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Evolutionary relationships of flavobacterial and enterobacterial endosymbionts with their scale insect hosts (Hemiptera: Coccoidea)

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Abstract

Flavobacteria and Enterobacteriaceae have been previously reported as scale insect endosymbionts. The purpose of this work was twofold: first, to screen different scale insect families for the presence of these endosymbionts by PCR analyses and second, to elucidate the history of cophylogeny between these bacteria and the insects by analysing a portion of 16S rRNA and 18S rRNA gene sequences by two reconciliation tools, CoRe-PA and Jane. From a survey of 27 scale insects within seven families, we identified Flavobacteria and Enterobacteriaceae as coexisting in ten species that belong to the Ortheziidae, Monophlebidae, Diaspididae and Coccidae families, and we frequently found two closely related enterobacteria harboured in the same individual. Analyses performed with CoRe-PA and Jane suggest that Flavobacteria from the scale insects analysed have a unique origin, except for *Candidatus* Brownia rhizoecola (Flavobacteria of Pseudococcidae, Phenacoccinae), which seems to come from a nonscale insect. Nevertheless, cospeciation between Flavobacteria and scale insects is suggested only within the families Monophlebidae, Ortheziidae and Diaspididae, and host switches seem to have occurred from the ancestors of Monophlebidae and Ortheziidae to insects from families Coccidae, Lecanodiaspididae, Eriococcidae and Pseudococcidae. Our analyses suggest that Enterobacteriaceae underwent more evolutionary events (losses, duplications and host switches), and their phylogenies showed a lower proportion of congruent nodes between host and bacteria, indicating a more relaxed relationship with scale insects compared with Flavobacteria.

Introduction

Insect bacterial endosymbionts live inside insect cells and are vertically transmitted (Moran *et al.*, 2008). Some endosymbionts provide their hosts with essential nutrients to complement the insect diet, especially insects that feed on plant sap or blood. Primary or obligate endosymbionts are considered essential to their hosts and are normally located inside specialized host cells called bacteriocytes. In addition, insects may have other endosymbionts that are not generally required for growth or reproduction; these are considered secondary or facultative endosymbionts and can live inside bacteriocytes or in body cavities (Baumann, 2005; Moran *et al.*, 2008). Secondary endosymbionts can also have beneficial roles like protection against fungi and parasitoid wasps, ameliorate the detrimental effects of heat or broaden the range of food plants (Oliver *et al.*, 2010).

Scale insects feed on plant sap (phloem or xylem; Gullan & Kosztarab, 1997). They belong to the superfamily Coccoidea of the suborder Sternorrhyncha within the Hemiptera. This suborder includes aphids, whiteflies and psyllids. The endosymbionts of scale insects have been studied in some families.

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Bacteroidetes from the class Flavobacteria have been reported from Monophlebidae (Matsuura *et al.*, 2009; Dhami *et al.*, 2012), Pseudococcidae (Gruwell *et al.*, 2010), Diaspididae and Eriococcidae (Zchori-Fein *et al.*, 2005; Gruwell *et al.*, 2007). Enterobacteriaceae have been documented in Pseudococcidae, Monophlebidae (Matsuura *et al.*, 2009; Dhami *et al.*, 2012), Putoidae and Coccidae (Gruwell *et al.*, 2010). As shown with fluorescent *in situ* hybridization, endosymbionts of Pseudococcidae (Kono *et al.*, 2008) and Monophlebidae (Matsuura *et al.*, 2009; Dhami *et al.*, 2012) are present inside bacteriocytes, which in turn are localized in bacteriomes. The presence of these endosymbionts have not been analysed in other families from scale insects.

Vertically transmitted endosymbionts suffer from population bottlenecks and altered selective pressures, because of the lack of recombination with other bacteria (Moran, 1996). Joint speciation with the host lineage (cospeciation) is commonly observed (Baumann *et al.*, 2000). This results in congruent phylogenies of host and bacteria. Occasionally, endosymbionts can be transferred laterally from one insect to another species (host shifts), decreasing the congruence of host and symbiont phylogenies. These events have been found mainly for secondary endosymbionts, often between closely related host species (Russell *et al.*, 2003; Degnan & Moran, 2008) but sometimes even in distantly related ones (Nováková *et al.*, 2009). The congruency of the phylogenies can also be reduced by other factors, such as speciation of a symbiont, where both new species are kept on the same host (duplication), or loss of a symbiont (losses). In most cases, cophylogeny has been analysed only by a visual assessment comparing the congruency between host and endosymbiont tree topologies (Chen *et al.*, 1999; Baumann & Baumann, 2005; Gruwell *et al.*, 2007). More recently, computational algorithms, called reconciliation tools, have been used in different systems (e. g. Ricklefs *et al.*, 2004; Downie & Gullan, 2005; Coulibaly-N'Golo *et al.*, 2011). In particular, three programs have been commonly applied: TreeMap 3b (Charleston, 2011), CoRe-PA (Merkle *et al.*, 2010) and Jane (Conow *et al.*, 2010; reviewed by Keller-Schmidt *et al.*, 2011).

The aims of this work are the following: first, to identify the bacterial endosymbionts belonging to Flavobacteria and to Enterobacteriaceae in different families of scale insects and second, to provide a possible scenario of the evolutionary history of each bacterium based on the host phylogeny.

Materials and methods

Insect sampling

We collected female insects in Mexico City and the states of Morelos and Chiapas. Some specimens were preserved in 70% ethanol, and others were freshly used for slide-mounting to allow subsequent identification. Vouchers were deposited in the Centro de Ciencias Genómicas of the Universidad Nacional Autónoma de México (CCG – UNAM). Most of the insects were identified by Douglass R. Miller, and these vouchers were also deposited in US National Museum of Natural History, Beltsville, MD (Tables 1 and S1).

The suprageneric classification within the family Margarodidae has undergone several modifications (Ben-Dov, 2005; Foldi, 2005). In the present work, we are adopting the classification from Koteja (1974) that has also been used in recent publications (Szklarzewicz *et al.*, 2005; Hodgson & Foldi, 2006; Gullan & Cook, 2007; Miller *et al.*, 2007; Unruh & Gullan, 2008) in which Monophlebidae (in this work the tribes Llaveiini and Iceryini) is recognized as a family. *Parasaissetia nigra* 8 in this study (Table 1) is the same insect as the unnamed *Parasaissetia* sp. in Ramírez-Puebla *et al.* (2010).

DNA extraction, PCR and cloning

Freshly collected female specimens from each insect species were sonicated (Bransonic 1510) for 10 min with sterile PBS (120 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, [pH 7.4]) to remove surface attached bacteria, washed with ethanol and rinsed several times with sterile PBS. Total DNA from whole insects was extracted from one to five specimens of each collected sample using Dneasy Blood & Tissue Kit (Quiagen, Hilden, Germany). When available, eggs or first instar nymphs that had just emerged were used to extract DNA. Insects that came from a single host plant were considered as belonging to the same sample.

Purified DNA was used as template in PCRs. We designed primers to amplify a 609-bp fragment of the 16S rRNA gene from Bacteroidetes, Bact362F (5′-GCAGCAGTGAGGAATATTGGT-3′) and Bact963R (5′-CGCGTATYATCGAATTAAACC-3′); a 550-bp fragment of the 16S rRNA gene from Enterobacteriaceae, SOPE437F (5′-AGCGCYATCGATTGACGTTAC-3′) and SOPE988R (5′-GAGCTCCCGAAGGCACCAAGG-3′); and a 630-bp region of *groEL* (which encodes the 60-kDa heat shock protein GroEL) from γ-Proteobacteria, FGa683 (5′-CCAAYATCCGYGAAATGCTGCC -3′) and RGa1336 (5′-GACGCAGHGGNGMTTCCATYGC -3′).

PCR conditions were as follows: an initial denaturation at 94 °C for 3 min, followed by 30 cycles of amplification (1 min at 94 °C, 1 min at 53 °C, 57 °C and 57 °C [for 16S rRNA of Flavobacteria, 16S rRNA and *groEL* genes of Enterobacteriaceae, respectively], and 1 min at 72 °C), and a final extension of 5 min at 72 °C. When no PCR product was obtained, annealing temperatures were gradually lowered up to 8 °C in 1 °C intervals.

For insects, a 620-bp fragment of the 18S rRNA gene was obtained with primers SSU2880F (5′-CTGGTTGAT

Table 1 Scale insect samples collected for this study.

Insect number identification	Endosymbionts*		Family	Subfamily	Tribe	Insect Species	Location inside Mexico
	F	E					
3	+	+	Monophlebidae		Llaveiini	*Llaveia axin axin* (Llave)	Tuxtla Gutiérrez, Chiapas
4	+	++	Monophlebidae		Iceryini	*Icerya purchasi* (Cockerell)	Cuernavaca, Morelos
5	+	++	Monophlebidae		Iceryini	*Crypticerya* sp. (Cockerell)	Tuxtla Gutiérrez, Chiapas
23	+	++	Ortheziidae			*Insignorthezia insignis* (Browne)	Cuernavaca, Morelos
31	+	+	Ortheziidae			*Insignorthezia* sp.	Cuautla, Morelos
10		++	Pseudococcidae	Pseudococcinae		*Pseudococcus longispinus* (Targioni-Tozzetti)	Cuernavaca, Morelos
28		+	Pseudococcidae	Pseudococcinae		*Ferrisia virgata* (Cockerell)	Cuernavaca, Morelos
18		++	Coccidae	Filippiinae		*Philephedra lutea* (Cockerell)	Cuautla, Morelos
33		+	Coccidae	Filippiinae		*Philephedra* sp.	Cuernavaca, Morelos
35		++	Coccidae			Not identified Coccidae	Cuautla, Morelos
8	+	+	Coccidae	Coccinae	Saissetiini	*Parasaissetia nigra* (Nietner)	Cuernavaca, Morelos
9		+	Coccidae	Coccinae	Saissetiini	*Parasaissetia nigra* (Nietner)	Cuernavaca, Morelos
36		+	Coccidae	Coccinae	Saissetiini	*Saissetia* sp.	Cuernavaca, Morelos
13		+	Coccidae	Ceroplastinae		*Ceroplastes sinensis* (Del Guercio)	Cuautla, Morelos
14	+	+	Coccidae	Ceroplastinae		*Ceroplastes* sp.	Cuautla, Morelos
16		+	Coccidae	Ceroplastinae		*Ceroplastes* sp.	Cuautla, Morelos
27		+	Coccidae	Ceroplastinae		*Ceroplastes* sp.	Cuernavaca, Morelos
30		++	Coccidae	Ceroplastinae		*Ceroplastes* sp.	Cuernavaca, Morelos
19		+	Coccidae	Coccinae	Coccini	*Coccus* sp.	Cuautla, Morelos
20		+	Coccidae	Coccinae	Coccini	*Coccus* sp.	Cuautla, Morelos
21		+	Coccidae	Coccinae	Coccini	*Coccus viridis* (Green)	Cuautla, Morelos
24	+	+	Coccidae	Coccinae	Coccini	*Coccus viridis* (Green)	Cuernavaca, Morelos
26			Eriococcidae			*Eriococcus euphorbiae* (Ferris)	Cuernavaca, Morelos
6	+		Lecanodiaspididae			*Lecanodiaspis prosopidis* (Maskell)	Mexico City, D.F.
34	+	+	Diaspididae	Diaspidinae	Diaspidini	*Aulacaspis* sp.	Cuernavaca, Morelos
38	+	+	Diaspididae	Aspidiotinae	Parlatoriini	*Parlatoria* sp.	Cuernavaca, Morelos
40	+		Diaspididae	Aspidiotinae	Aspidiotini	*Aonidiella* sp.	Cuernavaca, Morelos

*Symbionts detected by PCR, F, Flavobacteria; E, Enterobacteriaceae; +, one sequence obtained; ++, two sequences obtained with < 97% identity, either with 16S rRNA or with *groEL* directed primers.

CCTGCCAGTA-3′; Tautz *et al.*, 1988) and B- (5′-CCG CGGCTGCTGGCACCAG A-3′; von Dohlen & Moran, 1995), using PCR conditions described by Cook *et al.* (2002).

PCR products were cloned, and four individual plasmid clones were sequenced for each gene at Macrogen (Seoul, Korea). Sequences from different clones were submitted to GenBank. Accession numbers are indicated (Figs 1a,b, S1 and S2).

Phylogenetic analyses

Sequences from closely related organisms were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov). In the case of Pseudococcidae and Diaspididae, we included sequences from a representative of each major cluster from Flavobacteria (Gruwell *et al.*, 2007) and Enterobacteriaceae endosymbionts (Thao *et al.*, 2002), as well as from their host genes when available.

18S rRNA gene sequences from insects and 16S rRNA from bacteria were aligned independently using Muscle V3.6 (Edgar, 2004) and manually edited with JalView (Waterhouse *et al.*, 2009). The GTR+G model was chosen by FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html; Posada & Crandall, 2001) using Akaike Information Criterion (Akaike, 1974) for all ribosomal genes data sets. Numbers after the bacterial scientific names shown in all the figures indicate the insect number identification (Table 1). *groEL* gene sequences were translated with Transeq (part of the EMBOSS package; Rice *et al.*, 2000), aligned with ClustalW (Thompson *et al.*, 1994) and analysed with Prottest 2.4 (Abascal *et al.*, 2005) to obtain the best substitution model, which was LG + G + F.

Maximum likelihood (ML) analyses were performed using PhyML 3.0 (Guindon *et al.*, 2010) with 1000 bootstrap replicates and using the suggested models. Phylogenetic trees of 18S rRNA, 16S rRNA from Flavobacteria and 16S rRNA from Enterobacteriaceae

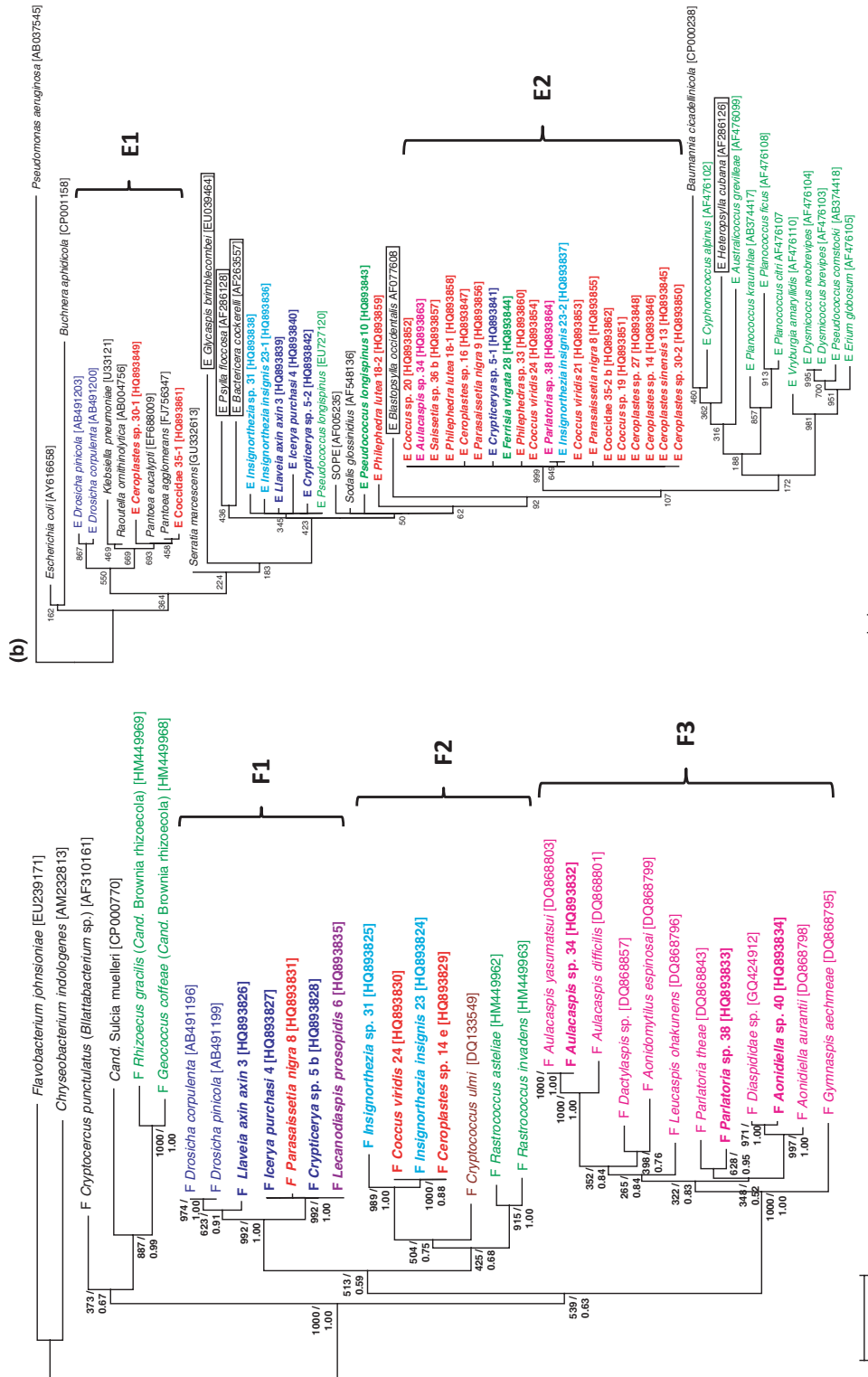

Fig. 1 Phylogenies from scale insect endosymbionts inferred with 16S rRNA gene sequences (Maximum likelihood). Sequences from this work are in bold. Numbers after scientific names are for sample identification. Different colours were used for each family: dark blue, Monophlebidae; light blue, Ortheziidae; green, Pseudococcidae; red, Coccidae; purple, Lecanodiaspididae; pink, Diaspididae. Accession numbers are shown in brackets. **e** indicates that the sequence was obtained from insect eggs, and **b** from first instar nymphs that had just emerged. Clade numbers are indicated. (a) Flavobacteria phylogeny constructed with 615-bp sequences. **F** indicates the flavobacterial endosymbiont of the respective insect species. Sequences from free-living bacteria (*Chryseobacterium indologenes* and *Flavobacterium johnsoniae*) and other closely related endosymbionts (*Blattabacterium* sp. from the wood roach *Cryptocercus punctulatus* and *Cand.* Sulcia muelleri GWSS) were used as outgroups. Bootstrap values for 1000 replicates and posterior probabilities from MrBayes analysis are shown adjacent to each node. (b) Enterobacteriaceae phylogeny constructed with 537-bp sequences. **E** indicates the enterobacterial endosymbiont of the respective insect species. Names inside a rectangle indicate endosymbionts from psyllids. SOPE indicates *Sitophilus Oryzae* Primary Endosymbiont. Sequences from the free-living bacteria *Pseudomonas aeruginosa* and *Buchnera aphidicola* were used as outgroups. Bootstrap values for 1000 replicates are shown adjacent to each node.

genes were also obtained by Bayesian inference using MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) with evolutionary model GTR+G with 2 000 000, 3 000 000 and 9 000 000 generations, respectively, and burning set to 7500. From the four sequenced clones of each DNA sample, we used one representative in the phylogenetic analysis, unless they had < 97% identity.

Cophylogenetic analyses

To study the evolutionary associations between scale insects and their endosymbionts, we employed the tree topologies obtained from 18S rRNA gene sequences from the insect hosts and 16S rRNA from the two endosymbionts. We used ribosomal genes to reconstruct species phylogenies to include the already existing sequences (taxa not in bold from Fig. 1) as it has been previously done (Thao *et al.*, 2000a,b; Downie & Gullan, 2005; Gruwell *et al.*, 2010). We used two reconciliation tools: CoRe-PA version 0.3a (Merkle *et al.*, 2010) and Jane (version 3, http://www.cs.hmc.edu/~hadas/jane/; Conow *et al.*, 2010). Of the recent bioinformatic tools that have been developed to reconciliate phylogenetic trees, it is worth mentioning that TreeMap can find optimal solutions and calculate costs like CoRe-PA. Nevertheless, Keller-Schmidt *et al.* (2011) demonstrated that the latter is more precise in predicting the cophylogeny. One of the problems with CoRe-PA is that some of the solutions could be chronologically invalid. For that reason, we decided to complement the analysis with Jane, to find consistent solutions from the previously retrieved reconstructions.

CoRe-PA and Jane assign costs to four events that are commonly used to describe host–parasite cophylogeny, namely cospeciation, duplication, host switch and sorting (or loss). In addition, Jane 3 also assigns costs to failure to diverge events (when a host speciates and the symbiont remains on both new host species). To find the fittest association between the host and the parasite (in this case the endosymbiont) tree, the programs implement algorithms that search the reconstruction where the total costs of all events that occur is minimal. Each one uses different cost models. CoRe-PA calculates the optimal cost values, whereas Jane requires the costs to be inputted.

For CoRe-PA and Jane analyses, the default options were used. Several minimum cost cophylogenies were retrieved. Our criteria for selecting a reconstruction result took into account the branch length shown in the host phylogeny (Fig. S1 and also shown in black in Figs 2 and 3), so that reconstructions were only accepted when they had host switches between existing hosts, and not to nonexisting hosts. This means that when a reconstruction was drawn on top of the host phylogeny (Figs 2 and 3), those with any arrow (host switches) pointing backwards were discarded.

For CoRe-PA analysis of Flavobacteria and their hosts, the costs obtained were cospeciation, 0.088; sorting, 0.248; duplications, 0.325; and host switch, 0.339. For Enterobacteriaceae and their hosts, the costs obtained were cospeciation, 0.192; sorting, 0.135; duplication, 0.198; and host switch, 0.475. For Jane analyses, we first used the same proportion of the costs obtained with CoRe-PA (but with integer numbers), and then we gradually moved the values until we fulfilled the criteria mentioned above (no host switches to a nonexisting host). For Flavobacteria and their hosts, we used cospeciation, 1; sorting, 2; duplication, 1; host switch, 1; and failure to diverge, 1. For Enterobacteriaceae and their hosts, we used cospeciation, 1; sorting, 2; duplication, 1; host switch, 5; and failure to diverge, 1.

To assess whether the reconstructions produce results different from those expected by chance, CoRe-PA and Jane make random trees of the symbiont. For both methods, 1000 random cycles were performed, using the same costs for the optimal reconstructions. Beta-splitting model minus one was used, as has been previously suggested (Aldous, 2001).

It should be considered that 16S rRNA sequences of Flavobacteria (F) *Icerya purchasi* 4, Enterobacteriaceae (E) *Icerya purchasi* 4, E *Ferrisia virgata* 28 and E *Dysmicoccus neobrevipes* (AF476104) were not obtained from the same DNA samples as sequences of 18S rRNA, although they were recovered from insects of the same species.

Results

Insect identification

We obtained 27 samples from scale insects belonging to the families Monophlebidae, Ortheziidae, Pseudococcidae, Coccidae, Diaspididae, Lecanodiaspididae and Eriococcidae. A fragment of the 18S rRNA gene was amplified for each of the scale insects. As a few samples from the same insect species did not have identical 18S rRNA sequences, we considered all of them for the analyses. These were *Parasaissetia nigra* 8 and *Parasaissetia nigra* 9 (99.5% identity), and *Coccus viridis* 21 and *Coccus viridis* 24 (97.5% identity). We identified only one sample at the family level (Coccidae 35; Table 1). 18S rRNA gene topology was similar to other scale insect molecular phylogenies (Cook *et al.*, 2002; Gullan & Cook, 2007; Yokogawa & Yahara, 2009). In addition, each taxonomic group was maintained in a separate cluster (Fig. S1).

Presence of endosymbionts in scale insects detected by PCR analysis

Symbiont 16S rRNA sequences were obtained by PCR with designed primers. The flavobacterial sequences had high identity to *Blattabacterium* spp. (89–92%), to *Candidatus* (subsequently abbreviated as *Cand.*) Uzinura

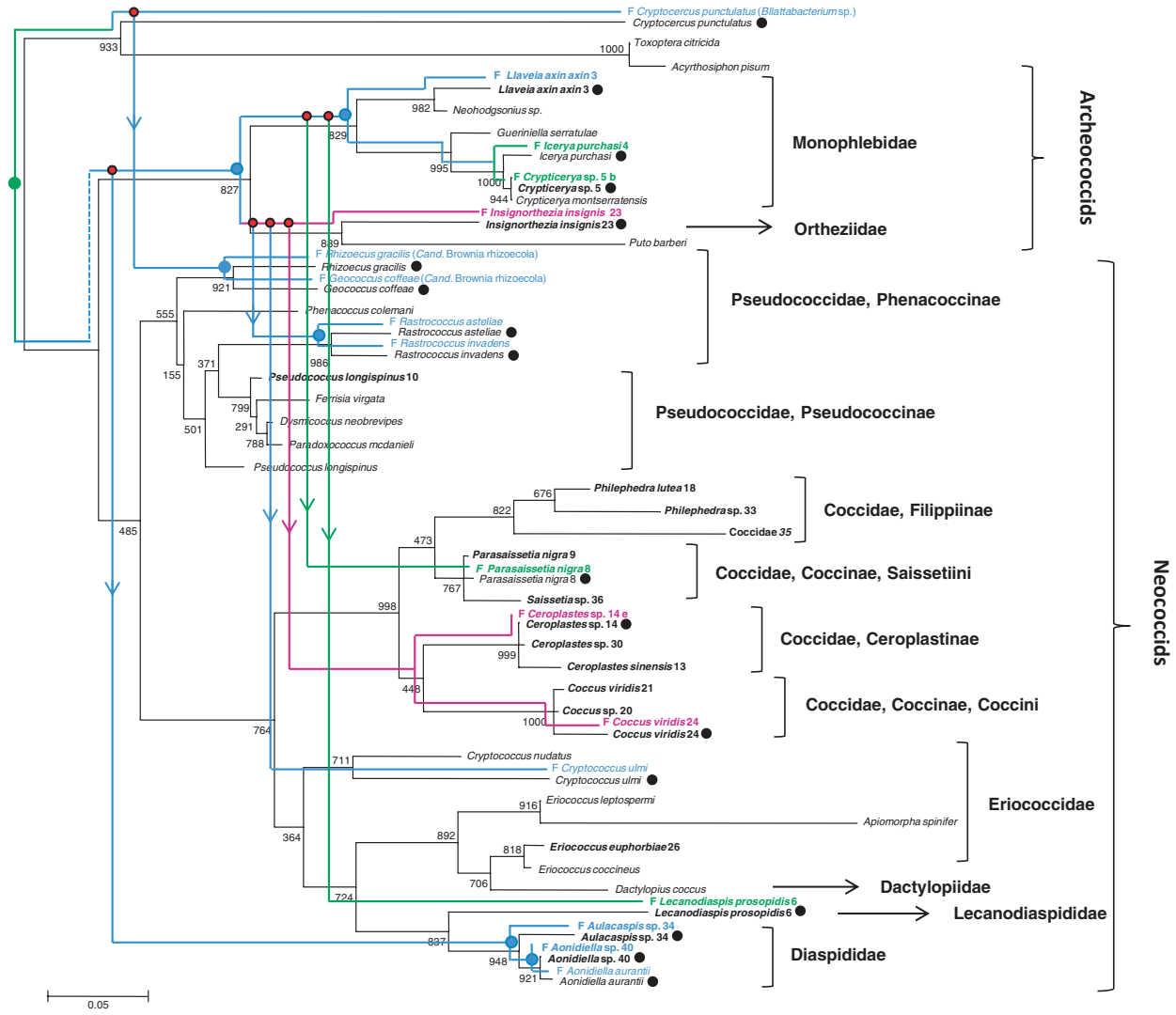

Fig. 2 Cophylogeny of scale insects and Flavobacteria. Phylogeny of scale insects (black) was inferred with 18S rRNA gene sequences (maximum likelihood) and rooted with nonscale insects (*Cryptocercus punctulatus*, *Toxoptera citricida* and *Acyrthosiphon pisum*). The phylogeny in black is the same shown in Fig. S1. In bold are species collected in this work. Bootstrap values for 1000 replicates are shown adjacent to each node. Blue, green and purple edges show the speciation process of 17 scale insect–associated Flavobacteria as it was inferred by CoRe-PA and Jane 3 softwares. Blue circles at the nodes indicate co-speciation events; red circles, duplications; arrows, host switch events; and dotted lines, sorting events (losses). Green and purple arrows indicate one of several possible host switch events equally weighted. Black circles at the end of each name represent sequences used for these analyses. Brackets and arrows to the right of the phylogeny indicate the family, subfamilies and tribes of the insects.

diaspidicola (87–90%) and to *Cand.* Sulcia muelleri (84–90%; Fig. 1a). Most Enterobacteriaceae had high identity to *Sitophilus oryzae* Primary Endosymbiont (SOPE; 97%) and to *Sodalis glossinidius* (97%). We obtained also two enterobacterial sequences (E *Ceroplastes* sp. 30-1 and E Coccidae 35-1) with 100% identity to the free-living *Pantoea agglomerans* (AY849936), and 97% identity to the enterobacterial endosymbiont of *Drosicha* spp. (Monophlebidae, Matsuura *et al.*, 2009; Fig. 1b).

Even though we were not able to amplify from all the samples with the 16S rRNA directed primers, we found that Flavobacteria and Enterobacteriaceae endosymbionts were present at least in one of the samples of Monophlebidae, Ortheziidae, Coccidae and Diaspididae families (Fig. 1a,b and Table 1).

From six insect samples belonging to Monophlebidae, Ortheziidae and Coccidae, we obtained two different 16S rRNA gene sequences of enterobacteria with an

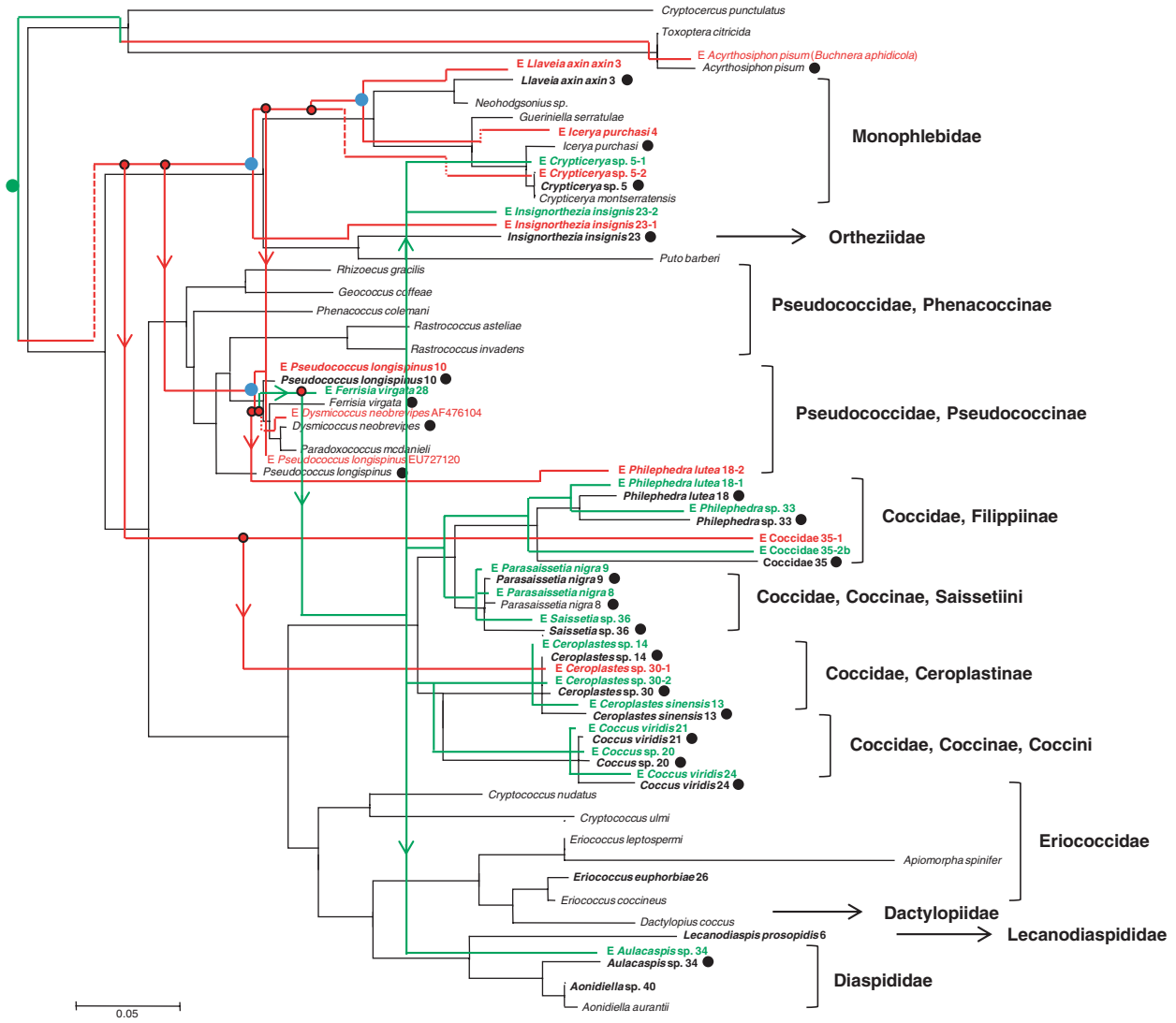

Fig. 3 Cophylogeny of scale insects and Enterobacteriaceae. Phylogeny of scale insects as in Fig. 2. Red and green edges show the speciation process of 27 scale insect–associated Enterobacteriaceae inferred by CoRe-PA and Jane 3 softwares. Green arrows indicate one of several possible host switch events equally weighted. Symbols and colours are as in Fig. 2.

identity below 97% (Fig. 1b and Table 1). If the different ribosomal gene sequences represent different bacterial species within a single insect sample, then we should be able to obtain different sequences of other core genes from the same samples as well. Therefore, we sequenced *groEL* from eight samples. This gene has a single copy in the enterobacterium endosymbiont of *Llaveia axin axin* 3 (determined by Southern blot hybridization, T. Rosas , M. Rosenblueth & E. Martínez, unpublished). The *groEL* phylogeny (Fig. S2) shows that *Icerya purchasi* 4 (Monophlebidae) and *Pseudococcus longispinus* 10 (Pseudococcidae) have two sequences of this gene with < 95% identity, indicating that there are indeed different enterobacterial species as cohabitants in single scale insects.

Insect/Flavobacteria cophylogeny analysis

Flavobacterial 16S rRNA gene ML phylogeny (Fig. 1a) had the same topology as the Bayesian phylogeny (posterior probabilities are shown in Fig. 1a). Three groups, designated F1, F2 and F3, can be observed in the phylogeny (Fig. 1a), with two of them (F1 and F3) being well supported. The F1 group includes Flavobacteria from Monophlebidae, *Parasaissetia nigra* 8 (Coccidae) and *Lecanodiaspis prosopidis* 6 (Lecanodiaspididae). F2

groups Flavobacteria from Ortheziidae, *Ceroplastes* sp. 14, *Coccus viridis* 24 (both Coccidae) and the Eriococcidae *Cryptococcus ulmi* (DQ133549). The F3 group contains Flavobacteria from Diaspididae (which have been named *Cand.* Uzinura diaspidicola; Gruwell *et al.*, 2007). *Cand.* Brownia rhizoecola (from Pseudococcidae) is more closely related to the endosymbionts of suborder Auchenorrhyncha (*Cand.* Sulcia muelleri) and of cockroaches (*Blattabacterium* sp.) as reported previously (Gruwell *et al.*, 2010).

Cophylogeny analyses of host and Flavobacteria were performed with CoRe-PA and Jane. CoRe-PA retrieved nine cophylogenies, but only one was congruent with the branch lengths of the host tree. To validate the significance of the results, we performed random reconstructions and compared the total cost values. With CoRe-PA and Jane, 99.7% and 99.8%, respectively, of a total of 1000 randomizations had a total cost value higher than that of our predicted result, suggesting that the reconstructions obtained were not attributed to randomness.

Both tools for determining the history of the cophylogeny between Flavobacteria and their hosts gave the same result (Fig. 2). In Fig. 2, the 18S phylogeny in black is the same as in Fig. S1. Cospeciation events within the individuals of a family were suggested in Archeococcids (Monophlebidae and Ortheziidae), and in Diaspididae. Cospeciation was also suggested in closely related species as in *Rhizoecus gracilis* and *Geococcus coffeae* and in *Rastrococcus astellae* and *Rastrococcus invadens* (all Pseudococcidae).

Insect/Enterobacteriaceae cophylogeny analysis

Maximum likelihood analysis of 16S rRNA gene phylogenies does not clearly resolve different enterobacterial clades because the sequences are highly conserved (Fig. 1b). In fact, the topology obtained with MrBayes was similar but had many polytomies (data not shown). Despite this, some groups were observed and designated E1–E3. E1 included enterobacteria from *Drosicha* sp. (Monophlebidae) and from two Coccidae (E Ceroplastes sp. 30-1 and E Coccidae 35), as well as sequences from free-living Enterobacteriaceae from the genus *Pantoea*, *Raoultella* and *Klebsiella*. E2 was a polytomous group with almost identical sequences formed by Enterobacteriaceae from all the families analysed (Ortheziidae, Monophlebidae, Pseudococcidae, Diaspididae, and Coccidae). E3 contained mainly secondary endosymbionts Pseudococcidae. Other sequences from Monophlebidae, Ortheziidae, Pseudococcidae and Coccidae were close to clade E2.

With enterobacteria, as with Flavobacteria, both methods (CoRe-Pa and Jane) gave the same result (Fig. 3). 55.4% (CoRe-PA) and 62.5% (Jane) of the randomizations had a higher total cost than the one of our predicted result, suggesting that cospeciation events between Enterobacteriaceae and their hosts were no more common than predicted by chance.

Discussion

Flavobacteria and Enterobacteriaceae have been reported in some families of scale insects. In this study, we analysed whether these bacteria were present in different scale insect families. In a survey of 27 scale insects within seven families, we frequently found that when an insect species harbours Flavobacteria, Enterobacteriaceae were also present. This was observed in ten species from the Monophlebidae, Ortheziidae, Coccidae and Diaspididae families. This could indicate a metabolic complementarity, as has been reported for other coexisting endosymbionts (Gosalbes *et al.*, 2008; McCutcheon & Moran, 2007; McCutcheon *et al.*, 2009; McCutcheon & Moran, 2010; McCutcheon & von Dohlen, 2011; Sloan & Moran, 2012). In the subfamily Phenacoccinae (Pseudococcidae), Flavobacteria have been reported (Gruwell *et al.*, 2010), members from this subfamily were not analysed here. Flavobacteria was not amplified from the subfamily Pseudococcinae (Pseudococcidae), which is in agreement with previous reports (Thao *et al.*, 2002). In the only sample that we had from Eriococcidae (*Eriococcus euphorbiae* 26), we were not able to amplify the genes of any endosymbiont. Although the genus *Cryptococcus* from this family harbours Flavobacteria (Gruwell *et al.*, 2005), our sample is not closely related to *Cryptococcus*; and as Eriococcidae has a paraphyletic origin (Cook *et al.*, 2002; Cook & Gullan, 2004; Fig. 1a), *Eriococcus euphorbiae* may have other endosymbionts.

From some individuals, we obtained two different enterobacterial 16S rRNA sequences with < 97% identity between them, as well as two *groEL* sequences with < 95% identity. These two sequences may represent different species according to Stackebrandt & Goebel (1994), who suggest a threshold of 97% identity to define a species. As we only analysed four clones from each sample, individual insects could hold even more than two enterobacterial species. Closely related bacteria may have similar metabolic functions and may undergo DNA recombination (Rosselló-Mora & Amann, 2001) (which usually does not take place in bacterial endosymbionts that are isolated inside the insects; Moran, 1996). The presence of two or more strains and recombination have been observed in *Wolbachia* (Werren, 1997; Baldo *et al.*, 2006). Other species that have been reported to harbour several endosymbiotic γ-Proteobacteria include dryophthoridae weevils (Lefèvre *et al.*, 2004) and the mollusc shipworm *Lyrodus pedicellatus* (Distel *et al.*, 2002). γ-Proteobacteria close to *Sodalis glossinidius* (from tsetse fly) tend to form associations with insects and are widely distributed in unrelated species (Charles *et al.*, 2001; Hypša & Nováková, 2009), as in Coleoptera (Lefèvre *et al.*, 2004; Grünwald *et al.*, 2010), Hemiptera (Spaulding & von Dohlen,

1998), Phthiraptera (Fukatsu *et al.*, 2007) and other Diptera (Nováková & Hypša, 2007).

We retrieved the same results with CoRe-Pa and Jane. In Flavobacteria, the fact that the analyses were statistically significant suggests cospeciation with their insect hosts in Monophlebidae and before the division of Monophlebidae and Ortheziidae (prior to divergence of Archeococcids). The evolutionary history of Flavobacteria with the rest of the scale insects involves an initial loss at the beginning of the scale insect diversification, where the neococcids lost Flavobacteria and obtained them subsequently, and several host switches: (1) Diaspididae acquired Flavobacteria (*Cand.* Uzinura diaspidicola) from the ancestor of Archeococcids, and cospeciation took place thereafter. This endosymbiont seems to be specific of the family Diaspididae. Cospeciation within this family has been reported previously (Gruwell *et al.*, 2007). (2) Flavobacteria from Pseudococcidae, Phenaoccinae, seem to have two origins: *Cand.* Brownia rhizoecola came from a nonscale insect (more related to *Blattabacterium* sp.) and Flavobacteria from *Rastrococcus* spp. arrived from the ancestor of Ortheziidae. (3) Flavobacteria from *Cryptococcus ulmi* (Eriococcidae) were also obtained from the ancestor of Ortheziidae.

The orientation of other host switches cannot be easily determined because sequences are identical with each other (purple and green edges in Fig. 2). Nevertheless, considering that the analyses suggest cospeciation in Archeococcids, and as other Flavobacteria from this group of insects (besides the ones used in the cophylogenetic analyses) are also in clades F1 and F2 (Fig. 1a), we propose that Flavobacteria from *Lecanodiaspis prosopidis* 6 (Lecanodiaspididae), *Parasaissetia nigra* 8, *Ceroplastes* sp. 14 and *Coccus viridis* 24 (all Coccidae) are derived from Archeococcids (Fig. 2).

It should be taken into account that both algorithms place the external group of the bacterial phylogeny into the host phylogeny, resulting in a cospeciation at the root of the tree (Fig. 2). This event could mean an independent origin instead of a true cospeciation.

Although analyses of Enterobacteriaceae and their insect hosts were not statistically significant, it can be observed that (1) identical enterobacteria can infect scale insects from different families, and (2) members from the same family can harbour different enterobacteria (Figs 1b and 3), revealing a plastic association and suggesting that horizontal transfer of these enterobacteria is an important way of spreading to other insects. The fact that all the insect families analysed possess enterobacteria from clade E2 could mean that this bacterium is a generalist, capable of using the nutrients obtained by each insect species who feed on different plants increasing host switches. We found that Pseudococcidae can harbour Enterobacteriaceae that do not belong to the previously reported clade (Thao *et al.*, 2002; group E3 in this work, Fig. 1b). We do not know whether these bacteria are also located inside the primary endosymbiotic bacteria from Pseudococcidae (*Cand.* Tremblaya princeps), as has been observed with enterobacteria from group E3 (Thao *et al.*, 2002).

Even though we found Flavobacteria and Enterobacteriaceae frequently together in the same individual, suggesting they are both essential to the hosts, they have been independently acquired and have evolved in different ways. Flavobacteria have cospeciated within some families, have been subjected to less host switches and exhibit a higher proportion of congruent nodes between host and bacteria than enterobacterial endosymbionts. On the other hand, enterobacteria have a more relaxed relationship with scale insects, presenting more evolutionary events (losses, duplications and host switches) and a lower proportion of congruent nodes in comparison with flavobacterial endosymbionts. Enterobacteria could have colonized this group of insects more recently or could have carried out more horizontal transfers within (and among) host species.

In future work, we recommend the use of different gene sequences and longer sequence fragments, especially in the case of Enterobacteriaceae, as it is known that the 16S rRNA gene from this group contains insufficient phylogenetic information to infer robust relationships (Williams *et al.*, 2010). To our knowledge, none of the existing tools for reconciliation analysis implement the use of branch lengths information and bootstrap support values. To discard proposed scenarios, we took them into account by manual inspection.

Unique origin and strict cospeciation between insect hosts and symbionts at a high phylogenetic level (higher than family level, such as superfamily or suborder) have been reported in very few cases, such as *Buchnera* – aphid (Clark *et al.*, 2000; Hypša & Nováková, 2009). It has been more frequent to find cospeciation at low phylogenetic levels (such as inside families; Schröder *et al.*, 1996; Chen *et al.*, 1999; Allen *et al.*, 2007), especially in primary endosymbionts, and multiple acquisitions, losses and replacements particularly in secondary endosymbionts (Thao *et al.*, 2000a,b; Nováková & Hypša, 2007; Lamelas *et al.*, 2008). Flavobacteria from Diaspididae (*Cand.* Uzinura diaspidicola) have already been suggested to be the primary endosymbionts (Gruwell *et al.*, 2007), and probably Enterobacteriaceae are secondary endosymbionts of scale insects.

In this work, we have contributed with new sequences of Flavobacteria and Enterobacteriaceae from the Monophlebidae, Ortheziidae, Pseudococcidae, Coccidae, Lecanodiaspididae and Diaspididae insect families. We have provided robust phylogenetic reconstructions for both bacteria phyla as well as the scale insects clade. Finally, we integrated this information to show different dynamics in the evolution of these endosymbionts with their associated hosts that support that Flavobacteria are old symbionts that have cospeciated with different groups of insects.

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References

Abascal, F., Zardoya, R. & Posada, D. 2005. ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* **21**: 2104–2105.

Akaike, H. 1974. A new look at the statistical model identification. *IEEE Trans. Autom. Control* **19**: 716–723.

Aldous, D.J. 2001. Stochastic models and descriptive statistics for phylogenetic trees, from Yule to today. *Statist. Sci.* **16**: 23–34.

Allen, J.M., Reed, D.L., Perotti, M.A. & Braig, H.R. 2007. Evolutionary relationships of "*Candidatus* Riesia spp.," endosymbiotic enterobacteriaceae living within hematophagous primate lice. *Appl. Environ. Microbiol.* **73**: 1659–1664.

Baldo, L., Bordenstein, S., Wernegreen, J.J. & Werren, J.H. 2006. Widespread recombination throughout *Wolbachia* genomes. *Mol. Biol. Evol.* **23**: 437–449.

Baumann, P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu. Rev. Microbiol.* **59**: 155–189.

Baumann, L. & Baumann, P. 2005. Cospeciation between the primary endosymbionts of mealybugs and their hosts. *Curr. Microbiol.* **50**: 84–87.

Baumann, P., Moran, N.A. & Baumann, L. 2000. Bacteriocyte-associated endosymbionts of insects. In: *The prokaryotes* (M. Dworkin, ed.), pp. 155–189. Springer, New York.

Ben-Dov, Y. 2005. *A Systematic Catalogue of the Scale Insect Family Margarodidae (Hemiptera: Coccoidea) of the World*. Intercept Ltd., Wimborne.

Charles, H., Heddi, A. & Rahbé, Y. 2001. A putative insect intracellular endosymbiont stem clade, within the Enterobacteriaceae, inferred from phylogenetic analysis based on a heterogeneous model of DNA evolution. *C. R. Acad. Sci. Paris* **324**: 489–494.

Charleston, M.A. 2011. TreeMap 3b. URL http://sites.google.com/site/cophylogeny

Chen, X., Li, S. & Aksoy, S. 1999. Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. *J. Mol. Evol.* **48**: 49–58.

Clark, M.A., Moran, N.A., Baumann, P. & Wernegreen, J.J. 2000. Cospeciation between bacterial endosymbionts (*Buchnera*) and a recent radiation of aphids (*Uroleucon*) and pitfalls of testing for phylogenetic congruence. *Evolution* **54**: 517–525.

Conow, C., Fielder, D., Ovadia, Y. & Libeskind-Hadas, R. 2010. Jane: a new tool for the cophylogeny reconstruction problem. *Algorithms for Mol. Biol.* **5**: 16.

Cook, L.G. & Gullan, P.J. 2004. The gall-inducing habit has evolved multiple times among the eriococcid scale insects (Sternorrhyncha: Coccoidea: Eriococcidae). *Biol. J. Linn. Soc.* **83**: 441–452.

Cook, L.G., Gullan, P.J. & Trueman, H.E. 2002. A preliminary phylogeny of the scale insects (Hemiptera: Sternorrhyncha: Coccoidea) based on nuclear small-subunit ribosomal DNA. *Mol. Phylogenet. Evol.* **25**: 43–52.

Coulibaly-N'Golo, D., Allali, B., Kouassi, S.K., Fichet-Calvet, E., Becker-Ziaja, B., *et al.* 2011. Novel Arenavirus sequences in *Hylomyscus* sp. and *Mus* (*Nannomys*) *setulosus* from Côte d'Ivoire: implications for evolution of Arenaviruses in Africa. *PLoS One* **6**: e20893.

Degnan, P.H. & Moran, N.A. 2008. Diverse phage-encoded toxins in a protective insect endosymbiont. *Appl. Environ. Microbiol.* **74**: 6782–6791.

Dhami, M.K., Turner, A.P., Deines, P., Beggs, J.R. & Taylor, M. W. 2012. Ultrastructural and molecular characterization of a bacterial symbiosis in the ecologically important scale insect family Coelostomidiidae. *FEMS Microbiol. Ecol.* **81**: 537–546.

Distel, D.L., Beaudoin, D.J. & Morrill, W. 2002. Coexistence of multiple proteobacterial endosymbionts in the gills of the wood-boring bivalve *Lyrodus pedicellatus* (Bivalvia: Teredinidae). *Appl. Environ. Microbiol.* **68**: 6292–6299.

von Dohlen, C.D. & Moran, N.A. 1995. Molecular phylogeny of the Homoptera: a paraphyletic taxon. *J. Mol. Evol.* **41**: 211–223.

Downie, D.A. & Gullan, P.J. 2005. Phylogenetic congruence of mealybugs and their primary Endosymbionts. *J. Evol. Biol.* **18**: 315–324.

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**: 1792–1797.

Foldi, I. 2005. Ground pearls: a generic revision of the Margarodidae *sensu stricto* (Hemiptera: Sternorrhyncha: Coccoidea). *Ann. Soc. entomol. Fr. (n.s.)* **41**: 81–125.

Fukatsu, T., Koga, R., Smith, W.A., Tanaka, K., Nikoh, N., Sasaki-Fukatsu, K., *et al.* 2007. Bacterial endosymbiont of the slender pigeon louse, *Columbicola columbae*, allied to endosymbionts of grain weevils and tsetse flies. *Appl. Environ. Microbiol.* **73**: 6660–6668.

Gosalbes, M.J., Lamelas, A., Moya, A. & Latorre, A. 2008. The striking case of tryptophan provision in the cedar aphid *Cinara cedri*. *J. Bacterial.* **190**: 6026–6029.

Grünwald, S., Pilhofer, M. & Höll, W. 2010. Microbial associations in gut systems of wood- and bark-inhabiting longhorned beetles [Coleptera: Cerambycidae]. *Syst. Appl. Microbiol.* **33**: 25–34.

Gruwell, M.E., Dohlen, C.D., Patch, K. & Normark, B.B., 2005. Preliminary PCR survey of bacteria associated with scale insects (Hemiptera: Coccoidea). In: *Proceedings of the Tenth International Symposium for Scale Insect Studies*, pp. 101–116. Adana, Turkey.

Gruwell, M.E., Morse, G.E. & Normark, B.B. 2007. Phylogenetic congruence of armored scale insects (Hemiptera: Diaspididae) and their primary endosymbionts from the phylum Bacteroidetes. *Mol. Phylogenet. Evol.* **44**: 267–280.

Gruwell, M.E., Hardy, N.B., Gullan, P.J. & Dittmar, K. 2010. Evolutionary relationships among primary endosymbionts of the mealybug subfamily phenacoccinae (Hemiptera: Coccoidea: Pseudococcidae). *Appl. Environ. Microbiol.* **76**: 7521–7525.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. 2010. New algorithms and methods to

estimate Maximum-likelihood phylogenies: assessing the performance of PhyML. *Syst. Biol.* **59**: 307–321.

Gullan, P.J. & Cook, L.G. 2007. Phylogeny and higher classification of the scale insects (Hemiptera: Sternorrhyncha: Coccoidea). *Zootaxa* **1668**: 413–425.

Gullan, P.J. & Kosztarab, M. 1997. Adaptations in Scale Insects. *Annu. Rev. Entomol.* **42**: 23–50.

Hodgson, C. & Foldi, I. 2006. A review of the Margarodidae *sensu* Morrison (Hemiptera: Coccoidea) and some related taxa based on the morphology of adult males. *Zootaxa* **1263**: 1–250.

Hypša, V. & Nováková, E. 2009. Insect symbionts and molecular phylogenetics. In: *Insect Symbiosis*, vol. **3** (K. Bourtzis & T. A. Miller, eds), pp. 1–31. CRC Press, Taylor & Francis Group, Boca Raton, FL.

Keller-Schmidt, S., Wieseke, N., Klemm, K. & Middendorf, M. 2011. Evaluation of host parasite reconciliation methods using a new approach for cophylogeny generation. Working paper from Bioinformatics Leipzig. URL http://www.bioinf.uni-leipzig.de/working/11-013

Kono, M., Koga, R., Shimada, M. & Fukatsu, T. 2008. Infection dynamics of coexisting beta- and gammaproteobacteria in the nested endosymbiotic system of mealybugs. *Appl. Environ. Microbiol.* **74**: 4175–4184.

Koteja, J. 1974. On the phylogeny and classification of the scale insects (Homoptera: Coccinea). *Acta Zool. Cracoviensia.* **19**: 267–325.

Lamelas, A., Pérez-Brocal, V., Gómez-Valero, L., Gosalbes, M. J., Moya, A. & Latorre, A. 2008. Evolution of the secondary symbiont "*Candidatus* Serratia symbiotica" in aphid species of the subfamily Lachninae. *Appl. Environ. Microbiol.* **74**: 4236–4240.

Lefèvre, C., Charles, H., Vallier, A., Delobel, B., Farrell, B. & Heddi, A. 2004. Endosymbiont phylogenesis in the dryophthoridae weevils: evidence for bacterial replacement. *Mol. Biol. Evol.* **21**: 965–973.

Matsuura, Y., Koga, R., Nikoh, N., Meng, X.-Y., Hanada, S. & Fukatsu, T. 2009. Huge symbiotic organs in giant scale insects of the genus *Drosicha* (Coccoidea: Monophlebidae) harbor flavobacterial and enterobacterial endosymbionts. *Zool. Sci.* **26**: 448–456.

McCutcheon, J.P. & Moran, N.A. 2007. Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. *Proc. Natl. Acad. Sci. USA* **104**: 19392–19397.

McCutcheon, J.P. & Moran, N.A. 2010. Functional convergence in reduced genomes of bacterial symbionts spanning 200 My of evolution. *Genome Biol. Evol.* **2**: 708–718.

McCutcheon, J.P. & von Dohlen, C.D. 2011. An interdependent metabolic patchwork in the nested symbiosis of mealybugs. *Curr. Biol.* **21**: 1366–1372.

McCutcheon, J.P., McDonald, B.R. & Moran, N.A. 2009. Convergent evolution of metabolic roles in bacterial co-symbionts of insects. *Proc. Natl. Acad. Sci. USA* **106**: 15394–15399.

Merkle, D., Middendorf, M. & Wieseke, N. 2010. A parameter-adaptive dynamic programming approach for inferring cophylogenies. *BMC Bioinformatics* **11**: S60.

Miller, D.R., Rung, A., Venable, G.L. & Gill, R.J. 2007. *Scale Insects: Identification Tools for Species of Quarantine Importance. Scale Families*. ARS & APHIS, USDA. URL http://www.sel.barc.usda.gov/ScaleKeys/ScaleInsectsHome/ScaleInsectsFamilies.html

Moran, N.A. 1996. Accelerated evolution and Muller's rachet in endosymbiotic bacteria. *Proc. Natl. Acad. Sci. USA* **93**: 2873–2878.

Moran, N.a., McCutcheon, J.P. & Nakabachi, A. 2008. Genomics and evolution of heritable bacterial symbionts. *Annu. Rev. Genet.* **42**: 165–190.

Nováková, E. & Hypša, V. 2007. A new *Sodalis* lineage from bloodsucking fly *Caterina melbae* (Diptera, Hippoboscoidea) originated independently of the tsetse flies symbiont *Sodalis glossinidius*. *FEMS Microbiol. Lett.* **269**: 131–135.

Nováková, E., Hypša, V. & Moran, N.A. 2009. *Arsenophonus*, an emerging clade of intracellular symbionts with a broad host distribution. *BMC Microbiol.* **9**: 143.

Oliver, K.M., Patrick H. Degnan, P.H., Burke, G.R. & Moran, N.A. 2010. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu. Rev. Entomol.* **55**: 247–266.

Posada, D. & Crandall, K.A. 2001. Selecting the best-fit model of nucleotide substitution. *Syst. Biol.* **50**: 580–601.

Ramírez-Puebla, S.T., Rosenblueth, M., Chávez-Moreno, C.K., Catanho Pereira De Lyra, M.C., Tecante, A. & Martínez-Romero, E. 2010. Molecular phylogeny of the genus *Dactylopius* (Hemiptera: Dactylopiidae) and identification of the symbiotic bacteria. *Environ. Entomol.* **39**: 1178–1183.

Rice, P., Longden, I. & Bleasby, A. 2000. EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet.* **16**: 276–277.

Ricklefs, R.E., Fallon, S.M. & Bermingham, E. 2004. Evolutionary relationships, cospeciation, and host switching in avian Malaria parasites. *Syst. Biol.* **53**: 111–119.

Ronquist, F. & Huelsenbeck, J.P. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Rosselló-Mora, R. & Amann, R. 2001. The species concept for prokaryotes. *FEMS Microbiol. Rev.* **25**: 39–67.

Russell, J.A., Latorre, A., Sabater-Muñoz, B., Moya, A. & Moran, N.A. 2003. Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Mol. Ecol.* **12**: 1061–1075.

Schröder, D., Deppisch, H., Obermayer, M., Krohne, G., Stackebrandt, E., Hôlldobler, B., *et al.* 1996. Intracellular endosymbiotic bacteria of *Camponotus* species (carpenter ants): systematics, evolution and ultrastructural characterization. *Mol. Microbiol.* **21**: 479–489.

Sloan, D.B. & Moran, N.A. 2012. Genome reduction and co-evolution between the primary and secondary bacterial symbionts of psyllids. *Mol. Biol. Evol.*, doi:10.1093/molbev/mss180.

Spaulding, A.W. & von Dohlen, C.D. 1998. Phylogenetic characterization and molecular evolution of bacterial endosymbionts in psyllids (Hemiptera: Sternorrhyncha). *Mol. Biol. Evol.* **15**: 1506–1513.

Stackebrandt, E. & Goebel, B.M. 1994. Taxonomic Note: a Place for DNA-DNA Reassociation and 16s rRNA Sequence Analysis in the Present Species Definition in Bacteriology. *Int. J. Syst. Bacteriol.* **44**: 846–849.

Szklarzewicz, T., Kedra, K. & Niznik, S. 2005. Ultrastructural studies of the ovary of *Palaeococcus fuscipennis* (Burmeister) (Insecta, Hemiptera, Coccinea: Monophlebidae). *Folia Biol.* **53**: 45–50.

Tautz, D., Hancock, J.M., Webb, D.A., Tautz, C. & Dover, G.A. 1988. Complete sequences of the rRNA genes of *Drosophila melanogaster*. *Mol. Biol. Evol.* **5**: 366–376.

Thao, M.L., Clark, M.A., Baumann, L., Brennan, E.B., Moran, N.A. & Baumann, P. 2000a. Secondary endosymbionts of psyllids have been acquired multiple times. *Curr. Microbial.* **41**: 300–304.

Thao, M.L., Moran, N.A., Abbot, P., Brennan, E.B., Burckhardt, D.H. & Baumann, P. 2000b. Cospeciation of psyllids and their primary prokaryotic endosymbionts. *Appl. Environ. Microbiol.* **66**: 2898–2905.

Thao, M.L., Gullan, P.J. & Baumann, P. 2002. Secondary (γ-Proteobacteria) endosymbionts infect the primary (β-Proteobacteria) endosymbionts of mealybugs multiple times and coevolve with their hosts. *Appl. Environ. Microbiol.* **68**: 3190–3197.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673–4680.

Unruh, C.M. & Gullan, P.J. 2008. Molecular data reveal convergent reproductive strategies in iceryine scale insects (Hemiptera: Coccoidea: Monophlebidae), allowing the re-interpretation of morphology and a revised generic classification. *Syst. Entomol.* **33**: 8–50.

Waterhouse, A.M., Procter, J.B., Martin, D.M.A., Clamp, M. & Barton, G.J. 2009. Jalview Version 2 – a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**: 1189–1191.

Werren, J.H. 1997. Biology of *Wolbachia*. *Annu. Rev. Entomol.* **42**: 587–609.

Williams, K.P., Gillespie, J.J., Sobral, B.W.S., Nordberg, E.K., Snyder, E.E., Shallom, J.M., *et al.* 2010. Phylogeny of gammaproteobacteria. *J. Bacterial.* **192**: 2305–2314.

Yokogawa, T. & Yahara, T. 2009. Mitochondrial phylogeny certified PGL (Paternal Genome Loss) is of single origin and haplodiploidy *sensu stricto* (arrhenotoky) did not evolve from PGL in the scale insects (Hemiptera: Coccoidea). *Genes Genet. Syst.* **84**: 57–66.

Zchori-Fein, E., Ben-Dov, Y., Portnoy, V. & Katzir, N. 2005. Distribution of the endosymbiont *Cardinium hertigii* in scale insects (Hemiptera: Coccoidea). In: *Proceedings of the Tenth International Symposium on Scale Insect Studies*, Adana, Turkey, 19–23 April 2004. (L.B. Erkılıç & M.B. Kaydan, eds), pp. 101–116. Scientific and Technical Research Council of Turkey, Ankara.

Supporting information

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

Table S1 Characteristics of scale insect samples collected for this study.

Figure S1 Maximum likelihood topology of 18S rRNA gene (658 bp) of scale insects, rooted with non-scale insects

Figure S2 Maximum likelihood topology based on the *groEL* gene (631 bp) from the enterobacterial endosymbiont of scale insects.

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