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ABSENCE OF *WOLBACHIA* ENDOBACTERIA IN *CHANDLERELLA QUISCALI*: AN AVIAN FILARIAL PARASITE

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Abstract

Chandlerella quiscali is a filarial nematode parasite of the common grackle (*Quiscalus quiscula*), a widespread bird species found throughout most of North America. Worms collected from wild-caught birds were morphologically identified as *C. quiscali* and tested for the presence of *Wolbachia*, an alphaproteobacterial endosymbiont required for reproduction and maturation by many filarial species. Although various methods, including PCR, in situ hybridization and immunohistology, were employed, we were unable to detect evidence of colonization with *Wolbachia*. Due to the widespread distribution of the grackle host, localization within the host, and high-infection rates, *C. quiscali* may be among the most easily obtainable of *Wolbachia*-free filarial species. Further studies of *C. quiscali* and other *Wolbachia*-free filarial species may help to shed light on the reason(s) that some filarial species require *Wolbachia* while others do not.

Filarial nematodes are a biologically diverse superfamily of parasitic worms that infect a wide array of vertebrates including amphibians, reptiles, birds, mammals, and humans. The causative agents of lymphatic filariasis (LF), *Wuchereria bancrofti* and *Brugia malayi*) and onchocerciasis (*Onchocerca volvulus*) infect approximately 150 million people in tropical and subtropical regions (Remme et al., 2006; 2009). New treatments are being sought to prevent and eliminate these infections, as they are important causes of long-term disability (2009).

Attention has recently shifted to *Wolbachia* endobacteria as a target for anti-filarial drugs. *Wolbachia* are common reproductive parasites of insects, but they behave as mutualists in many filarial nematode species (Werren et al., 2008). Some of the most important filarial pathogens such as *B. malayi, W. bancrofti, Dirofilaria immitis*, and *O. volvulus*, are dependent on *Wolbachia* endobacteria (order Rickettsiales, family Anaplasmataceae) for growth, fertility, and sometimes even survival (Bandi et al., 1999; Hoerauf et al., 1999; Casiraghi et al., 2002; Chirgwin et al., 2003). Other filarial species are naturally *Wolbachia*-free and thrive in the absence of a bacterial partner (Plenge-Bonig et al., 1995; Bandi et al.,

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1998; Chirgwin et al., 2002; Büttner et al., 2003; McGarry et al., 2003; Ferri et al., 2011). The biological mechanisms responsible for this disparity are poorly understood.

Phylogenetic studies may provide insight into the evolutionary history of *Wolbachia*-filaria relationships and improve understanding of the biological basis of *Wolbachia* dependence in some species. Early surveys focused mainly on medically and economically important and readily available parasites of humans and domestic animals. Most of these species are *Wolbachia*-dependent, but most also belong to just 2 of the 10 filarial subfamilies, the Onchocercinae and the Dirofilariinae (Bandi et al., 1998; Casiraghi et al., 2004). A recent study by Ferri et al. examined 35 species from 6 filarial subfamilies for *Wolbachia* infection, most of which were *Wolbachia*-free (Ferri et al., 2011). Their findings suggest that *Wolbachia*-dependent species may be in the minority in many subfamilies and that *Wolbachia* may be entirely absent in filarial parasites of lizards, frogs and birds.

Here we report results of studies to determine whether *Wolbachia* is present in *Chandlerella quiscali* (subfamily Splendidofilariinae), a parasite of the common grackle (*Quiscalus quiscula*) that was first described by Von Linstow in 1904 (Linstow, 1904). Grackles are widespread in North America east of the Rocky Mountains. This makes *C. quiscali* accessible throughout most of the United States and Canada. PCR studies, in situ hybridization and immunohistology were performed to detect evidence of colonization with *Wolbachia* endobacteria, but none was found. Our results offer *C. quiscali* as a readily available model in the study of *Wolbachia*-free filarial parasites.

MATERIALS AND METHODS

Parasite materials

Adult *C. quiscali* were dissected from the cerebral ventricles of euthanized *Q. quiscula* trapped in North Dakota, USA (Odetoyinbo, 1960). Trapping was conducted under USFW Permit MB072162, ND Game & Fish Permit GNF02344201, and University of North Dakota IACUC protocol 0705-3c. Adult *B. malayi* and *Acanthocheilonema viteae* were obtained from experimentally infected Mongolian gerbils as previously described (Ash et al., 1970; Lucius et al., 1995). Adult *O. volvulus*, adult *Onchocerca flexuosa* and *W. bancrofti* microfilariae (mf) were available from previous studies (Fischer et al., 1993; Weil et al., 2008; McNulty et al., 2010). Captive bred field crickets (*Gryllus bimaculatus*) were purchased from a pet shop in Hamburg, Germany.

For PCR studies, harvested worms were frozen in PBS or Trizol (Invitrogen, Carlsbad, California) at −80 C until use. For in situ hybridization and immunohistology, adult worms and crickets were fixed for 24–72 hr in 4% buffered formaldehyde, embedded in paraffin, and sectioned using standard histological procedures. For morphological examination, adult worms were killed with heated saline, preserved in 70% ethanol, and cleared in lactophenol. Worms were studied using an Olympus BX-51 compound light microscope equipped with DIC optics and a digital imaging system. Measurements were obtained using Rincon software (v. 7.1.2, Imaging Planet, Goleta, California).

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DNA isolation

DNA was isolated from *B. malayi, O. volvulus, O. flexuosa, A. viteae* and *C. quiscali* adult worms, *W. bancrofti* mf and *G. bimaculatus* formalin-fixed, paraffin embedded histological sections. The DNeasy Blood and Tissue Kit was used to isolate DNA from adult worms according to the manufacturer's suggested protocol (Qiagen). For *G. bimaculatus*, paraffin was removed from the tissue by 2, 30 minute incubations with 100% xylene, 2, 30 minute incubations with 100% ethanol, 2, 30 minute incubations with 75% ethanol, and 2, 15 minute incubations with 1× PBS. After removing the final PBS wash, tissue was re-suspended in 15 μl 1× PBS and subjected to alkaline lysis. The same alkaline lysis protocol was used to obtain DNA from *W. bancrofti* mf. Briefly, tissue was brought to a final volume of 15 μl in sterile water. Fifteen μl alkaline lysis buffer (400 mM KOH, 100 mM DTT and 10 mM EDTA) was added, and the mixture was heated to 95 C for 30 min before chilling on ice for 10 min. The solution was neutralized with 15 μl of neutralization buffer (400 mM HCl and 600 mM Tris-HCl pH 7.5). One μl of lysate or 10 ng purified DNA served as template in PCR reactions.

PCR reactions

Primers used to amplify the 5S ribosomal intergenic spacer were S2 5'-GTTAAGCAACGTTGGGCCTGG-3' and S16 5'-TTGACAGATCGGACGAGATG-3' (Fischer et al., 1998). Primers used to detect *Wolbachia* endobacteria are reported in Table I. *Wolbachia* primers are designed to match portions of the most highly conserved *Wolbachia* genes (Holman et al., 2009). The 5s intergenic spacer was cloned using the TOPO-TA cloning kit for sequencing (Invitrogen) and sequenced.

In situ hybridization

A 424bp segment of the 16S rDNA gene of the *Wolbachia* endosymbiont of *B. malayi* was amplified from *B. malayi* genomic DNA using the following primers: 16sF 5'-CAGCTCGTGTCGTGAGATGT-3' and 16sR '5-CCCAGTCATGATCCCACTT-3' (Fischer et al., 2011). Biotinylated antisense and sense (negative control) RNA probes were constructed from this sequence as previously described (Fischer et al., 2011).

For hybridization, 5μ histological sections were deparaffinized, digested in pepsin HCl for 7 min and hybridized overnight at 37 C in humid chambers with 1 μg of probe in hybridization buffer (50% formamide, 5× SSC, 0.3 mg/ml yeast tRNA, 100 μg/ml heparin, 1× Denhardt's solution, 0.1% CHAPS, and 5 mM EDTA). A stringency wash was performed at 60 C for 30 min, and detection was carried out using the 'In situ Hybridization Detection System' (Dako, Carpinteria, California). Sections were incubated with streptavidin-AP conjugate for 20 min at room temperature and with BCIP/NBT for 10–20 min to localize bound probe.

Immunohistochemistry

The alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was used for immunostaining according to the recommendations of the manufacturer (Dako) and as described previously (Rao et al., 2009; Fischer et al., 2011). A monoclonal antibody directed against *Wolbachia* surface protein (WSP) of *B. malayi* was used as the primary antibody at a dilution of 1:100. A monoclonal antibody directed against human heat shock protein 60

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(mAb HSP 60 LK2, Sigma, St. Louis, Missouri) was used as a positive control at a dilution of 1:5, as our prior studies have shown that it binds to *Wolbachia* and filarial mitochondria.

RESULTS

Based on morphological examination of adult and mf stages (Fig. 1), the grackle parasites that were used in this study belong to the genus *Chandlerella*. Details of adult morphology, such as the rounded shape of the anterior end (Fig. 1A,B), relative position of the nerve ring (Fig. 1A), position of the vulva relative to the end of the esophagus (Fig. 1A), presence of characteristic large cells at the junction between the esophagus and the intestine (Fig. 1E), and, most importantly, the configuration of the distal portion of the uterus (Fig. 1A,C), confirmed that the worms were *C. quiscali*. The distal portion of the uterus does not extend anteriorly beyond the vulva in *C. quiscali*. In contrast, the uterus extends anteriorly well beyond the vulva and then loops posteriorly to reach the vulva in closely related species,. Likewise, the length of spicules in males in our material (left spicule 75 μm, right spicule 82 μm) is identical to that of *C. quiscali* and different from the length of spicules in closely related species (Fig. 1D) Bartlett et al., 1980). Microfilariae in the blood were long (182–197 μm) and sheathed, with blunt, very slightly tapered tails (Fig. 1F,G), and this corresponds to the description of *C. quiscali* mf reported by Bartlett and Anderson (Bartlett et al., 1980). Localization of adult worms in the grackle brain also supports the identity of these parasites as *C. quiscali*.

DNA samples from worms taken from separate birds were tested by PCR with primer sets targeting *Wolbachia* 16s rRNA and other highly conserved *Wolbachia* genes, including the DNA-directed RNA polymerase (Rpo B/C), translation elongation factor GTPase (FusA), and succinate dehydrogenase (SucDyd) (Fig. 2). Primer sets previously used to demonstrate the absence of *Wolbachia* in *L. loa* were also tested (data not shown) (McGarry et al., 2003). All primer sets employed in this study amplified *Wolbachia* sequences from infected species (crickets, *B. malayi, W. bancrofti*, and *O. volvulus*) but not from *Wolbachia*-free filarial species (*O. flexuosa* and *A. viteae*) (Plenge-Bonig et al., 1995; Bandi et al., 1998) or *C. quiscali*. The filarial 5s rRNA intergenic spacer was amplified and sequenced as a positive control to ensure the quality of the *C. quiscali* genomic DNA. This sequence was deposited in Genbank under the accession number HM641830.

An in situ hybridization probe designed to bind highly conserved portions of *Wolbachia* 16s rRNA was also used to test for the presence of the endobacteria in *C. quiscali* (Fig. 3). This probe is able to detect a wide array of *Wolbachia* strains, including those in *B. malayi* (Figs. 3A, D, F) and in the cricket *G. bimaculatus* (Fig. 3B). Although dozens of sections were examined, no staining was detected in *C. quiscali* (Figs. 3C, E).

Likewise, a monoclonal antibody against a *Wolbachia* surface protein was used to stain the *Wolbachia* in *B. malayi* (Fig. 4A) and *G. bimaculatus* (Fig. 4C), but this antibody did not produce a signal in *C. quiscali* (Figs. 4D, E). A positive control antibody against hsp60 that labels filarial mitochondria did produce a signal in *C. quiscali* (Figs. 4B, F). This positive control indicates that the *C. quiscali* material was intact and suitable for antibody staining.

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DISCUSSION

The worms used in this study were identified as *C. quiscali* based on their morphologic features, host species, and localization within the host (Odetoyinbo, 1960; Anderson et al., 1976; Bartlett et al., 1980). These characteristics place species of the genus *Chandlerella* in the subfamily Splendidofilariinae (Anderson et al., 1976). *C. quiscali* is one of only two species of this subfamily to have been formally examined for *Wolbachia* infection (Ferri et al., 2011).

PCR with different primer sets, in situ hybridization, and immunohistology all indicated that *C. quiscali* does not contain *Wolbachia*. The genes targeted by PCR were among the most highly conserved among all *Wolbachia* strains, as indicated by the fact that they were able to amplify *Wolbachia* sequences present in a diverse array of host species including both insects and filarial nematodes. The in situ probe against *Wolbachia* 16s rRNA and the antibody against WSP also detected highly conserved targets in a wide array of filarial and insect *Wolbachia* strains. Similar methods have been used to confirm the *Wolbachia*-free nature of other filarial species, including *Loa loa* and *O. flexuosa* (Plenge-Bonig et al., 1995; Büttner et al., 2003; McGarry et al., 2003).

Our understanding of the co-evolutionary dynamics of *Wolbachia* in filarial parasites will benefit from wider sampling of parasite species representing different subfamilies of Onchocercidae. To-date, 54 species of Onchocercid species have been examined for *Wolbachia*, of which 26 (48%) harbor *Wolbachia* (McLaren et al., 1975; Taylor et al., 1999; Casiraghi et al., 2004; Ferri et al., 2011). Most (72%) of the species examined to-date belong to a single subfamily, Onchocercinae. Because there is good representation within this subfamily, some patterns can be discerned when comparing parasite genera. For example, 13 of 14 (93%) species tested within the genus *Onchocerca* harbor *Wolbachia*, whereas only 1 of 9 (11%) species tested within the genus *Cercopithifilaria* harbor *Wolbachia*. This difference in proportions is statistically significant difference (Fisher's exact test, p=0.002). The two subfamilies that contain most avian filarioid species (Splendidofilarinae and Lemdaninae) have not been extensively examined for *Wolbachia* symbionts. Avian filarioids (ca. 15 genera) may be among the most widely distributed of the filarioid parasites because of their hosts' unique ability to fly, yet only two avian species, an unidentified *Aproctella* species (Ferri et al., 2011) and *Chandlerella quiscali*, both Splendidofilarinae, have been studied. Both of these species are *Wolbachia* free. More species will need to be examined in order to determine if this is true of all avian filaria.

Future studies should not only include examination of a wider array of filarial species, but a more in-depth examination of species that have already been characterized as *Wolbachia*-free. Our previous studies uncovered evidence of horizontal gene transfer from *Wolbachia* endobacteria to two species that are currently *Wolbachia*-free (*O. flexuosa* and *A. viteae*,). This indicates that their ancestors must have been infected with *Wolbachia* (McNulty et al., 2010). Genomic surveys of other *Wolbachia*-free species like *C. quiscali* will help to determine whether *Wolbachia* was present in their lineage in the past.

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Now that *C. quiscali* has been experimentally shown to lack *Wolbachia*-endobacteria, it can be used as a model for studies of *Wolbachia*-free filarial parasites. *C. quiscali* has certain attributes that may make it an attractive model system for such studies. First, its host, the common grackle, is abundant and synanthropic, it is easy to trap, and it does well in captivity. Local infection rates, particularly in forested regions where vector [ornithophilic *Culicoides*] populations are high) can approach 100% (Welker, 1962; Granath, 1980; Johnson, 1984). Moreover, unlike many avian filarids whose adult stages are small and often difficult to locate within their hosts, *C. quiscali* adults are easily found within the ventricles of the brain. In depth studies of readily available *Wolbachia*-free species like *C. quiscali* may help to determine why some filarial nematodes require *Wolbachia* for fertility and maturation while others do not.

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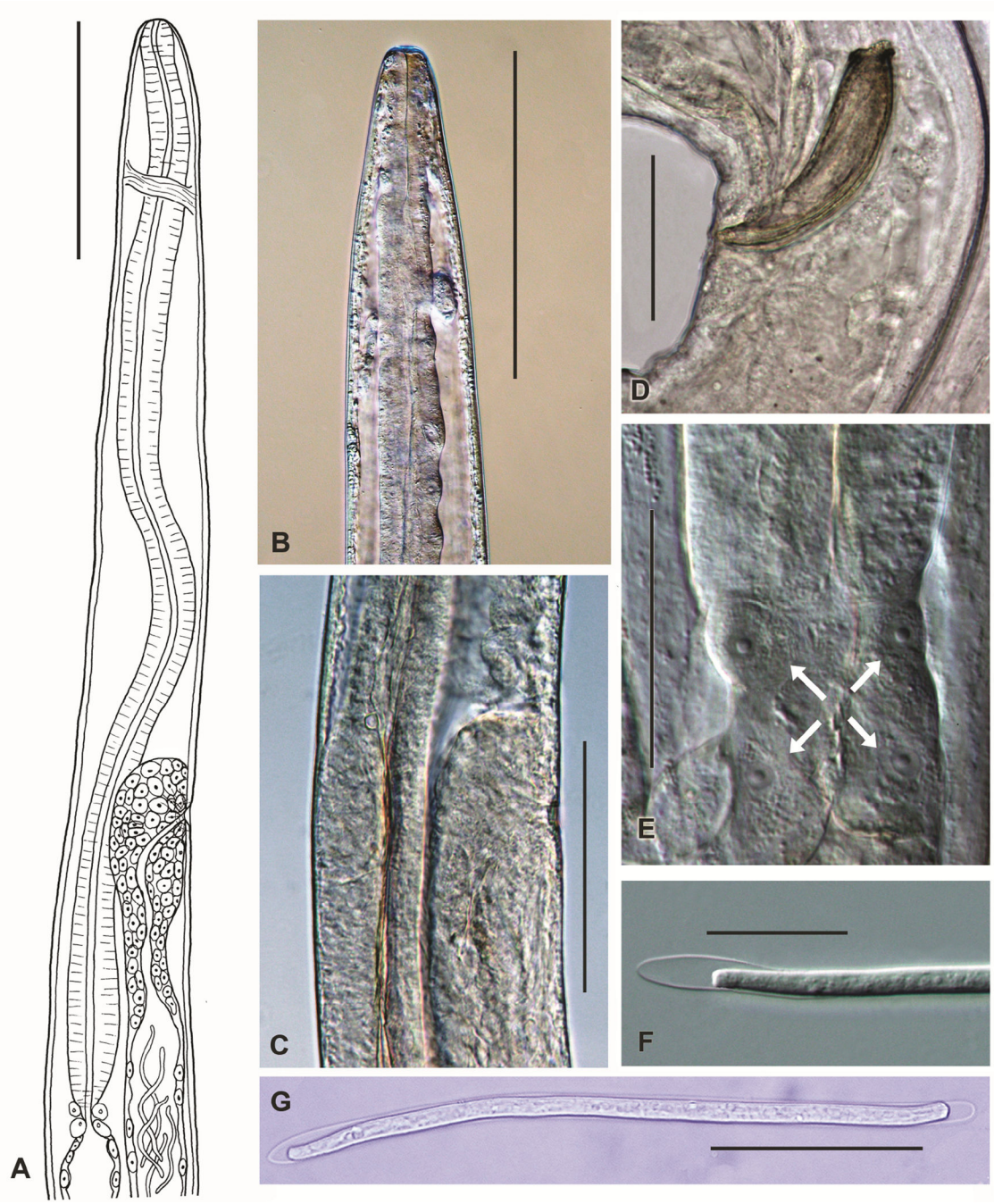

FIGURE 1.

Morphological characteristics of *C. quiscali*. Figure depicts a line drawing of the anterior end of the female demonstrating the relative position of the vulva, esophagus and nerve ring (A), the anterior end of *C. quiscali* (B), the region of the vulva (C) (note that the uterus does not make a loop anterior to the vulva), the posterior end of male with spicules (D), the of junction between esophagus and intestine (E) with characteristic large cells indicated with arrows, and sheathed microfilaria of *C. quiscali* from blood of common grackle (F,G). Scale-bars: A, B = 250 μm, C = 150 μm, D, E, G = 50 μm, F = 25 μm.

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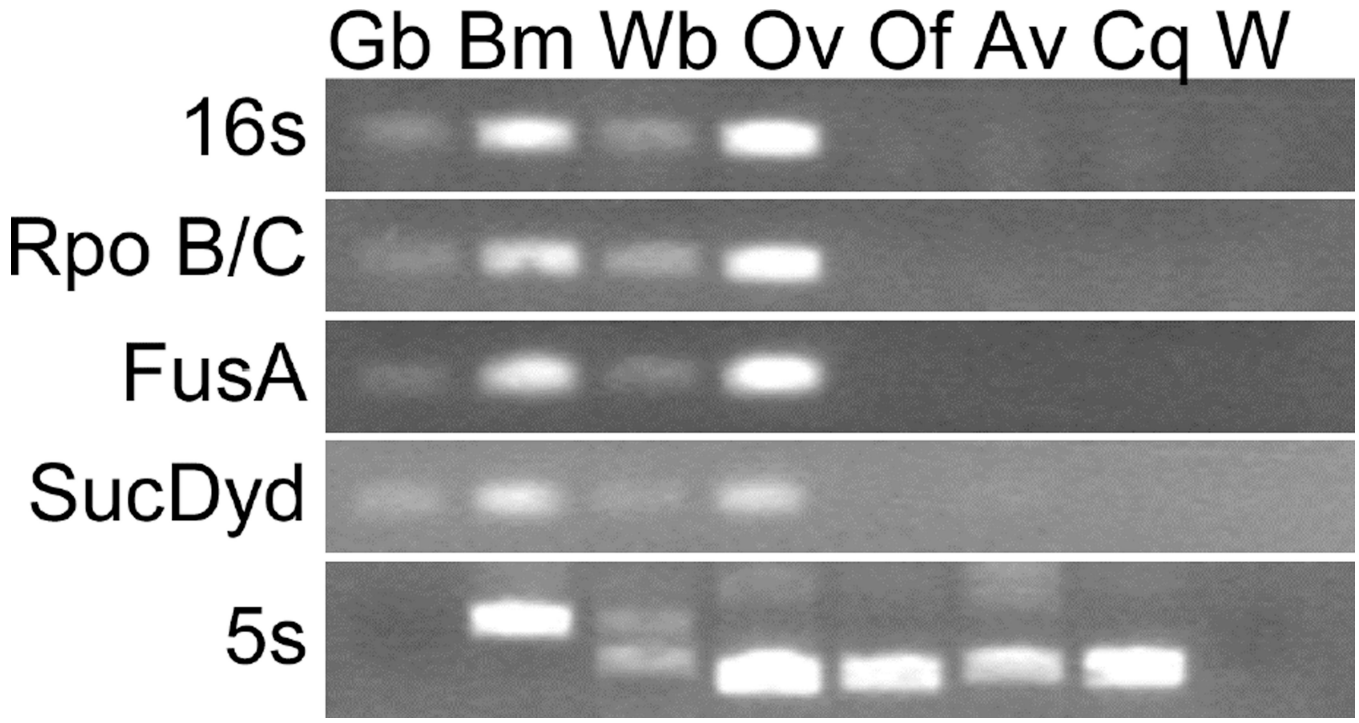

FIGURE 2.

Results of a PCR-based search for evidence of colonization with *Wolbachia* endobacteria in various species. Template species are designated as follows: Gb, field cricket *Gryllus bimaculatus*; Bm, *B. malayi*; Wb, *W. bancrofti*; Ov, *O. volvulus*; Of, *O. flexuosa*; Av, *A. viteae*; Cq, *C. quiscali*; W, water only (a no template control).

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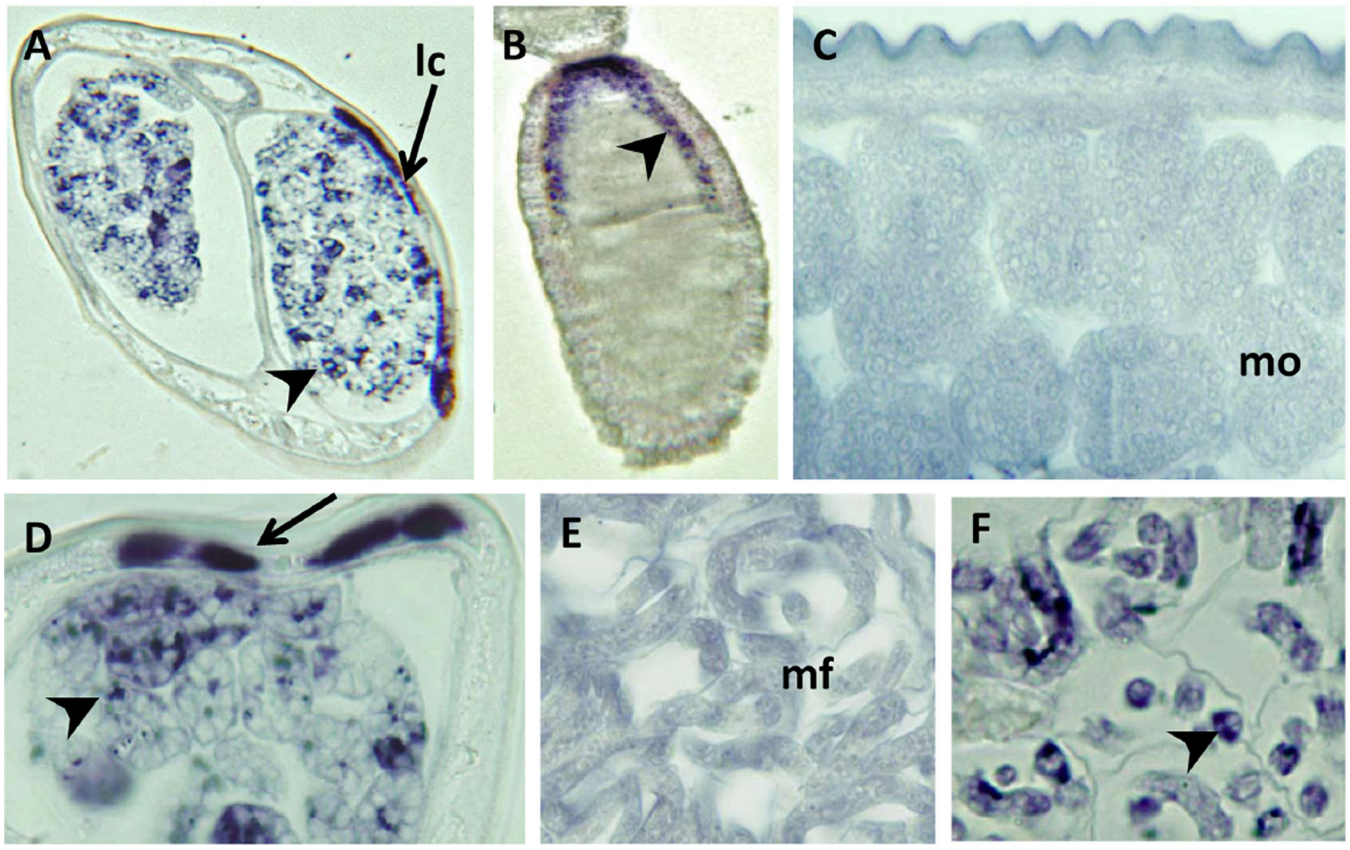

FIGURE 3.

In situ hybridization using a probe for the 16s rRNA of *Wolbachia* endobacteria. The 16s probe bound to *Wolbachia* in the lateral chords (A,D), early (A) and late (D) morulae, and microfilariae (F) of *B. malayi*. It also bound *Wolbachia* in the developing eggs of the cricket *G. bimaculatus* (B). No staining was detected in the late morulae (C) or microfilaria (E) of *C. quiscali*. Scale bars are all 25 μm. Species are designated within the figure as follows: Bm, *B. malayi*; Gb, *G. bimaculatus*; Cq, *C. quiscali*.

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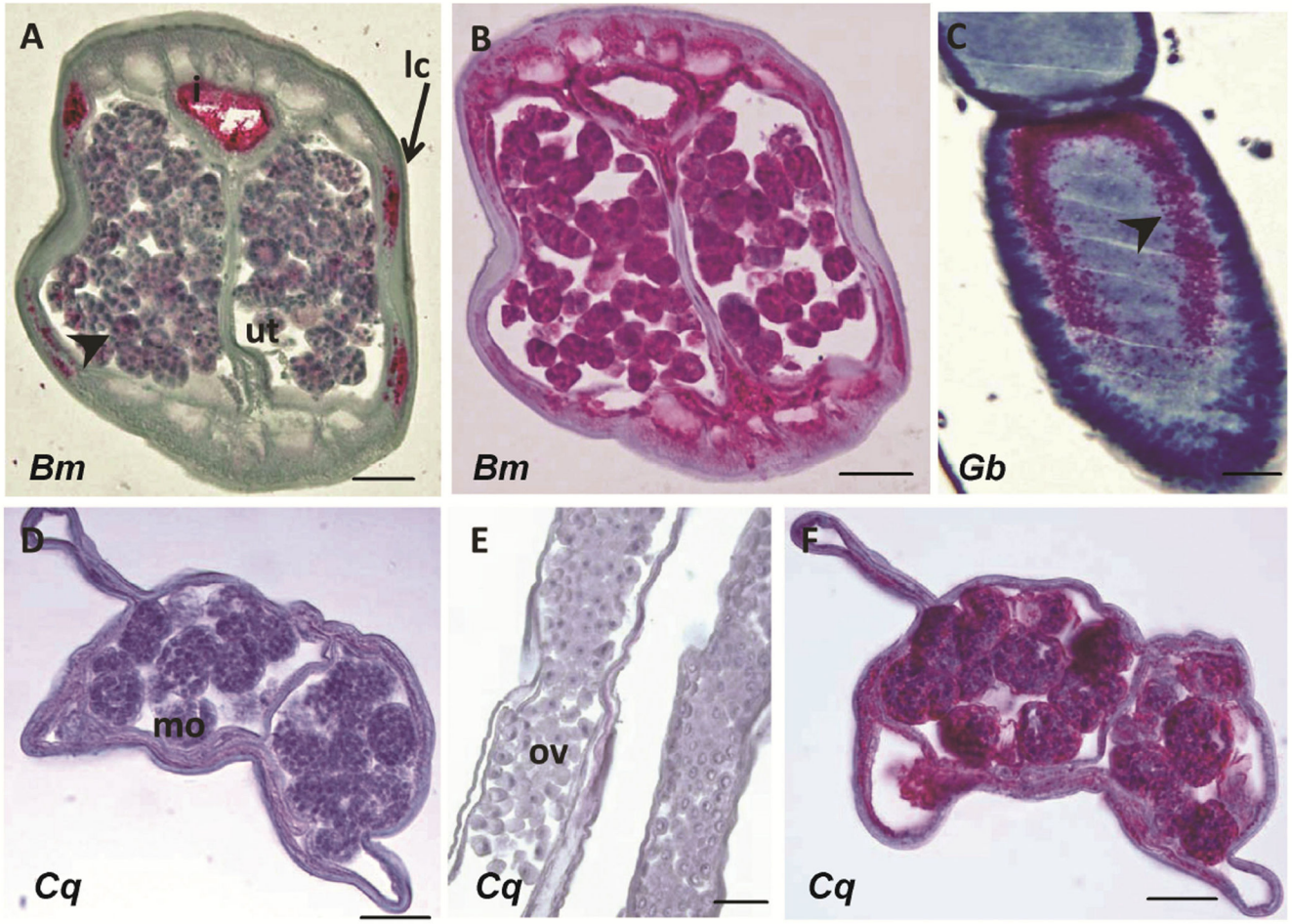

FIGURE 4.

Immunohistological detection of *Wolbachia* surface protein (WSP). WSP antibody labeled *Wolbachia* in the lateral chords (lc) and morula stage embryos in the uterus (ut) of *B. malayi* (A) and in developing eggs of *G. bimaculatus* (C). No staining was detected in morula stage (mo) embryos (D) or the ovaries (ov) (E) of *C. quiscali.* A positive control Hsp60 antibody labeled mitochondria in both *B. malayi* (B) and *C. quiscali* (F). Scale bars are all 25 μm. Species are designated within the figure as follows: Bm, *B. malayi*; Gb, *G. bimaculatus*; Cq, *C. quiscali*.

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Table I

PCR primer sequences used to detect *Wolbachia* endobacteria (5'-3')

16s Universal	CCAGTGGCGAAGGCGTCTAT	CCCCGTCAATTCCTTTGAGTTT
RpoB/C	TTCTGGCTCTGGTGCTGTAG	AACCTTGCCAAGACATAAAAGC
FusA	GATGGTGCAGCTTCTATGGAT	GCAACTCCATCAAATACAGCAA
SucDyd	CAGGTGGATATGGACGTGTT	CCATATATTCCTGTTGGATGAAA