EvoDevo final paper

Molecular and Physical Relationships among Individuals in a *Phoronis vancouverensis* Clump

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Evolution and Developmental Biology
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I. Introduction - Species: *Phoronis vancouverensis*

Within the bilateria, in the group Lophotrochozoa, there are at least two phyla that have evolved coloniality: Bryozoa and Entoprocta. Most of the phyla are solitary, and reproduce only sexually (Davidson *et al*. 2004). However, several other phyla**,** such as annelids and flatworms, have the ability to reproduce asexually (Yoshida et al. 2010) (Kobayashi *et al*. 2009). Phylogenetic analysis of Phoronida and Brachiopoda showed it to form a monophyletic clade (Cohen *et al*. 1998), but some species of phoronids such as *Phoronis vancouverensis* form a clump. People have long questioned if this clump is colonial, yet I have been unable to find scientific reports that indicate how individuals of *P. vancouverensis* are related to one another within a clump. There are three possibilities, or combinations thereof, for the relationship among the clump of *P. vancouverensis*. The first possibility is that individuals of *P. vancouverensis* within a clump are solitary and reproduce only sexually. The second possibility is that *P. vancouverensis* has the ability to produce asexually by budding or fission, but individuals do not maintain connections in a clump. The third possibility is that *P. vancouverensis* is truly colonial, thus physically connected to one another and exhibits asexual reproduction in addition to sexual reproduction.

Colonial animals by definition are physically connected to one another within a colony and perform asexual (Jackson *et al.* 1977) and sexual reproduction. What causes organisms to choose in a colonial life history verses solitary is still controversial, however predation, disease and environmental disturbances are the major factors, which may cause severe damage to organisms. Under such circumstances, colonial forms sometimes seem to provide more survival benefits than solitary since a loss of a significant part of colony can be repaired and supported by the other healthier parts of the colony (Winston. 2010). On the other hand, similar damage to solitary organisms can often lead to death.

Within the colonial invertebrates, organisms my have different kinds of colonial connections. We investigated how individuals of gregarious *P. vancouverensis* were connected to each other and if they colonize, which types of colonial structure they form compared to other colonial phyla. For example, hydrara in cnidarian has been reported to form three different colony structures: *Podocoryna – Stylactaria, Podocoryna, and Podocoryna – Hydractinia* (Boero *et al*. 1998) (See Figure 1). They are comprised of perisarc (the outmost layer), coenosarc (the soft tissue that lies over the stony skeleton), and a connective part. Individuals are connected with these components but these components associate differently in the different types of colonies (Fig 1: Boero *et al*, 1998). *P. vancouverensis* was examined to see if it has one of three above mentioned hydra colonial structures.

In *P. vancouverensis*, the inner u-shaped digestive tract is enclosed by epidermal tissue. *Phoronis vancouverensis* lives and moves within the tubes they form. This tube may be attached to or embedded in sand or other kind of substrates (Santagata and Cohen, 2009). If the clump structure of *P. vancouverensis* is similar to hydra's colonial structure, *P. vancouverensis* individuals may be connected by stolon at epidermis layer; therefore, these individuals can exchange nutrition and blood vessels.

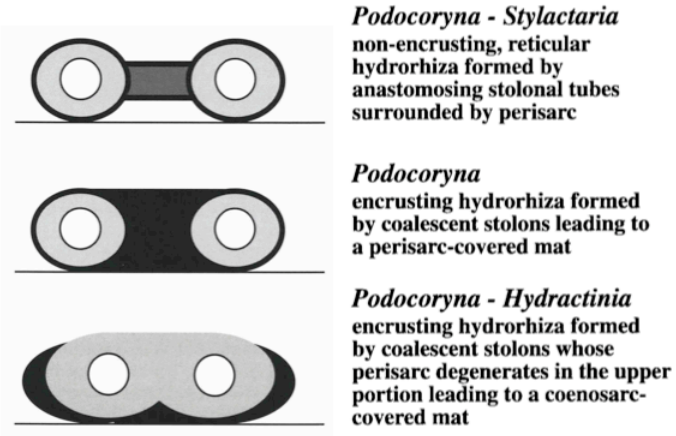

Fig. 1. Schematic representation (in section) of hydrorhizal features of the three main genera of the Hydractiniidae. Black: perisarc; dark grey: surface of transverse hydrorhizal tube connecting two main hydrorhizal tubes; light grey: coenosarc.

Figure 1. Cross section of *hydrara*, *Cnidaria* from the paper by Boero *et al* (1995)

Phoronis vancouverensis may seem to appear colonial due to its gregarious nature, yet it still has a high possibility of being solitary. The question is why it chooses to live in aggregations. It could be that the gregarious structure has unknown advantages over solidarity. Or it may be that the ancestor of phoronida was colonial and *P. vancouverensis* evolved to be solitary. However, the structure of *P*. *vancouverensis* may be reminiscent of its colonial ancestor.

II. Material and Methods

As Evolution and Developmental Biology course at the Friday Harbor Laboratories, we investigated how the individuals of *P. vancouverensis* are related to each other by comparing conserved DNA sequences from multiple *P. vancouverensis* individuals in a clump. Additionally, we examined if there was a direct physical connection between individuals by observation under a dissection microscope. Then, we compared DNA sequences of *P. vancouverensis* DNA in the clump to the sequences of other phoronids, which were available at GenBank. We also compared new sequences generated from *P*. *harmeri.*

Specimens and Genes

Phoronis vancouverensis was collected in Garrison Bay, WA and *Phoronis harmeri* was collected in False Bay in Friday Harbor, WA. A mitochondrial gene, Cytochrome *c* oxidase subunit I (COI) was used as an individual genetic marker for *P. vancouverensis* and *P. harmeri*. Cytochrome *c* oxidase I is a transmembrane protein complex and it is the enzyme found in the respiratory electron transport chain. We chose this gene because its mutation rate is fast enough to distinguish both conspecific (within the same species: individuals of *P. vancouverensis*) and heterospecific (between different species: *P. vancouverensis*, *P. vancouverensis* and other phoronis in NCBI GenBank) molecular relationships (Folmer *et al.*, 1994).

PCR

After collecting the animals, we dissected individuals and extracted DNA using a DNeasy Kit (Qiagen). Polymerase Chain Reaction (PCR) was carried out as described in Cohen and Weydamann (2005) using the universal primers HCO and LCO (Folmer *et al*., 1994). Since the PCR was successful, we isolated and purified the PCR products using gel purification kit (GE Illustra GFX kit). The purified DNA was sent for sequencing and the obtained data was used for alignment using MEGA 5 (Tomura *et al.*, 2011). We built a phylogenetic tree based on my alignment of the gene COI for individuals in *P. vancouverensis*, *P. harmeri*, and all phoronids whose sequences were available from NCBI GenBank.

DNA Mini-prep

10 DNA samples (9 individuals from *P. vancouverensis* and 1 *P. harmeri*) were purified using illustra^{TM} GFX^{TM} PCR DNA and Gel Band Purification kit. Then they were used to prepare samples for DNA sequencing. 10 μl of the individual DNA and $5\mu l$ of 5M primers were placed in each well of a sequencing plate. A total of 20 samples of DNA (10 samples for forward primer, LCO, and another 10 for reverse primer, HCO) were sent to a sequencing company.

Gene tree

The DNA sequences were saved as FASTA files under the Text Wrangler program and processed in the MEGA5 program to generate trees. Two gene trees were built for the purpose of this project. One gene tree was generated using COI from all phoronida found in GenBank; *Phoronis vancouverensis*, *Phoronis ijimal*, *Phoronis hippocrepia*, *Phoronis muellei*, *Phoronis viridis*, *Phoronis ovalis*, *Phoronis californica*, *Phoronis harmeri,* and *Phoronis australis*. All the phoronis' COI sequences obtained from a company including *P. harmeri* LCO, *P. vancouverensis* HCO and the individual *P. vancouverensis* 2, 3, 4, 5, 6, 8, and 9 were also included in this tree. I also included one species from Brachiopoda (*Terebratu*), which is monopyletic to Phoronida. For the out-groups, I used a species from Bryzoa (*Cristatella mucedo*). The second gene tree was built using just the sequence. I included only LCO reads for DNA sequence of *P. vancouverensis* individuals for this. *P. harmeri* was the out-group for the second gene tree.

Embedding and staining

To identify the relationship between the individuals, we looked for evidence of budding, or connections between individuals under a dissecting microscope. The identified tube connections and budding parts were removed from the clump using forceps and photographed using the ELMO camera and the program, BTB Pro. The isolated small pieces of phoronids in the tubes were embedded in PolyesterWax for sectioning. Sectioning and staining protocol were modified from the Animal Tissue Technique, 5th edition (1997). Prior to embedding, we used 7% $MgCl_2$ in R.O. H_2O for an hour to relax the animals, then fixed them in 4% formaldehyde in PBT for overnight. After a series of dehydration (ethanol in dH2O: 30%, 50%, 70%, 80%, 95%, 100%, 100%

each for 10mins), the animals were incubated in a 1:1 ratio of 100% ethanol to 100% polyester wax for one hour and incubated again in only 100% polyester wax at 40°C for another hour. Finally, the specimens were embedded in 100% polyester wax in the embedding mold. The embedded block was sectioned using a microtome at 7μm and first mounted in R. O. H_2O on Gelatin-Subbed slides (a manual for Gelatin-Subbed was provided at Dr. Swalla' s lab). The specimens were dried overnight. Then they were stained with trichrome (Milliagan Trichrome stain) overnight. Millgan Trichrome stain colors used were collagen green, nuclei and muscle magenta, and red blood cells orange to orange-red. The mounted slides were first hydrated with 100% ethanol twice and then 95% ethanol twice for 5mins each. Then, the staining method was continued based on Milligan Trichrome staining method, which was also provided at Dr. Swalla's lab. Finally, the slides were mounted with Promount solution under a hood, and slide observation was resumed the following day.

III. Results

PCR

Ten individuals of *Phoronis vancouverensis* were used to extract DNA for PCR but only nine individuals and one *Phoronis harmeri* were successfully purified with COI primers as a result. The Gel photo (Figure 2) clearly showed the nine bands from individuals of *P. vancouverensis* (missing one from lane 8, the *P. vancouverensis* individual 7) and *P. harmeri* at the lane 12, all at the size of 710-bp.

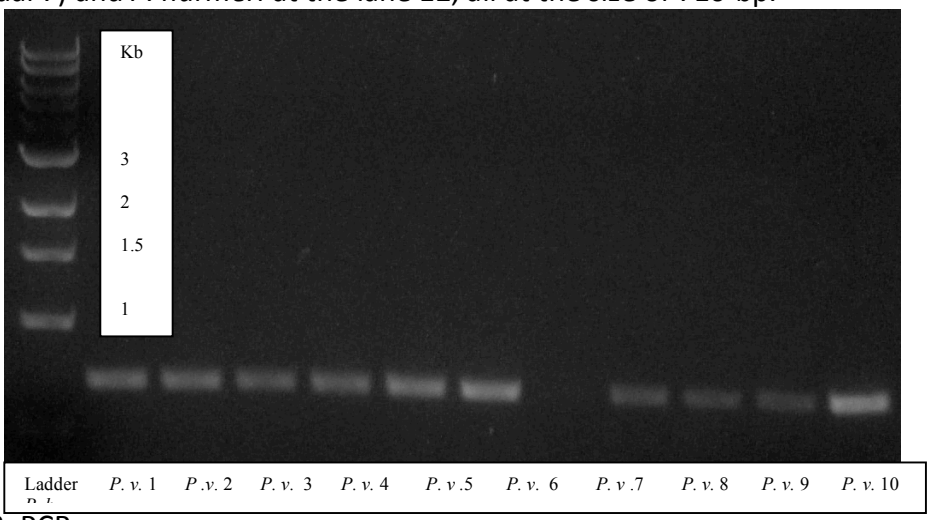

Figure2. PCR

Alignment

The obtained DNA sequences were aligned using the program MEGA5. Unfortunately, the DNA mini-prep wells were partially damaged. Thus, I was not able to obtain DNA sequences of *P. harmeri* and almost all *P. vancouverensis* (except individual number 2) from reverse primer HCO. The rest of DNA sequences were blasted to see if the sequences were reliable to use for further alignment. The DNA sequence of *P. harmeri* had a 64% match with *P. harmeri* COI in NBCI GenBank database. The DNA sequence of *P. vancouverensis* individual 1 from the forward primer (LCO) was not obtained from a company. The *P. vancouverensis* individual 2, on the other hand, had a

91% match. The *P. vancouverensis* individual 3 had 88%, the *P. vancouverensis* individual 4 had 95%, the *P. vancouverensis* individual 5 had 95%, the *P. vancouverensis* individual 6 had 88%, the *P. vancouverensis* individual 8 had 85%, and the *P. vancouverensis* individual 9 had 65% match with the *P. vancouverensis* COI in GenBank database. The *P. vancouverensis* individual 10 had a 95% match, but was excluded from gene tree generations because it was congruent with another vector. The alignment of the total 7 *P. vancouverensis* individuals showed differences in COI sequences. Alignments were used further for tree generation (Figure 4 and 5).

Tree generation

First, the gene tree was built based on COI of phornida species in GenBank (Figure 3). The gene tree of the total 7 *P. vancouverensis* individuals in a clump was generated (Figure 4). The alignment for *P. vancouverensis* was added to other phyla as outlined earlier (Figure 5).

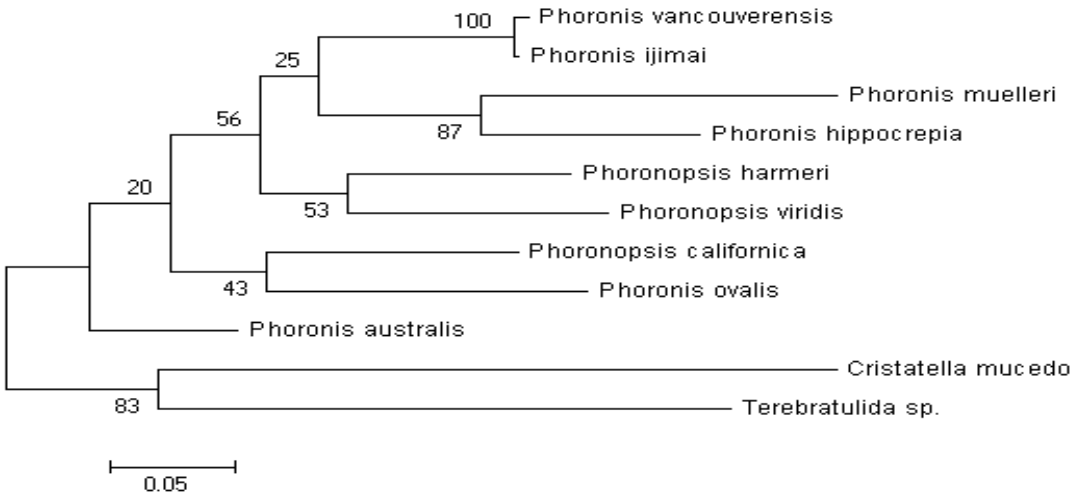

Figure 3. Heterospecific Gene tree for phoronida from NBCI GenBank including brachiopoda (*Cristatella mucedo*) and bryzoa (*Terebratulisa*)

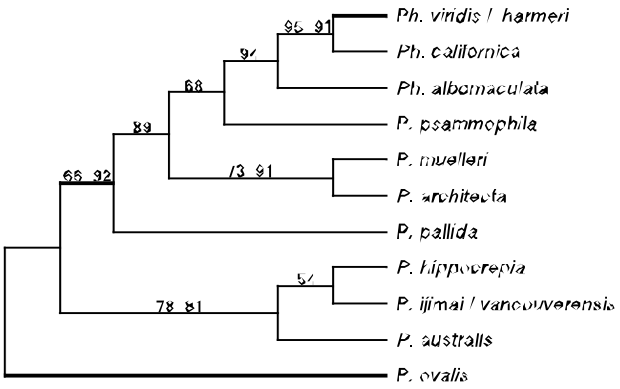

Figure 1. Phoronid phylogeny. Reweighted parsimony cladogram (length = 36.75, consistency index = 0.907, retention index = 0.899) based on a 24-character morphological data matrix with bootstrap support (%; 500 pseudoreplicates: first figure unweighted, second figure reweighted).

→figure 3_2

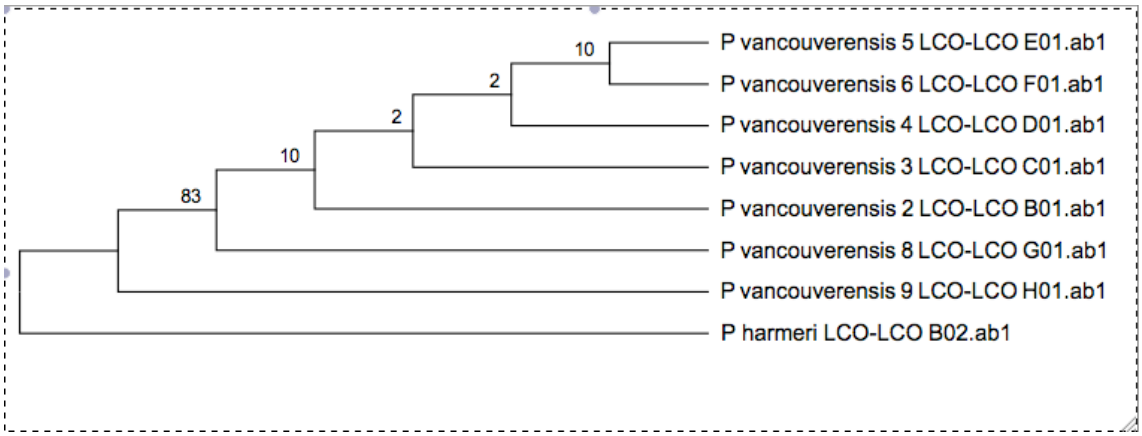

Figure 4. Conspecific Gene tree based on COI forward sequences of the individual *Phoronis vancouverensis* in a clump

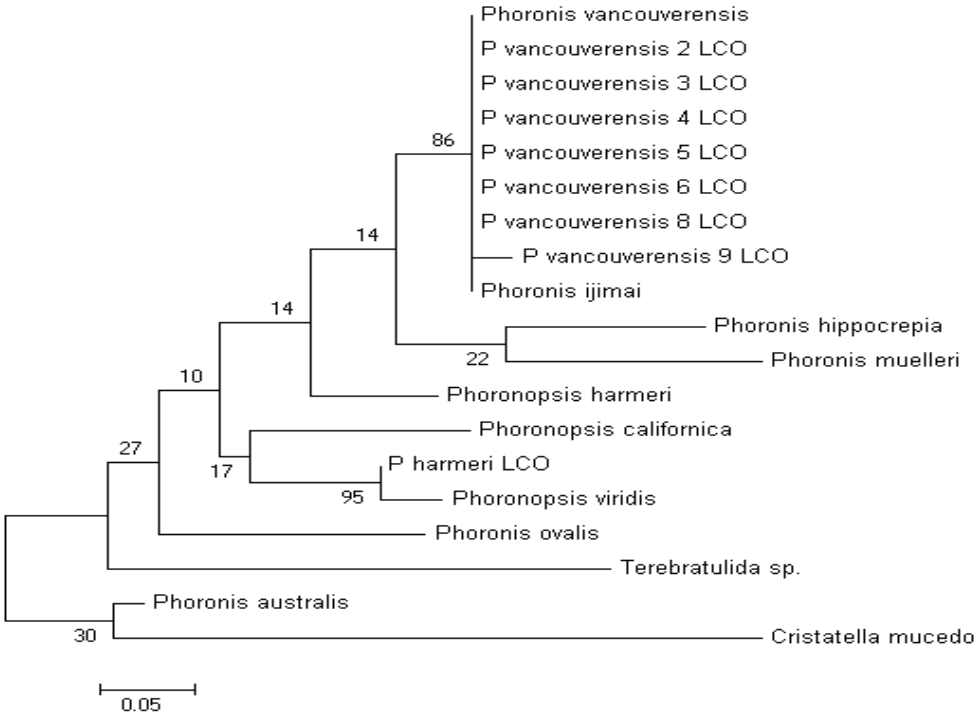

Figure 5. Heterogenic COI Gene tree among phoronida from NCBI GenBank, *P.vancouverensis* individual and outliers

Live Observation

Individual adults were thoroughly examined to identify the types of structural connections they retained. For colonial observation, I wanted to find evidence of budding or physical and direct connections between the individuals. Clumps of *Phoronis vancouverensis* were examined under a dissecting microscope. The buddings and the tubes directly connected to each other and the parts of interest were carefully cut using forceps. The pictures (Figure 6, 7, 8) showed the evidence of buddings (Figure 6, 7) and the direct connections between tubes (Figure 8).

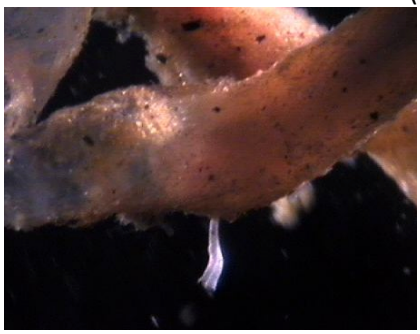

Figure 6. Evidence of budding

Figure 7. Evidence of budding

Figure 8. Evidence of tube connection

Sections

One of the polyester blocks containing budding traces in a *P. vancouverensis* tube was dissected and embedded in polyester wax. We observed more than one small elliptically-shaped structure inside of a stained *P. vancouverensis* body. However, we unfortunately did not have a time to figure out the detailed structures in each section. I did not have enough series of sections in one block to determine the structure.

III. Discussion

This project lent additional informational data to currently known phylogenetic studies. The gene tree in Figure 3 for different species of phoronids was generated by us to practice and compare the existing tree and to assess their similarities and differences. Figure 3 was generated to give a rough idea of how phoronids from NCBI GenBank were related to each other in the COI gene. The blast from the sequences of COI with LCO resulted in high percentages with both *P. vancouverensis* and *P. ijimai*. These two species were grouped together with a yield of 100% similarity in Figure 3. *Cristatella mucedo* (bryozoans) and *terebratulida sp* (brachiopoda) were used as out-groups for the gene tree (Figure 3). Both bryozoans and brachiopods are categorized under lophotrochozoa. Bryozoans have been reported to be colonial (Davidson *et al*., 2004). Brachiopoda, which is monophyletic to phoronida is, in contrast, not colonial. Those species were placed in a different group from phoronids in Figure 3. Figure 3_2 was obtained from the paper by Santagata and Cohen (2004). Compared to Figure 3_2, the numbers conveying similarity in Figure 3 were a lot lower. The effect responsible for causing the difference in numbers and the order differences between phoronids in Figure 3 and 3_2 is difficult to identify. Perhaps the locations in which the specimens were collected may contribute to this difference.

There were some initial concerns about the gene I selected to use for this project. My colleagues had pointed out that COI might be so conserved throughout the

taxa that it would not be able to provide enough information for determining *P. vancouverensis* coloniality. There was a possibility that the COI gene of one individual and the other would not show obvious genetic differences even though the individuals were genetically not identical. On the contrary, the sequence differences of COI was evidenced to be in alignment. From that prospective genetic analysis we concluded that individuals in a clump were solitary and not colonial. Unfortunately, however, the DNA with HCO (reverse primer) was not successfully sequenced, so we were therefore unable to completely align COI for each individual of *P. vancouverensis* and the *P. harmeri*. For that reason, we cannot yet conclude that the individuals in the *P. vancouverensis* clump are not colonial, since we do not know the actual COI sequences of the individuals.

The blast assured that the DNA fragments with LCO (forward primer) were indeed from the COI of *P. vancouverensis*. The blast result provided me with the high percentage match with *P. vancouverensis* in the GenBank database. For some individuals though, there were high- match percentages with *Phoronis ijimai* as well. This may have happened because current information on the morphology and reproductive characteristics of *P.* vancouverensis and *P*. *ijimai* (Wu, Chen & Sun, 1980; Zimmer, 1991) suggests that they could be a single species (Santagata and Cohen, 2009).

Figure 4 shows the relationship between the individuals of COI sequenced with LCO. *P. harmeri* was used as an out-group and was placed in different branch from *P. vancouverensis* in a gene tree. Thus, this gene tree (Fig.4) was considered valid. Figure 5 contained COI of different spices of phoronids, compared to the individuals of COI in a clump with out-grouping of bryozoans and brachiopods. We have purified COI from *P. harmeri* and sequenced the DNA with only LCO. The COI data of *P. harmeri* was also incorporated into the gene tree in Figure 5. *Phoronis harmeri* and *Phoronis viridis* were placed in a same group with the similarity of 95% in the gene tree of Figure 5. Since *P. harmeri* and *P. viridis* are morphologically similar and are easily confused, it is possible that I may have made mistakes in cueing the species. It is not impossible that I may have accidentely designated *P. viridis* as *P. harmeri*. However, *P. harmeri* LCO and *P. viridis* were placed in the same group, so the tree still retained accuracy. Also, Santagata and Cohen (2009) stated that *P. harmeri* (from Vladivostock) and *P. viridis* (from California) are genetically close enough to be conspecific.

Section

Figure 6 and 7 show there to be potential evidence of budding. We have considered them to be buddings because the portions that come out from the main phoronid tube were relatively small compared with the actual phoronid tube. This size difference might indicate that a brooding had just come out from the main phoronid body. Figure 8 shows the connections between a phoronid's tubes. The connections between tubes suggest coloniality of *P. vancouverensis* because it shows how the individual tubes were actually connected.

We have made several different types of *P. vancouverensis* blocks, including the block with the P. vancouverensis in figure 2, the blocks with a cluster of a portion of P. vancouverensis clump, and several more blocks with the portion of budding observed under a dissecting microscope. However, I did not have enough time to make sections of

all the blocks we made. Even if I made sections, with the limited time and undesirable climate (high humidity), I would only be able to obtain the sections in parts of a whole series. We needed to have a whole series of ribbons so that we could understand the structure of phoronids. Even though we did not identify every structure in the sections, we found more than one elliptically-shaped structure, which resembled a brooding.

Summary

The molecular experiment provided the result that the individuals of *P. vancouverensis* caused the sequence differences in COI. This result may be interpreted as evidence that individuals in a clump are not identical to each other. However, the possibility still exists that the individuals are colonial. It is feasible that I picked the individuals which just happened to disconnected and different from each other. LCO sequencing might not be enough to give an entire evaluation of the similarities and differences in the individuals in *P. vancouverensis* clump.

The morphological approach showed that some parts of the individuals of *P. vancouverensis* were physically connected (see Figure 8) and provided evidence of buddings like those shown in the Figures 6 and 7. Therefore, the results from the molecular approach and the physical approach may contradict one another.

There are three possibilities to explain the contradiction of *P. vancouverensis* being solitary in the molecular approach and colonial in the morphological approach. The first possibility is that the buddings we have seen were indeed the sign of coloniality. However, the COI alignment revealed many differences within the individuals because the reverse primer (HCO) required for a better alignment did not work for nine individuals out of ten. It is reasonable to assume there is a more reliable reverse primer for this procedure. The second possibility is that the individuals are solitary. The reason we evidenced the results in Figures 6 and 7 is that the little portion coming out of the tube is a larva. After a larva gets born, it may emerge and settle directly on to the parental phoronids. The last possibility is that the individuals are solitary and the little portion observed in Figures 6 and 7 are the result of regeneration. Since P. vancouverensis has an ability to regenerate, the damaged portions from transpiring may have been repaired, resulting in a little protrusion like the one displayed in Figures 6 and 7. In conclusion, whether the individuals in a *P. vancouverensis* clump are colonial or solitary has not been determined from this experiment. To investigate this matter further, the COI alignment needs to be successful with both forward and reverse primers. Additionally, a sectioning for the different polyester blocks for the P. vancouverensis (such as a cluster of a portion of a clump and one from an individual such as the subject of Figure 7) is required to determine what is contained in each section. Moreover, there are possibilities for lateral connections between P. vancouverensis tubes in a clump. Figure 8 showed the connection of the tubes as if they are extensions of buddings. The connections I monitored under a dissection microscope, did not quite show how those tubes were connected parallel to each other. Therefore the connections between tubes identified were unlike those in the cross section of *hydrara* (Figure 1). On the other hand, we still do not know how *P. vancouverensis* are connected to each other and can still not eliminate the possibility that *P. vancouverensis*

may connect like a form such as the *hydrara*. There were parallel connections among different tubes in a clump as seen from personal observations. Further investigation of the parallel and perpendicular connectivity may solve how *P. vancouverensis* is actually connected.

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V. Figure legend

Figure 1. Cross section of *hydrara*, *Cnidaria* from the paper by Boero *et al* (1995) Three possible connections in *hydrara*.1) Podocoryna, 2) Podocoryna, 3) Podocoryna.

Figure 2. Gel photo: PCR. The individuals of *Phoronis vancouverensis* DNA were amplified. The *P . vancouverensis* individual 7 was failed to amplify. The last lane was for *Phoronis harmeri* amplification.

Figure 3 Heterospecific Gene tree for phoronida from NBCI GenBank including brachiopoda (*Cristatella mucedo*) and bryzoa (*Terebratulisa*)

Figure 3_2 Phoronid Phylogeny. This figure taken from the paper by Santagata and Cohen (2004). This figure was used for the comparison to the gene tree I made using the data in NCBI GenBank.

Figure 4 Conspecific Gene tree based on COI forward sequences of the individual *Phoronis vancouverensis* in a clump. The individuals were shown not identical.

Figure 5 Heterogenic COI Gene tree among phoronida from NCBI GenBank, *P.vancouverensis* individual and outliers. Figure 5 was generated by combining Figure 3 and 4.

Figure 6. *Phoronis vancouverensis*. The evidence of budding. After removal of mud which surrounded P. vancouverensis clump, the individual P. vancouverensis was examinedcarefully to look for the budding evidence.

Figure 7. *Phoronis vancouverensis*. The evidence of budding. This individual P. vancouverensis was also fixed and embedded for the further sectioning.

Figure 8. *Phoronis vancouverensis*. The connective part of tube in *Phoronis vancouverensis* clump. Two tubes of P. vancouverensis were connected and gave Y shape structure.