Coevolution of Xiphinema americanum plant parasitic nematodes and their bacterial endosymbionts

by
McKinley S. Smith

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biology
(Honors Scholar)

Presented June 2, 2015
Commencement June 2015

AN ABSTRACT OF THE THESIS OF

McKinley S. Smith for the degree of Honors Baccalaureate of Science in Biology presented on June 2, 2015. Title: Coevolution of Xiphinema americanum plant parasitic nematodes and their bacterial endosymbionts.

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Dee Denver

Symbiosis takes place across the domains of life. In the plant-parasitic nematode, *Xiphinema americanum* species complex, the bacterial endosymbiont, identified as Candidatus Xiphinematobacter americanum, lives in the gut epithelia of mature female nematodes and moves to the ovaries and uterus where it is transmitted to the eggs. This suggests that the symbiont may be vertically transmitted. PCR followed by DNA sequencing was conducted on three loci, using nematode samples from across North America. Two loci were sequenced from the symbiont and the third from the mitochondrial DNA of the nematode. Using these sequences, phylogenetic trees were constructed for the nematode and the symbiont to provide new insights into the taxonomy of the nematode species, and to shed light on the potential for coevolution between the worm and its endosymbiont. A mitochondrial tree was constructed to examine nematode evolution. Mitochondrial phylogenetic analysis for the nematode built from work that suggested there are additional clades within the *X. americanum* species complex than previously known. The congruent phylogenies derived from these methods strongly suggest that the symbiont is primarily transmitted in a vertical fashion along with the germline of its nematode host.

Key Words: Nematode, endosymbiont, Xiphinema, species complex, symbiosis

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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.

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Coevolution between *Xiphinema americanum* plant parasitic nematodes and their bacterial endosymbionts

McKinley Smith

Supervisor Dee Denver

Honors Thesis

Introduction

Symbiosis occurs between many different species from among the domains of life. Eukaryotic life has its beginnings billions of years ago when protoeukaryotic cells engulfed a prokaryotic precursor of the modern-day mitochondria and chloroplasts (Sagan 1967). The relationship is known broadly as symbiosis, occurs between two or more species, and is characterized by a close association (Moya et al. 2008). This encompasses parasitic interactions, such as that between a tick and its host, as well as the commensal relationship between a remora and a shark and the mutualist relationship between corals and dinoflagellates (Moya et al. 2008). Symbiosis is further characterized by its relation to the host's cells, namely, whether it is an endosymbiont, living within the host's cells, or an ectosymbiont, living outside the host's cells (Moya et al. 2008).

Prokaryotes are commonly engaged in symbioses with eukaryotic hosts (Moran et al. 2008). Primary bacterial endosymbionts have typically evolved with their hosts for long periods of time to the point that they rely on the host for survival and are transmitted vertically, from parent to offspring (Moya et al. 2008). In contrast, facultative, secondary endosymbionts are less dependent on their hosts and may transmit horizontally, between members of a population (Moya et al. 2008). Because the relationship between bacterial symbionts and their hosts has often been intimately and inextricably tied to the latter, bacterial symbionts have been difficult to culture (Moran et al. 2008, Moya et al. 2008, Wernegreen 2002). The advent of modern molecular

biology, especially the polymerase chain reaction (PCR) and DNA sequencing, has made studying the genetics and evolution of these bacteria easier (Moran et al. 2008, Moya et al. 2008, Wernegreen 2002).

There are many examples of primary bacterial endosymbionts in invertebrates. Primary bacterial endosymbionts tend to be transmitted vertically through the maternal line, aggregate in specialized host cells, and play a role in nutrition (Wernegreen 2002). There are many examples of primary bacterial endosymbionts in insects, including *Curculio* weevils (Toju et al. 2010), whiteflies (Thao et al. 2004), and aphids (Martinez-Torres et al. 2001). Vesicomyid clams have bacterial endosymbionts that localize around the follicles, promoting vertical transmission and providing vital metabolic functions for the clams, which live around deep sea hydrothermal vents (Cary and Giovannoni 1993). Among filarial nematodes, the bacterial endosymbiont *Wolbachia* has been found in several species and observed to have a phylogeny that is congruent with its host (Brandi et al. 1998), suggesting vertical transmission. A plant parasitic nematode, *Radopholus similis*, was found to harbor *Wolbachia* endosymbionts in all individuals observed, with an endosymbiont observed with DAPI stains and immunolocalization in the ovaries and uterus (Haegeman et al. 2009), again, suggesting vertical transmission and the species' reliance on the endosymbiont.

Xiphinema americanum (Nematoda; Longidoridae) is a plant parasitic nematode, commonly called the dagger nematode, that is of agricultural importance, as it causes damage to economically important crops through feeding and subsequent damage by incidentally infecting the parasitized plant with nepovirus. *Xiphinema americanum* species live in the soil and are typically between 1.3 to 3 mm long as adults (Zasada et al. 2014) and have an odontostyle at the anterior end that they use to pierce into plant cells and feed on the cytoplasm (O'Bannon and

Inserra 1990). *Xiphinema americanum* species are found in Africa and North America (Lamberti et al. 2000) and can serve as a vector for cherry rasp leaf nepovirus, peach rosette mosaic nepovirus, tobacco ringspot nepovirus, and tomato ringspot nepovirus (Lamberi et al. 2000). The taxonomy of the *X. americanum* species complex is unresolved, so that it is difficult to predict may host the nepovirus and which may not, thwarting agriculturalists from using morphological or modern laboratory techniques to distinguish between problematic nematodes that may harbor the virus and selectively apply nematicides only when necessary (Zasada et al. 2014). Zasada et al. (2014) used PCR analysis of a multi-gene region of mitochondrial DNA to separate *X. americanum* populations into three different clades, one of which did not have the nepovirus.

Members of the *X. americanum* species complex have been shown using fluorescence in situ hybridization to contain a bacterial endosymbiont, *Candidatus* Xiphinematobacter americanum (hereafter *Xiphinematobacter*) aggregating within the gut epithelium and ovary epithelia in mature female nematodes (Vandekerckhove et. al 2002). *Xiphinematobacter*, a member of the Verrucomicrobia, is a rod-shaped, Gram-negative bacteria that is an obligate cytoplasmic endosymbiont of several *X. americanum* species complex populations, possibly diverging from its free-living lineage some 50 to 140 million years ago (Vandekerckhove et al. 2000). *Xiphinematobacter* does not have a peptidoglycan wall, similar to other obligate endosymbionts (Vandekerckhove et al. 2000). Not much is known about the function of *Xiphinematobacter* in the host, although there is some indication that it may cause parthenogenesis, which may transmit the endosymbiont to their offspring (Vandekerckhove et al. 2000); *X. americanum* species reproduce through thelytokous parthenogenesis (Coomans et al. 2000). *Xiphinematobacter* has not been cultured, and the role of *Xiphinematobacter* has not been demonstrated.

The purpose of this research was twofold. The first objective was to provide new insights into the phylogenetic relationships and taxonomy within the *X. americanum* species complex, using phylogenetic analysis of mitochondrial sequences derived from samples around North America. The second objective was to investigate the coevolution between the endosymbiont and nematode host by comparing maximum likelihood phylogenies derived from host and symbiont DNA sequences, with congruent phylogenies suggesting vertical transmission of *Xiphinematobacter* by *X. americanum*.

Methods and Materials

Sample Collection and Storage

Nematode samples were collected by Amy Peetz at the United States Department of Agriculture in 2014 from locations around the United States and Canada (Table 1.). Samples were chosen to maximize diversity and to include a range of predicted mitochondrial clades. Based on Zasada et al. (2014), populations from a diversity of geographic locations, host plants, predicted presence/absence of the nepovirus determined through symptoms of nepovirus infection or based on past occurrence at a given site were included in the study.

Table 1. *Xiphinema* species complex populations included in the study

Label	Location	Host	Predicted Clade	Predicted Virus
AR-1	Hope, AR	Blackberry	mtA	+
BC-2	Abbotsford, BC	Raspberry		-
CO-4	Mesa, CO	Cherry	mtA	+
CT-1	Windsor, CT	Apple	mtA	+
NC-1	Fletcher, NC	Blackberry	mtA	+
NY-1	Geneva, NY	Grape	mtA	+
OR-7	Alpine, OR	Grape	mtA	-
OR-8	Salem, OR	Grape	mtA/C	-
PA-1	Biglerville, PA	Apple	mtB	+
WA-2	Paterson, WA	Grape	mtC	-
WA-5	Mattawa, WA	Grape	mtC	-
WA-8	Woodland, WA	Raspberry	mtC	+
X. rivesi	Yakima, WA	Cherry	mtC	+
X. pachtaicum	Prosser, WA	Grape	mtC	-
X. bakeri	Lynden, WA	Raspberry	mtC	-

Table 1. Labels correspond to the place where the samples were collected, the type of host plant that was parasitized, the predicted mitochondrial clade, and the predicted absence or presence of the nepovirus.

Nematodes were isolated from soil by decant/sieving and sugar centrifugation as described by Zasada et al. (2014). Nematodes were stored at -20°C in 1 M NaCl.

DNA Extraction

Nematodes in 1 M NaCl were thawed on ice then pipetted onto a Petri dish and individually placed in separate wells of a glass slide using a worm pick. Wells contained 10µl lysis buffer (50mM KCl, 0.5% gelatin, 10mM Tris, 45% v/v Tween 20, 60 µg/ml Proteinase K, and 2.5 mM $MgCl_2$ in molecular grade water.) Nematodes were cut into at least three sections using a scalpel, then 10µl additional lysis buffer was added, and the entire mixture, including nematode pieces, was pipetted into a 0.2 mL strip tube on ice. Tubes containing nematode pieces and lysis buffer were incubated for 1.5 hours at 60°C, heated for 15 minutes at 95°C, and cooled to 10°C in the

Bio-RAD DNA Engine® or MJ Research PTC-200 thermal cycler. Extracted DNA was stored at -20°C.

PCR Primer Design

Primers were designed in the Denver lab based on previously published sequences in NCBI GenBank and on unpublished sequences obtained through Illumina MiSeq on a sample of *X.americanum* from a grape vineyard in Idaho, as follows. Primers were designed for *Xiphinematobacter* and *X. americanum.* Targets were chosen to identify sufficient genetic divergence to allow individuals from different regions to be distinguished.

Mitochondrial DNA (mtDNA) from the nematode was chosen because nucleic 18S genes are too slow evolving to be of use for comparing genetic divergence between individual nematodes from the *X. americanum* species complex (Zasada et al. 2014. Analysis of sequences from the mtDNA cytochrome c oxidase I (COXI) and 12S ribosomal RNA regions suggested suitably conserved primer targets with enough divergence in the intervening sequence to be useful for analysis.

Primers for the 16S ribosomal RNA region of *Xiphinematobacter* were chosen to be specific enough to bind preferentially to *Xiphinematobacter* over other bacteria and thus identify the presence of *Xiphinematobacter* in samples containing *X. americanum* species complex nematodes. The primers for the NAD (P) H dehydrogenase region of *Xiphinematobacter* were chosen because it was predicted to be a highly variable region of the genome (compared to the 16S rRNA region), comprised of parts of two genes, arginine decarboxylase and NAD (P) H dehydrogenase, and the non-coding intergenic region between them, providing an opportunity to compare sequence divergence between members of the same species.

Table 2. Primer Sequences

Primer type	5' to 3' Sequence	Genome position
Xiph_16S-F	TGCCAGCAGCCGCGGTAATACA	18096
Xiph_16S-R	GCAGCCTACAATCCGAACTGGGC	17309
xNAD-F	CGGTCCCGAAGATCYTGRAA	24095
xNAD-R	ACGCATTTCTTAAACCCTCAYTT	22930
XiphNAD1-F	CCCACGATGGCGGGCTTCATTTA	24037
XiphNAD2-R	TCCTCCACTAAGCCCTAGTACGC	22930
Xa_Cox1F	GAGCACAYCAYATRTTTAGACT	9861
Xa_12SR	CGACAAGGATYAGATACCCTTTT	12154

Table 2. Primers were created using unpublished genome data from *Xiphinematobacter* for symbiont primers and the mitochondrial genome for of *Xiphinema americanum.*

Polymerase Chain Reaction

PCR was performed in 25 µL and 50 µL reactions. In the 25 µL reactions, 18.7 µL of molecular grade water, 2.5 µL of Lucigen 10X Econo Taq® Buffer, 1 µL of 10µM forward primer, 1 µL of 10µM reverse primer, 0.5 µL of 10mM dNTPs, 0.3 µL at 5 U/µL of Lucigen Econo Taq® DNA Polymerase, and 1 µL of extracted DNA were added to each strip tube. In 50 µL reactions, 38.7 µL of molecular grade water, 5 µL of Lucigen 10X Econo Taq® Buffer, 2 µL of 10µM forward primer, 2 µL of 10µM reverse primer, 1 µL of 10mM dNTPs, 0.3 µL at 5 U/µL of Lucigen Econo Taq® DNA Polymerase, and 1 µL of extracted DNA were added to each strip tube.

Thermal cycler conditions were specific to each of the three regions. PCR52, which was used for the NAD and 16S regions, begins at 95°C for 2 minutes then cycles 36 times at 95°C for 30 seconds, at 52°C for 20 seconds and at 72°C for 2 minutes, ending at 72°C for 10 minutes. PCR56, which was used for the 16S region, begins at 95°C for 2 minutes then cycles 36 times at 95°C for 30 seconds, at 56°C for 20 seconds, and at 72°C for 2 minutes, ending at 72°C for 10 minutes. PCR52-3M, which was used for the mtDNA region, begins at 95°C for 2 minutes then

cycles 34 times at 95°C for 30 seconds, at 52°C for 20 seconds, and at 72°C for 3 minutes, ending at 72°C for 10 minutes.

Products were visualized using agarose gel electrophoresis. Five µL of PCR product was added to 2 µL of gel loading dye 6X blue (New England Biolab) and run on a 1% agarose gel alongside a mix of 3 µL 1Kb Plus DNA Ladder and 2 µL loading dye. The agarose gel was made by heating and cooling agarose with 1XTAE buffer. The gel was run at 100V and visualized using BioDoc-It™ Imaging System UV Transilluminator.

PCR Product Purification

PCR products were profiled using the Invitrogen ChargeSwitch® PCR Clean-Up Kit protocol. PCR products of 20-45 µL were mixed with 10 µL of Invitrogen ChargeSwitch® Magnetic Beads (25 mg/mL in 10 mM MES, pH 5.0, 10 mM NaCl, 0.1% Tween 20), and 25-50 µL of 0.5 M SPRI Hyb Buffer (0.5 M NaCl, 20% PEG8000) in strip tubes and left at room temperature for five minutes. Then the products were moved to a magnet until beads formed pellets along the sides of the tubes. The fluid was then aspirated and discarded. While the samples were on the magnet, two washes with 150 µL 80% ethanol were conducted before the samples were allowed to dry for 20-25 minutes. They were then removed from the magnet and mixed with 23 µL molecular grade water, disturbing the pellet in the process and mixing until the solution was well mixed, thus releasing the DNA into the water. The samples were retransferred to the magnet and pellets were allowed to form. Finally, 20 µL of the liquid containing DNA was removed and placed into new tubes without disturbing the pellet.

Purified products were visualized using agarose gel electrophoresis.

DNA Sequencing

Products were sequenced using 4 µL of purified PCR product, 1 µL sequencing buffer 1 µL of 2 µM reverse primers, 1 µL of BigDye® Terminator v. 3.1 (Applied Biosystems), and 3 µL molecular grade water. The samples were run in the Bio-RAD DNA Engine® or MJ Research PTC-200 thermal cycler, with 25 cycles of 96° for 30 seconds, 50° for 15 seconds, and 60° for 4 minutes. Samples were ethanol precipitated and sequencing was conducted using Economy Sanger Sequencing at the Center for Genome Research and Biocomputing (CGRB; Oregon State University, Corvallis, OR).

DNA Sequence Analysis

Sequences were analyzed using MEGA6 (Tamura et al. 2013). Chromatographs were visualized using MEGA6 software and were trimmed based on personal confidence in the reliability of the read (presence of discrete peaks), with most trimming occurring at the ends of the reads. Areas with excess terminator sequence artifacts were inspected to check for correct sequencer calls. Chromatographs were analyzed visually for evidence of single nucleotide polymorphisms, by identifying peaks that were about half as tall or more of the peaks above them.

Trimmed sequences were loaded into MEGA6 and aligned using MUSCLE (Edgar 2004) with default settings.

Maximum likelihood trees were produced for each sequence region (i.e., 16S, NAD, nematode mtDNA) in MEGA6 using data from alignments and a bootstrapping value of 1000 for high confidence. All other settings were default (Substitutions Type: nucleotide, Model/Method: Tamura-Nei model, Rates among Sites: Gamma Distributed, Number of Discrete Gamma Categories: 5, Gaps/Missing Data Treatment: complete deletion, ML Heuristic Method: Nearest-Neighbor-Interchange, Initial Tree for ML: Make initial tree automatically (Default – NJ/BioNJ),

Branch Swap Filter: Very Strong, Number of Threads:1). The only exception was that one phylogenetic analysis for the nematode mtDNA used a general time reversible model of molecular evolution (Fig. 1). Samples that produced successful sequences for all three primer sets were aligned again, this time excluding all samples that did not produce successful sequences for all three primer sets. Maximum likelihood trees were created for each sequence region and analyzed visually for deviations in branching between nematode and endosymbiont DNA.

Results

PCR Analysis

Three genetic loci were analyzed for this study. A mtDNA locus from the cytochrome oxidase I region that stretched across intergenic space to a 12S rRNA sequence from the nematode was used to resolve the nematode lineage and compare it to the endosymbiont phylogeny. A slow-evolving 16S rRNA locus from the endosymbiont was used to identify the endosymbiont and serve as a control to compare to the results from the fast-evolving endosymbiont NAD locus, which was used to more clearly resolve the endosymbiont phylogeny. Out of the 78 samples included in this research, differing levels of success were observed for each locus.

The mtDNA locus produced 37 successful sequences (Table 3, 4). Samples from CT-1 and NC-1 amplified, but no successful sequences were achieved. Samples from WA-5 and Xrivesi-1 were difficult to amplify and provided successful sequences half the time that PCR was successful. For the Fig. 5 alignment, sequences were trimmed to 594 base pairs. For the Fig. 1 alignment, sequences were trimmed to 596 base pairs.

The most successful locus was the *Xiphinematobacter* 16S region. 59 sequences were successful (Table 3, 4). For the Fig. 4 alignment, sequences were trimmed to 659 base pairs. For the Fig. 2 alignment, sequences were trimmed to 606 base pairs. There was only one instance in which 16S sequencing failed and another locus was successful, and that was with the sample NY-1-5, which sequenced successfully for the nematode mtDNA locus, but not the symbiont loci; however, PCR in all three cases was successful as verified by agarose gel electrophoresis.

The NAD locus was the second most successful with 49 successful sequences retrieved (Tables 3, 4). Several different combinations of primers were used. The NAD1F/NAD2R primer set was used for the Fig. 3 alignment, sequences were trimmed to 592 base pairs. Samples AR-1, CT-1, WA-5, and WA-7 were difficult to amplify for this locus, and WA-7 was difficult to sequence.

Table 3. DNA sequencing success with *Xiphinema americanum*

Sample location	**Number extracted**	**(Xiph_16S-F/ Xiph_16S-R)**	**(xNAD-F/xNAD-R)**	**(Xa_Cox1F/Xa_12SR)**
Arkansas	5	4	3	3
British Columbia	5	5	4	4
Colorado	5	5	4	3
Connecticut	5	5	4	0
Idaho	1	1	1	1
North Carolina	7	4	4	0
New York	5	5	3	4
Pennsylvania	5	4	4	4
Oregon	10	8	8	6
Washington	18	13	13	14
Totals	66	54	48	36

Table 3. Success rates for each locus differed, with the 16S primers being the most successful, the NAD primers being the second most successful, and the mitochondrial primers being the least successful.

Table 4. Sequencing success in other *Xiphinema* species

Species name	(Xiph_16S-F/ Xiph_16S-R)	(xNAD-F/xNAD-R)	(Xa_Cox1F/Xa_12SR)
X. rivesi	4	1	1
X. pachtaicum	1	0	0
X. bakeri	0	0	0

Table 4. Success rates for each locus differed between each species. *X. rivesi* sequenced the most easily, *X. pachtiacum* only worked with the 16S primer set, and *X. bakeri* didn't work at all.

Phylogenetic Analysis

Phylogenetic analysis was performed using the MEGA6 maximum likelihood program described in the methods to investigate the relationship of *X. americanum* clades and the coevolution of the nematode and the endosymbiont.

The tree (Fig. 1) produced using mtDNA data and the general time reversible model of molecular evolution option for a maximum likelihood test included data from the samples from Zasada et al. (2014) paper and samples from this study. Zasada et al. (2014) samples grouped together with samples from this study; other groupings did not contain any representatives from the Zasada et al. (2014) clades.

Figure 1. Maximum likelihood bootstrap analysis of aligned mitochondrial sequences

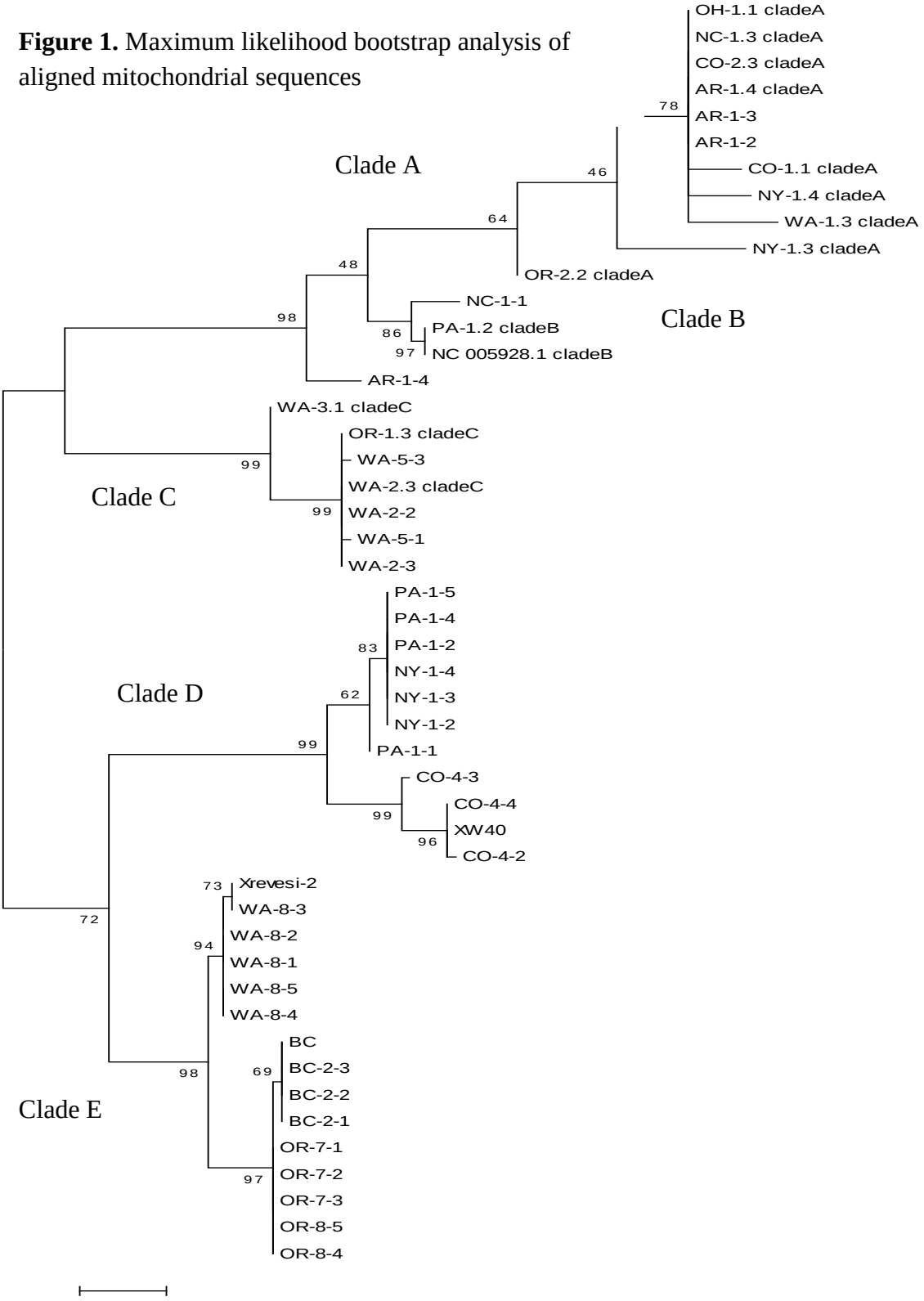

Figure 1. Maximum likelihood bootstrap analysis of aligned mtDNA sequences. 48 individuals from the *X. americanum* species complex were analyzed. Sequence names denote the location and individual as denoted in Table 1. Samples including clade notations provided courtesy of Zasada et al. 2014. Tree constructed using General time reversible model of molecular evolution in MEGA6.

Samples from clade A of Zasada et al. (2014) grouped together with samples from the same location, AR-1. Clade B samples from Zasada et al. (2014) grouped together with a NC-1 sample from this study, while clade C samples grouped with other regional samples from this study with 99% bootstrap support. Other samples did not group within the Zasada et al. (2014) clades, even samples from similar locations. Samples from PA-1, NY-1, CO-4, and XW40 grouped in a clade with 99% bootstrap support. Samples from WA-8, BC-2, OR-7, OR-8, and *X. rivesi* grouped together in another clade with 98% bootstrap support, with *X. rivesi* and WA-8 samples forming a subclade with 94% bootstrap support. Samples grouped geographically, with some exceptions. Not all samples from a particular state grouped with samples from the same state. OR-2.2 clade A from Zasada et al. (2014) grouped with samples from the Midwest and Northeast, and XW40 from Washington groups with the CO-4 samples.

The symbiont 16S rRNA phylogeny (Fig. 2) was less resolved than that for *X. americanum*. Samples from WA-2, WA-5, and one individual from OR-8 clustered as a clade outside of the bulk of the other samples. The tree then split into a clade containing one individual of *X. rivesi* and one from North Carolina as well as all Arkansas and Connecticut samples. The largest clade contained a clade with samples from Oregon, Washington, *X. rivesi*, and British Columbia and another clade containing several individuals from New York, Colorado, Pennsylvania, North Carolina as well as one individual each from Idaho and Washington. *Xiphinema pachtaicum* was also a part of this clade.

The phylogenetic trees for the three loci shared many similarities. In Figs. 3, 4, and 5, samples from Oregon and British Columbia grouped with a clade of Washington samples, while samples from Colorado, Washington, New York and Pennsylvania formed a sister clade. Two Washington samples from the same locations fell at the outside of the phylogeny along with a sample from North Carolina, and were most distantly related from the other samples. In the NAD tree, British Columbia samples formed a subclade from the Oregon samples with 64% bootstrap support (Fig. 3), but in the 16S rRNA tree (Fig. 4), they were all part of the same clade with 72% bootstrap support. Likewise, one Pennsylvania sample was more resolved in the NAD phylogenetic analysis (Fig. 3) as well as the mtDNA analysis (Fig. 5).

Figure 2. Maximum likelihood bootstrap analysis of aligned 16S sequences

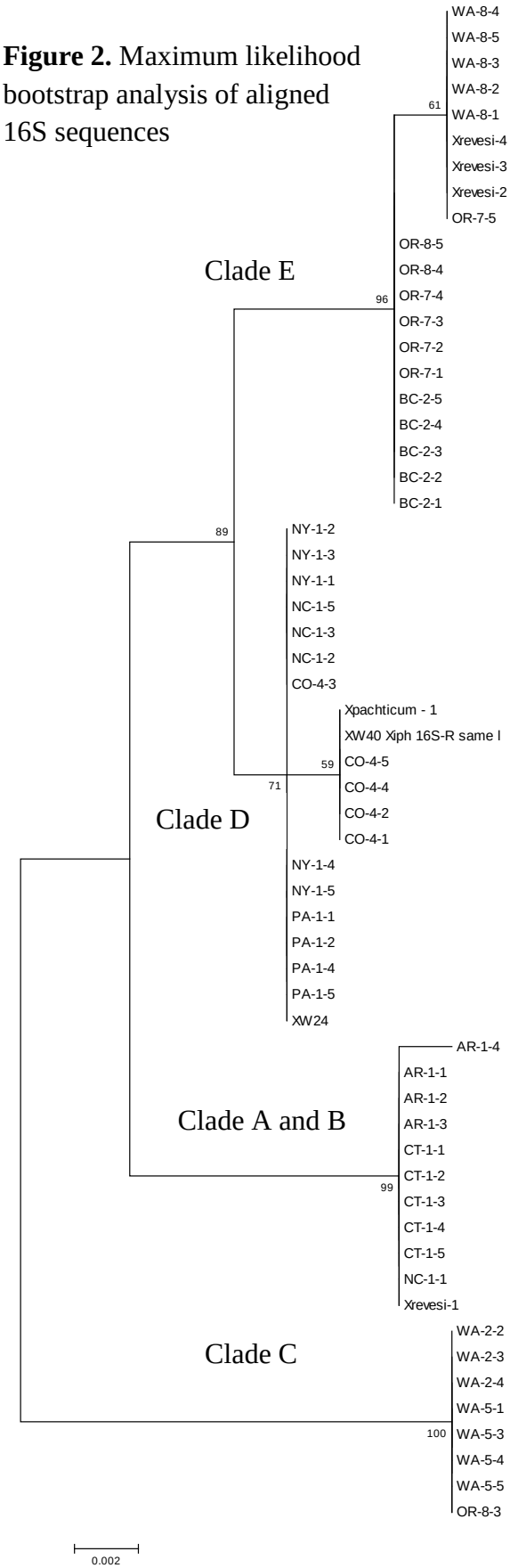

Figure 2. Maximum likelihood bootstrap of aligned 16S sequences. 59 sequences from *Xiphinematobacter* were analyzed. Sequence names denote the location and individual as denoted in Table 1.

Figure 3. Maximum likelihood bootstrap analysis of aligned NAD sequences

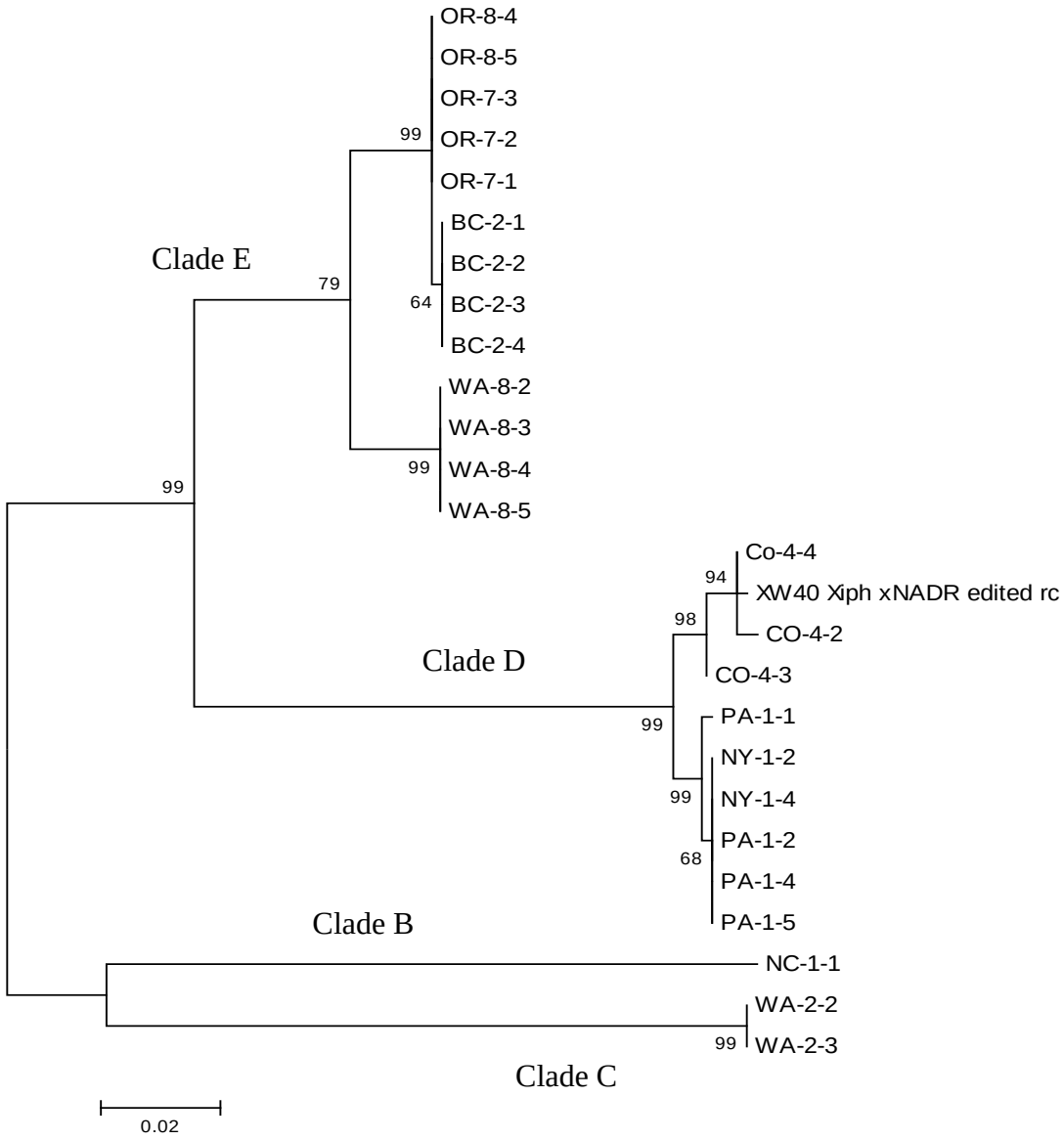

Figure 3. Maximum likelihood bootstrap of aligned NAD sequences. 26 individuals from the *Xiphinema americanum* species complex were analyzed. Sequence names denote the location and individual as denoted in Table 1.

Figure 4. Maximum likelihood bootstrap analysis of aligned 16S sequences

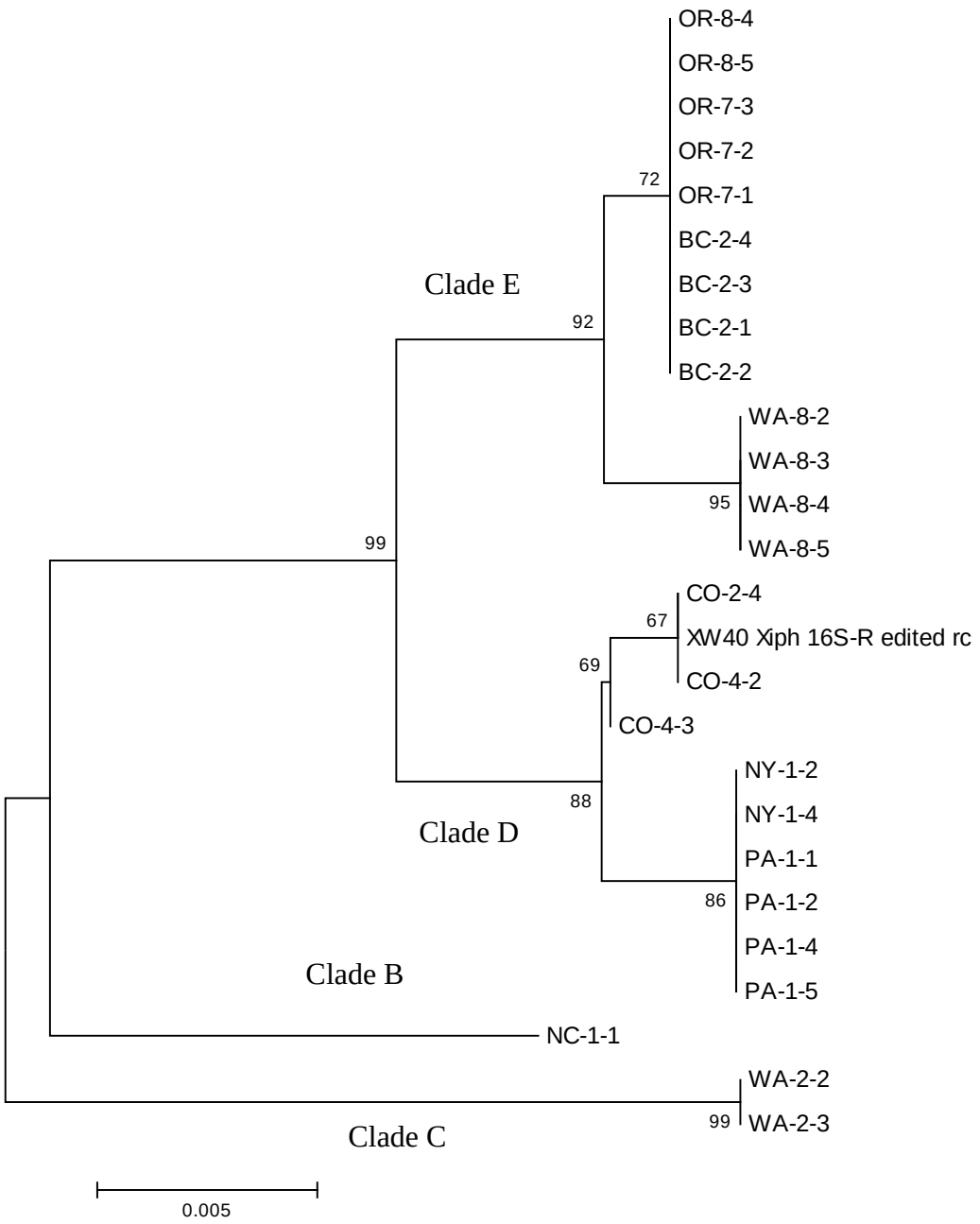

Figure 4. Maximum likelihood bootstrap of aligned 16S sequences. 26 individuals from the *Xiphinema americanum* species complex were analyzed. Sequence names denote the location and individual as denoted in Table 1.

Figure 5. Maximum likelihood bootstrap analysis of aligned mtDNA sequences

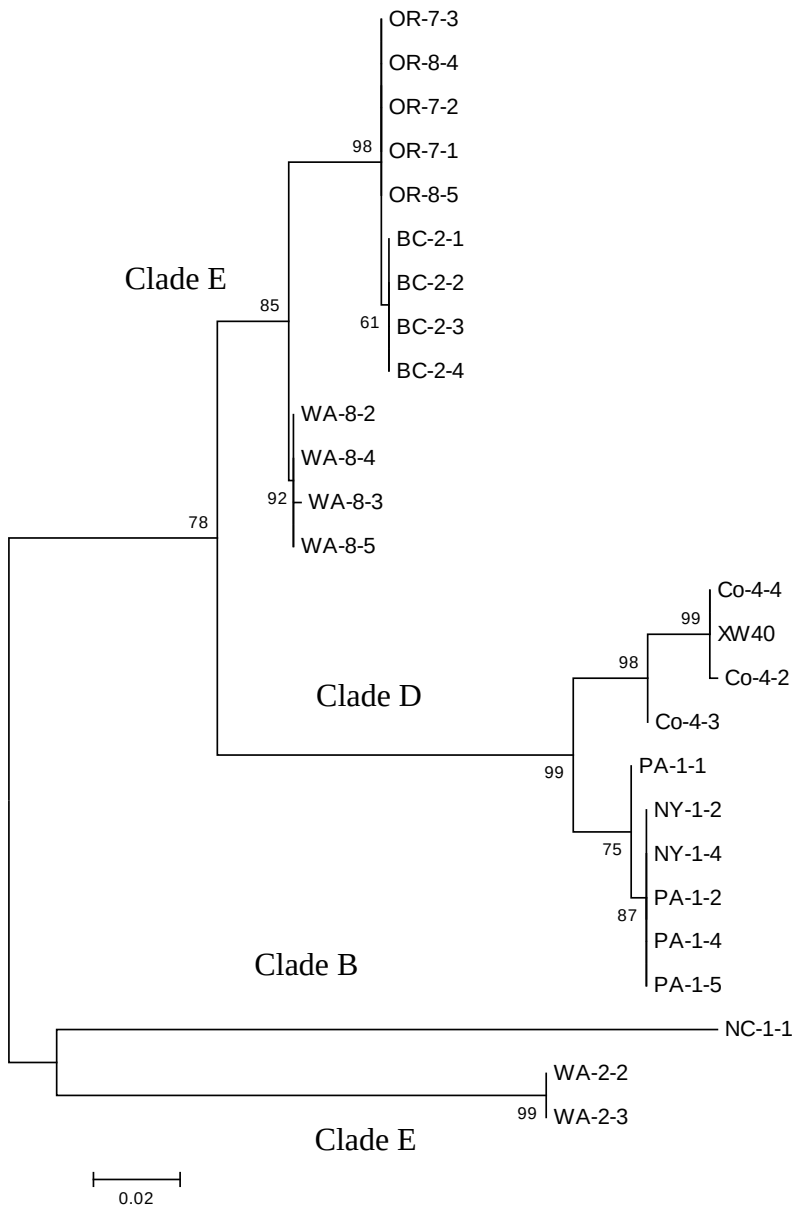

Figure 5. Maximum likelihood bootstrap of aligned mtDNA sequences. 26 individuals from the *X. americanum* species complex were analyzed. Sequence names denote the location and individual as denoted in Table 1.

Samples usually grouped with respect to geography. Oregon, Washington, and British Columbia samples formed a clade with the exception of a pair of highly diverged Washington sequences and a Washington sample that associated within a clade containing Colorado samples. Samples from Colorado, New York, and Pennsylvania grouped together. Only one sequence from North Carolina was available for all three loci. In all three phylogenetic analyses, the samples from Paterson, Washington (WA-2) formed a clade of two with a bootstrap of 99.

Discussion

This study provides new insights into the phylogenetic relationships of *X. americanum* and related species, as well as the coevolutionary patterns between the nematodes and their *Xiphinematobacter* endosymbionts. Primers for all three loci were effective for PCR, with mtDNA primers capable of identifying *X. rivesi* in addition to *X. americanum.* mtDNA primers were also capable of providing sequences sufficient to resolve the phylogeny of *X. americanum*. Primers for endosymbiont 16S rRNA and NAD sequences were also effective at demonstrating the presence of the endosymbiont.

mtDNA phylogenetic analysis supports the clades generated in the Zasada et al. (2014) paper and suggests that there are two additional clades previously unknown, preliminarily identified here as clades D and E. Samples from Zasada et al. (2014) grouped into predicted A, B, and C clades with samples from this study, but not all samples grouped with these clades. Bootstrap values of 99% and 98% support the existence of two novel clades: one clade containing samples from Pennsylvania, New York, Colorado, and Washington (clade D) and another containing samples from Washington, Oregon, and British Columbia (clade E). Clade E contained an individual of *X. rivesi* from Washington alongside other samples from Washington. The mtDNA sequence from one Washington sample was identical to that of the individual of *X. rivesi.* This grouping

suggests that these Washington samples are likely *X. rivesi*, as well as perhaps those from Oregon and British Columbia that share clade E. Sequences from morphologically identified outgroups such as *X. rivesi* and *X. bakeri* by expert nematologists are necessary to further resolve the phylogeny of the *X. americanum* species complex through inclusion in the current analysis.

The results (Fig. 1) suggest that there is some geographic dependence on relationships. Samples tended to group by broader geographical region, with a few notable exceptions. For example, XW40, a Washington sample, grouped with Colorado samples. It is possible that the Washington location from which XW40 was collected had been contaminated with worms from Colorado.

This paper builds on the work of several other researchers who have suggested the vertical transmission of *Xiphinematobacter* in *X. americanum* (Coomans et al. 2000, Vandekerckhove et al. 2012). The phylogenies of the endosymbiont (Figs. 3, 4) and nematode (Fig. 5) were largely congruent and showed only a few differences, including minor variations in supporting bootstraps values and resolution within clades, but are overall very similar. This evidence supports the conclusion that *Xiphinematobacter* is transmitted through vertical transmission and not horizontally. As an endosymbiont that is exclusively vertically transmitted, *Xiphinematobacter* is likely a primary obligate endosymbiont and can be counted among the endosymbionts of this category across many genera. It is likely based on the congruent phylogenies of the host and symbiont that *X. americanum* and *Xiphinematobacter* have coevolved in an obligate relationship, beginning perhaps 100-140 million years ago (Vandekerckhove et al. 2000). The hypothesis that this is an obligate relationship between both members is consistent with suggests that endosymbiont may have influenced *X. americanum's* parthenogenic propagation, ensuring its continued transmission through oocytes (Coomans et al. 2000).

Zasada et al. (2014) used an alignment of sequences with about 2.5 kb, while this analysis used 596 bp for each sequence to generate a phylogeny, so further research using longer regions could provide more support for these conclusions about additional clades and Zasada et al.'s (2014) clades of *X. americanum*. More research could also investigate the mechanisms for favoring parthenogenesis as well as what functions *Xiphinematobacter* might perform for the host.

Acknowledgements

I would like to thank Amy Peetz from the USDA for sharing her protocol for DNA extraction and for providing nematodes for this study. I would also like to thank Inga Zasada and colleagues for contributing several mitochondrial sequences from their 2014 paper. Thanks to Dee Denver, who directed my project; Amanda Brown and Dana Howe, who assisted me throughout; Amy Peetz, who taught me her technique for extraction; Inga Zasada, who provided insight; and the Denver and Zasada labs. Finally, I would like to acknowledge the support of the Center for Genome Research and Biocomputing, the University Honors College, and the Department of Integrative Biology. Without the assistance of all of these people, I would not have had the opportunity to experience research first-hand.

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