Applied and Environmental Microbiology, Nov. 2009, p. 6757–6763 Vol. 75, No. 21
0099-2240/09/$12.00 doi:10.1128/AEM.01583-09
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Prevalence of *Cardinium* Bacteria in Planthoppers and Spider Mites and Taxonomic Revision of "*Candidatus* Cardinium hertigii" Based on Detection of a New *Cardinium* Group from Biting Midges▽†‡

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Received 5 July 2009/Accepted 28 August 2009

***Cardinium* bacteria, members of the phylum *Cytophaga-Flavobacterium-Bacteroides* (CFB), are intracellular bacteria in arthropods that are capable of inducing reproductive abnormalities in their hosts, which include parasitic wasps, mites, and spiders. A high frequency of *Cardinium* infection was detected in planthoppers (27 out of 57 species were infected). A high frequency of *Cardinium* infection was also found in spider mites (9 out of 22 species were infected). Frequencies of double infection by *Cardinium* and *Wolbachia* bacteria (*Alphaproteobacteria* capable of manipulating reproduction of their hosts) were disproportionately high in planthoppers but not in spider mites. A new group of bacteria, phylogenetically closely related to but distinct from previously described *Cardinium* bacteria (based on 16S rRNA and *gyrB* genes) was found in 4 out of 25 species of *Culicoides* biting midges. These bacteria possessed a microfilament-like structure that is a morphological feature previously found in *Cardinium* and *Paenicardinium*. The bacteria close to the genus *Cardinium* consist of at least three groups, A, B, and C. Group A is present in various species of arthropods and was previously referred to as "*Candidatus* Cardinium hertigii," group B is present in plant parasitic nematodes and was previously referred to as "*Candidatus* Paenicardinium endonii," and group C is present in *Culicoides* biting midges. On the basis of morphological and molecular data, we propose that the nomenclature of these three groups be integrated into a single species, "*Candidatus* Cardinium hertigii."**

Compared to the *Wolbachia* bacteria, which belong to the alpha subdivision of the phylum *Proteobacteria* and are known as master manipulators of arthropod reproduction (48), the *Cardinium* bacteria, which belong to the phylum *Cytophaga-Flavobacterium-Bacteroides* (CFB), are relatively new to biological study. The phylum CFB includes many other bacteria associated with arthropods, such as symbionts in cockroaches (3) and termites (4) and the male-killing agents of ladybird beetles (21). *Cardinium* was first observed in tick cell cultures as an unknown intracellular prokaryote that was rod shaped and had an array of tubes extending from the cytoplasmic membrane (22). In 2001, related symbiotic bacteria were reported as manipulators of arthropod reproduction because they caused feminization, by which genetic males were converted into phenotypic females, in the false spider mite *Brevipalpas obovatus* (45) and parthenogenesis, in which haploid eggs were converted into viable diploid females, in the parasitoid wasp *Encarsia pergandiella* (50). Since the 16S rRNA gene sequences of these bacteria exhibited 96% to 98% similarity to the tick microorganism, they were classified in the phylum CFB. Subsequently, bacteria in this group were found to induce cytoplasmic incompatibility (CI), in which uninfected female hosts produce few offspring when mated with infected males in parasitic wasps of the genus *Encarsia* (20) and in two spider mites, *Eotetranychus suginamensis* and *Bryobia sarothamni* (14, 35). These bacteria were arbitrarily called CFB or *Cytophaga*-like organisms in earlier studies until the scientific name of "*Candidatus* Cardinium hertigii" was proposed by Zchori-Fein et al. (52). Since then, the bacteria have often been referred to as *Cardinium* for convenience. Recently, a bacterium related to *Cardinium* was found in plant parasitic nematodes, for which the scientific name "*Candidatus* Paenicardinium endonii" was proposed (31).

Three independent studies have shown that rates of *Cardinium* infection were consistently low in wide samplings of arthropods, i.e., 7.2% of 223 species (46), 6% of 99 species (51), and 4.4% of 136 species (11). However, the infection frequencies in mites and spiders were 31.6% (46) and 22% (12), respectively. *Cardinium* has previously been detected only in hymenopteran insects (20, 25, 46, 50, 51), hemipteran insects (6, 24, 37, 46, 51), mites (13, 14, 15, 19, 45, 46), and spiders (11, 12). Infection by *Wolbachia*, another group of bacteria belonging to the *Alphaproteobacteria* that are capable of manipulating arthropod reproduction, is more widespread among arthropods. A recent meta-analysis of published data on *Wolbachia* infection surveys demonstrated that the proportion of insect species with at least one infected individual is around 66% (16). Other arthropods, such as wood lice, spiders, and mites,

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 4 September 2009.

‡ The authors have paid a fee to allow immediate free access to this article.

are also infected with *Wolbachia*. Outside of arthropods, *Wolbachia* infection has been detected in filarial nematodes (2, 23). Compared to *Wolbachia*, *Cardinium* organisms have been found in more restricted taxonomic groups (11, 46, 51).

In this study, we performed PCR-based screening of various species of planthoppers (Hemiptera: Fulgoroidea), spider mites (Acari: Tetranychidae), and *Culicoides* biting midges (Diptera: Ceratopogonidae) for *Cardinium* infection by using primers that detect bacteria closely related to *Cardinium*. The frequencies of *Cardinium* infection were considerably higher in planthoppers and spider mites. In *Culicoides* biting midges, which are important vectors of arthropod-borne viruses pathogenic to livestock (27), some species were infected with *Cardinium*-like bacteria that had lower nucleotide sequence similarity to other *Cardinium* species, including those previously found in arthropods. Morphological characteristics and molecular phylogenetic analyses of these bacteria are reported, and their taxonomic classification is reconsidered.

MATERIALS AND METHODS

Sample collection and DNA extraction. Fifty-seven species (59 geographic strains) of planthoppers were collected in the field in Japan. Two adult females per species were mixed and treated as one sample. Planthoppers preserved in TRIzol reagent (Invitrogen, Carlsbad, CA) were homogenized in 100 μl of sodium chloride-Tris-EDTA (STE) buffer (100 mM NaCl, 1 mM EDTA [pH 8.0], 10 mM Tris-HCl [pH 8.0]). Planthopper DNA was purified with phenol and precipitated with ethanol. Twenty-two species (30 strains) of spider mites were sampled from laboratory populations. Mites were reared on detached leaves of the common bean *Phaseolus vulgaris* or, when necessary, on detached leaves of the original host plants in a climate-controlled room (25°C, 16 h/8 h light/dark cycle, relative humidity of 60%). Ten adult females per species were mixed and treated as one sample. Biting midges were collected using light traps in 11 geographic locations in Japan over a 2-year period (January 2002 to October 2004) (26). *Amoebophilus asiaticus*, a symbiotic bacterium closely related to *Cardinium*, was used as an outgroup in phylogenetic analyses. A freeze-dried culture of *Acanthamoeba castellanii*, a host species of *A. asiaticus*, was obtained from the American Type Culture Collection (ATCC 30868) and was dissolved in 1 ml of sterilized water. Then, 25 μl of the resuspended culture was mixed with 25 μl of STE buffer and 5 μl of proteinase K (20 mg/ml) and incubated at 37°C for 30 min, followed by inactivation at 95°C for 5 min. DNA was extracted from spider mites, biting midges, and *A. castellanii* using a DNeasy tissue kit (Qiagen, Valencia, CA) by following the manufacturer's protocol.

PCR screening for *Cardinium* and *Wolbachia* infection. Based on the alignment of 16S rRNA gene sequences in the GenBank database of *Cardinium* obtained from 35 arthropod species, a set of primers, i.e., Car-sp-F (5′-CGG CTT ATT AAG TCA GTT GTG AAA TCC TAG-3′) and Car-sp-R (5′-TCC TTC CTC CCG CTT ACA CG-3′), which amplify a 544-bp product was designed for highly conserved regions. To detect *Wolbachia* infection, a *Wolbachia*-specific primer pair that amplifies an approximately 900-bp DNA fragment of the 16S rRNA gene (32) was used.

PCR amplification was performed in 20 μl of reaction buffer containing 1× buffer, 0.16 mM of each deoxynucleoside triphosphate, 0.5 mM of each primer, 0.5 U of *Taq* DNA polymerase (Takara Bio Inc., Tokyo, Japan), and 2 μl of template DNA. DNA of *Cardinium* from *Ixodes scapularis* (22) was used as a positive control. The PCR conditions were as follows: 1 cycle of 95°C for 1 min; 35 cycles of 95°C for 30 s, 57°C (except 52°C for *Culicoides*) for 30 s, and 72°C for 1 min; and final extension at 72°C for 5 min. In order to validate the prepared templates, the mitochondrial cytochrome oxidase 1 (*cox1*) gene region was amplified in planthoppers (using primers COI-F [5′-ATA GCN TTY CCW CGA ATA AAT AAY ATA AGA TTY TG-3′] and COI-R [5′-GTT GRG GAA ARA AKG TTA AAT TTA CNC CNR NRA ATA-3′]) and in spider mites (29). The *cox1* and *cox2* regions were amplified in biting midges (26). All PCR products of the 16S rRNA gene from *Cardinium*-positive samples were cloned, and at least three clones were sequenced.

Sequencing. Typically, the full-length bacterial 16S rRNA gene (approximately 1,444 to 1,448 bp) was amplified from the DNA in *Cardinium*-positive samples by performing PCR using the fD1 and rP2 primers (47), and the PCR products were cloned and sequenced (26). A fragment of the *Cardinium* gyrase B subunit (*gyrB*) gene (1,267 bp) from planthoppers was amplified by using the primer pair Car gyrB2F (5′-GGK GTY TCB TGT GTA AAT GC-3′) and Car gyrB2R (5′-TAS TGY TCT TCT TTR TCT CG-3′). A fragment of the *Cardinium gyrB* gene (1,389 bp) from spider mites was amplified using the primer pair Car gyrB1F (5′-CAA AGA YAC CTA TAA RAT TTC TG-3′) and Car gyrB1R (5′-GTA ACG TTG TAC ARA KAC RGC AT-3′). Fragments of the *Cardinium gyrB* genes from biting midges and *A. asiaticus* (1,432 bp and 1,432 bp, respectively) were amplified using the primer pair UP-21 and 2Tr-SR1 (41). The temperature profile of the PCR was 1 cycle of 95°C for 1 min; 35 cycles of 95°C for 30 s, 52°C for 30 s (or 1 min), and 72°C for 1.5 min; and a final extension at 72°C for 5 min. Five clones per sample were usually sequenced, using the 3700 DNA analyzer (Applied Biosystems, Foster City, CA).

Phylogenetic analyses. The phylogenetic relationship of *Cardinium* bacteria from planthoppers, spider mites, and biting midges to related bacteria was analyzed using the nucleotide sequences of the 16S rRNA and *gyrB* genes. *A. asiaticus* was used as an outgroup. The sequences were aligned using Clustal X software (44). Gaps were treated as a fifth state. Neighbor-joining (NJ) analyses for 16S rRNA and *gyrB* genes were conducted using PAUP* version 4.0b10 software (42) under the Hasegawa-Kishino-Yano 85 distance model. Bootstrap replicates were calculated 1,000 times. Maximum-likelihood (ML) analyses were also conducted for the 16S rRNA gene. MODELTEST 3.7 software (34) was used to estimate the most appropriate substitution model for ML analyses. The Akaike information criterion model chosen for the 16S rRNA gene was general time reversible (GTR) + G (gamma distribution shape parameter = 0.6597) + I (including invariable site), Pinvr = 0.6157. Bootstrap pseudoreplications were performed 500 times.

Analysis of nucleotide variation. Numbers of polymorphic sites, nucleotide differences (*k*) (43), and nucleotide diversity (Pi) (30) within and among *Cardinium* groups were examined for a 1,442-bp sequence of the 16S rRNA gene and a 858-bp sequence of the *gyrB* gene by using DnaSP 4.5 software (36). The sequences were identical to those used in phylogenetic analyses, except for *Ixodes Cardinium*, which was excluded from the analysis of nucleotide variation because the sequence contained an unknown base. For the analysis of the *gyrB* gene, only one of the three sequences of *Tetranychus urticae* (red form A) was used.

Electron microscopy. The morphology of *Cardinium* was observed in the planthopper *Sogatella furcifera*; three species of spider mites, *Eotetranychus suginamensis*, *Panonychus mori*, and *Tetranychus pueraricola*; and the biting midge *Culicoides lungchiensis* by transmission electron microscopy. Ovaries of the planthoppers and whole bodies of the female spider mites and biting midges were fixed with a solution of 0.8% glutaraldehyde (Nakalai Tesque, Kyoto, Japan) and 1% paraformaldehyde in 0.06 M phosphate buffer for 1 to 2 h on ice. After brief rinsing with the same buffer, postfixation was performed with 2% osmium tetroxide for 1 h at room temperature, followed by dehydration with a graded ethanol series. The samples were replaced into propylene oxide and embedded in Spurr resin (Polysciences, Warrington, PA). Semithin sections were stained with 2% uranyl acetate and Sato's lead solution and observed under a JEM-1010 transmission electron microscope (JEOL, Tokyo).

RESULTS

Distribution of *Cardinium* bacteria among planthoppers, spider mites, and biting midges. The PCR screening using *Cardinium*-specific primers revealed that 27 out of 57 species (27 out of 59 strains) of planthoppers were positive for *Cardinium* infection (47.4% of the species and 45.8% of the strains) (see Table S1 in the supplemental material). Conversely, the PCR screening using *Wolbachia*-specific primers revealed that 29 out of 55 species (30 out of 57 strains) were infected with *Wolbachia* (52.7% of the species and 52.6% of the strains) (see Table S1 in the supplemental material). Double infection by *Cardinium* and *Wolbachia* was found in 18 species (18 strains) of planthoppers (32.7% of the species and 31.6% of the strains).

Cardinium infections were also widespread in spider mites. Out of 22 species (30 strains), 9 (11 strains) were positive for *Cardinium* infection (40.9% of the species and 36.7% of the strains) (see Table S2 in the supplemental material). In contrast, *Wolbachia* bacteria were detected in seven species (nine

strains) (31.8% of the species and 30.0% of the strains). Three species (three strains) of spider mites were doubly infected with *Cardinium* and *Wolbachia* (13.6% of the species and 10.0% of the strains).

In *Culicoides* biting midges, 4 out of 25 species were positive (16.0%) (see Table S3 in the supplemental material). On the other hand, only *C. paraflavescens* out of 25 species was infected with *Wolbachia* (4.0%), but this species was negative for *Cardinium*. Among four *Cardinium*-positive species, three (*C. arakawae*, *C. ohmorii*, and *C. peregrinus*) had high infection frequencies, since all the examined individuals were positive (16/16, 4/4, and 3/3, respectively). In contrast, in the case of *C. lungchiensis*, only 2 out of 10 individuals were positive for *Cardinium* infection.

16S rRNA gene. The bacterial 16S rRNA genes derived from three planthoppers (*Euides speciosa*, *S. furcifera*, and *Harmalia sirokata*) and four biting midges (*C. arakawae*, *C. lungchiensis*, *C. ohmorii*, and *C. peregrinus*) were sequenced. These sequences were analyzed together with the previously published sequences of *Cardinium* 16S rRNA genes derived from five spider mites (*Amphitetranychus quercivorus*, *E. suginamensis*, *Oligonychus ilicis*, *T. puearicola*, and *T. urticae*) (14). Four features, namely, the size (between fD1 and rP2 primers), G+C content, nucleotide similarity to the type strain *C. hertigii*, and identity to unique *Cardinium* sequences previously published by Zchori-Fein et al. (52), were examined for these sequences. First, the size of the 16S rRNA gene was 1,447 bp in planthoppers and spider mites, which was the same as that of 16S rRNA genes of *Cardinium* bacteria derived from other arthropods. Conversely, all the sequences of the bacteria derived from *Culicoides* were 1 bp larger (1,448 bp) than those from other *Cardinium* bacteria. The 16S rRNA gene of *A. asiaticus* was 1,444 bp, which was 4 and 3 bp shorter, respectively, than those of the *Cardinium* bacteria derived from *Culicoides* and from other arthropods. Second, bacterial 16S rRNA genes derived from planthoppers, spider mites, and biting midges consistently exhibited a G+C content of 49 mol%, which was exactly the same level for all *Cardinium* bacteria previously found in arthropods. Third, the nucleotide similarity shared with *C. hertigii* was 96% in planthoppers, 96% in spider mites (except for 97% in *T. urticae*), and 94% in biting midges. Fourth, these sequences were examined for identity with two unique *Cardinium* sequences, GCG GTG TAA AAT GAG CGTG and GGT CTT TAA CTG ACG CT, which are shared by *Cardinium* bacteria derived from *Encarsia* wasps, *Brevipalpus* mites, and *Ixodes* ticks (52). The bacteria detected from planthoppers and spider mites had identities in both sequences, but bacteria detected from *Culicoides* had nucleotide substitutions in both sequences; they exhibited 1- or 2-bp substitutions in the former sequence and 1-bp substitutions in the latter sequence. Moreover, bacteria found from *Culicoides* did not have either of the two sequences (CGC GAT ACA ATC GTG TGT GTCC and CAT CCC TAG AAA TAG GGA GTT CCG AAA) that are unique to *Paenicardinium* (31). They exhibited 9- or 10-bp substitutions in the former sequence and 12-bp substitutions in the latter sequence. Based on the differences in 16S rRNA gene sequence, *Cardinium*-like bacteria can be categorized into three groups: group A (previously named *Cardinium*), which has been found widely in arthropods; group B (previously named *Paenicardinium*), which has been found in plant-parasitic nematodes; and group C, which has been found in *Culicoides* biting midges.

***gyrB* gene.** The fragments of the bacterial *gyrB* gene (858 bp) derived from planthoppers (*E. speciosa* and *Indozuriel dantur*), spider mites (*A. quercivorus*, *E. suginamensis*, *O. ilicis*, *T. puearicola*, and *T. urticae*), and biting midges (*C. arakawae* and *C. ohmorii*) were aligned with that of *C. hertigii*. The G+C content was 41 mol% in both planthoppers, 40 to 42 mol% in the five spider mites and 40 mol% in the two biting midges. Nucleotide similarities shared with *C. hertigii* were 85% in planthoppers, 84% in spider mites (except for 87% in *T. urticae*), and 79% in biting midges.

Nucleotide variations. Molecular variation within sets of *Cardinium*-like bacteria containing group A bacteria, group B bacteria, and group C bacteria in various combinations was examined for 16S rRNA (1,442 bp) and *gyrB* (858 bp) gene sequences (see Table S4 in the supplemental material). Nucleotide variation in the 16S rRNA gene was highest for both nucleotide differences (*k*, 43.93) and nucleotide diversity (Pi, 0.03046) in the set that contained group A and C bacteria. In the *gyrB* gene, *k* was highest in the set that contained group A and B bacteria (216.70), whereas Pi was highest in the set that contained group B and C bacteria (0.19075).

Phylogenetic analyses based on molecular data. In order to elucidate the phylogenetic relationships of group A, B, and C bacteria, phylogenetic analyses were performed based on the nucleotide sequences of 16S rRNA genes (1,447 or 1,448 bp) (see Fig. S1 in the supplemental material) and the amino acid sequence of GyrB (286 amino acids [aa]). In the NJ tree based on 16S rRNA gene sequences, group C bacteria formed a monophyletic group (100% bootstrap support) (Fig. 1a). In the group A clade, bacteria derived from planthoppers formed a monophyletic group (100% bootstrap support), and bacteria derived from spider mites (except for *T. urticae*) formed a monophyletic group (80% bootstrap support). The ML tree of the 16S rRNA gene also supported the notion that *Cardinium*-like bacteria can be divided into three groups (Fig. 1b). Phylogenetic relationships within group A were similar to those for the NJ tree.

The topology of the NJ tree based on the deduced amino acid sequence of the *gyrB* gene was similar to that of the trees based on the 16S rRNA gene sequences (Fig. 2).

***Cardinium* and *Wolbachia* in biting midges.** As stated above, *Culicoides* biting midges were infected with a new group of *Cardinium* (group C). This led us to suspect that *Wolbachia* bacteria inhabiting the biting midges were also distinct from common strains of *Wolbachia*. However, the *Wolbachia* bacteria inhabiting *C. paraflavescens* were characterized as one of the common strains of *Wolbachia* (belonging to either the A or B supergroup), which is 99% identical to *Wolbachia* bacteria of the louse fly *Pseudolynchia canariensis* (10) and the wasp *Nasonia vitripennis* (8) in an approximately 900-bp DNA fragment of the 16S rRNA gene (32) (accession number AB506794).

Morphological characterization of *Cardinium*. *Cardinium*-like bacteria ($n = 32$) were observed in the planthopper *S. furcifera*; the three spider mites *E. suginamensis* ($n = 24$), *P. mori* ($n = 7$), and *T. puearicola* ($n = 67$); and the biting midge *C. lungchiensis* ($n = 106$). The bacteria were circular or rod shaped (major axis, 0.3 to 2.1 μm [$n = 236$; average ± standard deviation = 0.64 ± 0.32 μm]; minor axis, 0.2 to 0.87 μm [$n =$

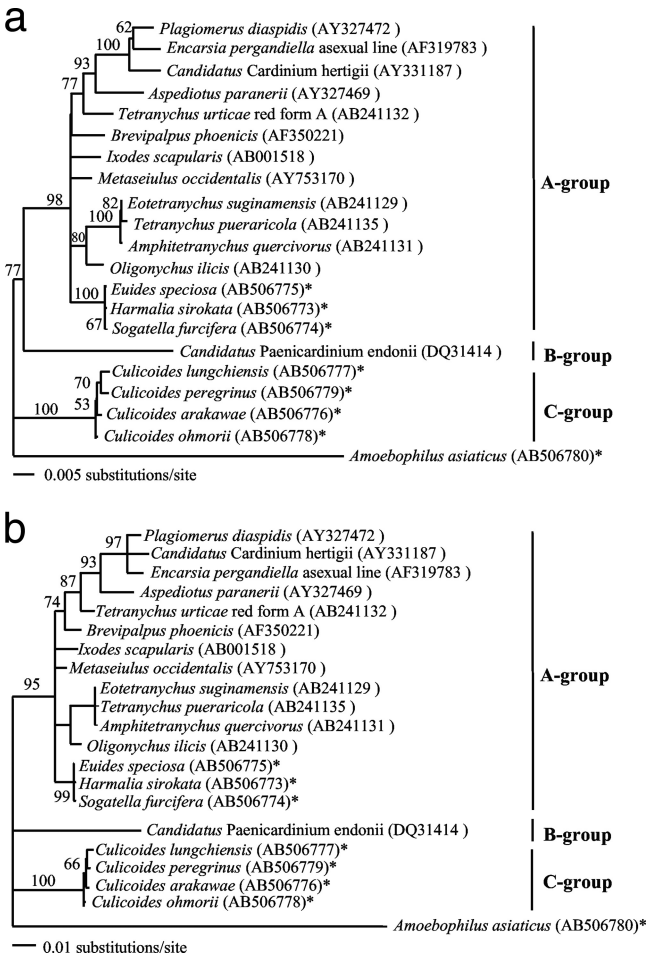

FIG. 1. Phylogenetic analyses based on nucleotide sequences of *Cardinium* 16S rRNA genes (1,444 to 1,448 bp). (a) NJ tree. (b) ML tree. Scientific names of host species are shown at the terminal nodes. *A. asiaticus* is defined as an outgroup. The numbers above the branches indicate bootstrap probabilities (percent). GenBank accession numbers are in parentheses. The examined species are denoted by asterisks.

236; average ± standard deviation = 0.37 ± 0.08 μm]). Microfilament-like structures, previously found in *Cardinium* and *Paenicardinium* (6, 22, 31, 37, 38, 52), were observed consistently in *Cardinium*-like bacteria inhabiting the five arthropod species (Fig. 3). Microfilament-like structures are considered to be a characteristic of *Cardinium*-like bacteria that are unlikely to be present in the close relative "*Candidatus* Amoebophilus asiaticus" (17). Microfilament-like structures of these bacteria were attached to the plasma membrane and extended vertically from an electron-dense plaque.

DISCUSSION

Cardinium infections have been reported to occur in taxonomically diverse groups of arthropods. In contrast to *Wolbachia* infections, *Cardinium* infections are not evenly distributed among arthropod species. Some taxonomic groups, such as parasitic wasps, mites, and spiders, show high frequencies of *Cardinium* infection (11, 46, 51), and the majority of arthropod groups are either free of or seldom harbor *Cardinium*. In this study, a high rate of *Cardinium* infection was detected in planthoppers. Among mites, a group previously reported to harbor *Cardinium*, spider mites were found to be particularly rich in *Cardinium* infections. In biting midges, a distinct group of bacteria closely related to *Cardinium* were found. On the basis of morphological observations and molecular phylogenetic analyses, we propose integration of the bacteria derived from biting midges into "*Candidatus* Cardinium hertigii." Following this proposal, "*Candidatus* Paenicardinium endonii," derived from plant-parasitic nematodes (31), should be revised to "*Candidatus* Cardinium hertigii" (Fig. 4).

Frequencies of *Cardinium* and *Wolbachia* infection. High frequencies of *Cardinium* infection were revealed in planthoppers and spider mites (47.4% and 40.9%, respectively). Ancient horizontal transfer of symbiont genes to the host genome has been reported (18). PCR amplification of the genes on the host genome would lead to erroneous estimation of

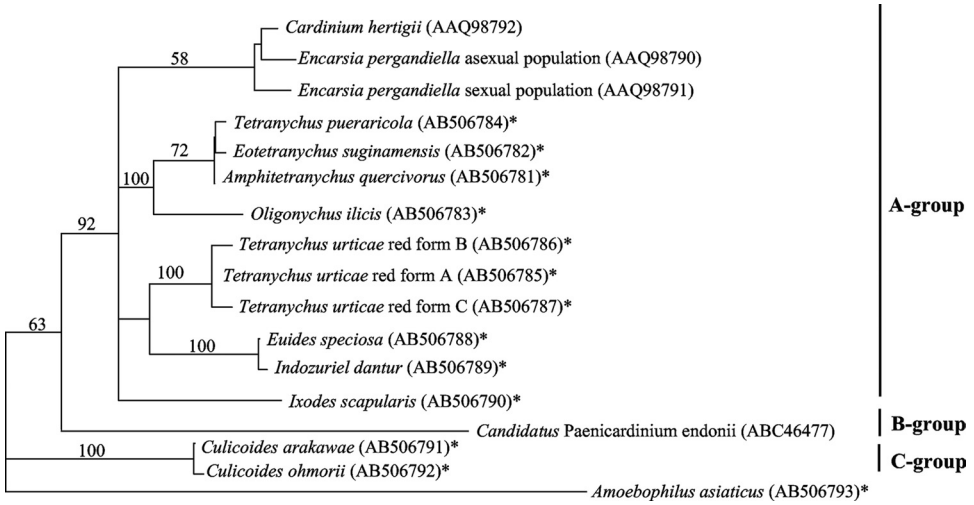

FIG. 2. NJ tree based on the amino acid sequences of *Cardinium* GyrB (286 aa). Scientific names of host species are shown at the terminal nodes. *A. asiaticus* is defined as an outgroup. The numbers above the branches indicate bootstrap probabilities (percent). GenBank accession numbers are in parentheses. The sequences of *A. quercivorus* and three forms of *T. uriticae* were determined from the samples in a previous study (14). The examined species are denoted by asterisks.

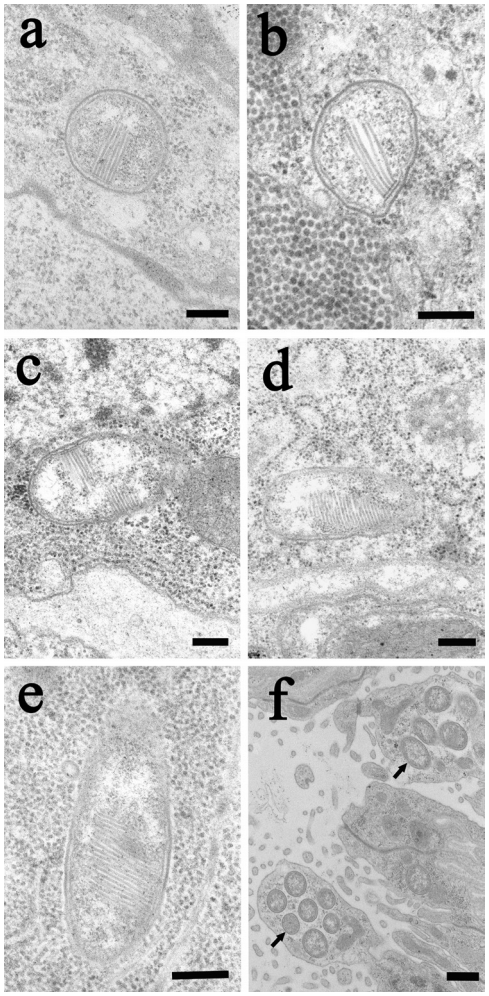

FIG. 3. Electron micrographs of *Cardinium*-like bacteria. (a) Follicle cell of the planthopper *S. furcifera*. (b) Oocyte of the spider mite *E. suginamensis*. (c) Follicle cell of the spider mite *P. mori*. (d) Epidermal cell of the spider mite *T. puerariicola*. (e and f) Oocyte and sheath, respectively, of the biting midge *C. lungchiensis. Cardinium* cells in anaplastic sheaths are indicated by arrows. Bars, 0.20 μm.

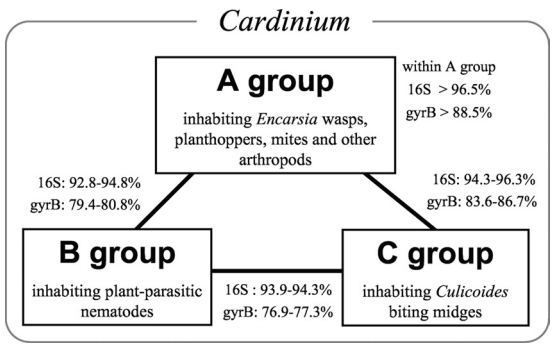

FIG. 4. Relationship of *Cardinium* groups A, B, and C. Each rectangular box represents a *Cardinium* group. Identities of 16S rRNA gene nucleotide sequences (1,447 or 1,448 bp) and in GyrB amino acid sequences (286 aa) are indicated on the lines which connect the groups. The nucleotide or amino acid identities were calculated with Genetyx version 9.1 software (Genetyx Corporation, Tokyo).

Cardinium infection rates. However, since antibiotic administration suppressed the *Cardinium*-positive PCR in five spider mites (14) and the planthopper *S. furcifera* (unpublished), horizontal transfer may not be responsible or may be only slightly responsible for the high rates of *Cardinium* infection in planthoppers and spider mites, and these findings can be attributed primarily to bacterial infection. High frequencies of *Cardinium* infection have been also reported for mites (31.6%) (46) and spiders (22%) (12). Considering the relatively low infection frequencies among arthropods in toto shown by three independent investigations (7.2%, $n = 223$ [46]; 6%, $n = 99$ [51]; and 4.4%, $n = 136$ [11]), mites, spiders, and planthoppers can be regarded as the arthropods with a high frequency of *Cardinium* infections.

Planthoppers and spider mites were also infected with *Wolbachia* at high frequencies, with 52.7% and 31.8% of the examined species, respectively, being infected. Double infections with *Cardinium* and *Wolbachia* in single individuals were found to be common; 32.7% of 55 planthopper species and 13.6% of 22 spider mite species were doubly infected. In planthoppers, 18 strains were doubly infected with *Cardinium* and *Wolbachia*, 7 strains were singly infected with *Cardinium*, 12 strains were singly infected with *Wolbachia*, and 20 strains were uninfected. Fisher's exact probability test showed that significantly higher numbers of strains were doubly infected ($P < 0.05$), which suggests mutualistic dependency of the two bacterial species in planthoppers. In spider mites, on the other hand, the numbers of strains doubly infected with *Cardinium* and *Wolbachia* were not significantly large (Fisher's exact probability test).

In contrast to the case for planthoppers and biting midges, *Cardinium* sequences from several spider mites were poorly amplified by PCR even after 35 cycles. Additionally, different PCR primers showed different positive and negative results in some samples, for example, between the primer pairs CFBf1/CFBr1 (28) and Car-sp-F/Car-sp-R in this study. The inconsistent results between primers probably reflect low infection titers of *Cardinium*. Extremely low infection titers of *Wolbachia* were recently reported for natural populations of the bark beetle *Pityogenes chalcographus* (49). In *Drosophila*, the severity of CI is associated with the density of *Wolbachia* bacteria (7). It is possible that, in some species of spider mites, *Cardinium* bacteria have the potential to cause CI but cannot do so since they are unable to proliferate to titers sufficient to cause strong CI.

In biting midges, a novel group of bacteria that we described as members of the genus *Cardinium* was found. The frequency of *Cardinium* infection among biting midges was 16.0% ($n = 25$). All of the PCR primers used for screening of *Cardinium* infection in earlier studies, i.e., CLOf1 and CLOr1 (46), ChF and ChR (51), and CFBf1 and CFBr1 (28), could amplify PCR products from the samples, which were proven positive for *Cardinium* infection (data not shown). The fact that this group of *Cardinium* bacteria had not been found in previous screenings strongly suggests that screening of a greater number of arthropod species will be required for a more complete understanding of the phylogenetic diversity of *Cardinium*.

Morphological observation. *Cardinium* bacteria derived from *C. lungchiensis* had a common type of microfilament-like structure, i.e., straight, parallel, and perpendicular tubules with

tips attached to the long dimension of the bacterial cell wall. Although the function of the microfilament-like structure remains unknown, this *Cardinium*-specific structure in bacterial cells is likely to play some important role in the survival of the bacteria. *Cardinium* was not observed in immature ovaries but was present in anaplastic sheaths adjacent to immature ovaries in young adult females of the biting midge *C. lungchiensis*. *Cardinium* bacteria have also been observed in terminal filaments and follicle cells. In every generation, *Cardinium* may be vertically transmitted during oogenesis by migrating from surrounding tissues into immature oocytes. Detailed systematic observations would be necessary to elucidate the mode of vertical transmission of *Cardinium* in future studies.

Nomenclature of *Cardinium*. We proposed a single species name, "*Candidatus* Cardinium hertigii," for three groups of bacteria, namely, the previously described "*Candidatus* Cardinium hertigii" and "*Candidatus* Paenicardinium endonii" and the bacteria derived from biting midges found in the present study (Fig. 4). It is generally assumed that a 3% substitution in the nucleotide sequence of the 16S rRNA is necessary to assign bacteria to a different species (1, 39). Particularly in the case of insect endosymbionts, bacteria having even more substitutions are conventionally assigned to a single species. For instance, *Wolbachia pipientis*, derived from *Trichogramma cordubensis* (group B) (40) and *Difilaria repens* (group C) (9), exhibited 7.2% substitution in 16S rRNA gene (1,395 bp). Likewise, "*Buchnera aphidicola*," a mutualistic endosymbiont of aphids, exhibits 9.3% substitution in the 16S-23S rRNA gene region, and *Carsonella*, *Portiera*, and *Termblaya* exhibit 8% to 10% differences in the 16S-23S rRNA gene region (5). At present, *Wolbachia* bacteria with more than 3% substitution in the 16S rRNA gene are assigned to different phylogenetic supergroups (23).

In *Cardinium*, the largest substitution in the 16S rRNA gene (1,447 bp) was 7.2% between *Cardinium* bacteria of *E. pergandiella* (50) and *Paenicardinium* (31), which is a value comparable to that for other insect symbionts. We therefore consider our taxonomic revision quite reasonable. Taking the data together, practical conditions for identifying *Cardinium* could be (i) the presence of microfilament-like structures and (ii) less than 7% to 10% substitution in 16S rRNA gene. Since every *Cardinium* organism found so far was present in ovaries (6, 31, 37, 38, 52), transovarial transmission could be included as a third condition.

Nomenclature of the symbiotic bacteria is still controversial. Considering the complex relationship and extensive recombination between various strains of *Wolbachia*, Lo et al. (23) have argued for the single species *Wolbachia pipientis*. Conversely, Pfarr et al. (33) claimed that *Wolbachia* should be divided into two species according to the host (arthropods versus filarial nematodes). In any case, a better understanding of their nature derived from further studies will allow more convincing classification of these symbionts.

ACKNOWLEDGMENTS

We are indebted to Timothy J. Kurtti of the University of Minnesota for offering *Ixodes Cardinium*.

This work is supported in part by Grants-in-Aid for Scientific Research (no. 19380038 and 19380032) from the Japan Society for the Promotion of Science.

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