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Caroline D. Keroack
Smith College

Jenna Irene Wurster
Smith College

Caroline G. Decker
Smith College

Kalini M. Williams
Smith College

Barton E. Slatko
New England Biolabs, Inc

See next page for additional authors

Authors
Caroline D. Keroack, Jenna Irene Wurster, Caroline G. Decker, Kalini M. Williams, Barton E. Slatko, Jeremy M. Forster, and Steven A. Williams

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ABSENCE OF THE FILARIAL ENDOSYMBIONT *WOLBACHIA* IN SEAL HEARTWORM (*ACANTHOCHEILONEMA SPIROCAUDA)* BUT EVIDENCE OF ANCIENT LATERAL GENE TRANSFER

Caroline D. Keroack*, Jenna I. Wurster*†, Caroline G. Decker*, Kalani M. Williams*, Barton E. Slatko‡, Jeremy M. Foster‡, and Steven A. Williams*

* Smith College, Department of Biological Sciences, Northampton, Massachusetts 01063. Correspondence should be sent to: *ckeroack@smith.edu*

ABSTRACT: The symbiotic relationship of *Wolbachia* spp. was first observed in insects and subsequently in many parasitic filarial nematodes. This bacterium is believed to provide metabolic and developmental assistance to filarial parasitic nematodes, although the exact nature of this relationship remains to be fully elucidated. While *Wolbachia* is present in most filarial nematodes in the family *Onchocercidae*, it is absent in several disparate species such as the human parasite *Loa loa*. All tested members of the genus *Acanthocheilonema*, such as *Acanthocheilonema viteae*, have been shown to lack *Wolbachia*. Consistent with this, we show that *Wolbachia* is absent from the seal heartworm (*Acanthocheilonema spirocauda*), but lateral gene transfer (LGT) of DNA sequences between *Wolbachia* and *A. spirocauda* has occurred, indicating a past evolutionary association. Seal heartworm is an important pathogen of phocid seals and understanding its basic biology is essential for conservation of the host. The findings presented here may allow for the development of future treatments or diagnostics for the disease and also aid in clarification of the complicated nematode–*Wolbachia* relationship.

The genus *Wolbachia* is comprised of intracellular α-proteobacteria within a wide range of arthropods and filarial nematodes (Serbus et al., 2008; Foster et al., 2013; Slatko et al., 2014). Since their discovery, *Wolbachia* spp. have been identified in every major insect order and in mites, crustaceans, and filarial nematodes (Hertig and Wolbach, 1924; McLaren, 1975; Werren et al., 1995; Bandi et al., 1998; Jeyaprakash and Hoy, 2000; Fenn et al., 2006; Pfarr et al., 2007; Werren et al., 2008). In arthropods, *Wolbachia* localize to the reproductive tract and exhibit reproductive parasitism through multiple mechanisms including induction of cytoplasmic incompatibility, feminization of males, male killing, or parthenogenesis (Bouchon et al., 1998; Stouthamer et al., 1999; Harris and Braig, 2003; Tram et al., 2003; Serbus and Sullivan, 2007; Werren et al., 2008). *Wolbachia* in nematodes appear not to have the same reproductive manipulations as in arthropods, as male killing, feminization, cytoplasmic incompatibility, and parthenogenesis have not been observed (Brattig, 2003; Fenn and Blaxter, 2004; Taylor et al., 2005a).

The dynamics of *Wolbachia*–nematode host interactions have been a topic of active investigation (Taylor and Hoerauf, 1999; Brattig, 2003; Taylor et al., 2005a; Foster et al., 2013; Landmann et al., 2014). Antibiotic depletion of *Wolbachia* leads to developmental defects in embryogenesis and nematode larvae, as well as killing of adult filarial worms, supporting the essential nature of the *Wolbachia*–nematode relationship (Hoerauf et al., 1999, 2000; Fenn and Blaxter, 2004; Taylor et al., 2001, 2005b; Strubing et al., 2010; Foster et al., 2013; McCall et al., 2014). There is also evidence that *Wolbachia* spp. may support or provide certain metabolic pathways, including heme and vitamin B2 metabolism, that are missing in their nematode hosts, which may explain why eliminating *Wolbachia* affects the nematode (Foster et al., 2005; Wu et al., 2009, 2013; Darby et al., 2012; Li and Carlow, 2012). Still, the nature of nematode–*Wolbachia* symbiosis remains to be elucidated (Foster et al., 2005; Wu et al., 2009; Strubing et al., 2010; Landmann et al., 2010, 2011, 2014; Desjardins et al., 2013; Melnikow et al., 2013).

Aside from the biological nature of the nematode–*Wolbachia* interaction, the phylogenetic relationship between the two also remains perplexing. In nematodes, *Wolbachia* spp. are only known to reside in the filarial nematodes (family: *Onchocercidae*) and mainly cluster in 2 families: *Dirofilariinae* and *Onchocercinae* (Taylor and Hoerauf, 1999; Bandi et al., 2001; Casiraghi et al., 2001; Chirgwin et al., 2002; Ferri et al., 2011; Landmann et al., 2014). However, *Wolbachia* distribution is not ubiquitous throughout the filarial nematodes. Notably, several species including *Loa loa*, *Acanthocheilonema viteae*, *Onchocerca flexuosa*, and all screened *Setaria* species lack *Wolbachia*, as demonstrated by various molecular analyses (Chirgwin et al., 2002; Bordenstein et al., 2003; McGarry et al., 2003; Casiraghi et al., 2004; McNulty et al., 2010; Ferri et al., 2011; Desjardins et al., 2013). Recently it has been shown that more filarial species lack *Wolbachia* than expected, including other species of the genus *Acanthocheilonema* (Ferri et al., 2011; Uni et al., 2013). These anomalies have generated contrasting opinions regarding the pattern of acquisition–loss of *Wolbachia* through filarial nematode evolutionary history. Two alternative hypotheses have been proposed: first, *Wolbachia* spp. were inherited by a distant common ancestor of filarial nematodes and were subsequently lost in some lineages or, second, that *Wolbachia* spp. were inherited independently multiple times (Casiraghi et al., 2001; Ferri et al., 2011; Blaxter and Koutsovoulous, 2015). Due to a small data set, this quandary persists. By adding data we hope to aid in elucidating the evolutionary history of the nematode–*Wolbachia* relationship.

This study focuses on the seal heartworm (*Acanthocheilonema spirocauda*), a filarial parasite of many phocid seals such as the charismatic harbor seal (*Phoca vitulina*) (Leidenberger et al., 2007). Seal heartworm has no singular definitive host seal species and is distributed throughout the Northern hemisphere (Measures, 2001). Clinical manifestations of infection include anorexia, fatigue, dehydration, coughing–bronchiospasm, and many other classical symptoms of heartworm infection (Daily, 2005). Complications include secondary bacterial infection, irreversible pathological changes, and death. Seal heartworm is a significant cause of morbidity in neonatal and juvenile seals, and its full host range is unknown (Geraci et al., 1981; Leidenberger et al., 2007).

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† Harvard Medical School, Department of Ophthalmology, Boston, Massachusetts, 02115.

‡ New England Biolabs, Inc, Genome Biology Division, Ipswich, Massachusetts 01938.

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Controlling the spread of the parasite between seals and potentially across species is likely essential for marine mammal health and conservation (Raga et al., 1997). In additional to potential conservational impacts, studying *A. spirocauda* has direct phylogenetic impact on the interpretation of the evolutionary history between *Wolbachia* and filarial nematodes.

Here, we present molecular evidence against *Wolbachia* endosymbiosis in seal heartworm (*A. spirocauda*), which is consistent with the general absence of *Wolbachia* in other *Acanthocheilonema* species such as *A. viteae* and *Acanthocheilonema delicata* (Casiraghi et al., 2004; McNulty et al., 2010; Uni et al., 2013). We also present data indicating lateral gene transfer (LGT) between *Wolbachia* and the nuclear genome of *A. spirocauda*, data which support the concept that *Wolbachia* was present in this genus at one point in its evolutionary history (McNulty et al., 2010; Blaxter and Koutsovoulous, 2015). This study bolsters the hypothesis that the acquisition of *Wolbachia* endosymbionts was an ancestral event and that loss of the endosymbiont is apomorphic. This study also reveals that *A. spirocauda* is a *Wolbachia*-free parasite that could be used in genomic comparisons with *Wolbachia*-containing parasites to attempt to decipher the biological relationship between the host and symbiont.

MATERIALS AND METHODS

Parasites

Whole adult seal heartworms (*A. spirocauda)* stored in glycerin were obtained from the National Marine Life Center (NMLC) in Falmouth, Massachusetts. Additional seal heartworm specimens frozen in saline were obtained from the North East Marine Fisheries Service (NEMFS) in Woods Hole, Massachusetts. Parasites were collected during necropsy of stranded or by-caught harbor seals (*P. vitulina*), and no live animals were involved or harmed in collection. Parasites were obtained with permission of the National Oceanic and Atmospheric Administration authorized under the regulations at 50 CFR 216.22(c)(5) and 216.37 of the Marine Mammal Protection Act, which allows transfer of marine mammal parts for scientific research purposes. Parasites used in this study were obtained from 2 stranded harbor seals (*P. vitulina*) identified as DO-5476 (NEMFS; seal by-caught off the coast of Gloucester, Massachusetts on 20 February 2000) and P Pr 13-104 (NMLC; seal found stranded in Eastham, Massachusetts on 1 January 2013). Five worms were obtained from DO-5476 and a single worm was obtained from P Pr 13-104. *Brugia pahangi* (females, *Wolbachia* positive) were provided by the Filariasis Research Reagent Resource Center (FR3), Athens, Georgia. *Acanthocheilonema viteae* DNA (positive control for the porphobilinogen deaminase pseudogene [*PBGD*] PCR), *Dirofilaria immitis*, and *Onchocerca volvulus* DNA (positive controls for *Wolbachia* surface protein [*WSP*] and 16s*Wolb* PCRs) and *Loa loa* (*PBGD* PCR negative control) were also provided by the FR3.

DNA isolation method

Intact worms (*A. spirocauda* worms [1 from DO-5476; 1 from P Pr 13-104]; 50 *B. pahangi* worms [verified by FR3]) were washed and resuspended in phosphate-buffered saline (250 μL) and homogenized using a sterile metal ball bearing (Qiagen, Venlo, Netherlands) in 250 μL lysis buffer (50 mM Tris-HCl pH 7.5, 0.1 M ethylenediaminetetraacetic acid, 0.2 M sodium chloride) using a Qiagen Retsch model TissueLyser II for 8 min at a frequency of 1/20 sec (total volume of homogenate was ~500 μL). Following homogenization, 60 μL of proteinase K (New England Biolabs, Ipswich, Massachusetts; 0.8 units/μL; 48 units total), 30 μL of 10% sodium dodecyl sulfate (final concentration 0.5%), and 2 μL β-mercaptoethanol (final concentration, 48 mM) were added to the sample and incubated at 56 C for 6–9 hr (total volume was 592 μL). The sample was treated with 1.5 μL RNaseA (Qiagen; 0.337 μg/mL final concentration) at 37 C for 1 hr. DNA was extracted using phenol followed by chloroform and then precipitated using absolute ethanol. DNA was resuspended in 50 μL 0.1X TE buffer (American Bioanalytical, 1X stock, Natick, Massachusetts) and quantified.

Species identification and DNA quality check

DNA isolated from *A. spirocauda* was subjected to barcode PCR and subsequent sequencing for identification. DNA provided for *Loa loa*, *O. volvulus*, *A. viteae*, and *D. immitis* was verified by the FR3 but subjected to the same PCR to ensure DNA was suitable for amplification. A partial cytochrome c oxidase subunit 1 (*cox1*) mitochondrial gene was used for identification. Primers used were 5′-TGATTGGTGGTTTTGGTAA-3′ and 5′-ATAAGTACGAGTATCAATATC-3′ with an expected product size between 500–700 base pairs (bp) (Casiraghi et al., 2001; Ferri et al., 2009). Amplification consisted of 40 cycles, an annealing temperature of 52 C, and an extension time of 1.5 min. Master mix was prepared following the manufacturer's protocols with 0.2 μM final primer concentrations (Phusion polymerase, New England Biolabs).

Polymerase chain reaction (PCR) amplification of target sequences

PCR was performed on both *A. spirocauda* from P Pr 13-104 and DO-5476 parasites in addition to controls. DNA was isolated from whole, single worms. All PCRs were performed using the following thermal cycling conditions, with any variations noted: 98 C for 3 min as an initial denaturing step followed by 35–40 cycles of 98 C denaturing, variable temperature for annealing, 72 C extension, followed by a final extension for 10 min at 72 C. The *WSP* was amplified using 5′-GTCCAATARST GATGARGAAAC-3′ and 5′- CYGCACCAAYAGYRCTRTAAA-3′ primers for an expected product size of ~590 bp (Bazzocchi et al., 2000). These primers were designed based on arthropod *Wolbachia* sequences and have been shown to amplify *WSP* from both *Brugia* spp. and *Litomosoides* sp. (Bazzocchi et al., 2000). Amplification consisted of 35 cycles of with an annealing temperature of 50 C for 45 seconds and an extension time of 1.5 min. The 16s ribosomal RNA subunit (*16s rRNA*) was amplified using primers specific to *Wolbachia* (*16swolb)* with 5′-GAAGATAATGACGGTACTCAC-3′ and 5′-GTCACTGATCC CACTTTAAATAAC-3′ for an expected product size of approximately 1,000 bp (Casiraghi et al., 2001). These primers were designed to amplify *Wolbachia* sequences from supergroups (clades) A–D and were previously demonstrated to amplify the 16s rRNA in a wide range of *Wolbachia* strains (Casiraghi et al., 2001). Amplification was for 40 cycles with an annealing temperature of 48 C for 30 sec and an extension time of 2 min. All reactions were prepared in nuclease-free water with the following final concentrations: deoxynucleotide triphosphate solution (0.2 mM; New England Biolabs), forward and reverse primer (0.2 μM each), dimethyl sulfoxide (New England Biolabs, 3% final concentration), 5X HF Phusion buffer (1X final concentration), and template DNA (~100 ng). The master mix was supplemented with an additional 0.5 mM magnesium chloride, bringing the final concentration to 2.0 mM. One unit of Phusion polymerase (New England Biolabs) was added to each reaction.

The *PBGD*, reported to be laterally transferred to the nuclear genome of *A. viteae* from a former *Wolbachia* colonization (McNulty et al., 2010; New England Biolabs, unpubl. data) was amplified as 3 different-sized fragments using 3 different forward primers and 1 reverse primer, Av-wPBGDr (Table I). Amplification used 35 cycles, an annealing temperature of 50 C, and an extension time of 1 min. Reactions were prepared as above, with the exclusion of additional magnesium chloride.

Controls for *WSP* and *16s rRNA* PCR were DNA samples extracted from *B. pahangi*, *O. volvulus*, and *D. immitis*, which are known to harbor *Wolbachia* (Casiraghi et al., 2004). The positive control for the *PBGD* PCR was *A. viteae* DNA and the negative control was *Loa loa* DNA. PCRs were performed in duplicate, and the results shown are representative of 2 independent experiments. All PCR products were electrophoresed on 1.5% agarose gels in 1X TAE buffer (50X Stock, American Bioanalytical).

DNA sequencing

Automated Sanger dideoxy terminator sequencing was performed at the Smith College core facility on an ABI 3130xl (Hitachi, Tokyo, Japan) following the BigDye© Terminator v3.1 cycle kit (Life Technologies, Carlsbad, California) protocols using the Av-PBGDf1 primer. Sequences were deposited in GenBank (*A. spirocauda* [P Pr 13-104] *PBDG:* KU19397; *A. spirocauda* [DO-5476] *PBDG:* KT369810; *A. viteae PBGD:*

TABLE I. Primer sequences for porphobilinogen deaminase pseudogene (*PBGD*) PCR.*

Name	Sequence	Amplicon size
Av wPBGDf1	5′-ACC TAA AAT CTG TGT GTC CAT ATG GTC-3′	400 bp
Av wPBGDf2	5′-TCG AAG TGC TTA AGA ATA ATA TAG AC-3′	300 bp
Av wPBGDf3	5′-TCA TTC GGT AAA GAT GTC TCT CC-3′	250 bp
Av wPBGDr	5′-AGC TAG AAT TAT TCC ATC AAA ATT GTG G-3′	—

* Primers were designed based on unpublished data from New England Biolabs and information generated by McNulty, et al. 2010.

KT369809; *A. spirocauda* [DO-5476] *cox1:* KT899871; *A. spirocauda* [P Pr 13-104] *cox1:* KU19398).

Western blot

Whole-worm lysates were prepared by freezing parasites in liquid nitrogen and homogenizing them using a mortar and pestle with 1X Cell lysis buffer (10X stock, Cell Signaling Technology, Danvers, Massachusetts, prepared according to the manufacturer's instructions) combined with SDS (6% w/v). Parasites tested were *A. spirocauda* (DO-5476; 1 adult worm, sex unknown) and *B. pahangi* (50 adult females, used as a control). The amount of parasite tissue used in both samples was roughly equal (~50 mg). Fourteen microliters of lysate were loaded into each well of a 4–20% polyacrylamide gel. Gels were wet-transferred onto nitrocellulose and blocked following a publicly available protocol (Cell Signaling Technology, 2013). Membranes were rocked overnight at 4 C in 5% non-fat dry milk in 1X TBS-T with the appropriate dilution of anti-*Wolbachia* surface protein primary antibody (Anti-*WSP*, 1:20,000 dilution; BEI Resources, Manassas, Virginia). This monoclonal antibody (purified IgG, produced in vitro; NR-31029) has been shown to bind to *WSP* in various systems including *Brugia malayi* and *Drosophila melanogaster* (Michalski et al., 2011; Newton et al., 2015). Anti α-tubulin (Cell Signaling Technology, Danvers, Massachusetts, #2125 1:2000) served as a control. Membranes were washed and then incubated with secondary antibody (anti-rabbit, Cell Signaling Technology, no. 7074, 1:2000) following the manufacturer's protocols (Cell Signaling Technology, 2013). Membranes were developed using SignalFire™ (Cell Signaling Technology).

Phylogenetic reconstruction

The phylogeny was constructed using MEGA 5.2 software (Tamura et al., 2011). *PBGD* sequences were aligned using MUSCLE (MEGA 5.2) with standard parameters. The maximum likelihood tree was constructed using a Tamura 3-parameter model, considering all nucleotide sites, with gamma distribution. *Neorickettsia risticii* (an α-proteobacterium) was used as an out-group to root the tree. In the tree, bootstrap confidence values after 500 iterations were displayed at the nodes (Fig. 5). Sequences not generated in this study were obtained from GenBank.

RESULTS

Species identification and DNA quality verification

PCR reactions were used for sequencing to confidently determine the species of the seal parasites. While parasites were identified morphologically by the providing agencies, PCRs were run to confirm these designations. PCRs also served as a control for all other DNA samples; these reactions served to show DNA quality and ability of the DNA samples to be amplified (Fig. 1). COI gene sequences were analyzed by BLASTn (National Center for Biotechnology Information [NCBI], http://www.blast.ncbi.nlm.nih.gov/Blast.cgi. Isolate P Pr 13-104 (KU19398) showed 99% identity with *A. spirocauda* (HF583266) with 100% coverage; isolate DO-5476 (KT899871) had 99% identity with *A. spirocauda* (HF583266) with 100% coverage. *Loa loa*, *O. volvulus*, *B. pahangi*, *D. immitis*, and *A. viteae* were not sequenced, as their identity was previously determined and confirmed by the providing agency (NIH).

PCR screen for *Wolbachia* presence

PCR reactions were used to determine the presence or absence of *Wolbachia* endosymbionts in *A. spirocauda*. Amplification was from total genomic DNA using *Wolbachia*-specific 16S ribosomal RNA subunit (*16swolb*) primers (Fig. 2) and *WSP* primers (Fig. 3). No amplicons were obtained from *A. spirocauda*, although correct-size amplicons (~1,000 bp, 16S rRNA; ~590 bp *WSP*) were generated from the *B. pahangi*, *O. volvulus*, and *D. immitis*-positive controls (Figs. 2, 3). Duplicate PCRs yielded identical results.

Western blot screen for *Wolbachia* presence

Western blots were performed using a monoclonal anti-*WSP* antibody. This antibody has been shown to react with protein from a wide range of *Wolbachia* strains (Michalski et al., 2011; Newton et al., 2015). No *WSP* was detected in the *A. spirocauda* samples. The antibody did detect the protein (*WSP*) in *B. pahangi*, indicated by the 25-kDa band (Fig. 4). To ensure intact protein

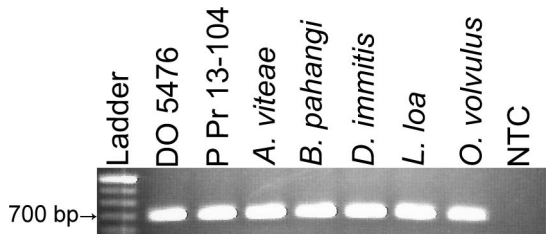

FIGURE 1. COI PCR for DNA quality verification. Lane 1: 100-bp ladder; Lane 2: DO 5476 (*Acanthocheilonema spirocauda*); Lane 3: P Pr 13-104 (*A. spirocauda*); Lane 4: *Acanthocheilonema viteae*; Lane 5: *Brugia pahangi*; Lane 6: *Dirofilaria immitis*; Lane 7: *Loa loa*; Lane 8: *Onchocerca volvulus*; Lane 9: no template control (NTC).

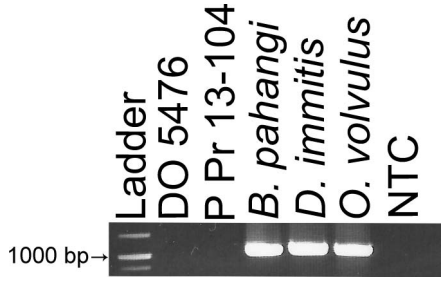

FIGURE 2. *16swolb* PCR. Lane 1: 100-bp DNA ladder (New England Biolabs); Lane 2: DO 5476 (*Acanthocheilonema spirocauda*) genomic DNA; Lane 3 P Pr 13-104 (*A. spirocauda*) genomic DNA; Lane 4: *Brugia pahangi* genomic DNA; Lane 5: *Dirofilaria immitis* genomic; Lane 6: DNA *Onchocerca volvulus* genomic DNA; Lane 7: no template control (NTC). Amplicons are at the expected size of 1,000 base pairs.

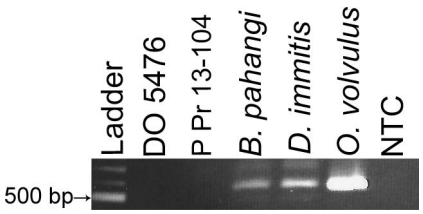

FIGURE 3. *Wolbachia* surface protein (*WSP*) PCR. Lane 1: 100-bp DNA ladder (New England Biolabs); Lane 2: DO 5476 (*Acanthocheilonema spirocauda*) genomic DNA; Lane 3 P Pr 13-104 (*A. spirocauda*) genomic DNA; Lane 4: *Brugia pahangi* genomic DNA; Lane 5: *Dirofilaria immitis* genomic; Lane 6: DNA *Onchocerca volvulus* genomic DNA; Lane 7: no template control (NTC). Amplicons are of expected size of approximately 590 bp.

was present in both samples, western blots using an anti–α-tubulin monoclonal antibody were also preformed. This protein was selected because it is a constitutively expressed housekeeping gene. Both lysates showed a band between 50 and 60 kDa on the western blot, indicating the presence of α-tubulin (52 kDa) and that the lysates were of suitable quality for western blots (Fig. 4).

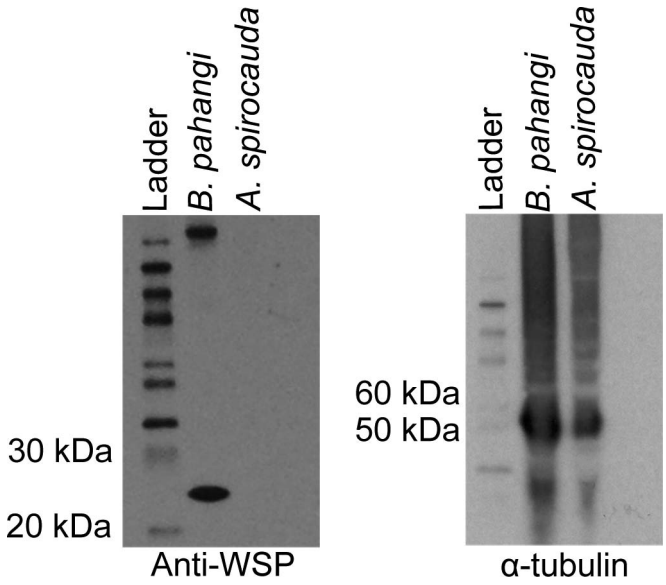

FIGURE 4. Left: Anti-*Wolbachia* surface protein (*WSP*) western blot. Left: *Wolbachia* surface protein appears between 20–30 kDa. Lane 1: Biotinylated ladder (Cell Signaling Technology no. 7727), Lane 2: positive control *Brugia pahangi* lysate, Lane 3: *Acanthocheilonema spirocauda* lysate (DO-5476, whole worm). Right: Control western blot. Alpha-tubulin bands appear between 50–60 kDa. Lanes are the same as *WSP* blot.

Screen for LGT

PCR was used to determine if the *Wolbachia*-like pseudogene *PBGD* was present in the *A. spirocauda* genome. The reaction captures a size-variable region of a pseudogene depending on the forward primer used (Table I). Primers were designed for specificity to the *Wolbachia*-like *PBGD* pseudogene of *A. viteae*, identified by McNulty et al. (2010). This gene was selected due to its relation to heme metabolism, which is one of the proposed functions of *Wolbachia* endosymbiosis in nematodes (Wu et al., 2009). This assay was developed based on a combination of data. First, screening of *A. viteae* cDNA revealed the presence of a *PBGD* pseudogene fragment even though the parasite is *Wolbachia*-free (Wu, Foster, and Slatko, unpubl. obs.). Second, low coverage genomic sequencing of the *A. viteae* genome revealed LGTs of heme-biosynthesis related gene fragments, including a *PBGD* sequence (McNulty et al., 2010), which overlapped and extended the first-identified fragment. The PCR generated amplicons of expected size for each of the different *PBGD* primer sets (Fig. 5, top). Sequencing of the largest PCR product (~400 bp) and subsequent NCBI BLAST identification showed that the *PBGD* sequences in both individuals of *A. spirocauda* (DO-5476: KT369810; P Pr 13-104: KU19389) and *A. viteae* (KT369809) were most similar to those of *Wolbachia* found in *B. malayi* and *O. volvulus*, both the filarial parasites known to harbor *Wolbachia* (Fig. 5, bottom).

The sequences generated using the *PBGD* primers contained no open reading frame, indicating these sequences are pseudogenes. The phylogeny of *PBGD* sequences shows that the sequences obtained in this study most closely resemble *Wolbachia hemC* (*hemC* is the gene designation for bacterial *PBGD*), which codes for porphobilingen deaminase in the bacterium (Klasson et al., 2009). More specifically, the *A. spirocauda PBGD* sequences matched *hemC* from extant *Wolbachia* that reside in other filarial parasites (McNulty et al., 2010) (Fig. 5, bottom). BLASTn analysis of the *PBGD* sequence of *A. spirocauda* (DO-5476) showed 80% sequence identity over 78% of the sequence with *Wolbachia hemC* of *O. volvulus* (e-value 6e-68), *A. spirocauda* (P Pr 13-104) showed 81% identity over 90% (e-value 2e-157), and the sequence from *A. viteae* similarly showed 83% sequence identity over 78% of the sequence (e-value 1e-76). This supports the conclusion that these pseudogenes derive from nuclear insertions of *Wolbachia* sequences similar to those found in the bacteria in other filarial parasites. Given that the *16s rRNA* and *WSP* PCR and western blot experiments showed the absence of *Wolbachia* in *A. spirocauda*, these results indicate an LGT occurred between a *Wolbachia* endosymbiont and *A. spirocauda* in the distant past, before the loss of the *Wolbachia* endosymbiont. Interestingly, the sequence similarity between *A. spirocauda* (DO-5476: P Pr 13-104) and *A. viteae PBGD* is 94/93% over 99/86% of the sequence lengths, indicating possible pressure to preserve the sequence over evolutionary time.

DISCUSSION

The molecular evidence presented here suggests that *A. spirocauda* lacks a current symbiotic relationship with *Wolbachia*. These results are consistent with the pattern of *Wolbachia* distribution in filarial worms, as members of the *Acanthocheilonema* genus including *A. viteae*, *A. reconditum*, and *A. delicata* also lack *Wolbachia* (Casiraghi et al., 2004; McNulty et al., 2010; Uni et al., 2013). These data might be thought to support the conclusion that the *Acanthocheilonema* genus diverged before filarial nematodes obtained *Wolbachia*. However, our data on LGT provide evidence to the contrary: specifically, that *A. spirocauda* has acquired *Wolbachia*-like genes, presumably through LGT. The close relationship of the laterally-transferred gene to *PBGD* genes found in *Wolbachia* present in other filarial worms implies that *A. spirocauda* once harbored *Wolbachia* endosymbionts and that the loss of the endosymbiont is a derived characteristic. The presence of *Wolbachia*-like sequences in the

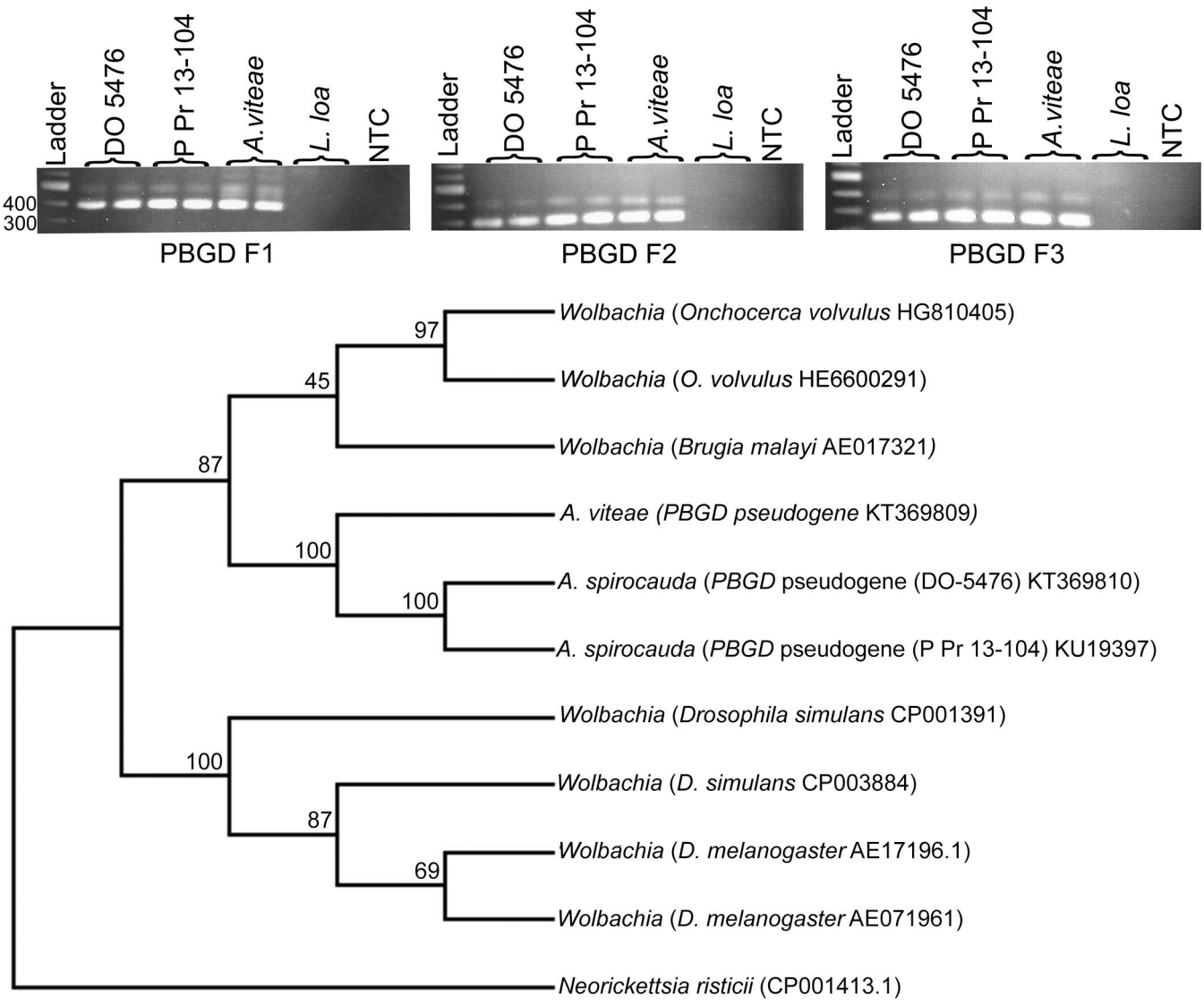

FIGURE 5. Top: porphobilinogen deaminase pseudogene (*PBGD*) gel images: Lane 1: 100-bp ladder (New England Biolabs); Lanes 2 and 3: *Acanthocheilonema spirocauda* genomic DNA (DO-5476); Lanes 4 and 5: *A. spirocauda* genomic DNA (P Pr 13-104); Lanes 6 and 7: *Acanthocheilonema viteae* genomic DNA; Lanes 8 and 9: *Loa loa* genomic DNA (negative control); Lanes 10 and 11: no template control. Bottom: Phylogeny of *hemC* (*PBGD*) pseudogene sequences. Bootstrap confidence values after 500 iterations are displayed at the nodes. Displayed are the *PBGD* sequences obtained in this study compared against the top 3 filarial parasite results indicated by BLASTn analysis as well as several insect *Wolbachia* sequences that showed similarity to the query sequence in BLAST.

nuclear genomes of both *A. spirocauda* and *A. viteae* suggests that the *Acanthocheilonema* genus once harbored *Wolbachia* and that divergence was preceded by endobacterial loss. This is counter to the hypothesis that *Acanthocheilonema* should be considered an out-group when examining *Wolbachia* phylogeny and coevolution with its nematode hosts (Bordenstein et al., 2003; Casiraghi et al., 2004).

The presented data support the hypothesis that multiple losses of *Wolbachia* have occurred, as seen in *Acanthocheilonema* spp., *O. flexuosa*, and possibly other nematode species. The clustering of the *PBGD* sequences with those from extant *Wolbachia* found in other filarial worms possibly suggests that they share a common origin (Fig. 5, bottom). However, due to low bootstrap values, this conclusion would need to be further supported by additional experimentation. A likely common origin between the *Acanthocheilonema PBGD* sequences is nonetheless supported by the similarity of the sequences from *A. spirocauda* and *A. viteae*, which additionally may imply that there is functional significance to the pseudogene.

In combination with reported filarial nematode phylogeny, the most parsimonious explanation for the existence of laterally transferred *Wolbachia* genes in the *A. spirocauda* and *A. viteae* nuclear genomes is that ancestral endosymbiont acquisition occurred in the last common ancestor of most filarial nematodes (Xie et al., 1994; Casiraghi et al., 2001, 2004; Desjardins et al., 2013). A counter example has been found in *Loa loa*, where genomic sequencing has shown an absence of obvious LGT between *Wolbachia* and the parasite; however, the evolutionary implications of this absence remain to be elucidated (Desjardins et al., 2013).

The *Wolbachia*-like *PBGD* sequences obtained in this study contained no functional open reading frame, meaning that the acquired *Wolbachia* sequence is not functionally utilized as a *PBGD* by the nematode host. We do not currently understand why this gene fragment has been maintained with high sequence identity in sister species following lateral transfer. As noted above, the *A. viteae PBGD* pseudogene fragment is transcribed (Wu, Foster, and Slatko, unpubl. obs.), as are many other gene fragments transferred from *Wolbachia* to their nematode hosts (Ioannidis et al., 2013). Perhaps certain laterally transferred genes are maintained for functions we have yet to elucidate, such as those of small regulatory RNAs for example. Further genomic and transcriptomic analysis of various *Acanthocheilonema* species would be required to elucidate these questions.

CONCLUSION

Evidence presented in this study indicates that *A. spirocauda* lacks the *Wolbachia* endosymbiont. However, *A. spirocauda* genomic DNA contains at least 1 gene fragment that appears to be of *Wolbachia* origin. A close relative, *A. viteae*, also harbors this same *Wolbachia*-like *PBGD* sequence amongst a number of other LGTs (McNulty et al., 2010). This suggests that *Acanthocheilonema* contained *Wolbachia* in the past and lost the endosymbiont after an LGT event. Future studies employing larger-scale genomic sequencing and analysis will be required to fully elucidate the nature of the evolutionary and symbiotic relationship between *Wolbachia* and filarial nematodes.

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