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journal homepage: [www.elsevier.com/locate/ijpara](http://www.elsevier.com/locate/ijpara)An endosymbiotic bacterium in a plant-parasitic nematode: Member of a new *Wolbachia* supergroup<sup>☆</sup>Annelies Haegeman<sup>a</sup>, Bartel Vanholme<sup>a,1</sup>, Joachim Jacob<sup>a</sup>, Tom T.M. Vandeckerckhove<sup>a</sup>, Myriam Claeys<sup>b</sup>, Gaetan Borgonie<sup>b</sup>, Godelieve Gheysen<sup>a,\*</sup><sup>a</sup> Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, B-9000 Ghent, Belgium<sup>b</sup> Department of Biology (Nematology Unit), Faculty of Sciences, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium

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## ABSTRACT

*Wolbachia* is an endosymbiotic bacterium widely present in arthropods and animal-parasitic nematodes. Despite previous efforts, it has never been identified in plant-parasitic nematodes. Random sequencing of genes expressed by the burrowing nematode *Radopholus similis* resulted in several sequences with similarity to *Wolbachia* genes. The presence of a *Wolbachia*-like endosymbiont in this plant-parasitic nematode was investigated using both morphological and molecular approaches. Transmission electron microscopy, fluorescent immunolocalisation and staining with DAPI confirmed the presence of the endosymbiont within the reproductive tract of female adults. 16S rDNA, *ftsZ* and *groEL* gene sequences showed that the endosymbiont of *R. similis* is distantly related to the known *Wolbachia* supergroups. Finally, based on our initial success in finding sequences of this endosymbiont by screening an expressed sequence tag (EST) dataset, all nematode ESTs were mined for *Wolbachia*-like sequences. Although the retained sequences belonged to six different nematode species, *R. similis* was the only plant-parasitic nematode with traces of *Wolbachia*. Based on our phylogenetic study and the current literature we designate the endosymbiont of *R. similis* to a new supergroup (supergroup I) rather than considering it as a new species. Although its role remains unknown, the endosymbiont was found in all individuals tested, pointing towards an essential function of the bacteria.

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## 1. Introduction

In 1924, Hertig and Wolbach discovered an endosymbiotic bacterium in the ovaries of the mosquito *Culex pipiens* (Hertig and Wolbach, 1924). The bacteria species was named *Wolbachia pipiens* and catalogued as a Rickettsiaceae member belonging to the Alphaproteobacteria. Since this first report, *Wolbachia* species have been found in numerous arthropods including insects, crustaceans, spiders and mites (Lo et al., 2007). Although widespread in arthropods, the distribution in nematodes is more restricted. Until a few years ago they were only described in filarial nematodes of the family Onchocercidae which includes the important animal parasites *Brugia malayi*, *Onchocerca volvulus* and *Wuchereria bancrofti* (Fenn and Blaxter, 2006). Molecular evidence for the presence of *Wolbachia* in the rat lungworm *Angiostrongylus cantonensis*, a non-filarial nematode, suggested a more widespread occurrence of *Wolbachia* among animal-parasitic nematodes (Tsai et al.,

2007), but this finding was afterwards claimed to be the result of contamination with DNA from arthropods and filarial nematodes (Foster et al., 2008). Bordenstein et al. (2003) investigated 21 non-filarial nematode species, but none were found to harbour *Wolbachia*.

In contrast to their symbiotic lifestyle in most arthropods, *Wolbachia* of nematodes are considered to be mutualists since all adult nematodes in species infected with *Wolbachia* have the endobacteria (Werren et al., 2008). Moreover, treatments with antibiotics that would eradicate *Wolbachia* have unsuspected and dramatic effects on the host, causing delayed moulting, reduced growth rates, aberrant embryogenesis and eventual death (Casiraghi et al., 2002; Fenn and Blaxter, 2006). Remarkably, similar treatments of insects have only minor or no effects. A possible explanation for their beneficial role in relation to nematodes was given upon sequencing of the *Wolbachia* genome of *B. malayi* (Foster et al., 2005). Careful annotation of the sequences revealed that *Wolbachia* could provide nematode-essential metabolites, such as riboflavin, heme, glutathione, glycolytic enzymes, and compounds necessary for the biosynthesis of purines and pyrimidines (Foster et al., 2005). Within filarial nematodes, *Wolbachia* occurs in the lateral chords of the hypodermis in both males and females, as well as throughout the whole reproductive tract of females (Kramer et al., 2003).

<sup>☆</sup> Sequences associated with this article have been submitted to the GenBank database (Accession Nos. EU833482, EU833483, EU833484, FJ168559 and FJ168560).

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The establishment of a conserved taxonomy is still a subject of controversy within the *Wolbachia* community. Lo et al. (2007) have proposed that all *Wolbachia* endobacteria be declared as one species, being *W. pipientis*. Pfarr et al. (2007) disagree with this idea mainly based on some remarkable differences in the genome sequences of *Wolbachia* of *B. malayi* and *Drosophila melanogaster* (wBm and wMel, respectively). Whereas wBm lacks genes necessary for recombination, resulting in little or no genomic recombination, wMel has an active recombination system (Wu et al., 2004). In combination with the biological differences between arthropod and nematode *Wolbachia*, Pfarr et al. (2007) state that there is enough evidence to consider the *Wolbachia* of filarial nematodes as a different species. However, it was recently shown that nematode *Wolbachia* are not monophyletic (Bordenstein et al., 2009). Evolutionary relationships between *Wolbachia* strains have been inferred mainly from 16S rRNA, *wsp* (*Wolbachia* surface protein), *ftsZ* (cell division protein) and *groEL* (heat shock protein 60) sequences. Based on these evolutionary trees, *Wolbachia* is divided into different clades, referred to as “supergroups” (Casiraghi et al., 2005). Supergroups A and B include most of the *Wolbachia* found in arthropods (Werren et al., 1995), whereas C and D harbour the majority of the *Wolbachia* found in filarial nematodes (Bandi et al., 1998). Supergroup E consists of *Wolbachia* from springtails (Collembola) (Vandekerckhove et al., 1999; Czarnetzki and Tebbe, 2004) and supergroup F comprises *Wolbachia* from termites, weevils, true bugs, scorpions (Lo et al., 2002; Rasgon and Scott, 2004; Baldo et al., 2007) and the filarial nematodes *Mansonella* spp. (Casiraghi et al., 2001; Keiser et al., 2008). Two relatively new supergroups are supergroup G with Australian spiders as hosts (Rowley et al., 2004), and H with Isopteran species (Bordenstein and Rosengaus, 2005). Finally, some *Wolbachia* strains such as those identified in *Dipetalonema gracile* (filarial nematode), *Ctenocephalides* spp. (flea) and *Cordylocheres scorpoides* (pseudoscorpion) could not be classified in any of these supergroups (Casiraghi et al., 2005; Zeh et al., 2005). Even a recent more detailed study could not classify those specific *Wolbachia* strains within one of the existing supergroups (Bordenstein et al., 2009). The latter phylogenomic study also revealed that the ancestry of mutualism and parasitism is not resolvable with the currently available sequence data.

During a recent screen of expressed sequence tags (ESTs) derived from the burrowing nematode *Radopholus similis*, a major parasite of banana, approximately 1% of the unigenes showed similarity to *Wolbachia* sequences (Jacob et al., 2008). However, it was not clear if these could be nematode genes acquired by lateral gene transfer from an ancient and long-lost endosymbiont (Dunning-Hotopp et al., 2007) or whether there was still a true endosymbiont present in *R. similis*.

In this article, we confirm the presence of a *Wolbachia*-like endosymbiont in this plant-parasitic nematode, using genetic and microscopic evidence. Other symbionts have already been identified in plant-parasitic nematodes: *Verrucomicrobia* species in *Xiphinema index* (Vandekerckhove et al., 2000), *Bacteroidetes* species in *Heterodera glycines* and *Globodera rostochiensis* (Noel and Atibalentja, 2006). This is, however, to our knowledge the first report on a *Wolbachia*-like bacterium in a plant-parasitic nematode.

## 2. Materials and methods

### 2.1. Searching for indications of *Wolbachia* in nematode ESTs

To look for *Wolbachia* ESTs in nematode EST databases, we used a reciprocal search approach utilising the BLAST algorithm. All 805 *Wolbachia* proteins from the *B. malayi* *Wolbachia* genome (WolBm, NC\_006833) were downloaded from the Genome division of Gen-

Bank (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome>). To identify nematode ESTs with significant homology to WolBm proteins, a tBLASTn search with the WolBm proteins as a query was done locally by StandAloneBLAST (<ftp://ftp.ncbi.nih.gov/blast/>) against all available nematode ESTs downloaded from GenBank (December 2007;  $n = 903,682$ ;  $E$ -value cut-off =  $1e-2$ ). All significant hits were retained and sequences with the term “*Caenorhabditis*” in the description line were removed to reduce the size of the dataset. To verify whether these selected nematode ESTs could be derived from *Wolbachia*, a BLASTx search with the identified ESTs was conducted against all known proteins using NetBLAST (blastcl3 network client, <ftp://ftp.ncbi.nih.gov/blast/>) (February 2008). Only when the top hit was a *Wolbachia* protein, was the nematode EST considered a putative candidate sequence originating from *Wolbachia*.

### 2.2. Culture of *R. similis* and sterile DNA extraction

*Radopholus similis* from Uganda was cultured on carrot discs (Jacob et al., 2007). All manipulations for DNA extraction were performed under sterile conditions. For surface sterilisation, nematodes were soaked for 30 s in 0.1% benzalkonium chloride (Sigma–Aldrich). Subsequently, nematodes were spun in a microcentrifuge (Eppendorf) at 1000g for 3 min and washed three times with sterile demineralised water. The nematodes were pelleted and used for genomic DNA extraction (Bolla et al., 1988) under sterile conditions. The DNA pellet was dissolved in 50 µl of sterile demineralised water.

### 2.3. Cloning of *Wolbachia* genes

To amplify the 16S rRNA gene of *Wolbachia*, PCR was performed on 150 ng of *R. similis* DNA. The 25 µl reaction mixture was prepared under sterile conditions and contained 0.5 µM of each universal bacterial 16S rRNA primer (16S-F and 16S-R) (Edwards et al., 1989) (Table 1), 4 mM of each dNTP, 1.5 mM MgCl<sub>2</sub>, 20 mM Tris–HCl (pH 8.3), 50 mM KCl and 1 U of Taq DNA polymerase (Invitrogen). The PCR consisted of 2 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 54 °C and 1 min 30 s at 72 °C. The PCR product was separated on a 0.5× TAE-buffer (20 mM Tris–acetate, 0.5 mM EDTA) 1.5% agarose gel, excised and purified using a QIAquick Gel Extraction kit (Qiagen). The purified fragment was ligated into pGEM-T (Promega) and transformed into heat shock competent *Escherichia coli* DH5α cells (Invitrogen). Transformed cells were selected on Luria-Bertani (LB) medium containing 100 µg/ml carbenicillin. Colony PCR with SP6 and T7 primers (Table 1) under conditions as described above confirmed the presence of an insert. Plasmids of the corresponding positive colonies were isolated using the Nucleobond AX kit (Macherey–Nagel) and sent for sequencing to the Flanders Institute for Biotechnology Genetic Service Facility (VIB–GSF, Antwerp, Belgium). An attempt was made to amplify the *wsp* gene using primers *wsp*-F and *wsp*-R (Braig et al., 1998). For *ftsZ* and *groEL*, part of the sequence was available as an EST (EY193345, EY195553). The full-length coding sequences were obtained by genome walking on *R. similis* DNA libraries using the Genome Walker Universal kit (Clontech) according to the manufacturer’s instructions. Briefly, genome walking makes it possible to isolate flanking genomic segments adjacent to a known sequence. Different pools or libraries are constructed of adapter-ligated genomic DNA fragments. Using nested primers based on these adapters combined with gene-specific primers, regions flanking the known sequence can be amplified by PCR. For both *ftsZ* and *groEL*, two successive primers were developed based on the EST sequences to obtain both up- and down-stream sequences (*ftsZ*-up1, *ftsZ*-up2, *ftsZ*-down1, *ftsZ*-down2, *groEL*-up1, *groEL*-up2, *groEL*-down1, *groEL*-down2), in combination with the

**Table 1**Primer sequences used for cloning of the 16S rRNA, *ftsZ* and *groEL* genes.

Primer	Primer sequence	Primer	Primer sequence
16S-F	AGAGTTTGATCCTGGCTCAG	16S-R	AAGGAGGTGATCCAGCCGCA
wsp-F	TGGTCCAATAAGTGATGAAGAACTAGCTA	wsp-R	AAAAATTAACGCTACTCCAGCTTCTGCAC
<i>ftsZ</i> -up1	ATTCCTCTGCCGACCTCTAC	<i>ftsZ</i> -up2	CATCAGGTAAAGCACCAGCA
<i>ftsZ</i> -down1	CCGGATTACCTGTACTACACC	<i>ftsZ</i> -down2	CTGTGGTGGAGTAGGTGGT
<i>ftsZ</i> -down3	GCTGATAATGTGCTGCATATAGG	<i>ftsZ</i> -down4	CTGATTTAATGGTTATGCCAGG
<i>groEL</i> -up1	CCAGCAGAAATGGGCTTTAAAGC	<i>groEL</i> -up2	ACACTGAGAACAACTTTGAGC
<i>groEL</i> -down1	GCTTGAGCGTGGTTATGCTTC	<i>groEL</i> -down2	GAGTGCCATACATCTTTAATACC
SP6	ATTTAGGTGACACTATAGAATACTCAAGC	T7	TAATACGACTCACTATAGGGCGAATTGG
AP1	GTAATACGACTCACTATAGGGC	AP2	ACTATAGGGCACGCGTGGT
Rs-act-F	TGTCCTGCTTTGTCAATTTGC	Rs-act-R	TGATCACCCTCGGAAACG
<i>ftsZ</i> -F	CTGTGGTGGGAGTAGGTGGT	<i>ftsZ</i> -R	AGCAGCCTTGTCTTCTTTG
<i>groEL</i> -F	AAAGCCCATTTCTGCTGGTA	<i>groEL</i> -R	GGAAGCATAACCACGCTCAA
<i>ftsZ</i> -F2	CCGGATTACCTGTACTACACC	<i>ftsZ</i> -R2	AGCAGCCTTGTCTTCTTTG

adapter primers from the Genome Walker Universal kit (AP1 and AP2) (Table 1). For the *ftsZ* gene, an additional nested PCR was required to obtain the downstream sequence (*ftsZ*-down3, *ftsZ*-down4) (Table 1). The longest resulting fragments were gel excised, purified, cloned and sequenced as described above. From the resulting coding sequences, guanine–cytosine (GC) percentages were calculated with an in-house perl script.

#### 2.4. Phylogenetic analyses

The CoreNucleotide database from the NCBI website (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucore>) was searched for *Wolbachia* sequences from different host species. In our analysis we included only *Wolbachia* strains for which sequences of all three genes (16S rRNA, *ftsZ* and *groEL*) were available. This approach ruled out *Wolbachia* strains belonging to supergroups E and G, but did include species of the different supergroups A, B, C, D, F and H. Accession numbers of the sequences used in this

study are shown in Table 2. ClustalW (Thompson et al., 1994) was used to construct a multiple alignment for the three genes and then manually edited. Two species of the genera most closely related to *Wolbachia* were added to the alignment (*Anaplasma marginale* and *Ehrlichia canis*). The chosen outgroup, *Rickettsia felis*, is a member of the same family as *Wolbachia*, i.e. the Rickettsiaceae. With these alignments as input, Bayesian analyses were conducted with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) for each gene separately and for a concatenated dataset of the three genes. The HKY model (Hasegawa et al., 1985) was used with 500,000 generations and resulting trees were visualized in TreeView 1.6.6 (Page, 1996).

#### 2.5. Detection of *Wolbachia* in individual nematodes and additional populations

Forty single adult female and 40 male nematodes were manually collected in 40 µl buffer (10 mM Tris–HCl pH 8.0, 1 mM DTT,

**Table 2**Host species, taxonomic classifications and GenBank accession numbers of sequences used for phylogenetic analyses based on 16S rRNA, *ftsZ* and *groEL* genes.

Host species	Phylum	Class	Order	16S rRNA	<i>ftsZ</i>	<i>groEL</i>	Super-group
<i>Brugia malayi</i>	Nematoda	Chromadorea	Rhabditida	AF051145	AJ010269	NC006833	D
<i>Brugia pahangi</i>	Nematoda	Chromadorea	Rhabditida	AJ012646	AJ010270	AJ609654	D
<i>Dirofilaria repens</i>	Nematoda	Chromadorea	Rhabditida	AJ276500	AJ010273	AJ609653	C
<i>Litomosoides sigmodontis</i>	Nematoda	Chromadorea	Rhabditida	AF069068	AJ010271	AF409113	D
<i>Mansonella</i> sp.	Nematoda	Chromadorea	Rhabditida	AJ279034	AJ628414	AJ628412	F
<i>Onchocerca gibsoni</i>	Nematoda	Chromadorea	Rhabditida	AJ276499	AJ010267	AJ609652	C
<i>Radopholus similis</i>	Nematoda	Chromadorea	Rhabditida	EU833482	EU833483	EU833484	?
<i>Tribolium confusum</i>	Arthropoda	Insecta	Coleoptera	X65674	U28194	AY714798	B
<i>Aedes albopictus</i>	Arthropoda	Insecta	Diptera	AF397408	DQ842305	DQ243927	A
<i>Drosophila melanogaster</i>	Arthropoda	Insecta	Diptera	DQ235275	DQ235339	DQ235379	A
<i>Drosophila simulans</i> wAu	Arthropoda	Insecta	Diptera	AF390865	DQ235342	DQ235382	A
<i>Drosophila simulans</i> wHa	Arthropoda	Insecta	Diptera	DQ235279	DQ235341	DQ235381	A
<i>Drosophila simulans</i> wMa	Arthropoda	Insecta	Diptera	AF390864	AY508999	AY714799	B
<i>Drosophila simulans</i> wNo	Arthropoda	Insecta	Diptera	DQ235288	DQ266426	AY714800	B
<i>Drosophila simulans</i> wRi	Arthropoda	Insecta	Diptera	DQ235278	U28178	AY714806	A
<i>Laodelphax striatellus</i>	Arthropoda	Insecta	Hemiptera	AB039036	AB039038	DQ356890	B
<i>Sogatella furcifera</i>	Arthropoda	Insecta	Hemiptera	AB039037	AB039039	DQ243918	B
<i>Trichogramma cordubensis</i>	Arthropoda	Insecta	Hymenoptera	X65675	U95749	AY714803	B
<i>Coptotermes acinaciformis</i>	Arthropoda	Insecta	Isoptera	DQ837197	DQ837186	AJ627384	F
<i>Kaloterms flavicollis</i>	Arthropoda	Insecta	Isoptera	Y11377	AJ292345	AJ609660	F
<i>Microcerotermes</i> sp.	Arthropoda	Insecta	Isoptera	AJ292347	AJ292346	AJ628411	F
<i>Zootermopsis angusticollis</i>	Arthropoda	Insecta	Isoptera	AY764279	AY764283	AY764278	H
<i>Zootermopsis nevadensis</i>	Arthropoda	Insecta	Isoptera	AY764280	AY764284	AY764277	H
<i>Ephesia kuehniella</i>	Arthropoda	Insecta	Lepidoptera	X65671	U62126	AB081644	A
<i>Hypolimnas bolina bolina</i>	Arthropoda	Insecta	Lepidoptera	AB052745	AB167352	AB167350	B
<i>Hypolimnas bolina jacintha</i>	Arthropoda	Insecta	Lepidoptera	AB085178	AB167399	AB167398	B
<i>Armadillidium vulgare</i>	Arthropoda	Malacostraca	Isoptoda	X65669	U28208	AM087231	B
<i>Chaetophiloscia elongata</i>	Arthropoda	Malacostraca	Isoptoda	AJ223241	AJ223246	AM087239	B
<i>Anaplasma marginale</i>	Proteobacteria	Alphaproteobacteria	Rickettsiales	CP000030	CP000030	CP000030	–
<i>Ehrlichia canis</i>	Proteobacteria	Alphaproteobacteria	Rickettsiales	CP000107	CP000107	CP000107	–
<i>Rickettsia felis</i>	Proteobacteria	Alphaproteobacteria	Rickettsiales	CP000053	CP000053	CP000053	–



0.45% Tween 20) and centrifuged for 5 min at 8000g. For DNA extraction, single nematodes were sonicated on ice twice for 5 s (Branson sonifier S-250). Proteinase K was added to a final concentration of 60 ng/ $\mu$ l, whereupon the nematode suspension was incubated for 30 min at 37 °C. Proteinase K was then inactivated by 5 min incubation in a boiling water bath. After centrifugation for 5 min at 8000g, 5  $\mu$ l of the supernatant was used for PCR. PCR conditions were as described above, with the exception of a higher number of cycles ( $n = 40$ ) and different primer pairs (Rs-act-F and Rs-act-R to amplify actin as a positive control, ftsZ-F and ftsZ-R to amplify the *ftsZ* gene and groEL-F and groEL-R for the *groEL* gene; Table 1). PCR mix without template served as a negative control. An additional population (*R. similis* from Indonesia) and related species (*Radopholus arabocoffeae* from Vietnam) were checked for the presence of *Wolbachia* by cloning the *ftsZ* gene with primers ftsZ-F2 and ftsZ-R2 (Table 1). DNA extraction and PCR conditions were as described above. PCR mix without template served as a negative control.

## 2.6. DAPI staining and immunolocalisation

To visualize genetic material from both the nematode and the endosymbiont, staining with DAPI (Invitrogen) was performed. Nematodes were fixed in 50% acetic acid and DAPI (dissolved in methanol) was added drop by drop to a final concentration of 100 ng/ml. Nematodes were mounted on a glass slide, washed and embedded in Vectashield (Invitrogen) to reduce photobleaching. Nematodes and fluorescence signals were visualized with a Nikon TE 2000-E inverted microscope, equipped with a 100 $\times$  oil objective (NA 1.2, Plan corrected) and a standard Nikon RGB camera. Excitation and detection was performed with filter cubes of the following composition (EX: excitation, DC: dichroic, EM: emission): EX 330–380; DC 400; EM 420 LP. Images were acquired using NIS-Elements software version 2.10 (<http://www.nis-elements.com/>).

Immunolocalisation was carried out with rabbit antiserum against purified *Wolbachia* bacteria from *Aedes albopictus* (kindly provided by Claude Nappes, Université de la Méditerranée, Marseille, France). Nematodes were fixed overnight at 4 °C in 4% paraformaldehyde in M9 buffer (42 mM Na<sub>2</sub>HPO<sub>4</sub>, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 86 mM NaCl, 1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, pH 7.0) followed by additional fixation for 4 h at room temperature. The fixative was removed and replaced by M9 buffer. Nematodes were cut and subsequently washed three times with M9 buffer by centrifuging for 1 min at 3000g. Incubation in 2 mg/ml proteinase K for 30 min at room temperature permeabilised the nematode sections. A washing step with M9 buffer removed the proteinase K. The nematode pellet was chilled on ice for 15 min, and incubated for 30 s on ice in 1 ml of cold methanol. Upon removing the methanol, one ml cold acetone was added and the pellet was incubated for 1 min on ice then washed with distilled water and M9 buffer. Blocking was done overnight at 4 °C with 1% blocking reagent (Roche) dissolved in M9 buffer. After removal of the blocking buffer, nematode fragments were incubated for another 3 h at 4 °C in blocking buffer to which primary antibodies were added (1:750). The nematodes were then washed with M9 buffer three times and incubated for 10 min in 0.5% blocking buffer at room temperature. The supernatant was removed after centrifuging for 4 min at 500g, whereupon 0.5% blocking buffer with 1:20 diluted secondary antibody (fluorescein-labeled goat anti-rabbit IgG (H+L), KPL, Guildford, UK) was added. After 3 h incubation at 4 °C, secondary antibodies were removed by three cycles of washing with M9 buffer and centrifuging for 4 min at 500g. As a negative control, the same procedure was executed without adding primary antibodies. Nematodes were mounted on a glass slide, embedded in Vectashield (Invitrogen), and viewed with a Nikon Eclipse TE300 epifluorescence microscope equipped with a Biorad Radiance 2000 confocal system.

Fluorescein was excited with a 488 nm Argon ion laser and detected with a photomultiplier tube (PMT) through a 528/50 nm HQ BP filter. To reduce aspecific signal, Kalman averaging ( $n = 3$ ) was applied during acquisition. The Argon laser was simultaneously used to acquire transmission images. The objective lens was as described above. Digital images were obtained with LaserSharp 2000 software and analysed using ImageJ (Abramoff et al., 2004).

## 2.7. Transmission electron microscopy

Female adult nematodes were fixed in Karnovsky solution (2% paraformaldehyde, 2.5% glutaraldehyde in 0.134 M sodium cacodylate buffer, pH 7.2) at 60 °C. After 30 min the heads and tails of the nematodes were removed. The fixed nematode pieces were then incubated in Karnovsky solution at 4 °C overnight on a rotator. After this primary fixation, nematodes were washed for 8 h at room temperature in 0.134 M sodium cacodylate buffer (pH 7.2). The washing solution was renewed three times during this period. Post-fixation was done in reduced osmium, a mixture of 1 ml OsO<sub>4</sub> (4%), 3 ml sodium cacodylate (0.134 M) and 66 mg K<sub>3</sub>Fe(CN)<sub>6</sub> overnight at 4 °C on a rotator. The nematode pieces were then washed with distilled water and subsequently dehydrated in a 50%, 70%, 90% and 100% ethanol series at room temperature three times at 10 min each. The samples were then transferred to absolute alcohol to which CuSO<sub>4</sub> bars were added to remove any remaining water. The specimens were then infiltrated with a low-viscosity embedding medium (Spurr, 1969) and polymerised at 70 °C for 8 h. Ultrathin (70 nm) longitudinal sections were cut on a Reichert Ultracut S Ultramicrotome (Leica, Vienna, Austria) with a diamond knife (Diatome Ltd., Biel, Switzerland). Formvar-coated single slot copper grids were used (Agar Scientific, Stansed, United Kingdom). The sections were post-stained with uranyl acetate and lead citrate stain (EM stain, Leica) and visualised with a Jeol JEM 1010 (Jeol Ltd., Tokyo, Japan) transmission electron microscope operating at 60 kV. Digital recordings were made with a DITABIS system (Pforzheim, Germany).

## 3. Results

### 3.1. *Wolbachia* sequences in nematode ESTs

A proportion of nematode EST sequences (30,909 out of 903,682) showed significant homology to one of the *Wolbachia* proteins of *B. malayi* *Wolbachia*. After removal of ESTs with the term “*Caenorhabditis*” in the description line ( $n = 17,606$ ) to reduce the number of sequences, the remaining 13,303 ESTs were searched for homology with all known proteins in GenBank (February 2008). The resulting ESTs with a *Wolbachia* sequence as the top hit ( $n = 110$ ; Supplementary Table S1) belonged to seven different nematode species as summarized in Table 3. Three species with *Wolbachia* ESTs have never been experimentally confirmed to contain *Wolbachia*: *R. similis*, *Ancylostoma caninum* and *Pristionchus pacificus*. However the latter two could be false positives as only one putative *Wolbachia* EST was found despite the large amount of available ESTs for these nematodes. The retained EST from *P. pacificus* (AW097859) also showed high homology to a *Caenorhabditis elegans* protein in a BLASTx search and can therefore be considered as a false positive. However, the EST retained from *A. caninum* (EX545240) only showed significant homology to *Wolbachia* proteins and may indicate the presence of *Wolbachia* in this nematode. Regardless of the low frequencies of *A. caninum* and *P. pacificus* putative *Wolbachia* ESTs, the percentage of *Wolbachia* sequences in the EST libraries of nematode species varies between 0.021% (*W. bancrofti*) and 0.623% (*R. similis*) (Table 3).

**Table 3**

Overview of the number of *Wolbachia* expressed sequence tags (ESTs) found among all nematode ESTs. Information provided for each species includes its total number of ESTs in the GenBank database (December 2007), whether or not *Wolbachia* has been described in literature, the number of *Wolbachia* ESTs found, and the percentage of *Wolbachia* ESTs in relation to the total amount of ESTs (December 2007).

Species	Lifestyle	Total ESTs	<i>Wolbachia</i> ?	<i>Wolbachia</i> ESTs	% <i>Wolbachia</i> ESTs
<i>Ancylostoma caninum</i>	APN	46965		1	0.0021
<i>Brugia malayi</i>	APN	26215	Yes	39	0.1488
<i>Onchocerca volvulus</i>	APN	14974	Yes	17	0.1135
<i>Wuchereria bancrofti</i>	APN	4847	Yes	1	0.0206
<i>Dirofilaria immitis</i>	APN	4005	Yes	5	0.1248
<i>Litomosoides sigmodontis</i>	APN	2699	Yes	–	
<i>Angiostrongylus cantonensis</i>	APN	1279		–	
<i>Brugia pahangi</i>	APN	28	Yes	–	
<i>Pristionchus pacificus</i>	FLN	14663		1?	0.0068
<i>Radopholus similis</i>	PPN	7380		46	0.6233
All other PPN species	PPN	157814		–	

APN, animal parasitic nematode; FLN, free-living nematode; PPN, plant-parasitic nematode.

### 3.2. Cloning of 16S rRNA, *ftsZ* and *groEL* sequences

A 1494 bp fragment of 16S rRNA was amplified from the sterile DNA extracted from *R. similis* (population Uganda), using universal bacterial primers. A BLASTn search revealed a 16S rRNA sequence of *Wolbachia* isolated from *Drosophila simulans* wRi as the top hit (supergroup A; 95% identity). Using primers based on EST fragments of the *ftsZ* and *groEL* genes, full-length coding sequences for both genes could be cloned. The full-length coding sequence of the *ftsZ* gene is 1161 bp. The cloning process revealed a 299 bp fragment of another gene, 270 bp upstream of the *ftsZ* gene. A BLASTx search revealed that the most homologous protein is a cytidine triphosphate (CTP) synthetase of *Wolbachia* from *D. melanogaster* (65% identity). Furthermore, the full-length coding sequence of the *groEL* gene is 1638 bp. No introns are present in full-length open reading frames of either *ftsZ* or *groEL*. GC percentages of the coding sequences of the 16S rRNA, *ftsZ* and *groEL* genes were, respectively, 48%, 38% and 37%. Despite efforts using different PCR conditions and nested PCR, amplification of *wsp* did not succeed. Additional *ftsZ* fragments were cloned from a different *R. similis* population (Indonesia), and from the closely related species *R. arabocoffeae* (Vietnam). In both cases a fragment of 369 bp was obtained, which differed in only two nucleotides from the *Wolbachia ftsZ* sequence amplified from the *R. similis* population Uganda. All obtained sequences were submitted to GenBank (Accession Nos. EU833482, EU833483, EU833484, FJ168559, FJ168560).

### 3.3. Phylogenetic analyses

16S rRNA, *ftsZ* and *groEL* genes from *Wolbachia* belonging to different supergroups (Table 2) were aligned with the corresponding sequences isolated from *R. similis Wolbachia* and used for Bayesian analyses. All constructed phylogenetic trees had the same topology and therefore only the tree of the combined dataset is shown (Fig. 1). The supergroups A, B, C, D, F and H are clustered with high posterior probability values. The *Wolbachia*-like symbiont from *R. similis* does not appear to belong to any of the known supergroups. Extra analyses with the limited data available for supergroups E (springtails), G (spiders) and the unclassified species *C. felis* and *D. gracile* also did not show any relatedness to these supergroups or species (data not shown). However it does form a monophyletic group with all other *Wolbachia* sequences with a high posterior probability value (1.00). Nevertheless, it remains difficult to say to which supergroup the *Wolbachia* of *R. similis* is most closely related.

### 3.4. The presence of *Wolbachia* in single nematodes

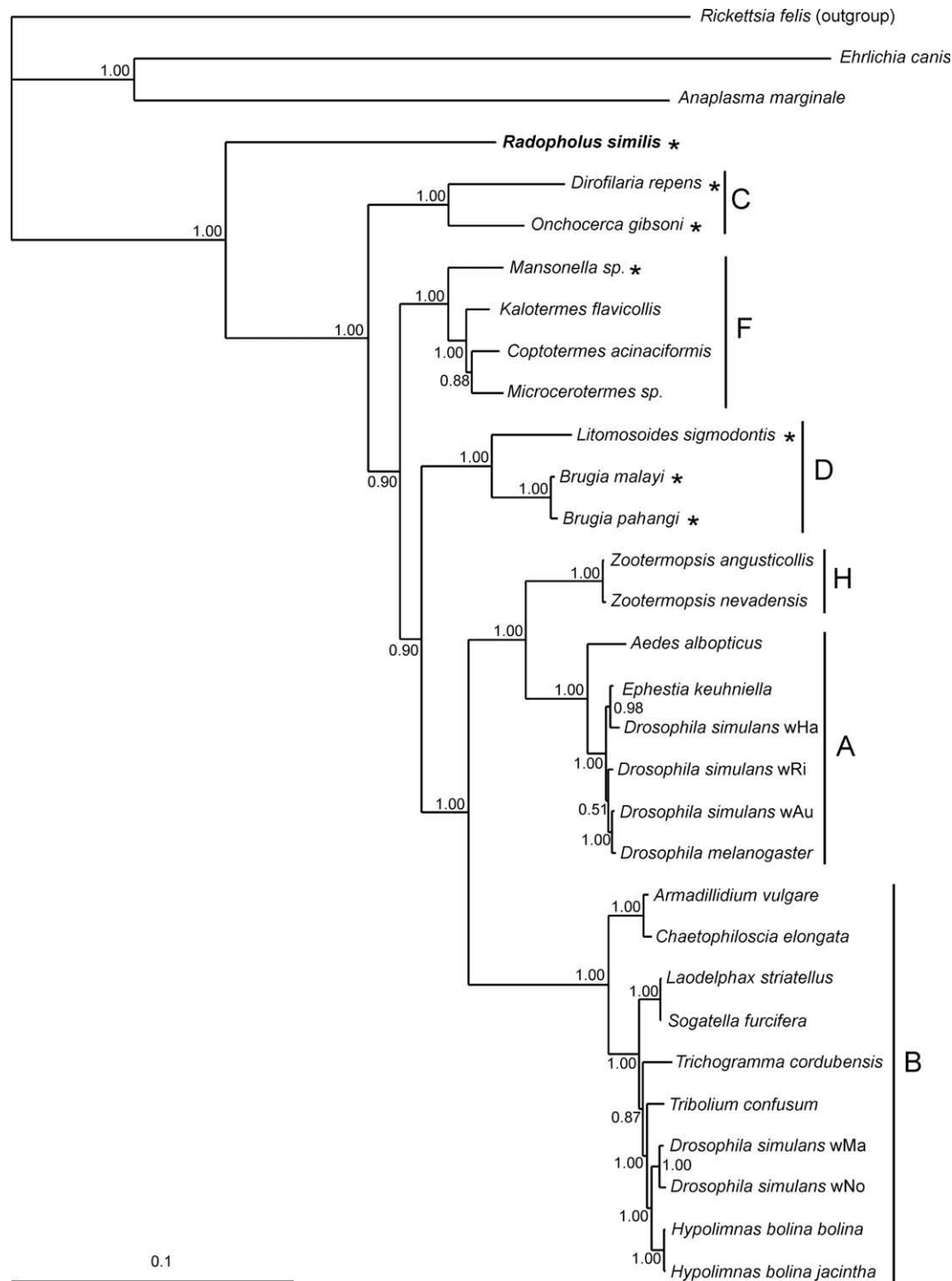
Forty adult females and 40 adult males were tested for the presence of the *Wolbachia*-like symbiont. A positive control PCR was performed with actin primers and a correct fragment was amplified from 28 females and 29 males. No amplification was obtained in the negative control. The actin-positive samples were subsequently tested for the presence of the symbiont, using both *ftsZ* and *groEL* primers. The PCRs resulted in amplification products of the expected lengths for all 28 females and 29 males, whereas no amplification product was detected in the negative controls. From these results we conclude that likely all *R. similis* individuals are infected, as is the case in animal-parasitic species.

### 3.5. Visualisation of the endosymbiont

DAPI staining showed strong signals for the different cell nuclei of the nematode. Around the oocyte nuclei in the ovaries, tiny fluorescent dots were observed, which may be of endosymbiotic origin (Fig. 2). Likewise, immunolocalisation using polyclonal antibodies against *Wolbachia* produced strongly stained particles in the female ovaries, both inside and surrounding the oocytes (Fig. 3). In some cases a signal was observed on the cuticle. Since the antibodies used are polyclonal and were raised against whole *Wolbachia* cells, these antibodies are not fully *Wolbachia*-specific and may also recognize other bacteria. The signal on the cuticle is most probably derived from external bacteria that are attached to the surface of the nematode. Transmission electron microscopy of female adults confirmed the presence of endosymbiotic bacteria in the ovary (Fig. 4). These bacteria had a large variety of shapes, ranging from rod-shaped to round, U-shaped and even club-shaped. The cross-sections of the observed endosymbionts ranged from approximately 350 to 900 nm. In many cases, the three membranes could be distinguished, two of bacterial origin and one of host origin. Inside the bacterial cell, ribosomes could be seen which typically appear smaller than ribosomes in the surrounding host tissue. Next to the ovary, bacterial cells inhabit the uterus, albeit in lower numbers.

## 4. Discussion

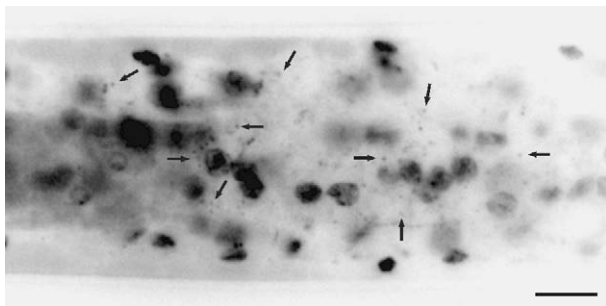
We investigated the presence of a *Wolbachia*-like bacterium in the plant-parasitic nematode *R. similis*. Despite past efforts (Bordenstein et al., 2003), we believe this is the first discovery of *Wolbachia* in a plant-parasitic nematode. A first indication came from a



**Fig. 1.** Phylogenetic tree inferred from 16S rRNA, *ftsZ* and *groEL* gene data with Bayesian statistics (500,000 generations). All *Wolbachia* taxa are represented by their host species names. Nematode hosts are marked with asterisks. Posterior probabilities are shown at branching points. *Wolbachia* supergroups A, B, C, D, F and H are indicated.

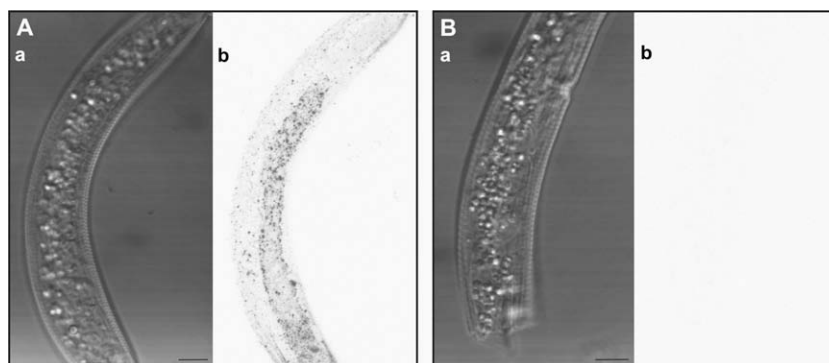
comprehensive analysis of an EST dataset derived from a mixed-stage population of *R. similis* (Jacob et al., 2008), revealing a subset of sequences (30 unigenes composed of 38 ESTs) with high similarity to *Wolbachia* proteins. Extending this in silico approach by exploring all nematode ESTs for putative *Wolbachia* material revealed seven nematode species with EST sequences reminiscent of *Wolbachia*. For four of those, the presence of *Wolbachia* was previously described in the literature, which indicates that our approach was adequate in retaining sequences derived from *Wolbachia*-infected nematode species. Although the presence of

*Wolbachia* was described in other nematode species as well (*Brugia pahangi* and *Litomosoides sigmodontis*) this could not be confirmed by our EST screen. It should however be noted that for these species the number of ESTs in the database is relatively low (less than 3000). For three nematode species, candidate *Wolbachia* tags were found whereas the endosymbiont itself has not yet been described. For two of these species only one tag was found among 14,663 (*P. pacificus*) or 46,965 (*A. caninum*) ESTs. The EST from *P. pacificus* is probably a false positive since it also showed high homology to a *C. elegans* gene. Nevertheless, the EST from the *A. caninum* library

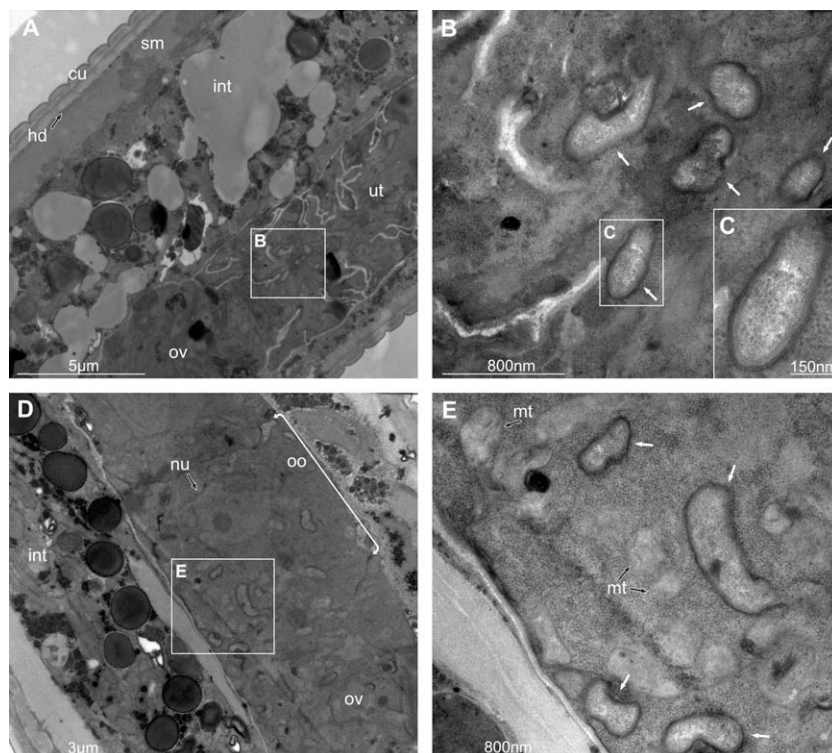


**Fig. 2.** Female adult nematode stained with DAPI, at the level of an ovary. Nuclei of the ovary cells show intense staining. Small dots (arrows) indicate genetic material of endosymbionts. Scale bar: 5  $\mu$ m.

showed high homology to *Wolbachia* proteins exclusively. In contrast to these few hits, for *R. similis* 46 putative *Wolbachia* sequences among 7380 ESTs were retained, adding eight ESTs to the sequences identified by Jacob et al. (2008) due to a higher *E*-value cut-off. The number of putative *Wolbachia* sequences found is too high to be considered false positives. Moreover, contamination of the cDNA library is highly unlikely since our laboratory has never worked with insects or filarial nematodes, so contaminating *Wolbachia*-containing organisms have never been present. Since the construction of most cDNA libraries is based on polyA tails, which are absent in bacterial transcripts, one would not expect bacterial sequences in EST libraries. This implies that the identified ESTs are not necessarily of bacterial origin, but could be derived from the nematode genome itself. It was shown that large pieces of the symbiont genome can be integrated into the host genome



**Fig. 3.** Female adult nematodes subjected to immunolocalisation with polyclonal antibodies against *Wolbachia*. (A) Nematodes treated with both primary and secondary antibody; (B) negative control (only secondary antibody). (a) Transmitted light; (b) epifluorescence. Scale bars: 10  $\mu$ m.



**Fig. 4.** Transmission electron microscopy sections of female adult nematodes. (B,C) Detailed pictures of (A) in the uterus (organ characterized by numerous invaginations), while (E) is a detail of (D) in the ovary. Endosymbionts are indicated with white arrows. cu: cuticle; hd: hypodermis; sm: somatic musculature; int: intestine; ut: uterus; ov: ovary; oo: oocyte; nu: nucleus; mt: mitochondria.



without much adaptation, and that the transferred genes can be transcriptionally active (Dunning-Hotopp et al., 2007). *Wolbachia*-like ESTs in the cDNA libraries are therefore not conclusive evidence of the presence of the symbiont itself. However, we were able to confirm the presence of an endosymbiont in *R. similis* by using different visualisation methods. DAPI staining and immunolocalisation showed signs of endosymbionts in the ovaries of adult females. Transmission electron microscopy confirmed the presence of endosymbionts surrounded by three membranes in the ovaries and in lower numbers in the uterus. This occurrence pattern is similar to that in filarial nematodes (Kramer et al., 2003), yet in our case no bacteria were found in the hypodermis. The endosymbionts could be clearly distinguished from other cell organelles such as mitochondria.

For different plant-parasitic nematodes, such as *H. glycines*, *G. rostochiensis*, *Bursaphelenchus xylophilus* and several *Meloidogyne* spp., substantial numbers of ESTs are available (over 10,000 per species). The fact that our approach could not identify *Wolbachia*-like sequences here most likely suggests that there is no *Wolbachia* present in these species. However it cannot be ruled out that other strains of these species do contain *Wolbachia*. Nonetheless, the occurrence of *Wolbachia* in plant-parasitic nematodes seems less widespread than in filarial nematodes.

Sequence analysis of the *ftsZ*, *groEL* and *16S rRNA* genes of the *R. similis* endosymbiont confirmed their similarity to *Wolbachia* genes from other hosts. However, the assumption that these genes are of bacterial origin should be made with caution because it cannot be ruled out that *Wolbachia*-like sequences have been integrated into the nematode host genome. An indication that these genes are indeed of bacterial origin is the average GC content (41%) which is lower than the average GC content of *R. similis* coding sequences (54%; Jacob et al., 2008). Moreover, the transcriptionally active genes (because those are present in ESTs) lack introns and have only a short untranslated region (UTR). The relatively high AT content could be one reason why bacterial transcripts emerged from the nematode cDNA library, since the oligo(dT) primer can bind to regions rich in adenine in addition to polyA tails of eukaryotic transcripts. It should however be noted that a recent horizontal transfer to the nematode genome would not yet reveal many eukaryotic features or changes in GC content. This was shown for *Drosophila ananassae*, where more than 90% nucleotide identity was found between the transferred fragment and the corresponding endosymbiont genes (Dunning-Hotopp et al., 2007). Nonetheless, with compelling microscopic evidence in support, it is clear that we are dealing with a full bacterial genome. After all, if the observed endosymbiont was not the origin of the sequences and was therefore not *Wolbachia*, we would expect other *16S rRNA* sequences to be amplified with the universal primers.

Phylogenetic analyses of the cloned sequences showed that the *Wolbachia* from *R. similis* does not cluster with one of the known supergroups. Independent analysis of the three genes confirmed its distant relationship to the other supergroups. Yet it still forms a monophyletic cluster with the other *Wolbachia* sequences, and shows less similarity to the closest known *Wolbachia* relatives *Ehrlichia* and *Anaplasma*. The relationships among the different supergroups in the phylogenetic tree should be handled with caution. It was recently shown that phylogenetic artifacts such as long branch attraction and the limitation of sequence models can lead to erroneous but highly supported tree reconstructions (Bordenstein et al., 2009). Nevertheless, it remains clear that the sequences from the *R. similis* *Wolbachia* are distantly related to sequences from the other supergroups. Based on the phylogenetic analysis, and the fact that a new host type (a plant-parasitic nematode) is involved, we propose the assignment of the endosymbiont of *R. similis* to a new *Wolbachia* supergroup, namely supergroup I. Baldo

et al. (2006) suggest that enough sequence information must be available to assign a *Wolbachia* strain to a new supergroup. Based on this constriction, Baldo et al. (2007) plead for the removal of supergroup G since it has only been based on the highly recombinant *wsp* gene and they propose a multilocus sequence typing (MLST) system to properly characterize *Wolbachia* strains based on five conserved genes. Despite the fact that only one of these (*ftsZ*) was found among the *Wolbachia* ESTs, we feel confident that in our specific case enough evidence is supplied to justify the establishment of a new supergroup, mainly because our phylogeny is based on three genes: *16S rRNA*, *groEL* and *ftsZ*. Nevertheless, it would be interesting to sequence the other genes of the MLST system for future comparisons. One might still argue that the identified endosymbiont is a different bacterial species closely related to *Wolbachia*. The phylogenetic analysis shows that the cloned sequences are quite distantly related to the other *Wolbachia* sequences, with *16S rRNA* similarities ranging from 93 to 97%. As a general rule in microbiology, a strain is considered as a new species when *16S rRNA* sequence similarity to its nearest neighbour is less than 97% (Janda and Abbott, 2007). Therefore the *Wolbachia* from *R. similis* could be considered as a different species. However, this 97% rule is not followed by the *Wolbachia* community due to unresolved relationships among the different supergroups. Recently, it has been suggested that the endosymbionts of filarial nematodes should be considered as a separate species (Pfarr et al., 2007). Nevertheless, even under the 97% criterion the question remains whether the *Wolbachia* strain of *R. similis* could be a member of the filarial nematode *Wolbachia* spp. group; strictly speaking, it should be considered as an independent *Wolbachia* sp. Attempts to clone the *Wolbachia* surface protein (*wsp*) failed, probably due to the high sequence divergence as opposed to other *Wolbachia* strains. This strengthens the idea that the use of the *wsp* gene is inappropriate for detection and phylogeny of *Wolbachia* as it is highly recombinant (Baldo and Werren, 2007).

Despite the apparent sequence divergence between *Wolbachia* strains, it is plausible that the strains have a common ancestor. Within the phylum Nematoda, animal parasitism and plant parasitism have originated independently multiple times (Blaxter et al., 1998). Both the filarial nematodes and some plant-parasitic nematodes including *R. similis* form a monophyletic clade, suggesting both groups had a common ancestor at some point in history (Holterman et al., 2006). If this ancestral nematode was infected with a *Wolbachia*-like endosymbiont, it is possible that this strain managed to survive in some species during evolution whereas it was lost in other nematodes. Perhaps the presence of *Wolbachia* is just an evolutionary relic from the common ancestor of plant-parasitic and animal-parasitic nematodes. In this respect, *Wolbachia* would have started to play an important role in animal-parasitic nematodes during evolution, whereas in plant-parasitic nematodes it became less important, accounting for its merely sporadic existence in only a few current species. An alternative hypothesis states that *Wolbachia* strains of filarial and plant-parasitic nematodes were acquired independently.

The role of *Wolbachia* in *R. similis* remains unknown. The high infection rate indicates that *R. similis* cannot survive without its endosymbiont, which is the case for all infected filarial nematodes. Additionally, it is present in another population and the closely related nematode species *R. arabocoffeae*. It is possible that *Wolbachia* provides its host with essential metabolites, as described for *B. malayi* (Foster et al., 2005). Whether it has any direct effect at all on reproduction could not be demonstrated; such effects have been reported for arthropods only. Although parthenogenesis and self-fertilisation have been described for *R. similis* (Brooks and Perry, 1962; Kaplan and Opperman, 2000), it is difficult to investigate whether *Wolbachia* plays a role in these processes.



The presence of *Wolbachia* in a plant-parasitic nematode is also interesting in the light of parasite evolution. For plant-parasitic nematodes, the origin of several genes involved in the parasitic interaction has been ascribed to horizontal gene transfer (Jones et al., 2005). In particular, genes encoding plant cell wall degrading enzymes have been suspected to be thus obtained. This hypothesis is based on the high similarity of these nematode sequences to prokaryotic proteins. The endobacteria in plant-parasitic nematodes could be considered as possible donors of horizontally transferred genes. Indeed, incorporation of pieces of the *Wolbachia* genome into the host genome has been proven for at least eight different host species (four insects and four nematodes), and these genes are transcriptionally active (Dunning-Hotopp et al., 2007). Although it seems very unlikely that cell wall degrading enzymes find their origin in *Wolbachia*, these genes could be traces of another, long lost endosymbiont. As such it would be interesting to investigate whether pieces of the *Wolbachia* genome have been inserted into the *R. similis* genome. Should this be the case, then it can serve as a model for how plant-parasitic nematodes may obtain genes from prokaryotes.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2009.01.006.

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