RESEARCH ARTICLE

Mealybugs with distinct endosymbiotic systems living on the same host plant

Ryuichi Koga[1], Naruo Nikoh[2], Yu Matsuura[1,3], Xian-Ying Meng[1] & Takema Fukatsu[1]

[1]National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan; [2]Department of Liberal Arts, The Open University of Japan, Chiba, Japan; and [3]Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan

Correspondence: Ryuichi Koga, National Institute of Advanced Industrial Science and Technology (AIST), Central 6, Higashi 1-1-1, Tsukuba 305-8566, Ibaraki, Japan. Tel.: +81 29 861 6087; fax: +81 29 861 6080; e-mail: r-koga@aist.go.jp

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Abstract

Mealybugs (Homoptera: Coccoidea: Pseudococcidae) possess a large bacteriome consisting of a number of bacteriocytes whose cytoplasm is populated by endosymbiotic bacteria. In many mealybugs of the subfamily Pseudococcinae, a peculiar endosymbiotic configuration has been identified: within the bacteriocytes, the primary betaproteobacterial endosymbiont *Tremblaya princeps* endocellularly harbor secondary gammaproteobacterial endosymbionts in a nested manner. Meanwhile, some mealybugs of the subfamily Phenacoccinae are associated only with a betaproteobacterial endosymbiont, designated as *Tremblaya phenacola*, which constitutes a distinct sister clade of *T. princeps*. However, cytological configuration of the endosymbiotic system in the phenacoccine mealybugs has not been established. Here, we investigated the endosymbiotic systems of the azalea mealybugs *Crisicoccus azaleae* (Pseudococcinae) and *Phenacoccus azaleae* (Phenacoccinae) living on the same host plants. *Crisicoccus azaleae* possessed a nested endosymbiotic system with *T. princeps* within the bacteriocyte cytoplasm and itself endocellularly harboring gammaproteobacterial cells, whereas *P. azaleae* exhibited a simple endosymbiotic system in which *T. phenacola* cells are localized within the bacteriocytes without additional gammaproteobacterial associates. Considering that these mealybugs live on the identical plant phloem sap, these different endosymbiotic consortia likely play similar biological roles for their host insects. The findings presented here should be helpful for future functional and comparative genomics toward elucidating evolutionary pathways of mealybugs and their endosymbionts.

Introduction

Homopteran insects, including aphids, scale insects, whiteflies, psyllids, planthoppers, cicadas, and many others, feed only on plant sap throughout their life. Plant sap is a nutritionally unbalanced food that is difficult to utilize for animals. Phloem sap is virtually devoid of lipids and proteins, and even carbohydrate is also lacking from xylem sap. Most lipids can be synthesized from the carbohydrates, but proteins cannot in the absence of nitrogenous precursors such as essential amino acids. Some amino acids are certainly present in plant sap, but they are mostly nonessential ones. Therefore, most homopteran insects depend on endosymbiotic microorganisms for the supply of essential amino acids and other nutrients, whereby they are able to live solely on the specialized food source (Baumann, 2005; Moran *et al.*, 2008).

Mealybugs (Homoptera: Coccoidea: Pseudococcidae) comprise one of the largest families of scale insects, some of which are notorious agricultural pests (Ben-Dov, 1994). In the abdomen of the mealybugs, there exists a large oval bacteriome consisting of a number of bacteriocytes, whose cytoplasm is densely populated by endosymbiotic bacteria (Buchner, 1965). Previous studies revealed that different lineages of mealybugs are associated with distinct lineages of bacterial endosymbionts. For example, many species of the subfamily Pseudococcinae harbor a betaproteobacterial endosymbiont *Tremblaya princeps* and an additional gammaproteobacterial endosymbiont (Fukatsu & Nikoh, 2000; von Dohlen *et al.*, 2001; Thao *et al.*, 2002; Kono *et al.*, 2008; McCutcheon & von Dohlen,

2011; Gatehouse *et al.*, 2012). The primary endosymbionts *T. princeps* are found in almost all pseudococcine species and exhibit host–symbiont co-speciation (Baumann & Baumann, 2005; Downie & Gullan, 2005), whereas the secondary gammaproteobacterial endosymbionts, including *Moranella endobia* whose genome was determined recently (McCutcheon & von Dohlen, 2011), are of polyphyletic evolutionary origins (Thao *et al.*, 2002). In the bacteriocytes of the pseudococcine mealybugs, strikingly, the secondary gammaproteobacterial endosymbionts are present within the cells of the primary *T. princeps* endosymbionts in a nested manner (von Dohlen *et al.*, 2001), which provides the only case of prokaryote–prokaryote endocellular symbiosis ever known. On the other hand, early histological works reported that mealybugs of the subfamily Phenacoccinae possess endosymbiotic systems distinct from those of pseudococcine mealybugs (Walczuch, 1932; Buchner, 1965). It was recently reported that some phenacoccine species representing the genera *Phenacoccus*, *Heliococcus*, *Heterococcus*, *Mirococcus*, *Oxyacanthus*, and *Peliococcus* harbor a betaproteobacterial endosymbiont *Tremblaya phenacola*. *Tremblaya phenacola* is a sister clade of *T. princeps* and constitutes a well-supported monophyletic clade together, suggesting a single evolutionary origin of the endosymbionts in the common ancestor of the pseudococcine and the phenacoccine mealybugs (Gruwell *et al.*, 2010); notably, however, additional gammaproteobacterial associates have not been detected from the phenacoccine mealybugs, and cytological configuration of the endosymbiotic system in the phenacoccine mealybugs, in particular *in vivo* localization and fine structure of *T. phenacola*, has not been established. It is of evolutionary interest to comparatively investigate the endosymbiont lineage *T. princeps* containing gammaproteobacterial endocellular associates and the sister endosymbiont lineage *T. phenacola* without such associates.

In Japan, we found that many plants of the giant purple azalea *Rhododendron pulchrum* cv. Oomurasaki are infested by two species of azalea mealybugs, *Crisicoccus azaleae* (Pseudococcinae) and *Phenacoccus azaleae* (Phenacoccinae). These mealybugs preferentially utilize the azalea as their plant (Ben-Dov, 1994) and frequently co-occur on the same leaves and stems of the plants and form mixed colonies. Although they are similar in size and shape with white waxy secretions, close inspection can easily distinguish purple *C. azaleae* from brownish *P. azaleae* (Fig. 1a and e). Here, we demonstrate that they possess distinct endosymbiotic systems on the same host plant: a nested endosymbiotic configuration in *C. azaleae* consisting of endocellular *T. princeps* that further harbors a gammaproteobacterium endocellularly vs. a simple endosymbiotic configuration in *P. azaleae* consisting of endocellular *T. phenacola* only. Considering that these mealybugs live on the identical food source of *R. pulchrum* phloem sap, it is expected that these different endosymbiotic consortia play similar biological roles for their host insects. The findings presented here should be helpful for future functional and comparative genomics toward elucidating evolutionary pathways of the mealybugs and their endosymbionts.

Materials and methods

Insect materials

Crisicoccus azaleae and *P. azaleae* were collected from the same bush of *R. pulchrum* cv. Oomurasaki in Tsukuba, Ibaraki, Japan, from April to May in 2008 and 2009. The collected insects were either directly subjected to molecular and histological analyses or preserved in acetone until analyzed later (Fukatsu, 1999).

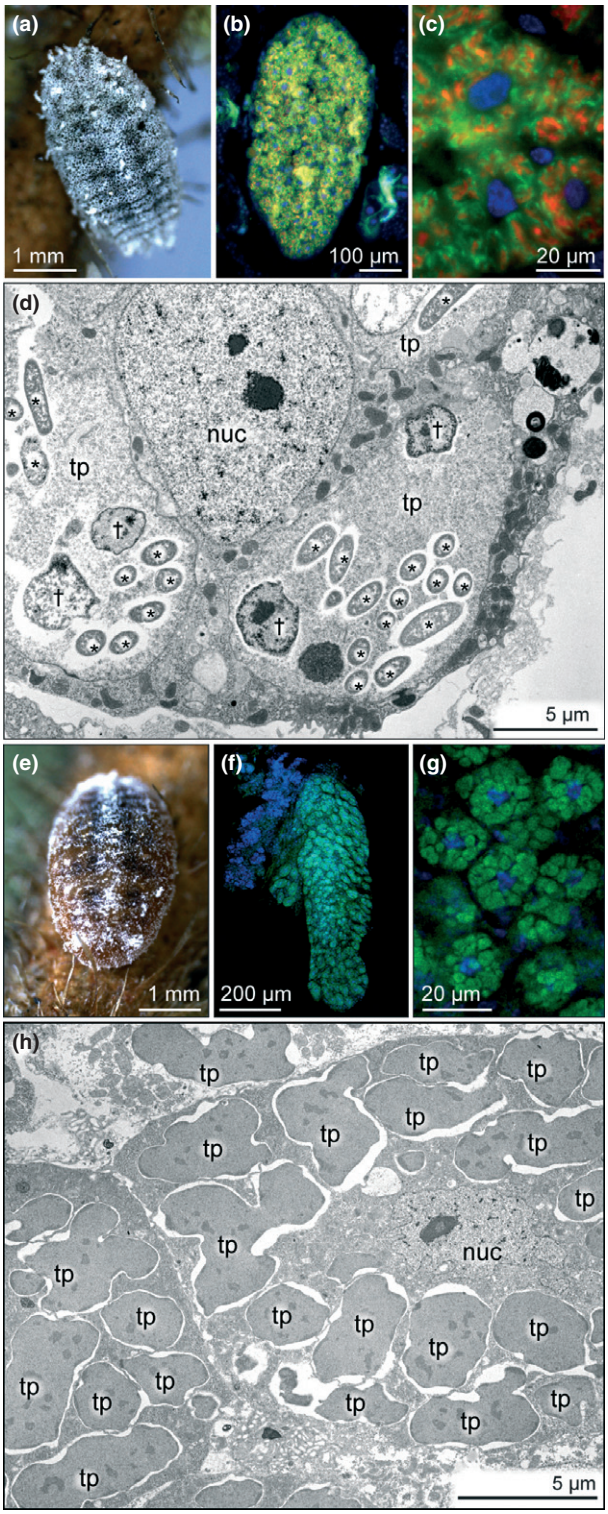
DNA extraction, PCR, cloning, and sequencing

Bacteriomes were dissected from five mealybug individuals for each species using a pair of fine forceps under a binocular microscope in a plastic Petri dish filled with a phosphate-buffered saline (PBS, 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4 [pH 7.5]) and were individually subjected to whole nucleic acid preparation by a conventional SDS–phenol extraction method. From the DNA samples, a 1.5-kb region of bacterial 16S rRNA gene was amplified by PCR as described (Fukatsu & Nikoh, 1998). About 0.3- and 0.4-kb regions of elongation factor 1α gene, a 0.6-kb region of 18S rRNA gene, and 0.8- and 0.3-kb regions of 28S rRNA gene of the host insects were also amplified by PCR as described (Hardy *et al.*, 2008). The PCR primers used in this study are listed in Supporting information, Table S1. The PCR products were cloned, and at least three clones for each PCR product were sequenced as described (Kaiwa *et al.*, 2010).

Molecular phylogenetic and evolutionary analyses

Multiple alignments of the nucleotide sequences were generated using the program MAFFT 5 (Katoh *et al.*, 2005). All aligned sequences of 18S rRNA gene, D2 and D10 regions of insect 28S rRNA gene, and 1st and 2nd codon positions of elongation factor 1α gene were used for the phylogenetic analyses of the host insect. The data were partitioned by gene for 18S rRNA and 28S rRNA genes and partitioned by codon for elongation factor 1α gene. We included sites with missing data to increase the nucleotide sites subjected to the phylogenetic analyses. The

GTR + I + G substitution model was selected using the program JMODELTEST (Posada, 2008). Phylogenetic analyses were conducted by maximum-likelihood and Bayesian methods using the programs RAXML version 7.0.0 (Stamatakis, 2006) and MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003), respectively. Relative rate tests were performed using the program RRTREE (Robinson-Rechavi & Huchon, 2000). Co-evolutionary analyses were performed using the program JANE 3.0 (Conow *et al.*, 2010). Default costs for evolutionary events were used. Significance values were calculated by comparing the actual cost and the expected distribution of costs, which was produced by 1000 randomly generated symbiont trees.



In situ hybridization

Adult insects preserved in acetone were transferred to 70% ethanol, and their legs were removed by forceps to facilitate the permeation of reagents into the insect tissues and cells. Then, the insects were fixed with Carnoy's solution (ethanol/chloroform/acetic acid = 6 : 3 : 1) for 3 days. In order to quench the autofluorescence of the insect tissues, the fixed materials were treated with 6% hydrogen peroxide in 80% ethanol for 2 weeks (Koga *et al.*, 2009) and then preserved in absolute ethanol until use. For specimens of *C. azaleae*, paraffin sections were prepared and subjected to fluorescence *in situ* hybridization with probes Al555-CazaTp1233R and Al647-CazaSS1235R (Table S2) as previously described (Koga *et al.*, 2003), while specimens of *P. azaleae* were directly subjected to whole-mount fluorescence *in situ* hybridization with a probe Al555-PazaBsym1238R (Table S2) as described (Koga *et al.*, 2009). The hybridized specimens were mounted in SlowFade antifade solution (Invitrogen) and observed under an epifluorescence microscope (Axiophoto; Carl Zeiss) and a laser confocal microscope (PASCAL5; Carl Zeiss).

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the DDBJ/EMBL/GenBank DNA

Fig. 1. (a–d) Endosymbiotic system of *Crisicoccus azaleae*. (a) An adult female. (b) Localization of the primary endosymbiont *Tremblaya princeps* (green) and the secondary gammaproteobacterial endosymbiont (red) in the bacteriome. Blue signals show host insect nuclei. (c) Localization of the endosymbionts in the bacteriocytes. (d) Transmission electron microscopy of a bacteriocyte, wherein *T. princeps* cells (tp) endocellularly contain rod-shaped gammaproteobacterial cells (*). Some gammaproteobacterial cells look degenerating (†). A bacteriocyte nucleus (nuc) is seen. (e–h) Endosymbiotic system of *Phenacoccus azaleae*. (e) An adult female. (f) Localization of the primary endosymbiont *Tremblaya phenacola* (green) in the bacteriome. Blue signals show host insect nuclei. (g) Localization of the endosymbiont in the bacteriocytes. (h) Transmission electron microscopy of a bacteriocyte, whose cytoplasm is full of *T. phenacola* cells (tp). A bacteriocyte nucleus (nuc) is seen.

database under accession numbers AB627016–AB627028 (Table S1).

Results and discussion

Crisicoccus azaleae is associated with a betaproteobacterial *T. princeps* and a gammaproteobacterial endosymbiont

Cloning of a bacterial 16S rRNA gene fragment from the bacteriome of different individuals of *C. azaleae* consistently yielded two major genotypes in terms of restriction fragment length polymorphism. DNA sequencing of the clones revealed that these two types represent distinct bacterial lineages. One sequence, 1473 bp in length, showed high sequence similarities to betaproteobacterial 16S rRNA genes. The best BLAST hit in the GenBank database was the primary endosymbiont *T. princeps* of a mealybug *Melanococcus albizziae* [98% (1446/1475) sequence identity; accession number AF476087]. The other sequence, 1479 bp in length, showed moderate sequence similarities to gammaproteobacterial 16S rRNA genes. The best BLAST hit in the GenBank database was the *Sodalis*-allied secondary endosymbiont of a weevil *Curculio sikkimensis* [92% (1348/1471) sequence identity; accession number AB517595].

Molecular phylogenetic analyses showed that the betaproteobacterial sequence from *C. azaleae* is placed within the clade of *T. princeps*, the primary endosymbionts of mealybugs of the subfamily Pseudococcinae, with high statistical supports (Fig. 2). Meanwhile, the gammaproteobacterial sequence from *C. azaleae* belonged to a clade of secondary endosymbionts of mealybugs such as *Melanococcus albizziae*, *Australicoccus greville*, *Amonostherium lichtensi*, *Antonina pretiosa*, and *Antonina crawii*, with high statistical supports (Fig. S1).

These results indicate that *C. azaleae* is associated with the primary endosymbiont *T. princeps* and an additional

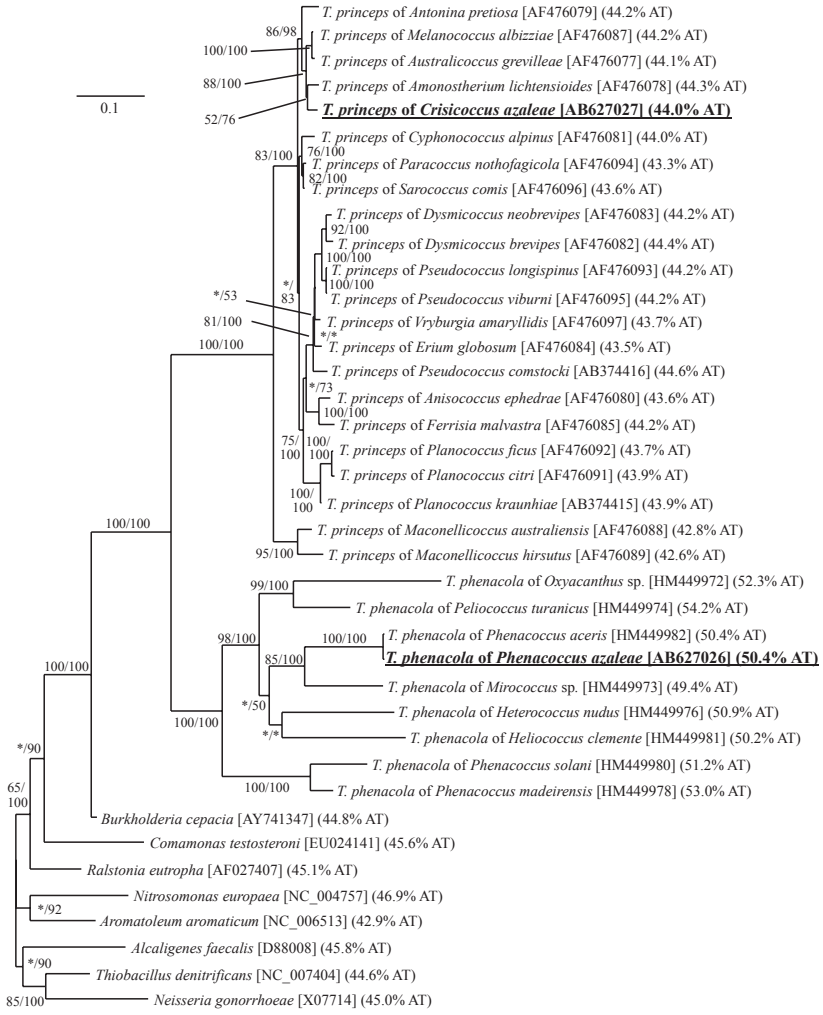


Fig. 2. Molecular phylogenetic analysis of betaproteobacterial endosymbionts, *Tremblaya princeps* from *Crisicoccus azaleae* and *Tremblaya phenacola* from *Phenacoccus azaleae*, on the basis of 16S rRNA gene sequences. A maximum-likelihood phylogeny inferred from 1256 unambiguously aligned nucleotide sites is shown, whereas a Bayesian analysis inferred essentially the same phylogeny (data not shown). Bootstrap values of maximum-likelihood analysis/Bayesian posterior probabilities are shown at the nodes. Asterisks indicate support values lower than 50%. Sequence accession numbers and adenine plus thymine contents are shown in brackets and parentheses, respectively.

gammaproteobacterial endosymbiont, as many mealybugs of the subfamily Pseudococcinae (Fukatsu & Nikoh, 2000; von Dohlen *et al.*, 2001; Thao *et al.*, 2002; Kono *et al.*, 2008; Gatehouse *et al.*, 2012).

Tremblaya princeps endocellularly contains the gammaproteobacterial endosymbiont in the bacteriome of *C. azaleae*

Fluorescent *in situ* hybridization revealed that both *T. princeps* and the gammaproteobacterial endosymbiont are localized within a large bacteriome in the abdomen of *C. azaleae* (Fig. 1b). Within the cytoplasm of the bacteriocytes constituting the bacteriome, rod-shaped gammaproteobacterial cells were co-localized with amorphous signals of *T. princeps* (Fig. 1c). Transmission electron microscopy unequivocally showed that the rod-shaped gammaproteobacterial cells are harbored within large pleomorphic cells of *T. princeps* in the bacteriocytes of *C. azaleae* (Fig. 1d). Some gammaproteobacterial cells looked degenerating within *Tremblaya* cells (daggers in Fig. 1d), which might favor the hypothesis of direct metabolite supply from the endocellular gammaproteobacterium to *T. princeps* via cell lysis (McCutcheon & von Dohlen, 2011).

These results indicate that *C. azaleae* possesses a nested endosymbiotic system wherein the gammaproteobacterial endosymbiont cells are harbored in *T. princeps* cells within the bacteriocytes, as has been reported from *Planococcus citri* and other mealybugs of the subfamily Pseudococcinae (von Dohlen *et al.*, 2001; Kono *et al.*, 2008; McCutcheon & von Dohlen, 2011; Gatehouse *et al.*, 2012).

Phenacoccus azaleae is associated with a betaproteobacterial *T. phenacola* only

Cloning of a bacterial 16S rRNA gene fragment from the bacteriome of different individuals of *P. azaleae* consistently yielded a single major genotype in terms of restriction fragment length polymorphism. Of 55 clones examined, 54 clones exhibited the same genotype, representing a 1477-bp sequence with high sequence similarities to betaproteobacterial 16S rRNA gene sequences. The best BLAST hit in the GenBank database was the primary endosymbiont *T. phenacola* of a mealybug *Phenacoccus aceris* [99% (1357/1363) sequence identity; accession number HM449982]. The single exceptional clone exhibited a high sequence similarity to 16S rRNA gene sequence of a chloroplast, suggesting its contaminant origin.

Molecular phylogenetic analyses showed that the betaproteobacterial sequence from *P. azaleae* is placed within the clade of *T. phenacola*, the primary endosymbionts of mealybugs of the subfamily Phenacoccinae, with high statistical supports (Fig. 2). This result indicates that *P. azaleae* is associated with the primary endosymbiont *T. phenacola* as reported for other mealybugs of the subfamily Phenacoccinae (Gruwell *et al.*, 2010).

Tremblaya phenacola contains no endocellular bacteria in the bacteriome of *P. azaleae*

Fluorescent *in situ* hybridization revealed that *T. phenacola* is localized within a large bacteriome in the abdomen of *P. azaleae* (Fig. 1f). Oval cells of *T. phenacola* were observed within the cytoplasm of the bacteriocytes constituting the bacteriome (Fig. 1g). Fluorescent *in situ* hybridization using a universal probe (Amann *et al.*, 1990) detected no bacterial signals in other tissues and cells of *P. azaleae* (data not shown). Transmission electron microscopy showed that the cytoplasm of the bacteriocytes was filled with pleomorphic cells of *T. phenacola*, in which no endocellular bacteria were seen (Fig. 1h).

These results indicate that, in *P. azaleae*, the primary endosymbiont *T. phenacola* is not associated with any gammaproteobacterial secondary endosymbionts therein. To our knowledge, this study is the first to describe *in vivo* localization and fine structure of *T. phenacola*.

Molecular evolutionary aspects in *T. princeps* and *T. phenacola*

Previous studies showed that the phylogeny of the primary endosymbionts *T. princeps* is congruent with the phylogeny of their host mealybugs, indicating host–symbiont co-speciation in the evolutionary course of the subfamily Pseudococcinae (Baumann & Baumann, 2005; Downie & Gullan, 2005). Meanwhile, although molecular phylogeny of *T. phenacola* endosymbionts was reported (Gruwell *et al.*, 2010), such a co-evolutionary analysis has not been conducted for *T. phenacola* endosymbionts and their mealybug hosts of the subfamily Phenacoccinae. Figure 3 shows a molecular phylogenetic comparison between phenacoccine representatives and their *T. phenacola* endosymbionts. The symbiont phylogeny was largely concordant with the host phylogeny, except for the placement of *Heliococcus clemente* and its symbiont. Considering that *H. clemente* constitutes a basal lineage in the host phylogeny with poor statistical support, it is unclear whether the apparent phylogenetic incongruence is substantial or not. To resolve the phylogenetic ambiguity, more sequence data for both the hosts and the symbionts are needed. Co-divergence analyses by the Jane algorism (Conow *et al.*, 2010) identified seven co-divergence events of eight nodes in the phylogenies. The level of phylogenetic congruence was statistically significant ($P < 0.001$).

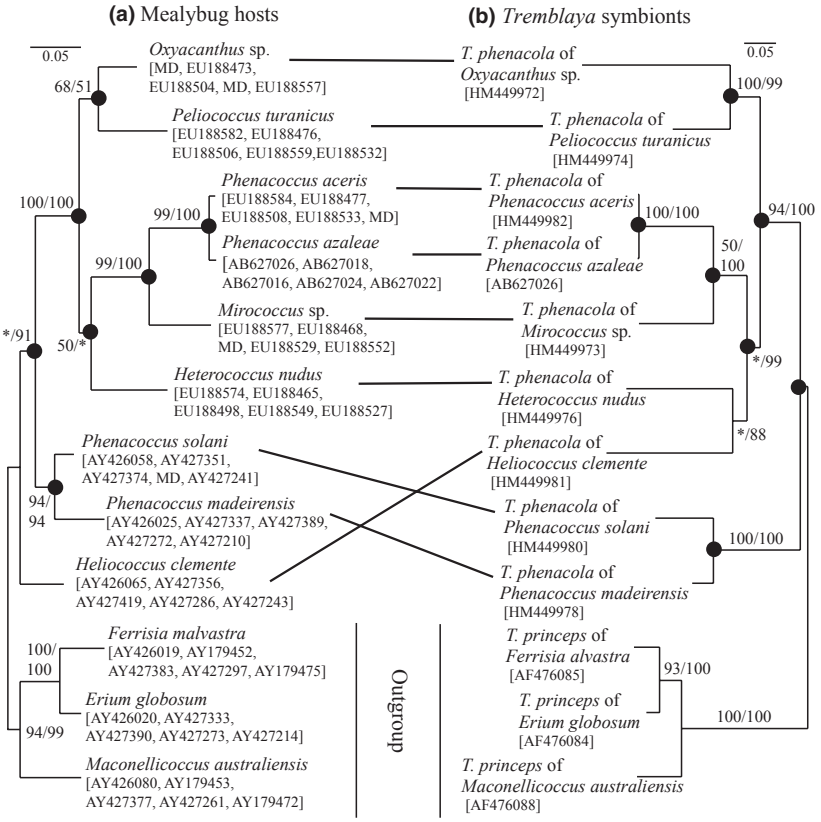


Fig. 3. Phylogenetic comparison between mealybugs of the subfamily Phenacoccinae and their endosymbionts *Tremblaya phenacola*. (a) A maximum-likelihood phylogeny of the host mealybugs inferred from 1642 unambiguously aligned nucleotide sites of concatenated sequences of 18S rRNA gene, 28S rRNA gene, and elongation factor 1α gene. (b) A maximum-likelihood phylogeny of their *T. phenacola* endosymbionts inferred from 1263 unambiguously aligned nucleotide sites of 16S rRNA gene sequences. Sequence accession numbers are shown in brackets. Dots on the nodes indicate co-divergence events inferred by the Jane algorithm (Conow *et al.*, 2010), while thick lines between the phylogenies indicate host–symbiont correspondence. Support values at the nodes are as shown in Fig. 2.

The 16S rRNA gene sequences of *T. princeps* of the pseudococcine mealybugs were GC-rich (39–46% AT) in comparison with those of free-living betaproteobacteria (43–47% AT; Fig. 2; Baumann *et al.*, 2002; López-Madrigal *et al.*, 2011; McCutcheon & von Dohlen, 2011). By contrast, the 16S rRNA gene sequences of *T. phenacola* of the phenacoccine mealybugs were AT-rich (49–54% AT) in comparison with those of free-living betaproteobacteria (43–47% AT; Fig. 2; Gruwell *et al.*, 2010). Relative rate tests revealed that molecular evolutionary rates in the lineage of *T. princeps* are significantly higher (3.4 times) than those in free-living betaproteobacteria, so are molecular evolutionary rates in the lineage of *T. phenacola* (4.6 times; Table 1). Similar acceleration of molecular evolutionary rates in *T. phenacola* was also estimated previously (Gruwell *et al.*, 2010). In addition, it was shown that molecular evolutionary rates in the lineage of *T. phenacola* are significantly higher (1.5 times) than those in the lineage of *T. princeps* (Table 1).

Host–symbiont co-speciation and accelerated molecular evolution in *T. princeps* and *T. phenacola*

These results suggest that the sister clades of the mealybug primary endosymbionts, *T. princeps* and *T. phenacola*, have both experienced stable vertical transmission and host–symbiont co-speciation in the evolutionary course of the respective host insect subfamilies. The accelerated evolutionary rates and skewed nucleotide compositions suggest that not only the lineage of *T. princeps* (López-Madrigal *et al.*, 2011; McCutcheon & von Dohlen, 2011) but also the lineage of *T. phenacola* may have experienced reductive genome evolution. These evolutionary patterns are typical of diverse vertically transmitted insect symbionts of obligate nature, which are attributed to their stable and nutrition-rich endocellular habitat and also to attenuated purifying selection because of the small population size and strong bottleneck associated with their symbiotic lifestyle (Wernegreen, 2002; McCutcheon & Moran, 2012). Meanwhile, it is notable that *T. princeps* and *T. phenacola* exhibit some differences in their nucleotide composition and molecular evolutionary rate, which may reflect biological and evolutionary differences between them represented by, for example, the presence/absence of the endocellular gammaproteobacterial co-symbionts.

Implication for biological function, genomics, and evolution of the endosymbionts of the mealybugs

In conclusion, we demonstrate that the phenacoccine azalea mealybug *P. azaleae* possesses a simple endosymbiotic

Table 1. Relative rate test for comparing the molecular evolutionary rates of 16S rRNA gene sequences between the lineages of *Tremblaya phenacola*, *Tremblaya princeps*, and their free-living betaproteobacterial relatives

Lineage 1	Lineage 2	Outgroup	K1*	K2†	K1 − K2	K1/K2	*P*-value‡
Tremblaya phenacola§	*Burkholderia cepacia* [AY741347]	*Comamonas testosteroni* [EU024141]	0.152	0.033	0.119	4.6	1.0×10^{-7}
Tremblaya princeps¶	*Burkholderia cepacia* [AY741347]	*Comamonas testosteroni* [EU024141]	0.111	0.033	0.078	3.4	1.0×10^{-7}
Tremblaya phenacola§	*Tremblaya princeps*¶	*Burkholderia cepacia* [AY741347]	0.117	0.076	0.041	1.5	1.9×10^{-4}

*Estimated mean distance between lineage 1 and the last common ancestor of lineages 1 and 2.

†Estimated mean distance between lineage 2 and the last common ancestor of lineages 1 and 2.

‡*P*-value was estimated by the program RRTREE (24) on the basis of 1231 unambiguously aligned nucleotide sites.

§16S rRNA gene sequences of *T. phenacola* from *Phenacoccus azaleae* (AB627026), *Phenacoccus aceris* (HM449982), *Heterococcus nudus* (HM449976), *Peliococcus turanicus* (HM449974), *Oxyacanthus* sp. (HM449972), and *Phenacoccus solani* (HM449980).

¶16S rRNA gene sequences of *T. princeps* from *Antonina pretiosa* (AF476079), *Australicoccus grevilleae* (AF476077), *Amonostherium lichtensioides* (AF476078), *Crisicoccus azaleae* (AB627027), *Cyphonococcus alpinus* (AF476081), *Paracoccus nothofagicola* (AF476094), *Sarococcus comis* (AF476096), *Dysmicoccus neobrevipes* (AF476083), *Dysmicoccus brevipes* (AF476082), *Pseudococcus viburni* (AF476095), *Pseudococcus longispinus* (AF476093), *Erium globosum* (AF476084), *Vryburgia amaryllidis* (AF476097), *Anisococcus ephedrae* (AF476080), *Ferrisia malvastra* (AF476085), *Planococcus ficus* (AF476092), *Planococcus citri* (AF476091), *Maconellicoccus australiensis* (AF476088), *Maconellicoccus hirsutus* (AF476089), *Melanococcus albizziae* (AF476087), *Pseudococcus comstocki* (AB374416), *Planococcus kraunhiae* (AB374415).

system consisting of the betaproteobacterial *T. phenacola* only, which is distinct from the nested endosymbiotic system of the pseudococcine azalea mealybug *C. azaleae* consisting of the betaproteobacterial *T. princeps* that further contains the endocellular gammaproteobacterium. The fact that *P. azaleae* and *C. azaleae* live on the same food source, phloem sap from leaves and stems of the great purple azalea *R. pulchrum*, provides insights into functional, genomic, and evolutionary aspects in the mealybug endosymbioses.

Recently, McCutcheon & von Dohlen (2011) determined the complete genome sequences of *T. princeps* and an associated gammaproteobacterial symbiont *M. endobia* from the citrus mealybug *Planococcus citri* (Pseudococcinae). Strikingly, *T. princeps* has the smallest bacterial genome of 0.14 Mb, exhibits a strikingly low coding capacity of 72.9% despite the tiny genome size, and lacks many of so-called essential genes that are highly conserved among almost all of the other bacterial genomes. Particularly notable is that the *T. princeps* genome retains 29 genes for essential amino acid biosynthesis, but does not encode complete pathways for any of the essential amino acids. Meanwhile, the genome of *M. endobia* is 0.54 Mb in size, which is four times larger than the *T. princeps* genome. The *M. endobia* genome contains 15 genes for essential amino acid biosynthesis, but also does not encode complete pathways for any of the essential amino acids. These genomic patterns suggest that interspersed gene products from *T. princeps*, *M. endobia*, and possibly the host insect are somehow recruited for the biosynthesis of major essential amino acids, constituting an intergenomic metabolic patchwork, in the mealybug.

Hence, we expect that, to compensate for the absence of gammaproteobacterial endocellular co-symbiont, *T. phenacola* should exhibit larger genome size and coding capacity for essential amino acid biosynthesis and other metabolic pathways than *T. princeps* (McCutcheon & von Dohlen, 2011). Whether the common ancestor of *T. princeps* and *T. phenacola* had already been associated with a gammaproteobacterial co-symbiont or not might have affected their genomic architecture. Genome sequencing of a *T. phenacola* strain from a phenacoccine mealybug would provide invaluable clues to understanding of these evolutionary issues.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Molecular phylogenetic analysis of gammaproteobacterial endosymbiont from *Crisicoccus azaleae* on the basis of 16S rRNA gene sequences.

Table S1. Target genes, PCR primers and sequence accession numbers.

Table S2. Oligonucleotide probes for in situ hybridization.

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