The Frequency of Ceratonova shasta Genotypes in Manayunkia speciosa Host Genotypes

by
Madison M. Messmer

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented May 24, 2016
Commencement June 2016

AN ABSTRACT OF THE THESIS OF

Madison M. Messmer for the degree of Honors Baccalaureate of Science in Microbiology presented on May 24, 2016. Title: *The Frequency of Ceratonova shasta* Genotypes in *Manayunkia speciosa* Host Genotypes .

Abstract approved: _____

Jerri Bartholomew

Ceratonova shasta is a myxozoan parasite that affects populations of salmonid species in the Pacific Northwest and is the cause of enteronecrosis. It has a complex life cycle involving two hosts; a definitive annelid host, *Manayunkia speciosa* (polychaete), and an intermediate salmonid host. Klamath River *M. speciosa* are genetically variable and genetic variability in at least one other invertebrate host has been correlated with parasite susceptibility: genetic variation in *Tubifex tubifex* is correlated with susceptibility to infection by the myxozoan *Myxobolus cerebralis*, the causative agent of salmonid whirling disease. This research looked at the genetic variation of *M. speciosa* in the Klamath River, Oregon with the aim of correlating *C. shasta* genotype to definitive host genotype in order to aid in risk assessment of the Klamath River system. This research found that *C. shasta* genotype II was more associated with *M. speciosa* infection than genotype I.

Key Words: salmonid, parasite, Klamath River, microbiology

Corresponding e-mail address: messmerm@oregonstate.edu

The Frequency of Ceratonova shasta Genotypes in Manayunkia speciosa Host Genotypes

by
Madison M. Messmer

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented May 24, 2016
Commencement June 2016

Honors Baccalaureate of Science in Microbiology project of Madison M. Messmer presented on May 24, 2016.

APPROVED:

Jerri Bartholomew, Mentor, representing Microbiology

Sascha Hallett, Committee Member, representing Microbiology

Kimberly Halsey, Committee Member, representing Microbiology

Toni Doolen, Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Madison M. Messmer, Author

The Frequency of *Ceratonova shasta* Genotypes in *Manayunkia speciosa* Host Genotypes

ABSTRACT

Ceratonova shasta is a myxozoan parasite that affects populations of salmonid species in the Pacific Northwest and is the cause of enteronecrosis. It has a complex life cycle involving two hosts; a definitive annelid host, *Manayunkia speciosa* (polychaete), and an intermediate salmonid host. Klamath River *M. speciosa* are genetically variable and genetic variability in at least one other invertebrate host has been correlated with parasite susceptibility: genetic variation in *Tubifex tubifex* is correlated with susceptibility to infection by the myxozoan *Myxobolus cerebralis*, the causative agent of salmonid whirling disease. This research looked at the genetic variation of *M. speciosa* in the Klamath River, Oregon with the aim of correlating *C. shasta* genotype to definitive host genotype in order to aid in risk assessment of the Klamath River system. This research found that *C. shasta* genotype II was more associated with *M. speciosa* infection than genotype I.

INTRODUCTION

Manayunkia speciosa (**Figure 1**) is a freshwater, polychaete worm that resides in tubes constructed of mucus and sediment attached to a variety of substrates[1]. It has been described from river systems in the eastern and western United States in addition to the Great Lakes Region of the United States[2]. *Manayunkia speciosa* vary in length from 2.6 to 4.9 millimeters and up to 0.3 millimeters in width. They are segmented with ciliated palps and a pair of eyespots on the anterior portion of the body[1]. There is scientific interest in *Manayunkia speciosa* because

it serves as the definitive host of *Ceratonova shasta*, a parasite that infects salmonid species in the Pacific Northwest region of the United States[3] (**Figure 2**).

Figure 1: photo of *M. speciosa* at 20X magnification. It is green in color and labeled on the polychaete are the eye spots, palps, and chetae

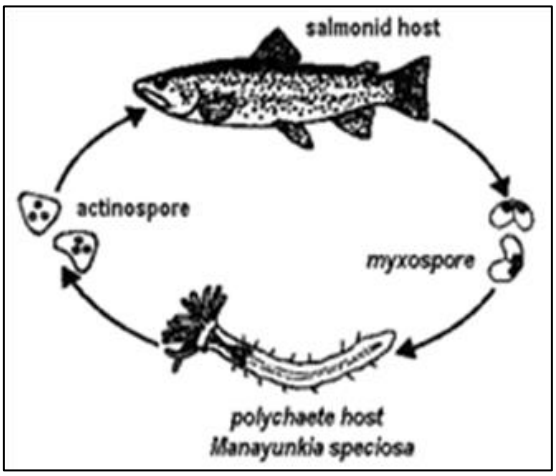

Figure 2: The *Ceratonova shasta* lifecycle is complex and involves salmonid and polychaete hosts and two waterborne spore stages, myxospores and actinospores.

Ceratonova shasta is a myxozoan parasite. Myxosporeans, a class of myxozoans, are microscopic and have a wide host range that can include both vertebrates and invertebrates. These parasites have two spore types, actinospores and myxospores[4]. The myxospores of *C. shasta* are released by infected salmonid hosts and infect *M. speciosa* while the worms filter feed[2]. Myxospores are kidney bean shape, about 22.4 um thick and 5.2 um long[5]. Actinospores

are released by infected *M. speciosa*. These spores float in the water column until they encounter, attach and penetrate the gills of the salmonid host. The parasite proliferates in the blood, migrates to the intestine and develops into myxospores which are released back into the water column following the death of the fish[6]. Four distinct genotypes of *C. shasta* have been described based on DNA sequencing and host specificity, referred to as genotypes I, II, III, and 0. Genotypes I and II occur in the Klamath River and can lead to mortality in Chinook and coho salmon, respectively[7].

Ceratonova shasta infects different species of salmon in the Pacific Northwest and is the causal agent of ceratomyxosis. The infection can present in different ways in different salmonid hosts. Clinical signs of ceratomyxosis include a distended abdomen, hemorrhages and swelling in the gut, as well as gross lesions on the kidney, liver and spleen. Infectious material is not limited to the gut but has been described from gut and liver in infected juvenile salmonids. Ceratomyxosis is usually diagnosed following observation of *C. shasta* myxospores in wet mounts of scrapings collected from the lower intestinal wall, gall bladder, or lesions[8]. This disease can be a problem in salmonids in the Pacific Northwest region of the United States, specifically in the Klamath River due to the temporal and spatial overlap of the parasite, the definitive host, *Manayunkia speciosa*, and the intermediate host, salmonids. The aim of this research is to look into host factors that may contribute to the spread and persistence of this serious salmonid parasite. The study of the definitive host and the factors that may or may not contribute to host susceptibility in this system are important so that there can be accurate information to asses risk and mitigate effects.

One mechanism to explain some of variation in disease risk among locations (or years) is susceptibility of the invertebrate host. Genetic variation in the obligate invertebrate host of

Myxobolus cerebralis, a different myxozoan that causes salmonid whirling disease, has been linked to susceptibility. Like *C. shasta*, *M. cerebralis* infects salmonid species and requires an annelid host, *Tubifex tubifex*[10]. This previously researched system can be used as a model for *C. shasta* and can guide in understanding the relationship between *C. shasta* and definitive host genotypes. *Tubifex tubifex* plays an important role in the transmission of *M. cerebralis* in terms of individual host factors, *T. tubifex* population factors including genetic and geographic variations, and ecological factors in addition to epidemiological parameters. Multiple studies have been conducted based on the life cycle of *M. cerebralis* and results were compiled by W.O. Granath, Jr. and M.A. Gilbert from the University of Montana Division of Biological Sciences. Previous research has shown that although *T. tubifex* serves as a definitive host for *M. cerebralis*, the "Great Lakes" *T. tubifex* was found to be an unsuitable host for the parasite, which may indicate that certain subsets or subspecies of *T. tubifex* more susceptible to *M. cerebralis* infection. Subsequent research found that there are certain genetic differences within and between populations of *T. tubifex* that could have an effect on *M. cerebralis* infections in freshwater systems. More so, the range of habitats that *T. tubifex* oligochaetes can occupy has led some to believe there could be multiple races within the *T. tubifex* species[11].

Research on genetic variation in Klamath River *M. speciosa* and *C. shasta* strain is limited to investigations by members of the J.L. Bartholomew Lab (Dept. Microbiology, OSU). Out of the 10 samples that tested positive for *C. shasta* via PCR and gel electrophoresis, 6 different genotypes were assigned to *M. speciosa*. The research looked at both infected and non-infected polychaetes and found no correlation between *C. shasta* genotypes and *M. speciosa* genotypes in the Upper Klamath River; however, more samples need to be examined.

I aimed to expand on this previous research in hopes of describing the relationship between polychaete host genotype and parasite genotype. Understanding the relationship between *M. speciosa* and *C. shasta* is important so we can inform our research of parasite biology as well as use the information in subsequent risk assessments in various river systems throughout the Pacific Northwest.

I hypothesize there are genotypes of *M. speciosa* with a higher frequency of *C. shasta* infections than other genotypes.

In the course of experimentation, I discovered that the DNA from previously processed *M. speciosa* was no longer viable. I developed several hypotheses to explain this result including that the DNA was degraded due to the extraction method used, which did not preserve DNA for long term storage. I determined that I needed to use polychaetes that had been stored for less than 6 months, and therefore changed my research focus to describing genetic variation in polychaetes that were cultured in our laboratory and experimentally exposed to *C. shasta*.

MATERIALS and METHODS

The general methodology is to identify polychaetes that are positive for infection of *C. shasta* via PCR and analyze the genotypes of both polychaete (*M. speciosa*) and parasite (*C. shasta*) DNA.

1. Field collected polychaetes:

Manayunkia speciosa samples that had been collected during monitoring efforts conducted from 2012-2015, crudely extracted and stored in the freezer at -20C were tested for *C. shasta*. A subset of the samples had previously tested positive for *C. shasta* infection by qPCR ('field positives') and a subset of the samples had previously tested negative for *C. shasta* by qPCR ('field negatives'). I began by trying to detect *C. shasta* in the 'field positives' by PCR so that I

could sequence the PCR product and describe the genotype of *C. shasta*. 'Field negatives' were not tested for *C. shasta* by PCR, but were included in the dataset so I could describe genetic variation in polychaetes that were not infected with *C. shasta* in addition to those that were infected.

The 'field positives' samples were assayed by several methods due to problems detecting *C. shasta* DNA. First, samples were diluted 1:100 and tested with the *C. shasta* assay (appendix 1.1). More polychaetes were tested that had higher Cq values according to previous qPCR data and more recent field collected samples were tested. Second, I modified the PCR program in an attempt to elongate the amount of time for the primers to anneal to the DNA. Third, I purified the DNA using Qiagen kit (appendix 1.2) to clean up DNA and eliminate impurities that could interfere with the PCR reaction. The purified samples were tested with PCR. Fourth, the *C. shasta* PCR water assay (appendix 1.3) was used because it is more sensitive and was thought to be able to detect smaller amounts of *C. shasta*. The *C. shasta* water PCR uses titanium taq polymerase, which is more sensitive than the standard taq polymerase. Lastly, detection of polychaete DNA was tested using PCR with no results (appendix 1.4). Finally, the samples were nanodropped to quantify the amount of DNA in a sample and found DNA seriously degraded.

2. DNA stability experiment:

I tested the stability of polychaete DNA because of problems with field collected polychaetes, see above. Fresh polychaetes were collected and held in the lab alive for one week. The polychaetes were picked and immediately individually crudely extracted (appendix 1.5) and assayed using the *Manayunkia speciosa* genotyping PCR to detect *M. speciosa* DNA.

3. Laboratory experiment polychaetes:

I was unable to move forward with conducting research on archived field collected samples, see above 1 and 2, and required fresh polychaetes. Therefore, I completed my research using polychaetes cultured in the laboratory that were exposed to *C. shasta*. Basic overviews of the methods are shown in **Figure 3:**

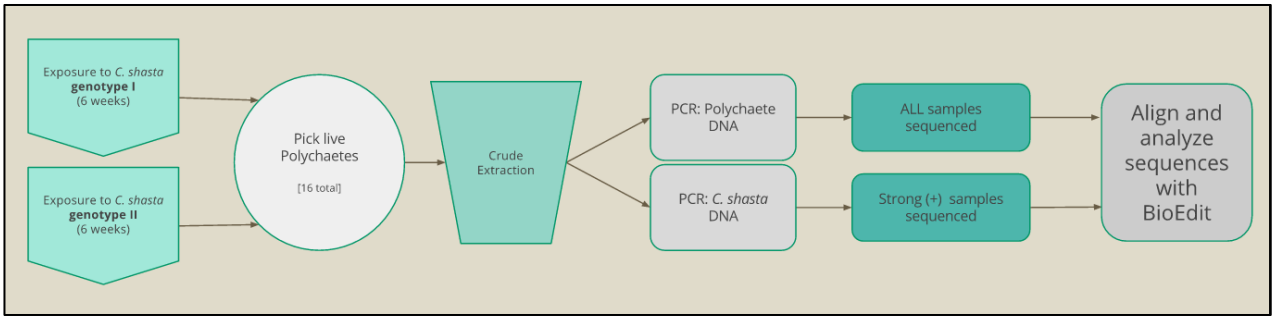

Figure 3: a schematic overview of the methods for "laboratory experiment polychaetes"

Manayunkia speciosa obtained from the Upper Klamath River and cultured at the John L. Fryer Aquatic Animal Health Laboratory on the Oregon State University Campus were used to provide live polychaetes. To obtain infected polychaetes, multiple groups were exposed to *C. shasta* myxospores genotypes I (n=3) and II (n=3) on January 18th, held for 6 weeks and then live worms were removed and transported to Nash Hall. In the Lab at Nash Hall they were kept in the refrigerator at 4˚C with a tube sending constant oxygen to the containers to stimulate constant flow of water. I picked 8 polychaetes from each group resulting in n=8 polychaetes that were exposed to genotype I and n=8 exposed to genotype II_R. DNA was extracted immediately by adding 5µl proteinase K and 95 µl ATL buffer, described as 'rough polychaete extraction' in appendix 1.5. DNA was diluted 1:100 and ran through PCR with manspec2F and manspec2R primers on the program FASTCOI targeting the COX2 gene with a positive control and a negative control (water). The PCR reactions were then visually confirmed using gel electrophoresis ran through a 2% agarose gel at 180 Volts for 15 minutes along a 1kb ladder,

positive control, and a negative control. A band at 550 bases confirmed that the sample was positive.

The crude extracted DNA from the live *M. speciosa* samples was diluted 1:100 assayed using the Cs1479F and Cs2067R primers targeting the ITS1 gene and the program was set on CSITS55B. This process amplifies a target region of *C. shasta* DNA within the sample if *M. speciosa* is infected. Gel electrophoresis was used to visualize the PCR product. Gels were run at 180 Volts for 15 minutes alongside a 1kb ladder, positive control, and negative control. A bright band at approximately 550 base pairs indicated the sample is positive for *C. shasta*.

To test the efficacy of the *C. shasta* assay designed for fish on the *M. speciosa* samples, the *C. shasta* assay used to test for presence in water samples was used for 8 of the 16 samples. The only difference in this protocol is the titanium taq polymerase being used instead of the standard taq polymerase in addition to a different PCR program being ran, TIT6860.

PCR products from the *M. speciosa* assay were purified according the protocol in appendix 1.6, diluted with water up to a volume of 10.8 µl, added to Manspec2F primer, and sent to The Center for Genome Research and Biocomputing (CGRB) for sequencing of the COX2 gene. The amplicons were sequenced in one direction. The sequences were then aligned using BioEdit and analyzed. The PCR products that were strong positives for *C. shasta* from the extracted sample DNA were purified using the protocol in appendix 1.2, diluted with water up to a volume of 10.8 µl, added to CS1479F primer, and sent to the CGRB for sequencing of the ITS1 gene and then analyzed using BioEdit. The sequences were lined up and analyzed using the Bioedit software. The positions of the sequences that did not match were noted and the lengths of

sequences that matched were deleted so that the sequences could be evaluated and placed into groups.

RESULTS

1. Field collected polychaetes:

Several attempts were made to detect *C. shasta* within previously collected samples that had been stored for periods of 6 months to 4 years and previously tested positive for *C. shasta* by qPCR. When the assays were run, there was no PCR product visible (**Figure 4**). Positive controls performed as expected suggesting it was a problem with the samples and not the PCR. Forty-six samples collected in 2012 (n=12), 2014 (n=12), and 2015 (n=22) were all negative for both *C. shasta* and *M. speciosa* DNA.

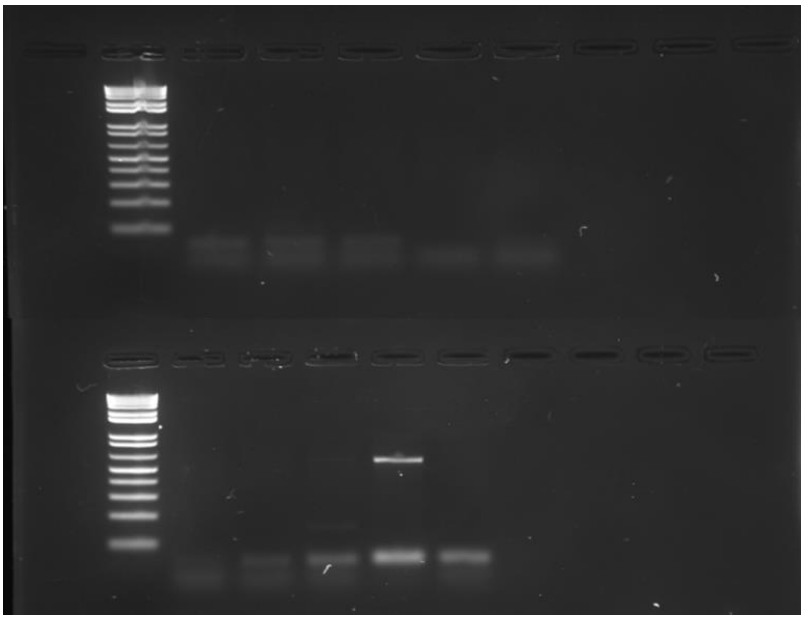

Figure 4: Image of gel under UV light showing results from testing October 2015 field *M. speciosa* samples for *C. shasta*. These samples had previously been determined to be positive for *C. shasta* infection by qPCR. The only positive result I detected was from the positive control, which indicated that the DNA in the samples had degraded.

2. DNA stability experiments:

I tested the stability of polychaete DNA because of problems with field collected polychaetes, see above. When the polychaete samples had been stored for long periods of time in ~95%

ethanol or previously processed, I was unable to detect polychaete or *C. shasta* DNA. When live polychaetes were freshly extracted, I was able to detect DNA. **Figure 5** is the gel photo from under the UV camera that shows all samples that were freshly collected and immediately processed had presence of *M. speciosa* DNA.

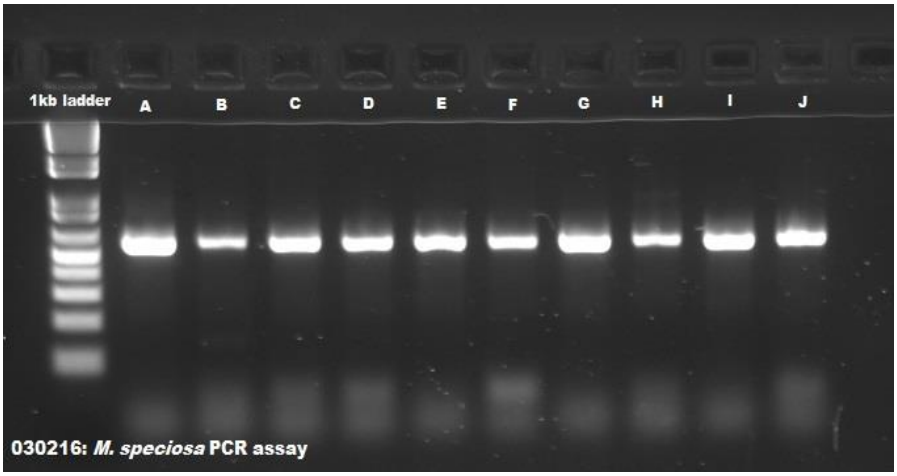

Figure 5: Image of gel under UV camera showing results from testing laboratory *M. speciosa* samples for *M. speciosa* DNA. All samples were positive which indicated that the DNA in the samples was intact and useable for sequencing.

3. Laboratory experiment polychaetes:

Sixteen individual polychaetes were exposed to *C. shasta* genotypes I and II_R that infect Chinook salmon and rainbow trout, respectively. The myxospores are collected from these hosts and are used to dose the polychaetes. Of the 16 samples, all were positive for *M. speciosa* DNA. Of the 16 samples tested for presence of *C. shasta,* 5 of the samples tested strong positives and all of them were from the groups of polychaetes exposed to IIR. No polychaetes exposed to genotype I tested positive for *C. shasta* DNA. **Figures 6 and 7** show the UV camera photos of the gel electrophoresis results for the *C. shasta* assays. Table 1 shows a description of the samples and the PCR results for both *M. speciosa* and *C. shasta* assays.

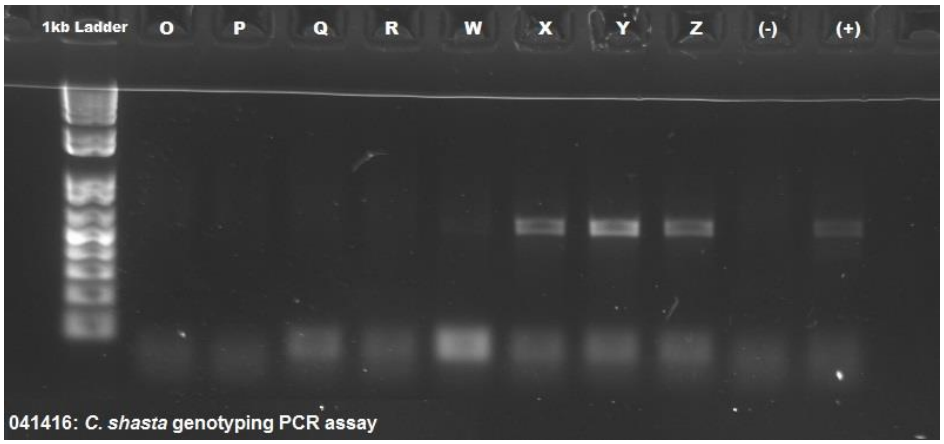

Figure 6: Image of gel under UV camera showing the samples are positive for *C. shasta* DNA after running through a PCR using Cs1479F and Cs2067R primers on the program CSITS55B.

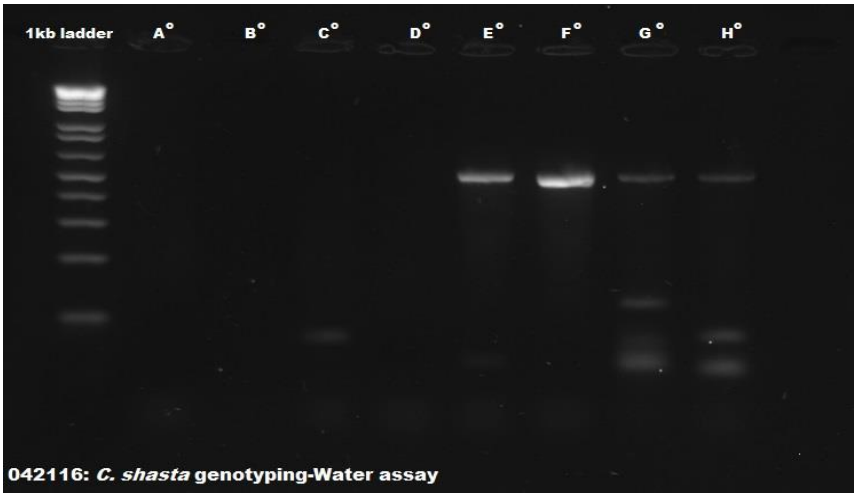

Figure 7: Image of gel under UV camera showing the samples are positive for *C. shasta* DNA after running through a PCR using Cs1479F and Cs2067R primers and titanium Taq on the program TIT6860B.

Table 1 shows the samples alongside the *M. speciosa* and *C. shasta* results. **Figure 8** is a graphic separating the two genotypes of *M. speciosa* observed from the 16 samples submitted. The asterisks indicate which samples tested positive for *C. shasta*. Of the 16 samples submitted to the CGRB, 15 returned with readable sequences approximately 550 bases long. There were 13 nucleotide positions relative to the forward primer Manspec2F where the sequences did not align. From these we found that 9 shared a T substitution for A at position 57, a G for A at position 334, an A for G at position 427. An additional sequence to those 9 previously stated had a C for T at position 393. There were other substitutions, but mostly single nucleotide polymorphisms (SNPs). Of the 8 polychaetes that were exposed to the IIR genotype of *C. shasta*, 5 tested positive. The samples that tested positive are asterisked in **Figure 8**.

Table 1 List of samples and corresponding description with the results of the *M. speciosa* and *C. shasta* PCR assays

Sample	Description	*M. speciosa* Result	*C. shasta* Result
O	Exposure to *C. shasta* genotype I	+	-
P	Exposure to *C. shasta* genotype I	+	-
Q	Exposure to *C. shasta* genotype I	+	-
R	Exposure to *C. shasta* genotype I	+	-
W	Exposure to *C. shasta* genotype II_R	+	-
X	Exposure to *C. shasta* genotype II_R	+	+
Y	Exposure to *C. shasta* genotype II_R	+	+
Z	Exposure to *C. shasta* genotype II_R	+	+
A°	Exposure to *C. shasta* genotype I	+	-
B°	Exposure to *C. shasta* genotype I	+	X-no result
C°	Exposure to *C. shasta* genotype I	+	-
D°	Exposure to *C. shasta* genotype I	+	-
E°	Exposure to *C. shasta* genotype II_R	+	+
F°	Exposure to *C. shasta* genotype II_R	+	+
G°	Exposure to *C. shasta* genotype II_R	+	+(weak)
H°	Exposure to *C. shasta* genotype II_R	+	+(weak)

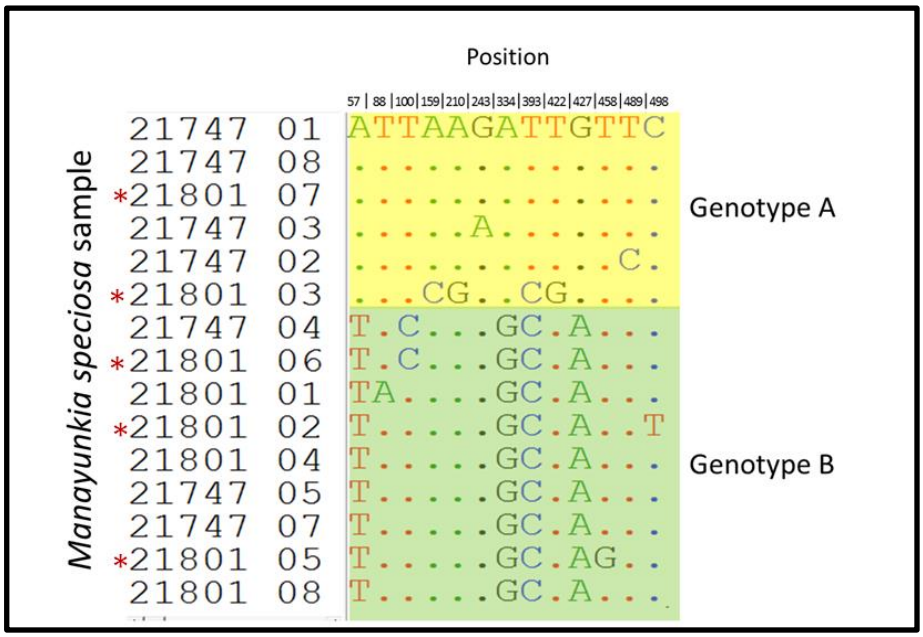

Figure 8: Sequences of COX2 gene from 15 of the 16 *M. speciosa* samples sent to the CGRB for sequencing, The samples are put into 2 groups, 'Genotype A' and 'Genotype B' based on the changes in sequences. The asterisks indicate the sample tested positive for *C. shasta* using PCR.

The sequencing of the *C. shasta* positive polychaetes had no result. The experimental design was such that the genotype of the *C. shasta* infection was known. The comparison of *M. speciosa* and *C. shasta* genotype infection is in **Figure 9.**

Infection and Polychaete Genotype

		C. shasta Genotype	
		I	II_R
M. speciosa genotype	A	0	3
	B	0	2

Figure 9: a schematic showing the number of *M. speciosa* polychaetes and their genotypes infected by *C. shasta* genotypes

CONCLUSION AND DISCUSSION

I determined that polychaete and *C. shasta* DNA in the field collected polychaete samples had degraded in a matter of about 6 months. The process of crude extraction and freezing at a temperature of -4˚C causes degradation of all the DNA in the sample and seems to decrease the amount of detectable DNA in the sample. When the previously collected samples were nanodropped at the CGRB, there was some DNA, but not enough to be detected via PCR. This has implications as far as processing goes for polychaete samples collected by the Bartholomew Lab at OSU for monitoring purposes. Clearly, the approach being used currently (rapid processing and qPCR) is sufficient for the initial assays, but any samples that will be retained for future research should be cleaned up and purified using a DNA extraction and purification kit such as the Qiagen DNeasy Kit. Currently, samples are extracted using a rough extraction protocol where the samples are not purified. My research demonstrated that the stability of the DNA in each sample needs to be taken into consideration if samples are to be used for future projects. My research also suggests that further research may be needed in order to create a protocol that can be implemented in the lab that keeps the collected sample DNA viable for future research. A possible route of study includes assessing the efficacy of immediate

processing of a few samples and preserving purified DNA in such a manner so that there would be little to no degradation of DNA over a period of time.

The lab-reared polychaetes were exposed to two genotypes of *C. shasta* (I and II_R) and sampled 6 weeks after exposure. All 16 samples tested positive for *M. speciosa* DNA and sequencing placed them into two genotypes, 'A' or 'B' (Figure 2). The COX2 gene of the polychaete samples was sequenced because this gene is highly conserved and can be used to differentiate populations. None of the polychaetes exposed to *C. shasta* genotype I were infected but both *M. speciosa* genotypes A and B were detected in roughly equal proportions. Only 5 of the 8 polychaetes exposed to *C. shasta* genotype II_R tested positive for *C. shasta*, and 3 of the 5 polychaetes were genotype B, suggesting that both genotypes are susceptible to *C. shasta* II_R infection (Table 1). The *C. shasta* genotyping did not return any results. The reason for this is because there was not enough DNA present in the purified PCR product. This was to be used as an extra step of confirmation for *C. shasta* genotype, but because I used a controlled environment and there were separate samples that had been exposed to either genotype I or II_R, we can assume that all the *M. speciosa* samples collected from the II_R exposure set were infected with *C. shasta* genotype II_R.

Previous research by the Bartholomew Lab that focused on the Klamath River found no association between polychaete genotype and *C. shasta* genotype. More samples need to be assayed to explore this idea further. Subsequent research could focus on temporal factors in addition to the infection of certain genotypes. Some questions that could not be answered in this study included why the type I exposed polychaetes tested negative for *C. shasta*; did the infection kill infected polychaetes during the 6 weeks following exposure, or did the infection

clear before the worms were sampled? These questions need to be answered in order for the effects to be understood.

One of the questions I asked at the beginning of this research was how the genetics of the polychaete host played a role in the transmission of *C. shasta* in order to do risk assessments at various sites. To do this, the previously collected samples from many different field sites were going to be used in order to get an overview of the differences in host genetics. Since the previously collected samples produced degraded DNA to be tested for either parasite or polychaete DNA, lab grown polychaetes had to be used instead. This had advantages and disadvantages. In addition to being able to obtain viable DNA from fresh polychaetes, an advantage of this was being able to expose these polychaetes to a certain parasite strain instead of retroactively genotyping *C. shasta* from the polychaetes that tested positive via qPCR. A disadvantage to this approach is that we are unable to assess the genetic diversity across different field sites to accurately get a picture of how the populations differ spatially. One way this research can expand is by looking at the differences between the lab grown strains and the field collected samples. This information could be crucial in understanding host-parasite interactions and aid in risk assessment that can be used in a practical manner in order to control *C. shasta*. The lab strains were cultured from a site in the Klamath River, but it is unknown whether or not the act of culturing has an effect on genetic diversity and whether growing samples in the lab artificially selects for certain genotypes that may not be as present in the wild.

ACKNOWLEDGMENTS

Thank you to Rich Holt, Ruth Milston Clements, and Ryan Craig at the Aquatic Animal Health Lab for the care of polychaetes and myxospore dosing of polychaetes prior to sampling. Thank you to Damian Barrett from the Bartholomew Lab for qPCR data. Thanks to Dr. Stephen Atkinson and Dr. Julie Alexander for help with lab bench experimental design, protocol help, and data interpretation. An additional thanks to Dr. Sascha Hallett for advising and help in writing and formatting. Thank you to my Honors Thesis Committee for advising my project. This project was funded in part by the Bureau of Reclamation.

REFERENCES

1. Pettibone, M. H.. (1953). *Fresh-Water Polychaetous Annelid, Manayunkia speciosa Leidy, from Lake Erie.* Biological Bulletin. 105(1).

2. Stocking, R. W. and Bartholomew J.L.. (2007). *Distribution and Habitat Characteristics of Manayunkia speciosa and Infection Prevalence with the Parasite Ceratomyxa shasta in the Klamath River, Oregon–California*. Journal of Parasitology. 93(1):78-88.

3. Bartholomew, J. L., Whipple, M. J., Stevens, D. G., & Fryer, J. L.. (1997). *The Life Cycle of Ceratomyxa shasta, a Myxosporean Parasite of Salmonids, Requires a Freshwater Polychaete as an Alternate Host*. The Journal of Parasitology. 83(5):859–868.

4. Ivan Fiala, Marie Hlavničková, Alena Kodádková, Mark A. Freeman, Pavla Bartošová-Sojková, Stephen D. Atkinson (2015). *Evolutionary origin of Ceratonova shasta and phylogeny of the marine myxosporean lineage.* Molecular Phylogenetics and Evolution. 86:75-89.

5. Atkinson, S. D. Foott, J. S. and Bartholomew. J. L. (2014). *Erection of Ceratonova n. gen. (Myxosporea: Ceratomyxidae) to Encompass Freshwater Species C. gasterostea n. sp. from Threespine Stickleback (Gasterosteus aculeatus) and C. shasta n. comb. from Salmonid Fishes*. Journal of Parasitology. 100 (5):640-645

6. Bjork, S.J., Bartholomew, J.L. (2010). *Invasion of Ceratomyxa shasta (Myxozoa) and comparison of migration to the intestine between susceptible and resistant fish hosts*. Journal of Parasitology. 40(9):1087-1095.

7. Hurst, C.N., Wong, P., Hallett, S.L., Ray, R.A., and Bartholomew, J.L. (2014). *Transmission and Persistence of Ceratonova shasta Genotypes in Chinook Salmon*. Journal of Parasitology 100(6):773-777.

8. Bartholomew, J.L. (2014). *Salmonid Ceratomyxosis*. Diagnostic Procedures for Finfish and Shellfish Pathogens. Section 3.2.4.

9. Koo, S.J., Horner, D.P., Hallett, S.L., Atkinson, S.D., and Bartholomew, J.L. *Correlation between Polychaete Host Genotype and Ceratomyxa shasta infection in a Klamath River, OR-CA*. Oregon State University Department of Microbiology.

10. Markiw, M.E., and Wolf, K. (1983). *Myxosoma cerebralis (Myxozoa: Myxosporea) Etiologic Agent of Salmonid Whirling Disease Required Tubificid Worm (Annelida: Oligochaeta) in its Life Cycle*. Journal of Protozoology. 36(3): 561-564.

11. Granath Jr, W.O., and Gilbert, M.A. (2002). *The Role of Tubifex tubifex (annelida: Oligochaeta: Tubificidae) in the Transmission of Myxobolus cerebralis (Myxozoa: Myxosporea: Myxobolidae).* American Fisheries Society Symposium. 29: 79-85.

APPENDIX

Appendix 1.1: ***Ceratonova shasta*** **genotyping assay from crude extracted fish tissue**

1. PREPARATION - In lab:

Thaw DNA samples (but leave in lab!)
Vortex or flick samples to mix
Take an ice bucket and fill with ice from autoclave room
If using Master Mix – remove appropriate number of strips from freezer, put in tray in your ice bucket, go to section 3.

2. PREPARATION OF MASTER MIX - In PCR-prep room:

Thaw your aliquots of reagents to room temperature (leave Taq in freezer)
Vortex briefly, then spin down. Vortexing is ESSENTIAL for buffer and $MgCl_2$
Calculate master mix based on following amounts X number of reactions + 8% for error.
For each 10ul reaction:

Reagent	Amount
DNA	2.0 ul
Cs1479F	0.4 ul
Cs2067R	0.4 ul
Taq	0.2 ul
dNTPs	0.2 ul
BSA	0.25 ul
Rediload	0.25 ul
$MgCl_2$	1.2 ul
Buffer	2.0 ul
H_2O	3.1 ul
Total	**10 ul**

Add reagents to 1.5mL tube, vortex, spin down
Label required number of 0.2mL strip tubes, in rack, on ice
Pipette 8ul master mix into each tube
Master mix can be made ahead of time and refrozen.

3. RUN PCR - In lab:

Add DNA to master mix tubes; add water to negative control (same water that you used in MM)
Flick or vortex tubes to mix; spin down
Place back in rack on **ice**
Turn on PCR machine
Start program: CSITS55B
Press PAUSE when block starts to heat up. Should hold at 94C
Place tubes in HOT block, press PAUSE again to start the program running. Look at display to confirm program is running. Program will run about 2.5 h

When complete, remove tubes and store in fridge; turn off PCR machine

4. GEL ELECTROPHORESIS – In lab:

Choose 50mL or 90mL with 1 or 2 combs based on number of samples
Tape ends of plastic gel former, add combs, confirm comb height
Make up 1% gel
e.g. 0.5g agarose + 50mL buffer from fridge (SybrSafe is already added)
Microwave 45s; swirl; microwave in additional 10s bursts until FULLY melted/clear

*****CAUTION WITH HOT GEL!*****

Cool gel on bench (about 5 minutes) or on ice (30s) until able to touch base of flask
Pour agarose; check for leaks; should set in 20min
Remove tape from gel former; remove combs
Place gel into correct bath; if necessary, fill gel bath with bulk TAE to JUST over gel surface; Replace entire buffer after 5-8 uses (down sink, rinse, refill)
Load gel with 4ul 1kb+ ladder (aliquot in fridge – dark green/blue); 4ul PCR products
Cover gel bath with plastic lid
Electrophorese at 160V for 15 minutes (BLACK electrode to back/top of gel)

5. VISUALIZE RESULTS – PHOTOGRAPH GEL – Room 502:

Turn on UV lamp (bottom switch) and computer up to 5 min before use to warm up
Carry gel in tray, in glass dish; transfer to plate in geldoc under WHITE light (top switch)
Start software, preview, adjust time to less than a second, move gel to center
Check bottom ring (focus) should be on 1
Adjust middle ring (zoom) to fill image with gel
Adjust top ring (aperture) to about 4 (turn to 1 for aligning gel at start)
Turn off white light, turn on UV
Adjust exposure time to about 7 sec, then as necessary to see ladder
Capture several images if bands are different brightnesses

Adjust contrast/brightness/gamma of image as needed
Label gel with PCR number using text tool ("A")
Print if needed; tear photo rapidly up and away – care not to jerk film roll crooked
Save image/s to your PCR folder
Shut down computer especially over weekends

6. ANALYZE RESULTS – Write up in your PCR book

A well-amplified PCR product should be as bright as the ladder. You can still purify & sequence a product that is about half as bright.

If bands are weak or do not appear, then use 1.0ul of the first round product in second round PCR.

7. SEQUENCING - In PCR prep room, then main lab

Thaw primer Cs1479F
Label a tube strip with PCR code and number each tube, one tube for each reaction you want to sequence; note sequence name/number in workbook
Add in each tube, 10.3ul H2O plus 1.2ul primer Cs1479f
Carry in to main lab
Add 0.5ul DNA from each reaction you want to sequence
Carry completed tubes over to CGRB lab in ALS, into freezer box "Sequencing To Do – Small Tubes"
Complete online sequence order form (only Stephen currently authorized to do this)

Appendix 1.2: **Qiagen purification for crude extracted DNA samples**

wipe down all surface area with RNAse Away or 5% bleach before this step
Make sure you can finish the rest of the extraction before continuing
(~2.5 hours for 30 samples)

1. PREPARATION

Increase water bath temperature to 70°C (dial position 7.25)
Dry tubes with paper towel & centrifuge sample briefly
Add 200µL Buffer AL
(repeat pipette 1=100ul dial set to 2)
Vortex & incubate at 70°C for 10 mins
This 70^{o}C step is TIME SENSITIVE
(Label your tubes now if you have not already see above listed tubes to label)
Remove samples from water bath and turn waterbath off
Dry tubes with paper towel & briefly centrifuge
Add 200µL ethanol (96-100%)
(can use repeat pipette from AL above)
Vortex then briefly centrifuge
Set pipette to 800 ul and pipette sample mixture into DNeasy spin column/collection tube.
(avoiding pellet but get ALL liquid)
Centrifuge at 8000 rpm 1 min
New collection tube

2. WASHING STAGE

(Use 1=250µL displacement tips and dial set to 2)

Add 500µL Buffer AW1
Centrifuge at 8000rpm 1 min,
New collection tube,
Add 500µL Buffer AW2 (reuse above repeater pipette)
Centrifuge at 8000rpm 1 min
New collection tube
Add 500µL Buffer AW2 (reuse above repeater pipette)
Centrifuge at 14,000rpm 3 mins
Carefully remove column from collection tube and - check that the column base is dry
if not, spin again 14000 rpm, 1 min
PLACE COLUMN INTO CLEAN 1.5ml LABELED ELUTION TUBE

3. ELUTION STAGE -spin column in clean 1.5mL centrifuge tube, Elute DNA in 2 steps:

(Use the 1=100ul displacement tip; program in 60ul)

Add 60ul Buffer AE to column (make sure liquid is on filter)
Incubate at room temperature 5 mins.
Centrifuge at 8000rpm 1 min

Add 60µL Buffer AE to column (make sure liquid is on filter)
Incubate at room temperature 5 mins
Centrifuge at 8000rpm 1 min

120ul of SAMPLE DNA IS NOW IN THE 1.5ML TUBE

Use sample directly in QPCR or place in freezer

Appendix 1.3: ***Ceratonova shasta*** **genotyping assay from collected water samples**

1. PREPARATION - In lab:

Thaw DNA samples (but leave in lab!)
Vortex or flick samples to mix
Take an ice bucket and fill with ice from autoclave room
If using Master Mix – remove appropriate number of strips from freezer, put in tray in your ice bucket, go to section 3.

2. PREPARATION OF MASTER MIX - In PCR-prep room:

Thaw your aliquots of reagents to room temperature (leave Taq in freezer)
Vortex briefly, then spin down. Vortexing is ESSENTIAL for buffer and $MgCl_2$
Calculate master mix based on following amounts X number of reactions + 8% for error.
For each 10ul reaction:

Reagent	Amount
DNA	2.0 ul
Cs1479F	0.4 ul
Cs2067R	0.4 ul
Titanium Taq	0.1 ul
dNTPs	0.2 ul
BSA	0.25 ul
Rediload	0.5 ul
$MgCl_2$	0.0 ul
Buffer	1.0 ul
H_2O	5.15 ul
Total	**10 ul**

Add reagents to 1.5mL tube, vortex, spin down
Label required number of 0.2mL strip tubes, in rack, on ice
Pipette 8ul master mix into each tube
Master mix can be made ahead of time and refrozen.

3. RUN PCR - In lab:

Add DNA to master mix tubes; add water to negative control (same water that you used in MM)
Flick or vortex tubes to mix; spin down
Place back in rack on **ice**
Turn on PCR machine
Start program: TIT6860
Press PAUSE when block starts to heat up. Should hold at 94C
Place tubes in HOT block, press PAUSE again to start the program running. Look at display to confirm program is running. Program will run about 2.5 h
When complete, remove tubes and store in fridge; turn off PCR machine

4. GEL ELECTROPHORESIS – In lab:

Choose 50mL or 90mL with 1 or 2 combs based on number of samples
Tape ends of plastic gel former, add combs, confirm comb height
Make up 1% gel
e.g. 0.5g agarose + 50mL buffer from fridge (SybrSafe is already added)
Microwave 45s; swirl; microwave in additional 10s bursts until FULLY melted/clear

*****CAUTION WITH HOT GEL!*****

Cool gel on bench (about 5 minutes) or on ice (30s) until able to touch base of flask
Pour agarose; check for leaks; should set in 20min
Remove tape from gel former; remove combs
Place gel into correct bath; if necessary, fill gel bath with bulk TAE to JUST over gel surface; Replace entire buffer after 5-8 uses (down sink, rinse, refill)
Load gel with 4ul 1kb+ ladder (aliquot in fridge – dark green/blue); 4ul PCR products
Cover gel bath with plastic lid
Electrophorese at 160V for 15 minutes (BLACK electrode to back/top of gel)

5. VISUALIZE RESULTS – PHOTOGRAPH GEL – Room 502:

Turn on UV lamp (bottom switch) and computer up to 5 min before use to warm up
Carry gel in tray, in glass dish; transfer to plate in geldoc under WHITE light (top switch)
Start software, preview, adjust time to less than a second, move gel to center
Check bottom ring (focus) should be on 1
Adjust middle ring (zoom) to fill image with gel
Adjust top ring (aperture) to about 4 (turn to 1 for aligning gel at start)
Turn off white light, turn on UV
Adjust exposure time to about 7 sec, then as necessary to see ladder
Capture several images if bands are different brightnesses
Adjust contrast/brightness/gamma of image as needed
Label gel with PCR number using text tool ("A")
Print if needed; tear photo rapidly up and away – care not to jerk film roll crooked

Save image/s to your PCR folder
Shut down computer especially over weekends

6. ANALYZE RESULTS – Write up in your PCR book

A well-amplified PCR product should be as bright as the ladder. You can still purify & sequence a product that is about half as bright.

If bands are weak or do not appear, then use 1.0ul of the first round product in second round PCR.

7. SEQUENCING - In PCR prep room, then main lab

Thaw primer Cs1479F
Label a tube strip with PCR code and number each tube, one tube for each reaction you want to sequence; note sequence name/number in workbook
Add in each tube, 10.3ul H2O plus 1.2ul primer Cs1479f
Carry in to main lab
Add 0.5ul DNA from each reaction you want to sequence
Carry completed tubes over to CGRB lab in ALS, into freezer box "Sequencing To Do – Small Tubes"
Complete online sequence order form (only Stephen currently authorized to do this)

Appendix 1.4**: *Manayunkia speciosa* genotyping assay from crude extracted polychaetes**

1. PREPARATION - In lab:

Thaw DNA samples (but leave in lab!)
Vortex or flick samples to mix
Take an ice bucket and fill with ice from autoclave room
If using Master Mix – remove appropriate number of strips from freezer, put in tray in your ice bucket, go to section 3.

2. PREPARATION OF MASTER MIX - In PCR-prep room:

Thaw your aliquots of reagents to room temperature (leave Taq in freezer)
Vortex briefly, then spin down. Vortexing is ESSENTIAL for buffer and $MgCl_2$
Calculate master mix based on following amounts X number of reactions + 8% for error.
For each 20ul reaction:

Reagent	Amount
DNA	2.0 ul
Manspec2F	1.0 ul
Manspec2R	1.0 ul
Taq	0.25 ul
dNTPs	0.4 ul
BSA	0.5 ul
Rediload	0.5 ul
$MgCl_2$	1.2 ul
Buffer	4.0 ul
H_2O	9.15 ul
Total	**20 ul**

Add reagents to 1.5mL tube, vortex, spin down
Label required number of 0.2mL strip tubes, in rack, on ice
Pipette 18ul master mix into each tube
Master mix can be made ahead of time and refrozen.

3. RUN PCR - In lab:

Add DNA to master mix tubes; add water to negative control (same water that you used in MM)
Flick or vortex tubes to mix; spin down
Place back in rack on **ice**
Turn on PCR machine
Start program: FASTCOI
Press PAUSE when block starts to heat up. Should hold at 94C
Place tubes in HOT block, press PAUSE again to start the program running. Look at display to confirm program is running. Program will run about 2.5 h
When complete, remove tubes and store in fridge; turn off PCR machine

4. GEL ELECTROPHORESIS – In lab:

Choose 50mL or 90mL with 1 or 2 combs based on number of samples
Tape ends of plastic gel former, add combs, confirm comb height
Make up 1% gel
e.g. 0.5g agarose + 50mL buffer from fridge (SybrSafe is already added)
Microwave 45s; swirl; microwave in additional 10s bursts until FULLY melted/clear

*****CAUTION WITH HOT GEL!*****

Cool gel on bench (about 5 minutes) or on ice (30s) until able to touch base of flask
Pour agarose; check for leaks; should set in 20min
Remove tape from gel former; remove combs
Place gel into correct bath; if necessary, fill gel bath with bulk TAE to JUST over gel surface; Replace entire buffer after 5-8 uses (down sink, rinse, refill)
Load gel with 4ul 1kb+ ladder (aliquot in fridge – dark green/blue); 4ul PCR products
Cover gel bath with plastic lid
Electrophorese at 160V for 15 minutes (BLACK electrode to back/top of gel)

5. VISUALIZE RESULTS – PHOTOGRAPH GEL – Room 502:

Turn on UV lamp (bottom switch) and computer up to 5 min before use to warm up
Carry gel in tray, in glass dish; transfer to plate in geldoc under WHITE light (top switch)
Start software, preview, adjust time to less than a second, move gel to center
Check bottom ring (focus) should be on 1
Adjust middle ring (zoom) to fill image with gel
Adjust top ring (aperture) to about 4 (turn to 1 for aligning gel at start)
Turn off white light, turn on UV
Adjust exposure time to about 7 sec, then as necessary to see ladder
Capture several images if bands are different brightnesses
Adjust contrast/brightness/gamma of image as needed
Label gel with PCR number using text tool ("A")
Print if needed; tear photo rapidly up and away – care not to jerk film roll crooked

Save image/s to your PCR folder
Shut down computer especially over weekends

6. ANALYZE RESULTS – Write up in your PCR book

A well-amplified PCR product should be as bright as the ladder. You can still purify & sequence a product that is about half as bright.

If bands are weak or do not appear, then use 1.0ul of the first round product in second round PCR.

7. SEQUENCING - In PCR prep room, then main lab

Thaw primer Cs1479F
Label a tube strip with PCR code and number each tube, one tube for each reaction you want to sequence; note sequence name/number in workbook
Add in each tube, 10.3ul H2O plus 1.2ul primer Cs1479f
Carry in to main lab
Add 0.5ul DNA from each reaction you want to sequence
Carry completed tubes over to CGRB lab in ALS, into freezer box "Sequencing To Do – Small Tubes"
Complete online sequence order form (only Stephen currently authorized to do this)

Appendix 1.5: **Rough polychaete extraction**

IMPORTANT NOTES:
*Change gloves anytime they contact a liquid.
*Bleach counter before and after processing.
*Use strip caps that fit; Foil seals are ok for some steps
no ETOH ASK!

1. DEHYDRATION- (red sticker)

Take a 96-well plate of worms from the fridge.
Write plate name, date and time in processing log.
Place in plate spinner and spin for ~10 seconds. Go down to CGRB in ALS to do this.
Slowly remove the strip caps. Be careful not to spill or splash any ethanol.
Place plate in hood and allow ethanol to evaporate. This will take several hours to several days, depending on how much ethanol was added to the wells
Put a blue sticker over the red one to indicate it is ready for step 2.

2. DIGESTION: ATL/Proteinase K- (blue sticker)

Once ALL ethanol has evaporated and samples are completely dry:

Turn flat bed rocker on, set 37°C.
Prepare ATL/PK Master Mix:

Reagent	Amount/sample+10%
ATL	95 ul
Proteinase K (PK)	5.0 ul
Total	**100 ul**

E.g., if you have 10 samples, place 950 µL ATL+50 µL PK into a 1.5 mL tube.

Use repeat pipettor to add 100µl of master mix to each well
***Make sure pipettor is set to 1 and you are using the 100µl size.
Seal plate with STRIP CAPS.
*Make sure: top is dry, blot with kimwipe if necessary.
the plate label is still readable. If not, make a new one.
Place a purple sticker on plate corner over the old blue one to indicate it is ready for
Place in oven overnight, or 2-4 hrs with regular inversions.

3. PURIFICATION: Proteinase K- (purple sticker)

Vortex briefly, spin briefly.
Place in PCR machine polychaete program **BAKEPOLY** (17 minutes @ 85C with heated lid)

Appendix 1.6**: Quiagen PCR product purification**

1. PREPARATION

Add ethanol (96-100%) to buffer PE before use

2. PURIFICATION

Add 5 volumes buffer PB to 1 volume of the PCR reaction and mix.
For *M. speciosa*: 10ul PCR Products
For *C. shasta*: 5ul PCR Products
Place a spin column in a 2mL collection tube
Apply prepared sample to the spin column
Centrifuge 30-60 seconds on 8000 RPM
New collection tube
Add 750ul buffer PE to spin column
Centrifuge 30-60 seconds on 8000 RPM
New collection tube
Centrifuge 30-60 seconds on 8000 RPM
Place into clean 1.5 mL microcentrifuge tube
Add 50ul buffer EB to center of spin column membrane
Centrifuge 30-60 seconds on 8000 RPM

Place in freezer until sequencing preparation