BioOne DIGITAL LIBRARY

Identification and Phylogenetic Analysis of Dirofilaria ursi (Nematoda: Filarioidea) from Wisconsin Black Bears (Ursus americanus) and its Wolbachia Endosymbiont

Authors: Michalski, Michelle L., Bain, Odile, Fischer, Kerstin, Fischer, Peter U., Kumar, Sanjay, et al.

Source: Journal of Parasitology, 96(2) : 412-419

Published By: American Society of Parasitologists

URL: https://doi.org/10.1645/GE-2208.1

The BioOne Digital Library (https://bioone.org/) provides worldwide distribution for more than 580 journals and eBooks from BioOne's community of over 150 nonprofit societies, research institutions, and university presses in the biological, ecological, and environmental sciences. The BioOne Digital Library encompasses the flagship aggregation BioOne Complete (https://bioone.org/subscribe), the BioOne Complete Archive (https://bioone.org/archive), and the BioOne eBooks program offerings ESA eBook Collection (https://bioone.org/esa-ebooks) and CSIRO Publishing BioSelect Collection (https://bioone.org/csiro-ebooks).

Your use of this PDF, the BioOne Digital Library, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Digital Library content is strictly limited to personal, educational, and non-commmercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne is an innovative nonprofit that sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

Downloaded From: https://complete.bioone.org/journals/Journal-of-Parasitology on 06 Jun 2025
Terms of Use: https://complete.bioone.org/terms-of-use

J. Parasitol., 96(2), 2010, pp. 412–419
© American Society of Parasitologists 2010

IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF *DIROFILARIA URSI* (NEMATODA: FILARIOIDEA) FROM WISCONSIN BLACK BEARS (*URSUS AMERICANUS*) AND ITS *WOLBACHIA* ENDOSYMBIONT

Michelle L. Michalski, Odile Bain*, Kerstin Fischer†, Peter U. Fischer†, Sanjay Kumar‡, and Jeremy M. Foster‡
University of Wisconsin Oshkosh, Oshkosh, Wisconsin 54902. *e-mail: michalsk@uwosh.edu*

ABSTRACT: *Dirofilaria ursi* is a filarial nematode of American black bears (*Ursus americanus* Pallas, 1780) that is vectored by black flies (Simuliidae) in many parts of the United States. In northwestern Wisconsin, the prevalence of microfilaremic bears during the fall hunting season was 21% (n = 47). Unsheathed blood microfilariae from Wisconsin bears possess characters consistent with the original description of *D. ursi*, as do adult worms observed histologically and grossly. Immunohistochemistry was used to identify the *Wolbachia* endosymbiont in the hypodermis and lateral cords of an adult female *D. ursi*. Amplification of *wsp*, *gatB*, *coxA*, *fbpA*, and *ftsZ* bacterial sequences from parasite DNA confirmed the presence of *Wolbachia*, and molecular phylogenetic analysis of the *Wolbachia ftsZ* gene groups the endosymbiont with *Wolbachia* from *D. immitis* and *D. repens*. Phylogenetic analysis of *D. ursi* 5s rDNA sequence confirms the morphological observations grouping this parasite as a member of *Dirofilaria*, and within the *Dirofilaria-Onchocerca* clade of filarial nematodes. This is the first report of *Wolbachia* characterization and molecular phylogeny information for *D. ursi*.

Filarial nematodes cause a variety of diseases of humans and other animals including onchocerciasis, lymphatic filariasis, and heartworm disease. The filarial worm life cycle involves maturation and sexual reproduction of diecious adults within a vertebrate host, followed by larval development within an arthropod. Transmission to the arthropod vector occurs during feeding, when the vector ingests the first-stage larva, or microfilaria (mf), from vertebrate blood, lymph, or tissue fluid. Subsequent development of the filarial worms to the third larval stage within the arthropod vector is necessary for transmission to the definitive host. Filarial nematodes are found naturally in a wide range of vertebrates including amphibians, reptiles, birds, and mammals and are vectored by acarines and insects (Bain and Babayan, 2003).

Dirofilaria Railliet and Henry, 1911 has at least 27 species, including *D. repens* Railliet and Henry, 1911 and *D. immitis* Railliet and Henry, 1911 of domestic and wild canids, *D. tenuis* Chandler, 1942 of raccoons, and *D. lutrae* Orihel, 1965 of otters and minks (Canestri Trotti et al., 1997), some of which have been implicated in zoonotic infection of humans (McCall et al., 2008). Most species of *Dirofilaria* develop to the third larval stage within the Malpighian tubules of their insect vector (Addison, 1980). *Dirofilaria ursi* Yamaguti, 1941 is vectored by black flies and is prevalent in American black bears (*Ursus americanus* Pallas, 1780) across North America, and Asiatic black bears (*Ursus thibetanus japonicus* G. Cuvier, 1823) in Japan (Yamaguti, 1941; Rogers, 1975; Addison and Pybus, 1978; Crum et al., 1978; Dies, 1979; Uni, 1983; Yokohata et al., 1990; Duffy et al., 1994). The parasite is ubiquitous and apparently comprises part of the primary helminth community of American black bears in northern regions of North America (Pence et al., 1983). Adults are found subcutaneously and in the connective tissues surrounding organs in the thoracic and abdominal cavities. Specimens from Japan and Ontario were studied by Anderson (1952), and found to be distinct compared to other *Dirofilaria* spp. Herein, we provide additional details on the morphological description of *D. ursi*, as well as phylogenetic data for this nematode and its *Wolbachia* endosymbiont that confirm placement of the worm in *Dirofilaria* (Wong and Brummer, 1978).

Received 12 June 2009; revised 13 September 2009; accepted 6 November 2009.
* Institut de Systématique, FR 1541, Muséum National d'Histoire Naturelle, 61 rue Buffon, F75231, Paris Cedex 05, France.
† Washington University School of Medicine, Infectious Disease Division, Campus Mailbox 8051, 660 S. Euclid Ave., St. Louis, Missouri 63110.
‡ Molecular Parasitology Division, New England Biolabs, Ipswich, Massachusetts 01938.
DOI: 10.1645/GE-2208.1

MATERIALS AND METHODS

Parasite collection and morphological identification

Sampling was conducted on hunter-killed bears processed at Wisconsin Department of Natural Resources check stations in Douglas, Bayfield, Ashland, Price, and Lincoln counties during the fall 2007 hunting season. Whole blood was aspirated from the body cavity of each bear into vacuum tubes containing EDTA to minimize clotting (Fisher Scientific, Pittsburgh, Pennsylvania). Blood smears were made in triplicate using 20 µl whole blood per slide, and air-dried, fixed in methanol for 5 min, then Giemsa stained for 1 hr (Harleco, Fisher Scientific, Waltham, Massachusetts). Whole blood was processed in this way because the degree of coagulation and field contamination of cavity-collected samples prevented centrifugation and filtration-based methods. Cover slips were mounted with Canada balsam and slides were microscopically examined for the presence of microfilariae (mf). Adult worms were field collected from the thoracic cavities of 2 bears in Bayfield County and stored in 70% ethanol for identification and DNA isolation. A representative pair of adults and a stained mf slide were submitted to the Museum of Natural History in Paris, France, for morphological study (MNHN no. 329JW). Adult worms were cleared in lactophenol, and transverse sections were made with a razor blade to observe the cuticular ornamentation, a diagnostic character of *Dirofilaria* spp.

Immunohistology

Midbody fragments of an adult female worm were fixed in 80% ethanol and embedded in paraffin using standard histological procedures. Adult *D. immitis* collected from naturally infected dogs were also embedded for comparison. Several different antibodies were used to screen *D. ursi* for the presence of *Wolbachia* endobacteria. First, polyclonal antibodies directed against the *Wolbachia* surface protein (WSP-1) of the endosymbiont of *D. immitis* (pab Di WSP,) or *Brugia pahangi* (pab Bp WSP) were used at dilutions of 1:500 to 1:1,000 (Kramer et al., 2003). Second, a monoclonal antibody raised against the *B. malayi wsp* (mab Bm WSP) was used at a dilution of 1:100 (Punkosdy et al., 2003). For comparison, a commercial monoclonal antibody directed against the human heat shock protein 60 (mab HSP 60 LK2, Sigma, St. Louis, Missouri) was used, which cross-reacts with the bacterial hsp-1 ortholog. This antibody was used at a dilution of 1:5.

For immunostaining, the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was applied according to the recommendations

Downloaded From: https://complete.bioone.org/journals/Journal-of-Parasitology on 06 Jun 2025
Terms of Use: https://complete.bioone.org/terms-of-use

of the manufacturer (DakoCytomation, Hamburg, Germany) as described previously (Buttner et al., 2003). Following the primary antibody, a mouse anti-rabbit immunoglobulin G (IgG) (1:25; DakoCytomation) was applied and this was followed by the application of a rabbit-anti mouse IgG (1:25; DakoCytomation) that binds the APAAP complex (1:50). As a substrate for the alkaline phosphatase, the chromogen Fast Red TR salt (Sigma) was used and hematoxylin (Merck, Darmstadt, Germany) served as the counter-stain. TBS with 1% albumin was used for a negative control instead of the primary antibody.

DNA isolation and sequence of filarial 5s rDNA intergenic region

Portions of a preserved adult female worm were excised using a razor blade and rinsed twice in 5-min changes of 1× phosphate-buffered saline, pH 7.2, at room temperature. Worm fragments were homogenized by vortexing with 2 zinc-coated BB shot pellets (Daisy, Rogers, Arkansas) in a 2-ml tube for 10 min at room temperature in tissue lysis solution (100 mM EDTA, 100 mM Tris, pH 7.5, 20 mM NaCl), and then were incubated at 65 C in the presence of 1% sodium dodecyl-sulfate, 2 µl 2-mercaptoethanol, and 2 mg/ml proteinase K for 1 hr, or until the samples were completely liquefied. Chromosomal DNA was purified by standard organic extraction and ethanol precipitation protocols (Sambrook and Russell, 2001). DNA was quantified using a NanoDrop Spectrophotometer (NanoDrop, Wilmington, Delaware) and integrity was verified by agarose gel electrophoresis (data not shown). Amplification of the 5s rDNA intergenic spacer region was performed as described (Xie et al., 1994), using *D. immitis* genomic DNA as a template for positive control, and substituting water for the template as a negative control. Amplicons from *D. ursi* and *D. immitis* templates were visualized by agarose gel electrophoresis (data not shown), cloned into the pCR4-TOPO vector, and transformed into OneShot Top 10 chemically competent cells following manufacturer's instructions (Invitrogen, Carlsbad, California). Recombinant plasmids were isolated from cultures of single-colony transformants using the QIAprep Spin Miniprep kit (Qiagen, Valencia, California), and DNA quantified as above. Plasmid inserts from 4 transformants for each species were sequenced from both sides using M13F and M13R primers at the University of Wisconsin Biotechnology Core DNA Sequence Laboratory (Madison, Wisconsin) using Big Dye Terminator chemistry (Applied Biosystems, Carlsbad, California). Forward and reverse sequences for each cloned fragment were aligned using ClustalW (www.ebi.ac.uk/clustalw) to verify sequence accuracy.

Sequencing of *Wolbachia* MLST and *wsp* loci

Genomic DNA was examined for the presence of *Wolbachia* by polymerase chain reaction (PCR). Amplification used standard primers developed for a multilocus sequence typing system (MLST) for *Wolbachia* (Baldo, Dunning Hotop et al., 2006) and contained M13 forward and reverse sequencing tags at the 5′ ends to serve as anchors for the degenerate primers during amplification (http://pubmlst.org/wolbachia/). PCR was performed essentially as described (Baldo et al., 2006a) by using Quick-Load Taq 2× Master Mix (New England Biolabs, Ipswich, Massachusetts), 1 µM of each primer, and 1 µl (~20 ng) DNA in 20-µl reactions. Additional degenerate primers, WSPintF and WSPintR (Bazzocchi et al., 2000), were used to amplify a fragment of the gene encoding *Wolbachia* surface protein (WSP-1). PCR was performed using Quick-Load Taq 2× Master Mix adjusted to 2.0 mM Mg^{2+}, 1 µM of each primer and 1 µl (~20 ng) of DNA in a 25-µl reaction. The thermal profile was 94 C for 4 min followed by 35 cycles at 94 C for 45 sec, 50 C for 45 sec, and 72 C for 1.5 min, followed by a final extension of 72 C for 10 min.

Products of the *Wolbachia* MLST primer pairs were verified by agarose gel electrophoresis, purified using a QIAquick PCR Purification Kit (Qiagen), then cloned. Plasmid DNA was isolated using the GenElute Plasmid Miniprep kit (Sigma) and inserts sequenced on both strands. The *ftsZ* amplicon was cloned into the *Psi*I site within the *Bam*HI restriction endonuclease gene of the positive selection vector, pPSV, and the construct was transformed into NEB 5-alpha $F'I^q$ chemically competent *Escherichia coli* (New England Biolabs). Inserts were sequenced with the M13R primer and a vector-specific primer: 5′ CAGATCGGAGAACATATAGACGTC. PCR products for all other MLST primer pairs were cloned into pCR2.1-TOPO and transformed into One Shot Top 10 competent cells (Invitrogen). Insert sequencing was with the M13F and M13R primers. For each gene fragment, a minimum of 6 independent clones were sequenced and an individual sequence matching the consensus sequence was analyzed further. The amplified *wsp* fragment was similarly purified, then sequenced bidirectionally, using the primers used for PCR. The sequences obtained were compared to sequences in NCBI databases using BLAST programs (Altschul et al., 1990). The *ftsZ* fragment was selected for multiple sequence alignment and phylogenetic analysis.

Sequence alignments and tree construction

Sequences were aligned using Muscle v3.6 (Edgar, 2004) with the '-noanchors' option (Supplements 1 and 2). Aligned sequences were trimmed at their termini to remove unaligned or ambiguously aligned regions using Jalview 9.5 (Waterhouse et al., 2009). The new *D. ursi* 5S rDNA (GenBank accessions GQ241942–GQ241945; and *Wolbachia ftsZ* (GenBank accession GQ217523) sequences were aligned with the following filarial 5S rDNA sequences and *Wolbachia ftsZ* sequences, respectively, as designated by NCBI gi and GenBank accession numbers. 5S rDNA: *Litomosoides sigmodontis* (975832, U31639.1), *Acanthocheilonema viteae* (975826, U31646.1), *Brugia timori* (975829, U31636.1), *Brugia malayi* (533165, L36060.1), *Wuchereria bancrofti* (975837, U31644.1), *Loa loa* (975831, U31638.1), *Mansonella perstans* (975833, U31640.1), *Onchocerca volvulus* (13661788, AF325539.1), *O. ochengi* (104345435, DQ523781.1), *O. cervicalis* (535354), *D. repens* (6006475, AJ242967.1), *D. immitis* (169641052, EU360965.1), and *Ascaris lumbricoides* (159683, M27961.1) (outgroup). *Wolbachia ftsZ*: *D. immitis* (44894817, AY523519.1), *D. repens* (4090332, AJ010273.1), *O. ochengi* (4090322, AJ010268.1), *O. volvulus* (9857237, AJ276501.1), *O. gutturosa* (4090318, AJ010266.1), *O. gibsoni* (4090320, AJ010267.1), *O. lupi* (23504732, AJ415416.1), *Brugia malayi* (113707539, DQ842341.1), *B. pahangi* (48476367, AY583315.1), *Wuchereria bancrofti* (70610294, DQ093835.1), *Litomosoides sigmodontis* (4090328, AJ010271.1), *Mansonella perstans* (60098023, AJ628414.1), *Kalotermes flavicollis* (18996128, AJ292345.2), *Falsomia candida* (19572717, AJ344216.1), *Drosophila melanogaster* (113707537, DQ842340.1), *Dr. simulans* (225591853, CP001391.1), *Tribolium confusum* (113707531, DQ842337.1), and *Armadilidium vulgare* (113707475, DQ842309.1). No outgroup was included in the *Wolbachia ftsZ* alignment and tree because recent data suggest that the standard outgroups from the Anaplasmataceae lead to erroneous reconstructions (Bordenstein et al., 2009). For the filarial sequence set, the total alignment length was 171 sites; of these, 60 were parsimony informative; for the *Wolbachia ftsZ* sequences, the total alignment length was 435 sites, with 115 sites being parsimony informative (data not shown). Trees were generated using Mega4 (Tamura et al., 2007) using the Minimum Evolution (ME) method (Rzhetsky and Nei, 1992) with the parameters listed below or by Maximum Parsimony (data not shown). Bootstrap confidence values, reported as percentages, were calculated based on 1,000 replicates (Felsenstein, 1985). Distances (base substitutions per site) were computed using the Kimura 2-parameter method (Kimura, 1980). The pairwise deletion option was used to eliminate positions containing alignment gaps and missing data. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 1.

RESULTS

Parasite prevalence and morphological analysis

Unsheathed mf were observed in the blood of 10 of 47 (21%) bears from 5 counties. Ten mf were examined morphologically (Fig. 1F–H) and measured (in µ) 198–242 long, 4–5.5 wide; head attenuated or not, depending on orientation; 1 or 2 first nuclei isolated in the cephalic space; nuclei tightly packed except in some regions; nerve ring, identified in all specimens; excretory pore and cell, identified in half of the specimens; anus identified once; R2–R4 nuclei identified in half of the specimens; R1 identified once. A very thin anucleated caudal filament 20–40 long was noted. With respect to the adult worm cuticle (Fig. 1A,B,E), females and males have the typical ornamentation marking of the subgenus *Nochtiella* Faust, 1937, made of successive longitudinal crests (Wong and Brummer, 1978). In the posterior ventral region of the

Downloaded From: https://complete.bioone.org/journals/Journal-of-Parasitology on 06 Jun 2025
Terms of Use: https://complete.bioone.org/terms-of-use

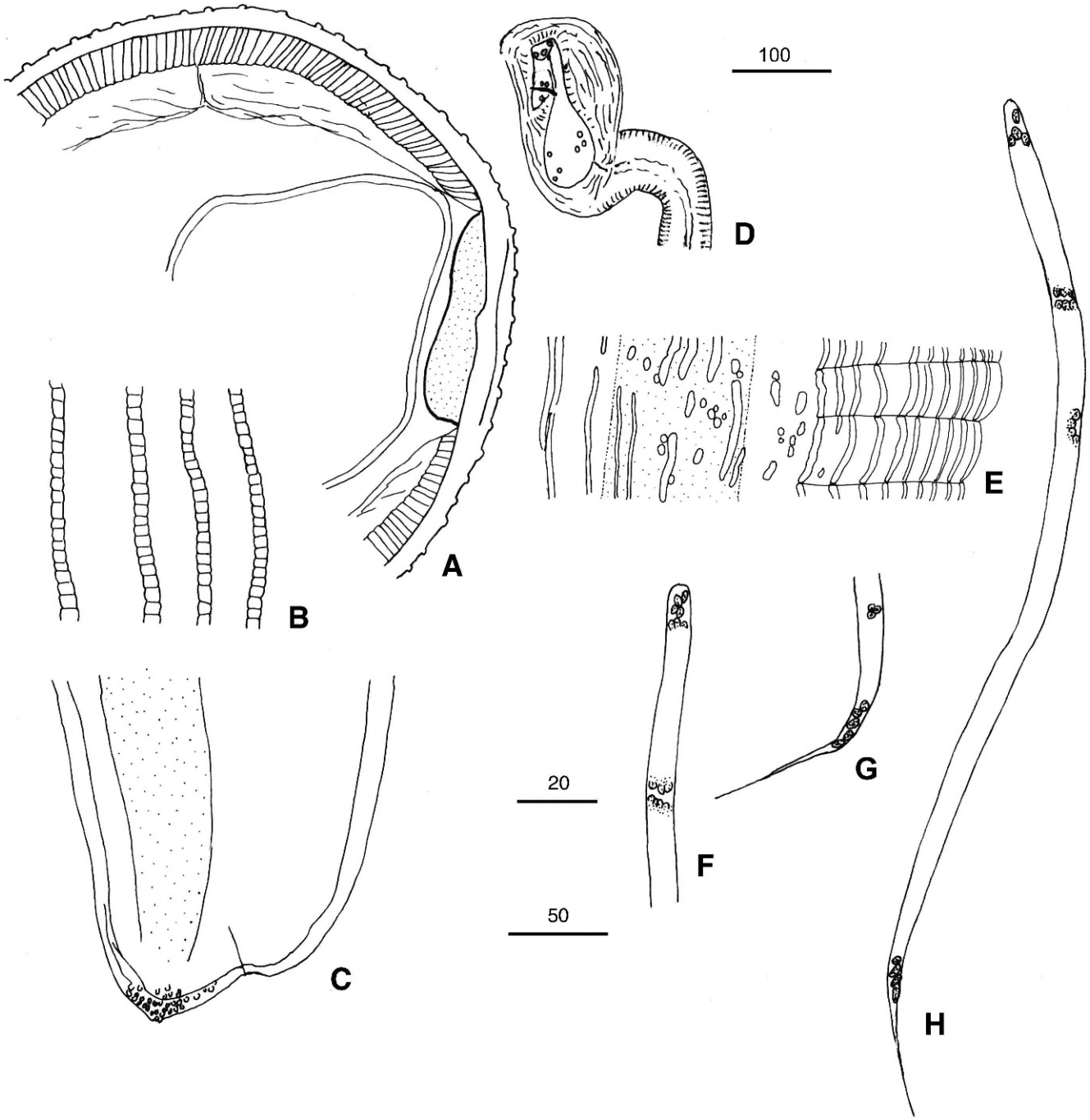

FIGURE 1. *Dirofilaria ursi* from *Ursus americanus*. (**A–D**) Female. (**A**) Transverse section at midbody, part of worm showing a lateral hypodermal chord, submedian muscles, and cuticular longitudinal crests. (**B**) Detail of 4 crests, longitudinal view. (**C**) Posterior region, tail with terminal rugosities, right lateral chord. (**D**) Vulva, ventral view, and vagina with 2 bends and chamber containing a few microfilariae in transverse sections. (**E**) Male, cuticular crests and area rugosa, right lateral view (pointed area is the lateral chord). (**F–H**) Blood microfilariae. (**F**) Anterior region, with nerve ring, orientation different from that of H. (**G**) Posterior region and anucleated filament; the R2, R3, and R4 are identified. (**H**) General morphology; nerve ring and excretory pore are identified. Scale bars in μ: A, C, D, 100; B, 30; E, 50; F, G, H, 20.

male, this ornamentation (Fig. 1E) has a particular aspect and forms the area rugosa (antislip apparatus for mating) (Bain and Chabaud, 1988). Other characters are those of *Dirofilaria* Railliet & Henry, 1910 (see Anderson and Bain, 1976), i.e., stout worms with a blunt anterior extremity, no buccal capsule; in females, tail short and caudal extremity with rugosities (Fig. 1C), vulva post-esophageal, relatively small vagina with 2 anterior bents and a posterior chamber (Fig. 1D); in males, caudal alae present, numerous bulky pedunculated precloacal papillae, spicules markedly dissimilar (data not shown). The female posterior region of this specimen is spirally coiled (4 coils), making the length measurement approximate. Measurements of female (in μ

Downloaded From: https://complete.bioone.org/journals/Journal-of-Parasitology on 06 Jun 2025
Terms of Use: https://complete.bioone.org/terms-of-use

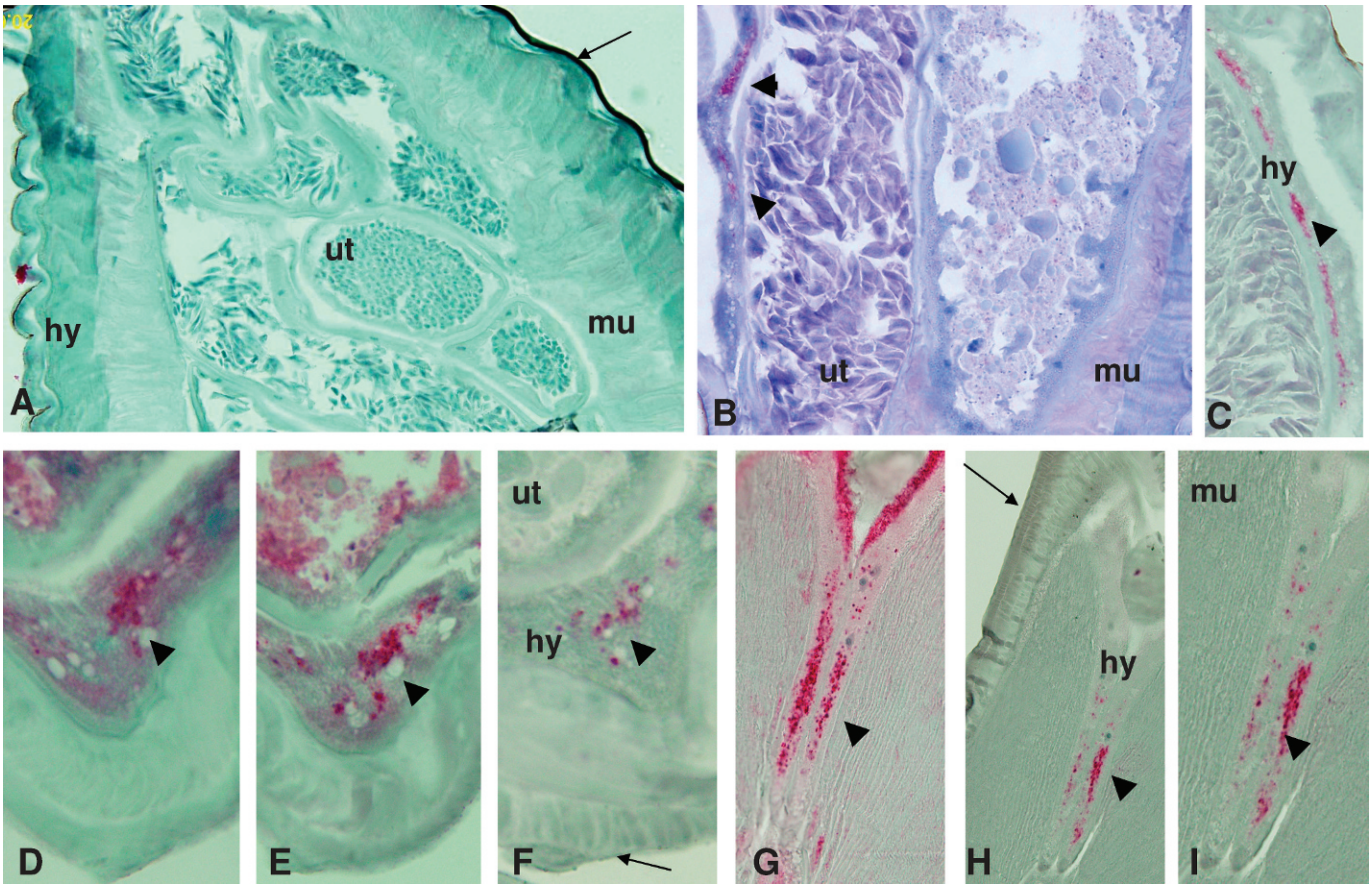

FIGURE 2. Immunohistological detection of *Wolbachia* endobacteria in the hypodermis of *Dirofilaria ursi* (**A–F**) from American black bear and in *Dirofilaria immitis* (**G–I**) for comparison. (**A**) Transversal section showing multiple uterus sections, hypodermis, and a thick musculature; negative control, no primary antibody. (**B**) Granular staining of *Wolbachia* in the hypodermis using polyclonal antibodies directed against the *Wolbachia* surface protein of the endosymbiont of *D. immitis* (pab Di WSP). (**C**) Strong labeling of *Wolbachia* in the hypodermis using a monoclonal antibody raised against the *Brugia malayi wsp* (mab Bm WSP). (**D–F**) Consecutive cross-sections showing *Wolbachia* in the hypoderms stained with different antibodies. (**D**) polyclonal antibodies directed against the *Wolbachia* surface protein of the endosymbiont of *Brugia pahangi* (pab Bp WSP); (**E**) pab Di WSP; (**F**) mab Bm WSP. (**G–I**) Consecutive longitudinal sections showing muscles and *Wolbachia* in the lateral chords using various antibodies. (**G**) mab HSP 60; (**H**) pab Di WSP; (**I**) mab Bm WSP. Arrow, cuticle; arrowhead, *Wolbachia*; hy, hypodermis; ut, uterus; mu, musculature.

unless otherwise stated): about 150 mm long (not precisely measurable because it is coiled); 600 wide; esophagus 1,225 long and relatively thin; tail 65 long; vulva 1,825 from head, vagina 170 long, 100 wide. Male: 50.65 mm long, 440 wide; esophagus 980; tail 75; left spicule 450 (handle 190); right spicule 165; area rugosa 2,000 long, extended from 3,400 to 1,300 from tail extremity.

Immunohistology

Polyclonal and monoclonal anti-wsp antibodies and a commercially available anti-hsp 60 antibody were used to examine *D. ursi* for *Wolbachia*. Histologically, *D. ursi* presented very similarly to *D. immitis*, with a broad hypodermis and thick musculature (Fig. 2A). Only unfertilized eggs and early embryos were observed in the uterine branches of the examined mid-body fragments of *D. ursi*. Numerous *Wolbachia* endobacteria were detected in the hypodermis and the lateral chords. No staining was observed in the uterus or the musculature (Fig. 2B–F). *Wolbachia* were labeled by all antibodies used, with differing levels of background labeling. The same was observed for *D. immitis* (Fig. 2G–I). The highest background was detected with the mab HSP 60 antibody (Fig. 2G), while the antibodies directed against *wsp* were more specific. Reactivity of all anti-*Wolbachia* antibodies tested indicated that *D. ursi* contains *Wolbachia* endobacteria that can be labeled by immunohistology with the same antibodies used to detect *Wolbachia* in *D. immitis*.

Sequence analysis and phylogenetic tree construction

Four 5s rDNA sequences amplified from *D. ursi* had 97–100% similarity and were distinguished by single nucleotide differences, mainly within the intergenic regions (data not shown), confirming previous observations of intergenic sequence variation within filarial species (Xie et al., 1994). Each sequence was most similar to orthologous sequences from *D. immitis* (E value $<6e^{-75}$) by sequence comparison using nucleotide level BLAST comparison (http://www.ncbi.nlm.nih.gov/). The experiment was controlled by amplification and sequencing of *D. immitis* 5s rDNA, which was confirmed to be identical to *D. immitis* 5s rDNA sequences present in GenBank (data not shown). We carried out a phylogenetic analysis using 1 of the *D. ursi* 5s rDNA sequences with orthologous sequences from other filarial genera, including *Dirofilaria*, *Onchocerca*, *Brugia*, *Wuchereria*, *Mansonella*, *Loa*, and *Litomosoides*, with the intestinal roundworm *Ascaris* as an

Downloaded From: https://complete.bioone.org/journals/Journal-of-Parasitology on 06 Jun 2025
Terms of Use: https://complete.bioone.org/terms-of-use

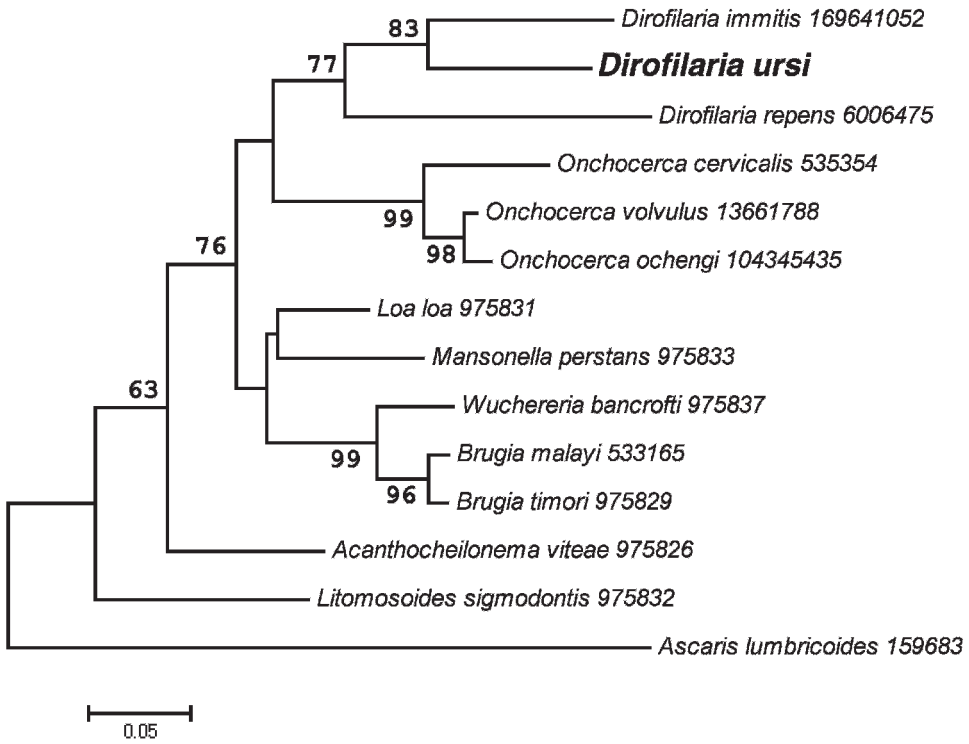

FIGURE 3. Minimum evolution tree based on an alignment of 5s rDNA intergenic sequences. Bootstrap confidence values (1,000 replicates) are shown as percentages. Values less than 50% are omitted. The units for the scale bar are substitutions per site. NCBI GI numbers are included after each species name. The *Dirofilaria ursi* sequence, with taxon name shown in bold font, was generated as part of this study. This analysis places *D. ursi* in the branching of *D. (D.) immitis*.

outgroup. The minimum evolution tree generated shows strong support for placement of the filarial worms collected from Wisconsin black bears into *Dirofilaria* (Fig. 3). A bootstrap consensus tree generated using maximum parsimony also grouped the *D. ursi* sequence with other *Dirofilaria* sequences (data not shown). As would be expected from their high sequence identity, the choice of *D. ursi* sequence did not alter the placement within the tree (data not shown). The *D. ursi* 5s rDNA sequences were deposited in GenBank, accessions GQ241942–GQ241945.

Gene fragments were successfully amplified using 4 of the 5 *Wolbachia* MLST primer pairs (*gatB*, *coxA*, *fbpA*, and *ftsZ*; GenBank accessions GQ217524, GQ217525, GQ217526, and GQ217523, respectively). No product was obtained with the standard *hcpA* primers or with alternative *hcpA* primers provided at the *Wolbachia* MLST website (http://pubmlst.org/wolbachia/). A fragment of the *wsp* gene was also amplified (GenBank accession GQ217527). In all cases, sequences matching the consensus of the individually sequenced clones gave greater BLAST similarity to *Wolbachia* sequences in GenBank than the variant sequences that deviated from the consensus. The *ftsZ* sequence from this filarial endosymbiont groups with *Wolbachia ftsZ* sequences from *D. immitis* and *D. repens*, with high bootstrap values in a minimum evolution tree (Fig. 4). Similar results were obtained using maximum parsimony (data not shown). With the exception of *ftsZ*, there are very few *Wolbachia* sequences from filarial nematodes in GenBank that correspond to the genes comprising the MLST set. This is particularly true for supergroup C, where sequences exist solely for *Wolbachia coxA* from *D. immitis* and *O. volvulus* and *Wolbachia fbpA* from *O. volvulus*. In contrast, the MLST primers have been used extensively on *Wolbachia* from arthropods (Baldo, Dunning Hotop et al., 2006). For this reason, the top BLAST hits for *gatB*, *coxA*, and *fbpA* were to supergroup C sequences where available, but otherwise to *Wolbachia* from *Brugia malayi* (supergroup D) and from arthropods. Nonetheless, the sequences that we obtained are clearly from *Wolbachia*. The *wsp* gene has been sequenced from *Wolbachia* that infect a large number of diverse arthropod and filarial nematode hosts (Bazzocchi et al., 2000; Jeyaprakash and Hoy, 2000; Baldo, Dunning Hotop et al., 2006; Baldo and Werren, 2007). The Wisconsin *D. ursi Wolbachia wsp* sequence had the greatest similarity to *wsp* orthologs from other supergroup C *Wolbachia* and clearly grouped with those from *D. immitis* and *D. repens* in neighbor joining phylogenetic trees generated as part of the BLAST result page (data not shown).

DISCUSSION

Dirofilaria ursi is a common filarial parasite of black bears in the United States upper Midwest and Canada. We calculated 21% prevalence of infection in our study region based on the presence or absence of microfilariae in body cavity blood from hunted bears, a figure that agrees closely with previous estimates from studies conducted in Wisconsin (Manville, 1978) and Quebec (Frechette and Rau, 1978). It is certainly possible that our study underestimated the true prevalence of *D. ursi* infection, because other studies with larger sample sizes have reported prevalences as high as 57–100% in the United States (Rogers, 1975; Frechette and Rau, 1977). It is also possible that seasonality affects

Downloaded From: https://complete.bioone.org/journals/Journal-of-Parasitology on 06 Jun 2025
Terms of Use: https://complete.bioone.org/terms-of-use

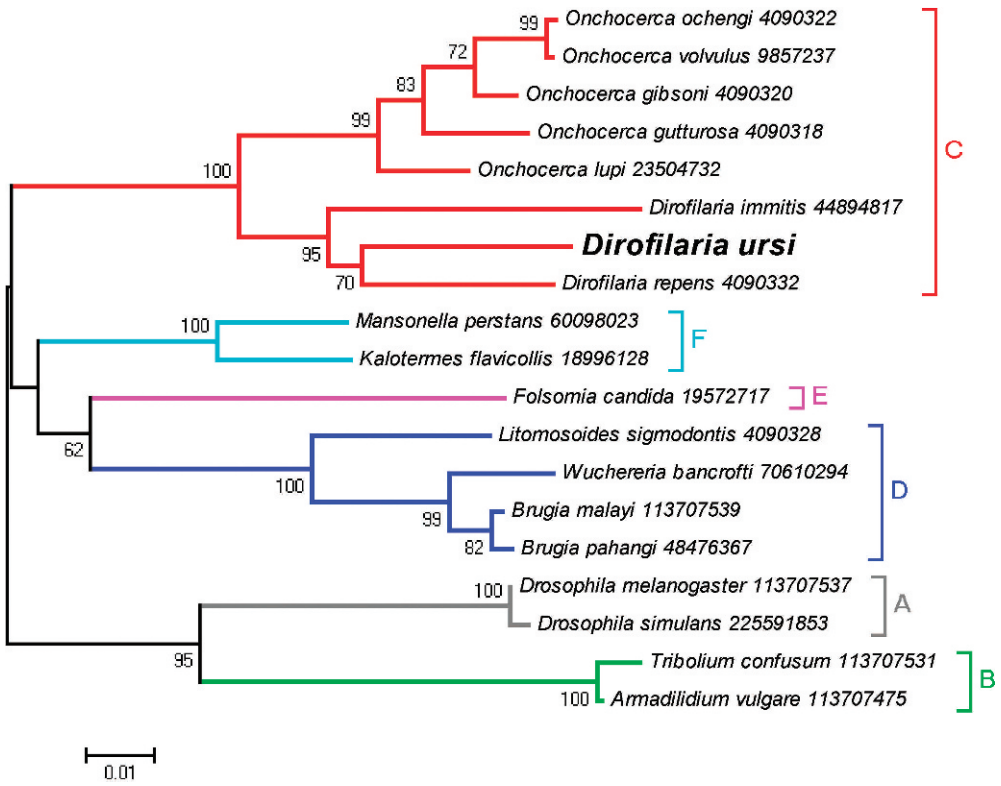

FIGURE 4. Minimum evolution tree based on an alignment of *Wolbachia ftsZ* nucleic acid sequences. Bootstrap confidence values (1,000 replicates) are shown as percentages. Values less than 50% are omitted. The units for the scale bar are substitutions per site. NCBI GI numbers are included after each species name. The *ftsZ* sequence from *Wolbachia* of *Dirofilaria ursi*, with taxon name shown in bold font, was generated as part of this study. Letters to the right of the bracketed branches denote the *Wolbachia* supergroup. Branches are color-coded to match the color of the supergroup letters. This analysis places *D. ursi* in the branching of *D. (N.) repens*.

microfilaremia, leading to bias in sampling. Bears undergo many physiological and metabolic adjustments in the fall that lead to metabolic depression and hypothermia in the winter denning period. Metabolic changes prior to denning impact the reproductive activity of intestinal helminths, resulting in increased cestode egg shedding and decreased ascarid egg shedding (Frechette and Rau, 1978), and may affect the reproductive activity of overwintering adult filarial worms by decreasing mf production during seasonal absence of the black fly vector. It is likely that adult *D. ursi* do overwinter within bears because the prepatent period of infection exceeds 6 mo (Addison, 1980), and mf have been recovered from bears sampled as early as May (Frechette and Rau, 1978).

Many filarial nematodes share a symbiotic relationship with alpha proteobacteria of the genus *Wolbachia*. Filarial *Wolbachia* are found intracellularly in the lateral hypodermal cords of adult worms and within oocytes of female worms, and are passed maternally to developing mf. Unlike the maternally inherited *Wolbachia* endosymbionts of insects, filarial *Wolbachia* are necessary for worm survival and are implicated in disease pathogenesis. Molecular phylogenetic analyses indicate that arthropod-derived *Wolbachia* are distinct from those found in filarial nematodes, and that those found in major filarial groups, for example, the *Brugia-Wuchereria* and *Dirofilaria-Onchocerca* groups, are distinct (Casiraghi et al., 2004; Taylor et al., 2005; Fenn et al., 2006). Morphological observations of sectioned worms revealed histology characteristic of the *Dirofilaria-Onchocerca* clade of filarial nematodes. We observed *Wolbachia* in the hypodermis of *D. ursi*, using immunohistology that employed a panel of different antibodies. Most informative were the antibodies directed against the *wsp*, because they showed high sensitivity and specificity. The distribution of the *Wolbachia* in the hypodermis and the lateral chords of *D. ursi* provides preliminary evidence that these bacteria play a similar role in development and reproduction, as was shown for its sister species *D. immitis* (Bandi et al., 1999; Bazzocchi et al., 2008). This is the first identification of *Wolbachia* in *D. ursi* and demonstrates by both immunohistochemistry and PCR/sequencing that this endosymbiont is indeed present in adult female worms. The examined worm sections contained only unfertilized eggs and early embryos, and did not have morula stages that usually harbor larger numbers of *Wolbachia* that are more easily detected by immunohistology.

This is also the first report to include sequences from *D. ursi* and its *Wolbachia* endosymbiont in molecular phylogenies of selected filarial and *Wolbachia* gene sequences. Our molecular phylogenetic analyses based on the *Wolbachia ftsZ* and filarial 5s rDNA loci were concordant and clearly indicate that *D. ursi* groups with *D. immitis* and *D. repens* into the *Dirofilaria* clade of filarial nematodes. Despite the availability of multiple *wsp* sequences from *Wolbachia* that are present in arthropods and filarial nematodes, we chose not to use *wsp* for tree construction because of its known high rate of recombination between *Wolbachia* strains infecting insects (Baldo, Bordenstein et al., 2006) that leads to unreliable phylogenies (Baldo and Werren,

Downloaded From: https://complete.bioone.org/journals/Journal-of-Parasitology on 06 Jun 2025
Terms of Use: https://complete.bioone.org/terms-of-use

2007). Instead, we used *ftsZ* because it also has been sequenced from diverse *Wolbachia* strains, including those in filarial worms, and is a component of the accepted MLST system (Casiraghi et al., 2005; Baldo, Dunning Hotop et al., 2006). This sequence classifies the *D. ursi* endosymbiont as group C *Wolbachia* characteristic of *Dirofilaria*, and the *Wolbachia*-bearing *Onchocera*, but not other Onchocercinae (Casiraghi et al., 2004). Sequence comparison of *D. ursi* 5s rDNA sequences to other nematode sequences in GenBank showed strong similarity to those reported for *D. immitis* and *D. repens* and, although there is disagreement in the literature as to the early branching of filarial parasite groups, the tree topology we generated is in overall agreement with previous studies of 5s rDNA and 12s rDNA sequences that define the major *Onchocerca-Dirofilaria*, *Brugia-Wuchereria*, and *Loa-Mansonella* clades (Xie et al., 1994; Casiraghi et al., 2004; Huang et al., 2009).

Our morphology and measurements of adults and mf, including the long, thin caudal filament, fit with those of *D. ursi* Yamaguti, 1941, redescribed by Anderson (1952) from *U. a. americanus* in Algonquin Park, Ontario, and expand on their previous descriptions with reference to male and female reproductive structures, i.e., area rugosa of adult males and vaginal morphology of adult females. With its distinctive longitudinal crests, the parasite we have redescribed (*D. ursi*) is clearly a *Dirofilaria* in the subgenus *Nochtiella*. It is quite interesting, however, that the groupings generated by molecular data slightly differed; *D. ursi* was aligned with *D. (D.) immitis* based on 5s rRNA comparison (Fig. 3) and with *D. (N.) repens* by *Wolbachia ftsZ* comparison (Fig. 4). A relatively recent scanning electron microscopy study (Uni and Takada, 1986) reported the presence of reduced longitudinal crests on the midbody of *D. immitis* males (not confused with the ventral area rugosa of *Dirofilaria* spp. and many filarial species) (Bain and Babayan, 2003), as well as on adult females. The presence of these anatomical features suggests that species of *Dirofilaria* and *Nochtiella* are not so strongly opposed. Adult worms of *D. (D.) immitis* are unique in that they live in blood vessels, contrary to other species, i.e., *D. ursi*, that live mainly in subcutaneous tissue; it appears that *Dirofilaria* species have a plesiomorphic character, i.e., "longitudinal cuticular crests present," that further develop during the adult stage, or do not, depending on the tissue parasitized.

ACKNOWLEDGMENTS

We are grateful to the Wisconsin Department of Natural Resources for cooperation in worm collection, Claudio Bandi (Milano, Italy) and Patrick Lammie (Atlanta, Georgia) for providing antibodies used for immunohistology, and Romas Vaisvila (NEB) for the gift of pPSV vector. Adult *D. immitis* were supplied by the Filariasis Research Reagent Repository Center, Athens, Georgia. This work was funded by NIH Grant 1R15AI067295-01A (Michalski), New England Biolabs, and a grant from the Barnes Jewish Hospital Foundation (Fischer).

LITERATURE CITED

Addison, E. M. 1980. Transmission of *Dirofilaria ursi* Yamaguti, 1941. (Nematoda:Onchocercidae) of black bear (*Ursus americanus*) by blackflies (Simuliidae). Canadian Journal of Zoology **58:** 1913–1922.

———, and M. J. Pybus. 1978. Helminth and arthropod parasites of black bear, *Ursus americanus*, in central Ontario. Canadian Journal of Zoology **56:** 2122–2126.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology **215:** 403–410.

Anderson, R. C. 1952. Description and relationships of *Dirofilaria ursi* Yamaguti, 1941, and a review of the genus *Dirofilaria* Railliet and Henry, 1911. Transactions of the Royal Canadian Institute **29:** 35–65.

———, and O. Bain. 1976. Keys to genera of the order Spirurida. Part 3. Diplotriaenoidea, Aproctoidea, and Filarioidea. *In* CIH keys to the nematode parasites of vertebrates, R. C. Anderson, A. G. Chabaud, and S. Willmott (eds.). CABI, Farnham Royal, U.K., p. 59–116.

Bain, O., and S. Babayan. 2003. Behavior of filariae: Morphological and anatomical signatures of their life style within the arthropod and vertebrate hosts. Filaria Journal **2:** 16.

———, and A. G. Chabaud. 1988. Un appareil favorisant l'accouplement des Filaires: Les renflements de la region anterieure du corps. Annales de Parasitologie Humaine & Comparee **63:** 376–379.

Baldo, L., S. Bordenstein, J. J. Wernegreen, and J. H. Werren. 2006. Widespread recombination throughout *Wolbachia* genomes. Molecular Biology and Evolution **23:** 437–449.

———, J. C. Dunning Hotop, K. A. Jolley, S. R. Bordenstein, S. A. Biber, R. R. Choudhury, C. Hayashi, M. C. Maiden, H. Tettelin, and J. H. Werren. 2006. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. Applied Environmental Microbiology **72:** 7098–7110.

———, and J. H. Werren. 2007. Revisiting *Wolbachia* supergroup typing based on WSP: spurious lineages and discordance with MLST. Current Microbiology **55:** 81–87.

Bandi, C., J. W. McCall, C. Genchi, S. Corona, L. Venco, and L. Sacchi. 1999. Effects of tetracycline on the filarial worms *Brugia pahangi* and *Dirofilaria immitis* and their bacterial endosymbionts *Wolbachia*. International Journal for Parasitology **29:** 357–364.

Bazzocchi, C., W. Jamnongluk, S. L. O'Neill, T. J. Anderson, C. Genchi, and C. Bandi. 2000. *wsp* Gene sequences from the *Wolbachia* of filarial nematodes. Current Microbiology **41:** 96–100.

———, M. Mortarino, G. Grandi, L. H. Kramer, C. Genchi, C. Bandi, M. Genchi, L. Sacchi, and J. W. McCall. 2008. Combined ivermectin and doxycycline treatment has microfilaricidal and adulticidal activity against *Dirofilaria immitis* in experimentally infected dogs. International Journal for Parasitology **38:** 1401–1410.

Bordenstein, S. R., C. Paraskevopoulos, J. C. Hotopp, P. Sapountzis, N. Lo, C. Bandi, H. Tettelin, J. H. Werren, and K. Bourtzis. 2009. Parasitism and mutualism in *Wolbachia*: What the phylogenomic trees can and cannot say. Molecular Biology and Evolution **26:** 231–241.

Buttner, D. W., S. Wanji, C. Bazzocchi, O. Bain, and P. Fischer. 2003. Obligatory symbiotic *Wolbachia* endobacteria are absent from *Loa loa*. Filaria Journal **2:** 10.

Canestri Trotti, G., S. Pampiglione, and F. Rivasi. 1997. The species of the genus *Dirofilaria*, Railliet & Henry, 1911. Parassitologia **39:** 369–374.

Casiraghi, M., O. Bain, R. Guerrero, C. Martin, V. Pocacqua, S. Gardner, A. Franceschi, and C. Bandi. 2004. Mapping the presence of *Wolbachia pipientis* on the phylogeny of filarial nematodes: Evidence for symbiont loss over evolution. Parasitology **34:** 191–203.

———, S. R. Bordenstein, L. Baldo, N. Lo, T. Beninati, J. J. Wernegreen, J. H. Werren, and C. Bandi. 2005. 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 **151:** 4015–4022.

Crum, J. M., V. F. Nettles, and W. R. Davidson. 1978. Studies on endoparasites of the black bear (*Ursus americanus*) in the southeastern United States. Journal of Wildlife Disease **14:** 178–186.

Dies, K. H. 1979. Helminths recovered from black bears in the Peace River region of Northwestern Alberta. Journal of Wildlife Disease **15:** 49–50.

Duffy, M. S., T. A. Greaves, and M. D. B. Burt. 1994. Helminths of the black bear, *Ursus americanus*, in New Brunswick. Journal of Parasitology **80:** 478–480.

Edgar, R. C. 2004. MUSCLE: A multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **5:** 113.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution **39:** 783–791.

Downloaded From: https://complete.bioone.org/journals/Journal-of-Parasitology on 06 Jun 2025
Terms of Use: https://complete.bioone.org/terms-of-use

FENN, K., C. CONLON, M. JONES, M. A. QUAIL, N. E. HOLROYD, J. PARKHILL, AND M. BLAXTER. 2006. Phylogenetic relationships of the *Wolbachia* of nematodes and arthropods. PLoS Pathogens **2:** e94.

FRECHETTE, J. L., AND M. E. RAU. 1977. Helminths of the black bear in Quebec. Journal of Wildlife Disease **13:** 432–434.

———, AND ———. 1978. Seasonal changes in the prevalence of ova of *Diphyllobothrium ursi* and *Baylisascaris transfuga* in the feces of the black bear (*Ursus americanus*). Journal of Wildlife Disease **14:** 342–345.

HUANG, H., T. WANG, G. YANG, Z. ZHANG, C. WANG, Z. YANG, L. LUO, L. LIU, J. LAN, AND X. HUANG. 2009. Molecular characterization and phylogenetic analysis of *Dirofilaria immitis* of China based on COI and 12S rDNA genes. Veterinary Parasitology **160:** 175–179.

JEYAPRAKASH, A., AND M. A. HOY. 2000. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. Insect Molecular Biology **9:** 393–405.

KIMURA, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution **16:** 111–120.

KRAMER, L. H., B. PASSERI, S. CORONA, L. SIMONCINI, AND M. CASIRAGHI. 2003. Immunohistochemical/immunogold detection and distribution of the endosymbiont *Wolbachia* of *Dirofilaria immitis* and *Brugia pahangi* using a polyclonal antiserum raised against WSP (*Wolbachia* surface protein). Parasitology Research **89:** 381–386.

MANVILLE, A. M. 1978. Ecto- and endoparasites of the black bear in northern Wisconsin. Journal of Wildlife Disease **14:** 97–100.

MCCALL, J. W., C. GENCHI, L. H. KRAMER, J. GUERRERO, AND L. VENCO. 2008. Heartworm disease in animals and humans. Advances in Parasitology **66:** 193–285.

NEI, M., AND S. KUMAR. 2000. Molecular evolution and phylogenetics. Oxford University Press, New York, New York, 348 p.

PENCE, D. B., J. M. CRUM, AND J. A. CONTI. 1983. Ecological analyses of helminth populations in the black bear, *Ursus americanus*, from North America. Journal of Parasitology **69:** 933–950.

PUNKOSDY, G. A., D. G. ADDISS, AND P. J. LAMMIE. 2003. Characterization of antibody responses to *Wolbachia* surface protein in humans with lymphatic filariasis. Infection and Immunity **71:** 5104–5114.

RZHETSKY, A., AND M. NEI. 1992. A simple method for estimating and testing minimum evolution trees. Molecular Biology and Evolution **9:** 945–967.

ROGERS, L. L. 1975. Parasites of black bears of the Lake Superior region. Journal of Wildlife Disease **11:** 189–192.

SAMBROOK, J., AND D. RUSSELL. 2001. Molecular cloning: A laboratory manual, 3rd ed. Cold Spring Harbor Press, Cold Spring Harbor, New York, 2,344 p.

TAMURA, K., J. DUDLEY, M. NEI, AND S. KUMAR. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution **24:** 1596–1599.

TAYLOR, M. J., C. BANDI, AND A. HOERAUF. 2005. *Wolbachia* bacterial endosymbionts of filarial nematodes. Advances in Parasitology **60:** 245–284.

UNI, S. 1983. Filarial parasites from the black bear of Japan. Annales de Parasitologie Humaine et Comparee **58:** 71–84.

———, AND S. TAKADA. 1986. The longitudinal cuticular markings of *Dirofilaria immitis* adult worm. Japanese Journal of Parasitology **35:** 191–199.

WATERHOUSE, A. M., J. B. PROCTER, D. M. MARTIN, M. CLAMP, AND G. J. BARTON. 2009. Jalview Version 2—A multiple sequence alignment editor and analysis workbench. Bioinformatics **25:** 1189–1191.

WONG, M. M., AND M. E. BRUMMER. 1978. Cuticular morphology of five species of *Dirofilaria*: A scanning electron microscope study. Journal of Parasitology **64:** 108–114.

XIE, H., O. BAIN, AND S. A. WILLIAMS. 1994. Molecular phylogenetic studies on filarial parasites based on 5s ribosomal spacer sequences. Parasite **1:** 141–151.

YAMAGUTI, S. 1941. Studies on the helminth fauna of Japan, Part 35. Japan Journal of Zoology **9:** 409–439.

YOKOHATA, Y., O. FUJITA, M. KAMIYA, T. FUJITA, K. KANEKO, AND M. OHBAYASHI. 1990. Parasites from the Asiatic black bear (*Ursus thibetanus*) on Kyushu Island, Japan. Journal of Wildlife Disease **26:** 137–138.

Downloaded From: https://complete.bioone.org/journals/Journal-of-Parasitology on 06 Jun 2025
Terms of Use: https://complete.bioone.org/terms-of-use