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Methods for Karyotyping and for Localization of Developmentally Relevant Genes on the Chromosomes of the Purple Sea Urchin, Strongylocentrotus purpuratus

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Abstract. The purple sea urchin, *Strongylocentrotus purpuratus*, is the only non-chordate deuterostome model with a fully sequenced genome. Chromosomal localization of individual genes and resulting gene maps are unavailable for this or for any sea urchin. As a result, the purple sea urchin genome has not been mapped onto specific chromosomes and remains inaccessible to genome-wide approaches addressing questions that require positional information for particular genes. Here we describe the first successful methods for karyotyping and localizing specific gene loci on chromosomes of *Strongylocentrotus purpuratus* and those of the phylogenetically related *Strongylocentrotus droebachiensis.* Both species have 42 chromosomes in their diploid genomes (n = 21). There are 2 large, 8 medium, and 10 small pairs, plus one putative sex pair. In both species, *bindin* genes were localized to 2 pair of homologous chromosomes by fluorescent *in situ* hybridization. Fluorescently labeled bacterial artificial chromosome clones generated from *S. purpuratus* for the functionally related genes *brachyury*, *foxa,* and *foxb* were localized to different chromosomes. Our protocols provide previously unavailable tools for developing a gene map for the purple sea urchin genome.

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Abbreviations: BAC, bacterial artificial chromosomes; BAC-FISH, fluorescently labeled bacterial artificial chromosome in situ hybridization; DAPI, 4′-6-diamidino-2-phenylindole; FISH, fluorescent *in situ* hybridization; FSW, filtered seawater; PABA, p-aminobenzoic acid; SSC buffer, 3.5% NaCl, 0.89% sodium citrate; PI, propidium iodide.

Introduction

The genome of the purple sea urchin, *Strongylocentrotus purpuratus*, has been fully sequenced, extensively annotated, and accompanied by a comprehensive analysis of when and where many genes are expressed during development (SUGSC, 2006). Genes characterized in this genome also indicate the potential importance of sea urchin genomics for comparative studies of genetics, immunology, olfaction, vision, and many other aspects of the life of embryonic and adult purple sea urchins. Until now, genes identified in this cloning effort have not been localized on chromosomes of this species, and only highly amplified ribosomal genes have been placed on the chromosomes of any sea urchin species (Caradonna *et al.*, 2007). This is principally because of the small size (1–6 μm) of sea urchin chromosomes and the technical difficulties involved in preparing them. The phylogenetically related green sea urchin from the eastern coast of the United States, *Strongylocentrotus droebachiensis* (Lee, 2003), also has highly conserved homologs for genes found in the purple sea urchin (*e.g.*, green sea urchin opsin, GenBank Accesson # DQ285097), and none of these has been localized on a specific chromosome.

We present techniques for the preparation of metaphase sea urchin chromosomal karyotypes and preliminary karyograms for both *S. droebachiensis* and *S. purpuratus*. Additionally, we have successfully localized *bindin* genes (that produce species-specific vitelline membrane-binding protein) by using fluorescent *in situ* hybridization (FISH) and three fluorescently labeled bacterial artificial chromosome

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(BAC-FISH) clones (*brachyury*, *foxa,* and *foxb)* from the Caltech BAC library on chromosomes from both species of strongylocentrotid sea urchins. Our protocols provide an important proof-of-concept for developing gene maps for the many interesting genes in the sea urchin genome.

Materials and Methods

Collection and fertilization of sea urchins

The specimens of *Strongylocentrotus purpuratus* used in this study from the coast of California were provided by Dr. Eric Davidson, California Institute of Technology; those of *S. droebachiensis* were collected intertidally at Odiorne Point on the New Hampshire coast; both species had ripe gonads containing ova and spermatozoa (Walker *et al.,* 2005). Intracoelomic injection of 0.5 mol l^{-1} KCl through the peristomial membrane (Auclair, 1965) resulted in spawning. Ova were washed and fertilized in 3–4 mmol l^{-1} p-aminobenzoic acid (PABA) in 0.2-μm filtered seawater (FSW) at pH 7.8.

Blastomere dissociation and chromosomal spreads

Cleavage-stage embryos (harvested after about 4 h at 18°C for *S. purpuratus* and 18 h at 4 °C for *S. droebachiensis* and with between 16 and 32 blastomeres for both species) were treated for 1 h with 1.0 mg/ml colchicine in FSW followed by resuspension in 1 mol l^{-1} urea. The resulting embryos were passed through a Nytex filter (120 μm) to physically remove the fertilization membrane and to dissociate the blastomeres. The resulting blastomeres were treated in 8% sodium citrate solution and washed three times with Carnoy's fixative (Saotome *et al.*, 2002); then 10-μl aliquots were placed on heated (46 °C), positively charged slides (Fisher Scientific, Colorfrost Plus slides). Blastomeres in each aliquot were spread (squashed) under coverslips manually using thumb pressure on a coverslip (22 × 22 mm).

Generating karyotypes for both strongylocentrotid species

Blastomere spreads were stained with Giemsa, 4′-6-diamidino-2-phenylindole (DAPI), or propidium iodide (PI) to visualize their chromosomes. CDP staining (specific for GC-rich regions) was conducted using 0.4 μg/ml PI and 0.8 μg/ml DAPI. Karyotypes were generated from clearly discernible chromosomal spreads by pairing the chromosomes on the basis of their constriction sites, centromere position, size, and faint DAPI banding patterns. All observations were made on a Zeiss Axioplan II MOT microscope equipped with epifluorescence, an AxioCam MR camera, and AxioVision 4.3 software (Carl Zeiss, Inc., Thornwood, NJ).

Preparation of chromosomes for FISH and BAC/FISH

Slides with chromosomal preparations (prepared as above) were submerged in liquid nitrogen and coverslips were removed using a razor blade (VWR). Resulting slides with chromosomal preparations were treated with 0.1% pepsin in 0.01N HCl at 37 °C for 10 min to remove histones and other chromosomal proteins. Following pepsin digestion, slides were rinsed in Dulbecco's modified phosphate buffered saline (PBS) for 10 min. Chromosomal preparations were denatured using 70% formamide in 2× SSC (3.5% NaCl, 0.89% sodium citrate) for 90 s at 80 °C. Finally, chromosomal preparations were dehydrated in an ethanol series (−20°C) and allowed to air dry.

Labeling of FISH and BAC/FISH probes

A 955-bp PCR product for *bindin* was amplified using primers (F:GTTGGGGTTCGGTGTGACTGAG and R: CCGCCCATCTGTTGAGGACT) developed from a sequence derived from *S. purpuratus* sperm (NCBI accession number AF077310). The resulting PCR product was purified using the High Pure PCR Cleanup Micro kit (Roche Applied Sciences) and was nick-translated by incubation for 1.5 h at 15 °C using the DIG-Nick Translation kit (Roche Applied Sciences, Indianapolis, IN). BAC clones provided by Caltech were nick-translated using the DIG-Nick Translation kit (Roche, Indianapolis, IN) and the BioNick kit (Invitrogen, Carlsbad, CA).

Hybridization and detection of genes on chromosomes

All probes resulting from protocols outlined above were prepared in hybridization buffer (50% dionized formamide, 10% dextran sulfate in 2× SSC), denatured at 90 °C for 10 min, and immediately placed on ice. Probes were delivered to slides of chromosomal preparations for both species, cover-slipped, and secured with rubber cement. Slides were incubated at 37 °C overnight in a moist chamber. Rubber cement was removed and coverslips were allowed to float off in 2× SSC. Slides were washed in 2× SSC for 10 min at 42 °C followed by 1× PBS for 10 min. Detection buffer containing anti-digoxigen rhodamine, streptavidin-Alexaflor 555, and/or anti-digoxigen fluorescein was placed over chromosomal preparations and incubated at 37 °C for 1 h in a moist environment. Slides were washed three times for 5 min in TNT buffer (0.1 mol l^{-1} Tris–HCl pH 7.6, 0.15 mol l^{-1} NaCl, 0.05% Tween-20) and placed in 1× PBS. Slides were drained, treated with Vectashield-DAPI, and observed on a Zeiss Axioplan II MOT microscope equipped with epifluorescence, an AxioCam MR camera, and AxioVision 4.6 software (Carl Zeiss, Inc., Thornwood, N.J).

Slides that yielded positive results for one gene were preserved at −20 °C prior to re-hybridization with alternative probes. Re-hybridization was accomplished by allowing coverslips to float off in 2× SSC followed by rinsing in Dulbec-

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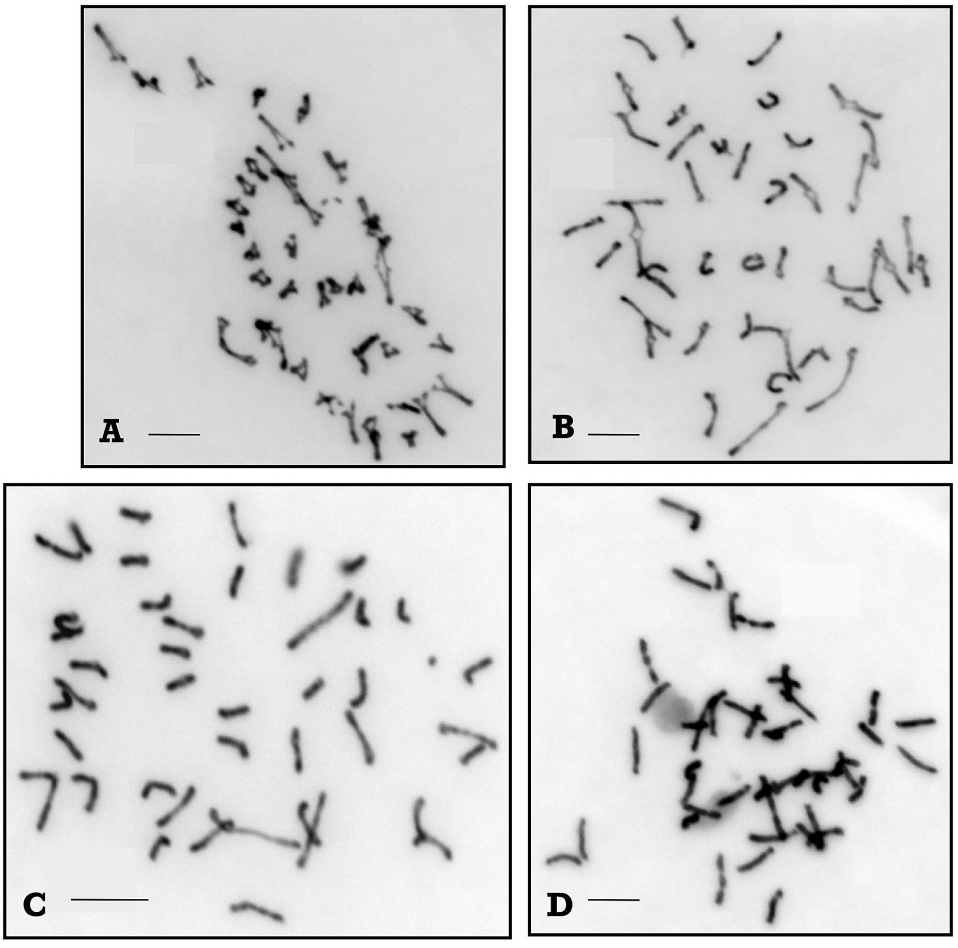

Figure 1. Metaphase chromosomes stained with DAPI for *Strongylocentrotus purpuratus* (**A**) heteromorphic spread and (**B**) homomorphic spread, and for *S. droebachiensis* (**C**) heteromorphic spread and (**D**) homomorphic spread. Scale bars = 5 μm.

co's modified PBS for 1 h. Chromosomal preparations and alternative hybridization probes were prepared as above.

Results

Established techniques described in the literature for chromosome preparation for other species of invertebrates (Shoguchi *et al.*, 2006) required modification when applied to sea urchins. We obtained excellent chromosomal preparations from a manual coverslip-squash technique. Use of heated slides in a moist environment as described in previous literature (Gerhart *et al.*, 1979) did not reliably yield interpretable chromosomal preparations. Excellent chromosomal preparations consistently resulted from protocols involving colchicine treatment, use of Nytex mesh for dissociation of blastomeres, and manual squashing of blastomeres under coverslips (Fig. 1). The most important procedures for generating cells for chromosomal squashes are complete removal of the fertilization membrane and complete separation of blastomeres from one another. Among a variety of concentrations tested (ranging from 10 μmol l^{-1} to 8 mmol l^{-1}), 3–4 mmol l^{-1} PABA thoroughly removed the fertilization membrane. Selected on the basis of its high cleavage rate, the optimum embryonic stage for generating chromosomal preparations was between 16 and 32 blastomeres. Application of colchicine at these developmental stages inhibits microtubule polymerization and results in the maximum accumulation of blastomeres in metaphase. Gentle shaking with urea followed by passage through Nytex mesh was necessary to fully dissociate blastomeres. Faint DAPI banding was more informative than Giemsa, PI, or CDP staining in establishing karyotypes for both species of strongylocentrotid sea urchins.

Both species have 21 chromosomal pairs—20 homomorphic (autosomal) and one heteromorphic (putative sex) pair (Fig. 2). In our preparations, the chromosomes of *Strongylocentrotus purpuratus* are structurally indistinguishable from those of *S. droebachiensis*. There are 2 large, 8 medium, and 10 small pairs of autosomal chromosomes in both

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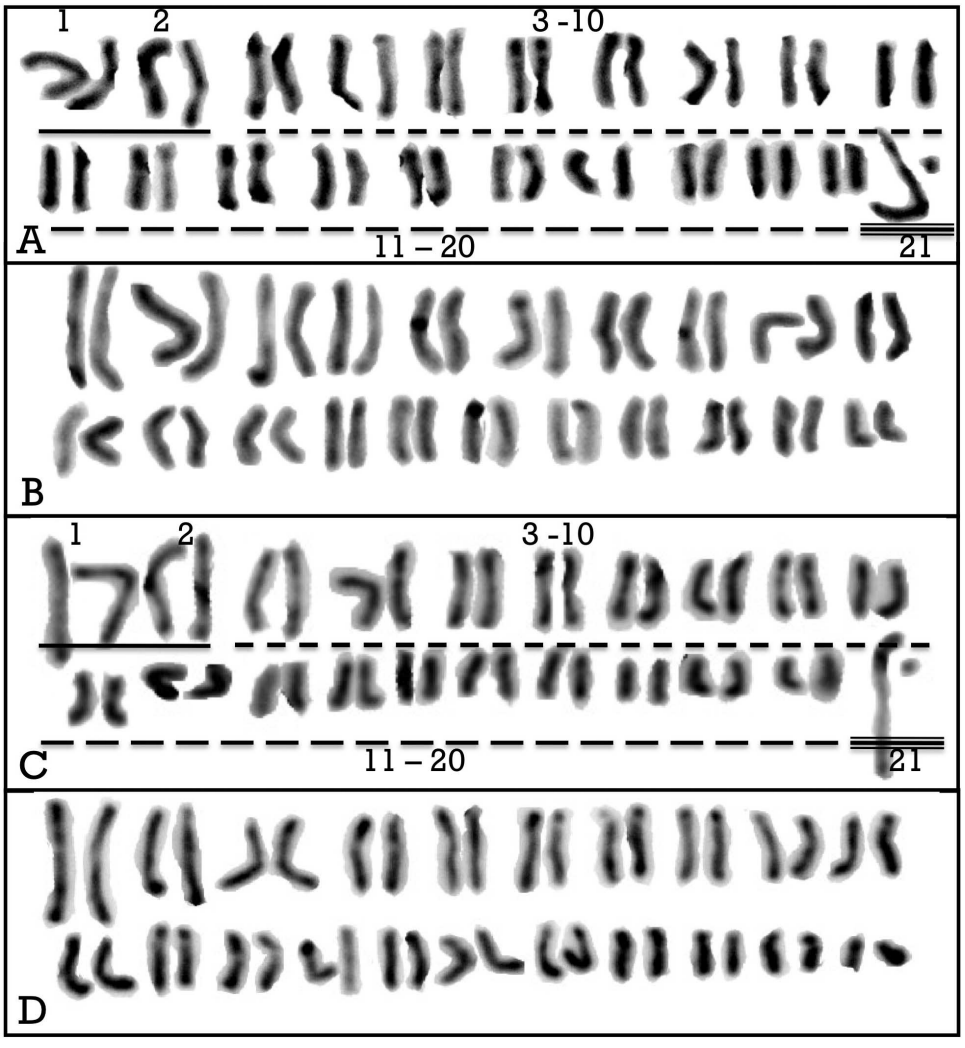

Figure 2. Karyograms based on constriction sites, centromere position, size, and faint DAPI banding patterns for *Strongylocentrotus purpuratus* (**A**) heteromorphic pair spread and (**B**) homomorphic pair spread, and for *S. droebachiensis* (**C**) heteromorphic pair spread and (**D**) homomorphic pair spread. In the heteromorphic spreads (A and C), 2 large chromosomes are indicated by a solid line; 8 medium chromosomes are indicated by small dashed line; 10 small chromosomes are indicated by large dashed lines; and the putative sex chromosomal pair is indicated by the divided line.

species. Among 34 clearly readable *S. purpuratus* chromosomal spreads, 19 had a heteromorphic pair and 15 had two of the longest chromosomes. Since preparations that lack the small chromosome have two large chromosomes, the additional large chromosome is believed to be the homolog for the small chromosome. On the basis of these spreads, we have generated a preliminary karyogram for both species of strongylocentrotid sea urchins. We feel confident in numbering chromosomal pairs 1, 2, and 21; precise pairing and localization of specific genes on small-(3–10) and medium -(11–20) sized chromosomal pairs 3–20 will depend upon more extensive use of our techniques.

F3 Figure 3a shows the *bindin* gene alleles localized on two pair of homologous chromosomes. *Bindin* genes were localized at the end of the long pair and also on a medium-sized chromosome in *S. purpuratus.* In separate preparations, use of BACs as probes for FISH gave a strong signal that can easily be visualized on specific chromosomes. The BAC clone for *brachyury* contains a single-copy gene that localized to a medium-sized chromosome that has a secondary constriction site. The BAC clones containing *foxa* and *foxb* both localized to different medium-sized chromosomes with no morphologically unique characteristics (Fig. 3b–d).

Discussion

Localizing genes on chromosomes of organisms with sequenced genomes is vital to assembling a map of the

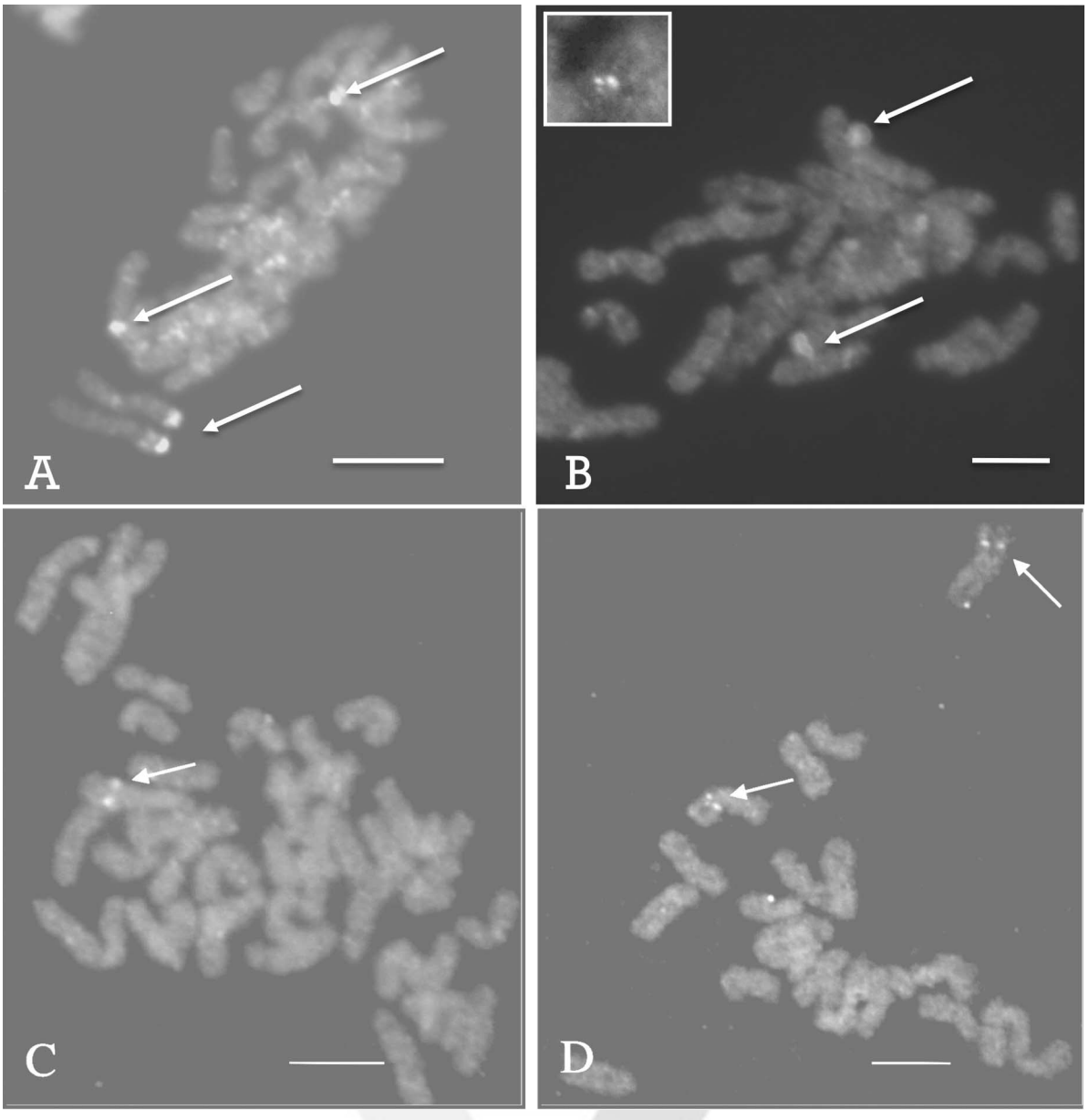

Figure 3. *Strongylocentrotus purpuratus* chromosomes counterstained with DAPI; arrows refer to FISH signals using DIG-labeled probes (anti-DIG rhodamine) for (**A**) *bindin*, (**B**) *foxb*, (**C**) *foxa*, and (**D**) *brachyury.* Inset in B at higher resolution for *foxb*. Scale bars = 5 μm.

genome sequences and also to understanding how genes may interact in intact interphase nuclei (Branco and Pombo, 2006; Shoguchi *et al.*, 2006). The availability of a complete catalog for the genes for *Strongylocentrotus purpuratus* provides important data necessary for localizing genes on the chromosomes of this sea urchin species. Data on the chromosomal locations of genes would permit an evaluation of their locations within chromosomal territories, where genes that are on different chromosomes may interact when they are located near each other (Levine and Davidson, 2005; Branco and Pombo, 2006).

Sea urchins have small chromosomes (1–6 μmol l^{-1}) that are highly similar, and conventional cytogenetic techniques have yielded preliminary karyograms for only three unrelated species: *Arbacia punctulata* (2n = 44—Auclair, 1965); *Pseudocentrotus depressus*, a strongylocentrotid urchin related to both species used in the current study (Lee, 2003; 2n = 42—Yamanaka *et al.,* 1989); and *Paracentrotus lividus* (2n = 36—Lipani *et al.,* 1996). We have localized bindin genes using FISH and single BACs on chromosomes using BAC-FISH. These are the first successful single-copy gene localizations on the chromosomes of the purple and

Orig. Op.	OPERATOR:	Session	PROOF:	PE's:	AA's:	COMMENTS	ARTNO:
1st Disk, 2nd	longd	7					z1n-3343

green sea urchins or any sea urchin species. Although we expect that a complete mapping of the genome of the purple sea urchin will provide extensive information, the data set we have generated in this study has already yielded previously unavailable information on the locations of specific genes:

Bindin. While a careful analysis of chromosomal preparations from the adult tissues of sexed individuals will be necessary to confirm the existence of sex chromosomes in sea urchins, another approach might be to determine the locations of genes encoding sex-specific proteins. At the molecular level, several proteins are differentially expressed in males and females of a variety of sea urchins. Numerous ovary-specific proteins are manufactured by the oocyte or the ovary and are eventually found in the cortical granules of ova (Walker *et al.,* 2007). Among others, these include hyalin (Wessel *et al.*, 1998), glycosaminoglycans (Schuel *et al.,* 1974), serine proteases (Haley and Wessel, 1999), and ovoperoxidase and proteoliaisin (Somers *et al.*, 1989; Nomura *et al.*, 1999). Proteins unique to the testis include histone H1, H2B-1, and H2B-2, which are produced in mitotically active spermatogonial cells (Poccia *et al.*, 1989); and bindin, which is produced by late spermatocytes and early spermatids (Cameron *et al.*, 1990). We hypothesized that a male-specific gene might be localized on one of the chromosomes in the heteromorphic (or putative sex chromosomal) pair. In an effort to test this hypothesis, we used the bindin gene because it is expressed only in the testis, and bindin mRNA is not present in female gonads or eggs (Gao *et al.*, 1986). Despite our initial hypothesis, we show that bindin is located on two sets of homologous chromosomes and that neither of these is the heteromorphic pair.

Brachyury, Foxa, and *Foxb. Brachyury* is a transcription factor that functions in gastrulation and endoderm development; downstream genes transcribed by *brachyury* are involved in morphogenesis of the gut (Rast *et al.*, 2002). *Foxa* represses the mesodermal fate in the veg2 endomesoderm, is required in postgastrular development for the expression of gut-specific genes, and is necessary for stomodeal formation (Oliveri *et al.*, 2006). *Foxb* transcription occurs in primary mesenchyme cells and in the endomesoderm; *foxb* is regulated by the gene products of *brachyury* and *foxa* (Levine and Davidson, 2005). We have shown that genes for *brachyury, foxa,* and *foxb,* which have interrelated functions and may be regulated by similar upstream gene products in the endomesodermal and other gene regulatory networks, are located on different chromosomes. These results make further analysis of gene locations within intact interphase nuclei possible and may yield positional information relevant to their documented interactions during sea urchin development (Levine and Davidson, 2005; Branco and Pombo, 2006).

Our methods provide a proof-of-concept for generating karyotypes of sea urchins and for use of FISH and BAC-FISH techniques on the chromosomes of two species of strongylocentrotid sea urchins. We hope that others will find these techniques useful in developing full gene maps for these strongylocentrotid sea urchins and that, as additional genomes are sequenced for other species of sea urchin, this information will be useful in determining interactions of genes during development and in adults of these model organisms.

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