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

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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 vitaeae, 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.
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 conservation 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 Koutsovoulos, 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 (NEFMS) 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 under the regulations at 50 CFR 216.22(c)(5) and 216.37 of the Marine of the National Oceanic and Atmospheric Administration authorized for scientific research purposes. Parasites used in this study were obtained from 2 stranded harbor seals (*P. vitulina*), which were 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 vitaeae* DNA (positive control for the porphobilinogen deaminase pseudogene [*PBGD*] PCR), *Dirofilaria immitis*, and *Onchocerca volvulus* DNA (positive controls for *Wolbachia* surface protein [*WSP*] and 16sWolb PCR) and *Luo 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 tissue Lyser 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 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. vitaeae* 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-wPBGDf (Table I). Amplification used 35 cycles, an annealing temperature of 50 C, and an extension time of 1 min. The 16s ribosomal RNA subunit (16s rRNA) was amplified using primers specific to *Wolbachia* (16swolb) with 5’-GAAGAATAAGCAGTTACTCAC-3’ and 5’-GTCACTGATCTAATGACTAAC-3’ primers for an expected product size of ~590 bp (Bazzocchi et al., 2000). These primers were designed based on arthropod *Wolbachia* sequences and used to amplify *Wolbachia* sequences from superfamilies (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: deoxyribonucleic acid 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). An additional 0.5 µM magnesium chloride, bringing the final concentration to 2.0 mM. One unit of Phusion polymerase (New England Biolabs) was added to each reaction.

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. vitaeae* DNA and the negative control was *Luo 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 (Bioanalytical). A cantho cheilonema *PBGD* primer. Sequences were deposited in GenBank (A. spirocauda [P Pr 13-104] *PBGD*: EU1939797; *A. spirocauda* [DO-5476] *PBGD*: KT369810; *A. viteae* *PBGD*: KU193979).

**DNA sequencing**

Automated Sanger dyeoxy 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] *PBGD*: EU1939797; *A. spirocauda* [DO-5476] *PBGD*: KT369810; *A. viteae* *PBGD*: KU193979).
Primers were designed based on unpublished data from New England Biolabs and information generated by McNulty, et al. 2010.

\[ \text{Av wPBGDf1} \ 5' \ - \text{ACC TAA AAT CTG TGT GTC CAT ATG GTC} - 3' \]
\[ \text{Av wPBGDf2} \ 5' \ - \text{TCG AAG TGC TTA AGA ATA TAG AC} - 3' \]
\[ \text{Av wPBGDf3} \ 5' \ - \text{TCA TTC GGT AAA GAT GTC TCT CC} - 3' \]
\[ \text{Av wPBGDr} \ 5' \ - \text{AGC TAG AAT TAT TCC ATC AAA ATT GTG G} - 3' \]

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

**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 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).

**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 identified morphologically by the providing agencies, PCRs 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 (16swoIb) primers (Fig. 2) and WSP primers (Fig. 3). No amplicons were obtained from *A. spirocauda* primers (Fig. 2).

**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 (16swoIb) 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.

**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 used 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).

**FIGURE 1.** COI PCR for DNA quality verification. Lane 1: 100-bp ladder; Lane 2: DO 5476 (*Acanthochilomena spirocauda*); Lane 3: P Pr 13-104 (*A. spirocauda*); Lane 4: *Acanthochilomena 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.** 16swoIb PCR. Lane 1: 100-bp DNA ladder (New England Biolabs); Lane 2: DO 5476 (*Acanthochilomena 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.
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).

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 1). 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 porphobilinogen 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

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
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 *PGBD* sequences obtained in this study contained no functional open reading frame, meaning that the acquired *Wolbachia* sequence is not functionally utilized as a *PGBD* 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* *PGBD* 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 *PGBD* 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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**LITERATURE CITED**


Ioannidis, P. K. L. Johnston, D. R. Riley, N. Kumar, J. R. White, K. T. Olarte, J. M. Foster, M. J. Taylor, and J. C. Dunnig Hotopp. 2013. Extensively duplicated and transcriptionally active recent lateral gene transfer from a bacterial *Wolbachia* endosymbiont to...


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