Evolution of a conserved gene regulatory network among echinoderms: A comparison of genes expressed in the skeletogenetic lineage of the ophiuroid Ophiocoma wendtii and the echinoid Strongylocentrotus purpuratus

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Evolution of a Conserved Gene Regulatory Network Among Echinoderms; a Comparison of Genes expressed in the Skeletogenetic Lineage of the Ophiuroid Ophiocoma Wendtii and the Echinoid Strongylocentrotus Purpuratus

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ABSTRACT

One of the most fundamental and critical functions of embryological development is the control and regulation of differential genes and gene networks. The study of the gene networks involved in development is a mechanism for understanding the developmental process at its most basic level. An evolutionary change in a morphological feature or features must depend on a reorganization or co-option of one or more developmental gene regulatory network just as retention of an ancestral morphological trait must rely on retention of a common gene regulatory network. Studying two closely related classes in the same phylum with the same essential morphological feature yet with unique developmental characteristics provides insight into the evolution of these evolutionarily resolute gene regulatory networks. We have developed a new model system using brittle stars to further these studies. In this investigation I have identified key genes of the gene regulatory network (GRN) found in embryonic endo-mesoderm development in the sea urchin, responsible for embryonic skeletogenesis, and compared
these key genes with homologues in the brittle star. From the examination of two closely related gene regulatory networks found in two related classes of Echinoderms insight can be gained into the foundation of morphological change over time.
Introduction

Since the time of Aristotle, Echinoderms have proven fascinating to biologists. While Aristotle’s description of a sea urchin’s bony mouth structure as resembling a lantern will forever link his early curious mind and name to the study of the phylum, this observational knowledge has been added to greatly in modern times. Almost a century ago Theodor Boveri used sea urchin embryos to determine that an embryo’s development is controlled by the genes contained in the embryonic cells following fertilization (Boveri, 1918). The process of fertilization still progresses as humbly as it did then. One egg surrounded by multiple motile sperm cells in any given volume of seawater. Each male gamete vies for the chance to pass its own version of genetic history to a future generation. Eventually fate in the form of random chance deems one sperm successful and egg and sperm fuse. This union becomes apparent almost immediately as a thin transparent line begins to rise from the surface of the zygote. This union and the fertilization envelope produced to prevent polyspermy are readily visible with light microscopy. This outward sign of sperm and egg union is only a small indicator of the myriad of processes that have begun and will continue on the inside of what will eventually become a fully functioning multi cellular organism.

One single cell, the fertilized egg, becomes two, two becomes four and so on as mitosis resumes. Eventually, cells begin to specialize and tissues begin to form. The one fertilized egg cell gives rise to hundreds of cell types that will eventually have many
varied cellular functions and will self organize to form what will become the adult body plan. For this to happen it must mean that contained within the egg cell are both the initial conditions and the program that processes these initial conditions eventually translating them into specification and differentiation. The initial conditions within the egg cell are comprised of particular proteins or mRNAs. The hardware and software that guide development stems from the genome (Davidson, 2007). For the sea urchin, this specialization is first apparent at the fourth cell division. What was once a ball of inconspicuous visibly identical cells now begins to specialize and develop structures that will play crucial roles as the organism continues through the process of growth and development. The instruction required to process all the information provided by the maternal mRNA and proteins and transform their influence into differential zygotic gene expression is contained in the genome. Because the egg, its predecessors and its cellular descendants contain the same genome, we should see the same pattern of gene expression repeated in all cells. However since this is not the case the question arises as to how does a similar static code enable the dynamic differential gene expression, what program regulates this expression and how does the dynamic nature of gene regulation lead to evolution? The regulatory apparatus is composed of regulatory genes and transcription factors that bind to specific sequences of DNA to activate or repress transcription of a gene.

The cells ability to control the gene expression of thousands of genes is of paramount importance and a fundamental task of the developing sea urchin. It is the systems of regulation that oversee this specialization and differentiation of cells and tissues that are specifically intriguing to us. The network of genes responsible for
embryonic skeletal formation within the phylum Echinodermata is of particular interest. Studying gene regulatory networks enables the understanding of the mechanism underlying the developmental process at the most fundamental level (Davidson et. al., 2006).

Echinoderms, as a Phylum, form a highly developed and well-defined group of Metazoans with a well-preserved fossil record. Echinoderms, like Hemichordata, Urochordata, Chephalochordata and Vertebrata, are deuterostomes. Echinoderms are subdivided into five classes with at least 7,000 extant species. The five classes of Echinoderm are, Crinoidea (sea lilies and feather stars), Ophiuroidea (basket stars and brittle stars), Echinoidea (sea urchins, sand dollars, and sea biscuits), Asteroidea (starfishes), and Holothuroidea (sea cucumbers). All members of the phylum Echinodermata contain a skeleton in the adult organism.

The class Echinodea is composed of sea urchins along with sand dollars, sea biscuits and heart urchins. Sea biscuits and heart urchins are grouped as irregular urchins. The more globular and radially symmetrically shaped sea urchin is referred to as a regular urchin (Hyman 1955). A typical adult echinoid contains 5 gonads arranged radially and attached to the interior side of the test (endoskeleton shell). The remaining majority of an adult echinoid is composed of skeleton and coelomic fluid. When in season the gonads swell to a size that almost fills the entire coelom (Nichols 1969, Hyman 1955). The test in echinoderms is composed of 5 skeletal plates (Hyman 1955). Possibly the most readily observable skeletal component of ecinoids is a jaw known as Aristotle’s lantern. This lantern creates their masticatory apparatus and contains 5 persistently growing teeth that are used to rasp both plant and animal matter (Nichols 1969, Birkland 1988). Sea urchins
can be found throughout the world’s oceans from tidal pools up to depths of about 5000 meters (Hyman 1955).

The class Ophiuroidea is one of the largest groups of Echinoderms containing approximately 1800 living species. Only the class Asteroidea contains more species. The class Ophiuroidea contains the brittle stars (Ophiuroidea) and the basket stars (Euryalida). Brittle stars usually have 5 arms and exhibit 5-segment pentaradial symmetry. Unlike the Asteroidea (true starfish) brittle stars have 5 long flexible arms. Where starfish crawl on hundreds of tube feet, brittle stars move fairly rapidly by movements of their flexible arms. An internal skeleton composed of calcium carbonate plates supports the arms of the brittle star. The basket star is similar structurally to the brittle star. The main distinguishing feature is the highly forked and branched arms of the basket star. Ophiuroids can be found in most of the world’s oceans and a few adaptable species can live in brackish waters. Ophiuroids are common in shallow water marine habitats as well as deep ocean beds. Ophiuroids can be scavengers by preying on small live animals such as crustaceans and worms. The basket stars in particular are adept filter feeders gathering plankton with their highly branched and web like arm structures.

Ophiuroids and Echinoids are the only members of the Phylum Echinodermata to posses a pluteus, bilaterally symmetrical larvae with a well-developed larval skeleton. The skeleton of Ophiuroidea and Echinoidea like all Echinoderms is composed of calcium carbonate. The embryonic skeleton unique to Echinoidea and Ophiuroidea and lacking in other Echinoderms appears to be formed by embryonic activation of genes that produce an adult skeleton in the other Echinoderms (Killian and Wilt 1996). Two mutually exclusive explanations are possible. 1) There could have been one common
ancestor to both brittle stars and sea urchins that contained this change in the skeletogenesis GRN. Or 2) Brittle stars and sea urchins could have evolved larval skeletons independently from one another.

The Genetic Basis of Development

Development begins from the time a haploid sperm fuses with a haploid egg to produce a diploid zygote at fertilization. From this single fused cell until adulthood there is a shared common progression among most animals. Shortly following fertilization, cleavage begins to occur and cell division resumes. During initial cleavage the rapid succession of cell divisions greatly reduces the cytoplasmic volume of the egg as it divides into numerous individual blastomeres. The blastomeres form what is collectively known as a blastula by the end of initial cleavage. As the mitotic divisions begin to slow in frequency a rearrangement of the cells begins called gastrulation. The embryo moves from blastula to gastrula as the cells rearrange into their three separate germ layers, ectoderm, endoderm and mesoderm. Once the cells have sorted themselves out and germ layers are defined organogenesis begins to proceed. In many organisms it is at this time when specialized germ cells begin to form at this time for future genetic inheritance. The process of meiotic germ cell formation is known as gametogenesis. From a single incomplete sperm and egg cell a genetically unique and complete organism is about to emerge.

The event that specifically demarcates the progression from fertilization to first cleavage is the activation of mitosis promoting factor (MPF). MPF is responsible for the
resumption of mitotic activity in a fertilized ovum. MPF continues to play a regulatory role by overseeing the biphasic cell cycle of early blastomeres as they pass from M phase to S phase (Gerhart et al., 1984) (Newport and Kirschner, 1984). The regulators of MPF are maternal in origin and stored in the cytoplasm of the ovum. As the cytoplasm is depleted during the initial rapid synchronous rounds of cell division these regulators are also depleted. At this point the embryo enters into the mid-blastula stage and the gap (G1 and G2) phases are added to the cell divisions, completing the cell cycle. Then mRNA’s are transcribed that will encode proteins to aid in gastrulation. The embryo must develop what will eventually be its three axis early in development. The blueprint for Anterior – Posterior, Dorsal – Ventral and left – right axis is initiated during gastrulation as the cells of the blastula are rearranged. The gastrula stage gives cells new arrangements from that which they had established during early cleavage. Gastrulation marks the point where the multilayered body plan of an organism is established. The Ectodermal layer covers what will be the internal structures created from endodermal and mesodermal layers now deep to the ectoderm. It is at this stage of development that a divergence of developmental paths and plans are enacted dictated by the specifics of the organism of interest.

Evolution of Development

As development proceeds the gene networks ensure that an organism is essentially the same as its predecessors. In contrast, evolutionary change in morphological features of an organism must be driven by a reorganization or modification of the very same gene regulatory networks. As genetic change occurs, essential gene regulatory networks can
also change and evolve. However, when looking at two closely related species with in the same phylum it is expected that there should be some recognizable similarity between homologous gene regulatory networks. The focus of this work is identifying one of these well-defined gene regulatory networks in sea urchins and comparing it to that found in the brittle star.

I have begun to characterize genes and their proteins that are involved in embryonic skeleton formation using two members of Echinodermata, an ophiuroid, or brittle star and an echinoid, or sea urchin. The representative ophiuroid I have chosen is *Ophiocoma wendtii*, and the representative echinoid is *Strongylocentrotus purpuratus*. The brittle star and sea urchin have been chosen because they represent the only two classes of the phylum Echinodermata that have a well developed larval skeleton and a free floating pluteus larvae. The remaining members of the phylum Echinodermata will possess a skeleton only when they are adult.

The sea urchin contains a skeleton composed of glycoproteins that have been previously well characterized and genes responsible for the proteins involved in skeleton formation have been isolated and cloned (reviewed by Killian and Wilt, 2008). The genes involved in skeletal formation comprise a gene regulatory network that includes at least, Dri, Ets1 and Alx1 gene homologues. My hypothesis is that this gene regulatory network has been conserved over time in both Ophiuroids and Echinoids. The resulting larval skeleton found in both classes of Echinodermata is the result of activation of this regulatory network.

I suggest that embryonic activation of the gene regulatory networks occurred independently and may include different mechanisms but the “kernel” required for
skeletal formation is the same in both ophiuroids and echinoids. A “kernel” is defined as, a class of gene regulatory network, which because of their developmental role and their particular internal structure, are most impervious to change (Davidson et al., 2006). A GRN kernel typically has the following five properties: 1) A kernel is composed of a network of subcircuits that consist of regulatory genes. 2) They execute the developmental patterning functions required to specify the spatial domain of an embryo in which a given body part will form. 3) Kernels are dedicated to given developmental functions and are not used elsewhere in development of the organism. 4) They have particular form of structure in that the products of multiple regulatory genes of the kernel are required for function of each of the participating cis regulatory modules of the kernel. 5) Interference with expression of any one kernel gene will destroy kernel function altogether (Davidson and Douglas, 2006). The resulting developmental gene kernel exhibits extraordinary conservation over evolutionary time. I have cloned partial cDNAs encoding proteins similar to the sea urchin skeletal gene kernel from the brittle star. Using whole mount in situ hybridization we have determined the pattern of expression of one of these sea urchin skeletal homologue in brittle stars.

**Sea Urchin Development**

**General Development**

The urchin has very little yolk in their zygote. Prior to fertilization the urchin egg’s animal vegetal axis is specified. Cell fate lines are oriented in the unfertilized egg according to animal vegetal axis. The anterior posterior axis is also laid out. The vegetal
region of the egg, through an unknown mechanism, sequesters all of the maternal components responsible for posterior development once a fertilization event occurs (Boveri 1901). The specification of dorsal ventral axis in sea urchins is not as well understood and appears to happen after fertilization. The first cleavage furrow generally defines the dorsal ventral axis. The oral (ventral) pole lies at a 45-degree angle clockwise to the first cleavage plane (Cameron et al. 1989).

Cleavage begins in these isolecithal (zygotes with little yolk) cells and the furrow extends throughout the entire cell. This form of cleavage is described as holoblastic. The first two divisions of the sea urchin zygote exhibit meridional division passing through the embryos vegetal and animal poles at perpendicular angles to each other. The third cleavage is perpendicular to the first two and passes through the cells equator. This third cleavage separates the animal and vegetal halves of the cell. One result of the fourth cleavage is the emergence of the first easily distinguishable cells within the embryo. The four cells of the animal half that separated from their vegetal counterparts during the last cleavage now divide meridionally into eight more blastomeres of equal size and volume. These eight cells are now referred to as mesomeres. The vegetal portion of the embryo does not divide evenly at the fourth division. An unequal cleavage produces four larger cells called macromeres and four smaller cells called micromeres. The micromeres are more vegetal situated than the macromeres, which are oriented toward the animal pole of the embryo (Summers et al., 1993). The embryo consists of 16 total cells broken down into groups of mesomeres, macromere and micromere cells.

The eight mesomeres divide along the equatorial plane to produce two layers of cells, an\textsubscript{1} and an\textsubscript{2}. The macromeres go on to divide meridionally producing eight identical
cells below the \( a_n \) mesomeres. The micromere division proceeds slower than both the mesomeres and macromeres. They produce eight smaller cells below the cluster of newly formed eight macromeres. A regular pattern of division continues for two more rounds. Animal cells that divide meridionaly and vegetal cells divide equatorially characterize the sixth division of the sea urchin embryo.

By the time the sea urchin embryo has reached 60 cells most embryonic cell fates are specified. However, specification is not irreversible at the 60-cell stage. For the most part, by the 60-cell stage specific blastomeres give rise to specific tissues or organs. Many cells remain pluripotent and can give rise to several other cell types. Normally the animal half of the embryo tends to give rise to the ectoderm. Specifically, animal hemisphere cells give rise to the larval skin and neurons. The vegetal cells give rise to the endoderm and mesoderm and a few ectodermal structures. Specifically, the \( v_1 \) cells give rise to ectodermal or endodermal organs. The \( v_2 \) cells give rise to the coelom and secondary mesenchyme, as well as, endoderm. Specification of vegetal cells is accomplished in a multi phase manner.

\( v_2 \) cells specification is accomplished by waves of specifying signal. First, B-catenin levels increase in \( v_2 \) cells and specify endoderm. Second, \( v_2 \) cells are exposed to the inductive signaling of neighboring micromeres. An early \( v_2 \) signal secreted by micromeres amplifies the mesoderm specification provided by B-catenin. The protein Delta next activates the Notch pathway in the adjacent \( v_2 \) cells. This notch pathway activation forces the adjacent cells to become secondary mesenchyme as opposed to endoderm. If either Notch or B-catenin signals are absent \( v_2 \) cells fail to make secondary mesenchyme or endoderm (Ransick and Davidson 1995). The first tier
micromeres of the vegetal hemisphere that give rise to mesenchyme cells that eventually produce the larval skeleton. These vegetal micromeres that are of interest to this thesis.

The skeletal first tier micromeres are the only cells of the embryonic sea urchin that are capable of autonomous development and determination. Skeletal forming micromeres give rise to skeletal spicules even when placed by themselves in a Petri dish or test tube and give rise to dislocated skeletal spicules if transplanted elsewhere in the blastula (Ransick and Davidson 1993). The second tier (called “small”) micromeres contributes to structures of the coelom and do not give rise to skeleton.

The seventh and last regularly definable division occurs in an opposite orientation to the sixth division. Animal hemisphere cells divide equatorially and vegetal cells divide meridionally. From the seventh division, or 128-cell stage, cell divisions become much less regular and cells begin to develop differently. At the 128-cell, blastocoel formation begins. The cells now begin to form a hollow space surrounding what will be the hollow cavity of the blastocoel. By this time the slower cleavage rate of the smaller micromeres and the faster mitotic rate of the larger cell types results in an embryo where all the cells are the same size. Each of the cells is now in contact with both the outer hyaline layer and the inner proteinaceous fluid filled blastocoel. By the 128-cell stage the blastocoel is surrounded by an epithelial sheet of cells connected to each other by tight junctions (Dan-Shokawa and Fujisawa 1980). The blastula remains one cell layer thick as cell division progresses. With each new round of cell division the cells thin out and expand by adhesion of the individual blastomeres to the hyaline layer. An influx of water fills and expands the size of the blastocoel (Dan 1960; Wolpert and Gustafson 1961; Ettensohn and Ingersoll 1992). Cell specification does not begin until after the ninth or tenth cell
cleavage depending on species. Following the tenth division specialized ciliated cells can begin to rotate the embryo within the fertilization envelope. Shortly following the tenth division differences can be seen that characterize individual cells. The vegetal plate now also begins to form as cells at the vegetal end on the embryo begin to thicken. The embryo finally becomes free swimming when cells at the animal pole synthesize and secrete a hatching enzyme that digests the fertilization envelope.

A hollow ball surrounded by a single layer of about one thousand cells characterizes the sea urchin blastula late in its development. The Vegetal end of the blastula is somewhat flattened by this time due to the thickening of the vegetal pole cells. Now each individual group of blastomeres can be distinguished from other groups by size, properties and region of origin. At the center of the vegetal plate the flattened cells begin to extend and contract into long thin processes called filopodia. These cells disassociate from the monolayer blastula and begin to ingress into the hollow cavity that is the blastocoel. These cells are the descendants of the micromeres and make up what is called the primary mesenchyme. These primary mesenchyme cells will make up what will eventually be the larval skeleton. Therefore theses cells are often referred to as skeletogenic mesenchyme cells. The other cells that make up the blastula retain their affinity for the adjoining cells and for the hyaline layer. Micromere descendants lose their affinity for their previous neighbors and gain an affinity for basil lamina and extracellular matrix (P. Oliveri 2002). This change in affinity results in the inward migration characteristic of these primary mesenchyme cells. Once inside the blastocoel these primary mesenchyme cells seem to be guided by the extracellular matrix and blastocoel wall. Movement happens as they extend and contract their filopodia in front of them.
Proteins such as fibronectin and sulfated glycoprotein are also attributed to guiding this ingression of the primary mesenchyme (Wessel et al. 1984). Ultimately these skeletogenic mesenchyme cells become localized within what will become the ventrolateral region of the blastocoel. Once at this location the cells fuse into syncytial cables that will serve as the foundation for the calcium carbonate spicules that are the first indicator of a larval skeleton.

Skeletal Development

Although the general development of the sea urchin is of interest to us, the focus of this study is on skeletal development. The first tier of micromere cells is responsible for the endomesoderm lineages and ultimately larval skeleton formation in the sea urchin.

The signaling molecule responsible for micromere specification, transplanted endodermal induction and normal larval skeletal development is B-Catenin (Ettensohn and Sweet 2000). B-Catenin is a transcription factor activated by the Wnt pathway. In canonical Wnt signaling, the Wnt family of factors interact with the Frizzled family of transmembrane receptors. Disheveled protein is activated by the Frizzled protein when Wnt binds to Frizzled. The activation of the Disheveled protein inhibits the activity of glycogen synthase kinase-3 enzyme (GSK-3). When active, GSK-3 prevents the dissociation of B-catenin protein from the APC protein. The APC protein targets B-catenin for degradation. The presence of the Wnt signal causes inhibition of GSK-3 and allows B-catenin to dissociate from APC protein. This disassociated B-catenin is now free to enter the nucleus. Once nuclear, B-catenin forms a heterodimer with an LEF or TCF DNA-binding protein and becomes a true transcription factor. The B-catenin
transcription factor complex now binds to and activates the Wnt genes (Behrens et al. 1996, Cadigan and Nusse 1997). A noteworthy item for this study is that in addition to nuclear signaling Wnt also is integral in actin and microtubular cytoskeleton (Shulman et al. 1998).

During normal sea urchin development B-catenin accumulates in the nuclei of any cell fated to become endoderm and mesoderm. This accumulation is autonomous and can occur even when the cells are removed from the embryo (Ransick and Davidson 1999). This indicates that the accumulation of B-catenin in the early blastomeres is independent of a Wnt signal, although the same cytoplasmic components are involved. It appears that this accumulation of B-catenin is what specifies mesodermal and endodermal fates of the vegetal cells. Experiments show that inhibition of the nuclear accumulation of B-catenin inhibits formation of mesodermal and endodermal cells (Logan et al. 1998; Wikramanayake et al. 1998). It is also clear that micromeres from embryos with blocked B-catenin also lose their inductive ability when transplanted to other embryos (Logan et al. 1998). Once in the nucleus the B-catenin binds to TCF transcription factors (Vonica et al. 2000). This binding allows B-catenin to activate genes. Pmar1 is the gene responsible for mediating micromere specification by B-catenin once it is bound to the TCF transcription factor. The repression of a recently discovered gene by Pmar1 allows expression of several genes required for primary mesenchymal cell development (Oliveri et al. 2002). This previously unknown repressor of the skeletogenic gene regulatory network has been recently isolated and named HesC (Revilla-i-Domingo et al., 2007). In the sea urchin it is the repression of a repressor that allows activation of the battery of genes responsible for embryonic skeletogenesis. At least some of the genes that are
repressed in the absence of Pmar1 are known to be Tbr, Ets, Alx1 and Dri. In the presence of Pmar1 these genes benefit from the repression of a repressor that prevents their expression. Each of these genes is responsible for directing a portion of skeletogenesis. Previous attempts in the laboratory have been made to isolate Pmar1 in brittle stars. Although it is present in the genome, it is not expressed during the embryonic development. This suggests that the gene responsible for the initial activation of the skeletogenic gene regulatory network in sea urchins does not play the same role in brittle stars.

_Hypothesis_

The hypothesis tested here is that both sea urchins and brittle stars contain a nearly identical regulatory cluster of genes associated with differentiation of skeletogenic mesoderm, but that the initial genes involved in activation of this network differs between the two groups.

When our representative species of Ophiuroid and Echinoid patterns of embryonic development are compared, ophiuroids, unlike echinoids, do not undergo an asymmetrical fourth division and do not produce micromere cells. However, even in the absence of micromere cells, mesenchymal cells appear at approximately the same developmental time point in Ophiuroid development. This suggests a conservation of essential genes responsible for skeletal formation throughout the phylum.

The composition of the sea urchin’s glycoprotein skeletal component has been well defined. Several of the echinoid genes that are responsible for this glycoprotein...
matrix have been identified (Benson et al., 1987, Livingston et al., 1991, Livingston et al., 1996). Some of the proteins associated with the spicule matrix have also been characterized (Benson et al., 1986, Killian and Wilt, 1996). A voluminous amount of information on these genes as well as others was generated by the Sea Urchin Genome Project (Sea Urchin Genome Consortium, 2006). Genes found to be expressed in the sea urchin mesenchyme are also found in the starfish. A similar understanding of the Ophiuriod skeleton has yet to be attained. By comparing two species from of the same phylum it will aid in our understanding of the evolution of these genes and proteins.

My hypothesis that there is a similar “kernel” (Davidson et al., 2006) of a gene regulatory network at work in both classes, ophiuroid and echinoid, during embryonic skeletal genesis. Conserved genes in this “kernel” are at least Dri, Alx1 and Ets1. We suggest that this embryonic activation of the gene regulatory networks occurred independently and may include different mechanisms but that the kernel required for skeletal formation is the same in both classes.

My research focuses on the characterization and expression patterns of Dri and Alx1 in the brittle star, *O. wendtii*. 
Research Design

Animal Husbandry / Spawning

A particular challenge when working with a non-model organism as is the case with the brittle star, Ophiocoma wendtii is that all procedures involving both molecular as well as animal husbandry have to be generated de novo or optimized from procedures used in related organisms. During the course of my project, research procedures used for urchins were often relied on as a starting point for procedural attempts and technical guidance. Animal collection was aided by our proximity to a site in the Florida Keys where O. wendtii can be found regularly. However, every aspect of animal collection, care and transportation had to be empirically derived. In addition to general collection and care, spawning an animal in which there is very little published data is a process that required much trial and optimization. Molecular techniques were initially derived from published echinoderm literature. However, most often variances from species to species or embryo type to embryo type created the need for modification to apply to the brittle star system.
**Animal Collection**

**Location**

Echinoderm collection was done in the shallow waters of the Florida Bay region of Long Key in the Florida Keys, USA. *O. wendtii* specimens were collected from the bay bottom near the Keys Marine Lab (KML) located in the city of Layton Florida (Figure 1).

![Map of Florida Bay and Long Key](image)

Figure 1. Animal collection site. Mile marker 65 U.S. highway 1, bridge piling 70 – 85, Florida bay, Florida Keys, Layton, Florida, USA

The Keys Marine Lab is operated with cooperation between The Florida Institute of Oceanography as well as the State University System. The KML facility is a 5-hour
drive from our lab at the University of South Florida. One of the major difficulties of working with *O. wendtii* lies in shipping and transport of the collected organism. Our proximity to the collection site and optimized capture and transportation techniques allows us a relatively high rate of survival when transporting collected specimen form Florida Bay to the University Of South Florida in Tampa. The brittle star *O. wendtii* was chosen due to their large size and abundance. *O. wendtii* are gravid form June to October (Hendler et al., 1995). *O. wendtii* are relatively abundant in the Florida Keys. *O. wendtii* possesses a central disk, which varies in size up to 1.5 inches. They have been found to inhabit several areas within a one-hour boat ride from the Keys Marine lab. *O.wendtii* congregate in debris piles or natural reef structures that provide a shelter from predation and harsh Florida bay tidal currents. Primary collection occurs under the U.S. highway 1 bridge (bridge pilings #’ 70 – 85) located at mile marker 65, between the islands of Long Key and Conch Key. It has been determined that *O. wendtii* are more concentrated near or directly at the bridge pilings that support the now unused Flagler.
Animals were found by gently raising and lowering large concrete scraps or rocky debris segments located at or near the bridge pilings (Figure 2).

**Time / Date of Dives**

It has been reported that sea urchins and other echinoderms are more prone to spawning at or near a full moon. This is also the case with several other species of marine animals that live near or in a tidal plain. We have also determined that in our specific dive location *O. wendtii* are gravid during a time period that begins in late spring (April) through early fall (Early October). Collection dives were planned during the spring to fall time that would allow us to dive and collect over a time period that covered one to two
days on either side of a full moon cycle to maximize the chances of finding gravid specimen that could be induce to or would naturally spawn in our collection tanks.

Dives were completed in 15-25 feet of water depending on the time of the month and high or low tide conditions.

Collection Protocols

For animal collection, we employed a device termed a bongo (Figure 3).

![Bongo Device](image)

A collection bongo consists of a 24-inch long, 18 inch diameter PVC tube with mesh netting encapsulating either end of the tube. A bungee corded lid flap was cut into the tube to allow for secure and safe insertion and removal of captured specimen. Once an
animal is located care must be used when removing it from its environment. To capture a brittle star a hand must be placed under the bulk of its body at least encompassing most of its central disk. With a gentle consistent motion the brittle star can be raised from the sea floor and placed into the hole in the bongo for storage. If grasped by one or more of its appendages, and not the bulk of its body, the brittle star will jettison the threatened limbs as a survival strategy.

In addition to this collection technique two alternative methods were deployed. In order to determine if gametes were being released in the bongos or in the 5 gallon buckets as a stress response while in transport back to the lab we attempted to isolate individual *O. wendtii* from each other immediately following capture. In one technique I would place collected specimen that were returned to the dive boat form the bongo into individual sealed Tupperware dishes containing seawater. Another attempt to further control or identify the release of gametes from collection to lab involved placing collected animals directly into Ziploc bags while underwater and then placing them into the bongo. This would ensure that we would be able to identify if gametes were released any time from capture to lab (Figure 4).
Collected animals were returned to the boat in the bongo and placed in a five gallon bucket of sea water and transported to the Keys Marine Lab for short term care and storage. At the dive site several carboys of seawater are collected. This water is used to house the animals once they are back at KML. This seawater is also filtered and used to house spawned developing embryos. Once at the KML animals are placed into large 30 gallon aquariums with heat and aeration (Figure 5).

Up to 30 animals have been shown to thrive in one 30-gallon tank of seawater. Using these techniques of collection and short-term storage we experienced a very high survival rate nearing 100%. By using these collection techniques over the past four years we have transported at least 423 healthy specimens to the Keys Marine Lab.
Transport to Tampa

Animals that will be transported to USF for further study and spawning attempts in Tampa are chosen from the specimens that appear the most vibrant and able to withstand the stress of transport. *O. wendtii* Animals chosen for transport are placed into a ¼ - 1 gallon Tupperware container that contains small slits or holes in it that allow for ventilation and water exchange. *O. wendtii* The Tupperware container is then placed in a large 75 gallon insulated cooler full of freshly collected seawater. Battery operated aerators are also placed in the coolers to allow for sufficient oxygen supply while in transport. Additional carboys of freshly collected and air supersaturated sea water are also transported with the collected specimens. During the 5 hour drive to Tampa at least once and usually twice water is drained form the coolers containing the animals and replenished with fresh sea water from the carboys. By using this collection and transportation technique we have consistently transported *O. wendtii* from the middle Florida Keys to USF, Tampa. Over the past four years we have transported at least 271 brittle stars to the lab in Tampa from the Keys Marine Lab.

Storage and Care of Brittle Stars in Tampa

Once the *O. wendtii* were transported to USF, Tampa they were checked for signs of stress or early stages of autolysis. Two 30 and two 15-gallon fish tanks were prepared with keys sea water prior to departure to the collection site. Healthy animals were dispersed evenly throughout the three tanks. It has been shown in our lab that the death and autolysis of one animal can trigger the death and autolysis of the remaining healthy animals in the tank in as early as one day. The use of a recovery tank for unhealthy
individuals eliminates some of the risk of placing an unfit *O. wendtii* triggering total tank death in an otherwise healthy specimen tank. This technique employed over the last three years has greatly increased specimen survival times and almost eliminated entire tank die-offs that were once a more frequent occurrence. Animals were feed frozen shrimp on an every third day basis. Tank water was regularly checked for salinity and signs of fouling. Salinity was kept at a specific gravity of 1.028. During the past three years we had several specimens that lived up to a period of one year or more.

*Spawning of O. wendtii*

Keys Marine Lab Spawning

Once animals are returned to the lab each specimen is examined for the presence of eggs and an attempt at gender identification is made. Females are discerned by the presence of visible swollen, purple gonads at the junction of the flexible arm and central disk. In the absence of this obvious sign of gender females tend to take on a puffed up appearance in the region of their central disk specifically the areas near the arm disk junction. Specimen that are thought to be female are separated and stored in individual tanks prior to spawning attempts. 30 gallon tanks containing freshly collected sea water are used for spawning attempts. Into each tank approximately 20 gallons of sea water is placed. Each tank also receives 3 – 5 air stones, depending on the final number of animals, for aeration prior to receiving animals. At least three tanks are regularly used with each spawning attempt. One tank is kept as close to current ocean temperature as possible, a second tank is slightly warmer (3-4 degrees C) than ambient ocean temperature and the last tank is kept warmer yet up to 90 degrees F at which temperature
the brittle stars start autolysis and die. Spawning does not seem to occur until the water temperature is at or near 85 degrees F.

In addition to varying the water temperature during the spawning attempts the addition of lab collected sperm also appears to induce spawning. Sperm can be obtained by .5M – 1 M KCL injection into males. Females however are not induced to spawn with the KCL injection method. Males injected at the appendage / central disk junction will regularly release sperm. Sperm generated by this method is collected in a beaker of seawater and this seawater / sperm solution is used to seed the tanks during spawning attempts. A system of light shock where specimens are maintained in dark environment and given intermittent exposure to simulated or actual moon light has proven to produce eggs while at the Keys Marine Lab as well. Once collected and sexed animals are placed into the appropriate 30-gallon tanks containing freshly gathered sea water and allowed to acclimate for a period of 1 -5 hours in a dark room. Once the initial period of acclimation is complete and generally at a time after sunset a systematic approach to light shock involving long periods (1 – 2 hrs) of absolute dark and shorter periods (15 -30 min) of light shock are employed. It has been observed that periodically immediately following light shock or light stimulus both or either male and female organisms will release gametes.

It has been our experience that a combination of moon phase, water temperature, sperm introduction and light shock produce a spawning event while at the Keys Marine Lab. There has been no one stimuli that has proven to definitively induce spawning and there has been no definitive combination of the stimuli that consistently produce a spawning event. However over the course of a spawning attempt if all variables are used...
and modified regular spawning can be induced. It is our experience that a spawning attempt one to two days prior to a full moon cycle in water that is approximately 85 degrees Celsius with a periodic light shock event and some freshly collected sperm / sea water solution added to the tanks will consistently produce a spawning of the brittle star *O. wendtii* while on site at the Keys Marine Lab.

**USF Tampa *O. wendtii* spawning**

A similar approach of light shock in a temperature-controlled environment during a full moon cycle and sperm solution added to the tanks has not proven successful when specimens are transported back to the lab at USF. Nighttime light shock has been attempted 14 times over the past three years and has yet to produce eggs from lab kept females.

A spawning attempt at USF involves the same variables of light, temperature, moon phase and sperm solution as are involved while attempting to induce spawning while at the Keys Marine Lab. I was able to induce spawning in male animals, but was not able to induce females to spawn.

Because of the troubles encountered with on site spawning all lab work has been done using fertilized eggs and RNA gathered from animals that spawned while at the Keys Marine Lab in Long Key.

*Fixation of Embryos*

Once a successful spawning is achieved time is noted and the seawater / embryo solution is removed from the tank and watched for development. During the course of the
night following a successful spawning, time points are taken at developmentally critical periods and the developing embryos are watched through out the night for general health and development. Time points are preserved from each developmentally critical time point with special consideration paid to time points critical to embryonic skeletal formation. Following time zero a time points of development are taken at late cleavage, 17 hr. blastula, 26 hour mesenchyme blastula, 39 hr. gastrula, 48 hr. late gastrula, and 75 hr + pluteus larvae. Lastly pluteus stage embryos are collected form approximately 100 hours of development and once daily until the embryo solutions stop development and die.

For each time point collected 50 ml of developing embryos in filtered seawater solution were placed into a sterile 50 ml conical vial. The 50 ml conical vial was spun at high speed in a bench top centrifuge for one minute. The liquid supernatant was aspirated off and 50 ml of additional embryo / sea water solution was added on top of the embryo pellet and spun again for one minute on high speed in a bench top centrifuge. This repeat of the collection procedure essentially doubled the size of the collected pellet. The liquid is aspirated off again down to approximately 4 ml of fluid remaining. 5 ml of 4X paraformaldehyde fix was added to the embryos. After a minimum of one day in fix embryos were washed into 1X PBS to a volume of 50 ml and stored at 4 degrees C for later use in either WMISH or photographic studies.

**Gene Regulatory Network and Molecular Isolation of a Fragment of O. wendtii Dri cDNA**

Homologues of the Alx1 and Dri skeletal genes have been isolated in the sea
urchin. By comparing the deduced sea urchin protein sequences to the protein sequence of homologous proteins from other organisms, we were able to locate sequences of amino acids that were highly conserved. From these regions of homology, degenerate primers were created. The degenerate primers were then used to amplify the brittle star cDNA prepared from extracted *O. wendtii* embryonic mRNA. Using this process sequences for both Alx1 and Dri have been identified in the brittle star.

**Design of Degenerate Primers**

*Alignment of Sea Urchin Dri Amino Acid Sequence With Those From Other Organisms to Determine Conserved Regions*

Degenerate PCR primers were initially created for the *O. wendtii* dead ringer gene using *S. purpuratus* sequence information that was aligned with 22 other organisms Dri amino acid sequences and analyzed for areas of homology. The degenerate primers are a convenient way to amplify the same gene from different organisms. In this case the degenerate primers are extremely valuable due to the fact that *O. wendtii* has very limited genomic information available. The degenerate primers were created by analyzing aligned Echinoderm as well as other Dri sequences found in GenBank. Both forward and reverse degenerate primers were generated as follows. See figure 6 for alignment and primer sequence generation.

In order to determine conserved regions within the Dri amino acid sequence 22 Dri amino acid sequences from 22 organisms were analyzed for regions of homology.

After analyzing the sequences of 22 organisms the list was narrowed to the sea urchin, mouse and ciona. The exclusion of protostomes revealed a higher degree of
conservation of sequences among deuterostomes. From the alignment of these three organisms areas of high homology were analyzed for hairpins, self-dimerization potential and melting point. The conclusions of this analysis lead to the creation of the initial forward and reverse Dri primers (Dri F1 and Dri R1) (Figure 6). In analyzing areas of homology and degenerate primer design care was taken to ensure that the echinoderm, sea urchin, was well represented in the area of homology. Due to the inter phylum relationship of sea urchin and brittle star it was presumed that there would be shared homology between the sea urchin and brittle star Dri sequences.

Figure 6. Dri degenerate primer design
Synthesis of *O. wendtii* cDNA

Into a sterile RNase free tube 0.5 micrograms of random primers were added to 1.5 micrograms of combined early gastrula, and gastrula stage *O. wendtii* RNA. These stages were used due to the fact that under microscopy embryonic skeleton can be seen forming in these stages. The tube was heated to 70 degrees C for five minutes to melt secondary structures within the template. The tube was cooled on ice for five minutes and briefly spun. The following were added to the tube in order of listing: RNase free water (to a volume of 25 micro-liters) 11.5 micro liters, M-MLV 5X buffer 5.0 micro liters, dATP 10 mM 1.25 micro liters, dCTP 10mM 1.25 micro liters, dGTP 10 mM 1.25 micro liters, dTTP 10 mM 1.25 micro liters, rRNAsin (40 units / micro liter) 25 units / .5 micro liters and M-MLV Reverse Transcriptase enzyme (200 units / micro liter) 1.0 micro liter. The tube was mixed gently and incubated for 60 minutes at 37 degrees C. A control PCR using Actin forward and reverse primers was conducted to confirm cDNA synthesis (Figure 12). Two separate cDNA reactions were used to generate two separate batches of *O. wendtii* cDNA (cDNA 1 and cDNA 2).

PCR Including Optimization and Gels of Degenerate Primer PCR

Degenerate PCR primers were used with the following optimized PCR cycle. 50 micro liter volumes were used for each reaction. Salt concentration was 1.5M. One tube was set up as experimental and contained a cDNA RT reaction and another was set up as a negative control and contained sterile PCR water and no cDNA template. To the experimental reaction tubes 5 micro liters of *O. wendtii* cDNA was added. To the negative control tube 5 micro liters of water was added. To each reaction tube 20.6 micro
liters of sterile PCR water, 5.0 micro liters of 10X TAQ buffer, 10 micro liters of 5X TAQ master, 1 micro liter of 0.3 mM dNTP mix, 0.4 micro liters of TAQ, 4 micro liters each of forward and reverse 0.8 mM degenerate primers was added. The following PCR program were completed: 95 degrees C for three minutes, [(39 cycles of) 95 degrees C for 40 seconds, 51 degrees C for 45 seconds, 72 degrees C for 45 seconds], 72 degrees C for eight minutes and hold at 4 degrees C indefinitely. Once PCR was completed visualization was done using a 1% agarose gel electrophoresis. Promising PCR products were visualized as bands of approximately 300 bp each for both PCR1 and PCR 2 cDNA templates (Figure 16).

**Purifying Degenerate Primer PCR Products**

Degenerate primer PCR products were purified using (Millipore, Tempecula Ca.) Montage filtration devices according to the manufacturers instructions. The purified PCR products were the visualized using a 1% agarose gel for confirmation (Figure 15). v.

**Degenerate Dri primer PCR product ligation and transformation**

Purified Dri degenerate PCR products were ligated and transformed using TOPO 4 vector cloning protocols and chemically competent E. coli from Invitrogen (Carlsbad, CA) using the manufacturers protocols.

**Crude Mini Plasmid Preparations of Degenerate Dri PCR Primer Products**

Using sterile technique, 3ml glass culture tubes were prepared with 3ml of LB broth and 12 micro liters of ampicillin. Tubes were labeled and a grid plate of LB-AMP was labeled in kind for a master control plate. A single colony from the overnight
incubated LB-AMP + TOPO reaction was selected with at autoclaved toothpick. The grid plate was scraped with one side of the toothpick and the toothpick was then dropped into the LB broth AMP tube that corresponded to the label on the grid plate. Following bacterial colony selection the tubes containing toothpicks were placed into the shaking incubator at 300 RMP and 37 degrees C for overnight incubation (12 – 18 hrs.).

Following overnight incubation 2 sets of 1.5 ml eppendorf tubes were labeled that corresponded to the 3 ml culture tubes. Half of the contents of each culture tube was decanted into the corresponding 1.5 ml eppendorf tubes. Eppendorf tubes were spun down in a bacterial centrifuge on max speed for one minute and the supernatant was removed. One set of the pair of eppendorf tubes was placed on ice to be used for perfect plasmid preparations once crude plasmid preparation had identified potential clones of interest.

On ice, the second set of eppendorf tubes containing bacterial pellets were re-suspended by pipetting 150 micro liters of Qiagen buffer P1 up and down until the entire pellet was re-suspended. To the re-suspended pellet 150 micro liters of Qiagen buffer P2 was added and the tubes were immediately inverted. Once inverted 150 micro liters of Qiagen buffer P3 was added to the tubes that were then vortexed thoroughly and placed on ice for 5 minutes. The tubes were then spun for 10 minutes on high at 4 degrees C. Another set of Eppendorf tubes was labeled and 1 ml of 100% EtOH was added to each newly labeled tube. The supernatant from the centrifuged tubes was poured off into the new tubes of corresponding label that contained 1 ml of 100% EtOH. The tubes were then incubated at -80 degrees C for one hour. Following -80 degree C incubation the tubes were spun at max speed at 4 degrees C for 15 minutes. The supernatant was poured
off and the pellet that remained was washed with cold 75% EtOH. The tube was next spun for 5 minutes on high speed at 4 degrees C. The pellet was air dried and re-suspended in 30 micro liters of 1X TE. Crude mini preps were next digested to separate the degenerate Dri PCR product insert from the TOPO 4 vector.

Digestions were done by labeling a corresponding set of 0.65 micro liter eppendorf tubes and adding to each; 1.0 micro liters of New England Biolabs Buffer H (10X), 0.1 micro liter of 10mg / ml BSA, 0.5 of New England Biolabs EcoR1 enzyme (8-12 units / micro liter), 3.5 micro liters of NP water and 5 micro liters of mini prepped DNA. The digests were incubated at 37 degrees C for 3 hours. The results were of the digests were confirmed on a 1% agarose gel.

Purified Plasmid Preparation of Degenerate Primer Dri PCR Products

To the second set of tubes that were placed at -20 degrees C from the crude prep the Qiagen procedure for [plasmid purification was applied in order to purify the plasmids with inserts representing the degenerate Dri PCR products to prepare for sequencing. The manufacturers protocols were followed. Products were visualized on a 1% agarose gel to confirm the presence of the expected degenerate Dri primer PCR bands.

Sequencing of Degenerate Dri Primer PCR Products

Sequencing was carried out using Macrogen Sequencing services, Seoul South Korea. 10 – 15 micro liters of purified DNA with a concentration of 50 nano grams per micro liter was prepared for each sample to be sequenced. All samples were sequenced
using both T3 and T7 primers provided by Macrogen. Samples were shipped via FedEx according to current state, local and Macrogen specifications for shipping biological samples. The sequence obtained from initial Dri degenerate primer PCR of *O. wendtii* cDNA was as follows.

*Obtaining Full-Length cDNA Sequences of Dri and Alx1*

Once initial incomplete *O. wendtii* sequences were obtained via degenerate Dri primers it became my goal to determine the full-length sequences of both Dri and Alx1. I employed the Ambion RACE (rapid amplification of cDNA ends) technique of cDNA sequence extension in both the 3’ and 5’ directions to extend both the Alx1 partial transcript and Dri partial transcript. The RACE cDNA extension approach entailed several rounds of varied primer combinations used along with the RACE treated cDNA and RACE specific primers. Several sequence specific primers were generated for each of Alx1 and Dri and used systematically in multiple combinations along with optimized PCR profiles in an attempt to generate complete transcripts that contained the 5’ start sequence and 3’ termination sequence of each respective skeletal gene. Following each RACE PCR attempt, PCR products would be ligated, transformed, plasmid prepped and sequenced to determine if indeed new sequence had been generated via our technique.

**RLM R.A.C.E.**

First Choice RLM-RACE is a protocol and Kit purchased from the Ambion Corporation (Austin, TX). RACE is an anachronism for Rapid Amplification of cDNA
Ends and is used to facilitate the cloning of full-length cDNA sequences when only a partial cDNA sequence is available. We followed the manufacturers protocol but used our gene specific primers. Temperatures were optimized for each primer set based on their sequence and the manufacturers recommendations.

Prior to using the newly processed and created *O. wendtii cDNA* with the designed *O. wendtii* sequence specific primers we generated a positive control mechanism that allowed for the confirmation of the processed *O. wendtii RNA*. This process allowed us to confirm that the RACE adapter had indeed been ligated to the cDNA sequence and therefore could be successfully used in the RACE procedure. Our method of positive control involved using a combination of the Actin reverse primer and the RACE 5’ outer primer. A combination of an Actin reverse primer and a 5’ RACE outer primer could be expected to generate a PCR product approximately 800 base pairs in size (figure 17).

![Diagram of RACE cDNA confirmation + Control using RACE outer and Actin Reverse primers](image)

Figure 7. RACE cDNA confirmation + Control using RACE outer and Actin Reverse primers
Or, roughly a 300 bp larger PCR product than the Actin forward and reverse primers produced. This result is expected due to the known size of 500bp that is produced by the Actin gene when an Actin forward and reverse primer combination is used and the additional size provided by the RACE adapter and Dri sequence provided by using the RACE outer primer in combination with the Actin reverse primer.

Once RACE outer and inner nested PCR are done, PCR products can be visualized via 1% agarose gel electrophoresis. A positive result is the presence of one to a few bands in the experimental lanes of the agarose gel. This is due to the variability of the transcription length for each cycle and transcript.

**Non-Degenerate PCR Primer Design and Generation**

Primers were designed to produce forward and reverse inner and outer non-degenerate primers using *O. wendtii* sequence obtained from the initial degenerate primer PCR (figure 16). These primers would be used in combination with RACE inner and outer primers to extend the ends of known transcripts using the Ambion RACE (Ambion, Austin, TX) described in the RACE section. Primers were created to have the most sequence specificity and highest melting point possible to increase primer binding specificity during PCR by allowing for higher annealing temperatures to be used. In addition to an inner and outer reverse RACE gene specific primer a second inner (2nddririn) was designed to further increase specificity effectiveness of the transcript end amplification of the Dri gene.

RACE forward and reverse perfect primers designed for Dri and Alx1:
Dead ringer perfect forward 3’ outer primers:

Sequence #1 (dripf) = 5’ GAG GAG CAA TTT AAG CAG CTC TAT GA 3’
Tm = 56.8 degrees C

Sequence #2 (drif41108) = 5’ CAG TGG CGT GAG ATC ACC AAG GGC C 3’
Tm = 65.6 degrees C

Dead ringer perfect forward 3’ inner primers:

Sequence #1 (dripfin) = 5’ TCT ATG AGT TAT CTG ATG ACC CTC A 3’
Tm = 54.5 degrees C

Sequence #2 (drifin41108) = 5’ GGG CCT CAA CCT ACC AGC ATC C 3’
Tm = 62.2 degrees C

Dead ringer perfect reverse 5’ outer primers:

Sequence #1 (driprev) = 5’ AGG TA C TTC ATA TAT TGG GTA TGA 3’
Tm = 50.7 degrees C

Sequence #3 (drir42108) = 5’ GAG CTG CTT AAA TTC CTC CTC 3’
Tm = 53.2 degrees C

Dead ringer perfect reverse 5’ inner primers:

Sequence #1 (dririn) = 5’ CCC TTG GTG ATC TCA CGC CAC T 3’
Tm = 61.5 degrees C

Sequence #2 (2nddririn) = 5’ CCG CTG AGG GTC ATC AGA TAA CT 3’
Tm = 58.3 degrees C
Sequence #3 (dririn42408) = 5’ CCT CTG AGG GTC ATC AGA TAA CTC 3’
Tm = 58.8 degrees C

Alx1 perfect reverse 5’ outer primers:
Sequence #1 (Ow Alx1 Out) = 5’ GAG TTG CTG AAA CCT TTC ACG C 3’
Tm = 57.0 degrees C
Sequence #2 (Ow Alx1 Out Spring 2008) = 5’ CTT TGG TCG ACT CAC TGC CTA TCG 3’
Tm = 59.2 degrees C

Alx1 perfect forward 5’ inner primers:
Sequence #1 (Ow Alx1 in) = 5’ TTC ACG CTT CCG CCA TTT GGC T 3’
Tm = 62.3 degrees C
Sequence #2 (Ow Alx1 In Spring 2008) = 5’ CGT TGT GTT GGA TGT TGA AAG CGG 3’
Tm = 59.2 degrees C

Alx1 perfect forward 3’ outer primer:
Sequence (Alx1 3’ Race Outer) = 5’ GGT GGA GAC AGG AGG ACA AGT AGT 3’
Tm = 59.6 degrees C

Alx1 perfect forward 3’ inner primer:
Sequence (Alx1 3’ Race Inner) = 5’ GCT CTG CGG TTG AGA GCA AAA GAA 3’
Tm = 59.9 degrees C
Race primer combinations and PCR profile optimization was achieved by multiple attempts at PCR using RACE kit primers with combinations of Dri and Alx1 gene specific primer combinations. In addition to varying combinations of primers used and optimized PRC profiles. The following list illustrates the optimized primer combinations and PCR conditions that produced the results that returned sequences as Dri. All other primer combinations and sequencing attempts did not produce positive results.

Empirically Derived Primer Combinations and PCR Protocols

**Dri degenerate primers:**
Using Dri degenerate primers, Dri forward and Dri reverse, with PCR profile; 95 degrees C – 3 min., 39 cycles of [95 degrees C – 40 sec, 51 degrees C – 45 sec, 72 degrees C – 45 sec], 72 degrees C – 8 minutes.

**Dri perfect primers 3’ RACE:**
3’ RACE using primer combinations drif40018 (outer 3’) and drifin41108 (inner 3’). Outer PCR profile = 94 degrees C - 3 minutes, 35 cycles of [94 degrees C – 30 sec, 65 degrees C – 30 sec, 72 degrees C – 30 sec], 72 degrees C – 30 sec.

**Alx 1 perfect primers 5’ RACE:**
5’ RACE using primer combinations OwAlx1Out (5’ outer) and OwAlx1In (5’ inner), Outer PCR profile = 94 degrees C – 3 minutes, 35 cycles of [94 degrees C – 30 sec, 54
degrees C – 30 sec, 72 degrees C – 2 minutes], 72 degrees C – 7 minutes. Inner PCR profile = 94 degrees C – 3 minutes, 40 cycles of [94 degrees C – 30 seconds, 54 degrees C – 30 sec, 72 degrees C – 2 minutes], 72 degrees C – 7 minutes.

**Alx1 perfect primers 5’ RACE:**

5’ RACE using primer combinations OwAlx1OutSpring2008 (5’ outer) and OwAlx1InSpring2008 (5’ inner), Outer PCR profile = 94 degrees C – 3 minutes, 40 cycles of [94 degrees C – 30 seconds, 54 degrees C – 30 sec, 72 degrees C – 30 sec], 72 degrees C – 7 minutes. Inner PCR profile = 94 degrees C – 3 minutes, 40 cycles of [94 degrees C – 30 seconds, 54 degrees C – 30 sec, 72 degrees C – 30 sec], 72 degrees C – 7 minutes.

**Alx1 perfect primers 3’ RACE:**

3’ RACE using primer combinations Alx13’RaceOuter (3’ outer) and Alx13’RanceInner (3’ inner), Outer PCR profile = 94 degrees C – 3 minutes, 35 cycles of [94 degrees C – 30 seconds, 58 degrees C – 30 sec, 72 degrees C – 1 minute], 72 degrees C – 7 minutes. Inner PCR profile = 94 degrees C – 3 minutes, 35 cycles of [94 degrees C – 30 seconds, 58 degrees C – 30 sec, 72 degrees C – 1 minute], 72 degrees C – 7 minutes.

*Ligation and Transformation of RACE PCR Products*

PCR products of appropriate size and interest were ligated into TOPO 4 vectors as described previously.
**Crude Mini Preparations of RACE PCR Products**

These were carried out as described previously.

**Digest of Crude Mini Preparations of RACE PCR Products**

For the digests of crude mini preps a master mix containing 1 micro liter 10X Buffer H (New England Biolabs, Stockton, New England), .1 mg/ml BSA (New England Biolabs), 0.5 micro liters EcoR1 Enzyme (New England Biolabs) and 3.5 micro liters sterile water per each reaction needed was created. 0.65 micro liter eppendorf tubes were labeled to correspond to each of the crude prepped bacterial samples. 5 micro liters of master mix was added to each 0.65 micro liter eppendorf tube. 5 micro liters of mini prepped DNA was added to each appropriately labeled 0.65-eppendorf tube. Reaction tubes were pulse spun in a micro centrifuge. Reactions were incubated at 37 degrees C for 2 – 3 hours. Following a one hour incubation at 37 degrees C the digests were run on a 1% agarose gel to identify positive clones.

**Perfect Plasmid Preparations of RACE PCR Products**

Perfect preparations were done using Qiagen quicklyse miniprep kits (Qiagen, Foster City, Ca.) as described previously.

**Sequencing of RACE Generated PCR Products**

Sequencing was carried out using Macrogen Sequencing services, Seoul South Korea. See sequencing results section for sequencing results.
Determining Spatial and Temporal Expression Patterns of O. wendtii Skeletal Genes
via Megascript RNA Probe Generation and Whole Mount In Situ Hybridization

Megascript Transcription Reactions and RNA Probe Generation

With the sequences provided by Macrogen DIG labeled sense and antisense RNA probes were made using Ambion Mega and Maxiscript transcription reactions and protocols (Ambion, Austin, Texas). Once generated these RNA probes are 1st tested for activity using Southern Blotting against linearized plasmid DNA to determine relative activity and strength of the probes intended for use in whole mount in situ hybridization. Sequencing is carried out with a T3 and T7 promoter. Once the T3 and T7 sequences are examined for insert orientation. T3 and T7 Megascript reactions are carried out producing both a sense and antisense RNA probe. Sequenced plasmids shown to contain either Alx1 or Dri were first linearized in a digest reaction. Each plasmid was digested in two separate restrictions enzymes in two separate restriction digests. This produced two versions of the same linearized plasmid. One restriction digest would produce a linearized plasmid with a T3 promoter site and the other would produce a linearized plasmid with a T7 promoter site. In one reaction plasmids were cut with NOT1 to produce a linearized plasmid with a T3 promoter site that would be used to produce a anti-sense Megascript probe for Southern Blotting and Whole Mount In Situ Hybridization. In the other restriction digest reaction purified DNA containing plasmids were linearized using restriction enzyme Spe1 to produce a linearized plasmid containing a T7 promoter that would be used to produce a control sense Megascript probe (figure 8).
Once restriction digests were complete a gel was run to confirm the linearization reaction that contained one lane of uncut plasmid as a control and two lanes that contained either NOT1 digested plasmid or Spe1 digested plasmid. The linearized plasmids should appear the same size when visualized with a 1% agarose gel. The uncut plasmid should present as a separate molecular weight when visualized (Figure 22).

**Southern Blotting**

In order to determine the activity of our Megascript generated DIG labeled probes all probes were first used with linearized plasmid DNA containing the specific sequence of DNA that was used to generate the RNA probes. This extra step was done to ensure
maximum likelihood that the probe and procedure could work prior to using valuable and scarce O. wendtii embryos with the RNA probes for whole mount in situ hybridization. A 1% Agarose gel was used to run two series of varying dilutions of linearized plasmid DNA (1/100, 1/1000 and 1/10,000) as well as DNA ladders for DNA size base pair determination (Figure 23).

These series of dilutions are then used to detect the sense and antisense RNA probes. Once the 1% agarose gel was run and photographed the DNA markers were cut away and the gel was denatured and neutralized. The gel was placed into 0.25 M HCl for 10 minutes and then rinsed with nanopure water. The gel was next placed into 1.5 M NaCl / 0.5 M NaOH for 20 minutes and then rinsed with nanopure water. Lastly the gel was placed into 1.5 M NaCl / 0.5 M Tris pH 7 for 20 minutes and then rinsed with nanopure water. Following gel denaturing and neutralization the DNA was transferred from the agarose gel to a nitro cellulose transfer membrane via capillary wicking action.

Once the DNA was transferred cutting the membrane separated the two dilution series contained on the membrane. One half of the membrane was used for antisense RNA probe detection and the other half was used for sense RNA probe detection. The membranes was first be pre-hybridized in 15 ml of pre-hybridization solution consisting of 7.5 ml formamide, 3.75 ml 20X SSC pH 7, 30 micro liters EDTA 0.5M, 750 micro liters 1 M NaPO4 buffer pH 6.8, 600 micro liters Denhardt’s solution, 316 micro liters 9.5 mg / ml salmon testes DNA and 2.054 ml nanopure water. The pre-hybridization solution was warmed to 68 degrees C in a warming oven. Each half of the blot was placed into a glass hybridization tube. 7 ml of warmed pre-hybridization was added to the
tubes. The tubes with pre-hybridization solution and membranes were incubated at 68 degrees while rotating for 30 minutes.

Prior to hybridization the probes were diluted to approximately 150 nano grams per micro liter by addition of appropriate probe RNA volume to 50 micro liters of DEPC water. The diluted probe was heated to 68 degrees C and immediately placed on ice. Following pre-hybridization and incubation of the membranes, 300 nano grams of RNA probe was added 3 ml of pre-warmed pre-hybridization solution. Appropriate sense and antisense probes were incubated at 68 degrees C overnight. A positive control was used when ever available that consisted of a probe proven to work in past experiments along with the corresponding linearized DNA at a concentration of 1/100. This control served as a reference for the sensitivity of the probes.

Membranes were washed in 100 ml of low stringency buffer consisting of 20 ml of 20X SSC pH 7, 1 ml 20% SDS and 179 ml nanopure water. Membranes were submerged and shook in low stringency buffer for five minutes. This low stringency wash was repeated 5 times. Following low stringency buffer washes the membranes were again transferred to glass hybridization tubes. 20 ml of high stringency buffer was added consisting of 200 micro liters of 20 X SSC, 200 micro liters 20% SDS and 39.6 ml of nanopure water and the blots were incubated at 68 degrees C for 15 minutes. This high stringency wash was repeated 4 times. The membranes were transferred to a tray containing 100 ml of wash buffer consisting of 300 ml maleic acid buffer (M.A.B.) and 900 micro liters of tween 20 and incubated at room temperature with gentle shaking for two minutes. The wash buffer was discarded and 100 ml of blocking buffer consisting of
12 ml 10X blocking reagent and 108 ml M.A.B. was added to the trays containing the membranes and incubated at room temperature with gentle shaking overnight.

The antibody used to detect the DIG-labeled RNA probes is Anti-DIG-Ab. The anti-DIG antibody was centrifuged at 10,000 RPM for five minutes. 4 micro liters of antibody was carefully pipetted from the surface of the antibody vial. The 4 micro liters of antibody was added to 40 ml of blocking solution to create the antibody solution. 20 ml of antibody solution was added to the blots. The blots were incubated for 30 minutes in antibody solution. Following antibody solution addition the blots were washed 2 times for 15 minutes each with gentle shaking in 100 ml of wash buffer. Using gloves CDP-Star was added to detection buffer in a 1:100 dilution (5.2 micro liters of CDP-star: 514.8 micro liters of detection buffer). For visualization the bag was placed into a developing cassette and exposed to film. Film is developed and examined for the expression of DIG-labeled tags associated with the Megascript RNA probes. If the sense and antisense probes at a dilution of 1/10,000 times produce visible bands (Data / Figure not shown) that correspond with the molecular weight of the linearized plasmid used to complementary base pair with the probes then the probes will be used with *O. wendtii* embryos in whole mount in situ hybridization.

*Whole Mount In Situ Hybridization*

Whole mount in situ hybridization was carried out on brittle star embryos of varying developmental time points from fertilization to pluteus larva (>75 hours post fertilization). Embryos used for whole mount in situ hybridization consist of the following time points; late cleavage, 17 hr. blastula, 26 hr. mesenchyme blastula, 39 hr.
gastrula, 48 hr. late gastrula and 75 + hr. pluteus larvae. Embryos used for whole mount in situ hybridization were generated during spawning attempts at the Keys Marine Lab on Long Key, Florida, USA. Embryos were fixed in 4X paraformaldehyde. Following paraformaldehyde fix embryos were washed into 1X DEPC treated PBS and stored at 4 degrees C until use in whole mount in situ hybridization. Prior to use in whole mount in situ hybridization embryos will be stepwise washed into 70% EtOH in DEPC treated 1X PBS as has been previously described in echinoderm whole mount in situ hybridization (T. Minokawa et al., 2004). We found that several smaller (4 – 5) volume washes work better than two larger washes as described in the Minokawa procedure. Other than this variation the Minokawa procedure was followed for washing embryos into EtOH was followed. Five 1 ml aliquots were prepared of each time point to be used. Each aliquot contains 1 ml 70% EtOH + embryos for that specific time point.

Once embryos form each time point are washed into EtOH they can be combined into slide chambers so that each slide chamber contains several of each developmental time point. For each DIG-labeled RNA probe there is a sense (negative control) and antisense (experimental) probe. Therefore two dishes were set up for use the whole mount procedure for every probe being used. One slide chamber was used for each sense and antisense DIG-labeled probe being used. In addition if embryos were plentiful enough one dish would be set up for use with the highly reproducible Alx1 labeled probe. This labeled probe would be used as a positive control. Pre-hybridization fluid was made as follows; for a total volume of 10 ml add; 7 ml formamide 1 ml 1M MOPS pH 7, 1 ml 5 M NaCl, 10 micro liters Tween 20, 1 micro liter 1 mg / micro liter BSA, 998 micro liter nanopure water. Embryos were washed into pre-hybridization fluid 2 – 4 times or until
slide chamber is nearly full. Pre-hybridization was for three hours at 50 degrees C.

Pre-hybridization fluid was replaced with an equal volume of hybridization solution (pre-hybridization solution + 500 ng/ml DIG-labeled RNA probes). Hybridization was for two and a half days at 50 degrees C. Pre-hybridization fluid was added as needed to replace fluid lost via evaporation over the course of the two and one half day hybridization.

Embryos were washed 5 times with MOPS buffer. MOPS = for a 200 ml volume add: 20 ml 1M MOPS, 20 ml 5M NaCl, 200 micro liter Tween 20 and 159.8 ml nanopure water. Blocking was done by incubating in 495.5 micro liters of MOPS buffer with 0.5 micro liters of 10 mg/micro liter BSA at room temperature for 20 minutes followed by incubation in 449.5 micro liters of MOPS buffer with 0.5 micro liters 1 mg/micro liter BSA and 10 % sheep serum (50 micro liters sheep serum) at 37 degrees C for 30 minutes. Addition of antibody: Remove blocking reagent from embryos and add 750 micro liters of antibody solution per slide chamber. 750 ul of antibody solution (494.17 micro liters MOPS, 0.5 micro liters 1 mg/micro liter BSA, 5 micro liters sheep serum and 25 units (.33 micro liters) Anti-DIG-Ab) was added And the embryos were incubated overnight at 4 degrees C without shaking. The O. wendtii embryos are very fragile and shaking overnight after the stress and manipulation involved in the whole mount in situ hybridization procedure result in damaged or destroyed embryos.

Antibody solution was removed and the embryos were washed with 400 micro liters of PBT (PBS with 1 mg/ml BSA and 0.02% Tween 20) at room temperature. This was repeated 5 times.

PBT was removed and replaced with AP buffer (100mM Tris-HCl, 100mM NaCl, 5mM
MgCl₂, 0.05% Tween 20, pH 9.5). Incubation was at room temperature for 5 minutes. AP buffer wash and incubation was repeated a total of three times.

300 micro liters of fresh AP buffer was added to each slide chamber followed by 200 micro liters of BM purple. BM purple was added in the dark. Slide chambers were covered with aluminum foil and placed in a desk drawer carefully. Staining has occurred in as little as 15 minutes and taken as long as 4 hours depending on probe strength. Embryos were periodically checked with a dissecting microscope to determine if adequate staining has occurred.

When the desired color develops staining was stopped by removing the BM purple with a 200 micro liter pipette. 600 micro liters of PBT was used to wash the embryos 3x. Staining was visualized with a Nikon Diaphot inverted microscope using a 20X phase contrast objective.
Results

*Animal Husbandry / Spawning*

*Animal Collection*

We have been 100% successful in obtaining animals from the location we identified under the bridge in Long Key, FL. The number of animals obtained varies somewhat, with an average of 18-20 per diver. Figure 9 shows the results per diver of a series of dives from August 2006. We generally had 2 – 4 divers collecting. As a result, an individual dive would yield 40 to 80 animals.

![Figure 9. The number of animals obtained/diver from a representative series of dives. In this case, 2 divers were collecting.](image-url)
Our ability to maintain the *O. wendtii* in the Keys Marine Lab and transport them to USF also approached a 100% success rate. We have also developed methods to maintain the adults in aquariums in the laboratory at USF.

It has been our experience that with minimal care and maintenance *O. wendtii* can be kept viable in a laboratory setting for a period of up to and possibly longer than one year. It becomes very difficult to tell apart different animals from different collection trips and therefore proper identification and dating of arrival times is a challenge. We have experienced cases where one tank of animals remains unchanged and no new *O. wendtii* are added for the period of one year.

A key to successful *O. wendtii* care and heath is identifying sick and or dying individuals before they foul the entire tank. *O. wendtii* are particularly sensitive to the death of one organism and respond quite rapidly to the death of one of their tank mates. It has been observed that following the death of one brittle star that a tank can foul and all individuals can be lost in as little as 2 days if the dead or dying *O. wendtii* is not removed from the tank. The use of a recovery tank where suspected sick or dying animals can be placed is crucial to the care and longevity of lab kept *O. wendtii*. We utilized a 15-gallon recovery tank that was maintained identical to the tanks that contained healthy individuals. Tank maintenance is very minimal when caring for *O. wendtii*. The salinity of the water is kept very high due to the high salinity of the Florida bay are of the Florida Keys. Salinity can range from 1.026 – 1.030 and is balanced by the addition instant ocean to raise salinity or autoclaved water to lower salinity. The most accurate way to keep the water salinity acceptable is to add collected Florida Keys seawater to account for lowered
water levels due to evaporation. Once the supply of Florida Keys collected seawater is exhausted, Instant Ocean and nanopure water are used to substitute. Animal health and mortality do not seem to be affected by the change from seawater to Instant Ocean.

Tanks were heated to an average temperature of 80 degrees in the lab. However, during frequent power failures or heat sources expiring water temperatures would drop to ambient air temperature. Never during any of these lower temperature situations was there any observed decrease in *O. wendtii* health or longevity.

In their natural environment *O. wendtii* are scavengers and opportunistic feeders. In the lab kept environment we fed them a diet of frozen aquarium shrimp and or standard aquarium fish food. Feeding would occur on average every third to fourth day. Animals would be fed by cutting small segments of shrimp and placing them in the general vicinity of each *O. wendtii* in the tank.

**O. wendtii Spawning (Keys Marine Lab, Layton, FL)**

Animals collected while at the Keys Marine Lab are found to routinely spawn under the following circumstances or conditions. Spawning attempts took place on a full moon night +/- 2 days. Outside of this 5-day period each month there has been little success with spawning of animals. Animals were brought to the lab and assessed for health and viability. Once unhealthy animals have been isolated an attempt to separate animals based on known females suspected females and probable males should be undertaken. This increased the chances of placing at least one animal of each gender in each spawning tank as well as decrease the chances of selecting a female for KCL
injection, which will kill the animal without producing gametes. Once animals have been placed in appropriate gender categories they can be added to spawning tanks in a gender equal fashion. At least three tanks were pre set up to hold animals. One tank was at ambient temperature and two tanks were equipped with heaters. One of the heated tanks should have its temperature elevated to at least 85 degrees F. The other heated tank should have its temperature elevated to 85 degrees F or hotter up to a temperature of 90 degrees F. It has been observed that almost all spawning occur in water that is at least 85 degrees F. As animals are being placed into tanks at least 2-3 suspected males should be reserved for KCL injection and sperm harvest. A syringe containing 0.5 – 1.0 M KCL should be inserted into the central disk / appendage junction of each suspected male. KCL should be slowly injected until there is a visible release of sperm (usually 1 – 3 ml of KCL). Often times it is necessary to spread the injections over several locations on the specimen. Once sperm is visible as a milky white film on the virtual surface of the animal the brittle star was placed into a beaker containing 50 – 100 ml of water and allowed to shed all sperm before being discarded. This procedure should be repeated with 1 – 2 more males depending on success and yield of sperm. The 100 ml beaker of sea water / sperm solution can be used directly to seed tanks with or it can be added to 50 ml conical vials and spun down and supernatant pouted off to concentrate the sperm for transport back to the university. Once animals are segregated into their respective tanks and sperm has been harvested animals should be allowed to acclimate for a period of at least one hour in the dark or until after sunset, which ever is longer, prior to light shock or sperm solution addition. Following a period of acclimation and after sunset the animals were light shocked by turning on a single bright light source in the room (bright desk lamp held high
over the tanks) for a period of at least 15 minutes. In addition to light shock a volume of 2 – 3 ml of seawater / sperm solution may be added to the tanks. Animals should be observed for signs of spawning (males release of milky white film that clouds tanks and female release of dark purple eggs that look like small debris on the floor of the tank). If no spawning event takes place the cycle of dark (45 – 60 minutes) and light shock (15 – 30 minutes) was repeated until dawn. This method if attempted over the course of 3 -5 nights routinely produces *O. wendtii* embryos. Once optimized this procedure proved to always produce fertilized eggs over the course of at most two evenings of attempted spawning. During the spawning events that took place while housed at the Keys Marine Lab it was impossible to accurately determine how many males or females were induced to release gametes since multiple animals were present in the containers. The success rates of various methods, as defined as a spawning event in a given container of animals is shown in figure 10.

<table>
<thead>
<tr>
<th>Method</th>
<th>% success</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCL injection/males</td>
<td>33</td>
</tr>
<tr>
<td>KCL injection females</td>
<td>0</td>
</tr>
<tr>
<td>Light shock alone</td>
<td>16</td>
</tr>
<tr>
<td>Heat alone</td>
<td>2</td>
</tr>
<tr>
<td>Light Shock and heat</td>
<td>22</td>
</tr>
</tbody>
</table>

**Figure 10. Spawning method success rates**

**O. wendtii spawning (U.S.F. Lab, Tampa, FL)**

Animal spawning at the U.S.F. lab was optimized to mimic that of the Keys Marine Lab as closely as possible. Animal spawning was attempted 17 times over the
course of the last four years. Any spawning attempt performed while at U.S.F. yielded only sperm. Sperm yielded from spawning attempts while in the lab Tampa lab at U.S.F. was derived under the following conditions: 2 – 4 *O. wendtii* were placed into 500 ml – 1 L beakers of Florida keys collected sea water according to 50% male and 50% female suspected rations on an evening +/- 2 days surrounding a full moon. The beakers of sea water themselves was heated in a water bath in a dark humid storage closet to simulate the conditions of the Keys Marine Lab. For successful release of male gamete, water temperature was raised up to at point between 85 and 90 degrees F for a period of no longer than two hours. Intense light shock was applied every 45 minutes that consisted of placing a desk lamp directly over each individual beaker of animals. 2 – 4 ml of seawater / sperm solution was added during the initial light shock period. Using this procedure male *O. wendtii* could be reliably induced to release sperm. Any attempt at light shock stimulus after a two-hour time point failed to yield any gamete release. Similarly no brittle star was recorded to release any gametes following 2 hours of this procedure. In addition any supplementation of seawater / sperm solution following the initial light shock not only failed to induce spawning but further seemed to increase the rate of specimen death and ill health. Using this method it could be expected that at least 50% of all animals used would fail to recover from the stimulus and die within 24 hours of a spawning attempt. The average amount of animals used during these attempts was 12. The most used was 30. The least used was 8. The average case of reported males releasing gametes was 2. The most males recorded to be induced to release sperm was 5. The least cases of males releasing gametes was 0. Never in any attempt was there a recorded case of a female *O. wendtii* releasing eggs.
Figure 11. *Ophiocoma wendtii* embryonic development. Panels proceed from fertilized egg in upper left and are labeled as follows: 8-8 cell, 16=16 cell, 32=32 cell and 64=64 cell cleavage stages. B= blastula, EG = early gastrula, MG = mid-gastrula, G= gastrula, P = pluteus. The arrow shows a mid-gastrula stage embryo that was squashed with a cover slip to show the forming skeleton.

**O. wendtii Embryonic Development**

Figure 11 shows the development of *Ophiocoma wendtii* from fertilized egg to pluteus larva. The zygote undergoes holoblastic, equal cleavages, including the fourth cell division (the products are labeled 16 in Figure 11), which is unequal in sea urchins. As a result, unlike sea urchins, no micromeres are formed. Ingression of mesenchyme cells into the blastocoels occurs at a time and stage similar to sea urchins, however a larger number of cells ingress (labeled EG in Figure 11). After invagination of the gut, the mesenchyme cells sort out into two positions in the embryos. One set becomes associated with the tip of the archenteron and will form the coelomic pouches. In sea urchins, three are mesenchyme cells associated with the tip of the archenteron, but they arise from the archenteron itself following invagination. Their fate is different as well,
since most form pigment cells, which are absent in brittle stars. The other set of
mesenchyme cells gather at the base and on either side of the archenteron. These are the
cells that will make the skeletal spicule. Their position and behavior appear identical to
the primary mesenchyme in sea urchins from this point on. Biomineralization begins as
these cells gather near the archenteron (see the squashed mid gastrula (MG) in Figure 11.

Gene Regulatory Network / Molecular

cDNA Synthesis and PCR Control

We tested our synthesis of embryonic cDNA synthesis using actin PCR primers.
Amplification of both early gastrula and gastrula cDNAs with actin primers generated a
product of the expected 300 bp size (Figure 12). The control reaction without cDNA
added yielded no product. From this we concluded that we had successfully generated
cDNA.
Figure 12. Positive control gel of *O. wendtii* cDNA using actin PCR primers. Lane 1; no cDNA control, Lane 2; early gastrula cDNA, Lane 3; gastrula cDNA, Lanes 5 and 6; DNA size markers.

**PCR Using Degenerate Dri Primers**

PCR with degenerate Dri primers using early gastrula and gastrula cDNA as target yielded very little product, but what was visible using a UV light box was of the expected size of around 300 bp (Figure 13). We purified the reaction and ligated into the vector as described in methods.
Figure 13. Result of Degenerate Dri primer PCR. Lane 1; early gastrula cDNA
Lane 2; gastrula cDNA, Lane 3; blank, Lanes 4 and 5, DNA size markers.

After ligation of the PCR products into vector and transformation into *E. coli*,
individual colonies were picked and crude minipreps of plasmid DNA were performed on
each. EcoR1 digests were performed on each plasmid and the results were separated on a
1% agarose gel (Figure 14). The sizes of the inserts clearly show that the products of the
PCR reaction were heterogeneous, indicating that more than one cDNA had
complementary sequences to the degenerate primers and that we likely had products that
were not from the Dri transcript.
Figure 14. Crude mini prep Dri degenerate primer PCR. Each lane contains an individual plasmid prep digested with EcoR1 to release the inserted cDNA. The lanes containing DNA size markers are indicated.

Plasmids in lanes where inserts are circled were then purified and sequenced.

We chose a number of individual clones within the size range that was expected based on comparative organisms. The plasmids chosen were purified as described so that they could be used for sequencing. The resulting plasmids are shown in Figure 15.
Figure 15. Purified plasmid preparation of Dri degenerate primer PRC products. Numbered lanes indicate individual plasmids that were purified for sequencing. The 100 bp and 1 KB are DNA size ladders.

**Sequence Analysis of Putative Dri Clones**

The plasmids were sequenced as described in methods. The sequences obtained were trimmed of vector sequences and then compared to NCBI sequence databases using BLAST. Of the eight clones sequenced, one had Dri sequences from *S. purpuratus* as the closest match. The nucleotide sequence is shown in figure 16.
Dri sequence from January 2006, T3+.F07_060125209G

AAAGCTTGTCAGGTAGTAGCAATTACCCTTTAGGTAATGCTATA
TATTTGCTATAGAGATTGAAAGCTGCACTTAGTGATGATGCT
GGTAGGTTGAGCCCTTTGATCTCACCAGCATTGCTCTTTTAT
TGATGACCTCTACTAAACCACCTTTATCTGTGACTAGATTGAA
GAGGCAATAGAGATCCAAAGACCTCTGGCGCTATATGGGAT
GCATCTGACTGGTGTGCCTCTTCTTCTGACATAAAAAGAGAAACAG
TCATCGAAAAACTCTTTCCGCTAGGGTCATAGATAACGCTCTATA
GAGCTGCTTAAAATTGCTCCTCAAGGGCGAATTCGCGGCCGCT
AAA

AAAAA EcoRI site
AAAAA (Drinw, Drinp) Dri perfect forward and reverse primers
AAAAA (Drinr, Drinrf) Dri internal forward and reverse primers
AAAAA New 2nd internal primer (3rd reverse primer)

Figure 16. Dri Nucleotide sequence of the O. wendii PCR fragment. Sequences where primers were made for RACE PCR reactions are highlighted as indicated on the key.

**Actin Controls for 5’ RACE PCR**

Following ligation of the adapter to mRNA and cDNA synthesis, actin 5’ RACE controls were run to ensure those processes were successfully completed. Figure 17 shows the result of two reactions. One set of reactions clearly worked, as the appropriate sized actin band was present (Figure 17). The other did not, indicating the either the adaptor ligation or the cDNA synthesis did not work. As a routine, we used this control to check the quality of our template for 5’ RACE prior to proceeding with the reactions.
Figure 17. + control gel for RACE treated and ligated cDNA

**Dri 3’ RACE**

3’ RACE was carried out for Dri sequences as described in Methods. When the products were separated on a 1% agarose gel, a faint, diffuse band was observed. These products were cloned into the TOPO TA vector as described in methods, and transformed into bacteria. Colonies were picked and crude digests indicated which had inserts and determined their sizes. Seven of these were then sequenced, and of these, one overlapped
with our initial fragment of *O. wendtii* Dri cDNA. The new sequences and their translated protein products are shown in figure 18.

![Image of nucleotide and amino acid sequences]

**A.**

| NNNNAACGGCTT AGATTCGCCTGGGCCTACCTACCACCATCCATCACTAG |
| TGCAGCTTTTCACTCTTCGTACACAATATATGAAAGTGACCTAGATCCATATG |
| AATGTGAGAAGAAAGTGTATAGCAGCACCAGGCTGAAATTACAAGCAGCAGG |
| CGAAGGGCAATGCAGGCACTCGTACTATACCATCATGCAGGTCCGGCAGC |
| GTGCTGCGGCATTTAGTCTCCATTCATTACAGGCTCTACCGCCACCTA |
| CCATGACCCACCACTCTCTGTACCTTTGCAAACCCACTTACTTCCTCA |
| CCACGCCAATTTAGCTGAGTCCACTGAGGAGGAGGAGGAGGAAGGAGGAGG |
| ACAACCGAGAATCTGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG |
| AVAAAAACCTATAGTGGATGTGTCATTAATTCGGATCCGGAAGGGCGAATTC |

**B.**

| RFAWAYLPASITSAAFTLRTQY Met KYLYPECEKKGLS |
| TPÄEQLAAIDNGRERGRPRPIYHHAAGPAAAASFLHSRASA |
| SPPT MetIPHPSRIPLGTLPLPSLPLSLSTSEELHALAAARR |
| QEFSDKHTL MetLERERERERERERERERERERERERERERERERERERERE |
| RERKRRKRE Stop |

**Figure 18.** Results of 3’ RACE extension of Dri sequence. A; nucleotide sequence, B. putative amino acid sequence. The key below identifies key sequences.

| AAAAAAAA = 3’ drifin4108 Primer |
| AAAAAAAA = Previously known sequence |
| AAAAAAAA = Start of new sequence |
| AAAAAAAA = 3’ inner race primer |

**Assembling of Dri Sequences Alignments of S. purpuratus**

Sequences generated for the *O. wendtii* Dri gene began by analyzing homologous regions of the Dri gene found in various other species and then designing degenerate
primers with which brittle star cDNA could be screened for a homologous Dri gene.

Using degenerate primers, perfect primers and the RACE technique of transcript end amplification a 1053 base pair segment of the *O. wendtii* Dri gene has been generated.

This segment contains what appears to be the 3’ end of the gene and the bulk of its body. The 5’ start has not yet been sequenced (Figure 18). Alignments of the nucleotide sequences with *S. purpuratus* show some conservation (Figure 19A). Comparison of the deduced amino acid sequence with that of *S. purpuratus* shows a much higher degree of similarity, as was expected. For the fragment assembled, there is 81% similarity between the two organisms and 60% identity.

A.

![Clustal W alignment](image)

**Figure 19.** *O. wendtii* Dri sequence alignment with *S. purpuratus*. A; Nucleotide sequences, B; Deduced amino acid sequences.
The initial Alx1 sequence obtained from *O. wendtii* by Mary Harmon was used to identify primers that could be used for 5' RACE. The position of the primers is shown in Figure 20. Target cDNA from Gastrula that had been verified by actin controls was used as template for 5' RACE PCR as described in methods. When separated on a 1% agarose gel, the products appeared primarily as a 500 bp band. When cloned and sequenced, 3 out of 6 cloned products were the 5' end of Alx1. The nucleotide and translated amino acid sequences obtained from these clones are shown in Figure 21. A potential start codon was
observed at the beginning of an extended open reading frame, and their were stop codons 5’ to that AUG.

A.

Q V W F Q N R R A K W R K R E R F Q Q L
CAGGGTTTGGTTTCAARAAATCGTCGAGCCAAATGGCGGAAGCGTGAAAGTTTCAGCAACTCCAG

Q G M R G
GGCATGCAGGGA

L G P G G Y E M P I A P R P D A Y A Q
ATCGGGCCAGGTGGGGCTATGAGATCCTATCGCCCCTCGTCGCCAGCATACCTATCGGCAGGTG

V S P P G
AGTCCCCCTGGTT

B.

MLGYTSEKIGLIIWTACADTTPHKEMTPPLSTNTTISETKVDAITAERENMG
TSPVSTKVEPDANANTPKTEDNNKDDAKSTDGDSDKSNGGDSPKRKRRNRTT
FTSFQLEEMERVFQKTHYPDVYCREQLALRCDLTEARY

Figure 20. 5’ RACE amplification of Alx1 5’ end. A; indicates 5’ end of the original PCR fragment of Alx1. The arrow indicates the sequence and direction of the PCR primer.

B; pututative protein product of new sequences obtained.

**Alx1 Assembly and Alignment With S. purpuratus Alx1**

The complete Alx1 coding sequence has been generated using the initial PCR product and the RACE technique. The DNA binding domains of Alx1 from the two species are nearly identical at the amino acid level (*O. wendtii* AA# 190-250). Outside the DNA binding domain there is less similarity with the exception of the carboxy terminal of the proteins where there is a putative trans-activating domain.


**Dig-Labeled Probe Preparation and Sensitivity Assay via Dot Blot**

DIG-labeled probe preparation for both Dri and Alx1 were carried out as described in methods. Plasmids were linearized to completion as seen on agarose gels (Figure 23). Probes were synthesized as described in the methods section.
Figure 22. Directional Plasmid Digest. Plasmids were digested with either Spe1 (lane 3) or Not1 (lane 4) and products were separated on 1% agarose gels. Undigested plasmid (Lane 5) was used for comparison.

Complete digestion was considered to be the absence of supercoiled and nicked circle forms of the plasmid and a corresponding appearance of a linearized plasmid band.

Dilutions of the same plasmid used to make the DIG-labeled RNA probes were spotted on membranes and cross-linked. Hybridization and visualization of the bound probe indicated that we had successfully synthesized probes. The relative sensitivity of the probes compared to probes that had been empirically tested experimentally was used as an assay for probe synthesis in sufficient quantity to be useful. Figure 23 shows one example of the assay.
Figure 23. Probe Quantification. Dri sense (S) and anti-sense (α) probes were compared to Alx1 probes used successfully for Southern blots (+ control).

*Whole Mount In Situ Hybridization Using Alx1 probes*

Alx1 whole mount in situ hybridization has produced results that are reproducible and appear to be exemplary of skeletal formation in at least gastrula and late gastrula stage embryos.
Figure 24. (BEST photo) Whole Mount In Situ Hybridization Alx1 expression. Spatial and temporal expression of *O. wendtii* Alx1 during embryogenesis. All views are lateral. Staining follows a similar pattern to that seen in the sea urchin.

*Temporal Expression During Development Using Semi-Quantitative PCR for Alx1*

1 = Egg  
2 = 16 - 32 cell  
3 = blastula  
4 = mesenchyme blastula  
5 = gastrula  
6 = pluteus

Figure 25. Semi-quantitative PCR *O. wendtii* Alx1. Lanes represent stages of development where total RNA was isolated and used as a template for limited-cycle PCR. Products were detected by Southern blotting and chemiluminescent detection. The stages are indicated on the legend.
To determine the relative levels of Alx1 gene expression semi-quantitative PCR was carried out. In this modified version of quantitative PCR a limited number of PCR cycles is performed. Alx1 levels are semi-quantified for developmental time points; egg, 16-32 cell stage, blastula, mesenchyme blastula, gastrula, and pluteus larva. The PCR cycle was halted during the exponential growth phase during semi-quantitative PCR of cDNA generated from developmentally critical time points of brittle star development. This visualization and semi-quantification results in a time course of expression for the Alx1 gene in the brittle star. A gel was run of the resulting PCR products. The gel was transferred to a nitrocellulose membrane and standard southern blotting is performed. The relative amounts of each band represents the relative levels of Alx1 expression found in that respective stage of development. In the case of the brittle star Alx1 expression is not seen until the blastula stage. Expression remains constant and level until a marked increase in transcription occurs during the gastrula phase of development. Once gastrulation is complete and the pluteus stage begins O. wendtii Alx1 expression decreases back to blastula / mesenchyme blastula amounts. This devised method provides us with a semi quantitative measure of the level of expression for each stage of development. From this result we have determined that Alx1 is expressed in skeletogenic mesenchyme cells. The expression pattern indicates that Alx1 is not expressed in all mesenchyme that ingresses indicating that possibly in brittle stars some of the mesenchyme that ingresses may give rise to the equivalent of what is secondary mesenchyme in the sea urchin. However, there are also no pigment cells in the brittle star unlike the sea urchin.
**Dri Whole Mount In Situ Hybridization**

DIG-labeled RNA probes designed for Dri gene expression in developing brittle star embryos have yet to produce consistent or validated results.

**Phylogenetic Analysis**

Figures 26 and 27 provide a visualize representation of the evolutionary relationship of *O. wendtii* Alx1 and Dri proteins to other Alx1 and Dri proteins. Sequences attained from brittle stars have been aligned using Clustal X in order to produce a phylogenetic alignment of Alx1 genes (figure 26) and separately Dri genes (figure 27). These aligned sequences have then been used to make MEGA neighbor joining trees. Bootstrap values (1000 replications) are given.

![Figure 26. Alx1 neighbor joining tree using complete Alx1 *O. wendtii* sequence](image-url)
The results of the phylogenetic analysis of the complete sequence of *O. wendtii* Alx1 skeletogenic gene is represented in the neighbor-joining tree found in figure 26. The neighbor-joining tree represents commonly used distance matrix method of calculating genetic distance from the alignment of the three sea urchin sequences (S. purpuratus, P. lividus, and L. lytechinus) as well as the brittle star Alx1 sequence and that found in Mice and humans. By calculating genetic distance and not directly implying an evolutionary model we can conclude from this tree that the brittle star groups outside the clade containing its fellow sequenced echinoderms represented here by the presence of three sea urchins.

The results were attained using the full length of each respective brittle star sequence aligned against equivalent sequences from the varying comparative species. The brittle star *O. wendtii* Alx1 groups with the three other members of the phylum Echinodermata. The two sea urchins (S. purpuratus and Lytechinus) form a node with each other as well. This suggests that the Alx1 gene is more similar or more closely conserved within the phylum Echinodermata than when compared to the other phylum. This result is consistent with the current consensus on deuterostome phylogeny.

![Neighbor-joining tree](image.png)

Figure 27. Dri neighbor joining tree using 1053bp *O. wendtii* dead ringer sequence
The results of the phylogenetic analysis of the partial (1053 bp) transcript of the
*O. wendtii* Dri dead ringer gene is represented in the neighbor joining tree found in figure
34. In this case the *O. wendtii* transcript groups more closely with the starfish, Asteria
miniata than with the sea urchin, *S. purpuratus*. This would lead one to believe that the
starfish and brittle star have a closer evolutionary relationship when compared by this Dri
gene sequence. Therefore, it would be a logical assumption that the sea urchin and the
brittle star may have developed the characteristic of the embryonic skeleton in two
separate events as apposed to the development of an embryonic skeleton occurring in a
shared common ancestor.
Conclusions

Both Alx1 and Dri sea urchin genes have *O. wendtii* homologues. The homologous sequences of *O. wendtii* Alx1 and Dri seem to show an expression pattern similar to those found in the sea urchin. Whole mount in situ hybridization show the expression of Alx1 consistently at gastrulation in the brittle star. This Alx1 expression is consistent with that seen in the sea urchin. Semi quantitatively we have shown that Alx1 is expressed in the skeletogenic mesenchyme of the brittle star (figure 25). However, not all of the skeletogenic mesenchyme that initially ingress in the brittle star appears to be expressing Alx1. It is therefore thought that some of the ingressing mesenchyme of the brittle star may give rise to the equivalent of the secondary mesenchyme found in the sea urchin. From our whole mount in situ hybridization and semi quantitative PCR results is was possible to construct a skeletal gene regulatory network comparison (figure 29). In addition to this gene regulatory network comparison an endomesodermal developmental comparison was developed (figure 28) using both photographic and gene expression data of Brittle Stars and Sea Urchins. Figure 28 displays the lack of micromeres at the 5th cleavage division, the apparent ingress of both primary and secondary mesenchyme at the 24th hour of development as well as the lack of pigmented cells found in the brittle star when compared to the development of the sea urchin. These traits compose the three distinct and apparent differences found through out our investigation.
Figure 28. Sea urchin and brittle star endomesoderm developmental comparison

<table>
<thead>
<tr>
<th>Time</th>
<th>Stage</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Eggs</td>
<td>Pigmented eggs (purple)</td>
</tr>
<tr>
<td>2h</td>
<td>1st cleavage</td>
<td>Radial holoblastic</td>
</tr>
<tr>
<td>5h</td>
<td>Fourth cleavage</td>
<td>Equal - no micromeres</td>
</tr>
<tr>
<td>16h</td>
<td>Hatching blastula</td>
<td>Flattened, disc-shaped</td>
</tr>
<tr>
<td>24h</td>
<td>Mesenchyme blastula</td>
<td>Both 1st and 2nd ingress?</td>
</tr>
<tr>
<td>26h</td>
<td>Gastrulation begins</td>
<td>1st mes cluster laterally at veget pole</td>
</tr>
<tr>
<td>40h</td>
<td>Gastrulation complete</td>
<td>Skeleton visible, 2nd mes join archenteron, more 2nd mes? Coelomic pouches form</td>
</tr>
<tr>
<td>48h</td>
<td>Bending of embryo</td>
<td>Lobes formed at animal and vegetal ends bend toward each other, ciliary band visible</td>
</tr>
<tr>
<td>70h</td>
<td>Pluteus</td>
<td>Pluteus complete, No pigment cells formed</td>
</tr>
</tbody>
</table>

Figure 29. Sea urchin and brittle star skeletal gene regulatory network comparison
Dri RNA probes have yet to yield reproducible results. However, in the several whole mount in situ attempts using RNA probes created from *O. wendtii* sequenced DNA there seems to be a consistent increase in staining from the gastrula stage embryos on into development. This would suggest a similar expression pattern to that fond in *O. wendtii* Alx1 (figure 29). Unfortunately, this result is not consistent or reproducible enough to make a concrete conclusion. In addition photographic evidence of this observed phenomenon has been elusive.

It appears that phylogenetically both brittle star Dri and Alx1 sequences group outside the clade of the sea urchin. This would lead one to believe that the development of a embryonic skeleton may have developed as a separate trait in each classes as apposed to once in a common ancestor.

Based on our sequencing, expression analysis and phylogenetic analysis it is our conclusion that there is a similar kernel of a gene regulatory network at work in both Ophiuroid and Echinoid classes during embryonic skeletal genesis. Conserved genes in this kernel are at least Dri and Alx1. We speculate that the embryonic activation of the skeletogenic gene network occurred independently and probably includes different mechanisms but the kernel required for skeletal formation in brittle stars and sea urchins is the same in both classes.

Furthermore, it can be speculated that activation of the gene regulatory network responsible for embryonic skeletogenesis in both the sea urchin and the brittle star would be the result of a separate mechanism. The mechanism in the sea urchin has been shown to be the activation of the repressor Pmar1 and its repression of the repressor HesC.
(figure 29). This repression of a repressor is responsible for activation of the gene regulatory network that results in the embryonic skeleton of the sea urchin. Pmar1 expression has yet to be identified in the brittle star. Therefore the mechanism of activation may be unique in the brittle star. It would be a logical next step to try and identify, in brittle stars, expression of the newly characterized skeletogenic gene regulatory network repressor HesC that has been identified in sea urchins (figure 29). If indeed a unique (non Pmar1) activation occurs in brittle stars determining if the activation includes repression of HesC would be valuable information.

Lastly, this investigation supports the idea of gene regulatory network kernel is a class of gene regulatory network, which because of their developmental role and their particular internal structure, are most impervious to change (Davidson et al., 2006). As exemplified here the skeletal gene regulatory network of the echinoderms, sea urchin and brittle star, contains conserved regulatory genes. Where interference with one or more of these regulatory genes would likely destroy kernel function, activation of these kernels can be a mechanism of evolutionary change in gene regulatory networks over time. The developing animal body plan is controlled by the action of gene regulatory networks. It is therefore concluded that evolution must be dependant on change in gene regulatory networks. This change can come in the form of disruption of or a change in the activation of a gene regulatory network producing changes in development, body plan, speciation and adult morphology. In echinoderms it is the brittle star’s and sea urchin’s embryonic activation of the skeletal gene regulatory network not seen until the adult form in Asteroideans, Holothuroideans and Crinoideans that give evidence for the evolution of the skeletal gene regulatory network in echinoderms.
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