Karyotyping and localization of genes on the chromosomes of the sea urchins, Strongylocentrotus drobachiensis and Strongylocentrotus purpuratus

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Karyotyping and localization of genes on the chromosomes of the sea urchins, Strongylocentrotus drobachiensis and Strongylocentrotus purpuratus

Abstract
The purple sea urchin, Strongylocentrotus purpuratus, is an important model for probing gene interactions during animal development and is the only non-chordate deuterostome for which a complete genome sequence is available. None of the genes cloned in this project have been localized on chromosomes, and maps of gene loci for S. purpuratus and for all sea urchin species do not exist. Here I provide a description of the first successful methods for karyotyping and localizing of specific gene loci on chromosomes of the purple sea urchin, Strongylocentrotus purpuratus and also of the related green sea urchin, Strongylocentrotus droebachiensis.

Both species have 42 chromosomes in their diploid genomes. Most of their chromosomal pairs are acrocentric or submetacentric with one telocentric pair. There are two large, eight medium and ten small pairs plus one putative sex specific pair. In both species, bindin genes were localized on two chromosomal pairs fluorescent in situ hybridization (FISH). Fluorescently labeled bacterial artificial chromosome (BAC) clones from the S. purpuratus genome were prepared for the developmentally important genes, brachyury, foxA, and foxB, and they were localized on chromosomes with both single and dual labeling. All three genes reside on different chromosomes. The protocols provide an important proof of concept for researchers in developmental and molecular biology as gene maps for the many interesting genes in the sea urchin genome are developed.

Keywords
Biology, Zoology

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KARYOTYPING AND LOCALIZATION OF GENES ON THE CHROMOSOMES OF THE
SEA URCHINS, STRONGYLOCENTROTUS DROBACHIENSIS AND
STRONGYLOCENTROTUS PURPURATUS.

BY

CELESTE CHLOE ENO
BS, University of New Hampshire, 2006

THESIS

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Master of Science
in
Zoology

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Department of Biological Sciences

5/11/09 Date
DEDICATION

I am extremely grateful to those who have motivated me to achieve this feat. From the beginning, I had a desire to incorporate art into my science. I have found microscopy achieves this desire. I dedicate my work to those who have given me the opportunity to achieve what I have achieved.

My parents, Lawrence and Susan Eno, gave me the gift of education and ability. They have instilled the importance of a good education in me. Without their support and push, I would not be in the place I am in now.
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Dr. Charles Walker took me into his laboratory as a freshman, and I am grateful. Thinking back, I was allowed to make mistakes and will always remember the lessons learned. Not only was he a research mentor, his teaching style is to be desired. I hope that my future students will think of me in a similar light.

Dr. Marianne Litvaitis showed me how to conduct myself in the science field. She taught me that women in science might be disadvantaged at points in their career, but they should never let that be an excuse. Her advice over the many years has kept me from just throwing in the towel when I faced daunting challenges.

Dr. Thomas Davis taught me that chromosomes are the packed form of DNA, and it is necessary to study them no matter the organism. I took basic genetics from him and learned that is what I wanted to pursue. Melanie Shields helped me with the corrections on my thesis.

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ABSTRACT

KARYOTYPING AND LOCALIZATION OF GENES ON THE CHROMOSOMES OF THE SEA URCHINS, STRONGYLOCENTROTUS DROBACHIENSIS AND STRONGYLOCENTROTUS PURPURATUS

By

Celeste Chloe Eno

University of New Hampshire, May, 2009

The purple sea urchin, Strongylocentrotus purpuratus, is an important model for probing gene interactions during animal development and is the only non-chordate deuterostome for which a complete genome sequence is available. None of the genes cloned in this project have been localized on chromosomes, and maps of gene loci for S. purpuratus and for all sea urchin species do not exist. Here I provide a description of the first successful methods for karyotyping and localizing of specific gene loci on chromosomes of the purple sea urchin, Strongylocentrotus purpuratus and also of the related green sea urchin, Strongylocentrotus droebachiensis.

Both species have 42 chromosomes in their diploid genomes. Most of their chromosomal pairs are acrocentric or submetacentric with one telocentric
pair. There are two large, eight medium and ten small pairs plus one putative sex specific pair. In both species, bindin genes were localized on two chromosomal pairs fluorescent in situ hybridization (FISH). Fluorescently labeled bacterial artificial chromosome (BAC) clones from the S. purpuratus genome were prepared for the developmentally important genes, brachyury, foxA, and foxB, and they were localized on chromosomes with both single and dual labeling. All three genes reside on different chromosomes. The protocols provide an important proof of concept for researchers in developmental and molecular biology as gene maps for the many interesting genes in the sea urchin genome are developed.
CHAPTER I

STRONGYLOCENTROTUS SP. CHROMOSOMES

INTRODUCTION

Sea Urchin

There are five extant classes in the phylum Echinodermata (invertebrate deuterostomes): Asteroidea, Ophiuroidea, Holothuroidea, Crinoidea, and Echinoidea. Sea urchins (Echinoidea) are of particular interest because they the basis for are an important commercial fishery and their development is easy to study in the laboratory. At one time, Maine and California had productive sea urchin industries. In America, this fishery developed in the 1980s based on the popularity of uni sushi in Japanese cuisine. There were 2,725 licensed divers and draggers in Maine by 1994, harvesting 38 million pounds (value $33 million that year). A dramatic decline in the urchin fishery occurred between 1997/98. Many regions of the coast of Maine have been unproductive for the past ten years and a moratorium on urchin collection has been established for most harvest zones to allow the stocks to recover (MDMR, 2004).

Sea urchins are not only important as a fishery, they also provide an excellent experimental system for the study of animal development. Since urchins are deuterostomes and share many genes with humans, sea urchins are
more closely related to us than are the protostomes, Drosophila or C. elegans. Additionally, since sea urchins are invertebrate deuterostomes, they provide an excellent outgroup for vertebrate deuterostomes in phylogenetic studies.

**Invertebrate Cytogenetics**

"Cytogenetics is the study of the structure, function, and evolution of chromosomes, the vehicles of inheritance that reside in the cell nucleus (Miller and Therman, 2001)." The study of cytogenetics has evolved over the past century from simple staining of chromosomes to the localization of individual cloned genes on specific chromosomes by *in situ* hybridization. Since many genomes have now been sequenced, an important next step is to locate groups of functionally related genes on chromosomes.

Sea urchin chromosomal preparations were first successfully produced by Auclair and German (1966). Auclair (1965) pointed out the difficulty of obtaining sea urchin chromosomes based on their small size and large numbers. In addition, it is difficult to accurately visualize complete sets of invertebrate chromosomes in single cells in histological sections.

Studies of sea urchin chromosomes have been hampered because of the tough fertilization membrane that develops immediately following fusion of sperm and egg. Before introduction of sperm, a vitelline membrane surrounds the egg.
Fusion of the gametes results in secretion of the contents of cortical vesicles (located in the cortical cytoplasm of the ovum). Included in the cortical granules are colloids, which cause the vitelline membrane to rise, and structural proteins, which bind to and harden the vitelline membrane that is subsequently, termed the fertilization membrane.

Auclair (1965) described a method to count chromosomes using embryos of sea urchins. He fertilized ova and immediately removed the fertilization membrane by using mercaptoethylgluconamide and passed the embryos through a mesh cloth. He then added colchicine 20 minutes prior to first cleavage (Auclair, 1965). Showman and Foerder (1979) found that it was possible to weaken the fertilization membrane using aminotrizole (ATA) during the first five minutes following fertilization. The embryo can be released from the weakened fertilization membrane by passage through Nytex mesh. ATA did not denature, reduce, or lyse proteins as did other commonly used reagents and no additional treatment was necessary before removal of the membrane (Showman and Foerder, 1979).

In previous studies, two different tissue preparations of sea urchin cells have resulted in successful chromosomal spreads: embryos and andromerogones. Saotome et al. (1982) prepared chromosomes from sea urchin embryos following fertilization of ova in Ca²⁺/Mg²⁺-free seawater, which loosens the thick and tough fertilization membrane. Once the membrane was weakened, it was removed by titration followed by washing the denuded embryos.
in full strength seawater. When the embryos reached a desired stage, they were treated with colchicine. Blastomeres were washed and dissociated by triturating in Ca$^{2+}$/Mg$^{2+}$-free seawater and exposure to a hypotonic solution of KCl. Dissociated blastomeres were fixed in freshly made Carnoy’s fixative, dropped onto slides and dried overnight. In this method, the membranes of blastomeres were broken open without squashing under a coverslip (Saotome, 1982).

Saotome et al. (2002) provided chromosome numbers for seven sea urchin species using andromerogones. Andromerogones are non-nucleated ova fertilized with a sperm. These preparations provide a count of the haploid male-derived chromosome complement. Saotome and Komatsu (2002) also published an account of the chromosomes of a Japanese starfish. They were able to establish a preliminary karyotype using Giemsa stain and locate nucleolar organizing regions (NORs) using silver nitrate. The authors noted that the karyotypes were preliminary because of the size and similar morphology of the chromosomes (Saotome and Komatsu, 2002).

Chromosomes of the European urchin, Paracentrotus lividus were characterized by Lipani et al. (1996). The diploid genome of this species comprises only 36 chromosomes, which is a relatively low number for sea urchins. Three chromosome pairs had NORs. A putative pair of sex chromosomes was identified with an XY configuration. These authors were the first to publish the hypothesis that sex chromosomes are present in sea urchins. Their justifications for this hypothesis were: a) one homologous pair was
heteromorphic in 50% of the blastulae as in other species with XY/XX and ZZ/ZW; b) the putative sex chromosomes were not only morphologically different (as with heteromorphisms from pericentric inversions), but also the size of the Y chromosome was dramatically smaller; and c) the blastomeres of all embryos were identical in chromosome morphology (Lipani et al., 1996).

Sex determination systems vary in invertebrates. For example, males may be the heterogametic sex (XY), females may be the heterogametic sex (ZW), or males may not have a corresponding sex chromosome (XO). Vitturi et al. (2003) found evidence for a male XO system in Pentodon bidens punctatum (scarab beetle). In a number of other insects, including some of those in the order Orthoptera and Caenorhabditis elegans, the male has the XO form and hermaphrodite has the XX form (John, 1990). Some insects, as well as birds, have the ZW sex-determination system in which the female has the differential pair and the male does not. The XY system is most commonly found in mammals.

Lipani et al. (1996) used G-banding followed by digestion with trypsin to identify chromosomal pairs. Perhaps as a result of their high degree of condensation, chromosomes lacked significant banding, a problem encountered in studies of many invertebrate chromosomes. These authors questioned whether other echinoids have sex chromosomes because species with heteromorphic chromosome sex systems tend to have fewer chromosomes. Auclair (1965) and German (1966) found that the diploid chromosome number for
Arbacia punctulata is 44, and Auclair (1965) presented a preliminary karyotype. For Echinarchnus parma, Auclair (1965) approximated the 2N genome to contain either 44 or 46 chromosomes.

The first report using the model species, Strongylocentrotus purpuratus was that of Gerhart (1979). He observed the chromosomes of Lytechinus variegatus and Strongylocentrotus purpuratus but did not publish any images. Gerhart (1979) determined the diploid chromosome number to be 42 in both species. Using aceto-orcein staining, he identified telocentric, acrocentric, and submetacentric chromosomes.

Banding methods have been used in the study of many species of sea urchins. Unfortunately, many banding techniques do not work with invertebrate chromosomes because of their size. Staining for ribosomal DNA using silver nitrate is a popular method for identifying NORs. Silver nitrate exists at secondary constrictions and will only stain transcriptionally active NORs (Hubbell et al., 1979).

G-C rich staining is performed using chromomyacin A3 (CMA₃) or propidium iodide (PI). Both solutions stain G-C regions preferentially; while the other popular intercalator stain, 4′, 6-diamidino-2-phenylindole (DAPI) preferentially stains A-T rich regions. When PI and DAPI are used together (a technique known as CDP “combined DAPI and PI” staining), chromosomal banding is often revealed (Kim et al., 2001). This technique has rarely been successful in invertebrates because of the lack of G-C rich regions.
Since invertebrate chromosomes are difficult to distinguish, researchers have relied heavily on more modern cytogenetic techniques to differentiate chromosomes, including in situ hybridization in which fluorescently labeled gene probes are used. Vitturi et al. (2003) used Giemsa and silver staining, CMA3, DAPI, and rDNA fluorescent in situ hybridization (FISH) to study chromosomes in the snail, Cerithium vulgatum. They found that CMA3 and DAPI staining were uninformative.

Torreiro et al. (2004) performed FISH on the bivalve, Brachidontes rodriguezi using probes for the 18S and 28S rDNA. They found the NOR locations (by silver staining) matched the locations of the rDNA probes.

Successful FISH experiments have been conducted with ascidian ribosomal DNA and telomeres (Castro and Holland, 2002), in which probes were easily placed on both metaphase and interphase chromosomes. Shoguchi et al. (2004) successfully mapped (physical) the entire genome of the ascidian, Ciona intestinalis. They pointed out that gene location is important to determine scaffold layout. Using BACs (bacterial artificial chromosomes) as probes, they demonstrated that all of the BACs labeled two chromosomes, as expected in a diploid genome. They followed this initial study by employing two-probe FISH, which allowed them to compare locations of two different BAC clones. Shoguchi (2005) demonstrated the value of using BAC clones in generating a karyotypes when compared to previous chromosome analysis techniques. He asserted that
mapping of genes onto chromosomes is necessary for future studies dealing with
genome-wide control of developmentally relevant gene expression.

Shoguchi et al. (2006) mapped 170 BAC clones to chromosomes of
Ciona intestinalis. These authors make the point that although cloning genome
sequence is vital, it is equally important to determine gene location. They
concluded that this study should be recreated for all species for which draft
genomes are available, including “such organisms as sea urchin and
amphioxus.”

Only two papers describe urchin chromosomes using in situ hybridization.
Gornung et al. (2005) hybridized rDNA to the chromosomes of Paracentrotus
lividus. Her group found that only one pair of chromosomes had 5S rDNA genes.
However, Caradonna et al. (2007) showed three different 5S rDNA clusters in P.
lividus. Their results were confirmed using co-hybridization experiments with two
different probes.

Chromosome Analysis of Strongylocentrotus sp.

The genome of Strongylocentrotus purpuratus (from the Western coast of
the USA) has been fully sequenced, extensively annotated and accompanied by
a comprehensive analysis of when and where many genes are expressed
(Ameniya et al., 2005). Genes characterized in this genome also indicate the
importance of urchin genomics for studies on genetics, immunology, olfaction
and vision, gene regulatory networks, etc. Among the interesting revelations
from this genome project is that sea urchins share a large number of orthologs for human diseases (e.g., those associated with Huntington's disease and muscular dystrophy). Research on the biology of sea urchins has lead to many important scientific advances (e.g., the importance of Ca$^{2+}$ in activating egg metabolism following fertilization, an understanding of sperm activation and flagellar motility, the importance of sperm-egg recognition, and structure and function of cytoskeletal components (SUGSC, 2006).

To date, no genes have been localized on chromosomes of *S. purpuratus*, and only one account of ribosomal genes have been placed on the chromosomes of any sea urchin species (Cardonna *et al.*, 2007). Again, this is mostly because of their small size and technical difficulties involved in preparing sea urchin chromosomes. The genome of the related green sea urchin of the East coast of the USA, *Strongylocentrotus droebachiensis*, contains highly conserved homologs for many genes found in the purple sea urchin, and again none of these have been localized to specific chromosomes.

In 2001, the Sea Urchin Genome Project developed a bacterial artificial chromosome (BAC) library, which contains about 100,000 clones (17.5X coverage, 140 kb average length) (SUGSC, 2006). Three other urchin species (*Paracentrotus lividus, Lytechinus variegatus*, and *Eucidaris tribuloides*) have BAC libraries available. Comparing the libraries led to the estimation that *L. variegatus* and *S. purpuratus* diverged 50 MYA from one another. Since this
project, there are 185 single-gene BAC clones available on the SUGP website with the average insert size of 40 kb.

In this thesis, I describe techniques for the successful preparation of metaphase sea urchin chromosomal karyotypes. Additionally, the bindin gene was localized on chromosomes in both species of strongylocentrotid sea urchins. Three single copy genes in BAC clones (brachyury, foxA and foxB) from the Caltech BAC library were also localized on chromosomes of the purple sea urchin.

Bindin is responsible for sperm-egg attachment and membrane fusion. The sequence of this protein is species-specific and results in reproductive isolation among sea urchin species (Zigler and Lessios, 2003). Bindin is of interest mainly because it is a male-specific protein. Gao et al. (1986) found that bindin was exclusively expressed in the testes. Bindin mRNA was not present in female gonads or eggs. Their findings show that there is differential expression of genes in males and females. Based on these observations, I hypothesized that bindin might be located on the male chromosome and allow the determination of the sex specific pair of chromosomes I identified were indeed sex chromosomes.

In vertebrates, brachyury is involved in notochord formation; it is also found in the sea urchin (Rast, 2002). Brachyury is a transcription factor that functions in gastrulation and endoderm development. Brachyury is a single copy
gene and I hypothesized that it would be located on two homologous chromosomes and would allow me to pair these chromosomes.

The Forkhead family of genes are expressed during embryonic development, cell fate specification, cell differentiation, morphogenesis, chromatin structure, and regulation of cell cycle and metabolism (Tu et al., 2006). Members of the Forkhead family, foxA and foxB are phylogenetically closely related. FoxA expression occurs in the endoderm and oral ectoderm and is involved in endoderm specification. FoxB is expressed in the skeletogenic mesenchyme cells. All genes of the Forkhead family are suggested to have undergone “cooption”, where the existing genes were tailored into a new functional gene group. It would be interesting to determine the location of these genes in relation to one another, particularly in interphase nuclei. Both foxA and foxB are single copy genes and I hypothesize that they should be located on two chromosomes each. Oliveri et al. (2006) found that the foxA gene can be repressed by the FoxA protein. FoxA protein also controls expression of many other genes, such as hedgehog and is required for formation of oral ectoderm.
AIMS and HYPOTHESES

Specific Aim 1: To generate karyotypes for both green and purple sea urchins.

Hypothesis 1: Using DAPI to match chromosomes by sites of constriction and size, a karyotype can be generated for both of these species.

Specific Aim 2: To localize bindin on sea urchin chromosomes using fluorescent in situ hybridization.

Hypothesis 2: Bindin is a sex specific gene and will be localized on one of the sex specific chromosomes identified in the karyotypes of both green and purple sea urchins.

Specific Aim 3: To use BAC clones as probes to localize three single copy genes on chromosomes of the purple sea urchin.

Hypothesis 3: Brachyury, foxA, and foxB will be located to specific chromosomes using single and dual probe FISH.
MATERIALS and METHODS

Collection and fertilization of sea urchins

*S. purpuratus* used in this study were collected from the coast of California and provided by Dr. Eric Davidson (Caltech); *S. droebachiensis* were collected intertidally at Odiome Point, New Hampshire coast; both species had ripe gonads containing ova and spermatozoa. Intracoelomic injection of 0.5 M KCl through the peristomial membrane resulted in spawning (Auclair, 1965). Ova were washed and fertilized in 3-4 mM p-aminobenzoic acid (PABA) in 0.2 μm filtered sea water at pH 7.8.

Blastomere dissociation and chromosomal spreads

Embryos at the early blastula stage (approximately 4 h at 18°C for *S. purpuratus* and 18 h at 4°C for *S. droebachiensis*, and between the 16 and 32 blastomeres for both species) were treated for 1 h with 1.0 mg/ml colchicine in filtered seawater (FSW) and then resuspended in 1 M urea. Resulting embryos were passed through a Nytex filter (120 μm) to mechanically remove the fertilization membrane and to dissociate the blastomeres. Resulting blastomeres were treated in 8% sodium citrate solution, washed three times with Carnoy’s fixative (Saotome *et al.*, 2002) and placed in aliquots of 10 μl on heated (46°C), positively charged slides (Fisher Scientific, Colorfrost Plus slides). Blastomeres in each aliquot were spread under coverslips manually using thumb pressure on a coverslip (22X22 mm).
Generating karyotypes for both strongylodentrotid species

Blastomere spreads were stained with Giemsa, 4'-6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) to visualize the 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 discernable chromosomal spreads by pairing the chromosomes based on their constriction sites, size and DAPI banding patterns. All observations were made on a Zeiss Axioplan II MOT 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. Resulting slides with chromosomal preparations were treated with 0.1% pepsin in 0.01 N HCl at 37°C for 10 min to remove histones and other chromosomal proteins. Following pepsin digestion, slides were rinsed in Dulbecco's modified PBS for 10 min. Chromosomal preparations on slides were denatured using 70% formamide in 2XSSC (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: 5'-GTTGGGGTTCGGTGTGACTGAG and R: 5'-CCGCCCATCTGTTGAGGACT) developed from S. purpuratus sperm DNA (NCBI accession number - AF077310). The resulting PCR product was purified using the High Pure PCR Cleanup Micro kit (Roche Applied Sciences, Indianapolis, IN) and was nick translated by incubating for 1.5 h at 15°C using the DIG-Nick Translation kit (Roche Applied Sciences, Indianapolis, IN). BAC clones of around 40 kb in size provided by Andy Cameron (Caltech) were nick translated using the DIG-Nick Translation kit (Roche Applied Sciences, Indianapolis, IN) and the BioNick kit (Invitrogen, Carlsbad, CA).

Primers for brachyury (F: 5'-ACACATCGACCATCAA, R: 5'-CATGGTGTGTATCTTGAAAG) and foxA (F: 5'-CCAACCGACTCCGTATCATC, R: 5'-CGTAGCTGCTCATGCTGTGT) (from Caltech) were used to obtain partial sequences of these genes using PCR with FITC-dNTPs (Roche, Indianapolis, IN) to yield FITC-labeled probes. Primers were not available for foxB (no FITC probes made).

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 2XSSC), denatured at 90°C for 10 min, and immediately placed on ice. Probes were
delivered to positively charged 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 2XSSC. Slides were washed in 2XSSC for 10 min at 42°C followed by 1XPBS for 10 min. Detection buffer containing anti-DIG rhodamine, streptavidin-Alexaflor 555 and/or anti-DIG Fluorescein was placed over chromosomal preparations and incubated at 37°C for 1 h in a moist environment. Slides were washed 3 X 5 m in TNT buffer (0.1 M Tris–HCl pH 7.6, 0.15 M NaCl, 0.05% Tween-20) and placed in 1XPBS. Slides were drained, treated with Vectashield-DAPI and observed on a Zeiss Axioplan II MOT 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 2XSSC followed by rinsing in Dulbecco’s modified PBS for 1 h. Chromosomal preparations and alternative hybridization probes were prepared as above.
RESULTS

In the current study, 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). Although I investigated tube feet and gut tissue, cells in these tissues were too small and had few cells involved in mitosis.

Figure 1: Spreads of Strongylocentrotus droebachiensis (A-B) and S. purpuratus (C-D) using DAPI. Scale bar = 5 µM

Accumulation of mitotic cells is important when performing chromosomal analyses. Treatment in colchicine for 1 to 1.5 h yielded the highest mitotic index
Concentrations of colchicine around 1 mg/ml stopped mitosis without resulting in mortality.

Most important for generating readable chromosomal preparations were complete removal of the fertilization membrane and complete separation of blastomeres from one another. Among a variety of concentrations tested (ranging from 10 μM to 8 mM), 3-4 mM PABA resulted in thorough removal of the fertilization membrane. Based on an apparent high cleavage rate, the optimum embryonic stage for generating chromosomal preparations was between the 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 allowed for full dissociation of blastomeres. Nytex mesh and pipetting sheared the fertilization membrane enough to denude the blastula. Urea caused blastomeres to dissociate. The cells were then washed and allowed to swell in a hypotonic solution.

Fixation was attempted using Carnoy’s, 70% ethanol, and paraformaldehyde. Paraformaldehyde fixation resulted in excellent preservation of whole blastomeres; however, it yielded poor squashes and FISH results. Carnoy’s fixative (3:1, methanol to acetic acid) gave the best results in all these areas. Since blastomeres degrade in acetic acid, Carnoy’s was replaced with 70% ethanol for long-term storage of blastomeres.
Many chromosomal spreading techniques were evaluated. Use of heated slides in a moist environment as described in previous literature (Gerhart et al., 1979) did not reliably yield readable chromosomal preparations. Dropping blastomeres onto slides from various heights resulted in chromosomal loss and did not effectively break the cells apart. The drop and steam technique also did not effectively burst the blastomeres (Saotome, 1982). In this study, excellent chromosomal preparations resulted from a manual coverslip-squash technique. Use of BACs as probes for FISH gave a strong signal that could easily be visualized on specific chromosomes. DAPI banding was used to establish karyotypes for both species of strongylocentrotid sea urchins and yielded superior results when compared to Giemsa, PI or CDP staining (results not presented).

Both species of strongylocentrotid sea urchins have 21 chromosomal pairs, 20 homomorphic (autosomal) and one heteromorphic (putative sex) pair (Figs. 2 and 3). Structurally, the chromosomes of *S. purpuratus* are indistinguishable from those of *S. droebachiensis*. There are two large, eight medium and ten small pairs of autosomal chromosomes in both species. Among 34 clearly readable *S. purpuratus* chromosomal spreads, 19 had a heteromorphic pair and 15 had two matching extra-long chromosomes. Since preparations lacking the small chromosome have two large chromosomes, the additional large chromosome is believed to be the homolog for the small chromosome.
Agarose gels were used to evaluate results of nick-translations for the bindin PCR product (Fig. 4) and BAC clones, which causes the DNA to be chopped into smaller (100-250 bp) labeled probe. The segment of DNA is cut and a dioxygenin molecule is added, which is the labeled probe to be used in FISH.
Figure 4: Agarose gel of DIG-Nick of bindin (955 bp) with PhiX174/HaeIII DNA marker.

Figure 5 shows the bindin gene alleles localized on two pair of homologous chromosomes. Bindin genes were localized at the end of the long pairs and also on a medium sized chromosome in S. purpuratus (Fig. 5 A and C). The probe did not yield excellent preparations with S. droebachiensis chromosomes (Fig. 5 D) as successfully because of the high amount of background fluorescence present.
The BAC clone for *brachyury* (Fig. 6 A) contains a single copy gene that localized to a medium sized chromosome that has a secondary constriction site. The BAC clones containing *foxA* (Fig. 6 B) and *foxB* (Fig. 6 C) both localized to different medium sized chromosomes with no morphologically unique characteristics.
The PCR-FITC labeled BAC segments for *brachyury* and *fox* were simultaneously labeled with nick-translated BAC probes (Fig. 7 A-C). The labels were difficult to decipher from the background, and therefore, a better method needs to be developed.
Figure 7: FoxA and foxB (A), brachyury and foxB (B), and brachyury and foxA (C) BAC probes on Strongylocentrotus purpuratus chromosomes.
DISCUSSION

Localizing genes on chromosomes of organisms with sequenced genomes is vital 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. Locating genes on the chromosomes of S. purpuratus could yield considerable information on gene interactions resulting from their proximity to one another on specific chromosomes, thus suggesting functional relationships in gene regulatory networks (Levine and Davidson, 2005). Also, by virtue of their locations within chromosomal territories, data on the chromosomal locations of genes would permit an evaluation of their positions relative to one another in intact nuclei. Genes that are on different chromosomes may interact when they are located near each other (Branco and Pombo, 2006).

Established techniques for chromosome preparation available in the literature for other species of invertebrates required modification when applied to sea urchins (Shoguchi et al., 2006). Sea urchins have very small chromosomes (1-6 μM) that are highly similar, and conventional cytogenetic techniques have yielded karyotypes for only two species (none for strongylocentrotids). Of the tissue types initially surveyed, only embryos resulted in successful chromosome analyses. The tube feet and gut tissue did not have large enough cells in active mitosis. Blastomere chromosomes were large and presented clear spreads.
The blastomere cells result from a dissociation of the heterogeneously mixed male and female embryos, making it difficult to correctly identify the sex by observing a cell's chromosome spread. Regenerating urchin test might also yield sufficient cells in mitosis to accomplish analysis of chromosomes.

Colchicine inhibits microtubule polymerization, resulting in the accumulation of cells in metaphase and not interphase. Treatment in colchicine was used to achieve mitotic cells, and I found that concentration of approximately 1 mg/ml stopped mitosis without resulting in mortality.

I have localized bindin using FISH and single and multiple BACs on chromosomes using single and dual (two color) BAC-FISH. These are the first successful single-copy gene localizations using BACs on the chromosomes of the purple and green sea urchins or any sea urchin species.

While I expect that a complete mapping of the genome of the purple sea urchin will provide extensive information, the data set generated in this study has already yielded previously unavailable information on specific genes and known gene interactions:

a) 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. Among others, these include: hyalin (Wessel et al., 1998), glycosaminoglycans (Schuel et al., 1974), serine proteases (Haley and Wessel, 1999), ovoperoxidase and proteolysin (Nomura et al., 1999, Somers et al., 1989). Proteins unique to the testis include histone H1, H2B-1 and H2B-2 and 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). I hypothesized that a male specific gene might be localized on the shorter chromosome in the heteromorphic pair. To demonstrate this, I 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). Contradicting my initial hypothesis, my results show that bindin is located on two sets of homologous chromosomes and that neither of these is the heteromorphic or putative sex chromosomal pair. It has become evident that, although a gene is not expressed in the female mRNA, it is not necessarily indicative of the gene's absence in the female DNA.

b) 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 postgastular development for the expression of gut-specific genes, and is necessary for stomodeal formation (Oliveri et al., 2006). FoxA 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). I have suggested 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. It seems that *brachyury* localized to a morphologically different chromosome pair than *foxA* or *foxB*, but I could not positively show that *foxA* and *foxB* are located on different chromosomes. Based on these results, further analysis of gene locations within intact interphase nuclei is now possible and may yield positional information relevant to their documented interactions during sea urchin development (Branco and Pombo, 2006, Levine and Davidson, 2005).

Shoguchi *et al.* (2006) stated that localizing gene markers on chromosomes of species with sequenced genomes is valuable since sequence in the absence of genetic context does not permit us to address core biological questions using modern genome-wide approaches. Given the availability of a complete catalog for the genes of *S. purpuratus*, it is important to develop methods for localizing genes on the chromosomes urchin using fluorescent *in situ* hybridization. Generation of a map of gene locations for *S. purpuratus* chromosomes could yield considerable information on gene interactions such as proximity to one another, which could lead to an increased knowledge of function in the gene regulatory network. The proximity determination would allow studies of linkage and recombination frequency for transmission genetic studies. Generated location data could allow for cross species comparisons with other
model organisms. Since there is an extensive BAC clone library, all 185 single-gene BACs could be localized in chromosomes in the karyotypes. The more chromosomes that are labeled will help to make an accurate map and perhaps identify errors in the karyotypes.

Final resolution of the nature of the putative sex chromosomal pair needs to be accomplished in those species where one differential pair has been demonstrated. The very small chromosome seen in both *S. purpuratus* and *S. droebachiensis* in this study may not be sex chromosome at all. For example, since about half of the spreads contained only 41 chromosomes, this may be an XO sex chromosome system. The Y chromosome in humans is homologous to the X chromosome except that the Y chromosome contains a few more genes (e.g., the *SRY* gene; Haqq *et al.* 1994). The *SRY*-gene is important because it allows for the different protein expression between the two sexes. If the very small chromosome in *Strongylocentrotus* sp. is a true sex chromosome in an XY system, it should contain a genetic feature important in the development of the sexes.

Biotin-labeling via the Bio-Nick Kit (Invitrogen) proved unsuccessful for these strongylocentrotid species. I was able to make biotin-labeled probes from the BACs, the background was too intense to allow discrimination of successful chromosomal labeling. Perhaps biotin or streptavidin (a biotin chelator) presents alternate substrates in sea urchin cytoplasm.
Future work based on these studies could include comparative genomic hybridization (CGH), fiber FISH, and interphase FISH. CGH is useful to compare similar genomes. One species genome is used as a probe and is hybridized to the chromosomes of another species. For example, since S. purpuratus genome is sequenced, it could be easily made into probes and hybridized to the genomes, such as S. droebachiensis or P. lividus. Another modern technique that is gaining prominence is fiber FISH. When in late interphase, the DNA is still connected. The long strands of DNA can be pulled across a slide and used for FISH. The results will have a higher resolution than that of metaphase FISH. Interphase FISH can lead to determining which genes are near each other during transcription. One gene might have an effect on another and showing the relative distance could provide insight to whether or not they function together (Branco and Pombo, 2006).

Scientists at Duke University have raised hybrids of S. purpuratus x P. lividus, which raises questions because S. purpuratus has 2N= 42 chromosomes and P. lividus has only 2N= 36. Further chromosome studies could uncover how the hybrids are able to survive by receiving 1N (18) and 1N (21) chromosomes from the parental generation. Since finding that S. purpuratus does have telocentric chromosomes, it is highly probable the hybrid is viable by means of Robertsonian translocation where two chromosomes with telocentric centromeres combine with one chromosome from the gamete with fewer chromosomes.
My 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. I hope that others will find these techniques useful in developing full cytological maps for these strongylocentrotid and as additional genomes are sequenced for other species of sea urchin and that this information will be useful in determining interactions of genes during development and in adults of these model organisms.
LITERATURE CITED


