Journal of Nematology 35(3):266–270. 2003.

Absence of *Wolbachia* in Nonfilariid Nematodes

Seth R. Bordenstein,[1] David H. A. Fitch,[2] and John H. Werren[3]

Abstract: Intracellular bacteria of the genus *Wolbachia* are among the most abundant endosymbionts on the planet, occurring in at least two major phyla—the Arthropoda and Nematoda. Current surveys of *Wolbachia* distribution have found contrasting patterns within these groups. Whereas *Wolbachia* are widespread and occur in all three major subphyla of arthropods, with estimates placing them in at least several million arthropod species, the presence of nematodes carrying *Wolbachia* is currently confined to the filariids, in which they occur at appreciable frequencies. It has been hypothesized that *Wolbachia* entered the ancestor of modern-day filariids in a single acquisition event, and subsequently cospeciated with their filariid hosts. To further investigate this hypothesis, we examined the broader distribution of *Wolbachia* in nematodes using a polymerase chain reaction (PCR) assay in a diverse set of nonfilariid species. The assay consisted of three different types of PCR screens on adults of 20 secernentean nematode species (14 rhabditids, 2 strongylid parasites of vertebrates; 1 diplogasterid; 3 cephalobid relatives, 1 myolaim, and 1 filariid) and two adenophorean species (plectids). Two PCR screens were specific to the 16S rDNA and *ftsZ* protein coding gene of *Wolbachia;* and the third screen was specific to the 18S rDNA of the nematodes. Based upon our results, we conclude that *Wolbachia* are absent in all 21 non-filariid species encompassing all the major groups of the Secernentea and two species of Adenophorea, from which the Secernentea derived. The absence of *Wolbachia* in these non-filariids is consistent with the hypothesis that *Wolbachia* entered the nematode phylum once, in an ancestral lineage of extant filariids.

Key words: endosymbiont, filaria, nematode, rhabditid, *Wolbachia*.

Wolbachia are a widespread and abundant group of alpha-proteobacteria closely related to the human pathogens *Ehrlichia* and *Rickettsia* and more distantly related to mitochondria. While *Wolbachia* are not known to occur in humans, they have an unparalleled host range in the endosymbiont world, occurring in the arthropod and nematode phyla at appreciable frequencies. *Wolbachia* infect a minimum of 20% of insect species, with estimates ranging up to 75% (Jeyaprakash and Hoy, 2000; Werren et al., 1995), and are nearly fixed throughout filariid nematodes (Bandi et al., 2001). Extrapolation of these percentages to the total number of insect and filariid species makes *Wolbachia* one of the most abundant endosymbionts in the world.

The *Wolbachia* in nematodes and arthropods form a monophyletic group comprised of six clades (A,B,C, D,E,F) that diverged 60 to 100 Mya (Bandi et al., 1998; Lo et al., 2002). Since then, these *Wolbachia* subgroups have traveled different evolutionary pathways, ultimately forming the basis of extraordinary phenotypic plasticity. Nematode *Wolbachia* (C and D) are strictly vertically transmitted and required for host embryogenesis (Bandi et al., 2001) and larval molting (Casiraghi et al., 2001). Arthropod *Wolbachia* (A and B) experience some lateral (interspecies) transmission, typically manipulate reproduction in their hosts, and are not required for host survival and reproduction (Werren, 1997). Some of the interesting reproductive alterations induced by *Wolbachia* in arthropods include male killing in various insects (Hurst and Jiggins, 2000), feminization of genetic males in isopods and moths (Fujii et al., 2001; Rigaud 1997), induction of parthenogenesis in wasps (Stouthamer et al., 1993), and a sperm-egg incompatibility in various arthropods (Hoffmann and Turelli, 1997). These parasitic phenotypes all impart a selective advantage to infected females (e.g., the transmitting host) and therefore enhance the spread of this maternally inherited bacterium through host populations. They also may profoundly affect the evolution of their hosts and may have important implications for the evolution of sex determination (Rigaud, 1997), speciation (Bordenstein, 2003; Bordenstein et al., 2001; Werren, 1998), and eusociality (Stouthamer et al., 1999; Wenseleers et al., 1998). Their presence in filarial nematodes also has made them a target for antibiotic therapy of human filarial diseases (Taylor and Hoerauf, 2001). Evidence suggests that inflammatory responses of vertebrate immune systems to *Wolbachia* antigens may be a major factor in the debilitating aspects of filarial disease (Cross et al., 2001; Saint Andre et al., 2002).

In contrast to their widespread distribution in arthropods, *Wolbachia* in nematodes have been reported to occur only in filariid nematodes (Bandi et al., 2001). Surveys based on polymerase chain reaction (PCR) or other detection methods (e.g., electron microscopy or immunohistology) place *Wolbachia* in 17 of 19 filariid species (Bandi et al., 2001; Chirgwin et al., 2002; Taylor and Hoerauf, 1999), with the infection nearly fixed within infected species. The evolutionary lineages leading to the two filariid species definitively lacking *Wolbachia—Acanthocheilonema viteae* and *Setaria equina*—are presumed to have diverged earlier than lineages leading to the rest of the infected species, which constitute

Received for publication 3 June 2002.

[1] The Josephine Bay Paul Center for Comparative Molecular Biology and Evolution, The Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543.

[2] Department of Biology, New York University, Room 1009 Main Building, 100 Washington Square East, New York, NY 10003.

[3] Department of Biology, The University of Rochester, Rochester, NY 14627.

For nematode strains, the authors thank the Caenorhabditis Genetics Center, which is supported by a grant from the NIH National Center for Research Support, James Baldwin, Manuel Mundo-Ocampo, and Paul and Irma De Ley (University of California, Riverside), and Steven Nadler (University of California, Davis). For comments on the paper, the authors thank Patrick Degnan, Brad Hyman, Karin Kiontke, Adam Lazarus, and two anonymous reviewers. This work was supported by a National Science Foundation grant to John Werren and a National Institutes of Health grant to Jennifer Wernegreen and in part performed while Seth Bordenstein held a National Research Council Research Associateship Award at The Marine Biological Laboratory.

E-mail: sbordenstein@mbl.edu

This paper was edited by Bradley Hyman.

the major group of filarial disease agents of humans. This pattern of infection distribution, along with the strict cospeciation observed between *Wolbachia* and their filariid hosts (Casiraghi et al., 2001), suggests that the establishment of the endosymbiosis preceded the evolutionary diversification leading to these major human filarial disease agents. However, more sampling within the filariids and other nematode groups is needed, especially outside of the filariids, in other secernenteans derived from the lineage that split from the filariids, or adenophoreans from which the secernenteans arose (Fig. 1). Similarly, more sampling of endosymbionts other than *Wolbachia* in non-filariids (e.g., the *Xiphinema* spp. endosymbionts in Dorylaimida and Longidoridae; Vandekerckhove et al., 2002) will also be important to understanding the distribution of endosymbiotic bacteria in the Nematoda.

To make an introductory assessment of the broader distribution of *Wolbachia* throughout other nematode groups and to determine whether *Wolbachia* occur in the model genetic system of *Caenorhabditis elegans* and its relatives, we screened for the presence of *Wolbachia* in all the major groups of the secernentean nematodes and two andenophorean outgroup representatives (plectids) using polymerase chain reaction (PCR) amplification of bacterial gene sequences. We focused part of the sampling in rhabditids because of the tantalizing potential to apply the genetic tools from *C. elegans* to genetic investigations of the *Wolbachia* endosymbiois in nematodes. We found that *Wolbachia* are absent in all 21 non-filariid species.

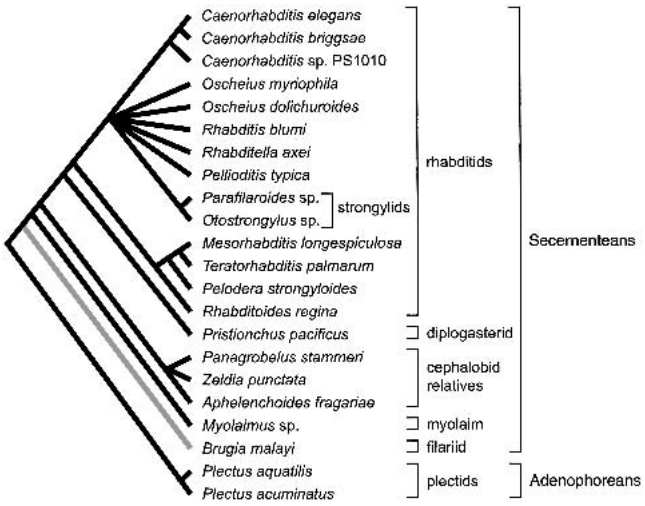

FIG. 1. Phylogenetic relationships of the Secernentean nematode taxa used in this study. Relationships depicted are based on previous analyses of 18S ribosomal RNA sequences (Blaxter et al., 1998; Sudhaus and Fitch, 2001). Informal names are applied to taxonomic groups because of the lability of current nematode taxonomy (De Ley and Blaxter, 2002). Polytomies represent uncertainty in the branching order (e.g., less than 68% bootstrap support), not simultaneous branching. Black lines represent lineages without *Wolbachia* endosymbiosis; the gray line represents the lineage to filariids (represented here by *Brugia malayi*) in which *Wolbachia* endosymbiosis was acquired.

MATERIALS AND METHODS

Nematode strains: Assays for the presence of *Wolbachia* were carried out on adults of the following rhabditid strains: *Rhabditella axei* (strain DF5006), *Rhabditis blumi* (strain DF5010), *Oscheius myriophila* (strain DF5020), *Rhabditoides regina* (strain DF5012), *Mesorhabditis longespiculosa* (strain DF5017), *Oscheius dolichuroides* (strain DF5018), *Teratorhabditis palmarum* (strain DF5019), *Pelodera strongyloides dermatitica* (strain DF5022), *Pellioditis typica* (strain DF5025), *Pristionchus pacificus* strain (PS312), *Caenorhabditis elegans* (strain N2), *Caenorhabditis briggsae* (strain AF16), and *Caenorhabditis sp.* (strain PS1010). All of these strains were obtained in culture from the Caenorhabditis Genetics Centr (CGC). Dates and locations of collection are provided by the CGC. Additional species included *Plectus aquatilis* (PDL18), *Plectus acuminatus* (strain JB126), *Panagrobelus stammeri* (strain PDL24), and *Zeldia punctata* (strain PDL3), kindly provided by Paul and Irma De Ley (University of California, Riverside); *Aphelenchoides figariae* (strain JB012), kindly provided by James Baldwin and Manuel Mundo-Ocampo (University of California, Riverside); and *Myolaimus* sp. (strain T-23), *Parafilaroides* sp. (sample 7-2756), and *Otostrongylus* sp. (sample 7-3129), kindly provided by Steve Nadler (University of California, Davis). One filarial nematode species, *Brugia malayi,* was also used in the *Wolbachia* screen as a positive control. Female larvae of this species were obtained frozen from TRS Laboratories (Athens, GA).

Insect strains: Infected (LBII) and uninfected (AsymC) single *Nasonia vitripennis* females were used as positive and negative PCR controls, respectively. LBII is doubly infected with A and B *Wolbachia;* AsymC was cured from LBII by heat shock in 1989. *Nasonia vitripennis* is a species of parasitoid wasp that has been used extensively for studies of *Wolbachia* (Bordenstein et al., 2001).

DNA extraction and amplification: From 0.2 ml of pelleted worms washed in double-distilled water three times, genomic DNA was extracted in 50 μl of a solution containing 5% Chelex resin (Bio-Rad) and 0.01% (w/v) Proteinase K in sterile, deionized water (Sigma), in a 0.5-ml microcentrifuge tube. After adding the solution, the microcentrifuge tube was placed in a heat block for 2 minutes at 90 °C. The nematodes were then ground with a pipet tip, and the extraction mix was vortexed for 10 seconds. The microcentrifuge tube was then placed in a water bath for 1 hour at 60 °C. After vortexing for 10 seconds, the microcentrifuge tube was placed again in a heat block for 20 minutes at 95 °C. The extract was finally spun at 14,000 rpm for 3 minutes and stored in a −20 °C freezer until used for PCR. DNA from *Nasonia vitripennis* females was extracted similarly, with the exception that single females were used.

Bacterial and host DNA were amplified using PCR in a volume of 25 μl (2 μl DNA, 2.5 μl 10× PCR buffer

(Gibco-BRL, Rockville, MD), 0.75 µl $MgCl_2$ (50 mM, final concentration 1.5 mM; Gibco), 0.5 µl nucleotide mix (10 mM each dNTP), 0.35 µl forward primer (20 µM), 0.35 µl reverse primer (20 µM), 0.25 µl Taq polymerase (5 U/µl; Gibco), and 20.3 µl of double-processed water (Sigma, St. Louis, MO). The PCR reaction mixes were prepared as a master mix and then added to each sample. A total of three primer pairs were used. The two *Wolbachia* primer pairs were designed for the *ftsZ* and 16S rDNA sequences and have been previously described, along with their PCR cycling conditions, in Lo et al. (2002) and Casiraghi et al. (2001), respectively. Both have been used to amplify nematode *Wolbachia* DNA and are the most conserved *Wolbachia* primers developed to date, amplifying DNA from all six major subgroups of *Wolbachia* (A–F). The rhabditid host primer pair was designed for host 18S rDNA sequences and described, along with PCR cycling conditions, in Fitch et al. (1995). The non-rhabditid host primer pair (G18s4a and DF12) also amplified host 18S rDNA as described in Fitch et al. (1995). After PCR, 5 µl of reaction product was run on a 1% agarose gel to determine presence and size of amplified DNA. A 1 Kb DNA Ladder (Gibco-BRL, Rockville, MD) was co-electrophoresed as a size standard.

RESULTS

A PCR survey of *Wolbachia* in a broad phylogenetic sampling of the nematode class Secernentea, as well as two representatives of the class Adenophorea, was undertaken using the same conditions that have revealed positive *Wolbachia* infections in filariid nematodes (Casiraghi et al., 2001). We used two different general primer pairs in separate reactions for amplification of the *Wolbachia ftsZ* and 16S rDNA gene sequences. Of the species screened, including several rhabditids, two strongylid parasites of vertebrates, a diplogasterid, three cephalobid relatives, a myolaim, a filariid, and two non-Secernentean plectid species (Fig. 1), none except the filariid showed amplification of either *Wolbachia* sequence (Table 1).

Positive controls from arthropod samples of infected *Nasonia vitripennis* females generated products of the predicted size in reactions with both these primer pairs, while negative controls from samples of uninfected *N. vitripennis* females did not amplify a product (Table 1). The possibility of false negatives (i.e., nematode samples testing negative for *Wolbachia* but actually infected) was minimized by lowering the annealing temperatures in reactions with the 16S rDNA primer pair (to 52 °C, 50 °C, and 48 °C from 60 °C, 55 °C, and 50 °C, respectively, in the three cycling rounds of the reaction). Lowering the annealing temperatures did not change the results. Ability of nematode lysates to yield PCR products was tested with 18S rDNA primers. All nematode lysates generated a positive amplification, except for one (*Panagrellus redivivus*—therefore not included in Table 1). Finally, PCR with the *Wolbachia* 16S rDNA primers was conducted using DNA template extracted from larvae of a filarial nematode (*Brugia malayi*) known to be infected. The larvae were approximately the same size as the average rhabditid adult used in the study. Amplification products of the expected size were obtained, indicating that the lysis method described previously is sufficient for small nematode samples. Taken together, the results indicate that the 21 non-filariid strains tested, representing both a specific investigation of rhabditids and a broader phylogenetic sample of other non-filariid nematodes (Fig. 1), do not harbor the endosymbiont *Wolbachia*.

TABLE 1. Presence (+) or absence (–) of PCR product for *Wolbachia* and host gene sequences.

Sample	*ftsZ Wolbachia*	16S Wolbachia rDNA	18S host rDNA
Caenorhabditis elegans	–	–	+
Caenorhabditis briggsae	–	–	+
Caenorhabditis sp. PS1010	–	–	+
Oscheius myriophila	–	–	+
Oscheius dolichuroides	–	–	+
Rhabditis blumi	–	–	+
Rhabditella axei	–	–	+
Pellioditis typica	–	–	+
Parafilaroides sp. 7-2756	–	–	+
Otostrongylus sp. 7-3129	–	–	+
Mesorhabditis longespiculosa	–	–	+
Tertorhabditis palmarum	–	–	+
Pelodera strongyloides	–	–	+
Rhabditoides regina	–	–	+
Pristionchus pacificus	–	–	+
Panangrobelus stammei	–	–	+
Zeldia punctata	–	–	+
Aphelenchoides figariae	–	–	+
Myolaimus sp. T-23	–	–	+
Plectus aquatilus	–	–	+
Plectus acuminatus	–	–	+
Controls			
Brugia malayi (infected)	+	+	n/a
Water	–	–	–
Lysate without worms	–	–	–
PCR cocktail only	–	–	–
Nasonia vitripennis 1 (infected)	+	+	–
Nasonia vitripennis 2 (infected)	+	+	n/a
N. vitripennis 1 (uninfected)	–	–	n/a
N. vitripennis 2 (uninfected)	–	–	n/a

DISCUSSION

Despite their widespread distribution in arthropod species, including mites (Breeuwer and Jacobs, 1996), terrestrial isopods (Bouchon et al., 1998), and all the major orders of insects (Werren et al., 1995) in nematodes, *Wolbachia* have been described only in the filariids (Bandi et al., 2001). We have addressed the question of whether *Wolbachia* exist in nematodes outside of the filariid clade. Our results indicate that *Wolbachia* are

not present in the 14 species representing the Rhabditidae and Strongylina, and 7 species representing a broader phylogenetic sample of the nematode phylum (Fig. 1).

Our sampling is phylogenetically broad, especially considering the paraphyly of Rhabditidae (De Ley and Blaxter, 2002; Sudhaus and Fitch, 2001). We cannot rule out the possibility that *Wolbachia* occur in other non-filariid nematodes. However, while the filariids show a high infection frequency for *Wolbachia* (17 of 19 species infected), the non-filariid species show a significantly lower level of infection (0 of 21 species infected) (Fishers Exact Test, $P < 0.0001$), with 95% confidence limits for infection frequency in the non-filariid nematodes of 0 to 13%.

There are several possible reasons for why *Wolbachia* would be absent from the strains we tested. First, there may have been a single acquisition of *Wolbachia* in the nematode phylum, likely to have occurred in some ancestral lineage of extant filariids. The congruence of filariid and *Wolbachia* phylogenies, along with the high infection rate within the filariid group, suggests that the endosymbiosis event predated the diversification of filariids and that *Wolbachia* have coevolved with these hosts for nearly 100 million years (Casiraghi et al., 2001). Furthermore, unlike many of the filariid species that are vectored by arthropods and are intimately in contact with *Wolbachia,* most associations of "free-living" nematodes with arthropods are temporary (e.g., phoretic), thus limiting the potential for exchange of *Wolbachia* between arthropods and nematodes. It has been hypothesized that such an exchange may have led to the early origin of *Wolbachia* in filariid nematodes (Lo et al., 2002). Second, exposure to antibiotics in nature or in laboratory cultures at the CGC could have cured some of these nematodes of their infection. This explanation seems unlikely because the media used to maintain the CGC cultures are not seeded with antibiotics, and filariid nematodes are unable to reproduce once cured of their *Wolbachia.* Third, while *Wolbachia* may flourish in the filariid cellular environment, they may not be able to tolerate the non-filariid cellular environment. There may be strong host specificity to the *Wolbachia* endosymbiosis in this phylum (as indicated by strict coevolution). Artificial transfer experiments from filariids to *C. elegans* via microinjection would help clarify this issue. Should such a transfer be successful, the genetic arsenal in *C. elegans* could be used to understand the prokaryotic-eukaryotic genome interactions that underlie the *Wolbachia* endosymbiosis. However, given the present results and the considerable evolutionary divergence between filariids and rhabditids, it seems unlikely that the *Wolbachia* of filariids could tolerate such a novel cellular environment.

We therefore conclude that the absence of *Wolbachia* in the phylogenetically broad taxa sampled in this study, in addition to the high infection rate and pattern of strict cospeciation observed in the filariids, supports a single origin of *Wolbachia* in the nematode phylum. The apparent confinement of *Wolbachia* to the flariids indicates that the endosymbiosis event occurred in an ancestral lineage of extant filariids, specifically after the divergence of this lineage from the rest of the secernenteans. *Wolbachia* then subsequently cospeciated with their filariid hosts. Further surveying the presence of *Wolbachia* within the filariids would help resolve when the endosymbiosis was established during the diversification of this group of nematodes.

The evolutionary patterns emerging from studies of the nematode *Wolbachia* strikingly contrast with the widespread distribution and frequent horizontal transmission of *Wolbachia* in arthropods. Just how *Wolbachia* entered the nematode host (i.e., a lateral transfer event from arthropods) and how their lifestyles diversified across the nematode and arthropod phyla remain important areas of research for future studies. We cannot rule out that other nematode species are infected with *Wolbachia,* perhaps by independent evolutionary acquisitions; however, the high frequency of infection and apparent cospeciation found in filariids appear not to occur in the other nematode taxa in our sample.

Literature Cited

Bandi, C., T. J. C. Anderson, C. Genchi, and M. L. Blaxter. 1998. Phylogeny of *Wolbachia*-like bacteria in filarial nematodes. Proceedings of the Royal Society of London Series B 265:2407–2413.

Bandi, C., A. J. Trees, and N. W. Brattig. 2001. *Wolbachia* in filarial nematodes: Evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. Veterinary Parasitology 98:215–238.

Blaxter, M. L., P. De Ley, J. R. Garey, L. X. Liu, P. Scheldeman, A. Vierstraete, J. R. Vanfleteren, L. Y. Mackey, M. Dorris, L. M. Frisse, J. T. Vida, and W. K. Thomas. 1998. A molecular evolutionary framework for the phylum Nematoda. Nature 392:71–75.

Bordenstein, S. R. 2003. Symbiosis and the origin of species. Pp. 283–304 *in* K. Bourtzis and T. A. Miller, eds. Insect symbiosis. New York: CRC Press.

Bordenstein, S. R., F. P. O'Hara, and J. H. Werren. 2001. *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia.* Nature 409:707–710.

Bouchon, D., T. Rigaud, and P. Juchault. 1998. Evidence for widespread *Wolbachia* infection in isopod crustaceans: Molecular identification and host feminization. Proceedings of the Royal Society of London Series B 265:1081–1090.

Breeuwer, J. A. J., and G. Jacobs. 1996. *Wolbachia:* Intracellular manipulators of mite reproduction. Experimental and Applied Acarology 20:421–434.

Casiraghi, M., T. J. C. Anderson, C. Bandi, C. Bazzocchi, and C. Genchi. 2001. A phylogenetic analysis of filarial nematodes: Comparison with the phylogeny of *Wolbachia* endosymbionts. Parasitology 122: 93–103.

Chirgwin, S. R., K. H. Porthouse, J. M. Nowling, and T. R. Kiel. 2002. The filarial endosymbiont *Wolbachia* sp. is absent from *Setaria equina.* Journal of Parasitology 88:1248–1250.

Cross, H. F., M. Haarbrink, G. Egerton, M. Yazdanbakhsh, and M. J. Taylor. 2001. Severe reactions to filarial chemotherapy and release of *Wolbachia* endosymbionts into blood. Lancet 358:1873–1875.

De Ley, P., and M. L. Blaxter. 2002. Systematic position and phylogeny. Pp. 1–30 *in* D. L. Lee, ed. The biology of nematodes. London: Taylor & Francis.

Fitch, D. H. A., B. Bugaj-Gaweda, and S. W. Emmons. 1995. 18S

ribosomal RNA gene phylogeny for some Rhabditidae related to *Caenorhabditis*. Molecular Biology and Evolution 12:346–358.

Fujii, Y., D. Kageyama, S. Hoshizaki, H. Ishikawa, and T. Sasaki. 2001. Transfection of *Wolbachia* in Lepidoptera: The feminizer of the adzuki bean borer *Ostrinia scapulalis* causes male killing in the Mediterranean flour moth *Ephestia kuehniella*. Proceedings of the Royal Society of London Series B 268:855–859.

Hoffmann, A. A., and M. Turelli. 1997. Cytoplasmic incompatibility in insects. Pp. 42–80 *in* S. L. O'Neill, A. A. Hoffmann, and J. H. Werren, eds. Influential passengers: Inherited microorganisms and arthropod reproduction. New York: Oxford University Press.

Hurst, G. D. D., and F. M. Jiggins. 2000. Male-killing bacteria in insects: Mechanisms, incidence, and implications. Emerging Infectious Diseases 6:329–336.

Jeyaprakash, A., and M. A. Hoy. 2000. Long PCR improves *Wolbachia* DNA amplification: Wsp sequences found in 76% of 63 arthropod species. Insect Molecular Biology 9:393–405.

Lo, N., M. Casiraghi, E. Salati, C. Bazzocchi, and C. Bandi. 2002. How many *Wolbachia* supergroups exist? Molecular Biology and Evolution 19:341–346.

Rigaud, T. 1997. Inherited microorganisms and sex determination of arthropod hosts. Pp. 81–101 *in* S. L. O'Neill, A. A. Hoffmann, and J. H. Werren, eds. Influential passengers: Inherited microorganisms and arthropod reproduction. New York: Oxford University Press.

Saint Andre, A. V., N. M. Blackwell, L. R. Hall, A. Hoerauf, N. W. Brattig, L. Volkmann, M. J. Taylor, L. Ford, A. G. Hise, J. H. Lass, E. Diaconu, and E. Pearlman. 2002. The role of endosymbiotic *Wolbachia* bacteria in the pathogenesis of river blindness. Science 295: 1892–1895.

Stouthamer, R., J. A. J. Breeuwer, and G. D. D. Hurst. 1999. *Wolbachia pipientis:* Microbial manipulator of arthropod reproduction. Annual Review of Microbiology 53:71–102.

Stouthamer, R., J. A. Breeuwer, R. F. Luck, and J. H. Warren. 1993. Molecular identification of microorganisms associated with pathenogensis. Nature 361:66–68.

Sudhaus, W., and D. H. A. Fitch. 2001. Comparative studies on the phylogeny and systematics of the Rhabditidae (Nematoda). Journal of Nematology 33:1–70.

Taylor, M. J., and A. Hoerauf. 1999. *Wolbachia* bacteria of filarial nematodes. Parasitology Today 15:437–442.

Taylor, M. J., and A. Hoerauf. 2001. A new approach to the treatment of filariasis. Current Opinion in Infectious Diseases 14:727–731.

Vandekerckhove, T. T. M., A. Coomans, K. Cornelis, P. Baert, and M. Gillis. 2002. Use of the *Verrucomicrobia*-specific probe EUB338-III and fluorescent in situ hybridization for detection of "*Candidatus* Xiphinematobacter" cells in nematode hosts. Applied and Environmental Microbiology 68:3121–3125.

Wenseleers, T., F. Ito, S. Van Borm, R. Huybrechts, F. Volckaert, and J. Billen. 1998. Widespread occurrence of the micro-organism *Wolbachia* in ants. Proceedings of the Royal Society of London Series B 265:1447–1452.

Werren, J. H. 1997. Biology of *Wolbachia*. Annual Review of Entomology 42:587–609.

Werren, J. H. 1998. *Wolbachia* and speciation. Pp. 245–260 *in* D. J. Howard and S. H. Berlocher, eds. Endless forms: Species and speciation. New York: Oxford University Press.

Werren, J. H., D. Windsor, and L. R. Guo. 1995. Distribution of *Wolbachia* among neotropical arthropods. Proceedings of the Royal Society of London Seires B 262:197–204.