Identification and Distribution of a Bone-eating Marine Worm (Annelida, *Osedax*)

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Deep Sea Biodiversity, Connectivity, and Ecosystem Function

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Keywords: Osedax, phylogenetic, whale fall, deep sea

ABSTRACT

Ephemeral reducing environments such as hydrothermal vents and whale falls create oases of chemosynthetic primary production in the nutrient poor deep sea. The polychaete family Siboglinidae has adapted to occupy these niches of chemical nutrition. This likely requires high levels of dispersal, either through long-living larvae or a "stepping-stone" dispersal method. Here we seek to identify recently found specimens of *Osedax* from whalebones off the coast of Oregon and Antarctica. Once species identity was determined, we sought to elucidate connectivity between known populations of *Osedax* from Monterey Bay and Sagami Bay. To accomplish this, we sequenced the mitochondrial marker COI and inferred relationships through a haplotype network and maximum likelihood tree.

INTRODUCTION

Siboglinidae are a family of annelids typically found in reducing environments such as hydrothermal vents and methane seeps, as well as deep-sea muds (Schulze and Halanych 2003). These organisms can be dominant fauna in such environments, and are often found in large groups close to vents or seeps, or on whalebones where they can absorb the dissolved chemicals. Interestingly, they lack a functional gut as adults and instead rely on endosymbiotic bacteria for nutrients (Cavanaugh et al. 1981, Hilário et al. 2011). In this mutualistic relationship, millions of bacteria can live within either a specialized internal organ called a trophosome or a root-like structure (Southward and Southward 1988, Goffredi et al. 2005).

Siboglinidae is comprised of four major lineages: frenulates, vestimentiferans, monoliferans, and *Osedax* (Rouse et al. 2004). Vestimentiferans include large tubeworms that can be found on hydrothermal vents, cold seeps, and whale falls (McMullin et al. 2003, Smith et al. 2015). Frenulates comprise 75% of the known siboglinid species and are thread-like and found within sediments of reducing environments (Hilario et al. 2010, Thornhill et al. 2008). Monilifera is a single genus, *Sclerolinum*, which shares similarities to frenulates, but can also be found on decaying organic material (Halanych et al. 2001). Finally *Osedax*, a recent discovery, are worms that colonize whalebones (Rouse et al. 2004).

Endosymbionts in most siboglinids are chemoautotrophic gamma proteobacteria (Thornhill et al. 2008). However in 2004, a new group of siboglinids, *Osedax*, was found on whalebones off the California coast (Rouse et al. 2004). *Osedax* use heterotrophic endosymbionts belonging to *Oceanospirillales*, instead of the chemoautotrophs that form symbioses with the other Siboglinids (Goffredi et al. 2005). There are approximately 31 known species of *Osedax* and earlier phylogenetic analysis placed them as sister to a moniliferan-vestimentiferan clade (Smith et al. 2015), but recent analyses suggest a position as sister to frenulate siboglinids (Glover et al. 2013). *Osedax* females do not contain a trophosome, like other siboglinids. Instead, they have a vascularized “root” system that pervades bones that they are colonizing. This root system houses endosymbiotic bacteria and allows them to process the fats and lipids within the whalebones (Rouse et al. 2004, Goffredi et al. 2005). Males of *Osedax* exhibit extreme sexual dimorphism and are microscopic, existing in harems inside females’ mucus tubes. This allows for eggs to be released and immediately fertilized (Rouse et al. 2009).

The genus *Osedax* is found in all the world's oceans (Rouse et al. 2004, Glover et al. 2005, Glover et al. 2013, Amon et al. submitted). To date, species have differed between oceans such that each ocean contains unique species of *Osedax*. In December 2013, an *Osedax* species was discovered in the Southern Ocean that did not match descriptions of endemic *Osedax*. Instead, it was morphologically similar to *Osedax rubiplumus*, endemic to the Pacific (Braby et al. 2007). Also, bone and wood landers off the Oregon coast recently brought up multiple *Osedax* individuals some of which were morphologically identified as *O. rubiplumus*. In this study, we sought to identify this Antarctic *Osedax* and individuals from the bone and wood landers from the Oregon coast using the mitochondrial marker COI. Once identified, we examined connectivity of the specimens through haplotype diversity analyses and generation of a Maximum Likelihood tree.

MATERIALS AND METHODS

Samples from Antarctica were collected from Flandres Bay off the West Antarctic Peninsula (Table 1). Two unidentified pieces of highly colonized bone were recovered using a Blake trawl. Samples of *Osedax* were taken and preserved in 100% ethanol after morphological comparison to earlier described species of Antarctic *Osedax* (Glover et al. 2013, Amon 2014). Identification did not match any previously described Antarctic *Osedax* species. The newly found *Osedax* resembled *O. antarcticus*, however the palps were covered in pinnules; a characteristic of *O. rubiplumus*. Therefore, molecular analysis was required to confidently determine species identity.

Table 1. Location data and amount of sequences retrieved. No latitude and longitude data was available for the Japanese samples.

Location	Number of Sequences	Unique Sequences	Latitude/Longitude
Monterey Bay	93	33	36.708˚N, 122.434˚W
Sagami Bay, Japan	4	2	N/A
Oregon Coast	4	2	43.909˚N, 125.171˚W
Antarctica	1	1	65.100˚S, 63.166˚W

DNA from one individual of the unidentified Antarctic *Osedax* was extracted using a Qiagen Blood/Tissue kit (Qiagen). DNA was sent to Hudson Alpha in Huntsville, AL for whole genome sequencing on an Illumina MiSeq and sequence assembly was performed with Ray (Boisvert et al. 2012). The mitochondrial genome was identified by BLAST (Zhang et al. 2000) searching against the genome of *Riftia pachyptila* before annotation by the MITOS web server (Bernt et al. 2013).

Samples from the northeast Pacific were taken off the Oregon coast (Table 1). Six bone and wood landers (BoWLs) were deployed in April 2013 and four were recovered in June-July 2014. Of these four, two contained bones that had been colonized by *Osedax*. Samples were taken and preserved in 100% ethanol. Morphological identification confirmed the presence of *O. rubiplumus* and other unknown species. Four samples, two individuals of *O. rubiplumus* and two unknown individuals, were taken from BoWLs station six for molecular analysis. Samples will be referred to as A3370, A3383, A3390, and A3391.

Palp tissue was taken and cleaned of mucus before DNA extraction using a DNeasy Blood & Tissue extraction kit protocol (Qiagen). PCR was performed using ddH_20, 2μL GoTaq Flexi Buffer, 5μL of magnesium chloride, 1μL of PCR nucleotide mix, 1μL of forward primer, 1μL of reverse primer, 1μL of extracted DNA and 0.25μL of GoTaq G2 Flexi DNA Polymerase for a total volume of 50μL (Promega) to amplify. Amplified DNA was purified using the QIAquick PCR Purification Kit (Qiagen). Purified DNA was sent to Genewiz in Seattle, WA for sequencing. Trace files were manually evaluated for any erroneous bases. Forward and reverse sequences were aligned and merged into consensus sequences using Geneious 7.1.7.

For Antarctic and Oregon samples, BLAST searches against NCBI's database were performed to determine identity of samples. COI was extracted from the Antarctic *Osedax* mitochondrial genome and aligned with samples from Oregon as well as all published *Osedax rubiplumus* COI sequences (Table 2) using the MUSCLE plugin for MEGA6.06 (Edgar 2004, Tamura et al. 2013). Missing data was trimmed from the beginning and end of the alignment. The resulting alignment was used to generate a TCS network showing inferred connections between haplotypes (Clement, Posada, and Crandall 2000). Due to the amount of identical haplotypes, this alignment was manually trimmed down to unique sequences and used to generate a Maximum Likelihood tree with 1,000 Bootstrap replicates using the RAxML blackbox web server (Stamatakis, Hoover, and Rougemont 2008).

Table 2. Accession number by location of all published *Osedax rubiplumus* sequences used in this study

Location	Accession Numbers
Monterey Bay, California	EU852420-EU852488 EU223297-EU223311 DQ996616-DQ996620
Sagami Bay, California	FM998060-FM998063

RESULTS

Four bone and wood landers were recovered off the Oregon coast. Two landers had *Osedax* colonization on the six bones. Samples from BoWLs station six were taken, four different individuals from four different bones. Testing of the DNA extraction, PCR, and PCR purification was performed with gel electrophoresis, which indicated high molecular weight DNA was extracted and PCR products were amplified. Sequencing of both the Antarctic and northeast Pacific samples was successful with the Antarctic sample producing a COI sequence of 1524 nucleotides, and the Oregon coast samples producing 804, 994, 883, and 749 for A3370, A3383, A3390, and A3391 respectively. BLAST results indicated strongly that specimens were of *O. rubiplumus.* Trimming of missing data resulted in an alignment of 535 base pairs.

The TCS network resulted in 36 unique haplotypes of the 98 used (Figure 1). A3370 and A3391 had the same COI haplotype, as well as A3383 and A3390. Reticulations were resolved by removing connections farthest from the ancestral haplotype (Templeton and Sing 1997). The two unique Oregon coast haplotypes differed by one single nucleotide polymorphism. The maximum likelihood tree resulting from the trimmed unique haplotypes has low support values for most nodes.

DISCUSSION

Through BLAST searches, all unknown individuals of *Osedax* were identified as *O. rubiplumus*. Antarctic vents have been discovered without siboglinid tubeworms, potentially indicating that the Antarctic Circumpolar Current (ACC) is a barrier to dispersal for Siboglinid larvae (Rogers et al. 2012). However, these data indicate that *O. rubiplumus* larvae are able to disperse across the ACC. On Monterey Canyon whale falls off central California, USA, *O. rubiplumus* has been shown to rapidly colonize and then be replaced by *O. frankpressi* (Goffredi et al. 2007). Perhaps *O. rubiplumus* uses a "stepping-stone" method of dispersal, colonizing whales quickly and spawning new cohorts that can then move on and colonize other whale falls. They may be more successful at this method of dispersal considering they have some of the largest larvae of all the *Osedax* species observed (Rouse et al. 2009). Alternatively, there could be insufficient hydrothermal vents forming a path to allow for vent Siboglinids to utilize this "stepping-stone" dispersal method. Without competition from endemic northeast Pacific species, it seems *O. rubiplumus* is able to colonize a bone for longer periods, due to the recovered whalebone in the Antarctic being highly degraded and dominated by *O. rubiplumus.*

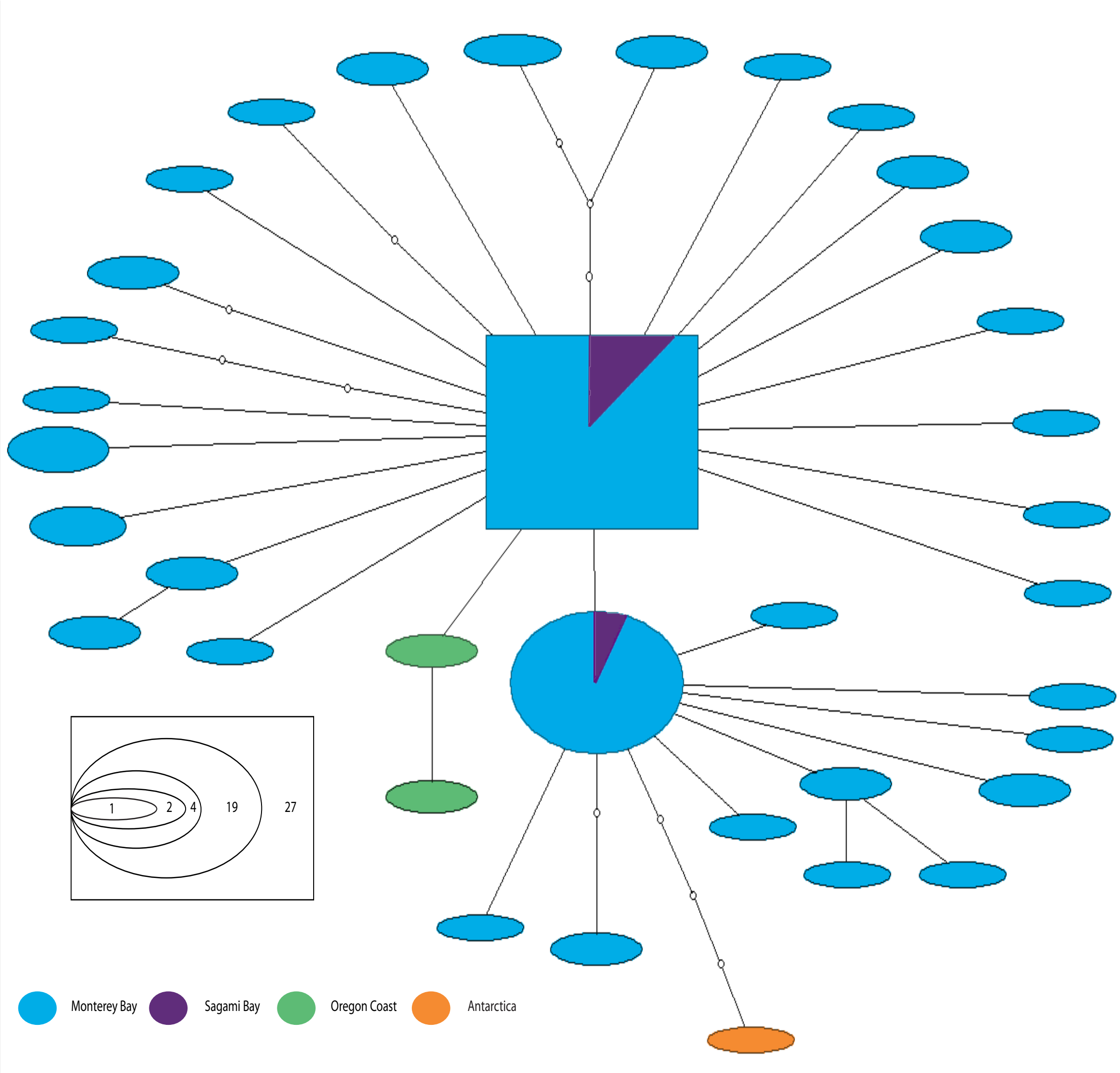

Figure 1 TCS network showing relationships between 36 unique haplotypes of *O. rubiplumus.* Missing haplotypes are represented by small open circles.

Our TCS network supports the high dispersal ability of *Osedax* species (Fig. 1). The four Japanese haplotypes were also found in Monterey Bay individuals. This suggests that there is no population structure between populations at these locations. This could be possible through larval transmission along the North Pacific gyre. Interestingly, individuals from off the Oregon coast did not share a haplotype with either the Japanese population or the Monterey Bay population. The Antarctic *O. rubiplumus* had the most divergent COI sequence. This can be explained by the absence of easy dispersal through the North Pacific gyre. Our maximum likelihood supports this finding with the Antarctic *O. rubiplumus* having the longest branch length of all *O. rubiplumus* analyzed (Fig. 2). However, the amount of signal from our data was low, resulting in a tree with poor support values. This likely stems from the low variability in the analyzed sequences. These results also must be taken in the context of the marker used. A marker with a higher mutation rate than COI could elucidate some of the observed findings.

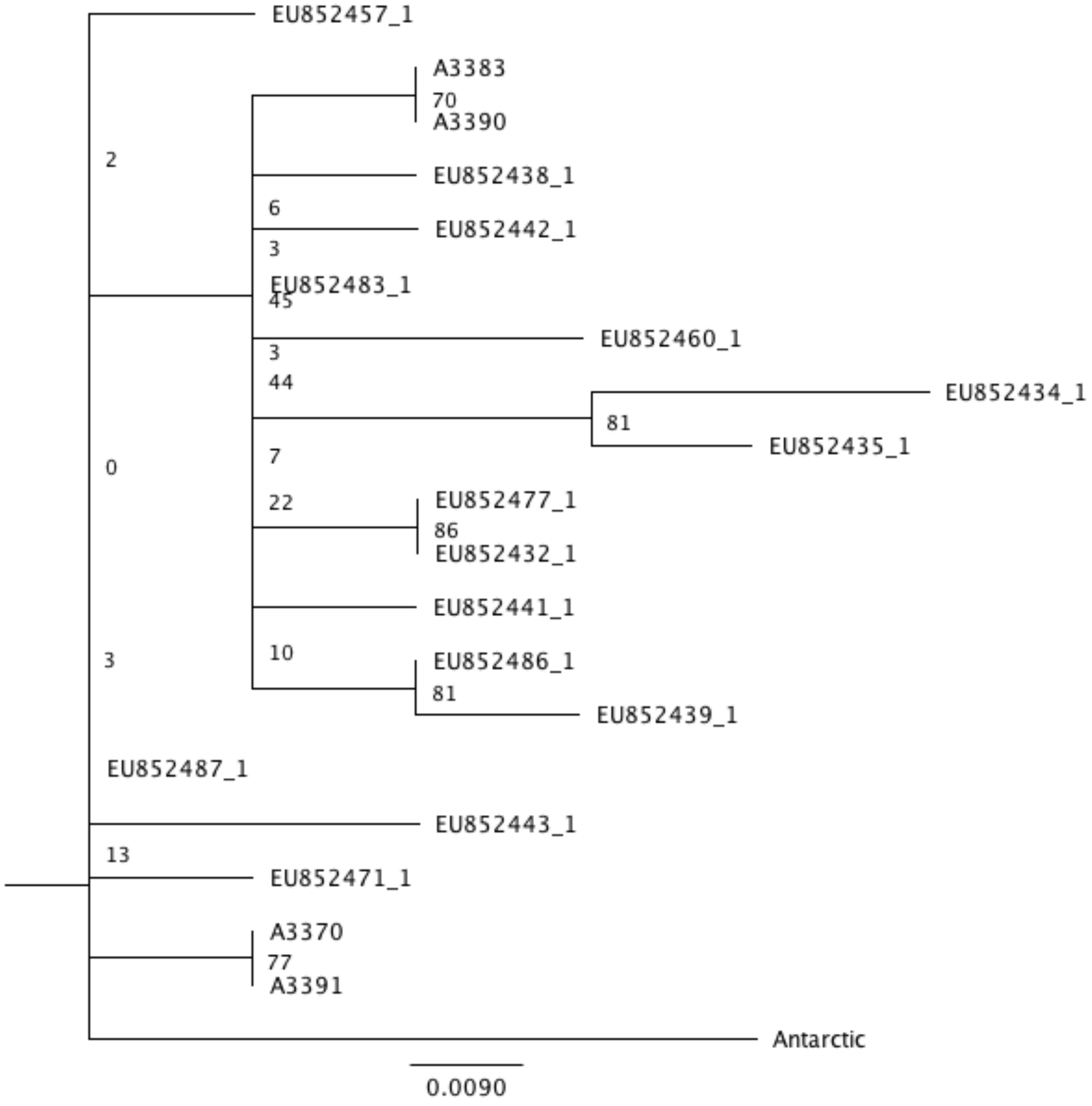

Figure 2. Maximum likelihood tree resulting from Antarctic, Oregon coast, and unique available *O. rubiplumus* COI sequences. Nodes EU852487_1 and EU852483_1 share a haplotype with the represented individual.

CONCLUSIONS

The results of our molecular analysis of an unknown Antarctic *Osedax* and two unknown *Osedax* individuals with new and previously sequenced *O. rubiplumus* supports the studies indicating *Osedax* has a high ability for dispersal. Our data suggests that the populations in Sagami Bay off Japan and in Monterey Bay off California are in fact one population. However, this conclusion is based off a marker with a relatively slow mutation rate. Further study, with more individuals and a marker with a faster mutation rate, could shed more light on population structure across the Pacific. Even taking into account possible “stepping-stone” dispersal from ephemeral whale falls, this level of dispersal from lecithotrophic larvae warrants further study.

ACKNOWLEDGEMENTS

The Bone and Wood Landers project as well as support for attendance and housing during the Deep Sea, Biodiversity, Connectivity and Ecosystem function course was funded by a NSF grant (OCE-1155188). Facilities and additional funding for attendance for the course was provided by Friday Harbor Laboratory, Friday Harbor, WA. Ken Halanych and Craig Smith were integral in their guidance and support during the development, execution, and analyses of this project. A special thank you to the captain and crew of the *R/V Oceanus* for keeping most of my blood from escaping through a hole in my head, as well as facilitating the deployment and recovery of the bone and wood landers.

REFERENCES:

Amon, D.J. “Bone-Eating Worms and Wood-Eating Bivalves: Characterising the Ecology of Deep-Sea Organic Falls from Multiple Ocean Basins.” Phd, University of Southampton, 2014. http://eprints.soton.ac.uk/361854/.

Bernt, M., A. Donath, F. Jühling, F. Externbrink, C. Florentz, G. Fritzsch, J. Pütz, M. Middendorf, P. F. Stadler. "MITOS: Improved de novo Metazoan Mitochondrial Genome Annotation." Molecular Phylogenetics and Evolution 2013, 69(2):313-319

Boisvert, Sébastien, Frédéric Raymond, Elénie Godzaridis, François Laviolette, and Jacques Corbeil. “Ray Meta: Scalable de Novo Metagenome Assembly and Profiling.” Genome Biology 13, no. 12 (2012): R122. doi:10.1186/gb-2012-13-12-r122.

Braby, Caren E., Greg W. Rouse, Shannon B. Johnson, William J. Jones, and Robert C. Vrijenhoek. “Bathymetric and Temporal Variation among Osedax Boneworms and Associated Megafauna on Whale-Falls in Monterey Bay, California.” Deep Sea Research Part I: Oceanographic Research Papers 54, no. 10 (October 2007): 1773–91. doi:10.1016/j.dsr.2007.05.014.

Cavanaugh, C.M., S.L. Gardiner, M.L. Jones, H.W. Jannasch, and Water-bury J.B. “Procaryotic cells in the hydrothermal vent tubeworm Riftia pachyptila Jones: possible chemoautotrophic symbionts.” Science, NY 213 (1981): 340±342

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

Edgar, Robert C. (2004), "MUSCLE: multiple sequence alignment with high accuracy and high throughput." Nucleic Acids Research 32(5), 1792-97.

Genious version 7.1.7 created by Biomatters. Available from http://www.geneious.com/

Glover, Adrian G, Bjorn Kallstrom, Craig R Smith, and Thomas G Dahlgren. “World-Wide Whale Worms? A New Species of Osedax from the Shallow North Atlantic.” Proceedings of the Royal Society B: Biological Sciences 272, no. 1581 (December 22, 2005): 2587–92. doi:10.1098/rspb.2005.3275.

Glover, Adrian G., Helena Wiklund, Sergio Taboada, Conxita Avila, Javier Cristobo, Craig R. Smith, Kirsty M. Kemp, Alan J. Jamieson, and Thomas G. Dahlgren. “Bone-Eating Worms from the Antarctic: The Contrasting Fate of Whale and Wood Remains on the Southern Ocean Seafloor.” Proceedings of the Royal Society B: Biological Sciences 280, no. 1768 (October 7, 2013): 20131390. doi:10.1098/rspb.2013.1390.

Goffredi, Shana K., Shannon B. Johnson, and Robert C. Vrijenhoek. “Genetic Diversity and Potential Function of Microbial Symbionts Associated with Newly

Discovered Species of Osedax Polychaete Worms." Applied and Environmental Microbiology 73, no. 7 (April 1, 2007): 2314–23. doi:10.1128/AEM.01986-06.

Halanych, Kenneth M., Robert A. Feldman, and Robert C. Vrijenhoek. "Molecular Evidence That Sclerolinum Brattstromi Is Closely Related to Vestimentiferans, Not to Frenulate Pogonophorans (Siboglinidae, Annelida)." The Biological Bulletin 201, no. 1 (August 1, 2001): 65–75.

Hilário, A., M. Capa, T.G. Dahlgren, K.M. Halanych, C.T.S. Little, et al. "New Perspectives on the Ecology and Evolution of Siboglinid Tubeworms." PLoS ONE 6(2)(2011): e16309. doi:10.1371/journal.pone.0016309

Hilário, A., S. B. Johnson, M. R. Cunha, and R. C. Vrijenhoek. "High Diversity of Frenulates (Polychaeta: Siboglinidae) in the Gulf of Cadiz Mud Volcanoes: A DNA Taxonomy Analysis." Deep Sea Research Part I: Oceanographic Research Papers 57, no. 1 (January 2010): 143–50. doi:10.1016/j.dsr.2009.10.004.

McMullin, EIUN R., STEPHANE Hourdez, STEPHEN W. Schaeffer, and CHARLES R. Fisher. "Phylogeny and Biogeography of Deep Sea Vestimentiferan Tubeworms and Their Bacterial Symbionts." Symbiosis 34, no. 1 (2003): 1–41.

Rogers, Alex D., Paul A. Tyler, Douglas P. Connelly, Jon T. Copley, Rachael James, Robert D. Larter, Katrin Linse, et al. "The Discovery of New Deep-Sea Hydrothermal Vent Communities in the Southern Ocean and Implications for Biogeography." PLoS Biol 10, no. 1 (January 3, 2012): e1001234. doi:10.1371/journal.pbio.1001234.

Rouse, G.W., S.K. Goffredi, and R.C. Vrijenhoek "Osedax: Bone-Eating Marine Worms with Dwarf Males." Science 305, no. 5684 (July 30, 2004): 668–671.

Rouse, Greg W., Nerida G. Wilson, Shana K. Goffredi, Shannon B. Johnson, Tracey Smart, Chad Widmer, Craig M. Young, and Robert C. Vrijenhoek. "Spawning and Development in Osedax Boneworms (Siboglinidae, Annelida)." Marine Biology 156, no. 3 (February 1, 2009): 395–405. doi:10.1007/s00227-008-1091-z.

Schulze, A., K.M. Halanych. "Siboglinid evolution shaped by habitat preference and sulfide tolerance." Hydrobiologia 496(2003): 199-205

Smith, C.R., A.G. Glover, T. Treude, N.D. Higgs, and D.J. Amon. "Whale-fall ecosystems: recent insights into ecology, paleoecology and evolution." Annu. Rev. Mar. Sci. 7:X-X (2015) doi: 10.1146/annurev-marine-010213-135144

Southward, A. J., and E. C. Southward. 1988 Pogonophora: Tube worms dependent on endosymbiotic bacteria ISI Atlas of Science, Animal & Plant Sciences 1 203–207

Stamatakis, A., P. Hoover, J. Rougemont. "A Rapid Bootstrap Algorithm for the RAxML Web-Servers. "Systematic Biology" 75, no. 5 (2008): 758-771

Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S "MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0." Molecular Biology and Evolution 30(2013): 2725-2729.

Templeton, A.R. and C.F. Sing. "A Cladistic Analysis of Phenotypic Associations with Haplotypes Inferred from Restriction Endonuclease Mapping. IV. Nested Analyses with Cladogram Uncertainty and Recombination." Genetics 145:659-669 (June, 1993).

Thornhill, Daniel J, Kevin T Fielman, Scott R Santos, and Kenneth M Halanych. "Siboglinid-Bacteria Endosymbiosis." Communicative & Integrative Biology 1, no. 2 (2008): 163–66.

Thornhill, Daniel J., Anne A. Wiley, Amy L. Campbell, Frank F. Bartol, Andreas Teske, and Kenneth M. Halanych. "Endosymbionts of Siboglinum Fiordicum and the Phylogeny of Bacterial Endosymbionts in Siboglinidae (Annelida)." The Biological Bulletin 214, no. 2 (April 1, 2008): 135–44.

Vrijenhoek, R. C., S. B. Johnson, and G. W. Rouse. "Bone-Eating Osedax Females and Their 'harems' of Dwarf Males Are Recruited from a Common Larval Pool." Molecular Ecology 17, no. 20 (October 1, 2008): 4535–44. doi:10.1111/j.1365-294X.2008.03937.x.

Vrijenhoek, R.C., B.J. Shannon, and G.W. Rouse. "A Remarkable Diversity of Bone-eating Worms (Osedax; Siboglinidae; Annelida)." BMC Biology 7, no. 1 (2009): 74.

Zhang, Z., S. Schwartz, L. Wagner, and W. Miller. "A Greedy Algorithm for Aligning DNA Sequences." Journal of Computational Biology: A Journal of Computational Molecular Cell Biology 7, no. 1–2 (April 2000): 203–14. doi:10.1089/10665270050081478.