

Origin of the Gonad:
A phylogenetic analysis of SoxB1
Tracing back human sex-determining gene Sry to an orthologue involved in
echinoid metamorphosis

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Abstract:

Until twenty-five years ago, the sex determining factor in humans had yet to be identified. The *Sox* family of transcription factors were identified after the human sex-determining gene *SRY* was sequenced and compared to other genomes (Bowles et al., 2000). *SOX* genes have been discovered across the Metazoans but with varying function. Looking at related invertebrate organisms can offer insights into the primary gene function and the evolution of the gene. Echinoderm life cycles begin with a larval, bilaterally symmetric shape which then transforms into a radially symmetric juvenile—a radical metamorphosis! The role of *Sox* transcription factors in the process of metamorphosis has been described in *Strongylocentrotus purpuratus* and in this project I will do research on metamorphosis with other echinoids to see if they are similar.

Introduction:

Sex determination in mammals is dictated by the presence or absence of the Y-chromosomal gene *SRY*. *SRY* functions as the primary sex-determining gene by activating formation of the testis (Kikuchi et al., 2013). Without *SRY*, an embryo will form ovaries. *SRY* is crucial for normal male development. For without it, Sertoli cells will not be produced. Sertoli cells are important as they secrete AMH to cause regression of the Mullerian duct (female reproductive system) (Zarkower, 2001). Mutations in the sequence of *SRY* is the most common cause of male infertility (Harley et al., 2003). The gene *SRY* contains a 79 amino acid sequence that codes an HMG (high mobility group) that is characteristic of the *Sox* gene family (Soullier et al., 1999). *SRY* exists solely in mammals; however, it evolved as a duplication of the *Sox* gene family which are found across Metazoans.

The *Sox* gene family has been implicated in various early developmental processes. In vertebrates, *Sox* genes are involved in sex determination, eye development, neurogenesis, pituitary development, carcinogenesis, neural crest formation, skeletogenesis, pancreas formation, and notochord cell formation (Lefebvre et al., 2007). Mammalian *Sox* genes are expressed during early development of gonads and are critical for neural crest development and neurogenesis (Barrionuevo and Scherer, 2010). Known functions of *Sox* genes have evolved as the gene family has been identified in more organisms.

Research on lamprey development by the Bronner Lab indicates that *Sox* genes are also expressed throughout early development in a basal and jawless vertebrate (Uy et al., 2012). In zebrafish, a distinct but similar version of the *Sox* gene plays a role developing neural and cardiac tissue (Rauch et al., 2003). In

Xenopus, *Sox* genes are expressed in neural plate formation (Cunningham et al., 2008).

In invertebrates, *Sox* genes are implicated in developmental processes such as metamorphosis, eye development, neural crest formation, and ectoderm formation (Luo & Su, 2012). Sequencing the sea urchin *S. purpuratus* genome demonstrated that *Sox* gene sequences are highly conserved even in radially symmetric body plans (Howard-Ashby et al. 2006). *SoxB1* (*S. purpuratus*) is expressed in the foregut during development and is closely related in sequence to the *Sox* genes (*Sox1*, *Sox2*, and *Sox3*) in mouse embryos (Wei et al., 2011).

For my research this quarter, I studied the evolution of the *Sox* genes across the metazoans and how they have evolved in sequence, function, and importance. The conservation of DNA and protein sequences in invertebrates offer insights into the genetic history of the *Sox* family. Looking at *Sox* genes in organisms more anciently diverged from the tree that have basal sequences can help us understand the primary functions of *Sox* genes. To elucidate the evolutionary history of the gene, I constructed a gene tree compiling the *SoxB1* sequences from 28 different species of predominately invertebrate organisms.

The phylum Echinodermata has potential for genetic research as they are the most basal phylum of the deuterostomes and have similar basic development as humans. Echinoderms undergo metamorphosis from a bilaterally symmetric swimming larvae to a radially symmetric juvenile, suggesting that they began with a bilaterally symmetric shape and regressed back into radially symmetric body form. With a development similar to our own, echinoderms offer a good model system for gene pathways during early development. *Sox* genes were found be expressed in *S.*

purpuratus in oral and arm development during metamorphosis (Duboc et al., 2005). They were also found to be expressed opposing *Nodal* in rudiment formation (Luo & Su, 2012). A goal of this project is to further examine the role of *Sox* transcription factors role in echinoid metamorphosis.

In addition, I compared the metamorphosis of *D. excentricus* and *S. droebachiensis* to compare and establish normal echinoid development patterns. I also experimented with external metamorphose cues by looking at relative metamorphosis rates of *D. excentricus* when presented with different sand types (sand formerly inhabited by *D. excentricus* and sand not formerly inhabited by *D. excentricus*) over a period of one week.

Methods:

Dendraster excentricus larvae

Adult specimens were collected off shallow waters of Orcas Island, WA during spring 2013. Larvae were obtained through artificial fertilization after combining gametes spawned with 0.5-1 ml of 0.5 M KCl. Experiment on metamorphosis conducted on animals fertilized April 20, 2013 started May 19, 2013 (one month after fertilization) with three different treatments of sand (no sand, sand collected from *D. excentricus* adult habitat, and sand untouched by *D. excentricus* (see Figure 2). On May 19, 2013 larvae with clear rudiments were selected and transferred 14 per well with different sand types in the well. Larvae were kept at Friday Harbor Labs in sea water tables at 10-12 °C.

Strongylocentrotus droebachiensis larvae

Adult specimens were collected off of San Juan Island, WA during spring 2013.

Larvae were obtained through artificial fertilization after combining gametes spawned with .5ml-5ml of 0.5 M KCl. Larvae were kept at Friday Harbor Labs in sea water tables at 10-12°C.

SOX alignments and trees

Sequences were obtained using the GenBank database and running BLAST searches with *S. purpuratus* to find *Sox* orthologs (28 different taxa *Sox* genes were used see Supp. Fig. 2) Multiple protein sequence alignments were made using MEGA 5.1 and Muscle to align the sequences. Gene phylogenetic trees were constructed using Maximum Likelihood algorithm after running MEGA's phylogenetic analysis with 500 bootstraps.

Results:

My first aim was to construct an invertebrate-rich alignment of gene *SoxB1*. I used 12 different taxa *SoxB1* sequences to construct my alignment. *Sox* gene sequences (see supp. Fig. 2) were obtained by Blasting GenBank with the *S. purpuratus* protein sequence (GenBank accession number NP_999639.1). A multiple sequence alignment was constructed by MEGA after running a phylogenetic analysis constructed with Maximum Likelihood Algorithm. Looking at the MEGA alignment, the HMG box is a DNA binding domain that is highly conserved across all sequences as a 79 amino acid sequence. Outside of the HMG box, *Sox* family sequences are more variable, especially when comparing such a broad spectrum of phyla.

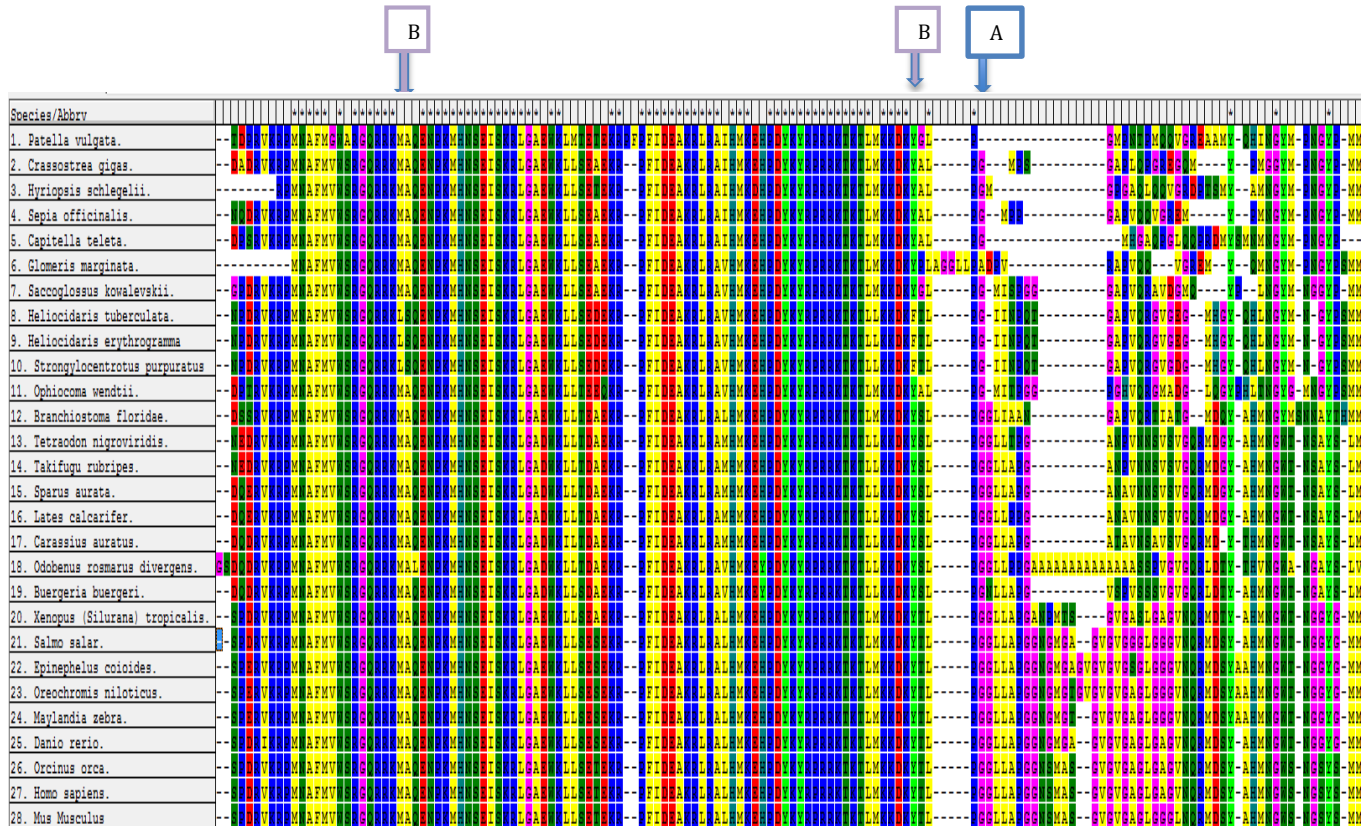


Figure 1: MEGA multiple protein sequence alignment of 28 taxa, ordered 1-28 by current position in tree of life. The conservation of the HMG box is shown in the Muscle generated protein alignment below (Fig. 2). The full MEGA alignment is shown in supplementary figure 3.

Sites: **A-** site of interest in which amino acid Glycine has been inserted after evolution of chordates. **B-** site of interest in which amino acid sequence variable in echinoderms

Although the HMG box is known to be almost perfectly conserved within the vertebrates, invertebrates, on the other hand, seem to have HMG boxes that have different conserved sites. *Patella vulgata* (limpet), *SoxB1* sequence on my alignment has the most divergent HMG domain with multiple insertions, substitutions, and deletions, which suggests sequencing or assembly errors. Most of the substitutions and additions are not conserved even throughout the protostome lineage.

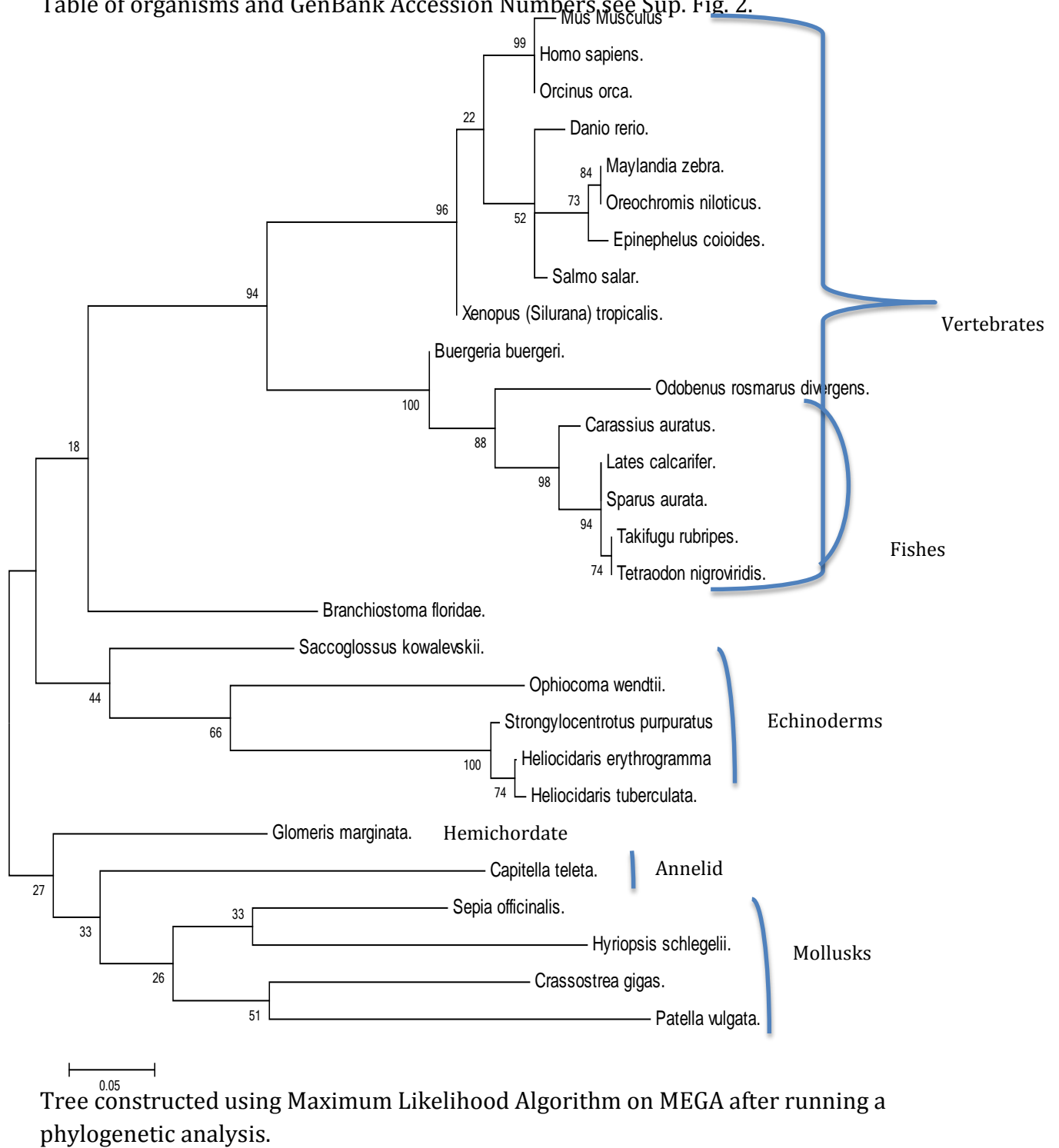
However, it is clear that there have been multiple additions and substitutions throughout the evolution of the HMG box. An example of this is at Site **A** (see Fig. 1), in which the addition amino acid Glycine seems to have been inserted after Phylum Echinodermata, or the ancestor of the phylum lost the site. There are a few other sites in the HMG domain similar to this.

One of the more unexpected results was the variation of the HMG domain within Phylum Echinodermata. On more than one occasion (see **B** sites), urchins have had a different protein than the brittle star *O. wendtii*. This is interesting as it suggests urchin *SoxB1* sequences are more divergent than other echinoderms. Non-HMG box coding regions are extremely variable and seem to evolve quickly, making it difficult to find regions of homology between animals in different phyla. If I were to analyze the evolution of these non-box regions I would need to narrow my scope to a single phyla with ample sequenced taxa.

My protein alignments produced the tree of the 28 taxa arranged to represent the evolution of *SoxB1*. In this tree (see Fig. 2), the evolution of *Sox* genes is shown from *Patella vulgata*, a limpet, up to the highly divergent human *SRY* gene.

Fig. 2: Gene Tree of SoxB1

Table of organisms and GenBank Accession Numbers see Sup. Fig. 2.

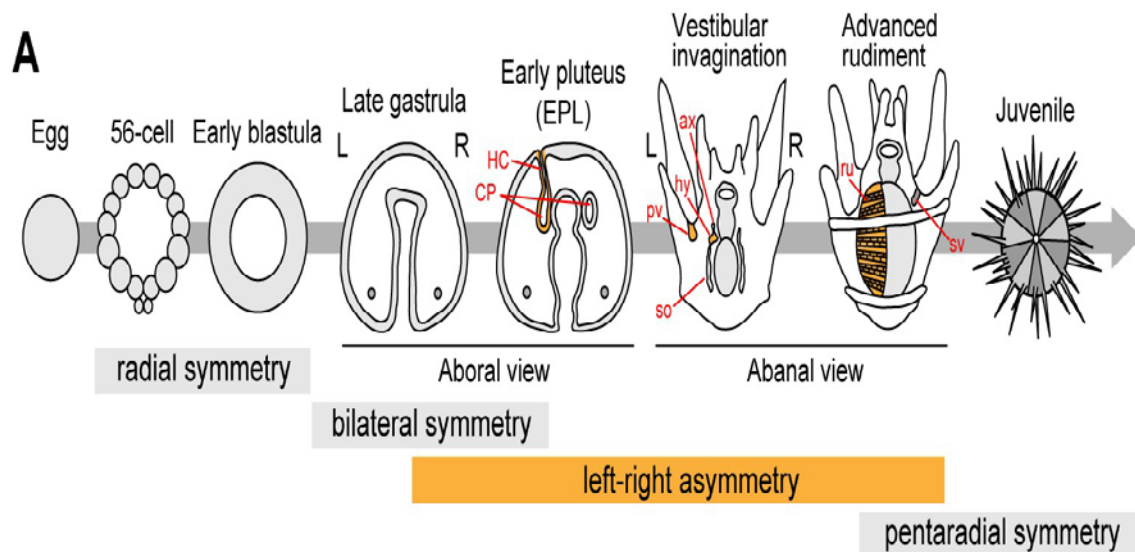


This tree mirrors the current view on the tree of life, indicating that gene *SoxB1* has evolved slowly.

The first split the tree recovers is the one between deuterostomes and protostomes. Within the deuterostomes, the tree provides good support for the phylum Echinodermata. The vertebrate evolutionary relationships on my tree are supported well with high bootstrap values.

The bootstrap values on my gene tree are not very high due to the fact that few invertebrate sequences of *SoxB1* exist. With an increased number of invertebrate sequences, more data would bring more support and evidence for evolutionary relationships between taxa.

Fig. 2 **Stages of Echinoid Metamorphosis** (Luo & Su, 2012)



In this figure from Luo and Su, the stages of development of *S. purpuratus* are shown. For the purpose of this project, I am concentrating on the transition from the advanced rudiment stage to the juvenile stage in *D. excentricus* and *S. droebachiensis*.

I have found that the average time it takes to metamorphosis from fertilization in lab conditions for *D. excentricus* takes 30 days and the average metamorphosis time from fertilization in lab conditions for *S. droebachiensis* is 33 days. I discovered rudiment formation typically occurs around 25 days post fertilization in *D. excentricus* and 27 days post fertilization in *S. droebachiensis*. These rates are representative of lab conditions.

Fig. 3 **Photos of Echiniod Metamorphosis**

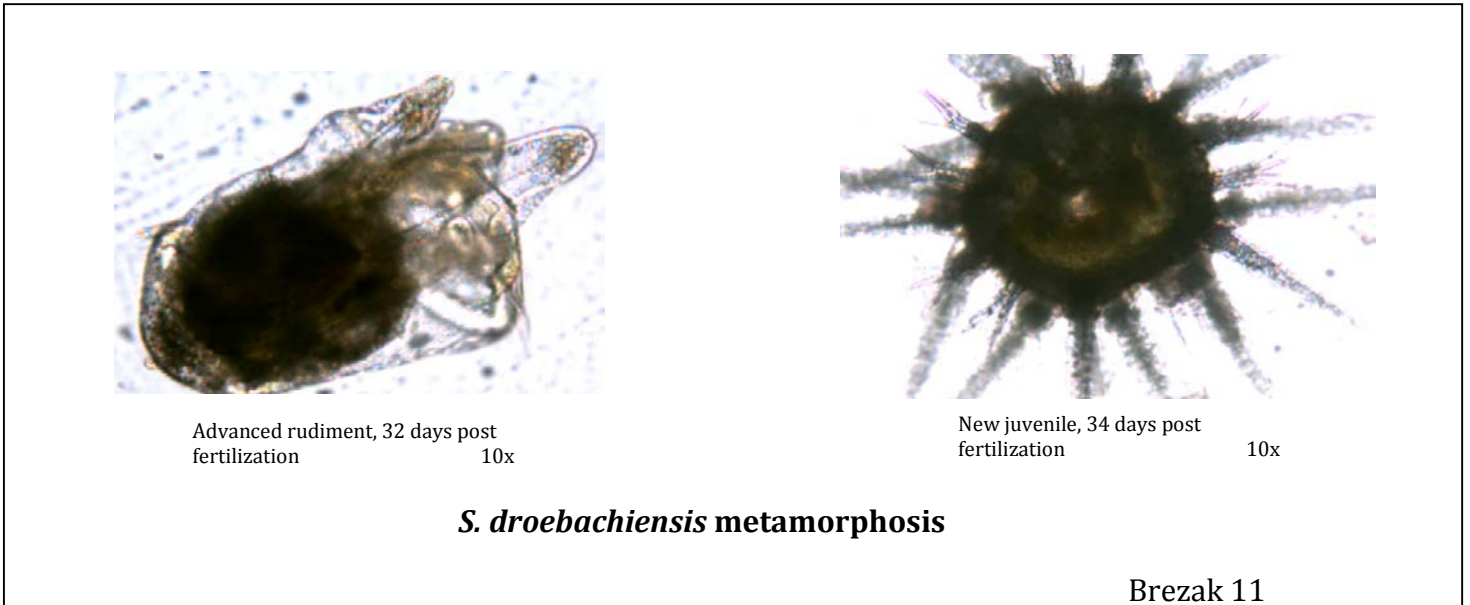
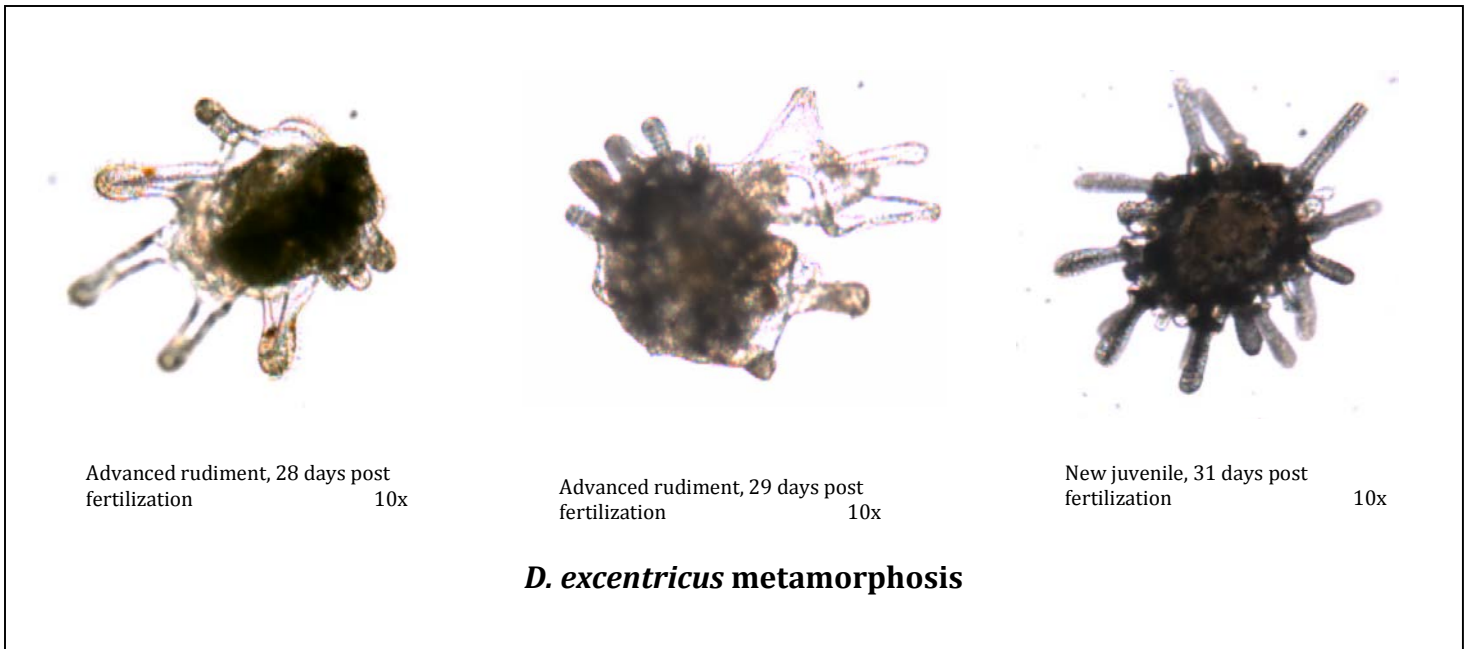
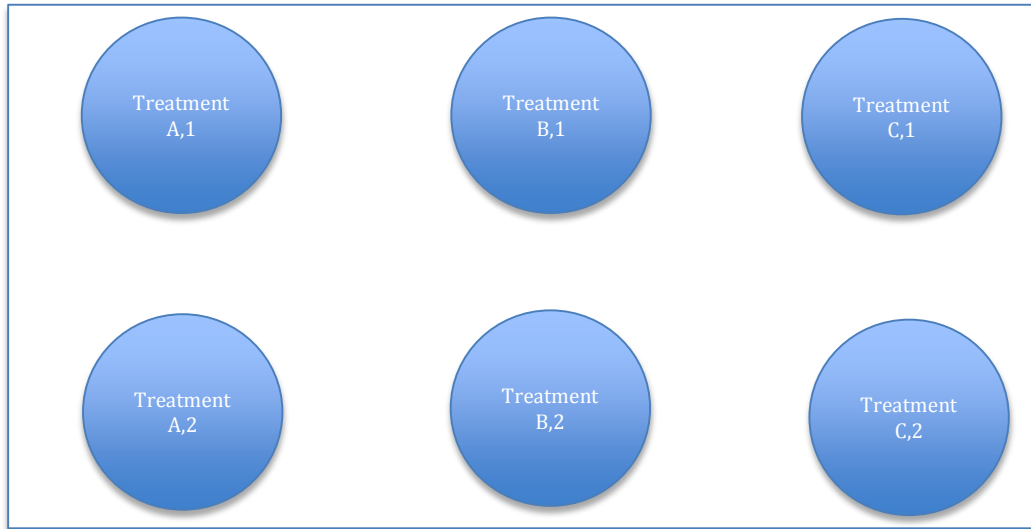


Fig. 4



















Metamorphosis Experiment



Treatment	A (1,2)	B (1,2)	C (1,2)
	no sand	sand from adult sand dollar tanks	Sand from beach (no sand dollars present)

Fig. 5

Table of Metamorphosis Rates of *D. excentricus*
(fertilized April 20, 2013)

	May 19		May 20		May 21		May 22		May 23		May 24		May 25		May 26		May 27	
																		
A1	14	0	13	1	13	1	13	1	13	1	12	2	12	2	12	2	12	2
A2	14	0	14	0	14	0	14	0	14	0	14	0	14	0	14	0	14	0
B1	14	0	13	1	13	1	11	3	11	3	11	3	11	3	11	3	10	4
B2	14	0	14	0	14	0	13	1	12	2	12	2	12	2	12	2	12	2
C1	14	0	14	0	14	0	13	1	13	1	13	1	13	1	12	2	10	4
C2	14	0	14	0	14	0	14	0	14	0	14	0	14	0	14	0	14	0

My experiment compared rates of metamorphosis when presented with different sand types. I explored the effect of three different sand types (sand previously inhabited by *D. excentricus* vs. sand not previously inhabited by *D. excentricus* vs. no sand) on metamorphosis rates within a one week period (see Fig. 4). In the experiment, I had six well plates and had two wells for each sand type. I transferred 14 *D. excentricus* larvae (fertilized April 20, 2013) of advanced rudiment stage (see Fig. 3) into each of the six wells on May 19, 2013. For treatment A, I did not introduce sand or any sort of organic material to the wells. In treatment B, I introduced the larvae to wells with a few granules of sand that was previously inhabited by *D. excentricus*. In Treatment C, I introduced the larvae to wells that had sand fresh from an area uninhabited by *D. excentricus*.

Within 24 hours of the experiment, one larva in well A1 and B1 had metamorphosed. The larva in well A1 (no sand) had metamorphosed on the water surface. The larva in well B1 (sand previously inhabited by *D. excentricus*) metamorphosed on one of the sand granules at the bottom of the well. At 24 hours, there seemed to be little real difference in response to the sand treatment.

As the week progressed, treatment B had the highest average of metamorphosed juveniles. No larvae metamorphosed in either of treatment C (sand uninhabited by *D. excentricus*) wells until the fourth day of exposure to the sand. At the end of the week, the metamorphosed percent of individuals in each well was:

WELL	A1	A2	B1	B2	C1	C2
% META	14%	0%	28%	14%	28%	0%

Treatment B had the highest combined percent of metamorphosed juveniles. Treatment C had the second highest percent of metamorphosed juveniles, followed by treatment A. This suggests that late rudiment larvae are more likely to metamorphosis when presented with sand rather than without sand, and that larvae are more likely to metamorphose with sand previously inhabited by their own species.

Discussion:

This project aimed to provide a better understanding of the early evolution of *Sox* genes. Previous *Sox* gene trees have included, at the most, five invertebrates. I have included the *Sox* sequences of twelve and proposed a path of evolution for gene *SoxB1*. My gene tree supports the Metazoan evolution tree, indicating that *SoxB1* has a conserved pattern of evolution. The most basal *Sox* gene family member according to my tree is that of *Patella vulgata* (limpet). From there, my tree has representatives of invertebrate phyla Mollusca, Annelida, Arthropoda, Echinodermata, and Hemichordata. The two clades my tree distinguishes between is deuterostomes and protostomes, and further differentiates between vertebrates and invertebrates. Human *SRY* is near the top of the tree along with mouse *SRY*, the first two sex-determining genes discovered. In my tree, the evolution of *SoxB1* matches the path of evolution taken through the tree of life. In the future I look forward to being able to elucidate the evolution of the gene within the phylum Echinodermata by sequencing *SoxB1* from *S. droebachiensis* and *D. excentricus*. With more organisms sequenced, I might be able to deduce when in the echinoderm lineage the gene evolved in sequence.

My metamorphosis experiment indicated that *D. excentricus* individuals of metamorphosing capability at the advanced rudiment stage are more likely to metamorphosis when exposed to sand previously inhabited by *D. excentricus* adults. Although I would like to reproduce results and conduct more trials to support my results, these preliminary results suggest larvae could use a number of factors to begin metamorphosis. As more *D. excentricus* larvae exposed to the sand from the adults metamorphosed than the other treatments, it seems that these larvae are recognizing the former presence of the adults. An experiment conducted by Robert Burke supported this finding. Burke found that advanced rudiment stage larvae ready to metamorphose respond to a pheromone released by adult sand dollars (Burke, 1984). This is a cue other organisms use to metamorphosis as well, as it indicates safety and increases chance of survival once in the juvenile stage.

Future directions:

Due to time constraints, I was not able to accomplish everything that I originally wanted to do. After three failed attempts of PCR (see sup. Fig. 4-7), I would like to do it again so that I am able to clone and sequence the *SoxB1* genes from *D. excentricus* and *S. droebachiensis*. With those sequences I can make probes and use them to track mRNA expression in in-situ hybridizations. I have fixed advanced rudiment phase larvae for in situs to see whether or not *SOX* is expressed in the rudiment formation in these echiniods as it was in *S. purpuratus* (Luo & Su, 2012).

Further questions I would like to explore are related to different facets of *Sox* genes. I would like to do further analysis of the evolution of non-box and HMG

regions within Phylum Echinodermata. I am interested whether function of gene SRY is conserved in echinoderms, or what their sex-determining genes and such mechanisms are.

I am curious as to what exactly the *D. excentricus* larvae are responding to as a metamorphose cue in the sand that was formerly inhabited by adults. I would like to do an analysis of the sand granules to extract the pheromone responsible for inducing metamorphosis. After sequencing this pheromone, I will compare it to metamorphosis cues across other metamorphosing organisms to see if this is a conserved function of this pheromone.

Acknowledgments:

I would like to thank my professors Dr. Billie J. Swalla and TA Elliott Jacobsen-Watts for their guidance throughout the course of this project. I would also like to thank my fellow classmates this quarter for helping me along the way—a special one to Sam LeDuc and our gremlin late nights in lab.

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AF157389.3      TACTCGGCCGCCTACAGCCAGGCGGCAATGATGCACCACGCACCCTGTCGGCACCTCAGCC
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AF157389.3      TCCGCTGGCGGGCGACCCATGCCCGGCGATTCGTTCGCTTCGTGATATGATCAACATGTAC
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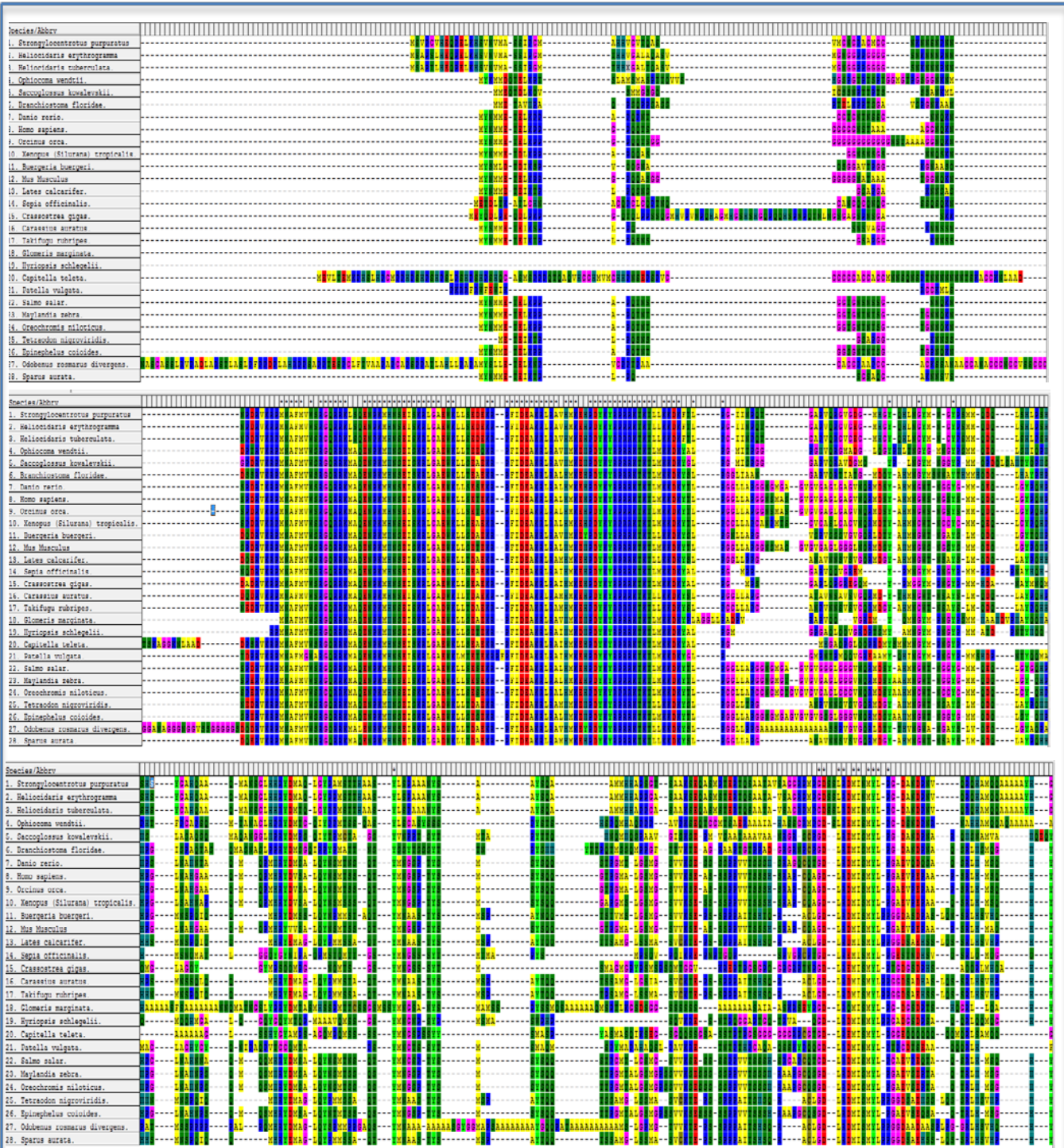
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Supplemental Figure 1

CLUSTALW alignment of *Heliocidaris tuberculata* (accession # AY532156.1), *Heliocidaris erythrogramma* (AY532155.1), and *Strongylocentrotus purpuratus* (AF157389.3) SOXB1 sequences to make PCR primers.

Organism	General name	GenBank Accession
<i>Strongylocentrotus purpuratus</i>	urchin	NP_999639.1
<i>Heliocidaris erythrogramma</i>	urchin	AAS59171.1
<i>Heliocidaris tuberculata</i>	urchin	AAS59172.1
<i>Ophiocoma wendtii</i>	brittle star	AFP99071.1
<i>Saccoglossus kowalevskii</i>	worm	NP_001158377.1
<i>Branchiostoma floridae</i>	lancelet	ABG66527.1
<i>Acipenser schrenckii</i>	sturgeon fish	ABG66527.1
<i>Danio rerio</i>	zebra fish	NP_998283.1
<i>Homo sapiens</i>	human	NP_003097.1
<i>Orcinus orca</i>	orca	XP_004278447.1
<i>Xenopus tropicalis</i>	frog	NP_998869.1
<i>Chelonia mydas</i>	turtle	EMP36471.1
<i>Buergeria buergeri</i>	frog	BAM11010.1
<i>Mus musculus</i>	mouse	P48432.2
<i>Tetraodon nigroviridis</i>	fish	CAF91617.1
<i>Sepia officinalis</i>	cuttlefish	AGL08098.1
<i>Crassostrea gigas</i>	pacific oyster	EKC24855.1
<i>Carassius auratus</i>	goldfish	ABM55677.1
<i>Takifugu rubripes</i>	bony fish	AAQ18496.1
<i>Glomeris marginata</i>	millipede	ABF21053.1
<i>Capitella teleta</i>	segmented worm	ELU12387.1
<i>Hyriopsis schlegelii</i>	bivalve	AEK27011.1
<i>Patella vulgata</i>	limpet	CAE30461.1
<i>Salmo salar</i>	salmon	NP_001135190.1
<i>Maylandia zebrafish</i>	zebra fish	XP_004562135.1
<i>Oreochromis niloticus</i>	tilapia fish	XP_003457401.1
<i>Tetraodon nigroviridis</i>	puffer fish	CAG09811.1
<i>Epinephelus coioides</i>	bony fish	ACJ61262.1
<i>Odobenus rosmarus divergens</i>	walrus	XP_004405962.1
<i>Sparus aurata</i>	bony fish	BAL14716.1

Supplemental Figure 1: Table of phyla and accession number from GenBank used in MEGA alignment and gene tree.



Supplementary Figure 3: MEGA multiple sequence protein alignment generated using Muscle

Supplemental Methods

PCR

Primers were constructed using GenBank in addition to BLAST searches to find conserved sequences among echinoderms. *Heliocidaris tuberculata* (accession # AY532156.1), *Heliocidaris erythrogramma* (AY532155.1), and *Strongylocentrotus purpuratus* (AF157389.3) SOXB1 sequences were used to construct primers after running a multiple alignment with CLUSTALW. Primers used were:

5' GCATTCTGTGAACGTCATGGC 3'

5' GTCATACCTGTGATGAAGTCC 3'

Note: see supplementary figure 1 for alignment

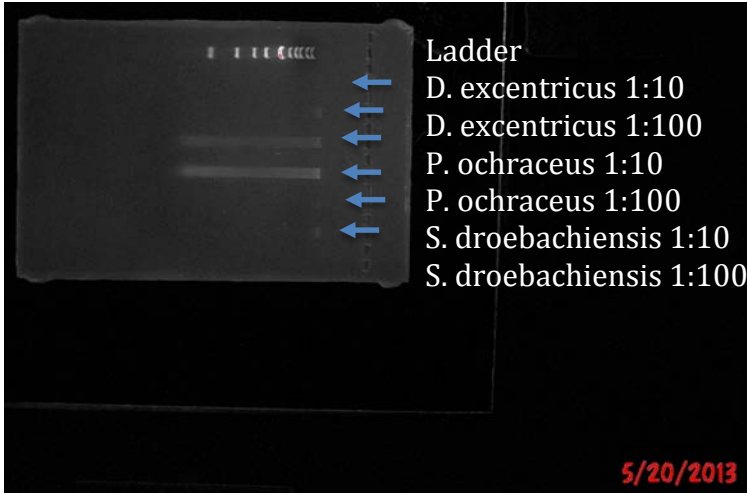
PCR design was:

1. Initial Denature 4 minutes at 94°C
2. Anneal 1 minute at 45°C
3. Extension 2 minute at 72°C
4. Denature 1 minute at 94°C
5. Repeat steps 2-4 for 30 cycles
6. Final extension 10 minutes at 72°C

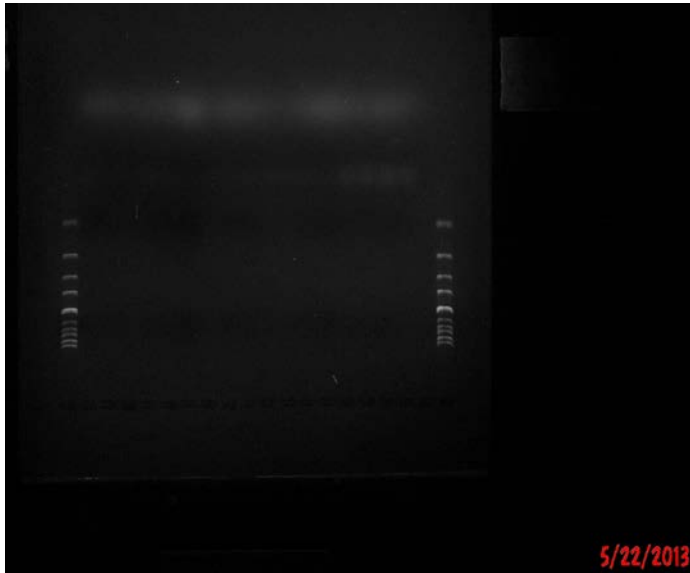
Store O/N at 4°C

Results of PCR:

Unsuccessful for unknown reason. However, DNA was verified to be in good condition through a DNA check gel (see Supp. Methods Fig. 1)



Supp. Methods Fig. 1
 DNA Check Gel w/ various dilutions



Supp. Methods Fig. 2-4
 PCR gel, no bands=
 inconclusive

