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Cospeciation of Psyllids and Their Primary Prokaryotic Endosymbionts

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Psyllids are plant sap-feeding insects that harbor prokaryotic endosymbionts in specialized cells within the body cavity. Four-kilobase DNA fragments containing 16S and 23S ribosomal DNA (rDNA) were amplified from the primary (P) endosymbiont of 32 species of psyllids representing three psyllid families and eight subfamilies. In addition, 0.54-kb fragments of the psyllid nuclear gene *wingless* were also amplified from 26 species. Phylogenetic trees derived from 16S-23S rDNA and from the host *wingless* gene are very similar, and tests of compatibility of the data sets show no significant conflict between host and endosymbiont phylogenies. This result is consistent with a single infection of a shared psyllid ancestor and subsequent cospeciation of the host and the endosymbiont. In addition, the phylogenies based on DNA sequences generally agreed with psyllid taxonomy based on morphology. The 3′ end of the 16S rDNA of the P endosymbionts differs from that of other members of the domain *Bacteria* in the lack of a sequence complementary to the mRNA ribosome binding site. The rate of sequence change in the 16S-23S rDNA of the psyllid P endosymbiont was considerably higher than that of other bacteria, including other fast-evolving insect endosymbionts. The lineage consisting of the P endosymbionts of psyllids was given the designation *Candidatus* Carsonella (gen. nov.) with a single species, *Candidatus* Carsonella ruddii (sp. nov.).

Insects within the families Aphididae (aphids), Psyllidae (psyllids), Aleyrodidae (whiteflies), and Pseudococcidae (mealybugs) feed predominantly or exclusively on plant phloem sap. These insects have a number of common structural properties (8) and constitute separate lineages within the suborder Sternorrhyncha (15, 54). The utilization of plant sap necessitates the penetration of tissue by flexible mouth parts (stylets) and the ingestion of the fluid. This mode of feeding is conducive to the transmission of viruses and other infectious agents, and members of these families are vectors of pathogens of agriculturally important plants (7, 28, 51). In addition, these insects may reach enormous populations, causing plant nutrient deprivation, leaf curling, and gall formation (8). Plant phloem sap, the diet of these insects, is rich in carbohydrates but deficient in nitrogenous compounds (21, 46). Due to this deficiency, a large amount of plant sap is consumed and is excreted as honeydew. This sticky material may cover the plant and serve as a substrate for fungal growth (8).

A common feature of organisms that live on diets containing an excess of one class of compounds and a deficiency in essential nutrients is the presence of prokaryotic intracellular symbionts (endosymbionts) that may provide the missing essential nutrients (6, 21, 37). Early histological studies indicated that these endosymbionts are housed within specialized cells (bacteriocytes) that form an aggregate (bacteriome) found within the body cavity (5, 10, 37). Typically, insects of a particular family or superfamily were found to have a common morphological type of endosymbiont within the bacteriocytes designated as the primary (P) endosymbiont. In addition, some insects have a morphologically distinct secondary (S) endosymbiont usually located in cells associated with the bacteriocytes. Phylogenetic studies using 16S ribosomal DNA (rDNA) have indicated that the P endosymbionts of aphids, psyllids, and whiteflies form distinct bacterial lineages within the γ subdivision of the division *Proteobacteria*, while the P endosymbionts of mealybugs are within the β subdivision (5, 17, 20, 25, 26, 37–39, 53). All of these endosymbionts are maternally transmitted to progeny.

The most extensive investigations of bacteriocyte associates have dealt with *Buchnera aphidicola*, the P endosymbiont of aphids, and this association serves as a paradigm for other studies (4–6, 22, 35–37). Results from molecular phylogenetic studies suggest that the association of *B. aphidicola* and aphids is the result of the establishment of a single infection 150 to 250 million years (MY) ago. There appears to be no exchange of P endosymbionts between different aphid lineages, suggesting cospeciation between the endosymbiont and the host. *B. aphidicola* has many of the genes required for housekeeping functions. Unlike other intracellular or fastidious pathogenic bacteria, it contains genes encoding enzymes of amino acid biosynthetic pathways. Some of these genes are amplified by being present on plasmids (4, 5, 49). These findings, coupled with nutritional studies, indicate that one of the functions of *B. aphidicola* is the synthesis of essential amino acids for the aphid host. Recently, it has been found that *B. aphidicola* also synthesizes the vitamin riboflavin (42). From the fossil record of the host and other information, it has been possible to obtain estimates of the time of divergence of some of the host lineages (36). These data in conjunction with analyses of sequence divergence of a wide variety of genes have indicated that *B. aphidicola* shows a major acceleration in the rate of sequence change compared to that of free-living bacteria that have been examined (18, 34, 56). The acceleration is concentrated in nonsynonymous substitutions in structural genes. This pattern of increased evolutionary rates has been hypothesized to be the consequence of the reduced effect of purifying selection due to the small genetic population size that results from bottlenecks during inoculation of progeny (18, 34).

The availability of extensive information on *B. aphidicola*

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and aphids made it interesting to initiate comparative studies of the endosymbionts of other insects feeding on plant phloem sap. Phloem sap is fairly uniform in nutritional composition across angiosperm families; all sampled phloem sap compositions have a deficit of essential amino acids relative to their proportions in plant and animal proteins (46). Consequently, it is probable that the endosymbionts of insects that feed on plant phloem sap perform a similar function, providing the host with missing essential nutrients. It is of interest to know how different bacterial lineages that entered into endosymbiotic associations became modified in order to solve similar problems. Recent work has provided some basic characterization of the endosymbionts of psyllids (25, 50), and we selected these organisms for comparative genetic studies. Like aphids, psyllids are widely distributed on angiosperm host plants, especially on members of woody families (23, 24).

Unlike aphids, psyllids have obligate sexual reproduction, and their rate of growth is much lower than that of most aphid species (30). They develop from eggs and as nymphs are relatively sessile, being attached to plants while feeding. As nymphs, they have five instars. The adult is winged and relatively active, with hind legs specialized for jumping, hence the common name "jumping plant lice" (8). Within psyllids is a bacteriome that has some structural variability (10). The most common bacteriome described consists of a large multinucleate syncytium within which are uninucleate bacteriocytes (10, 16, 25, 55). The latter contain a morphologically similar bacterium, designated the P endosymbiont; the syncytium may also contain an S endosymbiont, with different psyllids having different morphological types. Electron microscopic studies have indicated that, as in the case of *B. aphidicola*, both the P endosymbiont and the S endosymbiont of psyllids have a gram-negative cell wall and are enclosed in vesicles derived from the host cells (16, 55). Recently, the endosymbiont 16S rDNAs from four psyllid species were cloned and sequenced. It was found that the P endosymbiont was a distinct bacterial lineage that had 16S rDNA moles percent guanine-plus-cytosine (G+C) content lower than that previously found in bacteria (25, 54). Three of the four species had S endosymbionts which were within or related to the family *Enterobacteriaceae* (25, 54). Using specific probes and in situ hybridization, it was shown that the low-G+C-containing P endosymbiont was localized to the bacteriocytes while the S endosymbiont was within the syncytium (25).

We have initiated our studies on psyllid P endosymbionts by an examination of potential cospeciation between the endosymbiont and the host. For a total of 32 psyllid species, comprising a broad spectrum of the diversity of the superfamily Psylloidea, we cloned and sequenced a DNA fragment containing the 16S and 23S rDNAs of the P endosymbiont and compared the resulting tree with the taxonomy of the host. In addition, the phylogenetic tree for 26 species was compared with the tree obtained from a partial sequence of *wingless* (*wg*), a host nuclear gene (9).

MATERIALS AND METHODS

Collection and preservation of biological material. Table 1 presents the sources of the psyllids used in these studies together with information on the host plant, date of harvesting, and geographical location. Psyllids collected by D. Burckhardt were stored in 100% ethanol and shipped to P. Baumann's laboratory, where they were stored at 4°C. Psyllid species collected by other investigators were placed on dry ice and shipped to P. Baumann's laboratory, where they were stored at −80°C.

General molecular biology methods. Standard molecular biology methods that are described in detail in manuals (1, 45) as well as in past publications (39, 53) were used in most of the experiments. Only variations of these methods as well as additions will be described in detail.

DNA purification. DNA was purified from 30 to 100 mg (wet weight) of psyllids. Ethanol-preserved and frozen psyllid samples were pulverized in 1.5-ml microcentrifuge tubes by using a plastic pestle (Kontes Scientific Glassware/Instruments, Vineland, N.J.) while keeping the samples on dry ice. After thorough grinding of the specimens, 500 μl of prewarmed (75°C) lysis buffer (76 mM NaCl, 38 mM EDTA [pH 8], 1.8% [wt/vol] sodium dodecyl sulfate) was added and the tube was incubated at 75°C for 50 s. During this time, a pipette containing a plastic tip was used to break up the lumps and speed up lysis. Five hundred microliters of Tris-EDTA-saturated phenol (45) was added, and the tube was inverted about 150 times and then centrifuged for 2 min in a microcentrifuge. The aqueous phase was removed using wide-bore pipette tips to avoid shearing the high-molecular-weight DNA. The aqueous phase was extracted twice with chloroform-isoamyl alcohol to remove all traces of phenol. The aqueous sample was precipitated with a 1/10 volume of 3 M Na acetate (pH 5.2) and 2 volumes of cold 95% alcohol for 5 min on ice and then centrifuged at 4,000 × *g* for 5 min. After aspiration of all residual liquid, 1 to 2 ml of lysis buffer containing 0.5 to 1 mg of DNase-free RNase (Sigma, St. Louis, Mo.) per ml was added to the pellet. The sample was incubated at 37°C for 30 min with intermittent rocking to dissolve the DNA pellet. Proteinase K (Sigma) was added (0.5 mg per ml of lysis buffer), and incubation was continued for an additional 2 h with intermittent rocking. The preparation was extracted once with an equal volume of phenol, twice with phenol-chloroform, and twice with chloroform. The DNA was precipitated with a 1/10 volume of 3 M Na acetate (pH 5.2) and 2 volumes of cold 95% alcohol on ice. If high-molecular-weight DNA was visible after 5 min, the DNA was collected by centrifugation (4,000 × *g*, 15 min, 4°C), washed with 70% ethanol, dried under vacuum for 10 min, and resuspended in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. If no DNA was observed after 5 min, precipitation was allowed to continue at −20°C overnight.

Amplification, cloning, and sequencing of the 16S-23S rDNA. The 16S-23S rDNA fragment was amplified by PCR using the following synthetic oligonucleotides: A (*Pst*I and *Bam*HI sites; 5′-GCA CTG CAG GAT CCA GAG TTT GAT CAT GGC TCA GAT TG-3′) and B (*Kpn*I and *Not*I sites; 5′-GCA GGT ACC GCG GCC GCG CTC GCG TAC CAC TTT AAA TGG CG-3′). PCR amplification of the 16S-23S rDNA was carried out in a 25-μl reaction mixture containing 10 to 25 ng total of psyllid DNA, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mix, 1 pmol of primer A, 1 pmol of primer B, and 1 U of Bio-X-Act DNA polymerase in Opti buffer (Bioline, London, United Kingdom). The following conditions were used: denaturation at 94°C, 30 s; annealing at 55°C, 2 min; elongation at 70°C, 5 min; total of 30 cycles. The PCR-amplified DNA was purified by extraction with equal volumes of phenol-chloroform, followed by chloroform, and then precipitated as described above. The 16S-23S rDNA fragments were usually digested with *Bam*HI and *Not*I and ligated into pBluescript (Stratagene, La Jolla, Calif.). Clones carrying the insert of interest were identified by miniscreens (45). The DNA used for sequencing was purified using the Qiagen plasmid minikit according to the manufacturer's suggested protocol (Qiagen, Valencia, Calif.). The nucleotide sequence was determined at the University of Arizona (Tucson) Laboratory of Molecular Systematics and Evolution (LMSE) sequencing facility. Besides T3 and T7 primers, the oligonucleotide primers used for the sequencing reactions are given as nucleotide positions on the 16S-23S fragment of *Pachypsylla venusta*: downstream primers, 470 to 491, 1039 to 1059, 1456 to 1479, 1779 to 1801, 2334 to 2356, 2820 to 2848, 3374 to 3397, and 3705 to 3722; and upstream primers, 3727 to 3709, 3397 to 3374, 2848 to 2820, 2317 to 2293, 1801 to 1779, 1479 to 1456, 1059 to 1039, and 491 to 470.

Amplification and sequencing of *wg* DNA. The *wg* DNA fragments were 540 nucleotides (nt) in length and were amplified by PCR using the Insect Nuclear DNA Primer Oligonucleotide Set obtained from the University of British Columbia Nucleic Acid-Protein Service Unit. These primers were LEPWG1 (inDNA 22; 5′-GAR TGY AAR TGY CAY GGY ATG TCT GG-3′) and ModLEPWG2 (inDNA 23; 5′-ACT ICG CAR CAC CAR TGG AAT GTR CA-3′). Two taxa (*Trioza urticae* and *Diaphorina citri*) were amplified with oligonucleotide primers designed from the partial *wg* dataset: WGTryF (5′-AAA ACC TGT TGG ATG AGG C-3′) and WGTryR (5′-GTG GAA TGT GCA GGC ACA CCG-3′). All sequencing reactions were carried out in a 50-μl reaction mixture containing 20 to 40 ng total of psyllid DNA, PCR buffer (Gibco/BRL, Rockville, Md.), 0.25 mM (each) deoxynucleoside triphosphates, 0.4 pmol of each primer per ml, 2.5 mM $MgCl_2$, and 2 U of Platinum *Taq* DNA polymerase (Gibco/BRL). The following conditions were used: denaturation at 94°C, 1 min; annealing at 52°C, 1 min; and elongation at 72°C, 2 min, for a total of 30 cycles. The concentrated products of three 50-μl reaction mixtures were electrophoresed in 3% denaturing polyacrylamide gels (19:1 acrylamide/bisacrylamide ratio) at 350 V for 5 to 8 h. Gels were stained for 30 min with ethidium bromide, and bands corresponding to *wg* fragments were cut out from the gel. The DNA was eluted for 36 h in a 5 M NH_4 acetate–0.5 M EDTA buffer, ethanol precipitated, and resuspended in 50 μl of water. All PCR products were purified for sequencing using the CONCERT Rapid PCR Purification System (Gibco/BRL), and nucleotide sequences were determined at the University of Arizona LMSE sequencing facility. Sequencing reactions were carried out from both ends of the fragments using the same primers used for PCR amplification, except for the taxon *Psylla* sp., for which internal sequencing primers were designed (1, 5′-GCA CGG TCA AAA CCT GCT GG-3′; 2, 5′-GTG GAA TGT ACA AGC GCA CC-3′).

TABLE 1. Information concerning the psyllids used in this study and the accession numbers of the 16S-23S rDNAs of the P endosymbionts and the *wg* gene of the host

Psyllid species	GenBank accession no. for 16S-23S/*wg* sequences	Host plant/location/date (mo,yr)/collector[a]
Arytaina genistae	AF243136	*Cytisus scoparius*/France/7,99/D. Burckhardt
Aphalaroida inermis	AF211125	*Prosopis velutina*/AZ/6,99/N. Moran
Aphalara longicaudata	AF243137	*Coniferae*/Switzerland/10,99/D. Burckhardt
Acizzia uncatoides	AF211123/AF231367	*Acacia melanoxylon*/Chile/12,98/D. Burckhardt
Acizzia uncatoides	AF211124/AF231366	*Acacia* sp./El Cerrito, CA/4,99/E. Brennan
Bactericera cockerelli	AF211126	*Lycium* sp./AZ/12,98/N. Moran
Blastopsylla occidentalis	AF211127/AF231382	*Eucalyptus* sp./AZ/6,99/N. Moran
Boreioglycaspis melaleucae	AF211128/AF231383	*Melaleuca quinquenervia*/FL/9,99/S. Winewriter
Cacopsylla brunneipennis	AF243138	*Picea abies*/Switzerland/6,99/D. Burckhardt
Cacopsylla myrthi	AF211129/AF231368	*Rhamnus alaternus*/Malta/1,99/D. Mifsud
Cacopsylla peregrina	AF211130/AF231373	*Crataegus monogyna*/Switzerland/5,99/D. Burckhardt
Cacopsylla pyri	AF211131/AF231372	*Pyrus communis*/Switzerland/6,99/D. L. Schaub
Calophya schini	AF211132/AF231369	*Schinus molle*/El Cerrito, CA/7,99/E. Brennan
Ctenarytaina eucalypti	AF211133/AF231385	*Eucalyptus globulus*/El Cerrito, CA/4,99/E. Brennan
Ctenarytaina longicauda	AF211134/AF231386	*Lophostemon confertus*/El Cerrito, CA/7,99/E. Brennan
Ctenarytaina spatulata	AF211135/AF231384	*Eucalyptus globulus*/El Cerrito, CA/4,99/E. Brennan
Diaphorina citri	AF211136/AF231365	*Citrus* sp./FL/9,99/S. Winewriter
Glycaspis brimblecombei	AF211137/AF231381	*Eucalyptus camaldulensis*/Fremont, CA/7,99/E. Brennan
Heteropsylla cubana	AF211138/AF231376	*Leucaena leucocephala*/Kailua, HI/1,99/E. Brennan
Heteropsylla texana	AF211139/AF231375	*Prosopis velutiana*/AZ/9,98/N. Moran
Neotriozella hirsuta	AF211140/AF231363	*Vauquelinia* sp./AZ/4,99/N. Moran
Pachypsylla celtidis	AF211141/AF231379	*Hackberry/IL/9,99/D. Voegtlin*
Pachypsylla pallida	AF211142/AF231377	*Celtis reticulata*/AZ/2,99/N. Moran
Pachypsylla venusta	AF211143/AF231378	*Celtis reticulata*/AZ/11,98/N. Moran
Panisopelma fulvescens	AF211144	*Larrea nitida*/Chile/12,98/D. Burckhardt
Panisopelma sp.	AF211145/AF231387	*Larrea nitida*/Chile/1,99/D. Burckhardt
Psylla buxi	AF211146/AF231371	*Buxus sempervirens*/Switzerland/6,99/D. Burckhardt
Psylla sp.	AF211147/AF231389	Alder/UT/93/N. Moran
Russelliana intermedia	AF211148/AF231388	*Baccharis linearis*/Chile/12,98/D. Burckhardt
Spanioneura fonscolombii	AF211149/AF231374	*Buxus sempervirens*/Switzerland/6,99/D. Burckhardt
Tainarys sordida	AF211150/AF231380	*Schinus latifolius*/Chile/12,98/D. Burckhardt
Trioza eugeniae	AF211151/AF231362	*Syzygium paniculatum*/El Cerrito, CA/5,99/E. Brennan
Trioza urticae	AF211152/AF231364	*Urtica dioica*/Switzerland/6,99/D. Burckhardt

[a] Abbreviations: AZ, Arizona; CA, California; FL, Florida; HI, Hawaii; IL, Illinois; UT, Utah.

Analyses of the sequence data. The intergenic space between 16S and 23S rDNA was removed, and the resulting sequences were aligned using Pileup of the GCG package (version 9.1, 1997, Genetics Computer Group, Madison, Wis.). For reconstruction of phylogenies, we used maximum likelihood and neighbor-joining methods as implemented under "maximum likelihood" and "distance" optimality criteria in PAUP 4b.1 (D. L. Swofford, 1999, PAUP: phylogenetic analysis using parsimony, version 4.0; Sinauer Associates, Sunderland, Mass.). We performed analyses on the 16S and 23S regions separately and on the combined 16S-23S. The same model of sequence evolution was used for both neighbor-joining and maximum likelihood analyses. The model was based on the 16S-23S sequence evolution that was chosen by hierarchical likelihood ratio tests to be GTR+I+G, and substitution rate parameters, proportion of invariant sites, and gamma shape parameter were estimated (31; PAUP, version 4.0). These parameters along with empirical base frequencies were used in heuristic searches. In addition to one heuristic maximum likelihood search, 1,000 bootstrap replicates were run using the neighbor-joining analysis to assess support for individual nodes.

The *wg* sequences were translated, and the inferred amino acid sequences were aligned using Pileup. The DNA sequences were then fitted to the amino acid alignment. There were two indels in the alignment, one involving three amino acids (9 nt) and one involving one amino acid (3 nt); these were each coded as a single character with two states (present-absent). Otherwise, the alignment was unambiguous within the psyllid endosymbionts. For the *wg* data set, third positions of codons were not included in analyses for two reasons. First, these sites were highly divergent and largely saturated with substitutions. Second, nucleotides at third positions were heavily influenced by a shift in base composition and rate of substitution affecting some taxa. For the *wg* phylogenetic analysis, the same procedures were used as those for bacterial genes. In this case, the best model from the hierarchical likelihood ratio tests was "K3Puf+G" (PAUP, version 4.0), and this model, including estimated rate parameters and gamma parameter for the distribution of substitutions among sites, was used in both maximum likelihood and distance phylogeny reconstructions.

We used the "partition homogeneity" test in PAUP 4b.1 to determine if the 16S rDNA and 23S rDNA data were compatible with each other. We also used this test to determine if the combined 16S-23S rDNA data, reflecting endosymbiont phylogeny, were compatible with the *wg* data, reflecting host phylogeny. This analysis tests the null hypothesis that the two data "partitions" reflect the same phylogeny, under minimum evolution (parsimony) criteria. Parsimony-informative sites from both sources are assembled as partitions within a single alignment. Tree length for the most parsimonious tree is calculated for each partition and summed; this sum is then compared to a distribution of summed lengths based on similarly sized, random partitions of the total alignment. If the observed summed length is greater than 95% of the lengths based on random partitions, the null hypothesis can be rejected at the 5% level of significance. To obtain the distribution of random partitions, we performed 1,000 replications with 10 random additions per replication.

In some analyses, we included outgroup sequences for both insects (*wg*) and bacteria (16S+23S). The outgroups for *wg* were the aphid *Pemphigus obesinymphae* and the whitefly *Bemisia tabaci*. The bacterial outgroups were chosen on the basis of BLAST searches using psyllid P-endosymbiont 16S sequences to search the 16S rRNA databases (33). The two closest organisms in the database were the P endosymbiont of *B. tabaci* and *Zymobacter palmae*.

Relative rate tests. The rates of substitution of 16S and 23S rRNA were compared between several representative psyllid endosymbionts and a variety of related bacterial taxa, including *Escherichia coli*, *Z. palmae*, the P endosymbiont of *Bemisia argentifolii* (a very close relative of *B. tabaci*), and *B. aphidicola* (P endosymbiont of the aphid *Schizaphis graminum*) (Table 2). In each case, a more distant outgroup, *Acetobacter intermedius*, was used to perform a relative rate test of the null hypothesis that the rate of substitution in the lineage leading to the psyllid endosymbiont was the same as that leading to the related taxon. Distances were based on the Kimura two-parameter model (32), and tests were performed as described in the work of Muse and Weir (41). Accession numbers used in the tests were as follows: *A. intermedius*, Y14680 and Y14694; *B. aphidicola*, L18927 and U09230; and *E. coli*, U00096.

Absolute rate calculations. Using estimates of the age of psyllids, we calculated rates of substitution for the 16S rDNA gene and for the 23S rDNA gene. The psyllids were probably present by the late Cretaceous period (about 100 MY) and have been hypothesized to have been present in the late Permian period (about

TABLE 2. Rates of evolution of the combined 16S and 23S rDNA genes in psyllid symbionts relative to rates in other lineages in the γ subdivision of the *Proteobacteria*[a]

Organism to which compared	*K*(1-2)	*K*(1-3)	*K*(2-3)	*K*(0-1)/*K*(0-2)	*z*
Z. palmae	0.33	0.38	0.23	2.77	11.40[b]
E. coli	0.32	0.34	0.21	2.34	10.65[b]
P endosymbiont (whitefly)	0.30	0.35	0.24	2.07	7.56[b]
B. aphidicola	0.30	0.34	0.24	2.03	8.40[b]

[a] In all comparisons, the outgroup is *A. intermedius* (taxon 3), the psyllid symbiont is *P. venusta* (taxon 1), and the comparison is to a free-living or endosymbiotic γ-subdivision bacterium as listed (taxon 2). The null hypothesis is $K(1\text{-}3) = K(2\text{-}3)$.

[b] $P < 0.0001$.

250 MY) as the Protopsyllidae (29). Thus, we used 100 to 250 MY as a conservative range for the date of the common psyllid ancestor and as the basis for rate calibrations. Estimated rates were compared to values computed previously for enteric bacteria and for *B. aphidicola* (18).

Electron microscopy. Bacteriocytes were dissected from late instar nymphs of *P. venusta* collected in Tucson, Ariz., at the same site as the samples used for DNA sequence studies. Dissections were performed in fixative containing formaldehyde and gluteraldehyde (52), and samples were fixed using three cycles of microwave heating and cooling. Following dehydration in an ethanol series of increasing concentrations, bacteriocytes were embedded in Epon Araldite (Sigma) and subjected to microwave curing (27) before sectioning. Sections were viewed and photographed with a Philips 420 transmission electron microscope.

Nucleotide sequence accession numbers. All of the sequences were deposited in GenBank. The accession numbers for the 16S-23S rDNAs of the P endosymbionts of psyllids are given in Table 1. Additional sequences are those for the 16S-23S rDNAs of *B. argentifolii* (AF211870) and *Z. palmae* (AF211871) as well as *wg* of *P. obesinymphae* (AF231390) and *B. tabaci* (AF231391).

RESULTS

General properties of the cloned 16S-23S rDNA. Figure 1 presents the results of PCR amplification of 16S-23S rDNA fragments from the DNA of three species of psyllids. *Glycaspis brimblecombei* contained two bands (lane 2), a less intense band of 4.6 kb and a band of greater intensity at 4.0 kb. *Ctenarytaina eucalypti* and *Ctenarytaina spatulata* contained only the lower 4.0-kb band (lanes 3 and 4). The 4.0-kb band was digested by *Xho*I to two fragments of 1.4 and 2.6 kb (lanes 5 and 6); a *Xho*I site was not present in the upper band (lane 5). The upper 4.6-kb band was digested by *Apa*I to fragments

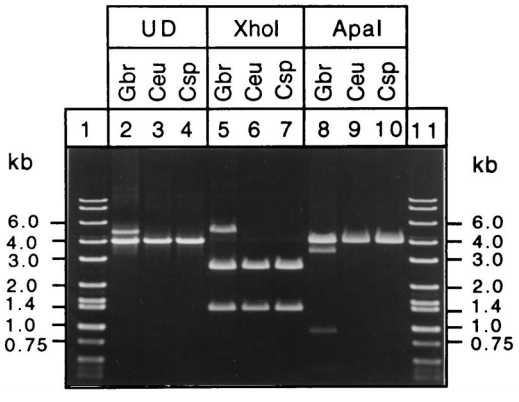

FIG. 1. Agarose gel electrophoresis of 16S-23S rDNA PCR products amplified from total DNAs of three species of psyllids. Lanes 1 and 11, molecular size standards; lane 2, 0.45 μg of DNA; lanes 3, 4, 6, 7, 9, and 10, 0.33 μg of DNA; lanes 5 and 8, 0.54 μg of DNA; UD, undigested DNA; *Xho*I and *Apa*I, restriction enzymes. A 0.4-kb band (lane 8) is not visible in the photograph. Gbr, *G. brimblecombei*; Ceu, *C. eucalypti*; Csp, *C. spatulata*.

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                end-16S                                    start 23S
Tso  GATTtttaatt----------------------tatattttaaataaaattat---TGAT
Ceu  GATAtaaattatatt-------------------ttttttttaaataaa----taaTGAT
Pce  GATAattatttttttaaaaaaaaaaaaaatatgtatattttaaaataaaaatttttTGAT
Aun  GATAttataa--------------------aat-atattttaaaat---ttttttttGAT
Cmy  GATAtaaaaaaaaaataa-------------ttatatttctaaatttaatttttttGAT
Hte  GATAattata----------------------t-atatttctcaat---attatatTGAT
Psp1 GATAaataaaa-----------------ta--tatttctcaattttt--tttttttGAT
Nhi  GATAtataaaa-----------------ta--tatatttcttaatat--tattttTGAT
Teu  GATAtataataat-----------------------atatttcttaat-attataaTGAT
Csc  GATAttataaa----------------------tatattttaaaataaaaaatta-TGAT
Bap  GATCACCTCCTTA
Eco  GATCACCTCCTTA
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FIG. 2. Comparison of the intergenic space between 16S rDNA and 23S rDNA of P endosymbionts from 10 species of psyllids. Capital letters, putative end of 16S rDNA and beginning of 23S rDNA; lowercase letter, intergenic space; double-underlined sequence, complement of the ribosome binding site in mRNA. Tso, *Tainarys sordida*; Ceu, *C. eucalypti*; Pce, *P. celtidis*; Aun, *A. uncatoides*; Cmy, *Cacopsylla myrthi*; Hte, *Heteropsylla texana*; Psp1, *Panisoplema* sp.; Nhi, *Neotriozella hirsuta*; Teu, *Trioza eugeniae*; Csc, *Calophya schini*; Bap, *B. aphidicola*; Eco, *E. coli*.

of 3.4, 0.8, and 0.4 kb (lane 8; the 0.4-kb fragment is not visible on the photograph); an *Apa*I site is absent in the lower band (lanes 8 to 10). Of the 33 psyllid DNA preparations from which the 16S-23S rDNA was amplified, all had the 4.0-kb fragment corresponding to the P endosymbiont. Of these, 23 had an additional larger fragment corresponding to a putative S endosymbiont. In all cases, the 4.0-kb fragment had an *Xho*I site at about 1.4 kb and lacked *Apa*I sites; the reverse was true for the larger fragment. Only the 4.0-kb DNA fragment was cloned and sequenced. The length of these fragments was 3,977 to 4,045 nt, and their G+C content was 33.3 to 35.6 mol%. The 16S rDNA portion was 1,487 to 1,529 nt in length and had a G+C content of 35.1 to 37.7 mol%, while the 23S rDNA portion was 2,453 to 2,497 nt in length and had a G+C content of 32.5 to 34.1 mol%. The 27- to 53-nt-long intergenic region had a much lower G+C content (0 to 12.9 mol%).

Figure 2 presents the end of the 16S rDNA sequence, the intergenic space, and the beginning of the 23S rDNA sequence for the P endosymbionts of 10 representative psyllids. The beginnings and ends are arbitrarily designated as the first and last nucleotides that are conserved in almost all P endosymbionts. Comparisons of the 3′ ends of 16S rDNAs of P endosymbionts with *B. aphidicola* and *E. coli* indicated an unusual feature, namely, the absence of a sequence in the P endosymbiont (CCTCC) corresponding to the complement of the mRNA ribosome binding site (Shine-Dalgarno sequence) (Fig. 2). Such a sequence is also absent in the 16S rDNA homolog of animal mitochondria (59).

Acizzia uncatoides samples 1 and 2 are psyllids of the same species; the former was collected in Chile, while the latter was collected in California. There are six sequence differences between the 16S-23S rDNAs from the P endosymbionts of these two strains; none of these differences are in the intergenic region.

Phylogenetic analyses of 16S-23S rDNA. For 16S, 23S, and combined 16S-23S analyses, the psyllid P endosymbionts formed a very strongly supported monophyletic group with respect to other bacteria, with 100% bootstrap support in neighbor-joining trees and strong support in maximum likelihood trees. BLAST searches of the 16S ribosomal databases (33) using psyllid endosymbiont 16S rDNA sequences revealed that the closest relatives were the P endosymbionts of whiteflies and *Z. palmae* (both in the γ subdivision of the *Proteobacteria*), and full 16S-23S rDNA sequences of these organisms were obtained for more complete comparisons. Based on these two outgroups plus *E. coli*, the position of the root of the

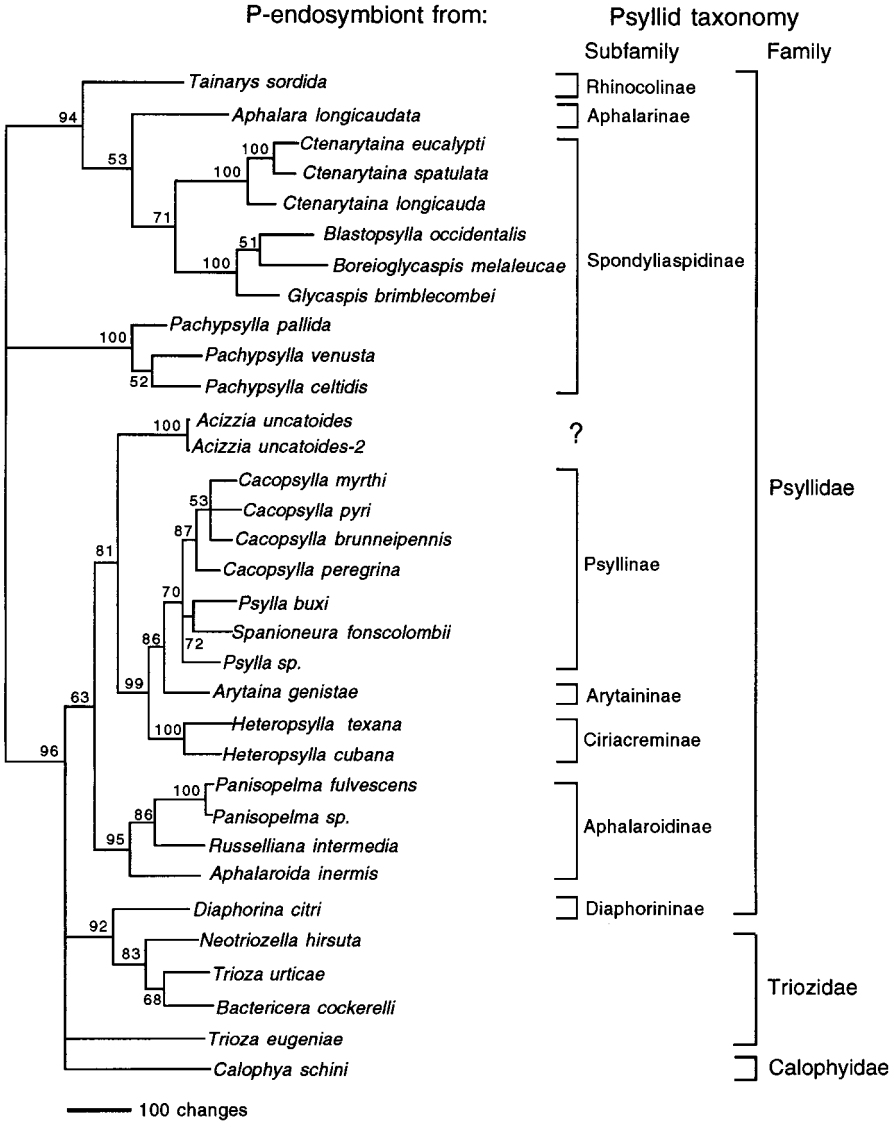

FIG. 3. Phylogenetic trees from neighbor-joining analyses of psyllid P-endosymbiont nucleotide sequences of combined 16S-23S rDNA and relationship of the results to the classification of psyllids based on morphology. Numbers at nodes are for bootstrap percentages from 1,000 replicates; only nodes supported by 50% or greater are shown. Designations refer to psyllid hosts, vertical lines indicate assignments of psyllids to subfamilies and families, and the question mark indicates uncertain taxonomic affiliation.

psyllid endosymbiont clade was not well resolved and in different analyses was placed on different basal internodes of the trees (results not shown). This lack of resolution of the outgroup position was due to the very long branch (high divergence) separating the psyllid endosymbionts from other bacteria and has been noted previously (50). Figure 3 presents an unrooted phylogenetic tree from the neighbor-joining method based on 16S-23S rDNA sequences. Maximum likelihood trees were very similar to those shown in Fig. 3. The only differences were in the branching order of some weakly supported nodes.

Although there were slight differences between the trees obtained for the 16S and 23S sequences (results not shown), the partition homogeneity test gave no evidence for statistically significant phylogenetic conflict between the data sets: the test failed to reject the null hypothesis that the 16S and 23S genes support the same phylogenetic tree. The sum of lengths for the 16S/23S partition was 3,546, a value lower than that for most of the random partitions [$P = 1 - (831/1{,}000) = 0.169$]. (Rejection of the null hypothesis requires that the sum be greater than that for most random partitions.)

Phylogenetic analysis of the host *wg* gene and congruence with 16S-23S rDNA. The phylogeny based on the host *wg* sequences showed overall agreement with the tree based on endosymbiont 16S-23S rDNA (Fig. 4). There was less resolution from the *wg* data set, presumably due to the fact that *wg* sequences were much shorter (540 nt, about 13% of the combined length of the 16S-23S rDNA sequences). Considering only nodes supported by at least 50% bootstrap values in neighbor-joining analyses for both 16S-23S and *wg* data sets, there was complete congruence between the host and endosymbiont trees (Fig. 4). Results from maximum likelihood analyses of *wg* sequences were almost identical to those for the neighbor-joining analyses and did not conflict at any of the nodes supported by >50% bootstrap values.

Phylogenetic congruence between host and endosymbionts was further supported by results from the partition homoge-

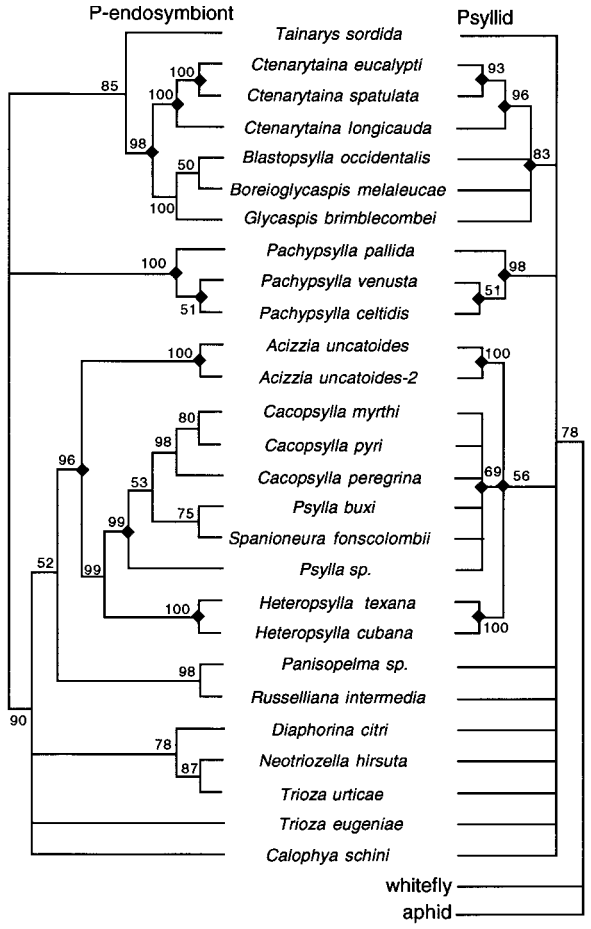

FIG. 4. Comparisons of phylogenetic trees derived from 16S-23S rDNA of psyllid P endosymbionts and the *wg* nuclear host gene. The tree for 16S-23S rDNA is the same as that in Fig. 3. Numbers at nodes of the host tree are for bootstrap percentages from 1,000 replicates; only nodes supported by 50% or greater are shown. ◆, nodes matching in the endosymbiont and host trees.

neity test, which failed to reject the null hypothesis that the 16S-23S rDNA and *wg* genes support the same phylogeny. The sum of lengths for the 16S+23S/*wg* partition was 6,181, yielding a *P* value of 0.69.

Relative rates. In all comparisons, the psyllid endosymbionts showed much higher rates of substitution. Both whitefly P-endosymbiont and *B. aphidicola* sequences have previously been shown to evolve relatively fast compared to sequences of free-living bacteria (34); however, sequences of psyllid endosymbionts evolve even faster. This extreme rate of substitution was previously noted for psyllid P endosymbionts (50). Our analyses show that the high rate extends to the 23S rDNA and to P endosymbionts of all psyllid lineages.

Absolute rates. The greatest divergences among psyllid endosymbionts for 16S rDNA and for 23S rDNA were similar at 0.13 and 0.12 substitutions/site, respectively. When calibrated using the minimum and maximum ages for the common ancestor of psyllids (100 and 250 MY, respectively), the rate of substitution for both 16S rDNA and 23S rDNA was 0.025 to 0.063 substitutions/site per 100 MY. These approximate values were slightly greater than those calculated for *B. aphidicola* (values from 0.019 to 0.054 substitutions/site per 100 MY) and substantially greater than those calculated for enteric bacteria (0.007 to 0.018 substitutions/site per 100 MY) (18).

Electron microscopy. Transmission electron micrographs of *P. venusta* bacteriocytes and endosymbionts (Fig. 5) were in agreement with past observations (16, 55). The P endosymbionts were pleomorphic bacteria that had the electron-dense structures previously observed (55).

DISCUSSION

The results of the phylogenetic analyses indicate overall agreement between the phylogenetic trees derived from psyllid P-endosymbiont 16S-23S rDNA and the host nuclear gene *wg* (Fig. 4). This agreement provides an indication of the nature of the history of the association between the host and the P endosymbiont and is consistent with a single infection of a psyllid ancestor with a bacterium followed by long-term cospeciation of the host and the P endosymbiont. The congruence of the trees derived from the endosymbiont and host sequences also indicates that P endosymbionts were not transferred between psyllid lineages; that is, there is vertical evolution of the host and the endosymbiont. Evidence for cospeciation between prokaryotic endosymbionts and their insect hosts has been previously obtained for *Buchnera* bacteria with aphids (18, 35, 36, 39), *Wigglesworthia* bacteria with tsetse flies, (17), *Blochmannia* bacteria with carpenter ants (47, 48), and *Blattabacterium* bacteria with cockroaches (2, 3). In addition, cospeciation has been observed between chemoautotrophic bacteria and deep-sea clams (44) and between luminous bacteria and sepiolid squids (43).

The results of the phylogenetic analyses of P-endosymbiont genes are in reasonably good agreement with the psyllid classification based on insect morphology (Fig. 3) (11, 12, 13, 15, 57). There is especially good agreement at the level of subfamilies. Most of the studied species are from the family Psyllidae, which in our analysis appears to be split into at least three major clusters.

The low G+C content of the 16S rDNA and 23S rDNA of the P endosymbiont is unique among prokaryotes (25, 33, 50), as is the absence of a sequence corresponding to the complement of the mRNA ribosome binding site at the 3′ end of 16S rDNA of the P endosymbiont. Both of these properties suggest a resemblance to animal mitochondria (59). Restriction enzyme and Southern blot analysis of DNA from *P. venusta* and *Pachypsylla celtidis* (psyllids that have only the P endosymbiont), using a probe for 16S rDNA, gave results consistent with a single copy of the rDNA operon per P-endosymbiont genome (M. L. Thao and L. Baumann, unpublished data). This result is similar to that observed for *B. aphidicola* as well as other endosymbionts and slow-growing bacteria (6).

It has been suggested elsewhere that partially characterized bacteria that have not been cultivated on laboratory media be given the designation "*Candidatus*" (40). Consequently, we propose to name the lineage corresponding to the P endosymbionts of psyllids as *Candidatus* Carsonella. Carsonella refers to Rachel Carson, an American naturalist and author of *Silent Spring*. *Candidatus* Carsonella consists of pleomorphic bacteria found in the bacteriocytes of psyllids (10) (Fig. 5). They have a gram-negative type of cell wall and are found within membrane vesicles derived from host cells (16, 55). Their 16S rRNA gene is directly upstream of the 23S rRNA gene. These genes have an unusually low G+C content in their DNA (35 to 38 mol%, 16S rDNA; 32 to 34 mol%, 23S rDNA). The 3′ end of their 16S rDNA lacks a sequence complementary to the mRNA ribosome binding site. Based on the sequence of the 16S and 23S rDNA, these organisms are members of the γ subdivision of the *Proteobacteria* (58). These organisms are transmitted ver-

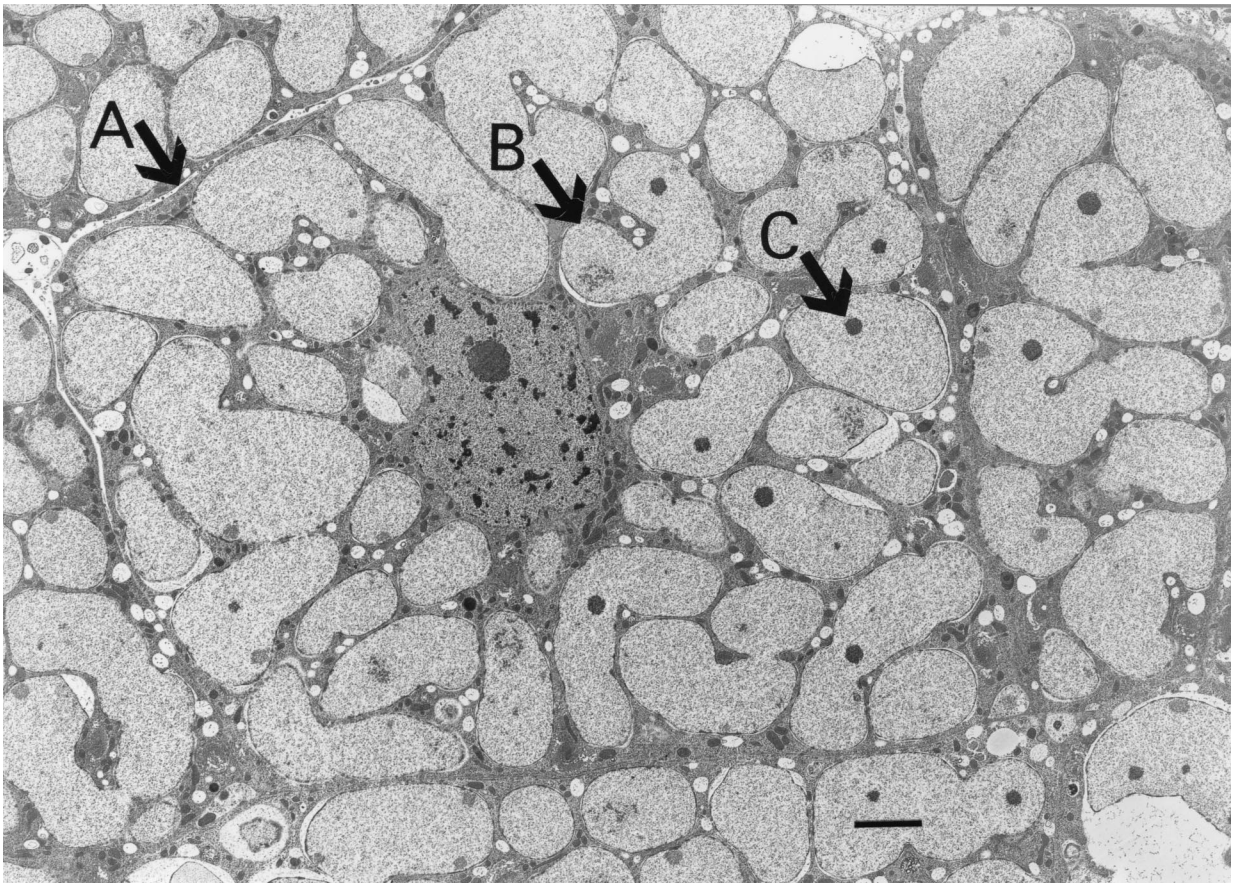

FIG. 5. Transmission electron micrograph of a bacteriocyte from *P. venusta*. A, bacteriocyte; B, endosymbiont; C, unidentified electron-dense aggregate. Bar, 2 μm.

tically to host progeny, as is indicated by cospeciation between the host and the endosymbiont.

Candidatus Carsonella contains a single species, *Candidatus* Carsonella ruddii. The species epithet refers to Robert L. Rudd, an American naturalist and author of *Pesticides and the Living Landscape*. The P endosymbiont from *P. venusta* (GenBank accession no. AF211143) is proposed as the type strain. The G+C content of 20 kb of *Candidatus* Carsonella ruddii is 20.0 mol% (M. A. Clark, L. Baumann, N. A. Moran, and P. Baumann, unpublished data). The following sequences are unique to *Candidatus* Carsonella ruddii: 16S rDNA, CAA ACT T(T/C)T AAG GAA GG; 23S rDNA, GAT GAA A(T/A)A GAA CCT TT(A/T) A(A/T)T AG.

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