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Centromere mapping in triploid families of the Pacific oyster *Crassostrea gigas* (Thunberg)

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ABSTRACT

Gene-centromere mapping, a tool for improving linkage maps, can be accomplished in families of triploid bivalve molluscs induced by inhibiting the second meiotic division (MII) of fertilized oocytes. Here, we report half-tetrad analyses of seven triploid families of the Pacific oyster *Crassostrea gigas*, using 56 microsatellite DNA markers and the *amylase* gene. Most MII segregations (88/93) show 1:1 ratios of homozygotes, as expected, and marker-centromere distance are calculated for these cases as one-half the proportion of heterozygotes, *y*. Heterogeneity of *y* is observed in nine of 25 cases in which markers were typed in more than one family, suggesting variation in gene-centromere recombination among families. Altogether, 64 statistically independent marker-centromere distances are obtained, 61 of which can be arranged onto previously published linkage groups (LG) and three of which are linked to a centromere but unlinked to any other marker. Differences in marker orders between linkage and centromere maps imply polymorphisms for chromosomal rearrangements. Six of the 10 centromeres of the Pacific oyster genome are closely linked to nine microsatellite DNA markers, facilitating future linkage assignments. Distances among markers on the centromere map correlate closely with distances among markers separated by 30 cM or less on the linkage map, supporting the assumption in centromere mapping of complete interference (i.e. one and only one cross-over between a marker and the centromere). High interference, reported in previous studies, is not supported here by a uniform distribution of *y*-values from zero to one. Detailed analysis of segregation patterns reveals more cross-over events than counted under the assumption of complete interference, with no or even partial negative interference calculated for pairs of markers on LG4.

1. Introduction

The Pacific oyster *Crassostrea gigas* has had the highest production of any farmed aquatic animal since 1998, reaching 4.5 mmt in 2005 (FAO, 2006). As a result of its importance in global aquaculture, as well as its status as a model species for physiological, genetic, ecological, and evolutionary studies, the Pacific oyster has been proposed as a candidate for whole genome sequencing (Hedgecock et al., 2005). Genomics, in concert with controlled crosses and inbred lines, will provide information and tools, such as markers, linkage and QTL maps, expression assays, and candidate genes for important traits. Knowledge of oysters genomics is still rudimentary, although many sequences are available in public databases for mitochondrial (Boudry et al., 2003; Pie et al., 2006) and genomic DNA, including 4.8 million Massively Parallel Signature Sequencing entries in the GEO database (Hedgecock et al., 2007). Different kinds of libraries have been constructed, such as full-length cDNA libraries (Cunningham et al., 2006; Durand et al., 2004; Jenny et al., 2002; Leung and Chu, 2001), suppression subtractive hybridization libraries (Boutet et al., 2004; Gueguen et al., 2003; Huvet et al., 2004), and a BAC library (Cunningham et al., 2006). However, genetic and physical mapping are essential for putting genomic or cDNA sequences into their context. Dense genetic maps allow a gene to be located on a specific linkage group (LG) or chromosome. Genetic maps have been developed for *C. gigas*, using microsatellite DNA markers (Hubert and Hedgecock, 2004) and AFLP markers (Li and Guo, 2004).

Linkage maps are typically constructed from recombination data and result in linear arrangements of markers on LG. Gene-centromere maps, on the other hand, are constructed by recovering two of four chromatids from a meiotic division (half-tetrad), following inhibition of meiosis II (MII) in the maternal parent. If the maternal parent is heterozygous at a particular marker, its reduction gametes are homozygous, in the absence of a cross-over between the centromere and the marker, but heterozygous, if a cross-over occurs during meiosis I (MI). The proportion of heterozygous progeny is a measure of the frequency of second division segregation, *y*, and marker-centromere

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distance in centimorgans can thus be estimated as $100\times(1/2)\times y$, assuming complete interference. The assumption of complete interference, the occurrence, if any, of one and only one cross-over per chromosome arm, can be examined by comparison of linkage and centromere maps and by detailed analyses of genotypes at multiple markers along a chromosome arm. Half-tetrad analyses thus permit a linkage map to be oriented with respect to the centromeres and allow investigations of recombination and interference. Centromere mapping also allows consolidation of linkage maps in some cases (Johnson et al., 1996). Finally, markers tightly coupled to centromeres permit the linkage group of new markers to be rapidly determined (Johnson et al., 1996).

Gene-centromere maps have been obtained for different fish species using allozyme markers (Allendorf et al., 1986; Guyomard, 1984; Liu et al., 1992; Seeb and Seeb, 1986; Thompson and Scott, 1984). Centromere maps have also been reported for bivalves, such as the Pacific oyster (Guo and Gaffney, 1993), dwarf surf clam (Guo and Allen, 1996) and mussel (Beaumont and Fairbrother, 1995) using allozyme markers. The development of microsatellite markers and moderately dense linkage maps have led more recently to the development of denser centromere maps in fish (Danzmann and Gharbi, 2001; Gharbi et al., 2006; Johnson et al., 1995, 1996; Lahrech et al., 2007; Martínez et al., 2008; Mohideen et al., 2000; Nomura et al., 2006; Sato et al., 2001). Recently a centromere map, using a small number of microsatellite markers has been published for *C. gigas* (Li and Kijima, 2006).

Triploid oysters have been studied for twenty years and are widely used in commercial oyster farming because of sterility, superior growth and meat quality, and increased survival and disease resistance (Guo, 2004; Nell, 2002). Triploids are widely cultivated on the west coast of the USA, but also in Australia, China, France and Chile. Most triploid Pacific oysters are currently produced by fertilizing the eggs of diploid females with sperm from tetraploid males (Eudeline et al., 2000; Guo et al., 1996). Though useful for investigating segregation from tetraploid males (Curole and Hedgecock, 2005), such mated triploid progeny are not useful for gene-centromere mapping, since the female parent contributes only a single chromosome. Induced triploids, on the other hand, are still made routinely by oyster breeders, as a step in producing new tetraploid male stocks. Triploidy is most commonly induced by treating fertilized oocytes with cytochalasin B to inhibit MII (Downing and Allen, 1987).

Many centromere maps in aquatic species have used gynogenetic offspring produced by fertilizing MII-blocked eggs with UV-irradiated sperm (Allendorf et al., 1986; Gharbi et al., 2006; Guyomard, 1984; Johnson et al., 1987, 1995, 1996; Liu et al., 1992; Seeb and Seeb, 1986; Thompson and Scott, 1984; Thorgaard et al., 1983). Gene-centromere recombination studies in bivalves have used either triploid or gynogenetic progenies, although both types of progenies have been examined in *Mulinia lateralis* (Guo and Allen, 1996).

In the present study, seven families of triploid *C. gigas* were produced by cytochalasin B treatment. Half-tetrad analyses of these families were carried out for 56 microsatellite DNA markers and an *amylase* marker. Microsatellite-centromere recombination rates were calculated and compared with the map distances obtained in the female linkage map of *C. gigas* based on many of the same microsatellite markers (Hubert and Hedgecock, 2004).

2. Materials and methods

2.1. Chemically induced triploid families

Seven families of Pacific oyster *C. gigas* (Table 1), in which triploidy had been induced with cytochalasin B (Downing and Allen, 1987), were supplied to us by Dr. Benoit Eudeline, Taylor Resources Inc., Quilcene, WA. The parents for these crosses came from five, full-sib families, four of which were produced and reared as cohort 4 of the Molluscan Broodstock Program (families 76, 91, 108, and 116; Langdon et al., 2003). The fifth family, 18, was produced by a brother–sister mating of cohort 4 family 111 and reared with MBP cohort 9. Families 76, 91, and 116 were produced by pair-wise crosses of oysters collected from Pipestem Inlet, B.C.; families 108 and 111 were produced by crosses of male oysters from Willapa Bay, WA, and females from Pipestem Inlet. Triploid families are given letter abbreviations for ease of reference (Table 1). Progeny were confirmed as 100% triploid by flow cytometry, within the detection limits of this method; we use genetic data below to evaluate the ploidy of progeny. Larvae from these families were harvested six to 10 days post-fertilization, killed with buffered formalin (three drops per 15 ml seawater), rinsed, and preserved in 70% ethanol.

Table 1
Number of loci, average number of individuals typed per locus, and percent triploid estimated from genetic data, in seven triploid Pacific oyster families

Family	Female	Male	No. loci	Ave *n*	% 3N
F76-1xM91-1	A	2	4	77.3	100.0
F76-3xM18-3	B	1	24	81.5	100.0
F91-1xM108-1	C	3	17	68.5	97.9
F91-3xM18-3	D	1	10	73.4	95.7
F108-3xM18-3	E	1	7	76.0	100.0
F116-1xM91-1	F	2	13	83.3	100.0
F116-3xM18-3	G	1	18	82.8	100.0

2.2. DNA extraction, markers, and PCR procedures:

DNA from the parents of the mapping families was extracted from adductor muscle using a CTAB extraction (Boutet et al., 2004). DNA from larvae was extracted with proteinase-K, at 1 mg/ml of buffer (1.5 ml Thermophilic DNA Polymerase 10× Buffer, Promega, Madison, USA), 75 µl Tween 20, adjusted to 15 ml with distilled water). Individual larvae were extracted with 200 µl of this solution, in 96-well trays, and then incubated at 55 °C for 3 h and at 95 °C for 30 min, using a Perkin-Elmer 9600 thermocycler. Aliquots of 50 µl of the extract were frozen to minimize DNA degradation. DNA was successfully extracted from larvae that were kept in 70% ethanol for more than one year.

Fifty-six microsatellite DNA markers were amplified for this study (Table 2), including 38 from the series *ucdCg107-204* described by Li et al. (2003), *uscCg*205 from Yamtich et al. (2005); 9 loci previously cloned in this laboratory (*ucdCg001*, *ucdCg002*, *ucdCg003*, *ucdCg006*, *ucdCg010*, *ucdCg014*, *ucdCg021*, *ucdCg028* (McGoldrick and Hedgecock, 1997), and *ucdCg024* (McGoldrick, 1997); 8 loci taken from the literature (*CG44*, *CG49*, and *CG108* from Magoulas et al. (1998), renamed here for consistency *imbCg044*, *imbCg049*, and *imbCg108*; *L10*, *L16* and *L48* from Huvet et al. (2000), renamed here *um2CgL10*, *um2CgL16* and *um2CgL48*; and *cmrCg005* (AF201464, unpublished) and *cmrCg141* (McGoldrick et al., 2000). We also amplified a microsatellite DNA marker in intron 4 of the *amylase* gene A, called here *AmyA* (Sellos et al., 2003).

Polymerase chain reactions (PCR) to amplify these DNA markers were performed in 96-well plates, with 1.5 µl of template DNA; 1 mM *Taq* buffer; 1, 1.25, 1.5, 2.0, or 2.5 mM $MgCl_2$, depending on the locus; 125 µM of dNTP; 400 µM of tetramethylrhodamine-6-dATP (NEL 470, NEN, Boston, MA 02118); 1.0 µM of each primer; 0.325 units *Taq* polymerase (Promega), and H_2O to a final volume of 7.5 µl. PCR was done with an initial denaturing at 92 °C for 2 min, followed by 30 cycles each of 30 s denaturing at 92 °C, 30 s annealing at locus-specific temperatures, 45 s extension at 72 °C, and concluding with a final extension at 72 ° C for 5 min. Products were separated on 8% acrylamide gels (acrylamide: bisacrylamide, 29:1, 7 M urea), using 1× TBE (Tris Borate EDTA) buffer, and imaged with a Hitachi FMBIO II scanner (MiraiBio, Alameda, CA). Each gel image was scored by at least two of the authors.

Table 2
Progeny genotypes at 57 markers in seven triploid families of Pacific oyster *Crassostrea gigas*

LG, locus	Family	Progeny 11	22	12	Total	*y*	$\chi^2_{11:22}$
LG1a							
imbCg044	G	46	36	0	82	0.000	1.220
cmrCg005	C	26	13	20	59	**−0.339***	4.333
cmrCg005	F	11	13	57	81	**−0.704***	0.167
ucdCg003	B	48	30	1	79	−0.013	4.154
ucdCg107	A	22	15	35	72	−0.486	1.324
ucdCg126	G	6	11	55	72	−0.764	1.471
LG1b							
ucdCg191	E	7	4	58	69	0.841	0.818
ucdCg191	G	10	8	71	89	0.798	0.222
ucdCg010	G	12	15	54	81	0.667	0.333
ucdCg006	A	16	21	28	65	0.431	0.676
ucdCg202	B	25	12	28	65	0.431	4.568
ucdCg185	F	44	42	0	86	0.000	0.047
ucdCg185	B	36	37	1	74	0.014	0.014
ucdCg157	F	35	37	16	88	−0.182	0.056
ucdCg187	B	26	24	32	82	−0.390	0.080
LG2							
um2CgL48	F	3	3	84	90	**0.933***	0.000
um2CgL48	C	22	17	43	82	**0.524***	0.641
ucdCg195	D	24	8	44	76	0.579	8.000*
ucdCg195	G	18	28	31	77	0.403	2.174
ucdCg148	C	23	20	17	60	0.283	0.209
ucdCg134	B	40	32	19	91	0.209	0.889
ucdCg001	G	40	28	21	89	0.236	2.118
ucdCg001	B	30	31	9	70	0.129	0.016
ucdCg160	D	17	7	35	59	−0.593	4.167
LG3							
ucdCg002	D	7	15	55	77	**0.714***	2.909
ucdCg002	C	24	24	28	76	**0.368***	0.000
ucdCg109	D	10	7	51	68	0.750	0.529
ucdCg109	B	18	11	44	73	0.603	1.690
ucdCg165	G	14	17	33	64	0.516	0.290
ucdCg165	C	16	16	17	49	0.347	0.000
imbCg049	A	46	35	2	83	0.024	1.494
imbCg049	C	43	38	0	81	0.000	0.309
imbCg049	F	43	43	0	86	0.000	0.000
LG4							
ucdCg139	B	17	17	56	90	0.622	0.000
ucdCg138	B	20	24	35	79	0.443	0.364
ucdCg117	C	23	10	17	50	0.340	5.121
ucdCg137	D	41	26	9	76	0.118	3.358
ucdCg137	B	48	28	2	78	0.026	5.263
ucdCg147	C	34	30	2	66	−0.030	0.250
ucdCg147	G	46	36	5	87	−0.057	1.220
ucdCg163	B	17	37	21	75	−0.280	7.407*
ucdCg164	B	28	14	26	88	**−0.523***	4.667
ucdCg164	F	26	29	22	77	**−0.286***	0.164
ucdCg119	B	28	23	42	93	−0.452	0.490
ucdCg112	B	33	11	45	89	−0.506	11.000*
ucdCg112	C	21	8	47	76	−0.618	5.828
ucdCg170	C	9	21	51	81	−0.630	4.800
ucdCg170	E	18	15	58	91	−0.637	0.273
ucdCg170	G	17	23	51	91	−0.560	0.900
LG5							
ucdCg021	F	14	18	53	85	**0.624***	0.500
ucdCg021	B	23	24	30	77	**0.390***	0.021
ucdCg014	F	24	18	45	87	**0.517***	0.857
ucdCg014	B	35	28	24	87	**0.276***	0.778
ucdCg151	E	46	22	19	87	0.218	8.471*
uscCg205	B	46	43	0	89	0.000	0.101
ucdCg141	F	9	23	52	84	−0.619	6.125
ucdCg141	D	10	11	56	77	−0.727	0.048
ucdCg130	C	9	10	63	82	−0.768	0.053
LG6							
ucdCg156	G	14	10	55	79	0.696	0.667
ucdCg156	B	21	16	49	86	0.570	0.676
ucdCg156	C	23	15	39	77	0.506	1.684
ucdCg028	G	45	45	3	93	0.032	0.000
ucdCg028	A	47	42	0	89	0.000	0.281
ucdCg197	F	42	31	0	73	0.000	1.658
ucdCg197	G	39	32	0	71	0.000	0.690
imbCg108	E	11	7	50	68	−0.735	0.889
LG7							
ucdCg024	B	3	7	68	78	0.872	1.600
ucdCg175	C	6	6	32	44	0.727	0.000
ucdCg175	G	8	13	41	62	0.661	1.190
ucdCg196	B	16	22	27	65	**0.415***	0.947
ucdCg196	E	24	29	14	67	**0.209**	0.472
ucdCg196	C	36	16	9	61	**0.148**	7.692*
ucdCg196	G	43	41	14	98	**0.143**	0.048
ucdCg203	G	27	32	29	88	0.330	0.424
cmrCg141	C	36	30	8	74	0.108	0.545
ucdCg149	D	35	26	22	83	−0.265	1.328
um2CgL16	E	18	17	23	58	−0.397	0.029
LG9							
ucdCg171	F	9	6	64	79	**0.810***	0.600
ucdCg171	C	14	20	30	64	**0.469**	1.059
ucdCg171	G	18	33	35	86	**0.407**	4.412
ucdCg140	B	32	46	15	93	0.161	2.513
ucdCg140	D	35	20	20	75	0.267	4.091
um2CgL10	F	29	21	34	84	**−0.405***	1.280
um2CgL10	C	22	16	44	82	**−0.537**	0.947
um2CgL10	B	17	16	59	92	**−0.641**	0.030
um2CgL10	G	25	11	55	91	**−0.604**	5.444
LG10							
ucdCg200	E	5	6	81	92	0.880	0.091
ucdCg181	B	11	14	63	88	0.716	0.360
Unlinked							
ucdCg179	D	15	6	39	60	0.650	3.857
ucdCg179	B	22	7	47	76	0.618	7.759*
ucdCg180	F	18	34	31	83	0.373	4.923
ucdCg180	D	25	29	25	79	0.316	0.296
AmyA	G	38	23	29	90	0.322	3.689

LG are numbered in Arabic numerals corresponding to the Roman numerals in Hubert and Hedgecock (2004), except that LG I is split into linkage groups, LG1a and LG1b. Families are designated by letters from Table 1. The proportion of heterozygotes in triploid progeny, *y*, is given a sign to indicate position above or below the centromeres in Fig. 3. When determined in two or more families, homogeneity of *y* was tested; bolded *y*-values with asterisks were heterogeneous ($P<0.01$) by Fisher exact test, in 2×2 cases, or by chi-square test with pseudo-probability estimates of significance, in cases involving more than two families (Zaykin and Pudovkin, 1993; bolded *y*-values not marked with an asterisk were homogeneous). $X^2_{11:22}$ is a 1 *d.f.* test of equal proportions of homozygotes (*, $P<0.01$).

2.3. Marker-centromere recombination

Parents were genotyped at 56 microsatellite markers and the amylase locus to determine informative loci for analysis in triploid progeny. Female parents must be heterozygous at a locus, and the male parent must be either homozygous or heterozygous for alleles distinguishable from those of the female. Paternal contributions are hereafter ignored, and the terms homozygous and heterozygous refer to individuals carrying one or two maternal alleles. Triploids produced by MII inhibition will be homozygous in the absence of a cross-over between the centromere and the marker, but heterozygous if such a cross-over occurs. The proportion of heterozygous progeny is a measure of the frequency of second division segregation (*y*), and marker-centromere distance in centimorgans is thus estimated as $100\times(1/2)\times y$, assuming complete interference.

We first used genetic data to confirm the ploidy of progeny. For every progeny that carries two different maternal alleles at any marker (i.e. we see ABC, where, say, A is the male allele), we have direct evidence against

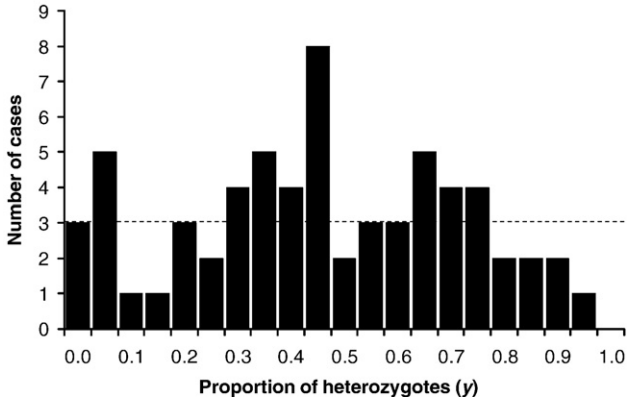

Fig. 1. Frequency distribution of 64, statistically independent estimates of the proportion of heterozygotes (y) observed in triploid progeny from seven heterozygous female parents. Dotted line gives the expectation for the uniform distribution, from which the observations do not differ (χ^2=21.97, 20 *d.f.*, P=0.342).

the hypothesis of diploidy. If a progeny shows only one maternal allele at a marker, we cannot be sure if this individual is a triploid, ABB, inheriting two B alleles from its maternal parent, or a diploid, AB. Looking across a series of genetic markers for this individual, however, we may find one or more markers that do show direct evidence of triploidy. Uncertainty about ploidy becomes an issue only for those individuals that show only one maternal allele at all markers typed. For all such individuals, we calculated the probability of a multi-loci homozygous genotype, under the hypothesis of triploidy, as $\prod_{1}^{k}(1-y_i)$, where k is the number of markers typed in that individual.

We next tested whether proportions of the two homozygotes, *11* and *22*, were equal with a 1 *d.f.* chi-square test. Owing to differences in y across cases, we did not correct significance levels for multiple testing, based on the cumulative number of tests, 93. We present the chi-square values themselves, calling attention only to those that were significant at the nominal 1% level, of which we would expect one (1/93=0.0108).

We next tested homogeneity of y (and its complement, $1-y$, the proportion of homozygotes), for all markers determined in multiple families, either by Fisher exact tests, for 2×2 cases, or by chi-square tests with pseudo-probability estimates of significance in 3×2 or 4×2 cases (Zaykin and Pudovkin, 1993). After testing significance of a 3×2 or 4×2 case, the homogeneity of y in 2×2 subsets was tested with the Fisher exact test.

Finally, markers from the same LG as described in (Hubert and Hedgecock, 2004) were grouped, and a centromere linkage map was created based on distance of each marker from the centromere, as described by Johnson et al. (1987).

2.4. Linkage analysis

A test for joint segregation was performed, by family, for every pair of loci, excluding loci that showed significant deviation from the expected 1:1 ratio of homozygotes, by comparing the numbers of individuals observed in each cell of a 3×3 table of possible two-locus genotypes with the numbers expected if the loci segregated independently (Thorgaard et al., 1983). The expected numbers in this table differ from those in a typical $r\times c$ contingency table, in that they are calculated as products of the frequencies of cross-over (y) and non-cross-over ($1-y$) gametes, assuming a 1:1 ratio of homozygotes. The significance threshold for these χ^2-tests was corrected for multiple testing.

Correlation of distances between markers obtained from the G–C analysis and distances between markers estimated from linkage analysis (Hubert and Hedgecock, 2004) was done, using a Spearman non-parametric test in SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

2.5. Centromere confidence interval

The 95% confidence interval around centromere markers for probable centromere location was calculated according to the formula $y/N\pm1.96\{[(y/N)(1-y/N)]/N\}1/2$, where y represents the number of heterozygous progeny for the indicated locus, N is twice the number of progeny, and $1/N$ substitutes in the second term if y is zero [Crown (1950) in Johnson et al. (1996)].

3. Results

3.1. Progeny genotypes

Seven triploid families were typed for four to 24 of 56 informative microsatellite markers and the *AmyA* gene; average sample size per locus ranged from 68.5 to 83.3 progeny across the families (Table 1). We first examined genetic evidence that individuals might be diploid rather than triploid. All individuals in families B, E, F, and G showed evidence of inheriting two maternal alleles for at least one locus and could be excluded as diploids. In family A, 46 individuals were homozygous at all markers assayed, but since a maximum of only four markers were typed in any individual and two of these markers had very few or no heterozygotes in any family (see Section 3.4 below), we could not exclude any of the individuals in family A as being triploids. In families C and D, however, we found 6 and 5 individuals, respectively, that appeared to be maternal homozygotes at all loci. In family C, two of these individuals were typed at 16 and 10 markers and had multilocus probabilities of being triploid and homozygous at all markers of 0.0002 and 0.00146, respectively. In family D, four individuals were typed at 12 to 16 markers and had probabilities of being triploid and homozygous at all markers that ranged from 0.019 to 0.002. We reject the hypothesis that these six individuals are triploids and exclude them from subsequent analyses. The adjusted triploid percentages for families C and D are, thus, 97.9% and 95.7%, respectively (Table 1).

Altogether, across all families and markers, we observed 93, progeny-genotype arrays (*11*:*22*:*12*, Table 2), comprising 7265 half-tetrad genotypes. We tested whether the two homozygous genotypes, *11* and *22*, were present in equal proportions in each case. Six of 93 tests were significant at the nominal 1% level: *ucdCg195*, in D; *ucdCg163*, in B; *ucdCg112*, in B; *ucdCg151*, in E; *ucdCg196*, in C; and *ucdCg179*, in B. We note that three of these significant deviations are in family B, two, involving markers on LG4, and the last, involving the unlinked marker *ucdCg179*. Of the 16 markers on LG4, six yielded tests significant at the nominal 5% level, four in family B (includng *ucdCg137* and *ucdCg164*) and two in family C (*ucdCg117* and *ucdCg112*). We excluded from further analyses estimates of y obtained in the six cases significant at the 1% level, since proportions of heterozygotes at these markers might have been affected not only by the probability of a cross-over between the centromere and the marker, but also by whatever caused deviation from the expected 1:1 ratio.

We next tested heterogeneity of y for loci tested in multiple families. In 19 cases, two families were typed for the same marker (and the ratio of the two homozygotes did not differ from 1:1 in either family); in another five cases, three families were typed for the same marker (and homozygotes were in 1:1 ratio), and in one case, four families were typed for *um2CgL10*. Homogeneity of y was rejected at the nominal 1% level of significance for nine loci: *cmrCg005*, *um2CgL48*, *ucdCg002*, *ucdCg164*, *ucdCg021*, *ucdCg014*, *ucdCg196*, *ucdCg171*, and *um2CgL10*. For the 16 loci with homogeneous y across families, we used the pooled estimate of y for further analyses. Among the four families typed for *um2CgL10* and three families typed for *ucdCg171* and *ucdCg196*, one family in each case had a statistically divergent y-value than the others; we calculated a second, pooled y for these loci, among the homogeneous families. All statistically independent y-values were retained for further analyses. The distribution of the resulting 64 statistically independent y-values is

uniform over the interval from zero to 1.0 (χ^2=21.97, 20 *d.f.*, P=0.342; Fig. 1).

3.2. New markers on the Pacific oyster map

To facilitate comparison of centromere and linkage maps throughout the remainder of this article, we retain Roman numerals for linkage groups (Hubert and Hedgecock, 2004) and use Arabic numerals for centromere-linkage groups. Microsatellite marker *uscCg205*, which Yamtich et al. (2005) reported to be 12 cM from *ucdCg130* on LGV was informative for family B. No heterozygotes were observed (n=89), and the 95% confidence interval for this centromere-linked marker is −1.10 cM to 1.66 cM. Tests for joint segregation show that it is significantly linked to *ucdCg14* and *ucdCg21* on LG5 ($P=2.4\times10^{-8}$ and 1.3×10^{-6}, respectively, both exceeding the Bonferonni adjusted significance threshold of $\alpha_{0.001}$; Table 3). In family C, *ucdCg130* is 36.9 cM from the centromere (and, by inference, from *uscCg205*), which appears to be significantly more than the distance previously reported by Yamtich et al. (2005). Marker *ucdCg010*, which was not previously mapped, was informative in family G and mapped 33 cM from the LG1a centromere, 7.5 cM proximal to marker *ucdCg191*.

Two unlinked markers reported by Hubert and Hedgecock (2004), *ucdCg179* and *ucdCg180*, were linked to centromeres in this study but remained unlinked to other markers. Informative in family D (homozygotes were not in 1:1 ratio in B), *ucdCg179* was 32.5 cM from a centromere that was unlinked to eight markers on seven different linkage groups. Also informative in two families, *ucdCg180* was 17.3 cM from a centromere but remained unlinked to eight markers on seven linkage groups in family D and 12 markers on eight linkage groups in family F. *AmyA* was 16.1 cM from a centromere that remained unlinked to 17 markers on 8 linkage groups in family G.

3.3. Marker-centromere recombination

The consensus linkage map for the Pacific oyster (Hubert and Hedgecock, 2004) was used to assign 53 markers to linkage groups. These were ordered by marker-centromere distances to produce a consensus centromere linkage map (Fig. 2). Again, nine loci are mapped to two positions on the marker-centromere map, owing to significantly different proportions of heterozygotes among families (see above).

Marker orders and distances among markers obtained by the half-tetrad analyses reported here are, in general, similar to those obtained in previous linkage analyses (Hubert and Hedgecock, 2004). One notable exception is rejection of LG I as a single linkage group (Hubert and Hedgecock, 2004). Markers linked to *imbCg044* and *ucdCg187* at either end of LG I were only weakly linked to each other in the female consensus linkage map, with a distance of 36.0 cM between *ucdCg191* and *ucdCg003* (Hubert and Hedgecock, 2004). At the same time, these two groups of markers were not linked in the male linkage map and were designated LG IA and LG IB by Hubert and Hedgecock (2004). In this study, *imbCg044* and four other markers were linked to one centromere, and *ucdCg187* and six other markers were linked to another centromere. Linkage mapping in an F_2 family unrelated to those studied by Hubert and Hedgecock (2004) and a re-analysis of the Hubert and Hedgecock (2004) data (G. M. L. Perry, D. Hedgecock, and M-L Voigt, unpubl.) also confirms that LG I comprises two subgroups, which correspond to centromere LG1a and 1b.

Distances among markers on LG2 of the centromere map are very close to those reported for LG II of the linkage map (Hubert and Hedgecock, 2004). The order of *ucdCg134* and *ucdCg148* is inverted in our map relative to the female consensus linkage map, and *um2CgL48* in family F is much more distal to *ucdCg195* than it is in family C, which has the tighter *um2CgL48–ucdCg195* linkage seen previously. LG3 also shows an inversion in the order of markers, relative to LG III, although the length of the *ucdCg002*, *ucdCg165*, *ucdCg109* and *imbCg049* subgroup is similar between linkage and centromere maps, 44 *vs.* 34.4 cM, respectively. LG4, on the other hand, shows two rearrangements of marker order relative to LG IV, as well as different distances between adjacent markers; this is addressed further in the linkage analysis section below.

LG5 is very similar to the male LG V and to subgroup LG VA of the female linkage map (shown in Fig. 2). We did not type the two markers, *ucdCg017* and *ucdCg173* that comprised female subgroup VB and thus lack direct evidence to consolidate VA and VB. Still, the addition of two loci, *ucdCg141* and *ucdCg130*, which were linked in male LG V but were not tested for the female linkage map, provides indirect evidence for a consolidated LG5.

LG6 is shorter than LG VI but has the same gene order. The distance between *ucdCg28*, which is tightly linked to the centromere (y=0.016 in 182 progeny from families A and G; Fig. 2) and *ucdCg156*, was 29.3 cM on the marker-centromere map but 44.9 cM on the linkage map. LG7, on the other hand, is longer than LG VII and differs from it by two inversions of gene order. The difference in length is mostly in the arm containing *ucdCg141–ucdCg203–ucdCg196–ucdCg175–ucdCg024*. On the centromere map, *ucdCg024* is distal to *ucdCg175* and *ucdCg196* is distal to *ucdCg203*, both of which represent inversions of their orders on LG VII (Fig. 2).

LG9 consolidates two subgroups of LG IX in the female consensus linkage map, by linking *ucdCg171*, which was on female LG IXA, across the centromere to *ucdCg140* and *um2CgL10*, which were both on LG IXB. (The two subgroups did form a single group in the male consensus linkage map.) The length of LG9 is greater than the sum of LG IXA and IXB. Marker *ucdCg171* has two locations on the centromere map, owing to heterogeneity of y among families; the distance from *ucdCg140* to the proximal location of *ucdCg171* is 31.6 cM, while the distance to the distal location of *ucdCg171* is 50.7 cM, which is consistent with the lack of linkage in the previous study. Finally, LG10 shows that *ucdCg200*, which is 19.5 cM from *ucdCg181* on the two-marker LG X, is 8.2 cM from *ucdCg181* on the marker-centromere map and 44 cM from the centromere.

3.4. Centromere confidence interval

Nine markers on six linkage groups—1a, 1b, 3, 4, 5, and 6—are closely linked to their centromeres (Fig. 2). The 95% confidence interval for marker-centromere distance ($d=y/2n$) includes the "zero" location of the centromere for seven of the nine markers (Table 4). Half-tetrad genotypes for four markers are available from two or three families; all of these markers show homogeneous proportions of heterozygotes (y). In LG1a, 4, and 6, two markers are located within the 95% confidence interval and further restrict centromere location.

3.5. Linkage analysis

Of the 618 pairwise tests of joint-segregation (Thorgaard et al., 1983), 38 were significant at the Bonferroni adjusted 5% level of

Table 3
Number of joint segregation tests, at three levels of significance, for unlinked and linked pairs of loci typed in seven triploid Pacific oyster families

Number of tests and results, for three levels of significance	Unlinked	Linked		Row sum
		Same LG	Same arm	
Number of loci pairs	569	17	32	618
$\alpha_{0.05}$, P=0.0000830	5	9	24	38
$\alpha_{0.01}$, P=0.0000163	5	8	24	37
$\alpha_{0.001}$, P=0.0000016	5	6	23	34

significance (P=0.000082). Of the significant cases, 33 were expected from evidence for linkage or centromere-linkage and 29 of these were significant at the 0.1% adjusted level of significance (Table 3). Only five of 569 joint segregation tests for markers known not to be linked were significant at the 0.1% level, eight times more than expected by chance. All of these unexpected significant cases, however, involved markers tightly linked to different centromeres (y=0; *imbCg049* vs. *ucdCg185* or *ucdCg197* in family F, *ucdCg 185* vs. *ucdCg157* in family F, *ucdCg 185* vs. *ucdCg003* in family B, and *imbCg044* vs. *ucdCg197* in family G). Owing to the absence of heterozygotes at these markers, expected values in five of nine cells in the 3×3 table are much less than one and the chi-square test is not valid. Near-equal numbers of individuals in the four categories of doubly homozygous individuals—*11,11*; *11,22*; *22,11*; and *22,22*—in all five tests show that these centromere-markers segregate independently.

Sixteen pairs of markers known to be linked did not yield significant joint segregation tests (Table 3). Markers in these pairs were significantly farther apart, on average 35.3 cM, than markers that showed significant joint-segregation, 21.4 cM (t=2.719, 47 *d.f.*, P=0.009).

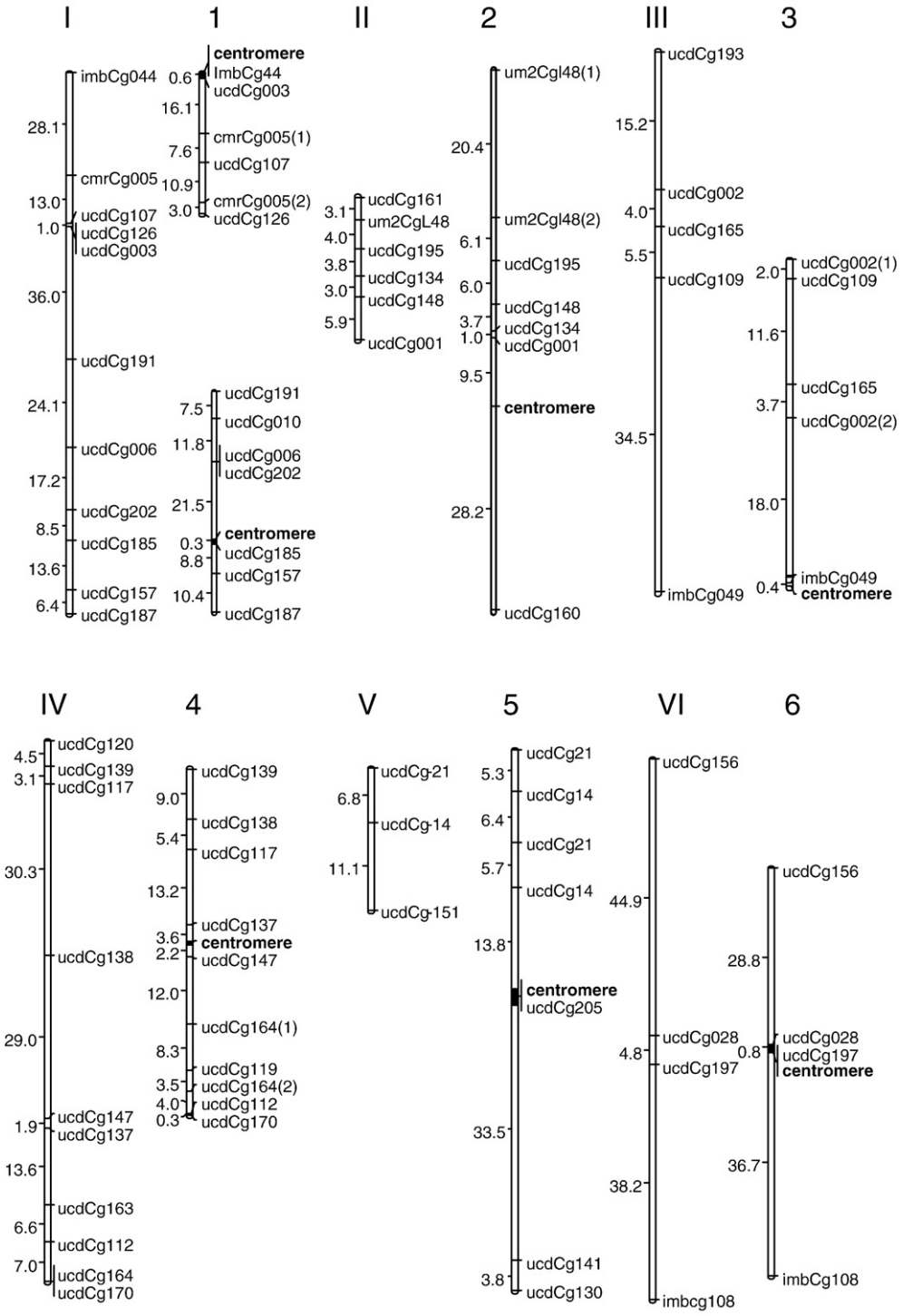

Fig. 2. Comparison between the female linkage map, left, LG in Roman numerals (Hubert and Hedgecock, 2004) and the centromere map, right, LG in Arabic numerals, for nine linkage groups of the Pacific oyster.

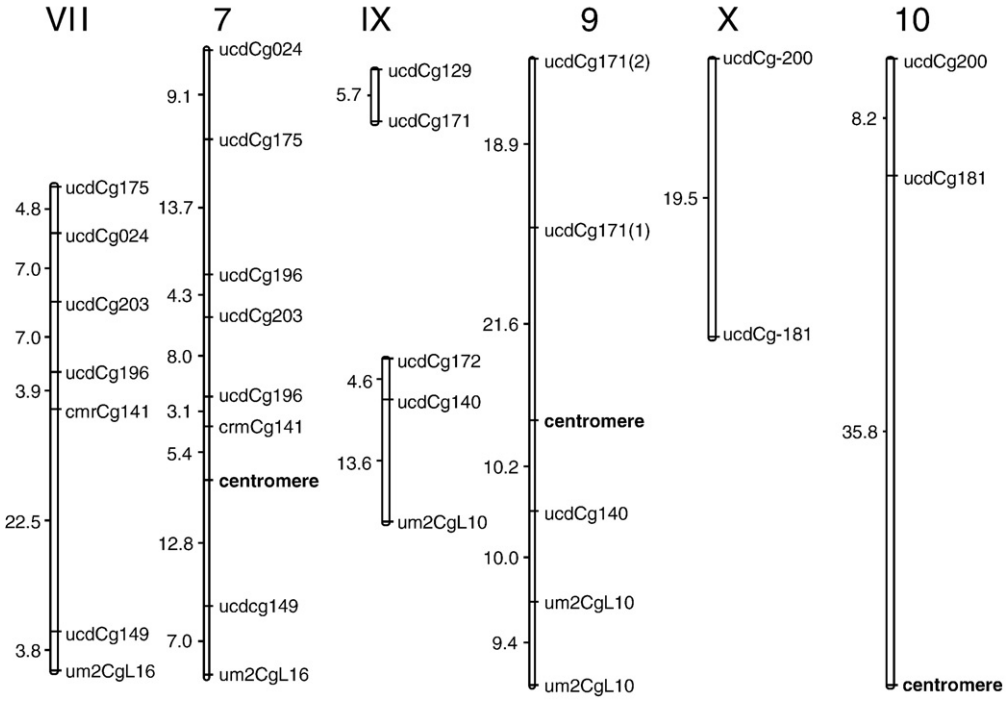

Fig. 2 (*continued*).

3.6. Interference and correlation of inter-marker distances from half-tetrad and linkage analyses

The frequency of heterozygotes in half-tetrad analysis, *y*, ranges from zero, if no cross-over occurs, to 1.0, with one and only one cross-over, and should be 0.67 for markers assorting independently of the centromere (Mather, 1935). Twelve of 64 independent *y*-values are greater than 0.67, suggesting interference. All linkage groups, except LG4, have at least one marker, with *y* greater than 0.67.

Assuming complete interference, distances between markers on the same linkage group can be estimated, either as the difference between the marker-centromere distances, if both markers lie on the same side of the centromere, or the sum of the marker-centromere distances, if markers lie on opposite sides of the centromere. Inter-marker distances were calculated in this manner for 25 pairs of markers, for which the joint segregation test was significant, at the adjusted α=0.05 level, and linkage information was available from the female consensus linkage map (Hubert and Hedgecock, 2004). A Spearman rank correlation between the two map distances is highly significant (r^2=0.866, P<0.000001, 23 *d.f.*), and correlation for marker pairs closer than 30 cM on the linkage map is even tighter (r^2=0.884, P<0.000001, 16 *d.f.*). Regression of marker-centromere distance on linkage distance, forced through {0,0}, has a slope of 1.06 for markers separated by 30 cM or less (Fig. 3). Six of seven marker-pairs separated by more than 30 cM on the linkage map fall well below the 1:1 regression line (open squares in Fig. 3), suggesting a lack of interference over longer distances. Five of these cases involve markers on LG4, however, which showed very large gaps on either side of *ucdCg138* on the consensus linkage map.

Detailed analysis of half-tetrad genotypes for LG4 allows reconstruction of the likely female haplotypes and reveals complex patterns of recombination, implying multiple cross-overs between markers,

Table 4
Confidence limits (95%) around markers closely linked to centromeres in triploid Pacific oyster families

LG	Locus	Families	Progeny genotypes		L 95% CL cM	U 95% CL cM
			11+22	12		
1a	*imbCg44*	G	82	0	−1.19	1.19
1a	*ucdCg003*	B	78	1	−0.60	1.87
1b	*ucdCg185*	F, B	159	0	−0.30	0.92
3	*imbCg049*	A, C, F	250	2	−0.15	0.95
4	*ucdCg137*	D, B	146	9	1.50	5.64
4	*ucdCg147*	C, G	148	2	0.61	3.96
5	*uscCg205*	B	89	0	−1.10	1.10
6	*ucdCg028*	A, G	179	3	−1.05	1.75
6	*ucdCg197*	F, G	144	0	−0.68	0.68

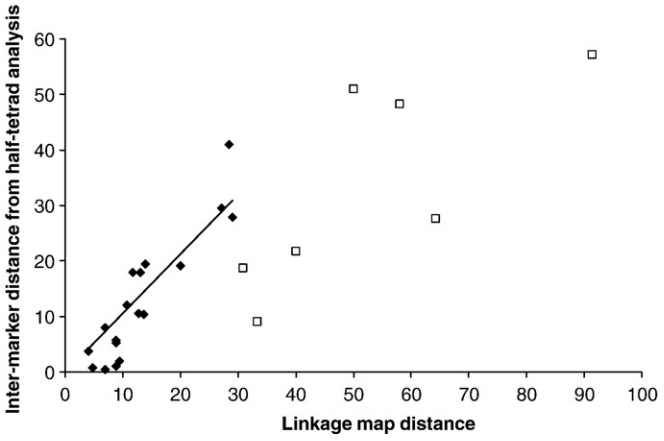

Fig. 3. Plot of inter-marker distances from half-tetrad analysis and from the female consensus linkage map (Hubert and Hedgecock, 2004), for 25 pairs of markers for which the joint segregation test was significant, at the adjusted α=0.05 level, and linkage information was available. Regression for 18 marker pairs closer than 30 cM on the linkage map (solid diamonds) has a slope of 1.06, when constrained to pass through {0,0}, and r^2=0.88; four of seven marker pairs separated by more than 30 cM on the linkage map (open squares) are on opposite sides of the LG4 centromere.

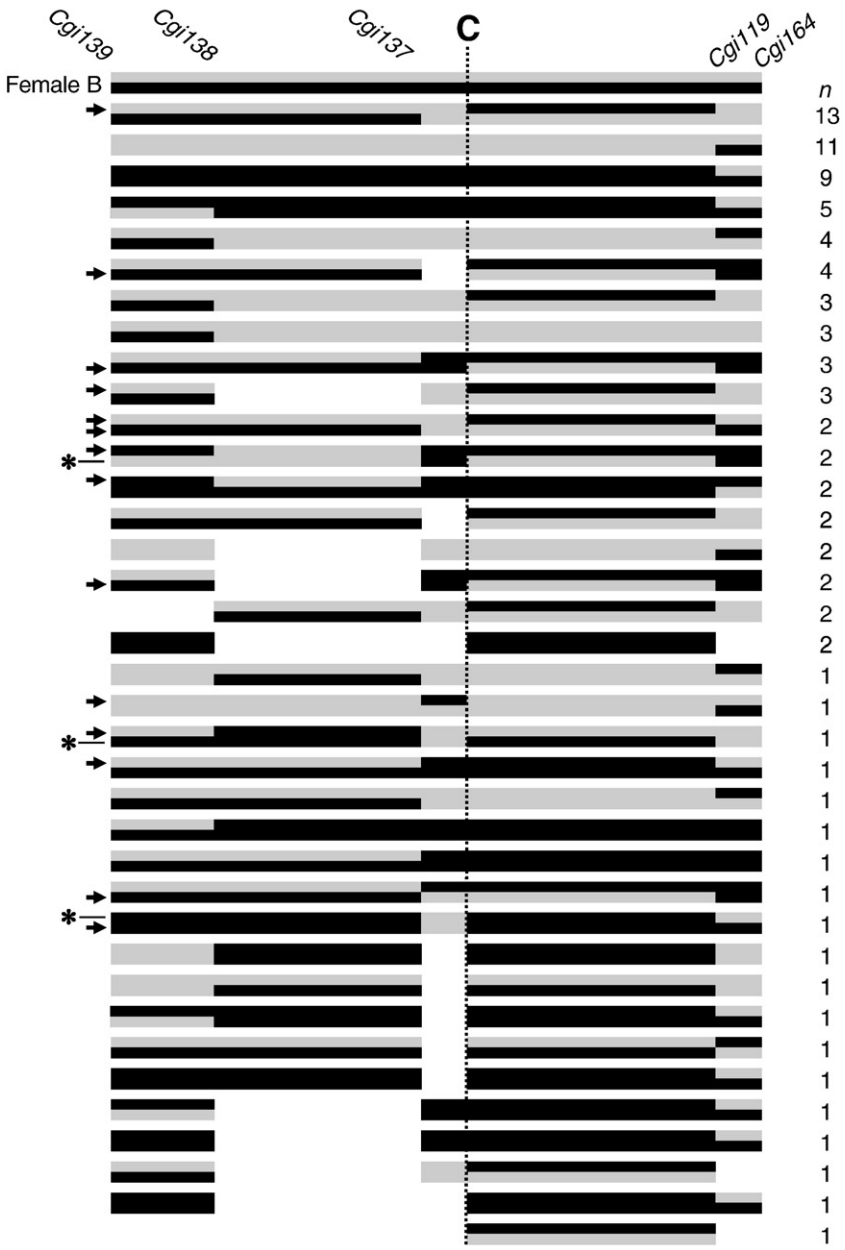

Fig. 4. Thirty-seven pairs of inferred non-recombinant and recombinant LG4 chromosomes recovered in triploid progeny of Pacific oyster female B. The linkage group is represented by five markers spanning 57.7 cM, with the centromere marked by a vertical dotted line, labeled "C." Column width is proportional to the distance between markers, estimated from marker-centromere distances, assuming complete interference. The two chromosomes present in female B are depicted as grey and black bars at the top of the figure. Each chromosome segment in female B's progeny is filled with grey or black to indicate genotype at the distal marker (gaps represent missing data); changes in color within a bar represent exchanges between non-sister maternal chromatids in the interval bounded by the markers. Double cross-overs are denoted by arrows at the left; triple cross-overs are denoted by asterisks. The frequency of each five-locus genotype in the sample of 93 individuals is shown on the right.

even markers on the same side of the centromere (Fig. 4). This reconstruction assumes an ordered likelihood of chromosomes—non-cross-over (NCO)>single cross-over (SCO)>double cross-over (DCO)>triple cross-over (TCO).

Inferring the number of cross-over events in each interval, following the methods of Thorgaard et al. (1983) or Danzmann and Gharbi (2001), generally increases the distance between markers, relative to that obtained by subtracting marker-centromere distances, which assumes complete interference. For example, the difference between the marker-centromere distances for *ucdCg138* and *ucdCg139* is 9.0 cM (Fig. 2), whereas an estimate of distance based on SCO between the markers plus inferred, two- or four-strand DCO is 18.2 cM (Table 5). The 3×3 joint segregation table for these two markers (Table 5) shows the significant association of the *1* allele at *ucdCg138* with the *2* allele at *ucdCg139* and conversely the *2* allele at the first locus with the *1* allele at the second. Eight DCO chromosomes are estimated from the observation of 4 individuals that are heterozygous at the proximal *ucdCg138* but homozygous at the distal *ucdCg139* (Table 5). Using information for the proximal, centromere-linked *ucdCg137*, four additional DCO may be inferred. For example, an individual heterozygous at the distal marker but homozygous at the proximal marker, which would normally be interpreted as the product of an SCO between the two markers, is homozygous at the proximal marker for an allele not in phase with the allele at the centromere marker, requiring one additional cross-over in each strand (Fig. 4). Likewise, the single individual in the upper left cell of Table 5, with the *11*, *11* genotype, had to have resulted from DCO in both sister chromatids. The total number of DCO enumerated is thus, 12, which is

Table 5
Joint segregation and recombination[a] between *ucdCg138* and *ucdCg139* in family B of triploid Pacific oysters

ucdCg139	ucdCg138			Totals
	11	12	22	
11	1 (4[b])	2 (6)	12 (4)	15
12	8 (14)	29 (21)	12 (14)	49
22	11 (4)	2 (6)	0 (4)	13
Totals	20	33	24	77

[a] Distance between the centromere and *ucdCg138* is estimated as 0.5×(33/77)×100=21.4 cM. Distance between *ucdCg138* and *ucdCg139* is estimated from inferred double and single cross-overs as 0.5×([(8+12)+2×(2+2)]/77)×100=18.2 cM.
[b] Expected numbers (in parentheses), under the null hypothesis of independent segregation, are calculated as the appropriate product of recombinant (33/77, 49/77) and non-recombinant frequencies ([1-(33/77)]/2, [1-(49/77)]/2) at each marker.

not different from the number expected with independent crossing over in each interval (calculated as the product of the recombination fractions in the two intervals and the number of individuals, 0.43×0.35×77=11.6). These recombinant phenotypes are unlikely to represent genotyping errors, since the gel images for these LG4 markers were very clear and were independently scored by at least two of the authors. There appears to be no evidence for interference on this arm of LG4; indeed, between other markers on LG4, we calculate negative interference, using these same methods (data not shown).

Across all intervals of LG4 in the triploid progeny of female B, we estimate that, of 186 chromosomes, 37 are NCO (19.9%), 102 are SCO (54.8%), 43 are DCO (23.1%), and four are TCO (2.2%). Similar analysis of 184 LG4 chromosomes in progeny of female C, which were typed for a different set of markers than family B—*ucdCg117*, *ucdCg147*, *ucdCg112*, and *ucdCg170*, spanning 46.9 cM—revealed a lower level of recombination, 88 NCO (47.8%), 80 SCO (43.5%), and 16 DCO (8.7%), but still substantially more recombination than expected with complete interference.

4. Discussion

4.1. Induced triploids as a mapping resource for bivalve molluscs

Triploids induced by inhibition of second meiosis in fertilized oocytes permit half-tetrad analyses. In our study, triploidy was induced by treatment of oocytes with cytochalasin B. Because such triploids are still made routinely by oyster breeders, as a step in producing new tetraploid male stocks, large families are readily available for centromere mapping studies. Triploid induction is not always 100%, however, which, if undetected, would bias downward estimates of gene-centromere recombination. Still, with enough markers, putative diploids, which show no evidence of having inherited both maternal alleles at any locus, can be reliably excluded. We found genetic evidence that two of seven families had small proportions of diploids, which were not detected by flow cytometry of early larvae.

An expected advantage of using induced triploid oysters, rather than gynogenetic diploids for half-tetrad analysis, should be a reduction in distortion of Mendelian segregation ratios caused by recessive deleterious mutations, which are common in Pacific oysters and other bivalves (Bierne et al., 1998; Launey and Hedgecock, 2001; Li and Guo, 2004). Unequal numbers of homozygotes, which are expected to be in a 1:1 ratio in half-tetrads, have been observed previously in shellfish (Guo and Gaffney, 1993; Guo and Allen, 1996; Li and Kijima, 2006) and fish (Allendorf et al., 1986; Guyomard, 1984; Johnson et al., 1987; Lindner et al., 2000; Seeb and Seeb, 1986; Thorgaard et al., 1983), but most of these studies used gynogenetic diploids, in which zygotic selection against linked recessive deleterious mutations was likely a major factor. Zygotic selection can also act against independent recessive deleterious mutations linked to both alleles at a marker, depressing the overall proportion of homozygotes but leaving, perhaps, the 1:1 ratio of homozygotes intact. Such selection would not be detected as a distortion of the 1:1 ratio of homozygotes but would inflate the proportion of heterozygotes, *y*, and thus the distance of the marker from the centromere. This effect of zygotic selection is discussed in the last section on interference. The presence of a third, unrelated genome in chemically induced triploids would presumably prevent selection against recessive alleles.

Our triploid oyster families were not free of distorted ratios of homozygotes; ratios of homozygotes in six of 93 (6.4%) half-tetrad analyses were significantly skewed, ranging from 1:1/2 to 1:1/3. Compared, though, with one-third to one-half of 40–60 markers commonly being distorted in first generation inbred or F_2 families (Bucklin, 2002; Launey and Hedgecock, 2001; McGoldrick and Hedgecock, 1997; G. M. L. Perry, D. Hedgecock, and M-L Voigt, unpubl.); the much lower level of distortion observed in our families supports the hypothesis that much of the genetic load in oysters owes to recessive mutations, which are suppressed in triploids. Six cases of skewed homozygote ratios out of 93 cases studied could be attributed to chance, but we expect only one such case. If not explained by chance, such observations could be explained by recessive mutations, acting during embryonic development, before paternal genes are expressed. Previous studies of F_2 families uncovered few distortions of Mendelian segregation ratios in early larvae (Launey and Hedgecock, 2001; Plough et al., 2008). Dominant mutations linked to the observed markers might also explain deviations from 1:1 homozygote ratios in triploid families, although the persistence of dominant mutations in the natural population, from which our stocks were derived, would be difficult to explain, considering their highly detrimental effects on early larval survival. Finally, linkage of markers to mutations affecting meiosis and segregation in the female, a phenomenon known as meiotic drive (see Crow, 1970; Ubeda and Haig, 2005), could explain skewed ratios of homozygotes. Mutations causing meiotic drive can persist in natural populations, when balanced by detrimental effects on viability and, especially, if they are involved in inversions (Dyer et al., 2007). Distortions of 1:1 ratios of homozygotes in induced triploids merit closer attention in future studies. Since the cause(s) of non-1:1 homozygote ratios could potentially affect the proportion of heterozygotes and compromise estimates of marker-centromere linkage, we eliminated these cases from further analyses.

4.2. Centromere mapping in the Pacific oyster

Our half-tetrad analyses suggest that two linkage subgroups previously reported for the female consensus linkage map, LG VA, VB and LG IXA, IXB (Hubert and Hedgecock, 2004), should be consolidated into LG5 and LG9, respectively, which accords with the male consensus linkage map. Consolidation of linkage subgroups, eventually to the number of chromosomes, is one of the chief benefits of half-tetrad analyses (Johnson et al., 1996). On the other hand, we confirmed that the LG I previously posited for the consensus female linkage map should be split into LG1a and LG1b, as in the male consensus linkage map and other F_2 maps generated since the linkage map was published (Hubert and Hedgecock 2001; G. M. L. Perry, D. Hedgecock, and M-L Voigt, unpubl.).

Six of the expected 10 centromeres of the Pacific oyster genome are linked to nine microsatellite DNA markers so closely that 95% confidence intervals (CI) around marker-centromere distances overlap zero for seven markers and for all LG but 4. No heterozygotes were observed at *imbCg044* (LG1a, $n=82$), *ucdCg205* (LG5, $n=89$), and *ucdCg197* (LG6, $n=144$); for four other markers, whose 95% CI included

zero, distances from marker to centromere ranged from 0.3 cM to 0.8 cM. Three of the six linkage groups, 1a, 4, and 6, have two markers closely linked to the centromeres (Fig. 2).

Centromere markers, in combination with half-tetrad analyses in induced triploid families, are a valuable genomic resource for the Pacific oyster, because they permit the rapid assignment of new markers to a linkage group (Johnson et al., 1996). From our data set, we estimate the "type II error" of obtaining a significant joint-segregation test (at a nominal α=0.01 level of significance) between one of these centromere markers and an unlinked marker as P=0.041 (7 of 171 tests). This error rate was homogeneous among the nine centromere markers. Screening new markers against these centromere markers in chemically induced triploid families should thus facilitate assignment to linkage group. Chances of incorrect assignment could be reduced to very low levels or eliminated by setting threshold significance levels lower; at α=0.001, for example, we obtained no incorrect assignments out of the 171 tests of unlinked markers. Incorrect assignments would quickly be determined in further linkage tests in any case. The availability of multiple markers for some centromeres also provides further protection against false assignment.

Despite the progress represented by the half-tetrad analyses reported here, present linkage and centromere maps of microsatellite DNA markers for the Pacific oyster are revealed as incomplete. Only six of 10 centromeres are marked, three markers remain unlinked (*ucdCg179*, *ucdCg180*, *AmyA*) despite linkage to a centromere, and three centromere linkage groups have only one arm, whereas cupped oysters, in general, have only metacentric and sub-metacentric chromosomes and the Pacific oyster, in particular, has only metacentric chromosomes (Leitao et al., 1999).

4.3. Map heterogeneity: polymorphism for chromosomal rearrangements?

Heterogeneity among families in the proportion of heterozygotes, *y*, at nine of 25 markers that were typed in more than one triploid family, substantiates previous evidence for variation in recombination rate in the Pacific oyster (Hubert and Hedgecock, 2004). These nine markers were distributed over seven linkage groups. Unfortunately, there were not enough informative markers in common among the various triploid families to test variation in gene order within a chromosome arm. However, by reference to the gene orders obtained in the previous linkage analysis, we infer that gene orders do vary for LG3, 4, and 7. More dense linkage and centromere maps are needed to confirm whether the Pacific oyster is polymorphic for chromosomal rearrangements.

4.4. Interference is less than previously believed

Linkage map distances are expected to correlate with marker-centromere distances if there is complete or nearly complete interference of crossing-over, although linkage map distances may exceed marker-centromere estimates for longer chromosome arms (Danzmann and Gharbi, 2001). The high correlation that we obtained between linkage and centromere distances, particularly for markers separated by 30 cM or less (Fig. 3) suggests that interference is high and that maps based on marker-centromere distances will be accurate when compared to linkage analysis. Similar correspondences have been observed in other species; for example, good agreement between map and centromere distances was found for salmon (Johnson et al., 1987). However, agreement of linkage and centromere maps may obtain, even when interference is less than complete or absent (Danzmann and Gharbi, 2001). Partial negative interference, in which a chromatid that has already undergone a cross-over event has an elevated probability of undergoing a second recombination event with the other non-sister chromatid, leaves distal markers heterozygous and gives the mistaken impression that only a single cross-over has occurred. Also, heterozygosity of distal markers is retained whenever there is an odd number of cross-overs between non-sister chromatids.

If the four chromatids produced in early MI (AAaa) separate at random, reductional separation (AA or aa) can only occur one way, while equational separation (Aa, Aa) can occur two ways. Therefore, the maximum recombination rate in relation to the centromere is 0.67 (Mather, 1935). Previous estimates of marker-centromere recombination have concluded that strong interference is present in bivalves, with second division segregation higher than 0.67 in all seven allozymes markers in *C. gigas* (Guo and Gaffney, 1993), in seven of eight microsatellite DNA markers in *C. gigas* (Li and Kijima, 2006) and in 11 out of 13 allozyme markers in *M. lateralis* (Guo and Allen, 1996). Most *y* values, moreover, were higher than 0.95 suggesting very strong interference. High interference was also reported in half-tetrad analyses of rainbow trout (Allendorf et al., 1986; Guo and Allen, 1996; Thorgaard et al., 1983). Our finding that 52 of 64 independent estimates of *y* are less than 0.67 does not agree with strong interference. The broad range in *y*-values seen in our study of triploid oysters does agree with a study in pink salmon, which showed a range of *y*-values, from 0 to 1.0, for allozymes and microsatellites (Lindner et al., 2000). Earlier, Stanley et al. (1984) reported no increase in heterozygosity at five enzyme loci in triploid *Crassotrea virginica* produced by blocking the second meiotic division, an unexpected result suggesting low marker-centromere recombination rates (Allendorf and Leary, 1984).

Mortality of identical-by-descent homozygotes, in the Pacific oyster and probably other bivalves, is explained by linkage of markers to deleterious recessive mutations (Launey and Hedgecock (2001). Because previous half-tetrad analyses in oysters used gynogens, selection against linked recessive mutations might have inflated the proportion of heterozygotes, leading to an overestimate of *y* and a false impression of high interference. Half-tetrad analyses of the dwarf surf clam, *M. lateralis*, however, used both gynogenetic and triploid individuals, which should have reduced this bias in *y* caused by selection. In their preliminary study of *M. edulis*, Beaumont and Fairbrother (1995) found a strong difference in gene-centromere recombination frequencies between triploid and gynogenetic mussels for two allozyme loci, which supports the ameliorating influence of the extra male chromosome in triploids.

Half-tetrad analysis of eight microsatellite markers in 24-h-old, gynogenetic *C. gigas* diploids yielded *y*-values ranging from 0.62 to 0.74 (Li and Kijima, 2006). However, heterogeneity of *y*-values among families is apparent in the raw data reported by these authors for *um2CgL48* and *imbCg108* (their Table 1), with statistically independent *y*-values of 0.783 and 0.533 for the former and 0.817 and 0.500 for the latter. Still, most of the *y*-values are greater than 0.67. Seven of eight microsatellite markers used by Li and Kijima (2006) were included in our study and six yield statistically heterogeneous *y*, when compared to our data (*um2CgL10*, *um2CgL16*, *um2CgL48*, *imbCg49*, *imbCg108*, and *ucdCg014*). For example, Li and Kijima (2006) reported a *y*-value of 0.62 (actually, 0.60 for their pooled data) for *imbCg049*, whereas we found this marker to be tightly linked (y=0.008) with the LG3 centromere. Differences in centromere mapping results cannot be attributed easily to the triploids *vs.* gynogens difference, since Li and Kijima (2006) sampled 1 day-old larvae, when few recessive mutations are expressed.

Under complete interference, the chromosomes recovered in half-tetrad progeny should appear to be homozygous near their centers and heterozygous near their tips. Thus, the chromosomes recovered in family B (Fig. 4) should have centers of the same color with bi-colored tips on one or possibly both arms. Although the tendancy for distal markers to be heterozygous is evident, more complicated cross-over patterns appear to be the norm. Direct estimates of interference for pairs of markers on this linkage group indicate that interference may be zero (Table 5) or even negative. This evidence against high interference is consistent with a discrepancy between cytological observations vs.

linkage estimates of genome size. An early study reported no more than one chiasma per chromosome arm in the Eastern oyster (Longwell et al., 1967), and Li and Guo (2004) subsequently reported averages of 1.1 to 1.2 chiasmata per chromosome in the Eastern and Pacific oysters. Genome size from the frequency of chiasmata was thus estimated to be 550–600 cM. Subsequent linkage mapping suggests, however, that the genome is 32% to 99% larger than this cytological estimate (Hubert and Hedgecock, 2004; Li and Guo, 2004). The greater recombination revealed by half-tetrad chromosomes suggests that cross-overs may be more frequent than direct observation of chiasmata would suggest. Chiasmata may be resolved too rapidly ("terminalized") for cytological observation.

5. Conclusion

Half-tetrad analysis of markers in induced triploid progeny is a valuable tool for genetic mapping in the Pacific oyster and other bivalve species. Most markers (88/93) show 1:1 ratios of homozygotes, as expected, and marker-centromere distance are calculated for these cases as one-half the proportion of heterozygotes, *y*. Heterogeneity of *y* is observed in 9 of 25 cases in which markers were typed in more than one family, suggesting variation in gene-centromere distances among families. Although inter-marker distances correlate closely with recombinational distances among markers separated by 30 cM or less on the published linkage map (Hubert and Hedgecock, 2004), gene orders are often different between the two maps, suggesting that the Pacific oyster genome may harbor chromosomal rearrangements. Nine markers are very tightly linked to six centromeres and permit rapid assignment of future markers to these linkage groups. High interference, inferred in previous studies from high *y*-values, was not supported by our large study, in which 64 estimates of *y* ranged uniformly over the interval from zero to 1.0. Detailed analysis of segregation patterns on LG4 reveals no or even partial negative interference. Interference of recombination appears not to be as high in the Pacific oyster as previously supposed.

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