yielded significant matches to entries in the protein database. Longer sequences around each of these clones will be required to confirm the identity of these matches. The 79 new markers comprised 38 di-, 20 tri-, 10 tetra-, 1 di-/tri- and 10 di-/tetranucleotide repeat motifs. There were 31 uninterrupted simple repeats, 22 interrupted simple repeats, nine uninterrupted compound or complex repeats and 17 interrupted compound or complex repeats. Interrupted repeat arrays comprised 49.4% (39/79) of the loci (Table 1).

The 79 new microsatellite markers varied widely in the degree of polymorphism (Table 1), with n_a ranging from 2

Table 1 Characterization of 79 microsatellite loci in *Crassostrea gigas*

Locus	GenBank accession no.	Repeat array	Primer sequence (5′–3′)	Size (bp)	$MgCl_2$ (mM)	T_m (°C)	n_a	H_O	No. null	Cross species
ucdCg-107	AF468524	$(TATC)_n$ $(CATA)_N$	TCCTGACTGGGCAGTTTTCT GGATGTGATGCTGGCTATGA	247	2.00	53	8	1.000	0	1, 2
ucdCg-109	AF468525	$(CAT)_n$	GCTATGGTTGTCATCCTCGAA TGCCTTTATCGGTTTTGCTT	202	1.25	53	7	0.875	0	1, 2
ucdCg-111	AF468526	$(TCA)_N$	TTTTCAACGGAATCTGAACAAA GGAAAATTGATGGATGAAACAGA	204	2.00	60	7	1.000	2	1, 2
ucdCg-112	AF468527	$(TCA)_n$ $(TCG)_N$	TCAGTCATCTGAATCCTCATCC CTGCCGCAGATTTAGACAAA	207	1.25	53	6	1.000	1	1, 2
ucdCg-117	AF468528	$(TC)_n$	CCAAGCTTGCACTCACTCAA GAGTGTTCTGGTGTGCCAAAT	290	1.25	57	6	0.75	0	1, 2, 3?
ucdCg-119	AF468529	$(TC)_N$	AGGATGCCAATCGATTTTATTT ACCATGCCGTCTTAGTGGAC	191	1.25	50	7	0.500	3	NA
ucdCg-120	AF468530	$(CA)_n$ $(GA)_N$	GGGTGAGATTTAGGGGGAGA CTCCATCAAACCTGCCAAAC	152	1.25	57	4	0.875	0	1, 2, 3
ucdCg-124	AF468531	$(CA)_n$	CACATCCAAACACACACACATC CAAAAACCAGTGTTTTGTCCA	230	1.25	55	4	1.000	1	1, 2
ucdCg-126	AF468532	$(TCTA)_N$	TGGATTTGATCACCCCTTACA CCTGGATTCTGTGCGAGATT	174	1.25	55	6	0.625	0	1, 2
ucdCg-128	AF468533	$(CT)_n$	CGGAGAGTCGTGTTCGTTTT TGAGAAACGGAGTTGCTTTTG	227	1.25	55	10	0.875	1	1, 2?
ucdCg-129	AF468534	$(GA)_N$	CGAATTTTTCGGACATCGTT GTGGTATGCCTGCATCATGT	232	1.25	57	6	1.000	0	1, 2
ucdCg-130	AF468535	$(CA)_N$	CCGACAGTCGTCACCTTTTT TTCCAGCATCACTGCAGATT	229	2.00	60	6	1.000	0	1, 2, 3, 4
ucdCg-131	AF468536	$(GA)_N$	GCACACGGATCTTGTGAAAA GCCAAGCTTGCTTGTAGGTT	211	2.00	55	5	0.250	1	1, 2
ucdCg-133	AF468537	$(CT)_N$	GTTGGCGCGAAATATAGGAA TGAACTTGTGATGTGCAGGA	293	1.25	55	6	1.000	0	1, 2
ucdCg-134	AF468538	$(CT)_n$ $(CGCT)_N$	TTTGTATGTCCGTCATCGTCA CCCTGCAAAATGGTGAAAAT	237	2.00	57	7	1.000	0	1, 2?

Table 1 *Continued*

Locus	GenBank accession no.	Repeat array	Primer sequence (5′–3′)	Size (bp)	$MgCl_2$ (mM)	T_m (°C)	n_a	H_O	No. null	Cross species
ucdCg-135	AF468539	$(TC)_n$	TTTGGTTGAATCAATCTCTCTCC TCCATGCGTTCTCTTCAATG	254	1.25	50	6	1.000	0	1, 2, 3?
ucdCg-136	AF468540	$(TATC)_N$ $(TA)_N$ $(GA)_N$	CTGGTGCAGGAAAGTCAGGT CATGTGGCACCTCTCTCTGT	254	1.25	57	6	0.625	2	1
ucdCg-137	AF468541	$(GATA)_n$	TGGGACAAGATCTCCCTTTTT CAAGCTTCTGGCGAAACATT	270	1.25	50	5	0.800	2	1, 2
ucdCg-138	AF468542	$(GA)_N$	CCTCGAACAGCACTCCAAAT TTCAGTTCAACGCTCTTGCT	271	1.25	57	5	0.750	0	1, 2, 3
ucdCg-139	AF468543	$(GA)_N$	CCCAAACACCTTTACATTCCA GGCAATTGTCGTTTTCATTCA	223	1.25	55	2	1.000	0	NA
ucdCg-140	AF468544	$(CT)_N$	TGCTCAATTCACAGCAATCAG TCTGACTGCTGAACAGCAAAAT	250	2.00	60	8	0.875	0	1, 2, 3
ucdCg-141	AF468545	$(TG)_n$	CTCAACGACTTTTTGCCTGA GTGTCTTCTATCCCGCAACG	243	1.25	57	4	0.625	1	1, 2
ucdCg-142	AF468546	$(GT)_N$	GCTCCTCAACCACCGATACA AATCGTGTTCGAAAGGGTTG	201	1.25	60	7	0.750	3	1?
ucdCg-145	AF468547	$(GA)_n$	CAGGTGTATGCGACGTGACT CAGGCTTTTAAGCGCATTTT	255	1.25	55	6	0.625	1	1, 2?
ucdCg-146	AF468548	$(CT)_N$	CGCTCTGGTCTTTGTTCCAT ACCCCAACAGATCACAATCC	218	1.25	57	5	0.500	1	1, 2
ucdCg-147	AF468549	$(TATC)_n$ $(TCTG)_N$	CAGGGATCCAAAACTACCAGA CACCATCGTACCATTCCAAA	326	1.25	55	6	0.500	3	1
ucdCg-148	AF468550	$(GA)_n$	TGTTGGTTGGTTGGTAGGTTG TGTCAAACGTCGAGAATTGG	249	1.00	57	6	0.857	0	1, 2, 3
ucdCg-149	AF468551	$(GA)_n$ $(GACA)_N$	TGATTAAACGTGGGTGATTCAG TTTCTGACTGTCCGTCTGTGA	232	1.25	60	8	1.000	1	1, 2, 3
ucdCg-150	AF468552	$(CT)_N$	CCTTTCATGTAGGATGACTCTTTTT TGAGGGGAGCATTGATTGAT	258	2.00	55	5	0.500	1	None
ucdCg-151	AF468553	$(GT)_N$	AGGTAATCCGCAAACCAGTG GCATTGCGTCAGGATTAGGT	265	1.50	60	5	0.750	0	1, 2, 3
ucdCg-152	AF468554	$(CAT)_n$	TGGTTTTGGAGCTTGGCTTA TCAAGCAAAGAAAGTCACCTCA	257	1.50	57	2	0.125	0	1, 3
ucdCg-153	AF468555	$(GAT)_n$	GCAGCAGCTTCAGAGTGAAA CAAAATCTGGTGGACCTTCG	257	1.50	57	9	0.833	1	1
ucdCg-155	AF468556	$(CA)_n$ $(CT)_N$	TGGCATCGGAAAGGTAAGAC TCGCGAGGCAAAATCTTAAT	236	1.00	55	2	1.000	1	1, 2
ucdCg-156	AF468557	$(GATA)_n$ $(GATG)_n$ $(TA)_n$	AGCAGACCTTGGCAAATACG CCGTCATCAGGTCCTGTTTT	325	1.50	50	7	0.857	1	1, 2
ucdCg-157	AF468558	$(GA)_N$ $(TAGA)_N$	GGGGGATGTCGGAGAAGTAT AACAGAGAAAGGTGGATTTTAGGA	244	1.00	58	8	0.857	1	1, 3
ucdCg-158	AF468559	$(TG)_N$ $(TG)_N$ $(TGCG)_N$	GGAGCTCCAACAAAAGTGGAT TGCGTTAAACATTTGGGACA	229	1.25	57	8	0.750	4	1
ucdCg-160	AF468560	$(GA)_n$ $(GACA)_n$	GGAGCCATTAACAACACCACA TCTCTCCCTTCCCCCTCTTA	251	1.50	57	6	0.875	0	1, 2, 4?
ucdCg-161	AF468561	$(GA)_N$	ATCACACGAGAGCACGAATG ACGTATGTGTCCGTCCGTTT	235	1.25	57	6	0.833	0	1, 2, 3
ucdCg-162	AF468562	$(TTCA)_N$ $(ATCT)_n$ $(GTCT)_N$	CCAAATCACCGTTTTAGTTTGTT AGCGACACAGAGACCACCTT	268	1.50	52	7	1.000	1	1, 2, 3, 4
ucdCg-163	AF468563	$(CAT)_N$	TTCAATGCTGCAAGAAGAGTC TTGACAAGTTCCACAATGCTG	242	1.50	52	8	0.800	1	1, 2, 3, 4
ucdCg-164	AF468564	$(CAT)_n$	ACTAGCGGCTGCTTTTATCG TCCTGTTCCCGTACCAGTTC	263	1.50	57	3	0.429	0	1, 2
ucdCg-165	AF468565	$(ATCT)_N$ $(CA)_N$	TTTTTACCAGCACTCGCTGT TCCGAATTTCACAAGTGTGTGT	241	1.50	57	5	0.667	2	NA
ucdCg-166	AF468566	$(TC)_N$	CATCGGAACTAAATCGGGTAA TTCCTTTGTGCTGTCTTACAGG	214	1.25	58	6	0.750	0	1, 2
ucdCg-167	AF468567	$(GATA)_N$	CAAAATGTGTGAAAGTGATGAAA CCAATTTCTATTCGCAAGCA	205	1.50	58	5	1.000	0	1, 2
ucdCg-170	AF468568	$(GA)_n$ $(GT)_n$	TGGTGGTCAGTGAATGTGAGA CGGACAGTAGCCTTTTAACACA	276	1.50	60	6	0.750	0	NA
ucdCg-171	AF468569	$(CAT)_N$	CCACTCATAAGGGAAAATGAAA TCGTCGACCTTAAAACTCGT	249	1.50	50	3	0.667	0	1, 2
ucdCg-172	AF468570	$(GAT)_n$	CCACCGTTAAACGTAGCATTG TTGTGTCCCTTTTCCGTCTC	246	1.50	57	3	0.375	1	1, 2, 3, 4
ucdCg-173	AF468571	$(CT)_N$ $(CA)_N$	AAAATGGGAATTCACTGTGTCA CGGCACCGGTTTGTTATCT	223	1.25	57	8	1.000	2	1, 2

Table 1 *Continued*

Locus	GenBank accession no.	Repeat array	Primer sequence (5′–3′)	Size (bp)	$MgCl_2$ (mM)	T_m (°C)	n_a	H_O	No. null	Cross species
ucdCg-174	AF468572	$(CAT)_n$	CTTCCTGCTGCAGAACCTGT AATGACGGGATGATGATGATG	200	1.50	55	4	0.800	0	1, 2
ucdCg-175	AF468573	$(CAT)_n$	GGGCATGGATCAACTCCTAA CCAACCAGCCCTAGTCTGTG	258	2.00	55	9	1.000	1	1, 2
ucdCg-176	AF468574	$(GAT)_n$	TTCCGATGATGATAGCGATG GGCTCGTGTTCCAATATGGT	258	1.50	57	2	0.250	0	1, 2, 3
ucdCg-177	AF468575	$(GA)_n$	GCTTCCGGGAATTAAACCAT TCAAGAAAAAGTCGACGGGTA	245	1.25	57	7	0.833	0	1, 2
ucdCg-178	AF468576	$(TAGA)_N$	AGGGAAACCGTGGCTTTAGT CAAGCTGTAGTAACCGGCTAAG	249	1.25	57	3	0.500	1	1, 2
ucdCg-179	AF468577	$(GA)_N$	CGTGATCACCTCCTTCTGGT GGTGCGAGGCTAGGTAAGAA	240	0.75	58	2	1.000	0	1, 2
ucdCg-180	AF468578	$(GAT)_N\ (GTT)_n$	TCACACGCAGCGAATTTTTA AATAACCACGCCGACAGC	277	2.00	55	5	0.500	1	1
ucdCg-181	AF468579	$(GT)_N\ (GA)_n$	CACCCCAAAGGACCACATAC TGTCAGCATGGGTAAGTCCA	239	1.25	60	6	0.750	0	1, 2, 3
ucdCg-182	AF468580	$(GT)_n\ (AT)_n$	TCAGACCTGAGAACGTGTGTG TTGGTAGCAAGATCGGGAAA	217	2.50	58	3	0.500	0	1, 2, 3, 4
ucdCg-183	AF468581	$(GA)_N$	CATTGTGGGGGTTTTCAGTT GACCCGATCTCCATTACACC	222	1.25	57	4	0.500	2	1, 2
ucdCg-184	AF468582	$(GA)_n$	TCAGTACAGGGCAGTCAACG GTTTGCGCCCATTGATATTT	244	1.25	54	5	0.667	1	1, 2, 3
ucdCg-185	AF468583	$(GA)_N$	CTGAAATGTCACGAACTGCAC AACCGGTTTGTGTATTGATGC	205	1.50	52	7	0.625	3	1, 2, 3, 4
ucdCg-186	AF468584	$(GA)_N$	GCCGCCGATTCTCTTAGATT GGGCTAGCTAGTCATCACCCTA	260	1.50	58	8	1.000	3	1, 2
ucdCg-187	AF468585	$(AG)_N\ (TG)_N$	GGCAATGGGCGTATCTTAAC CCGGTGCCATACTCCTTACA	288	1.50	58	6	0.833	0	1, 2, 3
ucdCg-188	AF468586	$(GA)_N$	ATTTTGTGGGGTGGTGGTAA TTATTTGCTTGCATTTGATCG	245	1.50	52	3	0.600	2	1, 2, 3
ucdCg-189	AF468587	$(GA)_N$	AATGCAGGGGTCACTGAGAG GGGATTAAGTTTGAACATGTGG	249	1.50	58	2	0.500	2	1, 2
ucdCg-190	AF468588	$(CT)_N$	TTGCCGTTGATCGTATGGTA GCATTGACGGGAAATCACTC	278	2.00	52	3	0.571	1	1, 2, 3
ucdCg-191	AF468589	$(GA)_n\ (CAGA)_N$	GCTTCCATGACACAACTACTGG ATGATGCATTGCCAAGTTGA	249	1.25	55	7	1.000	1	1, 2
ucdCg-192	AF468590	$(CAT)_n$	CGTCCCTCCAATCAAGTGTT GCCCGTCAAAGTGTTCTTTC	251	2.00	58	5	0.714	0	1
ucdCg-193	AF468591	$(GT)_N\ (TA)_N$	CTAGAAACGCTTCGGTCGAT TATCTTTCGCAAATCGCAGT	249	2.00	60	4	0.750	2	1, 2, 3
ucdCg-194	AF468592	$(GAT)_n\ (GAG)_N$	CCCAGTGAAAACTTGGAGACA TTTCGAATCGGGAAAATACG	253	2.00	52	9	0.714	1	1, 2
ucdCg-195	AF468593	$(CAT)_N$	CCAACAACAGGGCACCTACT GGTCCAGTTGGCATCCTCTA	280	2.00	58	5	0.714	0	1, 2
ucdCg-196	AF468594	$(GAT)_N\ (GAC)_N$	CCTTTCATTTGGAGGTTACATTG ATCTTGCCATTTGCTTTTGG	253	1.50	58	5	0.429	0	1, 2
ucdCg-197	AF468595	$(CTT)_n\ (TGA)_n\ (GA)_N$	AGCAGACCCACTGGAGGTAA GTCGCTTCACCCAGGAAAT	277	1.50	55	7	0.714	1	1, 2, 3
ucdCg-198	AF468596	$(CAT)_N$	GAAAGACACGACCGGAGAGA CTGATGATGTCCCACACCTG	230	1.50	58	5	1.000	0	NA
ucdCg-199	AF468597	$(CAT)_n$	GGGAAGAGTTGAATTCTGCAA AAACCGAGGCTCAGGAAAAT	270	2.00	55	3	0.800	0	1, 2, 3
ucdCg-200	AF468598	$(GAT)_N$	AAAGTTGCTTTGCTGTCGTC CGCTAACGTGCTTCATTCAA	254	2.00	58	5	1.000	0	1
ucdCg-201	AF468599	$(TAGA)_n$	CAGGGCGTTTATCTTGGTCT GCAAAGATAACCTTCCGTGA	268	1.25	58	6	0.714	1	1, 2
ucdCg-202	AF468600	$(GATA)_n$	AATGAAAATGATGATGAGGATGA TGCATGTCGAACAAATCCAT	256	1.50	50	5	0.333	0	2
ucdCg-203	AF468601	$(GATA)_N$	AAGCTTACCGGACTGGGTTA GGGTTCCGCCATTTACTTTA	205	2.00	55	3	0.333	0	NA
ucdCg-204	AF468602	$(GATA)_n\ (GA)_n$	CACATGCGCGTACAGATGAT CTCAATTTTTCTCTCACGCAAA	235	2.00	58	8	1.000	2	1, 2, 3, 4

Repeat array: *N*, pure; *n*, interrupted. Size (bp) is for allele sequenced. n_a, Number of alleles; H_O, observed heterozygosity; no. null, count of null alleles in independent families. Cross species: 1, *C. angulata*; 2, *C. sikamea*; 3, *C. ariakensis*; 4, *C. virginica*; NA, data not available.

to 10 (mean 5.71 ± 0.22) and H_O ranging from 0.125 to 1.0 (mean 0.705 ± 0.027). Neither measure of polymorphism varied significantly across motif-repeat or motif-complexity categories.

The PCR null alleles were found at 41 (51.9%) of the 79 new loci. Minimum counts of independent PCR null alleles per locus ranged from zero to four (Table 1) and summed to 64; the average frequency of null alleles was 0.093. Neither null allele count nor null allele frequency varied among motif-repeat or motif-complexity categories. Among the 73 new markers tested on congeneric species, 71 (97.3%) can be amplified from *C. angulata*, 62 (84.9%) from *C. sikamea*, 27 (37.0%) from *C. ariakensis* and only eight (11.0%) from *C. virginica* (Table 1).

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