

Pervasiveness of the symbiont *Serratia symbiotica* in the aphid natural environment: distribution, diversity and evolution at a multitrophic level

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One sentence summary: We highlight the presence of an insect symbiont at different levels of food webs, suggesting the ecological compartment existence for its acquisition in insects, as potential interfaces for symbioses emergence.

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Abstract

Symbioses are significant drivers of insect evolutionary ecology. Despite recent findings that these associations can emerge from environmentally derived bacterial precursors, there is still little information on how these potential progenitors of insect symbionts circulate in trophic systems. *Serratia symbiotica* represents a valuable model for deciphering evolutionary scenarios of bacterial acquisition by insects, as its diversity includes gut-associated strains that retained the ability to live independently of their hosts, representing a potential reservoir for symbioses emergence. Here, we conducted a field study to examine the distribution and diversity of *S. symbiotica* found in aphid populations, and in different compartments of their surrounding environment. Twenty % of aphid colonies were infected with *S. symbiotica*, including a wide diversity of strains with varied tissue tropism corresponding to different lifestyle. We also showed that the prevalence of *S. symbiotica* is influenced by seasonal temperatures. We found that *S. symbiotica* was present in non-aphid species and in host plants, and that its prevalence in these samples was higher when associated aphid colonies were infected. Furthermore, phylogenetic analyses suggest the existence of horizontal transfers between the different trophic levels. These results provide a new picture of the pervasiveness of an insect symbiont in nature.

Keywords: symbiosis, gut symbiont, horizontal transfer, insects, host plants, bacterial circulation

Introduction

Numerous studies have revealed the diversity of symbiotic associations in insects and their role in adapting to particular lifestyles or diets deficient in certain elements (Moran 2007, Douglas 2011). Many insects that have specialized on nutritionally unbalanced foods depend on obligate symbionts to synthesize deficient nutrients in their diets (Douglas 1998, Moran and Baumann 2000, Baumann et al. 2013). Obligate nutritional symbioses represent key evolutionary innovations, allowing insects to diversify in ecological niches otherwise inaccessible (Wernegreen 2017). Insects can also harbor facultative symbionts that are often involved in more recent associations and affect a wide range of host life history traits, even if they are not essential for host survival (Oliver et al. 2010, Feldhaar 2011, Heyworth and Ferrari 2015). Depending on the ecological context, facultative symbionts may act as mutualistic partners providing benefits to hosts, but may also produce fitness costs (Vorburger and Gouskov 2011, Oliver et al. 2014). This suggests that their persistence is determined by a balance of costs and benefits. In recent years, the origin of bacterial mutualism in insects has received increasing attention (Husník et al. 2011, Clayton et al. 2012, Sudakaran et al. 2017, Manzano-Maniñ et al. 2020), particularly in stinkbugs that pick up environmental bacteria that become mutualistic partners in specialized cavities of their digestive tract (Kikuchi and Yumoto 2013, Hosokawa et al. 2016, Takeshita and Kikuchi 2017). However, there is still little data

regarding how potential progenitors of insect symbionts circulate in the environment and to what extent these circulating bacteria can spread to different trophic levels.

Transmission mechanisms are known to be instrumental in the establishment and evolution of symbioses in host populations (Bright and Bulgheresi 2010). Although vertical transmission is the primary way by which symbioses are stably maintained (Salem et al. 2015), symbionts can also undergo occasional horizontal transfers or be acquired directly from the environment (Kikuchi et al. 2007, Gehrler and Vorburger 2012, Łukasik et al. 2015, Hosokawa et al. 2016). Novel associations could emerge through these latter mechanisms, subsequently allowing the rapid acquisition of new ecological traits by insects and the expansion of host ranges for symbiotic bacteria (Su et al. 2013, Oliver et al. 2014). In nature, the occurrence of bacterial strains capable of living freely outside of insects but related to insect symbionts suggests their potential role as source of symbiotic partners. For example, a strain of the bacterium *Sodalis* isolated from a hand wound in human but originated from a dead tree branch was found to share a very close relationship with *Sodalis glossinidius* and other members of *Sodalis*-allied clade of insect symbionts (Clayton et al. 2012, Chari et al. 2015). *Erwinia* bacteria, generally described as phytopathogens, are also found associated with insects, such as aphids where they were first described as gut associates (Harada and Ishikawa 1993, Harada et al. 1997, Nadarasah and Stavrinides

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2011). More recently, some *Cinara* aphids have been found to harbor *Erwinia*-related obligate symbionts that complement their ancestral obligate symbiont *Buchnera aphidicola* (Meseguer et al. 2017). Genomic analyses indicate that these associations originate from the acquisition of plant-associated strains, probably acquired horizontally and having evolved into an intracellular lifestyle (Manzano-Marín et al. 2020). Taken together, all these findings support the hypothesis of the existence of an environmental pool of bacteria from which new intimate partnerships with insects can emerge.

In addition to this, a growing number of studies have recently shown that symbioses between insects and symbionts evolve in a very dynamic fashion, involving the acquisition of new symbionts, and/or the loss and replacement of established bacterial partners, even in the context of obligate associations established for millions of years (Husnik and McCutcheon 2016, Sudakaran et al. 2017, Chong and Moran 2018, Matsuura et al. 2018, Mao and Bennett 2020). For example, genomic analyses in aphids indicate that the dependence of some species on co-obligate symbionts has arisen independently many times during their evolutionary history (Manzano-Marín et al. 2017, 2020, Meseguer et al. 2017, Monnin et al. 2020). The repeated replacement of preexisting symbionts by other microbial partners is now considered a ubiquitous evolutionary process that occurs in many insects, suggesting (i) the continuous formation of new mutualistic associations and (ii) the existence of a pool of environmental bacteria from which new associations are formed.

Serratia symbiotica is a valuable candidate to decipher the origin and evolution of bacterial mutualism in insects. In addition to the obligate symbiont *Buchnera aphidicola*, which houses in bacteriocytes and is essential for the correct development of their hosts, it is one of the most common facultative symbionts associated with aphids (Hansen and Moran 2011, Leclair et al. 2016, Pons et al. 2019a). *Serratia symbiotica* includes a great diversity of strains associated with very distinct biological features and reflecting various associations that bacteria can share with their aphid hosts (Burke and Moran 2011, Pons et al. 2019a, Monnin et al. 2020). The strains studied in the subfamily Aphidinae have been first described as intracellular facultative partners because they can invade host cells, and can be associated with protective phenotypes (against parasitoids and high temperatures) (Oliver et al. 2003, Burke et al. 2009, Heyworth and Ferrari 2015). *Serratia symbiotica* strains associated with the subfamilies Lachninae and Chaitophorinae are intracellular symbionts involved in co-obligate associations, compensating some metabolic capacities lost by their ancient obligate symbiont (Manzano-Marín and Latorre 2014, 2016, Manzano-Marín et al. 2018, Monnin et al. 2020). Moreover, the species *S. symbiotica* also includes strains that are gut-associates (Pons et al. 2019a, Perreau et al. 2020) and that can be cultivated freely on a pure artificial medium (Sabri et al. 2011, Grigorescu et al. 2018, Elston et al. 2021). These gut-associated strains, slightly pathogenic to aphids and that retain some free-living capacities (Pons et al. 2019a, Perreau et al. 2020), are extracellularly transmitted via contamination with honeydew (Pons et al. 2019a) and/or through the plant phloem (Pons et al. 2019b). They can be horizontally transferred between aphids through host plant, and their uptake by plant roots can induce new bacterial infections of aphids, as well as positive fitness effects on the host plant (Pons et al. 2019b). A field study also showed that *S. symbiotica* may reside in the gut of ants tending aphids (Renoz et al. 2018). Taken together, these data suggest that certain *S. symbiotica* strains have an ability to circulate from one compartment to another, with an aptitude to perform horizontal transfers between both phylogenetically close and dis-

tant species in the environment. We propose that these strains may thrive in the environment where aphids prosper and could provide an environmental source for the establishment of new symbiotic associations between aphids and bacteria.

While *S. symbiotica* strains with a broad spectrum of infection may represent ideal candidates to refine our understanding of evolutionary scenarios of symbiont acquisition, we still do not know how the different strains are distributed in nature, how they circulate and how they spread in aphid populations. To address these issues, we conducted a field study to investigate the distribution, diversity and evolution of *S. symbiotica* infections at a multitrophic level, including 58 aphid species, aphid-interacting insects and host plants. This study allowed us (i) to examine the prevalence of *S. symbiotica* in aphids and their environment, and to detect environmental and ecological factors that influence its prevalence, (ii) to determine the diversity of tissue tropism exhibited by *S. symbiotica* in natural insect populations and (iii) to investigate the propensity of *S. symbiotica* to circulate and be transferred horizontally in the aphid natural environment. Using diagnostic polymerase chain reaction (PCR), fluorescence *in situ* hybridization (FISH) and phylogenetic approaches, we provide a comprehensive picture of the diversity and the pervasiveness of *S. symbiotica* in a natural environment. Our study highlights the presence of *S. symbiotica* strains at different levels of food webs and suggests the existence of ecological compartments for the exchange and acquisition of the symbiont, serving as potential interfaces for the emergence of new symbioses.

Materials and methods

Sample collection

To get a comprehensive picture of the pervasiveness of *S. symbiotica* in the natural aphid environment, we sampled (i) aphid colonies of different species, (ii) insects potentially in interaction with aphids in the sampled colonies and (iii) the host plant. Sampling was carried out to maximize the diversity of aphid subfamilies and genera, representing 3 subfamilies of Aphididae, 27 genera and 58 species. Field specimens were sampled between May and August 2018 on various host plants at several locations in Belgium (Walloon Brabant province) (Table S1, Supporting Information). The sampling also includes some aphids sampled in Italy, France, Germany and Rwanda (2017). A total of 614 samples were collected, containing 250 aphid colonies, 203 insects associated with aphid colonies (tending ants, hoverfly larvae, larvae and adults of ladybugs, parasitoids, bugs, aphid midge larvae, moth larvae, fly larvae and lacewing larvae) and 161 host plant samples (Table S1, Supporting Information). To minimize the risk of pseudo-replicating, *S. symbiotica* infection was verified in only one pool of aphids per host plant. When insects were observed within aphid colonies, individuals were systematically collected and preserved in 90% ethanol at room temperature until use. Plant samples (stem and leaf pieces) associated with the sampled aphid colonies were also systematically collected and stocked at -80°C . For samples collected in Belgium, the mean daily temperatures and the maximum daily temperatures during the different months of sampling in Belgium were obtained via the database of IRM (Institut Royal Météorologique) of Belgium and the meteobelgique.be website (Table S1, Supporting Information).

DNA extraction

Insect DNA extraction was performed using a high salt-extraction method (Aljanabi and Martinez 1997). Extraction was carried out

on a pool of two to six aphids from each colony and on a pool of two to three individuals for associated tending ants and larvae (Jousselin et al. 2013, Renoz et al. 2018). Pools were carried out to avoid the risk of missing infection, when *S. symbiotica* is present. For the other associated insects, extraction was performed on a single individual. Plant DNA was extracted using the CTAB method (Doyle 1991). Prior to extraction, plant samples were surface-sterilized with 99% ethanol, 10% bleach, and rinsed with sterile water.

Insect identification

For insect species identification, the primers LepF and LepR (presented in Table S2, Supporting Information) were used to amplify the target 658-bp fragment of cytochrome c oxidase subunit I (COI) gene (D'acier et al. 2014). PCR reactions were conducted in a final volume of 15 μ l containing 1 μ l of the template DNA lysate, 0.5 μ M of each primer, 200 μ M dNTPs, 1 $\times$ buffer and 0.625 unit of Taq DNA polymerase (Roche, Basel, Switzerland). The thermocycling profile consisted of 94°C for 1 min; 6 cycles of 94°C for 1 min, 45°C for 1 min and 30 s and 72°C for 1 min and 15 s; followed by 36 cycles of 94°C for 1 min, 51°C for 1 min and 30 s and 72°C for 1 min and 15 s; with a final 5 min extension period of 72°C. Amplicons were then purified before sequencing (Macrogen Inc., Amsterdam). The resulting sequences were cleaned and aligned using Geneious® v9.1.5 (Kearse et al. 2012) and insects were identified by comparing resulting COI sequence data to the GenBank nucleotide database using BLAST.

Diagnostic screening for *S. symbiotica*

All samples were tested for the presence of *S. symbiotica* using PCR assays based on the 16S rRNA gene using the specific primers 16SA1 and PASScnp (presented in Table S2, Supporting Information). PCR amplification was performed as previously described (Pons et al. 2019a). PCR reactions were conducted in a final volume of 15 μ l containing 1 μ l of the template DNA lysate, 0.5 μ M of each primer, 200 μ M dNTPs, 1X buffer and 0.625 unit of Taq DNA polymerase (Roche, Basel, Switzerland). The thermocycling profile consisted of 95°C for 5 min; 35 cycles at 95°C for 30 sec, 55°C for 1 min 30 s and 72°C for 1 min 30 s; 72°C for 7 min. DNA from an infected line of the pea aphid *A. pisum* was used as a positive control (Burke et al. 2009) and in addition to the negative control (water), DNA from an uninfected line of the black bean aphid *A. fabae* was used as a negative control (Vorburger and Gouskov 2011). Amplicons were purified and sequenced in both directions (Macrogen Inc., Amsterdam). Sequence alignments were done using Geneious® v9.1.5 (Kearse et al. 2012) and compared with sequences on GenBank using BLAST. The sequences were deposited in GenBank.

Phylogenetic analyses

The diversity of *S. symbiotica* strains was characterized using the partial sequence of four housekeeping genes *accD*, *gyrB*, *murE* and *recJ* (Table S2, Supporting Information) (Henry et al. 2013, Łukasik et al. 2015). DNA samples that were found positive for *S. symbiotica* were subjected to PCR amplification under the following conditions: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 60–64°C depending on primers for 30 s, extension at 72°C for 1 min; a final extension at 72°C for 5 min (Renoz et al. 2018). Amplicons were then purified and sequenced in both directions (Macrogen Inc., Amsterdam). Sequences obtained were cleaned and aligned using Geneious® v9.1.5 (Kearse et al. 2012).

Phylogenetic associations were analyzed for 54 *S. symbiotica* strains obtained in this work, sequences from the three cultivable *S. symbiotica* strains that were isolated in our laboratory (Sabri et al. 2011, Foray et al. 2014, Grigorescu et al. 2018, Renoz et al. 2020), eight sequences from *S. symbiotica* that were found in a recent field study (from aphids collected in Belgium in summer 2015) (Renoz et al. 2018) and twelve sequences from *S. symbiotica* whose genomes have been sequenced and available in GenBank (Burke and Moran 2011, Manzano-Marín and Latorre 2014, Manzano-Marín et al. 2016, Meseguer et al. 2017, Manzano-Marín et al. 2018, Nikoh et al. 2019, Monnin et al. 2020). *Serratia proteamaculans* 568 was used as outgroup. All sequences are available in GenBank (Tables S3 and S4, Supporting Information). SeaView v4.6.1 was used to align sequences, and all ambiguously aligned regions identified by GBlocks v0.91b (Castresana 2000) were eliminated, as were regions of incomplete data at the 3' and 5' ends of the targeted regions. PartitionFinder v1.1.0 (Lanfear et al. 2012) was used to determine the substitution model for each gene. Phylogenetic analysis was performed by Bayesian inference methods with MrBayes v3.2.7a (Ronquist et al. 2012). Indels were treated as missing data. Four Markov chain Monte Carlo (MCMC) simulations were run independently for 10 000 000 generations. Trees and model parameters were sampled every 10 000 generations. Convergence of the MCMCs was estimated in three ways: (i) the standard deviation of split frequencies was <0.01, (ii) visual inspection of the plot of the log-likelihood score at each sampling point suggested that the four chains reached stationarity and (iii) the posterior probability plots of all splits for paired MCMC runs showed high correlation, which diagnosed convergence among the four chains (Nylander et al. 2008). The trees of the burn-in for each run were excluded from the tree set, and the remaining trees from each run were combined to form the full sample of trees assumed to be representative of the posterior probability distribution.

Localization of *S. symbiotica*

To determine the tissue tropism of *S. symbiotica* strains in aphids and in the other insects sampled, whole-mount fluorescence *in situ* hybridization (FISH) was carried out as described in Pons et al. (2019a) and Koga et al. (2009). All insect samples positive to *S. symbiotica* were tested. The following oligonucleotide probes were used: Cy5-ApisP2a (5'-Cy5-CCTCTTTTGGGTAGATCC-3') targeting 16S rRNA of *B. aphidicola* and Cy3-PASSisR (5'-Cy3-CCCGACTTTATCGCTGGC-3') targeting 16S rRNA of *S. symbiotica*. Insect tissues were stained with SYTOX Green. Samples (between 1 and 6 per colony) were whole mounted and observed under a Zeiss LSM 710 confocal microscope. Negative controls consisted of aphids not infected with *S. symbiotica* and stained with the two probes or infected aphids with no-probe staining. Positive controls comprised artificially and naturally infected aphids.

Statistical analyses

We analyzed whether the proportion of aphids harboring *S. symbiotica* differs according to their host plant range, as carried out in Henry et al. (2015): specialized (feeding on a single plant species or group of closely related species, $N = 13$), restricted (feeding on a single plant family, $N = 91$), polyphagous (feeding on various plant families, $N = 105$) or obligatory host plant alternating ($N = 31$). The presence of *S. symbiotica* in aphids was analyzed using generalized linear models (GLM) with a binomial error structure and a logit link function, and the degree of plant specialization by aphids was the explanatory variable. We also tested if the proportion of aphids infected by *S. symbiotica* differs in populations

collected from different plant species, using Pearson's Chi-square statistic. Aphids on plant species from which we had less than five collections, and where *S. symbiotica* symbiont infected <2% of the individuals on a plant, were excluded from the analysis as recommended in Henry et al. (2015). We also examined the effect of the temperature (mean daily and maximum daily) on the proportion of aphids harboring *S. symbiotica*. We tested if *S. symbiotica* infection was influenced by high seasonal temperatures, because this symbiont is known to protect aphids from heat shock, especially aphids collected from arid regions (Russell and Moran 2006, Henry et al. 2013). To perform analyses, temperature data were pooled into 6 balanced categories each (Table S1, Supporting Information). The presence of *S. symbiotica* in aphid colonies was thus analyzed on pooled data (Table S1, Supporting Information) using GLM with a binomial error structure and a logit link function, and the temperature was the explanatory variable. The relationship between mean daily temperatures or maximum daily temperatures and the proportion of aphid colonies harboring *S. symbiotica* was tested on pooled data using nonparametric Spearman's correlation coefficients. We also analyzed if the proportion of tending ants, associated other insects and host plants harboring *S. symbiotica* were correlated with the infection status of aphid colonies. The presence of *S. symbiotica* in these different samples was analyzed using GLM with a binomial error structure and a logit link function. The infection status of the aphid colonies was the explanatory variable and for the associated insects, the insect feeding specialization (parasitoids, phytophagous or predators) was also one explanatory variable. Statistical analyses were performed using the software R version 3.5.3 (R Development Core team 2019), using the GrapheR package.

Results

Distribution of *S. symbiotica* in natural aphid populations

The presence of *S. symbiotica* was examined in 250 aphid colonies from 58 species of the Aphididae family (Table S1, Supporting Information). These species were mostly part of the Macrosiphini (31 species) and Aphidini (23 species) tribes belonging to the Aphidinae subfamily (Table S1, Supporting Information). Other species were members of the subfamilies Chaitophorinae (two species) and Calaphidinae (two species) (Table S1, Supporting Information). The tribe Aphidini was the most collected group with 121 colonies belonging to 19 species of the genus *Aphis* (Table 1). Among the 250 colonies, 51 displayed a positive infection to *S. symbiotica* (20%), belonging to 22 aphid species (38%) (Table 1 and Table S1, Supporting Information). Within each infected aphid genus, the infection prevalence ranged from 13% to 100% (Table 1). The infection rate of *S. symbiotica* in *Aphis*, which is the most represented group in our sampling with 121 collected colonies, reached 29% (35/121) and 69% of infected aphid colonies belonged to the genus *Aphis* (35/51) (Table 1). More precisely, 27% (22/82) of *Aphis fabae* colonies, which is the most represented species of the genus *Aphis*, were found to be infected (Table S1, Supporting Information). We also found a high infection rate of *S. symbiotica* in the genera *Macrosiphum* (4/5) (Table 1). In contrast, we observed that in the genera *Uroleucon* and *Hyperomyzus*, the infection rate was zero (0/49 and 0/10, respectively; Table 1).

Environmental and ecological factors influencing *S. symbiotica* prevalence in aphids

We showed that among the 45 host plant genera collected (all containing aphids), 15 were associated with infected aphids (33%; Ta-

ble 2). Of the 37 plants associated with infected aphids (all genera included), 7 were members of the genus *Cirsium* (18%) (Table 2). We asked whether the degree of plant specialization by aphids was associated with *S. symbiotica* infection in aphid colonies. We found no significant differences in the proportion of aphids harboring *S. symbiotica* according to their degree of plant specialization (GLM, $df = 3$, $\chi^2 = 6.35$, $P = 0.096$; Fig. S1, Supporting Information). We also analyzed whether the infection of aphids with *S. symbiotica* that feed on multiple host plants was correlated with the species of plant on which they were collected. Sufficient data for analysis were available only for *A. fabae* that feeds on different host plants (*Cirsium arvense*, *Cirsium vulgare*, *Daucus carota*, *Heracleum sphondylium*, *Rumex obtusifolium* and *Sonchus asper*). We found that there were no significant differences in the proportion of *A. fabae* colonies harboring *S. symbiotica* collected from different plants ($N = 49$, $df = 5$, $\chi^2 = 3.59$, $P = 0.61$). The distribution of *S. symbiotica* in this sampling is thus uniform across collections of *A. fabae* feeding on different plant species.

To test the impact of temperature on *S. symbiotica* prevalence, we focused on the data sampled in July and August when most samples were collected (Table S1, Supporting Information). During these months, the mean daily temperatures ranged from 19.5°C to 25.8°C and the maximum daily temperatures ranged from 22.9°C to 32.9°C (Table S1, Supporting Information). To perform analyses, temperature data were pooled into 6 balanced categories each (Table S1, Supporting Information). We showed that *S. symbiotica* frequencies varied across Belgium aphid populations exposed to varying summer temperatures (Fig. 1). We found that the proportion of aphid colonies harboring *S. symbiotica* was significantly different between mean daily temperatures (GLM, $df = 1$, $\chi^2 = 2.53$, $P = 0.011$) and maximum daily temperatures in July and August (GLM, $df = 1$, $\chi^2 = 2.08$, $P = 0.038$) (Fig. 1A and B). We also showed a significant positive correlation between the proportion of aphid colonies harboring *S. symbiotica* and the maximum daily temperatures (Spearman's $r_s = 1$, $P = 0.017$; Fig. 1B). *Serratia symbiotica* frequency in aphid colonies was thus significantly higher when temperatures were higher at time of collection, ranging from 7% at 23°C to 31% at 30°C.

Distribution of *S. symbiotica* in insect populations associated with aphid colonies

Other insects associated with aphid colonies were collected to examine the presence of similar *S. symbiotica* strains in the trophic systems. Among 203 insects belonging to 52 species, 25 insects displayed a positive infection to *S. symbiotica* (12%) (Table 3).

Ants were the most frequent insects found in aphid colonies and were represented by 10 different species (Table S1, Supporting Information). Among the 98 tending ant samples, 10 exhibited a positive infection with *S. symbiotica*, representing an infection rate of ~10% (Table 3). These infected ants belonged to *Lasius niger*, *Linepithema humile* and *Myrmica rubra* species, with *L. niger* being the most represented species (7/10) (Table S1, Supporting Information). We found that the proportion of tending ants harboring *S. symbiotica* was significantly higher when collected in infected aphid colonies ($N = 8/27$) compared with uninfected colonies ($N = 2/71$) (GLM, $df = 1$, $\chi^2 = 13.55$, $P < 0.001$; Fig. 2A).

Among the 15 bug individuals representing 10 species, two (*Dictyla humuli* and *Coreus marginatus*) were infected with *S. symbiotica* (Table S1, Supporting Information). Twenty-two ladybugs were collected (larvae and adults), belonging to six species, and two adults were positive for *S. symbiotica* (*Harmonia axyridis* and *Coccinella septempunctata*; Table S1, Supporting Information). Nine-

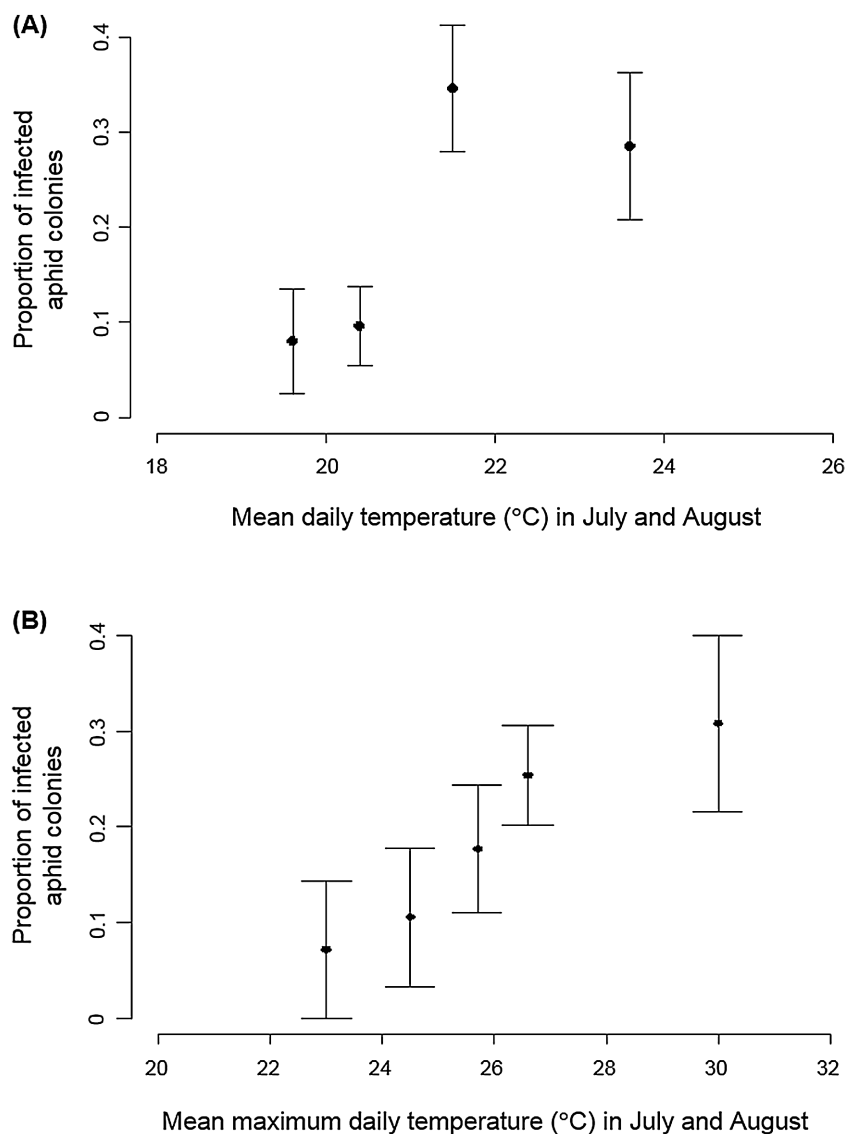


Figure 1. Relationships between *S. symbiotica* frequency across Belgian aphid populations and the mean daily temperature (A) or the maximum daily temperature (B) during sampling.

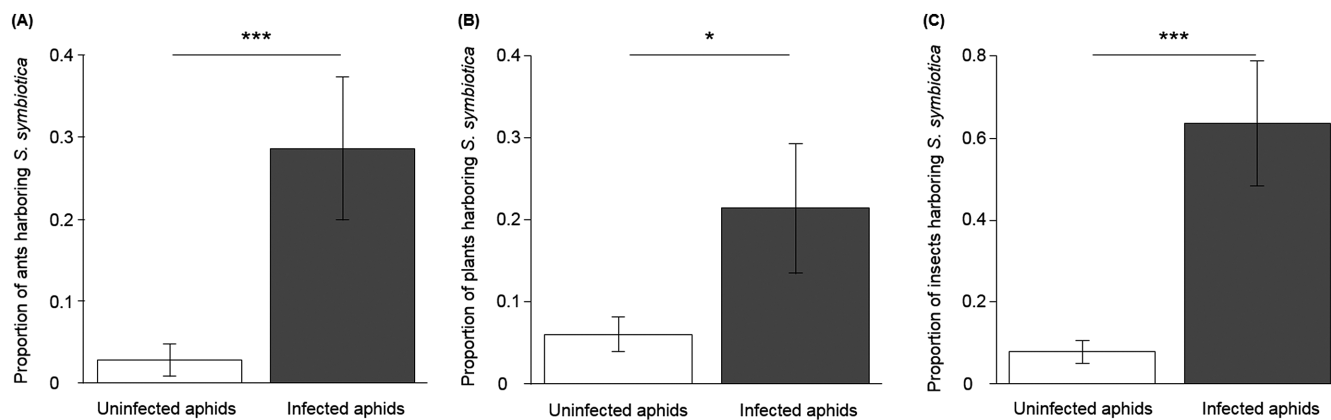


Figure 2. Prevalence of *S. symbiotica* in tending ants (A), plant samples (B) and associated insects (C) differs depending on the infection status of the aphid colonies. Columns represent the proportion of samples infected with *S. symbiotica* according to uninfected aphid colonies (white) or infected aphid colonies (dark gray). Error bars depict the standard error. Asterisks show significant differences (* $P < 0.05$; *** $P < 0.001$).

Table 1. Summary of the natural occurrence of *S. symbiotica* within 250 aphid colonies, across the different aphid genera.

Aphid genus	Number of colonies	Positive to <i>S. symbiotica</i>	Percentage of infected colonies (%)
Aphidinae			
Macrosophini			
<i>Acyrtosiphon</i>	3	0	0
<i>Brachycaudus</i>	3	1	33.3
<i>Capitophorus</i>	1	1	100
<i>Cavariella</i>	5	2	40
<i>Corylobium</i>	1	0	0
<i>Dysaphis</i>	1	0	0
<i>Hyadaphis</i>	1	0	0
<i>Hyalopteroides</i>	4	1	25
<i>Hyperomyzus</i>	10	0	0
<i>Macrosiphoniella</i>	8	1	12.5
<i>Macrosiphum</i>	5	4	80
<i>Megoura</i>	1	0	0
<i>Metopeurum</i>	2	0	0
<i>Metopolophium</i>	4	0	0
<i>Myzus</i>	1	0	0
<i>Sitobion</i>	8	1	12.5
<i>Straticobium</i>	1	0	0
<i>Uroleucon</i>	49	0	0
Aphidini			
<i>Aphis</i>	121	35	28.9
<i>Hyalopterus</i>	3	0	0
<i>Rhopalosiphum</i>	1	0	0
<i>Schizaphis</i>	3	2	66.6
<i>Toxoptera</i>	6	2	33.3
Chaitophorinae			
Chaitophorini			
<i>Chaitophorus</i>	1	0	0
<i>Periphyllus</i>	1	1	100
Calaphidinae			
Panaphidini			
<i>Pterocallis</i>	1	0	0
<i>Tinocallis</i>	1	0	0
Not identified	4	0	0
Total	250	51	20.4

teen adult parasitoids were also found in aphid colonies, mainly collected when laying eggs into aphids (Table S1, Supporting Information). Three individuals of three species (*Aphidius funebris*, *Lysiphlebus fabarum* and *Lysiphlebus hirticornis*) showed a positive infection (Table S1, Supporting Information). These three infected individuals were not sampled from the infected aphid colonies. Of the 34 hoverfly larvae that were sampled, 4 were infected with *S. symbiotica* (*Episyrphus balteatus* and *Scaeva pyrastris*) (Table S1, Supporting Information). Two aphid midge larvae (*Aphidoletes aphidimyza*) were infected out of seven, and one moth larva was infected out of six (*Eupithecia trisignaria*; Table S1, Supporting Information). Moreover, one fly larva was collected and was positive for *S. symbiotica* (*Chamaemyiidae* sp.) whereas the only lacewing larva collected was not positive (Table 3 and Table S1, Supporting Information). We found that the proportion of these associated insects harboring *S. symbiotica* was significantly higher when collected from infected aphid colonies ($N = 7/11$) compared with uninfected colonies ($N = 7/90$) (GLM, $df = 1$, $\chi^2 = 19.86$, $P < 0.001$; Fig. 2C). We also examined if the insect feeding specialization (parasitoids, polyphagous or predators) can influence its symbiotic infection. We found that the feeding specialization did not significantly affect the proportion of these insects harboring *S. symbiotica* (GLM [*S. symbiotica*], $df = 2$, $\chi^2 = 5.2$, $P = 0.074$).

Distribution of *S. symbiotica* in the natural aphid environment

To examine the presence of *S. symbiotica* in the surrounding environment of aphids, host plants associated with aphid colonies were collected. Among the 161 plant samples (11 leaves and 150 stems) collected from 52 plant species, 14 were positive (13 stems and 1 leaf) for *S. symbiotica* (~9%), belonging to 12 plant species (23%; Table S1, Supporting Information). The genera *Sonchus* ($N = 32$) and *Cirsium* ($N = 26$) were the most collected groups (Table 2). The latter exhibited the highest infection rate with 29% of infection (4/14), followed by plants of the genus *Rumex* with 14% of infection (2/14; Table 2). We showed that among the 14 positive plant samples, only 6 were associated with positive aphid colonies (belonging to the genus *Aphis*) (Table 2). However, we found that the proportion of plants harboring *S. symbiotica* was significantly higher when associated with infected aphid colonies ($N = 6/29$) compared with uninfected colonies ($N = 8/132$) (GLM, $df = 1$, $\chi^2 = 5.8$, $P = 0.016$; Fig. 2B).

Tissue tropism of *S. symbiotica* in insects

All aphid samples that showed a positive infection to *S. symbiotica* were observed, using whole-mount fluorescence *in situ* hybridization. A total of 21 aphid colonies have been examined and obser-

Table 2. Summary of the natural occurrence of *S. symbiotica* within 161 host plants. The number of plants infected with *S. symbiotica* associated with infected aphids was considered.

Plant genus	Number of samples	Positive to <i>S. symbiotica</i>	Plant associated with infected aphids		Infected plant associated with infected aphids	
			Number	Infected Aphid genus	Number	Infected Aphid genus
<i>Achillea</i>	1	0	1	<i>Macrosiphoniella</i>	0	/
<i>Artemisia</i>	5	0	0	/	0	/
<i>Campanula</i>	1	0	0	/	0	/
<i>Centaurea</i>	2	0	0	/	0	/
<i>Centranthus</i>	1	0	0	/	0	/
<i>Chaenomeles</i>	1	0	0	/	0	/
<i>Chenopodium</i>	2	0	0	/	0	/
<i>Cirsium</i>	26	4	7	<i>Aphis</i> , <i>Capitophorus</i> , <i>Macrosiphum</i>	3	<i>Aphis</i>
<i>Clematis</i>	1	0	1	<i>Aphis</i>	0	/
<i>Conyza</i>	1	0	0	/	0	/
<i>Corylus</i>	2	0	0	/	0	/
<i>Crataegus</i>	1	0	0	/	0	/
<i>Crepis</i>	1	0	0	/	0	/
<i>Daucus</i>	8	0	2	<i>Aphis</i>	0	/
<i>Epilobium</i>	10	0	2	<i>Aphis</i>	0	/
<i>Epipactis</i>	2	0	0	/	0	/
<i>Eupatorium</i>	2	0	1	<i>Aphis</i>	0	/
<i>Gaillet</i>	1	0	0	/	0	/
<i>Glebionis</i>	1	0	0	/	0	/
<i>Hedera</i>	1	0	1	<i>Aphis</i>	0	/
<i>Heracleum</i>	7	1	2	<i>Aphis</i> , <i>Cavariella</i>	0	/
<i>Hibiscus</i>	1	0	0	/	0	/
<i>Jacobaea</i>	6	1	2	<i>Aphis</i>	0	/
<i>Lactuca</i>	2	0	0	/	0	/
<i>Laphangium</i>	2	0	2	<i>Aphis</i> , <i>Brachycaudus</i>	0	/
<i>Leucanthemum</i>	3	0	2	<i>Aphis</i>	0	/
<i>Lythrum</i>	1	0	0	/	0	/
<i>Malus</i>	1	1	0	/	0	/
<i>Oenothera</i>	1	0	0	/	0	/
<i>Phragmites</i>	3	0	0	/	0	/
<i>Populus</i>	1	0	0	/	0	/
<i>Ranunculus</i>	1	1	0	/	0	/
<i>Robinia</i>	2	0	0	/	0	/
<i>Rosa</i>	3	0	2	<i>Macrosiphum</i>	0	/
<i>Rumex</i>	8	2	2	<i>Aphis</i>	1	<i>Aphis</i>
<i>Salvia</i>	1	0	0	/	0	/
<i>Sambucus</i>	1	1	1	<i>Aphis</i>	1	<i>Aphis</i>
<i>Solanum</i>	2	1	1	<i>Aphis</i>	1	<i>Aphis</i>
<i>Sonchus</i>	32	1	0	/	0	/
<i>Spirea</i>	2	0	0	/	0	/
<i>Symphytum</i>	1	0	0	/	0	/
<i>Tanacetum</i>	6	1	0	/	0	/
<i>Verbena</i>	2	0	0	/	0	/
<i>Vesca</i>	1	0	0	/	0	/
<i>Vicia</i>	1	0	0	/	0	/
Total	161	14	29	/	6	/

variations revealed the existence of different patterns: (i) presence of *S. symbiotica* in the digestive tract, (ii) in the sheath cells or (iii) in the secondary bacteriocytes (Fig. 3).

Gut-associated *S. symbiotica* were found either colonizing the midgut or the whole gut (Fig. 3A–D). The digestive tract is localized in the middle of the aphid body between the primary bacteriocytes hosting the obligate symbiont *B. aphidicola* and is composed of multiple loops (Pons et al. 2019a). Eleven aphid colonies observed exhibited an infection in the gut (Table S1, Supporting Information). Aphids that exhibited this pattern belonged to *Aphis*

sp. (mostly *A. fabae*), except one *Capitophorus elaeagni* individual. Infection of sheath cells pattern was also found in two colonies of *Macrosiphum* species (Table S1, Supporting Information). In this case, the symbionts were located around primary bacteriocytes enclosing *B. aphidicola* (Fig. 3E). *Serratia symbiotica* was also found in secondary bacteriocytes flanking the primary bacteriocytes containing *B. aphidicola* (Fig. 3F–H). This pattern was observed in eight aphid colonies belonging to four aphid species (*Macrosiphoniella millefolii*, *Periphyllus testudinaceus*, *Macrosiphum mordvilkoii* and *A. fabae*; Table S1, Supporting Information).

Table 3. Summary of the natural occurrence of *S. symbiotica* within 203 insects associated with aphid colonies. The number of insects infected with *S. symbiotica* associated with infected aphids was considered.

			Infected insect associated with infected aphids	
Insect genus	Number of samples	Positive to <i>S. symbiotica</i>	Number	Infected Aphid genus
Ants				
<i>Camponotus</i>	3	0	/	/
<i>Formica</i>	1	0	/	/
<i>Lasius</i>	78	7	6	Capitophorus, Aphis
<i>Linepithema</i>	2	2	2	Aphis
<i>Myrmica</i>	7	1	0	/
Not identified	7	0	/	/
Aphid Midge Larvae				
<i>Aphidoletes</i>	7	2	1	Aphis
Bugs				
<i>Coreus</i>	2	1	1	Aphis
<i>Deraeocoris</i>	1	0	/	/
<i>Dictyla</i>	2	1	0	/
<i>Dicyphus</i>	1	0	/	/
<i>Dolycoris</i>	3	0	/	/
<i>Graphosoma</i>	1	0	/	/
<i>Malacocoris</i>	1	0	/	/
<i>Orius</i>	1	0	/	/
<i>Orthops</i>	1	0	/	/
<i>Pinalitus</i>	1	0	/	/
Not identified	1	0	/	/
Fly Larvae				
<i>Chamaemyiidae</i>	1	1	1	Aphis
Hoverfly Larvae				
<i>Episyrphus</i>	6	1	1	Aphis
<i>Eupeodes</i>	3	0	/	/
<i>Paragus</i>	7	0	/	/
<i>Platycheirus</i>	2	0	/	/
<i>Scaeva</i>	11	3	1	Capitophorus
<i>Syrphus</i>	5	0	/	/
Lacewing Larvae				
<i>Chrysoperla</i>	1	0	/	/
Ladybugs				
<i>Coccinella</i>	8	1	0	/
<i>Harmonia</i>	7	1	1	Aphis
<i>Hippodamia</i>	2	0	/	/
<i>Propylea</i>	2	0	/	/
<i>Rhyzobius</i>	1	0	/	/
<i>Scymnus</i>	1	0	/	/
Not identified	1	0	/	/
Moth Larvae				
<i>Eupithecia</i>	3	1	1	Aphis
<i>Hecatera</i>	1	0	/	/
<i>Mompha</i>	1	0	/	/
<i>Pieris</i>	1	0	/	/
Parasitoids				
<i>Aphelinus</i>	1	0	/	/
<i>Aphidius</i>	2	1	0	/
<i>Binodoxys</i>	2	0	/	/
<i>Diglyphus</i>	1	0	/	/
<i>Eulophinae</i>	1	0	/	/
<i>Lysiphlebus</i>	7	2	0	/
<i>Pachyneuron</i>	1	0	/	/
<i>Pnigalio</i>	1	0	/	/
<i>Tetrastichinae</i>	3	0	/	/
Total	203	25	15	/

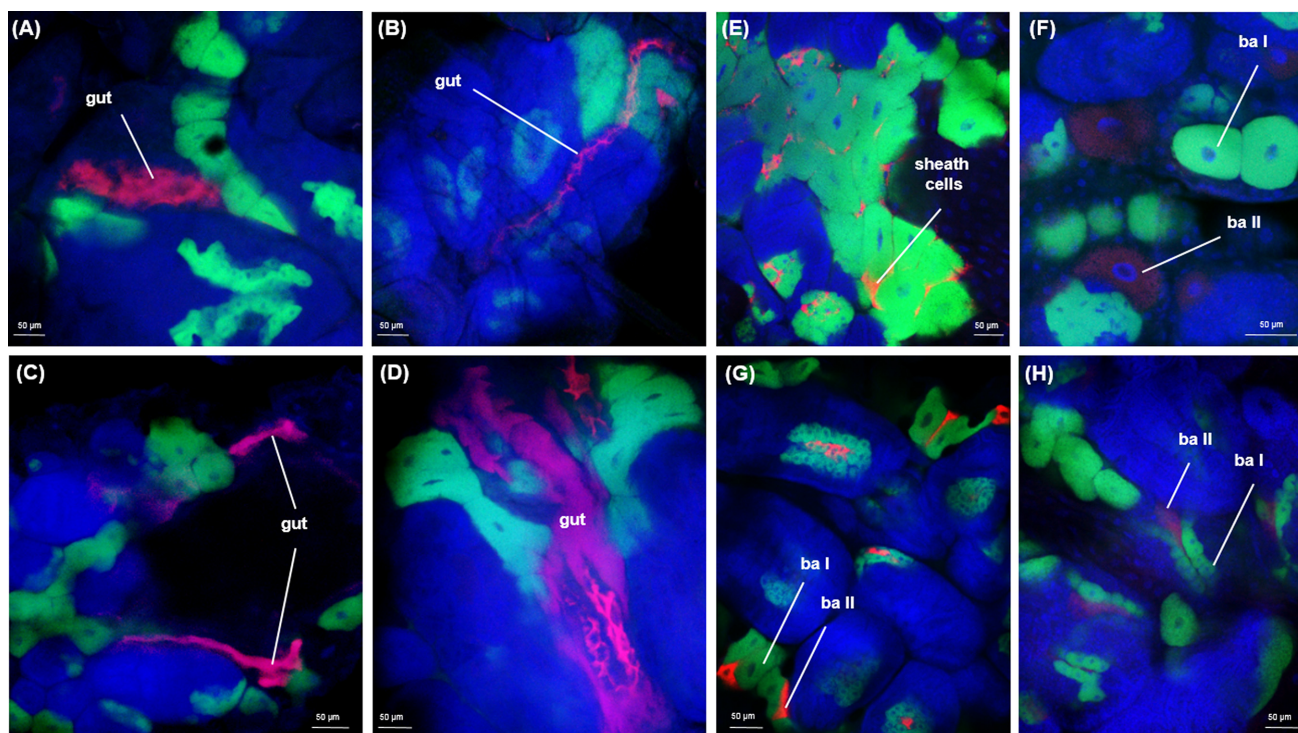


Figure 3. Whole-mount FISH of *S. symbiotica* in naturally infected aphids (ventral views). Red Cy3 signals are *S. symbiotica*, green Cy5 signals are *B. aphidicola* and blue SYTOX Green signals are aphid tissues. (A–D) *Serratia symbiotica* found residing at the gut level of aphids. (E) *Serratia symbiotica* found residing at the sheath cells level of aphids. (F–H) *Serratia symbiotica* found residing at the bacteriocytes level of aphids (ba I: primary bacteriocyte and ba II: secondary bacteriocyte). (A) *Aphis grossulariae* (ID 491), (B) *Capitophorus elaeagni* (ID 605), (C) *Aphis fabae* (ID 517), (D) *Aphis pomi* (ID 511), (E) *Macrosiphum rosae* (ID 263), (F) *Macrosiphum mordvilkoii* (ID 135), (G) *Periphyllus testudinaceus* (ID 1) and (H) *Aphis fabae* (ID 380).

All insect samples associated with aphid colonies showing a positive infection to *S. symbiotica* were observed using FISH. Unfortunately, due to the limited amount of material sampled, we were failed to properly observe *S. symbiotica* tissue tropism for the majority of aphid-associated insects, with the exception of hoverflies. In these predatory insects, our results clearly indicate that *S. symbiotica* is found in the prothorax of a hoverfly larva, at the beginning of the digestive tract (Fig. 4). Images suggest that *S. symbiotica* accumulates in the tentorial bar, in the junction of the pump chamber with the foregut, near the true mouth (Fig. 4). The bacterium has been observed in the proventriculus of ants previously, a specialized organ involved in food filtration (Renoz et al. 2018).

Diversity of *S. symbiotica* infections

Bacterial sequences of the *accD*, *gyrB*, *murE* and *recJ* genes were taken from all 90 positive samples for *S. symbiotica* infection. Sequences were easily readable in 54 *S. symbiotica* strains from aphids, host plants and other insects. We excluded the remaining samples that had either polymorphic sequences, sequences difficult to read or missing sequences (Table S2, Supporting Information).

The phylogenetic relationship between these 54 *S. symbiotica* strains and other already described *S. symbiotica* strains was estimated using sequences obtained in this study, as well as sequences available in GenBank (Table S2, Supporting Information). We also used *S. proteamaculans* 568, as outgroup. We found that the phylogeny of the *S. symbiotica* strains is strongly structured by the taxonomic identity of their host and the phylogenetic analyses established the existence of four distinct clades (Fig. 5). Co-obligate *S. symbiotica* strains from *C. cedri* (SCc), *C. fornicula* (SCf), *C. strobili* (SCs) and *T. salignus* (STs Pzieg) aphids that

were localized in secondary bacteriocytes form clade B (Burke and Moran 2011, Manzano-Marín and Latorre 2014, Manzano-Marín et al. 2017, Meseguer et al. 2017). The strains composing this clade are co-obligate symbionts of aphids and exhibit a long-term co-evolutionary history with their hosts. The clade A is composed of *S. symbiotica* strains that are generally considered as aphid facultative endosymbionts or recent co-obligates. This clade includes strains found in *A. pisum*: SAp Tucson strain localized in sheath cells (Burke and Moran 2011, Manzano-Marín et al. 2017) and SIS strain residing in secondary bacteriocytes and sheath cells (Nikoh et al. 2019). It also contains strains found in *C. tujaefolia* (SCt VLC) (Burke and Moran 2011, Manzano-Marín et al. 2017) and *A. urticae* (AURT-53S) that are localized in secondary bacteriocytes, and in *M. carnosum* (MCAR-56S) localized in sheath cells (Monnin et al. 2020). It is also composed of strains found in *M. rosae* and *M. mordvilkoii* and localized in secondary bacteriocytes or sheath cells (Fig. 3). The clade D forms a large monophyletic clade consisting of, among others, strains from aphids of the genus *Aphis*, and strains from the aphid species *M. absinthii*, *M. millefolii*, *B. cardui* and *T. aurantia*. This clade also includes *S. symbiotica* strains from the host plants and insects associated with the aphid colonies. Interestingly, these strains fall into the same clade as *S. symbiotica* strains associated with aphids sampled in the corresponding colony. Also included in this clade, the three strains previously isolated (SCWBI-2.3^T, SApA 8-A1, SAF 24.1) (Sabri et al. 2011, Grigorescu et al. 2018) and the strains previously detected in the aphid gut (FR65, FR28, FR35 and VP6; Table S4, Supporting Information) (Renoz et al. 2018). All *S. symbiotica* strains localized in the aphid gut are grouped within this clade. However, some strains localized in secondary bacteriocytes (ID 33, 380, 369, 197; Fig. 3) are also included in this clade. We also observed a polytomy with a group

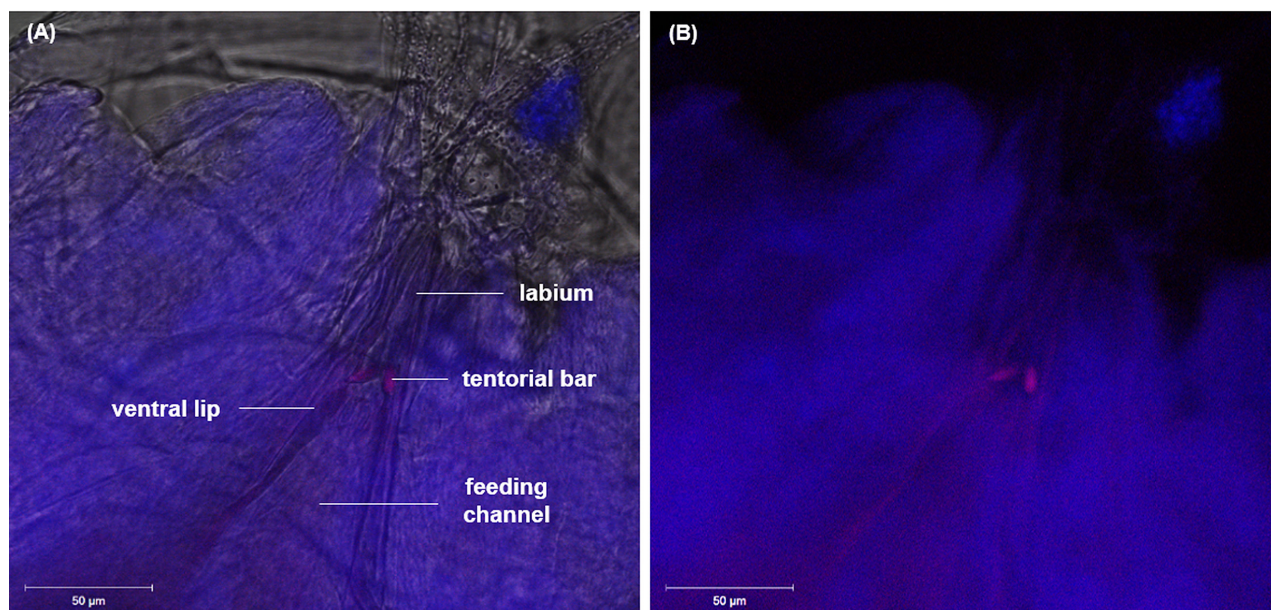


Figure 4. Whole-mount FISH of *S. symbiotica* in a naturally infected hoverfly larva (Prothorax, ventral view). Red Cy3 signals are *S. symbiotica* and blue SYTOX Green signals are insect tissues. Panel (A) is with bright field and panel (B) is without bright field. *Serratia symbiotica* is located at the level of the tentorial bar, near the true mouth (Reemer and Rotheray 2009).



Figure 5. *Serratia symbiotica* phylogeny constructed using MrBayes analysis based on concatenated sequences of the *accD*, *gyrB*, *murE* and *recJ* genes. The names given for each terminal node reflect the taxonomic identity of the host from which the *S. symbiotica* strain was sequenced, followed by the names of the *S. symbiotica* strains. Circles on branches indicate Bayesian posterior probabilities: black circles for probabilities greater than or equal to 0.95, gray circles for probabilities between 0.94 and 0.8, and white circles for probabilities between 0.79 and 0.7. The black names indicate the aphid samples, the green names indicate the host plant samples and the blue names indicate the insect samples associated with aphid colonies. Rectangles correspond to individuals belonging to the same colony. Clade D corresponds to the clade D of the study (Renoz et al. 2018) grouping the strains isolated and exhibiting a free-living capacity in laboratory conditions as well as strains exhibiting aphid gut infection.

containing strains from an aphid, an ant and a bug sampled in the same colony and another group containing the cultivable strain (SCWBI-2.3^T) and two strains from the aphid gut. The clade C includes *S. symbiotica* strains associated with aphids of the genus *Periphyllus* and a strain associated with *U. sonchi*. These strains are considered as nutritional co-obligate (Monnin et al. 2020). In *P. testudinaceus*, *S. symbiotica* was clearly localized in bacteriocytes (Fig. 3).

Discussion

In this study, we investigated the presence and distribution of *S. symbiotica* in wild aphid populations, as well as in the different compartments of their surrounding environment. Our results provide a comprehensive picture of the pervasiveness of *S. symbiotica* in the natural aphid environment. We first confirm that it exhibits different patterns of infection in aphids and that certain strains naturally transit through the gut of these insects. Our findings also demonstrate that its distribution is not limited to aphids but extends to host plants and other insects interacting with aphids, suggesting that they may act as vectors for horizontal transfers. In the light of these results, we discuss the multifaceted nature of *S. symbiotica* and the evolutionary scenarios of symbiont acquisitions in insects.

Our broad sampling highlights the great diversity of *S. symbiotica* strains in aphids with different tissue tropisms. We confirm the existence of strains that reside in the aphid gut, particularly in species of the genus *Aphis*, as already previously reported (Renoz et al. 2018). The existence of these strains that reside naturally within insect guts suggest that the feeding behavior is a likely source of new acquisition for these strains. Several studies support the idea that bacteria present in the aphid's diet are a source of endosymbiotic bacteria (Harada et al. 1996, Manzano-Marín et al. 2020). Although strict vertical transmission is the primary way by which symbioses are stably maintained (Salem et al. 2015), it is not always possible and not always necessary for the transmission of insect gut symbionts (Kikuchi et al. 2007, Kikuchi and Yumoto 2013). In the context of gut-associated *S. symbiotica*, a permanent environmental source of contamination (like host plants) will be also required to maintain the infection across aphid generations. These gut-associated strains were found to be grouped in a common phylogenetic clade (clade D), as previously shown in Renoz et al. (2018). At the exception of the oleaster aphid *Capitophorus elaeagni*, aphid species harboring gut-associated *S. symbiotica* were members of the genus *Aphis*. If experimental studies have previously highlighted the moderate pathogenic effects of gut-associated *S. symbiotica* (CWBI-2.3^T) (Pons et al. 2019, Perreau et al. 2020), their involvement in mutualistic associations with their insect host cannot be excluded. A recent study showed that the *S. symbiotica* strain (CWBI-2.3^T) infecting the aphid gut is capable to produce proteases including metalloproteases, which may facilitate the digestion of plant proteins by helping to suppress plant defenses (Skaljac et al. 2019). In light of this, it is possible that these gut-associated *S. symbiotica* contribute to improve the assimilation by aphids of some types of phloem sap or the elimination of toxins produced by host plants, allowing the aphid adaptation to feed on certain plants.

Our results offer the opportunity to investigate the different patterns of tissue tropism exhibited by *S. symbiotica* in aphids and beyond. In addition to the observed gut pattern, we showed that intracellular *S. symbiotica* strains exclusively compartmentalized into sheath cells surrounding the primary bacteriocytes have only been observed in individuals belonging to *Macrosiphum*

species, as already observed for several facultative symbionts of *Acyrtosiphon pisum* (Moran et al. 2005, Guo et al. 2017). *Serratia symbiotica* was also observed in secondary bacteriocytes in different aphid species, including *Periphyllus testudinaceus*, *Macrosiphoniella millefolii*, *Aphis fabae*, *Macrosiphum mordvilko* and *Macrosiphum rosae*. So far, this tissue tropism has mostly been reported in aphids where the symbiont is a co-obligate nutritional partner in Lachninae and Chaitophorinae subfamilies (Manzano-Marín et al. 2016, 2017, Monnin et al. 2020), or in *Aphis urticae* (Monnin et al. 2020) and in *A. pisum* after an artificial infection of the hemolymph (Fukatsu et al. 2000, Koga et al. 2003). Our observations demonstrate that a specific microscopic approach is definitively needed to clarify the nature of the associations in which bacteria and insects are involved. Indeed, the topology of the phylogenetic tree does not allow inferring the infection pattern displayed by *S. symbiotica*. The clade D remains diverse and includes strains that exhibit different tissue tropism patterns. This could be due to a lack of resolution of the analysis, which is based on a set of four housekeeping genes.

In a previous study, we experimentally demonstrated that a strain of *S. symbiotica* isolated from *A. fabae* aphids was able to invade phloem sap of *Vicia faba*, and that infected plants can serve as reservoirs for horizontal transmission of *S. symbiotica* in aphids (Pons et al. 2019a, Sabri et al. 2011). Our field study suggests that *S. symbiotica* can naturally reside in plants. To our knowledge, it is the first time that this symbiont was found in plants collected directly in the field. The presence of *S. symbiotica* in plants addresses major ecological issues including (i) the role of plants in the dissemination in insect populations of bacterial candidates, which may serve as environmental progenitors for the establishment of new associations and (ii) the nature of the interaction that certain *S. symbiotica* strains have with plants. When *S. symbiotica* were detected in both aphids and the corresponding host plant, they are found in the same phylogenetic clades (where the gut strains are located), indicating a close phylogenetic proximity that suggests frequent horizontal transfers of this bacterium between aphids and plants. The hypothesis of the existence of such transfers is strengthened by the fact that we found host plants harboring *S. symbiotica* significantly more frequently when aphids were also found infected on that plant. A study also exposed that gut-associated *S. symbiotica* can provide benefits to infected host plants (Pons et al. 2019b). The results raise questions whether *S. symbiotica* strains found in plants are aphid gut-associated that are horizontally transferred via plants or whether they are plant symbionts that are vectored by aphids. Among the 14 infected plants, only 6 were associated with infected aphids at the time of sampling, and the other 8 were associated with uninfected aphids. This may be because either aphids sampled had not yet been infected, we missed the infection or some strains only infect plants and fail to infect aphids. Indeed, the *Serratia* genus includes bacterial members that have the propensity to evolve in diverse environments (Grimont and Grimont 2006), and many *Serratia* species can establish mutualistic or parasitic partnerships with plants (Petersen and Tisa 2013). We also found that among the 29 plants associated with infected aphids, 23 remained uninfected. The hypothesis is that these aphid colonies only harbored *S. symbiotica* strains that are host restricted (localized to bacteriocytes and/or the hemolymph) and not likely to be transmitted to plants. All together, these results suggest that some *S. symbiotica* strains occur only in aphids, others occur in aphid guts as well as in host plants, and others are only associated with plants. Plants thus could serve as an environmental symbiont pool from which new intimate and restricted associations are formed. Our results are

in agreement with the following evolutionary scenario: (i) acquisition of bacterial progenitors originally inhabiting the plant, (ii) transition through the insect gut, an intermediate step before, and (iii) the possible establishment of more intimate associations.

While horizontal symbiont transfer is known to occur in the field, the rate at which it occurs is still unknown (Gehrer and Vorburger 2012, Henry et al. 2013, Jousset et al. 2013, Guyomar et al. 2018). Here, we demonstrated that *S. symbiotica* infection can occur in non-aphid insect species found in close proximity to aphid colonies and involved in trophic relationships with them (12% of the screened insects). We found that these insects were significantly more likely to harbor *S. symbiotica* when aphid colonies were infected. In addition, we showed that *S. symbiotica* strains associated with the non-aphid insects were in the same clades as strains found in aphids of the corresponding sampled colonies, suggesting that horizontal transfers of *S. symbiotica* can occur within natural insect communities and that these infections extend beyond aphids. Ant-tending is a possible route of transmission because ants feed on aphid honeydew that can be infected by *S. symbiotica* (Pons et al. 2019). With 10% of the sampled ants found infected, our results confirmed previous studies having reported its presence in these social insects (Sirviö and Pamilo 2010, He et al. 2011, Renoz et al. 2018). This is an important issue because, even if *S. symbiotica* is only an occasional passenger in ants, the social and feeding behavior of ants could promote the dissemination of certain strains and increase the environmental symbiont pool from which potential new associations could originate. Another possible route of symbiont transmission from aphids to non-aphid insects is predation, with ladybugs, hoverflies, aphid midges and fly larvae. We showed that *S. symbiotica* infect these insects and were able to localize it at the beginning of the digestive tract of a hoverfly larvae. A new study also found *S. symbiotica* in red-dragonfly that feed on aphids (Takemura et al. 2021). These results suggest that *S. symbiotica* can transit from one trophic level to another through predation and initiate an infection in non-aphid species. Further studies will be needed to determine whether the microbe may be transiently present as it passes through the gut or whether a more systemic and persistent infection may occur. We also found that parasitoids could be infected, but in this case, the tissue tropism was not studied due to a lack of insect material to perform all analyses. A study showed the role of parasitoids as vectors of facultative symbionts in aphids (Gehrer and Vorburger 2012). These insects could promote the emergence of symbioses because their mode of parasitism could allow symbionts to have a direct access to the aphid hemolymph where the cells specialized in symbiont sheltering are found. Ongoing studies should clarify the nature of this interaction at the developmental level, but also at the ecological level with the cascading effects that *S. symbiotica* infections could induce on parasitoid communities. Indeed, *S. symbiotica*, whether facultative intracellular or gut-associated strains, can severely hamper the development of parasitoids in aphids (Pons et al. 2019a, Attia et al. 2021). *Serratia symbiotica* was also detected in bugs, caterpillars and moth larvae that are sap-sucking insects or herbivores that do not directly interact with aphids but can feed on the same host plants. These results suggest, once again, that plants could be reservoir at the origin of new interactions between *S. symbiotica* and insects. All of these findings reveal the surprising pervasiveness of *S. symbiotica*, with strains that are likely capable of dispersing across multiple insect species. These insects could potentially serve as vectors for horizontal transfers, but in most cases it is unclear how the symbiont might return to the aphids. These results also suggest that a new relationship could emerge in certain insects. However, it

remains to be determined whether this symbiont is beneficial or pathogenic to these new hosts, or whether it is a transient commensal residing in multiple insect hosts. Some bacterial genera show a high propensity to infect diverse hosts, including *Spiroplasma*, *Sodalis* and *Arsenophonus* symbionts (Bressan et al. 2009, Clayton et al. 2012, Jousset et al. 2013, Schwarz et al. 2014, Santos-Garcia et al. 2017, Masson et al. 2018, Drew et al. 2021).

In our study, a total of 58 aphid species were sampled and the prevalence of *S. symbiotica* reaches 20% in aphid colonies, which is fairly similar to what has been recorded in previous studies (Henry et al. 2015, Renoz et al. 2018). Our results confirmed the high presence of *S. symbiotica* infections in the genus *Aphis* with 29% of aphids being infected. In addition to the aphid genus, the nature of the interaction can depend on abiotic and biotic factors including the presence of predators and parasitoids, the host plant and climatic factors (Henry et al. 2013, McLean Ailsa et al. 2016, Zytynska and Weisser 2016). Our results suggest that the *S. symbiotica* incidence increase with higher seasonal temperatures. Experimental studies have already shown that some endosymbiotic strains of *S. symbiotica* can provide a protection against heat shock in aphids (Montllor et al. 2002, Russell and Moran 2006, Burke et al. 2009). These protective effects are also supported by its high prevalence reported in arid regions (Henry et al. 2013). All these findings underlined a possible link between temperature seasonality and the prevalence of *S. symbiotica* in aphid populations, but its multifaceted nature must be considered. Indeed, an experimental study showed that gut-associated *S. symbiotica* strain does not confer benefits to aphids after heat stress (Pons et al. 2019), which agrees with the hypothesis that intracellular strains protect the aphid obligate symbiont by releasing metabolites as a result of cell lysis contrary to gut strains (Burke et al. 2009). Previous studies reported the influence of host plants on the presence of aphid facultative symbionts (Tsuchida et al. 2004, Brady and White 2013, Guidolin and Cônsoli 2017). In our sampling, *S. symbiotica* distribution is uniform across collections of *A. fabae* regardless of host plants. We showed that the aphid proportion (all aphid species included) harboring *S. symbiotica* is not different according to their degree of plant specialization. This result is not consistent with another study, which showed that *S. symbiotica* is most commonly found in specialist aphids (Henry et al. 2015). The distribution of *S. symbiotica* in natural aphid populations thus seems difficult to explain by considering only one or some factors, and is probably the result of the influence of multiple factors.

To conclude, our study provides a comprehensive picture of the pervasiveness of *S. symbiotica* in nature. It appears that some *S. symbiotica* strains occur only in aphids and are host restricted, others would only be associated with plants, and others would have a larger host spectrum ranging from the aphid guts to host plants and certain other insects. It is now established that intracellular host-dependent symbionts of insects can evolve from originally free-living bacterial lineages (Husník et al. 2011, Clayton et al. 2012, Manzano-Man'ñ et al. 2020). The presence of *S. symbiotica* at different trophic levels thus suggests the existence of an environmental pool of bacteria from which new intimate partnerships with insects may emerge. We hypothesize that the diversity of *S. symbiotica* includes strains that exhibit a generalist lifestyle, capable of developing cross-kingdom host jumps and exploiting multiple hosts before, under as yet unclear circumstances, potentially transitioning to a more specialized and host-dependent lifestyle. A key aspect is understanding the factors that favor such a transition, in particular the conditions that promote the passage from the digestive tract to the hemolymph, a required step for the adoption of an intracellular life. Another burning question concerns

the specific characteristics possessed by certain bacterial genera and species (e.g. members of the genera *Sodalis*, *Arsenophonus*, and members of *S. symbiotica*) to regularly engage in stable alliances with insects. The ecological versatility of *S. symbiotica* offers exciting avenues for answering these questions and refining our understanding of the evolution of bacterial mutualism.

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Supplementary data

Supplementary data are available at [FEMSEC](https://femsec.org) online.

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Data availability

The data underlying this article are available in the GenBank Nucleotide Database at [URL], and can be accessed with accession numbers (available

DNA sequences generated during this study have been submitted to GenBank, and the accession numbers are available in Table S3 (Supporting Information).

Author contributions

IP and TH designed the study; IP and NS did the sampling; IP, NS, LD and M-ER did the research; IP analyzed the data; IP wrote the first draft of the manuscript; and FR and TH revised the manuscript. All authors approved the manuscript for publication.

Conflict of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

- Aljanabi SM, Martinez I. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Res* 1997;**25**:4692–3.
- Attia S, Renoz F, Pons I et al. The aphid facultative symbiont *Serratia symbiotica* influences the foraging behaviors and the life-history traits of the parasitoid *Aphidius ervi*. *Entomol Gen* 2021. DOI: 10.1127/entomologia/2021/1274.
- Baumann P, Moran NA, Baumann LC. Bacteriocyte-associated endosymbionts of insects. In: E Rosenberg, EF DeLong, S Lory et al. (eds). *The Prokaryotes: Prokaryotic Biology and Symbiotic Associations*. Berlin, Heidelberg: Springer, 2013, 465–96.
- Brady CM, White JA. Cowpea aphid (*Aphis craccivora*) associated with different host plants has different facultative endosymbionts. *Ecol Entomol* 2013;**38**:433–7.
- Bressan A, Séméty O, Arneodo J et al. Vector transmission of a plant-pathogenic bacterium in the *Arsenophonus* clade sharing ecological traits with facultative insect endosymbionts. *Phytopathology* 2009;**99**:1289–96.
- Bright M, Bulgheresi S. A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol* 2010;**8**:218–30.
- Burke G, Fiehn O, Moran N. Effects of facultative symbionts and heat stress on the metabolome of pea aphids. *ISME J* 2009;**4**:242–52.
- Burke GR, Moran NA. Massive genomic decay in *Serratia symbiotica*, a recently evolved symbiont of aphids. *Genome Biol Evol* 2011;**3**:195–208.
- Castresana J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* 2000;**17**:540–52.
- Chari A, Oakeson KF, Enomoto S et al. Phenotypic characterization of *Sodalis praecaptivus* sp. nov., a close non-insect-associated member of the *Sodalis*-allied lineage of insect endosymbionts. *Int J Syst Evol Microbiol* 2015;**65**:1400–5.
- Chong RA, Moran NA. Evolutionary loss and replacement of *Buchnera*, the obligate endosymbiont of aphids. *ISME J* 2018;**12**:898.
- Clayton AL, Oakeson KF, Gutin M et al. A novel human-infection-derived bacterium provides insights into the evolutionary origins of mutualistic insect-bacterial symbioses. *PLoS Genet* 2012;**8**:e1002990.
- D'acier AC, Cruaud A, Artige E. DNA barcoding and the associated phylaphidb@se website for the identification of European aphids (Insecta: Hemiptera: Aphididae). 2014;**9**:e97620.
- Douglas AE. Lessons from studying insect symbioses. *Cell Host Microbe* 2011;**10**:359–67.
- Douglas AE. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annu Rev Entomol* 1998;**43**:17–37.
- Doyle J. DNA protocols for plants. *Molecular Techniques in Taxonomy*. Berlin, Heidelberg: Springer, 1991, 283–93.
- Drew GC, Budge GE, Frost CL et al. Transitions in symbiosis: evidence for environmental acquisition and social transmission within a clade of heritable symbionts. *ISME J* 2021;**15**:2956–68.
- Elston KM, Perreau J, Maeda GP et al. Engineering a culturable *Serratia symbiotica* strain for aphid paratransgenesis. *Appl Environ Microbiol* 2021;**87**:e02245–20.
- Feldhaar H. Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecol Entomol* 2011;**36**:533–43.
- Foray V, Grigorescu AS, Sabri A et al. Whole-genome sequence of *Serratia symbiotica* strain CWBI-2.3T, a free-living symbiont of the black bean aphid *Aphis fabae*. *Genome Announc* 2014;**2**:e00767–14.
- Fukatsu T, Nikoh N, Kawai R et al. The secondary endosymbiotic bacterium of the pea aphid *Acyrtosiphon pisum* (Insecta: Homoptera). *Appl Environ Microbiol* 2000;**66**:2748–58.
- Gehrer L, Vorburger C. Parasitoids as vectors of facultative bacterial endosymbionts in aphids. *Biol Lett* 2012;**8**:613–5.
- Grigorescu AS, Renoz F, Sabri A et al. Accessing the hidden microbial diversity of aphids: an illustration of how culture-dependent methods can be used to decipher the insect microbiota. *Microb Ecol* 2018;**75**:1035–48.
- Grimont F, Grimont PAD. The genus *Serratia*. In: M Dworkin, S Falkow, E Rosenberg et al. (eds). *The Prokaryotes*. New York: Springer, 2006, 219–44.
- Guidolin AS, Côté FL. Symbiont diversity of aphids (Homoptera: Citricidae) (Hemiptera: Aphididae) as influenced by host plants. *Microb Ecol* 2017;**73**:201–10.
- Guo J, Hatt S, He K et al. Nine facultative endosymbionts in aphids: a review. *J Asia Pac Entomol* 2017;**20**:794–801.
- Guyomar C, Legeai F, Jousset E et al. Multi-scale characterization of symbiont diversity in the pea aphid complex through metagenomic approaches. *Microbiome* 2018;**6**:181.

- Hansen AK, Moran NA. Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. *Proc Natl Acad Sci* 2011;**108**:2849–54.
- Harada H, Ishikawa H. Gut microbe of aphid closely related to its intracellular symbiont. *Biosystems* 1993;**31**:185–91.
- Harada H, Oyaizu H, Ishikawa H. A consideration about the origin of aphid intracellular symbiont in connection with gut bacterial flora. *J Gen Appl Microbiol* 1996;**42**:17–26.
- Harada H, Oyaizu H, Kosako Y et al. *Erwinia aphidicola*, a new species isolated from pea aphid, *Acyrtosiphon pisum*. *J Gen Appl Microbiol* 1997;**43**:349–54.
- He H, Chen Y, Zhang Y et al. Bacteria associated with gut lumen of *Camponotus japonicus* Mayr. *Environ Entomol* 2011;**40**:1405–9.
- Henry LM, Maiden MCJ, Ferrari J et al. Insect life history and the evolution of bacterial mutualism. *Ecol Lett* 2015;**18**:516–25.
- Henry LM, Peccoud J, Simon J-C et al. Horizontally transmitted symbionts and host colonization of ecological niches. *Curr Biol* 2013;**23**:1713–7.
- Heyworth ER, Ferrari J. A facultative endosymbiont in aphids can provide diverse ecological benefits. *J Evol Biol* 2015;**28**:1753–60.
- Hosokawa T, Ishii Y, Nikoh N et al. Obligate bacterial mutualists evolving from environmental bacteria in natural insect populations. *Nat Microbiol* 2016;**1**:15011.
- Husník F, Chrudimský T, Hypša V. Multiple origins of endosymbiosis within the enterobacteriaceae (γ -Proteobacteria): convergence of complex phylogenetic approaches. *BMC Biol* 2011;**9**:87.
- Husník F, McCutcheon JP. Repeated replacement of an intrabacterial symbiont in the tripartite nested mealybug symbiosis. *Proc Natl Acad Sci* 2016;**113**:E5416–24.
- Jousselin E, Coeur d'Acier A, Vanlerberghe-Masutti F et al. Evolution and diversity of *Arsenophonus* endosymbionts in aphids. *Mol Ecol* 2013;**22**:260–70.
- Kearse M, Moir R, Wilson A et al. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 2012;**28**:1647–9.
- Kikuchi Y, Hosokawa T, Fukatsu T. Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. *Appl Environ Microbiol* 2007;**73**:4308–16.
- Kikuchi Y, Yumoto I. Efficient colonization of the bean bug *Riptortus pedestris* by an environmentally transmitted *Burkholderia* symbiont. *Appl Environ Microbiol* 2013;**79**:2088–91.
- Koga R, Tsuchida T, Fukatsu T. Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. *Proc R Soc Lond B Biol Sci* 2003;**270**:2543–50.
- Koga R, Tsuchida T, Fukatsu T. Quenching autofluorescence of insect tissues for *in situ* detection of endosymbionts. *Appl Entomol Zool* 2009;**44**:281–91.
- Lanfear R, Calcott B, Ho SYW et al. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Mol Biol Evol* 2012;**29**:1695–701.
- Leclair M, Pons I, Mahéo F et al. Diversity in symbiont consortia in the pea aphid complex is associated with large phenotypic variation in the insect host. *Evol Ecol* 2016;**30**:925–41.
- Łukasik P, Guo H, Asch M van et al. Horizontal transfer of facultative endosymbionts is limited by host relatedness. *Evolution* 2015;**69**:2757–66.
- Manzano-Marín A, Coeur d'acier A, Clamens A-L et al. A freeloader? The highly eroded yet large genome of the *Serratia symbiotica* symbiont of *Cinara strobili*. *Genome Biol Evol* 2018;**10**:2178–89.
- Manzano-Marín A, Latorre A. Settling down: the genome of *Serratia symbiotica* from the aphid *Cinara tujafilina* zooms in on the process of accommodation to a cooperative intracellular life. *Genome Biol Evol* 2014;**6**:1683–98.
- Manzano-Marín A, Latorre A. Snapshots of a shrinking partner: genome reduction in *Serratia symbiotica*. *Sci Rep* 2016;**6**:32590.
- Manzano-Marín A, Simon J-C, Latorre A. Reinventing the wheel and making it round again: evolutionary convergence in *Buchnera-Serratia* symbiotic consortia between the distantly related Lachninae aphids *Tuberolachnus salignus* and *Cinara cedri*. *Genome Biol Evol* 2016;**8**:1440–58.
- Manzano-Marín A, Szabó G, Simon J-C et al. Happens in the best of subfamilies: establishment and repeated replacements of co-obligate secondary endosymbionts within Lachninae aphids. *Environ Microbiol* 2017;**19**:393–408.
- Manzano-Marín A, Coeur d'acier A, Clamens A-L et al. Serial horizontal transfer of vitamin-biosynthetic genes enables the establishment of new nutritional symbioses in aphids' di-symbiotic systems. *ISME J* 2020;**14**:259–73.
- Mao M, Bennett GM. Symbiont replacements reset the co-evolutionary relationship between insects and their heritable bacteria. *ISME J* 2020;**14**:1384–95.
- Masson F, Copete SC, Schüpfer F et al. *In vitro* culture of the insect endosymbiont *Spiroplasma poulsonii* highlights bacterial genes involved in host-symbiont interaction. *mBio* 2018;**9**:e00024–18.
- Matsuura Y, Moriyama M, Łukasik P et al. Recurrent symbiont recruitment from fungal parasites in cicadas. *Proc Natl Acad Sci* 2018;**115**:E5970–9.
- McLean Ailsa HC, Parker Benjamin J, Hrček Jan et al. Insect symbionts in food webs. *Philos Trans R Soc B Biol Sci* 2016;**371**:20150325.
- Meseguer AS, Manzano-Marín A, Acier AC et al. *Buchnera* has changed flatmate but the repeated replacement of co-obligate symbionts is not associated with the ecological expansions of their aphid hosts. *Mol Ecol* 2017;**26**:2363–78.
- Monnin D, Jackson R, Kiers ET et al. Parallel evolution in the integration of a co-obligate aphid symbiosis. *Curr Biol* 2020;**30**:1949–57.
- Montllor CB, Maxmen A, Purcell AH. Facultative bacterial endosymbionts benefit pea aphids *Acyrtosiphon pisum* under heat stress. *Ecol Entomol* 2002;**27**:189–95.
- Moran NA, Baumann P. Bacterial endosymbionts in animals. *Curr Opin Microbiol* 2000;**3**:270–5.
- Moran NA, Russell JA, Koga R et al. Evolutionary relationships of three new species of enterobacteriaceae living as symbionts of aphids and other insects. *Appl Environ Microbiol* 2005;**71**:3302–10.
- Moran NA. Symbiosis as an adaptive process and source of phenotypic complexity. *Proc Natl Acad Sci* 2007;**104**:8627–33.
- Nadarasah G, Stavrinides J. Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiol Rev* 2011;**35**:555–75.
- Nikoh N, Koga R, Oshima K et al. Genome sequence of “*Candidatus Serratia symbiotica*” strain IS, a facultative bacterial symbiont of the pea aphid *Acyrtosiphon pisum*. *Microbiol Resour Announc* 2019;**8**:e00272–19.
- Nylander JAA, Wilgenbusch JC, Warren DL et al. AWTY (are we there yet?): a system for graphical exploration of MCMC convergence in bayesian phylogenetics. *Bioinformatics* 2008;**24**:581–3.
- Oliver KM, Degnan PH, Burke GR et al. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu Rev Entomol* 2010;**55**:247–66.
- Oliver KM, Russell JA, Moran NA et al. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc Natl Acad Sci* 2003;**100**:1803–7.
- Oliver KM, Smith AH, Russell JA. Defensive symbiosis in the real world: advancing ecological studies of heritable, protective bacteria in aphids and beyond. *Funct Ecol* 2014;**28**:341–55.

- Perreau J, Patel DJ, Anderson H et al. Vertical transmission at the pathogen-symbiont interface: *Serratia symbiotica* and aphids. *mBio* 2020;**12**:e00359–21.
- Petersen LM, Tisa LS. Friend or foe? A review of the mechanisms that drive *Serratia* towards diverse lifestyles. *Can J Microbiol* 2013;**59**:627–40.
- Pons I, Renoz F, Hance T. Fitness costs of the cultivable symbiont *Serratia symbiotica* and its phenotypic consequences to aphids in presence of environmental stressors. *Evol Ecol* 2019;**33**:825–38.
- Pons I, Renoz F, Noël C et al. Circulation of the cultivable symbiont *Serratia symbiotica* in aphids is mediated by plants. *Front Microbiol* 2019b;**10**:764.
- Pons I, Renoz F, Noël C et al. New insights into the nature of symbiotic associations in aphids: infection process, biological effects and transmission mode of cultivable *Serratia symbiotica* bacteria. *Appl Env Microbiol* 2019a;**85**:e02445–18.
- R Core Team. R: A language and environment for statistical computing. 2019. <http://r.meteo.uni.wroc.pl/web/packages/dplR/vignettes/intro-dplR.pdf>.
- Reemer M, Rotheray G. Pollen feeding larvae in the presumed predatory syrphine genus *Toxomerus* Macquart (Diptera, Syrphidae). *J Nat Hist* 2009;**43**:939–49.
- Renoz F, Ambroise J, Bearzatto B et al. Draft genome sequences of two cultivable strains of the bacterial symbiont *Serratia symbiotica*. *Microbiol Resour Announc* 2020;**9**:e01579–19.
- Renoz F, Pons I, Vanderpoorten A et al. Evidence for gut-associated *Serratia symbiotica* in wild aphids and ants provides new perspectives on the evolution of bacterial mutualism in insects. *Microb Ecol* 2018;**78**:159–69.
- Ronquist F, Teslenko M, van der Mark P et al. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst Biol* 2012;**61**:539–42.
- Russell JA, Moran NA. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proc Biol Sci* 2006;**273**:603–10.
- Sabri A, Leroy P, Haubruge E et al. Isolation, pure culture and characterization of *Serratia symbiotica* sp. nov., the R-type of secondary endosymbiont of the black bean aphid *Aphis fabae*. *Int J Syst Evol Microbiol* 2011;**61**:2081–8.
- Salem H, Florez L, Gerardo N et al. An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proc R Soc Lond B Biol Sci* 2015;**282**:20142957.
- Santos-Garcia D, Silva FJ, Morin S et al. The all-rounder *Sodalis*: a new bacteriome-associated endosymbiont of the Lygaeoid Bug *Henestaris halophilus* (Heteroptera: Henestarinae) and a critical examination of its evolution. *Genome Biol Evol* 2017;**9**:2893–910.
- Schwarz RS, Teixeira ÉW, Tauber JP et al. Honey bee colonies act as reservoirs for two *Spiroplasma* facultative symbionts and incur complex, multiyear infection dynamics. *MicrobiologyOpen* 2014;**3**:341–55.
- Sirviö A, Pamilo P. Multiple endosymbionts in populations of the ant *Formica cinerea*. *BMC Evol Biol* 2010;**10**:335.
- Skaljac M, Vogel H, Wielsch N et al. Transmission of a protease-secreting bacterial symbiont among pea aphids via host plants. *Front Physiol* 2019;**10**:438.
- Su Q, Oliver KM, Pan H et al. Facultative symbiont *hamiltonella* confers benefits to *bemisia tabaci* (Hemiptera: Aleyrodidae), an invasive agricultural pest worldwide. *Environ Entomol* 2013;**42**:1265–71.
- Sudakaran S, Kost C, Kaltenpoth M. Symbiont acquisition and replacement as a source of ecological innovation. *Trends Microbiol* 2017;**25**:375–90.
- Takemura M, Maoka T, Koyanagi T et al. Elucidation of the whole carotenoid biosynthetic pathway of aphids at the gene level and arthropodal food chain involving aphids and the red dragonfly. *BMC Zool* 2021;**6**:19.
- Takeshita K, Kikuchi Y. *Riptortus pedestris* and *Burkholderia* symbiont: an ideal model system for insect-microbe symbiotic associations. *Res Microbiol* 2017;**168**:175–87.
- Tsuchida T, Koga R, Fukatsu T. Host plant specialization governed by facultative symbiont. *Science* 2004;**303**:1989.
- Vorburger C, Gouskov A. Only helpful when required: a longevity cost of harbouring defensive symbionts. *J Evol Biol* 2011;**24**:1611–7.
- Wernegreen JJ. In it for the long haul: evolutionary consequences of persistent endosymbiosis. *Curr Opin Genet Dev* 2017;**47**:83–90.
- Zytynska SE, Weisser WW. The natural occurrence of secondary bacterial symbionts in aphids. *Ecol Entomol* 2016;**41**:13–26.