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Characterization of Eastern Oyster (*Crassostrea virginica* Gmelin) Chromosomes by Fluorescence In Situ Hybridization with Bacteriophage P1 Clones

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Abstract

Chromosome identification is an essential step in genomic research, which so far has not been possible in oysters. We tested bacteriophage P1 clones for chromosomal identification in the eastern oyster *Crassostrea virginica*, using fluorescence in situ hybridization (FISH). P1 clones were labeled with digoxigenin-11-dUTP using nick translation. Hybridization was detected with fluorescein-isothiocyanate-labeled anti-digoxigenin antibodies and amplified with 2 layers of antibodies. Nine of the 21 P1 clones tested produced clear and consistent FISH signals when Cot-1 DNA was used as a blocking agent against repetitive sequences. Karyotypic analysis and cohybridization positively assigned the 9 P1 clones to 7 chromosomes. The remaining 3 chromosomes can be separated by size and arm ratio. Five of the 9 P1 clones were sequenced at both ends, providing sequence-tagged sites that can be used to integrate linkage and cytogenetic maps. One sequence is part of the bone morphogenetic protein type 1b receptor, a member of the transforming growth factor superfamily, and mapped to the telomeric region of the long arm of chromosome 2. This study shows that large-insert clones such as P1 are useful as chromosome-specific FISH probes and for gene mapping in oysters.

Key words: FISH — P1 clones — physical mapping — chromosome — genome — Mollusca

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Introduction

Chromosomes are basic units of eukaryotic genomes, and their characterization and identification are essential steps in genomic analysis. Chromosome identification is required for several areas of genetic research including studies on aneuploidy and chromosomal rearrangements, chromosomal assignment of genes, and the development of chromosome-specific libraries and markers. Although chromosome identification is routinely conducted in humans and other model organisms, it remains a challenge in oysters and other marine mollusks.

All species of *Crassostrea* oysters studied so far have a haploid number of 10 chromosomes (Nakamura, 1985). Although the haploid number is low, oyster chromosomes are small, and similar in size and arm ratio. Chromosome identification by karyotypic measurement is not feasible. Identification by traditional banding has also been difficult because of the lack of-cultured cells needed for the preparation of elongated chromosomes. C- and G-banded karyotypes have been produced in *Crassostrea* oysters (Rodriguez-Romero et al., 1979; Insua and Thiriot-Quiévreux, 1993; Leitão et al., 1999), but offered little help in the routine and reliable identification of oyster chromosomes. Banding differences among chromosomes are not outstanding, and the reproducibility is low. Nucleolar organizer region (NOR) staining with silver can identify the ribosomal RNA-bearing chromosome with variable accuracy (Thiriot-Quiévreux and Insua, 1992; Ladrón De Guevara et al., 1994).

Fluorescence in situ hybridization (FISH) offers new opportunities for the identification of oyster chromosomes. FISH has been widely used for chromosome identification, gene mapping, localization

of gene expression, and studies on chromosome rearrangement in a variety of organisms (Swiger and Tucker, 1996; Nath and Johnson, 1999). By DNA base pair hybridization, FISH provides specific and reproducible localization of genes and DNA sequences on chromosomes. FISH has recently been introduced to oyster cytogenetics. Using FISH, a repetitive element has been mapped to centromeric regions of several chromosomes in the Pacific oyster *Crassostrea gigas* (Clabby et al., 1996; Wang et al., 2001), and the vertebrate telomere sequence (TAAGGG)n has been mapped to telomeres of 3 *Crassostrea* oysters (Guo and Allen, 1997; Wang and Guo, 2001). The major ribosomal RNA genes (rDNA) have also been mapped using FISH in 6 species of *Crassostrea* oysters (Zhang et al., 1999; Xu et al., 2001; Cross et al., 2003; Wang et al., 2004). Similar FISH studies have been reported in other mollusks such as mussels (Martínez-Lage et al., 1997; Insua and Mendez, 1998; Torrerio et al., 1999; Gonzáles-Tizón et al., 2000; Vitturi et al., 2000), clams (Gonzáles-Tizón et al., 1998, 2000; Insua et al., 2001), cockle (Insua et al., 1999) and scallop (Insua et al., 1998).

So far, FISH in oysters and other marine mollusks has been limited to repetitive genes or sequences. The mapping of unique sequences depends on the size of the targeted DNA. In general, unique sequences shorter than 1 kb are difficult to map by FISH, although it is not impossible (Schriml et al., 1999; Guzzo et al., 2000). Large-insert clones from P1 (phage), cosmid, bacterial artificial chromosome (BAC), and yeast artificial chromosome (YAC) libraries have been successfully mapped using FISH (Lichter et al., 1990; Jiang et al., 1995; Zhu et al., 1996). The mapping of large-insert clones in most organisms used elongated chromosomes from cultured cells. Here we show for the first time that P1 clones can be mapped in the eastern oyster using metaphases from early embryos and Cot-1 DNA as a blocking agent.

Materials and Methods

P1 Library Construction, Clones, and DNA Extraction. A high molecular weight (HMW) DNA bacteriophage P1 genomic library was constructed for the eastern oyster *Crassostrea virginica* Gmelin using published methods (Pierce et al., 1992). The P1 library was organized into 49 glycerol-stock primary pools containing approximately 350 clones each. The average insert size was about 75 kb.

Random P1 clones were isolated from the library by plating dilutions of the primary pool stocks on Luria broth agar plates containing 25 μg/ml kanamycin and picking single colonies for overnight growth in LB media. P1 plasmid DNA was prepared from a 100-ml overnight culture and purified using the Plasmid Midi-100 purification system as described by the supplier (Qiagen) with minor modifications. The recommended buffer volumes were each increased by a factor of 2 for the entire protocol except for the final resuspension step. The DNA pellet containing P1 plasmid DNA was resuspended in a final volume of 100 μl TE buffer. For DNA sequencing purposes, we performed microdialysis of the P1 plasmid DNA by placing the resuspended DNA on a Millipore VSP 0.025-μM filter floating on a petri plate of TE buffer for 45 minutes. Yields of P1 plasmid DNA were approximately 5 to 20 ng/μl.

P1 DNA End-Sequencing and Analysis. P1 plasmid DNA was prepared for end-DNA sequencing by first digesting the plasmid DNA (approx. 200–400 ng) with the restriction enzyme *Bgl*II (New England Biolabs). The cut DNA was purified by phenol-chloroform extraction and concentrated by ethanol precipitation using standard protocols (Sambrook et al., 1989). The DNA pellet was resuspended in 10 μl of TE buffer and subjected to microdialysis for 30 minutes as described previously. DNA sequencing reactions were performed using 3 μl of the *Bgl*II-cut P1 plasmid DNA and 3 μl of oligonucleotide primers (0.2 μM) complementary to either the T7 or Sp6 binding sites of the P1 SacB II vector in a total volume of 20 μl using the FS *Taq* dideoxy, fluorescent DNA sequencing system as per supplier's instructions (Applied Biosystems). Sequencing reactions were analyzed on an ABI 310 Prism DNA Sequencer using a 61-cm capillary.

DNA sequence electropherograms were analyzed using the MacVector DNA analysis software program (Acclyres). Matches to the GenBank database were performed using the BLAST local sequence alignment program maintained at the National Center for Biotechnology Information (*www.ncbi.nlm.nih.gov*).

Probe Labeling. DNAs from P1 clones, with an average insert size of 80 to 100 kb, were labeled with digoxigenin-11-dUTP (Roche) by nick translation using the nick translation probe labeling kit (Invitrogen) following the manufacturer's instruction. Nick translation was performed in 50-μl reaction mix consisting of 1 μg of probe DNA, 0.1 mM dATP, dCTP, and dGTP, 36 μM digoxigenin-11-dUTP, and 66 μM dTTP (dTTP/dUTP ratio = 1.8) and 5 μl of enzyme mixture. The reaction lasted 3 hours at 15°C and was stopped by adding EDTA to a final concentration of 25 mM. Aliquots of 7-μl reaction mixture were sampled and run on 2% agarose gels to monitor fragment size.

Chromosome Preparation. The eastern oyster chromosomes were prepared from early embryos according to Guo and Alien (1997). Briefly, the eggs and sperm were obtained by stripping the gonad. Eggs were washed through a 60-μm Nytex screen to remove the large debris and rinsed on a 20-μm screen, then collected in a beaker with seawater. Sperm were collected after passing through a 20-μm screen. Fertilization was performed at 23°C. At 4 to 6 hours post-fertilization when embryos developed into the trochophore stage, larvae were collected into a 15-ml tube containing 0.005% colchicine. After 15 minutes the colchicine solution was replaced with a hypotonic solution (0.075 M KC1). The hypotonic treatment lasted for 12 minutes, after which the embryos were fixed with freshly made Carnoy's fixative (methanol–acetic acid, 3:1, v/v). The fixative was changed twice before storing the fixed embryos at 4°C. Slides were made by dropping 2 drops of embryos suspension onto a precleaned slide, then flooded by a layer of 1:1 methanol and acetic acid (v/v). The slides were air dried and stored at –20°C before use.

In Situ Hybridization. To condition metaphase chromosomes for FISH, slides were pretreated with 2× standard saline citrate (SSC, 0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0) at 37°C for 30 minutes, and dehydrated in 70%, 80%, and 95% ethanol series for 5 minutes each. After air-drying, slides and chromosomes were denatured with 70% formamide in 2 × SSC at 72°C for 2 minutes, and then dehydrated through a series of ethanol washes at –20°C. Digoxigenin-labeled probes (8–16 ng/μl, same for other two), unlabeled oyster Cot-1 DNA (50–75 ng/μl) and salmon sperm DNA (10 ng/μl, Sigma) were mixed in hybridization buffer, denatured at 72°C for 5 minutes, and annealed at 37°C for 15 minutes. The denatured probe was added to denatured slides for hybridization, and slides were covered with glass coverslips and sealed with rubber cement. Slides were then incubated in a humidified chamber at 37°C overnight.

Oyster Cot-1 genomic DNA was prepared by first shearing 400 μl (approx. 1 μg/μl) of HMW DNA by multiple passes through an 18-gauge needle using a sterile syringe followed by 2–3 rounds of sonication (1 minute each) in a Bransonic 220 water-bath sonicator. This treatment produced DNA fragments with an average size of approximately 500 bp. The DNA was denatured at 100°C for 10 minutes and then placed in a 65°C water bath for 4 minutes to allow temperature equilibration. The salt concentration of the DNA solution was adjusted to 0.3 M sodium chloride, and the sample was incubated for 10 minutes at 65°C for reannealing of repetitive sequences. Single-stranded DNA was digested for 30 minutes at 37°C with S1 nuclease (Invitrogen) at a concentration of 1 U/μg DNA as per supplier's instructions. The Cot-1 DNA was purified by standard phenol-chloroform extraction and ethanol precipitation. The DNA pellet was washed twice with 75% ethanol, air-dried, and then resuspended in 60 μl TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0).

Detection and Amplification. After hybridization, slides were washed in 2 × SSC at room temperature (RT) for 5 minutes (to remove coverglass), then transferred to 2 × SSC at 72°C for 5 minutes to wash off nonspecific hybridization signals. After being rinsed twice in 1 × PBT buffer (0.1 M NaH_2PO_4, 0.4% bovine serum albumin [BSA], 0.1% Tween-20, pH 7.4), slides were incubated, under plastic coverslip and at 37°C, with labeled with antidigoxigenin antibody labeled with fluorescein isothiocyanate (FITC) for 30 minutes, followed by 3 washes in 1 × PBT buffer at RT. After excess liquid was drained from the slides, slides were mounted in antifade containing 0.5 μg/ml propidium iodode (PI) as a counterstain. Probe signals were evaluated with an epifluorescence microscope (Nikon E600) equipped with a 3CCD camera. Chromosomes and the interphase cells were screened using the PI filter (Nikon, G-2A), and hybridization signals were obtained using the FITC/PI bipass filter (Chroma, 51005). FISH signals and karyotype were analyzed and documented using Image-Pro Plus software.

When hybridization signals were weak, fluorescence amplification and a detection kit were used (Oncor). For amplification slides were washed in 1 × PBT for 5 minutes for destaining. Signals were amplified by incubating slides with 20μl of rabbit antisheep antibody I at 37°C for 15 minutes, followed by incubation with FITC-labeled antirabbit antibody II for 15 minutes.

For the cohybridization, 2 DIG-labeled P1 probes were mixed in the hybridization buffer, and hybridization and detection were performed as described before.

Results

Probe Labeling and FISH. Probe labeling by nick translation was verified on agarose gels. DNA fragments generated by nick translation were visible as a faint smear after ethidium bromide staining. Duration of nick translation was adjusted so that the fragment size was mostly between 200 and 600 bp, which gave the best FISH results. Fragments larger

Table 1. Summary of P1 Clones Tested and Mapped by FISH in *C. virginica*

Category	*Number*
Total number of clones tested	21
Clones with no or weak signals	3
Clones with too much background	9
Clones successfully assigned	9
Chromosome 1	1
Chromosome 2	1
Chromosome 3	2
Chromosome 4	1
Chromosome 5	1
Chromosome 7	1
Chromosome 8	2

than 600 bp produced strong background signals, whereas those smaller than 200 bp resulted in low signal strength.

DNA from 21 P1 clones were labeled and tested for FISH. The use of Cot-1 DNA contributed to the successful suppression of background signals from repetitive sequences within the P1 clones. Initially, 7 P1 clones were tested without Cot-1 DNA. Only one clone (P0001) gave clear and strong signals and was successfully mapped. P0001 was the only clone that produced strong signals without Cot-1 and further amplification. The other 6 P1 probes gave strong background hybridization signals that were uniformly distributed throughout all chromosomes and interphase nuclei. After the addition of Cot-1 DNA as a blocking agent, 2 of the 6 clones produced clear and unique signals and were successfully mapped. Subsequently, Cot-1 DNA was used for all clones.

Among the 21 P1 clones tested, 9 produced stable and specific FISH signals, and the other 12 probes could not be mapped: 3 did not produce any stable FISH signals, and 9 produced too much background (Table 1). With the exception of P0001, all P1 that was eventually mapped required signal amplification. Without amplification, specific FISH signals were too weak to be separated from nonspecific background signals.

Chromosomal Assignment. Karyotypic analysis was conducted for each of the 9 P1 clones that produced strong and specific signals, in order to assign P1 clones to specific chromosomes. Fifteen or more metaphases were analyzed for each probe. Specific and paired signals were observed in over 80% of the metaphases analyzed. Two to 4 FISH signals, depending on cell cycle, were usually observed in interphase nuclei (Figure 1). When 2 probes were found on the same chromosome or chromosomes of similar size and arm ratio, cohybridization of 2 probes on the same metaphase was performed to confirm chromosomal assignment.

Clone P4604 was assigned to the long arm of chromosome 1, adjacent to the centromere (Figure 1, A). Chromosome 1 is the longest chromosome, which can be readily separated from the other chromosomes. It is metacentric with a centromeric index of 0.47 (Table 2), and actually sometimes it is difficult to tell which arm is longer. P4710 was assigned to the telomeric region of the long arm of chromosome 2 (Figure 1, B). Chromosome 2 has a lower centromeric index (0.39) relative to that of other chromosomes of similar size (Table 2). Two probes were mapped to chromosome 3: P4813 was located on the long arm near the centromere (Figure 1, C), and P0001 was found in the telomeric region of the short arm (Figure 1, D). P4810 was assigned to the long arm of chromosome 3, at a site about two fifths of the arm length from the centromere (Figure 1, E). P4801 was found on the long arm of chromosome 5, immediately adjacent to the centromere (Figure 1, F). P4911 was located about midway on the long arm of chromosome 7 (Figure 1, G). Chromosome 5, 6, and 7 are similar in size, but chromosome 6 had a higher centromeric index than the other two. P4411 and P4910 were both mapped to the long arm of chromosome 8. P4411 was located about one sixth of arm length from the telomere (Figure 1, H), while P4910 was next to the centromere (Figure 1, I). Chromosomes 7 and 8 are similar in size and centromeric index (Table 2). The chromosomal assignments of P4911 to chromosome 7 and P4910 to chromosome 8 were confirmed by cohybridization (Figure 1, J). Cohybridizations of P4801 with P4813 and P4801 with P0001 were also conducted, confirming their chromosomal assignments (not shown). No probes were assigned to chromosome 6, 9, and 10 (Figure 1, K).

Five of the 9 P1 clones were successfully sequenced from both ends, and the 10 sequences were deposited in GenBank (accession numbers AY619700–09). The other 4 clones did not produce high-quality sequences, and they are not presented here. A BLAST analysis of the 10 sequences against the GenBank nucleotide database showed only one significant sequence match. The sequence of P4710S (Sp6-end) matched to the bone morphogenetic protein (BMP) type 1B receptor gene from the Pacific oyster *Crassostrea gigas* (accession number AJ577293.1). The match generated an *E* value of $5e^{-08}$ with a similarity of 36 of 37 nucleotides within intron 10. If a single gap is inserted in the alignment immediately upstream of this region, an additional match of 24 (60%) of 40 nucleotides is observed. Clone P4710 was mapped to the telomeric region of the long arm of chromosome 2, providing a physical location for the BMP receptor gene.

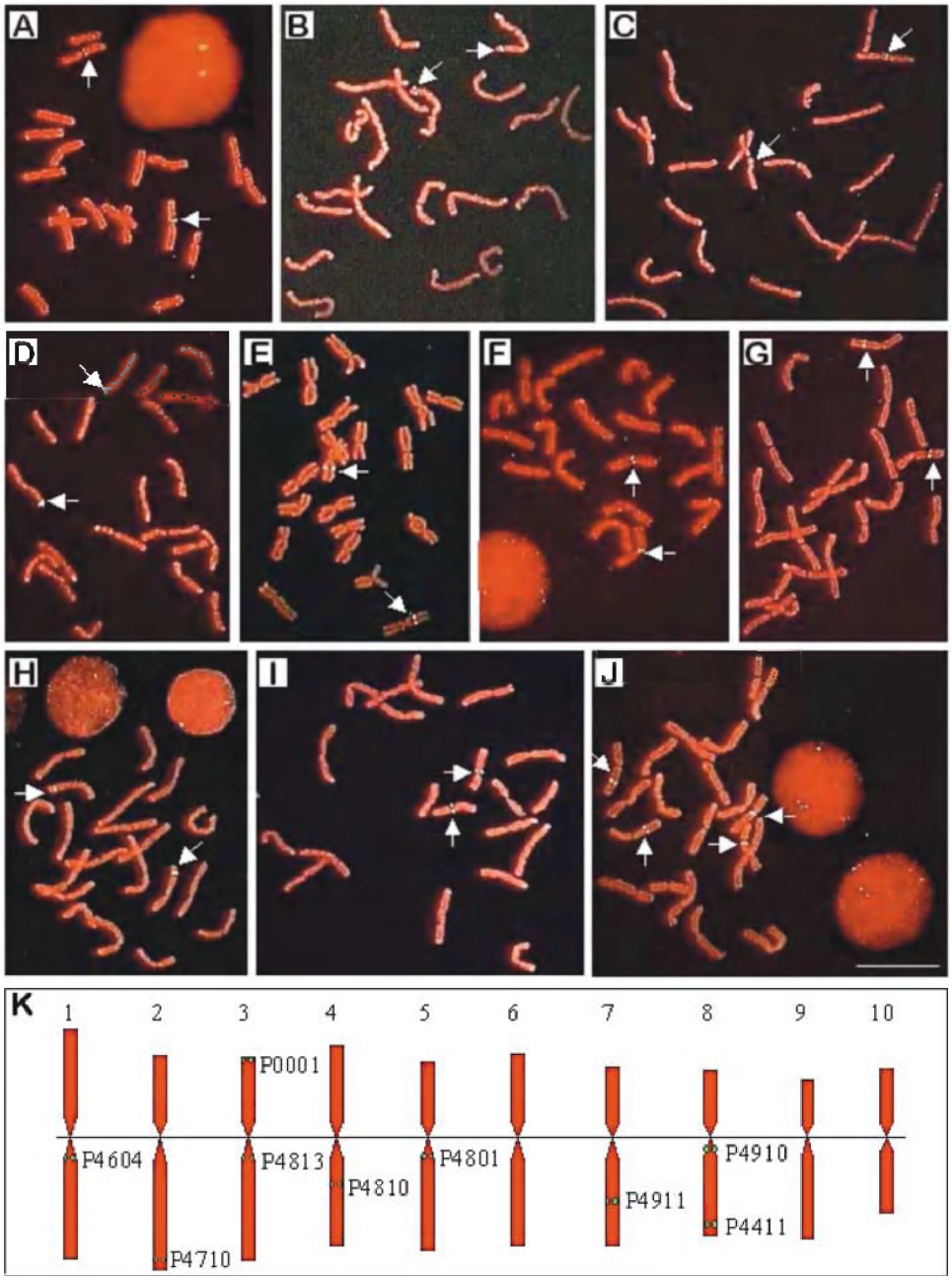

Fig. 1. Mapping of P1 clones to chromosomes of the eastern oyster: P4604 (**A**); P4710 (**B**); P4813 (**C**); P0001 (**D**); P4810 (**E**); P4801 (**F**); P4911 (**G**); P4411 (**H**); P4910 (**I**); cohybridization of P4911 and P4910 (**J**); karyotypic ideogram showing the chromosomal assignment of all 9 P1 clones (**K**). FISH signals are designated by arrows.

Discussion

Chromosome identification in oysters and other marine mollusks remains a challenge. Although FISH may offer new opportunities for chromosome identification, the number of probes available for FISH has been limited in oysters. So far, only repetitive DNA sequences and genes have been mapped using FISH to oyster chromosomes (Clabby et al., 1996; Guo and Allen, 1997; Zhang et al., 1999; Xu et al., 2001; Wang and Guo, 2001; Wang et al., 2001, 2004; Cross et al., 2003). As far as we can determine, this study is the first in which unique sequence probes have been mapped to specific chromosomes in the eastern oyster or any mollusks. Results of this study clearly demonstrated that large-insert clones such as P1 can be mapped to specific chromosomes using FISH. As the number of large-insert clones is virtually unlimited, chromosome-specific FISH probes can be potentially developed for all chromosomes.

In this study, 9 (43%) of the 21 P1 clones were successfully mapped to specific chromosomes. The 43% success rate in this study is higher than the 6% rate reported for YAC clones in soybean (Zhu et al. 1996), but lower than what was reported for some

Table 2. Karyotype and Chromosomal Assignment of Nine P1 Clones in *C. virginica*

Chromosome no.	*Relative length*[a] (±SD)	*Centromeric index*[b] (±SD)	*Classification*[c]	*P1 Clones mapped*
1	12.3 ± 0.2	0.47 ± 0.01	M	P4604
2	11.5 ± 0.2	0.39 ± 0.01	M	P4710
3	10.9 ± 0.1	0.40 ± 0.01	M	P0001, P4813
4	10.8 ± 0.2	0.47 ± 0.01	M	P4810
5	10.1 ± 0.1	0.39 ± 0.01	M	P4801
6	9.8 ± 0.1	0.46 ± 0.02	M	
7	9.5 ± 0.1	0.40 ± 0.01	M	P4911
8	8.9 ± 0.1	0.40 ± 0.01	M	P4910, P4411
9	8.4 ± 0.2	0.34 ± 0.01	SM	
10	7.7 ± 0.2	0.46 ± 0.02	M	

[a]Relative length of each chromosome as percentage of total length of the haploid complement.
[b]Length of short arm divided by total length.
[c]M indicates metacentric; SM, submetacentric.

other organisms with BAC and cosmid clones (Lichter et al., 1990; Jiang et al., 1995). Among the 12 clones that did not work, 3 had no or weak signals, probably owing to poor DNA or probe quality. Nine generated excessive background signals that interfered with real FISH signals. The background is presumably produced by repetitive sequences within the P1 clones. Several types of highly repetitive sequences have been identified in the eastern oyster and produced intense signals interspersed on all chromosomes (Gaffney et al., 2003; Y. Wang and X. Guo, unpublished results). Repetitive sequences are problematic for FISH with all large-insert clones and usually suppressed with the addition of total genomic or Cot-1 DNA (Lichter and Cremer, 1992; Matsuda and Chapman, 1995). Cot-1 is a fraction of genomic DNA that is rich in highly repetitive sequences. The unlabeled repetitive sequences in Cot-1 compete with labeled sequences within the P1 clones and reduce background signals. Our results confirm that Cot-1 DNA is effective in suppressing background signals in at least 2 of the 6 P1 clones tested.

Another factor that affects the success of FISH in oysters is the quality of metaphase material. Metaphases with elongated chromosomes and little background material (proteins or lipids) are ideal for FISH analysis. In humans and most other vertebrates, high-quality metaphases are easily obtained from cultured cells. In oysters and other marine mollusks, cultured cells are not available. Metaphases used in this study were obtained from early (4–6 hours postfertilization) embryonic cells. Embryonic cells yielded elongated chromosomes, but also contained yolk materials that interfere with FISH. The age of the embryos is important. At 4 to 6 hours postfertilization, the embryos had a balance of relatively high mitotic index and reduced yolk materials. High mitotic index allows shorter colchicine treatment and the production of elongated chromosomes (Figure 1). Cultured cell-lines, if developed, may greatly increase the sensitivity and efficiency of FISH in oysters.

The 9 P1 clones mapped in this study covered 7 of the 10 chromosomes of the eastern oyster. The chromosomal assignment was unambiguous and verified by cohybridization when necessary. The remaining 3 chromosomes, 6, 9, and 10, can be easily identified by size and arm ratio. Chromosome 7 is obviously larger than chromosome 9 and 10. Chromosome 9 is submetacentric with a markedly low centromeric index of 0.34, and chromosome 10 is metacentric. It is now possible to distinguish all 10 chromosomes in the eastern oyster. The ability to identify all oyster chromosomes may enable several types of cytogenetic analyses including studies on aneuploidy, chromosomal rearrangements, and development of chromosome-specific libraries and markers.

This study generated end sequences for 5 of the 9 clones mapped. The 5 clones were mapped to 5 chromosomes, providing sequence tagged sites (STSs) for chromosomes 2, 3, 4, 5, and 8. Single-nucleotide polymorphisms may be developed from these STSs and used for linkage analysis. A moderately dense genetic linkage map has been constructed for the eastern oyster (Yu and Guo, 2003), and genetic mapping of the STSs can facilitate the integration of linkage and cytogenetic maps. Interestingly, one of the P1 clones provided physical mapping of the BMP type 1B receptor gene to chromosome 2. BMPs are members of the transforming growth factor β superfamily and involved in the growth, differentiation, and morphogenesis of many tissue types (Parker et al., 2004). The BMP signaling pathway is evolutionarily conserved from *Drosophila* to human. It may play important roles in regulating growth and development in oysters. It would be interesting to deter-

mine if growth-related quantitative trait loci exist in the same chromosomal region.

FISH clearly has the potential to accelerate the pace of genomic discovery in oysters and other marine mollusks. This study represents the first step in the development of chromosome-specific probes and markers for all chromosomes and chromosomal arms in oysters. The number of clones mapped and sequenced is small. It would be ideal to have 2 to 4 FISH probes and STSs per chromosome arm. Further work is needed to increase chromosome coverage.

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