A population genetics pilot study of *Phoronis pallida* in Washington's Puget Sound

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Blinks-NSF REU-BEACON Research Fellowship 2012
Summer 2012

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Keywords: horseshoe worm, *Phoronis pallida*, COI, haplotype, divergent, False Bay

Abstract

Phoronida (Lophophorata) is the perplexing, small group of marine invertebrates that contains at least 10 species of lophophore-bearing horseshoe worms. One species, *Phoronis pallida*, is an inquiline commensal that aggregates in burrows made by the mud shrimp *Upogebia pugettensis*. Similar to other species of phoronids, there are questions surrounding the biogeography and reproductive habits of *Phoronis pallida*. The purpose of this study is to examine the *P. pallida* population level structure in Puget Sound, Washington, USA, and also infer from these data if the adults of this species are capable of reproducing asexually. Adult *P. pallida* specimens were collected from the shrimp burrows in the low intertidal mud flats of False Bay, Argyle Lagoon, and Padilla Bay in Washington's Puget Sound region. Twenty-seven samples of this phoronid were collected from 16 burrows among all sites and successfully analyzed using the mitochondrial cytochrome c oxidase subunit one gene (COI). Statistical parsimony identified 24 distinct haplotypes sorted into two networks. The networks showed no geographic pattern among our sites but were separated by a large *p*-distance of 0.06 indicating either a sequence divergence. No evidence for asexual reproduction was found since only genetically distinct individuals were found from the same burrows. Further sampling at larger spatial scales is necessary to find the total number of haplotypes, determine if the networks correlate to larger geographic areas, and investigate the sequence divergence in *P. pallida.*

Introduction

Phoronids are a perplexing small group of animals composed of marine invertebrate horseshoe worms. Though Phoronida only consists of 2 genera with at least 10 species, its individual species are cosmopolitan and they inhabit most oceans. Phoronids originally confounded scientists by their deuterostome and protostome traits (Zimmer, 1991); now molecular examinations regard phoronids in the Lophophorata grouping as protostomes with a closer relation with annelids and molluscs (Halanych et al., 1995; Mallatt and Winchell, 2002). The two assemblages under Lophophorata (Phoronida and Brachiopoda) exhibit radial cleavage (Emig, 1977; Nielsen, 1990; Santagata, 2004a) and

are primarily united by a lophophore: a ciliated tentacle appendage used for respiration and suspension feeding (Herrmann, 1997).

One species, *Phoronis pallida*, is reported from numerous coastal regions in the Pacific and Northern Atlantic oceans (Emig et al., 1977). This small species can be 200-400 mm long (Emig et al., 1977) and, similar to the interaction between *P. australis* and *Cerianthus maua* (Emig et al., 1972), *P. pallida* is an inquiline commensal with the thalassinid mud shrimps *Upogebia major* and *U. pugettensis* (Kinoshita, 2002; Santagata, 2004b). *U. pugettensis* creates "Y" or "U" shaped, mucus lined burrows in tidal mud flats where they generate a water current for their suspension feeding (Johnson, 1988; Kinoshita, 2002; Griffen, 2004; Santagata, 2004b). *P. pallida* larvae, or actinotroch larvae, settle on the interior tunnel walls deep in the mud shrimp burrow and rapidly metamorphose into its vermiform adult protected by a self-secreted chitin tube (Silén, 1952; Hyman, 1958; Santagata, 2004b).

Despite the few species in this grouping, a few aspects of phoronid reproduction remain unknown (Zimmer, 1991). Some species exhibit asexual fission or budding in addition to sexual cycles like *P. ovalis* (Marcus, 1949; Zimmer, 1991). Though *P. pallida* is confirmed to be a non-brooding hermaphrodite (Silén, 1952), there is curiosity if this species is also capable of asexual reproduction.

To date, no studies have examined the genetic structure of phoronid populations. Though Ayala et al. (1974) examined the biochemical variability for a population of *Phoronopsis harmeri*—formerly *P. viridis* (Emig, 1974; Santagata & Cohen, 2009), they did not consider its genetic variability or diversity over a large area (i.e. multiple bays). In this study, we examined the genetic diversity of *Phoronis pallida* in the Northeast Pacific. The goals of this study are to determine the *P. pallida* population level structure—which includes genetic diversity and size—as well as infer if *P. pallida* undergoes asexual reproduction by exploring if individuals within the same burrow are genetic clones.

Methods

Phoronid samples were collected from False Bay (Lattitude 48º29'04" North; Longitude 123º4'5" West), Argyle Lagoon (Latitude 48º31'12" North; Longitude 123º0'52" West),

and Padilla Bay (Latitude 48º29'38" North; Longitude 122º29'02" West) in the Pacific Northwest of Washington, USA (Figure 1) during the summer of 2012. *Phoronis pallida* collections were extracted from intertidal *Upogebia pugettensis* mudflat burrows at the low tide. Since the greatest densities of the phoronid occurred in the base of U-shaped or at the intersection of Y-shaped burrows, individual sampling was maximized by hand collecting sediment from these locations and filtering through a 1 mm sieve (see Santagata, 2004b).

To assess occurrences of asexual reproduction, the yields of *P. pallida* individuals from each burrow were recorded and kept isolated from other burrows for later genetic analysis. Specimens collected from the same burrow shared the numeric digit in their sample code but differed in the ensuing alphabetical character. Sampling sites were selected to best depict the genetic variability for the San Juan Island and the broader Northeast Pacific region. In Washington State, we collected phoronids from shrimp burrows in False Bay and Argyle Lagoon from San Juan Island, as well as Padilla Bay on the Washington mainland.

Using a dissecting microscope and sterilized forceps, the lophophores of individuals were separately removed from each sampled individual and placed into 1.5 ml centrifuge tubes while the remaining worm bodies were preserved in 1.5 ml tubes containing 0.65 ml of 100 % ethanol. DNA extraction protocol followed the DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, USA) protocol. Total genomic DNA was eluted into 100 µl of AE Buffer.

Genetic comparisons used the cytochrome c oxidase subunit one complex (COI) in the mitochondrial DNA with phoronid-specific forward and reverse primers. A Clontech Advantage® PCR Kit (Takara Bio Inc., Otsu, Shiga, Japan) supplied reagents for amplification reactions. In the PCR protocol, 1 µl of DNA was added to microcentrifuge tubes containing 2.5 µl of 10x reaction buffer (containing 10 Mm magnesium chloride), 0.5 µl 50x dNTPs mix (10 Mm each), 0.5 µl of the forward primer, 0.5 µl of the reverse primer, 0.5 µl of polymerase mix, and 19.5 µl of water. PCR was conducted in an Eppendorf Mastercycler® Gradient (Applied Biosystems, Hamburg, Germany) using the parameters of a 5 min 95 ˚C initial denaturation step followed by 35 cycles of denaturation steps at 95 ˚C for 40 sec, 50 ˚C annealing steps lasting for 45 sec,

72 ˚C extensions for 1 min, and an 8 min final extension step at 72 ˚C. PCR products were confirmed with an electrophoresis run through 1.8 % agarose gel using 30 ml of sodium borate buffer ($Na_2B_4O_7 + H_3Br_3$) (Jankowsky, 2010) for 8 minutes at 295 V. Negative controls for PCR used cocktail ingredients with water and were processed in parallel to monitor for contamination (Figure 2). Reactions were cleaned with using Invitrogen PureLink® (Life Technologies Corporation, Grand Island, New York, USA) purification reagents and manufacturer instructions. Fifteen microliters of cleaned PCR product was delivered to Macrogen© for sequencing.

Chromas Lite® Version 2.01 software (Technelysium Pty Ltd, Queensland, Australia) displayed data for visual inspection and alignments were performed by Muscle (Edgar, 2004). Molecular comparisons utilized *MEGA* version 5 (Tamura, Peterson, Stecher, Nei, and Kumar 2011) with 1,000 replicates in the bootstrapping. Asexual reproduction was based on the number specimens with identical haplotypes found in a single burrow. Distinct haplotypes and haplotype networks were determined using TCS (Clement et al., 2000) software.

Results

We collected 31 samples from the 3 sites in the Puget Sound, WA (Figure 1). False Bay on the Western side of San Juan Island yielded 16 *P. pallida*. Two of the burrows supplied 3 and 7 worms. Sampling from Argyle Lagoon on Eastern San Juan Island yielded 6 worms—three of which came from a single burrow. In Padilla Bay near the Padilla Bay National Estuarine Research Reserve Interpretive Center, we collected 9 worms from two burrows that yielded 3 and 6 worms. Of our collections, 30 samples allowed sufficient tissue for analysis with no contamination in the PCR product and each reaction producing consistently sized PCR product of about 1000 base-pairs in length (Figure 2).

Macrogen successfully sequenced 27 of the samples. Target sequence length was 951 bp with mean base frequencies of A = 0.2347, T = 0.3325, C = 0.2088, and G = 0.224. Of the 27 samples, 24 distinct haplotypes were detected based on the TCS analysis. We performed a Maximum Likelihood model test based on all three codons in MEGA to find the best nucleotide substitution model. According to the Bayesian

Information Criterion scores, the Hasegawa-Kishino-Yano (HKY+G) model described the best substitution pattern with a gamma distribution value equaling 0.22135. The statistical parsimony from the TCS operation also divided the haplotype data into 2 networks (Figure 3)—an arrangement similarly reflected by the HKY+G model (Figure 4B). The genetic distance within Network 1 is 0.0084, and the genetic distance within Network 2 is 0.0066. The networks are separated by a genetic distance of 0.059. See Table 1 for distances among sampled sites.

The phylogenetic trees did not commonly reflect a strong similarity between worms sampled from identical burrows except for specimens TF8A and TF8E from Padilla Bay as well as TF13B and TF13C from Argyle Lagoon. Though not genetic clones, these two pairs of adult phoronids reflected small *p*-distances of 0.001 between the TF8 specimens and 0.006 between the TF13 specimens.

Discussion

From this preliminary study, 27 sequenced samples produced 24 haplotypes for *P. pallida* collected from 3 sampling locations in the Puget Sound of Washington State. These haplotypes are grouped into 2 networks separated by a genetic *p*-distance of nearly 0.06.

It is certain we under-sampled the haplotype diversity for the Puget Sound. Chen et al. (2010) collected 150 cephalotrichid nemerteans to find 90 unique haplotypes that composed 15 networks. In another case, researchers recorded 121 distinct haplotypes from 279 polychaete worms, *Pygospio elegans* (Kesäniemi et al., 2012). Because we sampled 27 phoronids and found 24 haplotypes, it is reasonable to expand this project to further sampling within the San Juan Islands and identify more haplotypes. This will increase the genetic diversity resolution and determine more resolved population structures.

With regard to any population structure determined in this study, observations from the bootstrapped and maximum likelihood trees did not indicate a geographic and genetic correlation: the haplotypes appeared to occur indiscriminately over the sampled range. Though specimens from False Bay did compose the majority of Network 1, this probably represents a sampling bias in that we collected about twice as many phoronids from False Bay than the other sites.

This geographic mix most probably indicates a meeting of the two networks at a more extensive geographic extent. In one study, researchers found two genetically distinct clades of a brown hare to overlap in an area the size of Bulgaria (Kasapidis et al., 2004). Because it takes about 5-6 weeks for the *Phoronis pallida* larvae to reach metamorphic competency (Santagata, 2004a), it is possible that the larvae can drift over larger areas than just local bays in the San Juan Islands thus it more likely to find a resolved genetic and geographic correlation at a much broader spatial scale when considering sampling locations.

Due to the low occurrence of genetically similar adults found in this study and no genetic clones originating from the same mud shrimp burrow, there is no support for asexual reproduction in *P. pallida* from our sampling. The phylogenetic trees (Figure 3) do not indicate asexual reproduction. Though it is possible that sequencing error might be responsible for some genetic difference, such as the slight difference between TF8 and TF13 specimens, the occurrence of genetic similarities from the same burrows is rare in this data set. Therefore, even if by some chance asexual budding does occur in this phoronid, it does not appear to significantly influence the fecundity of *P. pallida* populations. Further study with a greater yield of *P. pallida* from more burrows is needed before any more consideration can be pursued regarding asexual reproduction.

A notable observation from this study was finding the 0.06 *p*-distance between the two networks. When examining the global diversity of cephalotrichid nemerteans, Chen et al. (2010) found between two morphologically different species (*Cephalothrix simula* and *C. fasciculus*) a genetic difference of 0.056 (Kimura-2-parameter distance between networks 8 and 11 was 5.8). Kasapidis et al. (2004) found two clades for a species of brown hare (*Lepus europaeus*) to be likewise defined by a *p*-distance of 0.066. However, one study of the genetic diversity for *Pygospio elegans* measured several *p*-distances as high as 0.065 between sampled populations from the Netherlands and from Maine, USA (Kesäniemi et al., 2012), but they did not conclude a separate species. So because these genetic divergences can be specific to each species, it likely possible this phoronid may have a more divergent COI sequence than other species. Typically mtDNA is more divergent than nuclear DNA with some few exceptions (Wiklund et al., 2009). Santagata and Cohen (2009) found a consistent yet unanticipated large genetic distance between

other phoronid species. This fact, combined with possible reproductive isolation from its lifestyle of living in deep mud burrows, could explain this large genetic distance between networks as molecular quality exhibited by *Phoronis pallida.* Hence the suggestion of cryptic speciation by the 0.06 distance in our study for now is ambiguous until further sampling and analysis addresses the variety of genetic *p*-distances existing in the *P. pallida* populations. In addition to analyzing this genetic divergence with mtDNA, it would behoove future studies to employ other genetic markers—such as nuclear 18S or 28S genes (Wiklund et al., 2009)—to support the conclusions from these COI analyses. If further molecular investigation supports the concept of *P. pallida* and a cryptic species, then it is recommended that subsequent studies employ environmental and physiological comparisons between these two networks before assumptions can be made regarding the potential for a cryptic species.

Future aspects of sampling could include Vancouver Island in Canada and several bays in Oregon (Dumbauld et al., 2011) to accommodate a broader geographic scale and investigate other populations. A drift card study conducted by Terrie Klinger found that cards released into the Puget Sound from Point Caution, San Juan Island, WA (Latitude 48º33'43.34" North; Longitude 123º01'01.65" West) only were recovered from sites south of Doughty Point, Orcas Island, WA (Latitude 48º42'39.03" North; Longitude 122º57'14.55" West). There could be genetically distinct *P. pallida* populations in the mudflats of more northern islands such as Sucia Island or Patos Island. Because *P. pallida* is a commensal with mud shrimp, gaps in *Upogebia* occurrence along the coastline could cause genetically isolated *P. pallida* populations. A study by Chapman et al. (2011) found some populations of *Upogebia* along the U.S. West Coastline to have collapsed or even gone extinct because of a parasitic bopyrid isopod, *Orthione griffensis* (Dumbauld et al., 2011). Within the *Upogebia* shrimp range from Vancouver Island, Canada, to Baja California are numerous bays that were formerly inhabited by *Upogebia* before the isopod devastated the mud shrimp populations and created breaks in their distribution (Chapman et al., 2011). Of these breaks, a notable region where mud shrimp populations are allegedly extinct is in Central California between Bodega Harbor and Elkhorn Slough. This large separation in *Upogebia* could allow for a northern population to be genetically distinct compared to any populations to the south of this area.

The results of this study should be taken with consideration to its small sampling size. From this study, *P. pallida* populations appear to express noticeable sequence divergence at its COI mtDNA. This is either the result of cryptic speciation or a regular but previously unrecorded attribute to its genetic behavior. Also, at the scale of sampling in the Puget Sound, there is not apparent population structure. Future work on broader sampling scales most likely will yield a genetic and geographic correlation for this species. With regard to investigating the phoronid genetic diversity in each mud shrimp burrow, there was little occurrence of genetic similarity and no indication of asexual reproduction due to the absence of genetic clones. Future work can further investigate these aspects by increasing the sampling range and sample size of sequenced *P. pallida* specimens.

Acknowledgements

Special thanks to Billie Swalla, Sophie George, and Jaquan Horton for summer project support. Very special thanks to Scott Schwinge, Adam Summers, and Emily Carrington for arranging for this summer undergraduate experience. And a special thanks to all of the undergraduate student researchers for their help and assistance to make this project possible.

References:

Ayala, F. J., Valentine, J. W., Barr, L. G., & Zumwalt, G. S. (1974). Genetic variability in a temperate intertidal phoronid, *Phoronopsis viridis*. *Biochemical Genetics*, 11(6), 413-427.

Chapman, J. W., Dumbauld, B. R., Itani, G., & Markham, J. C. (2011). An introduced Asian parasite threatens northeastern Pacific estuarine ecosystems. *Biological Invasions*. doi :10.1007/s10530-011-0151-3

Chen, H., Strand, M., Norenburg, J. L., Sun, S., Kajihara, H., Chernyshev, A. V., Maslakova, S. A., & Sundberg, P. (2010). Statistical parsimony networks and species assemblages in cephalotrichid nemerteans (Nemertea). *PLoS ONE*, 5(9).

Clement, M., Posada, D., & Crandall, K. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, 9(10), 1657-1660.

Dumbauld, R. D., Chapman, J. W., Torchin, M. E., & Kurls, A. M. (2011). Is the collapse of mud shrimp (*Upogebia pugettensis*) populations along the Pacific coast of North America caused by outbreaks of a previously unknown bopyrid isopod parasite (*Orthione griffenis*)? *Estuaries and Coasts*, 34, 336-350.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32(5), 1792-1797.

Emig, C. C. (1974). The systematics and evolution of the phylum Phoronida. *Zeitschrift für zoologishe Systematik un Evolutions-forschung*, 12, 128-151.

Emig, C. C. (1977). Embryology of Phoronida. *American Zoologist*, 17(1), 21-37.

Emig, C. C., Boesch, D. F., & Rainer, S. (1977). Phoronida from Australia. *Records of the Australian Museum*, 30(16), 455-474.

Emig, C. C., Herberts, C., & Thomassin, B. A. (1972). Sur l'association de *Phoronis australis* (Phoronida) avec *Cerianthus maua* (Ceriantharia) dans les zones récifales de Madagascar. *Marine Biology*, 15, 304-315.

Gilchrist, J. D. (1919). Reproductive by transverse fission in *Phoronopsis*. *Quarterly Journal of Microscopical Sciences*, 63, 493-507.

Griffen, B. D., DeWitt, T. H., & Langdon, C. (2004). Particle removal rates by the mud shrimp *Upogebia pugettensis*, its burrow, and a commensal clam: effects on estuarine phytoplankton abundance. *Marine Ecology Progress Series*, 269, 223-236.

Halanych, K. M., Bacheller, J. D., Aguinaldo, A. M. A., Liva, S. M., Hillis, D. M., & Lake, J. A. (1995). Evidence from 18S ribosomal DNA that the Lophophorates are Protostome animals. *Science*, 267, 1641-1642.

Herrmann, K. (1997). Phoronida. In F. W. Harrison, and R. M. Woollacott, (Eds), *Microscopic Anatomy of Invertebrates, Vol.13, Lophophorates, Entoprocta, and Cycliophora* (207-255). New York, NY: Wiley-Liss, Inc.

Hyman, L. H. (1958). The occurrence of chitin in the Lophophorate phyla. *Biological Bulletin*, 114(1), 106-112.

Jankowsky, J. (2010). *Buffers and Broths*. Retrieved from Baylor College of Medicine, The Jankowsky Lab in the Department of Neuroscience, Neurosurgery, and Neurology Web site: http://neuro.bcm.edu/jankowskylab/wp-content/uploads/2010/08/Buffers-and-Broths4.doc

Johnson, A. S. (1988). Hydrodynamic study of the functional morphology of the benthic suspension feeder *Phoronopsis viridis* (Phoronida). *Marine Biology*, 100, 117-126.

Kasapidis, P., Suchentrunk, F., Magoulas, A., Kotoulas, G. (2005). The shaping of mitochondrial DNA phylogeographic patterns of the brown hare (*Lepus europaeus*) under the combined influence of Late Pleistocene climatic fluctuations and anthropogenic translocations. *Molecular Phylogenetics and Evolution*, 34, 55-66.

Kesäniemi, J. E., Rawson, P. D., Lindsay, S. M., & Knott, K. E. (2012). Phylogenetic analysis of cryptic speciation in the polychaete *Pygospio elegans*. *Ecology and Evolution*, 2(5), 994-1007.

Kinoshita, K. (2002). Burrow structure of the mud shrimp *Upogebia major* (Decapoda: Thalassinidea: Upogebiidae). *Journal of Crustacean Biology*, 22(2), 474-480.

Lüter, C. (2000). The origin of the coelom in Brachiopoda and its phylogenetic significance. *Zoomorphology*, 120, 15-28.

Mallatt, J. & Winchell, C. J. (2002). Testing the new animal phylogeny: first use of combined large-subunit and small-subunit rRNA gene sequences to classify the protostomes. *Molecular Biology and Evolution*, 19(3), 289-301.

Nielsen, C. (1990). The development of the brachiopod *Crania* (*Neocrania*) *anomala* (O. F. Müller) and its phylogenetic significance. *Acta Zoologica* (Stockholm), 72(1), 7-28.

Nielsen, C. (2002). The phylogenetic position of Entoprocta, Ectoprocta, Phoronida, and Brachiopoda. *Integrative and Comparative Biology*, 42(3), 685-691.

Santagata, S. (2004a). Larval development of *Phoronis pallida* (Phoronida): implications for morphological convergence and divergence among larval body plans. *Journal of Morphology*, 259, 347-358,

Santagata, S. (2004b). A waterborne behavioral cue for the actinotroch larva of *Phoronis pallida* (Phoronida) produced by*Upogebia pugettensis* (Decapoda: Thalassinidea), *Biological Bulletin* 207(2), 103-115.

Santagata, S., & Cohen, B. L. (2009). Phoronid phylogenetics (Brachiopoda; Phoronata): evidence from morphological cladistics, small and large subunit rDNA sequences, and mitochondrial *cox1*. *Zoological Journal of the Linnean Society*, 157, 34-50.

Silén, L. (1952). Researches on Phoronidea of the Gullmar Fjord area (west coast of Sweden). *Arkiv för Zoologi*, 4(2), 95-140.

Silén, L. (1954). Developmental Biology of Phoronidea of the Gullmar Fjord area (west coast of Sweden). *Acta Zoologica*, 35(3), 215-257.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*, 28, 2731-2739.

Wiklund, H., Glover, A. G., Johannessen, P. J., & Dahlgren, T. G. (2009). Cryptic speciation at organic-rich marine habitats: a new bacteriovore annelid from whale-fall and fish farms in the North-East Atlantic. *Zoological Journal of the Linnean Society*, 155, 774-785.

Zimmer, R. L. (1991). Phoronida. In A. C. Giese, J. S. Pearse, and V. B. Pearse, (Eds), *Reproduction of Marine Invertebrates, Vol. 6, Echinoderms and Lophophorates* (1-45). Pacific Grove, CA: The Boxwood Press.

Figure 1. Map San Juan Island system and Padilla Bay composing sampling sites in Puget Sound as indicated by colored markers (False Bay in blue, Argyle Lagoon in red, and Padilla Bay in yellow). **Inset Map** Broader view of Washington State and Southwestern Canada.

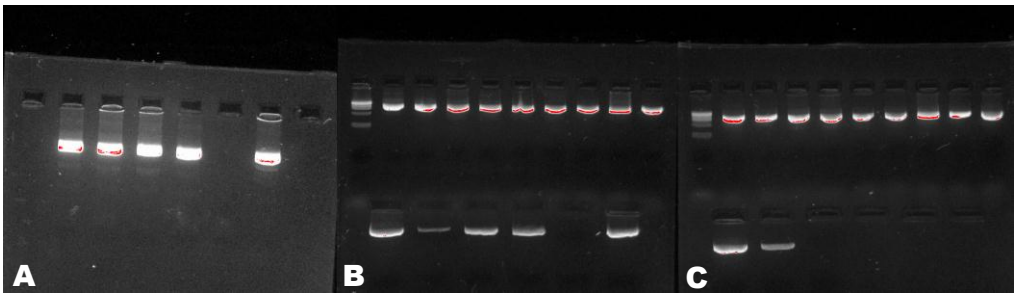

Figure 2. Electrophoresis reactions from PCR. **Gel A** was run on June 26 for samples TF1-TF3 (the sixth well was the water control). **Gel B** was run on July 9 for samples TF4-TF9A (TF8F did not amplify). **Gel C** was also run on July 9 for samples TF9B-TF14C with the first blank well containing the water control.

Table 1. Below are the number of sampled adult worms, number of haplotypes, the genetic distances for within each site, and between each site.

Sites	No. of *P. pallida*	No. of Haplotypes	*p*-Distance within Sites	*p*-Distance from False Bay Site	*p*-Distance from Argyle Lagoon Site	*p*-Distance from Padilla Bay Site
False Bay	16	12	0.0252	N/A	0.017	0.027
Argyle Lagoon	6	5	0.0065	0.017	N/A	0.022
Padilla Bay	9	7	0.0328	0.027	0.022	N/A

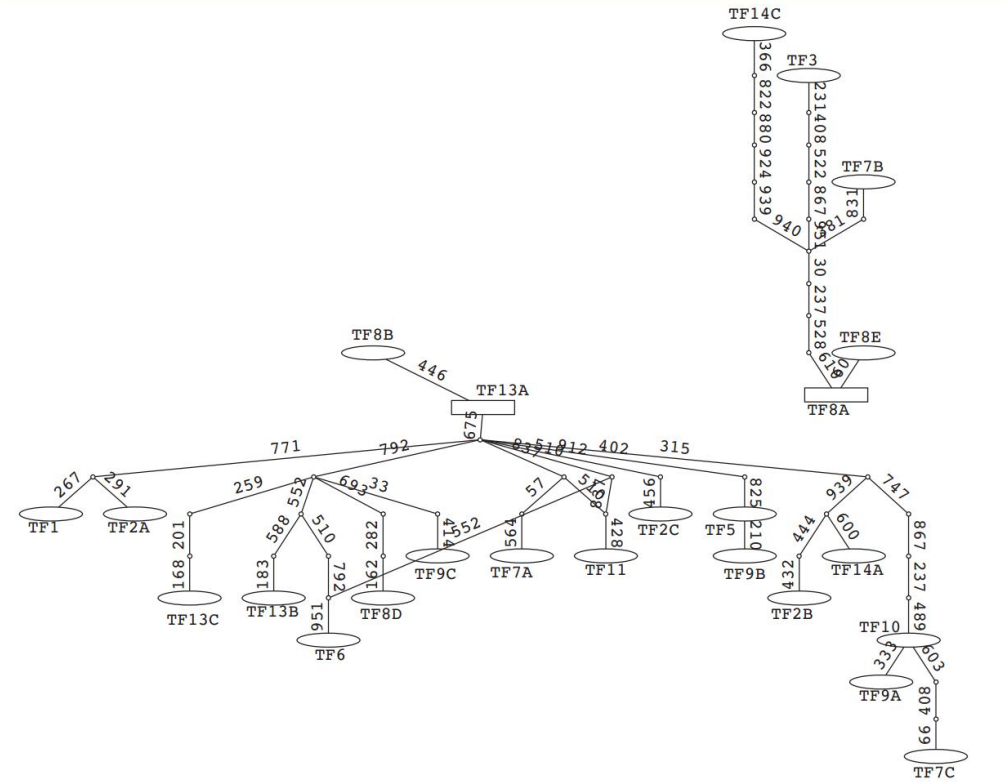

Figure 3. Output for the 24 haplotypes using TCS phylogenetic network estimation software v1.21. Smaller circles at nodes indicate intermediate haplotypes and number represent the position of the substitution within the alignment. The haplotypes are grouped into two different networks

Figure 4. A Maximum likelihood trees of phoronid relationships based on a HKY+G bootstrapped model with 1000 replicates using 3 codon sites displays the position for each sampled *P. pallida* adult in the phylogenetic analysis, **B** network labeled HKY+G maximum likelihood model differentiates the two network compositions in a similar fashion as the TCS output in Figure 3.