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Source: Zoological Science, 17(7) : 983-989

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.17.983>

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# Complex Endosymbiotic Microbiota of the Citrus Psyllid *Diaphorina citri* (Homoptera: Psylloidea)

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**ABSTRACT**—We investigated the endosymbiotic microbiota of the citrus psyllid *Diaphorina citri* that vectors *Liberobacter* sp., the causative agent of citrus greening disease. Eubacterial 16S rDNA fragments were amplified by PCR and cloned from the whole DNA of *D. citri*. RFLP analysis showed that the PCR products contained five major sequence types. Nucleotide sequences of the five types were determined and subjected to molecular phylogenetic analysis. The first sequence was a member of the  $\gamma$ -Proteobacteria, closely related to mycetocyte symbionts of other psyllids. The second sequence belonged to the  $\beta$ -Proteobacteria, related to *Oxalobacter* and *Herbaspirillum*. The third sequence, belonging to the  $\gamma$ -Proteobacteria, showed a significant similarity to the sequences of *Arsenophonus* spp., known as endosymbionts of a triatomine bug and a parasitoid wasp. The fourth sequence was almost identical to the sequences of *Liberobacter* spp. in the  $\alpha$ -Proteobacteria. The fifth sequence showed a high similarity to the sequences of *Wolbachia* spp. in the  $\alpha$ -Proteobacteria. *In situ* hybridization experiments using specific oligonucleotide probes confirmed that the first and second sequences originated from the mycetocyte and syncytium symbionts, respectively. The presence/absence of the five endosymbiotic bacteria in adult individuals of *D. citri* from an Indonesian natural population was examined by diagnostic PCR analysis. The mycetocyte symbiont and the syncytium symbiont exhibited 100% infection, whereas the *Arsenophonus*, *Liberobacter* and *Wolbachia* showed partial infections at a rate of 83.3%, 45.2% and 76.2%, respectively.

## INTRODUCTION

The Homoptera, including cicadas, planthoppers, aphids, scale insects, psyllids and others, is an insect group whose endosymbiotic system with microorganisms is highly developed. In many cases, the insects possess a large organ, a mycetome (or a bacteriome), composed of special cells for endosymbiosis, mycetocytes (or bacteriocytes), in which microorganisms are harbored intracellularly (Buchner, 1965). Because homopteran insects live on a nutritionally unbalanced diet of plant sap all through their life, it is believed that they need the help of endosymbiotic microorganisms to compensate for the nutritional deficiency. In fact, it has been demon-

strated that endosymbiotic microbes of homopterans are involved in metabolic processes such as the synthesis of essential nutrients and recycling of nitrogenous wastes (Baumann *et al.*, 1995; Douglas, 1989; Sasaki *et al.*, 1996; Dixon, 1998).

Psyllids have a large, yellow and bilobed mycetome in the abdomen. The mycetome is a complex of three types of cells; many round uninucleated mycetocytes, a syncytial tissue surrounded by them, and an envelope composed of many flattened cells encasing the whole. The cytoplasm of the mycetocytes is full of a specific bacterium, called X-symbiont, primary symbiont, or mycetocyte symbiont. The syncytial cytoplasm is filled with another type of bacterium, called Y-symbiont, secondary symbiont, or syncytium symbiont (Profft, 1937; Buchner, 1965; Chang and Musgrave, 1969; Waku and Endo, 1987; Fukatsu and Nikoh, 1998; Spaulding and Von Dohlen, 1998). The mycetocyte symbionts and the syncytium

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symbionts of psyllids were reported to belong to distinct lineages in the  $\gamma$ -*Proteobacteria*, respectively (Fukatsu and Nikoh, 1998; Spaulding and Von Dohlen, 1998).

In addition to the mycetome symbionts, many homopterans harbor various types of facultative endosymbiotic microorganisms such as *Rickettsia* in an aphid (Chen and Purcell, 1997), *Wolbachia* in a planthopper (Hoshizaki and Shimada, 1995), *Spiroplasma* in a scale insect (Fukatsu and Nikoh, 2000) and others, making their endosymbiotic microbiota more complex. In psyllids, however, no such endosymbiotic associates have been reported, except *Liberobacter* sp., a plant pathogen vectored by certain psyllids.

Citrus greening is one of the most severe diseases of oranges in Asia and Africa, caused by uncultured, gram-negative, walled, and phloem-limited bacteria, *Liberobacter* spp., belonging to the  $\alpha$ -subdivision of the *Proteobacteria* (Jagoueix *et al.*, 1994). The disease is transmitted from infected to healthy plants by psyllids, *Diaphorina citri* in Asia and *Trioza erythrae* in Africa (McClellan and Oberholzer, 1965; Capoor *et al.*, 1967; Schwarz *et al.*, 1970). To control the disease, therefore, it is important to know the infection rates and dynamics of *Liberobacter* sp. in natural populations of the psyllids.

In order to investigate the endosymbiotic microbiota of the citrus psyllid *D. citri* that vectors *Liberobacter*, we analyzed eubacterial 16S rDNA amplified from the insects which were collected from a citrus orchard in Indonesia where citrus greening disease is prevailing. Surprisingly, as many as five endosymbiotic bacteria were identified from the psyllid, including mycetocyte symbiont, syncytium symbiont, *Arsenophonus* sp., *Wolbachia* sp. and *Liberobacter* sp.

## MATERIALS AND METHODS

### Materials

Adults of *D. citri* were collected from the orange trees, *Citrus nobilis*, at a citrus orchard at Jatinom, Klaten district, central Java, Indonesia. The insects were preserved in glass vials filled with acetone until molecular and histological analyses (Fukatsu, 1999).

### DNA extraction, PCR, cloning, RFLP typing, and sequencing of 16S rDNA

The insects preserved in acetone were subjected to DNA extraction using QIAamp tissue kit (QIAGEN). For 16S rDNA cloning and sequencing, DNA was collectively extracted from several tens of insects. For diagnostic PCR, DNA was extracted individually. Fragments of bacterial 16S rDNA were amplified by PCR using AmpliTaq DNA polymerase (Perkin Elmer) under the temperature profile of 94°C for 2 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 70°C for 2 min. Primers 16SA1 [5'-AGAGTTTGATCMTGGCTCAG-3'] and 16SB1 [5'-TACGGYTACCTTGTACGACTT-3'] recognize eubacterial 16S rDNA universally. Primers GOIn fwd [5'-GCGCGTATGCAATACGAGCGGCA-3'] and GOrev [5'-GCCTCGGCACTTCGCAACCCAT-3'] are specific to *Liberobacter* spp. The PCR product was purified by GeneClean II kit (BIO 101 Inc.) and cloned with TA-cloning vector pT7Blue (Novagen) and *E. coli* JM109 competent cells (Takara) using ampicillin and X-gal blue-white selection system. White colonies that were expected to contain the inserted plasmid were directly subjected to PCR using the amplifying primers in order to check the length of the inserted DNA fragment. If the expected size of PCR product was obtained, the product was digested

by 4 bases-recognizing restriction endonucleases HinfI, RsaI, Sau3AI and TaqI, and electrophoresed in TAE-agarose gels for restriction fragment length polymorphism (RFLP) typing of cloned 16S rDNA. The white colonies identified to contain 16S rDNA clone were isolated and cultured in 1.5 ml LB medium with ampicillin overnight and subjected to plasmid extraction using QIAprep-Spin Miniprep Kit (QIAGEN). The purified plasmids, eluted with 30  $\mu$ l TE buffer, were subjected to DNA sequencing as previously described (Fukatsu and Nikoh, 1998). The 16S rDNA sequences determined in this study are deposited in the DDBJ/ EMBL/ GenBank nucleotide sequence databases with the accession numbers AB038366-AB038370.

### Molecular phylogenetic analysis

The 16S rDNA sequences were subjected to multiple alignment (Feng and Doolittle, 1987; Gotoh, 1993). The final alignment was inspected and corrected manually. Ambiguously aligned regions were excluded from the phylogenetic analysis. Nucleotide sites that included alignment gap(s) were also omitted from the aligned data set. The aligned data are available upon request to the corresponding author. Neighbor-joining trees (Saitou and Nei, 1987) were constructed with Kimura's two parameter distance (Kimura, 1980) using the program package Clustal W (Thompson *et al.*, 1994). Maximum parsimony trees were constructed using the program package PAUP 4.0b2 (Swofford, 1999). Bootstrap test (Felsenstein, 1985) was conducted with 1000 resamplings.

### Diagnostic PCR

Using specific reverse PCR primers, DC16SMycR, DC16SSynR, DC16STriR, DC16SWolR and DC16SLibR (Table 1A), in combination with universal forward primer 16SA2 [5'-GTGCCAGCAGCCGCGTAATAC-3'], diagnostic PCR detection of 16S rDNA of endosymbiotic bacteria was conducted. DNA samples extracted from individual insects were subjected to PCR using AmpliTaq DNA polymerase Gold (Perkin Elmer) under the temperature profile of 94°C for 10 min followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. Plasmids containing the cloned 16S rDNA fragment of each endosymbiont were used as positive/ negative control samples. The PCR products were resolved in 1.5 % agarose gels and stained with ethidium bromide.

**Table 1.** Specific primers and probes used for detection of the endosymbiotic bacteria of *D. citri* by diagnostic PCR and *in situ* hybridization, respectively. Nucleotide sites responsible for specificity are indicated.

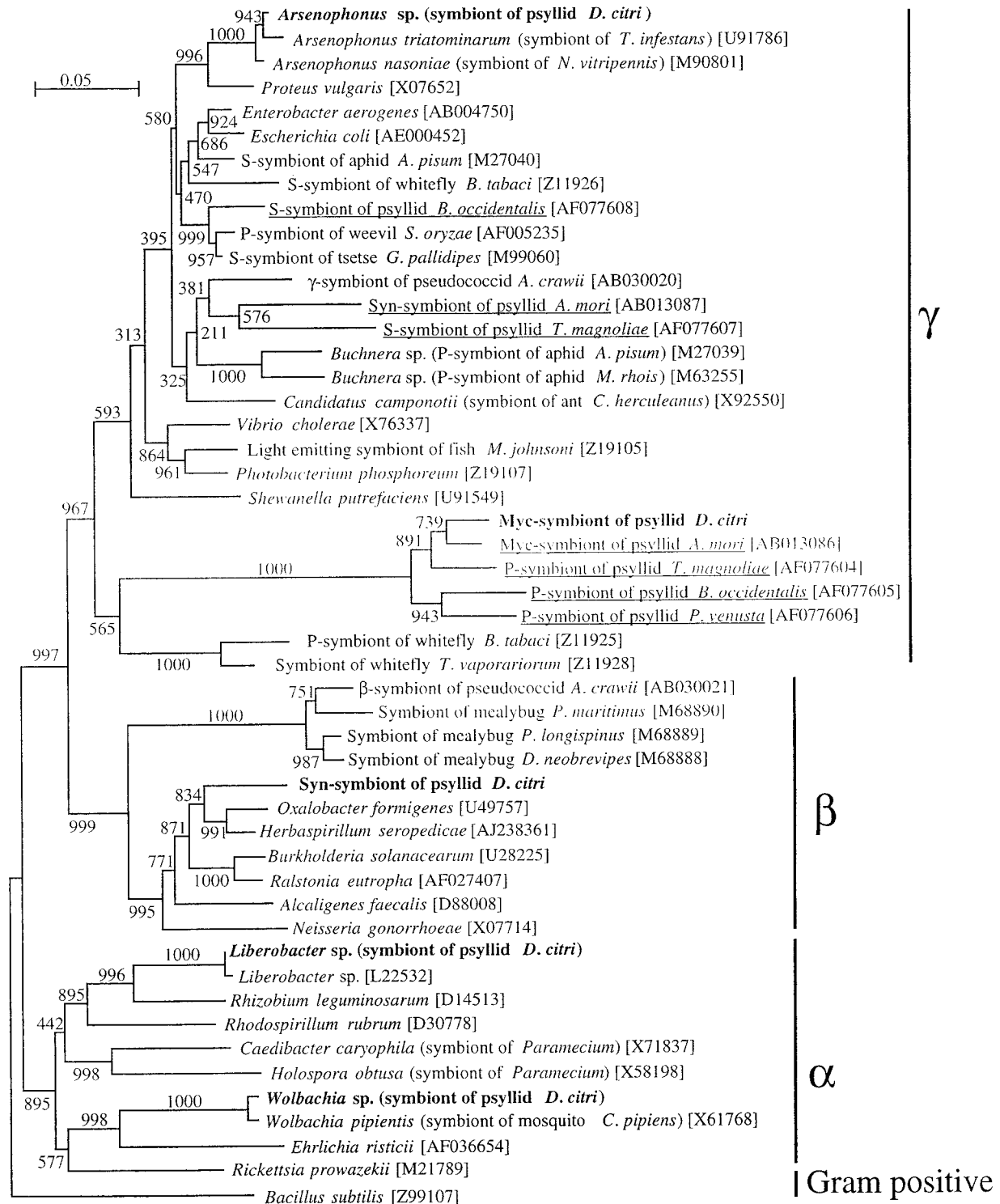
A. Primers	1	10	20
DC16SMycR	CACCTGT	CTC	AAAGCTAAA
DC16SSynR	CACCTGTGTT		TAGATTC
DC16STriR	CACCTGTCTC		ACCGCTCCCG
DC16SLibR	CACCTGTGTA		AAGCTCTCCG
DC16SWolR	CACCTGTGTG		AAATCCGGCC
B. Probes	1	10	20
DIG-DC16SMyc	AAC	TTT	TGT
DIG-DC16SSyn	GAC	CT	CTGT
DIG-DC16STri	CTC	TCT	CTGT
DIG-DC16SLib	AAC	CC	ATTGT
DIG-DC16SWol	AAC	CC	ATTGT
	AT	TACT	CATT
	AT	CACC	CATT
	AT	ACC	CATT
	AAC	CACC	CATT
	AG	AC	CACCATT

### *In situ* hybridization

Specific oligonucleotide probes used in this study, DIG-DC16SMyc, DIG-DC16SSyn, DIG-DC16STri, DIG-DC16SLib and DIG-DC16SWol, whose 5' end is labelled with digoxigenin, are listed in Table 1B. Histological preparation and *in situ* hybridization were

conducted as previously described (Fukatsu and Nikoh, 1998; 2000; Fukatsu *et al.*, 1998). About 150  $\mu$ l of hybridization buffer [20 mM Tris-HCl (pH 8.0), 0.9 M NaCl, 0.01% SDS, 30% formamide] containing 70 pmole/ml probe was applied to the tissue section, covered with

a coverslip, and incubated in a humidified chamber at room temperature overnight. After washing with 1xSSC, the tissue section was subjected to detection of bound probe using DIG Nucleic Acid Detection Kit (Böhringer Mannheim). To confirm the specificity of the hybridiza-



**Fig. 1.** Placement of the five endosymbiotic bacteria identified from *D. citri* in 16S rDNA phylogeny of the *Proteobacteria*. A total of 988 unambiguously aligned nucleotide sites were subjected to the analysis. A neighbor-joining tree is shown, whereas maximum parsimony analysis gave essentially the same result. The bootstrap values obtained with 1,000 resamplings are shown at the nodes. The numbers in brackets are accession numbers. The sequences determined in this study are shown in bold. The sequences of endosymbionts from other psyllids reported in previous studies are underlined.

tion, the following control experiments were conducted: no probe control, RNase digestion control, and competitive suppression control with excess unlabelled probe (Fukatsu *et al.*, 1998). Control experiments with a widely-used general eubacterial 16S rRNA probe, DIG-EUB338 (Amann *et al.*, 1995), were also conducted.

## RESULTS

From the total DNA of *D. citri*, eubacterial 16S rDNA sequences were amplified by PCR and cloned. The cloned DNA fragments were subjected to RFLP analysis. The PCR product with primers 16SA1 and 16SB1 contained three major types of sequences, tentatively designated A-, B- and C-types. The PCR product with primers GOINfwd and GOrev was composed of only one sequence species, designated D-type, which was expected to originate from *Liberobacter* sp. The PCR product with primers 16SA1 and GOrev, designated E-type, was also analyzed because its RFLP profile was unexpectedly distinct from that of D-type.

Four clones of A-type, three clones of B-type, three clones of C-type, two clones of D-type and two-clones of E-type were sequenced. The nucleotide sequences of the same type were almost identical. The lengths, without the regions of amplifying primers, were 1,457 bases for the A-type, 1,463 bases for the B-type, 1,465 bases for the C-type, 1,122 bases for the D-type and 1,185 bases for the E-type.

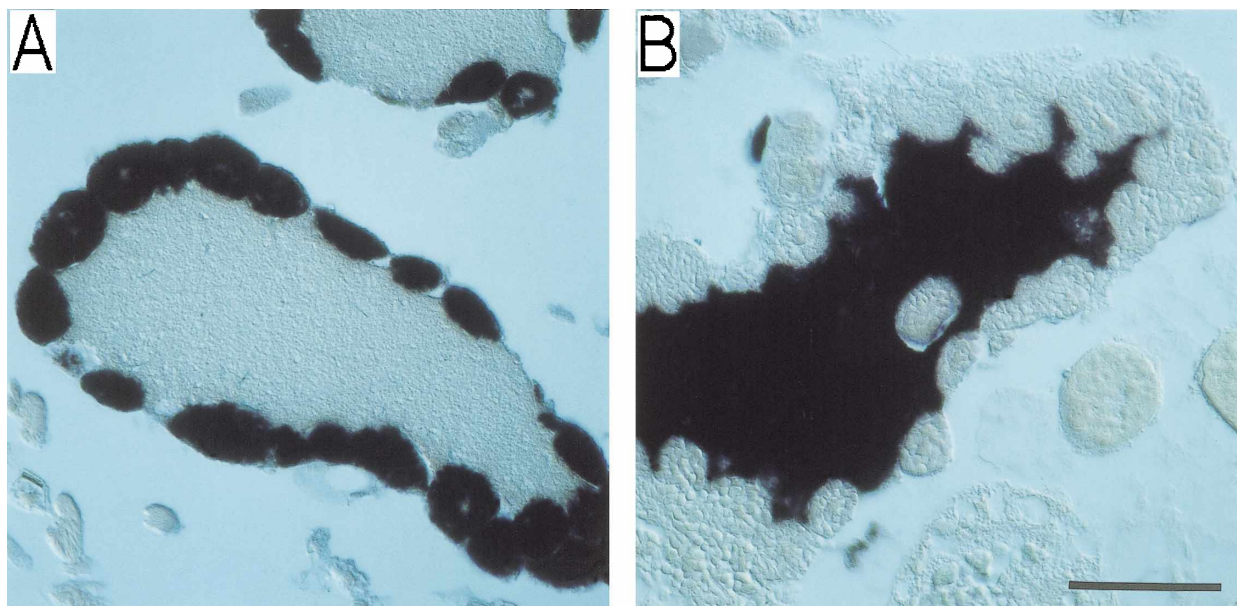
These sequences were subjected to homology search in DNA databases. The A-type sequence was a member of the  $\gamma$ -*Proteobacteria*, closely related to mycetocyte symbionts of other psyllids. The B-type sequence belonged to the  $\beta$ -*Proteobacteria*, related to *Oxalobacter* and *Herbaspirillum*. The C-type sequence, belonging to the  $\gamma$ -*Proteobacteria*, showed a significant similarity to the sequences of *Arsenophonus* spp.,

known as endosymbionts of a triatomine bug and a parasitoid wasp. The D-type sequence was almost identical to the sequences of *Liberobacter* spp. in the  $\alpha$ -*Proteobacteria*. The E-type sequence showed a high similarity to the sequences of *Wolbachia* spp. in the  $\alpha$ -*Proteobacteria*. Fig. 1 is the phylogenetic positions of the five 16S rDNA sequences in the *Proteobacteria*, illustrating the complex endosymbiotic microbiota of *D. citri*.

To specifically detect these 16S rDNA sequences, highly specific reverse PCR primers for them were designed (Table 1A). Using these primers, the presence/ absence of the five endosymbiotic bacteria in 42 field-collected adults of *D. citri*

**Table 2.** Diagnostic PCR detection of the five endosymbiotic bacteria from field-collected individuals of *D. citri*. Abbreviations: Myc, A-type symbiont harbored in the mycetocyte; Syn, B-type symbiont localized in the syncytium; Ars, C-type symbiont *Arsenophonus* sp.; Lib, D-type symbiont *Liberobacter* sp.; Wol, E-type symbiont *Wolbachia* sp.; +, detected; -, not detected

Bacteria Type	Myc (A)	Syn (B)	Ars (C)	Lib (D)	Wol (E)	No. of Insects	(%)
Infection Patterns	+	+	+	+	+	10	23.8
	+	+	+	-	+	16	38.1
	+	+	+	+	-	4	9.5
	+	+	+	-	-	5	11.9
	+	+	-	+	+	5	11.9
	+	+	-	-	+	1	2.4
	+	+	-	-	-	1	2.4
No. of Positive Insects	42	42	35	19	32	42	
Infection Rate (%)	100	100	83.3	45.2	76.2		



**Fig. 2.** *In situ* hybridization of intracellular symbiotic bacteria in the mycetome of *D. citri*. (A) Probe DIG-DC16SMyc targets the A-type sequence in the round mycetocytes. (B) Probe DIG-DC16SSyn targets the B-type sequence in the syncytium. Bar shows 50  $\mu$ m.

was examined by diagnostic PCR analysis (Table 2). The A- and B-type sequences showed 100% infection. The other types showed imperfect infection; 83.3% for the C-type *Arsenophonus* sequence, 45.2% for the D-type *Liberobacter* sequence, and 76.2% for the E-type *Wolbachia* sequence. Out of 42 individuals examined, 10 possessed all of the five symbionts whereas the others lacked either of the latter three bacteria.

To demonstrate that these 16S rDNA sequences were certainly derived from endosymbiotic bacteria of *D. citri*, digoxigenin-labelled specific oligonucleotide probes were designed (Table 1B). The tissue sections of *D. citri* were subjected to *in situ* hybridization using these probes. Using the probe DIG-DC16SMyc for the A-type sequence, the cytoplasm of uninucleate round mycetocytes arranged at the surface of huge mycetomes were specifically stained (Fig. 2A). Thus, it was confirmed that the A-type sequence was derived from a bacterium localized in the mycetocyte. Using the probe DIG-DC16SSyn for the B-type sequence, the large syncytial cytoplasm at the center of mycetomes surrounded by a number of mycetocytes was specifically visualized (Fig. 2B). Therefore, it was demonstrated that the B-type sequence certainly originated from a bacterium harbored by the syncytium. We also conducted *in situ* hybridization experiments using the probes DIG-DC16STri for the C-type, DIG-DC16SLib for the D-type, and DIG-DC16SWol for the E-type (data not shown). The results were not so convincing as those by DIG-DC16SMyc and DIG-DC16SSyn. A relatively small number of tiny round or pleomorphic signals were sporadically found in various tissues such as ovary, gut, fat body, muscle, salivary gland and others. Since the signals were in general not reproducible and differed from samples to samples, we could not be confident of the results. Further intensive investigations are needed to address the *in vivo* distribution of these facultative endosymbiotic microbes.

## DISCUSSION

In the present study, we identified five distinct proteobacterial endosymbionts in a natural population of *D. citri* (Fig. 1 and Table 2); a  $\gamma$ -proteobacterium harbored in the mycetocyte and related to endosymbionts of other psyllids, a  $\beta$ -proteobacterium located in the syncytium of the mycetome, a  $\gamma$ -proteobacterium related to *Arsenophonus* spp. endosymbiotic to a triatomine bug and a parasitoid wasp, an  $\alpha$ -proteobacterium of the genus *Liberobacter*, and an  $\alpha$ -proteobacterium of the genus *Wolbachia*. Hereafter we designate them as myc-symbiont, syn-symbiont, *Arsenophorus*, *Liberobacter* and *Wolbachia*, respectively.

There have been a number of histological works describing various insects with multiple types of endosymbiotic microorganisms, which are morphologically distinguishable from each other, in a variety of cells and tissues (for review, see Buchner, 1965). Because most of insect endosymbionts are difficult to culture, identity and taxonomic position of these microorganisms have been unspecified for a long time. How-

ever, owing to recent innovations in molecular phylogenetics, many endosymbiotic microorganisms of insects have come to be identified (Baumann and Moran, 1997). Thus far, two distinct endosymbiotic microbes in the same host have been characterized by molecular phylogenetic approaches in aphids (Unterman *et al.*, 1989; Chen *et al.*, 1996; Chen and Purcell, 1997; Fukatsu *et al.*, 2000), psyllids (Fukatsu and Nikoh, 1998; Spaulding and Von Dohlen, 1998), whiteflies (Clark *et al.*, 1992), pseudococcids (Fukatsu and Nikoh, 2000), a bedbug (Hypsa and Aksoy, 1997), weevils (Campbell *et al.*, 1992; Heddi *et al.*, 1999), tsetse flies (Aksoy *et al.*, 1995; 1997) and other insects. In a pseudococcid, even three endosymbiotic bacteria have been identified (Fukatsu and Nikoh, 2000). As far as we know, this is the first report in which as many as five distinct endosymbiotic bacteria were definitely identified from the same insect by a molecular phylogenetic approach. Notably, however, it is not certain whether this is the full picture of the endosymbiotic microbiota in *D. citri*. The possibility cannot be excluded that we might have failed to detect some minor endosymbiotic components.

In an Indonesian natural population of *D. citri*, the five endosymbionts varied in their infection rates (Table 2). The myc-symbiont and syn-symbiont exhibited 100% infection, suggesting that they may have some important biological roles for the host insect. The perfect infection rate may also reflect the situation that these symbionts are specifically harbored and integrated in a highly developed mycetomal endosymbiotic system (Fig. 2). In contrast, the *Arsenophorus*, *Liberobacter* and *Wolbachia* exhibited imperfect infection, suggesting that they are facultative guest microbes of a commensal or parasitic nature.

In the 16S rDNA phylogeny (Fig. 1), the myc-symbiont of *D. citri* formed a monophyletic group with mycetocyte symbionts of other psyllids, suggesting that the myc-symbionts are highly conserved in psyllids, just as the mycetocyte symbionts *Buchnera* spp. in aphids. At this stage, it is unknown whether the myc-symbionts and the host psyllids have co-specified as *Buchnera* and aphids (Moran and Baumann, 1994; Baumann *et al.*, 1995). Phylogenetic analysis of the host psyllids is required to confirm this idea.

In contrast, the syn-symbiont of *D. citri* was the first case of psyllid symbiont that belongs to the  $\beta$ -*Proteobacteria*, and did not show any phylogenetic affinity to other insect endosymbionts. As for the syncytium symbionts of other psyllids, Fukatsu and Nikoh (1998) demonstrated that the syn-symbiont of *Anomoneura mori* is a  $\gamma$ -proteobacterium related to endosymbionts of ants and aphids. Spaulding and Von Dohlen (1998) reported two presumable syn-symbiont sequences, one from *Trioza magnoliae* and the other from *Blastopsylla occidentalis*, that belong to distinct lineages in the  $\gamma$ -*Proteobacteria*. A significant similarity was found between the sequences from *A. mori* and *T. magnoliae*. Therefore, as far as examined so far, at least three distant proteobacterial lineages have symbiosed in the syncytium of psyllids, which strongly suggests that acquisitions and replacements of syn-symbiont have occurred repeatedly in the evolutionary his-

tory of psyllids. This evolutionary pattern, conserved mycetocyte symbionts and variable secondary symbionts, appears quite reminiscent of that found in aphids (Buchner, 1965; Fukatsu and Ishikawa, 1993; 1998; Moran and Baumann, 1994; Fukatsu *et al.*, 1998; 2000).

Biological functions of the mycetome endosymbionts of psyllids have not been investigated. However, the highly developed mycetome is conserved in all psyllids ever examined (Profft, 1937; Buchner, 1965; Chang and Musgrave, 1969; Waku and Endo, 1987), suggesting that the endosymbionts may play essential physiological and nutritional roles for the host psyllids, as has been demonstrated in aphids, planthoppers and other insects (Douglas, 1989; Baumann *et al.*, 1995; Sasaki *et al.*, 1996; Dixon, 1998). Considering their evolutionary stability, it appears that the myc-symbionts have more important roles than the syn-symbionts.

Thus far, only two members of the genus *Arsenophonus* have been reported, both of which were endosymbiont of insect. *A. nasoniae* is known to cause son-killer trait in the parasitoid wasp *Nasonia vitripennis* (Gherna *et al.*, 1991). *A. triatominarum* is found in various types of cells and hemolymph of the triatomine bug *Triatoma infestans* (Hypsa and Dale, 1997). Here we report the third member of *Arsenophonus* from the citrus psyllid *D. citri*, reinforcing the idea that *Arsenophonus* species constitute a bacterial group specialized for endosymbiotic life in insects. Sex ratios of *D. citri* with and without the *Arsenophonus* may be intriguing in the light of male-killing effect in the wasp.

Members of the genus *Wolbachia*, which are widely distributed among various groups of insects, mites, crustaceans and nematodes, are one of the most intensively studied facultative endosymbiotic bacteria (O'Neill *et al.*, 1997). This is the first identification of *Wolbachia* in psyllids. The infection rate of the *Wolbachia* was pretty high, 76.2% in the *D. citri* population. It has been shown that *Wolbachia* infection often causes various sex-related aberrations in their arthropod hosts such as cytoplasmic incompatibility, parthenogenesis and feminization of genetic males, which can be interpreted as the reproductive strategy of *Wolbachia* to increase the frequency of infected females in populations of the host (O'Neill *et al.*, 1997). It is quite interesting but not investigated yet whether the *D. citri* carrying the *Wolbachia* exhibit such reproductive symptoms.

In this study, we first presented the infection rate of *Liberobacter* in a natural population of *D. citri* using a diagnostic PCR approach. The infection rate, 45.2%, confirmed that *D. citri* can be a threatening vector of the citrus greening in the area. We expect that the specific primer set for the *Liberobacter* developed in this study (Table 1A) will be a useful tool to monitor the invasion and prevalence of the citrus greening in the field.

Regrettably, we could not surely identify the *in vivo* localization and distribution of the three facultative endosymbionts, *Arsenophonus*, *Liberobacter* and *Wolbachia*, in *D. citri*. Further *in situ* hybridization experiments with a sufficient amount of materials with and without these microbes are needed to

establish the final conclusion. Particularly we are interested in the biology of the *Liberobacter*. Whether the *Liberobacter* is localized in either salivary gland, gut, or body cavity will be an important point to understand the mechanism of disease propagation. Whether the *Liberobacter* can be vertically transmitted to the offspring via ovary or not must be confirmed. Various types of interactions between the *Liberobacter* and other endosymbionts might occur in the same host insect. Population dynamics of the *Liberobacter* in the wild populations of *D. citri* should be investigated to understand the propagation and transmission of the citrus greening disease.

## ACKNOWLEDGMENT

We thank A. Sugimura, S. Kumagai and K. Sato for their technical and secretarial assistance. This research was supported by the Bilateral Research Cooperation Project "Conservation and Sustainable Use of Tropical Bioresources" and the Industrial Science and Technology Frontier Program "Technological Development of Biological Resources in Bioconsortia" of the Ministry of International Trade and Industry of Japan, and by the Riset Unggulan Terpadu (RUT) VI, Indonesia. S. S. was under the Dissertation Ph.D. Program of Japan Society for the Promotion of Science.

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(Received February 24, 2000 / Accepted April 25, 2000)