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Endosymbiont diversity among sibling weevil species competing for the same resource

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Abstract

Background: Whereas the impact of endosymbionts on the ecology of their hosts is well known in some insect species, the question of whether host communities are influenced by endosymbionts remains largely unanswered. Notably, the coexistence of host species competing with each other, which is expected to be stabilized by their ecological differences, could be facilitated by differences in their endosymbionts. Yet, the composition of endosymbiotic communities housed by natural communities of competing host species is still almost unknown. In this study, we started filling this gap by describing and comparing the bacterial endosymbiotic communities of four sibling weevil species (*Curculio spp.*) that compete with each other to lay eggs into oak acorns (*Quercus spp.*) and exhibit marked ecological differences.

Results: All four species housed the primary endosymbiont *Candidatus Curculioniphilus buchneri*, yet each of these had a clearly distinct community of secondary endosymbionts, including *Rickettsia*, *Spiroplasma*, and two *Wolbachia* strains. Notably, three weevil species harbored their own predominant facultative endosymbiont and possessed the remaining symbionts at a residual infection level.

Conclusions: The four competing species clearly harbor distinct endosymbiotic communities. We discuss how such endosymbiotic communities could spread and keep distinct in the four insect species, and how these symbionts might affect the organization and species richness of host communities.

Keywords: Endosymbiosis, Host community, Curculio, Oak weevil, Infection pattern, Niche partitioning, Field study

Background

The last decade has seen a growing number of studies exploring microbial endosymbiosis among arthropods and its implication for the evolution of the host species. The impressive array and diversity of species harboring one or more type(s) of endosymbionts raises the possibility that such associations may play a predominant role in the ecology and evolution of both partners. Most studies that have considered symbiotic associations from the perspective of the host support the view that heritable symbionts offer their host an opportunity for rapid and deep evolutionary changes and for greater adaptation to a novel environment [1-3]. Symbionts are considered to be

a keystone in the diversification and expansion of the ecological niche of their host [3-5]. Thus, endosymbionts have been shown to assist their host in exploiting poor nutritional resources, notably by complementing its nutrition [6-8], often to the point that they become essential or obligatory symbionts for the host. From the differences observed between the endosymbiotic communities of various host populations, it has been suggested that symbionts may mediate the specialization of their hosts on distinct resources and thus promote the emergence of allopatric host races, which might subsequently lead to speciation [9-15].

Mutualistic facultative symbionts are also able to provide their host with other skills, such as broadening the range of temperatures they can tolerate [16], reinforcing their ability to resist natural enemies [17-20], or increasing their dispersal capacity [21-24]. These rapidly-acquired capacities are likely to promote the expansion of the host populations and the diversification of their habitat [1-5].

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Besides these mutualistic relationships, facultative endosymbionts are also known to manipulate the reproduction of the host. In response to selective pressures favoring their vertical transmission, many symbionts, such as *Wolbachia*, have been shown either to skew the sex-ratio of the offspring toward females or to induce sterilization of uninfected females [25-27]. Such effects can affect both the population dynamics and gene flow, notably by promoting the reproductive isolation of infected hosts.

While firm knowledge is accumulating about the symbiont-mediated interactions between given host species and their environments, the possibility that endosymbionts may influence the organization of communities composed of several competing host species has so far been seldom investigated. The first empirical evidence of an endosymbiont-mediated relationship between competing host species has been recently provided from artificial protist communities kept under highly-controlled laboratory conditions. It was suggested that a green algal symbiont is essential for competing protist species to coexist, notably because the allelochemicals and the nutritional resource provided by this microorganism seem to balance the competitive capacity of the hosts [28]. Moreover, the issue of the competition between two hymenopteran parasitoid species has been shown experimentally to be tightly dependent on the presence of an inherited virus in one of the insect species [29]. These findings highlight the fact that the influence of microbial symbionts on the organization of competing host communities may have been considerably underestimated. The paucity of empirical data about the role of endosymbionts on competing host communities contrasts with the body of knowledge that has accumulated about extracellular symbiosis that has been shown to play a key role in the organization and composition of plant communities [30-32]. Microbial communities present in the soil often mediate nutrient uptake of plants, and might facilitate the coexistence of plant species in various ways, notably by allowing resource partitioning (reviewed in [33]).

A first support to the hypothesis that endosymbiosis does impact communities of insect species competing with each other would be that endosymbiotic profiles vary with respect to the host species. To study this aspect, we investigated endosymbiosis in natural oak weevil communities composed of four *Curculio* species – *C. glandium* (Marsham), *C. elephas* (Gyllenhal), *C. pellitus* (Boheman), and *C. venosus* (Gravenhorst) (Coleoptera, Curculionidae). These weevils coexist on the same individual oak trees (*Quercus* spp.) in southern Europe [34-36], where they compete for oak acorns that constitute the sole food resource for larval development and that have been shown to be highly limiting some years [37]. This biological system is relevant for examining our proposal in that (i) the four oak weevil species are expected to host

endosymbionts, like the few other *Curculio* species already described [15,38] and (ii) they exhibit marked differences in their life history traits despite their recent divergence [36], suggesting that their coexistence is stabilized by means of ecological niche partitioning [37].

We provide an exhaustive description of the endosymbiotic meta-community harbored by the four oak weevil species in two distinct natural communities. Using a correlative and a quantitative approach, we found that endosymbiont communities differed across the four *Curculio* species, while being consistent within each species. We discuss the mechanisms possibly accounting for the contrasted infection patterns observed across these sibling species and the ways by which endosymbiosis may contribute to structuring communities of insect species competing with each other.

Methods

Study system

Four weevil species of the genus *Curculio* (*C. glandium*, *C. elephas*, *C. pellitus* and *C. venosus*) coexist on oak trees (*Quercus* spp.) in southern Europe [34]. In all four species, females lay eggs during summer within oak acorns, where larvae achieve their development before self-extracting and burrowing into the soil. Fully mature larvae then enter diapause during variable time periods -i.e., they spend from one to four years underground-, depending on the species or even on individuals within one species [37].

Insect sampling

Our study was conducted on two natural communities found in two sites in southern France (site 1: N45° 35'; E5° 01'; site 2: N45° 45'; E5° 16'). Adult weevils were live-trapped on oak trees at each site weekly throughout one breeding season (June to September 2009) by beating branches with a wooden stick [39]. Insects were collected on a white sheet laid under the tree and their species was identified from morphological traits [34].

DNA extraction

Ovaries of 391 adult females caught on one of the two study sites and belonging to one of four weevil species (see Table 1) were dissected in buffer A (KCL 25 mM, MgCl₂ 10 mM, Saccharose 250 mM, Tris-HCl 35 mM; pH = 7.5) and homogenized in 500µL of buffer STE (NaCl 100 mM, Tris-HCl 10 mM and EDTA 1 mM, pH = 8). These dissected tissue samples were individually subjected to DNA extraction by adding proteinase K (120 mg/L) and SDS (sodium dodecyl sulfate: 0.5% m/v), and were then incubated for 2 h at 55°C. RNA contamination was removed with the addition of RNaseA (60 mg/µL) for 1 h at 37°C. DNA from each sample was then purified with phenol-chloroform extraction and precipitated using

Table 1 Number of adult males and females of each weevil species collected at the two sites

Species	Females		Males	
	Site 1	Site 2	Site 1	Site 2
<i>C. elephas</i>	74	38	25	21
<i>C. glandium</i>	76	40	26	29
<i>C. pellitus</i>	78	7	36	7
<i>C. venosus</i>	64	14	15	12

isoamelic alcohol [40]. In addition, 171 adult males (Table 1) were automatically crushed individually with stainless steel beads shaken 20s at 20Hz (Tissue Lyser, Qiagen) [41] and their total DNA was extracted using NucleoSpin Tissue Kit (Macherey-Nagel).

Characterization of the endosymbiotic lineages and phylogenetic analyses

To get an overview of the endosymbionts harbored by the four *Curculio* species, bacterial 16S rRNA amplification was performed individually on the ovaries of 95 females among which 40 *C. elephas* (24 from Site 1 and 16 from Site 2) and 20 females of each of the three remaining species (*C. glandium*, *C. pellitus* and *C. venosus*) comprising 10 females per site, except for *C. pellitus* from the site 2 for which only 5 females could be sampled. A 1.5 kb fragment was amplified using eubacterial universal primers (Table 2). Reactions were carried out in a 50µL final volume consisting of 2.5 units of Taq DNA polymerase (UptiTherm, Interchim, France), 2 mM MgCl₂, 0.8 mM

dNTPs, 0.6 µM primers, and 200 ng DNA template. The polymerase chain reaction (PCR) started at 95°C for 5 min and was followed by 26 cycles, each of these running 95°C for 30s, 53°C for 1 min and 72°C for 2 min; the final step of the reaction was 7 min at 72°C. The PCR products were then pooled into eight groups, one per species and per site. Each of these pools was then subjected to cloning. In a first step, ten clones per pool were randomly selected and sequenced (Additional file 1; Beckman Coulter Genomics, Grenoble, France). Identifying these sequences revealed a predominance of three endosymbiont types (among which, mostly *Curculioniphilus buchneri*, and to a lesser extent, *Wolbachia* and *Rickettsia*; see below). Consequently, in a second step at least 130 clones were grown up for each pool (Additional file 1), and each clone was screened by diagnostic PCR using the specific primers corresponding to the three dominant bacterial types (Table 2). Clones that were grown successfully but that could be amplified by none of these three diagnostic PCRs (*i.e.*, *Curculioniphilus buchneri*, *Wolbachia* and *Rickettsia*) were then sequenced (30 clones; see Additional file 1).

We characterized the bacteria hosted by *Curculio* females by performing phylogenetic analyses using the sequences obtained in this study together with the most similar ones found by Blast in Genbank. Multiple sequences were aligned using MUSCLE software [46]. The appropriate model of evolution was estimated with jmodeltest [47] for each set of sequences considered. Phylogenetic analyses were performed using maximum likelihood (ML) inference with PhymI v3.0.1 [48]. The

Table 2 Primers used for PCR diagnostic

Target Species	Target gene	Primer name	Primer sequence (5'-3')	Product size (kb)	Temp.* (°C)	Refs.†
Host (<i>Curculio</i> sp.)	CytB rRNA	CBJ-10933	TATGTACTACCATGAGGACAAATATC	0.5	45	[42]
		CBN-11367	ATTACACCTCCTAATTTATTAGGAAT			[42]
Eubacterial Universal Primers	16S rRNA	008For	AGA GTT TGA TCA TGG CTC AG	1.5	53	[43]
		1487Rev	TAC CTT GTT ACG ACT TCA CC			(**)
<i>Candidatus</i> <i>Curculioniphilus buchneri</i>	16S rRNA	16S-F	AGAGATCTGGAGGAATATCA	0.4	52	(*)
		16S-R	CACTAAAGCATCTCTGCTAAAT			(*)
	<i>GroEL</i>	GroEL 2 F	ATG GGB GCT CAA ATG GTK AAA	0.9	55	[38]
		GroEL 2R	CTCTTTCATTCAACTTCNGTBGCA			[38]
<i>Wolbachia</i>	16S rRNA	W-Spec F	CATACCTATTCTGAAGGGATAG	0.4	60	[44]
		W-Spec R	AGCTTCGAGTGAAACCAATTC			[44]
<i>Rickettsia</i>	16S rRNA	RbF	GCTCAGAACGACGCTATC	0.9	58	[12]
		RbR	GAAGGAAAGCATCTCTGC			[12]
<i>Spiroplasma</i>	ITS and 16S rRNA	Spixo-16S F	TTAGGGGCTCAACCCTAACC	0.8	52	[45]
		Spixo-16S R	TCTGGCATTGCCAACTCTC			[45]
<i>Sodalis</i>	16S rRNA	Sodalis 370 F	CGRTRGCGTTAAYAGCGC	0.2	55	[38]
		16SSod590R	AACAGACCGCTGCGTACG			[38]

Annealing temperature. †References: () this study; (**) nucleotides numbering from *Escherichia coli*, GenBank accession number J01859.

models selected were *GTR + G* for *Rickettsia* (16S, 873 sites) as well as for *Spiroplasma* (16S + *ITS*, 1405 sites), *HKY85 + I* for *Serratia* (16S, 503 sites), *K80 + I* for *Wolbachia* (16S, 340 sites). The robustness of the nodes was assessed with 100 bootstrap replicates. Additionally, Bayesian phylogenies were performed with MrBayes v3.1.2 [49] using appropriate parameters leading to convergence between two runs. We then compared topologies obtained with the two methods using approximate unbiased (AU) [50] and Shimodaira and Hasegawa (SH) [51] tests available in the CONSEL program package [52]. No significant difference was detected between the two topologies for any of the bacteria.

To examine the possible co-diversification between *Curculioniphilus buchneri*, considered as the primary symbiont, and its hosts, we used the method previously described to build the phylogenies based on *COI* gene sequences retrieved from Genbank for the weevil phylogeny, and on two distinct markers, the 16S *rRNA* sequence and the *GroEL* gene, for the bacterial phylogeny. Nine host-symbiont pairs were included in the analysis, among which the four *Curculio* species studied here, the hazelnut weevil *C. nucum* that we collected in a French population near Lyon, and four Japanese *Curculio* species whose primary symbiont has already been described (*C. cameliae*, *C. sikkimensis*, *C. robustus* and *C. dentipes*; [38]). The models selected were *TPM2 + I* for *C. buchneri* (16S, 267 sites), *GTR + I + G* for *Curculioniphilus buchneri* (*GroEL* gene, 915 sites) and for *Curculio* spp. (*COI* gene, 375 sites). For *Curculioniphilus buchneri* bacteria, the congruence of the 16S *rRNA* and the *GroEL* topologies was rejected by the reciprocal AU and SH tests (AU: $P = 0.004$; SH: $P = 0.014$) probably because the 16S topology was weakly resolved, which precluded the concatenation of both sequences. Then, we tested the global congruence between the symbiont and host phylogenies, using 16S and *GroEL* gene for the bacterial tree separately, with the CopyCat program (The Cophylogenetic Analysis Tool, version 2.00.02 [53]). After checking the occurrence of global co-diversification, we further tested the association of each of the nine individual host-symbiont pairs using the *COI* and the *GroEL* phylogeny that provided the best resolved bacterial tree. Patristic distances were used as input matrix and the AxParafit program was run with 999 permutations [54].

Diagnostic PCR

After checking the quality of the DNA of each insect sample (by amplifying a 487 bp fragment of the *cytochrome b* gene of the insect; Table 2), 562 adult weevils (males or females from one of the four species; see Table 1) were screened for each of the five endosymbionts found in the first part of the study using their specific primers (*i.e.*, *C. buchneri*, *Wolbachia*, *Rickettsia*, *Spiroplasma* and *Sodalis*; Table 2). Although diagnostic PCR might fail to detect an

endosymbiont that might be too rare in the host individual, we considered in subsequent analyses that the insect was free of this bacterium. To check whether the successful PCRs were actually specific of the targeted symbiont, two DNA amplicons randomly selected per symbiont and per weevil species were sequenced. To discriminate between the two possible *Wolbachia* strains detected in our samples (see Results), we performed a Restriction Fragment Length Polymorphism following the PCR assay based on the 16S *rRNA* sequence (PCR-RFLP) on each *Wolbachia*-infected weevil. For that purpose, 17 μ l of each PCR product was digested at 37°C for 3 h with 5U of *AluI* (Fermentas, Villebon/Y., France) and was separated on a 2% agarose gel electrophoresis during 1 h at 100 V. One DNA sample from each *Wolbachia* strain and each host species was randomly selected and sequenced to check the accuracy of the bacterial lineage identified by PCR-RFLP.

Data analysis

In each weevil species, we used a generalized linear model (GLM) fitted with a binomial error and a logit-link function to test whether the probability for an insect to be infected by the predominant facultative endosymbiont depended on its sex, on the locality it came from, or both. The best-fitted model was selected for each endosymbiont according to the AIC criterion. Further, the effects of each variable selected in the model and the possible interactions between variables were tested by an analysis of deviance. All the analyses were performed with the R software v.2.12.0 (<http://www.r-project.org/>).

Results

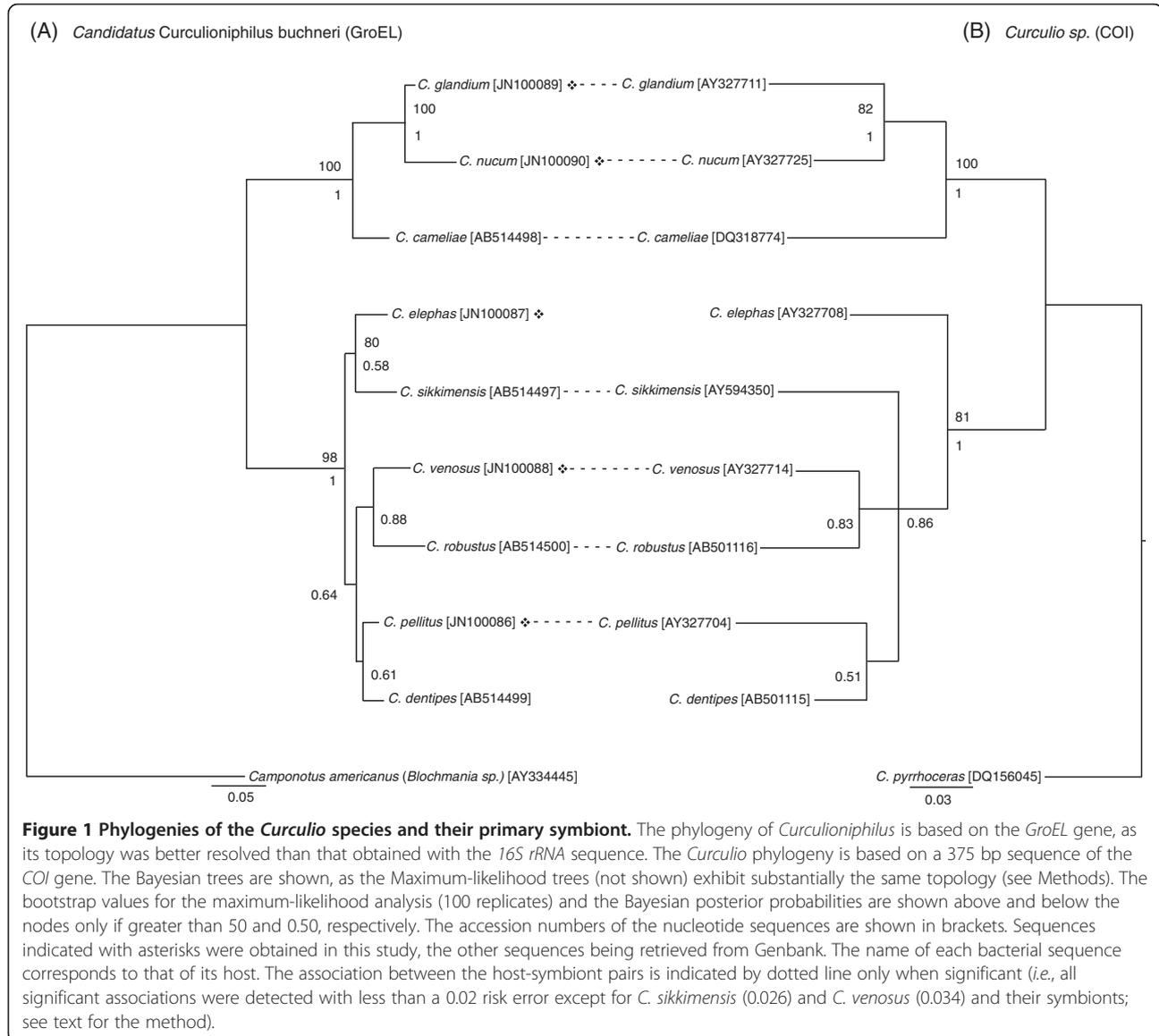
Endosymbiotic communities in the oak weevil communities

On the basis of 1,358 16S *rRNA* clones derived from the ovaries of females of the four oak weevil species collected at the two study sites, ten distinct bacterial lineages were identified (Additional file 1): *Curculioniphilus buchneri*, *Wolbachia*, *Rickettsia*, *Spiroplasma*, *Sodalis*, *Serratia*, *Xenorhabdus*, *Brevundimonas*, *Erwinia* and *Escherichia*. *Xenorhabdus*, *Brevundimonas* and *Erwinia* are known to be entomopathogenic and/or free-living bacteria. The identified strain of *Escherichia* corresponds to the vector used for the cloning step. The phylogenetic analysis of *Serratia* reveals that it is part of a monophyletic group of non-symbiotic bacteria that are distant from those known as symbionts (data not shown). Furthermore, this bacterium was shown to infect very few insect individuals at any site and irrespective of the weevil species, suggesting that the *Serratia* strain identified in this study was likely to be an insect pathogen: we discarded it from further analysis. A single clone among 1,358 ones was identified as a *Sodalis*-like endosymbiont (97,77% of similarity with the *Sodalis* strain found in *C. sikkimensis*). It

was detected in the pool of *C. elephas* females sampled at the site 2, and could be *a posteriori* detected by means of individual PCR diagnostic in one of the 16 females of this pool. We could not detect this symbiont in any of the 562 oak weevils individually screened with the same method. Consequently, we conducted phylogenetic analyses on the five endosymbionts identified (i.e. *Curculioniphilus*, *Wolbachia* strains 1 and 2, *Rickettsia*, *Spiroplasma*) (Figures 1 and 2).

For the *Curculioniphilus* endosymbiont, four distinct sequences were obtained, one for each weevil species. We found statistical evidence of global co-diversification between weevil hosts and their primary symbiont on each dataset ($P = 0.002$ for *COI-GroEL* comparison, $P = 0.005$ for *COI-16S* comparison). In addition, the association of seven of the nine host-symbiont pairs individually tested

was found significant (see Figure 1). The insignificant associations corresponded to *Curculio elephas* and *C. dentipes* with their symbionts, and could be explained by their weakly supported positions in the phylogenetic trees generated either by bootstrap or by Bayesian posterior probabilities. This finding suggests co-diversification between *Curculioniphilus* and its weevil hosts and implies that this endosymbiont has been acquired by a common ancestor of *Curculio* spp. and subsequently strictly vertically transmitted. The *Wolbachia 16 s rRNA* sequences revealed at least two distinct strains. The corresponding phylogeny (Figure 2A) indicates that one of them belongs to the *Wolbachia* supergroup A while the other one seems rather positioned at the edge of the *Wolbachia* supergroup B, according to the way *Wolbachia* sequences are annotated in Genbank. While we detected *Rickettsia* in all four



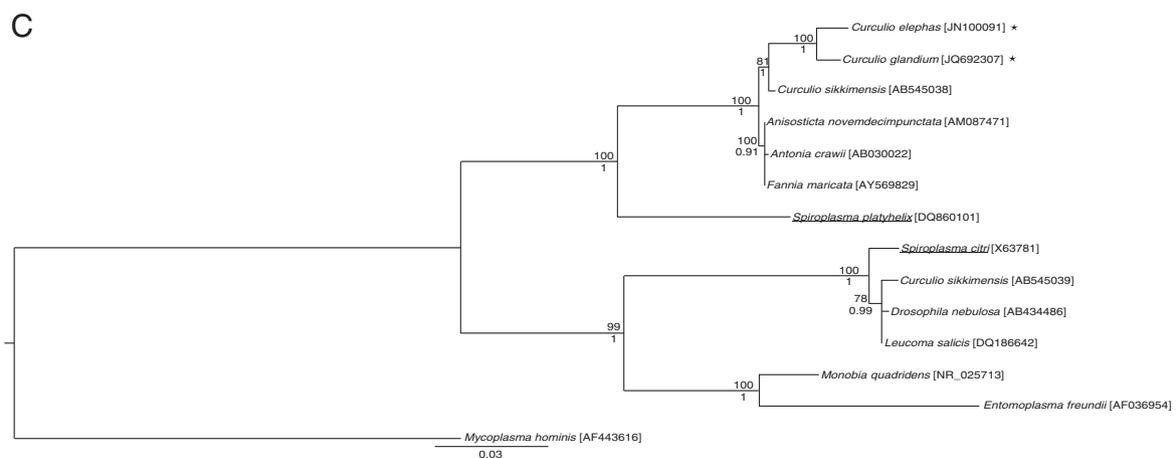
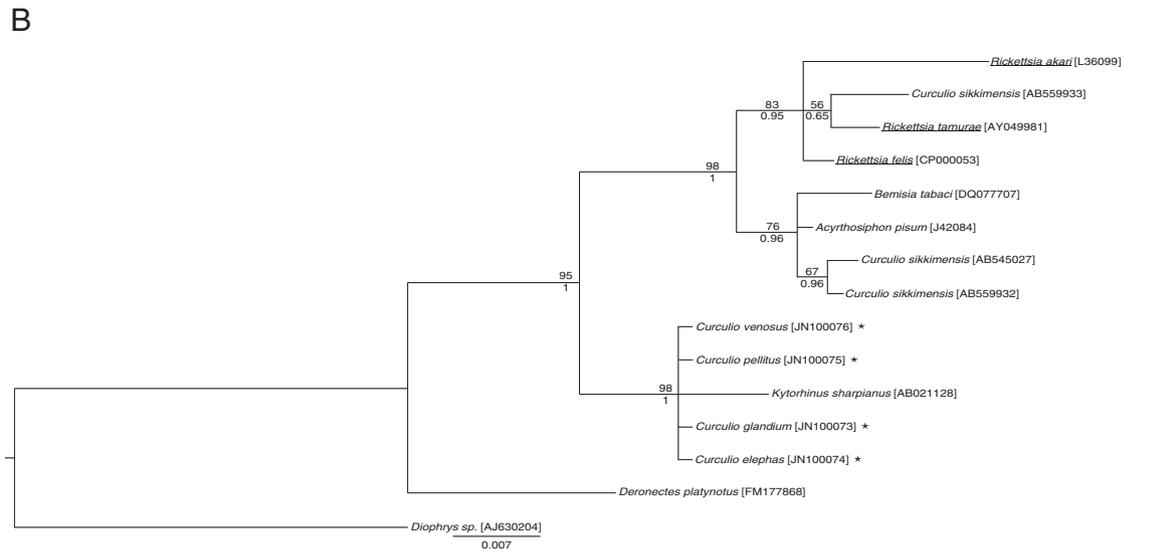
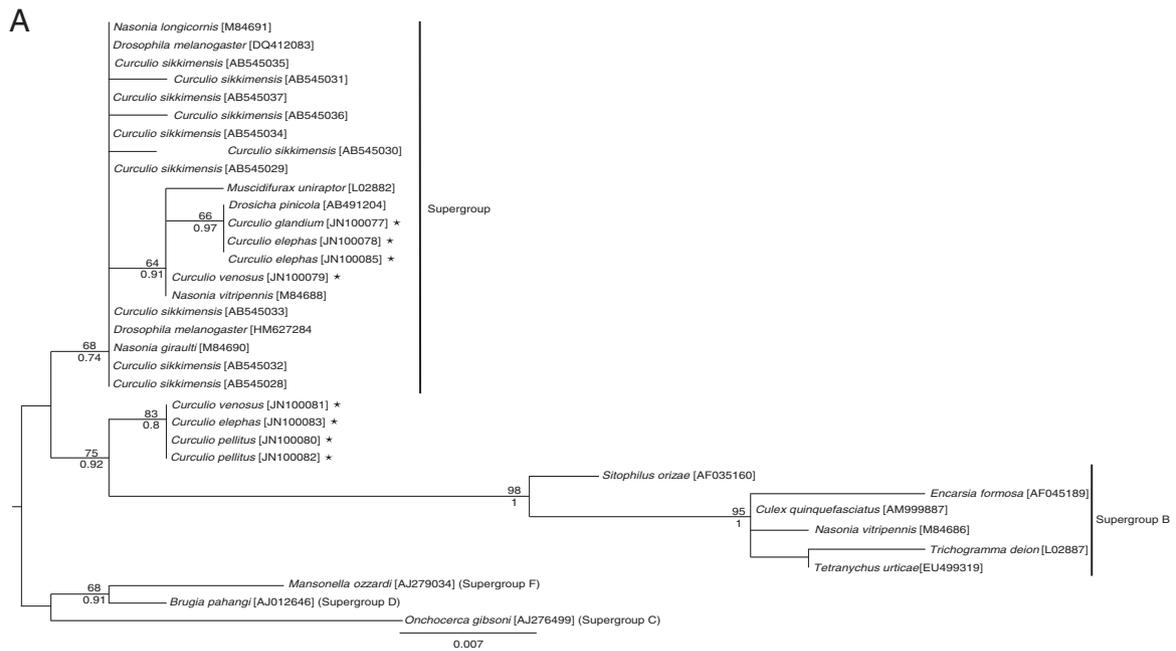


Figure 2 (See legend on next page.)

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Figure 2 Phylogenies of the facultative endosymbionts identified in *Curculio* spp. (A) *Wolbachia* (340 bp unambiguously-aligned nucleotide sites, 16S rRNA sequences) is shown with indication of the supergroups A and B based on the annotation of sequences found in Genbank; (B) *Rickettsia* (873 sites, 16S rRNA sequences); (C) *Spiroplasma* (1405 sites, ITS and 16S rRNA sequences). The Bayesian trees are shown, as the Maximum-likelihood trees (not shown) exhibit substantially the same topology (see Methods). The bootstrap values for the maximum-likelihood analysis (100 replicates) and the Bayesian posterior probabilities are shown above and below the nodes only if greater than 50 and 0.50, respectively. The accession numbers of the nucleotide sequences are shown in brackets. Sequences obtained from this study are indicated by asterisks. The name of each bacterial sequence corresponds to that of its host. Bacteria reported as non-endosymbiotic ones in the literature are underlined.

weevil species, we failed to discriminate them from one host species to the next. The phylogenetic analysis (Figure 2B) suggests that this bacterium is closely related to the *Kytorhinus sharpianus* endosymbiont (Coleoptera: Bruchidae, [55]). Finally, 3.33% and less than 1% of the bacterial clones derived from *C. elephas* and *C. glandium* females contained *Spiroplasma*, respectively: this bacterium was allied to the *Curculio sikkimensis* endosymbiont (Figure 2C, [15]).

Individual and multiple infection status of the oak weevils

We have screened *Curculioniphilus* as well as the three other endosymbionts in each of 562 weevils collected (Table 1). *Curculioniphilus* is highly prevalent in adults from the four species irrespective of the sex or the study site (it was found in 90.5% *C. elephas*, 84.2% *C. glandium*, 98.4% *C. pellitus* and 97.1% *C. venosus*; see Figure 3). The few uninfected individuals were essentially males (37 out of 44 uninfected individuals). Besides this so-called primary symbiont, three weevil species were shown to house their own predominant facultative symbiont (see Figure 3): 97.1% of 171 *C. glandium* individuals were infected by the *Wolbachia* strain belonging to the supergroup A, 98.5% of 128 *C. pellitus* individuals were infected by the *Wolbachia* strain at the basis of the supergroup B, and 77.1% of 105 *C. venosus* individuals hosted *Rickettsia*. In none of these species did the prevalence of the predominant secondary symbiont significantly vary according to either the sex or the study site (Table 3). A significant effect of the sex:locality interaction was statistically detected, however, on the prevalence of the supergroup A *Wolbachia* strain in *C. glandium*. This effect seems to be due to the unequal infection rate observed mostly in males between the two sites (infected males: 87% (site 1, n = 26) vs 100% (site 2, n = 29); infected females: 100% (site 1, n = 76) vs 95% (site 2, n = 40); Figure 3). This finding might stem from an unbalanced distribution of aged males in the two sites sampled (see discussion), and seems to be of low relevance owing to the small sample size of males as compared with that of females. In the two oak weevil communities studied, *C. elephas* differed from the three other species in that it lacked a predominant symbiont and exhibited *Spiroplasma* in addition to the three other secondary symbionts, all four endosymbionts being detected at a

residual frequency. The three other weevil species, in addition to their predominant secondary symbiont, also harbored the other endosymbionts -except *Spiroplasma*- at a residual frequency. Considering the individual host level, multiple infections commonly occurred in the four species, mostly due to the co-occurrence of the primary symbiont and the predominant secondary symbiont (Figure 3). Interestingly, the two strains of *Wolbachia* were never detected simultaneously in any individual despite their marginal co-occurrence within the same species and study site.

Discussion

This study thoroughly describes the endosymbiotic communities hosted simultaneously by four weevil sibling species that coexist, compete with each other for a limiting resource (i.e., oak acorns as egg-laying sites) [37,56,57] and display contrasted ecological traits [37,58]. We found that while all four species hosted the same primary endosymbiont, they harbored markedly distinct communities of secondary endosymbionts. Even if our results are strictly correlative, since we did not investigate the actual role of each symbiont on its host, this finding is compatible with our proposal that stable coexistence of host species competing with each other, which is expected to be ensured by their ecological differences, might be mediated by their endosymbionts.

Curculioniphilus buchneri was identified in all four weevil species using an *a priori*-free method based on 16S rRNA cloning and sequencing. This symbiont is known to be part of a distinct clade of Gammaproteobacteria, and is hosted by as many as 9 *Curculio* species. These insects feed on achenes or capsules and are specialist of perennial plants, including Theaceae (*Camellia* spp.) and Fagaceae (*Corylus*, *Castanea* and *Quercus* spp.) [36,38]. The congruence observed between the phylogenies of *C. buchneri* and that of their host (Figure 1) suggests that this symbiont has been ancestrally acquired [4], which sustains its primary status. *C. buchneri* was found at a very high infection rate in the weevils screened in this study, and the few insects that did not carry *C. buchneri* were almost exclusively males (Figure 3). As primary endosymbionts are most often involved in complementing the host's diet, this finding suggests that this bacterium might have

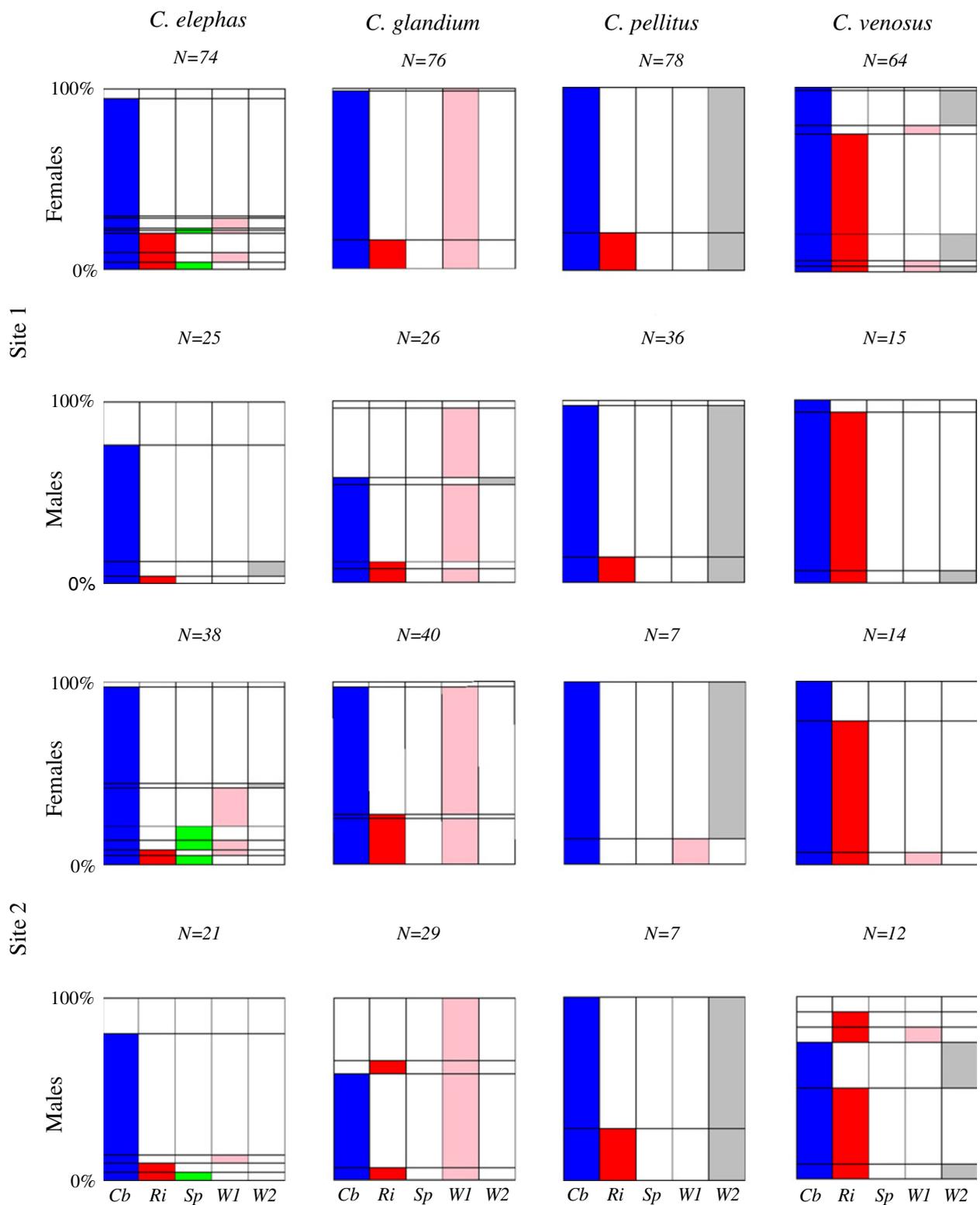


Figure 3 Individual multiple infection status observed in two French communities of acorn weevils. Five different endosymbionts have been detected in the four *Curculio* species studied: (Cb) Blue - *Candidatus Curculioniphilus buchneri*, (Ri) Red - *Rickettsia*, (Sp) Green - *Spiroplasma*, (WA) Pink - *Wolbachia* strain A and (WB) Gray - *Wolbachia* strain B. The geographical origin and the sex of the weevils are indicated on the left of the figure. The number of individuals tested is shown at the top of each graph. Horizontal reading informs us about the proportion of individuals sharing a given infection status. The prevalence of a given bacteria in a sample (of a given sex, site, species) is obtained by summing vertically the corresponding color.

Table 3 Probability for a predominant facultative symbiont to infect an individual host as a function of its sex and of the study site

Variable	Deviance	df	P
<i>Rickettsia</i> (<i>C. venosus</i>)			
Site	0.31589	1	0.57409
Sex	0.66578	1	0.41453
Site:Sex	2.75129	1	0.09718
<i>Wolbachia</i> A (<i>C. glandium</i>)			
Site	0.0003	1	0.98704
Sex	1.746	1	0.18635
Site:Sex	8.9503	1	0.00277
<i>Wolbachia</i> B (<i>C. pellitus</i>)			
Site	1.9356	1	0.16415
Sex	0.0747	1	0.78467
Site:Sex	3.7132	1	0.05398

Each analysis was performed on the weevil species shown in brackets. For each bacterium, the best-fitted generalized linear model (logistic regression) is shown, and was selected on the basis of the Akaike Information Criterion (AIC =117.2 for *Rickettsia*, 42.48 for *Wolbachia* A and 22.88 for *Wolbachia* B). The P-values for respective explanatory variables were obtained with chi-square statistics.

been lost during adulthood by a few males and hence, that it might be essential to the larval rather than the adult stage [59].

Three of the secondary symbionts (two distinct clades of *Wolbachia* and one *Rickettsia*) were commonly detected in all four species, whereas the fourth one, *Spiroplasma*, was found exclusively in *C. elephas*. This pattern resembles that of an endosymbiotic metacommunity, in which the endosymbiotic communities of each host population/species comprise all the bacterial types and are connected to one another by means of horizontal transfers [2,4,60-65]. Horizontal transfers have been reported to occur between parasitoid species sharing the same host [65], and are likely to occur between the four weevil species under study here, because of the concomitant presence of larvae from several species occasionally feeding on the same individual acorns (unpublished results). Evidence of such transfers remains to be established, however, notably through the analysis of more variable molecular sequences of the symbionts.

While at first sight the set of secondary endosymbionts detected was found to be identical across the four weevil species, a finer-tuned, quantitative analysis revealed that these ones had clearly distinct endosymbiotic communities. The four insect species differed from one another in that, with the exception of *C. elephas*, each had its own major secondary symbiont. Two questions remain to be addressed: (i) why not all symbionts have spread to all four weevil species, and (ii) why different symbionts predominate in the different host species? Multiple infections are likely to be counter-selected since at the individual

host level, competition between endosymbionts for the resource, might lead to competitive exclusion and/or to increased cost for the host [66,67]. Although we observed the presence of a particular endosymbiont correlating with a weevil species, we cannot infer at this time its actual impact on host phenotype. However, assuming that symbionts have spread in response to selective pressures, two alternatives can be envisaged, depending on whether each of the major secondary symbionts engages in a mutualist interaction with its host or manipulates its reproduction [2,4].

First, mutualistic endosymbionts provide their hosts with higher benefits than costs and are expected to invade the population rapidly [5,20]. The net benefit for a host to house a given endosymbiont might greatly depend on its physiological or ecological requirements. These needs might differ from one host species to the other, notably when these ones interact in different ways with the same environment [11,64]. In the oak weevil communities, the marked ecological differences observed between the four species [37,58] would coincide with unequal probability for a given symbiont to spread. Conversely, we cannot rule out that the marked ecological differences between the four weevil sibling species might be due to the major symbionts having a distinct impact on their host. Hence, the different symbionts might facilitate the partitioning of ecological niches between their hosts by providing them with expanded or new skills, thereby possibly leading to unequal nutritive assistance [6,7,68-71], dispersal capacity [21-24], or ability to resist natural enemies among host species [17-20,72].

Second, the probability for reproductive parasites to successfully invade their host heavily relies on the efficiency of their transmission from mother to offspring. In each of the four weevil species, males and females were frequently and equally infected, which makes male-killing or feminization processes unlikely. For instance, the infection patterns observed would rather be compatible with cytoplasmic incompatibility (CI) [25-27]. Notably, the two strains of *Wolbachia* identified on the basis of *16 s rRNA*, that are found to be the major secondary symbionts of *C. pellitus* and *C. glandium*, show almost complete mutual exclusion at the individual host level (Figure 3), suggesting mutual bidirectional CI. Should this mechanism actually occur, these *Wolbachia* strains would have been prevented from invading *C. elephas* and *C. venosus* populations because of their too low infection rate (reviewed in [73]). Interestingly, endosymbiont-mediated CI has theoretically been shown to facilitate and reinforce reproductive isolation and speciation [25], and such endosymbiont-mediated species enrichment would therefore be expected particularly in communities composed of sibling host species. However, empirical evidence for this mechanism is still scarce and much debated [73-77].

Conclusions

Our findings raise the question of whether intracellular micro-organisms might contribute to the structure of natural communities of host species competing with each other, either by accelerating their ecological divergence or by reinforcing their reproductive isolation, possibly leading to speciation. Exploring this proposal calls for both theoretical development and empirical investigations describing the endosymbiotic communities housed by various arthropod communities composed of sibling and competing host species. It should then be possible to test the predictions that communities harboring diverse secondary symbionts have greater species richness and include more closely related species than those deprived of symbionts. It would be worth running experiments in parallel to elucidate the actual impact endosymbionts have on their host, which still remains largely unknown in most natural systems.

Data accessibility

DNA sequences have been deposited at Genbank with the accession numbers AB514497-AB514504, AB507714-AB507716, and JN100059-JN10090. Phylogenetic trees have been deposited at TreeBase (<http://purl.org/phylo/treebase/phylovs/study/TB2:S13815>).

Additional file

Additional file 1: Identification of the clones sequenced in the four weevil species. To identify the endosymbiotic diversity, clones were sequenced in adult females belonging to the four weevil species and live-trapped in two sites (see Methods). We described the best hit blast of each clone and the ratio of the number of similar nucleotides to the total number of nucleotides (called Identities).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

This study is part of the PhD thesis of AM who is focused on the implication of endosymbionts on the structure of sibling insect communities. MCBV and AH supervised this PhD at the interface between community ecology and endosymbiosis interactions. MCBV and SV suggested the exploration of endosymbiotic communities within competing species, supervised and contributed to the ecological research. FM provided his experience about weevil ecology and contributed to the experimental scheduling. AH and FV gave their expertise in the interactions existing between insects and their bacterial endosymbionts. AV conducted the fine dissections of the female ovaries and molecular analyses from DNA purification, cloning, up to sequencing. HH contributed to the methodological setting up of diagnostic PCRs and supervised the phylogenetic analyses. All authors read and approved the final manuscript.

Authors' information

MCBV, SV and FM are focused on evolutionary and community ecology. FV, AH, HH and AV have interest in associations between insects and bacteria endosymbionts. This work is part of the PhD of AM who develops an integrative research project linking ecological and symbiotic aspects.

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