

Attempted Cloning of a *Wnt* gene from *Botrylloides violaceus*

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Introduction

Botrylloides violaceus is a sessile, colonial ascidian, also called a “sea squirt” for their filter-feeding behavior. *Botrylloides* along with other ascidians are grouped into a category known as tunicates. Tunicates are organisms that form a thin membranous tissue layer containing cellulose, which covers their bodies, a feature rarely found in the animal kingdom. Mobile, tadpole-like larvae are formed through sexual reproduction and establish new colonies by settling and attaching to hard, open surfaces. Newly established colonies expand through asexual reproduction, beginning with settled larvae (or oozoid). New but genetically identical members of the colony (zooids) are added through the asexual reproductive process called palleal budding (Oka and Watanabe, 1957 & 1959), characterized by the formation of walled vesicles, or buds, from the side of the parent organism. The new organism begins as a growth on the side of the adult, and as the palleal bud continues to grow, development includes differentiation of tissues to develop different organs. These organs include a simple neural cord and brain, gonads, a pharynx, endostyle, esophagus, stomach, and cardiac tissue (Abbot and Newberry, 1980; Berrill 1947). Fully developed adults also have an incurrent siphon used to pull water into its branchial basket (which leads to the esophagus) in order to filter feed. Colonial species like *Botrylloides violaceus* share atrial siphons through which to release excess water and waste (Berrill 1947).

Botrylloides violaceus have come to be an excellent model organism as it is small, grows rapidly, and is readily available, which are crucial in terms of housing these organisms. Tractability, the ease at which they can be manipulated, is also another useful attribute. In past experiments, the family *Botryllus* (a close, tunicate relative of

Botrylloides) has been collected in Vostok Bay and pieces of the colony were fixed in Bouin fluid, dehydrated, and embedded in paraffin for histological study (Akhmadieva, A. V. et al, 2007). Also, other tunicates, such as *Polyandrocarpa misakiensis* (another related tunicate), have been shown to be attach to glass plates and reared in the bay near the Shimoda Marine Research Center at the University of Tsukuba, Japan (Mukai, H. et al, 1990). Similarly, Joly, J. S. et al, 2007 improved culture methods of *Ciona intestinalis*. In the experiment, multiple factors are taken into account including seawater, temperature, food, light, shade, and contamination of unfavorable animals, animal collection and reproduction (Joly, J. S. et al, 2007).

Botrylloides and other related organisms are used in a variety of biological fields including immunology, physiology, ecology, histology, developmental biology, evolutionary biology, and aging (Epelbaum et al. 2009 and references therein). One study showed how they compared vasa expression in a solitary ascidian and a closely related colonial ascidian, in an effort to uncover developmental mechanisms important during the evolution of these contrasting life histories, including the ability to reproduce by budding (Brown, F. D. et al. 2007). Another study showed attention directed towards the introduction of colonial ascidians in coastal and estuarine areas in the Gulf of Maine. The study focused on the invasion of the Gulf of Maine by the colonial ascidians *Botryllus schlosseri*, *Botrylloides violaceus*, *Diplosoma listerianum*, and *Didemnum* species. The goal of the study was to document the arrival, changes in abundance and space occupied throughout the year to compare seasonal variability among the four ascidian species (Dijkstra. J. A. et al. 2005).

The development and regeneration of *Botrylloides* is especially interesting because it is an invertebrate chordate and therefore closely related to vertebrates. Moreover, it is a tunicate, which was found to be the closest living relatives of vertebrates rather than cephalochordates as originally thought (Delsuc, F. et al. 2006). The ability to regenerate interests the scientific community as it poses the possibility to harness such ability for medical benefit.

In a study done in 2009, colonial ascidians were shown to be the only chordates to undergo whole body regeneration (WBR), the ability to form an entirely new individual from the peripheral vasculature. The study reports the differing presence of Piwi-positive, a class of genes originally identified as encoding regulatory proteins responsible for maintaining incomplete differentiation in stem cells and maintaining the stability of cell division rates in germ line cells that are highly conserved across evolutionary lineages and are present in both plants and animals, and proliferating cell nuclear antigen (PCNA)-positive cells in circulatory hemocytes in late regenerates and in differentiating tissues during vascular budding. PCNA and Piwi weren't regularly found in differentiating tissues during vascular budding, which suggested tissues formed during budding and WBR were mostly from circulatory hemocyte precursors. They proposed that multiple stem cell types are circulating within *Botrylloides violaceus* and that they undergo proliferation in the peripheral vasculature before differentiating into epithelial tissues for all three germ layers during WBR. In summary, the authors provided a reliable colonial ascidian system to study WBR that are positive for at least one stem cell maintenance marker, have the ability to proliferate, and likely contribute to the formation of tissues during regeneration. (Brown, F. D. et al. 2009)

Solitary ascidians such as *Ciona* have been studied much more thoroughly, including full genome sequences of two *Ciona* species (Dehal et al. 2002). Gene expression (Hamada, M. et al 2007), embryonic development (Kumano, G., Nishida, H. 2007), notochord morphogenesis (Jiang, D., Smith, W. C. 2007), culturing (Joly, J. S. et al 2007) and proteomics (Inaba, K. et al. 2007) of *Ciona* have also been explored. With so much research done on *Ciona* and its close relation with *Botrylloides*, it serves as a great reference organism because *Ciona* does not have the regeneration ability of colonial ascidians. The adult body plan is relatively unstudied at a molecular level, but several examples of extensive regeneration following surgical ablation of different tissues have been described. In addition, within this chordate subphylum two distinct adult body plans exist: solitary (e.g. *Ciona*) and colonial (e.g. *Botrylloides*). Following larval metamorphosis, solitary species grow into an adult that can range from several millimeters to tens of centimeters in length. In addition, colonial species grow not by increasing in size, but by asexually propagating, eventually creating a colony of genetically identical individuals that can cover areas of several square meters. Thus colonial ascidians (e.g. *Botrylloides*) have two independent developmental pathways to create an identical adult body plan, and are the only chordates with this regenerative ability. Taken together, the ascidians represent an excellent system to study the biology of both embryonic and adult stem cells.

Further studies on development and regeneration of *Botrylloides* will require optimized laboratory culture conditions, since variables such as food availability, degree of water filtration, and presence/absence of aeration can be important for survival and developmental rates. One study has been done to test some of these variables for the

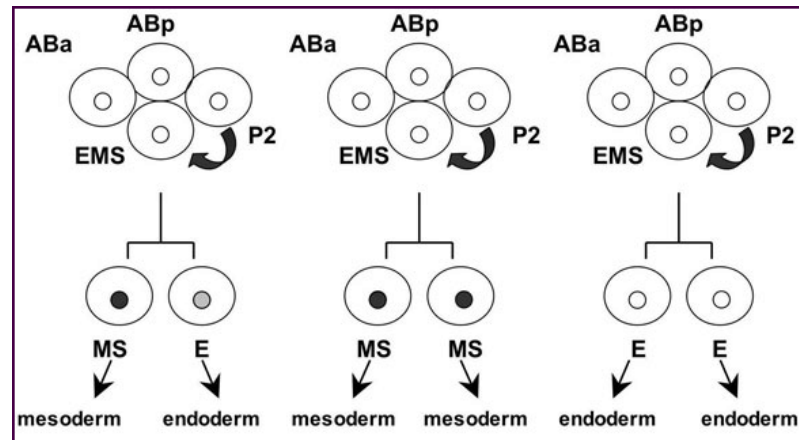
related species *Ciona intestinalis*. Joly, J. S. et al. 2007 discuss the effects of several closed-system conditions on the growth and survival of the solitary ascidian, *Ciona intestinalis*. A small-tank system was used to ensure that tanks and systems were reasonably equipped, water exchange was rapid, and animals separated to minimize the risk of infection. They also tried to determine the optimal conditions to limit handling operations, and to save artificial seawater by avoiding water pollution. A mixture of at least two types of live algae was found to be better than any single-organism diet. With these maintenance protocols, they were able to obtain several generations of *Ciona intestinalis* making it easier to rear *Ciona intestinalis* in laboratories and increase the potentialities of this model organism for research.

Maintenance of healthy laboratory cultures makes it possible to conduct long-term experiments and study seasonal variations in colony availability, growth rate, and sexual reproduction. Laboratory cultures also make it possible to directly and accurately assess the effects of various physical, chemical, and biological factors on colony survivorship, growth, and reproduction. (Epelbaum et al. 2009) Most studies of *Botrylloides* focused on specific experimental aspects and described culture methods only briefly.

Boyd et al. (1986) provided the most detailed description of laboratory methods and conditions for culturing colonies of *B. schlosseri*, but knowledge gaps still exist. (Epelbaum et al. 2009) The purpose of this investigation is to optimize and summarize some techniques for culturing *Botrylloides violaceus* and perhaps *Botryllid* tunicates in general in controlled lab conditions to be used for future experiments. With the success of culturing *Ciona intestinalis* in closed-systems and its close relation to *Botrylloides violaceus*, we hope to similarly optimize culturing techniques for this particular species.

In order to understand the processes of development and regeneration, it is important to identify key molecules involved and study their roles. One candidate group of signaling proteins is the *Wnt* family. Interestingly, the *Wnt* gene was discovered as two different genes in two different organisms. According to Rijsewijk, F., et al. 1987, the gene, *Int* was first discovered as an oncogene in *Mus musculus*. Previously, the gene *Wingless*, was found in *Drosophila* and served as a segment polarity gene (Nusslein-Volhard, C. Weischaus, E. 1980). Rijsewijk, F., et al 1987, performed an alignment of *Int* and *Wingless* and found them to be homologous. Hence they constituted this gene as one and we know it as *Wnt*. Sometimes this gene is also referred to as “wingless-type MMTV integration site family.” This stands for wingless type mouse mammary tumor virus integration site family, a way to show that this gene existed in two organisms with two functions.

Wnt has been known to have two pathways, canonical and non-canonical. According to Eisenmann DM, 2005, the canonical pathway exists when the *Wnt* signaling protein interacts with *Frizzled* receptors to trigger the B-catenin pathway. In *C.elegans* the canonical *Wnt-B-Catenin* pathway has been attributed to play a role in gene specification especially in Anterior/Posterior axis specification. (Eisenmann, DM, 2005). *Wnt* proteins not interacting with B-catenin (non-canonical pathway) are best known in embryonic development.

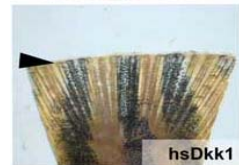


On left, normal embryonic development is shown. The arrow from P2 to EMS indicates the *Wnt* gene interaction. The EMS cell polarizes into both mesoderm and endoderm. The second two pathways are mutations in the non-canonical *Wnt* pathway. (Eisenmann, DM, 2005).

During mesoderm/endoderm formation, at the 4-cell embryo *Wnt* is secreted by the P2 blastomeres to the EMS blastomere and polarizes the EMS cell to become endoderm and mesoderm (Eisenmann, DM, 2005). Mutations in the *Wnt*'s non-canonical pathway will cause the EMS cell to divide into both mesoderm cells or both endoderm cells.

Currently *Wnt* has been annotated in Mice, Humans, *Xenopus*, Chicken, Zebrafish, *Drosophila*, *C. elegans*, *Ciona*, and *Dictyostelium*. *Wnt* proteins are highly conserved in evolution; there are 13 subfamilies containing 19 *wnt* genes in humans. (Guder et al. 2006). Twelve shared gene families exist in vertebrates, 11 are found in *cnidarians* (*Hydra* and sea anemones) (Carrol, SB 2008). Carrol, 2008 deems it as one of the genes that must have been highly conserved through all genetic diversification, otherwise known as a toolkit gene. Our study relies on this principle, because if it is present in *cnidarians*, an ancestral organism and also present in modern mammals such as mice and humans, then it must be conserved in the chordate lineage as well.

The regenerative potential held by *Wnt* and their related families are of particular interest to us. Using the model organism, *Danio rerio* (Zebrafish), which possesses 15 *Wnt* genes, Ueno, S. 2007, was able to track the regenerative capabilities of Zebrafish fins post ablation. When they cut the ends of the zebrafish fins, they noticed that the fin would form a blastema, or a mass of undifferentiated tissue. The fin would fully regenerate within a few days. When they cut the fins and supplemented the fish's diet with *hsDkk1*, an inhibitor of B-catenin, blastema formation did not occur and the fins wouldn't regenerate (Ueno, S. 2007). Because B-catenin interaction occurs through the canonical pathway, Ueno, S. 2007 have hypothesized that through the canonical pathway, *Wnt* plays a large role in regeneration.



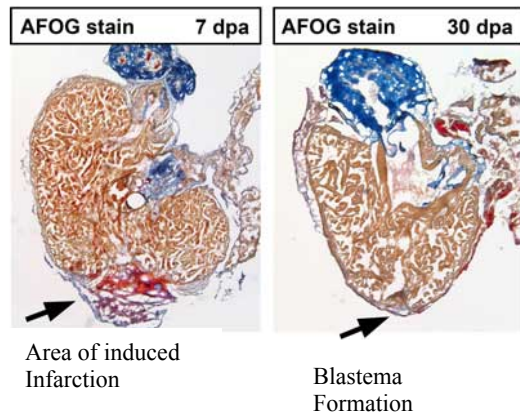
hsDKK=β-Catenin Inhibitor

Fin Regeneration:

- Arrow indicates point of ablation
- Wnt pathway is necessary to form the blastema
 - Blastema-contains stem cell progenitors
- Inhibition of β-Catenin prevents blastema formation

Ueno, S. 2007 has also observed a unique property in Zebrafish hearts. After a myocardial infarction, zebrafish hearts have regenerated to 100% capacity. In the lab, they induced an infarction in zebrafish hearts and removed the damaged tissues. After an infarction typically scar tissue forms around the damaged areas. However these hearts successfully formed a blastema and regenerated fully in 30 days without any scar formation. Most importantly, after the induced infarction Ueno, S. 2007 found high

expression of *Wnt* and B-catenin.



Heart Regeneration:

- Arrow indicates point of infarction
 - Normally; scarring
 - In zebrafish-Heart regenerates in ~ 30 days. And no Scar
 - Virtually complete regeneration
 - Only works if <20% of the heart is removed
 - **Found high expression of *Wnt* and β -Catenin early after induced infarction**

The involvement of *Wnt* in such important developmental processes such as segment polarization, regeneration and differentiation have led us to believe that this gene is key in regulating the regenerative potential in *Botrylloides*.

In our attempts to target a *Wnt* gene from *Botrylloides* we first had to utilize the bioinformatic resources on the internet. We had to learn and understand these resources to correctly process and understand these resources because *Botrylloides* has not been sequenced.

Bioinformatics is a modern approach to science that blends the worlds of computational analysis and biology. The purpose of this technology is to radically change the way researchers perform their experiments. These advances not only store large amounts of data, from whole genomes to single intron sequences, it can also be applied towards preventing, diagnosing and treating diseases, especially inheritable diseases. Research labs now have the power to analyze numerous genomes in a blink of an eye,

and analyze biological knowledge, from evolution to medicine development. We harness this power in our project to try and obtain primers for an un-sequenced organism.

Using the advances in bioinformatics, we are able to investigate the *Wnt* gene and its involvement not only in *Botrylloides*, but in other organisms as well. Using databases such as the National Center for Biotechnology Information (NCBI) we were able to search out organisms that would also share the *Wnt* gene, as it would reveal conservation across the species line. In our alignment we searched out organisms that belonged to the vertebrate, cephalochordates, echinoderms, cnidarians, and the chordate families. To perform an alignment, the NCBI database based BLAST, or Basic Local Alignment Search Tool (Shown below).

The theory of alignment that BLAST is based upon is aligning small segments of genomic sequences to the larger genome. Using the metaphor of words, the book would represent the genome while the individual searches would be words or sentences. Nucleotide alignments are simple in principle. One would be able to align one sequence with another, and based on how much the sequences match indicate a good alignment, much like below, with words reprimanded and demanded and mandated

R	E	P	R	E	M	A	N	D	E	D			
			D	E	M	A	N	D	E	D			
					M	A	N	D	A	T	E	D	

It's easy to see that this alignment is very close between the first two words, except or the first three letters. The last word, mandated, does not match as well as the other two, as only a few letters are matching. Now if a penalty is introduced, it will weed out which words are better than the others. If we were to use a simple +1/-1 score, +1

meaning there is a positive match and -1 if there is no match we can see the results

between each word

	R	E	P	R	E	M	A	N	D	E	D
				D	E	M	A	N	D	E	D
	0	0	0	-1	+1	+1	+1	+1	+1	+1	+1
Total score =	6										

	R	E	P	R	E	M	A	N	D	E	D		
						M	A	N	D	A	T	E	D
	0	0	0	0	0	+1	+1	+1	+1	-1	-1	0	0
Total score =	2												

This is the basis of BLAST alignment; those with larger scores are considered better alignments. Although this example was staged we can perform the same analysis with small genomic sequences such as with the human *Wnt 4* gene with a segment from *Drosophila Wnt* and a mouse *Wnt 4* gene.

Human *Wnt 4* (NG_008974.1) : TTGCCAAATCTCCCATTTCTCA
Drosophila Wnt 4 (NM_057624.2): AGTCGAGTGCCGAACGAGCTG
 Mouse *Wnt 4* (NM_009523.1): CGGGAGCCTTGCGGCCGCTGC

Alignment of human and *Drosophila*
 TTGCCAAATCTCCCATTTCTCA
 AGTCGAGTGCCGAACGAGCTG

Alignment of *Drosophila* and mouse
 AGTCGAGTGCCGAACGAGCTG
 CGGGAGCCTTGCGGCCGCTGC

-1-1-1+1-1+1-1-1-1+1-1-1-1-1-1-1-1-1-1+1-1-1
 Total Score= +3

-1+1-1-1-1-1-1-1-1-1-1-1-1-1-1+1-1-1-1-1-1
 Total Score=2

Alignment of Human and Mouse

TTGCCAAATCTCCCATTTCTCA
 CGGGAGCCTTGCGGCCGCTGC

-1-1+1-1-1-1-1-1-1+1-1-1-1-1-1-1-1-1-1+1-1-1
 Total Score= +3

*Scores are normalized to not go below zero, and positive matches are scored.

With this simple alignment, one could say that human and mice are more similar than *Drosophila* and mouse sequences. However much longer sequences are used in sequences and compared so scores are usually more significantly different.

In reality, these sequences would contain long stretches of nucleotide sequences that contains gaps, that are designed to compensate for plausible insertions or deletions in a sequence. These gaps are designated as ----. So a very non-specific alignment would look like this:

```
TTGCGCCAAAGTCCGATTACTAAACA
TTGC----CAAATCTCCCATTCT----CA
```

Although there are mismatching pairs, there are more matching pairs when gaps are included. While these +1/-1 scores are easy to compare and analyze, modern computer alignments use a +1/-3 scoring, where +1 is or a positive match and -3 for a mismatch.

These modern alignments also introduced Gap penalties which penalize the overall score if a gap is introduced and a further penalty if that gap is needed to continue. These gap penalties are penalized and subtracted from the alignment score highly because it is changing how the sequence is aligned. More gaps means more manipulation of the sequence to make it fit, hence increasing the likelihood that these sequences do not match (lower score value). So BLAST uses the calculation:

$$G + Ln$$

Where G= Gap introducing penalty
L=Gap extension penalty
N=length of gap.

The G and L penalties vary on how stringent the alignment needs to be. Typically BLAST will use 10-15 as G, and 2 for the L. So a sequence that has three gaps,

each 3 nucleotides long, means a $1(10)+2(3)=16(x\ 3)$ which gives. Whatever the resulting alignment score is, these gaps decrease the score by 48 points.

The nucleotide alignment is quite simple because there are only a few changes that can happen, and it is easy to score those mis-matches, however protein alignments are more complicated, because of the degeneracy of the codons.

Serine codons, AGU or AGC
Glycine codons: GGU or GGC
Arginine codons: AGG or AGA

Unlike the nucleotides sequences, amino acid alignments will be harder to score as there are a lot more changes that can occur to produce the same amino acid. However this makes it easier for us to find homology between sequences, because natural selection only affects the phenotype, in this case the protein structure. Synonymous mutations should end up neutral as they do not affect the phenotype. These changes can be scored, however but requires penalties based on the number of changes needed to go from amino acid to amino acid. For example the change from serine to glycine is only one nucleotide in each case (the A to G change). Glycine to Arginine requires two changes in both cases. Therefore the likelihood of serine changing to glycine is more likely than glycine changing to arginine.

There are also more conserved amino acids in biology, some that have R side chains that are more complicated and serve a more specific purpose. For example, the amino acid tryptophan has the single codon, UGG. Tryptophan has no alternate codon sequence, meaning any type of mutation will cause a change in amino acid. If the last codon was point mutated, the sequence would either create a cysteine, or a stop codon. However looking at something like alanine, having the codons, GCU, GCC, GCA, or

GCG, the chance of the last base pair change creating a new amino acid is dramatically decreased, virtually impossible. In this case, a change in tryptophan is valued higher than alanine. Biochemically this makes sense-alanine only possesses a methyl R group while tryptophan possesses a complicated R side chain, likely involved in higher levels of protein structure

One of the first methods of scoring was done by Dayoff et al in 1978. They based it on substitution probability. They called this method, PAM or Percent Accepted Mutation. This type of substitution scoring was based upon the fact that each change in a sequence is independent of previous states. In scoring, they counted the number of changes in each amino acid and divided by a subjective 'normalization factor' or what they called exposure to mutations. In their analysis, 1PAM was supposed to represent 1% change in a sequence, one amino change per 100 amino acids. These matrices could be expanded to PAM50 or PAM 200. This type of substitution model doesn't take into account biochemistry so a new type of scoring was developed.

In 1992, Henikoff and Henikoff came up with what they called Blocks Amino Acid Substitution Matrix (BLOSUM). This method is not based on phylogenetic trees, but does take into biochemistry. They looked at the protein families and identified conserved motifs in the sequence. Using computer software they were able to analyze the pattern of amino acids in these motifs. Protein families were created and defined by the presence of such a motif in every member of the family. They pulled what they called blocks from these organisms, which consisted of the conserved motifs and the nearby regions. Cleverly they aligned these sequences without introducing gaps. Because the motifs were assumed to be present in all organisms, they were assumed to be homologous. They then

counted the number of substitutions within each of the amino acids and these scores were the basis of scoring. To control for substitutions in closely related proteins, they aligned sequences that were at least 60% similar, and we know this method as BLOSUM60 (seen below). More stringent scoring can be used, such as with BLOSUM80 or BLOSUM90.

Ala	4																						
Arg	-1	5																					
Asn	-2	0	6																				
Asp	-2	-2	1	6																			
Cys	0	-3	-3	-3	9																		
Gln	-1	1	0	0	-3	5																	
Glu	-1	0	0	2	-4	2	5																
Gly	0	-2	0	-1	-3	-2	-2	6															
His	-2	0	1	-1	-3	0	0	-2	8														
Ile	-1	-3	-3	-3	-1	-3	-3	-4	-3	4													
Leu	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4												
Lys	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5											
Met	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5										
Phe	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6									
Pro	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7								
Ser	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4							
Thr	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5						
Trp	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11					
Tyr	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7				
Val	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4			
	Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val			

As many types of scoring can exist, Lobo, I (2008) have created an example of how a matrix can be made. For example, aligning the words Pelican and Coelacanth can be used as a model matrix. The word Coelacanth is placed on top of a grid with the word Pelican placed vertically down the left side as seen below.

	C	O	E	L	A	C	A	N	T	H
P										
E										
L										
I										
C										
A										
N										

The grid corresponds to each letter and adds in other columns to accommodate for the extra letters in coelacanth. The next step is to assign a score based on this alignment. In this case the perfect alignment would be a diagonal line down from the top left down to bottom right. This alignment is a 'forced' alignment in order to serve a purpose. Coelacanth and Pelican could easily be aligned by aligning the 'E.' However as we will see later on, the method of scoring comes to this same conclusion.

Using this assumption, scores would start at zero on the top left and get progressively less as its read to the right, or down. Anytime the sequence deviates from this diagonal, it is penalized. Essentially, every letter in the alignment is given a score based on its position from 'zero.' So a simple scoring method would look like this:

	C	O	E	L	A	C	A	N	T	H	
	0	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10
P	↑										
E	↑										
L	↑										
I	↑										
C	↑										
A	↑										
N	↑										

The next step in this alignment is to fill in the rest of the table. To start the analysis, the letters C and P are aligned and scored. The scoring is based upon probability. The method uses first the alignment from the C and the P, $-1 + -1$ which is -2 . Like stated before, the perfect forced alignment exists in a diagonal from the top left to the bottom right. To force that alignment they made the scores of each of the letters in their respective alignment to represent that score. This means, the box of alignment of C and P is -1 (the same for both C and P). The -2 score is compared to the negative -1 and the higher score is left there. The same procedure is performed throughout the alignment and the ideal alignment is shown by the direction of the arrows:

	C	O	E	L	A	C	A	N	T	H	
P	0	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10
E	-1	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10
L	-2	-2	-2	-1	-0	-3	-4	-5	-6	-7	-8
I	-3	-3	-3	-2	-2	-1	-2	-3	-4	-5	-6
C	-4	-4	-4	-3	-1	-1	-2	-1	-4	-5	-6
A	-5	-3	-4	-4	-2	-2	-0	-1	-2	-3	-4
N	-6	-4	-4	-5	-3	-1	-1	-1	-0	-1	-2
	-7	-5	-5	-5	-4	-2	-2	-0	-2	-1	-0

As with the nucleotide alignment the highest score has the most similar sequence, hence is the one with the least penalties. As said previously the perfect alignment exists in the diagonal in the top left to the bottom right, and it is clearly seen below.

	C	O	E	L	A	C	A	N	T	H	
P	0	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10
E	-1	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10
L	-2	-2	-2	-1	-0	-3	-4	-5	-6	-7	-8
I	-3	-3	-3	-2	-2	-1	-2	-3	-4	-5	-6
C	-4	-4	-4	-3	-1	-1	-2	-1	-4	-5	-6
A	-5	-3	-4	-4	-2	-2	-0	-1	-2	-3	-4
N	-6	-4	-4	-5	-3	-1	-1	-1	-0	-1	-2
	-7	-5	-5	-5	-4	-2	-2	-0	-2	-1	-0

The blue boxes are telling where the lowest score exists and as one could tell the blue boxes have shifted to the right, basically telling us to align the C of coelacanth with nothing. P in Pelican would be aligned with O and then the best alignment would be seen.

Instead of doing these alignments by hand, computer programs can integrate all these algorithms and principles while analyzing large sequences.

The traditional way to use BLAST is to input the desired genomic sequence into the tool and the tool will search the genome database of choice. For example, one would be able to investigate whether or not the sequence ACGGTCCACGTA existed in the fly genome.

The input sequence looks similar to this:

BLAST *Drosophila melanogaster* Sequences.

Enter an accession, gi, or a sequence in FASTA format:

ACGGTCCACGTA

Or, choose a file to upload

Choose File No file chosen

Set subsequence: (optional)

From: To:

Database:

genome (reference only) 6 sequences

Program:

megaBLAST: Compare highly related nucleotide sequences

Optional parameters

Expect	Filter	Descriptions	Alignments
0.01	default	100	100

Advanced options:

Begin Search Clear Input

The accession input box is where the query is added in. BLAST provides the option of using an accession number, gi, or simply a sequence of nucleotides will do.

For example for the Wnt 4 gene in *Homo sapiens* you have the choice of entering the following:

Homo sapiens wingless-type MMTV integration site family, member 4 (WNT4) on chromosome 1

[Comment](#) [Features](#) [Sequence](#)

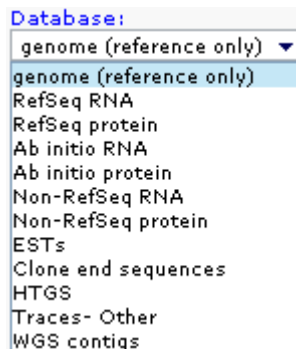
LOCUS NG_008974 32722 bp DNA linear PRI 26-JUL-2009
 DEFINITION Homo sapiens wingless-type MMTV integration site family, member 4
 (WNT4) on chromosome 1.
 ACCESSION NG_008974
 VERSION NG_008974.1 GI:212549725
 KEYWORDS RefSeqGene.
 SOURCE Homo sapiens (human)

1. Accession Number: NG_008974
2. GI: GI:212549725
3. Entire Sequence (in FASTA* format, the “>” indicates where the sequence begins)

>ttgcaaattctcccatttctcaagagaagctggagatctagcttctcatg tgaatctcc

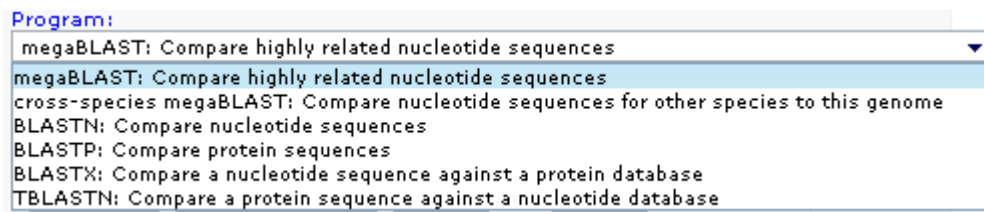
*FASTA= “FAST-Any sequence. Also Available, FASTP (protein), FASTN (Nucleotide)

Underneath the query box is a option to search not just genome databases by other databases as well.



- RefSeq (reference sequence) - contains non redundant set of sequences, including genomic DNA, transcripts and proteins. It is best used during gene identification and characterization, as well as expression studies, in this case one could search either RNA or protein collections
- EST (expressed sequence tags) – contains short sequences of cDNA. These are designed to be a very rapid analysis of what is being expressed in the organism. These are best used if one would like to quickly analyze what is being expressed at the time.
- WGS (Whole genome shotgun) – contains sequences produced by the shotgun analysis, as done by the Celera corporation. These sequences are not necessarily completely contiguous however.
- Traces- Other- refers to sequences that aren't WGS or ESTs.

Below the database section is 'program.' Typically one would compare a nucleotide sequence to the huge genome database. Below is shown the options one could search for.



BLAST as a default performs the search alignment using these details;

Expect: 0.01 Filter: default Descriptions:100 Alignment: 100

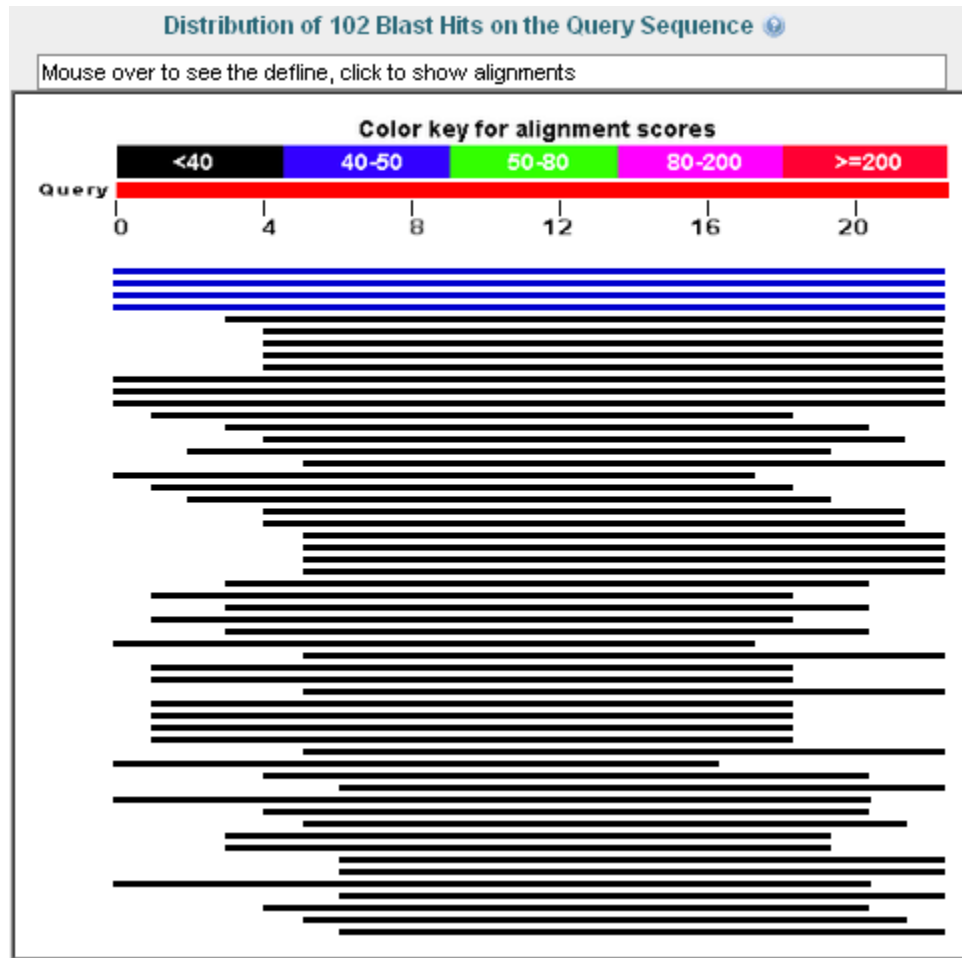
These will give a general alignment but if you wish to be more specific you can change the values for these scores, and descriptions for these scores are provided below, according to the Human Genome project resource page.

Expect score indicates how closely the desired matching sequence is to your sequence. Selecting scores that are zero or close to zero is equivalent to choosing sequences that are not random, or significantly different. This value is also equivalent to probability scores. In other words the score indicates how likely the alignment pair is occurring randomly.

Filter options allow one to select for regions that contain areas that contain a complex set of nucleotides vs a simple set. For example, the sequence CCCC is less complex than the sequence CTGTCA. Filtering low-complexity sequences will mean that the more complex sequences such as GTGTCA will be allowed through sequences like CCCC will not be used.

Descriptions indicate how many matches one can observe, while Alignments restricts how much data is shown.

Once the desired settings are used, this is the output, using an alignment of human *Wnt4* as a model:



The lines correspond to the areas of matching sequences matched to the query sequence. The sequences in blue are showing an exact match while the others only match at certain locations. Looking at more detail, we can see what the blue lines correspond to.

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NG_008974.1	Homo sapiens wingless-type MMTV integration site family,	44.1	44.1	100%	0.005	100%
AF335591.1	Homo sapiens WNT4 (WNT4) gene, partial cds	44.1	44.1	100%	0.005	100%
AL445253.13	Human DNA sequence from clone RP4-660K3 on chromos	44.1	44.1	100%	0.005	100%
AY033056.1	Homo sapiens secreted glycoprotein Wnt4 (WNT4) gene, pr	44.1	44.1	100%	0.005	100%
AL160273.9	Human DNA sequence from clone RP11-366I5 on chromos	38.2	38.2	86%	0.29	100%
FJ362362.1	Caenorhabditis brenneri contig JD10, partial sequence	36.2	36.2	81%	1.1	100%
AC008415.6	Homo sapiens chromosome 5 clone CTC-283O18, comple	36.2	36.2	81%	1.1	100%
AC092440.5	Homo sapiens BAC clone RP11-453O5 from 4, complete s	36.2	36.2	81%	1.1	100%
AC080089.5	Homo sapiens BAC clone RP11-785J10 from 4, complete s	36.2	36.2	81%	1.1	100%
BS000062.1	Pan troglodytes chromosome 22 clone:PTB-045C17, map	36.2	36.2	100%	1.1	95%
AP001679.1	Homo sapiens genomic DNA, chromosome 21q, section 23	36.2	36.2	100%	1.1	95%
AP001251.1	Homo sapiens genomic DNA, chromosome 21q21.1-q21.2,	36.2	36.2	100%	1.1	95%
XM_002429589.1	Pediculus humanus corporis microtubule associated protei	34.2	34.2	77%	4.5	100%
AC189407.2	Brassica rapa subsp. pekinensis clone KBrB059G16, comp	34.2	34.2	77%	4.5	100%
AC232504.1	Brassica rapa subsp. pekinensis clone KBrB057N22, comp	34.2	34.2	77%	4.5	100%
XM_001949851.1	PREDICTED: Acyrthosiphon pisum similar to Tripeptidyl-p	34.2	34.2	77%	4.5	100%
CU633157.2	PTB-088N14, complete sequence	34.2	34.2	77%	4.5	100%
AC196783.3	MACACA MULATTA BAC clone CH250-311C18 from chr	34.2	34.2	77%	4.5	100%
CR786577.16	Zebrafish DNA sequence from clone CH211-285C10 in lin	34.2	34.2	77%	4.5	100%

At the top, we see the first four sequences are an exact match to human *Wnt4*. The way we can determine this is by looking at the E-value and the max score. The lowest E-value score corresponds to the least random sequences and together with the highest total score the best sequences can be chosen.

In our project, we created primers based on *Wnt* sequences from related organisms, determined suitable lab culturing techniques, optimized tissue and DNA isolations, and performed Polymerase Chain Reactions.

Methods

Bioinformatics

I. Obtaining Raw *Wnt* sequences.

1. Search genome databases by first visiting NCBI database online.
(<http://www.ncbi.nlm.nih.gov/sites/entrez?db=nuccore&itool=toolbar>)
2. This opens the Genome database, providing the nucleotide sequences from organisms. In the search box, search “*Ciona Wnt*”
3. Find the sequences that have ‘complete cds’ in their name. Choose the data set from *Wnt7*. Once clicked, scroll down to find the heading called “Features.” Look for the sequence following the words “Translation.” (in FASTA format) Copy this sequence (should begin with Methionine) into Word/Notepad and save sequences. Repeat with other desired organisms [*N. vectensis*, *S.purpuratis*, *H. erythrogramma*, *B. floridae*, *M. musculus*]
4. If protein sequences are not available, obtain nucleotide sequences instead by scrolling to the bottom and copying the sequence into a word document and translate the sequence by using the ExPASy translate tool:

(<http://ca.expasy.org/tools/dna.html>). Copy the sequence in the text box, and on the result page, choose the sequence that starts with M.

- Optional: Select one sequence from this organism and use in BLAST to verify identity of the sequence, and related organism.

II. Alignment of sequences

- Open the CLUSTALW alignment program by going to <http://www.ebi.ac.uk/Tools/clustalw2/index.html>. Leave the settings at the default settings.
- In the text box below enter the sequences in desired order as follows. And be sure to enter the sequences as indicated below

>NAME OF SEQUENCE (e.g. *M. musculus Wnt5*)

```
MMYQGFAGEY EAPSSRCSSA SPAGDSLTYT PSPADSFSSM GSPVNSQDFC TDLAVSSANF
VPTVTAISTS PDLQWLQPT LISSVAPSQN RGHPYGVPA APPAAYS RPA VLKAPGGRGQ
SIGRRGKVEQ LSP EEEEEKRR IRRERNKMAA AKCRNRRREL TDTLQAETDQ LEE EK SALQA
EIANLLKEKE KLEFILAAHR PACKMPEELR FSEELAAATA LDLGAPSPAA AEEAFALPLM
TEAPPAVPPK EPSGSGLELK AEPFDELLFS AGPREASRSV PDMDLPGASS FYASDWEPLG
AGSGGELEPL CTPVVTCTPC PSTYTSTFVF TYPEADAFPS CAAHRKGSS SNEPSSDSL
SPTLLAL
```

***These are not counted in the analysis: Spaces and numbers.**

- Enter the rest of the sequences by leaving a space between the sequences and starting each sequences with a ">"
- The input should look like this:

```
>FOSB_MOUSE Protein fosB
MFQAFPGDYD SGSRCS SSPS AESQYLSSVD SFGSPPTAAA SQECAGLGEM PGSFVPTVTA
ITTSQDLQWL VQPTLISSMA QSQGQPLASQ PPAVDPYDMP GTSYSTPGLS AYSTGGASGS
GGPSTSTTTS GPVSARPARA RPRRPREETL TPEEEEEKRRV RRERNKLA AA KCRNRRRELT
DRLQAETDQL EEEKAELESE IAELQKEKER LEFVLVAHKP GCKIPYEEGP GPGPLAEVRD
LPGSTSAKED GFGWLLPPPP PPPLPFQSSR DAPPNLTASL FTHSEVQVLG DPFVPSY
TSSFVLTCP E VSAFAGA QRT SGSEQPSDPL NSP L L L L
```

```
>FOSB_HUMAN Protein fosB
MFQAFPGDYD SGSRCS SSPS AESQYLSSVD SFGSPPTAAA SQECAGLGEM PGSFVPTVTA
ITTSQDLQWL VQPTLISSMA QSQGQPLASQ PPVVDPYDMP GTSYSTPGMS GYSSGGASGS
GGPSTSGTTS GPGPARPARA RPRRPREETL TPEEEEEKRRV RRERNKLA AA KCRNRRRELT
```

```
DRLQAETDQL EEEKAELESE IAELQKEKER LEFVLVAHKP GCKIPYEEGP GPGPLAEVRD
LPGSAPAKED GFSWLLPPPP PPPLPFQTSQ DAPPNLASL FTHSEVQVLG DPFVNVNPSY
TSSFVLTCPV VSAFAGAQRT SGSDQPSDPL NSPSLLAL
```

>FOS_CHICK Proto-oncogene protein c-fos

```
MMYQGFAGEY EAPSSRCSSA SPAGDSLTYT PSPADSFSSM GSPVNSQDFC TDLAVSSANF
VPTVTAISTS PDLQWLQPT LISSVAPSN RGHPYGVPA APPAAYSIPA VLKAPGGRGQ
SIGRRGKVEQ LSPEEEEKRR IRRERNKMAA AKCRNRRREL TDTLQAETDQ LEEEKALQA
EIANLLKEKE KLEFILAAHR PACKMPEELR FSEELAAATA LDLGAPSPAA AEEAFALPLM
TEAPPAVPPK EPSGSGLELK AEPFDELLFS AGPREASRSV PDMDLPGASS FYASDWEPLG
AGSGGELEPL CTPVVTCTPC PSTYTSTFVF TYPEADAFPS CAAHRKGSS SNEPSSDSL
```

5. After performing the alignment, Click “Start JalView” which is an interactive input to the alignment and shows colors and regions of conservation. Save screenshots of the alignment for later use.
6. Copy and paste the raw text alignment and scores into a Word document and Save.

III. Primer Design

1. Using both the JalView output and the raw text amino acid alignments, scroll through the sequence until areas of conservation are found. The best regions are ones in which a majority (if not all) of the organisms share the same amino acids. Create a ‘consensus’ sequence that would most likely bind to a related genomic sequence.
2. Once regions are found, obtain the amino acid sequence and reverse translate into nucleotide sequences, using ExPASy reverse translate tool, (http://www.bioinformatics.org/sms2/rev_trans.html)
3. Make sure to understand the directionality of the regions in the alignment (i.e. if it is in a 3’ to 5’ orientation or the opposite). Use these sequences and the following principles to design degenerate primers.
 1. Primers should be between 18-22 nucleotides long

2. Primers preferably should end with GC (3' end)
3. Primers should not bind to itself (non complementary)
4. Introduce degeneracy by using keys that introduce random nucleotides or random pyrimidines/purines.

4. Send to external source for primer creation

IV. Analysis of Primer fragment size

1. Obtain whole genome of desired organism, (eg *C.intestinalis*), and save in Word document.
2. Using the Align (BLAST2) program in NCBI Enter the forward primer in the first box and enter the whole genome in the bottom box. Press Submit
3. In the alignment, note the nucleotide position at the BEGINNING of where the primer binds to. Write this number down.
4. Repeat step 2, but instead enter the reverse primer. In this alignment, note the nucleotide position at the END of this sequence. Subtract this number from the number obtained in step 3. This is the expected fragment size.

Culturing and Larvae Manipulations

I. Tank Design

Method 1: Tank Attempt #1

From initial observations, we attempted to re-create an artificial environment where our organisms (*Botrylloides violaceus*) could thrive in a lab setting. The main components involved in this manipulation were maintenance of water level height, inflow/outflow, storage of organisms safely while in the system, and prevention of unwanted variables (e.g. algae).

1. To maintain a water level height suitable for *B. violaceus*, a tub was chosen deep enough to contain it. This particular tub was purchased from Home Depot.
2. To maintain a constant flow rate, spouts on either end were carved out, placed, and secured. The decision as to how many spouts were needed was arbitrary. Here, we decided to use two outflow spouts shown in the picture below.
3. For inflow, a PVC pipe was used.
4. This was then punctured with a heated needle to create holes on one side of the pipe used in allowing water into the system and distributing the flow evenly across the system.
5. A water source was then attached to the pipe with holes. We used a combination of raw seawater and filtered seawater in this particular manipulation.

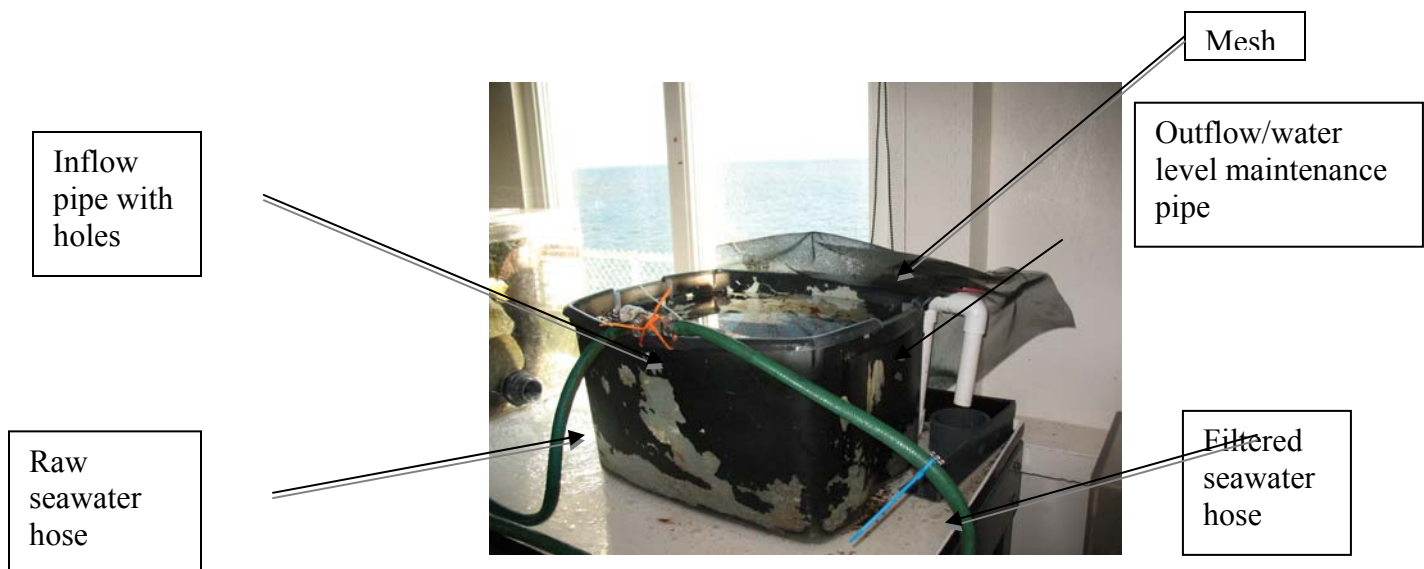
6. To maintain the proper water level height, a third spout was made out of PVC pipe so that the height of the pipe was the same height as the desired water level.
7. As shown below, we placed this third spout on the same side as the outflow spouts.
8. To store the organisms safely while in the system, we attached colonies to pieces of Styrofoam so they could float freely.
 - a. *B. violaceus* is very durable so don't be afraid of handling it liberally while attaching it to the Styrofoam or when transferring anywhere else.
9. The prevention of the unwanted variables was carried out by a few components of the tank.
10. One used a mesh screen atop the tank so as to discourage algal growth (algae will enter the system regardless but at a slower rate).
 - a. It is important to clean the tanks at least twice a week. A build up of algae interferes with *B. violaceus* by taking up desired space.
Reducing this factor will allow *B. violaceus* to succeed in the system.



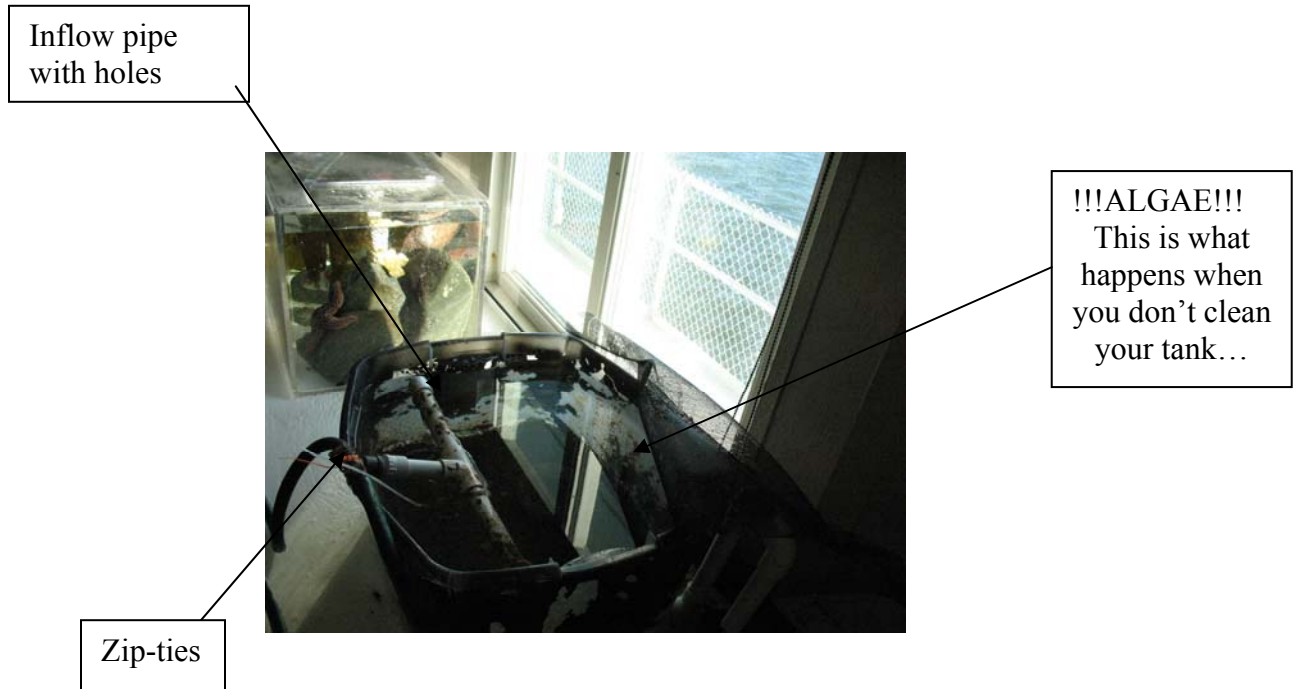
Method 2: Tank Attempt #2

Using a deeper tank, a similar set-up was constructed as shown below.

1. Each component was replicated to accommodate the larger apparatus.
2. One outflow spout was used as opposed to two from the previous set-up.
3. Zip-ties were used to secure any risk of displacement of components.



Alternate view of larger tank



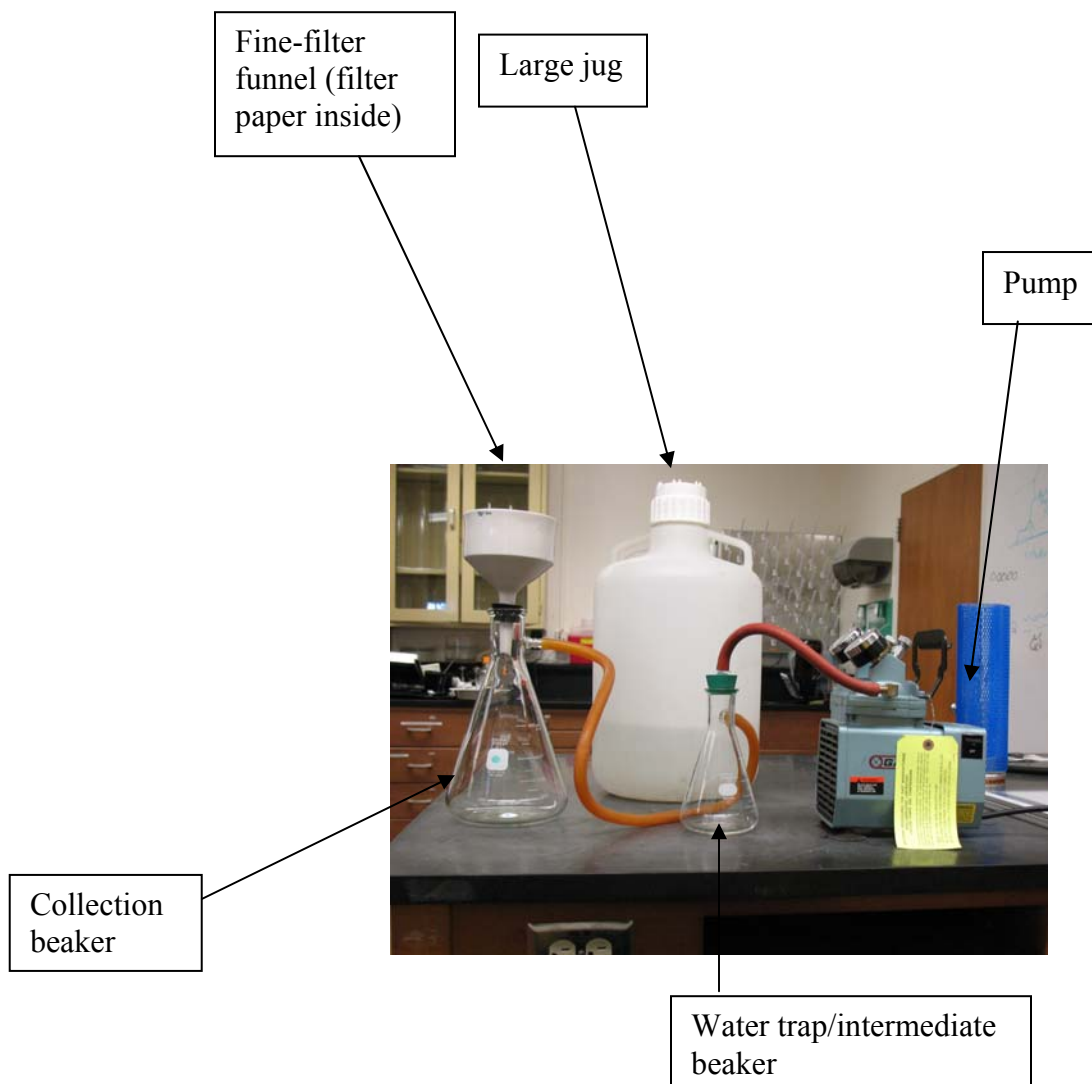
These tanks were used to house *Botrylloides violaceus* after retrieval from a natural site (Morro Bay). These colonies would eventually be examined later for embryos and brooding larvae.

II. Lab culturing

Fine-filtering seawater obtained from pier

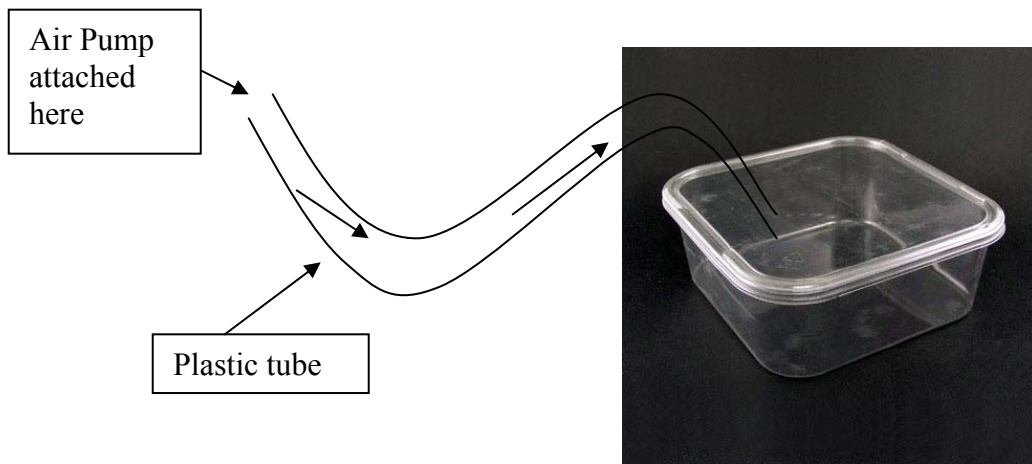
A large jug was used to transport filtered seawater from the pier into lab as shown below.

1. The following filtering system was set up using a pump, a water trap/intermediate beaker (to ensure water does not enter the pump), and a collection beaker along with a fine-filter funnel (obtain from Grad lab).



1. Attach all components as shown and turn on pump.
2. Pour water from jug into fine-filter funnel with filter paper inside being sure not to overfill the funnel.
3. Allow water to be pumped through the filter and into the collection beaker. This water will be used to test the effects of water quality on *Botrylloides* colonies in later experiments.

Aeration Apparatus



Two tanks were used to test the effect of aeration on the development of *Botrylloides*.

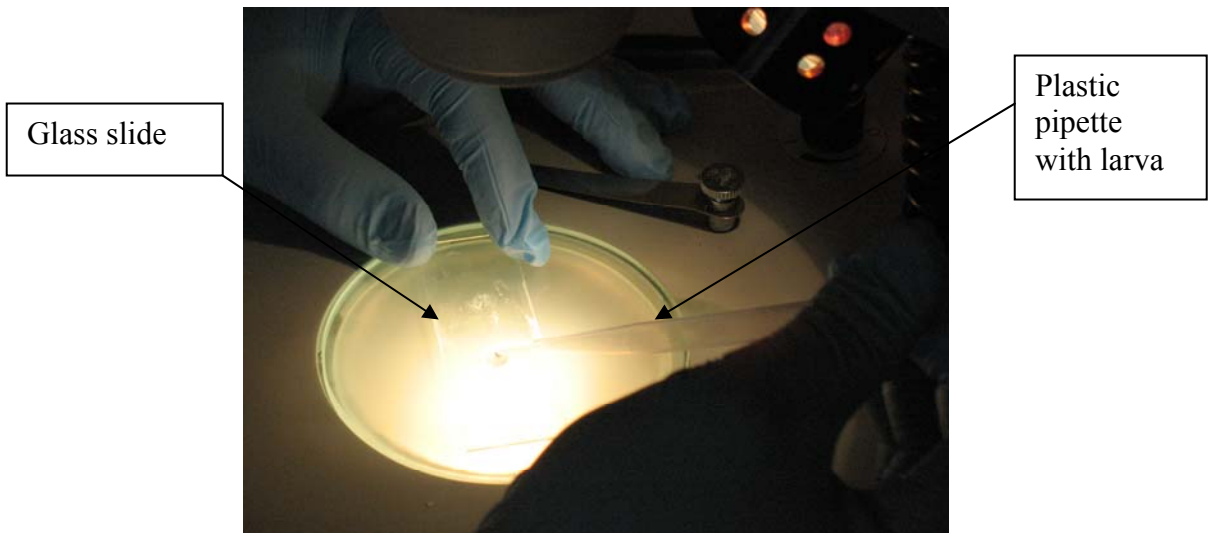
1. A small air pump was placed in the refrigerator (37°C) with the glass slides of settled larvae and tubing attached the air pump to the small tank system.

2. These were checked every few days to determine survival rates, colony takeover, etc.

III. Manipulation

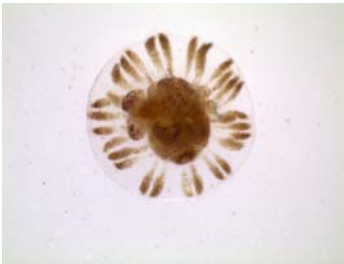
Application of larvae on glass slides

1. Obtain released larvae from colonies in tank system and manually pipette the larvae from the system onto a glass slide as shown on the next page.
2. Be sure to orient the larvae so the ampullae are radially symmetric with their tail relatively straight up (doesn't necessarily have to perfectly point at you).
3. Allow to settle for ~20 minutes to allow them to attach themselves properly to the glass. Examples of good and bad orientations are shown on the next page.

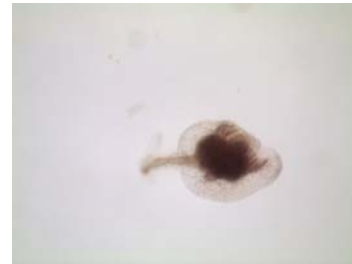


Larvae slide orientation

Good



Bad



Safely storing larvae in test conditions

1. Place slides of settled larvae, shown below into plastic containers (one set for each condition)



2. Observe and record the health and survival of each larva every day or every other day being sure to change out the water every three days and re-establish experimental conditions.
3. Remove larva/colonies that are obviously dead.
4. Take pictures using Leica microscope cam.
5. When finished observing, store containers in environmental chamber at 17°C.

Experimental Conditions

1. Food Availability
 - a. One tank with 800 μ L
 - b. One tank with 400 μ L
2. Water Purity
 - a. One tank with Filter Seawater from Pier
 - b. One tank with Fine Filtered Seawater purified in lab as discussed in Method 3 above.
3. Aeration
 - a. One tank aerated
 - i. Tube with air pumped through it into the container where colonies are maintained (shown in Method 4)
 - b. One tank not aerated

Tissue/DNA isolations

I. Isolation of Tissues

- Isolation of tissue from human cheek cells:
 1. Cotton swabs were used to obtain cheek cells and then washed off with 400uL PBS buffer into 1.5mL tube.
 2. 400uL Quiagen AL buffer added
 3. 20uL proteinase K added, vortexed 15-20 seconds.
 4. Tube incubated in 56 C heat block for 10 minutes, vortexing periodically
 5. 400uL pure EtOH added to tube and vortexed for 15 seconds.
 6. 700uL removed and placed into quiagen spin column. Centrifuged for 1 min at 8K RPM
 7. Filtrate (bottom) was discarded and spin filter placed in fresh tube
 8. 400uL AW1 buffer added and spun for 1 min at 8000 RPM
 9. Filtrate discarded and spin filter placed into fresh tube.
 10. 500uL AW2 buffer added *careful not to touch the rim.* centrifuged 3 minutes at 14K RPM
 11. Filtrate discarded, spin column placed into fresh tube. 200uL elution buffer added and tube was centrifuged for 3 minutes at 14K RPM
 12. Spin column discarded and the filtrate (now containing DNA) capped and placed in freezer.

- Isolation of Tissues from *Ciona*:

Tissue samples were provided by the Tomanek Lab, and tunic was removed by using high concentrations of urea. We cut portions of the sample and followed the directions of Qiagen DNeasy Kit

Qiagen DNeasy Tissue Isolation Procedure.

1. Obtain no more than 25mg tissue from desired sample. Homogenize the sample by Ground Glass homogenization or Physical Homogenization.
2. Add 25mg of homogenized tissue sample to a 1.5mL centrifuge tube containing no more than 80uL PBS.
3. Add 100uL ATL buffer, 20uL proteinase K and vortex the sample before incubation at 56 degrees until tissue has lysed.
4. Briefly centrifuge the tube to remove drops on the side
5. Add 200uL AL buffer and vortex for 15s and incubating at 70C for 10 minutes.
6. Add 200uL 100% EtOH and vortex for 15s. Centrifuge briefly.
7. Transfer mixture to mini spin column and centrifuge 6000g for one min. Discard the filtrate and transfer the filter to a clean tube.
8. Add 500uL AW1 buffer and centrifuge 6000g for one min. Discard filtrate and transfer to clean tube
9. Add 500uL AW2 buffer and centrifuge 20000g for 3 min.
10. Perform another centrifugation one min to remove trace chemicals.
11. In a clean tube, add 200uL AE buffer or DI water and centrifuge 6000g for one min.

12. Repeat again. Save the filtrate, discard the filter.

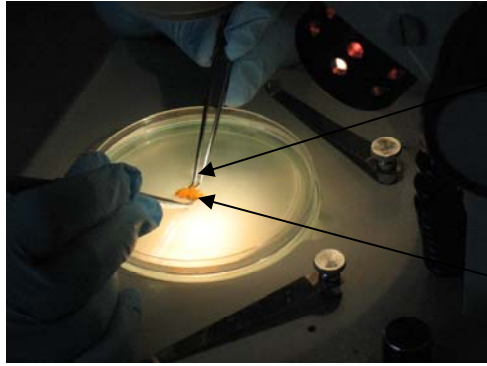
- Isolation of Tissue from *Botrylloides*:

Isolating tissue from *Botrylloides* was very difficult because of the tunic that surrounds the tissue. We determined multiple ways to try and isolate the tissues, including commercial products. We based our tissue yield on the presence of orange tissue, because of the pigments that exist in the actual tissue. The tunic is cellulosed based and doesn't have the orange pigment.

Method 1: Physical homogenization of tissue

To try and isolate the tissue, we first began to simply cut a piece of the colony and cut it into many different pieces. The intention was to 'set free' all the actual tissue and have it be separate from the tunic.

1. In a microscope, place a colony piece on a Petri dish under a microscope.
Avoid water as it is easier to manipulate dry.
2. Using forceps in one hand, hold the colony firmly. With a razor blade or another set of forceps in the other hand, pry apart the colony. The presence of orange pigments in the water is a sign that the tunic is breached. Obtain a dime sized portion of intact colony (less liquid as possible) and set aside.
3. Repeat if desired.
4. Place the small portion in a 1.5mL centrifuge tube and pull off the extra water.
5. If desired, centrifuge the sample and remove the water.
6. Keep at 0C if not using immediately



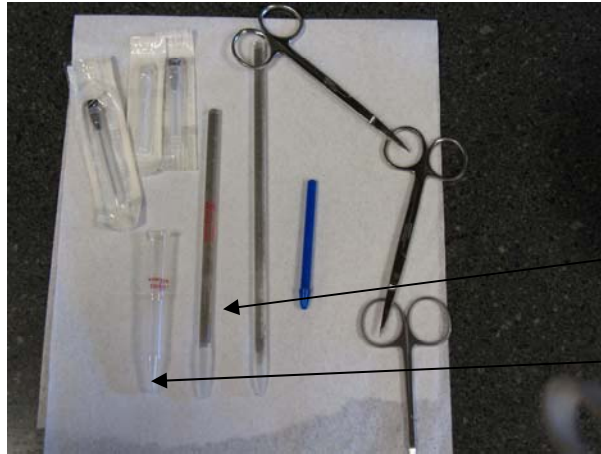
Holding the colony down, we used another pair of forceps to pull part the colony into many pieces.

Sometimes a razor blade was used in place of forceps.

Method 2: Ground glass homogenization

Ground glass homogenization is an isolation procedure intended to be used in conjunction with liquid nitrogen freezing. After freezing in liquid N₂, the subsequent homogenization would fracture all the ice crystals and eventually homogenize most of the tissue.

1. Obtain Liquid N₂. In the homogenization chamber, place a portion of the colony. Remove as much water as possible
2. Pour the LN₂ in the chamber and allow the sample to freeze completely.
3. With the homogenizer, grind the sample until it becomes soft. If desired, add more LN₂ and repeat grinding.
4. Sequester homogenized sample and place in 1.5mL centrifuge tube and store at 0C if not using immediately.



Used to physically grind the tissue, much like a mortar and pestle

Colony pieces are placed in here, frozen in Liquid N₂ and then ground using the tool on its right

Method 3: Commercial DNA isolation (MoBio PowerSoil DNA kit)

-Obtained from ASCB conference, 2008

This method utilized two principles in its isolation; Physical isolation and chemical isolation. The colony was placed into a tube containing chemically coated pebbles and together they are vortexed for up to 10 minutes.

1. Perform procedure as stated in MoBio protocol
2. For *Botryllodes* use close to 50mg tissue as most of it is aqueous
3. Note: One does not have to use the MoBio DNA isolation, after centrifugation and EtOH addition, the solution can be used in any other method of isolation.

Tube containing
chemically coated pebbles



Method 4: Microscopic Tissue isolation

Because we understood the tunic sometimes inhibits DNA isolation, we next tried using finely pulled needles to get under the tunic and suck out actual tissues. This was done under a microscope and could be performed two different ways.

Version 1: Creating a Mouth Pipette

Aspirating through a latex tube attached to a pipette allowed us to only use one hand to manipulate the needle, and allowed us the other hand to adjust the focus or hold onto the colony. We would locate an area in the colony that was densely orange and then insert the needle at an oblique angle to get between the two tunic layers. We blew

into the needle to see if a bubble appeared in the colony, which meant we had successful penetration of the tunic. Afterwards, we would try to aspirate to try and pull out some tissue. We knew if it was successful when we would see orange tissue come through the needle.

1. Create a mouth pipette by attaching a syringe to one side of a latex tube. Use the smallest needle possible (20cc) to start out with, but move to larger sizes if needed.
2. In the microscope, hold the colony down by holding the colony firmly with a pair of forceps
3. Insert the needle at an oblique angle, to penetrate through only one tunic layer
4. Aspirate by sucking on the latex tube, careful not to aspirate heavily but rather gradually.
5. Blow out isolated tissue into a 1.5mL centrifuge tube. Save at 0C if not using immediately.

Version 2: Syringe Aspiration

This is similar to the mouth pipette in terms of trying to isolate tissue, but differed in how to manipulate the needle. In this case we needed to use both hands to first insert the needle, and second pull back the syringe to try and aspirate the tissue within.

1. Attach a syringe to a plunger and perform the same procedure as described above

II. DNA isolation:

We used many types of kits in our DNA isolation procedures. While they were all similar in principle, some were better than others.

Isolation Kit 1: Qiagen DNeasy Tissue Kit



1. Perform Isolation as described by Qiagen Tissue Isolation Protocol
2. More DNA can be obtained by using more than 25mg tissue in the beginning of the procedure.

Isolation Kit #2: PowerSoil DNA kit



1. Perform Isolation as described by MoBio Power Soil DNA kit.

PCR Reactions

We began the project using GE puReTaq ready-to-go PCR beads then transitioned to using Master Mixes. We first determined our controls with various control primers such as GPDH, NS1/2 and B-actin. We also tried to manipulate MgCl₂ in the beads. Our biggest variable in our PCR reactions were determining annealing temperature, so we utilized the gradient PCR to test many annealing temperatures.

27-9559-01

Lot 314232

Pack W 315421

Exp 31 Dec 06

PuReTaq Bead Components:

Contains: dATP,dCTP,dGTP, dGTP,
reaction buffer, stabilizers and 2.5units
PureTaq DNA polymerase.

When constituted to 25mL:

- dNTP-200mM
- Tris-HCl-20mM (pH 9.0)
- KCl-50mM
- 1.5 mM MgC

1. Typical PCR reaction components and protocol using PCR beads:

Place in order, the following components into PCR bead, while on ice:10uL DNA

5uL F-primer

5uL R-primer

5uL diH₂O

25uL Total



Protocol Name: **std55/45**

Initial warm up: 94 C (5 min)

Denature: 94 C (45 sec)

Anneal: 55 C (45 sec)*

Extend: 72 C (45 sec)

x 30 cycles

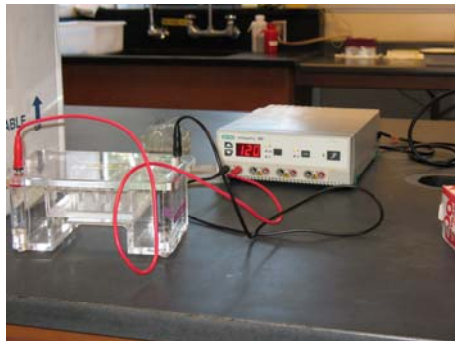
Final Extension: 72 C (7 min)

Hold: 4 C

*All PCR reactions were performed in the same method as described before, only

Annealing Temperatures were changed*

Typical Gel Electrophoresis Protocol



- 1% gel: 40mL sterile TAE buffer + 0.40g Agarose, microwaved until boiling.
- Add 4mL EtBr before adding to Gel tray
 - Gel tray prepared with borders of tape and addition of a comb (typically a 10 lane comb)

- Agarose sometimes cooled under water before adding to tray
(minimizes cooling time)
- Once cool (determined by if comb can pull out cleanly), load gel as follows:
 - Lane 1 and Lane 10 (if applicable):
4uL 100bp ladder + 1uL Loading Dye
 - Lanes 2-9:
8uL PCR sample* + 1uL loading Dye
- Run 120Volts for approximately 1 hour or until loading dye reaches 2/3 down the gel.

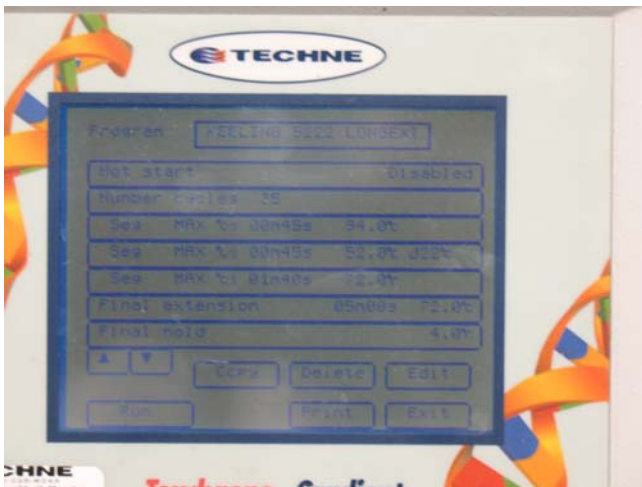
*Sometimes we changed this value depending on the experiment.

2. Typical Gradient PCR protocol

Prepare PCR reactions in the same way as described above.

- Gradient PCR relies on one basic requirement:
The Range of Annealing Temperatures
-Determined via Gradient Calculator.
- To set up desired annealing temperature, first use a median annealing temperature calculate a range
 - $\Delta 30$ means the differences between the lowest and highest temp is 30 degrees. **Note: The median of this range is your annealing temperature.**

- Depending on how low or how high the annealing temperatures should be, adjust the range and annealing temp accordingly
- In our notation, 52 Δ 30 means there is a 30 C range between the highest and lowest annealing temp (among a set of 13).
The middle tube will have an annealing temperature of 52, the lowest annealing temperature will be at 22 C, and the highest annealing temperature will be at 82 C
- Run PCR in the same way as described above.



Protocol name: **Keeling
52 Δ 22LongExt**

Initial warm up: 94 C (5 min)

Denature: 94 C (45 sec)

Anneal: 52 C (45 sec)*

Gradient: Δ 22 C*

(lowest tube is at 30 C, middle tube is at 52, highest is at 74 C)

Extend: 72 C (90 sec) **

x 35 cycles

Final Extension: 72 C (7 min)

*All Gradient PCRs were performed in a manner similar to what is described but we altered the gradient and annealing temperatures

**Longer extensions were used to attempt to obtain longer fragments from the PCR

3. Increasing MgCl₂ Concentration

25uL PCR tube-MgCl₂ concentration 1.5uM4. Master Mix Set up

Stock Solution	Stock Concentration	Desired Concentration
10X PCR buffer	10X	1X
dNTP	10uM	1uM
MgCl ₂	25uM	10uM
Primers	1uM or 10uM	0.1 (eg Ns1/Ns2, B-Actin) 0.2 /0.25 (Degenerate)
Water	NA	(only to fill up to 25uL)
Taq (add last)	5Uunits/uL	0.02uM

-Aliquot 25ul into each PCR tube

-Tube(s) needs to be centrifuged before placing into PCR machine

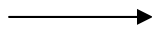
5. Nested PCR

Because we designed the primers as we did, we had the opportunity to perform nested PCR. Nested primers defined by, Haff, LA., 1994 are a widely accepted and generally effective solution to PCR nonspecificity is nested primer PCR. (3) Nested primer PCR involves an initial set of thermal cycles with a pair of primers spaced relatively far apart. Then, an additional primer or pair of primers, located between the outer primers, is added, followed by a second round of thermal cycling. The set of inner

primers is added at a higher concentration than the outer primers. After a few thermal cycles, inner primer PCR -product predominates.

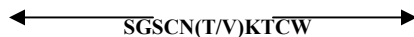
Essentially, nested primers are PCR reactions within PCR reactions. Going back to our primer design, we were fortunate enough to perform not one, but two potential nested primers.

Forward Primer 1



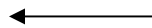
QFRYSKWNC

Reverse Primer 1 / Forward Primer 2



SGSCN(T/V)KTCW

Reverse Primer 2

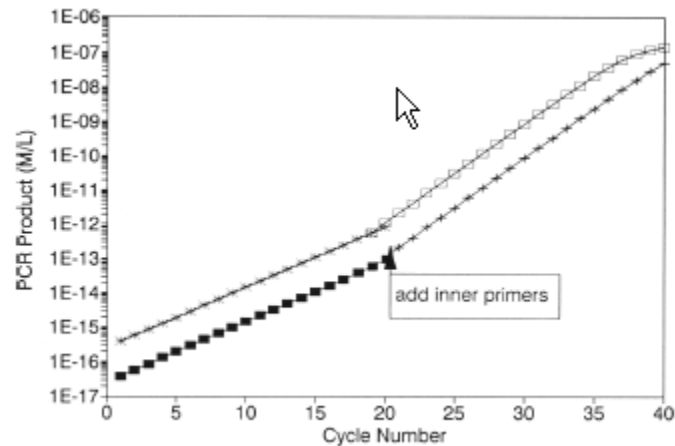


CCWFFKC|JC

When we performed a nested primer, we first used the Forward 1 / Reverse 2 primer combination in a PCR reaction. During gel electrophoresis we only used 8uL of PCR product in our analysis, leaving 17uL of amplified F1/R2 DNA. Sometimes we were not able to see this in the gel, because the concentration of amplified product is not high enough to be observed. After performing PCR clean-up with MoBio PCR clean up kit, we were able to perform two nested PCRs. The first could be performed by applying the

Forward 1 / Reverse 1 primer combination in a PCR reaction using this amplified DNA as our template. The second nested primer could be performed by using the Forward 2 / Reverse 2 primer combination.

The mathematical model provided by Haff, L.A. 1994, shows how much PCR product can be further obtained when using a second set of 'inner' primers.



6. Control primer sets:

- GPDH: Glycerol-3-phosphate dehydrogenase. Functions in metabolism to catalyze the reaction of Dihydroxyacetone phosphate to glycerol-3-phosphate. Will not appear in early embryonic states (pre-morula). **450 base pairs long.**

- Forward: 5'-TGAAGGTCGGTGTGAACGGTATTTGGC-3' Tm: 54 C
Reverse: 5'-CA-TGTAGGCCATGAGGTCCACCAC-3' Tm: 54 C

(Pan, G. et al. 2009)

- NS1/NS2: 18s Ribosomal subunits, non structural. First found in plant cells. **550 base pairs long**

- Forward: 5'-GTAGTCATATGCTTGTCTC-3' Tm: 49 C

Reverse: 5'-GGCTGCTGGCACCAGACTTGC-3' Tm: 65 C

(Pastey, K.M, Samal, S.K, 1995)

- B-Actin: Non muscular Cytoskeleton Component. **297 base pairs long.**

- Forward: 5'CTATACGCTTCCGGCAGAAC 3' Tm: 63.5 C

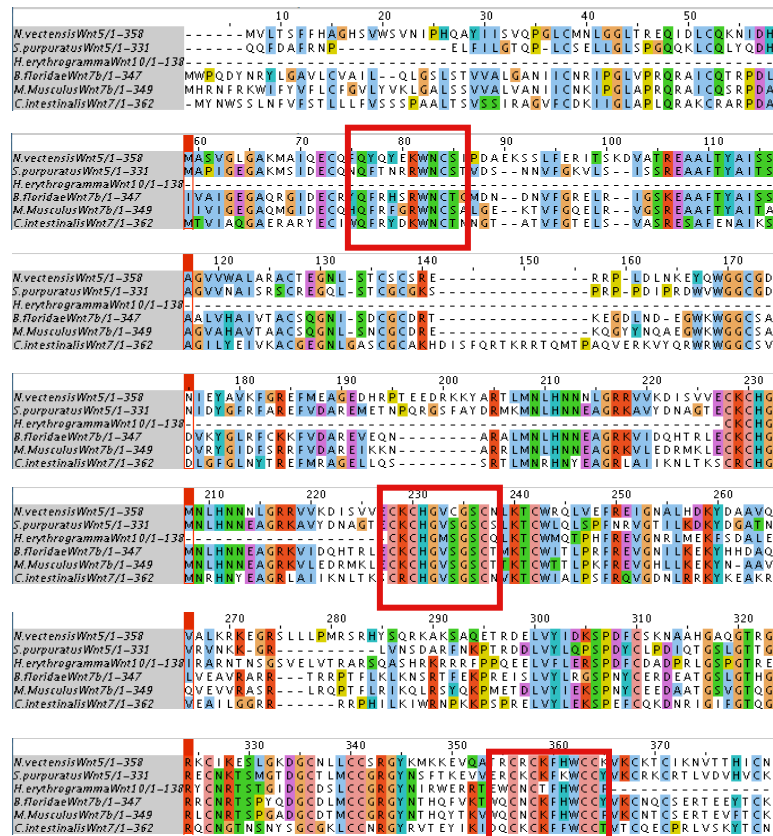
- Reverse: 5'-GGCTGCTGGCACCAGACTTGC-3' Tm: 63.7 C

Control primers were used at 1mM concentrations unless otherwise stated.

Results

Bioinformatics

I. Alignment



Output of CLUSTALW multiple sequence alignment. Colors indicate areas of conservation among species. Gaps (---) indicate the introduced gap to create the best alignment. Notice that alignment was performed with amino acid sequences to allow for better indication of conservation.

To create suitable primers, we had to look through the amino acid alignment above to see regions that were conserved (i.e. possessed large region of similar amino acids). We noticed three major regions in the sequences, indicated by the red boxes. The next step was to determine 'consensus sequences' in which we decide the best fit for this alignment. Creating an exact match was not favorable because it would create a more specific primer and be less likely to bind to our un-sequenced *Botrylloides* DNA. Instead, we chose to keep the amino acids that were exactly matching across the six species but leave the others varied. We did this by allowing the primers to be designed with random pyrimidines or purines or even completely random nucleotides. The intention would be to create a large population of primers, with the hope that some are very good primers and will actually bind the *Botrylloides* DNA, and that some will not be so good and not bind at all. These are known as degenerate primers. Degenerate in the sense that they are not very specific primers-not that they will degrade over time. As primers require both a forward and reverse primers, we chose the first consensus sequence to be the forward primer (read left to right). Naturally we chose the third consensus sequence to be the reverse primer (read right to left) to try and obtain the largest fragment possible. However in case there was a presence of a very large intron or an insertion, we used the second consensus sequence as a 'dual primer.' That is we read the sequence in two ways, forwards (to be our forwards primer) and reverse (to be a reverse primer). Arrows on the bottom of these sequences show the direction of the primers.

(Reading top to bottom)

First consensus sequence:

QFRYSKWNC


Second consensus sequence:

SGSCN(T/V)KTCW
 

*T or V were used because of its complicated R group and hence most likely conserved.

Third consensus sequence:

CCWFFKC[]C


*[] was used because there is no conserved amino acid

To actually create the primers, we first needed to reverse translate the amino acid sequences to nucleotide sequences. Using a codon table we assigned the amino acids to a codon. Because there are multiple codons per amino acid, we sometimes left the third base pair unspecific* (using a random pyrimidine or purine). In the case of the second consensus sequence, we read it backwards to create a reverse primer and forwards for a forwards primer.

“Forward 1” Primer

Amino Acid conservation

QFRYSKWNC

Degenerate Primer

5' CARTTYCGNTAYAGYAARTGG 3'

“Reverse 1” Primer

Amino Acid conservation

SGSCN(T/V)KTCW

Degenerate Primer

3' GGTGTNCARAAGTAYAANTCYGA 5'

“Forward 2” Primer

Amino Acid conservation

SGSCN(T/V)KTCW

Degenerate Primer

5' AGYCTNAAAYATGAARACNTGYTGG 3'

“Reverse 2” Primer

Amino Acid conservation

CCWFFKC[]C

Degenerate Primer

5' YGTNNNYGTRAAAYTTYTTGG 3'

*NNN symbolizes “any” nucleotide.
 Y symbolizes any Pyrimidine nucleotide
 R symbolizes any Purine nucleotide

Using these primer sequences along with the larger sequence-we calculated the potential lengths of each the fragments we could obtain:

Forward 1 primer → Reverse 2 primer: 821 base pairs
 Forward 1 primer → Reverse 1 primer: 243 base pairs
 Forward 2 primer → Reverse 2 primer: 374 base pairs

To serve as a control, we also used β -Actin primers which are present in all cells in the cytoskeleton.

II Preparation and Dilution of Primers

<u>Bactin F</u> 5'CTATACGCTTCCGGCAGAAC 3' 65.1 nmol Tm=63.5C	<u>Bactin R</u> 5'CAAGAGCGACATAGCACAGC 3' 74.5nmol Tm=63.7C	<u>WntF1 (“forward 1”)</u> 5' CARTTYCGNTAYAGYAARTGG 3' 77.4nmol Tm=53.2C
<u>WntF2</u> 5' AGYCTNAAYATGAARACNTGYTGG 3' 56nmol Tm=54.9C	<u>WntR1</u> 3' GGTYGTNCARAAGTAYAANTCYGA 5' * reverse primer of WntF2 69.0nmol Tm=54.9C	<u>WntR2</u> 5' YGTNNNYGTRAAATTTTGG 3' 78.7nmol Tm 54.4C

These were stock primers, and came completely free of water. Below are procedures and methods we used to dilute and prepare the primers.

*All procedures involved prolonged centrifugation at each step. TAE was used as a buffer to preserve integrity of the primers.

<p><u>Bactin F</u></p> <p>Stock: 65.1nmol</p> <p>Add 651uL solution (1/2 sterile H2O, ½ sterile TAE)</p> <p>Divide into 6 x 100uL tubes (100uM) ← store at -80 C</p> <p>Take 51uL+200uL diH2O to make 20uM ←Store at -20C</p>	<p><u>Bactin R</u></p> <p>Stock: 74.5nmol</p> <p>Add 745uL solution (1/2 sterile H2O, ½ sterile TAE)</p> <p>Divide into 7 x 100uL tubes (100uM) ←store at -80 C</p> <p>Take 45uL+180uL diH2O to make 20uM ←store at -20 C</p>
<p><u>Wnt F1</u></p> <p>Stock: 77.4nmol</p> <p>Add 774uL solution (1/2 sterile H2O, ½ sterile TAE)</p> <p>Divide into 7 x 100uL tubes (100uM) ←store at -80 C</p> <p>Take 74uL+74uL diH2O to make 50uM←store at -20 C</p>	<p><u>Wnt F2</u></p> <p>Stock: 56nmol</p> <p>Add 560uL solution (1/2 sterile H2O, ½ sterile TAE)</p> <p>Divide into 5 x 100uL (100uM) ← store at -80 C</p> <p>Take 60uL+60uL diH2O ←store at -20 C</p>
<p><u>Wnt R1</u></p> <p>Stock: 69nmol</p> <p>Add 690uL solution (1/2 sterile H2O, ½ sterile TAE)</p> <p>Divide into 6 x 100uL tubes (100uM)</p> <p>1 x 40uL tube (100uM) ←store at -80 C</p> <p>Take 50uL+50uL diH2O to make 50uM ←store at -20 C</p>	<p><u>Wnt R2</u></p> <p>Stock: 78.7nmol</p> <p>Add 787uL solution (1/2 sterile H2O, ½ sterile TAE)</p> <p>Divide into 7 x 100uL (100uM)</p> <p>1 x 40uL (100uM) ←store at -80 C</p> <p>Take 47uL+47uL diH2O to make 50uM ←store at -20 C</p>

I. Tank Design

Method 1: Tank attempt #1

Benefits: Most factors of environmental replication were accounted for and successful in maintaining *B. violaceus* colonies.

Limitations: One major limitation associated with this particular tank included shallowness that affected the colonies ability to thrive. Colonies were dying at an alarming rate. To correct this, we decided to use a larger tub with the same design concept. Our theory was that with a larger tank, algal growth could be decreased and organisms would be able to grow without spatial restrictions.

Method 2: Tank attempt #2

Benefits: Colonies were more successful in this set-up than previously attempted ones. Larger colonies were maintained for a longer period of time. Cleaning of tank can be extended to once a week.

Limitations: Larger tanks require larger area of clean-up but no other apparent limitations were identifiable for our research.

II. Lab Culturing

Fine-filtering seawater obtained from pier

Benefits: Great way to purify crude seawater obtained from pier. System is easily set-up, used, cleaned, and disassembled.

Limitations: None observed

Aeration Apparatus

Benefits: Small enough to be used in the environmental chamber and not disrupt colonies that were attached to the slides. Supplies were cheap. Easily disassembled and accessible.

Limitations: Need more reliable pump (wasn't strong enough to get air through at times). Pump was borrowed. Purchasing one would be ideal.

III. Manipulation

Application of larvae on glass slides

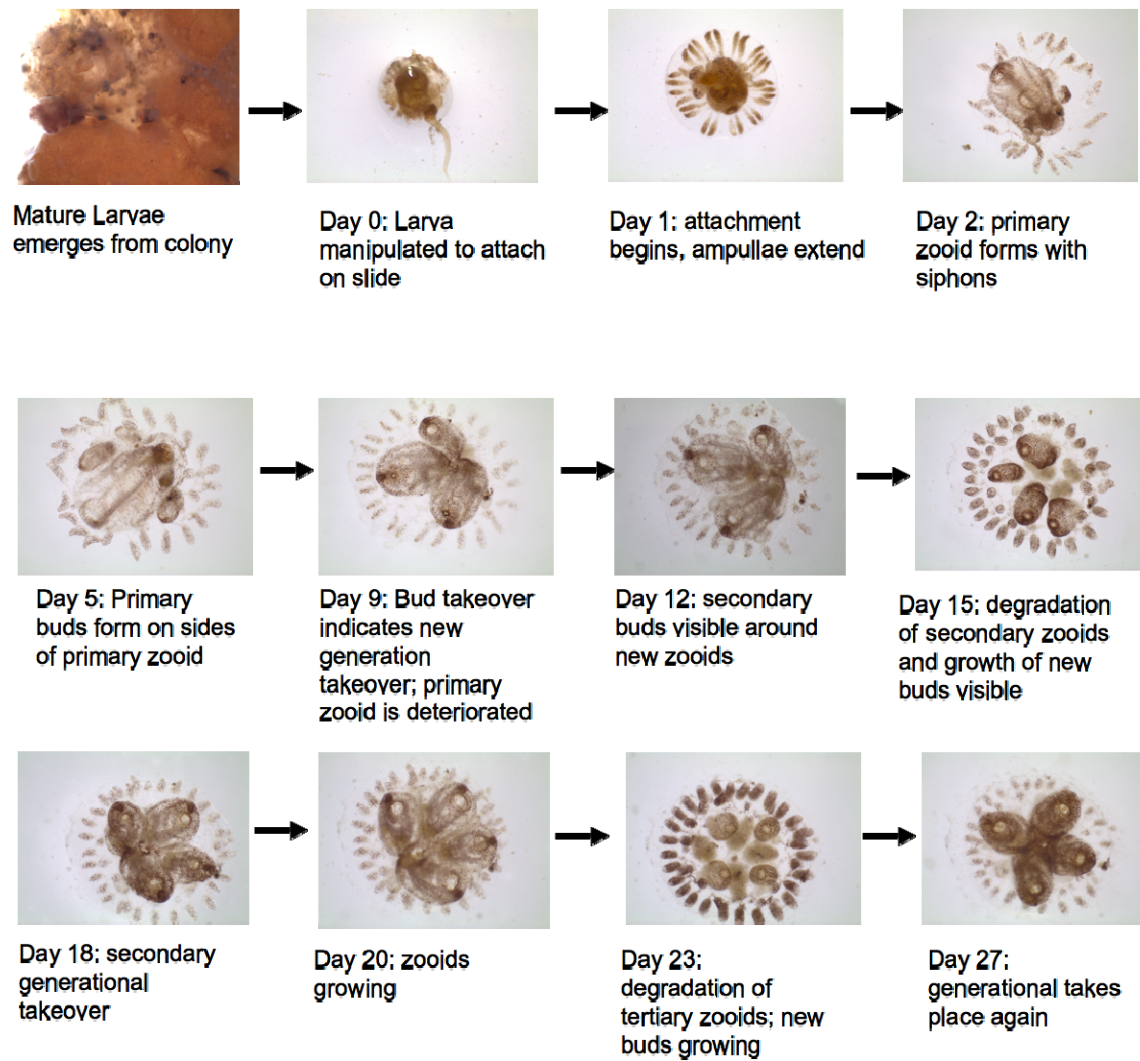
Benefits: Simple/easy to do. Doesn't take an extensive amount time. Organism is durable, easy to manipulate to establish proper orientation, shown in Method 5.

Limitations: None observed

Safe storage of larvae/colonies in experimental conditions

Benefits: Accessible and easy to manipulate. Kept colonies healthy enough to survive 3+ weeks.

Limitations: None observed.



Progression of *Botrylloides violaceus* development and growth are shown.

Survival after one and two weeks under each condition are shown.

Aeration/No Aeration	Category	- Aeration	+ Aeration
	Survival after two weeks	28.9% (n=38)	42.9% (n=14)
	Survival after one week	60.5% (n=38)	81.8% (n=11)
	Mean Rate of development	~6.9 days/generation	~4 days/generation
Crude/Filtered Seawater	Category	Crude	Filtered
	Survival after two weeks	28.6% (n=14)	28.6% (n=42)
	Survival after one week	40% (n=15)	71.4% (n=42)
	Mean Rate of Development	~6.7 days/generation	~7.6 days/generation

	Category	400 μ L	800 μ L
Less/More Food	Survival after two weeks	32.2% (n=38)	22.2% (n=18)
	Survival after one week	44.7% (n=38)	12/18 = 66.7% (n=18)
	Avg. Rate of Development	~7.8 days/generation	~6.3 days/generation

- Aeration: more survival & faster rate of development in + aeration
- Filtration: more survival in filtered but faster rate of development in crude
- Food Volume: more survival in 800 μ L after one week and faster rate of development but more survival in 400 μ L after two weeks

Tissue/DNA Isolations

I. Isolation of Tissues

Method 1. Physical Homogenization

Benefits: This method works beautifully when done correctly. To perform tissue isolation, we needed a suitable size of colony to work with. The perfect size of a colony for this method needed to be about the size of a post-it or approximately twice the size of

the colony in the picture. To be able to perform this method correctly, it needed to be done in the absence of water. Performing the isolation in water seemed to create a drifting effect, and ‘washed’ away potential tissues. When kept dry, the tissues became easier to cut and separate.

Limitations: Whereas this method was simple, we needed to control how much a pair of forceps came into interaction with the tissue. The more times we tear, the more times *Botrylloides* tissues came into contact with the forceps and became easily lost. This was especially in the case when we used razor blades. While we are not sure of the DNA yield, the sample we actually transferred appeared quantitatively smaller than other tissue isolation methods. As mentioned before, this method didn’t appear to work well with colony sizes smaller than a post it note. When it became this small, the likelihood of the piece sticking to the razor blade or forceps grew and more often than not we would lose a large portion of the colony.

Method 2: Ground Glass Homogenization

Benefits: Using this method, we were able to homogenize any size of colony. We weren’t limited to how much raw tissue to start with, any size we were able to homogenize. Furthermore this method seemed to work more significantly than the first method, just by the appearance of more orange solution in the homogenate.

Limitations: Liquid N₂ presence, without it the homogenization will not work as designed. Sometimes the heat of friction would melt the ice to a point where the material became so aqueous that homogenization could not happen. Also, this method

homogenizes everything on the colony, so bacteria and other organisms are also getting homogenized and placed into the tissue sample for DNA isolation.

Method 3: Commercial DNA isolation (MoBio PowerSoil DNA kit)

Benefits: This method easily produced the largest yield of tissue and DNA. After vortexing and centrifugation, we would easily see two layers in the tube. In the bottom part (where the pebbles were) we could clearly see such things like dirt and non-orange tissues. On more than one occasion we were able to see clear tissues, which we hypothesized to be tunic.

Limitations: Expensive, costs around 200 dollars for a 15-kit reaction. The pebbles aren't intended to be recycled. We tried reusing the pebbles, with a subsequent proteinase K digestion, and seemed to get similar results. However, the pebbles do retain detergents from the added lysis buffer, so care had to be used not to wash in or add water (causes bubbles).

Method 4: Microscopic Tissue isolation

Benefits: Very little, sometimes we got lucky and isolated ~5uL tissue.

Limitations: Method was very complicated and smaller needles prevented stronger suction, so only a small amount of tissue was pulled out. Using this method was dependent on the 'thickness' of the colony because on the thinner sections of the colony the needle would always push through the other side.

II. DNA isolations

Isolation Kit #1: Qiagen DNeasy Tissue Kit

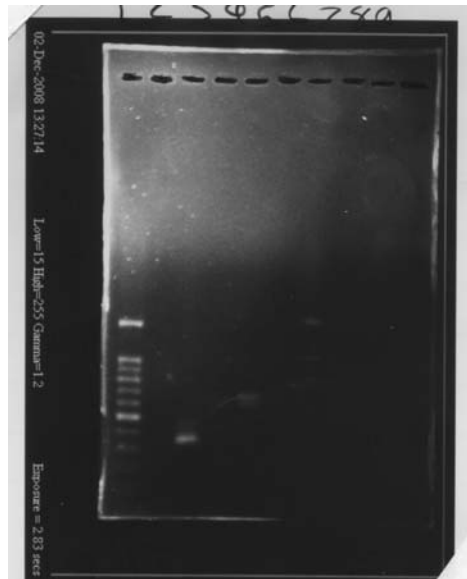
Using Qiagen was the simplest method of isolation we possessed. However we sometimes did not get the results we expected, and this in part we thought was due to the reagent quality. Aside from the actual kit we also had other bottles of ‘old’ reagents from previous kits. Sometimes using an old reagent with a new reagent produced a better result than using completely new reagents. What we generally concluded over many experiments is that the quality of the lysis buffer was important while the elution buffer quality was less important.

<p>Benefits:</p> <ul style="list-style-type: none"> • Easy to use • Usually produces a good yield of DNA from only 25-50mg tissue 	<p>Limitations:</p> <ul style="list-style-type: none"> • Doesn't work well with less than 25mg tissue samples • Only 200uL elution samples • Reagents sometimes don't work well
---	--

Isolation Kit #2: PowerSoil DNA kit

Using the MoBio PowerSoil DNA kit gave us the highest yield of DNA. We suspect this came from the actual tissue isolation (i.e. the pebbles). Other than that, the reagents in the kit were no different from the Qiagen reagents (they are only listed differently). For example, the Lysis buffer in MoBio is the same as Qiagen's lysis buffer. Solution 1 in MoBio is equivalent to AW1 in Qiagen. When MoBio reagents were running low we started using Qiagens' without any dramatic change in yield.

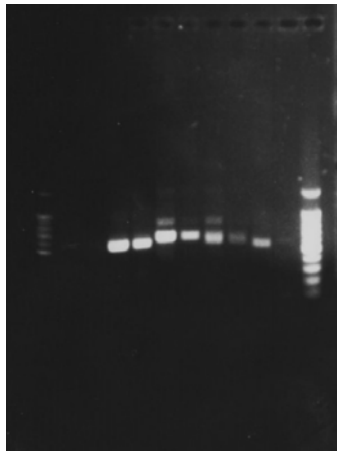
Benefits:	Limitations
<ul style="list-style-type: none"><li data-bbox="310 268 781 373">• High yield from DNA even from small amounts of tissue<li data-bbox="310 415 781 520">• No Proteinase K digestion (when using fresh pebbles)<li data-bbox="310 562 675 594">• Can use Qiagen reagents	<ul style="list-style-type: none"><li data-bbox="857 268 1252 300">• Only 50uL elution samples<li data-bbox="857 342 1365 447">• Low efficiency of recycled pebbles, without proteinase K digestion

PCR Reactions

Date: 12/02/08, with PCR beads

First PCR reaction we ran, tried to determine the correct stock of Ns1/2 primers to use in our experiments. None of them worked.

<u>Lane(L→R)</u>	<u>Identity</u>
1	4uL 100bp Ladder
2	5uL Alu primer with cheek cell DNA
3	Ns1/2 (100uM stock) and cheek cell DNA
4	Ns1/2 (10uM stock) and cheek cell DNA
5	GPDH (100uM stock) and cheek cell DNA
6	(-) control
7	100bp Ladder



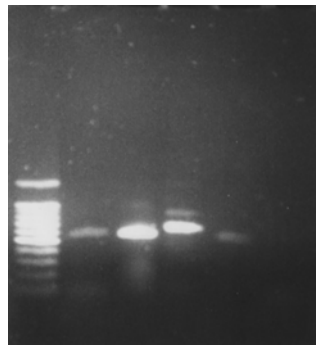
Date: 1/13/09, with PCR beads.

*PCR was run at 45 second extension instead of 30 seconds.

We tried to obtain DNA from *Botrylloides*, *Ciona*, and Human DNA using Ns1/2 primers. Successful isolation of *Botrylloides* DNA and use of Ns1/2, at the correct size.

<u>Lane(R→L)</u>	<u>Identity</u>
1	100bp Ladder
2	Bot DNA + Ns1/2 (100uM stock)
3	“ “ (10uM stock)
4	Ciona + Ns1/2 (100uM stock)
5	Ciona + Ns1/2 (10uM stock)
6	Human + Ns1/2 (100uM stock)
7	Human + Ns1/2 (10uM stock)
8	“Old human” + Ns1/2 (100uM stock)
9	“Old human” + Ns1/2 (10uM stock)
10	Human + Alu Primers

11	(-) control
12	100 bp ladder



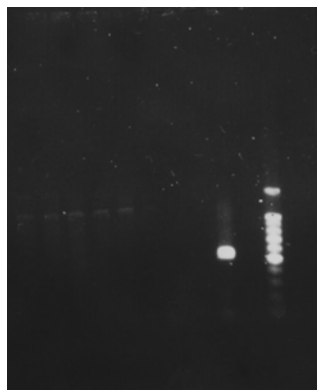
Date: 1/14/09, using PCR beads

Closer Analysis of the difference of Ns1/2 between *Ciona*, human and *Botrylloides* DNA.

Ciona and human seem to both possess another band, around the same size as Ns1/2.

However the last row has a band, and because it is a negative control this result is not conclusive.

<u>Lane(L→R)</u>	<u>Identity</u>
1	100bp Ladder
2	*15uL DNA Bot + Ns1/2(100uM)
3	*15uL DNA Ciona + Ns1/2 (100uM)
4	*15uL DNA human + Ns1/2 (100uM)
5	(-) control

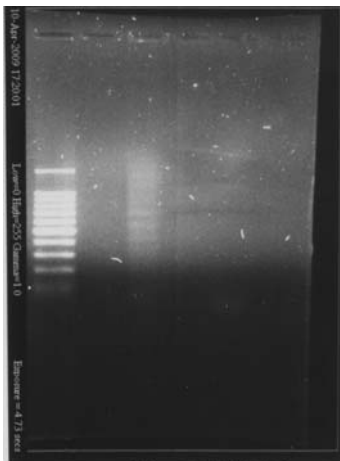


Date: 2/13/09, Gradient PCR using Increased MgCl₂ procedure (see methods)

Test of *Wnt* primers on *Botrylloides*, and use of increased MgCl₂ on Ns1/2 primer set.

Bands present do not match the expected size of *Wnt F2/R2*.

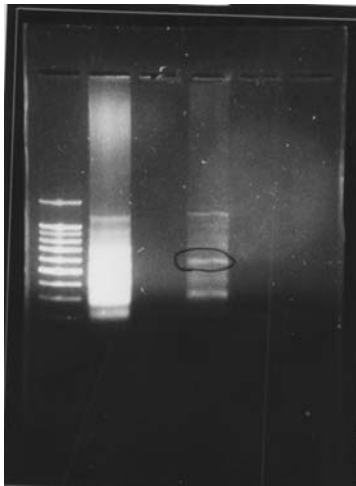
<u>Lane(L→R)</u>	<u>Identity</u>
1	WF2/R2 with Bot DNA 35.9C
2	“ “ 37.6C
3	“ “ 40.1C
4	“ “ 42.6C
5	“ “ 45.2C
6	“ “ 47.8C
7	“ “ 49.7C
8	“ “ 52.5C
9	Ns1/2 with MgCl ₂ (increased to 3uM)
10	(-) control



Date: 4/9/09, with PCR Clean Up and Nested Primers, combining previous reactions.

We tried to see if we could isolate fragments from Bot DNA. The first reaction was done by using the F1/R2 primers which would isolate the largest fragment. We did a PCR clean up and combining the tubes, the hope was that there were some F1/R2 fragments, and by adding the F1/R1 and F2/R2 primer sets we might be able to get a band.

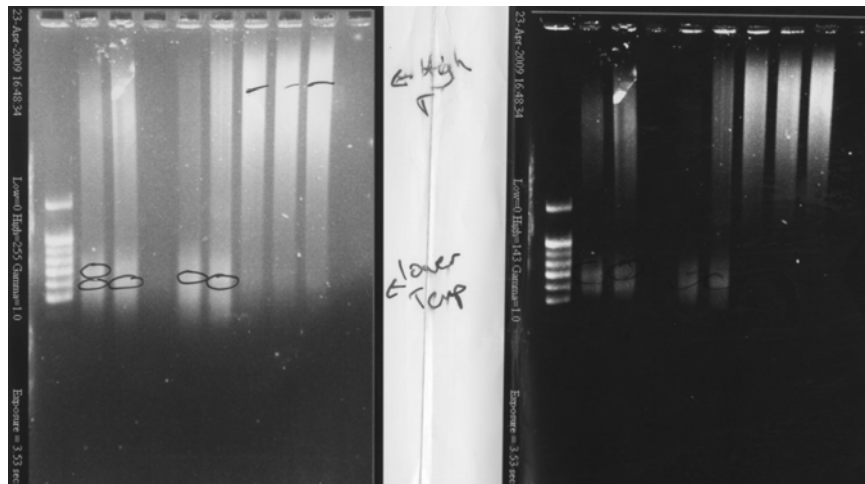
<u>Lane(L→R)</u>	<u>Identity</u>
1	100 bp ladder
2	NA
3	PCR Clean up of 4/8 (WF1/R2) with 4 tubes (no bands shown) + WF1/R1 Primers
4	“ “ + WF2/R2 primers
5	F1/R1 (-) controls
6	F2/R2 (-) controls



Date: 4/15/09, using PCR beads, and Long extension PCR.

Performed clean up of 4/9 reaction and performed another PCR with the same primer sets as above, but used 45 second extension instead of 30 (LONGEXT). Band circled represents the most likely identity of the F2/R2 fragment

<u>Lane(L→R)</u>	<u>Identity</u>
1	100bp ladder
2	4/9 clean up (F1/R1) + F1/R1 again
3	F1/R1 (-) control
4	4/9 clean up (F2/R2) + F2/R2 again
5	F2/R2 (-) control



Date: 4/22/09, using PCR beads and Gradient PCR

Instead of doing nested PCR we ran the same primer sets after PCR clean up, and then we ran two sets at different temperatures. Interestingly, higher MW bands were obtained at higher temperatures compared to the lower temperatures. The lower temperature with the F1/R1 fragment, (meaning more specific binding) produced bands that close to the expected size for an F1/R1 fragment.

<u>Lane(L→R)</u>	<u>Identity</u>
1	100bp ladder
2	Prev. Reaction with WF1/R1 after 3X PCR clean up + WF1/R1 primers (again) 42.4C Annealing
3	“ “ 43.6C
4	“ “ 45.5C
5	“ “ 47.3C
6	Prev. Reaction with WF2/R2 after 3X PCR clean up + WF2/R2 primers 54.6C

7	“ “ 56.7C
8	“ “ 58.6C
9	“ “ 60.4C
10	F1/R1 (-) control
11	F2/R2 (-) control

Discussion

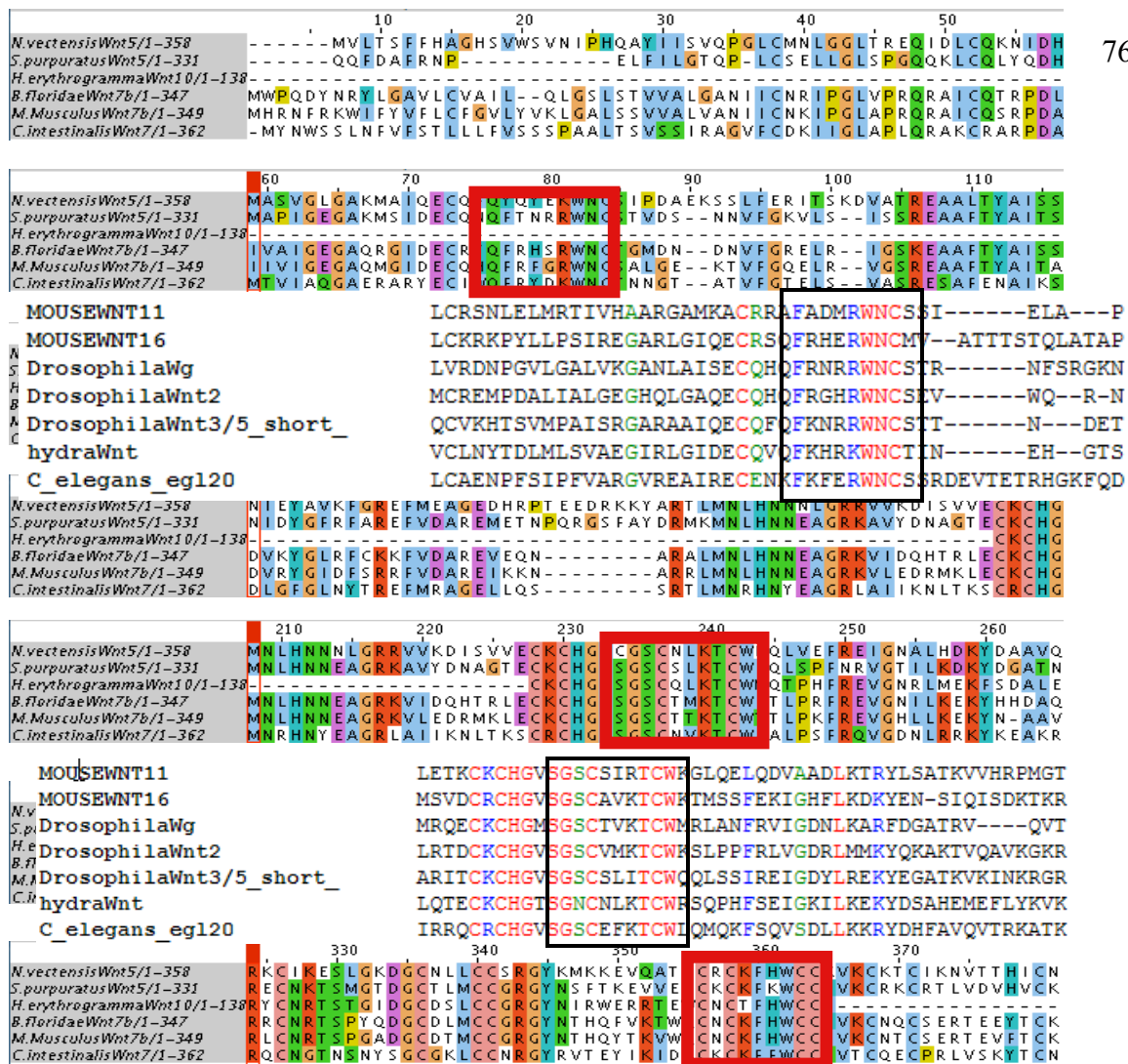
Bioinformatics

Our experiences with bioinformatics and primer design have been the most insightful and powerful. We thought we did an adequate job in aligning multiple *Wnt* genes and finding areas of conservation between them. It might have benefited us to use another organism as part of our alignment as it might reveal an aspect of conservation we may have not observed. At first we did try to target a specific isoform of *Wnt*, *Wnt7*. Our research showed that it was present in many species so it became our candidate gene. The primers were designed with these isoform sequences in mind. However we were fortunate in *Wnt7* shared the same conserved regions as all the isoforms. When we would use these primers, not only would it pull our hypothetical *Wnt7* gene, it would also pull out every *Wnt*-related gene. We were also fortunate to design our primers in such an orientation that we could perform Nested PCR.

In the actual primer design, we later found that there might be a reason why we had less success with the primer binding. In the R2 primer, towards the 5' end, we couldn't determine a consensus sequence so we allowed it contain random nucleotides. In the 5' end there is a series of very degenerate nucleotides so it may contribute to lack of

bands in our PCR reactions. However the hope is that the 3' end would play a larger significance, and we were lucky to have two glycines at the end, providing for a stronger hydrogen bond.

In the future, we suggest redesigning the primers with new consensus sequences. We know now that we could obtain DNA from *Botrylloides* so with more specific degenerate primers, there might be some that actually bind and produce a fragment. We wish we had known this before, but the Nusse lab in Stanford has also produced a *Wnt* alignment with different organisms. Using their sequences and resources, and not to mention advice, one could create a better primer set, and may be able to have another set of nested primers. During our progress into this project, unbeknownst to us, a technology came out to test where one's primers would bind in a sequence, essentially performing a virtual PCR. We encourage anyone progressing in this project to look into this resource. As a side note, proving that we did something right, the Stanford's lab alignment and our alignment result in the same regions of conservation, as seen below. (Nusse Lab in white, ours in color).



Culturing and Larvae Manipulations

Tank manipulation was the most creatively flexible. As long as the essential properties are present as described above, tank design is arbitrary. The bigger tub was the most successful because it produced the most visibly healthy colonies probably due to the extra real estate and lack of competition for space. The pier is an ideal set-up for this experiment as it is closest to *Botrylloides*' natural habitat. Maintaining them at Cal Poly in the environmental chambers worked to an extent and kept them healthy enough to observe the effects of the various conditions. The direct application of the larvae on the slides worked best. We tried to hang slides on the sides of the tubs at the pier in hopes of larvae attaching themselves but had no luck. We attributed this to the glass not being an

ideal surface for them to settle if other surface options are available. We concluded that if we limit that option to just the glass, the larvae have no choice but to settle there.

The aeration apparatus was slightly cumbersome as it was hard to keep in place while still pumping air through the tube. A more powerful pump may be the solution but should still be small enough to fit in the environmental chamber. Also, a device to keep the tube in place will be helpful. Be sure to acquire a lot of seawater from the pier so frequent trips are reduced. Another troublesome area of the investigation was the algae build-up. Reducing the algae helps create an optimum environment for *Botrylloides*. Though *Botrylloides* does encounter algae in the wild, the aim is to reduce any kind of competition for space. Further, the algae inhibit larvae from swimming as freely as they could and also creates problems with flow, a property important in maintaining healthy colonies. Clean pipes and tank regularly.

Those interested in continuing this optimization/investigation could try to test salinity. Some research has shown that this is an important variable in ascidians. Our tests assumed salinity was rather constant but quantifying it and maybe discovery optimum salinity could allow *Botrylloides* to survive longer in lab conditions. This could be easily tested with the general outline from the other factors we tested previously.

Tissue/DNA Isolation

Tissue without the tunic was most successful in DNA isolation. Details on how to achieve this are outlined above. We attribute the strong discrepancy in DNA isolation to the fact that the tunic is made of cellulose and the cells containing the most DNA are protected by this cellulose layer. Since the tunic is made of cellulose, plant DNA

isolation kits are just as useful. This was evident in the use of our Power Soil Kit from MoBio.

Sterility was an important issue we'd like to correct. Contaminants in the water the colonies were kept in, was likely the cause. Age of reagents does affect results, so be wary of this. Volume of tissue was at times too small. This could easily be corrected by acquiring more but be sure to have plenty of colonies in stock. This puts more emphasis on the success of colony maintenance in the lab.

Those pursuing this area may look into other commercial means that produce the highest yield of DNA. The enzyme cellulase could also be explored to degrade the tunic while maintaining the integrity of the DNA.

PCR Reactions

In our experience, PCR reactions have not only been the most time consuming, but also the most challenging. We were lucky in the beginning to be able to use PCR beads, prepared PCR reactions, and we could run up to 20 reactions a week if we wanted to. As we were dealing with degenerate primers, our biggest challenge was trying to determine annealing temperatures, particularly with the *Wnt* primers. During our optimization of these primers, we tried nearly every range of temperatures possible, from 20C up to 60C and 70C.

In addition to trying to determine the annealing temperature, we also experimented with MgCl₂ concentrations and longer extension times. Both seemed to increase PCR results and are very good techniques to utilize in the future. In the end of our project we started using Master Mixes, which while decreasing our rate of reactions, allowed us more flexibility in controlling master mix components.

Our biggest accomplishment was through the use of Nested PCR. Through this process we were able to get the closest we ever could to getting an actual fragment of the gene. In our case Nested PCR, especially when done more than once, would produce the greatest yield. To get even more of a band using the same primers after a PCR clean up would produce an even more significant band.

In the future, we would suggest more experimentation with MgCl₂ and longer extension times. RNA isolation at specific times of regeneration of *Botrylloides* something we unable to do, and we feel it holds promise in determining if a *Wnt* gene is indeed involved in regeneration. We also suggest further investigation with Nested PCR. We were never able to do this, but we hoped to try and elute DNA from a specific band in our gels and use that in a Nested PCR reaction. Unfortunately we were unable to try clone and sequence a *Botrylloides* gene in our project and feel that it is an important part of this experiment. We highly suggest performing a sequencing reaction on any band close to the expected size of a desired fragment.

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