FINAL YEAR NARRATIVE FOR R/A-120

MEIOSIS IN TETRAPLOID PACIFIC OYSTERS, THEIR TRIPLOID MOTHERS, AND DIPLOID GRANDMOTHERS

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INTRODUCTION

The Pacific oyster occurs on all continents but Antarctica (Mann 1979) and for the past several years has had the highest annual production of any freshwater or marine organism (4.2 million metric tons in 2002, worth $3.5 billion; FAO 2004). About 92 million pounds of Pacific oyster meat were produced on the West Coast in 2000, with a farm-gate value of nearly $70 million (Toba and Chew 1999). West Coast production exceeds the harvest of Eastern oysters on either the Gulf or Atlantic Coasts, primarily because diseases have wiped out those natural stocks. As the Pacific oyster does not naturally reproduce along much of the West Coast, seed was imported from Japan for decades (Chew 1984). In the early 1980s, however, the industry adopted hatchery techniques and now relies mainly on hatchery seed. This sets the stage not only for commercial breeding programs, which industry identifies as a top priority (Pacific Shellfish Institute 1999), but also for development in the future of a mature seed industry servicing global markets.

The hatchery-based Pacific oyster industry on the U.S. West Coast adopted triploid seed technology nearly two decades ago, as a means of producing oysters in all seasons (Allen et al. 1989). Triploid oysters, which are here symbolized as 3n, where n is the haploid chromosome number, 10, are important in aquaculture because of near sterility, superior growth and meat quality, and sometimes-increased survival and disease resistance.

Tetraploid oysters (4n) are revolutionizing commercial production of triploid oyster seed, because a tetraploid-by-diploid cross (4n × 2n) yields all-triploid (3n) offspring. This eliminates dependence on cytotoxins to induce triploidy and greatly reduces ecological risk of hatchery stocks spawning in the wild. West Coast hatcheries have now developed tetraploid brood stocks through collaboration with 4Cs Breeding Technologies, Inc. (4Cs), which has exclusive license for tetraploid technology from Rutgers University.   The next stage in tetraploid development is to breed and manage the mature tetraploid brood stocks that exist. Simple crosses of tetraploid oysters, 4n × 4n, will produce tetraploid offspring, but industry needs a breeding plan for tetraploid broodstocks. Should tetraploid populations be inbred and hybridized to capture the hybrid vigor seen in diploid Pacific oysters, or should tetraploids be selected from pedigreed breeding populations? Is inbreeding depression going to be a problem? Will selection work? Answers to these and other questions on how to breed tetraploid stocks depend greatly on knowing how genetic segregation proceeds during meiosis in 4n oysters and their 3n mothers and 2n grandmothers.

OBJECTIVES

The goal of this project was to support the development of commercial tetraploid brood stocks, by contributing to fundamental understanding of meiosis and genetic segregation. The project tested two hypotheses: (1) H_0: Complete interference of secondary crossovers at the first meiotic division (MI) retards inbreeding of distal markers in triploid offspring produced by chemically blocking the second meiotic division (MII; Fig. 1A), as previously hypothesized by

Guo and Gaffney (1993); and (2) H_0: Segregation of quadrivalent chromosomes at the first meiotic division (MI) in tetraploid oysters is balanced (*i.e.* two pairs of chromosomes segregate to opposite poles) and random (*i.e.* crossovers and chromosomal exchanges are random among chromatids (Fig. 1B). We were unable to test a third hypothesis, that segregation of trivalent chromosomes at MI in a triploid female oyster is random, leading to unbalanced division. The project objectives were: (1) to make triploid progeny from replicated, controlled, pairwise crosses between chemically induced and naturally produced tetraploid males and inbred diploid females; (2) to make triploid backcross and outcross progeny, by chemical inhibition of second polar body formation in eggs of pedigreed hybrid females fertilized with sperm from related and unrelated inbred males; (3) to begin genotyping these two categories of triploid progeny for microsatellite DNA markers, in order to check segregation patterns in the tetraploid male parents of (1) and the diploid female parents of (2).
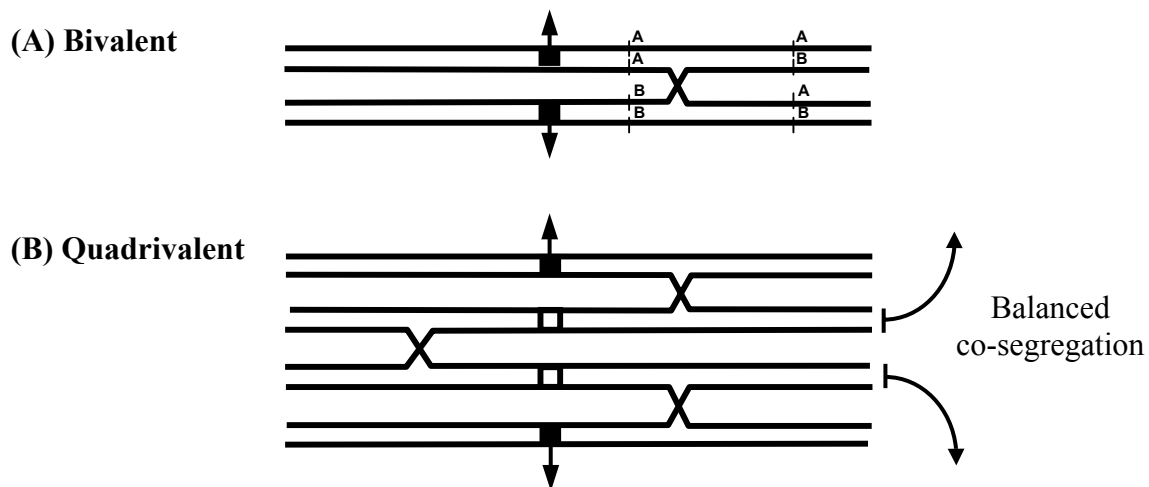

Fig. 1. Schematic segregations of chromosomes at anaphase of meiosis I in diploid and tetraploid Pacific oysters (after Guo and Allen 1997). (A) Bivalents segregating in normal diploids; heterozygous proximal and distal markers on one arm in the diploid chromosome illustrate the different consequences of crossing over and recombination. (B) Balanced segregation of two extra chromosomes from a quadrivalent in an autotetraploid.

METHODS

The approach used to understand details of meiosis in oysters was genetic rather than cytological, because chromosome-specific markers are not available nor would the localization of crossovers be as precise as that afforded by already mapped markers. Meiotic segregation and recombination of chromosomes was followed by means of mapped microsatellite DNA markers in the Pacific oyster (Hubert and Hedgecock 2004), assayed by methods described (*op. cit.*) and elsewhere (Launey and Hedgecock 2001; Li et al. 2003).

Chemically Induced Triploid Families. Eight triploid families were obtained for this study from Dr. Benoit Eudeline of Taylor Resources Inc., who induced triploidy chemically, by application of cytochalasin B to inhibit second meiosis (MII) in fertilized Pacific oyster eggs. That each family was 100% triploid was confirmed by flow cytometry. To determine the frequency of crossing over between chromosome centromeres and mapped markers and to detect whether or not there was interference of crossing over in MI, we need female parents to be heterozygous at a locus and the male parent to be either homozygous or heterozygous but for

alleles that could be distinguished from those of the female. Triploids produced by MII inhibition are homozygous in the absence of a crossover between the centromere and the marker, but heterozygous if such a crossover occurs (Fig. 1A). The proportion of heterozygous progeny is a measure of the frequency of crossing over followed by second division segregation (y) and thus gene-centromere distance can be estimated by $(1/2)\,y$ (or $100y/2$ centimorgan). With no crossing-over, y is zero; with complete interference, which means one and only one crossing-over between the centromere and the marker, $y=1.0$, which is what was observed by Guo and Gaffney (1993) for allozyme loci.

Segregation in Tetraploid Male. Tetraploid parents were second-generation, mated tetraploids, which were created from a chemically induced tetraploid founder (see Guo et al. 1996). In 1996, tetraploid founders were created from the eggs of two triploid females and 4 diploid males. In 1998, A and B lineages were created by crossing 1 tetraploid female and 2 males for lineage A and 1 female and 3 males for lineage B. In 2000, individuals from each lineage were crossed to generate the second-generation, mated tetraploid males used for this experiment. For this study, eight tetraploid males were each crossed with a diploid female to produce triploid progeny. Progeny were sampled at 11 days post-fertilization and stored in 70% ethanol. To investigate genetic differences between the A and B lineages or among the parents within these lineages, we tallied levels of polymorphism for each parent and subjected these data to a nested analysis of variance, with the four individual parents nested within their respective lineages. Variance between lineages was tested by variance among parents within lineages, and variance among parents was tested with the residual variance.

To determine whether segregation of alleles from the tetraploid male is balanced and random, we require that a tetraploid male parent have four alleles at a marker, each of which is distinguishable from the others and from alleles present in the diploid mother of the triploid offspring. Balanced segregation should always result in two of the four male alleles being transmitted to offspring; furthermore, balanced and random segregation from the male ABCD should yield six types of offspring, categorized from the standpoint of male gametic contributions, AB-, AC-, AD-, BC-, BD-, and CD-, in equal proportions. Since proportions of the six gametic types may be affected by random deviations in the frequencies of male alleles in the progeny, we first test whether male allele frequencies in the offspring conform to expected 1:1:1:1 proportions. We then calculate expected gametic frequencies, using observed allele frequencies and conditional probabilities, e.g. $P(AB) = P(A)*P(B|A) + P(B)*P(A|B)$, where $P(AB)$ is the expected probability or frequency of the AB male gamete in the progeny and $P(B|A)$ is calculated by dividing the frequency of the observed B allele by the sum of the observed non-A allele frequencies. We then used a chi-square goodness-of-fit test to see if observed gamete frequencies fit those expected under the hypothesis of random assortment of male alleles into gametes.

RESULTS

Gene-centromere recombination. We typed eight female parents and their triploid progeny for 92 microsatellite DNA markers, of which 56 were heterozygous in at least one female parent. Fifty-two of these markers are located on nine of the 10 linkage groups of the Pacific oyster (Hubert and Hedgecock 2004); four markers are unlinked (Table 1). Tests of the equality of the two homozygous classes reveal only a single case, in which these deviate from a 1:1 ratio, at *Cgi-112* on linkage group 1. This deviation is observed in two families, which are homogenous

(*G*-test = 2.10, *P*=0.35), with the pooled data being 55 AA: 20 BB: 90 AB. In this case, we follow Thorgaard et al's (1983) advice to estimate the proportion of heterozygous progeny, *y*, as 90/(2*55) rather than 90/(55+20). Twenty-eight other markers were also studied in two or more families. Testing the heterogeneity of genotypic proportions among families, we find nine markers that show significant heterogeneity among families, which is consistent with evidence for linkage heterogeneity and chromosomal inversion polymorphism presented by Hubert and Hedgecock (2004). For these cases, we keep separate the estimates of gene-centromere recombination; for the remaining 20 loci, we pool data over families to estimate gene-centromere recombination frequency.

Gene-centromere recombination frequencies, estimated from 68 independent observations of the proportion of heterozygous progeny (*y*), range from 0% to 93% (Fig. 1). The large range in *y* falsifies the general hypothesis of complete interference that was based on a study of allozyme segregation (Guo and Gaffney 1993), although 15 values of *y* are above the 0.67 expected for freely recombining markers. This may mean that there is a tendency for only one chiasma to form per chromosome arm, as observed cytologically (e.g. Longwell et al. 1967), and that the small number of allozyme markers studied by Guo and Gaffney are located, on average, more distal on chromosome arms than the average microsatellite DNA marker. This hypothesis can be tested ultimately by mapping the allozyme loci. The implication of this result for tetraploid development is that complete interference will not retard the inbreeding that results from chemical inhibition of second meiosis in the eggs of diploid grandmothers of tetraploid lineages.

Five markers – two on linkage group 1, one each on linkage groups 4 and 6, and one unlinked – show no recombination with the centromere. These markers should help localize centromeres or other markers on these linkage groups (Johnson et al. 1996); in the case of linkage group 1, the markers are on opposite ends of the linkage group, which may indicate either a rare double recombinant for one of these markers or a mistake in the gene order for this linkage group.

Meiotic segregation in tetraploid males. We typed 41 loci in the oyster genome, distributed across seven linkage groups, in eight tetraploid males and their female mates. An average of 35.125 loci were sampled for each individual, with an overall level of polymorphism of 2.39 alleles per locus. Levels of polymorphism do not differ significantly between lineages (Table 1). Lineage A has an overall level of polymorphism of 2.29 alleles per locus, whereas lineage B is modestly greater, with 2.45 alleles per locus. Differences among parents within lineages are marginal significant (*P* = 0.065). All parents have an average of at least two alleles per locus, with five of the parents having a level of polymorphism greater than 2.4 alleles per locus. Two of the remaining three parents are moderately close with 2.2 and 2.3 alleles per locus; parent 4 of lineage A is the least polymorphic parent with 2.03 alleles per locus. The average number of alleles per locus in these tetraploid males is about twice what we have observed in unrelated diploid Pacific oysters and is thus consistent with their tetraploid status.

Progeny were genotyped in 12 cases, for which a given male parent had four alleles at a particular microsatellite DNA marker (Table 2); an average of 74.6 progeny were genotyped in each case. The 12-locus/family cases are distributed across six parents and four linkage groups (Hubert and Hedgecock 2004). Parents A3 and B4 were each polymorphic for four alleles at a

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locus that could not be scored in the offspring. Differences between observed and expected allele frequencies are not significant for any of the loci genotyped (Table 2); allele frequencies range from conforming nearly perfectly to expectations (χ^2=0.13, P=0.99) to being marginally distorted (χ^2 = 5.82, P=0.12).

We detected one or three, rather than the expected two paternal alleles in a few progeny, for nine of the 12-locus/family cases studied. These progeny suggest a low level of transmission of unbalanced, aneuploid gametes from tetraploid males; however, this is a much lower rate than that previously reported for chemically induced tetraploids (18-35%; Guo and Allen 1997). The maximum number of aneuploid offspring observed in any family in this study was three, with the average proportion of aneuploid offspring being 0.013. Three family/locus cases showed no aneuploidy. Two potential causes for the lower rate of aneuploidy observed in this study, compared to Guo and Allen (1997), are the use of mated rather than chemically induced tetraploids and the analysis of 11-day old larvae rather than 2-cell stage embryos.

Segregation is random at the 12 family/locus pairs we sampled. Observed haplotype frequencies in all tests are in agreement with expectations, taking into account the observed frequencies of alleles (Table 2). Two families do show relatively high, though non-significant χ^2 goodness-of-fit values at locus *ucdCgi-194*. The test, yielding the highest χ^2 value (B3/*ucdCgi-194*), shows a pattern of segregation suggestive of preferential chromosome pairing. In this case, there is a hierarchical pattern, with two gametes (AD and BC) showing the highest frequencies, one set of reciprocal gametes (AB and CD) showing the lowest frequencies and the remaining set with somewhat intermediate frequencies. Though not statistically significant, this pattern may indicate weak non-random segregation. The second family (A2/*ucdCgi-194*) does not show any clear pattern of preferential segregation.

Meiotic segregation in polyploids is generally correlated with the origin of the polyploid. Autopolyploids, formed by a doubling of the diploid chromosome complement within a species, generally exhibit tetrasomic inheritance with random segregation of chromosomes, whereas allopolyploids, formed by interspecific hybridization followed by chromosome doubling, show preferential pairing of homologous chromosomes. That tetraploid Pacific oysters exhibit random segregation at all loci examined is thus consistent with their autotetraploid origin. All loci examined conform statistically with the expected random segregation pattern. Locus *ucdCgi-194*, was slightly distorted in two of three families. Family B3 exhibited a pattern of inheritance suggestive of preferential pairing, but family A2 did not. Thus, although preferential pairing may occur in these tetraploids, any such pairing is weak.

Tetrasomic inheritance suggests that the response of tetraploid Pacific oysters to inbreeding and selection will not differ greatly from the responses of normal diploid stocks, although the rate of response may be somewhat slower in tetraploids. Of particular concern at this point in the development of tetraploid stocks is the danger of inbreeding and inbreeding depression, because oysters carry a very large load of deleterious recessive mutations (Launey and Hedgecock 2001). The tetraploid stocks that are being used by commercial hatcheries have all been derived from a narrow genetic base, the two lineages represented in our study, which were in turn derived from two triploid females and four diploid males. While allelic diversity in these lineages seems appropriate to their tetraploid status, at this point in time, allelic diversity

and heterozygosity could well decline in the future, unless care is taken to avoid consanguineous crosses and to broaden the genetic base of these stocks by creating new tetraploid lineages.

DISSEMINATION OF RESULTS

Preliminary data on gene-centromere mapping was presented to the International Plant and Animal Genome Conference in 2002 by Dr. Hedgecock (abstract online).

Hedgecock, D., S. Hubert and K. Bucklin. 2003. Linkage and gene-centromere maps of the Pacific oyster *Crassostrea gigas.* http://www.intl-pag.org/11/abstracts/W05_W36_XI.html

A manuscript to be submitted to a peer-reviewed journal is in preparation by Dr. Sophie Hubert, the first postdoctoral scholar to work on this project. Results of our analysis of meiotic segregation in tetraploid oysters have been and will continue to be discussed with Taylor Shellfish Farms and other commercial oyster hatcheries and with Dr. Stan Allen, whose company, 4Cs Breeding Technologies, Inc., holds an exclusive license for the tetraploid technology. A manuscript on the tetraploid results to be submitted to a peer-reviewed journal is also in preparation by Dr. Jason Curole, the second postdoctoral scholar to work on this project.

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Table 1. Genomic distribution of 56 loci assayed in three sets of triploid progeny from heterozygous females. For loci tested in two or more families (column 3), segregation ratios are tested for heterogeneity, with the number of significant results ($P<0.05$) shown in column 4. Only one locus on linkage group 4 showed a significant inequality in the ratio of the two homozygotes.

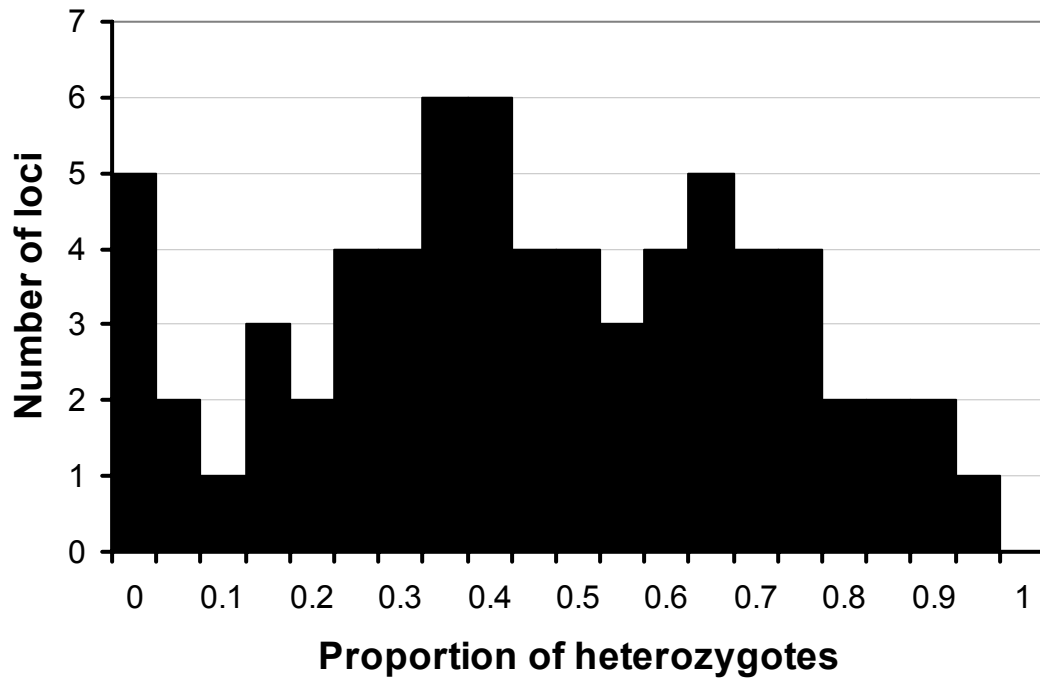| Linkage group	No. of loci	No. loci tested in ≥2 families	No. loci hetero- geneous among families	No. loci at which AA≠BB
1	11	3	1	
2	6	3	1	
3	4	4	1	
4	10	5	1	1
5	5	3	2	
6	4	3	1	
7	7	2	0	
8	0	0	0	
9	3	2	2	
10	2	0	0	
Unlinked	4	1	0	
Totals	56	26	9	1

Fig. 1. Distribution of the proportion of heterozygotes (*y*) observed in triploid progeny from heterozygous female parents.

Table 2. Allelic diversity at 41 microsatellite DNA markers surveyed in eight tetraploid Pacific oysters derived from tetraploid × tetraploid crosses.

(A) Descriptive statistics								
Lineage		A				B		
Parent	1	2	3	4	1	2	3	4
No. loci surveyed	27	38	39	35	34	37	38	33
Avg. no. alleles/parent	2.3	2.42	2.41	2.03	2.5	2.73	2.21	2.45
Avg. no. alleles/lineage				2.3				2.46

(B) ANOVA results					
Variable	df	Sum Sq.	Mean Sq.	F value	P
Lineage	1	2.1973	2.1973	1.5156	0.26
Lineage:parent	6	8.6990	1.45	2.0031	0.065
Residuals	273	197.5950	0.724		

Table 3. Male allele and gametic frequencies observed in triploid offspring of tetraploid males crossed to diploid females. See text for description of statistical tests.

Male/Locus	No.	AB	AC	AD	BC	BD	CD	$\chi^2_{(gametic)}$	Prob	$\chi^2_{(allelic)}$	Prob
B1/*ucdCgi-194*	Obs.	17	12	9	17	19	13				
	Exp.	15.73	11.91	11.59	17.63	17.15	13	0.903	0.97	2.966	0.40
B3/*ucdCgi-194*	Obs.	7	11	16	16	15	8				
	Exp.	11.75	10.67	12.12	12.15	13.79	12.53	6.139	0.29	0.466	0.93
A2/*ucdCgi-194*	Obs.	19	10	12	7	25	16				
	Exp.	15.86	9.6	16.62	12.43	21.45	13.03	5.558	0.35	5.820	0.12
B1/*ucdCgi-126*	Obs.	13	14	13	11	14	12				
	Exp.	13.22	12.82	13.62	12.07	12.83	12.44	0.358	1.0	0.130	0.99
B1/c*mrCgi-1*	Obs.	9	12	16	10	13	21				
	Exp.	9.29	13.05	15.66	11.07	13.3	18.63	0.513	0.99	4.469	0.22
A1/*cmrCgi-1*	Obs.	9	12	16	10	13	21				
	Exp.	9.29	13.05	15.66	11.07	13.3	18.63	0.170	1.0	1.522	0.68
B3/*ucdCgi-126*	Obs.	17	16	10	11	17	15				
	Exp.	15.12	13.95	13.95	14.71	14.71	13.57	0.849	0.97	3.586	0.31
B2/*ucdCgi-197*	Obs.	17	16	10	11	17	15				
	Exp.	15.12	13.95	13.95	14.71	14.71	13.57	3.096	0.68	0.140	0.99
B2/*ucdCgi-156*	Obs.	9	10	16	10	11	10				
	Exp.	10.56	10.56	13.49	8.82	11.28	11.28	1.036	0.96	1.152	0.76
B4/*ucdCgi-14*	Obs.	12	13	11	14	17	15				
	Exp.	12.44	12.1	12.44	14.86	15.28	14.86	0.495	0.99	0.829	0.84
B4/*ucdCgi-156*	Obs.	14	7	13	12	21	9				
	Exp.	14.38	7.87	12.9	11.57	18.89	10.38	0.541	0.99	1.152	0.76
B1/*ucdCgi-133*	Obs.	15	14	11.5	7	7	12.5				
	Exp.	11.87	14.01	12.81	9.46	8.65	10.21	2.424	0.79	2.254	0.52