



**HAL**  
open science

## **Buchnera has changed flatmate but the repeated replacement of co-obligate symbionts is not associated with the ecological expansions of their aphid hosts**

Andrea Sanchez Meseguer, Alejandro Manzano-Marin, Armelle Coeur d'Acier, Anne Laure Clamens, Martin Godefroid, Emmanuelle Jousselin

### ► To cite this version:

Andrea Sanchez Meseguer, Alejandro Manzano-Marin, Armelle Coeur d'Acier, Anne Laure Clamens, Martin Godefroid, et al.. Buchnera has changed flatmate but the repeated replacement of co-obligate symbionts is not associated with the ecological expansions of their aphid hosts. *Molecular Ecology*, 2017, 26 (8), pp.2363 - 2378. 10.1111/mec.13910 . hal-02623952

**HAL Id: hal-02623952**

**<https://hal.inrae.fr/hal-02623952>**

Submitted on 31 May 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

1 Original article

2

3 **Title: *Buchnera* has changed flatmate but the repeated replacement of co-obligate symbionts is not**  
4 **associated with the ecological expansions of their aphid hosts**

5

6 **Running title: Cosymbiont replacements is not linked with their aphid hosts ecological expansions**

7

8 **Authors: A.S. Meseguer, A. Manzano-Marín, A. Coeur d'Acier, A-L. Clamens, M. Godefroid, E.**  
9 **Jousselin**

10

11 **Addresses:** INRA, UMR 1062, Centre de Biologie pour la Gestion des Populations CBGP (INRA, IRD,  
12 CIRAD, Montpellier SupAgro), Montferrier-sur-Lez, 34980, France.

13

14 **Corresponding author:** Andrea Sánchez Meseguer. INRA, UMR 1062, Centre de Biologie pour la  
15 Gestion des Populations CBGP (INRA, IRD, CIRAD, Montpellier SupAgro), Montferrier-sur-Lez, 34980,  
16 France ; Telephone: +33 4 99 62 33 11; email: [asanchezmeseguer@gmail.com](mailto:asanchezmeseguer@gmail.com); Orcid ID:  
17 <http://orcid.org/0000-0003-0743-404X>  
18

19

20 **Key words:** Endosymbiosis, aphids, *Cinara*, bacteria, comparative phylogenetic analyses, ecological  
21 niche.

22

## 23 **Abstract**

24 Symbiotic associations with bacteria have facilitated important evolutionary transitions in insects and  
25 resulted in long-term obligate interactions. Recent evidence suggests that these associations are not  
26 always evolutionarily stable and that symbiont replacement and/or supplementation of an obligate  
27 symbiosis by an additional bacterium has occurred during the history of many insect groups. Yet, the  
28 factors favoring one symbiont over another in this evolutionary dynamic are not well understood;  
29 progress has been hindered by our incomplete understanding of the distribution of symbionts across  
30 phylogenetic and ecological contexts. While many aphids are engaged into an obligate symbiosis with  
31 a single Gammaproteobacterium, *Buchnera aphidicola*, in species of the Lachninae subfamily, this  
32 relationship has evolved into a “*ménage à trois*”, in which *Buchnera* is complemented by a co-  
33 symbiont, usually *Serratia symbiotica*. Using deep sequencing of 16S rRNA bacterial genes from 128  
34 species of *Cinara* (the most diverse Lachninae genus), we reveal a highly dynamic dual symbiotic  
35 system in this aphid lineage. Most species host both *Serratia* and *Buchnera* but, in several clades,  
36 endosymbionts related to *Sodalis*, *Erwinia* or an unnamed member of the Enterobacteriaceae have  
37 replaced *Serratia*. Endosymbiont genome sequences from four aphid species confirm that these co-  
38 resident symbionts fulfill essential metabolic functions not ensured by *Buchnera*. We further  
39 demonstrate through comparative phylogenetic analyses that co-symbiont replacement is not  
40 associated with the adaptation of aphids to new ecological conditions. We propose that symbiont  
41 succession was driven by factors intrinsic to the phenomenon of endosymbiosis, such as rapid  
42 genome deterioration or competitive interactions between bacteria with similar metabolic  
43 capabilities.

44

## 45 **Introduction**

46 Symbiotic associations with bacterial partners have facilitated important evolutionary transitions in  
47 the life histories of eukaryotes and have probably driven species diversification. Some groups of  
48 plant-eating insects have made use of the metabolic versatility of bacteria to feed on plant parts

49 lacking certain essential nutrients (Hansen & Moran 2014). They generally shelter their bacterial  
50 partners within specialized cells and transmit them from mother to offspring (Buchner 1965). This  
51 type of nutritional endosymbiosis was first described in aphids (Hemiptera: Aphididae), a group of  
52 about 5000 species that feed on the phloem of their host plants. Almost all aphids host a  
53 Gammaproteobacterium, *Buchnera aphidicola*, which provides them with essential amino acids and  
54 vitamins that are rare in their diet (Douglas 1998; Wilson *et al.* 2010). Aphids may also be associated  
55 with facultative endosymbiotic bacteria that are not required for survival; their prevalence varies  
56 across populations (Oliver *et al.* 2010).

57 Obligate endosymbionts provide net benefits to their hosts, but reliance on long-term symbiotic  
58 associations can sometimes lead to evolutionary “dead-ends” (Bennett & Moran 2015). The maternal  
59 transfer of bacteria causes severe bottlenecks in bacterial populations, leading to genetic drift and  
60 the fixation of slightly deleterious mutations (Moran 1996; Risper & Moran 2000; Toft & Andersson  
61 2010). This process may alter symbiotic functions (McCutcheon & Moran 2012) and limit the thermal  
62 tolerance of bacteria (Wernegreen 2012), ultimately having a deleterious effect on the host  
63 dependent on these bacteria. One possible outcome of this situation is the replacement or the  
64 supplementation of the ancestral symbiont by a new one. Since the biosynthesis of amino acids are  
65 ubiquitous capabilities in bacteria, in some insect species, some of the facultative endosymbionts  
66 have become more than occasional partners, either entirely replacing the ancestral primary symbiont  
67 (Conord *et al.* 2008; Koga & Moran 2014; Smith *et al.* 2013; Toju *et al.* 2013), or persisting alongside  
68 it whilst taking on a subset of its functions (McCutcheon & Moran 2010; McCutcheon & von Dohlen ;  
69 Takiya *et al.* 2006; Wu *et al.* 2006). An increasing body of evidence now shows that symbiont  
70 replacements have occurred repeatedly in insects, yet the factors favoring one symbiont over  
71 another in this evolutionary dynamic are still not well understood. It has been suggested that the  
72 acquisition of a new symbiont may not only provide the insect with a way of coping with the  
73 degradation of the genome of the primary symbiont, but may also confer new metabolic capabilities  
74 on the insect host (Koga & Moran 2014; Toenshoff *et al.* 2012). The acquisitions of these bacteria

75 would then represent key innovations that allow their hosts to diversify in ecological niches that  
76 would otherwise be unavailable to them (Moran & Telang 1998). Studies on facultative symbionts in  
77 aphid populations, and insects in general, support this hypothesis; they generally increase the fitness  
78 of their hosts in specific environments and mediate ecological interactions (Frago *et al.* 2012; Henry  
79 *et al.* 2015; Oliver *et al.* 2010; Oliver & Martinez 2014; Russell & Moran 2006). However, because  
80 obligate nutritional symbioses remain more stable over evolutionary times than facultative ones,  
81 investigating the ecological factors that govern obligate symbiont turnover requires a wide  
82 taxonomic coverage and a solid phylogenetic framework.

83 Recent studies have reported the presence of labile di-symbiotic systems in aphids of the Lachninae  
84 subfamily. In *Cinara cedri*, *Cinara tujafilina* and *Tuberolachnus salignus*, *B. aphidicola* has lost the  
85 ability to synthesize the essential compounds riboflavin and biotin (in *C. cedri* and *T. salignus* it has  
86 also lost the ability to synthesize tryptophan), and these functions are now fulfilled by a former  
87 facultative endosymbiont, *Serratia symbiotica* (Gosalbes *et al.* 2008; Lamelas *et al.* 2011a; Lamelas *et*  
88 *al.* 2011b; Manzano-Marin & Latorre 2014; Manzano-Marín *et al.* 2016a). *Serratia* has thus become a  
89 co-obligate partner with a nutritional role complementary to that of *Buchnera* (Manzano-Marín *et al.*  
90 2016a). It has been suggested that the riboflavin biosynthetic capability of *Buchnera* was lost in the  
91 ancestor of the Lachninae (Lamelas *et al.* 2011b; Manzano-Marín *et al.* 2016a). This implies that a co-  
92 resident symbiont is now required by all members of the Lachninae for the system to survive.

93 Characterizations of endosymbiotic bacteria in members of the subfamily including some *Cinara* spp.  
94 have shown that all the specimens studied so far harbor at least one additional bacterial  
95 endosymbiont alongside *Buchnera* (Burke *et al.* 2009; Jousselin *et al.* 2016; Lamelas *et al.* 2008)—  
96 while most species host *Serratia symbiotica*, some are associated with an alternative member of the  
97 Enterobacteriaceae. Observations of endosymbiont morphology and location in their hosts lend  
98 further support to the obligate aspect of the association with this new bacterial partner (Manzano-  
99 Marín *et al.* 2016b). Altogether these results also suggest that symbiont replacement has occurred in  
100 Lachninae. However, these studies were conducted on relatively few species and usually a single

101 specimen per species. Results from a few samples represent mere snapshots of the ongoing  
102 evolutionary dynamics of these associations. A full understanding of the factors mediating symbionts  
103 replacements requires the analysis of the distribution of obligate symbionts across wide phylogenetic  
104 and ecological contexts. The aphid genus *Cinara* (Lachninae) might be an ideal model to conduct such  
105 a study. *Cinara* accounts for more than half of the Lachninae species diversity and is the second most  
106 diverse genus of aphids. It has diversified on various conifer genera, giving rise to more than 240  
107 species (Chen *et al.* 2015; Meseguer *et al.* 2015). Species of this genus are distributed throughout the  
108 Holarctic and originated about 45 Mya, surviving all climatic changes that occurred through the  
109 Cenozoic (Zachos *et al.* 2008). Therefore, *Cinara* spp. have experienced a wide range of ecological  
110 conditions during their evolution. This long evolutionary history might have been accompanied by  
111 major changes in symbiotic interactions.

112 To elucidate the long-term evolution and maintenance of symbiotic associations in *Cinara*, we carried  
113 out an extensive survey of endosymbionts on a sample encompassing 50% of the genus' known  
114 species diversity. We deep sequenced 16S rRNA genes, and modeled their distribution across the  
115 aphid phylogeny. We also sequenced the paired *Buchnera* and— *Serratia*, *Erwinia*, *Sodalis* or *Type-X*  
116 genomes (the main endosymbionts identified in our study) of four *Cinara* species to search for the  
117 presence/absence of the Riboflavin biosynthetic genes. We found that *Cinara* species have acquired  
118 different companion symbionts alongside *Buchnera* during the course of their diversification and that  
119 those have become obligate partners of the association complementing *Buchnera* in its nutritional  
120 role. We then explored the evolutionary pathways leading to the replacements of symbionts in this  
121 obligate dual symbiosis, by investigating whether the variation of host life-history traits and the  
122 climatic conditions experienced by the aphid were correlated with changes in co-obligate symbiont  
123 identity.

124

## 125 **Experimental Procedures**

### 126 **16S rDNA Endosymbiont characterization**

127 *DNA samples and 16S rDNA amplification.* We sampled 366 colonies of *Cinara* and 5 outgroup  
128 colonies from the Lachninae and Mindarinae in the field. We sampled several colonies per species  
129 (from 1 to 17) to represent the species geographic distribution and diversity of host-plants. Aphids  
130 were kept in 70% ethanol at 6 °C immediately after collection. They were identified in the laboratory  
131 using different keys (Blackman & Eastop 2000; Favret & Voegtlin 2004). Collection details are given in  
132 Appendix 1. A single individual per colony was washed three times in ultrapure water and total  
133 genomic DNA was extracted from whole individuals with the DNeasy Blood & Tissue Kit (Qiagen,  
134 Germany), according to the manufacturer's recommendations. The DNA was eluted in 40 µL of  
135 elution buffer. During the extraction procedure a negative control (i.e. a 'blank template' of ultrapure  
136 water) was processed with the same extraction kit. All DNA samples were stored at -20 °C. We  
137 amplified a 251bp portion of the V4 region of the 16S rRNA gene (Mizrahi-Man *et al.* 2013), and used  
138 targeted sequencing of indexed bacterial fragments on a MiSeq (Illumina) platform (Kozich *et al.*  
139 2013), following the protocol described in (Jousselin *et al.* 2016). DNA extracts were amplified twice  
140 along with negative controls. PCR replicates were conducted on distinct 96-well microplates. As  
141 positive DNA controls, we used DNA extracts from three pure bacterial strains and three arthropod  
142 specimens with known bacterial endosymbionts.

143 We obtained a total of 749 PCR products, which were pooled and submitted for paired-end  
144 sequencing on a MISEQ (Illumina) FLOWCELL equipped with a version 2, 500-cycle reagent cartridge.

145  
146 *Sequence analyses and Taxonomic assignation.* We used Mothur v1.3.3 (Schloss & Westcott 2011)  
147 implemented on a Galaxy workbench (Goecks *et al.* 2010) ([http://galaxy-](http://galaxy-workbench.toulouse.inra.fr/)  
148 [workbench.toulouse.inra.fr/](http://galaxy-workbench.toulouse.inra.fr/)) to assemble paired-end reads and filter out sequencing errors and  
149 chimeras from the results. The overlapped paired-end reads were assembled with the *make.contigs*  
150 function of MOTHUR, and the contigs exceeding 280 bp in length and/or containing ambiguous base  
151 pairs were filtered out and excluded from further analyses, since the V4 region is expected to have  
152 about 251 pb. A FASTA file containing unique contigs and a file reporting the occurrence of these

153 sequences in each sample were created. Unique sequences from the FASTA file were then aligned  
154 with the V4 portion of reference sequences from the SILVA 16S reference database (v119) (Quast *et*  
155 *al.* 2013). Sequences that did not align with the V4 fragment were excluded from further analyses.  
156 After this filtering step, a new file containing unique sequences was created. The number of reads  
157 resulting from sequencing errors was then reduced by merging rare unique sequences with frequent  
158 unique sequences with a mismatch of no more than 2 bp relative to the rare sequences (*pre.cluster*  
159 command in MOTHUR). We then used the UCHIME program (Edgar *et al.* 2011) implemented in  
160 MOTHUR to detect chimeric sequences and excluded them from the data set. For each sequence, the  
161 number of reads per sample was transformed into percentages using an R script (Jousselin *et al.*  
162 2016) and used to compile a contingency table (Appendix 2). We removed individual sequences  
163 representing less than 0.5 % of the reads in each sample. Sequences represented by such a small  
164 proportion of the reads were often found in negative controls, were generally not arthropod  
165 endosymbionts and, in most cases, were not found across PCR replicates of the same sample,  
166 suggesting that they could represent contaminants or spurious sequences (Jousselin *et al.* 2016). For  
167 each sample, we then eliminated all the sequences that did not appear in both PCR replicates.  
168 Taxonomic affiliations of each unique sequence were obtained using the RDP Classifier in Qiime  
169 (Caporaso *et al.* 2010), with the Silva database, and leBIBI<sup>QBPP</sup> (Flandrois *et al.* 2014), with the 16S  
170 SSU-rRNA-TS-stringent database. In addition, a neighbour-joining tree was reconstructed with all  
171 unique sequences combined with sequences of aphid endosymbionts identified in previous studies;  
172 sequences of *Hamiltonella*, *Phlomobacter*, *Erwinia*, *Dickeya*, *Edwardsiella*, *Sodalis*, *Pantoea*,  
173 *Klebsiella*, *Spiroplasma* and *Cardinium* were retrieved from Silva. Sequences of *Regiella* identified in a  
174 previous study (Smith *et al.* 2015), and sequences of *Arsenophonus*, *Buchnera*, *Hamiltonella*, the  
175 secondary symbionts of *Cinara* spp. identified by (Burke *et al.* 2009) were retrieved from NCBI. We  
176 then checked the coherence of the phylogenetic clusters obtained with the NJ tree and the  
177 taxonomic assignation from RDP and leBIBI.  
178



179 *Assessing endosymbiont diversity and specificity across samples.* We added the frequencies of unique  
180 sequences assigned to a particular bacterial genus (or higher rank when genus assignment was not  
181 available) in a sample. We assessed the replicability of our results by plotting the percentage of reads  
182 assigned to each bacterium in one PCR replicate against the other for all *Cinara* samples, and  
183 calculated the Pearson correlation coefficient. We then plotted the bacterial community (phylum and  
184 frequency as estimated by read abundances) of each *Cinara* specimen/species to the tips of the  
185 *Cinara*'s phylogenetic tree (see below) using the R package *gplots v2.23* (Warnes *et al.* 2015); for the  
186 species-level analyses, we only considered the bacteria that were found in all the specimens of a  
187 given species. We calculated the mean prevalence of each endosymbiont in each *Cinara* species as  
188 the percentage of specimens per species that harboured a particular symbiont against the total  
189 number of individuals collected for that aphid species, excluding outgroups and species that were  
190 represented by a single specimen to avoid overestimation of mean values.

191 We represented the specificity of the aphid-endosymbiont associations by plotting the links between  
192 16S bacterial sequences and the *Cinara* specimens/species in which they were found using *bipartite*  
193 (Dormann *et al.* 2008).

194

## 195 **Symbiont genome**

196 *Endosymbiont DNA extraction and sequencing.*

197 In order to obtain genome data of putative co-obligate endosymbionts of *Cinara*, for four species  
198 (*Cinara confinis*, *C. pseudotaxifoliae*, *C. strobi*, *C. fornacula*), we prepared DNA samples enriched with  
199 bacteria following a slightly modified version of the protocol by Charles and Ishikawa (Charles &  
200 Ishikawa 1999) as described in Jousselin *et al.* (2016). For this filtration procedure, for each aphid  
201 colony, 7 to 15 aphids were pooled together. DNA libraries were then prepared using the Nextera XT  
202 Library Kit (Illumina) and each library was multiplexed and sequenced as a combination of 300bp  
203 paired-end and/or 250 paired-end read on MiSeq (Illumina) flowcells and/or 100bp paired end read

204 on one fourth of an Illumina Hiseq2000 lane (see supplementary information for details on the  
205 sequencing efforts used for each sample).

206 *Draft genome assembly and annotation of riboflavin biosynthetic genes.* Before assembly, reads were  
207 quality-trimmed using FASTX-toolkit v0.0.14 ([http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)) and reads  
208 shorter than 75 bps were filtered out. Additionally, reads containing undefined nucleotides ("N")  
209 were discarded using PRINSEQ-lite v2.24.0 (Schmieder & Edwards 2011). Remaining paired reads  
210 were used for *de novo* assembly in SPAdes v3.8.0 (Bankevich *et al.* 2012) with kmer lengths of 33, 55,  
211 77 for samples "2801" and 33, 55, 77, 99, 127 for samples "3056" and "3249". The resulting contigs  
212 were then taxonomically assigned to a putative symbiont through blastx searches against a database  
213 composed of the proteome of the pea aphid (ABLF00000000), diverse aphid endosymbiont bacterial  
214 strains (*Buchnera* spp. [BA000003, AP001070-1, AE013218, AE016826, AF492591, CP000263,  
215 AY438025, EU660486], *Hamiltonella defensa* [CP001277-8], *Serratia symbiotica* [CP002295,  
216 CCES00000000, FR904230-48, HG934887-9], *Regiella insecticola* [ACYF00000000]), *Sodalis* spp.  
217 (CP006569-70, AP008232-5, CP006568), *Wolbachia* spp. (AM9998877, AP013028), and *Erwinia* spp.  
218 (FN666575-7, FP236842, FP236827-9, FP928999) strains, followed by manual curation. Afterwards,  
219 the resulting references were used for mapping with bowtie v2.2.5 (Langmead & Salzberg 2012) and  
220 reassembled using SPAdes (as previously described). Draft *Buchnera* chromosome assemblies were  
221 scaffolded using *Buchnera* from *Cinara tujafilina* (GenBank:CP001817.1) as reference. Final contigs  
222 were manually curated to remove spurious sequences (resulting from misassemblies or  
223 contamination). Riboflavin biosynthetic genes were searched for using the online tblastn server,  
224 followed by manual curation.

225

## 226 **Phylogenetic relationships in *Cinara***

227 *DNA sequences and phylogenetic reconstruction.* We used five DNA fragments to reconstruct the  
228 phylogeny of the 371 aphids used in the endosymbiont survey. We used three DNA fragments from  
229 the aphid's genome (cytochrome c oxidase subunit I "*COI*"; cytochrome b "*Cytb*"; and the elongation

230 factor “*EF*”) and two from the DNA of *Buchnera aphidicola* (a chaperonin assisting in the folding of  
231 proteins “*GroEL*”; and “*His*”, which includes the ATP phosphoribosyltransferase (HisG) gene, the  
232 histidinol dehydrogenase (HisD) gene and the intergenic region). Total genomic DNA was extracted  
233 from a single individual from the same aphid colony as used in the endosymbiont survey. DNA was  
234 extracted, amplified and sequenced as in previous studies (Jousselin *et al.* 2013). A total of seventy-  
235 five specimens were newly sequenced for this study, while the other sequences were retrieved from  
236 GenBank (Appendix 1). Contigs were assembled from forward and reverse reads and corrected with  
237 Geneious 8.1.7 (Drummond *et al.* 2010). Alignments were generated with *mafft* v.6 (Katoh & Toh  
238 2008), with the default option L-INS-I, and were manually adjusted with *Se-AL* 2.0a11 Carbon  
239 (Rambaut 2002).

240 We concatenated all the markers in a single matrix and inferred phylogenetic relationships using  
241 MrBayes v3.2 (Ronquist *et al.* 2012) in a dataset including the sequences of 371 aphid individuals  
242 (*specimens dataset*) and a reduced dataset (*species dataset*) including only one specimen per  
243 phylogenetic cluster retrieved in the delineation test (128 species; see below). We evaluated  
244 different partitioning strategies of the datasets using Bayes factor comparisons of the harmonic  
245 mean to determine the best-fit partitioned scheme: *UnPart* (single data partition), *GenePart*  
246 (partitioned by gene), *PartFind* (partitioned following Partition Finder results), and *MixedPart* (the  
247 mixed model described below). For the *PartFind* scheme, we used the partitioning scheme and  
248 across-site rate variation suggested by PartitionFinder v1.1 (Lanfear *et al.* 2012); we inferred the best  
249 substitution model for each partition among those available in MrBayes, using the Bayesian  
250 Information Criterion (BIC) metric under a *greedy* algorithm. For the *MixedPart* scheme, instead of *a*  
251 *priori* applying a specific substitution model for each partition, we sampled across the substitution  
252 model space using a reversible-jump Markov Chain Monte Carlo (rj-MCMC), with the option  
253 *nst=mixed*. This procedure integrates the uncertainty concerning the correct structure of the  
254 substitution model (Huelsenbeck *et al.* 2004). The best-fit partitioned scheme was the *PartFind*  
255 (Supplementary information Table S1), which was used for subsequent analyses. To prevent the

256 overestimation of branch lengths when mutation rates differ between partitions of different genes  
257 (Brown *et al.* 2010) and between regions of single genes (Brown *et al.* 2010; Meseguer *et al.* 2013),  
258 we set the value of the shape parameter  $\lambda$ , which controls the exponential prior for branch lengths,  
259 to  $\lambda = 100$ , assigning greater probability to short branches. We conducted 2 independent runs of 4  
260 Metropolis-coupled chains each for 40 million generations, sampling every 1000 generations and  
261 discarding 20% as burnin.

262

263 *Aphid species delimitation.* Aphids show considerable overlap in their morphological characters;  
264 consequently, their identification often relies on biological traits such as host-plant associations,  
265 which renders further investigations on the evolution of aphid life-history traits tautological (Coeur  
266 d'acier *et al.* 2014). To avoid this bias, we complemented the morphological identifications of  
267 specimens with DNA-based species delimitation analyses. We used the Bayesian implementation of  
268 the Poisson tree processes (BPTP) model (Zhang *et al.* 2013) to delimit putative *Cinara* species. We  
269 ran the analysis for 500.000 generations, thinning every 100, and discarding 0.1 % as burnin. We  
270 considered the clusters retrieved in this analysis as “phylogenetic species” and repeated the  
271 phylogenetic analyses including only one individual per phylogenetic species.

272

### 273 **Phylogenetic comparative analyses**

274 We tested whether endosymbiotic associations were phylogenetically conserved using the  $\lambda$  of *Pagel*  
275 (Pagel 1994). It is a quantitative measure that varies between 0 (when there is no phylogenetic signal  
276 in the trait) and 1 (when there is phylogenetic signal). We optimized the value of lambda for the  
277 presence/absence of each bacterial lineage found in our samples onto the species tree using  
278 maximum-likelihood (ML) in *geiger* (Harmon *et al.* 2008). The presence/absence of *Serratia*, *Erwinia*,  
279 *Sodalis*, *Wolbachia*, *Hamiltonella*, *Type-X*, and *Acetobacteraceae* were modelled as binary traits. We  
280 did not analyse other endosymbionts detected in our study since they were poorly represented in  
281 our samples nor fixed within *Cinara* species (i.e. they never infected all the individuals of the same

282 species). The species-level analysis excluded *Rickettsia*, *Regiella* and *Arsenophonus* that were fixed in  
283 few species. To test if  $\lambda$  was significantly different from 0 we compared a model with the observed  
284 value of  $\lambda$  to a model with a fixed  $\lambda$  of zero using a likelihood ratio test.

285 We inferred ancestral associations of *Cinara* with each bacteria showing phylogenetic signal using ML  
286 in *ape* (Paradis *et al.* 2004) over the species phylogeny. Symbiotic associations were treated as a  
287 discrete character with 7 states: *Serratia*, *Erwinia*, *Sodalis*, *Type-X*, *Wolbachia*, *Hamiltonella* and “No  
288 cosymbiont”, for species in which no bacterium, apart from *Buchnera*, was fixed in the species.

289 Transition probabilities between character states were estimated under two models; equal rates  
290 “ER” and all-rates different “ARD” model, where all possible transitions between states receive  
291 distinct parameters. A likelihood ratio test was used to select the most appropriate model.

292 We evaluated the factors that were correlated with the presence of symbionts across *Cinara* species  
293 using logistic phylogenetic regressions (Ives & Garland 2010) in *phylolm* (Ho & Ané 2014). We fitted  
294 five different regression models for each of the bacteria exhibiting phylogenetic signal, with each  
295 time its presence/absence as the dependent variable and one explanatory variable. We assessed the  
296 significance of the correlations by comparing these models with a null model using AIC. We chose  
297 explanatory variables that reflect several dimensions of the aphid’s ecological niches and are  
298 generally used to explain the distribution of secondary symbionts across aphid populations. We  
299 included three life-history traits related to host-plant use: (i) *host-plant genera*; i.e. *Pinus*, *Picea*,  
300 *Cupressaceae*, *Larix*, *Pseudotsuga*, *Abies* or *Cedrus*, (ii) *feeding range*; whether species were  
301 monophagous (feeding on a single plant species or a few closely related species) or polyphagous, and  
302 (iii) *feeding site*; whether species fed on lignified parts of the plant (branches and trunks) or not  
303 (needles, shoots, young twigs or at the base of new cones). These characters likely reflect the panel  
304 of variations in the metabolic needs of aphid species. We also tested (iv) the role of *aphids’ life habit*;  
305 i.e. whether aphids lived solitarily or in dense colonies. Differences in life habit as well as variations in  
306 host-plant use are likely associated with variations in the communities of natural enemies of aphids,  
307 which might favour alternative defensive symbionts (Cayetano & Vorburger 2015; Henry *et al.* 2015;

308 Oliver *et al.* 2008; Smith *et al.* 2015). We assigned character states by combining information  
309 available in the literature for each recognized *Cinara* species (Blackman & Eastop 1994; Jouselin *et*  
310 *al.* 2013) and information recorded from the field in the course of aphid sampling. We did not  
311 explore the effect of aphid life cycle nor the association of aphids with ants as all *Cinara* species are  
312 monoecious and almost all are attended by ants. Recent studies underlined that expansion to new  
313 geographic areas could favour the acquisition of new bacterial partners in aphids (Zytyńska &  
314 Weisser 2016), we therefore investigated whether the *(v) aphids' geographic distribution*– i.e.  
315 whether species were distributed in the western or eastern parts of the Nearctic and the Palearctic–  
316 could explain variations in symbiont partnerships. Widespread geographic ranges in species of *Cinara*  
317 mostly resulted from recent dispersal events; we thus coded the distribution of these species  
318 according to the distribution of their most recent ancestor estimated in a previous study (Meseguer  
319 *et al.* 2015). The prevalence, distribution and abundance of symbionts (both obligate and facultative)  
320 across aphids can also vary with the temperature (Russell & Moran 2006), suggesting that symbiont  
321 turnover could be driven by climatic variations. We thus tested the effect of climatic variables in the  
322 distribution of symbionts. Climatic values of specimen records were extracted from 6 raster layers,  
323 Worldclim (Hijmans *et al.* 2005), at a resolution of 30 arc-seconds: annual mean temperature,  
324 temperature of the coldest and the warmest month, annual precipitation, and precipitation of the  
325 wettest and driest month. Multiple occurrences of a symbiont in the same grid cell were reduced to a  
326 single occurrence. We ran between-group principal component analysis (PCA) (Dolédec & Chessel  
327 1987) to compare climatic envelopes of symbionts on *ade4* (Dray & Dufour 2007). We tested the  
328 significance of the between-groups structure using Monte-Carlo permutation tests with 999  
329 replications. Temperature and precipitation ranges of symbionts were also visualized with barplots.

330

## 331 **Results**

332 *16S rDNA dataset description*

333 High-throughput sequencing of 16S rRNA bacterial genes from 371 individual aphids generated  
334 8.412.145 reads passing Illumina stringent quality control (mean number of reads per  
335 sample=11.246, standard deviation=9113; excluding negative controls). After all sequence filtering  
336 steps, we obtained 8.403.870 reads, corresponding to 182.913 unique sequences (Appendix 3). After  
337 discarding sequences accounting for less than 0.5% of the reads in each sample, we obtained 630  
338 unique sequences. Overall, 88.7% of these sequences were attributed to Enterobacteriaceae  
339 (*Arsenophonus*, *Buchnera*, *Edwardsiella*, *Erwinia*, *Hamiltonella*, *Regiella*, *Serratia*, *Sodalis*), 2.7% to  
340 Rickettsiaceae (*Rickettsia*, *Wolbachia*), 1.6% to Acetobacteraceae, 0.6% to *Acinetobacter* and 0.3% to  
341 *Spiroplasma*. The remaining 6% of the sequences were assigned to families containing water- and  
342 soil-borne bacteria (e.g. Comamonadaceae, Flavobacteriaceae or Methylobacteriaceae), each of  
343 which occurred at very low frequency (Appendix 2). The removal of sequences accounting for less  
344 than 0.5% of the reads in aphid samples eliminated most of the sequences common to negative  
345 controls (Fig. S1). The bacterial taxonomic compositions of aphid samples were highly similar across  
346 PCR replicates ( $r^2 > 0.99$ ; Fig. S2), except for *Acinetobacter* ( $r^2 < 0.8$ ) which did not appear in similar  
347 proportions in replicates.

348

#### 349 *Diversity of symbionts associated with Cinara*

350 Of the 371 aphid specimens examined here, 218 hosted two bacteria: *B. aphidicola* and a second  
351 partner belonging to various lineages: *Serratia*, *Erwinia*, *Sodalis*, *Wolbachia* or a non-described  
352 lineage of Enterobacteriaceae. The 16S rDNA sequence of the latter is highly similar to the one of the  
353 secondary symbiont found associated with *Acyrtosiphon pisum*, named *Type-X* (Guay *et al.* 2009). We  
354 thus referred to this symbiont as *Type-X* throughout the manuscript (Fig. 1, S3; Table S2). 146  
355 specimens hosted *Buchnera* alongside with 3–5 other bacterial partners). *Serratia*, *Erwinia*, *Sodalis*,  
356 *Wolbachia* and *Type-X* tended to be present in all the individuals of the species they infected, with an  
357 overall mean prevalence for each bacterium over 70% (i.e. each bacterium appeared, on average, in  
358 more than 70% of the specimens studied) (Fig. S4). Conversely, *Arsenophonus*, *Hamiltonella*, *Regiella*,

359 *Rickettsia*, *Acinetobacter* and *Acetobacteraceae* were generally detected in some, but not all of the  
360 individuals of the species they infected (the overall mean prevalence for each of these bacteria was  
361 below 60%). Seven *Cinara* specimens did not contain an alternative bacteria along with *Buchnera*: *C.*  
362 *laricis* (1 specimen out of the 3 studied was associated only with *Buchnera*), *C. kochi* (1/1), *C.*  
363 *brevispinosa* (2/11), *C. cf coloradensis* (1/2), *C. close piceae* (1/4) and *C. sp\_3210* (1/1).

364

### 365 *Symbiont specificity*

366 The association between *Serratia* and *Cinara* was generally species-specific, with 68% of *Cinara*  
367 species hosting a single *Serratia* 16S rRNA gene sequence, and 74% of *Serratia* 16S rRNA gene  
368 sequence found associated with a single *Cinara* species (Fig. S5). These patterns of species specificity  
369 were very similar to those observed for *Cinara* and *Buchnera* (Table 1; Fig. S6). For the species in  
370 which the presence of more than one *Serratia* haplotype was reported, some cases resulted from  
371 different bacterial haplotypes being present in individuals from different populations, whereas in  
372 other cases, up to two *Serratia* haplotypes were found in a single *Cinara* specimen (Appendix 2).  
373 These situations may represent cases of co-infection of an aphid with different *Serratia* strains or  
374 they might stem from slight divergences in 16S rDNA sequences from a single *Serratia* strain (slightly  
375 divergent bacterial chromosomes can sometimes occur within a single bacteriocyte (Komaki &  
376 Ishikawa 1999). More than half of the *Cinara* species infected with *Sodalis*-related bacteria or *Type-X*  
377 were found associated with more than one haplotype of these endosymbionts (Table 1; Fig. S5), a  
378 single specimen could host up to 7 different 16S rDNA sequences (Appendix 2) (again indicating co-  
379 infections or multiple 16S rDNA sequences in a single bacterial strain) whereas about 90% of these  
380 bacteria were specific to their host (i.e. found associated with a single *Cinara* species). Overall, 85%  
381 of the *Cinara* species infected with *Erwinia* contained a single haplotype, and 50% of the *Erwinia*  
382 haplotypes were host-specific (Fig. S5). For all these bacteria, there were cases in which the same  
383 haplotype was found to be present in different *Cinara* species. Shared haplotypes were generally  
384 found in closely related *Cinara* species, but a few haplotypes were well represented in distantly



385 related species (green lines in Fig. S5). We identified one *Serratia* 16S rRNA gene sequence that was  
386 present in 34 distantly related species of *Cinara*.

387

### 388 *Endosymbiont genomes*

389 In order to corroborate the role of *Buchnera*'s co-resident symbionts as the riboflavin providers, we  
390 sequenced the genomes of *Buchnera* and the secondary symbiont of *C. strobili* (*Sodalis*-like), *C.*  
391 *fornacula* (*S. symbiotica*), *C. pseudotaxifoliae* (*Erwinia*), and *C. confinis* (*Type-X*). The genomes of all  
392 *Buchnera* symbionts were assembled into one single circular scaffold plus the typical *leucine* and  
393 *tryptophan* plasmid. T genome of the *Erwinia*-like symbiont was assembled into one contig plus one  
394 circular plasmid and the genome of the *Serratia* was assembled into 1 contig. The genomes of the  
395 *Sodalis*-like and *Type-X* secondary symbionts were highly fragmented, given the high presence of  
396 mobile genetic elements. All four secondary symbionts hold small genomes, when compared to their  
397 free-living relatives, with the genomes of both *Erwinia* (circa 1.09Mb) and *Serratia* (ca. 1.16Mb)  
398 symbionts being the smallest. Full statistics for the genome assemblies can be found in Table S3.

399 Blastx searches for *riboflavin*, *tryptophan* and *biotin* biosynthetic genes revealed that none of the  
400 *Buchnera* strains is able to synthesize riboflavin while all co-resident symbionts preserve intact routes  
401 for the biosynthesis of this compound (Fig. 2).

402

### 403 *Phylogenetic reconstruction of Cinara and species delimitation*

404 The phylogeny of 371 aphid individuals was well resolved and highly sustained ( $pp >95$ ) (Fig. S7).  
405 Species delimitation analyses with BPTP identified 128 putative *Cinara* species (acceptance  
406 rate=0.18; range: 121–147) among the total of 371 specimens analyzed, separating morphological  
407 species into several clusters in a few cases (Fig. S7). The phylogeny including one specimen of each of  
408 the 128 species retrieved in the delineation test was also well resolved and strongly supported (Fig.  
409 S8).

410

411 *Comparative phylogenetic methods*

412 *Pagel's*  $\lambda$  test indicated a significant ( $p < 0.01$ ) phylogenetic signal over the species tree for the  
413 association with *Serratia*, *Erwinia*, *Type-X*, *Wolbachia* and *Sodalis* (Table S4). The ancestral character  
414 state reconstructions suggested that *Serratia* was acquired in the common ancestor of all Lachninae  
415 (Fig. S9). *Serratia* was then independently lost in eight lineages, and recently reacquired in *Cinara*  
416 *glabra*. *Sodalis*, *Wolbachia*, *Erwinia* and *Type-X* were acquired more recently in different lineages of  
417 *Cinara*, following the loss of *Serratia*, although *Type-X* and *Wolbachia* persisted alongside *Serratia* in  
418 a small group of species.

419 The presence of different cosymbionts across *Cinara* species could not be explained by any of the  
420 aphid traits examined here, as the null models always fitted the data better than the models  
421 including explanatory variables (Table S5)—all of the obligate cosymbionts identified here were found  
422 in aphid species using a wide range of ecological niches (Fig. 3). The climatic envelopes of the main  
423 co-symbionts were not significantly different (inertia: 0.009;  $P = 0.298$ ; Fig. 4, S10)

424

425 **Discussion**

426 *Repeated evolution of dual symbiosis in Cinara*

427 The evolutionary history of *Cinara* has been accompanied by major changes in its symbiotic partners.  
428 We show that, during the course of its diversification, *Cinara* has acquired different cosymbionts that  
429 reside with their primary symbiont, *Buchnera aphidicola*. In 79% of the 128 species studied here,  
430 *Buchnera* was found to coexist with *Serratia*, whereas, in the remaining 21%, *Serratia* was replaced  
431 by a bacterium related to *Erwinia*, *Sodalis* or an unknown member of the Enterobacteriaceae  
432 referred to here as *Type-X* (Fig. 1, Table S2). The tendency of these bacteria to be present in all the  
433 specimens of the species they infect (Fig. S4), throughout their geographic range and on their various  
434 host-plants, together with the phylogenetic conservatism of the associations (Table S4), suggests that  
435 they have established long-term relationships with their aphid hosts. The analyses of the  
436 endosymbiont genomes confirm that none of the *Buchnera* strains newly sequenced here is able to

437 synthesize *riboflavin*, while all secondary symbionts preserve intact routes for the biosynthesis of this  
438 compound and would be capable of putatively complementing the truncated *biotin* biosynthetic  
439 pathway (Fig. 2). Altogether, these results, along with recent studies within Lachninae (Manzano-  
440 Marín *et al.* 2016b) add to the mounting evidence that all *Cinara* species and probably all Lachninae  
441 shelter a di-symbiotic system: *Serratia*, *Erwinia*, *Sodalis* and Type-X coexist with *Buchnera* as co-  
442 obligate partners. The absence of *riboflavin* biosynthetic genes in all currently sequenced *Buchnera*  
443 from *Cinara* aphids (Lamelas *et al.* 2011b; Manzano-Marín *et al.* 2016a; Perez-Brocal *et al.* 2006)  
444 suggests that the *Buchnera* from the *Cinara* last common ancestor (CLCA) was probably dependent  
445 on a secondary endosymbiont for the biosynthesis of this essential vitamin. Also, given the *Buchnera*  
446 gene content, it is highly likely that the CLCA developed a *biotin* auxotrophy: the biosynthesis of this  
447 vitamin could be split between *Buchnera* and its companion symbiont (Fig. 2). The finding that  
448 *Buchnera* seems to lack a co-resident symbiont in a few specimens (7 individuals in our sampling, Fig.  
449 S3) could challenge this interpretation— it could suggest that some individuals rely only on *Buchnera*  
450 and thus that the primary symbiont genome is still fully functional or that the aphid can fulfill the  
451 functions lost in *Buchnera*; it could have acquired this ability through horizontal gene transfer.  
452 Alternatively, the failure to detect a co-symbiont in these individuals could be the result of  
453 bacteriocyte size reduction and/or symbiont degradation that occurs with aphid aging. This  
454 phenomenon has been described for *Buchnera* bacteriocytes in the pea aphid (Simonet *et al.* 2016).  
455 It is also possible that individuals lose their co-obligate symbiont during their development; recent  
456 studies have shown that the cereal weevil *Sitophilus pierantonius*, eliminates its obligate symbiont  
457 (*Sodalis pierantonius*) when it no longer needs it (Vigneron *et al.* 2014). A thorough investigation of  
458 symbiont cell localization within their host and dynamics throughout the aphid development will be  
459 needed to validate any of these interpretations. Specimens without a co-symbiont mostly belonged  
460 to the *Cinara* clade associated with *Erwinia*; it will be interesting to follow bacteria cell population  
461 dynamics of this symbiotic association.

462 The obligate association of *Serratia* with *C. tujaefilina*, *C. cedri* and *Tuberolachnus salignus* has already  
463 been demonstrated through genome-based metabolic inference (Lamelas *et al.* 2011b; Manzano-  
464 Marin & Latorre 2014; Manzano-Marín *et al.* 2016a). Associations of aphids and bacteria related to  
465 *Sodalis* have rarely been documented. However specific PCR assays and histological work in  
466 Lachninae (Burke *et al.* 2009; Manzano-Marín *et al.* 2016b) have shown that *Sodalis*-related bacteria  
467 were associated with several species, and suggested that these bacteria could have replaced *Serratia*  
468 in different Lachninae lineages. *Sodalis*-related bacteria are actually ubiquitous in insects and have  
469 seemingly established obligate associations with various hemipterans (Husnik & McCutcheon 2016;  
470 Koga *et al.* 2013; Koga & Moran 2014; Oakeson *et al.* 2014; Snyder *et al.* 2011). *Type-X* has also been  
471 detected in members of the Lachninae and its presence was interpreted as a replacement for  
472 *Serratia* in some lineages (Manzano-Marín *et al.* 2016b). Bacteria related to *Erwinia* are generally  
473 free-living plant pathogens. However, it has been suggested that they can act as symbiotic partners  
474 of aphids (Clark *et al.* 2012; Harada *et al.* 1997), though it is usually assumed to be an arthropod gut  
475 symbiont. This is the first study to show an *Erwinia* related lineage to be an obligate symbiotic  
476 partner of aphids.

477 Most *Cinara* species host only two obligate partners. However, in some species, *Type-X* was found  
478 with both *Serratia* and *Buchnera* in all the individuals sampled (Fig. 1). This either suggests that some  
479 *Cinara* species have more than two obligate symbionts, as observed in other arthropods (Koga *et al.*  
480 2013), or that these species are currently in a transitional state, in which the ancestral *Serratia* (Fig.  
481 S9) has not yet been eliminated. A third possibility is that one of the co-symbionts represents a more  
482 recent facultative infection. This may apply to the species hosting *Wolbachia*. Although this  
483 bacterium is usually found in all individuals of the species it infects (Fig. S4) and associated with  
484 *Serratia*, *Wolbachia* has not established a long-term association with *Cinara*, as it is not fixed in any  
485 particular clade of the genus. *Wolbachia* may probably be a facultative symbiont that is widespread  
486 in aphids' populations thanks to its ability to manipulate reproduction, as previously suggested  
487 (Augustinos *et al.* 2011; Gomez-Valero *et al.* 2004). We have detected many individuals in which the

488 cosymbionts described above are present together with other bacteria (*Arsenophonus*, *Edwardsiella*,  
489 *Regiella*, *Rickettsia*, *Hamiltonella* or *Spiroplasma*; Fig. 1, S3). These additional partners are probably  
490 facultative infections, because they occur sporadically within the species and their association with  
491 *Cinara* is not evolutionarily stable (Fig. S4, Table S4).

492

#### 493 *Evolutionary history of symbiotic associations*

494 Two evolutionary scenarios could explain the distribution of co-symbionts observed here. *Serratia*  
495 may have infected an ancestor of the Lachninae (Fig. S9), a subfamily that originated more than 70  
496 million years ago (Chen *et al.* 2015; Meseguer *et al.* 2015). The division of labor in the nutrition of  
497 *Cinara* may have been established then (Fig. 2) (Lamelas *et al.* 2011a; Lamelas *et al.* 2011b). At  
498 various times points between the Oligocene and the present, *Serratia* may then have been lost from  
499 several clades of *Cinara* (Fig. S9). These losses were associated with the acquisition of an alternative  
500 co-resident, *Erwinia*, *Type-X* or *Sodalis*. This scenario implies a long history of cospeciation between  
501 *Cinara* and *Serratia*, followed by further cospeciation between *Cinara* and its more recently acquired  
502 cosymbionts. Alternatively, there may have been multiple independent colonizations of an already  
503 diversified *Cinara* genus by several lineages of *Serratia*, *Erwinia*, *Type-X* and *Sodalis*.

504 We cannot conduct robust cospeciation tests using endosymbiont phylogenies based on the short  
505 16S rRNA marker sequenced here. However, the patterns of specificity revealed here shed some light  
506 on the history of the symbiotic associations between *Cinara* and its bacterial symbionts. The  
507 distribution of 16S *Serratia* haplotypes across *Cinara* species suggests that the codiversification  
508 history of *Serratia* and *Cinara* probably involved both cospeciation and multiple infections. On the  
509 one hand, the interaction between the two partners is generally species-specific (Fig. S5, Table 1),  
510 which is consistent with cospeciation scenarios. On the other hand, several *Serratia* strains are  
511 common to distantly related aphid species (e.g. one *Serratia* haplotype is present in 34 unrelated  
512 *Cinara* species), and most of the species containing these haplotypes are also infected with another  
513 *Serratia* strain. Furthermore, previous studies suggest that the *Serratia* strains associated with *C.*

514 *cedri* and *C. tujafilina* could belong to two distantly related lineages (Manzano-Marin & Latorre 2014;  
515 Manzano-Marín *et al.* 2016a). Altogether, these findings demonstrate that, during the course of its  
516 evolution, *Cinara* has experienced multiple infections with different lineages of *Serratia*. Some  
517 lineages may have infected the ancestors of *Cinara*, establishing an obligate association and partly  
518 cospeciated with them, whereas other *Serratia* lineages may have infected different species of the  
519 genus more recently. Thus obligate and facultative *Serratia* strains might even be found co-infecting  
520 the same aphid (Fig. S5). This would explain why previous studies based on phylogenetic analyses of  
521 *Serratia* 16S rDNA sequences rejected the hypothesis of cospeciation between *Serratia* and *Cinara*  
522 (Burke *et al.* 2009; Lamelas *et al.* 2008).

523 Similarly, the overall patterns of *Type-X* and *Sodalis* specificity for their hosts suggest that these  
524 associations may result from a combination of cospeciation and host-switches (Fig. S5).  
525 Codiversification scenarios will be difficult to unravel for these associations, as the presence of  
526 multiple, slightly divergent, bacterial haplotypes in each aphid specimen (Appendix 2) suggests some  
527 co-infections by several strains, and/or that the 16S rRNA gene is present in multiple copies in a  
528 single symbiont type (Koga *et al.* 2013). The association of *Cinara* and *Erwinia* is not species-specific,  
529 although each *Cinara* species generally contains only one *Erwinia* haplotype, closely related aphid  
530 species harbor *Erwinia* strains of the same haplotype. This suggests a lack of differentiation in *Erwinia*  
531 during the speciation of aphids, or that the DNA fragment studied here may not have been variable  
532 enough to reflect interspecific variation.

533 The patterns of symbiont-aphid specificity depicted here show that future studies should make use  
534 of several single-copy bacterial DNA markers and take into account coinfections by several strains  
535 and possibly bacterial cell polyploidy— *i.e.* the presence of many, slightly divergent, bacterial  
536 chromosomes in a single bacteriocyte (Komaki & Ishikawa 1999)— in the investigation of  
537 codiversification history between *Cinara* and its obligate symbionts.

538

539 *Which factors drive symbiont replacement?*

540 The repeated evolution of dual symbiosis in *Cinara* provides us with a unique opportunity to  
541 investigate the factors favoring one co-resident symbiont over another in symbiotic associations  
542 between aphids and bacteria. The presence of facultative symbionts in aphid populations, and  
543 insects in general, is generally explained by the ecological niche occupied by their hosts (Henry *et al.*  
544 2015; Liberti *et al.* 2015; Oliver *et al.* 2010). However, little is currently known about the forces  
545 governing the evolution of these associations when the symbionts are essential for the system to  
546 survive. We found that changes in obligate co-symbionts were not correlated with evolutionary  
547 transitions in *Cinara* (Table S5). All of the obligate co-symbionts identified here were found in aphid  
548 species using a wide range of ecological niches (Fig. 3, 4). This implies that the acquisition of new co-  
549 symbionts did not trigger the adaptation of the host aphid to environmental conditions. The addition  
550 of a new partner to symbiotic associations could be explained by a degradation of the functions of  
551 the existing partner (e.g. due to genetic drift) (Bennett & Moran 2015). In a dual symbiotic systems in  
552 leafhoppers (Bennett *et al.* 2014), the most recently recruited symbiont has been shown to possess a  
553 less stable genome than the ancient symbiont and has been repeatedly replaced. A similar situation  
554 may apply to our model system; a loss of symbiotic functions in *Serratia* may have favored the  
555 establishment of an alternative bacterium, while *Buchnera* persisted in the association. Alternatively,  
556 the relatively rapid turnover of cosymbionts alongside *Buchnera* may result from competitive  
557 interactions between bacteria with similar metabolic capabilities. Essential functions have been lost  
558 from *Buchnera* in an ancestor of Lachninae (Fig. 2, S9) (Manzano-Marín *et al.* 2016a). The delegation  
559 of these symbiotic functions of *Buchnera* to a bacterial partner may have paved the way for  
560 colonization by any bacterium with similar metabolic capacities. In this scenario, the identity of the  
561 new partner depends on the outcome of competition between bacteria, regulated by their  
562 population dynamics (*i.e.*, demographic advantages due to higher replication rates) rather than the  
563 selective advantages they confer on the host.

564

565 **Conclusions**

566 We report here a highly dynamic di-symbiotic system in the second most diverse aphid genus. An  
567 additional endosymbiotic partner is required in *Cinara* to complement *Buchnera* in its nutritional  
568 role. Interestingly, this new "flatmate" has been repeatedly replaced during the diversification of the  
569 group but it never replaces *Buchnera*. This mirrors findings for other sap-feeding insects where the  
570 primary symbiont is supplemented by an additional bacterium. In Auchenorrhyncha, the ancestral  
571 symbiont, *Sulcia*, coexists with another obligate symbiont, the identity of which differs between  
572 lineages (Koga *et al.* 2013). In some mealybugs, the primary symbiont shelter within its cells another  
573 Gammaproteobacterium symbiont that has also been repeatedly replaced (Husnik & McCutcheon  
574 2016). The aphid sister group (Adelgidae), has not established a long-term association with a primary  
575 symbiont, but is found associated with a diverse set of obligate bacterial partners throughout its  
576 evolutionary history (Toenshoff *et al.* 2012; Toenshoff *et al.* 2014). Our results suggest that the  
577 succession of essential symbionts does not necessarily result from the adaptation of their hosts to  
578 changing ecological conditions. It might therefore be driven by factors intrinsic to the evolutionary  
579 dynamics of endosymbionts, such as rapid genome deterioration or the competitive displacement of  
580 symbionts providing similar benefits to the host.

581

#### 582 **Authors Contributions**

583 **EJ, ASM, ACA** designed the study. **ASM** and **EJ** wrote the paper with contributions of **AMM**. **ASM**,  
584 **AMM** and **MG** analysed the data. **ALC** performed the molecular work. **ACA** identified the aphid  
585 specimens. **ACA** and **EJ** collected the specimens.

586

#### 587 **Acknowledgements**

588 Research funding was provided by a Marie-Curie FP7-COFUND (AgreenSkills fellowship-26719) to  
589 ASM, the ANR PhyloSPACE and the project ("*Cinara's* microbiome") from the Agropolis  
590 foundation/Labex Agro to EJ. The authors are grateful to the CBGP-HPC computational platform, J.



591 Abbat and S. Joly for help with statistical analyses. To F. Condamine, F. Kjellberg and the three  
592 anonymous reviewers for comments on the manuscript.

593

#### 594 **Data Accessibility:**

595 The 16S rRNA sequence data produced in this study are available from the Dryad Digital Repository  
596 (doi:10.5061/dryad.9jn6r). Appendices 1–3 are also accessible in Dryad (doi:10.5061/dryad.sk130);  
597 Appendix 1 includes collection details for aphid samples and GenBank accession numbers. Appendix  
598 2 shows a contingency table reporting bacterial sequence occurrences across aphid samples, and  
599 Appendix 3 includes a summary of the number of reads and unique sequences per sample. DNA  
600 sequences for the aphid phylogeny are available in Genbank accessions KY064183–KY064504. Reads  
601 used for genome assembly as well as draft assemblies for the endosymbionts' genomes have been  
602 deposited in the European Nucleotide Archive under the project numbers PRJEB15400 (*C. fornacula*),  
603 PRJEB15504 (*C. confinis*), PRJEB15506 (*C. pseudotaxifoliae*) and PRJEB15507 (*C. strobi*).

604

#### 605 **References**

606 Augustinos AA, Santos-Garcia D, Dionyssopoulou E, *et al.* (2011) Detection and characterization of  
607 *Wolbachia* infections in natural populations of Aphids: Is the hidden diversity fully  
608 unraveled? *Plos One* **6**, e28695.  
609 Bankevich A, Nurk S, Antipov D, *et al.* (2012) SPAdes: a new genome assembly algorithm and its  
610 applications to single-cell sequencing. *Journal of Computational Biology* **19**, 455–477.  
611 Bennett GM, McCutcheon JP, MacDonald BR, Romanovicz D, Moran NA (2014) Differential genome  
612 evolution between companion symbionts in an insect-bacterial symbiosis. *Mbio* **5**.  
613 Bennett GM, Moran NA (2015) Heritable symbiosis: The advantages and perils of an evolutionary  
614 rabbit hole. *Proceedings of the National Academy of Sciences* **112**, 10169–10176.  
615 Blackman RL, Eastop VF (1994) *Aphids on the world trees: an identification and information guide* The  
616 Natural History Museum, London, UK.  
617 Blackman RL, Eastop VF (2000) *Aphids on the world's crops: an identification and information guide*,  
618 Second edition edn. Willey and sons, New York.  
619 Brown JM, Hedtke SM, Lemmon AR, Lemmon EM (2010) When trees grow too long: Investigating the  
620 causes of highly inaccurate bayesian branch-length estimates. *Systematic Biology* **59**, 145-  
621 161.  
622 Buchner P (1965) *Endosymbiosis of animal with plant microorganisms* Interscience, New York.  
623 Burke GR, Normark BB, Favret C, Moran NA (2009) Evolution and diversity of facultative symbionts  
624 from the aphid subfamily Lachninae. *Applied and Environmental Microbiology* **75**, 5328-5335.  
625 Caporaso JG, Kuczynski J, Stombaugh J, *et al.* (2010) QIIME allows analysis of high-throughput  
626 community sequencing data. *Nature Methods* **7**, 335-336.  
627 Cayetano L, Vorburger C (2015) Symbiont-conferred protection against Hymenopteran parasitoids in  
628 aphids: how general is it? *Ecological Entomology* **40**, 85-93.

- 629 Charles H, Ishikawa A (1999) Physical and genetic map of the genome of *Buchnera*, the primary  
630 endosymbiont of the pea aphid *Acyrtosiphon pisum*. *Journal of Molecular Evolution* **48**, 142-  
631 150.
- 632 Chen R, Favret C, Jiang L, Wang Z, Qiao G (2015) An aphid lineage maintains a bark-feeding niche  
633 while switching to and diversifying on conifers. *Cladistics* DOI: [10.1111/cla.12141](https://doi.org/10.1111/cla.12141).
- 634 Clark EL, Daniell TJ, Wishart J, Hubbard SF, Karley AJ (2012) How conserved are the bacterial  
635 communities associated with aphids? A detailed assessment of the *Brevicoryne brassicae*  
636 (Hemiptera: Aphididae) using 16S rDNA. *Environmental Entomology* **41**, 1386-1397.
- 637 Coeur d'acier A, Cruaud A, Artige E, et al. (2014) DNA barcoding and the associated  
638 PhylAphidB@se website for the identification of European aphids (Insecta:  
639 Hemiptera: Aphididae). *Plos One* **9**, e97620.
- 640 Conord C, Despres L, Vallier A, et al. (2008) Long-term evolutionary stability of bacterial  
641 endosymbiosis in curculionoidea: Additional evidence of symbiont replacement in the  
642 dryophthoridae family. *Molecular Biology and Evolution* **25**, 859-868.
- 643 Dolédec S, Chessel D (1987) Rythmes saisonniers et composantes stationnelles en milieu aquatique. I:  
644 Description d'un plan d'observation complet par projection de variables. *Acta Oecologia* **8**,  
645 403-426.
- 646 Dormann CF, Gruber B, Fruend J (2008) Introducing the bipartite package: analysing ecological  
647 networks. *R news* **8**, 8-11.
- 648 Douglas AE (1998) Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic  
649 bacteria *Buchnera*. *Annual Review of Entomology* **43**, 17-37.
- 650 Dray S, Dufour A-B (2007) The ade4 package: implementing the duality diagram for ecologists.  
651 *Journal of Statistical Software* **22**, 1-20.
- 652 Drummond AJ, Ashton B, Buxton S, et al. (2010) Geneious v5.5. Available from  
653 <http://www.geneious.com>. Biomatters, Auckland, New Zealand.
- 654 Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of  
655 chimera detection. *Bioinformatics* **27**, 2194-2200.
- 656 Favret C, Voegtlin DJ (2004) A revision of the *Cinara* species (Hemiptera: Aphididae) of the United  
657 States Pinyon pines. *Annals of the Entomological Society of America* **97**, 1165-1197.
- 658 Flandrois J-P, Perrière G, Gouy M (2014) leBIBIQBPP: A set of databases and a webtool for automatic  
659 phylogenetic analysis of prokaryotic sequences. [http://umr5558-bibiserv.univ-  
660 lyon1.fr/lebibi/lebibi.cgi](http://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi)
- 661 Frago E, Dicke M, Godfray HCJ (2012) Insect symbionts as hidden players in insect-plant interactions.  
662 *Trends in Ecology & Evolution* **27**, 705-711.
- 663 Goecks J, Nekrutenko A, Taylor J, Team TG (2010) Galaxy: a comprehensive approach for supporting  
664 accessible, reproducible, and transparent computational research in the life sciences.  
665 *Genome Biology* **11**, R86.
- 666 Gomez-Valero L, Soriano-Navarro M, Perez-Brocal V, et al. (2004) Coexistence of *Wolbachia* with  
667 *Buchnera aphidicola* and a secondary symbiont in the aphid *Cinara cedri*. *Journal of*  
668 *Bacteriology* **186**, 6626-6633.
- 669 Gosalbes MJ, Lamelas A, Moya A, Latorre A (2008) The striking case of tryptophan provision in the  
670 cedar aphid *Cinara cedri*. *Journal of Bacteriology* **190**, 6026-6029.
- 671 Guay JF, Boudreault S, Michaud D, Cloutier C (2009) Impact of environmental stress on aphid clonal  
672 resistance to parasitoids: Role of *Hamiltonella defensa* bacterial symbiosis in association with  
673 a new facultative symbiont of the pea aphid. *Journal of Insect Physiology* **55**, 919-926.
- 674 Hansen AK, Moran NA (2014) The impact of microbial symbionts on host plant utilization by  
675 herbivorous insects. *Molecular Ecology* **23**, 1473-1496.
- 676 Harada H, Oyaizu H, Kosako Y, Ishikawa H (1997) *Erwinia aphidicola*, a new species isolated from pea  
677 aphid, *Acyrtosiphon pisum*. *Journal of General and Applied Microbiology* **43**, 349-354.
- 678 Harmon LJ, Weir JT, Brock CD, Glor RE, Challenger W (2008) GEIGER: investigating evolutionary  
679 radiations. *Bioinformatics* **24**, 129-131.

- 680 Henry LM, Maiden MCJ, Ferrari J, Godfray HCJ (2015) Insect life history and the evolution of bacterial  
681 mutualism. *Ecology Letters* **18**, 516–525.
- 682 Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A (2005) Very high resolution interpolated climate  
683 surfaces for global land areas. *International Journal of Climatology* **25**, 1965-1978.
- 684 Ho LST, Ané C (2014) A linear-time algorithm for Gaussian and non-Gaussian trait evolution models.  
685 *Systematic Biology* **63**, 397-408.
- 686 Huelsenbeck JP, Larget B, Alfaro ME (2004) Bayesian phylogenetic model selection using reversible  
687 jump Markov chain Monte Carlo. *Molecular Biology and Evolution* **21**, 1123–1133.
- 688 Husnik F, McCutcheon JP (2016) Repeated replacement of an intrabacterial symbiont in the tripartite  
689 nested mealybug symbiosis. *Proceedings of the National Academy of Sciences* **113**, E5416-  
690 E5424.
- 691 Ives AR, Garland T (2010) Phylogenetic logistic regression for binary dependent variables. *Systematic*  
692 *Biology* **59**, 9-26.
- 693 Jousselin E, Clamens AL, Galan M, *et al.* (2016) Assessment of a 16S rRNA amplicon Illumina  
694 sequencing procedure for studying the microbiome of a symbiont-rich aphid genus.  
695 *Molecular Ecology Resources*, n/a-n/a.
- 696 Jousselin E, Cruaud A, Genson G, *et al.* (2013) Is ecological speciation a major trend in aphids?  
697 Insights from a molecular phylogeny of the conifer-feeding genus *Cinara*. *Frontiers in Zoology*  
698 **10**, 56-73.
- 699 Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program.  
700 *Briefings in Bioinformatics* **9**, 286-298.
- 701 Koga R, Bennett GM, Cryan JR, Moran NA (2013) Evolutionary replacement of obligate symbionts in  
702 an ancient and diverse insect lineage. *Environmental microbiology* **15**, 2073-2081.
- 703 Koga R, Moran NA (2014) Swapping symbionts in spittlebugs: evolutionary replacement of a reduced  
704 genome symbiont. *Isme Journal* **8**, 1237-1246.
- 705 Komaki K, Ishikawa H (1999) Intracellular bacterial symbionts of aphids possess many genomic copies  
706 per bacterium. *Journal of Molecular Evolution* **48**, 717-722.
- 707 Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD (2013) Development of a dual-index  
708 sequencing strategy and curation pipeline for analyzing amplicon sequence data on the  
709 MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology* **79**, 5112-  
710 5120.
- 711 Lamelas A, Gosalbes MJ, Manzano-Marin A, *et al.* (2011a) *Serratia symbiotica* from the aphid *Cinara*  
712 *cedri*: a missing link from facultative to obligate insect endosymbiont. *Plos Genetics* **7**.
- 713 Lamelas A, Gosalbes MJ, Moya A, Latorre A (2011b) New clues about the evolutionary history of  
714 metabolic losses in bacterial endosymbionts, provided by the genome of *Buchnera aphidicola*  
715 from the aphid *Cinara tujafilina*. *Applied and Environmental Microbiology* **77**, 4446-4454.
- 716 Lamelas A, Perez-Brocail V, Gomez-Valero L, *et al.* (2008) Evolution of the secondary symbiont  
717 "*Candidatus Serratia symbiotica*" in aphid species of the subfamily Lachninae. *Applied and*  
718 *Environmental Microbiology* **74**, 4236-4240.
- 719 Lanfear R, Calcott B, Ho SYW, Guindon S (2012) PartitionFinder: combined selection of partitioning  
720 schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*  
721 **29**, 1695–1701.
- 722 Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nature Methods* **9**, 357-  
723 U354.
- 724 Liberti J, Sapountzis P, Hansen LH, *et al.* (2015) Bacterial symbiont sharing in Megalomyrmex social  
725 parasites and their fungus-growing ant hosts. *Molecular Ecology* **24**, 3151–3169.
- 726 Manzano-Marin A, Latorre A (2014) Settling down: the genome of *Serratia symbiotica* from the aphid  
727 *Cinara tujafilina* zooms in on the process of accommodation to a cooperative intracellular  
728 life. *Genome Biol Evol* **6**, 1683-1698.
- 729 Manzano-Marin A, Simon J-C, Latorre A (2016a) Reinventing the Wheel and Making It Round Again:  
730 Evolutionary Convergence in Buchnera – Serratia Symbiotic Consortia between the Distantly

- 731 Related Lachninae Aphids *Tuberolachnus salignus* and *Cinara cedri*. *Genome Biology and*  
732 *Evolution* **8**, 1440–1458.
- 733 Manzano-Marín A, Szabo G, Simon J-C, Horn M, Latorre A (2016b) Happens in the best of subfamilies:  
734 Replacement and internalisation of co-obligate *Serratia* endosymbionts in Lachninae aphids.  
735 *bioRxiv.org*, 059816.
- 736 McCutcheon JP, Moran NA (2010) Functional convergence in reduced genomes of bacterial  
737 symbionts spanning 200 My of evolution. *Genome Biology and Evolution* **2**, 708-718.
- 738 McCutcheon JP, Moran NA (2012) Extreme genome reduction in symbiotic bacteria. *Nature Reviews*  
739 *Microbiology* **10**, 13-26.
- 740 McCutcheon John P, von Dohlen Carol D An interdependent metabolic patchwork in the nested  
741 symbiosis of Mealybugs. *Current Biology* **21**, 1366-1372.
- 742 Meseguer AS, Aldasoro JJ, Sanmartin I (2013) Bayesian inference of phylogeny, morphology and  
743 range evolution reveals a complex evolutionary history in St. John's wort (*Hypericum*).  
744 *Molecular Phylogenetics and Evolution* **67**, 379-403.
- 745 Meseguer AS, Coeur d'acier A, Genson G, Jousset E (2015) Unravelling the historical biogeography  
746 and diversification dynamics of a highly diverse conifer-feeding aphid genus. *Journal of*  
747 *Biogeography* **42**, 1482-1492.
- 748 Mizrahi-Man O, Davenport ER, Gilad Y (2013) Taxonomic Classification of Bacterial 16S rRNA Genes  
749 Using Short Sequencing Reads: Evaluation of Effective Study Designs. *Plos One* **8**.
- 750 Moran NA (1996) Accelerated evolution and Muller's ratchet in endosymbiotic bacteria. *Proceedings*  
751 *of the National Academy of Sciences of the United States of America* **93**, 2873-2878.
- 752 Moran NA, Telang A (1998) Bacteriocyte-associated symbionts of insects - A variety of insect groups  
753 harbor ancient prokaryotic endosymbionts. *Bioscience* **48**, 295–304.
- 754 Oakeson KF, Gil R, Clayton AL, *et al.* (2014) Genome degeneration and adaptation in a nascent stage  
755 of symbiosis. *Genome Biology and Evolution* **6**, 76-93.
- 756 Oliver KM, Campos J, Moran NA, Hunter MS (2008) Population dynamics of defensive symbionts in  
757 aphids. *Proceedings of the Royal Society B-Biological Sciences* **275**, 293-299.
- 758 Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative symbionts in aphids and the  
759 horizontal transfer of ecologically important traits. *Annual Review of Entomology* **55**, 247-  
760 266.
- 761 Oliver KM, Martinez AJ (2014) How resident microbes modulate ecologically-important traits of  
762 insects. *Current Opinion in Insect Science* **4**, 1-7.
- 763 Pagel M (1994) Detecting correlated evolution on phylogenies: a general method for the comparative  
764 analysis of discrete characters. *Proceedings of the Royal Society B-Biological Sciences* **255**, 37-  
765 45.
- 766 Paradis E, Claude J, Strimmer K (2004) APE: Analyses of phylogenetics and evolution in R language.  
767 *Bioinformatics* **20**, 289-290.
- 768 Perez-Brocal V, Gil R, Ramos S, *et al.* (2006) A small microbial genome: The end of a long symbiotic  
769 relationship? *Science* **314**, 312-313.
- 770 Quast C, Pruesse E, Yilmaz P, *et al.* (2013) The SILVA ribosomal RNA gene database project: improved  
771 data processing and web-based tools. *Nucleic Acids Research* **41**, D590-D596.
- 772 Rambaut A (2002) Se-AL: sequence alignment editor. Available:  
773 <http://tree.bio.ed.ac.uk/software/seal/>.
- 774 Rispe C, Moran NA (2000) Accumulation of deleterious mutations in endosymbionts: Muller's ratchet  
775 with two levels of selection. *American Naturalist* **156**, 425–441.
- 776 Ronquist F, Teslenko M, Van Der Mark P, *et al.* (2012) Mrbayes 3.2: Efficient bayesian phylogenetic  
777 inference and model choice across a large model space. *Systematic Biology* **61**, 539-542.
- 778 Russell JA, Moran NA (2006) Costs and benefits of symbiont infection in aphids: variation among  
779 symbionts and across temperatures. *Proceedings of the Royal Society B-Biological Sciences*  
780 **273**, 603-610.

- 781 Schloss PD, Westcott SL (2011) Assessing and improving methods used in operational taxonomic unit-  
782 based approaches for 16S rRNA gene sequence analysis. *Applied and Environmental*  
783 *Microbiology* **77**, 3219-3226.
- 784 Schmieder R, Edwards R (2011) Quality control and preprocessing of metagenomic datasets.  
785 *Bioinformatics* **27**, 863-864.
- 786 Simonet P, Duport G, Gaget K, *et al.* (2016) Direct flow cytometry measurements reveal a fine-tuning  
787 of symbiotic cell dynamics according to the host developmental needs in aphid symbiosis.  
788 *Scientific Reports* **6**, 19967.
- 789 Smith AH, Łukasik P, O'Connor MP, *et al.* (2015) Patterns, causes and consequences of defensive  
790 microbiome dynamics across multiple scales. *Molecular Ecology* **24**, 1135-1149.
- 791 Smith W, Oakeson K, Johnson K, *et al.* (2013) Phylogenetic analysis of symbionts in feather-feeding  
792 lice of the genus *Columbicola*: evidence for repeated symbiont replacements. *BMC*  
793 *Evolutionary Biology* **13**, 109.
- 794 Snyder AK, McMillen CM, Wallenhorst P, Rio RVM (2011) The phylogeny of *Sodalis*-like symbionts as  
795 reconstructed using surface-encoding loci. *FEMS Microbiology Letters* **317**, 143-151.
- 796 Takiya DM, Tran PL, Dietrich CH, Moran NA (2006) Co-cladogenesis spanning three phyla: leafhoppers  
797 (Insecta: Hemiptera: Cicadellidae) and their dual bacterial symbionts. *Molecular Ecology* **15**,  
798 4175-4191.
- 799 Toenshoff ER, Gruber D, Horn M (2012) Co-evolution and symbiont replacement shaped the  
800 symbiosis between adelgids (Hemiptera: Adelgidae) and their bacterial symbionts.  
801 *Environmental microbiology* **14**, 1284-1295.
- 802 Toenshoff ER, Szabo G, Gruber D, Horn M (2014) The pine bark adelgid, *Pineus strobi*, contains two  
803 novel bacteriocyte-associated Gammaproteobacterial symbionts. *Applied and Environmental*  
804 *Microbiology* **80**, 878-885.
- 805 Toft C, Andersson SGE (2010) Evolutionary microbial genomics: insights into bacterial host  
806 adaptation. *Nature Reviews Genetics* **11**, 465-475.
- 807 Toju H, Tanabe AS, Notsu Y, Sota T, Fukatsu T (2013) Diversification of endosymbiosis: replacements,  
808 co-speciation and promiscuity of bacteriocyte symbionts in weevils. *Isme Journal* **7**, 1378-  
809 1390.
- 810 Vigneron A, Masson F, Vallier A, *et al.* (2014) Insects recycle endosymbionts when the benefit is over.  
811 *Current Biology* **24**, 2267-2273.
- 812 Warnes GR, Bolker B, Bonebakker L, *et al.* (2015) gplots: various R programming tools for plotting  
813 data. R package.
- 814 Wernegreen JJ (2012) Mutualism meltdown in insects: bacteria constrain thermal adaptation.  
815 *Current Opinion in Microbiology* **15**, 255-262.
- 816 Wilson ACC, Ashton PD, Calevro F, *et al.* (2010) Genomic insight into the amino acid relations of the  
817 pea aphid, *Acyrtosiphon pisum*, with its symbiotic bacterium *Buchnera aphidicola*. *Insect*  
818 *Molecular Biology* **19**, 249-258.
- 819 Wu D, Daugherty SC, Van Aken SE, *et al.* (2006) Metabolic complementarity and genomics of the dual  
820 bacterial symbiosis of sharpshooters. *PLoS Biology* **4**, 1079-1092.
- 821 Zachos JC, Dickens GR, Zeebe RE (2008) An early Cenozoic perspective on greenhouse warming and  
822 carbon-cycle dynamics. *Nature* **451**, 279-283.
- 823 Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with  
824 applications to phylogenetic placements. *Bioinformatics* **29**, 2869-2876.
- 825 Zytynska SE, Weisser WW (2016) The natural occurrence of secondary bacterial symbionts in aphids.  
826 *Ecological Entomology* **41**, 13-26.

827

828

## 829 Tables

830 **Table 1.** Aphid-symbiont specificity. Only well represented haplotypes (representing >1% of the reads  
831 in a sample) are considered.

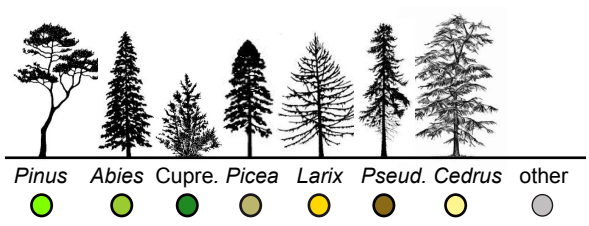
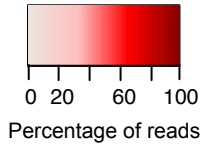
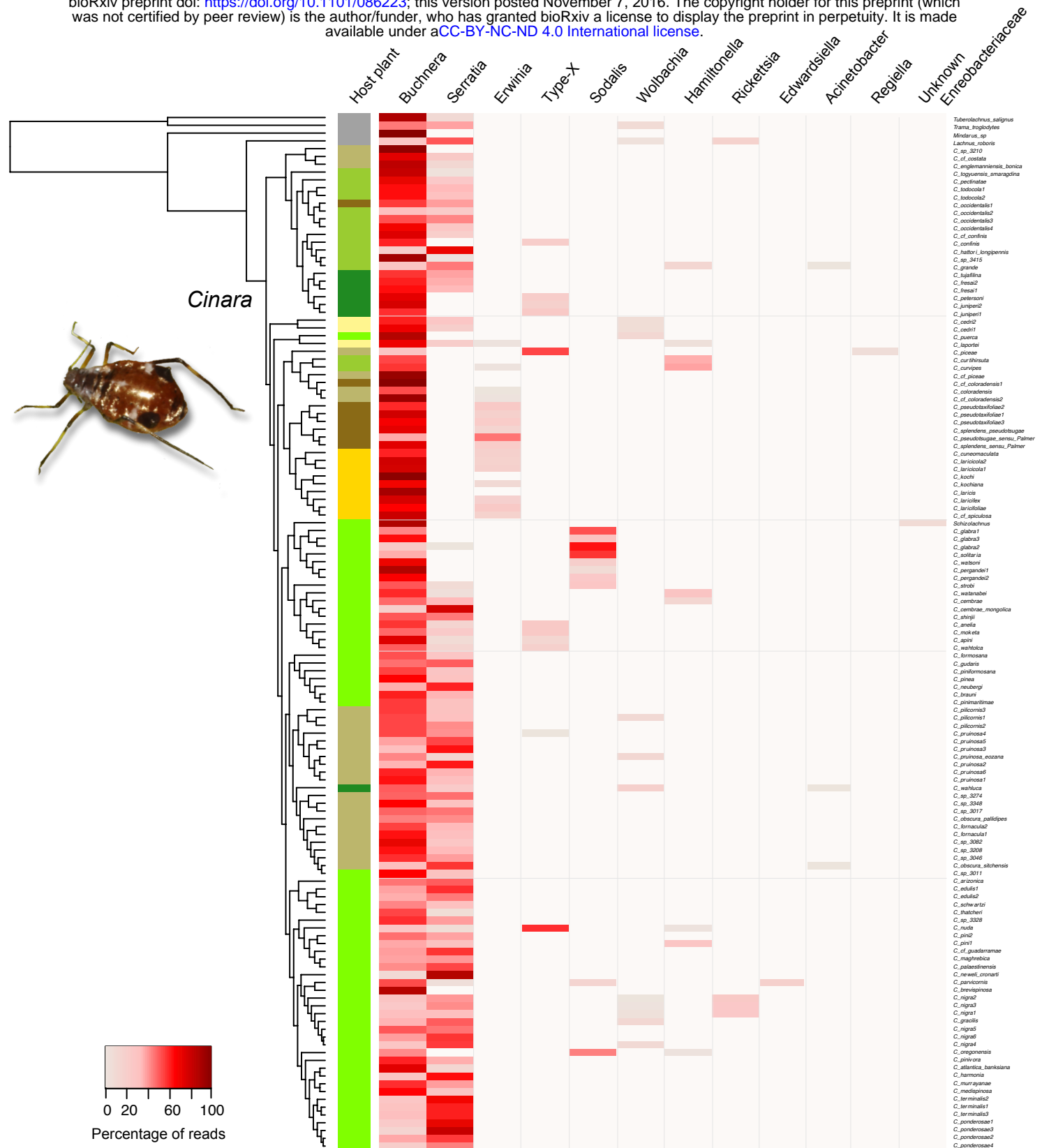
---

*Buchnera* *Serratia* *Sodalis* *Erwinia* Type-X

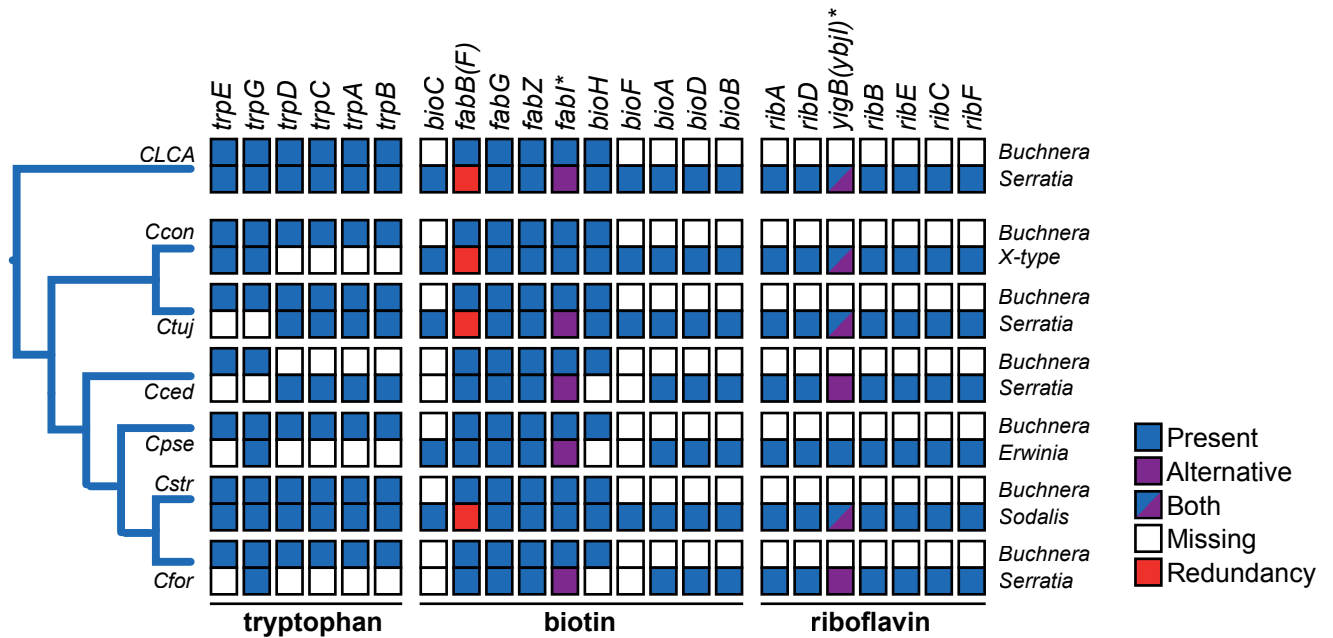
---

<b>% <i>Cinara</i> species hosting a single bacterial haplotype</b>	67	68	8.3	85	50
<b>% Bacterial haplotypes infecting a single <i>Cinara</i> species</b>	85	74	88	50	89

832



**Figure 1.** Bacterial community (phylum and frequency as estimated by read abundances) of *Cinara* species mapped onto the tips of the ultrametric species tree of *Cinara*. For the total number of reads found in an aphid specimen, we have calculated the percentage of reads belonging to each bacterium. In this figure, only endosymbionts that are fixed within *Cinara* species (bacteria found in all specimens of a given species) are plotted. Therefore, the frequency of each endosymbiont (the red bars) in a species represents the mean percentage of reads obtained in all the specimens of that species (including PCR replicates). Colours in the figure correspond to the colour circles and conifer silhouettes in the inset legend. Black circles in the inset map show the collection sites of the specimens of *Cinara* (N=371). The photo shows *Cinara ponderosae* (col: Coeur d’acier).



**Figure 2.** Diagram representing the metabolic complementation found in the biosynthesis of tryptophan, biotin and the riboflavin biosynthesis take-over/rescue between *Buchnera* and– *Serratia*, *Erwinia*, *Sodalis* or *Type-X* obligate symbionts in the endosymbiotic systems of *Cinara*. Gene names are used as column names. On the left, a schematic representation of the phylogenetic relationships of *Cinara* species. Abbreviations for the different species are: CLCA= *Cinara* last common ancestors; Ccon = *Cinara confinis*, Ctuj = *C. tujhafilina*; Cced = *C. cedri*; Cpse = *C. pseudotaxifoliae*; Cfor = *C. fornacula*. Data from *C. tujhafilina* and *C. cedri* comes from Gosalbes et al (2008), Lamelas et al. (2011a,b) and Manzano-Marín & Latorre (2014).



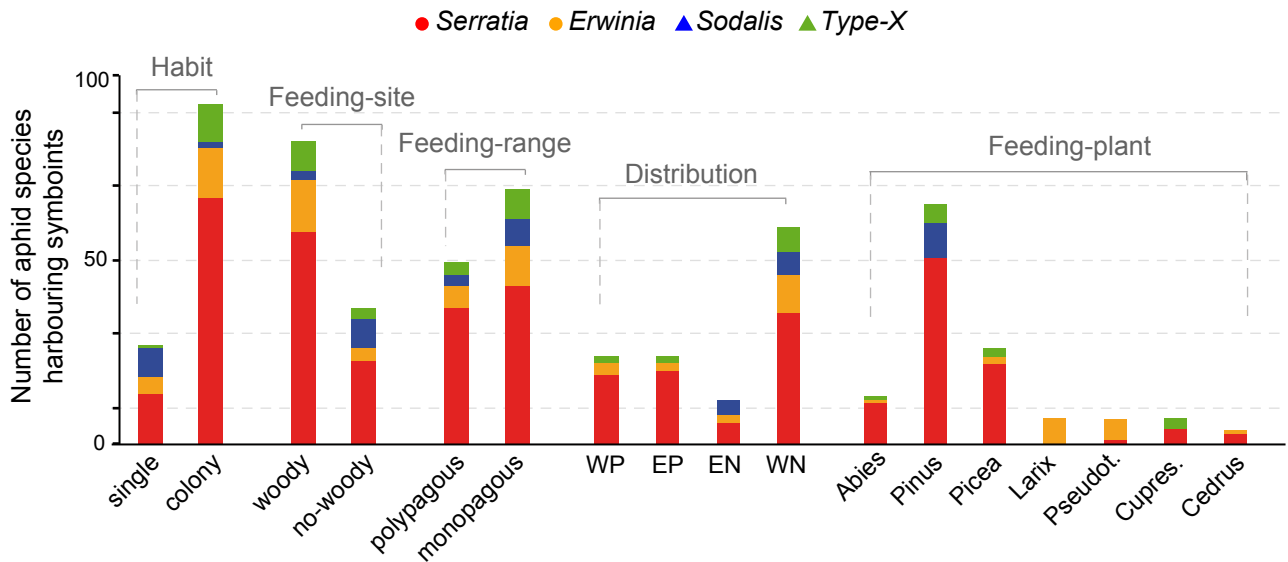


Figure 3. Distribution of cosymbionts across aphid's ecological contexts. For several aphids characters, the histograms show the number of species hosting each cosymbionts. Abbreviations: Pseudot.= Pseudotsuga, Cupre.=Cupressaceae, WP= Western Palearctic, EP= Eastern Palearctic, WN= Western Nearctic, EN= Eastern Nearctic.

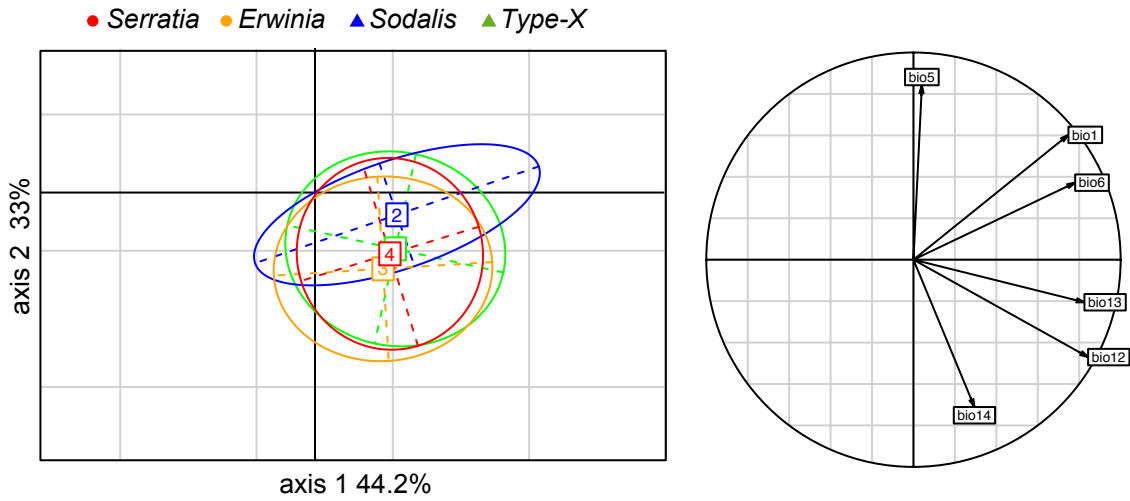


Figure 4. Circle of correlation and factor scores obtained from a principal component analysis performed on bioclimatic variables. Ellipses delimit the climatic envelopes of aphids hosting the different cosymbionts. Climatic values were extracted from the localities where aphid specimens carrying the essential symbionts were found. The percentages of variance explained by the first two principal components are indicated in the axis labels. Abbreviations; BIO1 = Annual Mean Temperature; BIO5 = Max Temperature of Warmest Month; BIO6 = Min Temperature of Coldest Month; BIO12 = Annual Precipitation; BIO13 = Precipitation of Wettest Month; BIO14 = Precipitation of Driest Month