

Evolutionary Relationships among Primary Endosymbionts of the Mealybug Subfamily Phenacoccinae (Hemiptera: Coccoidea: Pseudococcidae)[∇]

Matthew E. Gruwell,^{1*} Nate B. Hardy,² Penny J. Gullan,³ and Katharina Dittmar⁴

Penn State Erie School of Science, P-1 Prischak Building, 4205 College Drive, Erie, Pennsylvania 16563-0203¹;
Queensland Primary Industries and Fisheries, Entomology, 80 Meiers Road, Indooroopilly, QLD 4068,
Australia²; UC Davis, Department of Entomology, One Shields Avenue, Davis, California
95616-8584³; and Department of Biological Sciences, 109 Cooke Hall,
SUNY at Buffalo, Buffalo, New York 14260⁴

Received 7 June 2010/Accepted 8 September 2010

Mealybugs (Coccoidea: Pseudococcidae) are sap-sucking plant parasites that harbor bacterial endosymbionts within specialized organs. Previous studies have identified two subfamilies, Pseudococcinae and Phenacoccinae, within mealybugs and determined the primary endosymbionts (P-endosymbionts) of the Pseudococcinae to be *Betaproteobacteria* (“*Candidatus Tremblaya princeps*”) containing *Gammaproteobacteria* secondary symbionts. Here, the P-endosymbionts of phenacoccine mealybugs are characterized based on 16S rRNA from the bacteria of 20 species of phenacoccine mealybugs and four outgroup *Puto* species (Coccoidea: Putoidae) and aligned to more than 100 published 16S rRNA sequences from symbiotic and free-living bacteria. Phylogenetic analyses recovered three separate lineages of bacteria from the Phenacoccinae, and these are considered to be the P-endosymbionts of their respective mealybug hosts, with those from (i) the mealybug genus *Rastrococcus* belonging to the *Bacteroidetes*, (ii) the subterranean mealybugs, tribe Rhizoecini, also within *Bacteroidetes*, in a clade sister to cockroach endosymbionts (*Blattabacterium*), and (iii) the remaining Phenacoccinae within the *Betaproteobacteria*, forming a well-supported sister group to “*Candidatus Tremblaya princeps*.” Names are proposed for two strongly supported lineages: “*Candidatus Brownia rhizoecola*” for P-endosymbionts of Rhizoecini and “*Candidatus Tremblaya phenacola*” for P-endosymbionts of Phenacoccinae excluding *Rastrococcus* and Rhizoecini. Rates of nucleotide substitution among lineages of *Tremblaya* were inferred to be significantly faster than those of free-living *Betaproteobacteria*. Analyses also recovered a clade of *Gammaproteobacteria*, sister to the P-endosymbiont lineage of aphids (“*Candidatus Buchnera aphidicola*”), containing the endosymbionts of Putoidae, the secondary endosymbionts of pseudococcine mealybugs, and the endosymbionts of several other insect groups.

Many insect species possess specialized cells that contain obligate mutualist bacteria, primary endosymbionts (P-endosymbionts). P-endosymbionts usually show an evolutionary history of codiversification with an insect host and are thought to be essential to the survival of host species (4, 27). Insects with P-endosymbionts are phylogenetically and ecologically heterogeneous but commonly have a nutritionally unbalanced diet. Endosymbiotic bacteria can provide nutritional supplements to their host, permitting exploitation of otherwise inadequate food sources. For example, tsetse fly P-endosymbionts (*Wigglesworthia glossinidia*) synthesize B vitamins that are lacking in vertebrate blood (1, 3, 32, 33). Several additional examples of blood-feeding insect groups that depend upon P-endosymbionts are known, but the greatest diversity of P-endosymbionts is found in insects that feed on plant sap, a food source poor in amino acids. More than 80% of the approximately 63,000 described insect species with P-endosymbionts feed on plant sap and depend on P-endosymbionts to enrich their diet with amino acids (4, 34, 35, 45, 46).

Insect P-endosymbionts are vertically transmitted (4, 9, 43), and in general, the relationships among the bacteria are highly congruent with those among host taxa due to codiversification. Diverse clades of sap-sucking insects are associated with specific P-endosymbiont lineages. Major sap-sucking groups occur in the hemipteran suborders Auchenorrhyncha (11) and Sternorrhyncha (18). Auchenorrhynchan bugs have codiversified with a *Bacteroidetes* P-endosymbiont lineage, *Sulcia muelleri*, with just a few exceptions that are interpreted as losses (28). In addition, a few auchenorrhynchan groups, mainly the sharpshooters, house a second P-endosymbiont, *Baumannia cicadellincola* (*Proteobacteria*), in the same bacteriome as *S. muelleri* (24, 27, 28, 38). Sternorrhynchan insect groups follow the pattern of old and persistent associations with P-endosymbiont lineages but are more heterogeneous, with each of several family level or other higher taxa characterized by unique P-endosymbiont lineages: Aphididae hosts with *Buchnera aphidicola* P-endosymbionts (23), Diaspididae with *Uzinura diaspidicola* (16), Pseudococcidae with *Tremblaya princeps* (5, 13), Psylloidea with *Carsonella ruddii* (40), and Aleyrodoidea with *Portiera aleyrodidarum* (39).

Mealybugs (Pseudococcidae) are the second largest family of scale insects (Hemiptera: Coccoidea), with ca. 2,000 identified species in more than 270 genera (<http://www.sel.barc.usda>

* Corresponding author. Mailing address: Penn State Erie School of Science, P-1 Prischak Building, 4205 College Drive, Erie, PA 16563-0203. Phone: (814) 898-6276. Fax: (814) 898-6213. E-mail: meg26@psu.edu.

[∇] Published ahead of print on 17 September 2010.

TABLE 1. Primers designed for 16S rRNA gene sequences from bacteria^a

Primer name	Description	Sequence
8FBAC	Universal bacteria	AGAGTTTGCATCCTGGCTCAG
1492RBac	Universal bacteria	GTTACCTTGTACGACTT
S30 Buch16S	Universal bacteria	GGCGGCAAGCCTAACACATGCAAGTCG
A1446 Buch16S	Universal bacteria	CTCCCATGGTGTGACGGGCGGTGTG
s688DIASP	Universal bacteria	GGAATGTATGGTGTAGCGGTGAAATGC
TremintF	<i>Tremblaya</i> internal	CTACTGCCAGCAGCAGCCGCGG
TremintR	<i>Tremblaya</i> internal	CCGCGGCTGCTGTGGCAGTAG
RHIZINTF	<i>Brownia</i> internal	GCAAACAGGATTAGATACCCTGG
RHIZINTR	<i>Brownia</i> internal	CCAGGGTATCTAATCCTGTTTGC
RastintF	<i>Rastrococcus</i> internal	GCCGTAACGAATGATCACTAG
RastintR	<i>Rastrococcus</i> internal	CTAGTGATCATTCGTTTACGGC

^a Universal primers were used for amplification, and internal primers were used for sequencing.

.gov/SCALENET/classif.htm). They have a worldwide distribution and feed on a broad range of hosts but are concentrated in the tropics and favor grasses, legumes, and composites (6, 12). Several mealybug species are serious pests, especially of grapes, figs, apples, citrus, dates, bananas, avocados, and mangos (25). Phylogenetic analyses have identified two primary subgroups within mealybugs, the subfamilies Pseudococcinae and Phenacoccinae (19). Munson et al. (30) and Thao et al. (41) reported monophyly for the mealybug P-endosymbionts, and Downie and Gullan (13) inferred mealybug-P-endosymbiont codiversification, but these studies included samples from only the Pseudococcinae (19). Buchner (8, 9) observed microscopic anatomical differences between the P-endosymbionts of mealybug genera that today are placed in either Pseudococcinae or Phenacoccinae and also considerable variation among phenacoccine species. Hardy et al. (19) hypothesized that a bacteriome containing *Tremblaya* P-endosymbionts is a synapomorphy (shared feature due to common ancestry) of the Pseudococcinae and that a bacteriome with *Tremblaya* P-endosymbionts containing *Gamma-proteobacteria* S-endosymbionts (44) is a synapomorphy of the Pseudococcinae, excluding *Maconellicoccus* and the *Ferisia* group. Here, we estimate relationships among 16S rRNA sequences from the bacterial endosymbionts of Phenacoccinae and other insects, as well as some free-living bacteria.

MATERIALS AND METHODS

Nomenclature. Most symbiotic bacteria of insects cannot be named formally because the difficulty of culturing them outside their host means that characteristics required for description according to the Bacteriological Code 1990 Revision (22) are not known. Instead, they are given names in the category “*Candidatus*” (22, 27). For example, the P-endosymbiont of Pseudococcinae should be referred to as “*Candidatus Tremblaya princeps*.” However, most authors drop the “*Candidatus*” part of the name and italicize the genus and species name (e.g., *Tremblaya princeps*), which is a practice that we have followed in this paper.

Taxa sampling. The data set included 140 sequences, of which 27 were bacterial sequences from 23 species of Phenacoccinae and Putoidae. *Puto*, the only genus in Putoidae, once was included in Pseudococcidae but now is recognized as a distantly related group (10, 17). One host insect (*Puto albicans*) is represented by three different bacterial sequences. The Phenacoccinae and Putoidae sequences resulted from direct sequencing and cloning of PCR products obtained using general bacterial primers. Voucher specimens of the insects from which the bacterial DNA was extracted are deposited in the Bohart Museum of Entomology, University of California, Davis, and voucher numbers for new data and GenBank (7) accession numbers for all sequences are given in Table S2 at <http://bio.bd.psu.edu/gruwel/supplemental/>.

The remaining 113 samples (see Table S2 at <http://bio.bd.psu.edu/gruwel/supplemental/>) were included to help identify bacteria associated with species of

Phenacoccinae and Putoidae. These data include 16S rRNA sequences from all known groups of scale insect endosymbionts (15, 16, 41), a diversity of other insect endosymbionts, and various free-living bacteria.

DNA extraction and gene amplification. All genomic DNA samples of Phenacoccinae and Putoidae were the same as those used by Hardy et al. (19). Sequences of 16S rRNA were amplified with two sets of general PCR primers, 8fBAC-1492BAC and 16SA1-16SB1, which cover almost the entire gene (ca. 1,200 bp). Two PCR protocols were used: a standard three-step PCR protocol with a 52°C annealing temperature, and the EFTD touchdown protocol of Moulton and Wiegmann (29). If initial amplifications did not produce bands that could be sequenced, reamplifications were done using PCR products as the DNA template and the internal primers S30Buch and a1271Diap (15, 16). Internal primers were used for double coverage in sequencing: TremINTF, TremINTR (*Tremblaya*-like bacteria), RhizINTF, RhizINTR (bacteria from Rhizoecini), RastINTF, RastINTR (bacteria from *Rastrococcus*), and S688DIASP (designed from *Uzinura diaspidicola*, the primary symbiont of armored scale insects). All primer sequences are given in Table 1.

Each PCR was run with a total volume of 25 μ l made up of 13 μ l double-distilled water (ddH₂O), 2 μ l 50 mM MgCl₂, 3 μ l 10 \times buffer, 2.5 μ l of 10 mM deoxynucleoside triphosphate (dNTP), 2.5 μ l loading dye, 1 μ l each primer (10 pmol/ μ l), 0.1 μ l *Taq* polymerase, and 1 μ l of genomic DNA. Each PCR was run with negative controls. Reactions were confirmed as successful using agarose gel electrophoresis and sent to the High Throughput Genomics Unit (Seattle, WA) for cleaning and DNA sequencing.

The samples PG48, NH12, NH59, PG96, and NH57 were cloned using a TOPO One Shot cloning kit (Invitrogen Corp.) and sequenced with M13 forward and reverse primers. The samples were chosen for cloning to obtain coverage across the Phenacoccinae and Putoidae. Only PG48 returned multiple distinct sequences during cloning.

Sequences and alignment. Sequences were edited, assembled, and proofread in Sequencher 4.2 (GeneCodes Corp., 2003). Sequences were aligned using MAFFT (version 6), with default parameter values under the E-INS-i strategy that is optimized for sequences with multiple conserved domains and long gaps. The alignment was edited by eye, and ambiguous regions were excluded using MacClade (version 4.08; Sinauer Associates).

Phylogenetic analysis. Phylogenies were estimated using maximum likelihood (ML), maximum parsimony, and Bayesian methods via the Cipres Portal (<http://www.phylo.org/>). Bayesian analyses were run in MrBayes 3.04b (21) using a general time-reversible (GTR) model with gamma-distributed among-site rate variation, as selected by MrModeltest (version 2; distributed by J. A. A. Nylander, Evolutionary Biology Centre, Uppsala University). Two runs of 10,000,000 generations were completed. Burn-in (250,000 generations) was determined by visual inspection of a graph of likelihood values. Trees sampled before burn-in were discarded using Mesquite (version 2.6; <http://mesquiteproject.org>). RAXML (37) ML searches consisted of 1,500 bootstrap pseudoreplicates under GTR with CAT approximation of gamma-distributed among-site rate variation. Every fifth bootstrap tree was used as a starting tree for ML inferences on the original alignment under GTR + γ . Parsimony runs were completed under Fitch optimization using heuristic searches implemented with a ratchet algorithm (31) in PAUP* (version 4.0b10; Sinauer Associates, Sunderland, MA) as a batch file in PAUPRAT (version 1; distributed by D. S. Sikes and P. O. Lewis, Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT). For each of 2,500 iterations, 20% of characters were reweighted. Trees were filtered for length and duplication.

Estimated branch lengths among samples of *Tremblaya* were conspicuously greater than those among closely related free-living lineages of *Betaproteobacteria*. To test if the rate of 16S rRNA evolution was significantly different between *Tremblaya* and its free-living sister group, we compared two molecular clock models: a null 1-rate model and an alternative 2-rate model in which *Tremblaya* and its sister group were each assigned a unique rate (as in reference 36). A rooted ML topology was inferred from an alignment of *Betaproteobacteria* 16S rRNA sequences with RAxML (detailed phylogenetic tree available; see Fig. S1 posted at <http://bio.bd.psu.edu/gruwell/supplemental>) (37). The RAxML analysis consisted of 500 bootstrap replicates under GTR + CAT, with every fifth bootstrap tree used as a starting tree for more thorough optimization under GTR + γ . We used the baseml module of PAML 4.2b (47) to estimate branch lengths under HKY + γ and to calculate the likelihoods of the null and alternative clock models. We evaluated the difference between the likelihoods of our clock models with a likelihood ratio test (14).

Assigning P-endosymbiont status. It was unclear that the amplified 16S rRNA sequences were from bacteria occurring within the host bacteriome. Direct assessment of endosymbiotic properties of bacteria was not undertaken. Endosymbiont status was allocated to bacterial sequences on the basis of three criteria: (i) relationships among P-endosymbiont sequences should reflect those of their hosts, (ii) P-endosymbiont lineages should be related to other known P-endosymbiont lineages, and (iii) it should be possible to amplify P-endosymbiont sequences consistently from closely related specimens. These premises assume that (i) P-endosymbionts are vertically transmitted, (ii) the likelihood of a host's successful acquisition of an endosymbiont increases if the candidate bacterium is already an endosymbiont, and (iii) P-endosymbionts are always present in the host bacteriome.

RESULTS

P-symbiont identities. Three groups of bacteria were recovered from phenacoccline mealybugs (Fig. 1), as detailed in the next three paragraphs.

Bacterial sequences from the Phenacocclinae, excluding *Rastrococcus* and the Rhizoecini, belonged within the *Betaproteobacteria*, as sister to *Tremblaya princeps*, the pseudococcine endosymbiont lineage (bootstrap support [BS] = 100; posterior probabilities [PP] = 100). Relationships inferred among these bacterial sequences reflected those inferred among their hosts (19). We propose the designation "*Candidatus Tremblaya phenacola*" for the P-endosymbiont of mealybug species in the Phenacocclinae, excluding *Rastrococcus* and Rhizoecini. This lineage has unique sequences at the following 16S rRNA sites that distinguish it from *Tremblaya princeps* (homologous to the *Escherichia coli* positions): positions 466 to 469 are AATT (ACCA in *T. princeps*), 817 is G (C in *T. princeps*), 831 is A (G in *T. princeps*), 848 and 849 are TT (AA in *T. princeps*), and 1257 to 1261 are AGTT (GGTC in *T. princeps*).

Bacterial sequences from the mealybug tribe Rhizoecini were recovered as a clade within *Bacteroidetes*. A sister relationship with cockroach endosymbionts (*Blattabacterium*) was weakly supported. We propose the designation "*Candidatus Brownia rhizoecola*" for the P-endosymbiont of mealybug species in the tribe Rhizoecini. The genus name is in honor of Spencer W. Brown, who was a pioneer of scale insect cytogenetics, and the species name is in reference to the host taxon. This bacterial lineage has sequences that differ from its sister taxon *Blattabacterium* at the following loci, which correlate to the *E. coli* positions of 16S rRNA: positions 729 to 736 are GGCAAGCT (AGCAGGT in *Blattabacterium*), 1037 to 1048 are AACGCAAG (AGTACAAG in *Blattabacterium*), 1117 to 1121 are GTCG (ATTG in *Blattabacterium*), and 1138 to 1140 are GCT (GTC in *Blattabacterium*).

Bacterial sequences from the genus *Rastrococcus* were re-

covered within *Bacteroidetes*, in a clade containing a bacterial sequence from *Cryptococcus ulmi* (Eriococcidae) (15). Strong support (BS = 76; PP = 100) was discovered for a sister relationship between this clade and a group of symbiont sequences from two other scale insect families (Monophlebidae and Diaspididae). In this case, relationships among the bacteria do not mirror those among hosts. Therefore, we do not propose a name for this putative P-endosymbiont of *Rastrococcus*.

Most sequences from species of Putoidae were recovered from within the *Gammaproteobacteria*, in a weakly supported group comprised of symbiont sequences from *Tetropium castaneum* (Cerambycidae) (S. Gruenwald, unpublished data), *Sitophilus oryzae* (Dryophthoridae) (20), *Glossina austeni* (the symbiont *Wigglesworthia glossinidia*) (2), *Paromenia isabellina* (the symbiont *Baumannia cicadellinicola*) (38), and Pseudococcinae mealybugs (secondary symbionts) (41). Sequences from a psyllid secondary symbiont (*Arsenophonus*) (40, 42) and an uncharacterized soft scale (Coccidae) symbiont were sisters to this group with weak support. Monophyly was not identified for the putoid-associated bacterial sequences within this group. One sequence from *Puto albicans* was quite distantly related; it was recovered as sister to a *Spiroplasma* (*Mollicutes*) lineage from *Drosophila* with an unknown function.

Rate differences. A significant difference ($P < 0.0001$) in the rate of 16S rRNA evolution between *Tremblaya* lineages and free-living *Betaproteobacteria* was inferred from comparison of the null 1-rate clock model (log likelihood score [lnL] = -10,007.11) to an alternative 2-rate model (lnL = -9,942.20) in which the rates of *Tremblaya* and its sister group were independent. The estimated local clock rate for *Tremblaya* was more than 7-fold faster than that of the included free-living *Betaproteobacteria*.

DISCUSSION

Sap-sucking insect lineages have had relationships with P-endosymbionts characterized by persistence and stability. Nearly all of Auchenorrhyncha share a P-endosymbiont in *Sulcia muelleri*, and three of four Sternorrhyncha superfamilies are each thought to have a single lineage of P-endosymbiont. In contrast, among scale insects (Coccoidea), mealybugs alone have three distantly related P-endosymbiont lineages.

Contrary to the hypothesis of Hardy et al. (19) that *Tremblaya* P-endosymbionts were a synapomorphy of the Pseudococcinae, many species in the Phenacocclinae also possess *Tremblaya* P-endosymbionts. It is the specific lineage of *Tremblaya princeps* that is synapomorphic of the Pseudococcinae, with *Tremblaya phenacola* occurring in all Phenacocclinae mealybugs other than *Rastrococcus* and the Rhizoecini. This suggests an endosymbiotic relationship between *Tremblaya* and mealybugs that evolved in conjunction with or predates the mealybug radiation.

The drastically elevated rates of 16S rRNA evolution observed among *Tremblaya* samples in comparison to rates among closely related *Betaproteobacteria* lineages are consistent with the results of other empirical studies (e.g., references 26 and 42) and with theoretical predictions centered on small populations sizes and a lack of lateral gene transfer in endosymbiont lineages (reviewed in reference 45).

This work further establishes the mealybugs and in particular the Phenacocclinae as a system in which to study compara-

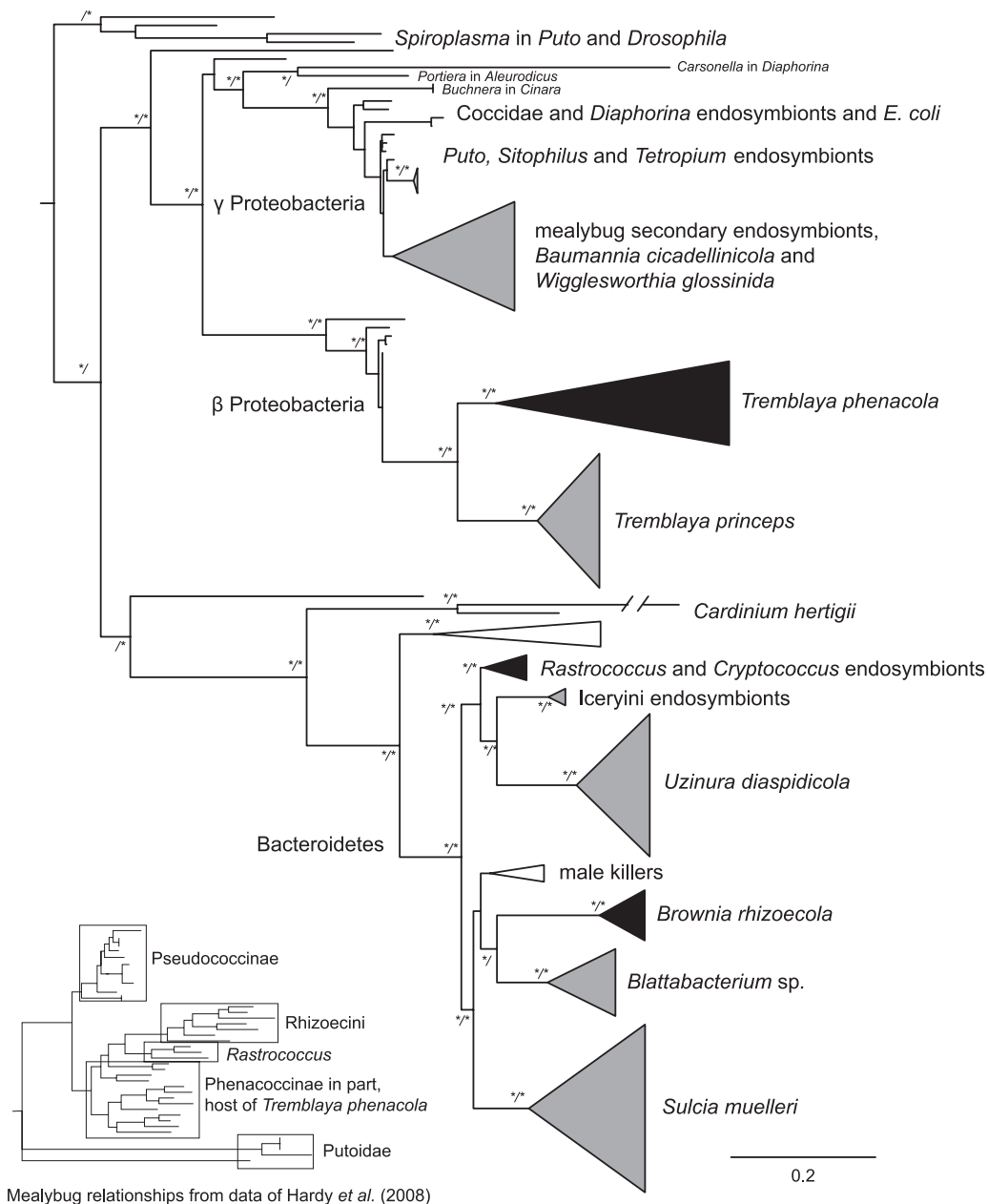


FIG. 1. Salient features of the maximum likelihood estimate of relationships among 16S rRNA sequences from free-living and endosymbiotic bacteria. Nodes having posterior probabilities (PP) $\geq 95\%$ and/or bootstrap support (BS) $\geq 80\%$ are denoted with asterisks (PP/BS). Free-living bacteria lineages are not named. Endosymbiont clades of the Phenacoccinae are collapsed and in black; the bacterial groups *Tremblaya phenacola* and *Brownia rhizoecola* are designated in this paper. Endosymbiont lineages of other insect groups are in gray.

tive endosymbiosis. Obligate relationships with P-endosymbionts have been more fundamental in the evolutionary history of mealybugs than historical biogeography or host associations (19). Therefore, mealybugs should be an ideal model for researchers wishing to isolate the history of endosymbiosis from other historical and ecological processes.

ACKNOWLEDGMENTS

We thank the generous support from the scale insect community in obtaining specimens. We gratefully acknowledge the laboratory support provided by undergraduates Doug Brummell and Emily DiBlasi (SUNY at Buffalo). P.J.G. thanks Paul Baumann for initiating her

interest in mealybug endosymbionts. M.E.G. thanks Benjamin B. Normark for inspiring interest in scale insects and endosymbionts and for continued support and direction during the development of this project. This paper benefited from the critical comments of two anonymous reviewers.

This research was supported by funds from the State University of New York at Buffalo, Pennsylvania State University at Erie, Behrend College, and Hatch funding from the California Agricultural Experiment Station.

REFERENCES

1. Akman, L., A. Yamashita, H. Watanabe, K. Oshima, T. Shiba, M. Hattori, and S. Aksoy. 2002. Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. *Nat. Genet.* 32:402–407.

2. Aksoy, S. 1995. *Wigglesworthia* gen. nov. and *Wigglesworthia glossinidia* sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of tsetse flies. *Int. J. Syst. Bacteriol.* **45**:848–851.
3. Aksoy, S. 2000. Tsetse—a haven for microorganisms. *Parasitol. Today* **16**: 114–118.
4. Baumann, P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu. Rev. Microbiol.* **59**:155–189.
5. Baumann, L., and P. Baumann. 2005. Cospeciation between the primary endosymbionts of mealybugs and their hosts. *Curr. Microbiol.* **50**:84–87.
6. Ben-Dov, Y. 1994. A systematic catalogue of the mealybugs of the world (Insecta: Homoptera: Coccoidea: Pseudococcidae and Putoidae) with data on geographical distribution, host plants, biology and economic importance. Intercept Ltd., Andover, Hants, United Kingdom.
7. Benson, D. A., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, and D. L. Wheeler. 2004. GenBank: update. *Nucleic Acids Res.* **32**:D23–D26.
8. Buchner, P. 1957. Endosymbiosestudien an schildläusen. 5. Die gattung *Rastrococcus Ferris* (*Ceropto* Sulc). *Z. Morphol. Ökol. Tiere.* **46**:111–148.
9. Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience Inc., New York, NY.
10. Cook, L. G., P. J. Gullan, and H. E. Trueman. 2002. A preliminary phylogeny of the scale insects (Hemiptera: Sternorrhyncha: Coccoidea) based on nuclear small subunit ribosomal DNA. *Mol. Phylogenet. Evol.* **25**:43–52.
11. Dietrich, C. H. 2009. Auchenorrhyncha (cicadas, spittlebugs, leafhoppers, treehoppers, and planthoppers), p. 56–64. *In* V. H. Resh and R. T. Cardé (ed.), *Encyclopedia of insects*, 2nd ed. Elsevier, San Diego, CA.
12. Downie, D. A., and P. J. Gullan. 2004. Phylogenetic analysis of mealybugs (Hemiptera: Coccoidea: Pseudococcidae) based on DNA sequences from three nuclear genes, and a review of the higher classification. *Syst. Entomol.* **29**:238–259.
13. Downie, D. A., and P. J. Gullan. 2005. Phylogenetic congruence of mealybugs and their primary endosymbionts. *J. Evol. Biol.* **18**:315–324.
14. Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.* **17**:368–376.
15. Gruwell, M. E., C. D. von Dohlen, K. Patch, and B. B. Normark. 2005. Preliminary PCR survey of bacteria associated with scale insects (Hemiptera: Coccoidea), p. 101–115. *In* L. Erkiñç and M. B. Kaydan (ed.), *Proceedings of the X International Symposium on Scale Insect Studies* (2004). Adana Zirai Müscadele Arastirma Enstitüsü, Adana, Turkey.
16. Gruwell, M. E., G. E. Morse, and B. B. Normark. 2007. Phylogenetic congruence of armored scale insects (Hemiptera: Diaspididae) and their primary endosymbionts from the phylum Bacteroidetes. *Mol. Phylogenet. Evol.* **44**:267–280.
17. Gullan, P. J., and L. G. Cook. 2007. Phylogeny and higher classification of the scale insects (Hemiptera: Sternorrhyncha: Coccoidea). *In* Z. Q. Zhang and W. A. Shear (ed.), *Linnaeus tercentenary: progress in invertebrate taxonomy*. *Zootaxa* **1668**:413–425.
18. Gullan, P. J., and J. H. Martin. 2009. Sternorrhyncha (jumping plant-lice, whiteflies, aphids, and scale insects), p. 957–967. *In* V. H. Resh and R. T. Cardé (ed.), *Encyclopedia of insects*, 2nd ed. Elsevier, San Diego, CA.
19. Hardy, N. B., P. J. Gullan, and C. J. Hodgson. 2008. A subfamily-level classification of mealybugs (Hemiptera: Pseudococcidae) based on integrated molecular and morphological data. *Syst. Entomol.* **33**:51–71.
20. Heddi, A., C. Hubert, C. Khatchadourian, G. Bonnot, and P. Nardon. 1998. Molecular characterization of the principal symbiotic bacteria of the weevil *Sitophilus oryzae*: a peculiar G + C content of an endocytobiotic DNA. *J. Mol. Evol.* **47**:52–61.
21. Huelsenbeck, J. P., and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**:754–755.
22. Lapage, S. P., P. H. A. Sneath, E. F. Lessel, V. B. D. Skerman, H. P. R. Seeliger, and W. A. Clark. 1990. International code of nomenclature of bacteria: bacteriological code 1990 revision. International Union of Microbiological Societies, ASM Press, Washington, DC.
23. Martinez-Torrez, D., C. Buades, A. Lattore, and A. Moya. 2001. Molecular systematics of aphids and their primary endosymbionts. *Mol. Phylogenet. Evol.* **20**:437–449.
24. McCutcheon, J. P., and N. A. Moran. 2007. Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. *Proc. Natl. Acad. Sci. U. S. A.* **104**:19392–19397.
25. Miller, D. R., G. L. Miller, and G. W. Watson. 2002. Invasive species of mealybugs (Hemiptera: Pseudococcidae) and their threat to U.S. agriculture. *Proc. Entomol. Soc. Washington* **104**:825–836.
26. Moran, N. A. 1996. Accelerated evolution and Muller's ratchet in endosymbiotic bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **93**:2873–2878.
27. Moran, N. A., J. P. McCutcheon, and A. Nakabachi. 2008. Genomics and evolution of heritable bacterial symbionts. *Annu. Rev. Genet.* **42**:165–190.
28. Moran, N. A., P. Tran, and N. M. Gerardo. 2005. Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterium phylum *Bacteroidetes*. *Appl. Environ. Microbiol.* **71**:8802–8810.
29. Moulton, J. K., and B. M. Weigmann. 2004. Evolution and phylogenetic utility of CAD (rudimentary) among Mesozoic-aged Eremoneuran Diptera (Insecta). *Mol. Phylogenet. Evol.* **31**:363–378.
30. Munson, M. A., P. Baumann, and N. A. Moran. 1992. Phylogenetic relationships of the endosymbionts of mealybugs (Homoptera: Pseudococcidae) based on 16S rRNA sequences. *Mol. Phylogenet. Evol.* **1**:26–30.
31. Nixon, K. C. 1999. The parsimony ratchet, a new method for rapid parsimony analysis. *Cladistics* **15**:407–414.
32. Nogge, G. 1981. Significance of symbionts for the maintenance of an optimal nutritional state for successful reproduction in hematophagous arthropods. *Parasitology* **82**:299–304.
33. Pais, R., C. Lohs, Y. Wu, J. Wang, and S. Aksoy. 2008. The obligate mutualist *Wigglesworthia glossinidia* influences reproduction, digestion, and immunity processes of its host, the tsetse fly. *Appl. Environ. Microbiol.* **74**:5965–5974.
34. Ramsey, J. S., S. J. MacDonald, G. Jander, A. Nakabachi, G. H. Thomas, and A. E. Douglas. 2010. Genomic evidence for complementary purine metabolism in the pea aphid, *Acyrtosiphon pisum*, and its symbiotic bacterium *Buchnera aphidicola*. *Insect Mol. Biol.* **19**(Suppl. 2):241–248.
35. Sandstrom, J., A. Telang, and N. A. Moran. 2000. Nutritional enhancement of host plants by aphids—a comparison of three aphid species on grasses. *J. Insect Physiol.* **46**:33–40.
36. Schuettelpelz, E., and K. M. Pryer. 2006. Reconciling extreme branch length differences: decoupling time and rate through the evolutionary history of filmy ferns. *Syst. Biol.* **55**:485–502.
37. Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**:2688–2690.
38. Takiya, D. M., P. L. Tran, C. H. Dietrich, and N. A. Moran. 2006. Co-cladogenesis spanning three phyla: leafhoppers (Insecta: Hemiptera: Cicadellidae) and their dual bacterial symbionts. *Mol. Ecol.* **15**:4175–4191.
39. Thao, M. L., and P. Baumann. 2004. Evolutionary relationships of primary prokaryotic endosymbionts of whiteflies and their hosts. *Appl. Environ. Microbiol.* **70**:3401–3406.
40. Thao, M. L., M. A. Clark, D. H. Burckhardt, N. A. Moran, and P. Baumann. 2001. Phylogenetic analysis of vertically transmitted psyllid endosymbionts (*Candidatus Carsonella ruddii*) based on *atpAGD* and *rpoC*: comparisons with 16S-23S rDNA-derived phylogeny. *Curr. Microbiol.* **42**:419–421.
41. Thao, M. L., P. J. Gullan, and P. Baumann. 2002. Secondary (*Gammaproteobacteria*) endosymbionts infect the primary (*Betaproteobacteria*) endosymbiont of mealybugs multiple times and coevolve with their hosts. *Appl. Environ. Microbiol.* **68**:3190–3197.
42. Thao, M. L., N. A. Moran, P. Abbot, E. B. Brennan, D. H. Burckhardt, and P. Baumann. 2000. Cospeciation of psyllids and their prokaryotic endosymbionts. *Appl. Environ. Microbiol.* **66**:2898–2905.
43. Tremblay, E. 1989. Coccoidea endocytobiosis, p. 145–173. *In* W. Schwemmler and G. Gassner (ed.), *Insect endocytobiosis: morphology, physiology, genetics, evolution*. CRC Press Inc., Boca Raton, FL.
44. von Dohlen, C. D., S. Kohler, S. T. Alsop, and W. R. McManus. 2001. Mealybug β -proteobacterial endosymbionts contain γ -proteobacterial symbionts. *Nature* **412**:433–436.
45. Wernegreen, J. J. 2002. Genome evolution in bacterial endosymbionts of insects. *Nat. Rev. Genet.* **3**:850–861.
46. Wilson, A. C. C., P. D. Ashton, F. Caleviro, H. Charles, S. Colella, G. Febvay, G. Jander, P. F. Kushlan, S. J. Macdonald, J. F. Schwartz, G. H. Thomas, and A. E. Douglas. 2010. Genomic insight into the amino acid relations of the pea aphid, *Acyrtosiphon pisum*, with its symbiotic bacterium *Buchnera aphidicola*. *Insect Mol. Biol.* **19**(Suppl. 2):249–258.
47. Yang, Z. 2007. PAML 4: a program package for phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* **24**:1586–1591.