Published in final edited form as:
FEMS Microbiol Ecol. 2011 July ; 77(1): 50–56. doi:10.1111/j.1574-6941.2011.01089.x.

Distribution and molecular characterization of *Wolbachia* endosymbionts and filarial nematodes in Maryland populations of the lone star tick (*Amblyomma americanum*)

Xing Zhang[1], **Douglas E. Norris**[1,2], and **Jason L. Rasgon**[1,2]

[1]The W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, USA

[2]The Johns Hopkins Malaria Research Institute, Baltimore, MD, USA

Abstract

The lone star tick *Amblyomma americanum* is host to a wide diversity of endosymbiotic bacteria. We identified a novel *Wolbachia* symbiont infecting *A. americanum*. Multilocus sequence typing phylogenetically placed the endosymbiont in the increasingly diverse F supergroup. We assayed a total of 1031 ticks (119 females, 78 males and 834 nymphs in 89 pools) from 16 Maryland populations for infection. Infection frequencies in the natural populations were approximately 5% in females and <2% (minimum infection rate) in nymphs; infection was not detected in males. Infected populations were only observed in southern Maryland, suggesting the possibility that *Wolbachia* is currently invading Maryland *A. americanum* populations. Because F supergroup *Wolbachia* have been detected previously in filarial nematodes, tick samples were assayed for nematodes by PCR. Filarial nematodes were detected in 70% and 9% of *Wolbachia*-positive and *Wolbachia*-negative tick samples, respectively. While nematodes were more common in *Wolbachia*-positive tick samples, the lack of a strict infection concordance (*Wolbachia*-positive, nematode-negative and *Wolbachia*-negative, nematode-positive ticks) suggests that *Wolbachia* prevalence in ticks is not due to nematode infection. Supporting this hypothesis, phylogenetic analysis indicated that the nematodes were likely a novel species within the genus *Acanthocheilonema*, which has been previously shown to be *Wolbachia*-free.

Keywords

Wolbachia; *Amblyomma americanum*; tick; nematode; *Acanthocheilonema*; MLST

Correspondence: Jason L. Rasgon, The W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, E4626, 615 N. Wolfe Street, Baltimore, MD 21205, USA. Tel.: +1 410 502 2584; fax: +1 410 955 0105; jrasgon@jhsph.edu.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phylogenetic analysis of *Wolbachia gatB* sequences.
Fig. S2. Phylogenetic analysis of *Wolbachia coxA* sequences.
Fig. S3. Phylogenetic analysis of *Wolbachia hcpA* sequences.
Fig. S4. Phylogenetic analysis of *Wolbachia fbpA* sequences.
Fig. S5. Phylogenetic analysis of *Wolbachia ftsZ* sequences.

Introduction

Amblyomma americanum (the lone star tick) is broadly distributed, with a host range spanning the Midwest to the eastern United States (Childs & Paddock, 2003). *Amblyomma americanum* is a vector of pathogens that cause diseases of humans and domestic animals such as granulocytic ehrlichiosis, tularemia and borreliosis, and is associated with Southern tick-associated rash illness (Goddard & Varela-Stokes, 2009). Several endosymbiotic bacterial species (including *Coxiella, Rickettsia* and *Arsenophonus*) have been detected previously in *A. americanum* (Clay *et al*., 2008). *Wolbachia* is a common endosymbiotic associate of arthropods and filarial nematodes (Rowley *et al*., 2004; Baldo *et al*., 2007; Hilgenboecker *et al*., 2008; Werren *et al*., 2008) and has been described previously in the ticks *Ixodes ricinus* (Hartelt *et al*., 2004) and *Ixodes scapularis* (Benson *et al*., 2004). In this report, we identified a novel *Wolbachia* symbiont in *A. americanum*. The infection was found only in southern Maryland at a low frequency in female ticks and pooled nymphs, but was never observed in males. Multilocus sequence typing (MLST) placed the infection into the F supergroup. Filarial nematode infection was observed in some tick samples, but concordance between *Wolbachia* infection and nematode infection was not observed. Nematodes were phylogenetically placed into the genus *Acanthocheilonema*, members of which were previously shown to be *Wolbachia*-free.

Materials and methods

Tick collection information and sample sizes are listed in Table 1 and Fig. 1. All ticks were collected in 2008 using tick drags. Individual adult ticks and pooled nymphs (10 per pool) were homogenized using a TissueLyser II bead mill (Qiagen, Valencia, CA) with 5-mm stainless-steel bead. Genomic DNA was isolated using the MasterPure DNA purification kit (Epicentre Biotechnologies, Madison, WI) and eluted in 30 μL water. Samples were screened for *Wolbachia* infection using *Wolbachia*-specific 16S primers WSpecF and WSpecR (Sakamoto *et al*., 2006; Werren & Windsor, 2000) using a PTC thermocycler (Bio-Rad, Hercules, CA) with a program of 95 °C for 5 min; 40 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; and a final extension of 72 °C for 5 min. Each 40 μL reaction consisted of 2.0 μL template DNA, 1 μM concentrations of all forward and reverse primers, 0.4mM dNTPs and 2.0U Taq polymerase. Infections were confirmed using *wsp* gene amplification using primers *wsp*F1, *wsp*R1 (*Wolbachia* MLST website: http://pubmlst.org/wolbachia/). The PCR protocol used was 94 °C for 2 min, followed by 37 cycles of 94 °C for 30 s, 59 °C for 45 s and 72 °C for 1.5 min and a final extension of 72 °C for 10 min (http://pubmlst.org/wolbachia/). Template DNA from a *Wolbachia*-infected mosquito cell line was used as a positive control; a sample containing deionized water instead of template DNA was used as a negative control. For *Wolbachia* MLST, the four MLST genes (*coxA, gatB, hcpA* and *fbpA*) were amplified using nested PCR. Primary PCR amplification was performed using F3/R3 ~64-fold degenerate primer sets (http://pubmlst.org/wolbachia/). Each 40 μL reaction consisted of 2 μL template DNA, 1 μM concentrations of all forward and reverse primers, 0.4mM dNTPs and 1.0U HotStar Taq polymerase (Qiagen). Primary PCR cycling conditions followed the ‘alternative protocols’ listed on the MLST website. Secondary PCR amplification was performed using the MLST website ‘standard protocols’ and F1/R1 primer sets for these four MLST genes (Baldo *et al*., 2006). The *fts*ZuniF/

*fts*ZuniR (Lo *et al*., 2002) primer pair was used for the *ftsZ* gene PCR. PCR products were directly sequenced in both directions using an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences were deposited in GenBank under accession numbers HM061157–HM061163.

Thirty-nine concatenated MLST sequences were retrieved from the *Wolbachia* MLST database. The *Wolbachia* strains are listed by host species name and concatenated MLST sequence ID number in Fig. 2. Sequence alignment and neighbor-joining phylogenetic analyses were conducted using MEGA version 4 (Tamura *et al*., 2007). Tree support was evaluated by bootstrapping with 1000 replications. Phylogenies were produced for concatenated and gene-specific data sets.

Tick samples were tested for the presence of filarial nematodes using specific 12S primers as described (Casiraghi *et al*., 2004). Template DNA from a *Wuchereria bancrofti*-infected *Culex pipiens* mosquito was used as a positive control; a sample containing deionized water instead of template DNA was used as a negative control. The positive amplicons obtained were directly sequenced. Phylogenetic placement of nematode sequences was conduced as described above.

Results and discussion

Out of 16 sampled Maryland populations, *Wolbachia*-infected ticks were found in eight populations. Infection rates within populations were low, ranging from 3.5% to 25% in females, and a minimum infection rate (MIR) from 1% to 7% in pooled nymphs. *Wolbachia* was not detected in males. The overall statewide infection rate was approximately 5% in females and 1.4% (MIR) in pooled nymphs (Table 1). *Wolbachia* infection was spatially structured; all infected populations were located in southern Maryland, while infection was not detected in central and northern populations (Fig. 1). This distribution suggests the possibility that *Wolbachia* is currently invading *A. americanum* populations in Maryland. Future sampling efforts will be required to confirm or refute this hypothesis.

The *coxA, gatB, hcpA, ftsZ* and *fbpA* gene amplicons were produced by PCR from sample #366 from Dorchester County. Additionally, the *fbpA* gene was amplified and sequenced from all positive samples, while the *coxA, gatB, hcpA* and *ftsZ* genes were amplified and sequenced from selected samples (Table 2). All sequences were identical to those obtained from sample #366. According to the phylogenetic analysis of these concatenated sequences, the *Wolbachia* strain found in *A. americanum* was placed into supergroup F and was 99% identical to *Wolbachia* from the ant *Ocymyrmex picardi* (Russell *et al*., 2009). Gene-specific phylogenetics analyses showed the same result (Supporting Information). This is the first report of supergroup F *Wolbachia* in ticks. Up to this point, F supergroup*Wolbachia* infections have been detected in nematodes, scorpions and several insect hosts (termites, weevils, bush crickets, bedbugs, lice, louse flies, cockroaches and antlions) (Lo *et al*., 2002; Casiraghi *et al*., 2005; Dunn & Stabb, 2005; Sakamoto *et al*., 2006; Baldo *et al*., 2007; Covacin & Barker, 2007; Panaram & Marshall, 2007; Vaishampayan *et al*., 2007).

F supergroup *Wolbachia* have been identified in filarial nematodes (genus *Mansonella*) in addition to arthropods. Although ticks collected for this study were collected by dragging, and were unlikely to be blood fed, we investigated the hypothesis that *Wolbachia* infection in *Amblyomma* was due to filarial nematode infection. All *Wolbachia*-positive tick samples and a random selection of *Wolbachia*-negative tick samples were assayed for filarial nematode infection by specific 12S PCR. Filarial nematodes were detected in both *Wolbachia*-positive and *Wolbachia*-negative adult female and nymphal ticks. Nematode infection was more common in *Wolbachia*-infected ticks (70% vs. 9%), but strict concordance of infection types was not observed (both *Wolbachia*-positive, nematode-negative ticks and *Wolbachia*-negative, nematode-positive ticks were identified) (Table 2). Phylogenetic analysis indicated that the filarial nematode infecting *A. americanum* was most closely related to nematodes in the genus *Acanthocheilonema* (formerly *Dipetalonema*) (Fig. 3). *Acanthocheilonema* nematodes have not been described previously from *Amblyomma*, but their transmission by both soft ticks (*Ornithodoros*) and hard ticks (*Rhipicephalus*) has been demonstrated (Londoño, 1976; Olmeda-García *et al*., 1993). *Acanthocheilonema* nematodes have also been shown previously to lack *Wolbachia* infection (Casiraghi *et al*., 2004). In total, these results indicate that *Wolbachia* prevalence in *A. americanum* is not explained by nematode infection.

The frequency of *Wolbachia* infection is low in *A. americanum*. Some *Wolbachia* reproductive phenotypes, such as cytoplasmic incompatibility, tend to equilibrate at high frequencies in populations (Turelli & Hoffmann, 1999). In contrast, phenotypes such as male killing tend to equilibrate at low frequencies (Hurst & Jiggins, 2000).We did not detect any infected males in our study – this could reflect sex-specific infection differences (possibly related to male killing) or could simply be due to sampling. The reproductive phenotype of *Wolbachia* in *A. americanum* is unknown and needs further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was funded by NIH/NIAID grants R21AI067386 and R03AI079297 to D.E.N., and R21AI070178 to J.L.R. X.Z. was partially supported by the JHSPH Ralph and Sylvia Barr fellowship. We thank Timothy Shields and the Johns Hopkins Environmental Surveillance Core for assistance in producing Fig. 1.

References

Baldo L, Dunning Hotopp JC, Jolley KA, Bordenstein SR, Biber SA, Choudhury RR, Hayashi C, Maiden MCJ, Tettelin H, Werren JH. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis* . Appl Environ Microb. 2006; 72:7098–7110.

Baldo L, Prendini L, Corthals A, Werren JH. *Wolbachia* are present in southern African scorpions and cluster with supergroup F. Curr Microbiol. 2007; 55:367–373. [PubMed: 17676427]

Benson MJ, Gawronski JD, Eveleigh DE, Benson DR. Intracellular symbionts and other bacteria associated with deer ticks (*Ixodes scapularis*) from Nantucket and Wellfleet, Cape Cod, Massachusetts. Appl Environ Microb. 2004; 70:616–620.

Casiraghi M, Bain O, Guerrero R, Martin C, Pocacqua V, Gardner SL, Franceschi A, Bandi C. Mapping the presence of *Wolbachia pipientis* on the phylogeny of filarial nematodes: evidence for symbiont loss during evolution. Int J Parasitol. 2004; 34:191–203. [PubMed: 15037105]

Casiraghi M, Bordenstein SR, Baldo L, Lo N, Beninati T, Wernegreen JJ, Werren JH, Bandi C. Phylogeny of *Wolbachia pipientis* based on *gltA*, *groEL* and *ftsZ* gene sequences: clustering of arthropod and nematode symbionts in the F supergroup, and evidence for further diversity in the *Wolbachia* tree. Microbiology. 2005; 151:4015–4022. [PubMed: 16339946]

Childs JE, Paddock CD. The ascendancy of *Amblyomma americanum* as a vector of pathogens affecting humans in the United States. Annu Rev Entomol. 2003; 48:307–337. [PubMed: 12414740]

Clay K, Klyachko O, Grindle N, Civitello D, Oleske D, Fuqua C. Microbial communities and interactions in the lone star tick, *Amblyomma americanum* . Mol Ecol. 2008; 17:4371–4381. [PubMed: 19378409]

Covacin C, Barker SC. Supergroup F *Wolbachia* bacteria parasitise lice (Insecta: Phthiraptera). Parasitol Res. 2007; 100:479–485. [PubMed: 17048002]

Dunn AK, Stabb EV. Culture-independent characterization of the microbiota of the ant lion *Myrmeleon mobilis* (Neuroptera: Myrmeleontidae). Appl Environ Microb. 2005; 71:8784–8794.

Goddard J, Varela-Stokes AS. Role of the lone star tick, *Amblyomma americanum* (L.), in human and animal diseases. Vet Parasitol. 2009; 160:1–12. [PubMed: 19054615]

Hartelt K, Oehme R, Frank H, Brockmann SO, Hassler D, Kimmig P. Pathogens and symbionts in ticks: prevalence of *Anaplasma phagocytophilum* (*Ehrlichia* sp.), *Wolbachia* sp., *Rickettsia* sp., *Babesia*. sp. in Southern Germany. Int J Med Microbiol. 2004; 293(suppl 37):86–92. [PubMed: 15146989]

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH. How many species are infected with *Wolbachia*? – A statistical analysis of current data. FEMS Microbiol Lett. 2008; 281:215–220. [PubMed: 18312577]

Hurst GD, Jiggins FM. Male-killing bacteria in insects: mechanisms, incidence, and implications. Emerg Infect Dis. 2000; 6:329–336. [PubMed: 10905965]

Lo N, Casiraghi M, Salati E, Bazzocchi C, Bandi C. How many *Wolbachia* supergroups exist? Mol Biol Evol. 2002; 19:341–346. [PubMed: 11861893]

Londoño I. Behavior of *Dipetalonema viteae* (Filarioidea) during escape from the vector tick, *Ornithodoros tartakowskyi* (Argasidae). J Parasitol. 1976; 62:596–603. [PubMed: 986435]

Olmeda-García AS, Rodríguez-Rodríguez JA, Rojo-Vázquez FA. Experimental transmission of *Dipetalonema dracunculoides* (Cobbold 1870) by *Rhipicephalus sanguineus* (Latreille 1806). Vet Parasitol. 1993; 47:339–342. [PubMed: 8333138]

Panaram K, Marshall JL. F supergroup *Wolbachia* in bush crickets: what do patterns of sequence variation reveal about this supergroup and horizontal transfer between nematodes and arthropods? Genetica. 2007; 130:53–60. [PubMed: 16924406]

Rowley SM, Raven RJ, McGraw EA. *Wolbachia pipientis* in Australian spiders. Curr Microbiol. 2004; 49:208–214. [PubMed: 15386106]

Russell JA, Goldman-Huertas B, Moreau CS, Baldo L, Stahlhut JK, Werren JH, Pierce NE. Specialization and geographic isolation among *Wolbachia* symbionts from ants and lycaenid butterflies. Evolution. 2009; 63:624–640. [PubMed: 19054050]

Sakamoto JM, Feinstein J, Rasgon JL. *Wolbachia* infections in the Cimicidae: museum specimens as an untapped resource for endosymbiont surveys. Appl Environ Microb. 2006; 72:3161–3167.

Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol. 2007; 24:1596–1599. [PubMed: 17488738]

Turelli M, Hoffmann AA. Microbe-induced cytoplasmic incompatibility as a mechanism for introducing transgenes into arthropod populations. Insect Mol Biol. 1999; 8:243–255. [PubMed: 10380108]

Vaishampayan PA, Dhotre DP, Gupta RP, Lalwani P, Ghate H, Patole MS, Shouche YS. Molecular evidence and phylogenetic affiliations of *Wolbachia* in cockroaches. Mol Phylogenet Evol. 2007; 44:1346–1351. [PubMed: 17350292]

Werren JH, Windsor DM. *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? Proc Biol Sci. 2000; 267:1277–1285. [PubMed: 10972121]

Werren JH, Baldo L, Clark ME. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008; 6:741–751. [PubMed: 18794912]

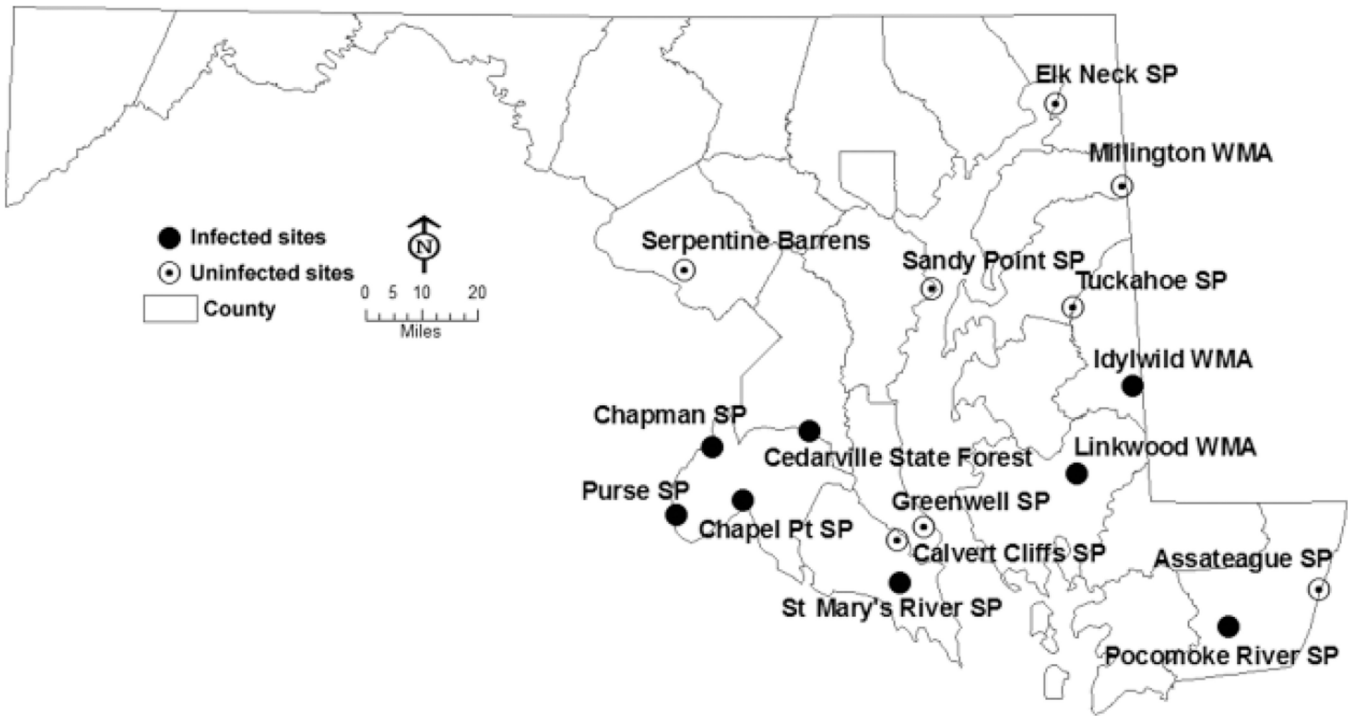

Fig. 1.

Map of Maryland *Amblyomma americanum* collection sites. Closed circles indicate populations with infected ticks; open circles indicate populations where no infected ticks were collected.

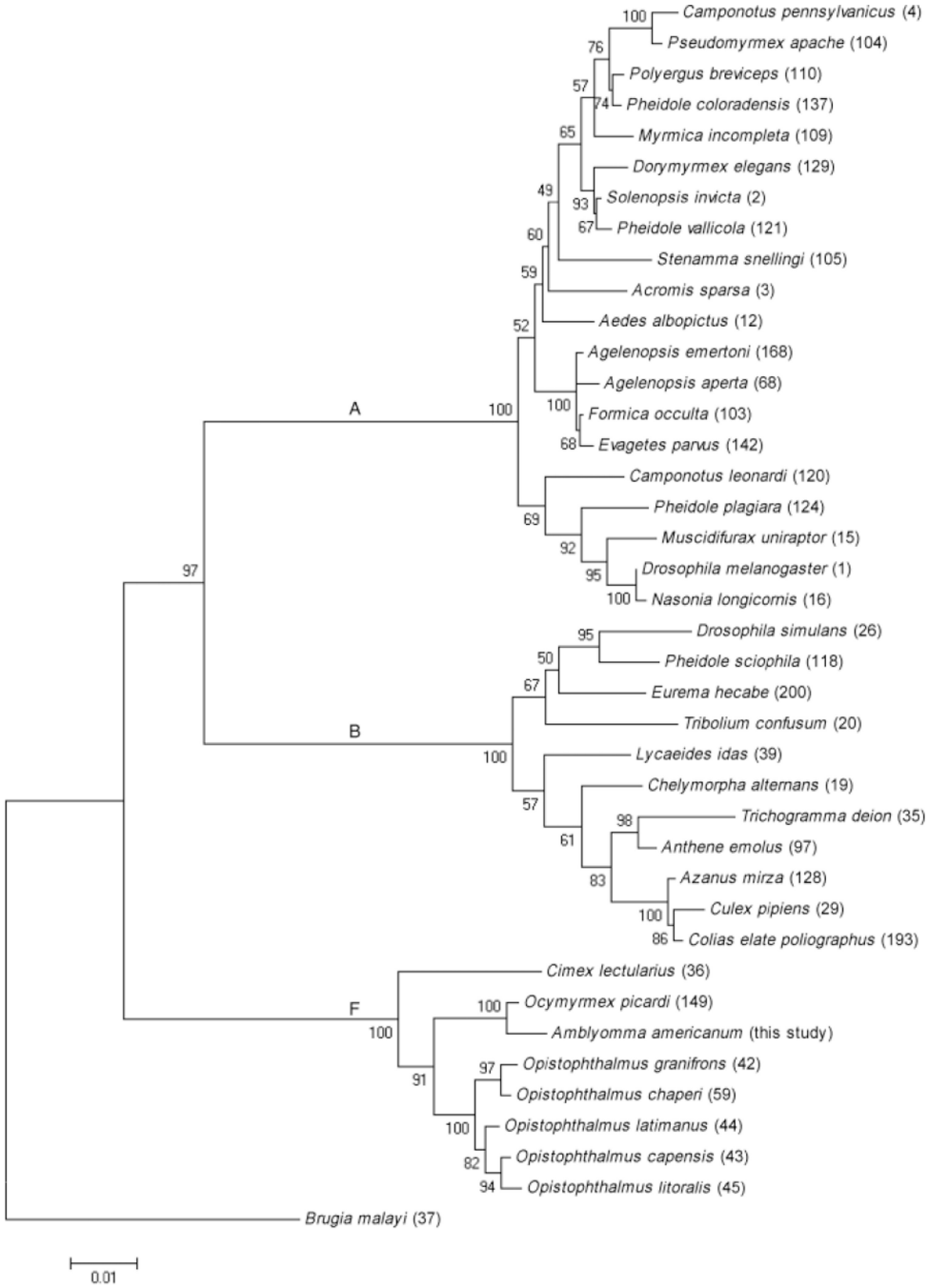

Fig. 2.

Neighbor-joining phylogenetic analysis of a 2079 bp alignment of five *Wolbachia* MLST concatenated gene sequences (*coxA*, *gatB*, *hcpA*, *ftsZ* and *fbpA*). Numbers at nodes indicate bootstrap support values (1000 replicates). Taxon names are host species. Values in parentheses represent MLST sequence ID numbers (searchable at http://pubmlst.org/wolbachia/). Letters represent *Wolbachia* supergroup designations. The tree is rooted by the supergroup D strain from *Brugia malayi*.

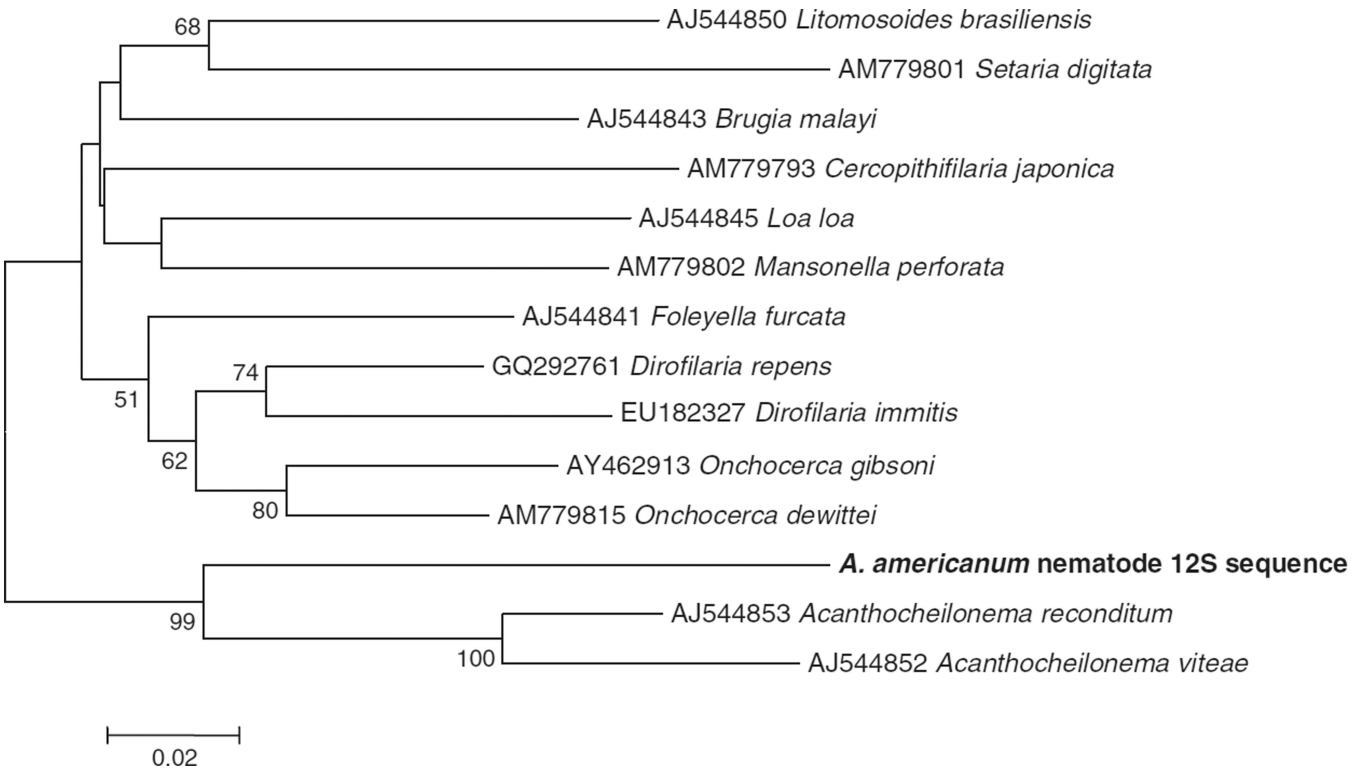

Fig. 3.

Unrooted neighbor-joining phylogenetic analysis of of nematode 12S gene sequences. *Amblyomma americanum* nematode sequence has been deposited in GenBank under accession number JF732757. Alphanumeric codes represent GenBank ID numbers. Numbers at nodes indicate bootstrap support values (1000 replicates). Bold indicates nematode sequence identified in this study.

Table 1

Collection site, tick stage and infection rate

Location	County	Stage	*N*	# Positive	% Infected
Idylwild WMA	Caroline	Female	19	2	10.5%
		Male	10	0	0
		Nymph	5 pools (44)	0	0
Linkwood WMA	Dorchester	Female	29	1	3.5%
		Male	19	0	0
		Nymph	3 pools (27)	0	0
Assateague SP	Worchester	Female	9	0	0
		Male	7	0	0
		Nymph	7 pools (68)	0	0
Pocomoke SP	Worchester	Nymph	7 pools (63)	3	4.76%*
Cedarville SF	PG and Charles	Female	2	0	0
		Male	1	0	0
		Nymph	6 pools (51)	3	5.88%*
Chapel Point SP	Charles	Female	3	0	0
		Nymph	6 pools (56)	4	7.14%*
Chapman SP	Charles	Female	17	2	11.8%
		Male	9	0	0
		Nymph	11 pools (103)	1	0.97%*
Purse SP	Charles	Nymph	5 pools (46)	1	2.17%*
Serpentine	Montgomery	Female	15	0	0
		Male	17	0	0
		Nymph	4 pools (40)	0	0
St Mary's SP	St Mary's	Female	4	1	25%
		Male	1	0	0
		Nymph	3 pools (29)	0	0
Greenwell SP	St Mary's	Female	3	0	0
		Male	1	0	0

Location	County	Stage	*N*	# Positive	% Infected
Sandy Point SP	Anne Arundel	Female	8	0	0
		Male	9	0	0
		Nymph	4 pools (38)	0	0
Millington WMA	Kent	Female	2	0	0
		Male	3	0	0
		Nymph	1 pool (11)	0	0
Calvert Cliffs SP	Calvert	Female	7	0	0
		Nymph	19 pools (183)	0	0
Elk Neck SP	Cecil	Female	1	0	0
		Nymph	3 pools (28)	0	0
Tuckahoe SP	Queen Anne's	Male	1	0	0
		Nymph	5 pools (47)	0	0
	Total	Female	119	6	5.04%
		Male	78	0	0
		Nymph	89 pools (834)	12	1.44%*

* Infection rate of nymphs is reported as the MIR; the number of infected pools divided by the total number of ticks tested.

Table 2

Sequences obtained from assayed ticks

Specimen	Stage assayed	*Wolbachia*	FbpA	CoxA	GatB	HcpA	ftsZ	Nematode
366	Adult female	+	×	×	×	×	×	Not tested
294	Adult female	+	×		×	×		−
295	Adult female	+	×			×		+
995	Adult female	+	×		×			+
998	Adult female	+	×					+
1178	Adult female	+	×					+
445–454	Pooled nymphs	+	×	×				−
465–474	Pooled nymphs	+	×					+
485–494	Pooled nymphs	+	×					+
790–799	Pooled nymphs	+	×					−
817–826	Pooled nymphs	+	×					−
827–836	Pooled nymphs	+	×					+
868–877	Pooled nymphs	+	×					+
927–932	Pooled nymphs	+	×					+
1016–1025	Pooled nymphs	+	×					+
1032–1041	Pooled nymphs	+	×					+
1042–1051	Pooled nymphs	+	×					+
1062–1071	Pooled nymphs	+	×					−
1337	Adult female	−						−
1338	Adult female	−						−
255–258	Pooled nymphs	−						−
475–484	Pooled nymphs	−						+
837–840	Pooled nymphs	−						−
907–916	Pooled nymphs	−						−
917–926	Pooled nymphs	−						−
997	Adult female	−						−
999	Adult female	−						−
1000	Adult female	−						−

Specimen	Stage assayed	*Wolbachia*	FbpA	CoxA	GatB	HcpA	ftsZ	Nematode
1001	Adult female	–					–	