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HIGH-RESOLUTION ANALYSIS OF KARYOTYPES PREPARED FROM DIFFERENT TISSUES OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*

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ABSTRACT Establishment of chromosome identity is the first step of physical genome mapping. This step is hindered by a lack of banding techniques and size homogeneity in the chromosomes of the eastern oyster, *Crassostrea virginica*. In this study, chromosomes prepared from adult gill, ripe gonad tissues, and embryos were analyzed with a computer-based measurement system. Chromosomes from embryos were elongated with excellent morphology and identifiable secondary constrictions, although homologous pairs were difficult to establish because of asymmetric sizes found between homologues. Meiotic chromosomes at the stages of diakinesis (testis) and pachytene (ovary) offered distinct advantages for karyotyping. These chromosome bivalents possessed a haploid chromosome number (n = 10) with transverse chromomere bands analyzable by microdensitometry. Chromosomes derived from gill tissue were highly condensed and few spreads were analyzable. Idiograms of each chromosome were developed in this study based on size, centromere position, and chromomere bands. These results indicate that mitotic and meiotic cells are each important for the study of chromosomes of the eastern oyster and that computer-assisted analysis will be useful for establishment of karyotypes and idiograms.

KEY WORDS: Chromomere bands, microdensitometry, karyotype, *Crassostrea virginica*

INTRODUCTION

Identification of individual chromosomes is the prerequisite step for *in situ* detection and location of specific DNA sequences. The genome of most oyster species is composed of 10 metacentric and submetacentric chromosomes, comprising three groups based on size (Longwell and Stiles 1967). Techniques need to be developed for further identification of specific chromosomes within the oyster genome. Identification of individual mammalian chromosomes has been accomplished by techniques such as Giemsa (G)-banding or reverse (R)-banding; however, these techniques do not produce consistent banding patterns in lower vertebrates (Zhang and Tiersch 1998a) and invertebrates, including oysters.

It is well established that the quality of chromosome preparations is directly related to the source material. Embryos and somatic tissues (such as gill filament) are most commonly used for cytogenetic analysis in oysters. Karyotypes of the eastern oyster, *Crassostrea virginica,* based on somatic (Rodriguez-Romero et al. 1978) and meiotic chromosomes (Longwell et al. 1967), have been described. However, none of these studies examined structural markers on the chromosomes; therefore, chromosomes of similar size could not be distinguished in these studies. Although "G-like" chromosome bands were studied in this species (Rodriguez-Romero et al. 1979), routinely useful banding patterns and analysis techniques have not been established.

In fish, meiotic chromosomes (at early prometaphase I) without colchicine treatment, are typically extended with knot-like structural markers (chromomeres), which are useful for identifying individual chromosomes (Yu et al. 1994). Most spreads prepared from ripe gonad tissue are composed of chromosome bivalents, and no pairing is required to establish a karyotype. With the assistance of microdensitometric methods (Zhang and Tiersch 1998b), these chromomere bands could be identifiable as markers for chromosomes of oysters.

In the present study, we developed haploid karyotypes for the eastern oyster from meiotic chromosomes (in diakinesis and pachytene stages). By computer-based measurements and statistical analysis, we compared these karyotypes with diploid karyotypes prepared from gill and embryonic cells, and documented naturally occurring chromomere structures useful for chromosome identification.

MATERIALS AND METHODS

Materials

Eastern oysters were obtained from Grand Isle, Louisiana, and were maintained in an indoor recirculating system until use (Buchanan et al. 1998). Twenty ripe females and 15 ripe males were used to sample gonad and gill tissues in this study. Embryos were produced by artificial fertilization based on methods described in Paniagua-Chavez et al. (1998).

Chromosome Preparation

Gonad Tissue

About 0.2 g of testis or ovary tissue were removed and cut into ~2-mm^2 pieces, which were placed in 0.9% sodium citrate (prepared in deionized water) with continuous swirling for 5–7 h. The tissue fragments were fixed twice for 20 min with cold Carnoy's fixative I (methanol:chloroform:acetic acid; 6:3:1), and fixed three times for 20 min in cold Carnoy's fixative II (methanol:acetic acid; 3:1). The tissues were ground and passed through a 70-μ cell strainer. Cells were collected, pelleted, and resuspended in Carnoy's fixative II overnight. Chromosomes were prepared on microscope slides using standard air-drying methods.

Embryos

Embryonic cells collected 5 h after fertilization were used in this study. Techniques for preparation of chromosomes of oyster embryos have been described in Guo and Allen (1997). Embryos were concentrated on a 15-μ nylon filter after the colchicine treat-

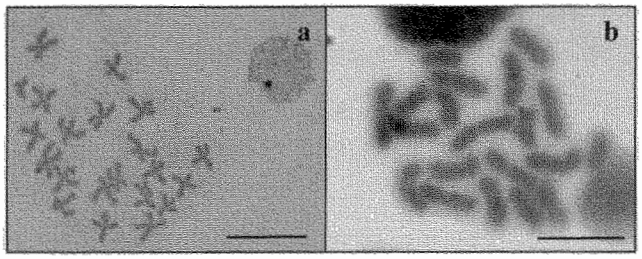

Figure 1. Preparation of mitotic (somatic) metaphase chromosomes from eastern oyster (*C. virginica*); (a) gill; and (b) male gonad tissue; bar = 10 µ.

ments. Chromosomes were prepared using standard air-drying methods.

Gill Filaments

Gill tissues were incubated for 3 h in the medium JL-ODRP-4 developed for culture of oyster cells (J. LaPeyre pers. comm.). Colchicine was added to the medium at a final concentration of 0.01%. Chromosomes were prepared with the same method used for gonad tissues, except that hypotonic (sodium citrate) treatment was 30 min.

Computer-Assisted Karyotyping

Chromosome images were digitized with a 24-bit video capture board (Imaging Technology Inc., Bedford, MA) using a light microscope (Microphot-SA, Nikon Inc.) equipped with a high-resolution RGB color video camera (model A206A, Microimage Video Systems Co., Inc., Boyertown, PA). The Optimas® computer software packages (Bioscan, Inc., Edmonds, WA), a Windows™ based application, was used to capture and process the chromosomal images. Total length, arm length, and banding patterns (chromomeres) of chromosomes were measured with the linear functions of Optimas (Zhang and Tiersch 1998b). Meiotic chromosomes (n = 10) were arranged by order of descending size. For comparisons, mitotic chromosomes (2n = 20) were ordered by size, and pairs were established based on relative length and ratio of short arm to entire length (i.e., centromeric index) (Levan et al. 1964). Idiograms were created using Microsoft Powerpoint™ for each chromosome based on size, centromere position, and banding patterns.

Repeated measurements (n = 10) were taken from representative spreads, and statistical analysis (see below) was performed to estimate technical variation using data derived from individual spreads, and for biological variation using data derived from different spreads of a particular tissue type or from different tissue types.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed to compare: (1) 10 repeated measurements of total length of chromosomes within a representative spread, used to examine the accuracy of the image analysis system; (2) relative length of individual chromosomes derived from different spreads from the same tissue source (embryo); and (3) relative length of individual chromosomes derived from four different tissue sources (gill, embryo, testis, and ovary). The relative length data were transformed to arcsine square root values before analysis. Data were collected

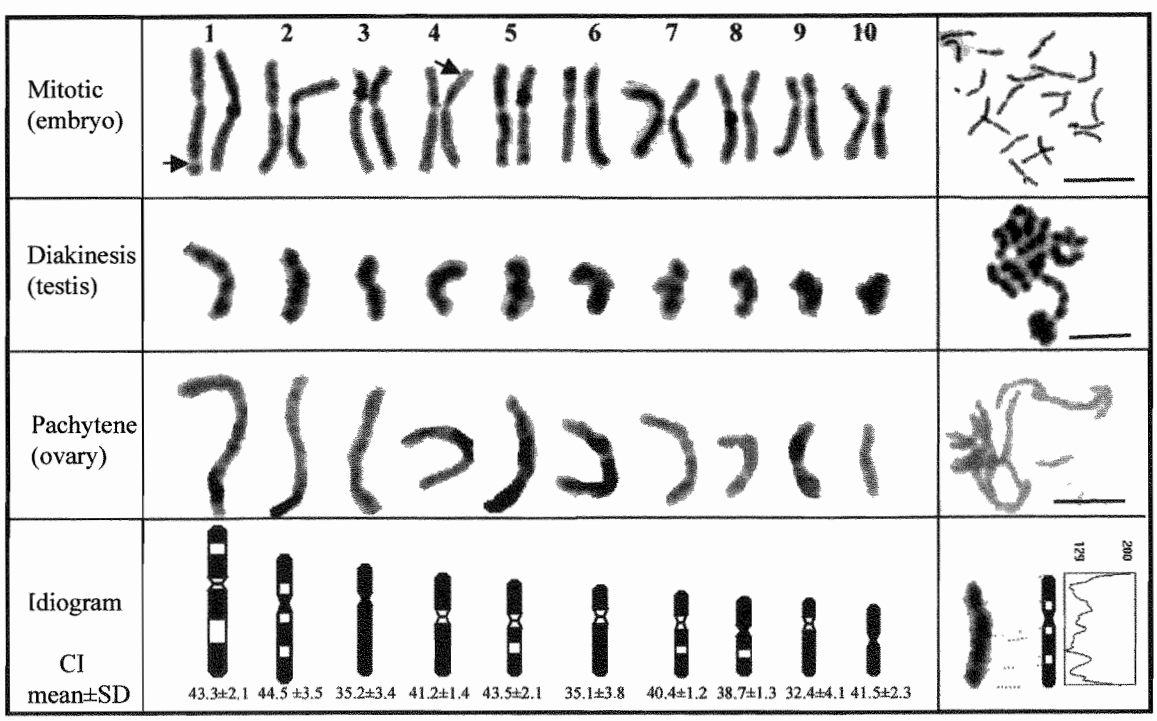

Figure 2. Karyotypes developed from different tissues of the eastern oyster; arrowheads point to secondary constrictions; insets to the right for mitotic, diakinesis, and pachytene chromosomes were the original spreads used for karyotyping; to the right of the idiogram is a demonstration of the method of microdensitometry; CI: centromeric index; bar = 10 µ.

TABLE 1.

Repeated measurements (expressed in arbitrary computer units) of total length of chromosomes (in descending order of size) within a representative spread derived from eastern oyster embryos; one-way ANOVA was used to examine the variation among these measurements (p = 1.00).

	Measurements (Replicates)												
Chromosome	1	2	3	4	5	6	7	8	9	10	Mean ± SD	Range	Percentage Variation[a]
1	4.36	4.44	4.34	4.32	4.37	4.34	4.35	4.43	4.34	4.33	4.36 ± 0.04	0.12	2.75
2	4.09	4.14	3.96	4.02	4.14	4.10	4.00	4.02	4.15	4.12	4.07 ± 0.07	0.19	4.66
3	4.02	4.04	3.95	4.02	4.04	3.95	4.00	4.02	4.03	4.00	4.01 ± 0.03	0.09	2.24
4	3.86	3.93	3.89	3.91	3.88	3.89	3.86	3.92	3.89	3.90	3.89 ± 0.02	0.07	1.80
5	3.69	3.75	3.62	3.69	3.70	3.62	3.69	3.75	3.62	3.69	3.68 ± 0.05	0.13	3.53
6	3.65	3.67	3.65	3.65	3.67	3.65	3.66	3.65	3.67	3.65	3.66 ± 0.01	0.02	0.55
7	3.62	3.66	3.53	3.62	3.66	3.62	3.53	3.62	3.66	3.62	3.61 ± 0.05	0.13	3.60
8	3.53	3.59	3.56	3.55	3.55	3.53	3.56	3.55	3.55	3.53	3.55 ± 0.02	0.06	1.69
9	3.53	3.64	3.57	3.55	3.64	3.53	3.57	3.55	3.64	3.53	3.58 ± 0.05	0.11	3.07
10	3.46	3.52	3.42	3.46	3.47	3.42	3.46	3.51	3.42	3.43	3.46 ± 0.04	0.10	2.89
11	3.41	3.45	3.36	3.37	3.41	3.30	3.41	3.45	3.35	3.40	3.39 ± 0.07	0.15	4.42
12	3.25	3.24	3.22	3.22	3.25	3.22	3.27	3.24	3.22	3.22	3.24 ± 0.02	0.05	1.54
13	3.13	3.14	3.17	3.15	3.14	3.13	3.18	3.16	3.14	3.13	3.15 ± 0.02	0.05	1.59
14	3.00	3.03	3.00	3.01	3.03	3.00	3.01	3.01	3.03	3.02	3.01 ± 0.01	0.03	1.00
15	2.96	3.07	3.04	3.02	2.95	3.04	3.00	3.08	3.03	3.02	3.02 ± 0.04	0.13	4.30
16	2.88	2.91	2.83	2.86	2.89	2.83	2.89	2.91	2.85	2.86	2.87 ± 0.03	0.08	2.79
17	2.77	2.84	2.77	2.78	2.84	2.78	2.77	2.78	2.83	2.78	2.79 ± 0.03	0.07	2.51
18	2.69	2.72	2.59	2.63	2.70	2.69	2.60	2.63	2.68	2.69	2.66 ± 0.05	0.13	4.89
19	2.63	2.70	2.66	2.65	2.63	2.66	2.64	2.70	2.66	2.66	2.66 ± 0.02	0.07	2.63
20	2.53	2.57	2.51	2.51	2.53	2.51	2.51	2.53	2.53	2.52	2.53 ± 0.02	0.06	2.37
Average ±SD	3.35 ±0.27	3.40 ±0.27	3.32 ±0.26	3.35 ±0.27	3.37 ±0.28	3.34 ±0.26	3.35 ±0.27	3.38 ±0.27	3.36 ±0.27	3.35 ±0.27			

[a] Percentage variation = (range/mean) × 100.

TABLE 2.

Variation analysis of relative lengths of chromosomes derived from embryos of the eastern oyster; one-way ANOVA was used to compare data from five representative spreads with 10 repeated measurements for each chromosome; the measurements were expressed as percentage of total length of the entire chromosome complement.

Chromosome	Spread 1	Spread 2	Spread 3	Spread 4	Spread 5	Mean ± SE	p value	Range	Percentage Variation[a]
1	6.43 ± 0.00	6.29 ± 0.00	6.51 ± 0.00	6.48 ± 0.01	6.33 ± 0.01	6.43 ± 0.10	<0.001	0.22	3.42
2	6.13 ± 0.00	6.29 ± 0.00	6.16 ± 0.01	6.20 ± 0.01	6.18 ± 0.01	6.19 ± 0.07	0.001	0.16	2.58
3	6.06 ± 0.01	5.83 ± 0.00	6.06 ± 0.00	6.11 ± 0.00	6.16 ± 0.01	6.01 ± 0.12	<0.001	0.33	5.49
4	6.01 ± 0.01	5.66 ± 0.01	5.68 ± 0.00	5.95 ± 0.01	5.90 ± 0.00	5.83 ± 0.18	<0.001	0.35	6.00
5	5.57 ± 0.01	5.33 ± 0.01	5.49 ± 0.01	5.71 ± 0.01	5.67 ± 0.01	5.52 ± 0.16	<0.001	0.38	6.88
6	5.42 ± 0.00	5.33 ± 0.00	5.43 ± 0.01	5.64 ± 0.01	5.45 ± 0.01	5.46 ± 0.13	<0.001	0.31	5.68
7	5.50 ± 0.00	5.24 ± 0.00	5.40 ± 0.01	5.64 ± 0.01	5.45 ± 0.01	5.44 ± 0.17	<0.001	0.40	7.35
8	5.49 ± 0.00	5.17 ± 0.00	5.35 ± 0.01	5.29 ± 0.01	5.21 ± 0.01	5.33 ± 0.13	<0.001	0.32	6.00
9	5.36 ± 0.00	5.07 ± 0.00	5.32 ± 0.01	5.00 ± 0.01	5.15 ± 0.00	5.19 ± 0.18	<0.001	0.36	6.94
10	4.93 ± 0.01	5.05 ± 0.00	5.07 ± 0.01	4.82 ± 0.00	5.08 ± 0.01	5.02 ± 0.07	<0.001	0.26	5.18
11	4.82 ± 0.01	4.93 ± 0.00	4.86 ± 0.01	4.82 ± 0.00	4.91 ± 0.01	4.86 ± 0.05	0.001	0.11	2.26
12	4.75 ± 0.00	4.82 ± 0.00	4.78 ± 0.01	4.79 ± 0.00	4.75 ± 0.01	4.78 ± 0.03	0.020	0.07	1.46
13	4.74 ± 0.01	4.81 ± 0.00	4.66 ± 0.01	4.79 ± 0.01	4.72 ± 0.01	4.75 ± 0.07	0.001	0.15	3.16
14	4.33 ± 0.01	4.74 ± 0.01	4.48 ± 0.01	4.53 ± 0.01	4.57 ± 0.00	4.52 ± 0.17	<0.001	0.41	9.07
15	4.44 ± 0.00	4.68 ± 0.00	4.45 ± 0.00	4.38 ± 0.01	4.37 ± 0.00	4.49 ± 0.13	<0.001	0.31	6.90
16	42.8 ± 0.01	4.51 ± 0.00	4.39 ± 0.00	4.27 ± 0.01	4.24 ± 0.01	4.36 ± 0.11	<0.001	0.27	6.19
17	4.18 ± 0.01	4.36 ± 0.01	4.11 ± 0.00	4.05 ± 0.01	4.19 ± 0.01	4.17 ± 0.13	<0.001	0.31	7.43
18	3.93 ± 0.01	4.26 ± 0.00	3.95 ± 0.01	4.10 ± 0.01	4.09 ± 0.01	4.06 ± 0.15	<0.001	0.33	8.13
19	3.90 ± 0.01	4.02 ± 0.01	3.97 ± 0.01	3.87 ± 0.01	3.82 ± 0.01	3.94 ± 0.07	0.001	0.20	5.08
20	3.83 ± 0.01	3.62 ± 0.00	3.67 ± 0.00	3.71 ± 0.01	3.76 ± 0.01	3.71 ± 0.07	<0.001	0.21	5.66

[a] Percentage variation = (range/grand mean) × 100.

TABLE 3.

Relative lengths of individual chromosomes derived from gill, embryo, testis, and ovary of the eastern oyster were compared by one-way ANOVA (n = 10 spreads for each tissue type); the measurements were expressed as percentage of total length of the entire chromosome complement.

Chromosome Number	Gill	Embryo	Testis (Diakinesis)	Ovary (Pachytene)	p value
1	12.70 ± 0.54	12.62 ± 0.01	16.84 ± 0.12	15.15 ± 0.32	0.001
2	12.20 ± 0.43	11.53 ± 0.03	11.63 ± 0.32	12.63 ± 0.07	0.001
3	11.10 ± 0.37	10.98 ± 0.02	11.20 ± 0.24	11.06 ± 0.08	0.09
4	10.87 ± 0.40	10.77 ± 0.01	10.83 ± 0.13	10.53 ± 0.11	0.07
5	10.45 ± 0.51	10.04 ± 0.03	9.64 ± 0.09	10.40 ± 0.10	0.001
6	9.83 ± 0.36	9.56 ± 0.01	9.30 ± 0.05	10.36 ± 0.07	0.001
7	9.65 ± 0.32	9.36 ± 0.02	8.42 ± 0.10	9.83 ± 0.08	0.001
8	8.10 ± 0.27	8.84 ± 0.04	7.84 ± 0.08	7.34 ± 0.12	0.001
9	8.00 ± 0.46	8.13 ± 0.03	7.04 ± 0.05	6.63 ± 0.10	0.001
10	6.42 ± 0.52	7.63 ± 0.02	6.48 ± 0.07	5.90 ± 0.05	0.001

based on measurements of 10 complete chromosome spreads for each source material.

RESULTS

Ten pairs of metaphase chromosomes were observed in cells obtained from gill (Fig. 1a) and embryos (Fig. 2) with seven pairs of metacentrics and three pairs of submetacentrics. Embryonic chromosomes were arranged in descending order by size (Fig. 2) for comparison with the karyotypes prepared from meiotic spreads. Two pairs of chromosomes from the embryos typically had visible secondary constrictions (arrowheads in Fig. 2).

Chromosome spreads in different phases of meiotic division were found in gonadal tissues (Fig. 2), including diakinesis and pachytene bivalents, and a few mitotic (somatic) chromosome spreads were found (Fig. 1b). In ovary, most spreads (~45%) were pachytene chromosomes; whereas, in testis, most spreads (~70%) were in diakinesis. Haploid karyotypes (Fig. 2) were developed for chromosomes in diakinesis and pachytene stages, in which chromosomes were arranged by size in descending order. Chromomere bands were distinct on diakinesis chromosomes and less distinct on pachytene chromosomes, and were reproducible for each of the 10 chromosomes. Idiograms were prepared for each chromosome based on size, centromeric index (calculated from the chromosomes produced from embryonic tissues), and chromomere bands of diakinesis chromosomes (Fig. 2).

There was no difference ($p = 1.0$) among 10 repeated measurements of total length for a given chromosome spread (Table 1). However, relative lengths of chromosomes were significantly different ($p < .05$) among different spreads prepared from the same tissue type (Table 2). The relative lengths of chromosomes were significantly different ($p < .05$) among the four tissue types (gill, embryo, testis, and ovary) except for chromosomes 3 and 4 ($p > .05$) (Table 3).

DISCUSSION

In this study, we found that chromosomes derived from four tissue types had distinct morphological features providing different options for year-round genetic studies of the eastern oyster (Tables 4, 5). The mitotic activity of oyster somatic cells is low, and there are no methods available for stimulation of mitosis in oyster somatic cells *in vivo* or *in vitro* (Cornet 1993). Although an increase of colchicine concentration increased the number of spreads observed, the resultant chromosomes were short, less distinct in size, and not suitable for use in physical mapping. Chromosomes prepared from embryos were elongated and provided images of high resolution. Chromosome structures such as secondary constrictions were recognizable on these chromosomes. However, heavy background caused by adherent materials limited subsequent analysis. Techniques have been developed for removing yolk materials from preparations of oyster chromosomes derived from cleaving eggs (Longwell and Stiles 1968). In this study, we found that 50% acetic acid could eliminate most background materials (data not shown). Nevertheless, the effects of these treatments on the quality of chromosomes for use in physical gene mapping needs to be evaluated.

In this study, a new protocol was introduced for preparation of bivalent chromosomes from ripe gonad tissue of the eastern oyster, which omitted colchicine treatment and included a prolonged hy-

TABLE 4.

Ploidy level, availability, division stage, and spreads per slide of four tissue types used in this study; the number of spreads observed on each microscope slide was used to estimate mitotic index of each tissue type of eastern oyster.

Tissue	Ploidy	Availability	Division Stage	Spreads per Slide[a]
Gill	2N	Year-around	Mitotic metaphase	Low
Embryo	2N	Spawning season	Mitotic metaphase	High
Testis	1N, 2N	Spawning season	Mostly diakinesis	——
Ovary	1N, 2N	Spawning season	Mostly pachytene	——

[a] Low, <5 spreads per slide; high, >30 spreads per slide, and ——, cell division dependent on season.

TABLE 5.

Suitability of chromosomes derived from different tissues of eastern oyster for physical genome mapping.

Tissue	Elongation	Dispersal	Resolution	Centromeres Identified	Chromomere Bands	Pairs Identified	Mapping
Gill	Short	Separated	Low	Yes	No	3–5	Not suitable
Embryo	Long	Separated	High	Yes	No	7	Suitable
Testis (diakinesis)	Intermediate	Less separated	High	No	Yes	10	Suitable
Ovary (pachytene)	Very long	Overlapping	Highest	No	Yes	10	Suitable

potonic treatment step. Bivalent haploid spreads yielded the advantages of reduced chromosome numbers, no need for pairing of homologous chromosomes, and identifiable chromomere bands on most chromosomes. However, these chromosomes were difficult to disperse, especially for the pachytene bivalents. Although incubation of gonad tissue with colchicine would facilitate dispersal of bivalent chromosomes, the chromomere bands on these chromosomes would become less identifiable (Yu et al. 1994). Further identification of the advantages and disadvantages of chromosomes from different source tissues is essential for analysis of oyster chromosomes and establishment of a representative karyotype in this species for such activities as physical mapping of genes.

In this study, the computer-based image analysis system provided a reproducible and objective method for measurement of chromosomes. The difference (percentage variation) among measurements of individual chromosomes was less than 5%, which accounted for technical variation including manual error. Variation caused by biological factors was analyzed by comparing different spreads of the same tissue type or spreads of different tissue types. The difference in average relative length was found to be as high as 9% among different spreads from individual embryos. These differences were largely derived from the continuous changes of chromosome morphology produced during the cell cycle. On the other hand, results of this study demonstrated that our measurement system is highly sensitive and capable of detecting minor differences among individual chromosomes. These results suggest that methods need to be developed (such as use of marker chromosomes) for standardization of the eastern oyster karyotype.

Pairing of homologous chromosomes based solely on size and centromeric index was difficult for oyster chromosomes prepared from embryonic cells. Several chromosome pairs were asymmetric in size, centromeric index, and secondary constrictions. A typical example was chromosome 1, the largest metacentric chromosome, which displayed differences in relative length between homologues of 0.2 to 0.4% and in presence or absence of secondary constriction. These asymmetric features could be attributable to normal development of embryos, abnormal development, or integration of features of the genomes of the parents. We did not observe such asymmetric features on chromosomes of other cells, although these chromosomes were highly condensed.

Some differences were found between the karyotype developed previously for embryos of *C. virginica* (Longwell and Stiles 1968) and the one developed in this study. Only three submetacentric chromosomes were found in this study, as compared with four submetacentrics described previously. Also, the second largest chromosome was found to be metacentric rather than submetacentric, as described previously. Reasons for these differences include differences in measurement techniques, variation in the contraction of chromosomes used in each study, and genetic polymorphism among populations of the eastern oyster. Development of such methods as C-banding (for identification of constitutive heterochromatin) would be helpful for resolving these differences by identifying the location of centromeres.

In summary, most chromosomes (seven or eight) prepared from embryos could be identified based on morphological measurements; however, the pairing of homologous chromosomes was difficult. Thus, embryos are a convenient source material for chromosome preparation, although their utility for high-resolution analysis is limited. Meiotic chromosomes, although less frequently studied, were useful, given their reduced numbers and the presence of unique, naturally occurring bands on each diakinesis chromosome.

This study provided information about the composition of the eastern oyster genome. Techniques developed for meiotic chromosomes and idiograms based on these chromosomes have proved to be valuable for physical genetic mapping of oysters in our laboratories (unpublished data). Integrative analysis of mitotic metaphase chromosomes and meiotic bivalent chromosomes will be a useful tool for specific identification of chromosomes in the oyster genome until other techniques become available for mollusks.

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