Polymerase chain reaction and gyrA nucleotide sequence analysis of *Wolbachia* endosymbionts (Rickettsiales: Anaplasmataceae) in various species of Culicidae, *Cimex lectularius* (Hemiptera: Cimicidae) and *Dirofilaria immitis* (Rhabditida: Onchocercidae)

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Abstract

Wolbachia Hertig and Wolbach (Rickettsiales: Anaplasmataceae) are non-culturable, bacterial endosymbionts that have been found in a broad range of arthropods and other invertebrate species. They have been implicated in human and veterinary pathologies, and may play a major role in embryonic development and evolution of host species. Given the apparent ubiquity of *Wolbachia* in certain animal taxa suggested by previous studies, there are still many unanswered questions about its biology. Like other obligate intracellular bacteria, they are difficult to cultivate outside of their host and often are analyzed using molecular methods. Polymerase chain reaction (PCR) assays have been developed previously for *Wolbachia* detection within host species, and several genes have been explored for strain typing and phylogenetic reconstruction. However, given the expansive host range and biological complexity of symbiotic relationships between *Wolbachia* and its host species, new methods could help accelerate the pace of *Wolbachia* research. As part of an overarching goal to study the distribution of *Wolbachia* in local mosquitoes and in the heartworm, *Dirofilaria immitis* (Leidy) (Rhabditida: Onchocercidae), we aimed to develop cost-effective methods that can be used in strain identification and analysis. We developed a novel PCR assay targeting the gyrA gene of *Wolbachia* and explored various methods of sample preparation. Presumptive *Wolbachia* were detected in mosquito specimens from several genera, as well as from *D. immitis* samples obtained from canine necropsy. DNA sequence analysis of the PCR products confirmed the identity of *Wolbachia* and revealed variability within some regions of the gyrA gene that correspond to host species. Consequently, this gene could be useful for future phylogenetic and population studies.

Key Words: heartworm; symbiosis; *Mansonia*; evolution; mosquito; parasite

Resumen

Wolbachia Hertig y Wolbach (Rickettsiales: Anaplasmataceae) son endosimbiontes bacterianos no cultivables que se han encontrado en una amplia gama de artrópodos y otras especies de invertebrados. Se han implicado en patologías humanas y veterinarias y pueden jugar un papel importante en el desarrollo embrionario y la evolución de las especies hospederas. Dada la aparente ubicuidad de *Wolbachia* en ciertos taxones de animales sugerida por estudios previos, indican que quedan muchas preguntas sin respuesta sobre su biología. Al igual que otras bacterias intracelulares obligadas, son difíciles de cultivar fuera de su hospedero y son a menudo analizadas mediante métodos moleculares. Los ensayos de reacción en cadena de la polimerasa (PCR) se han desarrollado previamente para la detección de *Wolbachia* dentro de especies hospederas, y se han explorado varios genes para la tipificación de cepas y la reconstrucción filogenética. Sin embargo, dada la amplia gama de hospederos y la complejidad biológica de las relaciones simbióticas entre *Wolbachia* y su especie hospedera, el tener nuevos métodos podrían ayudar a acelerar el paso de la investigación de *Wolbachia.* Como parte de un objetivo general para estudiar la distribución de *Wolbachia* en mosquitos locales y en el gusano del corazón, *Dirofilaria immitis* (Leidy) (Rhabditida: Onchocercidae), nuestro objetivo fue el desarrollar métodos rentables que se puedan utilizar en la identificación y análisis de cepas. Desarrollamos un ensayo de PCR novedoso dirigido al gen gyrA de *Wolbachia* y exploramos varios métodos de preparación de muestras. Se detectaron presuntas *Wolbachia* en muestras de mosquitos de varios géneros, así como en muestras de *D. immitis* obtenidas de necropsias caninas. El análisis de la secuencia de ADN de los productos de la PCR confirmó la identidad de *Wolbachia* y reveló variabilidad dentro de algunas regiones del gen gyrA que corresponden a la especie hospedera. En consecuencia, este gen podría ser útil para futuros estudios filogenéticos y poblacionales.

Palabras Clave: gusano del corazón; simbiosis; *Mansonia*; evolución; mosquito; parásito

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Wolbachia Hertig and Wolbach (Rickettsiales: Anaplasmataceae) is a Gram-negative bacterium and common endosymbiont of insects and nematodes. Its role in insect and nematode evolution has been reviewed previously (Werren et al. 2008; Werren & Windsor 2000; Bouchery et al. 2013). *Wolbachia* antigens have been implicated as a factor in the inflammatory process associated with nematode infections, highlighting their medical importance (Brattig et al. 2000). Mosquitoes and other hematophagous arthropods are sometimes vectors of parasitic nematodes. As such, mosquitoes and nematodes have symbiotic relationships that may add to the complexity of *Wolbachia* research. Consequently, learning more about the prevalence and phylogenetic distribution of *Wolbachia* in both taxa may aid in understanding ecological interactions and the evolution of symbiotic relationships. The use of *Wolbachia* infection of mosquitoes as a means to control vector borne diseases such as dengue and malaria has been proposed by Zabalou et al. (2004). *Wolbachia* infected mosquitoes have been released previously to help control mosquito populations in South Florida as well as other countries (Waltz 2017). Consequently, the biology of *Wolbachia* is critical in basic entomological science as well as biomedical applications, and further research on its ecology and evolution would be beneficial.

Because it is non-culturable, typical microbiological methods are not as useful for studying the taxonomy of *Wolbachia*. Therefore, systematic characterization of *Wolbachia* is based largely on molecular methods and has even resulted in reclassification of some *Wolbachia* species (Larson et al. 2016). Specifically, the wsp gene and Multi-Locus Sequence Typing methods have been investigated for strain typing (Baldo et al. 2006). Alignment of wsp gene sequences along with the use of variable loci have revealed some higher taxonomic grouping but little about the distribution and coevolution of *Wolbachia* in various host species. Given the estimated number of insects and other species that are infected (Werren & Windsor 2000; Weinert et al. 2015), there could be many host-specific *Wolbachia* types, each having a different evolutionary and ecological impact. Pietri et al. (2016) have reviewed several characteristics of *Wolbachia* in host tissues and ways in which infection can exert its effects on the host. Studies on genetic variation of *Wolbachia* as it relates to host species may shed light on its diversification and speciation as an endosymbiont. Furthermore, it may help to elucidate the levels of host-specificity and possible routes of horizontal transfer between hosts.

Various housekeeping genes have been proposed for use in bacterial systematics (Owen 2004). The objective of our project was to find a single gene that could be used for Polymerase chain reaction (PCR) detection and subsequent sequence analysis of diverse *Wolbachia* strains in various arthropod and nematode host species. Based on previous studies in bacteria, the gyrA gene was shown to be useful for phylogenetics (Chun and Bae 2000; Abdelbaqi et al. 2007; Lück et al. 2010; Ménard et al. 2016), though gyrA has not been explored specifically for *Wolbachia*. Working under the hypothesis that gyrA could be a useful target for detection and sequence analysis, PCR assays were developed and used to explore *Wolbachia* in arthropod and heartworm samples. Given the complexity of Multi-Locus Sequence Typing, this method could provide a simplified approach to identifying host-specific subtypes while studying the ecological distribution of *Wolbachia* in host species and provide additional information for more extensive typing methods.

Materials and Methods

COLLECTION

Arthropods from various regions of Tampa, Florida, USA, were obtained over a 7-yr period from 2012 to 2019 using several methods. Several hundred individual mosquitoes representing 7 different species were donated from Hillsborough County Mosquito Control in Tampa. These specimens had been identified but originally were not intended for laboratory analysis. Consequently, they originally were not maintained at −20 °C. Given there was likely some DNA degradation from the point of collection to lab analysis, we considered PCR detection less likely from these samples. Many of these were used in the developmental stages of this study, but were not used to estimate infection prevalence. Mosquitoes also were collected specifically for this study using both traps and manual collection. Mosquito traps were placed in areas near foliage and water sources and monitored every few d for specimens. A New Standard Miniature Incandescent Light Trap, Model 1012 (John W. Hock Co., Gainesville, Florida, USA) was baited with an octanol-soaked cotton ball placed near the intake fan. These traps are battery powered and may be placed in remote locations. A DynaTrap3 DT1050-CST-DEC insect trap (Dynamic Solutions Worldwide, Milwaukee, Wisconsin, USA) also was used to collect mosquitoes. This trap required a 110 volt power outlet and was used in residential areas. If no mosquitoes were collected, traps were relocated. Alternatively, mosquitoes were collected manually with a collection jar opportunistically as they attempted to feed on humans. Mosquitoes were kept alive, if possible. However, some remote-trapped mosquitoes may have been dead in the trap for up to 3 d until storage at −20 °C, which was done as soon after collection as possible. Ethanol was not used to preserve specimens. Other arthropods were collected opportunistically in specimen jars, and stored at −20 °C. Specimens were collected alive and intact with the exception of *Eacles imperialis* Drury (Lepidoptera: Saturniidae), the remains of which was collected from an automobile windshield after impact and stored on a sterile swab on ice until freezing at −20 °C. Heartworms, *Dirofilaria immits* (Leidy) (Rhabditida: Onchocercidae), were collected from canines during veterinary necropsy procedures and presumptively identified based on morphology and circumstantial evidence. Heartworms were kept on ice or frozen until processing. Specimens used in this study are listed in Tables 1 and 2.

IDENTIFICATION

Mosquitoes were identified morphologically to the extent possible based on the physical condition of the sample using a dichotomous key (Darsie & Ward 1981). Mosquito identity also was confirmed using PCR amplification and DNA barcoding methods described by Chan et al. (2014). Other arthropods and representative heartworms were identified first based on morphological features and then confirmed genetically using PCR and DNA barcoding methods described by Folmer et al. (1994). Species-specific PCR (Kronefeld et al. 2014) also was used to test for the presence of *D. immitis*, especially if heartworm *Wolbachia* were suspected in mosquito samples.

PREPARATION OF DNA TEMPLATE FOR PCR

Two methods were used to prepare samples for PCR, pulverization of specimen bodies to expose soft tissues and cells, and DNA extraction. Whole mosquitoes or nematodes were placed individually in separate 1.5 mL sterile Eppendorf tubes (Fisher Scientific, Hampton, New Hampshire, USA). A separate, sterile toothpick was used to crush each mosquito specimen. For whole cell PCR, a small amount of tissue homogenate (the amount that covered only the tip of a toothpick) was placed in the PCR reaction. Prior to DNA extraction, a sterile toothpick was used to dismember mosquitoes, whereas heartworms were homogenized using a PowerGen500 (Fisher Scientific, Hampton, New

Table 1. Results of PCR amplification indicating the *Wolbachia* infection rate and 95% confidence interval for host species with at least 1 positive individual. Confidence intervals are not reported for samples of 1 specimen (0 degrees of freedom). Total frequency is not reported as it is not a predictive measure of infection rate.

Host species		Template	PCR assay type	Target	Number of individual samples tested	Number of PCR positive samples	Freq	95% CI lower	95% CI upper
Order: family	Genus species								
Diptera: Culicidae	*Aedes taeniorhynchus*	Whole cell	nested wsp	wsp	10	3	0.30	0.02	0.58
		DNA	nested wsp	wsp	10	7	0.70	0.42	0.98
	Aedes albopictus	DNA	gyrA inside	gyrA	3	2	0.16	0.13	1.00
	Aedes aegypti	DNA	gyrA inside	gyrA	6	1[a]	0.17	0.00	0.47
	Culex coronator	Whole cell	wsp	wsp	1	1	1.00	NA	NA
	Coquillettidia perturbans	Whole cell	wsp	wsp	1	1	1.00	NA	NA
		DNA	gyrA inside	gyrA	4	0	0.00	0.00	0.00
	Mansonia titillans	Whole cell	nested wsp	wsp	10	3	0.30	0.02	0.58
		DNA	gyrA inside	gyrA	24	14	0.50	0.28	0.72
Hemiptera: Cimicidae	*Cimex lectularius*	DNA	gyrA inside	gyrA	1	1	1.00	NA	NA
Rhabditida: Onchocercidae	*Dirofilaria immitis*	Whole cell	nested wsp	wsp	5	0	0.00	0.00	0.00
		DNA	nested wsp	wsp	5	1	0.35	0.00	0.55
		DNA	gyrA inside	gyrA	25	25	1.00	1.00	1.00
	Total				106	58	NA	NA	NA

[a]unable to confirm PCR results using GenBank due to ambiguous nucleotide sequence results.

Hampshire). Heartworms were homogenized with 500 µL sterile DI H_2O. To improve detectability of *Wolbachia*, a DNeasy Tissue extraction kit (Qiagen, Hilden, Germany) was used when available.

POLYMERASE CHAIN REACTION AMPLIFICATION

Several primers were used throughout this study (Table 3). Novel primers were designed using Primer Blast (Altschul et al. 1990) and constructed by Integrated DNA technologies (Coralville, Iowa, USA). All reactions were performed using PCR MasterMix (Promega, Madison, Wisconsin, USA) and an Eppendorf Mastercycle (Hamburg, Germany). Initially, PCR to detect *Wolbachia* was performed using primers targeting the wsp gene (Baldo et al. 2006). An additional, high-temperature step (5 min at 94 °C) was added to the PCR program to lyse cells. In an attempt to improve detection from whole tissue, a nested assay was developed using novel primers targeting regions outside the wsp gene in aligned reference sequences, including the genomes of *Wolbachia* Pel strain wPip from the mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae) (Klasson et al. 2008; Salzberg et al. 2009) and the nematode *Onchocerca volvulus* O'Neil (Spirurida: Onchocercidae) (Darby et al. 2012). For the nested wsp assay, the outside primers, out_wsp_F and out_wsp_R (Table 3) were used in a final concentration of 1 µM to amplify a 1,451 bp product containing the wsp gene region using the following program: denaturation at 94 °C for 1 min, primer annealing at 55 °C for 45 s, and extension at 72 °C for 2 min. Subsequently, 1 µL of the reaction mixture was used in the wsp assay according to the method of Baldo et al. (2006).

Novel PCR assays targeting the gyrA gene were developed using reference sequences for *Wolbachia* (Klasson et al. 2008; Salzberg et al. 2009; Darby et al. 2012). Target primer binding sites were identified that contain sequences unique to the genus *Wolbachia* yet are conserved among various *Wolbachia* reference sequence subtypes. Outside primers, gyrA_out_F and gyrA_out_R, were designed to amplify a relatively large fragment (1,268 bp) of the *gyrA* gene. A second set of inside primers, gyrA_in_F and gyrA_in_R, was designed to bind internally on the gene and amplify an 817 bp product (Table 3). These inside primers could be used in a subsequent, nested PCR reaction using 1 µL of the product from the outside PCR amplification. The following temperature cycles were used for amplification of gyrA using the outside primer pair: denaturation at 94 °C for 1 min, primer annealing at 55 °C

Table 2. Additional host species screened from which *Wolbachia* gyrA DNA was not detected by PCR.

Order: family	Species screened for *Wolbachia*	Number of individuals screened
Diptera: Culicidae	*Anopheles crucians* (Wiedemman)	5
	Deinocerites sp.	1
	Uranotaenia iowii (Theobald)	1
Hemiptera: Cicadidae	*Megatibicen resonans* (Walker)	1
Hemiptera: Coreidae	*Acanthocephala declivis* (Say)	2
Lepidoptera: Lymantriidae	*Orgyia* sp.	1
Lepidoptera: Nymphalidae	*Limenitis archippus* (Cramer)	2
Lepidoptera: Saturniidae	*Eacles imperialis* (Drury)	1
Acari: Ixodidae	*Amblyomma americanum* (L.)	1
	Rhipicephalus sanguineus (Latreille)	2

Table 3. PCR primers used to detect *Wolbachia* from samples of mosquitoes and heartworms.

Primer	Sequence	Product
out_wsp F	5'CGCACTTCCTCTTCTTGGGA	1,451 bp
out_wsp R	5'TGATGGGGTTACCACCTGGA	
wsp_F1[a]	5'GTCCAATARSTGATGARGAAAC	606 bp
wsp_R1[a]	5'CYGCACCAAYAGYRCTRTAAA	
gyrA_out_F	5'GCAGAAGTTCTTTTACCAAAGCCA	1,268 bp
gyrA_out_R	5'GGGCTCTACATTGCGGTCTT	
gyrA_in_F	5'CGCCATCTGAATTGCGACTT	817 bp
gyrA_in_R	5'ACTGAGCTGCAAACGAAAGC	

[a]primers from Baldo et al. 2006.

for 45 s, and extension at 72 °C for 2 min. For the inside assay, extension was the same except that the extension was for only 1 min. For whole cell PCR, an initial step of 5 min at 94 °C was added to lyse cells.

PCR assays included a negative control to indicate the presence of contamination. For the whole cell PCR method outlined above, a sterile toothpick was inserted into a PCR tube as would be for samples with tissue. For PCR assays that used purified DNA as a template, the negative control included sterile water instead of DNA. A positive control consisting of *Wolbachia* DNA was not available at the onset of the study. However, at later stages, gyrA DNA that was amplified and sequenced from heartworms and confirmed as *Wolbachia* was used as a positive control to assess the repeatability of PCR amplification. Batches of whole cell/tissue samples that all tested negative for PCR without a positive control were excluded from further analysis. No inferences were made regarding *Wolbachia* infection frequency from those samples. However, PCR-negative samples from purified DNA were retested for *Wolbachia* gyrA DNA, and also were tested for host DNA using PCR detection of the Cytochrome Oxidase gene (Folmer et al. 1994; Chan et al. 2014). A nanodrop spectrophotometer also was used to confirm the presence of template DNA. Samples were excluded from analysis if no host DNA was detected.

All amplification products were visualized by gel electrophoresis on 2% agarose gels containing ethidium bromide and viewed by an ultraviolet transilluminator (Bio-Rad, Hercules, California, USA).

DNA SEQUENCING AND ANALYSIS

PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and the resulting DNA concentrations were measured using 1 µL of product in a ThermoFisher NanoDrop 2000c spectrophotometer (ThermoFisher, Waltham, Massachusetts, USA). Purified PCR samples were sent to MacrogenUSA (Rockville, Maryland, USA) for nucleotide sequencing. Sequence identities were confirmed using a nucleotide Blast search tool (Altschul et al. 1990) in National Center for Biotechnology Information (Bethesda, Maryland, USA). MEGA X (Kumar et al. 2016) was used for DNA sequence alignment and phylogenetic analysis. Sequences were aligned with *Wolbachia* gyrA from previously published genomes (Wu et al. 2004; Foster et al. 2005; Klasson et al. 2008; Salzberg et al. 2009; Darby et al. 2012; Scott et al. 2012). Using the aligned *Wolbachia* gyrA sequences, a best fit model was determined (TN93 + G + I) and a Maximum Likelihood tree with 1,000 bootstrap replicates was generated in MEGA (Felsenstein 1985; Kumar et al. 2016; Tamura & Nei 1993). Representative gyrA sequences obtained during this study were submitted to National Center for Biotechnology Information, GenBank using the BankIt submission portal and were assigned the accession numbers MT040622, MT040623, MG948326, MG948325, MG948324, MH304223, MH304222, MH304221, MH304220, MG948329, MG948328, MG948327, MG948323, MG948322, MT081966.

Results

During the preliminary screening of mosquitoes for *Wolbachia* in the early stages of this study, wsp PCR (Baldo et al. 2006) was used on mosquito tissue. Approximately 100 mosquitoes were screened during this phase using homogenized tissue as a template and only 2 positive samples were detected from *Culex coronator* (Dyar & Knab) and *Coquillettidia perturbans* (Walker) (both Diptera: Culicidae). These were subsequently confirmed by sequencing based on the nearest matching *Wolbachia* sequence (99%) in GenBank at the time (2013), which were from the *Wolbachia* endosymbiont of *Culex quinquefasciatus* Pel strain wPip (Klasson et al. 2008). It was this low detection rate (2% of mosquitoes tested) that spurred the development of a wsp nested PCR assay to use on whole tissues, which improved the detection rate to 6 positive out of the 20 subsequent mosquitoes that were screened using this new assay (3 out of 10 *Aedes taeniorhynchus* [Wiedmann], and 3 out of 10 *Mansonia titillans* [Walker]; both Diptera: Culicidae). Using the wsp as a PCR target, *Wolbachia* was found in a total of 4 different host mosquito species.

The yield of DNA varied depending on the specimen size and state of preservation, but typically ranged from 0.6 ng per µL to 574.4 ng per µL. Even after extracting DNA from heartworms, nested wsp PCR amplification was relatively weak compared to previous PCR results from mosquitoes. Furthermore, though nucleotide sequences from wsp PCR-positive heartworm samples aligned with *Wolbachia* accessions in GenBank, they were from taxonomically disparate insect hosts. The top matching sequence had a 96% Identity Score and was *Wolbachia* wPup1 (GenBank accession DQ493917.1) from the host *Pteromalus puparum* L. (Hymenoptera: Pteromalidae). Weak PCR amplification of wsp from heartworms along with taxonomically ambiguous GenBank alignments led us to develop the gyrA PCR assay in an effort to improve detectability and classification of *Wolbachia* from various hosts.

The novel PCR primers designed to target gyrA yielded 43 *Wolbachia*-positive individuals in total. *Wolbachia* was detected in 5 out of 15 total arthropod species screened by this PCR assay (30% of species). *Wolbachia* was detected in all 25 heartworm (*D. immitis*) specimens that were screened (Table 1). PCR amplicons that were sequenced produced significant alignments with *Wolbachia* entries when queried in National Center for Biotechnology Information using the default Blastn search settings, verifying the specificity of the PCR. Amplification from purified DNA samples resulted in improved overall detection of *Wolbachia*, especially from heartworms, from which detection was not achieved using whole tissues and a PCR template. The improvement in detection rate from mosquitoes was less obvious. However, considering 20 *Ae. taeniorhynchus* specimens (Table 1), the detection rate appears to be 2.3 times greater using purified DNA. An added advantage of DNA extraction is that it also allowed for retesting of stored DNA. A subset of 10 mosquito DNA samples, including some positive and negative samples, were tested again to determine if amplification was repeatable. The results were the same and demonstrated that either gyrA primer pair (inside or outside) worked equally well to detect positive samples out of the subset, and that both positive and negative results were reproducible. The nested reaction for gyrA PCR did not yield additional positives out of the subset of samples that were retested when compared to using only 1 primer pair. This demonstrated that it was likely unnecessary to use nested PCR to detect the gyrA target sequence from purified DNA samples. Nested PCR requires additional time and reagents, and also increases the opportunity for cross contamination. Consequently, only the gyrA inside PCR was deemed practical for detection of *Wolbachia* throughout the remainder of the study.

When gyrA nucleotide sequences from mosquito hosts were aligned in National Center for Biotechnology Information Blast, they

matched *Wolbachia* sequences (*C. quinquefasciatus* Pel strain wPip) in GenBank with 94% identity score, but there were no entries from the same host species such as *M. titillans* (at the time) to confirm the utility of gyrA to genotype *Wolbachia* by host species. Sequences amplified from heartworm specimens matched 91% with *Wolbachia* wOo of *Onchocerca ochengi* (Spirurida: Onchocercidae) (HE660029.1), a nematode in the same family (Darby et al. 2012). Analysis of gyrA sequences aligned in MEGA revealed distinct clades for *Wolbachia* from different host species (Fig. 1). Once some of our sequences were accessioned in GenBank, new sequences that were obtained subsequently from the same host species (from spatially and temporally different host populations) aligned with our gene bank accessions when using National Center for Biotechnology Information Blast.

The gyrA sequences compared here (Fig. 1) form a tree in which taxonomically more divergent *Wolbachia* are from divergent hosts (nematode as compared to arthropod), and those sequences from the same host are clustered together. The lack of complete phylogenetic congruency with host taxa occurs in the middle taxonomic ranges (e.g., host suborder, family) and highlights the question of how often new symbiotic associations are established between *Wolbachia* and host species.

Discussion

Laboratories with limited funds may be able to amplify *Wolbachia* DNA directly from insect tissue using whole cell PCR, reducing the cost and time associated with DNA extraction. However, this method likely would not yield accurate estimations of *Wolbachia* infection rate in host tissues. It also prevents repeat testing of the template DNA for quality control or with other subsequent assays. Desiccation of internal soft tissues limited the detection rate using whole cell PCR, necessitating rapid storage at −20 °C. However, in this study it proved to be a viable starting point to find representative samples of *Wolbachia* during a period of limited funding and time. While the use of soft body tissue as a PCR template was an economical way to look for *Wolbachia* in mosquitoes in the early phases of this research, it was not useful for heartworms because they have a much more durable cuticle, and mesodermal tissues were not exposed as easily. DNA extraction, although more costly and time-consuming, enhanced the detectability from heartworms and arthropods. We found that processing the specimen with a homogenizer is an effective method of releasing cells in preparation for DNA extraction.

Nested PCR appeared to enhance detection of *Wolbachia* from the whole cells in soft tissue from mosquitoes but was not needed to improve detection from extracted DNA based on our results. Considering the added time, expense and risk of cross contamination associated with nested PCR assays, our observations might be helpful to those considering it as an option.

Our results suggest *Wolbachia* can be studied in arthropod and heartworm populations using PCR amplification and analysis of the gyrA gene. At the time of this study, only a limited number of *Wolbachia* gyrA sequences were available in GenBank for comparison as part of genome projects such as Klasson et al. (2008), Salzberg et al. (2009), Darby et al. (2012), and Nikoh et al. (2014). Out of the host species from which *Wolbachia* was detected in this study, *Cimex lectularius* L. (Hemiptera: Cimicidae) was the only one with its symbiotic *Wolbachia* gyrA sequence in GenBank. Although the queried gyrA sequence aligned with the correct host source in this case (*C. lectularius* with 100% match), the lack of available sequences made it impossible to assess the species-specificity of gyrA sequences using Blast alignments alone.

Phylogenetic analysis of *Wolbachia* gyrA sequences revealed distinct monophyletic grouping (Fig. 1) which corresponded to the host species from which they were detected (Table 1) supporting the existence of host-specific variation in gyrA sequences. The individual *Aedes albopictus* Skuse (Diptera: Culicidae), *M. titillans*, and *D. immitis* specimens used here represent Florida populations of the respective species collected from Hillsborough County, Tampa, Florida. Consequently, we cannot claim definitive host-specific gyrA genotype of *Wolbachia* is consistent across members of the host species throughout their entire geographic range (although that might be a subject of future studies). However, specimens of *M. titillans* were collected from 2 locations on opposite sides of the county (approximately 29 miles apart). Since *D. immitis* were collected from dogs with no known background, it was not possible to determine the parameters of their population. However, they were collected from 3 different dogs over a 3 yr period and could have come from locations throughout the county (or even neighboring counties). Consequently, the analysis presented here suggests that gyrA variation is at least useful for tracking genetically distinct *Wol-*

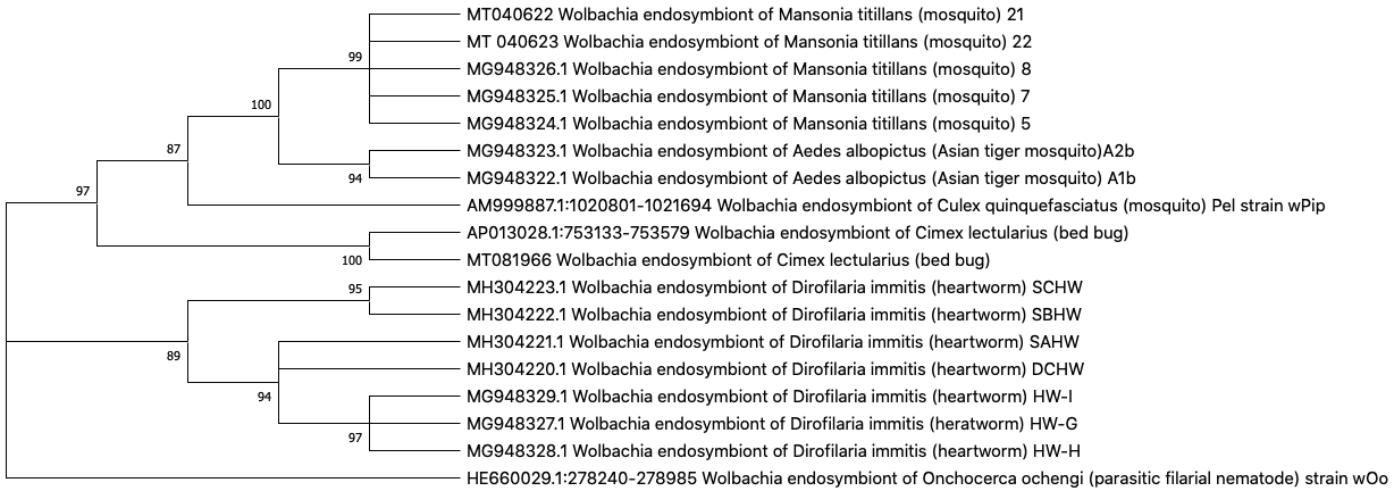

Fig. 1. Phylogenetic tree based on Maximum Likelihood depicting the grouping of *Wolbachia* from various hosts based on analysis of the gyrA gene. The numerical value displayed on branches is the bootstrap value (1,000 replicates), and branches with values below 50% are collapsed. The tree illustrates that gyrA sequences distinguish *Wolbachia* subtypes based on host taxonomy, demonstrating that this gene may contribute to *Wolbachia* strain typing projects and future phylogenetic analysis.

bachia lineages regionally. Two deductions can be surmised from these results and tested with further research: one possibility is that gyrA will reveal host species-specific genotypes when tested across broader geographic range. A second possibility is that distant populations of the same host do not have similar *Wolbachia* gyrA sequences, indicating less host species-specificity of *Wolbachia* and suggesting population specificity that may arise after a recent transmission. In the latter case, gyrA variation would help identify and track cases of horizontal transfer and subsequent establishment of regional *Wolbachia* infections.

Pietri et al. (2016) reviews the likelihood of horizontal transmission of *Wolbachia* and describes its establishment in the host somatic tissues, beyond infection of gametes (through which vertical transmission occurs). We believe that increased scrutiny of genetic markers such as gyrA may help answer some important questions about the evolution of *Wolbachia* endosymbiosis such as how the occurrence of mutualism (as in some nematodes) might correspond to host-specificity and whether genetic markers can be used to identify the level of *Wolbachia* transience. Even with a modest number of samples, as in this case, detection of *Wolbachia* across various diverse ecdysozoan hosts is noteworthy. The intimate ecological relationship between nematodes, arthropods, and *Wolbachia* is worth exploring to help understand the evolution of *Wolbachia* as well as host species. Moreover, the absence of *Wolbachia* in some individual host samples in this study (Table 2) should not be taken as evidence that it does not exist in the respective species, since that is difficult to prove (Weinert et al. 2015) and it is not our intention to imply that from the given results. Given the high rate of *Wolbachia* infection of lepidopteran species (Ahmed et al. 2015), it might be surprising that it was not detected in the 4 individuals from 3 species that were opportunistically collected and screened here. However, as noted by Ahmed et al. (2015), only a third to a quarter of individuals are infected, and low infection rates contribute to the underestimate of the prevalence across various species.

We believe the gyrA PCR and sequence analysis developed in our study is an additional method to help detect *Wolbachia* and characterize its host-associations. Although much has been accomplished using Multi-Locus Sequence Typing methods (Baldo et al. 2006), investigations using gyrA may supplement that and other genotyping methods to help resolve phylogenetic relationships and identify cases of horizontal transfer.

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