'*Candidatus Paenicardinium endonii*', an endosymbiont of the plant-parasitic nematode *Heterodera glycines* (Nemata: Tylenchida), affiliated to the phylum *Bacteroidetes*

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Bacteria-like endosymbionts of females of the plant-parasitic nematodes *Globodera rostochiensis* and *Heterodera goettingiana* and juveniles of *Heterodera glycines* were first observed during transmission electron microscopy (TEM) studies conducted in the 1970s. These organisms were characterized as being rod-shaped, ranging in size from 0.3 to 0.5 µm in diameter and 1.8 to 3 µm in length and containing structures labelled as striated inclusion bodies or tubular structures. A population of *H. glycines* was obtained from the soybean field where infected nematodes were first discovered in order to conduct TEM studies of females and males and to determine the phylogenetic position of the *H. glycines* endosymbiont among bacteria by studying the 16S rRNA and *gyrB* gene sequences. The bacterium was observed in the pseudocoelom and intestine of juveniles, females and males, in hypodermal chords of juveniles and males, in ovary walls and in oocytes and spermatozoa. The bacterium was polymorphic, measuring 0.4–0.8 × 2.5–4.5 µm, and many specimens contained an array of microfilament-like structures similar to those observed in '*Candidatus Cardinium hertigii*', the endosymbiont of *Encarsia* spp. wasps. Phylogenetic analysis of the 16S rRNA and *gyrB* genes of the *H. glycines*-infecting bacterium revealed 93% and 81% sequence identity, respectively, to the homologous genes in '*Candidatus C. hertigii*'. Thus, the name '*Candidatus Paenicardinium endonii*' is proposed for the bacterial endosymbiont of the plant-parasitic nematode *H. glycines*.

INTRODUCTION

The occurrence of bacterial endosymbionts in invertebrates is well documented and, among the endosymbionts of nematodes, the genus *Wolbachia* is perhaps the most studied (Taylor, 2003). *Wolbachia* species are associated with the animal-parasitic nematodes *Brugia malayi* and *Wuchereria bancrofti* (both Spiruvida: Filariidae), which cause lymphatic filariasis and are vectored by mosquitoes, and *Onchocerca volvulus* (Spiruvida: Onchocercidae), which causes onchocerciasis or river blindness and is vectored by black flies (Taylor, 2003; Saint André *et al.*, 2002). *Wolbachia* species are believed to be the primary cause of acute inflammatory filarial disease and the inflammatory response of the cornea.

Abbreviation: MLS, microfilament-like structure.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *gyrB* sequences of '*Candidatus Paenicardinium endonii*' are DQ314214 and DQ314215.

Maximum-likelihood trees based on 16S rRNA gene and *gyrB* sequences showing the position of '*Candidatus Paenicardinium endonii*' within the *Bacteroidetes* are available as supplementary material in IJSEM Online.

Members of the nematode genera *Steinernema* and *Heterorhabditis* have a mutualistic association with endosymbiotic bacteria of the genus *Xenorhabdus* in which the nematode host vectors the bacterium into the haemocoel of the insect host, where the bacterium is deposited in the haemolymph, multiplies and kills the insect by inducing septicaemia (Kaya, 1993). Another group of nematode endosymbionts includes three candidate verrucomicrobial species of '*Candidatus Xiphinematobacter*' associated with three species of plant-parasitic nematodes of the genus *Xiphinema* (Dorylaimida: Longidoridae) (Vandekerckhove *et al.*, 2000). These nematodes reproduce by thelytokous parthenogenesis and the '*Candidatus Xiphinematobacter*' organisms are inherited maternally. Another group of bacterial endosymbionts, which have characteristic microfilament-like structures (MLSs) similar to those of '*Candidatus Cardinium hertigii*', infect the cyst-forming, plant-parasitic nematodes *Globodera rostochiensis* (potato cyst nematode), *Heterodera glycines* (soybean cyst nematode) and *Heterodera goettingiana* (pea cyst nematode) (all Tylenchida: Heteroderidae). These endosymbionts were first observed in females and males of two populations of *G. rostochiensis*

and one population of *H. goettingiana* during transmission electron microscopy (TEM) studies (Shepherd *et al.*, 1973) and subsequently in *H. glycines* juveniles (Endo, 1979). The endosymbionts have not been encountered in other populations of these species or in other genera of nematodes. Unlike their allies that infect arthropods (Zchori-Fein *et al.*, 2004), the cyst-nematode-infecting endosymbionts are not known to affect host preference or reproduction or to cause other pathological manifestations (Shepherd *et al.*, 1973).

The objectives of our study were twofold. The first was to confirm observations of infection of second-stage juveniles (J2) of *H. glycines* and to identify sites of infection of the endosymbiont in females and males. The second objective was to characterize the endosymbiont and to determine its phylogenetic position within the bacterial domain by using 16S rRNA and *gyrB* gene sequences.

METHODS

Culture of *H. glycines*. Soil infested with *H. glycines* was obtained from the Ames Plantation near Grand Junction, TN, USA. This population was obtained from the same field as the population studied by Endo (1979). Cysts (dead bodies of females containing eggs) were extracted from soil and added to pots containing steam-sterilized sand in which soybean seeds were planted. The cultures have been maintained in a greenhouse for many years by repeating the extraction and replanting process as needed.

Electron microscopy. Male nematodes were obtained from infected soybean roots placed in a mist chamber. Vigorous individuals were selected with the aid of a dissecting microscope and placed in distilled water in a BPI dish. The water was drawn off with a micropipette and replaced with cold 2% glutaraldehyde in 0.08 M sodium phosphate buffer, pH 7.2. Nematodes were cut in half, fixed for 1 week and rinsed with buffer. Heads and tails were placed in separate vials and post-fixed overnight in 1% osmium tetroxide. Sections were selected individually, placed in triangular slits of excised agar approximately the size of the tip of a beam capsule and then covered with warm agar. Agar blocks containing nematode pieces were excised, dehydrated in an ethanol series (10, 25, 50, 75, 95 and 100%) and placed in a 50% solution of ethanol and *n*-butyl glycidyl ether (BGE) for 1 h. The 50% solution was replaced with 100% BGE and held overnight under vacuum. The sections were infiltrated under vacuum for several hours with 50, 75 and 100% Quetol 654 (Electron Microscopy Sciences). Specimens were placed in fresh 100% Quetol 654 and infiltrated overnight under vacuum and then embedded in Quetol 654 for 72 h at 70 °C. Females and J2 were prepared as for males except that nematodes were not cut. Ultrathin sections (90–100 nm) were cut with an ultramicrotome (Reichert) and mounted on uncoated grids. Sections were stained with aqueous saturated uranyl acetate for 40 min and with Sato's triple lead stain (Hanaichi *et al.*, 1986) for 1 min. Sections were observed and photographed with a Hitachi H-600 transmission electron microscope.

PCR amplification, cloning and sequencing of the 16S rRNA and *gyrB* genes. J2 presumably infected with the bacterial endosymbiont were hatched from eggs and rinsed by repeated centrifugation in sterile water. Nematodes were pipetted onto a column of sterile, moist silica and allowed to migrate through the 5-cm-long column into a reservoir of sterile water. Nematodes were concentrated by centrifugation under sterile conditions and used for extraction of DNA as described by Atibalentja *et al.* (2004). Briefly, several

thousand nematodes in 200 µl sterile water were added to an equal volume of acid-washed 0.150–0.212 mm diameter glass beads (Sigma-Aldrich) in a screw-capped vial and subjected to vibration in a Mini-BeadBeater (BiosPec Products) at 5000 r.p.m. for 2 min. The nematode plus silica slurry was centrifuged at 16 000 *g* for 5 min and the supernatant was transferred into a 1.6 ml microfuge tube and stored at –20 °C. Combinations of universal primers 27f/1492r (Atibalentja *et al.*, 2004; Lane, 1991) and UP-1/UP-2r (Yamamoto & Harayama, 1995) were used for PCR amplification of the 16S rRNA and *gyrB* genes, respectively, following procedures described previously (Atibalentja *et al.*, 2004), except that the annealing temperature for the *gyrB* gene was set at 56 °C. Cloning and sequencing of PCR products were also conducted as reported previously (Atibalentja *et al.*, 2004). Both DNA strands were sequenced from each of three and four clones, respectively, for the 16S rRNA and *gyrB* genes, and the resulting DNA fragments were assembled with the computer program SEQUENCHER (Gene Codes).

Phylogenetic analysis. The consensus DNA sequences for the 16S rRNA and *gyrB* genes were subjected to sequence-similarity searches, using the BLAST algorithm (Altschul *et al.*, 1997). The ORF finder utility of GenBank also was used to identify the putative open reading frame of the *gyrB* gene, and the deduced amino acid sequence was also submitted to BLAST. Homologous sequences from a selection of bacteria suggested by the BLAST results were retrieved from public databases and aligned with the corresponding sequence from the endosymbiont of *H. glycines*, using CLUSTAL X (Thompson *et al.*, 1997). The alignments were edited manually to remove terminal and internal gap-rich regions. Phylogenetic analyses were conducted with PAUP* 4.0b10 (Swofford, 2003) under the maximum-parsimony (MP), maximum-likelihood (ML) and distance optimality criteria, following the procedures described by Zchori-Fein *et al.* (2004) unless indicated otherwise. Specifically, the HKY85 distance and the weighted least-squares objective function were used to construct the neighbour-joining (NJ) tree, which was used to estimate the parameters for ML reconstruction under a general time reversible (GTR) model of nucleotide substitution, with rate heterogeneity among sites (Γ) and a given proportion of invariable sites (I). The robustness of the inferred clades was assessed through bootstrap analysis, which, for MP and NJ trees, involved 10 000 replicates (of 100 random-addition sequence replicates each for MP). For ML trees, bootstrap analysis involved 1000 replicates of 10 random-addition sequence replicates each for the 16S rRNA gene and 100 replicates of one random-addition sequence replicate each for the *gyrB* gene.

We also sought to identify signature sequences for the 16S rRNA gene of the *H. glycines* endosymbiont, using a slight modification of the method described by Zchori-Fein *et al.* (2004). Briefly, candidate oligonucleotides suggested by MacClade (Maddison & Maddison, 2001) were submitted to probe match in the Ribosomal Database Project (RDP II Release 9, <http://rdp.cme.msu.edu/html>) (Cole *et al.*, 2003) and to BLAST search for short, nearly exact matches in GenBank only after they were selected by PRIMROSE (Ashelford *et al.*, 2002) as oligonucleotide probes that exclusively targeted the 16S rRNA gene sequence of the *H. glycines* endosymbiont.

RESULTS AND DISCUSSION

Morphology

The endosymbiont was observed in the pseudocoelom of J2, females and males. Additionally, bacteria were observed in oocytes, ovaries, spermatozoa and the intestine of both genders (Fig. 1). For the most part, our observations confirmed those of Shepherd *et al.* (1973), who reported

Fig. 1. Electron micrographs of 'Candidatus Paenicardinium endonii' in *H. glycines* males and females. (a) Longitudinal section of a male in which the presence of bacteria in the intestine (Int), pseudocoelom (Pc) and spermatzoa within a testis (T) is indicated by arrows. The bacterium was not observed in somatic muscles (Sm), the hypodermis (Hd) or the cuticle (C). (b) Higher magnification of bacteria (arrows) in spermatzoa. MLSs are indicated by arrowheads. (c) Presence of bacteria in ovary and oocytes is indicated by arrows. (d) Bacteria in pseudocoelom of female. MLSs are indicated by arrowheads. Bars, 5 µm (a), 1 µm (b, d) and 3 µm (c).

that the bacterium was most common in reproductive tissues of females including the ovary, oviduct wall and oocytes. We did not observe bacteria in the hypodermis of females, but found them in the hypodermal chords of males. Our study of J2 was done to confirm the presence of the bacterium in the second-stage infective juveniles. Bacteria were observed in the hypodermal chords, intestinal tissue and pseudocoelom. The research of Endo (1979) was an exhaustive study of the oesophageal region of infective juveniles. Bacteria observed in that study were most abundant in the sarcoplasm of stylet protractor muscles and muscles of the median bulb and the oesophageal glands. In our study, the bacterium was polymorphic in appearance due to the random orientation of individuals within nematode tissues and cells and sectioning of bacteria that often was tangential, especially along the length of the organisms. We found that the diameter of the bacteria ranged from 0.4 to 0.8 µm and the length ranged from 2.5 to 4.5 µm. These values are similar to those reported by Endo (1979), but greater than the values reported by Shepherd *et al.* (1973) for the endosymbionts in the nematodes *G. rostochiensis* and *H. goettingiana* and for 'Candidatus *C. hertigii*' (Zchori-Fein *et al.*, 2004). MLSs were not observed in all bacteria (Fig. 2). It is unclear whether they were absent or in a plane or section that was not studied (Zchori-Fein *et al.*, 2004). In our study

and previous studies, some MLSs extended across the width of the cell, whereas some extended part way. The width of MLSs that we determined (17 nm) was similar to that of Endo (1979), but wider than the 14–15 nm reported by Shepherd *et al.* (1973). These differences might be due to artefacts of fixation and embedding or calibration of instruments. We observed what appeared to be bundles of MLSs in cross section (Fig. 2). However, cell walls were not apparent with these structures. Endo (1979) observed a cross section of MLSs inside a cell with a clearly defined cell wall.

Sequence and phylogenetic analysis

16S rRNA gene. PCR amplification, using the universal primers 27f and 1492r, of crude DNA extracts prepared from *H. glycines* J2 harbouring the unidentified bacterial endosymbiont resulted in a single band of the expected size (1.5 kb). Cloning and sequencing of the gel-eluted DNA fragment yielded a 1490 bp DNA sequence (G + C content 50.1 mol%) that shared 94 % nucleotide identity with the 16S rRNA gene of the endosymbiont of *Meta-seiulus occidentalis* (GenBank accession no. AY635291), the best fit returned by BLAST searches, and 93 % identity with 'Candidatus *C. hertigii*' (accession no. AY331187). The alignment of 16S rRNA gene sequences used to infer

Fig. 2. Electron micrograph of '*Candidatus Paenicardinium endonii*' in an *H. glycines* male. MLSs are indicated by arrows. Two possible cross sections of MLSs appear as bundles of microfilaments (arrowheads). Bar, 0.5 μ m.

the phylogeny of the *H. glycines* endosymbiont included 12 taxa and 1467 characters, 1208 of which were constant and 173 of which were parsimony-informative. MP analysis produced two most-parsimonious trees, each 369 steps in length (consistency index 0.79). The two MP trees placed the *H. glycines* endosymbiont in a lineage that had diverged much earlier from the common ancestor of acari and insect endosymbionts, a finding that was also supported by the NJ and ML trees (Fig. 3 and Supplementary

Fig. S1 available in IJSEM Online). Using the NJ tree under the ML criterion, the nucleotide substitution parameters were estimated as follows: A–C, 1.96; A–G, 7.26; A–T, 1.33; C–G, 0.08; C–T, 10.25; and G–T, 1.00. The proportion of invariant sites (I) was estimated at 0.56 and the shape parameter (α) of the gamma distribution was equal to 0.71.

Of the two oligonucleotide sequences previously reported as unique to '*Candidatus Cardinium*' (Zchori-Fein *et al.*, 2004), only one, 5'-GGTCTTTAACTGACGCT-3', is present in the 16S rRNA gene sequence of the *H. glycines* endosymbiont (position 728). This oligonucleotide did not meet the PRIMROSE selection criteria for a probe (Ashelford *et al.*, 2002). In contrast, we have identified two oligonucleotide sequences, 5'-CGCGATACAATCGTGTGTGTCC-3' (position 822) and 5'-CATCCCTAGAAATAGGGAGTTCCGA-AA-3' (position 991), that are unique to the *H. glycines* endosymbiont.

gyrB gene. A single band of the expected size also was obtained when DNA extracts from *H. glycines* J2 carrying the unidentified bacterium were amplified by PCR using the primers UP-1 and UP-2r. Cloning and sequencing of the resulting DNA fragment produced a 1237 bp sequence (G+C content 41.7 mol%) that contained a 397 aa partial ORF. BLAST searches using the DNA sequence or the deduced amino acid sequence of the partial ORF retrieved the *gyrB* gene of the '*Candidatus Cardinium*' endosymbiont of *Encarsia pergandiella* (GenBank accession nos. AY332002 and AAQ98790) as the best match with the query sequence, sharing 83 and 81% nucleotide and amino acid identity, respectively. Phylogenetic analyses using either the nucleotide or the amino acid sequence of the *gyrB* gene produced similar results for the position of the *H. glycines* endosymbiont. Therefore, the following

Fig. 3. NJ tree of the bacterial endosymbiont of *H. glycines*, '*Candidatus Paenicardinium endonii*', based on HKY85 distances among 16S rRNA gene sequences from selected members of the phylum *Bacteroidetes*. The tree was rooted using two isolates of '*Candidatus Amoebophilus asiaticus*' and an endoparasite of *Acanthamoeba* sp. as outgroup taxa. The NJ tree is similar to the two trees produced by the MP analysis; numbers above branches indicate bootstrap support (%) from MP analysis, whereas numbers below branches show bootstrap support (%) for the NJ topology. Bar, 0.01 substitutions per site.

discussion focuses only on the DNA-based analyses. The alignment of *gyrB* gene sequences used comprised 17 taxa and 861 characters, of which 263 were constant and 524 were parsimony-informative. MP analysis yielded two most-parsimonious trees, each 2742 steps long (consistency index 0.43). As was the case with the 16S rRNA gene, the two MP trees not only agreed between themselves but they also agreed with the NJ and the ML trees by placing the *H. glycines* endosymbiont in a lineage that had diverged earlier from the branch leading to the bacteria in the recently proposed 'Candidatus Cardinium' (Fig. 4 and Supplementary Fig. S2). Apparently, the speciation event was followed by a more rapid evolution in the branch containing the *H. glycines* endosymbiont than in the one leading to the cardinia. Estimates of nucleotide substitution parameters under the ML criterion, based on the NJ tree, were as follows: A–C, 2.36; A–G, 3.75; A–T, 1.20; C–G, 2.17; C–T, 7.66; and G–T, 1.00. The proportion of invariant sites (I) was estimated at 0.26 and the shape parameter (α) of the gamma distribution was equal to 1.27.

Proposal of 'Candidatus Paenicardinium endonii'

The name *Paenicardinium* (Pae.ni.car.di'ni.um. L. adv. *paene* nearly, almost; N.L. n. *Cardinium* the name of an organism in the *Candidatus* category; N.L. n. *Paenicardinium* nearly *Cardinium*) is chosen to acknowledge the morphological similarity between 'Candidatus Paenicardinium' and 'Candidatus Cardinium', both of which contain characteristic arrays of MLSSs. The specific epithet *endonii* (en.do'ni.i. N.L. gen. masc. n. *endonii* of Endo) is chosen to honour the memory of Dr Burton Yoshiaki Endo, a colleague and friend, who was the first to study this organism.

The status of *Candidatus* is required by the International Committee on Systematics of Prokaryotes for prokaryotic organisms that are not culturable and thus cannot be characterized according to the *International Code of Nomenclature of Bacteria* (Murray & Stackebrandt, 1995). 'Candidatus Paenicardinium endonii' is Gram-negative and rod-shaped and is classified in the phylum *Bacteroidetes*, class *Sphingobacteria*, order *Sphingobacteriales* and family 'Flexibacteraceae' (Garrity & Holt, 2001).

Two 16S rRNA gene oligonucleotide sequences were reported as unique to 'Candidatus Cardinium' (Zchori-Fein *et al.*, 2004), but only the sequence 5'-GGTCTTTAA-CTGACGCT-3' occurs in the *H. glycines* endosymbiont (position 728). Two additional oligonucleotide sequences, 5'-CGCGATACAATCGTGTGTGTCC-3' (position 822) and 5'-CATCCCTAGAAATAGGGAGTTCCGAAA-3' (position 991), are unique to the bacterium that infects *H. glycines* and, therefore, are diagnostic of 'Candidatus Paenicardinium endonii'. The G + C content of the 16S rRNA gene of the bacterium is 52 mol%, compared with 48 mol% for 'Candidatus C. hertigii' (Zchori-Fein *et al.*, 2004). 'Candidatus Paenicardinium endonii' shares 94 % 16S rRNA gene nucleotide sequence identity with the endosymbiont of *Metaseiulus occidentalis* and 93 % identity with 'Candidatus C. hertigii'. The *gyrB* nucleotide sequence shares 83 % identity with the 'Candidatus Cardinium' endosymbiont of *Encarsia pergandiella* and 81 % identity with 'Candidatus C. hertigii'. These levels of identity for the 16S rRNA and *gyrB* genes are indicative of a distinct taxon (Ludwig *et al.*, 1998). The name 'Candidatus Paenicardinium endonii' is proposed on the basis of the 16S rRNA gene sequence (GenBank accession no. DQ314214), the *gyrB* gene sequence (accession no. DQ314215), the signature sequences listed above and the presence of MLSSs. The bacterium found in the population of *H. glycines* from the Ames Plantation near Grand Junction, TN, USA, is the proposed type strain.

Fig. 4. NJ tree of the bacterial endosymbiont of *H. glycines*, 'Candidatus Paenicardinium endonii', based on HKY85 distances among *gyrB* gene sequences from selected members of the phylum *Bacteroidetes*. The tree was rooted using the bacterium *Chitinophaga pinensis* IFO 15969 as the outgroup taxon. The NJ tree is similar to the two trees produced by MP analysis; numbers above branches indicate bootstrap support (%) from MP analysis, whereas numbers below branches show bootstrap support (%) for the NJ topology. Bar, 0.1 substitutions per site.

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REFERENCES

- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Ashelford, K. E., Weightman, A. J. & Fry, J. C. (2002). PRIMROSE: a computer program for generating and estimating the phylogenetic range of 16S rRNA oligonucleotide probes and primers in conjunction with the RDP-II database. *Nucleic Acids Res* **30**, 3481–3489.
- Atibalentja, N., Noel, G. R. & Ciancio, A. (2004). A simple method for PCR-amplification, cloning, and sequencing of *Pasteuria* 16S rDNA from small numbers of endospores. *J Nematol* **36**, 100–105.
- Cole, J. R., Chai, B., Marsh, T. L. & 8 other authors (2003). The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* **31**, 442–443.
- Endo, B. Y. (1979). The ultrastructure and distribution of an intracellular bacterium-like microorganism in tissues of larvae of the soybean cyst nematode, *Heterodera glycines*. *J Ultrastruct Res* **67**, 1–14.
- Garrrity, G. M. & Holt, J. G. (2001). The road map to the *Manual*. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 119–166. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrrity. New York: Springer.
- Hanaichi, T., Sato, T., Iwamoto, T., Malavasi-Yamashiro, J., Hoshino, M. & Mizuno, N. (1986). A stable lead by modification of Sato's method. *J Electron Microsc* **35**, 304–306.
- Kaya, H. K. (1993). Entomogenous and entomopathogenic nematodes in biological control. In *Plant Parasitic Nematodes in Temperate Agriculture*, pp. 565–591. Edited by K. Evans, D. L. Trudgill & J. M. Webster. Wallingford, UK: CAB International.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. New York: Wiley.
- Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. & Schleifer, K. H. (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**, 554–568.
- Maddison, D. R. & Maddison, W. P. (2001). MacClade version 4.0.1. Sunderland, MA: Sinauer Associates.
- Murray, R. G. E. & Stackebrandt, E. (1995). Taxonomic note: implementation of the provisional status *Candidatus* for incompletely described procaryotes. *Int J Syst Bacteriol* **45**, 186–187.
- Saint André, A. V., Blackwell, N. M., Hall, L. R. & 9 other authors (2002). The role of endosymbiotic *Wolbachia* bacteria in the pathogenesis of river blindness. *Science* **295**, 1892–1895.
- Shepherd, A. M., Clark, S. A. & Kempton, A. (1973). An intracellular micro-organism associated with tissues of *Heterodera* spp. *Nematologica* **19**, 31–34.
- Swofford, D. L. (2003). PAUP* – Phylogenetic Analysis Using Parsimony* and other methods, version 4. Sunderland, MA: Sinauer Associates.
- Taylor, M. J. (2003). *Wolbachia* in the inflammatory pathogenesis of human filariasis. *Ann N Y Acad Sci* **990**, 444–449.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Vandekerckhove, T. T. M., Willems, A., Gillis, M. & Coomans, A. (2000). Occurrence of novel verrucomicrobial species, endosymbiotic and associated with parthenogenesis in *Xiphinema americanum*-group species (Nematoda, Longidoridae). *Int J Syst Evol Microbiol* **50**, 2197–2205.
- Yamamoto, S. & Harayama, S. (1995). PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl Environ Microbiol* **61**, 1104–1109.
- Zchori-Fein, E., Perlman, S. J., Kelly, S. E., Katzir, N. & Hunter, M. S. (2004). Characterization of a '*Bacteroidetes*' symbiont in *Encarsia* wasps (Hymenoptera: Aphelinidae): proposal of '*Candidatus Cardinium hertigii*'. *Int J Syst Evol Microbiol* **54**, 961–968.