A population genetics study of the wood-boring bivalve, *Xylophaga washingtona,* from wood-fall experiments along OR and WA, USA

Amanda Ziegler[1]

Deep-Sea Biodiversity, Connectivity and Ecosystem Function
Summer 2014

[1] Department of Oceanography, University of Hawaii at Manoa, Honolulu, HI, 96822

Contact information:
Amanda Ziegler
Department of Oceanography
University of Hawaii at Manoa
1000 Pope Rd.
Honolulu, HI 96822
ziegler8@hawaii.edu

Keywords: Xylophaga washingtona, population genetics, connectivity, wood-boring

ABSTRACT

A widely distributed species of wood-boring bivalve, *Xylophaga washingtona*, was collected from three sites along the Washington-Oregon margin and assessed with molecular techniques. 16S gene sequences revealed low differentiation between individuals from the two sites on the outer continental slope suggesting that these sites harbor a single population. However, greater genetic distance was observed between the slope individuals and those collected from a shallow-water site in the San Juan Islands, Washington. These results suggest the presence of at least two distinct populations of *Xylophaga washingtona* along the Washington-Oregon margin and the potential for species-level differences. The juxtaposition of the two hydrographic settings supports the hypothesis that isolation of the shallow-water population has resulted in sufficient genetic differentiation over fairly short geographic distances. Further research is needed to verify these observed patterns and to explore whether morphological differences support the proposed population and species structure.

INTRODUCTION

Wood falls represent understudied reducing habitats created by large falls of organic-rich substrate. In previous experimental deployments of various wood substrates (Distel and Roberts, 1997, Voight, 2007, Bienhold et al., 2013, McClain and Barry, 2014) specialized fauna has been recovered including wood-boring bivalves of the families Teredinidae and Pholadidae (*Xylophaga* spp.), as well as isopods (Limnoridae), gastropods, and many polychaetes. These fauna are important ecosystem engineers acting to transform highly refractory terrigenous debris that is otherwise nutritionally unavailable to most

organisms. *Xylophaga* (Pholadidae, Bivalvia) is known to harbor bacterial endosymbionts which enable it to consume large volumes of wood, actively remineralizing this substrate back to a form available to microbial communities (Distel and Roberts, 1997). Members of the genus *Xylophaga* have been documented from the subtidal to 7000m and are quite tolerant of dissolved oxygen levels as low as 0.3 ml/L (Turner, 2002). Further, *X. washingtona* is known to occur from the west coast of British Columbia to Santa Barbara, CA (Voight, 2007) with a type locality of San Juan Island, Washington (Bartsch, 1921). This species is distinct from others of the same genus as well as other closely related members of the Pholadidae family by the presence of a truncated excurrent siphon, a fairly triangular mesoplax, and the *in situ* presence of fecal material both within the burrows and forming a fecal chimney at the borehole opening (Turner, 2002).

The specialized nature of the Xylophagainae subfamily suggests that their distributions would be heavily constrained by the presence and delivery of woody material to the seafloor. The patchy distribution of these organic-substrate islands brings into question genetic connectivity and population level relationships of these fauna. It was noted by Turner (1973) that colonization of juvenile *Xylophaga* spp. occurred up to 48 days after deployment of wood substrates at 2032m suggesting that this species has the potential for wide dispersal and rapid, opportunistic colonization. However, Tipper (1968) hypothesized that the larvae of *X. washingtona* would not likely disperse greater than 3-8m (10-25ft) above the seafloor based on adult and borehole distributions. These observations emphasize the need for population characterization of deep-sea wood-borers.

Though *X. washingtona* has a vast latitudinal distribution along the west coast of the United States, the degree to which individual populations disperse and are genetically connected across several hundred kilometers remains unknown. Generally, the genetic connectivity of benthic organisms is limited by dispersal capability across large distances (e.g. by pelagic larval duration) and the isolation of populations via physical barriers. Dispersal barriers can be large topographic features (e.g. fracture zones), water mass structures (e.g. semi-permanent to permanent fronts, large currents), or patchy habitat distribution. Depth is also recognized to structure populations as it covaries with many other environmental parameters including light, POC flux, temperature and salinity (Costantini et al., 2011). The presence of dispersal barriers can become more apparent through the use of genetic techniques. Comparison of 16S mitochondrial DNA gene sequences from individuals of the same species at different sites can reveal if there is sufficient genetic diversity between populations to indicate limited gene flow between populations. To date, there have been very few population genetics studies of wood-fall fauna, most of which have explored microbial rather than macrofaunal diversity (Fagervold et al. 2013), phylogenetics (Johnson et al. 2008, Wiklund et al. 2012) or investigations of non-specialist, associated fauna (Thubaut et al. 2013). It is often difficult to interpret connectivity data because a very small amount of gene flow between populations may be sufficient to prevent large genetic structuring that would otherwise occur via isolation (Palumbi, 2003). Several connectivity models have been proposed including the 'Island Model' and the 'Stepping-Stone Model' (Palumbi, 2003). These models predict the mechanism by which populations may show varying levels of genetic connectivity between habitats that are spatially isolated. Therefore, this study aimed to

determine whether deep-sea populations of *X. washingtona* along the Oregon and Washington margin of the United States exhibit genetic exchange through the use of molecular analyses. It is hypothesized that individuals collected from the type locality of *X. washingtona* (San Juan Island, WA) will be genetically distinct from individuals collected at two deep sites along the Washington and Oregon margin. These differences are hypothesized to exist as a result of complex flow and bathymetry in the San Juan Island region causing sufficient isolation of these populations, likely resembling the "stepping-stone" model of connectivity.

METHODS

Study Sites

To study the genetic connectivity of dominant wood-fall fauna, 5-10 individuals of the shallow-water, wood-boring bivalve, *Xylophaga washingtona,*were collected at each of the three sites along the Pacific Northwest of the United States. Two sites, BoWL 2 (47° 57.462' N, 126° 2.118'W) and BoWL 6 (43° 54.522'N, 125° 10.238'W) (Figure 1), were part of an NSF-funded experimental deployment of whale bone (*Megaptera novaeangliae*), wood, (*Pseudotsuga menziesii*) and artificial substrates at depths of 1596m and 1624m, respectively. These substrates remained at depth for a 15-month period until they were recovered. The third site, FHL BoWL (48° 32' 41.71"N, 123° 0' 42.58"W), was located roughly 20m from the pier at Friday Harbor Laboratories, Friday Harbor, WA (Figure 1) in a water depth of approximately 15-17m depending on tidal cycle. This experimental deployment was similar to those deployed as part of the Bone and Wood Landers (BoWLs) project, however all specimens from FHL BoWL in this

study were collected from a natural wood fall located <5m from the experimental deployment which was opportunistically found and sampled by divers. The type of wood and duration of submergence was undetermined. All individuals sampled were identified

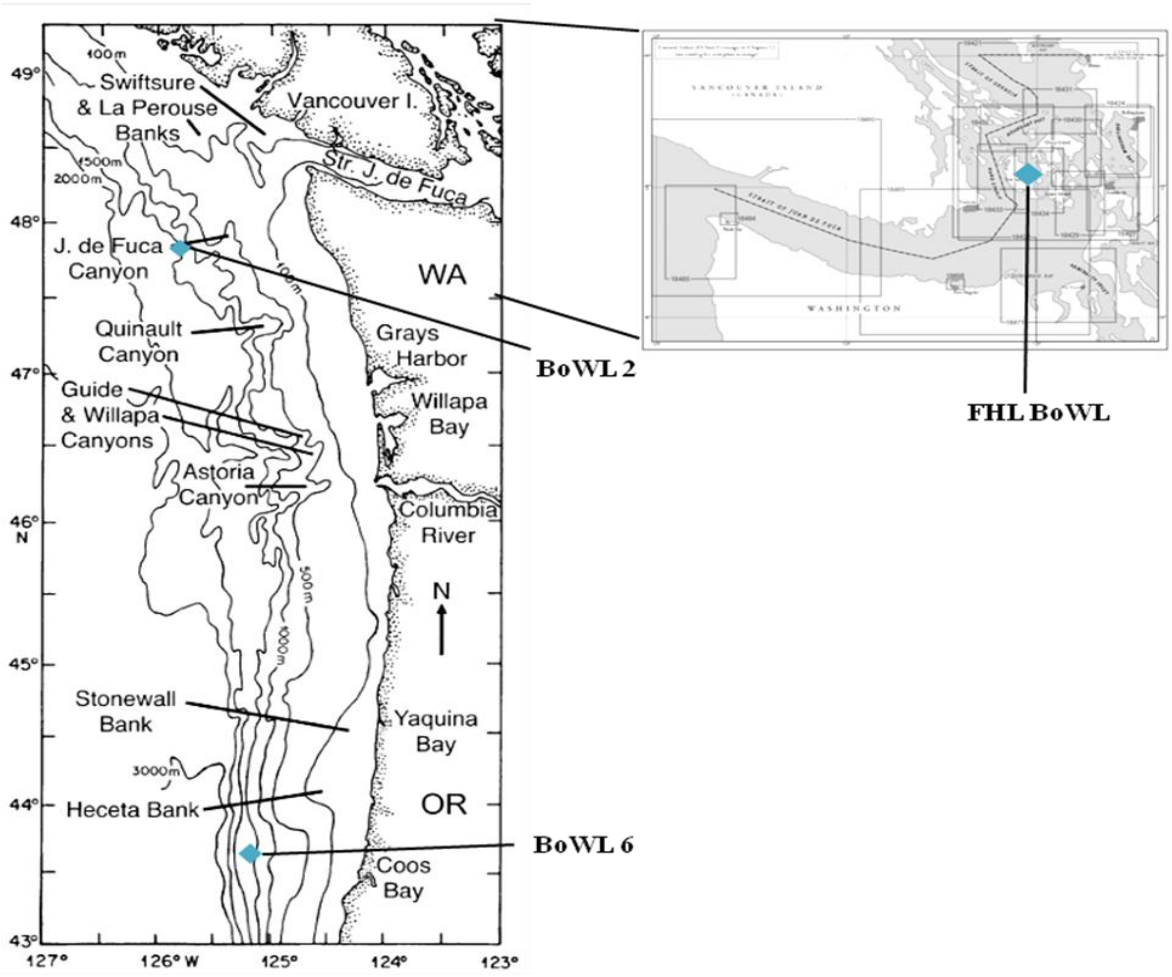

Figure 1.
Locations of experimental wood deployments at BoWL 2, BoWL 6, and FHL BoWL indicated by blue diamonds. (modified from Hickey and Banas 2003 and Nauticalcharts.NOAA.gov)

as *Xylophaga washingtona* using identification literature and comparison with expert-identified voucher specimensrecovered from the same sites (Turner, 2002, Voight, 2007, and Voight, pers. com.). The main identification characteristics utilized were the overall shape of the mesoplax and a truncated siphon (Turner, 2002) (Figure 2).

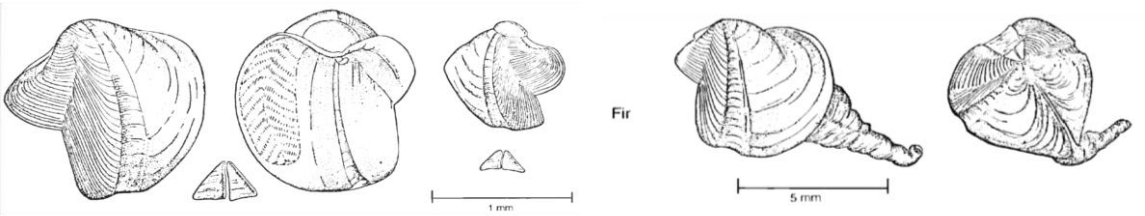

Figure 2.
Morphology of *Xylophaga washingtona* as referenced in Turner, 2002. Key distinguishing features include the truncated siphon (right) and triangular mesoplax (left).

DNA Extractions, PCR and Sequencing

Following collection of the experimental wood substrates, visible epifauna was removed from the surface and the blocks were sectioned into 4 identical pieces. At least one of the quarters was preserved whole in 95% ethanol while others were preserved in 4% formalin, dried, or frozen. Only pieces stored in ethanol were utilized for this study. Wood blocks from the two shallow BoWLs and the FHL BoWL were destructively sampled for *Xylophaga washingtona.*5-10 individuals were removed and identified as outlined above while in 95% ethanol. DNA was extracted from *X. washingtona* siphon tissue using DNeasy blood and tissue kits (QIAGEN). Body tissue was extracted for those collected from the shallow FHL BoWL as siphons were not intact. This could account for some differences in extraction product quality. Extractions were run on a 60µl agarose gel (0.6 mg agarose/60ml water, 1%) stained with 6µl of SYBERSAFE dye in 1X TBE at roughly 100 volts to verify that extractions yielded DNA. Polymerase chain reaction (PCR) was performed in 50µl reactions via Promega kits (Figure 3) and run on a heated-lid thermocycler.

Figure 3. Reagent volumes used per 50µl PCR reaction. Note that DNA template was diluted 1:10 with nuclease-free water.

Component	Volume
5x Green or Colorless GoTaq® Flexi Buffer	10µl
$MgCl_2$ Solution, 25mM	8µl
PCR Nucleotide Mix, 10mM each	1µl
Upstream primer (16Sa, universal primer)	0.5µl
Downstream primer (16Sb, universal primer)	0.5µl
GoTaq® G2 Flexi DNA Polymerase (5u/µl)	0.25µl
Template DNA	4µl
Nuclease-Free water	25.75µl

PCR began with a 2-minute heating period to 94°C, followed by a denaturation period of 30 seconds at 94°C. Annealing was carried out for 1 minute at 40°C, followed by a 1-minute period of extension at the optimal operating temperature of Taq polymerase of 72°C. The denaturation, annealing and extension steps were then repeated for 35 cycles total and a final 5-minute extension at 72°C ended the program. PCR products were held in the thermocylcer at 6°C at the end of the program until they were moved to a 4°C refrigerator prior to gel electrophoresis. PCR products were run on a 1% agarose gel at roughly 60-80 volts to verify amplification of the proper base pair length expected for the 16S gene (470-500bp). Successful PCR products were purified using a PCR purification kit (QIAGEN) or a gel extraction kit (QIAGEN) prior to sequencing which was conducted by GENEWIZ Inc., Seattle, Washington.

Sequence Processing and Tree Building

Sequences were inspected and trimmed using Geneious® version 7.1.7 (available from Geneious.com) and those sequences that were of poor quality were removed from further

analyses (Figure 4). Consensus sequences for each individual were generated from De Novo assemblies of forward and reverse sequences returned from GENEWIZ Inc. The potential identities of these consensus sequences were checked utilizing the nucleotide BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) which helped reveal contamination of some samples. Multi-alignments using MUSCLE with 12 iterations (Edgar, 2004) were conducted to align consensus sequences of *X. washingtona* individuals as well as a closely related outgroup, *Bankia fimbriatula*, whose sequence was retrieved from GenBank (AY633503.1, 16S). Bayesian inference and maximum likelihood trees were generated using MRBAYES on XSEDE v3.2.2 (Huelsenbeck and Ronquist, 2001, Ronquist and Huelsenbeck, 2003) and RAxML-HPC BlackBox v8.0.24 (Stamatakis et al., 2008) with 1000 bootstrap replicates in the CIPRES Science Gateway V. 3.3 (http://www.phylo.org).

Sample ID	Location	Comments
AZ_SB_1	FHL BoWL	
AZ_SB_2	FHL BoWL	Sequencing failed
AZ_SB_3	FHL BoWL	
AZ_SB_4	FHL BoWL	PCR failed - not sequenced
AZ_SB_5	FHL BoWL	De Novo alignment failed
AZ_W15_1	BoWL 2	De Novo alignment failed
AZ_W15_2	BoWL 2	De Novo alignment failed
AZ_W15_3	BoWL 2	PCR failed - not sequenced
AZ_W15_4	BoWL 2	PCR failed - not sequenced
AZ_W15_5	BoWL 2	
AZ_W15_6	BoWL 2	
AZ_W22_1	BoWL 6	Poor alignment
AZ_W22_2	BoWL 6	De Novo alignment failed
AZ_W22_3	BoWL 6	De Novo alignment failed
AZ_W22_4	BoWL 6	PCR failed - not sequenced
KM_W22_1	BoWL 6	Contamination
KM_W22_2	BoWL 6	De Novo alignment failed
KM_W22_4	BoWL 6	
KM_W22_5	BoWL 6	Contamination
KM_W22_6	BoWL 6	Poor alignment
KM_W22_8	BoWL 6	Contamination

Figure 4. Sample details. Grey highlighting indicates samples that were used in tree assembling.

RESULTS

Twenty one sequences were received from GENEWIZ inc. but after inspection of the sequences, it was determined that only five would be utilized for further analyses (Figure 4). BLAST comparison revealed contamination of a few samples

and others suffered from very low quality. Several sequences were particularly noisy and it was clear that the confidence in nucleotide reads was quite low. After trimming the forward and reverse reads several individuals (again listed in Figure 4) did not contain contiguous regions between them and therefore would not undergo proper De Novo assembly. Inspection of single direction reads revealed poor quality sequences and it was decided that these individuals would be fully removed from the tree-building analyses. Only five individuals appeared to assemble and align properly to allow for tree-building and those results are presented here.

The combined Bayesian inference and maximum likelihood tree shows two samples retrieved from BoWL 2 and one sample from BoWL 6 grouping more closely to each other than to the two samples from the FHL BoWL with posterior probability values of 100 and 90, respectively (Figure 5). The same relationships were observed in the maximum likelihood tree with 99% and 100% of the best trees retaining the grouping of both BoWL 2 and BoWL 6 samples as well as 91% of trees maintaining a separation between the FHL BoWL individuals. Branch lengths between the two major clades of the trees are quite long representing substantial genetic differences.

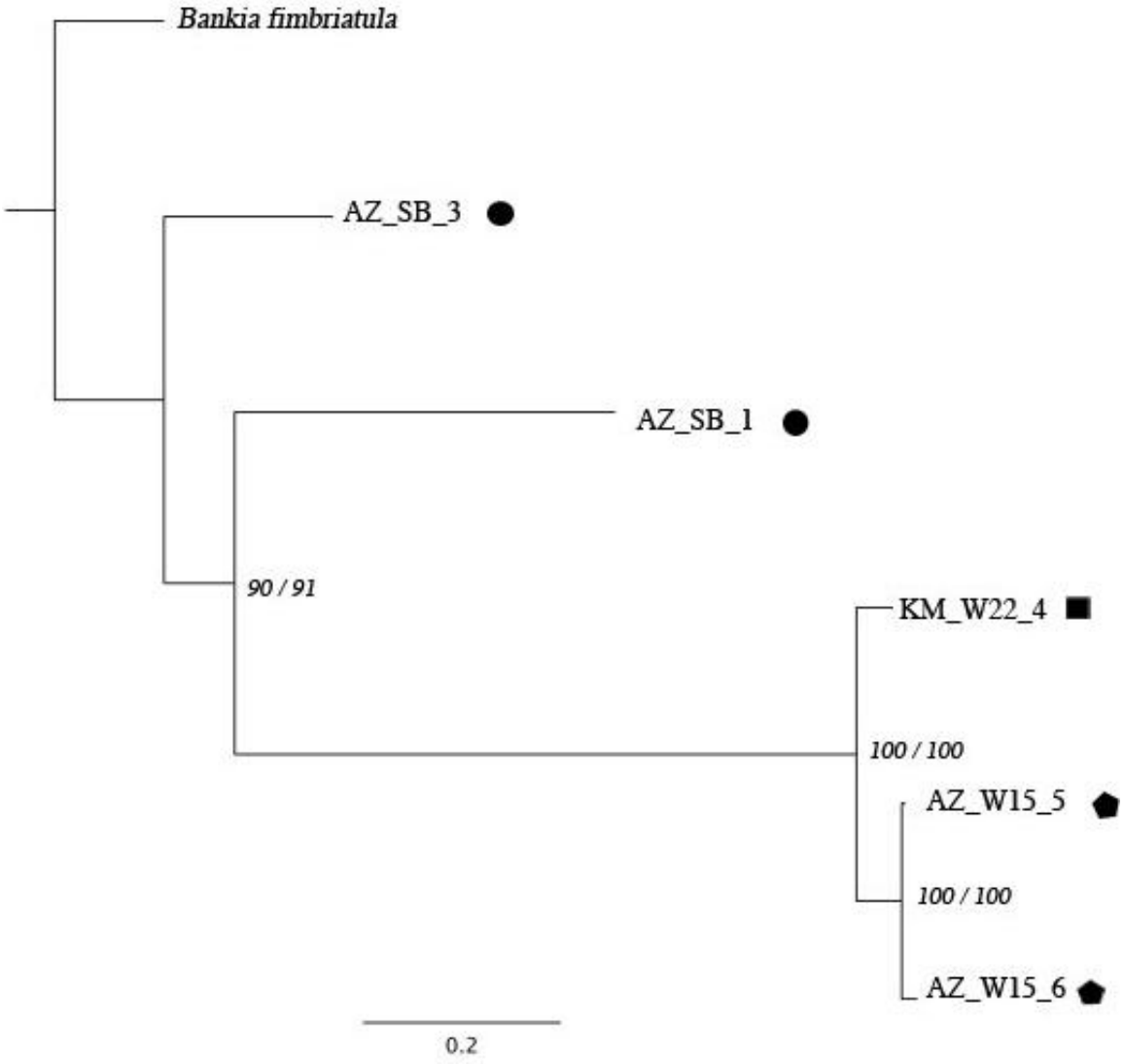

Figure 5. Combined Bayesian inference and maximum likelihood tree displaying (posterior probability values / bootstrap values). ● FHL BoWL ■ BoWL 6 ⬟ BoWL 2

DISCUSSION

Clustering of sequences from BoWL sites 2 and 6 suggests that these locations experience sufficient genetic exchange to be considered a single population. The individuals from the FHL BoWL clustered more closely to each other than to those from

BoWL 2 and 6 suggesting that these populations are distinct and do not exhibit sufficient gene flow to inhibit genetic changes. Despite the poor quality of the sequence data, the observed relationships were maintained across both analyses and were always supported with high (≥90%) bootstrap and posterior probability values. The branch lengths separating the individuals from FHL BoWL were quite long compared to those in the clade of BoWL 2 and 6 indicating that there is greater genetic distance between the shallow-water individuals which could suggest further population structure within this region. These results also suggest the potential for species-level differences between the shallow water *Xylophaga washingtona* and those found at deep sites within the observed latitudinal range of the species.

Hydrography and connectivity

BoWL 2 and 6 were <500km apart along a seemingly uninterrupted hydrographic setting, while the FHL BoWL was located <300km from the northern-most BoWL site (6) and separated by the Olympic Peninsula, narrow Strait of Juan de Fuca, and complicated bathymetry of the Salish Sea region. Dispersal of *Xylophaga* larvae would be expected to differ between these two regions as a result of the different hydrographic settings. The major surface flow at BoWL 2 and 6 is generally to the south in the California Current though there is evidence of a slight seasonal shift to the north in the Davidson and California Counter Currents (Hickey and Banas, 2003).It is unlikely that surface flow will influence the dispersal of deep-dwelling *Xylophaga* spp. as greatly as flow at depth considering there have been no reports of ontogenetic migration by the species or retrieval of larvae at the surface, however limited research exists on reproduction and

dispersal of this taxon. Flow at depth in this region has been previously modeled and revealed that it is slow (<1cms^{-1}) and anticyclonic in nature which causes a predominantly southward flow along the eastern boundary of Cascadian Basin (Hautala et al., 2009). In addition, the Strait of Juan de Fuca undergoes seasonal flow changes in which major storm events and changes in the prevailing winds can result in an onshore transport of water rather than a flushing of the strait (Hickey and Bana, 2003). This could deliver larvae into the Salish Sea sporadically which may be enough to maintain gene flow. However, the Salish Sea region is further complicated by the presence of many persistent fronts, eddies, fjord circulation, and much greater freshwater influx as it represents a very large estuarine habitat (Cannon, 1978) all of which likely prevent larval exchange between oceanic and estuarine waters in this area. Further, the extreme depth difference between these sites (FHL BoWL at <20m and BoWL 2 and 6 at >1500m) alone may contribute greatly to the observed genetic variation as mentioned previously (Costantini et al., 2011). The juxtaposition of these two hydrographic settings and hypothesized phenomena affecting dispersal support the observed differences between sites for *X. washingtona* in this study.

Future Studies

To fully characterize the population structure of *Xylophaga washingtona* along the Pacific Northwest coast, extensive collection and observation of wood-boring bivalves must be conducted. Both natural and experimental wood falls should be utilized as these each provide insights into specialized wood-fall fauna as well as the potential to conduct genetic analyses. This study suffered from poor sequence quality which limited the

number of individuals that could be included in the population analyses. This is likely a result of non-specific binding of primers during PCR reactions, contamination of samples, or high temperatures used in the sequencing process. Future studies should aim to collect and analyze more individuals to enhance confidence in observed relationships as well as to investigate within-population variation. The results of this study suggest that there are at least two distinct populations of *Xylophaga washingtona* along the Washington-Oregon margin, but it is likely that there are species-level differences between these populations which should be verified by extensive morphological comparisons. Furthermore, the genetic distances between individuals within the shallow-water population from FHL BoWL indicate the poor resolution of the 16S gene. It would be useful to compare genes that evolve on faster time scales such as CO1or nuclear DNA markers. Finally, though a "stepping-stone" model of connectivity is supported by the findings of this study, targeted research of the larval dispersal of *Xylophaga washingtona* is needed. Determining the population structure and connectivity of this species is important as this taxon represents a link in oceanic carbon cycling and acts as an ecosystem engineer in the deep sea.

ACKNOWLEDGEMENTS

Funding for this study was provided as part of an NSF supported project (OCE-1155188). The captain and crew of the *R/V Oceanus* made the deployment and recovery of the bone and wood landers possible. Friday Harbor Laboratories, Friday Harbor, WA provided facilities and scholarship funding for attendance to the Deep-Sea Biodiversity, Connectivity and Ecosystem Function course as well as during construction of the

landers. Kirsty McQuaid supplied additional sequences (denoted by ‘KM’ in sample names). Dr. Craig Smith and Dr. Kenneth Halanych provided expertise and guidance during this project.

REFERENCES

Bartsch, P. (1921). A new classification of the ship-worms and descriptions of some new wood boring mollusks. Proceedings of the Biological Society of Washington, (34): 25-32.

Bienhold, C., Ristova, P. P., Wenzhöfer, F., Dittmar, T., & Boetius, A. (2013). How deep-sea wood falls sustain chemosynthetic life. PloS one, 8(1), e53590.

Cannon, G. A. (1978). Circulation in the Strait of Juan de Fuca: some recent oceanographic observations (Vol. 399). Department of Commerce, National Oceanic and Atmospheric Administration, Environmental Research Laboratories.

Costantini, F., Rossi, S., Pintus, E., Cerrano, C., Gili, J. M., & Abbiati, M. (2011). Low connectivity and declining genetic variability along a depth gradient in Corallium rubrum populations. *Coral reefs*, *30*(4), 991-1003.

Distel, D. L., & Roberts, S. J. (1997). Bacterial endosymbionts in the gills of the deep-sea wood-boring bivalves Xylophaga atlantica and Xylophaga washingtona.The Biological Bulletin, 192(2), 253-261.

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

Fagervold, S.K., Bessette, S., Romano, C., Martin, D., Plyuscheva, M., Le Bris, N., Galand, P.E. (2013). Microbial communities associated with the degradation of oak wood in the Blanes submarine canyon and its adjacent open slope (NW Mediterranean). Progress in Oceanography (118): 137-143.

Hautala, S. L., Paul Johnson, H., & Hammond, D. E. (2009). Bottom water circulation in Cascadia Basin. *Deep Sea Research Part I: Oceanographic Research Papers*, *56*(10), 1688-1707.

Hickey, B. M., & Banas, N. S. (2003). Oceanography of the US Pacific Northwest coastal ocean and estuaries with application to coastal ecology.Estuaries, 26(4), 1010-1031.

Huelsenbeck, J. P., & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics, 17(8), 754-755.

Johnson, S. B., Warén, A., & Vrijenhoek, R. C. (2008). DNA barcoding of Lepetodrilus limpets reveals cryptic species. Journal of Shellfish Research,27(1), 43-51.

McClain, C., & Barry, J. (2014). Beta-diversity on deep-sea wood falls reflects gradients in.

Palumbi, S. R. (2003). Population genetics, demographic connectivity, and the design of marine reserves. Ecological applications, 13(sp1), 146-158.

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19(12), 1572-1574.

Stamatakis, A., Hoover, P., Rougemont, J. (2008). A Rapid Bootstrap Algorithm for the RAxML Web-Servers. Systematic Biology, 75(5): 758-771.

Thubaut J, Corbari L, Gros O, Duperron S, Couloux A, et al. (2013) Integrative Biology of Idas iwaotakii (Habe, 1958), a 'Model Species' Associated with Sunken Organic Substrates. PLoS ONE 8(7): e69680. doi: 10.1371/journal.pone.0069680

Tipper, R. C. (1968). Ecological aspects of two wood-boring molluscs from the continental terrace off Oregon.

Turner, R. D. (1973). Wood-boring bivalves, opportunistic species in the deep sea. Science, 180(4093), 1377-1379.

Turner, R. D. (2002). On the subfamily Xylophagainae (Family Pholadidae, Bivalvia, Mollusca). Bull.../Museum of comparative zoology (Cambridge, Mass.),157(4).

Voight, J. R. (2007). Experimental deep-sea deployments reveal diverse Northeast Pacific wood-boring bivalves of Xylophagainae (Myoida: Pholadidae).Journal of Molluscan Studies, 73(4), 377-391.

Wiklund, H., Altamira, I. V., Glover, A. G., Smith, C. R., Baco, A. R., & Dahlgren, T. G. (2012). Systematics and biodiversity of Ophryotrocha (Annelida, Dorvilleidae) with descriptions of six new species from deep-sea whale-fall and wood-fall habitats in the north-east Pacific. Systematics and Biodiversity, 10(2), 243-259.