東海大學生命科學系碩士論文

論文題目：沫蟬內共生物之親緣多樣性及共演化

Phylogenetic Diversity and Cospeciation of Spittlebug's Endosymbionts

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ABSTRACT

Symbiotic life style is a major adaptation of organisms that can increase their diversity. Many insects, especially hemipterans, are associated with the primary as well as secondary endosymbionts. The primary endosymbiont (bacteriome-associated) of insects provides their hosts with nutrition whereas the function of secondary endosymbionts is not clear. Here we used molecular phylogenetic analyses to describe the characteristics of bacterial endosymbionts in the red striped spittlebug, *Okiscarta uchidae*, and to investigate their phylogenetic placement within the eubacteria. We also evaluated relative evolutionary rates of endosymbionts and their free-living and pathogenic relatives. TEMs suggested that there were at least two types of endosymbionts in the bacteriomes, one of them are bacterial symbionts and the other are yeast-like organisms. Phylogenetic results suggested that there are four distinct symbiont lineages, three of them belonging to γ-proteobacteria and the other one clustered witnin the phylum *Bacteroidetes*. Results obtained from *in situ* hybridization suggested that five of the isolated endosymbionts were located inside the bacteriomes. In this study, we found that the evolutionary rates of identified symbionts were frequently the lowest among free-living, pathogenic and symbiont bacteria of insects, suggesting that they are most likely to be the "secondary" endosymbionts of *O. uchidae*.

Keywords: Phylogeny, spittlebugs, bacteriomes, endosymbionts, Bayesian relative rates, TEM, *in situ* hybridization.

紅紋沫蟬內共生物之親緣多樣性及分子特徵

共生關係的生活型態能增加多樣性的一種主要適應方式。許多昆蟲，尤其是半翅目，體內具有初級及次級內共生物。初級內共生物 (存在於懷菌體內) 提供寄主昆蟲所缺乏之養份，然而次級內共生物的功能尚不清楚。我們利用分生技術及親緣分析來描述紅紋沫蟬（*Okiscarta uchidae*）的內共生物特徵及他們在 eubacteria 內的親緣關係。我們也估算共生物的演化速率並且與自營菌的速率做比較。穿透式電子顯微鏡的照片顯示至少有兩種不同型態的內共生物在沫蟬的懷菌體內，而且其中一種內共生物的外部型態與酵母菌相似。16S rDNA 的分析結果顯示分離出的內共生物分為四個類群，其中三個類群位於 γ-proteobacteria 內，一個類群在 *Bacteroidetes* 內。*In sit*u Hybridization 的結果顯示五種不同的內共生物位於懷菌體內。自營菌通常具有較快的演化速率，但紅紋沫蟬內共生物的演化速率往往是所有分析的菌種中最慢的，我們推測，這些內共生物應該屬於次級內共生物。

關鍵字：親緣關係樹、沫蟬、懷菌體、內共生物、貝氏相對速率、穿透式電子顯微鏡、原位雜合實驗。

INTRODUCTION

Symbiosis represents one of the major innovations in the history of life and allows diversification of interacting organisms by expanding ecological niches (Margulis & Fester 1991; Maynard Smith & Szathmary 1995). Symbiotic interactions between prokaryotes and multicellular eukaryotes are important evolutionary forces in generating phenotypic complexity of both hosts and symbionts (Moran 2007). It is widely accepted that endosymbionts of insects play a nutritional role and are beneficial to their hosts (Buchner 1965; Houk & Griffiths 1980; Douglas 1994; Baumann *et al.* 1995; Moran & Telang 1998; Douglas 1998). Prokaryotic endosymbionts of insects in general can be recognized as either primary or secondary symbionts (Buchner 1965; Moran & Telang 1998). Primary or obligate endosymbionts are restricted to the cytoplasm of host cells, called bacteriocytes. They are located at characteristic positions of hosts and form specialized tissues, called bacteriomes for housing bacteria (Buchner 1965). The secondary or facultative endosymbionts coexist in the same individual hosts with the primary endosymbionts but occur in midguts, sheath-like cells bordering the bacteriocytes, or other thoraxic (Buchner 1965; Moran & Telang 1998; Fukatsu *et al*. 1998; Fukatsu *et al*. 2000). The primary endosymbionts of a variety of insects were shown to provide their hosts with essential amino acids (Buchner 1965; Baumann *et al.* 1995; Subandiyah *et al*. 2000; Moran *et al*. 2003), whereas the biological role of the secondary bacteria is largely unknown (Buchner 1965; Moran & Telang 1998). Nevertheless, recent studies demonstrated that the secondary endosymbionts could affect the fitness (growth and reproduction) of insect hosts, and the dregree of effects depending on temperatures and ages of hosts (Chen *et al*. 2000; Fukatsu *et al*. 2001). Other researches suggested that the secondary endosymbionts are associated with plant preference of the insect

host (Tsuchida *et al.* 2004), and they are involved in the resistance to thermal stress in pea aphid hosts (Oliver *et al.* 2003).

Molecular genetic and phylogenetic studies of primary insect endosymbiosis have revealed that these symbiotic associations were ancient and involved strict vertical transmission within host lineages (Moran & Telang 1998; Moran & Baumann 2000; Moran *et al.* 2003; Baumann 2005). For examples, origins of endosymbiosis in weevils and aphids were estimated to occur at least 50 and 150 million years ago, respectively (Moran *et al*. 1993; Dale *et al*. 2002). Congruent topologies on the phylogenies of symbionts and hosts were frequently observed in diverse insects (Chen *et al.* 1999; Clark *et al*. 2000; Sauer *et al.* 2000; Hosokawa *et al.* 2006). These phylogenies suggested that intimate interaction between hosts and symbionts often lead to one on one cospeciation of both partners. A recent molecular study also suggested that the cospeciation pattern could exist for multiple co-inheritant endosymbionts in leafhoppers (Takiya *et al*. 2006). These molecular genetic results reinforce the idea that the origin of endosymbiosis was an integral factor driving diversification of these organisms (Baumann 2005). Comparative genomic analyses of insect endosymbionts also revealed shared characteristics of genetic repertoire across insect taxa to enhance the survival of their hosts (Moran & Wernegreen 2000; Zientz *et al*. 2001; Wernegreen 2002; Gil *et al*. 2004). Therefore, endosymbiosis probably has a massive effect on insect diversification (Margulis & Fester 1991; Maynard Smith & Szathmary 1995; Moran & Telang 1998). Although endosymbiosis appears to facilitate diversification via expanding ecological niches, an endosymbiont may simultaneously enforce restrictions on host evolution, thus imped insect diversification (Margulis & Fester 1991; Moran & Telang 1998).

Symbiotic associations with prokaryotes are prevalent in sap-feeding hemipteran suborder of Sternorrhyncha (aphids, psyllids, whiteflies, and mealybugs) and Auchenorrhyncha (cicadas, spittlebugs, leafhoppers, treehoppers, and planthoppers) (Buchner 1965; Baumann 2005). Spittlebugs or froghoppers (Cercopoidea) are phytophagous insects feeding primarily on the xylem, with a preference of nitrogen-fixing angiosperms (Thompson 1994). These insects are equipped with midgut filter chambers that allow extraction of nutrients (mainly amino acids and carbohydrates) from the plant sap (Chapman 1998). The plant sap contains a large amount of carbohydrates, but is deficient in essential amino acids and vitamins necessary for the insect's growth and development (Baumann *et al*. 1995; Douglas 1998; Sandström & Moran 1999; Thao *et al.* 2000; Moran 2001; Moran *et al.* 2003). Many insects with restricted diets such as blood, plant sap, or wood, harbor bacterial endosymbionts for additional nutrients that are absent in their food sources or can not be synthesized in the host (Buchner 1965; Moran & Telang 1998; Baumann 2005; Dale & Moran 2006). Through their biosynthetic activities, these prokaryotes were hypothesized to provide their insect hosts with the essential amino acids (Buchner 1965; Moran & Telang 1998). An early detailed histological study by Buchner (1965) suggested that in most spittlebug species there are at least two biotypes of morphologically distinct endosymbionts. However, symbiotic bacteria frequently have different morphologies depending on life history stages and environments, therefore histological characterizations must be regarded as tentative and examined together with molecular and the phylogenetic studies. A recent molecular study identified a symbiont species in spittlebugs, "*Candidatus* Sulcia muelleri", belonging to the bacterial phylum of *Bacteroidetes* and with an ancient history of acquisition dating back to at least 260 million years ago (Moran *et al.* 2005). However, the majority of

endosymbiotic diversity in spittlebugs is unexplored. To what extent are these endosymbiots related to each other and to free-living prokaryotes, and their molecular characteristics are still unknown.

The purpose of this study is using the red striped spittlebug, *Okiscarta uchidae*, as an example to identify bacteriome-associated endosymbionts in spittlebugs, and to estimate the phylogenetic diversity of eubacterial endosymbionts associated with *O. uchidae* on the basis of 16S rDNA sequences. Phylogenetic analyses were conducted to evaluate their relationships to other known insect endosymbionts, pathogenic and free-living prokaryotes within the eubacteria. We also characterized the ultrastructure of the endosymbionts using transmission electron microscopy (TEM). In additions, *in situ* hybridization with specific 16S rDNA probes was used to associate the location of endosymbionts inside bacteriomes with amplified nucleotide sequences.

One of the major molecular characteristics of primary endosymbiont evolution is an increase in evolutionary rates compared to free-living relatives. As a result of maternal cytoplasmic inheritance and asexual reproduction, primary endosymbionts have experienced frequent cycles of population bottlenecks and expansion that lead to increased accumulation of mildly deletions mutations and accelerated sequence evolution (Moran 1996; Woolfit & Bromham 2003). Compared to closely related free-living bacteria, DNA sequences of insect endosymbionts often exhibit higher percentage of A+T (Moran 1996; Clark *et al.* 1999) and an increase in nonsynomonous substitutions in protein-coding genes (Wernegreen *et al.* 2001; Spaulding & von Dohlen 2001). In this study, relative nucleotide substitution rates of 16S rDNA sequences from isolated symbionts and their closely related bacteria were compared to exam whether these isolated endosymbionts exhibit an elevated

evolutionary rate, which is a common characteristic of the insect primary endosymbionts.

MATERIALS AND METHODS

Study organism, collecting, and rearing. The red striped spittlebug, *Okiscarta uchidae* is a cercopid species commonly occurring in the subtropical lowland forests of Taiwan. The insect usually occurs in moist habitats near streams, and a large number of adults frequently appear in June and July. Adults of *O. uchidae* are black in coloration with two red stripes across the center of forewings, and an adult size is around 13-16 mm (Fig 1A). Nymphs of this species cover themselves with air bubbles trapped within secretions of glands, and frequently feed on *Oreocnide pedunculata* (Shirai) Masamune and *Boehmeria densiflora* Hook. & Arn. (Urticaceae). We collected 30 adults and 50 nymphs of *O. uchidae* near Ping-Deng of Mt. Yang-Ming, approximately 30 nymphs of *O. uchidae* near Wai-Shung River of northern Taiwan, and 15 adults near Ku-Kuan of Central Taiwan, between August of 2005 to July of 2007. Insects were brought back to the laboratory and reared on potted *Or. pedunculata* in a greenhouse with the room temperature kept around 27°C.

TEM. The whole insect was immersed into the fixative solution [4% paraformaldehyde (PFA) and 7% gluteraldehyde in 0.1 M cacodylate buffer] at 4°C overnight. Then, the specimen was dissected with the aid of a microscope, and the dissected bacteriomes were placed in the fixative solution for two hours. The bacteriomes were then bathed within 1% tannic acid for two hours, and 15 minutes of 0.1M cacodylate buffer was used six times to wash the tissue. We then stored the bacteriomes in 1% osmium tetroxide for one hour at room temperature. After fixation, specimens were stained *en bloc* with 2% uranyl acid for one hour. After that, the bacteriomes were dehydrated with an ethanol series of increased concentrations from 50% to 100% and then embedded in Spurr's resin. Embedded tissues were sectioned by a Leica Ultracut R ultramicrotome (Leica, Heerbrugg, Switzerland) into ultra-thin

sections (80 nm) and collected on copper grids for observation using a TEM (H-7500, Hitachi High-Technologies Co., Tokyo, Japan) at 80 kV.

DNA extraction and PCR. The dissected bacteriomes from nymph were kept in phosphate- buffered saline (PBS) (0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4 and 0.007 M KH_2PO_4 at pH 7.4) before DNA extraction. Each DNA extraction and subsequent PCR was done using the bacteriome isolated from an individual insect. The DNA from whole abdomen of the insect was also extracted to exam additional symbionts found in the insect's abdomen. DNAs from the white and light yellow portions of bacteriomes were extracted separately using DNeasy Tissue Kit (Qiagen, Hilden, Germany). The extracted DNA was eluted in 30 µl of elution buffer. A fragment of approximately 1500 bps of 16S rDNA gene were amplified using universal primers for eubacteria, 10F (5'-AGT TTG ATC ATG GCT CAG ATT-3') and 1507R (5'-TAC CTT GTT ACG ACT TCA CCC CAG-3') (Moran *et al.* 2003). PCR reactions contained 35 µl of ddH_2O, 5 µl of 10X *Taq* buffer, 4 µl of 1 mM dNTP, 2 µl of each primers (10 mM), 1 µl of the DNA template, and 1 µl of 2 u/ul *Taq* polymerase (PRO tech, Taiwan) in a total volume of 50 µl. The PCR amplification was performed in a sequence for one cycle, denaturating step (94°C for one minute), annealing step (52°C for 1.5 minute), and extension step (72°C for two minutes). After 35 cycles, an added extension step at 72°C for ten minutes was done. PCR products were kept in 4°C and examined by electrophoresis in agarose gel. All PCR products were purified by Geneaid PCR/GEL purification Kit (Geneaid, Taipei, Taiwan), and eluted in 30 µl of elution buffer. The 16S rDNA genes of symbionts in the phylum *Bacteroidetes* were amplified with a primer set, 10_CFB_FF (5'-AGA GTT TGA TCA TGG CTC AGG ATG-3') and 1515_R (5'-GTA CGG CTA CCT

TGT TAC GAC TTA G-3') (Moran *et al.*, 2005). The annealing temperature of PCR for this primer pair was set to 58°C (Moran *et al.* 2003).

Cloning and colony selection. The amplified PCR products were cloned into vectors using TOPO Ligation Kits (Invitrogen, CA, USA) to get a single clone for sequencing. Competent cells of *Escherichia coli* (XL1 blue) with ampicillin (100 mg/ml) and 1 mM X-gal blue-white selection system were used to identify the positive inserts. White colonies with the inserted DNA fragments were selected for plasmid extraction. The plasmid DNA was extracted with Plasmid Miniprep Purification Kit (GeneMark, Taichung, Taiwan) accoding to the manufacturer's instruction and eluted in 30μl of elution buffer for subsequent RFLP analysis.

RFLP (Restriction Fragment Length Polymorphisms). An amplification, about 1700 bps were produced with the M13R/ M13F primer set. Six restriction enzymes (*AluI*, *HaeIII*, *RsaI*, *HindIII*, *BglII* and *EcoRI*) were used. but used *RsaI* and *BglII* to digest amplified PCR fragments from selected colonies for better resolution. The total volume of the RFLP reaction was 20 μl including 16.5 μl of ddH_2O, 2 μl of 10× buffer, 0.2 μl of BSA (10 μg/μl), 1 μl of PCR products (1 μg/μl), 0.5 μl of *RsaI* or *BglII* (10 μ/μl). The reaction was incubated in 37°C water bath for two and a half hours. The fragment lengths of digested DNAs for selected colonies were compared after gel electrophoreses.

Sequencing, sequence alignment and representative taxa. Plasmids containing DNA fragments of different lengths were sequenced on an ABI PRISM^{TM} 377 automatic sequencer (Perkin Elmer, Forst, CA, USA) by Mission Biotech (Taipei, Taiwan). The 16S sequences of endosymbionts were used query sequences in GenBank to search the closely related bacteria, and sequences of representative taxa in all 14 families of γ-proteobacteria and the sequences with the highest similarity

were downloaded from GenBank. We also downloaded 16S sequences of exemplars from insect symbionts, free-living bacteria, and pathogens for comparison (Table 1). Based on a secondary structure model of 16S rDNA in *E. coli* (Mueller & Brimacombe 1997), each sequence was partitioned into 168 stem and 158 loop regions, and manually aligned in MegAlign program of the DNASTAR package (version 5.01, DNASTAR Inc., Madison, USA).

Preparation of *in situ* hybridization probes. To associate endosymbionts with amplified 16S sequences in bacteriomes of insects, we developed specific probes using 16S rDNA sequences. We first aligned all amplified sequences and chose the most variable region to design oligonucleotide probes. There are six specific probes of proximately 25 bps, including Ba 309 (5'-GAT GAC CAG CCA CAC TGG AAC T-3'), Wh 21 (5'-GAT GAT CAG CCA CAG TGG AAC T-3'), Wh07 (5'-GAT GAT CAG CCA CAC TGA AAC T-3'), Wh02 (5'GAT GAT CAG CCA CAC TGG AAC T-3'), Ba104 (5'-GAT GAC CAG CCA CAC TGG AAC T-3'), and Ba12 (5'-GAT GAC CAG CCA CAC CGG AAC T-3'). We used eubacterial universal primer EUB338 (5'-GCT GCC TCC CGT AGG AGT-3') (Fukatsu *et al*. 1998) as positive controls. The negative control probe was designed from the rbcL gene of the chloroplasts of plants, C331F (5'-TCT ACG TAG TAA ATC AAC AAA GCC TAA A -3') (Jeong *et al.* 1997). Oligonucleotide probes were labeled with digoxygenin (Dig) at the 5' end of sequences by the Mission Biotech (Taiwan).

Whole-mount *in situ* hybridization. The bacteriomes were dissected in PBS and fixed using 4% paraformaldehyde (PFA) in PBS at 4°C overnight. After that, the bacteriomes were bleached in 20% H_2O_2 in methanol for three hours and washed with 100% methanol twice. Then the tissues were re-hydrated in PTw (0.1% Tween 20 in PBS), and then incubathed with proteinase K (10 μg/ml) for three minutes at room

temperature. The treated bacteriomes were washed with PBS for five minutes. The DNAs in the target cells were denatured by immersion in denaturation solution (70% formamide, 0.3 M NaCl, and 30 mM sodium citrate at pH 7) for two minutes at 70°C. The tissues were then fixed with 4% PFA in PBS for 20 minutes and washed by PBS for five minutes. The hybridization mixture [a total volume of 10 µl consisting of 50% formamide, 0.3 M NaCl/ 30 mM sodium citrate, 10% dextran sulfate, 50 µg of sonicated salmon sperm DNA (Calbiochem, Darmstadt, Germany, cat #262012) per ml, and 8 µg of oligonucleotide probes] was then denatured for five minutes at 70°C. Then, the hybridization mixture was placed on the bacteriomes at 37°C overnight. After hybridization steps, the samples were washed in hybridization buffer (50% formaide, 0.3 M NaCl and 30 mM sodium citrate at pH7) for five minutes three times at 45°C, and in 1× TNT [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl and 0.05% Tween 20] for 30 minutes twice at room temperature. For anti-Dig immunohistochemistry, the bacteriomes were treated with TNB (0.1 M tris-HCl at pH7.5, 0.15 M NaCl, and 0.05% Tween 20) for 30 minutes, and then anti-Dig antibody mix solution (Jackson ImmunoResearch Laboratories, Inc., Baltimore, USA, cat # 71769) was placed in each tube (150 U/ml antibody : TNB = 1:100), and the tissues were kept at 4°C overnight. Then the tubes were washed in PBS for five minutes three times, and the Nova Red Substrate working solution (Vector, Burlingame, CA) was placed in tubes for one and a half hours. The tissue was washed in PBS for five minutes and re-fixed in 4% PFA for 20 minutes. The bacteriomes were then washed with PBS for five minutes at room temperature. Finally, we mounted the bacteriomes with 80% glycerol/ PBS and the samples were embedded using OCT (optimal cutting temperature solution) overnight. Embedded tissues were sectioned using a cryostat (Leica, Germany) into 40 µm sections () and visualized using a microscope.

Phylogenetic analyses. We performed maximum parsimony (MP) analyses using PAUP* (version 4.0b10, Swofford 2003). Branch support was calculated using nonparametric bootstrapping (Felsenstein 1985) with 100 replicates of tree-bisection-reconnection (TBR) and the starting trees obtaining via random stepwise addition of 10 replications. The number of rearrangements was limited to 1×10^8 for each bootstrap replicate. We used MODELTEST (version 3.7, Posada & Crandall 1998) to select the best-fitting model for stems and loops of 16S separately based on Bayesian Information Criterion (BIC) (Posada & Buckley 2004; Alfaro & Huelsenbeck 2006). Prior settings for nucleotide substitution model were derived from the results of MODELTEST. These models were then used in the Bayesian analyses. Bayesian analyses were done using MrBayes (version 3.1.2, Huelsenbeck & Ronquist 2001). The Markov chain Monte Carlo (MCMC) searches were run for 1×10^7 generations with trees sampled for every 100 generations. Two separated runs each with four Markov chains were run simultaneously. After removal of 2,5000 burnin trees, the remaining trees were used to compute a 50% consensus tree in PAUP*.

Bayesian estimate of evolutionary rates. We used branch lengths and phylogenetic trees obtained from the Bayesian analyses to compare relative rates among lineages. The closest relatives of isolated endosymbionts including pathogens, free-living bacteria, and insect symbionts were identified and used as outgroups for rate comparision. The branch lengths from the most recent common ancestor (MRCA) of isolated endosymbionts and outgroups to each of the terminal taxa were obtained using Cadence program (version 1.08beta, Wilcox *et al.* 2004). The 95% posterior probability distribution of estimated branch length from terminal taxa to the MRCA were calculated using the Bayesian trees. The test is significant when the confidence

interval of the estimated branch length from the MRCA to a given taxa does not overlap with that for the other taxa. Relative rates of ingroup and outgroup taxa were plotted using OriginPro (version 75, MA, USA).

RESULTS

General morphology of bacteriomes. The bacteriomes of *O. uchidae* were located laterally beneath the tergites from second to fourth abdominal segments (Fig 1A, 1B). There were six bacteriomes on average in one individual. Bacteriomes of adult cercopids were composed of about 13 substructures, whereas that of nymphs were nearly 22. Bacteriomes of nymphs were more abundant than those in adults. Two types of bacteriomes co-existed in *O. uchidae*, one was light yellow and the other was white in coloration. The yellow bacteriome was oval, more translucent and assembled in the shape of the grapes (Fig 1C). The white bacteriome was comprised of closely aggregated tissues in a shape of balloons (Fig 1D). The yellow bacteriome in general was more abundant than the white one.

TEM and whole-mount *in situ* hybridization. Two morphologically distinct endosymbionts of the yellow bacteriomes were observed using the TEM (Fig 2A). One of them contained yeast-like symbionts with nucleus, mitochondria, endoplasmic reticulum, secretory granule or peroxisome, suggesting that they are eukaryotes (Fig 2B & C). Within the host cells, these endosymbionts were surrounded by numerous lipids droplets. These symbionts had lipid bilayer nuclear membrane and their inner mitochondrial membranes did not develop well. The other type of endosymbionts were located inside bacteriocytes with a large nucleus in the center, and the bacteriocytes were surrounded by sheath cells (Fig 2D). These sheath cells consisted of an enlarged cytoplasm and a nucleus which was smaller than that of the bacteriocytes (Fig 2E). Endosymbionts were numerous and densely packed within bacteriocytes. Morphologically, these symbionts are likely to be the primary endosymbionts of *O. uchidae*. The cytoplasm of these endosymbionts contained a large number of mitochondria. The slender bacteriocyte's cytoplasm could be seen

between two symbionts. Three lipid bilayer membranes can be observed in the envelope of these endosymbionts (Fig 2F). Five out of six labeled probes representing symbiont lineage (A-C) showed positive signals when hybridized with bacteriomes (Fig 3). The oligonucleotide probe, wh07 did not show positive signals (Fig 3M & N). Three probes, ba309, ba104, and ba12, showed positive signals in symbiont lineages C and D: wh02 and wh21 probes in symbiont lineage B showed positive signal (Fig 3E-L, O & P).

Phylogenetic relationships. Phylum *Bacteroidetes* sequences were successfully amplified by raising annealing temperature to 58°C. The symbiont sequences of the γ-subdivision could be amplified with a variety of annealing temperature below 58°C. Using the digestions of *BglII* and *RsaI*, a total of 34 distinct RFLP patterns from 94 colonies could be distinguished (Fig 4). Sequencing the selected colonies of these different RFLP patterns resulted in 24 distinct 16S rDNA sequences of approximately 1.5 kb. For phylogenetic analyses, sequence alignment contained 111 taxa and 1694 characters, in which 458 characters were constant and 941 variable characters were parsimony informative. Using the secondary structure model of *E. coli*, we identified a total of 883 bp in stem and 811 bp in loop regions. There were 45.5% A+T (37.6% in *O. uchidae*) in the stem region, but was biased toward AT-rich (62%; 60.8% in *O. uchidae*) in the loop region. Based on the Bayesian information criterion (BIC), TVM + I + G and TIM + I + G model was selected as best-fitted model for stem and loop region respectively. Four monophylytic symbiont lineages of *O. uchidae* were identified in the phylogeny (Fig 5). They all grouped phylogenetically within the γ division of the Proterobacteria within the eubacteria, except the "*Candidatus* Sulcia muelleri" (symbiont A), which was clustered within the phylum *Bacteroidetes*. The symbiont D lineage was closely

related to pathogenic *Shigella* sp. (Hershberg *et al.* 2007) and *E. coli*. The monophyletic symbiont C lineage was closely related to *Pectobacterium* which contains phytopathogenic species (Gardon *et al.* 2003). The sequences of symbiont B lineage derived from the whole abdominal extraction, was closely related to *Aeromonas* sp., and facultatively anaerobic bacteria in aquatic environments (Holmes *et al.* 1996; Demarta *et al.* 1999). The symbiont A lineage belonged to the phylum *Bacteroidetes* and was likely a related species of "*Candidatus* Sulcia muelleri" which grouped with endosymbionts of cockroaches (*Blattabacterium*) and lady beetles (*Coleomegilla maculate*).

Relative evolutionary rates. Results of Bayesian analyses using site-specific rate (SSR) model results showed that loop regions ($0.71272 \pm 8.13444 \times 10^{-5}$ subsitutions/site) evolved slower, approximately one half, than stem regions of 16S rDNA ($1.26385 \pm 7.47115 \times 10^{-5}$). Transversion rate (A-C) in the stem region (0.08) was lower than that in the loop region (0.12), transversion rate (A-G) in the stem region (0.24) was higher than that in the loop region (0.17). The other types of nucleotide substitution rate were equal in both regions. The estimated Bayesian evolutionary rates revealed that within the identified bacterial lineages, free living bacteria in general exhibited a higher evolutionary rate and the rates of the symbionts of *O. uchidae* were frequently lower (Fig 6). In the symbiont A lineage, the obligate anaerobe in digestive system of human, *Bacteroides fragilis* evolved in the same rate with that of the obligate symbiotic species of cockroaches, *Blattabacterium* sp. (Fig 6A). Within the same lineage, the other three symbiotic species, "*Candidatus* Sulcia muelleri" of cercopids (*Mahanarva costaricensis*), "*Candidatus* Sulcia muelleri" of *O. uchidae*, and *Coleomegilla maculate*, showed significant slower evolutionary rates than the other two species (Fig 6A). In the symbiont B lineage, the free living

bacterium, *Tolumonas auensis*, had a significantly faster evolutionary rate than that of the symbionts found within *O. uchidae* and the two pathogens, *Aeromonas sobria* and *A. media*; whereas within these three species, no significant difference in rate could be detected (Fig 6B). Within the symbiont C lineage, the symbiont of *O. uchidae* had the slowest rate which was significantly slower than that of two phytopathogens, *Brenneria nigrifluens*, and *Pectobacterium wasabiae* (Fig 6C). Evolutionary rates of the bacteria within the symbiont D lineage did not significantly differ from each other. Nevertheless, estimated relative rates of symbionts in the *O. uchidae* were among the lowest (Fig 6D). When analyzing the stem and loop regions separately, within the stem region of symbiont C lineage, the evolutionary rates of pathogen, *Yokenella regensburgei* were significant higher than other phytopathogen and symbionts of *O. uchidae*. However, this phenomenon was not observed in other lineages.

DISCUSSION

Our phylogenetic analyses of stem and loop regions of bacterial 16S rDNA revealed that the loop on average had a higher A+T content but slower substitution rate than that of the stem region. This result is not consistent with a positive correlation between AT-rich and highly elevated nucleotide substitution rates in insects (Lin & Danforth 2004; Danforth *et al.* 2005) and other organisms. For example, relative rates in loop region of 16S rDNA sequences of mammals evolved nearly 0.5 times faster than that in stem region (Burk *et al*. 2002). However, the substitution rate at stem region of genus *Streptomyces* evolved about twofold faster than that in loop region (Ueda *et al.* 1999), and this result is similar to our study. One possible reason for a slower rate in loop regions is that mutations in loop regions frequently make important structural changes (configuration of binding sites) and are expected to be under strong selection. (Ueda *et al.* 1999).

Other than fitting a single model of nucleotide substitution for the entire 16S rDNA as in earlier studies of bacterial evolution (Takiya *et al.* 2006), applying region-specific substitution model to stems and loops of 16S rDNA revealed that there was substantial rate heterogeneity among sites in these two regions. The pattern of nucleotide substitution was biased toward transversion in stems and transition in loops. Our results suggested that the rate heterogeneity among stem and loop regions of ribosomal genes, such as the frequently used bacterial 16S rDNA, should be taken into consideration in phylogenetic analyses by applying region-specific substitution models.

Within bacteriocytes of *O. uchidae*, two types of intracellular symbionts with distinct morphology were found in the detailed TEM observations. The irregular overall shape, size, and the electron-dense structures of the prokaryotic symbiont of *O.*

uchidae are similar to that of the primary endosymbiont of psyllids (Thao *et al.* 2000). These putative primary bacterial symbionts were also observed to have three lipid bilayer membranes as in the primary endosymbionts of mealybugs (von Dohlen *et al.* 2001). The morphological similarity in ultra-structure between these prokaryotes resided within bacteriocytes of *O. uchidae* and the primary endosymbionts of the closely related insects suggests that they constitute a group of primary or obligate endosymbionts with possible common ancestry. The eukaryotic endosymbionts found in *O. uchidae* inhabit in loosely structured bacteriocytes with abundant lipids and resemble the yeast-like endosymbionts (YLS) found in closely related hemipteran insects, planthoppers and aphids (Fukatsu & Ishigawa 1996; Suh *et al.* 2001), and anobiid beetles (Suh *et al.* 2001). The YLS was located in the mycetocytes (bacteriocytes) and had fewer and smaller lipid droplets than those in the fat body cells (Noda 1977). These fungal obligate gut endosymbionts were found to have metabolic function of utilizing sterol and recycling nitrogen for the insect hosts (Wetzel *et al.* 1992; Sasaki *et al.* 1996; Hongoh & Ishikawa 1997). Molecular phylogenetic studies suggested that some of them have independent origins other than the true yeasts (Saccharomycetes). The intracellular YLS in planthoppers (family Delphacidae) was derived from the filamentous ascomycetes (Euascomycetes) (Suh *et al*. 2001), whereas the intercellular YLS in abdominal hemocoel of aphids (Fukatsu & Ishigawa 1996) belongs to the Pyrenomycetes. Like other obligate fungal symbionts of insects, the YLS of *O. uchidae* identified in this study may play an important role in the host's nutrition by expanding the range of available resources or supplying enzymes for degradation and detoxification of plant saps. The nutritional role and phylogenetic affinity of this YLS of cercopids require further study.

We identified the proteobacterial symbionts of cercopids using the molecular

phylogenetic analyses of the bacterial 16S rDNA sequences for the first time. Here we have demonstrated that three distantly related symbiont species are all belong to the gram-negative, γ-Proteobacteria where most endosymbionts of phloem-feeding insects were originated (Munson *et al*. 1991b; Clark *et al*. 1993; Spaulding & von Dohlen 1998; Moran 2001; Baumann 2005). The remaining symbiont species is closely related to *Candidatus Sulcia muelleri* (phylum *Bacteroidetes*) of cercopids (Moran *et al*. 2005). The phylogenetic distribution of identified bacteria in *O. uchidae* strongly suggested that all four of these prokaryotic symbionts had independent origins.

The "primary" endosymbiont of insects frequently exhibited an elevated evolutionary rate compared to those of the secondary, facultative symbionts (Moran 1996; Woolfit & Bromham 2003; Baumann 2005). For example, the evolutionary rate of *Buchnera* species, primary endosymbiont of aphid, is 1.7 to 2.7 times faster than the free-living *E. coli* and *Salmonella enterica* serovar Typhimurium (Moran 1996). The endosymbionts of whiteflies, *Siphoninus phillyreae*, evolved nearly four times faster than the closest nonendosymbiotic species, *Zymobacter palmae* (Woolfit & Bromham 2003). However, in this study Bayesian estimation of evolutionary rates of isolated symbiont sequences are not significantly higher than that of the closely related free-living and pathogenic bacteria. On the contrary, the estimated relative rates of symbionts were frequently among the lowest of all bacteria compared. This result implied that the identified bacterial lineages are likely the "secondary "or facultative symbionts of *O. uchidae*, except the "*Candidatus* Sulcia muelleri" lineage, which has been shown to have an ancient origin among all sap-feeding hemipterans in the suborder Auchenorrhyncha and are likely to be obligate, vertically transmitted bacterial symbionts that provided hosts with nutrients (Baumann 2005; Moran *et al.* 2005). The A+T contents in these isolated bacteria are not as high as those that have

been isolated from other insects in previous studies (Fukatsu and Nikoh 1998; Spaulding & von Dohlen 1998, 2001). The average 53 % GC content of 16S rDNA sequences of *O. uchidae* symbionts is in the same range as that described for free-living bacteria (Moran & Telang 1998; Moran & Baumann 2000). Together the molecular characteristics observed for *O. uchidae*'s symbionts suggested that they may be not obligate, and are probably acquired more recently and not essential for this spittlebug's living.

Our study is among the few that focused on the bacterial symbiotic fauna of the xylem-feeding spittlebug. Buckner (1965) and Müller (1962) suggested that the symbiosis of hemipteran suborder Auchenorrhyncha, which includes cicadas (Cicadoidea), spittlebugs (Cercopoidea), leafhoopers and treehoppers (Membracoidea) and planthoppers (Fulgoroidea), contain the most morphologically diverse symbiont types among all insects. The spittlebugs primarily feed on xylems of plants and are considered to possess relatively more symbiontic species than phloem-feeder because xylem saps are nutritionally limited with far less organic carbon and nitrogen than those of phloem sap (Andersen *et al.* 1989; Redak *et al*. 2004). In conclusion, our study found one previously described "primary" bacterial endosymbionts, and characterized one intracellular eukaryotic yeast-like symbiont, and at least three putative γ-proteobacterial "secondary" symbiont species/lineages. The secondary endosymbionts of independent origins within the spittlebug's bacteriomes are in consistent with Buchner (1965) and Muller's (1962) view. In addition, our results confirm the existence of an additional phylum *Bacteroidetes* primary endosymbiont, "*Candidatus* Sulcia muelleri" in *O. uchidae*.

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FIGURE LEGENDS

Figure 1 (A) Adult of *O. uchidae*. (B) The third star nymphs of *O. uchidae*. (C) Light yellow bacteriomes. (D) White bacteriomes.

Figure 2 Transmission electron micrographs (TEM) of endosymbionts in *O. uchidae*. (A) Bacteriocytes. (B) Yeast-like symbiont surrounded by lipids. (C) Organelles of yeast-like symbiont. (D) Putative "primary" endosymbionts within a bacteriocyte. (E) Cell boundary of bacteriocytes. (F) Cell membranes of putative "primary" endosymbiont. Arrows indicat three lipid bilayer membranes. E is endosymbiotic bacteria, Y is yeast-like symbiont, N is the nucleus, L is lipid, M is mitochondria, ER is endoplasmic reticulum, G is secretory granule or peroxisome.

Figure 3 Specific detection of endosymbionts by whole-mount *in situ* hybridization. Probes were labeled with Dig. The red color is positive signal. Results of whole-mount (A, C, E, G, I, K, M, O) and tissue sections (B, D, F, H, J, L, N, P) with light yellow bacteriomes (A-D, G-L) and white bacteriomes (E, F, M-P). Positive signal were detected when tissues were probed with positive control probes (A & B), ba12 (E & F), ba309 (G & H), ba104 (I & J), wh02 (K & L), wh21 (O & P); no signal was detected when tissues were probed with negative control probes (C & D) and wh07 (M & N).

Figure 4 Representatives of RFLP analysis of symbiont's 16S rDNA amplified from bacteriomes of *O. uchidae*. Lanes M contained DNA size markers (3,000, 2,000, 1,500, 1,000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp, from top to bottom). Lanes 1 through 9 contained cloned 16S rDNA fragments digested by *Bgl II* (A) and *Rsa I* (B) and resolved in 2% agarose gel. (A) Results were from restriction enzyme, *Bgl II*. (B) RFLP patterns with restriction enzyme, *Rsa I*.

Figure 5 Bayesian phylogram based on 16S rDNA sequences of bacteria. Blue letters indicate identified endosymbionts of *O. uchidae*, green letters are endosymbionts of insects, red letters are obligate and facultative pathogens, and black letters are free-living bacteria. The numbers above the branches are parsimony bootstrap values followed by the Bayesian posterior probabilities.

Figure 6 Bayesian relative rate test. (A) Symbiont A lineage, *Bacteroides fragilis* evolves in a rate that is not significantly higher than the obligate *Blattabacterium* sp.. Primary symbiotic species, "*Candidatus* Sulcia muelleri", "*Candidatus* Sulcia muelleri" of *O. uchidae*, and *Coleomegilla maculate*, showed significantly smaller evolutionary rates than the former. (B) Symbiont B lineage, *Tolumonas auensis* had a higher evolutionary rate than the symbiotic species found within *O. uchidae* as well as the two pathogens, *Aeromonas sobria* and *A. media*. (C) Symbiont C lineage, *Brenneria nigrifluens*, had a higher evolutionary rate than the symbiotic species found within *O. uchidae* and phytopathogen, *Pectobacterium carotovorum*. The evolutionary rates of three phytopathogens, *Pectobacterium wasabiae*, *P. carotovorum*, and *P. cacticida*, were higher than *P. carotovorum*. (D) Symbiont D linage, *E. coli* and two isolated bacterial sequences from *O. uchidae* had overlapped 95% intervals; however, the obligate pathogen, *Shigella* sp. exhibited the highest evolutionary rate.

TABLE

Table 1 Bacterial 16S rDNA sequences used in this study.

Table 2 RFLP analyses of bacterial 16S rDNA amplified and coloned from endosymbionts of *O. uchidae*.

Figure 1

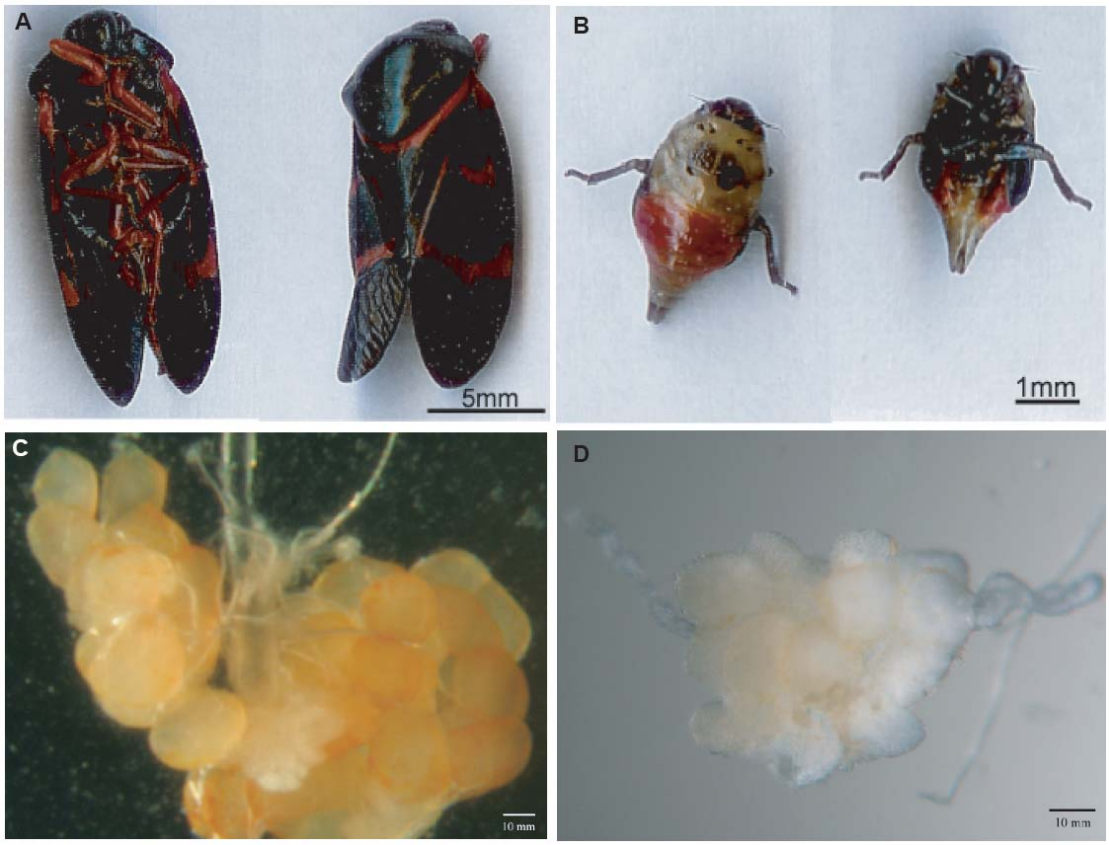

Figure 2A

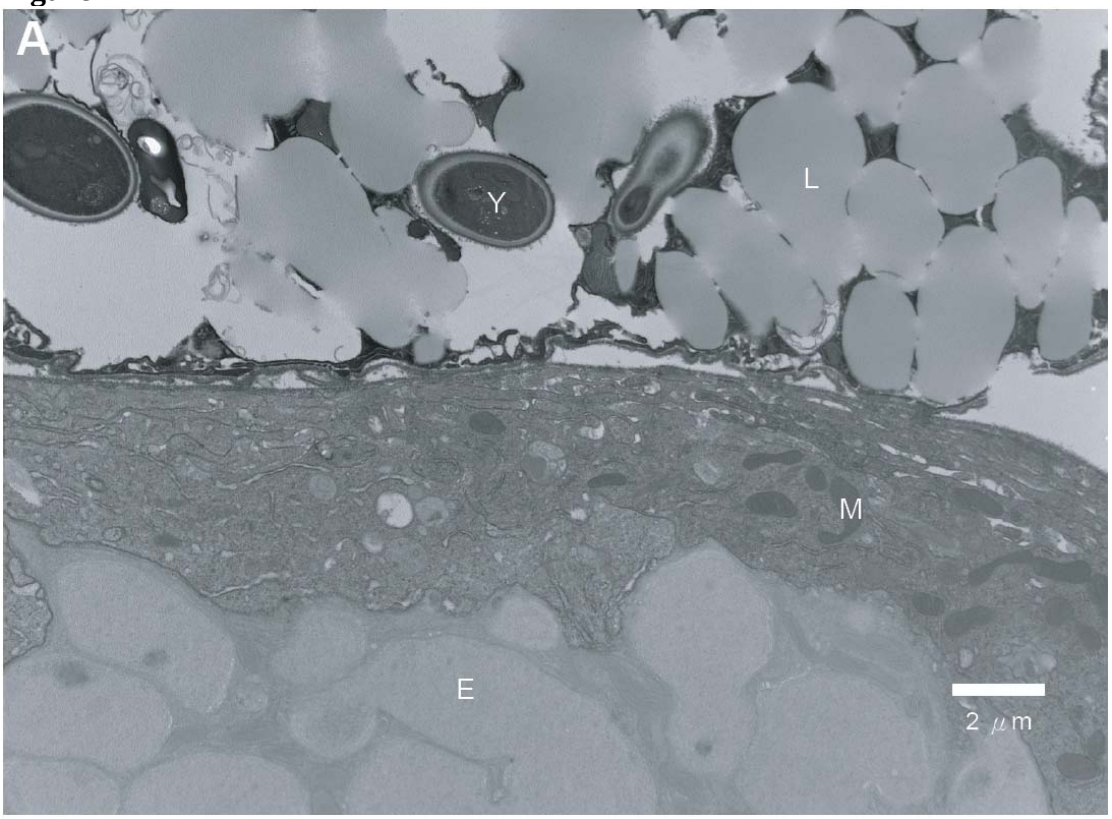

Figure 2B

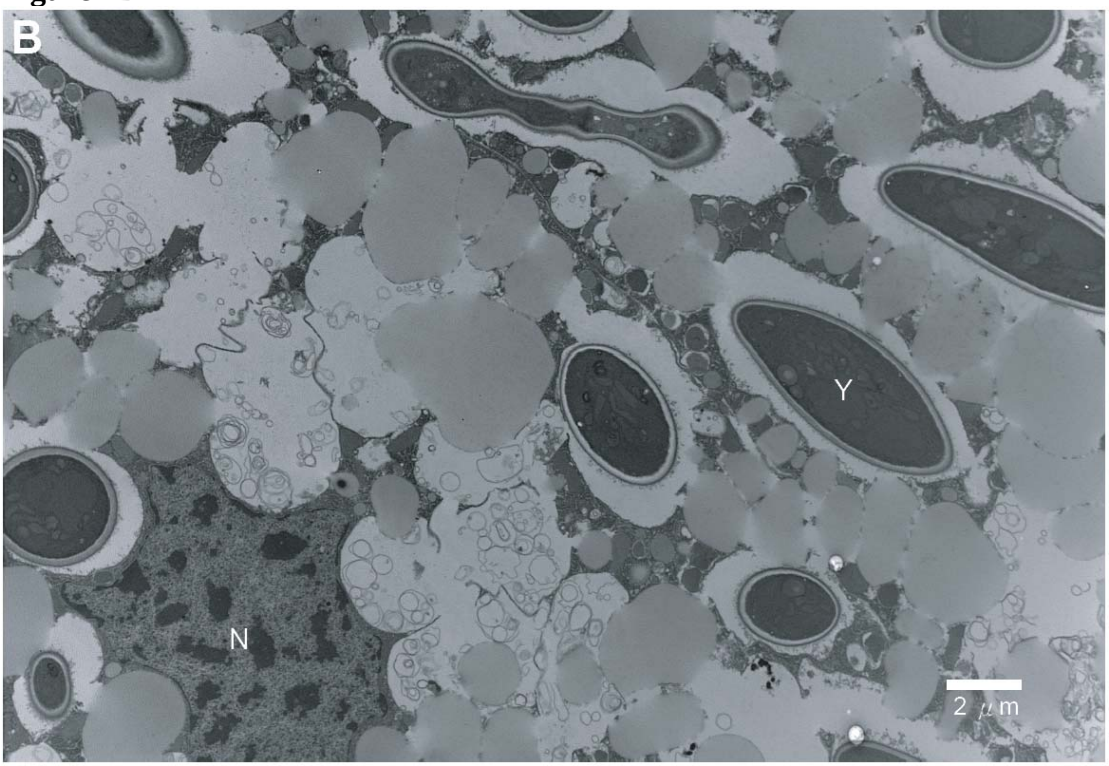

Figure 2C

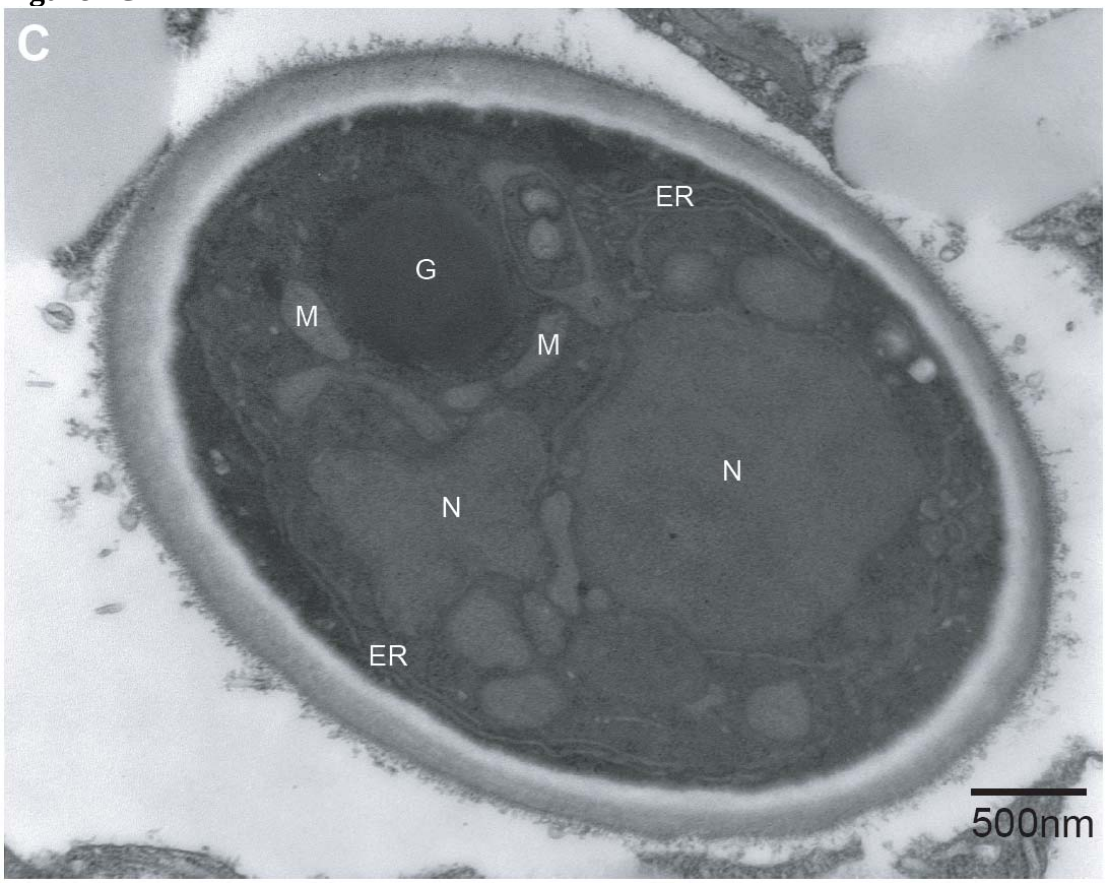

Figure 2D

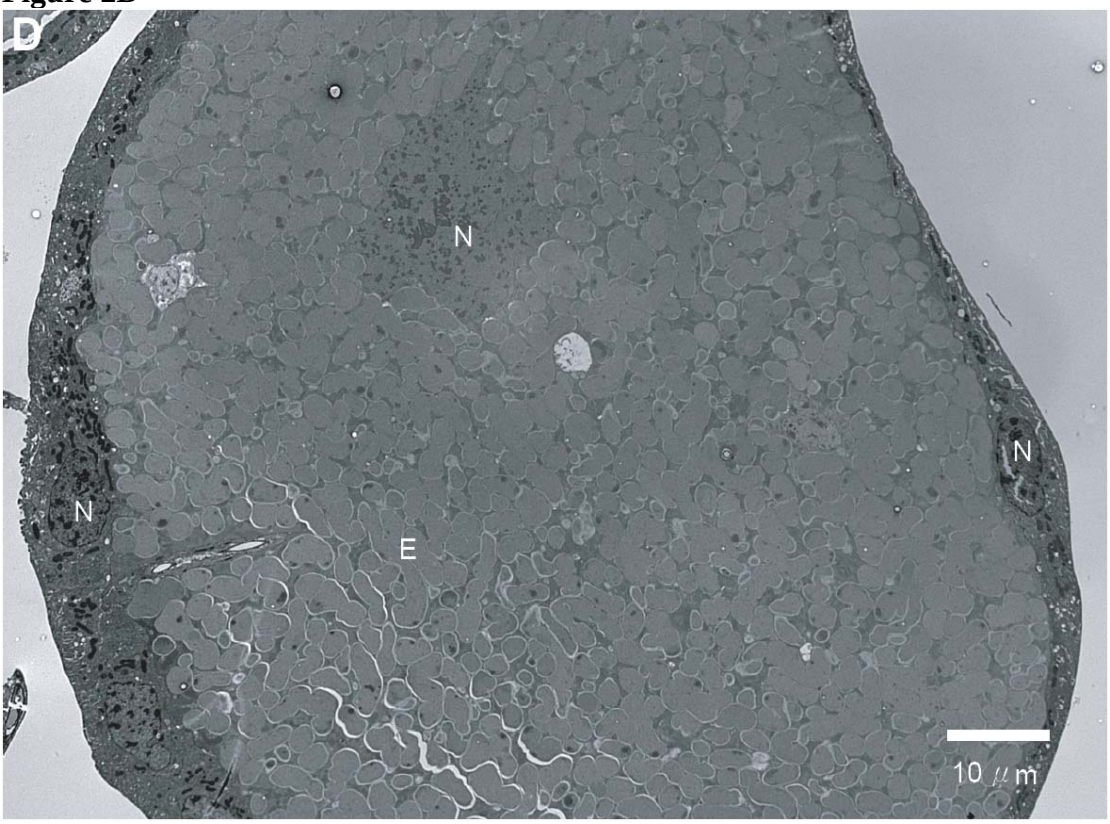

Figure 2E

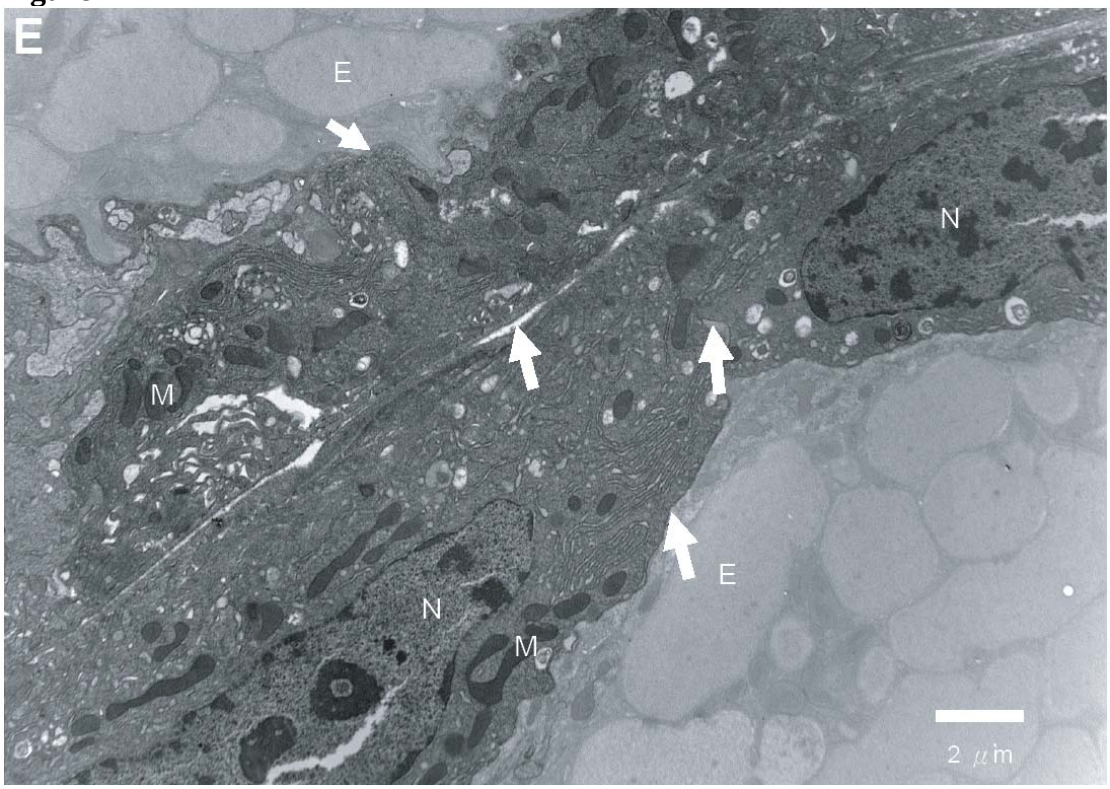

Figure 2F

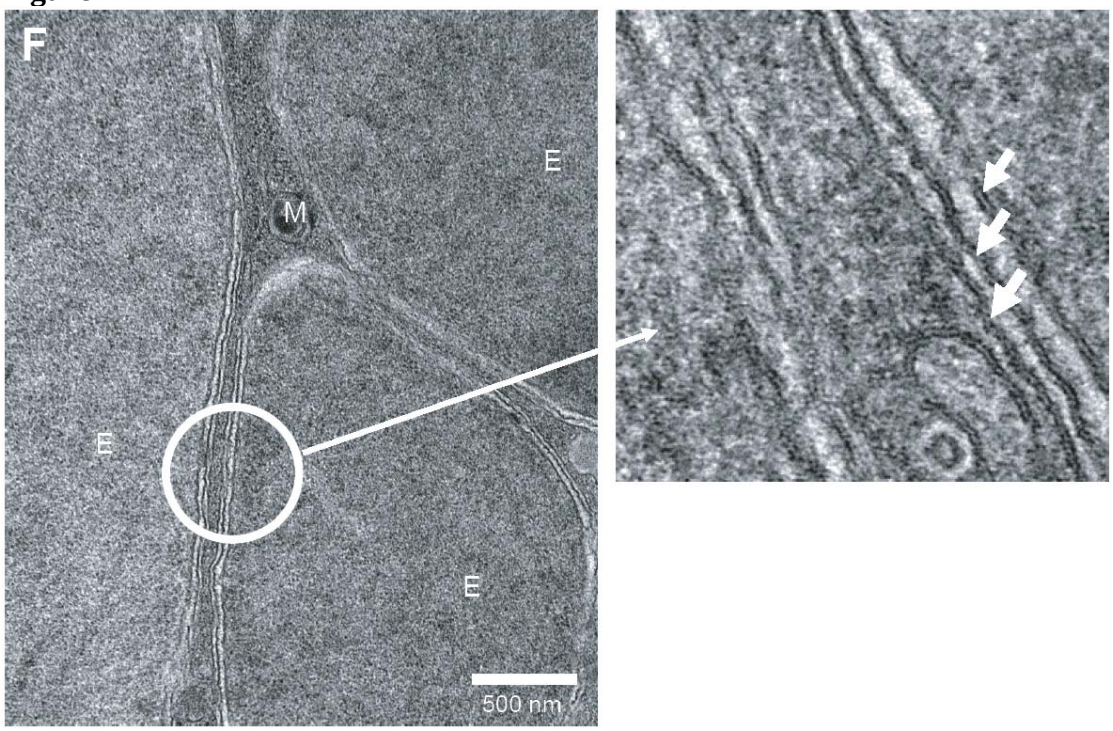

Figure 3

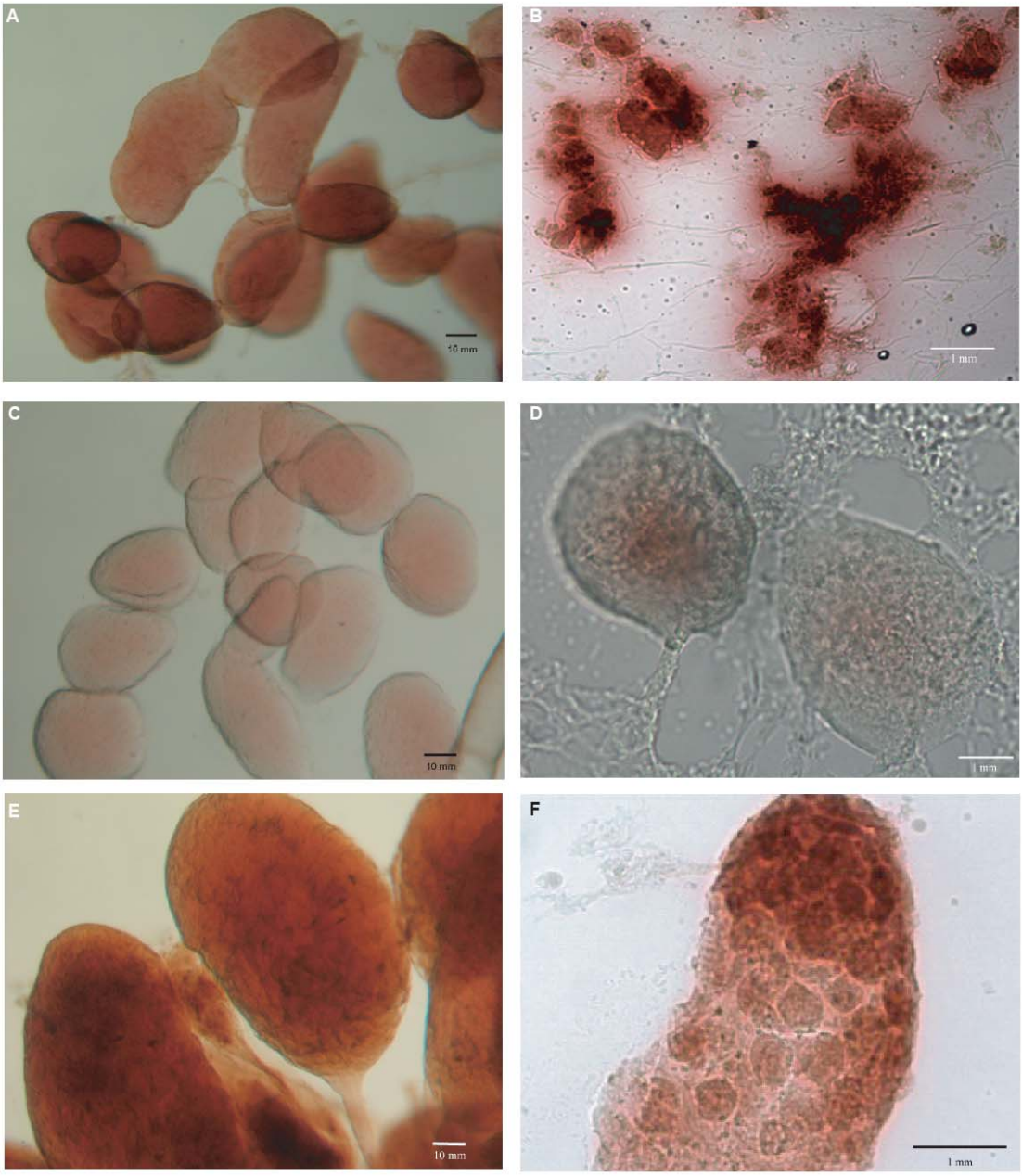

Figure 3 (contd')

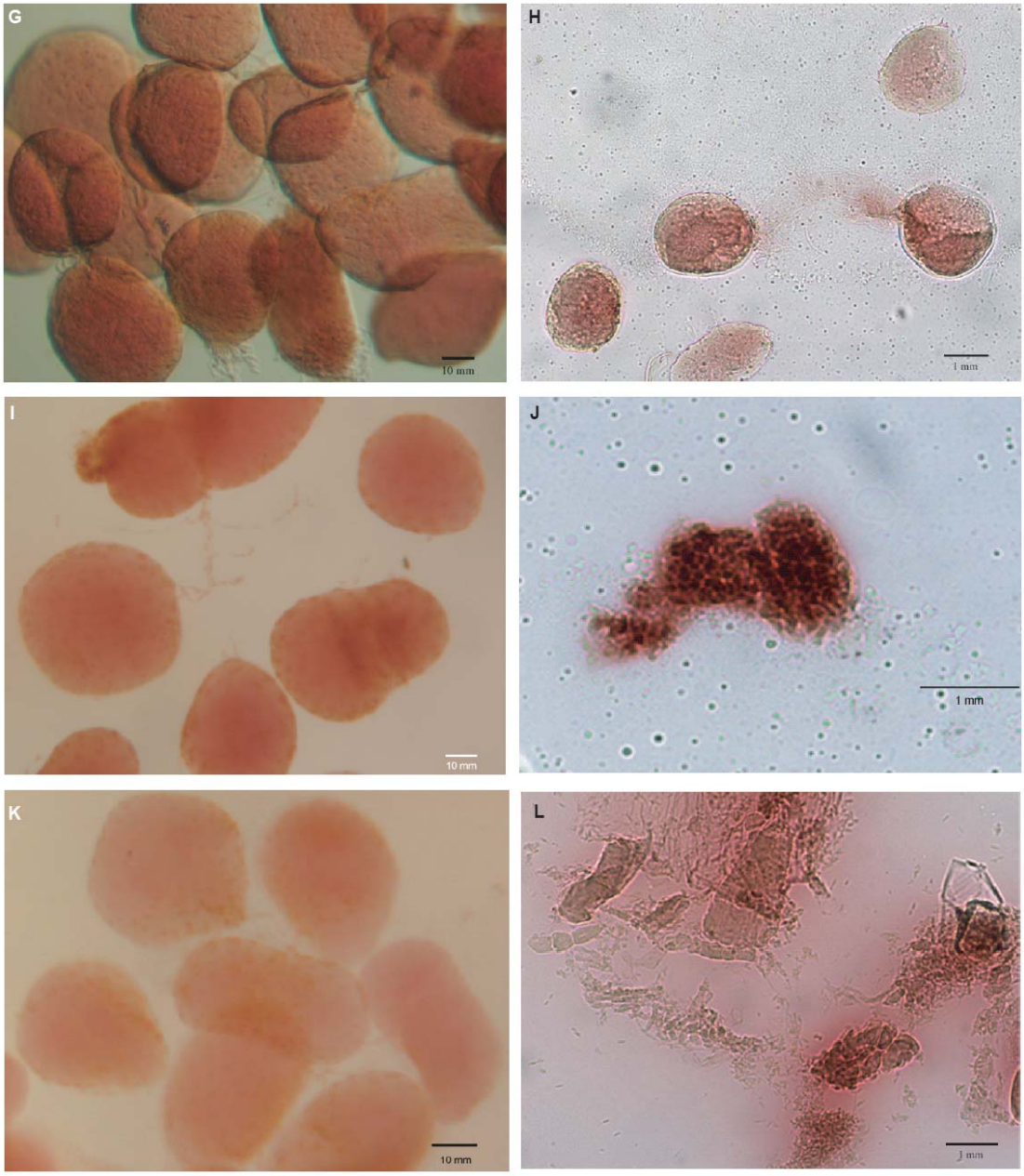

Figure 3 (contd')

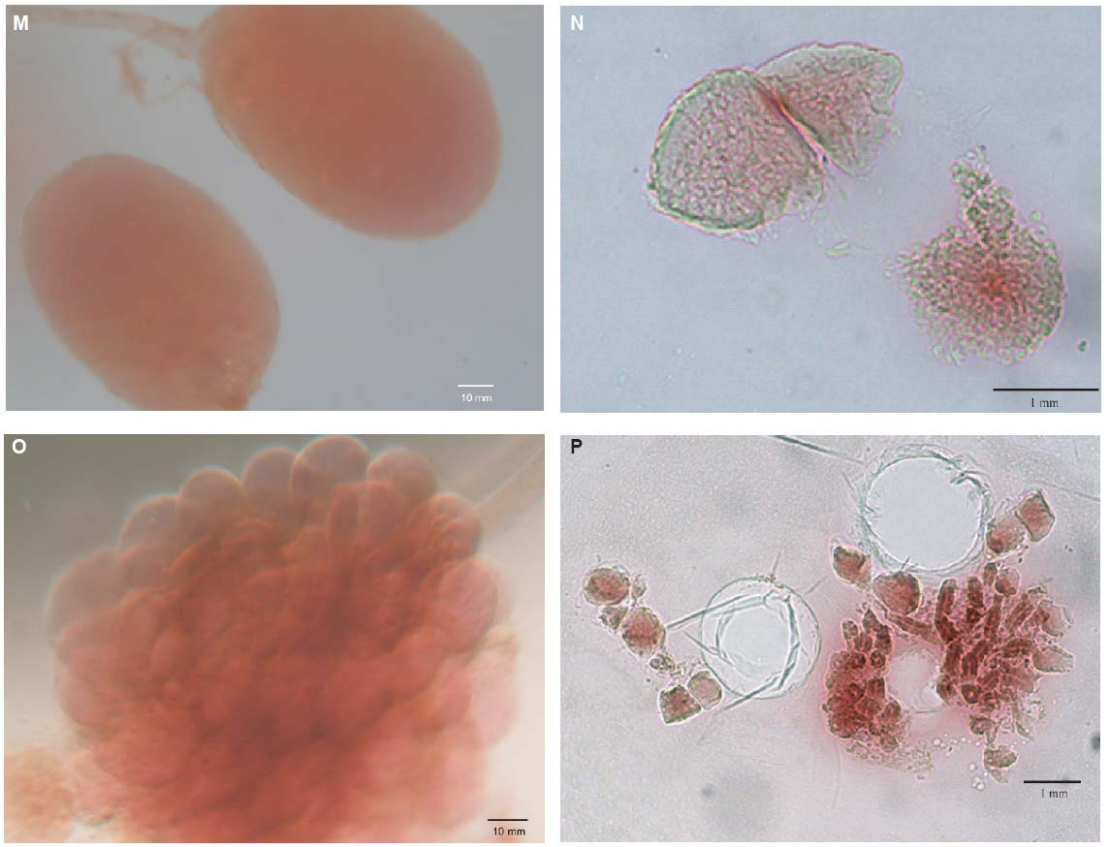

Figure 4

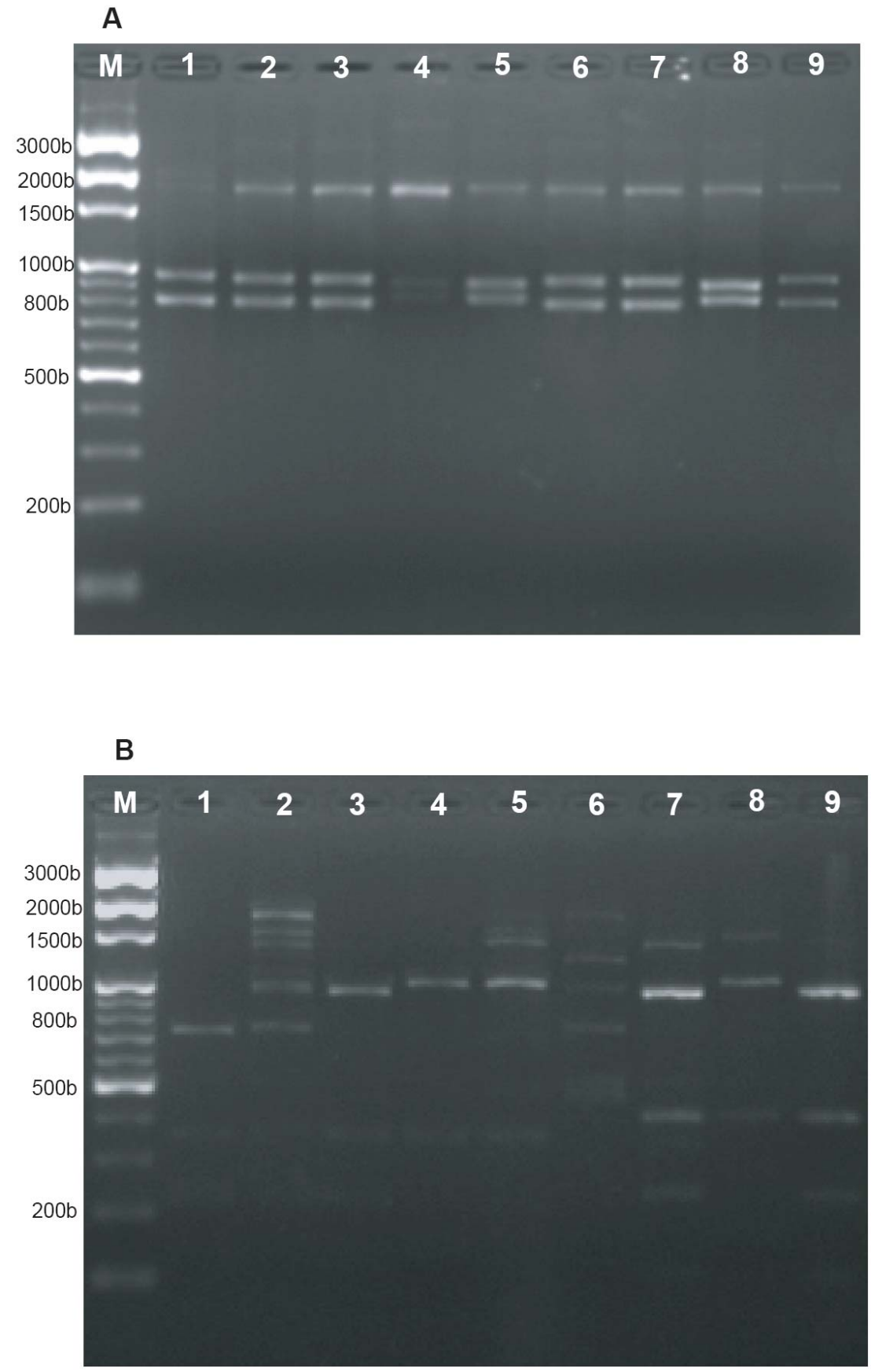
A
M
1
2
3
4
5
6
7
8
9
3000b
2000b
1500b
1000b
800b
500b
200b
B
M
1
2
3
4
5
6
7
8
9
3000b
2000b
1500b
1000b
800b
500b
200b

Figure 5

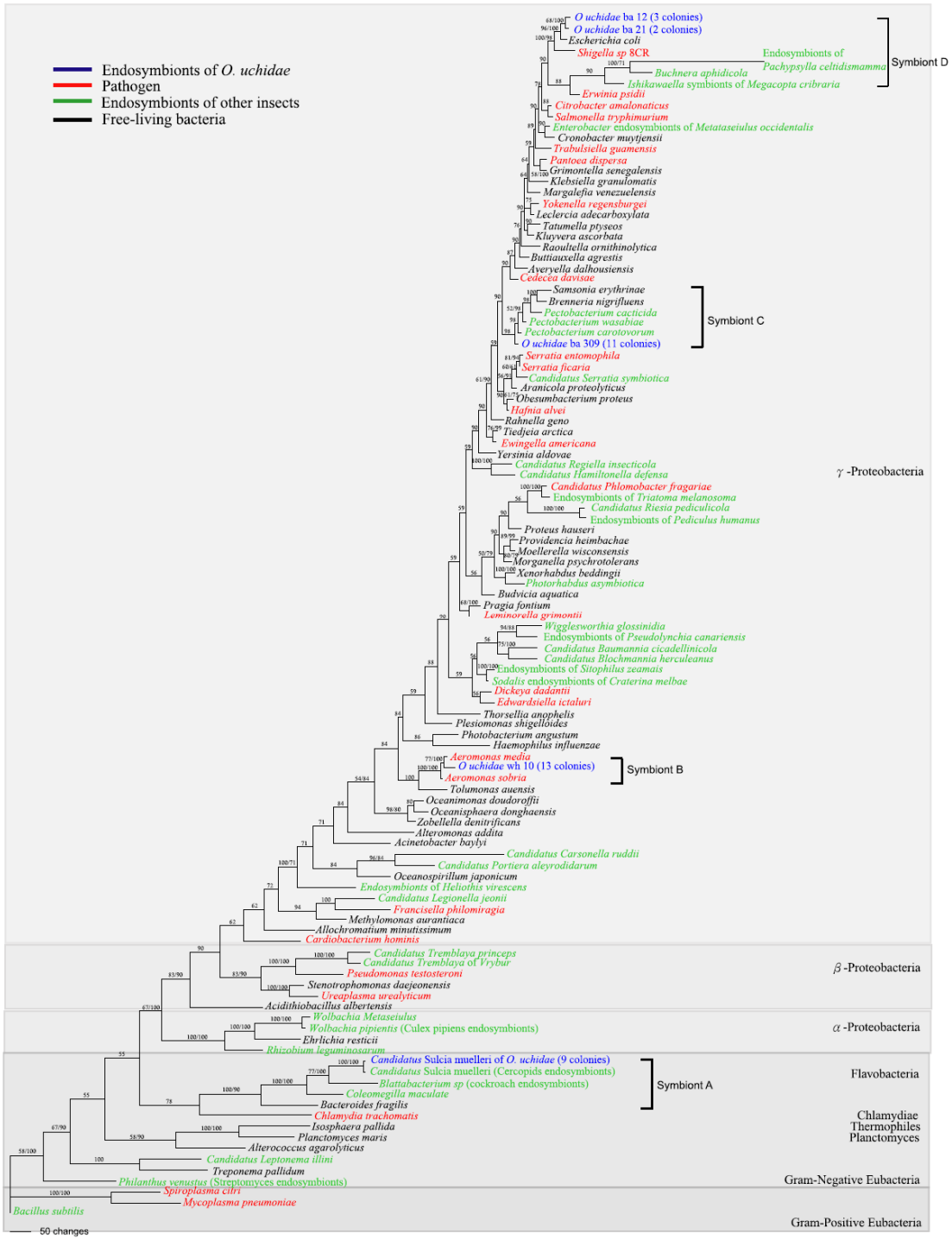
Endosymbionts of O. uchidae
Pathogen
Endosymbionts of other insects
Free-living bacteria
O uchidae ba 12 (3 colonies)
O uchidae ba 21 (2 colonies)
Escherichia coli
Shigella sp 8CR
Endosymbionts of Pachypsylla celtidismamma
Buchnera aphidicola
Ishikawaella symbionts of Megacopta cribraria
Symbiont D
Erwinia psidii
Citrobacter amalonaticus
Salmonella tryphimurium
Enterobacter endosymbionts of Metataseiulus occidentalis
Cronobacter muytjensii
Trabulsiella guamensis
Pantoea dispersa
Grimontella senegalensis
Klebsiella granulomatis
Margalefia venezuelensis
Yokenella regensburgei
Leclercia adecarboxylata
Tatumella ptyseos
Kluyvera ascorbata
Raoultella ornithinolytica
Buttiauxella agrestis
Averyella dalhousiensis
Cedecea davisae
Samsonia erythrinae
Brenneria nigrifluens
Pectobacterium cacticida
Pectobacterium wasabiae
Pectobacterium carotovorum
O uchidae ba 309 (11 colonies)
Symbiont C
Serratia entomophila
Serratia ficaria
Candidatus Serratia symbiotica
Aranicola proteolyticus
Obesumbacterium proteus
Hafnia alvei
Rahnella geno
Tiedjeia arctica
Ewingella americana
Yersinia aldovae
Candidatus Regiella insecticola
Candidatus Hamiltonella defensa
Candidatus Phlomobacter fragariae
Endosymbionts of Triatoma melanosoma
Candidatus Riesia pediculicola
Endosymbionts of Pediculus humanus
Proteus hauseri
Providencia heimbachae
Moellerella wisconsensis
Morganella psychrotolerans
Xenorhabdus beddingii
Photorhabdus asymbiotica
Budvicia aquatica
Pragia fontium
Leminorella grimontii
γ -Proteobacteria
Wigglesworthia glossinidia
Endosymbionts of Pseudolynchia canariensis
Candidatus Baumannia cicadellinicola
Candidatus Blochmannia herculeanus
Endosymbionts of Sitophilus zeamais
Sodalis endosymbionts of Craterina melbae
Dickeya dadantii
Edwardsiella ictaluri
Thorsellia anophelis
Plesiomonas shigelloides
Photobacterium angustum
Haemophilus influenzae
Aeromonas media
O uchidae wh 10 (13 colonies)
Aeromonas sobria
Symbiont B
Tolumonas auensis
Oceanimonas doudoroffii
Oceanisphaera donghaensis
Zobellella denitrificans
Alteromonas addita
Acinetobacter baylyi
Candidatus Carsonella ruddii
Candidatus Portiera aleyrodidarum
Oceanospirillum japonicum
Endosymbionts of Heliothis virescens
Candidatus Legionella jeonii
Francisella philomiragia
Methylomonas aurantiaca
Allochromatium minutissimum
Cardiobacterium hominis
Candidatus Tremblaya princeps
Candidatus Tremblaya of Vrybur
Pseudomonas testosteroni
Stenotrophomonas daejeonensis
Ureaplasma urealyticum
Acidithiobacillus albertensis
β -Proteobacteria
Wolbachia Metaseiulus
Wolbachia pipientis (Culex pipiens endosymbionts)
Ehrlichia resticii
Rhizobium leguminosarum
α -Proteobacteria
Candidatus Sulcia muelleri of O. uchidae (9 colonies)
Candidatus Sulcia muelleri (Cercopids endosymbionts)
Blattabacterium sp (cockroach endosymbionts)
Coleomegilla maculate
Symbiont A
Flavobacteria
Bacteroides fragilis
Chlamydia trachomatis
Isosphaera pallida
Planctomyces maris
Alterococcus agarolyticus
Chlamydiae
Thermophiles
Planctomyces
Candidatus Leptonema illini
Treponema pallidum
Philanthus venustus (Streptomyces endosymbionts)
Gram-Negative Eubacteria
Spiroplasma citri
Mycoplasma pneumoniae
Bacillus subtilis
Gram-Positive Eubacteria
50 changes

Figure 6

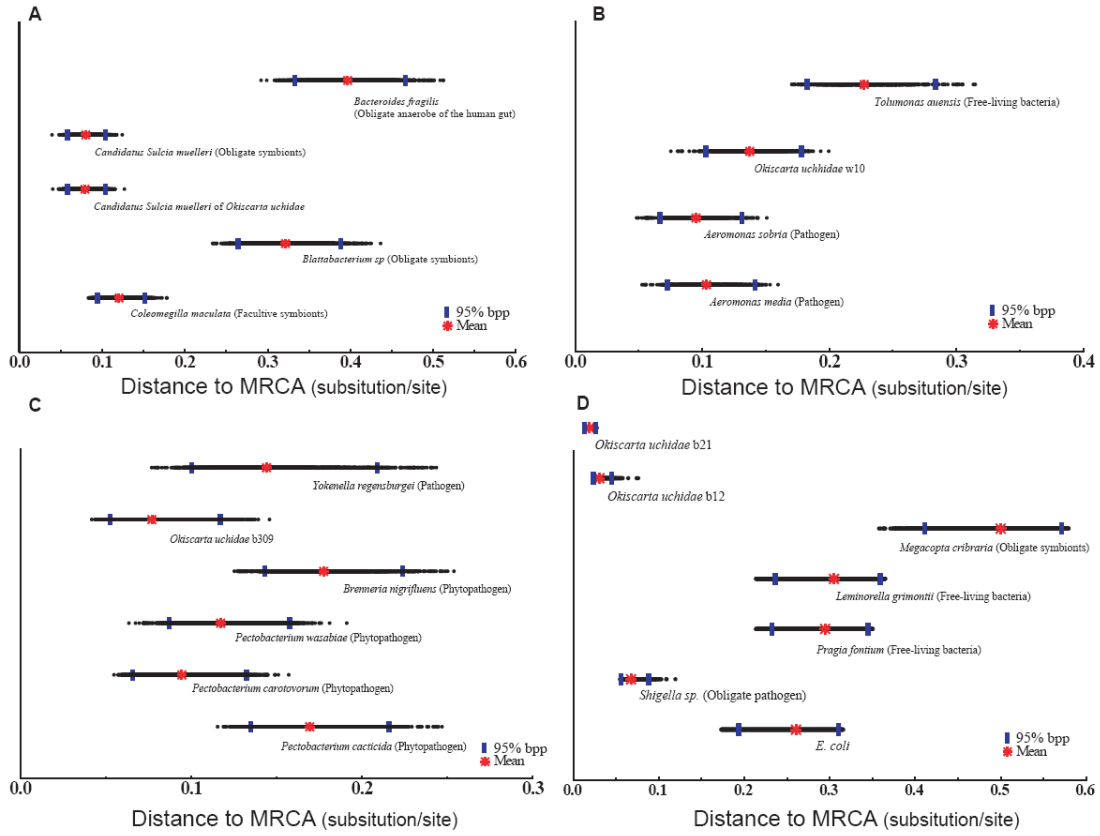
A
Bacteroides fragilis
(Obligate anaerobe of the human gut)
Candidatus Sulcia muelleri (Obligate symbionts)
Candidatus Sulcia muelleri of Okiscarta uchidae
Blattabacterium sp (Obligate symbionts)
Coleomegilla maculata (Facultive symbionts)
95% bpp
Mean
0.0 0.1 0.2 0.3 0.4 0.5 0.6
Distance to MRCA (subsitution/site)
B
Tolumonas auensis (Free-living bacteria)
Okiscarta uchidae w10
Aeromonas sobria (Pathogen)
Aeromonas media (Pathogen)
95% bpp
Mean
0.0 0.1 0.2 0.3 0.4
Distance to MRCA (subsitution/site)
C
Yokenella regensburgei (Pathogen)
Okiscarta uchidae b309
Brenneria nigrifluens (Phytopathogen)
Pectobacterium wasabiae (Phytopathogen)
Pectobacterium carotovorum (Phytopathogen)
Pectobacterium cacticida (Phytopathogen)
95% bpp
Mean
0.0 0.1 0.2 0.3
Distance to MRCA (subsitution/site)
D
Okiscarta uchidae b21
Okiscarta uchidae b12
Megacopta cribraria (Obligate symbionts)
Leminorella grimontii (Free-living bacteria)
Pragia fontium (Free-living bacteria)
Shigella sp. (Obligate pathogen)
E. coli
95% bpp
Mean
0.0 0.1 0.2 0.3 0.4 0.5 0.6
Distance to MRCA (subsitution/site)

Table 1

Classification	Species	GenBank Accession
Gram-Positive Eubacteria		
Low G+C	*Bacillus subtilis*	Z99107
	Mycoplasma pneumoniae	M29061
	Spiroplasma citri	AM157769
Gram-Negative Eubacteria		
Chlamydiae	*Chlamydia trachomatis*	DQ019310
Flavobacteria	*Blattabacterium sp.* (cockroach symbiont)	AF322473
	Candidatus Sulcia muelleri	DQ066635
	Bacteroides fragilis	AB050106
	Coleomegilla maculate (male-killing endosymbionts)	Y13889
Planctomyces	*Planctomyces maris* (strain DSM 8797T)	AJ231184
Thermophiles	*Isosphaera pallida* (strain DSM 9630T)	AJ231195
Spirochaeta	*Treponema pallidum*	AF426102
Leptospiras	*Leptonema illini*	M88719
α-Proteobacteria	*Ehrlichia resticii*	AF036654
	Rhizobium leguminosarum	D14513
	Philanthus venustus (*Streptomyces* endosymbionts)	AY854956
	Wolbachia pipientis (*Culex pipiens* endosymbionts)	X61768
β-Proteobacteria	*Candidatus Tremblaya* (mealybug symbiont)	AF476098
	Pseudomonas testosteroni	M11224
γ-Proteobacteria		
Acidithiobacillales		
Acidithiobacillus	*Acidithiobacillus albertensis*	AJ459804
Aeromonadaceae		
Aeromonas	*Aeromonas media*	AY987773
	Aeromonas sobria	X60412
Oceanimonas	*Oceanimonas doudoroffii*	AB019390
Oceanisphaera	*Oceanisphaera donghaensis*	DQ190441
Tolumonas	*Tolumonas auensis*	X92889
Zobellella	*Zobellella denitrificans*	DQ195675
Alteromonadales		
Alteromonas	*Alteromonas addita*	AY682202
Cardiobacteriales		
Cardiobacterium	*Cardiobacterium hominis*	M35014
Chromatiales		
Allochromatium	*Allochromatium minutissimum*	Y12369

Table 1 (contd.)

Classification	Species	GenBank Accession
Enterobacteriaceae		
Alterococcus	*Alterococcus agarolyticus*	AF075271
Aranicola	*Aranicola proteolyticus*	APU93263
Arsenophonus	*Arsenophonus* (*Triatoma melanosoma* endosymbionts)	DQ508172
Averyella	*Averyella dalhousiensis*	DQ481464
Brenneria	*Brenneria nigrifluens*	AJ233415
Buchnera	*Buchneria aphidicola*	M27039
Budvicia	*Budvicia aquatica*	AJ233407
Buttiauxella	*Buttiauxella agrestis*	DQ440549
Candidatus Ishikawaella	*Megacopta cribraria* (*Ishikawaella* symbiont)	AB240158
Candidatus Phlomobacter	*Candidatus Phlomobacter fragariae*	AB246669
Candidatus Riesia	*Candidatus Riesia pediculicola*	EF110572
Cedecea	*Cedecea davisae*	AF493976
Citrobacter	*Citrobacter amalonaticus*	AF025370
Cronobacter	*Cronobacter muytjensii*	EF059887
Dickeya	*Dickeya dadantii*	AF520707
Edwardsiella	*Edwardsiella ictaluri*	EF015475
Enterobacter	*Metaseiulus occidentalis* (Enterobacter endosymbionts)	AY753173
Erwinia	*Erwinia psidii*	Z96085
Escherichia	*Escherichia coli*	NC000913
Ewingella	*Ewingella americana*	DQ383802
Grimontella	*Grimontella senegalensis*	AY217653
Hafnia	*Hafnia alvei*	DQ412565
Klebsiella	*Klebsiella granulomatis*	AF010251
Kluyvera	*Kluyvera ascorbata*	AM184232
Leclercia	*Leclercia adecarboxylata*	AJ277978
Leminorella	*Leminorella grimontii*	AJ233421
Margalefia	*Margalefia venezuelensis*	AY702662
Moellerella	*Moellerella wisconsensis*	AM040754
Morganella	*Morganella psychrotolerans*	DQ358143
Obesumbacterium	*Obesumbacterium proteus*	DQ223874
Pantoea	*Pantoea dispersa*	AY227805
Pectobacterium	*Pectobacterium cacticida*	Z96092
	Pectobacterium carotovorum subsp. *brasiliensis*	AY207086
Photorhabdus	*Photorhabdus asymbiotica* subsp. *asymbiotica*	Z76755
Plesiomonas	*Plesiomonas shigelloides*	X60418

Table 1 (contd.)

Classification	Species	GenBank Accession
Providencia	*Providencia heimbachae*	AM040490
Rahnella	*Rahnella geno*sp. 3	RGU90758
Raoultella	*Raoultella ornithinolytica*	AJ630277
Salmonella	*Salmonella typhimurium* LT2	NC003197
Samsonia	*Samsonia erythrinae*	AF273037
Serratia	*Candidatus Serratia symbiotica*	AY296732
	Serratia entomophila	AJ233427
Shigella	*Shigella* sp. 8CR	DQ376908
Sodalis	*Craterina melbae* (*Sodalis* endosymbiont)	EF174495
Tatumella	*Tatumella ptyseos*	AJ233437
Thorsellia	*Thorsellia anophelis*	AY837748
Tiedjeia	*Tiedjeia arctica*	DQ107523
Trabulsiella	*Trabulsiella guamensis*	AY373830
Wigglesworthia	*Wigglesworthia glossinidia*	AF022879
Xenorhabdus	*Xenorhabdus beddingii*	X82254
Yersinia	*Yersinia aldovae*	AJ871363
Yokenella	*Yokenella regensburgei*	AY269192
Legionellales		
Legionella	*Candidatus Legionella jeonii*	AY598719
Methylococcales		
Methylomonas	*Methylomonas aurantiaca*	X72776
Oceanospirillales		
Pseudospirillum	*Oceanospirillum japonicum*	AB006766
Pasteurellales		
Pasteurellaceae	*Haemophilus influenzae* Rd KW20	NC000907
Pseudomonadales		
Acinetobacter	*Acinetobacter baylyi*	EF178435
Thiotrichales		
Francisella	*Francisella philomiragia*	EF153479
Vibrionales		
Photobacterium	*Photobacterium angustum*	AY900628
Xanthomonadales		
Stenotrophomonas	*Stenotrophomonas daejeonensis*	DQ109037

Table 1 (contd.)

Classification	Species	GenBank Accession
Unclassified		
Cardomonas	*Candidatus Carsonella ruddii*	AF211143
	Pachypsylla celtidismamma	AF286122
	Candidatus Blochmannia herculeanus	X92550
	Candidatus Hamiltonella defensa	AY296733
	Candidatus Regiella insecticola	AY296734
Portiera	*Candidatus Portiera aleyrodidarum*	AY268081
Trembleya	*Candidatus Tremblaya princeps*	AF476098
	Pseudolynchia canariensis	DQ115535
Baumannia	*Candidatus Baumannia cicadellinicola*	AY676895
	Pediculus humanus	DQ076660
	Sitophilus zeamais	M85269
	Heliothis virescens (testis endosymbionts)	L22481

Table 2

Individual	Restriction Enzymes	# bands	Fragment Lengths (bp)
wh02	*HaeIII*	2	3000, 1500
	RsaI	3	900, 350, 150
wh05	*HaeIII*	2	3000, 1500
	RsaI	2	1000, 350
wh06	*HaeIII*	2	3000, 1501
	RsaI	3	700, 350, 200
wh07	*HaeIII*	2	3000, 1502
	RsaI	6	2500, 1500, 1000, 600, 300, 150
wh10	*HaeIII*	2	3000, 1500
	RsaI	3	900, 350, 150
wh11	*HaeIII*	2	3000, 1500
	RsaI	3	1000, 350, 150
wh12	*HaeIII*	2	300, 150
	RsaI	3	900, 350, 150
wh15	*AluI*	2	200, 180
	RsaI	3	800, 350, 200
wh21	*HaeIII*	2	3000, 1500
	RsaI	3	900, 350, 150
wh22	*HaeIII*	2	3000, 1501
	RsaI	4	900, 350, 250, 150
wh213	*BglII*	3	300, 200, 150
	RsaI	4	550, 400, 250, 150
wh214	*BglII*	3	300, 200, 150
	RsaI	3	800, 350, 250
wh218	*BglII*	2	300, 200, 151
	RsaI	2	1000, 400
ba02	*BglII*	3	1800, 950, 800
	RsaI	3	1000, 500, 200
ba11	*BglII*	2	900, 850
	RsaI	2	1000, 500
ba12	*BglII*	2	900, 850
	RsaI	3	550, 500, 450
ba14	*BglII*	2	950, 850
	RsaI	3	550, 500, 450
ba21	*BglII*	5	1100, 900, 500, 400, 300
	RsaI	4	1500, 900, 700, 400
ba22	*BglII*	3	300, 250, 150
	RsaI	2	800, 400

Table 2 (contd')

Individual	Restriction Enzymes	# bands	Fragment Lengths (bp)
ba302	*BglII*	3	2000, 1000, 900
	RsaI	6	2500, 1800, 1000, 700, 400, 150
ba308	*BglII*	3	2000, 900, 850
	RsaI	5	2500, 1800, 900, 700, 400
ba309	*BglII*	3	2000, 900, 850
	RsaI	3	900, 400, 250
ba311	*BglII*	3	2000, 900, 850
	RsaI	3	950, 400, 150

II. 沫蟬與其初級內共生物的共種化

Cospeciation of Spittlebugs and their Primary β-Proteobacterial Endosymbionts

ABSTRACT

Most organisms have evolved mutualistic relationships with other genetic entities. Many insects hemipterans, harbor primary or obligate endosymbionts found inside the bacteriomes, which are tissues specialized for housing bacteria. Spittlebugs are unique among hemiptran insects because they primarily feed on xylem sap, which is the most nutritionally limited diet. Thus spittlebugs were considered to host a large number of diverse bacterial symbionts in bacteriomes. As a consequence of their vertical transmission, the primary endosymbionts were expected to cospeciate with their insect hosts.Our study examined the pattern of cospeciation between a group of newly identified obligate endosymbionts and their cercopid hosts using phylogenetic analyses. According to our analyses, the primary endosymbionts of spittlebugs formed a monophyletic group were clustered phylogenetically within gram-negative, β-proteobacteria. Five cospeciation events occured non-randomly when comparing the phylogenies of endosymbionts and their hosts. Bayesian relative rate analyses indicated hosts and their endosymbionts evolve at the same rate and likely to speciate at the same time. Our results suggest that this lineage of symbiont species in spittlebugs is the primary endosymbionts which cospeciated with their insect hosts.

Keywords: Cercopoidea, 16S rDNA, phylogeny, *COI*, *wingless*, Bayesian evolutionary rates, bacteriomes, endosymbiotic association.

沫蟬與其初級共生物的共種化

大部分的生物均演化出和其他基因體互利共生的關係。尤其是半翅目昆蟲體內的懷菌體存在著初級共生物。在半翅目昆蟲中，沫蟬的食性比較獨特，他們吸食具有較少養分的木質部汁液。我們的研究利用親緣分析來測試沫蟬與其初級共生物之間是否有共演化的模式。我們發現這群沫蟬的共生菌屬於格蘭氏陰性菌 β- proteobacteria 結果顯示沫蟬與其共生菌的親緣關係樹有五次的共演化事件發生，且顯著的不為隨機發生。貝氏分析指出內共生菌與沫蟬的相對演化速率呈正相關且演化的時間相同，結果支持共生菌與沫蟬具有共演化的歷史。由我們的結果推論，這一系群的內共生物應為 ”初級 ”內共生菌。

關鍵字：沫蟬總科、16S rDNA、親緣關係樹、*COI*、*wingless*、貝氏演化速率、懷菌體、內共生關係。

INTRODUCTION

Most organisms have mutualistic relationships with other genetic entities (Maynard Smith & Szathmary 1995). Among these interactions, symbiotic life style represents one of the major adaptations to overcome organism's own physiological limitations, and allows them to explore new ecological niches (Margulis & Fester 1991; Maynard Smith & Szathmary 1995; Moran & Telang 1998; Moran 2007). Symbiotic bacteria of insects were found to persist for hundreds of millions of years, and play an important role in host's biology including development (Braendle *et al.* 2003; Koropatnick *et al.* 2004), nutrition (Buchner 1965; Baumann *et al.* 1995; Subandiyah *et al*. 2000; Moran *et al*. 2003; Baumann 2005; Bäckhed *et al.* 2005), reproduction (Stouthamer *et al.* 1999; Hurst & Jiggins 2000; Bandi *et al.* 2001), speciation (Hurst & Jiggins 2000; Hurst & Werren 2001), and defense (Piel 2002; Oliver *et al.* 2003; Scarborough *et al.* 2005).

Unlike extra-cellular bacteria, endosymbionts of insects live intracellularly within specialized host tissues, called bacteriomes or mycetomes (Buchner 1965). Bacteriomes are groups of cells that have characteristic locations within the insect body (usually abdomenal region), and appear to function for housing bacteria (Buchner 1965; Fukatsu *et al.* 1998; Moran & Telang 1998; Fukatsu *et al.* 2000). Endosymbiotic bacteria of bacteriomes can provide nutrition for their insect hosts which usually have narrow or nutritionally unbalanced diet, such as wood, blood or plant saps (Moran *et al.* 1993; Wernegreen 2002; Moran *et al.* 2003). Many insects are associated with primary (bacteriome-associated), as well as secondary endosymbionts (Baumann 2005). The secondary endosymbionts are located in sheath cells around the bacteriomes or other locations (Baumann 2005). The primary endosymbionts of insects were found to supply essential amino acids for their hosts (Douglas 1998; Dale & Moran 2006). The nutritional function of insect primary

symbionts, including *Buchnera* (Lai *et al.* 1994; Shigenobu *et al.* 2000; Baumann 2005), *Wigglesworthia* (Zientz *et al.* 2004), *Blochmannia* (Zientz *et al.* 2004), and *Baumannia* (Wu *et al.* 2006), has been confirmed recently by whole-genome sequencing and microarray analyses (Dale & Moran 2006). Functions of the secondary endosymbionts are still unclear (Buchner 1965; Moran & Telang 1998; Russel *et al.* 2003). Nevertheless, a few secondary endosymbionts of insects have been successfully cultured (Darby *et al.* 2005), and recent studies suggest that the secondary endosymbionts may have an effect on host plant preference of their insect hosts (Tsuchida *et al.* 2004), make aphid host more resistant to thermal stress (Montllor *et al.* 2002; Oliver *et al*. 2003), and protect host from virus in the aphid and whitefly (ven den Heuvel *et al.* 1997; Morin *et al.* 2000).

The primary endosymbionts of insects are maternally transmitted from the mother to developing eggs or embryos (Moran & Telang 1998; Moran *et al.* 2003; Baumann 2005). As a consequence of their vertical transmission, the primary endosymbionts were expected to cospeciate with their insect hosts. Phylogenetic analyses of the primary endosymbionts and their insect hosts often resulted in congruent phylogenies with ancient correlated evolutionary history (Moran & Telang 1998; Spaulding & von Dohlen 2001; Moran *et al.* 2003; Baumann 2005). On the contrary, secondary symbionts were considered to be mostly horizontal transmitted, and appeared to be associated with phylogenetically diverse host lineages with relatively short evolutionary histories. Phylogenetic studies of secondary endosymbionts often indicate no concordant phylogenies between insect hosts and symbionts (Thao *et al*. 2000; Thao & Baumann 2004; Baumann 2005).

Spittlebugs, or froghoppers are plant-sucking insects belong to the insect superfamily Cercopoidea (Hemiptera). Nymphs, or immatures of the majority of spittlebugs embedded themselves into small patches of foamy structure, which is

attached to plant stems. These bubble-like structures resulted from foaming plant saps can protect the insects from predation and prevent further dehydration (Weiss 2006). Spittlebugs are unique among hemiptran insects that feed on plant saps. They are among the few insect taxa that feed exclusively on the xylem which are the most nutritionally limited diets and contain the most diluted concentration of nitrogen and carbohydrates than any other plant tissues (Redak *et al.* 2004). Therefore, spittlebugs were considered to host a large number of diverse bacterial symbionts in bacteriomes that supplement the nutritional need of the insects (Müller 1962, Buchner 1965). However, the biology of endosymbiont fauna in spittlebugs was little known. Much of our current knowledge of spittlebug's endosymbiont was still based on extensive histological studies by Paul Buchner (1965). The migration of the symbionts from the bacteriome to other locations, such as body cavity and oocytes in spittlebugs was observed and the morphology of the spittlebug's bacteriomes are considered the most variable among hemipteran insects (Buchner 1965).

Since the primary endosymbionts of insects are currently unculturable outside their hosts, molecular genetic methods were increasingly used to study the properties of these prokaryotes. For most primary or obligate endosymbionts, the characteristic and phylogenetic relationships of bacteria in these bacteriomes could not be established until the development of cloning and DNA sequencing techniques. So far only one widespread primary endosymbiont, "*Candidatus* Sulcia muelleri" in the bacterial phylum *Bacteroidetes*, was identified in spittlebugs and related insects in Auchenorrhyncha of the order Hemiptera (Moran *et al*. 2003; Moran *et al*. 2005). Phylogenetic relationships of *Sulcia* species in diverse auchenorrhynchan hosts are congruent with the insect host phylogenies, and the results suggested that this symbiotic association was obligatory and have an ancient history dated back to a

common ancestor of Auchenorrhyncha at least 260 million years ago (Moran *et al*. 2005).

In this study, we address the question of whether the newly identified primary bacteria have undergone long-term codiversification with their insect hosts. The specific aim was to characterize this group of obligate endosymbionts, and to test the pattern of cospeciation between symbionts and their spittlebug hosts using molecular phylogenetic analyses. Phylogenetic analyses were performed based on bacterial 16S rDNA, and on nuclear *wingless* and mitochondrial cytochrome oxidase I (*COI*) sequences of insects to independently reconstruct phylogenetic hypotheses of endosymbionts and insects. These data sets and phylogenies were then compared to assess the extent of support for a history of codiversification between host species and their two microbial associates. Specifically, tree topologies and relative evolutionary rates of the data sets were compared to assess the degree of congruence. Our results identified the first group of β-subdivision proteobacterial endosymbionts in spittlebugs, and the finding suggested that they are likely to represent the primary endosymbionts that maintain a long-term coevolutionary history with their insect hosts.

MATERIALS AND METHODS

Taxon sampling. Using the current classification and phylogenetic hypotheses of the spittlebugs (Cercopoidea) (Metcalf 1960; Cryan 2005), specimens of 17 species representing four major cercopid families, Aphrophoridae (8 spp.), Cercopidae (5 spp.), Clastopteridae (2 spp.), and Machaerotidae (2 spp.) were obtained from various locations (Table 1). The specimens were preserved in 95% ethanol immediately after capture and kept at -20°C until molecular works. The voucher specimens were subsequently preserved in 95% ethanol and stored at -80°C in the insect collection of Tunghai University.

DNA sequencing. The whole genomic DNA was extracted from bacteriomes using DNeasy Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The final products were eluted in 30 µl of elution buffer. For spittlebugs, mitochondrial cytochrome oxidase I (*COI*) and nuclear *wingless* (*Wg*) genes were amplified using primer sets, Ron and 3014R (Simon *et al.* 1994) and wg1a and wg2a (Brower & DeSalle 1998) respectively. The amplified *COI* and *Wg* fragments were about 1300 and 428 bps. For endosymbionts, a fragment of approximately 1500 bps of 16S rDNA gene was amplified using universal primers for eubacteria, 10F and 1507R (Moran *et al.* 2003). The PCR cycles included an initial denaturating step (94°C for one minute), followed by 35 cycles of 94°C for one minute, 58°C for one minute, 72°C for two minutes, and a final extension step of 72°C for ten minutes. When the annealing temperature was set from 50℃ to 52℃, the 16S rDNA sequences of endosymbionts in different insect hosts can be amplified. PCR products were kept at 4°C after the reactions were done. PCR products were gel purified and then cloned into vectors using TOPO Ligation Kits (Invitrogen, CA, USA). Plasmid DNAs containing desired fragment lengths were selected for sequencing on an ABI PRISM^{TM} 377 automatic sequencer (Perkin Elmer, USA) by the Mission Biotech,

Taiwan. *COI* and *Wg* sequences were manually aligned in MegAlign program of the DNA STAR package (version 5.01, DNA STAR Inc., Madison, USA). The 16S sequences of the primary endosymbionts were aligned based on the secondary structure model of 16S rDNA in *E. coli* (Mueller & Brimacombe 1997).

Phylogenetic analyses. We performed maximum parsimony (MP) analyses using PAUP* (version 4.0b10, Swofford 2003). Heuristic tree searches were performed using 10 random sequence additions and TBR (tree-bisection-reconnection) branch swapping. The branch support was calculated using non-parametric bootstrapping (Felsenstein 1985) with 1000 replicates of TBR branch-swapping with starting tees obtaining via stepwise addition of 10 replications. For Bayesian phylogenetic analyses, we used Modeltest (version 3.7, Posada & Crandall 1998) to select the best-fitted model for *COI* , *Wg* and 16S genes separately based on Bayesian information criteria (BIC) (Posada & Buckley 2004; Alfaro & Huelsenbeck 2006). Bayesian analyses were performed using MrBayes (version 3.1.2, Huelsenbeck & Ronquist 2001). Prior settings of model parameters for Baysesian analyses were obtained from results of Modeltest. Four Markov chains of two separated runs were performed simultaneously at each analysis. The Markov chain Monte Carlo (MCMC) search was set to run for 1×10^6 generations initially, and the resulting trees were sampled for every 100 generations. The MCMC runs were monitored for every 1×10^5 generations and were terminated after the average split frequencies falling below 0.01 (1×10^6 of generations). The Bayesian posterior probability was calculated by summarizing 7,500 trees excluding burnin of 2,500 trees.

Testing for pattern of cospeciation. To assess whether species of spittlebugs and their endosymbionts have undergone parallel speciation, we used topology-based statistical methods implemented in TreeMap (version 1.0 beta, Page 1994) to compute the fit between the host and symbiont phylogenies, and to find the optimal

reconstructions of the history of the association by maximizing cospeciation and minimizing host-switching events (Page 1994). A randomization test was performed in TreeMap to assess whether the number of reconstructed cospeciation events is significantly higher than created by chance alone. The test procedure was done by generating 10,000 of random symbiont trees and comparing the number of cospeciation events in these random trees to the observed number of reconstructed associations on the host trees.

Testing for correlation of coalescence times. The hypothesis of correlated sequence divergence was tested by comparing the relative evolutionary rates of corresponding hosts and symbionts. We plotted the sequence divergence of hosts against that of endosymbionts, and then fitted a line to the plot to exam the departure of the slope of the line from one. A slope of one in the fitted line suggested equal rate of host and symbiont evolution (Hafner & Page 1995; Hughes *et al.* 2007). The intercept of the fitted line represented the divergence of symbiont when their insect host speciated. An intercept of zero would suggest synchronous cospeciation in the hosts and symbionts. Likelihood methods with molecular model and parameter values derived from Modeltest were first used to obtain the ultrametric trees in PHYLIP (version 3.5c, Felsenstein 1989). The ultrametric trees were searched using maximum likelihood option with molecular clock. The resulted trees were imported into TreeMap to calculate the coalescences times of hosts and their endosymbionts. The coalescence time is corresponding to the distance between the ancestral node and any one of its descendants on the ultrametric trees. We then tested the expectation of cospeciation in which the speciation depth of nodes in the spittlebug and endosymbiont tree would be highly correlated. The correlation between copaths in the spittlebug and endosymbiont phylogenies were calculated in TreeMap using reduced major axis regression. Branch lengths from the sampled Bayesian trees of

phylogenetic analyses were used to obtain posterior probability of the distance from the most recent common ancestor (MRCA) of endosymbionts and their insect hosts. These Bayesian branch lengths were estimated for each cospeciation events using Cadence program (version 1.08beta, Wilcox *et al*. 2004). The resulted Bayesian estimates of MRCA of cospeciated spittlebugs and their endosymbionts were plotted against each other using OriginPro (version 75, MA, USA) to test the pattern of correlated coalescence times.

RESULTS

Sequence variation. For spittlebugs, three nuclear *Wg* copies of 389 bp, 426 bp and 428 bp long were identified. We used the more abundant 428 bp copy for phylogenetic reconstruction of spittlebugs. The 426 bp copy had four indels each with three base pair long in the sequence alignment, implying potentially four amino acid substitutions in this coding region. For 428 bp copy, the nucleotide base frequency was 42.5% A+T (22.8% A, 19.8 % T, 25.97% C, and 31.5% G). For *COI*, approximately 1034 bp in the cording region were obtained, and the sequence alignment contained no gaps. The nucleotide base frequency for *COI* was biased toward A+T (71.2%) that is frequently observed in most insect mitochondrial genomes (Simon *et al.* 1994, 2006). Bacterial 16S rDNA sequences of approximately 1.5 kb were obtained for the endosymbionts. Representative sequences of selected reference bacteria taxa for phylogenetic analyses were downloaded from GenBank. Using the secondary structure model of *E. coli*, the sequence alignment was partitioned into 163 stem and 173 loop regions. There were a total of 721 bp for stem and 684 bp long for loop regions. A+T content of 16S rDNA was 57.6% in stem regions and 69.3% in loop regions. For testing the monophyly of identified endosymbionts, a sequence alignment was constructed which contained 124 taxa and 1698 characters, in which 438 characters were constant and 980 variable characters were parsimony informative.

Phylogenetic distribution of the primary endosymbionts. Using the best-fitted TVM + G and GTR + G model for stem and loop region of 16S rDNA, reconstructed phylogenetic relationships based on the Bayesian and parsimony methods resulted in congruent phylogeny and a well resolved tree (Fig 1). This tree suggested that the identified endosymbionts of spittlebugs were all grouped phylogenetically within the β-subdivision of the Proteobacteria in the eubacteria. All endosymbionts formed a

well-supported monophyletic lineage (bootstrap/Bayesian posterior probability = 95/100) sister to a free-living *Pseudomonas testosteroni* and endosymbiont species of mealybugs, *Candidatus* Tremblaya. Whereas the other primary symbionts of spittlebugs, *Sulcia muelleri* were located within the phylumn Bacteroidetes bacteria as previously suggested (Moran *et al.* 2005), and closely related to *Blattabacterium* that is the symbiont of cockroaches (Fig 1).

Spittlebug and endosymbiont phylogenies. Based on BIC, the GTR + G and TVMef + G was selected as the best-fitted model of sequence evolution for *COI* and *Wg* data sets of spittlebugs. The reconstructed phylogenies of spittlebug species using parsimony and Bayesian methods were congruent, and the tree was well resolved and supported with high bootstrap values and posterior probability (Fig 2). Species from the same spittlebug family were each clustered into a monophyletic lineage. We used *Machaerota* and *Hindola* in the family Machaerotidae as outgroups to polarize the tree. The resulted tree suggested that the Cercopidae was a basal lineage and the Clastopteridae and Aphrophoridae were sister groups. However, the phylogenetic relationships of the represented four major cercopid families were not in concordant with the previous spittlebug phylogenies based on nuclear rDNA and histone genes (Cryan *et al.* 2005), which may be resulted from the choice of genes and taxon sampling. Parsimony and Bayesian phylogenetic analyses of endosymbiont's 16S rDNA sequences revealed one well resolved tree with strong bootstrap support and high posterior probability (Fig 3). Symbiont's sequences were grouped into the same family lineages where their spittlebug hosts were classified.

Spittlebug-endosybmiont association. Analyses of cospeciation pattern was performed in TreeMap using the optimal parsimony and Bayesian tree topologies derived from the combined *COI* and *Wg* data of spittlebug hosts and 16S rDNA of endosymbionts. The tanglegrams of the Bayesian tree of spittlebug versus that of

endosymbiont were reconstructed to compare the congruence of the two tree topologies (Fig 4). The optimal solution of TreeMap had to invoke five cospeciation, two duplication, and 21 sorting events without host switching to reconcile the Bayesian host trees and endosymbiont tree. The randomization test on this data set suggested that the optimal five cospeciation events were significantly higher (P = 0.045) than would have occurred randomly.

Correlated evolutionary rates. Bayesian relative rates of spittlebug hosts versus that of their symbionts were plotted to measure the degree of correlation between coalescence times to MRCA (Fig 5). The regression line had a slope of 1.03 (R = 0.84, P = 0.0023) which suggested the coalescence times were significantly correlated, and hosts and their symbionts evolved at the same rates. The intercept of the slope was positive (0.0052) but not significantly different from zero that suggested synchronous cospeciation in the hosts and symbionts. We also tested the correlation of evolutionary rates by plotting the coalescence times of spittlebug and endosymbionts using ultrametric trees generated in TreeMap (Fig 6). The reduced major axis method found a regression line with a slope of 0.70 (R = 0.70, P = 0.26) which suggested the evolutionary rates of hosts evolved faster than that of their endosymbionts. The intercept of the slope was positive (2.28×10^{-4}) but not significantly different from zero that suggested synchronous cospeciation in the hosts and symbionts.

DISCUSSION

The primary endosymbionts were identified in at least 15% of insect taxa (Baumann 2005), including aphids (Unterman *et al.* 1989; Munson *et al.* 1991; Moran *et al.* 1993; Clark *et al.* 2000), psyllids (Fukatsu & Nikoh 1998; Thao *et al.* 2000), sharpshooters (Moran *et al.* 2003; Takiya *et al.* 2006), whitefies (Thao & Baumann 2004), mealybugs (von Dohlen *et al.* 2001; Baumann 2005), tsetse flies (Chen *et al.* 1999), weevils (Lefevre *et al.* 2004), carpenter ants (Schröder *et al.* 1996; Degnan *et al.* 2004), and cockroaches (Lo *et al.* 2003). This is the first study reporting on a cospeciation pattern of the spittlebugs and their primary endosymbionts. The nine identified endosymbiont species/lineages belongs to the same β- proteobacterial group with ratively long branches. Analyzing the topological congruence between the host and endosymbiont phylogeny suggests that there is significantly non-random association between them. Bayesian relative rate analyses indicated hosts and their endosymbionts evolve at about the same rate. Furthermore, the coalescent times estimated for hosts and symbionts are significantly different, suggesting hosts had a higher evolutionary rate. All of these results together indicated a tight cospeciation pattern between those spittlebugs and their endosymbionts.

The group of endosymbiont identified in our study shows several characteristics suggesting that they are "primary" or obligate endosymbionts of spittlebugs. First, these endosymbionts evolved faster than closely related free-living bacteria, the secondary, facultative symbionts (Moran 1996; Woolfit & Bromham 2003; Baumann 2005). For example, comparing the evolutionary rates in the same lineage of extracellular symbionts and intracellular symbionts, the primary endosymbionts of aphids, *Buchnera* were significantly higher (about 6.3 fold) than that of free-living bacteria (Hosokawa *et al.* 2006). Secondly, the identified primary endosymbionts of spittlebugs were located phylogenetically within the β-proteobateria

clade and were monophyletic suggesting they shared a common ancestory within spittlebugs. However, different from pervious studies of phloem-feeding insects, whose endosymbionts belonged to the *γ*-Proteobacteria (Munson *et al*. 1991; Clark *et al*. 1993; Spaulding & von Dohlen 1998; Moran 2001; Baumann 2005). Thirdly, the congruence between phylogenies of hosts and their endosymbionts indicate that the mutualistic association between hosts and their primary endosymbionts was ancient and the endosymbionts were transmitted vertically through maternal germlines.

Studies of cospeciation patterns using different methodologies (MP, ML, and Bayesian inference) may generate various topologies, and thus result in different interpretations (Downie & Gullan 2005). In our study, using ML topologies derived from Bayesian analyses resulted in four cospeciation events; when applying ML analyses, however, five cospeciation events were found. There are a total of 21 estimated sorting events estimated in our analyses. Sorting event represents that the endosymbionts did not follow the lineage diversification of their hosts and thus some host lineages did not possess symbionts (Thao *et al*. 2000; Page 2003). This problem can be resolved by more adequate sampling of additional endosymbiont species. On the other hand, sorting events may occur if the host species diverged from the ancestral lineage with only a small founder population and occasionally lost their symbionts. This process can easily take place since the endosymbionts of spittlebugs are maternally inherited and thus may gradually lost their genetic diversity during the time as mothers passed them to the next generations. In our study, number of host species is greater than that of identified endosymbionts. Moreover, two species of spittlebugs, *Phinia* sp. and *Clovia puncta*, are classified into the same tribe, but they are grouped in different lineage in *COI* and *wg* phylogeny. The above reasons may explain the unusual large number of sorting events.

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FIGURE LEGENDS

Figure 1 Bayesian phylogeny of endosymbionts of spittlebugs and other bacteria reconstructed using 16S rDNA. Endosymbionts of spittlebugs are labeled in blue, Green letters represent insect endosymbionts, red letters are obligate and facultative pathogens, and black letters are free-living bacteria. Numbers on the left side of slash are parsimony bootstrapping values (bootstrap values smaller than 50% are not shown). Numbers on the right side of slash are Bayesian posterior probability. The endosymbionts of spittlebugs are shown within dash-block.

Figure 2 Bayesian tree based on mitochondrial *COI* and nuclear *wg* gene from spittlebugs. Parsimony bootstrap values are labeled on the left and Bayesian posterior probability on the right.

Figure 3 Phylogram with the highest likelihood value phylogram based on 16S rDNA sequences of identified endosymbionts of cercopids. Maximum parsimony bootstrap value are indicated on the left and Bayesian posterior probability on the right.

Figure 4 Tanglegram for spittlebugs and their endosymbionts. Spittlebug phylogeny was reconstructed using *COI* and *wg* genes, and the symbiont phylogeny was reconstructed using 16S rDNA. The black dots were cospeciation evens. The red lines connected the parallel lineages between spittlebugs and their endosymbionts.

Figure 5 Plot of Bayesian relative rates. The X-axis and Y-axis represent the time to MRCA for spittlebugs and endosymbionts respecttively. Error bars are the 95% posterior probability distribution of estimated time to the MRCA. The regression line with a slope of 1.03 (SE = 0.25, R = 0.84, $P = 0.0023$) was found using the reduced major axis method. The intercept of regression line is 0.0052 (SE = 0.033). The data labels refer to node letters as following: A [*Eoscarta botelensis* - *E. botelensis* b1], B [*Clastoptera sp1* - *C. sp1* b9], C [*Clastoptera sp2* - *C. sp2* b9], D [*Ariptyelus*

auropilosus - *A. auropilosus* b12], E [*Aphrophora taiwana* - *A. taiwana* b2], F [*Clovia puncta* - *C. puncta* b2], G [*Phinia sp.* - *P. sp.* b6], H [*Philagza sp.* - *P. sp.* b24], and I [*Machaerota sp.* - *M. sp.* b6].

Figure 6 Plot of estimated coalescence times for spittlebugs and their endosymbionts using the ML tree in TreeMap. Data labels refer to node numbers in Figure 4. The X-axis is host coalescence time, and the Y-axis is that for endosymbionts. The regression line with a slope of 0.70 (SE = 0.511, R = 0.70, $P = 0.26$) was found using the reduced major axis method. The intercept of regression line is close to zero (2.28×10^{-4}, SE = 0.046).

TABLE LEGENDS

Table 1 The collecting data of spittlebug specimens used in this study.

Table 2 Downloaded bacterial 16S rDNA sequences used in this study.

Figure 1

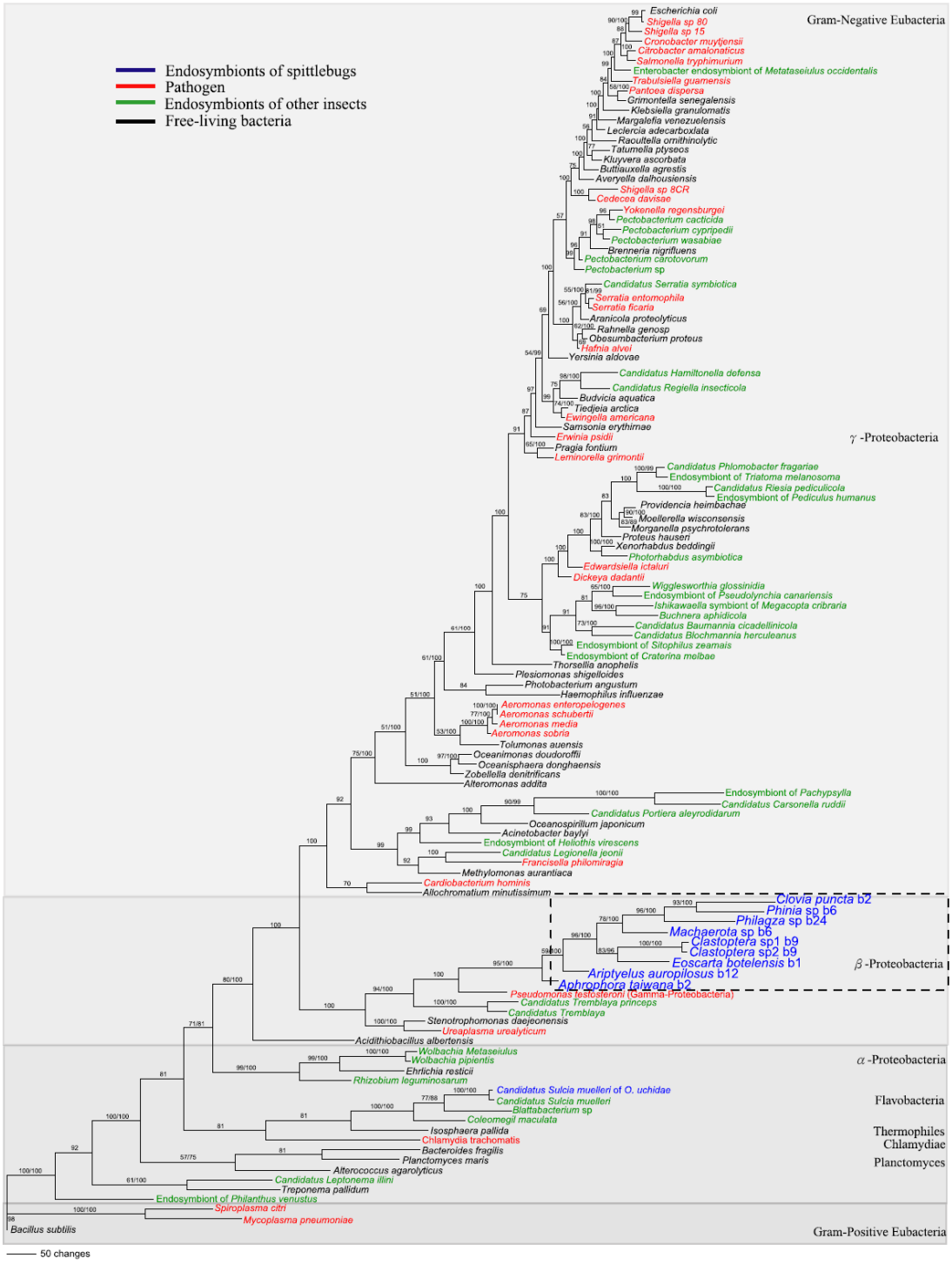
Endosymbionts of spittlebugs
Pathogen
Endosymbionts of other insects
Free-living bacteria
Gram-Negative Eubacteria
Escherichia coli
Shigella sp 80
Shigella sp 15
Croncbacter muytjensii
Citrobacter amalonaticus
Salmonella tryphimurium
Enterobacter endosymbiont of Metataseiulus occidentalis
Trabulsiella guamensis
Pantoea dispersa
Grimontella senegalensis
Klebsiella granulomatis
Margalefia venezuelensis
Leclercia adecarboxlata
Raoultella ornithinolytic
Tatumella ptyseos
Kluyvera ascorbata
Buttiauxella agrestis
Averyella dalhousiensis
Shigella sp 8CR
Cedecea davisae
Yokenella regensburgei
Pectobacterium cacticida
Pectobacterium cypripedii
Pectobacterium wasabiae
Brenneria nigrifluens
Pectobacterium carotovorum
Pectobacterium sp
Candidatus Serratia symbiotica
Serratia entomophila
Serratia ficaria
Aranicola proteolyticus
Rahnella genosp
Obesumbacterium proteus
Hafnia alvei
Yersinia aldovae
Candidatus Hamiltonella defensa
Candidatus Regiella insecticola
Budvicia aquatica
Tiedjeia arctica
Ewingella americana
Samsonia erythrinae
Erwinia psidii
Pragia fontium
Leminorella grimontii
γ -Proteobacteria
Candidatus Phlomobacter fragariae
Endosymbiont of Triatoma melanosoma
Candidatus Riesia pediculicola
Endosymbiont of Pediculus humanus
Providencia heimbachae
Moellerella wisconsensis
Morganella psychrotolerans
Proteus hauseri
Xenorhabdus beddingii
Photorhabdus asymbiotica
Edwardsiella ictaluri
Dickeya dadantii
Wigglesworthia glossinidia
Endosymbiont of Pseudolynchia canariensis
Ishikawaella symbiont of Megacopta cribraria
Buchnera aphidicola
Candidatus Baumannia cicadellinicola
Candidatus Blochmannia herculeanus
Endosymbiont of Sitophilus zeamais
Endosymbiont of Craterina melbae
Thorselia anophelis
Plesiomonas shigelloides
Photobacterium angustum
Haemophilus influenzae
Aeromonas enteropelogenes
Aeromonas schubertii
Aeromonas media
Aeromonas sobria
Tolumonas auensis
Oceanimonas doudoroffii
Oceanisphaera donghaensis
Zobellella dentrificans
Alteromonas addita
Endosymbiont of Pachypsylla
Candidatus Carsonella ruddii
Candidatus Portiera aleyrodidarum
Oceanospirillum japonicum
Acinetobacter baylyi
Endosymbiont of Heliothis virescens
Candidatus Legionella jeonii
Francisella philomiragia
Methylomonas aurantiaca
Cardiobacterium hominis
Allochromatium minutissimum
Clovia puncta b2
Phinia sp b6
Philagra sp b24
Machaerota sp b6
Clastoptera sp1 b9
Clastoptera sp2 b9
Eoscarta botelensis b1
Ariptyelus auropilosus b12
Aphrophora taiwana b2
β -Proteobacteria
Pseudomonas fluorescens (Gamma-Proteobacteria)
Candidatus Tremblaya princeps
Candidatus Tremblaya
Stenotrophomonas daejeonensis
Ureaplasma urealyticum
Acidithiobacillus albertensis
Wolbachia Metaseiulus
Wolbachia pipientis
Ehrlichia resticii
Rhizobium leguminosarum
α -Proteobacteria
Candidatus Sulcia muelleri of O. uchidae
Candidatus Sulcia muelleri
Blattabacterium sp
Coleomegil maculata
Flavobacteria
Isosphaera pallida
Chlamydia trachomatis
Thermophiles
Chlamydiae
Bacteroides fragilis
Planctomyces maris
Planctomyces
Alterococcus agarolyticus
Candidatus Leptonema illini
Treponema pallidum
Endosymbiont of Philanthus venustus
Spiroplasma citri
Mycoplasma pneumoniae
Bacillus subtilis
Gram-Positive Eubacteria
50 changes

Figure 2

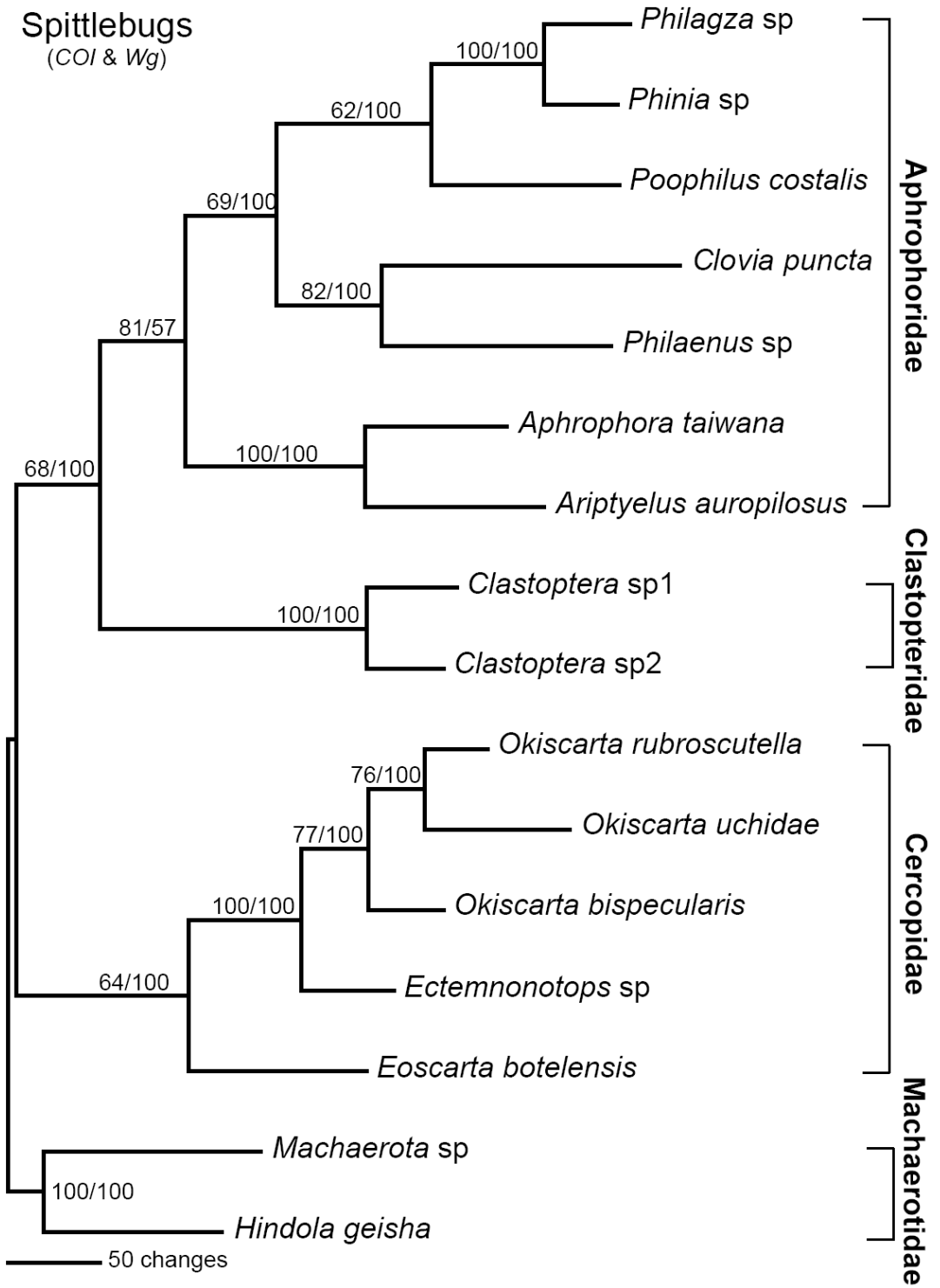
Spittlebugs
(COI & Wg)
Philagza sp
Phinia sp
Poophilus costalis
Clovia puncta
Philaenus sp
Aphrophora taiwana
Ariptyelus auropilosus
Clastoptera sp1
Clastoptera sp2
Okiscarta rubroscutella
Okiscarta uchidae
Okiscarta bispecularis
Ectemnonotops sp
Eoscarta botelensis
Machaerota sp
Hindola geisha
100/100
62/100
69/100
82/100
81/57
68/100
100/100
100/100
76/100
77/100
100/100
64/100
100/100
50 changes
Aphrophoridae
Clastopteridae
Cercopidae
Machaerotidae

Figure 3

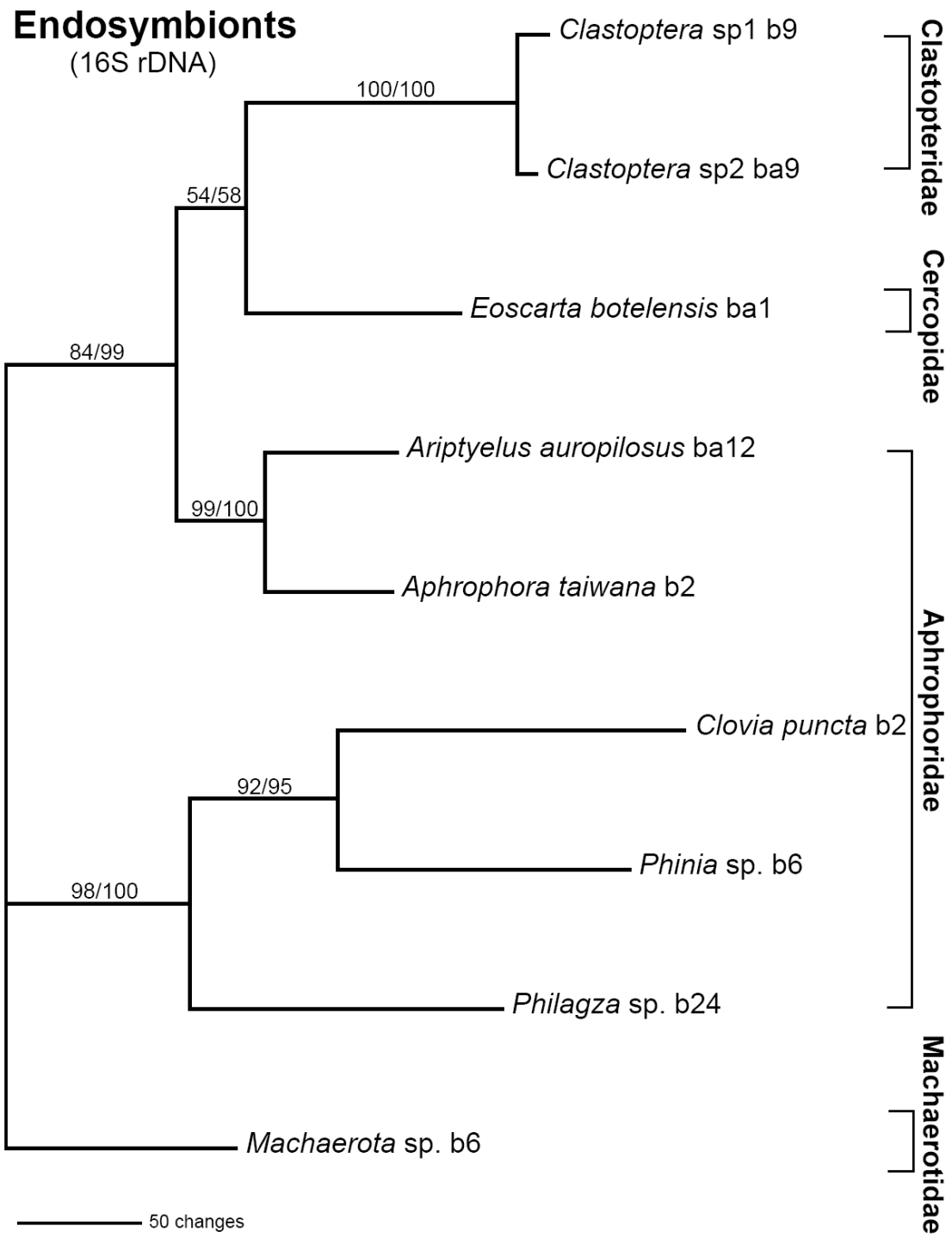
Endosymbionts
(16S rDNA)
Clastoptera sp1 b9
Clastoptera sp2 ba9
Eoscarta botelensis ba1
Ariptyelus auropilosus ba12
Aphrophora taiwana b2
Clovia puncta b2
Phinia sp. b6
Philagza sp. b24
Machaerota sp. b6
100/100
54/58
84/99
99/100
92/95
98/100
50 changes
Clastopteridae
Cercopidae
Aphrophoridae
Machaerotidae

Figure 4

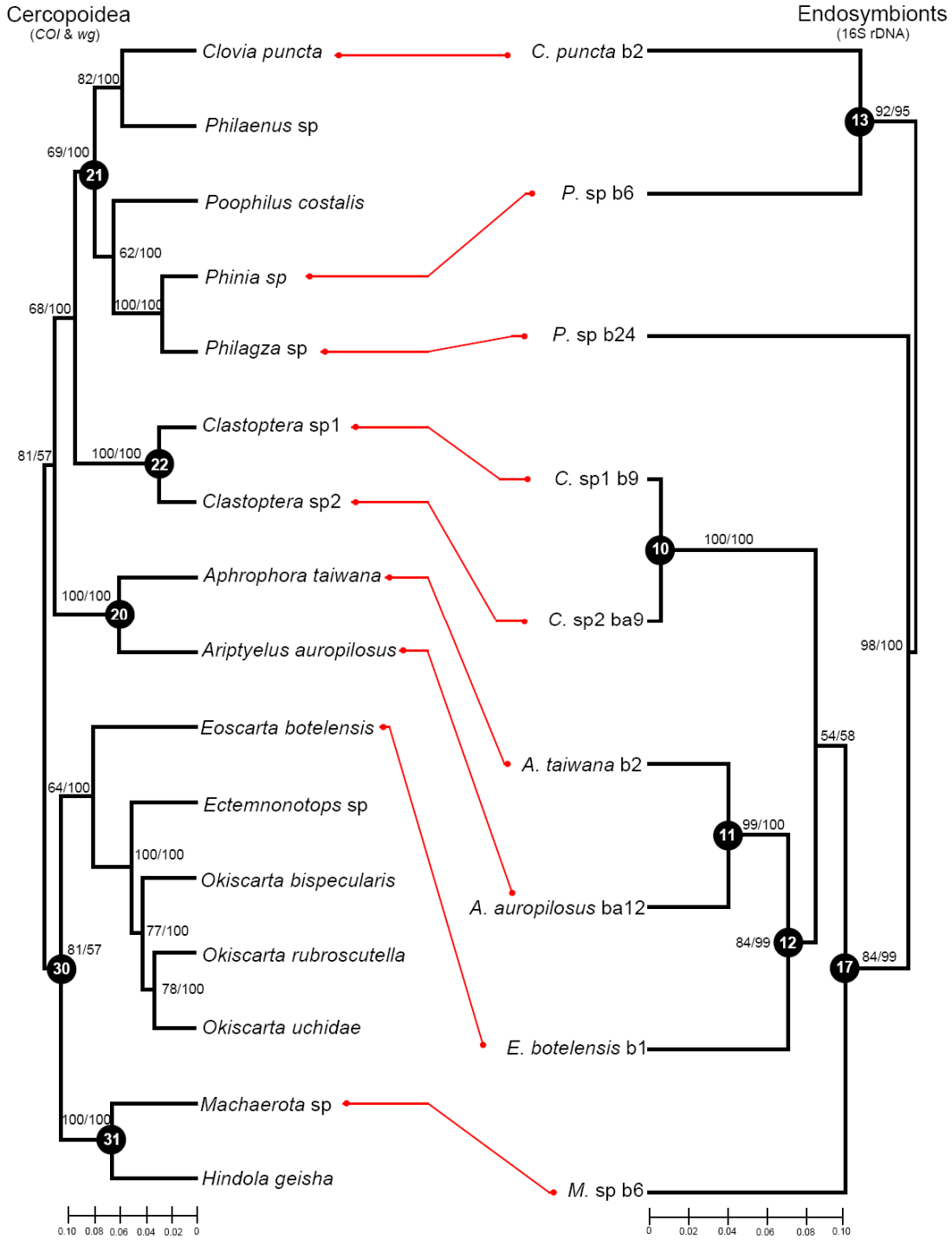
Cercopoidea
(COI & wg)
Endosymbionts
(16S rDNA)
Clovia puncta
Philaenus sp
Poophilus costalis
Phinia sp
Philagza sp
Clastoptera sp1
Clastoptera sp2
Aphrophora taiwana
Aripтyelus auropilosus
Eoscarta botelensis
Ectemnonotops sp
Okiscarta bispecularis
Okiscarta rubroscutella
Okiscarta uchidae
Machaerota sp
Hindola geisha
C. puncta b2
P. sp b6
P. sp b24
C. sp1 b9
C. sp2 ba9
A. taiwana b2
A. auropilosus ba12
E. botelensis b1
M. sp b6
82/100
69/100
62/100
100/100
68/100
81/57
100/100
100/100
64/100
100/100
77/100
78/100
81/57
100/100
92/95
100/100
98/100
54/58
99/100
84/99
84/99
0.10 0.08 0.06 0.04 0.02 0
0 0.02 0.04 0.06 0.08 0.10

Figure 5

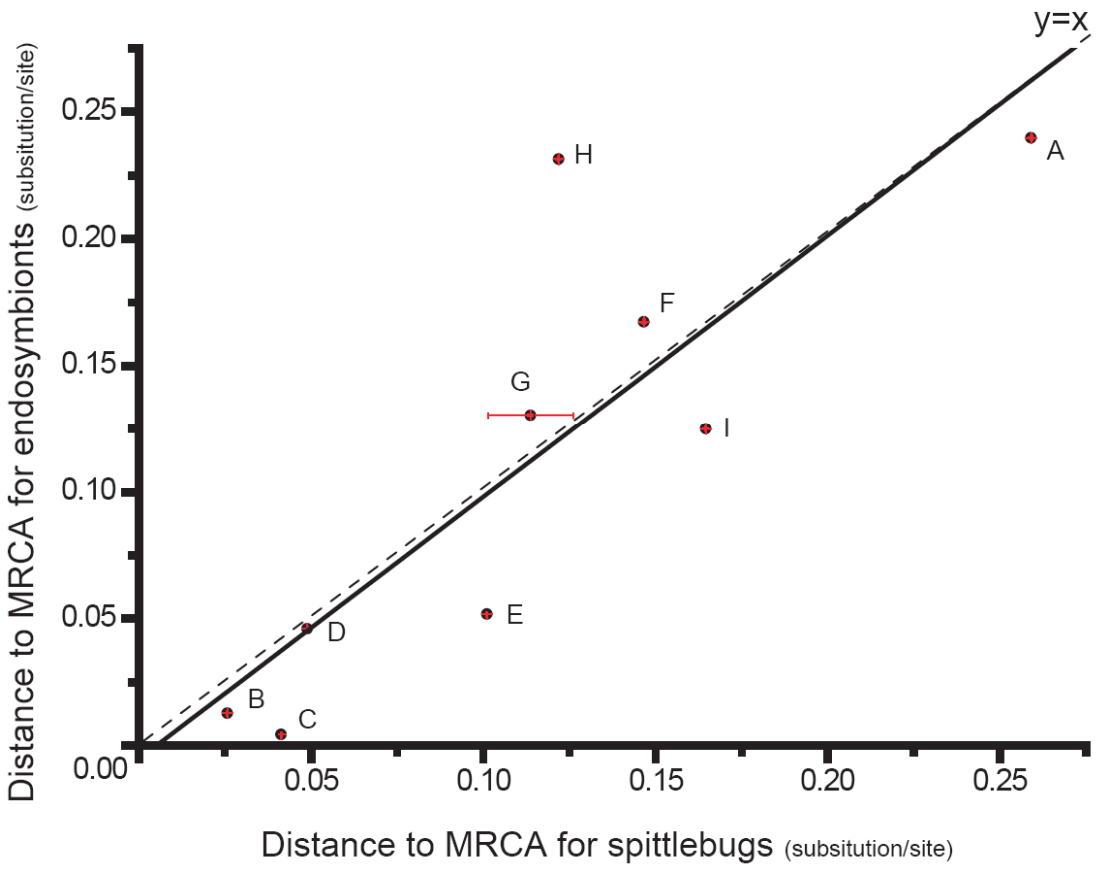
y=x
Distance to MRCA for endosymbionts (substitution/site)
0.25
0.20
0.15
0.10
0.05
0.00
H
A
F
G
I
E
D
B
C
0.05
0.10
0.15
0.20
0.25
Distance to MRCA for spittlebugs (subsitution/site)

Figure 6

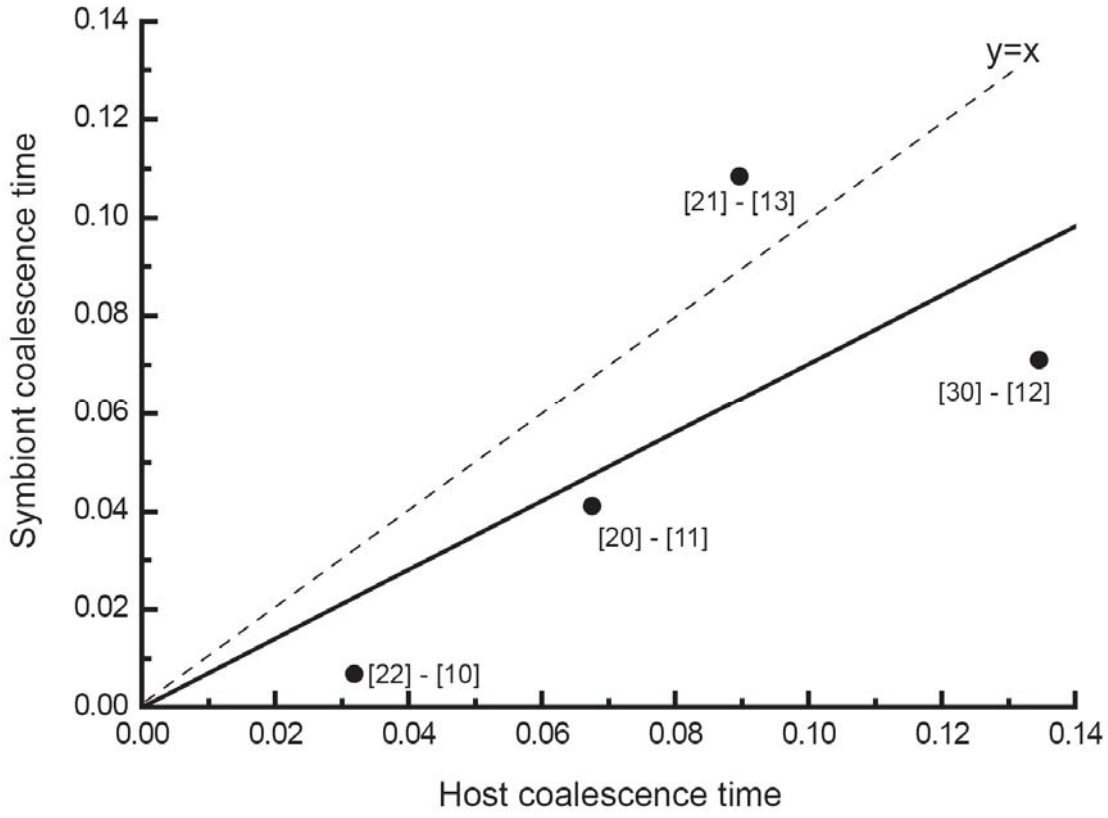
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Symbiont coalescence time
y=x
[21] - [13]
[30] - [12]
[20] - [11]
[22] - [10]
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
Host coalescence time

Table 1

Family	Tribe	Species	Location	GPS	Collectors	Date
Aphrophoridae	Lepyroniini	Ariptyelus auropilosus	Lian Hwa Chih, Nantou, Taiwan	23˚ 55' 50" N 120˚ 52' 30" E	WY Chen & CP Lin	Mar. 27, 2005
	Aphrophorini	Aphrophora taiwana	Dasyueshan, Taichung, Taiwan	24˚ 16' 00" N 121˚ 00' 00" E	WY Chen	Mar. 4, 2005
	Ptyelini	Poophilus costalis	Taiwan Agricultural Research Institute, Taichung, Taiwan	24˚ 02' 10" N 120˚ 41' 00" E	WY Chen & CP Lin	Mar. 21, 2006
	Cloviini	Phinia sp.	Kubah National Park, Sarawak, Malaysia	1˚ 36' 33.37" N 110˚ 13' 52.66" E	CP Lin	Aug. 1, 2000
		Clovia puncta	Taiwan Agricultural Research Institute, Taichung, Taiwan	24˚ 02' 10" N 120˚ 41' 00" E	WY Chen & CP Lin	Mar. 21, 2006
	Philaenini	Philaenus sp.	Livingstone, New York, USA	42˚ 08' 32.26" N 73˚ 46' 41.33" W	CP Lin	Aug. 13, 1999
	Philagrini	Philagza sp.	Shuangliou, Pingtung, Taiwan	22˚ 12' 50" N 120˚ 47' 00" E	WY Chen & CP Lin	Mar. 25, 2006
Cercopidae	Eoscartini	Eoscarta botelensis	Tianchih, Taitung, Taiwan	22˚ 02' 02" N 121˚ 34' 15" E	CP Lin	Jul. 13, 2005
	Cosmoscartini	Okiscarta uchidae	Mt. Yang-Ming, Taipei, Taiwan	25˚ 08' 30" N 121˚ 34' 30" E	WY Chen	Aug. 1, 2005
		Okiscarta rubroscutella	Shuangliou, Pingtung, Taiwan	22˚ 12' 50"N 120˚ 47' 00" E	WY Chen & CP Lin	Apr. 20, 2006
		Okiscarta bispecularis	Mt. Yang-Ming, Taipei, Taiwan	25˚ 08' 30" N 121˚ 34' 30" E	WY Chen	Mar. 20, 2007
		Ectemnonotops sp.	Mt. Banahaw, Kinabuhagah, Australia	14˚ 02' 37.1" N 121˚ 26' 35.7" E	CP Lin	Aug. 30, 2005
Clastopteridae	Clastopterini	Clastoptera sp.1	Creosote, USA	47˚ 36' 53.73" N 122˚ 30' 19.67" W	CP Lin	Jul. 29, 1999
		Clastoptera sp.2	Robinic, New York, USA	43˚ 98' 21.23" N 77˚ 35' 08.05" W	CP Lin	Sep. 10, 1998
Machaerotidae	Hindolini	Hindola geisha	Lian Hwa Chih, Nantou, Taiwan	23˚ 55' 50" N 120˚ 52' 30" E	WY Chen & CP Lin	Mar. 7, 2005
	Machaerotini	Machaerota sp.	Taman Negera National Park, Malysia	4˚ 19' 50.23" N 102˚ 23' 48" E	CP Lin	Aug. 10, 2000

Table 2

Classification	Species	GenBank Accession
Gram-Positive Eubacteria		
Low G+C	*Bacillus subtilis*	Z99107
	Mycoplasma pneumoniae	M29061
	Spiroplasma citri	AM157769
Gram-Negative Eubacteria		
Chlamydiae	*Chlamydia trachomatis*	DQ019310
Flavobacteria	*Blattabacterium sp.* (cockroach symbiont)	AF322473
	Candidatus Sulcia muelleri	DQ066635
	Bacteroides fragilis	AB050106
	Coleomegilla maculate (male-killing endosymbionts)	Y13889
Planctomyces	*Planctomyces maris* (strain DSM 8797T)	AJ231184
Thermophiles	*Isosphaera pallida* (strain DSM 9630T)	AJ231195
Spirochaeta	*Treponema pallidum*	AF426102
Leptospiras	*Leptonema illini*	M88719
α-Proteobacteria	*Ehrlichia resticii*	AF036654
	Rhizobium leguminosarum	D14513
	Philanthus venustus (*Streptomyces* endosymbionts)	AY854956
	Wolbachia pipientis (*Culex pipiens* endosymbionts)	X61768
β-Proteobacteria	*Candidatus Tremblaya* (mealybug symbiont)	AF476098
	Pseudomonas testosteroni	M11224
γ-Proteobacteria		
Acidithiobacillales		
Acidithiobacillus	*Acidithiobacillus albertensis*	AJ459804
Aeromonadaceae		
Aeromonas	*Aeromonas media*	AY987773
	Aeromonas sobria	X60412
Oceanimonas	*Oceanimonas doudoroffii*	AB019390
Oceanisphaera	*Oceanisphaera donghaensis*	DQ190441
Tolumonas	*Tolumonas auensis*	X92889
Zobellella	*Zobellella denitrificans*	DQ195675
Alteromonadales		
Alteromonas	*Alteromonas addita*	AY682202
Cardiobacteriales		
Cardiobacterium	*Cardiobacterium hominis*	M35014
Chromatiales		
Allochromatium	*Allochromatium minutissimum*	Y12369

Table 2 (contd.)

Classification	Species	GenBank Accession
Enterobacteriaceae		
Alterococcus	*Alterococcus agarolyticus*	AF075271
Aranicola	*Aranicola proteolyticus*	APU93263
Arsenophonus	*Arsenophonus* (*Triatoma melanosoma* endosymbionts)	DQ508172
Averyella	*Averyella dalhousiensis*	DQ481464
Brenneria	*Brenneria nigrifluens*	AJ233415
Buchnera	*Buchneria aphidicola*	M27039
Budvicia	*Budvicia aquatica*	AJ233407
Buttiauxella	*Buttiauxella agrestis*	DQ440549
Candidatus Ishikawaella	*Megacopta cribraria* (*Ishikawaella* symbiont)	AB240158
Candidatus Phlomobacter	*Candidatus Phlomobacter fragariae*	AB246669
Candidatus Riesia	*Candidatus Riesia pediculicola*	EF110572
Cedecea	*Cedecea davisae*	AF493976
Citrobacter	*Citrobacter amalonaticus*	AF025370
Cronobacter	*Cronobacter muytjensii*	EF059887
Dickeya	*Dickeya dadantii*	AF520707
Edwardsiella	*Edwardsiella ictaluri*	EF015475
Enterobacter	*Metaseiulus occidentalis* (Enterobacter endosymbionts)	AY753173
Erwinia	*Erwinia psidii*	Z96085
Escherichia	*Escherichia coli*	NC000913
Ewingella	*Ewingella americana*	DQ383802
Grimontella	*Grimontella senegalensis*	AY217653
Hafnia	*Hafnia alvei*	DQ412565
Klebsiella	*Klebsiella granulomatis*	AF010251
Kluyvera	*Kluyvera ascorbata*	AM184232
Leclercia	*Leclercia adecarboxylata*	AJ277978
Leminorella	*Leminorella grimontii*	AJ233421
Margalefia	*Margalefia venezuelensis*	AY702662
Moellerella	*Moellerella wisconsensis*	AM040754
Morganella	*Morganella psychrotolerans*	DQ358143
Obesumbacterium	*Obesumbacterium proteus*	DQ223874
Pantoea	*Pantoea dispersa*	AY227805
Pectobacterium	*Pectobacterium cacticida*	Z96092
	Pectobacterium carotovorum subsp. *brasiliensis*	AY207086
Photorhabdus	*Photorhabdus asymbiotica* subsp. *asymbiotica*	Z76755
Plesiomonas	*Plesiomonas shigelloides*	X60418

Table 2 (contd.)

Classification	Species	GenBank Accession
Providencia	*Providencia heimbachae*	AM040490
Rahnella	*Rahnella genos*p. 3	RGU90758
Raoultella	*Raoultella ornithinolytica*	AJ630277
Salmonella	*Salmonella typhimurium* LT2	NC003197
Samsonia	*Samsonia erythrinae*	AF273037
Serratia	*Candidatus Serratia symbiotica*	AY296732
	Serratia entomophila	AJ233427
Shigella	*Shigella* sp. 8CR	DQ376908
Sodalis	*Craterina melbae* (*Sodalis* endosymbiont)	EF174495
Tatumella	*Tatumella ptyseos*	AJ233437
Thorsellia	*Thorsellia anophelis*	AY837748
Tiedjeia	*Tiedjeia arctica*	DQ107523
Trabulsiella	*Trabulsiella guamensis*	AY373830
Wigglesworthia	*Wigglesworthia glossinidia*	AF022879
Xenorhabdus	*Xenorhabdus beddingii*	X82254
Yersinia	*Yersinia aldovae*	AJ871363
Yokenella	*Yokenella regensburgei*	AY269192
Legionellales		
Legionella	*Candidatus Legionella jeonii*	AY598719
Methylococcales		
Methylomonas	*Methylomonas aurantiaca*	X72776
Oceanospirillales		
Pseudospirillum	*Oceanospirillum japonicum*	AB006766
Pasteurellales		
Pasteurellaceae	*Haemophilus influenzae* Rd KW20	NC000907
Pseudomonadales		
Acinetobacter	*Acinetobacter baylyi*	EF178435
Thiotrichales		
Francisella	*Francisella philomiragia*	EF153479
Vibrionales		
Photobacterium	*Photobacterium angustum*	AY900628
Xanthomonadales		
Stenotrophomonas	*Stenotrophomonas daejeonensis*	DQ109037

Table 2 (contd.)

Classification	Species	GenBank Accession
Unclassified		
Cardomonas	*Candidatus Carsonella ruddii*	AF211143
	Pachypsylla celtidismamma	AF286122
	Candidatus Blochmannia herculeanus	X92550
	Candidatus Hamiltonella defensa	AY296733
	Candidatus Regiella insecticola	AY296734
Portiera	*Candidatus Portiera aleyrodidarum*	AY268081
Trembleya	*Candidatus Tremblaya princeps*	AF476098
	Pseudolynchia canariensis	DQ115535
Baumannia	*Candidatus Baumannia cicadellinicola*	AY676895
	Pediculus humanus	DQ076660
	Sitophilus zeamais	M85269
	Heliothis virescens (testis endosymbionts)	L22481

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