Wnt Pathway in Sand Dollar *Dendraster excentricus* Clones

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Marine Genomics Research Experience Spring 2013

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Keywords: *Dendraster excentricus*, *Wnt* expression, cloning, development

Abstract

Although regeneration has been described in many deuterstomes, little is known about the molecular pathways involved in regeneration and cloning in the echinoderms (Rychel and Swalla, 2008). Larval cloning occurs in the sand dollar *Dendraster excentricus* in the presence of predatory threat and food stress (Vaughn, 2007). The molecular pathways involved in the process of limb patterning in development and in cloning may be analogous (Mueoka and Bryant, 1982). The goal of this research project is to determine the role of *Wnt* expression in newly cloned larval *Dendraster excentricus*.

Introduction

Regeneration has been described in many of the deuterostome group Echinodermata (Rychel and Swalla, 2008). Echinoderm regeneration has been heavily studied, from repair of broken or lost limbs across many species to regenerating entire halves of their body in adults when damaged in *Asteroidea* to complete organogenesis in larval *Pisaster ochraceus* and *Luidia foliolata* (Vickery et al.1998, Vickery et al. 2001). It is known that in *Dendraster excentricus*, full larval cloning has been observed in the presence of predators (Vaughn, 2007). Although much is known about the capabilities of these metazoans, little is known about the molecular pathways involved in this regeneration process. Limb patterning in regenerated limbs share many similarities with body patterning in development (Muneoka and Bryant, 1982). If we consider the process of cloning as analogous to regeneration, the same molecular pathways should be involved. The purpose of this study is to investigate the molecular pathways involved in *Dendraster excentricus* larval cloning.

In the process of development, genes are turned on and off by transcription factors and their regulatory networks. The turning on and off of genes in specific locations and times creates a patterning in the embryo that is responsible for body axis formation, cell fate and tissue formation (Drager et al. 1989). The *Wnt* signaling pathway is involved in proper body patterning, regulating cell differentiation in many tissues and control of cell death (Church and Francis-West, 2002). Two *Wnt* orthologs, *Smwnt8* and *Pjwnt8* were previously isolated the two sand dollar species *Scaphechinus mirabilis* and *Peronella japonica*, respectively, both very closely related

to *Dendraster excentricus*. It is known the *Wnt* pathway plays a key role in cell proliferation and differentiation in development and is evolutionarily conserved (Nakata and Minokawa, 2008), but it is not known how the *Wnt* pathway and its components contribute to cloned larvae. The *Wnt* signaling pathway cascade occurs when the Wnt ligand binds to the transmembrane receptor Frizzled. After the Wnt ligand is bound to Frizzled and LRP, Dischevelled is activated, causing Glycogen Synthase Kinase-3 (GSK-3) to be inhibited and allowing ß-catenin to build up in the cytoplasm. Once the level of ß-catenin is high enough, it will go into the nucleus and activate specific genes through binding of DNA. This causes the *Wnt* pathway to be active and an upregulation in the transcription of *Wnt* responsive genes (Church, 2002; Konikoff et al, 2010).

A study of the temporal expression of the *Wnt* pathway will help to understand the interaction of this gene network in early cloned larvae. This study will also help determine if the patterned expression of *Wnt* and the time points for nerve tissue, gonads and stem cells in larval cloning in *Dendraster* is analogous to regeneration and development.

The goal of this project is to examine expression of the components of the *Wnt* pathway in order to determine sites of expression, perform QPCR and gene assays for expression levels at various times of development, as well as construct a gene tree to examine genetic conservation of these genes in the Class Echinodermata.

Methods and Materials

Spawning and Culturing:

Dendraster excentricus were spawned using 0.5 mL of 0.5 M KCl via intracoelomic injection. Eggs and (dry) sperm were collected from the gonopores by putting them gonopore-down in filtered seawater beakers and dry watch glasses, respectively. After activation of the sperm and fertilization, we fed and monitored the sand dollars (Vaughn, 2010; Vickery and McClintock, 2000). It has been shown that by keeping the embryos in high food levels (5 X 10^4 cells/ml), the rate of death is much less than in low or medium food levels (Flores and Swalla, 2010).

LiCl Cloning:

Three *Dendraster excentricus* larvae were put into each well of 12-well plates for monitoring. Once at the larval stage, I induced cloning by treatment with 25mM LiCl for 3 hours (modified from Flores and Swalla, 2010). Post-LiCl treatment, larvae were washed using Filtered Sea Water twice. Larvae were observed and monitored for 7 days. After collecting induced clones, I observed the body patterning and gut formation process in clones.

PCR and Primers:

Wnt8 in *Dendraster excentricus* was previously unavailable, so we designed primers to clone and sequenced the gene. This was used to determine the position of *Dendraster excentricus* in a phylogenetic analysis.

Primer alignments were made using GenBank Blast sequence data for *Wnt8* in *Hemicentrotus pulcherrimus* (ACC38467.1), *Heliocidaris erythrogramma* (AY532155.1), and *Strongylocentrotus purpuratus* (AF157389.3) (Figure 1).

Sequences were aligned using ClustalW and Muscle in MEGA 5.1 and selected for G/C-rich areas.

Wnt8 sequences were used to construct primers after running a multiple alignment with MEGA 5.1. Primers used were:

F: 5' ACG AGA TCA AGC GCA AGT ACT TCA 3'

R: 5' ACA GTT CGC CGA CAG GTG TCG CAC 3'

PCR Reagent mix was performed using a 1:10, 1:100 and 1:1000 DNA dilutions.

PCR Protocol Design:

Initial Denature	4 min	94°C
Anneal	1 min	45 °C
Extension	2 min	72°C
Denature	1 min	94°C
REPEAT 2-4 for 30 cycles		
Final Extension	10 min	72°C

Store O/N at 4°C

Table 2: PCR Design for *Wnt8*, *SoxB* and *18S* (control).

Whole Mount Antibody Staining:

I performed whole mount antibody staining using ß-catenin antibody to probe for *Wnt* expression and *Smad4* (ab137861) probe for a control in newly formed clones. Individuals of interest were fixed in 4% paraformaldehyde for 2 hours then stored in 0.5 mL PBS. Once all individuals were collected, 1:500 dilutions of primary antibodies were used in standard Goat anti-Rabbit protocol. Larvae were allowed to soak in primary antibody and secondary antibody overnight to obtain optimal staining. Once rinsed, photos were taken using fluorescent light and a

compound microscope.

Phylogenetic tree and genomic analysis

Using a BLAST search for *Wnt8* in *Hemicentrotus pulcherrimus* (ACC38467.1), sequences were obtained on the NCBI GenBank database. Seventeen sequences of available echinoderms, hemichordates and chordates were used to construct a gene tree using ClustalW and Muscle alignment tools in MEGA 5.1.

Results

LiCl Cloning:

Preliminary trials for cloning have been successful (Table 1.), showing a 22% success rate of cloning after 7days. Second trial of cloning has shown a similar trend of 5% clone success 1 day post-treatment.

PCR:

Attempts to run PCR and sequence *Wnt8* in *Dendraster excentricus* were unsuccessful thus far (Figure 3). Prior to performing PCR, a DNA check gel was ran for *Dendraster excentricus, Strongylocentrotus droebachiensis* and *Pisaster ochraceous*, showing our DNA isolations were successful (Figure 2).

Whole Mount Antibody Staining:

Whole mount antibody staining was successful. Nuclear expression for ß-catenin is seen. My control, Smad4, antibodies also showed expression, but in different areas of the newly formed clone (Figure 5).

Phylogenetic tree and genomic analysis:

Attempts to clone and sequence *Wnt8* in *Dendraster excentricus* were unsuccessful; as a result, *Dendraster excentricus* is omitted from my tree. The gene tree constructed with the available sequences shows a phylogenetic following normal phylogenetic patterning you would expect with relations of echinoderms, hemichordates and chordates supported with high bootstrap values (Figure 9).

Discussion

LiCl Cloning:

As my attempts to induce larval cloning in *Dendraster excentricus* were at a very high rate, we can conclude that the *Wnt* pathway must be activated for 3 hours in order to induce cloning reliably. Clones were observed as being very small, averaging 115 μm in size, whereas the larvae they were cloned from averaged 352μm in size (Figures 6, 7 and 8).

PCR, Phylogenetic Tree and Genomic Analysis:

Attempts at PCR were unsuccessful. However, because we did run a successful DNA check gel, poor DNA extraction is probably no to blame. Condensation was found on the sides of PCR tubes; potential problem with PCR machine hot top. Numerous attempts to right the issues with PCR were made. In the future, I would like to finish PCR and get the sequence for *Wnt8* in *Dendraster excentricus*. I predict that *Dendraster excentricus* would fall in as sister taxa to *Scaphechinus mirabilis* and *Peronella japonica.*

Whole Mount Antibody Staining:

Nuclear expression of ß-catenin, the indicator for the *Wnt* pathway was seen. Previously, it was not know if the Wnt pathway was even activated in the cloning process. There also appears to be a patterning of *Wnt* in the clones, suggesting that although the fluorescing clones do not appear to have a polarity, there is an anterior-posterior nuclear memory in the clones. With this information, along with the fact that clones develop from a gastrula-like stage to pluteus in 24 hours, instead of 72 hours in normally developing larva, it is likely that the cells making up the clone have memory in their nucleus and know how to self-organize.

Future Directions:

Continuing this research to gain greater insight into the expression of *Wnt* in *Dendraster excentricus* clones would be very beneficial to understanding the molecular pathways involved in cloning. I would like to watch the developing clones and perform antibody staining in all stages in order to get a full picture of the *Wnt* pathway's involvement in cloning. As attempts at PCR and sequencing did not yield results, I would like to see the sequence of Wnt8 in *Dendraster excentricus* be completed. *In situ* hybridization and QPCR were also not performed to determine the timeline of stem cells, nerve tissue and gonads in the cloned larvae. Completeing this and probing for stem cells, nerve tissue and gonads in the *In situ* test would also provide good information on the eventual development of the clones.

Acknowledgements

I would like to thank my instructor Dr. Billie J Swalla and Teaching Assistant Elliott Jacobsen-Watts for all their guidance along the way in this project. Thanks to Henry and Holly Wendt Endowment to support FHL research apprenticeships, UW Provost support of FHL apprenticeships and UW Friday Harbor Laboratory. I would also like to thank Dr. Kevin Kocot for his instruction on MEGA. Also, huge thanks to my MGRE and ZooBot classmates, especially Audrey Brezak for the late gremlin nights in lab, Dave Haas for his words of wisdom and help with my gene trees and to Tessa Putz and Chase O'Neil for keeping it real.

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Figures

Figure 1. Alignment data for forward and reverse primers used in PCR for the three echinoid species *Hemicentrotus pulcherrimus* (ACC38467.1), *Heliocidaris erythrogramma* (AY532155.1), and *Strongylocentrotus purpuratus* (AF157389.3).

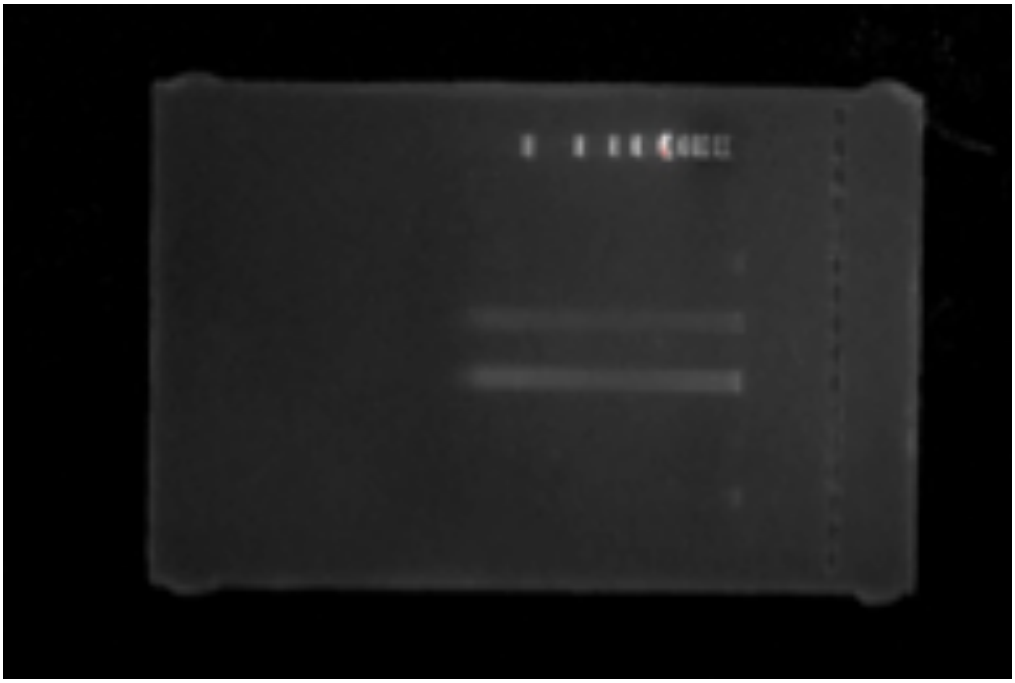

Figure2. Lanes 2 and 3 show quality DNA of *Dendraster*, Lanes 4 and 5 show extraction of S. Droebachiensis, Lanes 6 and 7 show *Pisaster*.

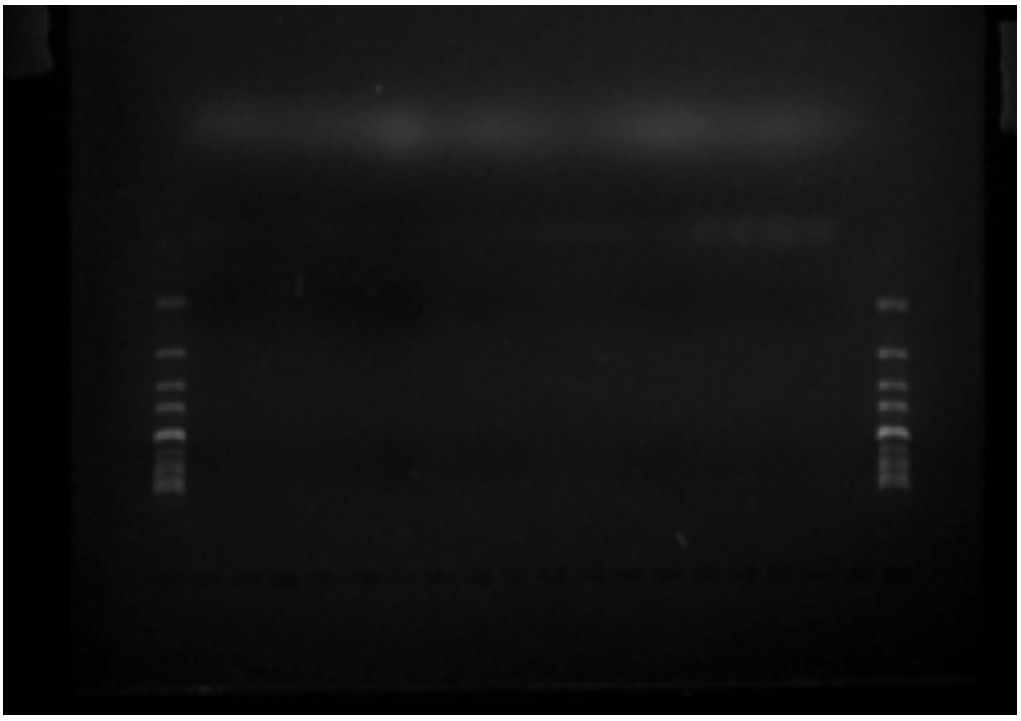

Figure 3. *Wnt 8*: Lanes 2 and 3: *Dendraster*, Lanes 4 and 5 *Droebachiensis*, Lanes 6 and 7 *Pisaster. SoxB*: Lanes 8 and 9: *Dendraster*, Lanes 10 and 11: *Droebachiensis*, Lanes 12 and 13: *Pisaste*r. *18S* (control): Lanes 14 and 15: *Dendraster*, lanes 16 and 17: *Droebachiensis*, lanes 18 and 19: *Pisaster*.

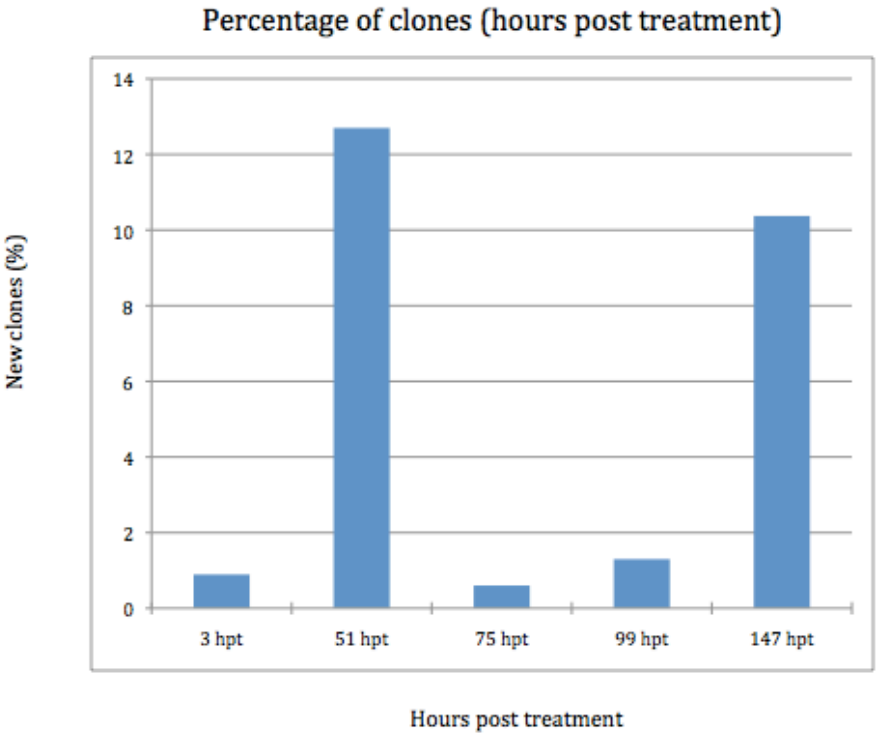

Figure 4. Summary of clone results for preliminary trial.

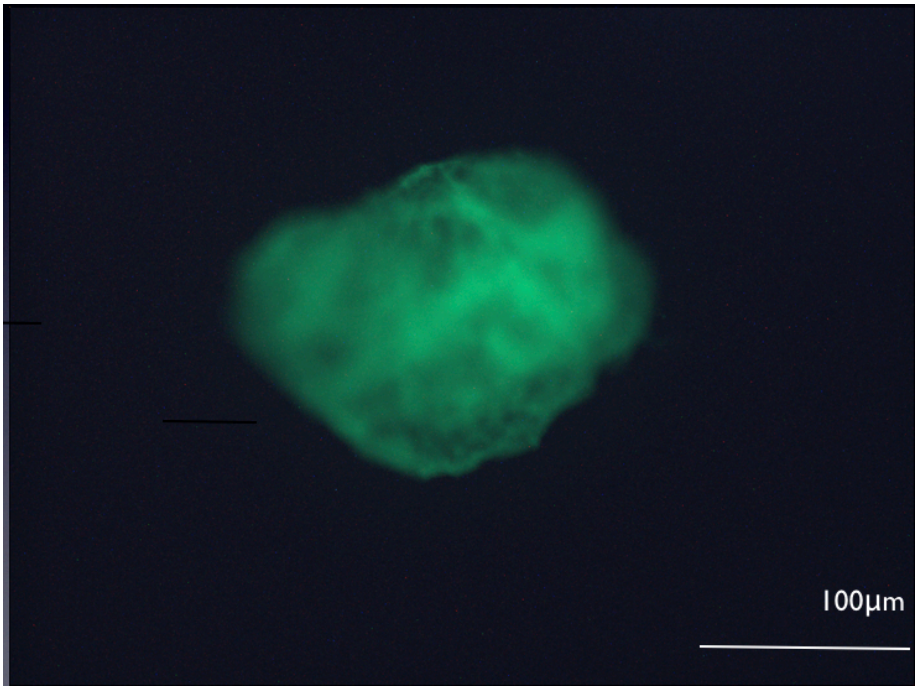

Figure 5: Antibody staining for *Wnt8* expression in *Dendraster excentricus* clone.

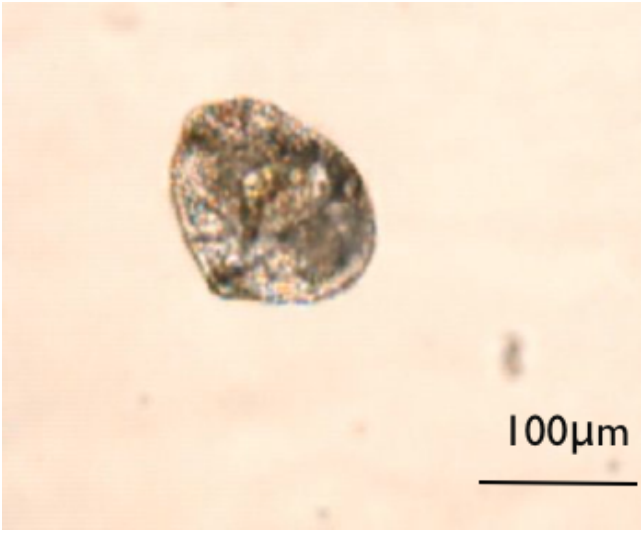

Figure 6: Newly budded off clone in early gastrula-like stage.

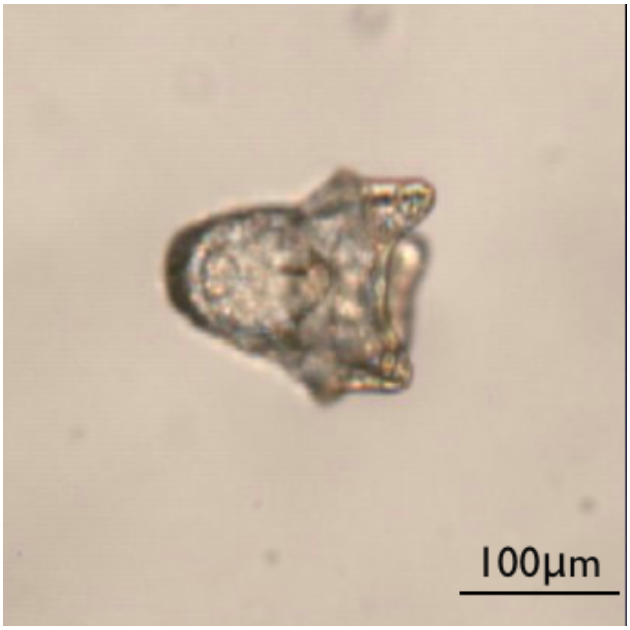

Figure 7: Partially developed clone in early pluteus stage.

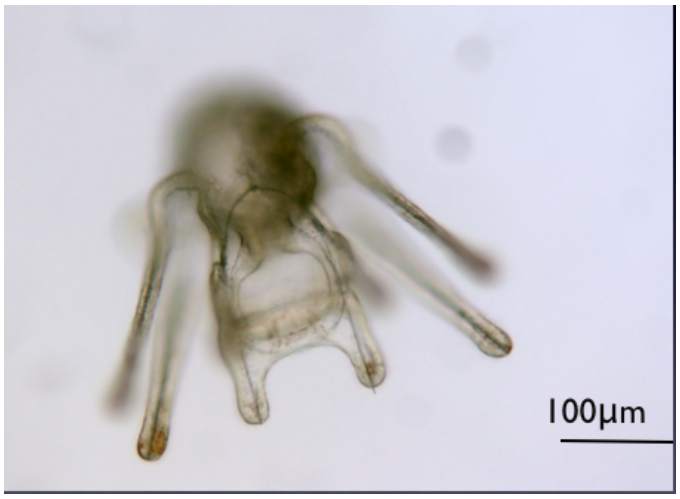

Figure 8: Normal *Dendraster excentricus* larva.

Gene Tree of *Wnt8*

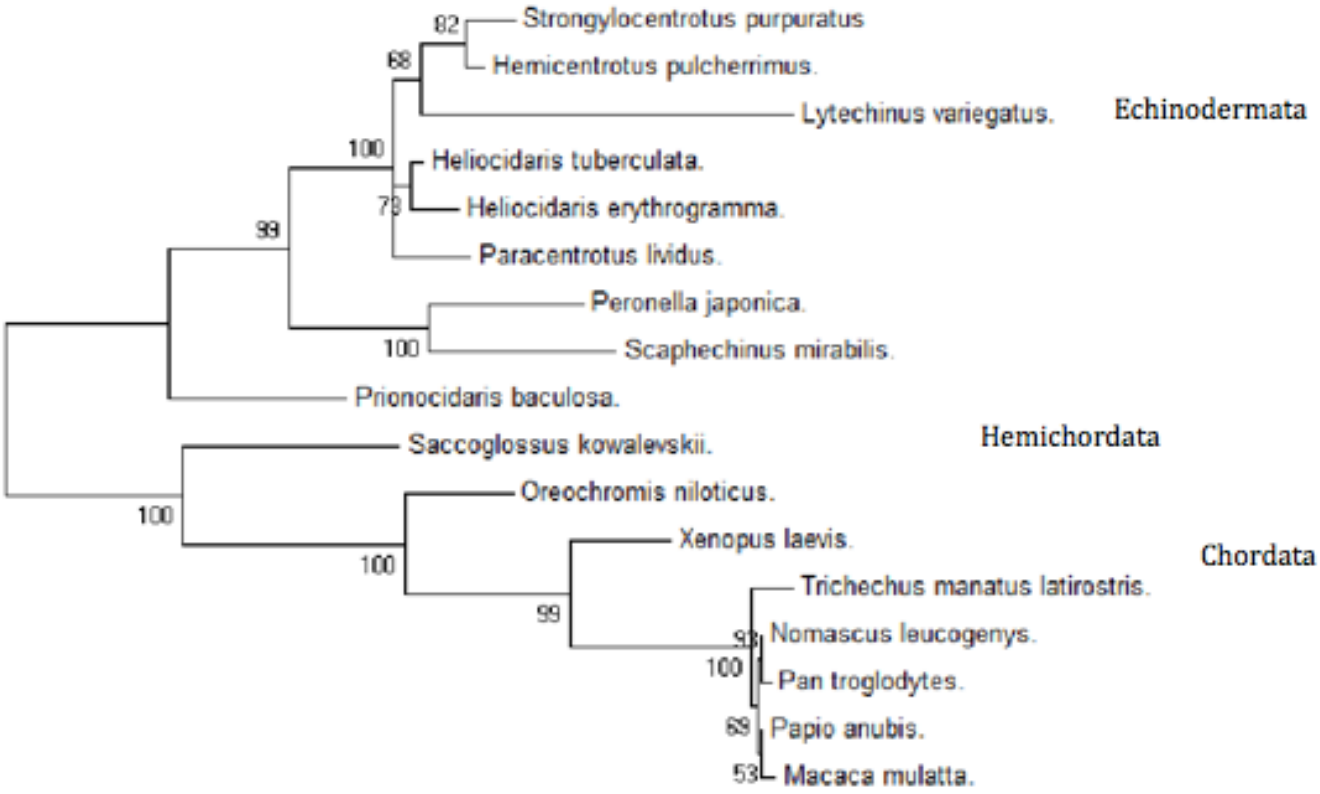

Figure 9: *Wnt8* gene tree. Tree constructed using Maximum Likelihood through MEGA 5.1.

Summary:	Starting larvae	Dead (final)	Clones	% Cloned
Plate A	68	32	6	8.823529412
Plate B	59	23	17	28.81355932
Plate C	67	14	20	29.85074627
Plate D	30	4	0	0
Total (treated)	194	69	43	22.16494845

Table 1.1 Results summary of Preliminary Cloning Trial after 10 days. See Supplementary Figures Tables 1.2-1.6 for more information.

Supplementary Figures

Day 1:	Average size (um)	Alive	Dead	Clones	% Cloned
Plate A	271.16	68	0	0	0
Plate B	298.3	59	0	0	0
Plate C	288.3	67	0	2	0.029850746
Plate D (Control)	332	30	0	0	0
Total:	297.44	224	0	2	0.892857143

Table 1.2 Preliminary Cloning Trial Day 1.

Day 3:	Alive	Dead	Clones	% Cloned
Plate A	62	2	4	6.451612903
Plate B	60	0	12	20
Plate C	67	0	8	11.94029851
Total:	189	2	24	12.6984127

Table 1.3 Preliminary Cloning Trial Day 3.

Day 4:	Alive	Dead	Clones	% Cloned
Plate A	51	16	1	1.960784314
Plate B	51	6	0	0
Plate C	64	8	0	0
Total:	166	30	1	0.602409639

Table 1.4 Preliminary Cloning Trial Day 4.

Day 5:	Alive	Dead	Clones	% Cloned
Plate A	60	2	0	0
Plate B	39	11	0	0
Plate C	55	4	2	3.636363636
Plate D (Control)	27	2	0	0
Total (treated):	154	17	2	1.298701299

Table 1.5. Preliminary Cloning Trial Day 5.

Day 7:	Alive	Dead	Clones	% Cloned
Plate A	49	12	1	2.040816327
Plate B	33	6	5	15.15151515
Plate C	53	2	8	15.09433962
Plate D (control)	25	2	0	0
Total (treated)	135	22	14	10.37037037

Table 1.6. Preliminary Cloning Trial Day 7.