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Authors: Cardoso, Anabela, and Gómez-Zurita, Jesús

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Food Resource Sharing of Alder Leaf Beetle Specialists (Coleoptera: Chrysomelidae) as Potential Insect–Plant Interface for Horizontal Transmission of Endosymbionts

Anabela Cardoso¹ and Jesús Gómez-Zurita^{1,2}

¹Institute of Evolutionary Biology (CSIC-University Pompeu Fabra), Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain, and ²Corresponding author, e-mail: j.gomez-zurita@csic.es

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Abstract

Recent studies suggest that endosymbionts of herbivore insects can be horizontally transferred to other herbivores feeding on the same host plants, whereby the plant acts as an intermediate stage in the chain of transmission. If this mechanism operates, it is also expected that insect communities sharing the same host plant will have higher chances to share their endosymbionts. In this study, we use a high-throughput 16S rRNA metabarcoding approach to investigate the presence, diversity, and potential sharing of endosymbionts in several species of leaf beetles (Coleoptera: Chrysomelidae) of a local community specialized on an alder diet in North America. *Rickettsia* and *Wolbachia* were predominant in the sample, with strong evidence for each species having their own dominant infection, of either or both types of bacteria. However, all species shared a much lower proportion of a particular *Wolbachia* type, compatible with the same strain dominant in one of the species of leaf beetles. Crucially, the same 16S rRNA haplotype of *Wolbachia* was found on alder leaf extracts. The combined evidence and the absence of this strain in a syntopic species of leaf beetle feeding on a different host plant support the hypothesis that at least the initial stages of the mechanism that would allow horizontal transmission of endosymbionts across species feeding on the same plant is possible. The accessibility and characteristics of endosymbiont associations of this system make it suitable for deeper analyses of their diversity and transmission in natural conditions.

Key words: biodiversity, natural transmission, trophic network, *Wolbachia*, *Rickettsia*

Many insects and other arthropods show stable associations with endosymbiotic microorganisms (Werren et al. 2008). Adaptation to an endosymbiotic lifestyle, whereby the intracellular environment of these invertebrate cells has become the niche of a number of bacteria, including *Wolbachia*, *Rickettsia*, *Spiroplasma*, *Cardinium*, and others (Duron et al. 2008), implies that efficient transmission mechanisms are required to maintain these associations (Dale and Moran 2006, Moran et al. 2008, Kikuchi 2009, Bright and Bulgheresi 2010). In particular, the specialization of the bacteria colonizing the right cells in the female germline helps achieving this goal, with both benefits for the symbiont and the host (Frank 1996, Engelstädter and Hurst 2009, Martínez et al. 2017). The role of these tissues and organs to allow for vertical inheritance of the host aligns well with the interest of the intracellular bacteria (Frost et al. 2014, Pietri et al. 2016), although intracellularity and vertical transmission are not strictly correlated (Fisher et al. 2017). This specialization has led to some astounding effects induced by the bacteria on their hosts to make their vertical transmission more efficient, including reproductive and developmental effects (Werren et al. 2008, Engelstädter and Hurst

2009). However, theory predicts that it is selectively advantageous for symbioses of this nature to retain at least in part some potential for horizontal transmission between hosts, i.e., acquisition from the environment instead of from the mother, as a strategy to increase reproductive rate and reduce competition within the host (Frank 1994). Indeed, the obvious discordance between host and bacterial genealogies, with the presence of endosymbionts of the same or related strains in distantly related hosts, soon pointed at the importance of horizontal transmission processes in the spread of these bacteria as well (Werren et al. 1995, 2008; Bright and Bulgheresi 2010; Morrow et al. 2014).

Up to three different mechanisms have been hypothesized and confirmed in most cases that allow for horizontal transmission of endosymbionts across taxa (Morrow et al. 2014): 1) interspecific hybridization; 2) physical contact through occupation of the same niche; and 3) food intake with viable bacteria. When the trophic association is directly on prey infected by the endosymbionts, as in the case of parasitoids and predators, these organisms are directly exposed to the potential source of bacteria. However, evidence

accumulates supporting that other food or substrates that in principle are not normal hosts of the bacteria can also act as source of the infection in some cases (Chiel et al. 2009, Gehrher and Vorburger 2012). The generality of this hypothesis needs to be evaluated, particularly in natural conditions.

In a recent review, Chrostek et al. (2017) examined the mechanisms by which host plants in particular may contribute to the colonization of new herbivore hosts. The mechanism is simple enough in terms of stages considered, but each has specific challenges that contribute complexity to the process. In a first stage, the host has to inoculate or spread the bacteria in or on plant tissues, which may be facilitated when the pattern of host infection includes other tissues apart from the ovaries, particularly those in contact with food, such as salivary glands, or when the bacteria exist extracellularly in the host gut and can occur in feces (e.g., Brumin et al. 2009, Espino et al. 2009, Pietri et al. 2016, and references therein). But there is also a critical requirement of the bacteria, after all adapted to intracellular life, to be able to survive in or on the plant. There is some evidence on the actual viability of endosymbionts like *Wolbachia* and *Rickettsia* outside the host and also within plant tissues, which may even provide nutrients for the survival of the bacteria (Rasgon et al. 2006, Caspi-Fluger et al. 2012, Li et al. 2017). However, despite of findings supporting the resilience of these bacteria outside of the host cells (up to 1 wk in studies in vitro; Pietri et al. 2016), or in the transitory cellular environment of plant cells, it is assumed that their survival is seriously compromised (Purcell et al. 1994). Finally, a potential host has to ingest the endosymbiont, which makes this mechanism particularly likely for herbivorous insects. Still, the bacteria have to endure the rigors of the digestive tract, the host immunity, potential competition with other bacteria in the host, and a trip into the body cavity and through important tissue barriers to reach suitable cells in the germline to ensure vertical transmission (Vallet-Gely et al. 2008, Ratzka et al. 2012, Pietri et al. 2016, Russell et al. 2019).

One corollary of this potential mechanism is that communities of insects that share resources, for example, a community of insects feeding on the same host plant, may have a high chance to share also the type and strain of endosymbiont infection. In other words, if the food or substrate have the potential to supply passively endosymbionts to the species feeding on or using these resources, it would be reasonable to assume that insect communities sharing the resource may share their endosymbiotic composition too. Very few studies address this hypothesis, but there are some exceptions, including both herbivore and predatory insects. For example, Sintupachee et al. (2006) found that a community of insects feeding on pumpkin leaves in Thailand had very closely related *Wolbachia* types and hypothesized that one of the insect species, the whitefly *Bemisia tabaci*, could be the source of the infection to representatives of other insect orders through their shared host plant. Similarly, Morrow et al. (2014) found data supporting different mechanisms of horizontal transfer among Tephritidae fruit flies in Australia, whereby sharing of food resources could not be discarded as a predominant transmission mechanism. Among predatory insects, Bili et al. (2016) investigated the communities of bacteria, including endosymbionts, associated with two beetles and one wasp attacking the same prey, the cabbage root fly. The study showed that these predatory insects actually shared some bacteria, but laboratory artifacts were not discarded, and differences in associations were explained by life-history peculiarities of each species.

The mechanisms described and available data lend support to the idea that host plants could facilitate horizontal spread of

endosymbionts among herbivore insects. However, we ignore how frequently endosymbionts are shared across unrelated species in a trophic network, or what is the actual role of host plants in favoring these shared associations through horizontal transfer. The generality of the hypothesis requires further testing, and there is a need to expand this research to other plants and the arthropod communities they sustain, particularly in natural conditions. Here, we will test the hypothesis that food sharing may favor horizontal transfer of endosymbionts by providing a first approach on a natural and reasonably accessible and diverse trophic system, offered by the leaf beetles feeding on North American alders (Betulaceae in the genus *Alnus*). Alders are deciduous shrubs or trees common in damp meadows and lacustrine, riverine, and swampy environments of northern and temperate areas. In North America, there are up to 10 species, of which the speckled alder, *Alnus incana* ssp. *rugosa* (Du Roi) R.T. Clausen, is common in Northeastern North America, from Saskatchewan to northern Illinois, to Newfoundland and Pennsylvania (Fralish and Franklin 2002). Alders, and in particular grey alders (*A. incana*), have relatively few threats by pests and diseases (e.g., Arhipova et al. 2011), but they sustain a rich community of phytophagous, specifically folivorous insects (e.g., Gharadjedaghi 1997). Only among the leaf beetles in North America, there are alder-feeding records for more than 30 species in some 15 genera (Clark et al. 2004). This rich and taxonomically diverse community and its accessibility, together with mounting evidence for their usual association with endosymbionts (Kajtoch and Kotásková 2017), thus constitute a suitable system to investigate aspects related to endosymbiont transmission in relation to the occupation of identical niches and links in a particular food web. We will examine some of the leaf beetles representative of the community associated with alders in North America, and explore their association with endosymbiotic bacteria through high-throughput sequencing methods. The analysis will address specifically the existence of patterns of potentially shared endosymbionts among these species and the plausibility of the hypothesis of their transmission through the nexus offered by identical choice of host plant.

Materials and Methods

Samples

We sampled several species of leaf beetles specialized on alder, specifically found on speckled alder, *A. incana* ssp. *rugosa*, in three localities in the area between the counties of Franklin and Essex, in the Whiteface Mountain region, upstate New York (Fig. 1; Table 1). In particular, we obtained samples of *Calligrapha alni* Schaeffer, *C. confluenta* Schaeffer, *Chrysomela mainensis* ssp. *mainensis* Bechyně, and *Altica ambiens* ssp. *alni* LeConte. None of these species has been tested before for the presence of endosymbionts in any part of their range. Moreover, in two of the localities, we also obtained samples of leaves from the trees where the beetles were captured to investigate the diversity of bacteria on their surface. In the case of the insects, we avoided manipulation and they were transferred with forceps from the leaf where they were sitting to individual tubes containing absolute ethanol; in turn, fragments from central parts of some leaves were cut with scissors, while holding the fragment with clean forceps, and also directly transferred into a vial with ethanol. The sample was completed for exploratory purposes with one specimen of *Calligrapha philadelphica* (Linnaeus) from one of the localities (Table 1), a sympatric species but with a completely different trophic selection, since it is exclusively associated with dogwood, *Cornus* spp. (Cornaceae).

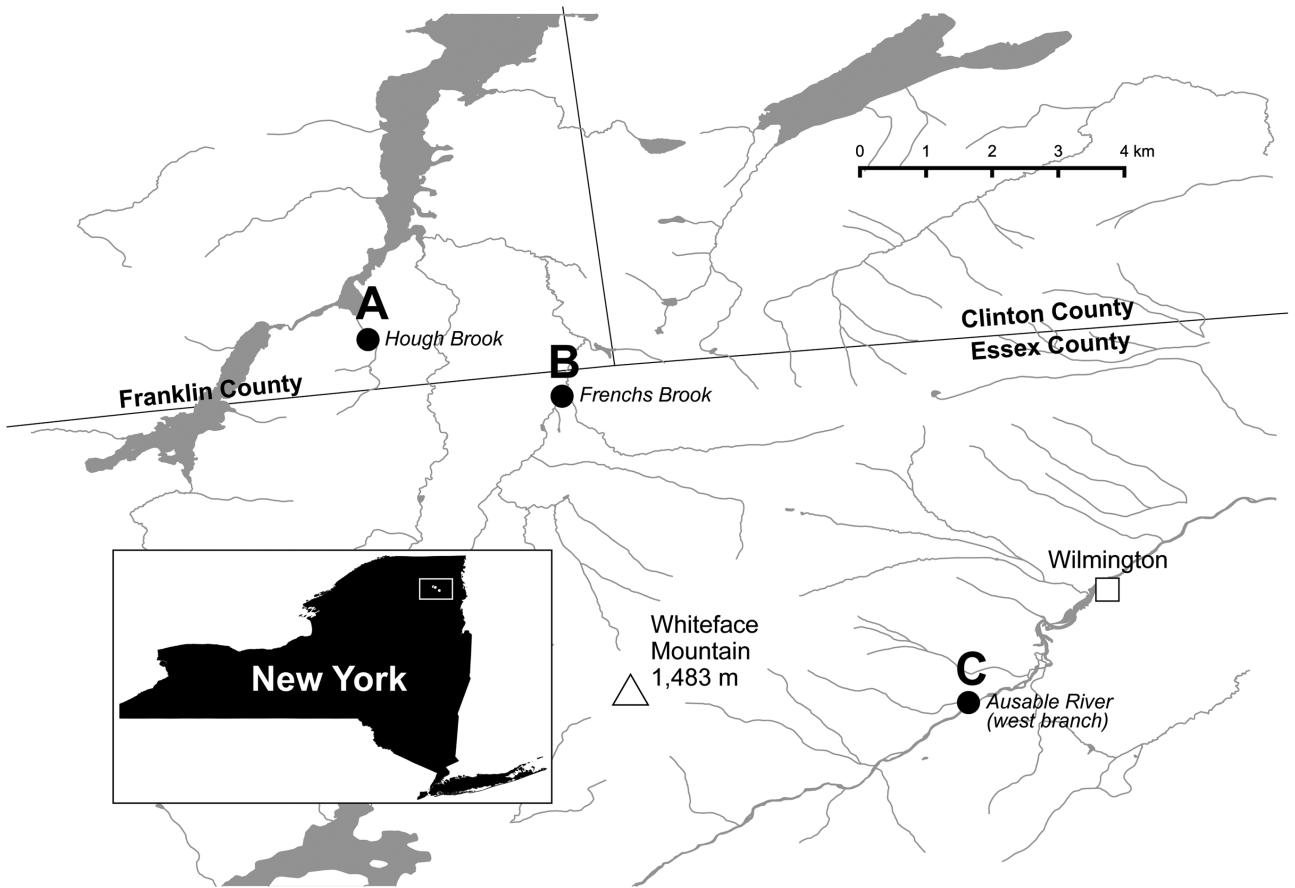


Fig. 1. Geographic location of the study sites. Locality codes and collection data are the same given in Table 1.

16S rRNA Gene Metabarcoding Library Preparation and Sequencing

Owing to the effects of ethanol, including hardening and loss of structure and color of internal organs, it was not possible to recognize features of the internal anatomy of the leaf beetles. Thus, we dissected under the microscope and in sterile conditions tissue fragments of the abdomen of each specimen, hoping to extract enough tissue typically carrying endosymbiont colonies, preferentially the gonads (Pietri et al. 2016). The dissection was facilitated soaking the tissues in PBS 1× or the first buffer (AHL) of the DNA extraction kit. In particular, microbial genomic DNA was obtained from each leaf beetle sample individually using the QIAamp DNA Microbiome Kit (Qiagen Iberia S.L., Barcelona, Spain) and following the manufacturer’s instructions. In the case of the leaf samples, we used approximately 1 cm² of preserved leaf fragments from Hough Brook and the Flume Trail system, which were subject to the same bacterial DNA extraction protocol as the beetle tissues.

The 16S rRNA gene metabarcoding libraries targeted the V3 and V4 regions of the large ribosomal RNA subunit and using the primers of Klindworth et al. (2013) with Illumina adapter overhang nucleotide sequences (in italics) as follows: Forward Primer, 5′-TCGTCCGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; and Reverse Primer, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. The PCR used some 10–15 ng of microbial DNA (except in the negative control), 0.2 μM final concentration of each primer, and the KAPA HiFi HotStart ReadyMix (Roche Diagnostics, Rotkreuz, Switzerland), with a standard PCR cycling protocol: 95°C for

3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 5 min. PCR products were purified and cleaned off primers and primer dimers with AMPure XP beads (Beckman Coulter, Indianapolis, IN), and 5 μl of cleaned PCR products used as template for an indexing PCR with the Nextera XT Index kit (Illumina, San Diego, CA) to attach dual indices and Illumina sequencing adapters to each library. Indexed libraries were cleaned again with the AMPure XP beads and 1 μl of each library checked for quality and size on a Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA). Libraries were

Table 1. Sampling localities, species studied, and number of individuals

Code	Locality	Species	N
A	Franklin Co., Hough Brook, 44.43498N 73.95645W, 462 m, 26.vi.2015	<i>Calligrapha confluens</i>	1
B	Essex Co., Frenchs Brook, 44.42424N 73.91965W, 519 m, 2.vii.2015	<i>Alnus incana</i>	2
		<i>Altica ambiens alni</i>	2
		<i>Calligrapha alni</i>	3
		<i>Calligrapha confluens</i>	1
		<i>Chrysomela mainensis</i>	3
C	Essex Co., Ausable River, Flume Trail system, 44.36617N 73.84258W, 344 m, 2.vii.2015	<i>Alnus incana</i>	1
		<i>Calligrapha alni</i>	1
		<i>Calligrapha confluens</i>	2
		<i>Calligrapha philadelphica</i>	1
		<i>Chrysomela mainensis</i>	2

normalized to a final concentration of 4 nM with 10 mM Tris pH 8.5, and 5 µl of each indexed library pooled for a single MiSeq run. Prior to sequencing, DNA of pooled libraries was denatured with 0.2 N NaOH, the amplicon library diluted with HT1 hybridization buffer according to Illumina protocols and combined with 5% of the PhiX Control v3 library. The pooled sample of 19 libraries was loaded on a MiSeq for a 2 × 300 bp paired-end sequencing run in the Genomics Core facility at the University Pompeu Fabra (Barcelona, Spain). Haplotypes discussed in the text have been deposited in the European Nucleotide Archive (EMBL-EBI, Hinxton) under sequence accession numbers LR877032–LR877144.

Microbiome Characterization

The sequencing procedure generated paired FASTQ files for each library that were sorted based on their indices, excluding all individual sequences with quality and indexing issues. Amplicon 16S rRNA gene primers and adapters were removed from each sequence after this initial filtering using the Python tool Cutadapt 2.6 (Martin 2011). Trimming ends of low-quality, fragment length filtering ($\text{truncLen}=\text{c}(225,205)$, $\text{maxEE}=\text{c}(2,2)$, $\text{truncQ}=2$), merging of paired ends, removal of length outliers by retaining fragments of 400–414 nucleotides, and removal of chimeras (based on the consensus method) were done using the R package DADA2 v1.14.0, which uses a procedure that models and corrects sequenced amplicon errors in Illumina (Callahan et al. 2016). The taxonomic identification of the resulting data sets was done based on the March 2018 update of the Silva 132 reference database, the one currently with highest taxonomic coverage (Quast et al. 2013), and the IDTAXA algorithm for classification (Murali et al. 2018), as implemented in the R package DECIPHER v2.14.0 (Wright 2016). Among the advantages of DECIPHER, it handles sequence data as a database, instead of reading sequences to the computer memory, making it possible to handle high-throughput sequence data efficiently even with computers that are not high-performance (see below). In turn, IDTAXA exploits machine-learning algorithms which reduce misclassification errors by identifying sequence diagnostic features of specific ranks in the training set, which are also statistically rare in others. These traits are used to reassess the success in the classification of the actual training set and assign confidence values to the classification procedure by traversing the nodes of the classification hierarchy from the root to the tips. The same procedure is applied to query sequences by requiring strict (bootstrap support = 98%) associations of a query within a particular level of the classification to yield that result as the final taxonomic assignment.

Phylogenetic Identification Pipeline

The previous taxonomic identification pipeline allowed sorting the sequences in major taxonomic groups and singling out the generally abundant endosymbiont sequences. However, in order to find finer taxonomic structure in the endosymbiont data, we used an adaptation of our automated DNA-based phylogenetic identification pipeline, BAGpipe (Papadopoulou et al. 2015), on the full availability of bacterial sequences from GenBank. This pipeline was originally designed to study *psbA-trnH* cpDNA plant sequences, but it can be relatively easily adapted to other markers and other organisms (Papadopoulou et al. 2015). The first stage of the pipeline consists of creating a reference database from GenBank data for the marker of interest, matching orientation and length of the studied barcode, and removing taxonomic redundancy, while retaining relevant taxonomic

information for downstream identification and filtering steps. We followed the pipeline instructions for database construction with some modifications and all the analyses were run on an iMac OS X 10.7.5, with 2.7 GHz Intel Core i5 processor and two 2 GB memory modules (1333 MHz DDR3). The 387 flatfiles of the latest (November 2019) bacteria GenBank release (gbtct) and the associated GenBank taxonomy (NCBI taxon number for bacteria = 2) were downloaded to automatically parse all the available V3–V4 16S rRNA gene fragments. Identification of the homologous fragment of interest was based on an anchor query text file with a selection of V3–V4 16S rRNA gene sequences from our own libraries, as sequenced and identified in the previous steps. Some steps for the preparation of the reference database, namely reorientation and trimming of sequences using USEARCH (steps 1.6 to 1.8 of the original pipeline description), required splitting the original files that included all the available sequences into smaller files due to processing memory limitations in our setup, possibly because of many entries corresponding to complete genomes, thus also including the V3–V4 16S rRNA gene region of interest. In particular, in this implementation of the pipeline, we used USEARCH v5.2.32 (Edgar 2010) because the version recommended in the description of the pipeline (v4.66) was deprecated and no longer available.

The second stage of the pipeline consists of the identification of query sequences by comparison with the previous reference database based on either phenetic, phylogenetic, or both criteria, by reducing the problem to manageable groups of similar sequences in the query group. In our case, we split the endosymbiont sequences and homologous sequences from the reference database in 90% similarity clusters, which fundamentally distinguished between two types of endosymbiont, and submitted them to phylogenetic clustering and identification. Query and reference sequences were aligned with the G-INS-i algorithm in MAFFT 7 (Katoh and Standley 2013), and the resulting matrices used for maximum likelihood phylogenetic inference using RAxML 7.2.8 (Stamatakis 2006) implementing a GTR+CAT model on a heuristic hill-climbing search based on 100 random most parsimonious initial trees, and assessing node support based on 100 bootstrap pseudoreplicates. Also, the relationships among the empirically obtained haplotypes of the two endosymbionts present in our sample were investigated using statistical parsimony networks based on the software TCS 1.21 (Clement et al. 2000).

Results

Characteristics and Quality of Library Reads

16S rRNA gene amplification and library preparation using the multi-tagged primers yielded in every case fragments of approximately the expected size (592–607 bp) and relatively low, but usable concentrations (2.74–16.31 ng/µl) (Table 2). Index-based sorting produced on average nearly 875,000 reads per amplicon library, ranging between a low ~565,000 reads in one sample of *Ca. alni*, and the expected ~1,110,000 reads in two samples of *Ch. mainensis* (Table 2). Filtering based on sequence end-quality threshold, denoising based on the explicit error rate models of the procedure that we followed, merging of paired ends, and removing length outliers retained 43.9–68.0% of the initial reads. Finally, 0.33% of the surviving reads were automatically recognized as chimeras and also excluded from subsequent analyses (Table 2).

Table 2. Parameters of 16S rDNA library synthesis based on the Bioanalyzer results and of the sequence-filtering procedure with DADA2 (Callahan et al. 2016)

Species	Ind./ Loc.	Size (bp)	Conc. (ng/μl)	Input	Filtered	de- noisedF	denoise- dR	merged	nochim	contam- ination	useful
<i>Alnus incana</i>	Ai07/B	602	6.23	1056929	678331	678209	678263	677441	676501	676152	349
	Ai15/B	606	9.81	1081656	725776	725673	725690	725386	724458	724443	15
	Ai25/C	595	10.72	951669	614157	613996	614064	613605	612590	612488	102
<i>Altica ambiens</i>	Aa03/B	603	7.29	826142	501981	501795	501188	497217	474478	28477	446001
	Aa09/B	596	4.70	1024015	598344	598131	598230	591208	565220	58850	506370
<i>Calligrapha alni</i>	Ca01/B	607	12.11	564561	382351	382290	382284	382220	381635	381620	15
	Ca02/B	601	11.44	964454	656655	656515	656593	656429	655748	655669	79
	Ca12/B	598	10.02	965457	553102	553038	553039	552928	552377	552299	78
	Cc18/C	599	15.88	672008	427249	427158	427176	426938	426468	426348	120
<i>Calligrapha confluens</i>	Cc11/A	599	14.87	936424	615162	614991	614941	613746	610359	610221	138
	Cc05/B	603	13.70	715138	452144	452059	452106	451978	450965	450362	603
	Cc16/C	599	16.31	762414	500996	500665	500817	499557	498435	498286	149
	Cc17/C	599	15.81	812653	534961	534884	534925	534765	534192	534142	50
<i>Calligrapha philadelphica</i>	Cp23/C	598	14.51	1167727	778026	776950	777918	776362	771435	770544	891
<i>Chrysomela mainensis</i>	Cm04/B	597	9.80	587430	336105	335993	336048	334912	333480	222473	111007
	Cm10/B	592	11.53	1102771	649385	649024	649258	647791	645460	1540	643920
	Cm13/B	596	10.46	907151	548158	548077	548052	547922	547054	546840	214
	Cm19/C	593	6.99	881222	489797	489600	489743	386794	382157	1892	380265
	Cm20/C	592	2.74	1112097	643667	643533	643596	533831	496570	89243	407327

Composition of Libraries

One striking result after the initial taxonomic assignment of all sequences based on the reference database Silva 132 was finding that more than half of the libraries were overwhelmed with contaminant plant mitochondrial, but mainly plastid V3–V4 DNA sequences. Interestingly, this problem did not affect samples randomly, and it fundamentally showed in all *Calligrapha* and leaf samples, while most samples of the other two leaf beetle species included normal values between 0.2 and 66.7% of plastid and mitochondrial sequences (Table 2).

Up to 36 sequences (7.47% of all bacterial reads) were not assigned any taxonomic rank or yielded very high ranks in the taxonomic hierarchy (e.g., Gammaproteobacteria). Most (98.45%) of these unidentified reads actually corresponded to a unique sequence obtained from one of the libraries of *A. ambiens* (and just eight reads from one of the alder leaf samples), which showed maximum similarity (approximately 94%) with environmental sequences of saline habitats inhabited by natural populations of *Artemia* in Israel (Tkavc et al. 2011). Of the remaining 35 unidentified sequences, 15 (197 reads) were recognized in the alignments or with additional BLAST searches as chimeras of highly divergent taxa, and eight (89 reads) were confirmed as plastid sequences affected by a particular sequencing problem: stretches of 50–100 bp where all G residues were replaced by T (or C to A, in the reverse strand). More worrying, although always relatively considering their minimal representation in the sample (61 reads), were five sequences which were attributed family ranks in the Lactobacillaceae (Bacilli) and the Porticoccaceae (Gammaproteobacteria). Multiple sequence alignments of all ingroup 16S rRNA gene sequences and BLAST searches helped recognizing them as chimeras between sequences of *Wolbachia* and of bacteria of the groups mentioned.

After excluding plant contaminant sequences and the obvious artifacts mentioned above, we found evidence for 115 different microbial 16S rRNA gene sequences in the whole data set (42 operational taxonomic units [OTUs] at a 97% identity threshold), of which 58 (corresponding to 91.51% of all bacterial reads) were

unambiguously identified as Rickettsiales of the genera *Wolbachia* and *Rickettsia*. Apart from the abundant *Wolbachia* and *Rickettsia*, the highest diversity of bacterial identifications corresponded to the samples of *Ch. mainensis*. In this species, Actinobacteria of the Frankiales (*Nakamurella*), Micrococcales (*Brachybacterium* and *Kocuria*), and Propionibacteriales (*Cutibacterium*), and Alphaproteobacteria of the Acetobacterales (e.g., *Acetobacter*) and of the Rhizobiales (*Afipia* and *Rhodopseudomonas*) dominated the sample (Fig. 2; Supp Table S1 [online only]). The next most diverse sample, albeit deduced from a reduced number of observations and again by excluding *Wolbachia*, corresponded to the bacteria characterized from *Calligrapha confluens*. In this case, we also found evidence for associations with *Kocuria* and *Cutibacterium* among the Actinobacteria, Rhizobiales of three different families (A0839, Hyphomicrobiaceae and Rhizobiaceae), as well as Clostridia and different types of Gammaproteobacteria (Fig. 2). In the case of *Altica*, and excluding the Rickettsiales, the sample was dominated by Actinobacteria of the Mycobacteriaceae (*Mycobacterium*), Micrococcaceae, and Propionibacteriaceae (*Cutibacterium*), but it also had evidences for Cyanobacteria and Proteobacteria of the Rhizobiales (*Methylobacterium*). Finally, *Alnus* leaf surfaces yielded a rather diverse set of inferences, mainly Alphaproteobacteria of the Acetobacterales (Acetobacteraceae, including *Acidiphilium* and *Endobacter*) and of the Rhizobiales (Beijerinckiaceae, including *Methylobacterium* and the unidentified genus 1174-901-12).

Diversity of *Wolbachia* and *Rickettsia*

The sample yielded 36 sequence variants unambiguously assigned to *Wolbachia* and 22 to *Rickettsia*, with the former retrieved from all beetle species, and the latter predominantly in *A. ambiens*, but with some reads in the libraries from *Ch. mainensis* and *Ca. alni*. Interestingly, for one of the leaf samples, most of the bacteria characterized also belonged to *Wolbachia*, and specifically with the same most abundant 16S rRNA haplotype as retrieved from the beetle samples. Despite the relatively large number of 16S rRNA

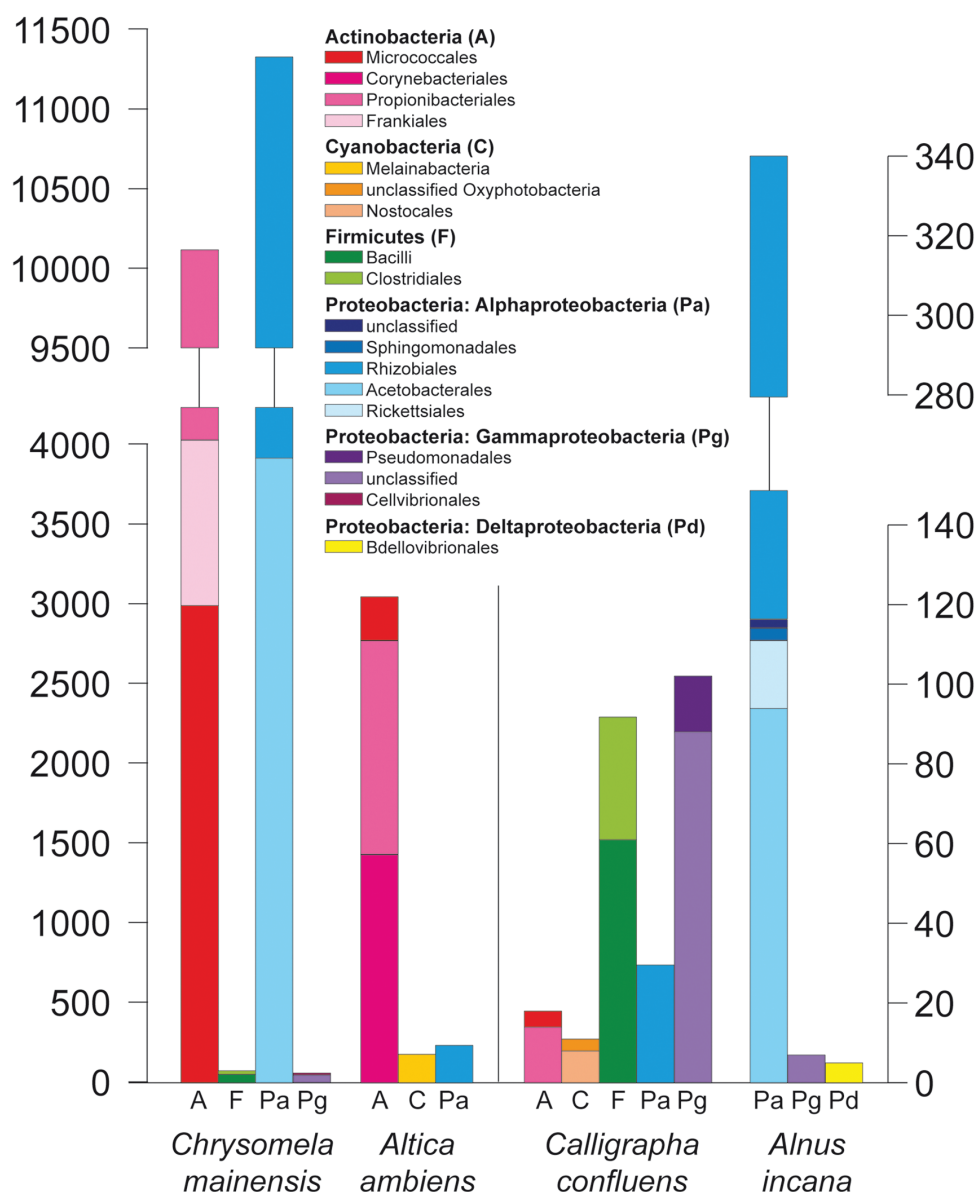


Fig. 2. Taxonomic diversity and abundance of nonendosymbiotic bacteria deduced from 16S rRNA haplotypes from the libraries of four species of the study. The samples of *Calligraphaalni* and *Ca. philadelphica* only yielded sequences of *Rickettsia* and *Wolbachia* and are not shown here. Frequency axes apply to *Chrysomela mainensis* and *Altica ambiens* (left) and to *Ca. confluens* and *Alnus incana* (right).

haplotypes, four haplotypes (W1–W4) represented 98.5% of all *Wolbachia* sequences, and a single haplotype 95.9% of the *Rickettsia* contigs. Most parsimonious networks relating these haplotypes revealed a number of interesting patterns (Fig. 3). In the case of *Rickettsia* (Fig. 3a), the dominant haplotype (R1) was the only one also found, if at very low frequencies, in *Ch. mainensis* and *Ca.alni*, and this haplotype was the only one shared between the two specimens of *A. ambiens* tested, which otherwise had their discrete sets of haplotypes (R2–R4 in one individual, and all the others in the other). For *Wolbachia*, 16S rRNA genetic diversity was higher than for *Rickettsia* and haplotypes belonged to two main groups separated by 10 mutational steps, one with haplotypes W4 (one of the most abundant in the sample) and W36, exclusively found in *A. ambiens*, and a group with all the other haplotypes found in all species tested (Fig. 3b). The last group showed a central core with the other three dominant haplotypes from which radiated all others in star-shaped patterns, particularly from the most abundant W1. Each of these

haplotypes defined nonetheless three domains, one derived from W3 and nearly exclusive of *A. ambiens*, one derived from W1 for *Ch. mainensis*, and one derived from W2, dominated by *Ch. mainensis*, but with haplotypes W28 and W29 exclusively found in *Calligrapha* spp. Interestingly and for the reasons that will be elaborated in the discussion, the latter haplotypes were the only ones recovered from the library of *Ca. philadelphica*.

The 16S rRNA gene phylogeny of *Rickettsia* showed very little resolution, but four main groups were distinguishable: one of them only characterized in the whitefly *B. tabaci* and other hemipterans; one mainly found in weevils; one in diverse organisms, but predominantly water beetles and leeches; and a last one, highly diverse, poorly structured and characterized from all sorts of insects and arachnids (Supp Fig. S1 [online only]). The *Rickettsia* haplotypes characterized from *A. ambiens* and in lower frequencies in two other species of leaf beetles belong in the latter group. The only *Rickettsia* haplotype in our sample that was already present

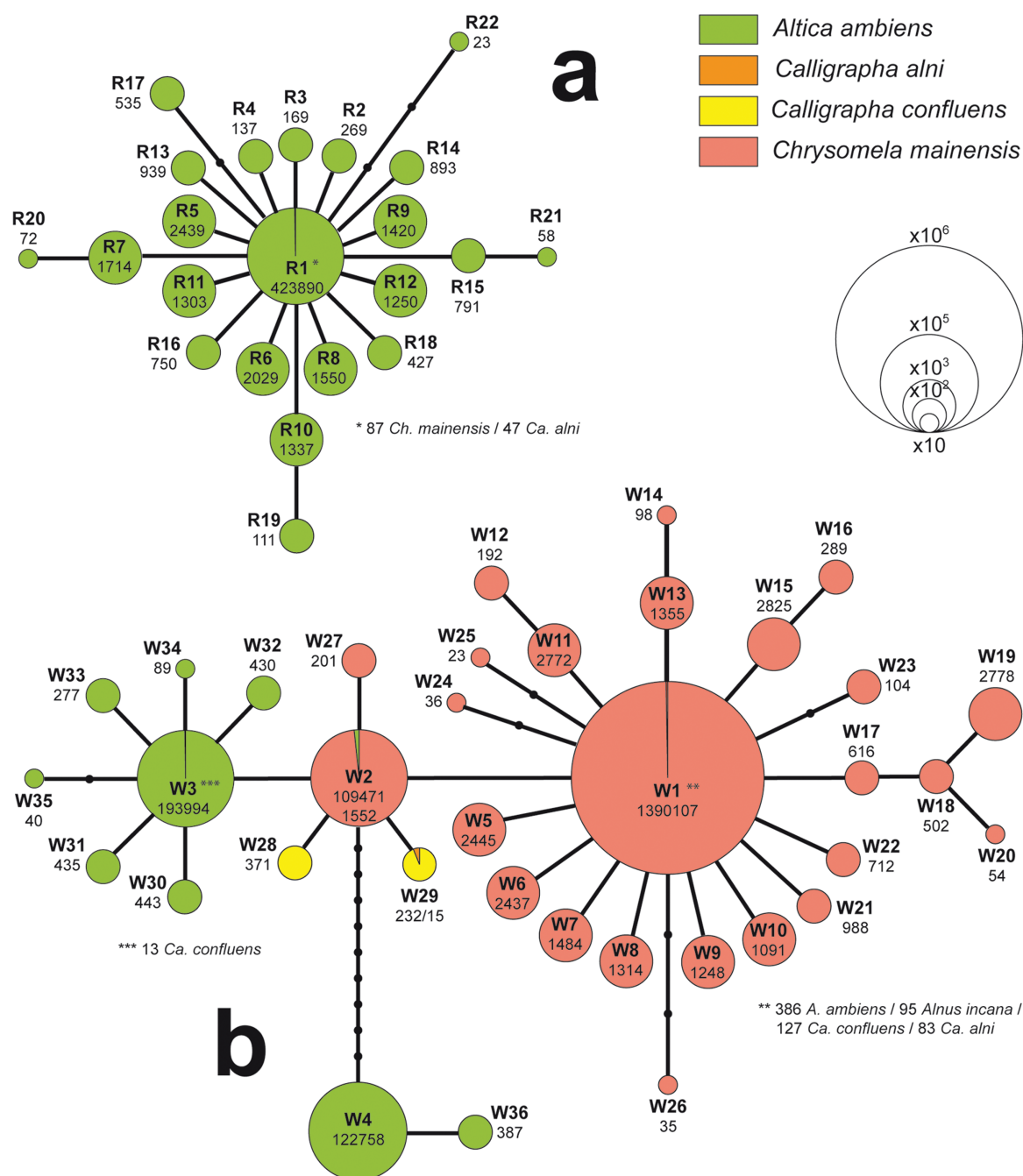


Fig. 3. Unrooted statistic parsimony networks of 16S rRNA haplotypes of *Rickettsia* (a) and *Wolbachia* (b) obtained for four species of alder specialists of the Chrysomelidae. Each colored node represents an observed haplotype and its code and absolute frequency are given, and the size of the node is proportional to the order of magnitude of this frequency. Edges represent mutational steps and small black circles missing intermediate states.

in the database was the most abundant R1, identical to sequences previously characterized in mites, lacewings, different genera of true bugs, fleas, and all major groups of holometabolous insects, including several groups of beetles. This same 16S rRNA haplotype was the one characterized from the type strains of *R. bellii* and *R. australis*.

The diversity and phylogenetic structure of the 16S rRNA gene for *Wolbachia* as deduced from public sequence databases was higher than for *Rickettsia*, but still not enough for high resolution of supported phylogenetic groups (Supp Fig. S2 [online only]). But relevant for the purposes of the current study, this taxonomic

marker allowed discriminating between two supergroup clusters of the bacterium, including an assemblage of representatives in supergroups A, B, and E, where the haplotypes W4 and W36 exclusive of *A. ambiens* were found, and another assemblage with representatives of supergroups C, D, and F where all the other *Wolbachia* haplotypes found in this study clustered. Haplotypes W4 and W36 appeared in a large, poorly resolved group of haplotypes described from numerous insect, arachnid, and isopod groups, and specifically recognized as members of supergroup B in *Wolbachia* (e.g., Mateos et al. 2006). The remaining haplotypes appeared in a lineage within the supergroup F of *Wolbachia* with many endosymbionts

predominantly characterized from ants and beetles, including weevils and other leaf beetles in the genera *Basilepta*, *Galeruca*, and *Lema*.

Discussion

Technical Issues of Metabarcoding and the Reliability of Inferences

Before extracting the main conclusions from our study, we consider that it will be useful for other researches working in similar systems to be aware of and have some context for a number of artifacts that plagued our results. The problems that we detected in our study belong to two main, well-known drawbacks of metabarcoding studies (Kunin et al. 2010, Pinto and Raskin 2012, Tamošiūnė et al. 2019), including 1) artifacts derived from PCR, and 2) sequencing problems, with some subsequent issues in automatic identification procedures. A third type of problem, in part related to the PCR-based approach as well, was the amplification of nontarget sequences, which will be discussed in the next section.

Among the common problems of PCR-based approaches to metabarcoding, we could not assess here biases in species detection due to primer mismatches or bacterial contamination (e.g., von Wintzingerode et al. 1997, Hong et al. 2009). However, in the specific case of the study of *Wolbachia* and *Rickettsia* sequences, their analysis using genealogical approaches revealed patterns consistent with at least a certain proportion of PCR errors being resurfaced by the power of the sequencing technology used here. Theory predicts that PCR produces erroneous sequence variants due to point mutations, with their frequencies diminishing as they accumulate more changes (Weiss and von Haeseler 1995). These PCR errors typically result in star-like tree topologies with the target sequence as a more abundantly represented core and de novo sequences resulting from polymerase mistakes radiating from it, most of them by a single mutation (Smart et al. 2019). This is exactly the pattern that we retrieved, suggesting that a great deal of lower frequency haplotypes may indeed represent PCR artifacts and not true diversity of *Wolbachia* and *Rickettsia* in the sample. This idea is strengthened by the fact that these putative artificial sequences are absent in public sequence databases (with the exception of W28, common in weevils and also found in one leaf beetle; Toju et al. 2013, Jeong et al. 2019). Conversely, our core haplotypes have been characterized already in other organisms and are shared among species in our sample (W29 is also shared between three different *Calligrapha* species, thus suggesting that it may be real too). Focusing on the most abundant core haplotypes and not on the whole diversity revealed by the ultrasequencing approach used here, even though some of it may represent true endosymbiont diversity, makes our conclusions conservative and equally valid.

Some of the point mutations discussed above may be also the result of wrong base calling in the sequencing stage, rather than PCR artifacts. However, the most obvious sequencing problem that we detected, if for a reduced number of sequences, was the complete transformation of G residues in the original sequence to T residues in the deduced sequence for a relatively long stretch of nucleotides (and mirrored by changes from Cs to As at the opposite end of the sequence, thus in the paired read). The effect was observed in one *Wolbachia*, one *Rickettsia*, and several plastid sequences. Given that this specific problem affected both paired ends of the same sequence, we interpret it as a topological artifact, a local saturation with T fluorophore that outshined the G fluorophore, as their emission spectra overlap in the 4-channel technology used by MiSeq systems

(fluorophore cross talk; Sheikh and Erlich 2012). This effect lasted until the excess of T was successfully washed away, and sequencing resumed normally. What is important, however, is that these sequences, even though they had a very low representation (0.005% of reads), passed the pipeline quality filters and reached the identification steps of the analysis, contributing 10 haplotypes (7%) and a number of unidentified OTUs to the global results.

Another problem derived from PCR was the formation of chimeras (Haas et al. 2011), which can be approached analytically in most cases, although it was revealed here because of some limitations in the quality control of library composition. We had evidence that a number of objective chimeras survived the quality-filtering steps of the identification pipeline, and only a meticulous analysis of individual 16S rRNA sequences in multiple sequence alignments, phylogenetic trees, and by BLAST searches against public sequence databases allowed us to effectively remove the most obvious cases. Using this approach we could filter out a number of obvious chimeric sequences (0.008% of reads; 10.5% of haplotypes) and identify their potential sources. Interestingly, even though most of these artifacts were not identified below the rank of order, in some cases, when most of the chimera corresponded to a particular identifiable taxon, they yielded a positive taxonomic identification at least at family level. As with the T-saturation problem mentioned above, only a manual examination of data made it possible to detect these sequences, often with partial *Wolbachia* sequences, and remove them from subsequent interpretations of data.

The Challenge of Metabarcoding Libraries of Herbivorous Insects

Although not a technical problem per se, the main setback that we found in our study was the hyperabundance of contaminant plastid sequences, which nearly obliterated target bacterial sequences in some libraries. Finding these specific plant contaminants is common and a permanent source of concern in metabarcoding studies of microorganisms from certain environments (e.g., Sakai and Ikenaga 2013). For example, metabarcoding studies of plant microbiomes, research of soil samples and of herbivorous insects have shown that organellar sequences can account for more than 80% of data retrieved from these samples (as reported by Lundberg et al. 2013). Conversely, studies on nonphytophagous beetles show that the proportion of these contaminants stays very low (Hammer et al. 2015). In our case, working with abdominal contents of herbivorous beetles and plant tissue, finding them was certainly expected. However, with a 99.9% rate of retrieval of these contaminant sequences in a number of samples, this surpassed any original expectation. Similar studies focusing on herbivore beetles typically report that chloroplast (and/or mitochondrion) sequences were detected and removed. However, the figures to assess the importance of these findings are often not shown or are buried in supplements (Hanshaw et al. 2013). The few explicit data that we could find show that these figures are erratic, even within a single study, and it is difficult to recognize a pattern. For example, Hammer et al. (2015) found around 20% of chloroplast sequences in samples of phytophagous *Epilachna* ladybugs; Kelley and Dobler (2011) reported about half the sequences of their *Longitarsus* flea beetle microbiome libraries being of chloroplastic origin; or Blankenchip et al. (2018), in a study of microbial profiles in *Cephaloleia* leafminers, found up to 93.6% nontarget sequences in one of their libraries, and 60.6% (SD = 19.2%) on average in their whole data set. Such lack of consistency possibly responds to idiosyncratic factors from each sample and/or study, in part related to practical choices (e.g., tissues used for library preparation),

incomparability of approaches (e.g., PCR primers), metabolic state of the individual (e.g., food quantity and time since last meal), or ecological determinants (e.g., host plant species), among others.

Beckers et al. (2016) reviewed this problem and the strategies that researchers use to avoid it. These include 1) DNA extraction protocols that minimize organellar DNA co-extraction, 2) use of blocking agents to prevent PCR amplification of specific targets, or 3) the method of choice in most studies, based on mismatch primers specifically designed to avoid the amplification of organellar genes. We opted for the latter strategy, using V3–V4 mismatch primers supposedly excluding eukaryotic versions of the 16S rRNA gene, at least in environments with low plastid input (Klindworth et al. 2013, Beckers et al. 2016). Yet, this strategy proved inefficient in our hands, at least for some of the samples, specifically all *Calligrapha* individuals tested and one of *Ch. mainensis*. In those cases where we obtained a high representation of plastid genes, it is likely that they were preferentially amplified because they were already in a much higher proportion relative to bacterial genes in the extracted DNA (Sakai and Ikenaga 2013). In retrospective, it seems possible that our dissections of abdominal tissue in the larger *Calligrapha* beetles ended up taking gut tissue, and unfortunately also gut contents, e.g., recently consumed and partially digested plant tissue. A lesson directly derived from the study of Fitzpatrick et al. (2018) on the efficiency of peptide nucleic acid clamps to prevent nontarget coamplification depending on the organelle gene sequence is that the efficiency of mismatch primers may also depend on the plant species. We cannot discard that the unintended presence of plant tissue coupled with a reduced mismatch efficiency in alder contributed to exacerbate the nontarget amplification problem.

Regardless of the actual explanation for such a high representation of nontarget sequences in some of our libraries, our main concern was whether the idiosyncratic composition of these libraries could diminish the reliability of the comparatively few endosymbiont (or

other bacterial) inferences retrieved. Several lines of evidence suggest that these sequences may indeed represent trustworthy associations. One of the *Ch. mainensis* libraries showed the same nontarget near exclusivity as seen in all samples of *Calligrapha*, but it also showed evidences for a relatively small number of *Wolbachia* sequences. These contigs belonged without exception to haplotype W1, the most abundant by far in all the other *Chrysomela* samples. In turn, *Calligrapha* libraries had a low representation of *Wolbachia* and/or *Rickettsia* sequences, but they were either identical to the most abundant sequences characterized in other species or were shared between *Calligrapha* species, enormously reducing the odds of being an artifact. The same would be true for the bacteria characterized on the leaf samples, particularly those of *Wolbachia*, again belonging to the W1 haplotype, the most abundant in the entire sample.

Implications of Endosymbiont Diversity and Their Distribution

Some remarkable results from our study include 1) finding that all samples, except for two of the three leaf extracts, had evidence for *Wolbachia*, for *Rickettsia*, or for both, 2) that these bacteria were clearly dominant in all cases, and 3) that more than one type of endosymbiont were present in every sample (Fig. 4). The rate of infection with *Wolbachia* in Coleoptera varies considerably, depending on the host taxonomy, with Chrysomelidae showing intermediate values (Kajtoch and Kotásková 2017). However, our study precisely focused on two genera, *Altica* and *Calligrapha*, which have been shown to have particularly high infection rates with *Wolbachia* among the leaf beetle groups tested (Jäckel et al. 2013, Gómez-Zurita 2019). *Rickettsia* has received much less attention than *Wolbachia* in studies of associations of bacteria with Coleoptera. Yet, although it is less prevalent than *Wolbachia*, it is not rare to find this association too (Kolasa et al. 2019). The fact that these endosymbiont

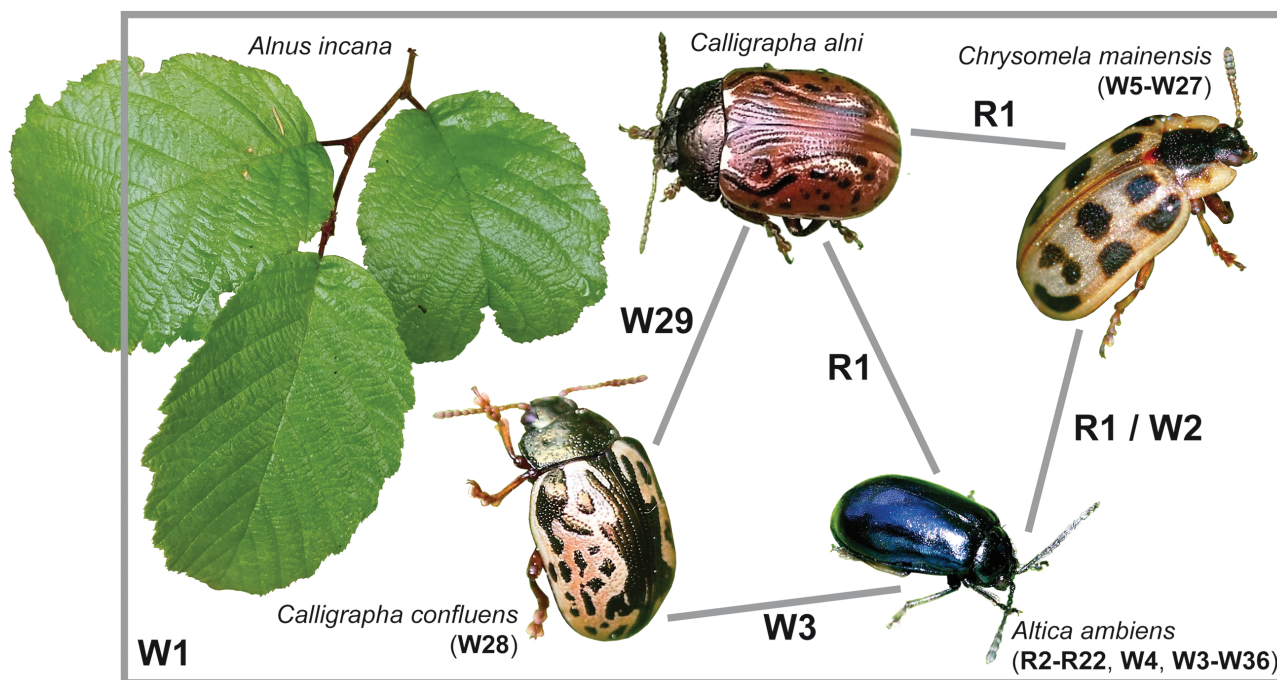


Fig. 4. Schematic representation of endosymbiont diversity and shared associations of *Rickettsia* (R haplotypes) and *Wolbachia* (W haplotypes) among alder specialists of the study. Haplotype W1 of *Wolbachia* is shared among all species, including samples of the host plant, *Alnus incana*, and haplotype R1 of *Rickettsia* was detected in the three of the studied genera of Chrysomelidae. [Pictures used with permission from the authors: see Acknowledgments.]

Rickettsiales are prevalent in the specimens studied could explain in part the low diversity observed for other bacteria (Kolasa et al. 2019). Also in support for this idea, Blankenchip et al. (2018) empirically found that beetles infected by *Rickettsia* and *Rickettsiella* had lower levels of other bacterial groups, while beetle species free from *Rickettsia* presented microbiomes more diverse. This particular effect of endosymbionts modulating the diversity of other bacterial associations could be coupled with several other characteristics of our system that have been related with lower numbers of host associations with bacteria. For example, gut bacterial diversity has been explained by a number of factors including the taxonomic group (Yun et al. 2014), and in this respect, leaf beetles seem to have typically lower diversity in their microbial profiles compared to other beetle groups (Kolasa et al. 2019). Also, generalist species have been proposed to harbor more diverse microbiota, perhaps related to their exposure to a wider range of bacterial sources (Engel and Moran 2013, Hansen and Moran 2014; but see Blankenchip et al. 2018). However, this latter explanation may be questionable, since individuals of generalist insect species are nonetheless most likely exposed to a single or very few potential host plants through their lives. In any case, the species studied here, known alder specialists (Clark et al. 2004), have necessarily restricted diets and exposure.

Lessons About Endosymbiont Transmission Among Alder Specialists

The presence of more than one endosymbiont in each sample and information on their relative proportions represent the findings with more relevant implications relative to the mode of transmission of these bacteria. It appears that each species has their own typical endosymbiont strains of either *Wolbachia* and/or *Rickettsia*: W1 and W2 *Wolbachia* strains in *Chrysomela*; W28 and W29 *Wolbachia* strains in *Calligrapha*; and W3 and W4 *Wolbachia* and R1 *Rickettsia* strains in *Altica*. Given the taxonomic conservatism and their prevalence in every case, we can assume that these associations represent the stable, vertically inherited germline infections for these individuals or populations. Differences in the life histories of these insects and the ways they relate with the environment could explain the patterns of endosymbiont exclusivity and also potential sources of transmission from external sources in the trophic community. In principle, one could speculate that these differences would have a major impact if they affected larvae, when individuals are first exposed to external infections. However, the most notable difference among these species has to do with pupation, occurring underground in the case of *Altica* and *Calligrapha*, and on the host leaves in the case of *Chrysomela*. Otherwise, these beetles lay eggs on the host plant; develop as larvae feeding on leaves (*Altica* are rather an exception among flea beetles, which usually develop as larvae in the soil; Doguet 1994); and live as adults associated with the host plant (Woods 1917, Whitehead 1919, Brown 1956). There is not much information on endosymbiont turnover across these developmental stages in insects, but it is possible that contrary to gut bacteria (Yun et al. 2014), pupation and the drastic disintegration of larval tissues do not necessarily involve a break in the association with endosymbionts, which are carried along from larval to adult stages (e.g., Goto et al. 2006, Zhukova et al. 2017). If this were the case for these leaf beetles, life-history similarities would rule out most species-specific external sources of endosymbiont transmission (except, perhaps, the contribution of parasitoids; Ahmed et al. 2015).

Endosymbiont exclusivity contradicts in part our initial expectation that a tight ecological network like the one portrayed in this

study, where different species can be found contemporaneously on a same plant, may have higher chances to share their endosymbionts as well. These results mirror those obtained for other phytophagous beetles in equally close trophic communities, competing for identical resources (Merville et al. 2013). But, what do the lower frequencies of these *Wolbachia* and *Rickettsia* haplotypes represent in other species? Considering that these endosymbionts could reflect either stable infections or transient associations with the host (e.g., Chiel et al. 2009, Chrostek et al. 2017, Joubert and O'Neill 2017), can we discard their acquisition from the environment and challenge the hypothesis that common food choice favors sharing of endosymbionts? The strain represented by haplotype W1 in *Wolbachia* is the most informative to advance an answer to these questions thanks to a couple of circumstantial evidences: 1) this strain of *Wolbachia* was also confidently found on a leaf extract, while it was not supposed to be there because at the time of collection or during processing it had not been in touch with any of the leaf beetles; and 2) the only sample outside of the chrysomelid-alder system, i.e., the sample of the dogwood specialist *Ca. philadelphica*, was also the only one without W1 *Wolbachia*, although it objectively shared the W28/W29 strains, considered vertically transmitted and stable associations with the other two species of *Calligrapha* in the study. The latter is interesting, since it relates this finding with the other only study on *Calligrapha* endosymbionts, because we know that individuals of *Ca. philadelphica* in the locality of the study and in the general area are infected by two *Wolbachia* strains that were named *wCalA1* and *wCalC2*, with *wCalA1* shared with at least three other species, two of them specialized on yet a different host plant, willows (Gómez-Zurita 2019). These data are compatible with the hypothesis that host plants favor shared endosymbiont associations, suggesting a number of interesting ideas, worth testing with a more extensive study. These include, for example, that W1 in this system is in a stable association with *Ch. mainensis* based on its high representation and in all individuals analyzed. Also, that these bacteria are transitorily present in the environment (e.g., *Alnus* leaves), most likely propagated from their usual host. And, finally, that other herbivore beetle species living in the same environment get exposed to and introduce in their bodies these abundant bacteria, simply by feeding. Similar statements could be proposed for the R1 variant of *Rickettsia*, characteristic of *A. ambiens* and found at low frequencies in *Ca. alni* and *Ch. mainensis*; however, we prefer to be more cautious about interpretations on this endosymbiont, since we did not find it on *Alnus* leaves.

Based on metabarcoding studies, particularly using powerful next-generation sequencing (NGS) methodologies and a low-resolution taxonomic marker such as the 16S rRNA gene, we cannot know whether the secondary associations that we detected also respond to secondary infections of new hosts, thus accomplished horizontal transfers, or just a transient stage in the gut of the studied individuals after they fed upon contaminated food (e.g., Chiel et al. 2009). However, even if we could prove that endosymbionts like the one represented by the W1 strain of *Wolbachia* in our system were not transferred horizontally and infected alternative hosts, that it was a transient association, data from other studies support that this mechanism is possible (e.g., Mitsuhashi et al. 2002, Sintupachee et al. 2006, Fenton et al. 2011, Caspi-Fluger et al. 2012, DeLay et al. 2012, Gonella et al. 2015, Li et al. 2016, Kolasa et al. 2017). In this context, we can still contribute additional understanding about this transmission mechanism. At least the first necessary stages in the chain of events leading to horizontal transfer via food, i.e., the transmission from an original host to the environment and the acquisition from this external source, a host plant in this particular case, by a new insect host is certainly a possibility (Chrostek et al. 2017). Relative to the presence of the

bacteria on the plant, we still favor a viewpoint that considers this to be accidental and temporary, e.g., through feces or laid eggs. However, new evidence suggests that endosymbionts may have some potential to survive in environments outside of the insect host, including plant tissues (Caspi-Fluger et al. 2012, Gonella et al. 2015, Li et al. 2016). In any case, this possibility only increases the chances to acquire endosymbionts through ingestion even from phylogenetically distant hosts that use the same host plant.

Conclusions

The accessibility, abundance, and taxonomic diversity of insects associated with *Alnus* and the high natural prevalence of *Wolbachia* and *Rickettsia* in species assemblages like the one studied here contribute to this trophic network being a suitable system to investigate how these associations occur and how endosymbionts may be acquired and transmitted in nature. In our exploratory study of these associations, we found some analytical problems that may be specific for the system, including species that seem more recalcitrant to yield data from their microbiota and a pervasive problem with the use of our choice of V3–V4 primers in an alder-centric environment. However, this experience also helps pointing the way as well as new questions for subsequent approaches to investigate this system, including the use of methodologies that effectively avoid the amplification of organelle sequences (e.g., Hanshew et al. 2013, Beckers et al. 2016), and also investigating causes that could explain the nonrandom efficiency of library preparation comparing *Calligrapha* with the other species analyzed. We found high and low frequency associations of *Wolbachia* and *Rickettsia* with each species investigated, which have been interpreted as stable and transient associations, respectively, with the host. Moreover, we proposed the low frequency, transient associations to be probable because all the species share the same host plant with the particular donor of this strain, *Ch. mainensis* in this case. However, some elements of this hypothesis need to be verified with population level analyses of these hosts and their endosymbiont associations, also with genetic markers that allow for higher discrimination of bacteria strains, and in localities where these species do not co-exist, to exclude the possibility of contemporary, transient transmission. Moreover, as hinted by the results on *Ca. philadelphica*, other congeneric species with the same and different host plant choices and sympatric with the species studied here would likely provide a useful comparative context for the observed associations with bacteria, including endosymbiont species. Indeed, there are close relatives of *A. ambiens* and *Ch. mainensis* that also feed on *Alnus* (e.g., *A. alni* Harris, *Ch. interrupta* F.; Clark et al. 2004), and others that are specialized on *Salix* (e.g., *A. subplicata* LeConte, *A. bimarginata* Say, *Ch. knabi* Brown; Clark et al. 2004). Relative to the low frequency associations, retrievable thanks to the use of NGS approaches, they have been important for a deeper understanding of the system. In our case, we scored them preferentially as transient stages (possibly in the gut) upon ingestion in the case of the beetle samples, or upon smearing from a *Ch. mainensis* donor in the case of the leaf samples. These transient stages are recognized as a necessary but insufficient condition to allow for horizontal transmission to occur (Chrostek et al. 2017). However, it would be interesting to verify if the presence of these low frequency strains actually represents a historical infection of these hosts and not contemporaneous to this study. Or, even if the infection were contemporary, discovering whether the infection found its way to the germinal line or to somatic tissues only, which could explain both stable coinfections and differential population densities of the endosymbiont (Pietri et al. 2016). The ecological and evolutionary

prospects of these studies are enticing and the analysis of more natural systems like the one we put forward in this work can help a better understanding of the mechanisms behind the association and transmission of endosymbionts to new hosts.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

Table S1. Number of surviving reads and their taxonomic identification using DADA2 (Callahan et al. 2016) and the identification algorithm IDTAXA of DECIPHER (Wright 2016, Murali et al. 2018). Sample codes and localities are the same as in Tables 1 and 2 of the main article.

Figure S1. Maximum likelihood tree of *Rickettsia* 16S rRNA V3–V4 haplotypes obtained from our study (marked in bold) and the homologous sequences retrieved from GenBank using BAGpipe (Papadopoulos et al. 2015). The host from which the particular sequence was obtained is given after each accession number and color-coded according to their taxonomic group, typically Order. Bootstrap supports higher than 50% are shown.

Figure S2. Maximum likelihood tree of *Wolbachia* 16S rRNA V3–V4 haplotypes obtained from our study (marked in bold) and the homologous sequences retrieved from GenBank using BAGpipe (Papadopoulos et al. 2015). The host from which the particular sequence was obtained is given after each accession number and color-coded according to their taxonomic group, typically Order. Bootstrap supports higher than 50% are shown and two major haplotype assemblages corresponding to different supergroups of *Wolbachia* are highlighted.

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