Ubiquity of the Symbiont *Serratia symbiotica* in the Aphid Natural Environment: Distribution, Diversity and Evolution at a Multitrophic Level

Inès Pons[1*], Nora Scieur[1], Linda Dhondt[1], Marie-Eve Renard[1], François Renoz[1], Thierry Hance[1]

[1] Earth and Life Institute, Biodiversity Research Centre, Université catholique de Louvain, 1348, Louvain-la-Neuve, Belgium.

*** Corresponding author:**

Inès Pons

Croix du Sud 4-5, bte L7.07.04, 1348 Louvain la neuve, Belgique

ines.pons@uclouvain.be

ABSTRACT

Bacterial symbioses are significant drivers of insect evolutionary ecology. However, 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 circulates in the trophic systems. The aphid symbiont *Serratia symbiotica* represents a valuable model for deciphering evolutionary scenarios of bacterial acquisition by insects, as its diversity includes intracellular host-dependent strains as well as gut-associated strains that have retained some ability to live independently of their hosts and circulate in plant phloem sap. These strains represent a potential reservoir for the emergence of new and more intimate symbioses. Here, we conducted a field study to examine the distribution and diversity of *S. symbiotica* found in aphid populations, as well as in different compartments of their surrounding environment. A total of 250 aphid colonies, 203 associated insects, and 161 plant samples associated with aphid colonies were screened for *S. symbiotica*. Twenty percent of aphids were infected with *S. symbiotica*, and the symbiont includes 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. For the first time, we found that *S. symbiotica* was present in non aphid species and in host plants, and that the prevalence of the bacterium 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 examined. These results provide a completely new picture of the ubiquity of an insect symbiont in nature. They suggest that ecological interactions promote the dissemination of strains that are still free-living and poorly specialized, and for which plants are a proabable reservoir for the acquisition of new bacterial partners in insects.

Keywords: Symbiosis, Gut symbiont, Horizontal transfer, Insects, Host plants.

INTRODUCTION

Numerous studies have revealed the diversity of microbial symbiotic associations in insects and their role in adapting to particular lifestyles or diets deficient in certain elements (Moran 2007; Douglas 2011; McFall-Ngai et al. 2013). Many insect species 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 (Russell and Moran 2006; Vorburger and Gouskov 2011; Oliver et al. 2014; Zytynska et al. 2021). They suggest that their persistence is determined by a balance of costs and benefits. In recent years, the origin of bacterial mutualism in insects has been well studied (Husník et al. 2011; Clayton et al. 2012; Sudakaran et al. 2017; Manzano-Marín et al. 2020), particularly in stink bugs that pick up environmental bacteria that become mutualistic partners in specialized cavities of their digestive tract (Kikuchi et al. 2007; 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.

Several successive evolutionary steps can be considered in the establishment of mutualistic symbioses between insects and bacteria: 1) an infection by a generalist bacterium that circulates in the trophic chain from the environment, 2) if this infection results in a loss of fitness, a reduction of virulence by selection of the most resistant hosts and the least virulent bacteria in successive coevolutionary processes, 3) a contribution of benefits in particular circumstances, benefits greater than fitness costs for the host, and which allow the establishment of a profitable association for both

partners under specific conditions, 4) the perpetuation of the association by a permanent reacquisition via a circulation of the symbiont in the trophic chain, 5) the establishment of horizontal and/or vertical transfers, and 6) the development of an increasingly tight relationships between the partners with a reduction in the size of the bacterial genome (Hosokawa et al. 2016; Latorre and Manzano-Marín 2017; Takeshita and Kikuchi 2017; Gil and Latorre 2019). Depending on the degree of evolution of these systems, each of these stages should still be observed or in many cases, the intermediate stages have disappeared over time, making the reconstruction of this evolutionary history more complex. In this evolutionary scheme, transmission mechanisms are known to be instrumental in the establishment and evolution of symbioses in natural 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; Gehrer and Vorburger 2012; Caspi-Fluger Ayelet et al. 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 endosymbionts suggests their potential role as source of insect symbionts. For example, a strain of the bacterium *Sodalis* isolated from a hand wound in a human host but originated from a dead tree branch was found to share a very close relationship with *Sodalis glossinidius* and other menbers of Sodalis-allied clade of insect symbionts (Clayton et al. 2012; Chari et al. 2015). *Arsenophonus* bacteria are known to infest plants while members of the genus are widely distributed in insect populations (Bressan et al. 2009; Jousselin et al. 2013). *Erwinia* bacteria, generally described as phytopathogens, are also found associated with insects, such as aphids where it was first described as a gut associate (Harada and Ishikawa 1993; Harada et al. 1997; Nadarasah and Stavrinides 2011). More recently, some *Cinara* aphids have been found to harbor *Erwinia*-related obligate symbionts that

complement the ancestral obligate symbiont *Buchnera aphidicola* (Meseguer et al. 2017). Genomic analyses indicate that these associations originate from the acquisition of free-living *Erwinia* strains, likely acquired horizontally throught plants, and have 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 symbiotic associations between insects and bacteria 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 sometimes for millions of years (Koga and Moran 2014; Hosokawa et al. 2016; Husnik and McCutcheon 2016; Manzano-Marín et al. 2017; 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 symbiotic bacteria has arisen independently many times during their evolutionary history (Manzano-Marín et al. 2016, 2017; Meseguer et al. 2017; Manzano-Marín et al. 2020; Monnin et al. 2020). The repeated replacement of pre-existing symbionts by other microbial partners is now considered a redundant evolutionary process that occurs in many insect species, suggesting 1) the continuous formation of new mutualistic associations in nature, and 2) the existence of a pool of environmental symbionts from which new intimate, facultative, or obligate associations are formed in nature.

Serratia symbiotica, one of the most common symbionts in aphids (Oliver et al. 2010; Henry et al. 2015; Zytynska and Weisser 2016; Monnin et al. 2020), is a valuable candidate to decipher the origin and the evolution of bacterial mutualism in insects. It includes a great diversity of strains associated with very distinct biological features and reflecting the various associations that bacteria can share with insects (Burke and Moran 2011; Manzano-Marín and Latorre 2016; Pons et al. 2019a; Monnin et al. 2020; Perreau et al. 2020). The strains studied in aphids of the subfamily *Aphidinae* have been first described as intracellulaire facultative partners because they can invade host cells (including

bacteriocytes and sheath 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). *S. symbiotica* strains associated with aphids of the subfamilies Lachninae and Chaitophorinae are intracellular symbionts involved in co-obligate associations, compensating some metabolic capacities lost by the ancient obligate symbiont *B. aphidicola* (Lamelas et al. 2011; 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 (Renoz et al. 2018; Pons et al. 2019a; Perreau et al. 2020) and which can be cultivated freely on a pure artificial medium (Sabri et al. 2011; Grigorescu et al. 2017). These strains, slightly pathogenic to aphids, are considered to be at the pathogen-symbiont interface (Pons et al. 2019a; Perreau et al. 2020). We recently showed that these gut-associated strains that retain some free-living capacities, 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 suggest that *S. symbiotica* may reside in the gut of ants tending aphids (Renoz et al. 2018). Taken together, these results indicates that certain *S. symbiotica* strains have a tremendous ability to circulate from one compartment to another, with an aptitude to perform horizontal transfers between both phylogenetically close and distant species in the environment. We suggest that these strains may thrive in the environment where aphids prosper and could provide an environmental source for the establishment of new symbiotic asociations 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 *S. symbiotica* 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 provide comprehensive picture of the ubiquity of *S. symbiotica* across the food web. We investigated the distribution, diversity and

evolution of *S. symbiotica* infections at a multitrophic level, including 58 aphid species, aphid-interacting insects (i.e. predators, parasitoids, etc.), and host plants. This study allowed us 1) to examine the prevalence of *S. symbiotica* in aphids and their environment, and to detect environmental and ecological factors that influence its prevalence, 2) to determine the diversity of tissue tropism exhibited by *S. symbiotica* in natural insect populations, and 3) to investigate the propensity of *S. symbiotica* to circulate and be transferred horizontally in the natural environment of aphids. Using diagnostic PCR, fluorescence *in situ* hybridization and phylogenetic approaches, we provide a picture of the diversity and the ubiquity 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 *S. symbiotica* in insects, serving as potential interfaces for the emergence of new symbiotic associations.

MATERIALS AND METHODS

Sample collection

To get a compregensive picture of the ubiquity of *S. symbiotica* in the natural aphid environment, we sampled 1) aphid colonies of different species, 2) insects potentially in interaction with aphids in the sampled colonies, and 3) 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). 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). 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).

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) 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 µl containing 1µl of the template DNA lysate, 0.5 µM of each primer, 200 µM dNTPs, 1 × buffer and 0.625 unit of Taq DNA polymerase (Roche). 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 GeneBank 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 PASScmp (presented in Table S2). PCR amplification was performed as previously described (Pons et al. 2019a). PCR reactions were conducted in a final volume of 15 µl containing 1µl of the template DNA lysate, 0.5 µM of each primer, 200 µM dNTPs, 1X buffer and 0.625 unit of Taq DNA polymerase (Roche). The thermocycling profile consisted of 95°C for 5min; 35 cycles at 95 °C for 30 sec, 55 °C for 1 min 30 s and 72 °C for 1 min 30s; 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, 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 (Macorgens Inc., Amsterdam). Sequence alignments were done using Geneious® v9.1.5 (Kearse et al. 2012) and compared to sequences on GenBank using BLAST. The sequences were deposited in GenBank.

Phylogenetic analyses

The diversity of *S. symbiotica* strains was also characterized using the partial sequence of four housekeeping genes *accD*, *gyrB*, *murE* and *recJ* (Table S2) (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 30s, 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. 2017; 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; Lamelas et al. 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 (Table S3). 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: (1) the standard deviation of split frequencies was < 0.01, (2) visual inspection of the plot of the log-likelihood score at each sampling point suggested that the four chains reached stationarity, and (3) 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.

248 **Localization of *S. symbiotica***

249 To determine the tissue tropism of *S. symbiotica* strains in aphids and in the other insects sampled in
250 the colonies, whole-mount fluorescence *in situ* hybridization (FISH) was carried out as described in
251 (Koga et al. 2009; Renoz et al. 2018; Pons et al. 2019a). All insect samples positive to *S. symbiotica*
252 were tested. The following oligonucleotide probes were used: Cy5-ApisP2a (5'-Cy5-
253 CCTCTTTTGGGTAGATCC-3') targeting 16S rRNA of *B. aphidicola* and Cy3-PASSisR (5'-Cy3-
254 CCCGACTTTATCGCTGGC-3') targeting 16S rRNA of *S. symbiotica*. Insect tissues were stained
255 with SYTOX Green. Samples (between 1 to 6 per colony) were whole mounted and observed under a
256 Zeiss LSM 710 confocal microscope. Negative controls consisted of aphids not infected with *S.*
257 *symbiotica* and stained with the two probes or infected aphids with no-probe staining. Positive controls
258 comprised of artificially and naturally infected aphids.

259

260 **Statistical analyses**

261 We analyzed whether the proportion of aphids harboring *S. symbiotica* differs according to their host
262 plant range, as carry out in (Henry et al. 2015): specialized (feeding on a single plant species or group
263 of closely related species, N=13), restricted (feeding on a single plant family, N=91), polyphagous
264 (feeding on various plant families, N=105), or obligatory host-plant alternating (N=31). The presence
265 of *S. symbiotica* in aphids was analyzed using generalized linear models (GLM) with a binomial error
266 structure and a logit-link function, and the degree of plant specialization by aphids was the explanatory
267 variable. We also tested if the proportion of aphids infected by *S. symbiotica* differs in populations
268 collected from different plant species, using Pearson's Chi-square statistic. Aphids on plant species
269 from which we had less than five collection, and where *S. symbiotica* symbiont infected < 2% of the
270 individuals on a plant, were excluded from the analysis as recommended in (Henry et al. 2015). We
271 also examined the effect of the temperature (mean daily and maximum daily) on the proportion of
272 aphids harboring *S. symbiotica*. We tested if *S. symbiotica* infection was influenced by high seasonal

temperatures, because this symbiont is known to protect its host from heat shock, especially aphids collected from arid regions (Russell and Moran 2006; Henry et al. 2013). The presence of *S. symbiotica* in aphids was analyzed on pooled data using generalized linear models (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 aphids harboring *S. symbiotica* was tested on pooled data using non-parametric 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 generalized linear models (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 population

The presence of *S. symbiotica* was examined in 250 aphid colonies from 58 species of the Aphididae family (Table S1). These species were mostly part of the Macrosiphini (31 species) and Aphidini (23 species) tribes belonging to the Aphidinae subfamily (Table S1). Other species were members of the subfamilies Chaitophorinae (2 species) and Calaphidinae (2 species) (Table S1). 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). 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

298 69% of infected aphid colonies belonged to the genus *Aphis* (35/51) (Table 1). More precisely, 27%
299 (22/82) of *Aphis fabae* colonies, which is the most represented species of the genus *Aphis*, were found
300 to be infected (Table S1). We also found a hight infection rate of *S. symbiotica* in the genera
301 *Macrosiphum* (4/5), *Capitophorus* (1/1) and *Periphyllus* (1/1) (Table 1). In contrast, we observed that
302 in the genera *Uroleucon* and *Hyperomyzus*, the infection rate was zero (0/49 and 0/10, respectively,
303 Table 1).
304
305 **Environmental and ecological factors influencing *S. symbiotica* prevalence**
306 We showed that among the 45 host plant genera collected (all containing aphids), 15 were associated
307 with infected aphids (33%, Table 2). Of the 37 plants associated with infected aphids (all genera
308 included), 7 were members of the genus *Cirsium* (18%) (Table 2). We asked whether the degree of
309 plant specialization by aphids was associated with *S. symbiotica* infection in aphids. We found no
310 significant differences in the proportion of aphids harboring *S. symbiotica* according to their degree of
311 plant specialization (GLM, df = 3, $\chi^2 = 6.35$, p = 0.096, Figure S1). We also analyzed whether the
312 infection of aphids with *S. symbiotica* that feed on multiple host plants was correlated with the species
313 of plant on which they were collected. Sufficient data for analysis were available only for *A. fabae* that
314 feed on different host plants (*Circium arvense*, *Circium vulgare*, *Daucus carota*, *Heraclum*
315 *sphondylium*, *Rumex obtusifolium*, and *Sonchus asper*). We found that there were no significant
316 differences in the proportion of *A. fabae* harboring *S. symbiotica* collected from different plants (N=49,
317 df = 5, $\chi 2 = 3.59$, p = 0.61). The distribution of *S. symbiotica* in this sampling is uniform across
318 collections of *A. fabae* feeding on different plant species.
319 During the sampling in Belgium from May through August 2018, the mean daily temperatures
320 ranged from 12.9 °C to 25.8 °C and that maximum daily temperatures ranged from 15.9 °C to 32.9 °C
321 (Table S1). To test the impact of temperature on *S. symbiotica* prevalence, we focused on the data
322 sampled in July and August when most samples were collected (Table S1). 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). To perform analyzes, temperature data were pooled into 6 balanced categories each (Table S1). We showed that *S. symbiotica* frequencies varied across Belgium aphid populations exposed to varying summer temperatures (Figure 1). We found that the proportion of aphids 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) (Figure 1A-B). We also showed a significant positive correlation between the proportion of aphids harboring *S. symbiotica* and the maximum daily temperatures (Spearman's rs = 1, p = 0.017, Figure 1B). *S. symbiotica* frequency in aphid populations 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 also collected to examine the presence of similar *S. symbiotica* strains in the trophic systems. Among 203 insects composed of 52 species, 25 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). Among the 98 tending ant samples, 10 exhibited a positive infection with *S. symbiotica*, representing an infection rate of about 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). We found that the proportion of tending ants harboring *S. symbiotica* was significantly higher when collected in infected aphid colonies (N = 8/27) compared to uninfected colonies (N = 2/71) (GLM, df = 1, $\chi^2 = 13.55$, p < 0.001, Figure 2A).

Among the 15 bug individuals representing 10 species, two (*Dictyla humuli* and *Coreus marginatus*) were infected with *S. symbiotica* (Table S1). Twenty-two ladybugs were collected (larvae and adults), belonging to 6 species, and two adults were positive to *S. symbiotica* (*Harmonia axyridis*

and *Coccinella septempunctata*; Table S1). Nineteen adult parasitoids were also found in aphid colonies, mainly collected when laying eggs into aphids (Table S1). Three individuals of three species (*Aphidius funebris, Lysiphlebus fabarum* and *Lysiphlebus hirticornis*) showed a positive infection (Table S1). 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 pyrastri*) (Table S1). Two aphid midge larvae (*Aphidoletes aphidimyza*) were infected out of 7, and one moth larva was infected out of 6 (*Eupithecia trisignaria*, Table S1). 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). 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 to uninfected colonies (N = 7/90) (GLM, df = 1, $\chi^2 = 19.86$, $p < 0.001$, Figure 2C). We also examine 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 plant associated with aphid colonies were collected. Among the 161 plant samples (11 leaves and 150 stems) collected from 52 plant species, 14 were positive for *S. symbiotica* (about 9%), belonging to 12 plant species (23%; Table S1). 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 = 6/27) compared to uninfected colonies (N =8/132) (GLM, df = 1, $\chi^2 = 5.8$, p = 0.016, Figure 2B).

Tissue tropism of *S. symbiotica* in insects

All insect 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 observations revealed the existence of different patterns: 1) presence of *S. symbiotica* in the digestive tract, 2) in the sheath cells, and 3) in the secondary bacteriocytes (Figure 3).

Gut-associated *S. symbiotica* were found either colonizing in the midgut or into the whole gut (Figure 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). 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). In this case, the symbionts were located around primary bacteriocytes enclosing *B. aphidicola* (Figure 3E). *S. symbiotica* was also found in secondary bacteriocytes flanking the primary bacteriocytes containing *B. aphidicola* (Figure 3F-H). This pattern was observed in 8 aphid colonies belonging to 4 aphid species (*Macrosiphoniella millefolii, Periphyllus testudinaceus, Macrosiphum mordvilkoi,* and *A. fabae*, Table S1).

Regarding insects associated to aphid colonies, *S. symbiotica* has already been detected among ants, in the proventriculus, a specialized organ involved in food filtration (Renoz et al. 2018). Here, only one observed hoverfly larva showed an infection to *S. symbiotica* in the prothorax, at the beginning of the digestive tract (Figure 4). Images clearly suggest that *S. symbiotica* is accumulate into the tentorial bar, in the junction of the pump chamber with the foregut, near the true mouth (Figure 4).

398 **Diversity of *S. symbiotica* infections**

399 Bacterial sequences of the *accD*, *gyrB*, *murE*, and *recJ* genes were taken from all 90 positive samples
400 for *S. symbiotica* infection. Sequences were easily readable in 54 samples and for analyses, we
401 excluded the remaining samples that had either polymorphic sequences, sequences that were difficult
402 to read, or missing sequences (Table S2, Supplementary Information).

403 The phylogenetic relationship between these 54 *S. symbiotica* strains and other already
404 described *S. symbiotica* strains was estimated using sequences obtained in this study, as well as
405 sequences available in Genbank (Table S2). We also used *S. proteamaculans* 568, as outgroup. We
406 found that the phylogeny of the *S. symbiotica* strains is strongly structured by the taxonomic identity
407 of their host and the phylogenetic analyses established the existence of four distinct clades (Figure 5).
408 Co-obligate *S. symbiotica* strains from *C. cedri* (SCc), *C. fornacula* (SCf), *C. strobi* (SCs), and *T.*
409 *salignus* (STs Pazieg) aphids that were localized in secondary bacteriocytes form clade B (Burke and
410 Moran 2011; Manzano-Marín and Latorre 2014; Manzano-Marín et al. 2017; Meseguer et al. 2017).
411 The strains composing this clade are co-obligate symbionts of aphids and exhibit a long-term co-
412 evolutionary history with their hosts. The clade A is composed of *S. symbiotica* strains that are
413 generally considered as aphid facultative endosymbionts or recent co-obligates. This clade includes
414 strains found in *A. pisum*: SAp Tucson strain localized in sheath cells (Burke and Moran 2011;
415 Manzano-Marín et al. 2017) and SIS strain residing in secondary bacteriocytes and sheath cells (Nikoh
416 et al. 2019). It also contains strains found in *C. tujafilina* (SCt VLC) (Burke and Moran 2011;
417 Manzano-Marín et al. 2017) and *A. urticata* (AURT-53S) that are localized in secondary bacteriocytes,
418 and in *M. carnosum* (MCAR-56S) localized in sheath cells (Monnin et al. 2020). It is also composed
419 of strains found in *M. rosae* and *M. mordvilkoi* and localized in secondary bacteriocytes or sheath cells
420 (Figure 3). The clade D forms a large monophyletic clade consisting of, among others, strains from
421 aphids of the genus *Aphis*, and strains from the aphid species *M. absintii*, *M. millefolii*, *B. cardui* and
422 *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. 2017) and the strains previously detected in the aphid gut (FR65, FR28, FR35 and VP6; Table S4) (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; Figure 3) are also included in this clade. We also observed a polytomy with a group 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 asociated with aphids of the genus *Periphullus* 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 (Figure 3).

DISCUSSION

In this study, we investigated the presence and the distribution of *S. symbiotica* in wild aphid populations, as well as in the different compartiments of the surrounding environment of these sap-feeding insects. Our results provide a comprehensive picture of the ubiquity of *S. symbiotica* in the natural aphid environment including ants, predators, parasitoids and plants. They first confirm that *S. symbiotica* exhibit different patterns of infection in aphids and that certain strains naturally transit through the digestive tract of these insects (Renoz et al. 2019). Our findings also demonstrate that the distribution of *S. symbiotica* is not limited to aphids but extends to other organisms that may interact with them and host plants. Finally, our results suggest that some *S. symbiotica* strains may be able to jump from one host to another, including plants, and could undergo frequent horizontal transfers. In the light of these results, we discuss the multi-faceted nature of *S. symbiotica* and the evolutionary scenarios of symbiont acquisitions in insects.

In a previous study, we experimentally demonstrated that a strain of *S. symbiotica* previously isolated from the aphid *Aphis fabae* (Sabri et al 2014) was capable to invade phloem sap of *Vicia faba* and that infected plants can then serve as reservoirs for horizontal transmission of *S. symbiotica* in aphids (Pons et al. 2019). Our field study suggests that *S. symbiotica* can naturally reside in plants. To our knowledge, it is the first time that this symbiont species was found in plants collected directly on the field. The presence of *S. symbiotica* in plants addresses major ecological issues including 1) the role of plants in the dissemination in insect populations of bacterial candidates, which may serve as environmental progenitors for the establishment of new mutualisms, and 2) 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, indicating a close phylogenetic proximity whose suggests horizontal transfers 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. However, in some cases, we did not detect *S. symbiotica* in aphids when the host plant was positive. 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. Our results thus raise questions about the nature of the interaction between *S. symbiotica* and plants. The *Serratia* genus includes bacterial members that have the propancy to evolve in diverse environments including water, soil, plants, humans and invertebrates (Grimont and Grimont 2006), and many *Serratia* species can establish mutualistic or parasitic partnerships with plants (Petersen and Tisa 2013). In the context of our study, one assumption is that certain *S. symbiotica* strains are associated with plants, either in commensalistic associations, or in more intimate associations (pathogenic or mutualistic). Our previous study suggests that *S. symbiotica* can provide benefits to the infected plants, and that, in exchange, plants provide a suitable ecological niche mostly composed of sugar for *S. symbiotica* (Pons et al. 2019b). Ongoing studies should provide a better understanding of the nature of the interaction between certain bacterial strains and plants.

Another assumption is that *S. symbiotica* strains residing in plants could serve as an environmental symbiont pool from which new intimate associations are formed. This is supported by phylogenetic analyses showing that the *Cinara*-associated *Erwinia* co-obligate symbionts initially derived from plant associates (Manzano-Marín et al. 2020). Our field study thus suggests that *S. symbiotica* in aphids evolved from bacteria that originally inhabited plants, and highlights this bacterium as a valuable model for understanding how bacteria develop cross-kingdom host jumps and exploit multiple hosts before becoming, under still unclear circumstances, long-term mutualists in insects.

The broad sampling in our study highlights the great diversity of *S. symbiotica* strains in aphids with different tissue tropisms. We confirms 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 gut 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). Our results support this hypothesis and the following scenario: 1) acquisition of bacterial progenitors originally inhabiting the plant, 2) transition through the insect digestive tract, an intermediate step before, and 3) the possible establishment of more intimate associations. Experimental works have demonstrated that gut strains are unable to establish persistent maternal transmission (Pons et al. 2019, Perreau et al. 2020). A strict vertical transmission is, however, not always possible and in fact, not always necessary for the transmission of some gut symbionts. One of the most remarkable cases is the relationship between bean bug *Riptortus pedestris* and the beneficial gut symbiont *Burkholderia*. For successful inoculation of the next generation, the offspring must feed on inoculated or contaminated material from the adult's gut flora, and the symbiont must be environmentally re-acquired with each generation through nymph feeding (Kikuchi et al. 2007; Kikuchi and Yumoto 2013). In the context of the interaction between aphid and these gut-associated *S. symbiotica*, a permanent environmental source of contamination is required to maintan the infection across aphid

498 generations (Pons et al. 2019a). It is possible that infected plants provide such a source. These gut-
499 associated strains were found to be grouped in a common phylogenetic clade (clade D), as previously
500 shown in (Renoz et al. 2018). At the excpetion of the oleaster aphid *Capitophorus elaeagni*, aphid
501 species harboring gut-associated *S. symbiotica* were members of the genus *Aphis*. If experimental
502 studies have previously highlighted the moderate pathogenic effects of gut-associated *S. symbiotica*
503 (CWBI-2.3^T) (Pons et al. 2019, Perrau et al. 2020), their involvement in mutualistic associations with
504 their insect host cannot be excluded. A recent study showed that the *S. symbiotica* strain (CWBI-2.3^T)
505 infecting the aphid gut is capable to produce proteases including metalloproteases, which may facilitate
506 the digestion of plant proteins by helping to suppress plant defense (Skaljac et al. 2019). In light of
507 this, it is possible that these gut-associated *S. symbiotica* contribute to improve the assimilation by
508 aphids of some types of phloem sap or the elimination of toxins produced by host plants, allowing the
509 aphid adaptation to feed on certain plants.

510 Our results offer the opportunity to investigate the different patterns of tissue tropism exhibited
511 by *S. symbiotica* in aphids and beyond. It was previously established that intracellular *S. symbiotica*
512 strains could be housed either in secondary bacteriocytes and in sheath cells in the case of facultative
513 strains (Koga R. et al. 2003; Moran et al. 2005; Koga et al. 2012) and solely in bacteriocytes (subfamily
514 Aphidinae subfamily) in the case of nutritional co-obligate strains (subfamilies Lachninae and
515 Chaitophorinae subfamilies) (Manzano-Marín et al. 2016, 2017; Monnin et al. 2020). In parallel to
516 these infection patterns, the aphid gut that evolve exclusively, to our knowledge, at the extracellular
517 level (Renoz et al. 2018; Pons et al. 2019a; Perreau et al. 2020; Elston et al. 2021). All of these patterns
518 could be observed from the sampling we carried out in this study. Intracellular *S. symbiotica* strains
519 exclusively compartmentalized into sheath cells surrounding the primary bacteriocytes hosting *B.*
520 *aphidicola* have only been observed in individuals belonging to *Macrosiphum* species. *S. symbiotica*
521 was also observed in secondary bacteriocytes in different aphid species, including *Periphyllus*
522 *testudinaceus, Macrosiphoniella millefolii, Aphis fabae, Macrosiphum mordvilkoi* and *Macrosiphum*

523 *rosae*. So far, this tissue tropism of *S. symbiotica* has mostly been reported in aphids where the
524 symbiont is a co-obligate nutritional partner (Manzano-Marín et al. 2016, 2017; Monnin et al. 2020).
525 In our study, this is the case of *Periphyllus testudinaceus*, a member of the subfamily Chaitophorinae
526 where *S. symbiotica* has evolved as a nutritional co-obligate partner (Monnin et al. 2020). This tissue
527 tropism pattern has also been described in *A. pisum* but mainly after an artificial infection of the
528 hemolymph (Fukatsu et al. 2000; Koga R. et al. 2003) and in *Aphis urticata* (Monnin et al. 2020). Our
529 observations demonstrate that a specific microscopic approach is definitively needed to clarify the
530 nature of the associations in which bacteria and insects are involved because the topology of the
531 phylogenetic tree does not allow inferring the infection pattern displayed by *S. symbiotica*: the clade
532 D remains diverse and includes strains that exhibit different tissue tropism patterns, suggesting a lack
533 of resolution. It would thus be necessary to target additional housekeeping genes to increase the
534 resolution of the phylogentic analyses, discriminate differences within clade D, and fully resolve the
535 phylogeny of *S. symbiotica*.

536 While horizontal symbiont transfer is known to occur in the field, the rate at which it occurs is
537 still unknown (Russell et al. 2003; Darby and Douglas 2003; Gehrer and Vorburger 2012; Jousselin et
538 al. 2013; Henry et al. 2013; Guyomar et al. 2018). Here, we demonstrated that *S. symbiotica* infection
539 can occur in non-aphid insect species found in close proximity to aphid colonies and involved in
540 trophic relationships with them (12% of the screened insects were found positive to *S. symbiotica*).
541 We found that these insects were significantly more likely to harbor *S. symbiotica* when aphid colonies
542 were infected by the bacterium. In addition, we showed that *S. symbiotica* strains associated with the
543 non-aphid insects were in the same clades as strains found in aphids of the corresponding sampled
544 colonies. These results suggest that horizontal transfers of *S. symbiotica* can occur within natural insect
545 communities and that *S. symbiotica* infections extend beyond aphids. This can be an adaptive
546 characteristic allowing a wide dispersion of a fairly generalist bacterium although dependent on sugar
547 rich diet. Ant-tending is a possible route of transmission of symbiotic bacteria from aphids to ants and

548 vice versa, especially because ants feed on honeydew that can be infected by *S. symbiotica* (Pons et al
549 2019). With 10% of the sampled ants found infected by *S. symbiotica*, our results confirmed previous
550 studies having reported the presence of the bacterium in these social insects (Sirvio and Pamilo 2010,
551 Het et al. 2014, Renoz et al. 2019). This is an important issue because, even if *S. symbiotica* is only an
552 occasional passenger in ants, the social and feeding behavior of these insects could promote the
553 dissemination of certain *S. symbiotica* strains in the aphid colonies and increase the environmental
554 symbiont pool from which potential new associations could originate. Another possible route of
555 symbiont transmission from aphids to non-aphid insects is predation by entomophagous insects such
556 as ladybugs, hoverflies, aphid midges and fly larvae. We showed that *S. symbiotica* infect these insects
557 and we were able to localize the infection at the beginning of the digestive tract of a hoverfly larvae.
558 This suggest that *S. symbiotica* can transit from one trophic level to another through predation and
559 initiate an infection in non-aphid species. Further studies will be needed to determine the impact of *S.*
560 *symbiotica* in these new hosts and the extent to which more systemic and persistent infection may
561 occur. We also found that parasitoids could be infected, but in this case, the tissue tropism of *S.*
562 *symbiotica* was not studied due to a lack of insect material to perform all analyses. A previous study
563 showed the role of parasitoids as vectors of facultative bacterial symbionts in aphids (Gehrer and
564 Vorburger 2012). These insects could promote the emergence of symbioses because their mode of
565 parasitism could allow symbionts to have a direct access to the aphid hemolymph where the cells
566 specialized in symbiont sheltering are found. Ongoing studies should clarify the nature of this
567 interaction at the developmental level, but also at the ecological level with the cascading effects that
568 *S. symbiotica* infections could induce on parasitoid communities. Indeed, *S. symbiotica*, whether
569 facultative intracellular or gut-associated strains, can severely hamper the development of parasitoids
570 in aphids (Pons et al. 2019a). *S. symbiotica* was also detected in bugs, caterpillars and moth larvae that
571 are sap-sucking insects or herbivores that do not directly interact with aphids but can feed on the same
572 host plants. These results suggest, once again, that plants could be reservoir at the origin of new

573 interactions between *S. symbiotica* and insects. All these findings reveal the surprising ubiquity of *S.*
574 *symbiotica*, with strains that are likely able to disperse through multiple insect species via horizontal
575 transmission. Some bacterial genera show a high propensity to infect diverse hosts, including
576 *Spiroplasma*, *Sodalis* and *Arsenophnus* symbionts (Fukatsu et al. 2001; Bressan et al. 2009; Clayton
577 et al. 2012; Jousselin et al. 2013; Schwarz et al. 2014; Santos-Garcia et al. 2017; Ballinger et al. 2018;
578 Masson et al. 2018). These exemples stresses that putative progenitors of host-beneficial symbionts
579 circulate throughout the trophic system, with potential consequences not only for the ecology and
580 evolution of their primary host, but also for those of transitive hosts (Moran and Yun 2015). In the
581 case of *S. symbiotica* (especialy gut-associated strains), whether it is an established symbiont or
582 whether a transient commensal resident in multiple insect hosts, is yet to be elucidated.

583 In our study, a total of 58 aphid species were sampled and the prevalence of *S. symbiotica*
584 reaches 20% in aphids, which is fairly similar to what has been recorded in previous studies (Henry et
585 al. 2015; Renoz et al. 2018). Our results confirmed the high presence of *S. symbiotica* infections in the
586 genus *Aphis* with 29% of aphids being infected. In addition, *S. symbiotica* was identified in a
587 significant proportion in other aphid genera, such as in *Macrosiphum sp.* and *Capitophorus sp.*, while
588 no infection was detected in some aphid genera, such as in *Uroleuchon sp.*. The prevalence of *S.*
589 *symbiotica* in aphid populations may depend on the aphid genus, but this assumption should be taken
590 with caution. Indeed, one study reported no general effect of aphid phylogeny on the symbiont
591 presence (Henry et al. 2015), although another study, which considered all aphid symbionts, suggests
592 a genus-specific effect (Zytynska and Weisser 2016). The nature of the interaction between insects and
593 symbiotic bacteria can also depend on abiotic and biotic factors including the presence of predators
594 and parasitoids, the host plant and climatic factors (Henry et al. 2013; Oliver et al. 2014; Zytynska and
595 Weisser 2016; McLean Ailsa H. C. et al. 2016). Our results suggest that the incidence of *S. symbiotica*
596 infection increase with higher frequencies at higher seasonal temperatures. Some experimental studies
597 have already shown that some 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 associated with *S. symbiotica* are also supported by the high prevalence of *S. symbiotica* 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. The multifaceted nature of *S. symbiotica* must, however, be considered (a diversity that includes strains ranging from pathogens to strict mutualists). Previous studies reported the influence of host plants on the presence of facultative symbionts in aphids (Tsuchida et al. 2004; Brady and White 2013; Guidolin and Cônsoli 2017). In our sampling, the distribution of *S. symbiotica* is, however, uniform across collections of *A. fabae* regardless of host plants. More generally, we showed that the proportion of aphids (all 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. In some cases, it appeared to be random and in other cases as a consequence of selection acting on specific associations.

To conclude, our study provides a comprehensive picture of the ubiquity of *S. symbiotica* in nature. 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-Marín et al. 2020). Genetic analyses indicate that these intimate relationships between insects and bacteria can evolve in a very dynamic fashion involving the recruitment of new bacterial partners and the repeated replacement of pre-existing intracellular symbionts (Koga and Moran 2014; Husnik and McCutcheon 2016; Meseguer et al. 2017; Monnin et al. 2020; Mao and Bennett 2020). Symbiont switching is an important evolutionary mechanism, which is not limited to insects (e.g., in reef organisms such as corals and sponges) (Webster and Reusch 2017), and by which maladaptive symbionts are replaced by new functional ones (Sudakaran et al. 2017). The presence of *S. symbiotica* at different trophic levels suggests the existence of an environmental pool of bacteria from which new intimate partnerships with

623 insects may emerge. We hypothesize that the *S. symbiotica* diversity includes strains that exhibit a
624 generalist lifestyle, still capable to develop cross-kingdom host jumps, before, under specific
625 conditions, potentially transiting to a more specialized, and host-dependent lifestyle. A key aspect is
626 understanding the factors that favor such a transition, in particular the conditions that promote the
627 passage from the digestive tract to the hemolymph, a required step for the adoption of an intracellular
628 life and for access to vertical transmission. Another burning question concerns the specific
629 characteristics possessed by certain bacterial genera and species (e.g., members of the genera *Sodalis*,
630 *Arsenophonus*, and members of *S. symbiotica*) to regularly engage in stable alliances with insects. The
631 ecological versatility of *S. symbiotica* offers exciting avenues for answering these questions and
632 refining our understanding of the evolution of bacterial mutualism.
633

634 **ACKNOWLEDGMENTS**

635 We thank Charlotte Tinel, Aurélien Kaiser and Anastase Harelimana for some samples collection. We
636 also thank Alain Vanderpoorten (University of Liège) for his help in phylogenetic analyses. This work
637 was supported by the Fonds de la Recherche Scientifique (FNRS) through a Fonds pour la Formation
638 à la Recherche dans l'Industrie et dans l'Agriculture (FRIA). This paper is publication BRC 348 of the
639 Biodiversity Research Center (Universtité catholique de Louvain).
640

641 **REFERENCES**

642 Aljanabi SM, Martinez I (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR-based
643 techniques. Nucleic Acids Res 25:4692–4693. https://doi.org/10.1093/nar/25.22.4692

644 Ballinger MJ, Moore LD, Perlman SJ (2018) Evolution and Diversity of Inherited Spiroplasma Symbionts in
645 Myrmica Ants. Appl Env Microbiol 84:e02299-17. https://doi.org/10.1128/AEM.02299-17

646 Baumann P, Moran NA, Baumann LC (2013) Bacteriocyte-Associated Endosymbionts of Insects. In:
647 Rosenberg E, DeLong EF, Lory S, et al. (eds) The Prokaryotes: Prokaryotic Biology and Symbiotic
648 Associations. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 465–496

649 Brady CM, White JA (2013) Cowpea aphid (Aphis craccivora) associated with different host plants has
650 different facultative endosymbionts. Ecol Entomol 38:433–437. https://doi.org/10.1111/een.12020

651 Bressan A, Sémétey O, Arneodo J, et al (2009) Vector Transmission of a Plant-Pathogenic Bacterium in the
652 Arsenophonus Clade Sharing Ecological Traits with Facultative Insect Endosymbionts.
653 Phytopathology 99:1289–1296. https://doi.org/10.1094/PHYTO-99-11-1289

654 Bright M, Bulgheresi S (2010) A complex journey: transmission of microbial symbionts. Nat Rev Microbiol
655 8:218–230. https://doi.org/10.1038/nrmicro2262

656 Burke G, Fiehn O, Moran N (2009) Effects of facultative symbionts and heat stress on the metabolome of pea
657 aphids. ISME J 4:242–252. https://doi.org/10.1038/ismej.2009.114

658 Burke GR, Moran NA (2011) Massive Genomic Decay in Serratia symbiotica, a Recently Evolved Symbiont of
659 Aphids. Genome Biol Evol 3:195–208. https://doi.org/10.1093/gbe/evr002

660 Caspi-Fluger Ayelet, Inbar Moshe, Mozes-Daube Netta, et al (2012) Horizontal transmission of the insect
661 symbiont Rickettsia is plant-mediated. Proc R Soc B Biol Sci 279:1791–1796.
662 https://doi.org/10.1098/rspb.2011.2095

663 Castresana J (2000) Selection of Conserved Blocks from Multiple Alignments for Their Use in Phylogenetic
664 Analysis. Mol Biol Evol 17:540–552. https://doi.org/10.1093/oxfordjournals.molbev.a026334

665 Chari A, Oakeson KF, Enomoto S, et al (2015) Phenotypic characterization of Sodalis praecaptivus sp. nov., a
666 close non-insect-associated member of the Sodalis-allied lineage of insect endosymbionts. Int J Syst
667 Evol Microbiol 65:1400–1405. https://doi.org/10.1099/ijs.0.000091

668 Chong RA, Moran NA (2018) Evolutionary loss and replacement of Buchnera , the obligate endosymbiont of
669 aphids. ISME J 12:898. https://doi.org/10.1038/s41396-017-0024-6

670 Clayton AL, Oakeson KF, Gutin M, et al (2012) A Novel Human-Infection-Derived Bacterium Provides Insights
671 into the Evolutionary Origins of Mutualistic Insect–Bacterial Symbioses. PLOS Genet 8:e1002990.
672 https://doi.org/10.1371/journal.pgen.1002990

673 D'acier AC, Cruaud, Artige (2014) DNA Barcoding and the Associated PhylAphidB@se Website for the
674 Identification of European Aphids (Insecta: Hemiptera: Aphididae).
675 https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0097620. Accessed 12 Apr 2019

676 Darby AC, Douglas AE (2003) Elucidation of the Transmission Patterns of an Insect-Borne Bacterium. Appl
677 Environ Microbiol 69:4403–4407. https://doi.org/10.1128/AEM.69.8.4403-4407.2003

678 Douglas AE (1998) Nutritional Interactions in Insect-Microbial Symbioses: Aphids and Their Symbiotic
679 Bacteria Buchnera. Annu Rev Entomol 43:17–37. https://doi.org/10.1146/annurev.ento.43.1.17

680 Douglas AE (2011) Lessons from Studying Insect Symbioses. Cell Host Microbe 10:359–367.
681 https://doi.org/10.1016/j.chom.2011.09.001

682 Doyle J (1991) DNA Protocols for Plants. In: Molecular Techniques in Taxonomy. Springer, Berlin, Heidelberg,
683 pp 283–293

684 Elston KM, Perreau J, Maeda GP, et al (2021) Engineering a Culturable Serratia symbiotica Strain for Aphid
685 Paratransgenesis. Appl Environ Microbiol 87:. https://doi.org/10.1128/AEM.02245-20

686 Feldhaar H (2011) Bacterial symbionts as mediators of ecologically important traits of insect hosts. Ecol
687 Entomol 36:533–543. https://doi.org/10.1111/j.1365-2311.2011.01318.x

688 Foray V, Grigorescu AS, Sabri A, et al (2014) Whole-Genome Sequence of Serratia symbiotica Strain CWBI-
689 2.3T, a Free-Living Symbiont of the Black Bean Aphid Aphis fabae. Genome Announc 2:e00767-14.
690 https://doi.org/10.1128/genomeA.00767-14

691 Fukatsu T, Nikoh N, Kawai R, Koga R (2000) The Secondary Endosymbiotic Bacterium of the Pea Aphid
692 Acyrthosiphon pisum (Insecta: Homoptera). Appl Environ Microbiol 66:2748–2758.
693 https://doi.org/10.1128/AEM.66.7.2748-2758.2000

694 Fukatsu T, Tsuchida T, Nikoh N, Koga R (2001) Spiroplasma Symbiont of the Pea Aphid, Acyrthosiphon pisum
695 (Insecta: Homoptera). Appl Environ Microbiol 67:1284–1291.
696 https://doi.org/10.1128/AEM.67.3.1284-1291.2001

697 Gehrer L, Vorburger C (2012) Parasitoids as vectors of facultative bacterial endosymbionts in aphids. Biol Lett
698 rsbl20120144. https://doi.org/10.1098/rsbl.2012.0144

699 Gil R, Latorre A (2019) Unity Makes Strength: A Review on Mutualistic Symbiosis in Representative Insect
700 Clades. Life 9:21. https://doi.org/10.3390/life9010021

701 Grigorescu AS, Renoz F, Sabri A, et al (2017) Accessing the Hidden Microbial Diversity of Aphids: an
702 Illustration of How Culture-Dependent Methods Can Be Used to Decipher the Insect Microbiota.
703 Microb Ecol 1–14. https://doi.org/10.1007/s00248-017-1092-x

704 Grimont F, Grimont PAD (2006) The Genus Serratia. In: Dworkin M, Falkow S, Rosenberg E, et al. (eds) The
705 Prokaryotes. Springer New York, pp 219–244

706 Guidolin AS, Cônsoli FL (2017) Symbiont Diversity of Aphis (Toxoptera) citricidus (Hemiptera: Aphididae) as
707 Influenced by Host Plants. Microb Ecol 73:201–210. https://doi.org/10.1007/s00248-016-0892-8

708 Guo J, Hatt S, He K, et al (2017) Nine facultative endosymbionts in aphids. A review. J Asia-Pac Entomol.
709 https://doi.org/10.1016/j.aspen.2017.03.025

710 Guyomar C, Legeai F, Jousselin E, et al (2018) Multi-scale characterization of symbiont diversity in the pea
711 aphid complex through metagenomic approaches. Microbiome.
712 https://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-018-0562-9. Accessed 25
713 Nov 2019

714 Harada H, Ishikawa H (1993) Gut microbe of aphid closely related to its intracellular symbiont. Biosystems
715 31:185–191. https://doi.org/10.1016/0303-2647(93)90048-H

716 Harada H, Oyaizu H, Ishikawa H (1996) A Consideration About the Origin of Aphid Intracellular Symbiont in
717 Connection with Gut Bacterial Flora. J Gen Appl Microbiol 42:17–26.
718 https://doi.org/10.2323/jgam.42.17

719 Harada H, Oyaizu H, Kosako Y, Ishikawa H (1997) Erwinia aphidicola, a new species isolated from pea aphid,
720 Acyrthosiphon pisum. J Gen Appl Microbiol 43:349–354. https://doi.org/10.2323/jgam.43.349

721 Henry LM, Maiden MCJ, Ferrari J, Godfray HCJ (2015) Insect life history and the evolution of bacterial
722 mutualism. Ecol Lett 18:516–525. https://doi.org/10.1111/ele.12425

723 Henry LM, Peccoud J, Simon J-C, et al (2013) Horizontally Transmitted Symbionts and Host Colonization of
724 Ecological Niches. Curr Biol 23:1713–1717. https://doi.org/10.1016/j.cub.2013.07.029

725 Heyworth ER, Ferrari J (2015) A facultative endosymbiont in aphids can provide diverse ecological benefits. J
726 Evol Biol 28:1753–1760. https://doi.org/10.1111/jeb.12705

727 Hosokawa T, Ishii Y, Nikoh N, et al (2016) Obligate bacterial mutualists evolving from environmental bacteria
728 in natural insect populations. Nat Microbiol 1:15011. https://doi.org/10.1038/nmicrobiol.2015.11

729 Husník F, Chrudimský T, Hypša V (2011) Multiple origins of endosymbiosis within the Enterobacteriaceae (γ-
730 Proteobacteria): convergence of complex phylogenetic approaches. BMC Biol 9:87.
731 https://doi.org/10.1186/1741-7007-9-87

732 Husnik F, McCutcheon JP (2016) Repeated replacement of an intrabacterial symbiont in the tripartite nested
733 mealybug symbiosis. Proc Natl Acad Sci 113:E5416–E5424.
734 https://doi.org/10.1073/pnas.1603910113

735 Jousselin E, Cœur d'Acier A, Vanlerberghe-Masutti F, Duron O (2013) Evolution and diversity of
736 Arsenophonus endosymbionts in aphids. Mol Ecol 22:260–270. https://doi.org/10.1111/mec.12092

737 Kearse M, Moir R, Wilson A, et al (2012) Geneious Basic: An integrated and extendable desktop software
738 platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649.
739 https://doi.org/10.1093/bioinformatics/bts199

740 Kikuchi Y, Hosokawa T, Fukatsu T (2007) Insect-Microbe Mutualism without Vertical Transmission: a Stinkbug
741 Acquires a Beneficial Gut Symbiont from the Environment Every Generation. Appl Environ Microbiol
742 73:4308–4316. https://doi.org/10.1128/AEM.00067-07

743 Kikuchi Y, Yumoto I (2013) Efficient Colonization of the Bean Bug Riptortus pedestris by an Environmentally
744 Transmitted Burkholderia Symbiont. Appl Environ Microbiol 79:2088–2091.
745 https://doi.org/10.1128/AEM.03299-12

746 Koga R, Meng X-Y, Tsuchida T, Fukatsu T (2012) Cellular mechanism for selective vertical transmission of an
747 obligate insect symbiont at the bacteriocyte–embryo interface. Proc Natl Acad Sci 109:E1230–E1237.
748 https://doi.org/10.1073/pnas.1119212109

749 Koga R, Moran NA (2014) Swapping symbionts in spittlebugs: evolutionary replacement of a reduced
750 genome symbiont. ISME J 8:1237–1246. https://doi.org/10.1038/ismej.2013.235

751 Koga R., Tsuchida T., Fukatsu T. (2003) Changing partners in an obligate symbiosis: a facultative
752 endosymbiont can compensate for loss of the essential endosymbiont Buchnera in an aphid. Proc R
753 Soc Lond B Biol Sci 270:2543–2550. https://doi.org/10.1098/rspb.2003.2537

754 Koga R, Tsuchida T, Fukatsu T (2009) Quenching autofluorescence of insect tissues for *in situ* detection of
755 endosymbionts. Appl Entomol Zool 44:281–291. https://doi.org/10.1303/aez.2009.281

756 Lamelas A, Gosalbes MJ, Manzano-Marín A, et al (2011) Serratia symbiotica from the Aphid Cinara cedri: A
757 Missing Link from Facultative to Obligate Insect Endosymbiont. PLoS Genet 7:e1002357.
758 https://doi.org/10.1371/journal.pgen.1002357

759 Lanfear R, Calcott B, Ho SYW, Guindon S (2012) PartitionFinder: Combined Selection of Partitioning Schemes
760 and Substitution Models for Phylogenetic Analyses. Mol Biol Evol 29:1695–1701.
761 https://doi.org/10.1093/molbev/mss020

762 Latorre A, Manzano-Marín A (2017) Dissecting genome reduction and trait loss in insect endosymbionts. Ann
763 N Y Acad Sci 1389:52–75. https://doi.org/10.1111/nyas.13222

764 Łukasik P, Guo H, Asch M van, et al (2015) Horizontal transfer of facultative endosymbionts is limited by host
765 relatedness. Evolution 69:2757–2766. https://doi.org/10.1111/evo.12767

766 Manzano-Marín A, Coeur d'acier A, Clamens A-L, et al (2018) A Freeloader? The Highly Eroded Yet Large
767 Genome of the Serratia symbiotica Symbiont of Cinara strobi. Genome Biol Evol 10:2178–2189.
768 https://doi.org/10.1093/gbe/evy173

769 Manzano-Marín A, Latorre A (2016) Snapshots of a shrinking partner: Genome reduction in *Serratia*
770 *symbiotica*. Sci Rep 6:32590. https://doi.org/10.1038/srep32590

771 Manzano-Marín A, Latorre A (2014) Settling Down: The Genome of Serratia symbiotica from the Aphid
772 Cinara tujafilina Zooms in on the Process of Accommodation to a Cooperative Intracellular Life.
773 Genome Biol Evol 6:1683–1698. https://doi.org/10.1093/gbe/evu133

774 Manzano-Marín A, Simon J-C, Latorre A (2016) Reinventing the Wheel and Making It Round Again:
775 Evolutionary Convergence in Buchnera–Serratia Symbiotic Consortia between the Distantly Related
776 Lachninae Aphids Tuberolachnus salignus and Cinara cedri. Genome Biol Evol 8:1440–1458.
777 https://doi.org/10.1093/gbe/evw085

778 Manzano-Marín A, Szabó G, Simon J-C, et al (2017) Happens in the best of subfamilies: establishment and
779 repeated replacements of co-obligate secondary endosymbionts within Lachninae aphids. Environ
780 Microbiol 19:393–408. https://doi.org/10.1111/1462-2920.13633

781 Manzano-Marín A, Coeur D'acier A, Clamens A-L, et al (2020) Serial horizontal transfer of vitamin-
782 biosynthetic genes enables the establishment of new nutritional symbionts in aphids' di-symbiotic
783 systems. ISME J 14:259–273. https://doi.org/10.1038/s41396-019-0533-6

784 Mao M, Bennett GM (2020) Symbiont replacements reset the co-evolutionary relationship between insects
785 and their heritable bacteria. ISME J 14:1384–1395. https://doi.org/10.1038/s41396-020-0616-4

786 Masson F, Copete SC, Schüpfer F, et al (2018) In Vitro Culture of the Insect Endosymbiont Spiroplasma
787 poulsonii Highlights Bacterial Genes Involved in Host-Symbiont Interaction. mBio 9:.
788 https://doi.org/10.1128/mBio.00024-18

789 Matsuura Y, Moriyama M, Łukasik P, et al (2018) Recurrent symbiont recruitment from fungal parasites in
790 cicadas. Proc Natl Acad Sci 115:E5970–E5979. https://doi.org/10.1073/pnas.1803245115

791 McFall-Ngai M, Hadfield MG, Bosch TCG, et al (2013) Animals in a bacterial world, a new imperative for the
792 life sciences. Proc Natl Acad Sci 110:3229–3236. https://doi.org/10.1073/pnas.1218525110

793 McLean Ailsa H. C., Parker Benjamin J., Hrček Jan, et al (2016) Insect symbionts in food webs. Philos Trans R
794 Soc B Biol Sci 371:20150325. https://doi.org/10.1098/rstb.2015.0325

795 Meseguer AS, Manzano-Marín A, d'Acier AC, et al (2017) Buchnera has changed flatmate but the repeated
796 replacement of co-obligate symbionts is not associated with the ecological expansions of their aphid
797 hosts. Mol Ecol 26:2363–2378. https://doi.org/10.1111/mec.13910

798 Monnin D, Jackson R, Kiers ET, et al (2020) Parallel Evolution in the Integration of a Co-obligate Aphid
799 Symbiosis. Curr Biol 30:1949–1957.e6. https://doi.org/10.1016/j.cub.2020.03.011

800 Montllor CB, Maxmen A, Purcell AH (2002) Facultative bacterial endosymbionts benefit pea aphids
801 Acyrthosiphon pisum under heat stress. Ecol Entomol 27:189–195. https://doi.org/10.1046/j.1365-
802 2311.2002.00393.x

803 Moran NA (2007) Symbiosis as an adaptive process and source of phenotypic complexity. Proc Natl Acad Sci
804 104:8627–8633. https://doi.org/10.1073/pnas.0611659104

805 Moran NA, Baumann P (2000) Bacterial endosymbionts in animals. Curr Opin Microbiol 3:270–275.
806 https://doi.org/10.1016/S1369-5274(00)00088-6

807 Moran NA, Russell JA, Koga R, Fukatsu T (2005) Evolutionary Relationships of Three New Species of
808 Enterobacteriaceae Living as Symbionts of Aphids and Other Insects. Appl Environ Microbiol
809 71:3302–3310. https://doi.org/10.1128/AEM.71.6.3302-3310.2005

810 Moran NA, Yun Y (2015) Experimental replacement of an obligate insect symbiont. Proc Natl Acad Sci
811 112:2093–2096. https://doi.org/10.1073/pnas.1420037112

812 Nadarasah G, Stavrinides J (2011) Insects as alternative hosts for phytopathogenic bacteria. FEMS Microbiol
813 Rev 35:555–575. https://doi.org/10.1111/j.1574-6976.2011.00264.x

814 Nikoh N, Koga R, Oshima K, et al (2019) Genome Sequence of “Candidatus Serratia symbiotica” Strain IS, a
815 Facultative Bacterial Symbiont of the Pea Aphid Acyrthosiphon pisum. Microbiol Resour Announc 8:.
816 https://doi.org/10.1128/MRA.00272-19

817 Nylander JAA, Wilgenbusch JC, Warren DL, Swofford DL (2008) AWTY (are we there yet?): a system for
818 graphical exploration of MCMC convergence in Bayesian phylogenetics. Bioinformatics 24:581–583.
819 https://doi.org/10.1093/bioinformatics/btm388

820 Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative Symbionts in Aphids and the Horizontal
821 Transfer of Ecologically Important Traits. Annu Rev Entomol 55:247–266.
822 https://doi.org/10.1146/annurev-ento-112408-085305

823 Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer
824 resistance to parasitic wasps. Proc Natl Acad Sci 100:1803–1807.
825 https://doi.org/10.1073/pnas.0335320100

826 Oliver KM, Smith AH, Russell JA (2014) Defensive symbiosis in the real world – advancing ecological studies
827 of heritable, protective bacteria in aphids and beyond. Funct Ecol 28:341–355.
828 https://doi.org/10.1111/1365-2435.12133

829 Perreau J, Patel DJ, Anderson H, et al (2020) Vertical transmission at the pathogen-symbiont interface:
830 Serratia symbiotica and aphids. bioRxiv 2020.09.01.279018.
831 https://doi.org/10.1101/2020.09.01.279018

832 Petersen LM, Tisa LS (2013) Friend or foe? A review of the mechanisms that drive Serratia towards diverse
833 lifestyles. Can J Microbiol 59:627–640. https://doi.org/10.1139/cjm-2013-0343

834 Pons I, Renoz F, Noël C, Hance T (2019a) New Insights into the Nature of Symbiotic Associations in Aphids:
835 Infection Process, Biological Effects and Transmission Mode of Cultivable Serratia symbiotica
836 Bacteria. Appl Env Microbiol AEM.02445-18. https://doi.org/10.1128/AEM.02445-18

837 Pons I, Renoz F, Noël C, Hance T (2019b) Circulation of the Cultivable Symbiont Serratia symbiotica in Aphids
838 Is Mediated by Plants. Front. Microbiol.

839 Reemer M, Rotheray G (2009) Pollen feeding larvae in the presumed predatory syrphine genus Toxomerus
840 Macquart (Diptera, Syrphidae). J. Nat. Hist.

841 Renoz F, Ambroise J, Bearzatto B, et al (2020) Draft Genome Sequences of Two Cultivable Strains of the
842 Bacterial Symbiont Serratia symbiotica. Microbiol Resour Announc 9:.
843 https://doi.org/10.1128/MRA.01579-19

844 Renoz F, Pons I, Vanderpoorten A, et al (2018) Evidence for Gut-Associated Serratia symbiotica in Wild
845 Aphids and Ants Provides New Perspectives on the Evolution of Bacterial Mutualism in Insects.
846 Microb Ecol. https://doi.org/10.1007/s00248-018-1265-2

847 Ronquist F, Teslenko M, van der Mark P, et al (2012) MrBayes 3.2: Efficient Bayesian Phylogenetic Inference
848 and Model Choice Across a Large Model Space. Syst Biol 61:539–542.
849 https://doi.org/10.1093/sysbio/sys029

850 Russell JA, Latorre A, Sabater-Muñoz B, et al (2003) Side-stepping secondary symbionts: widespread
851 horizontal transfer across and beyond the Aphidoidea. Mol Ecol 12:1061–1075

852 Russell JA, Moran NA (2006) Costs and benefits of symbiont infection in aphids: variation among symbionts
853 and across temperatures. Proc Biol Sci 273:603–610. https://doi.org/10.1098/rspb.2005.3348

854 Sabri A, Leroy P, Haubruge E, et al (2011) Isolation, pure culture and characterization of Serratia symbiotica
855 sp. nov., the R-type of secondary endosymbiont of the black bean aphid Aphis fabae. Int J Syst Evol
856 Microbiol 61:2081–2088. https://doi.org/10.1099/ijs.0.024133-0

857 Salem H, Florez L, Gerardo N, Kaltenpoth M (2015) An out-of-body experience: the extracellular dimension
858 for the transmission of mutualistic bacteria in insects. Proc R Soc Lond B Biol Sci 282:20142957.
859 https://doi.org/10.1098/rspb.2014.2957

860 Santos-Garcia D, Silva FJ, Morin S, et al (2017) The All-Rounder Sodalis: A New Bacteriome-Associated
861 Endosymbiont of the Lygaeoid Bug Henestaris halophilus (Heteroptera: Henestarinae) and a Critical
862 Examination of Its Evolution. Genome Biol Evol 9:2893–2910. https://doi.org/10.1093/gbe/evx202

863 Schwarz RS, Teixeira ÉW, Tauber JP, et al (2014) Honey bee colonies act as reservoirs for two Spiroplasma
864 facultative symbionts and incur complex, multiyear infection dynamics. MicrobiologyOpen 3:341–
865 355. https://doi.org/https://doi.org/10.1002/mbo3.172

866 Skaljac M, Vogel H, Wielsch N, et al (2019) Transmission of a Protease-Secreting Bacterial Symbiont Among
867 Pea Aphids via Host Plants. Front Physiol 10:. https://doi.org/10.3389/fphys.2019.00438

868 Su Q, Oliver KM, Pan H, et al (2013) Facultative Symbiont Hamiltonella Confers Benefits to Bemisia tabaci
869 (Hemiptera: Aleyrodidae), an Invasive Agricultural Pest Worldwide. Environ Entomol 42:1265–1271.
870 https://doi.org/10.1603/EN13182

871 Sudakaran S, Kost C, Kaltenpoth M (2017) Symbiont Acquisition and Replacement as a Source of Ecological
872 Innovation. Trends Microbiol 25:375–390. https://doi.org/10.1016/j.tim.2017.02.014

873 Takeshita K, Kikuchi Y (2017) Riptortus pedestris and Burkholderia symbiont: an ideal model system for
874 insect–microbe symbiotic associations. Res Microbiol 168:175–187.
875 https://doi.org/10.1016/j.resmic.2016.11.005

876 Tsuchida T, Koga R, Fukatsu T (2004) Host plant specialization governed by facultative symbiont. Science
877 303:1989. https://doi.org/10.1126/science.1094611

878 Vorburger C, Gouskov A (2011) Only helpful when required: a longevity cost of harbouring defensive
879 symbionts. J Evol Biol 24:1611–1617. https://doi.org/10.1111/j.1420-9101.2011.02292.x

880 Webster NS, Reusch TBH (2017) Microbial contributions to the persistence of coral reefs. ISME J 11:2167–
881 2174. https://doi.org/10.1038/ismej.2017.66

882 Wernegreen JJ (2017) In it for the long haul: evolutionary consequences of persistent endosymbiosis. Curr
883 Opin Genet Dev 47:83–90. https://doi.org/10.1016/j.gde.2017.08.006

884 Zytynska SE, Tighiouart K, Frago E (2021) The benefits and costs of hosting facultative symbionts in plant-
885 sucking insects: a meta-analysis. Mol Ecol n/a: https://doi.org/https://doi.org/10.1111/mec.15897

886 Zytynska SE, Weisser WW (2016) The natural occurrence of secondary bacterial symbionts in aphids. Ecol
887 Entomol 41:13–26. https://doi.org/10.1111/een.12281

888

889

890

891

892

893

894

895

896

897

898

899

900

901

902

FIGURES AND TABLES

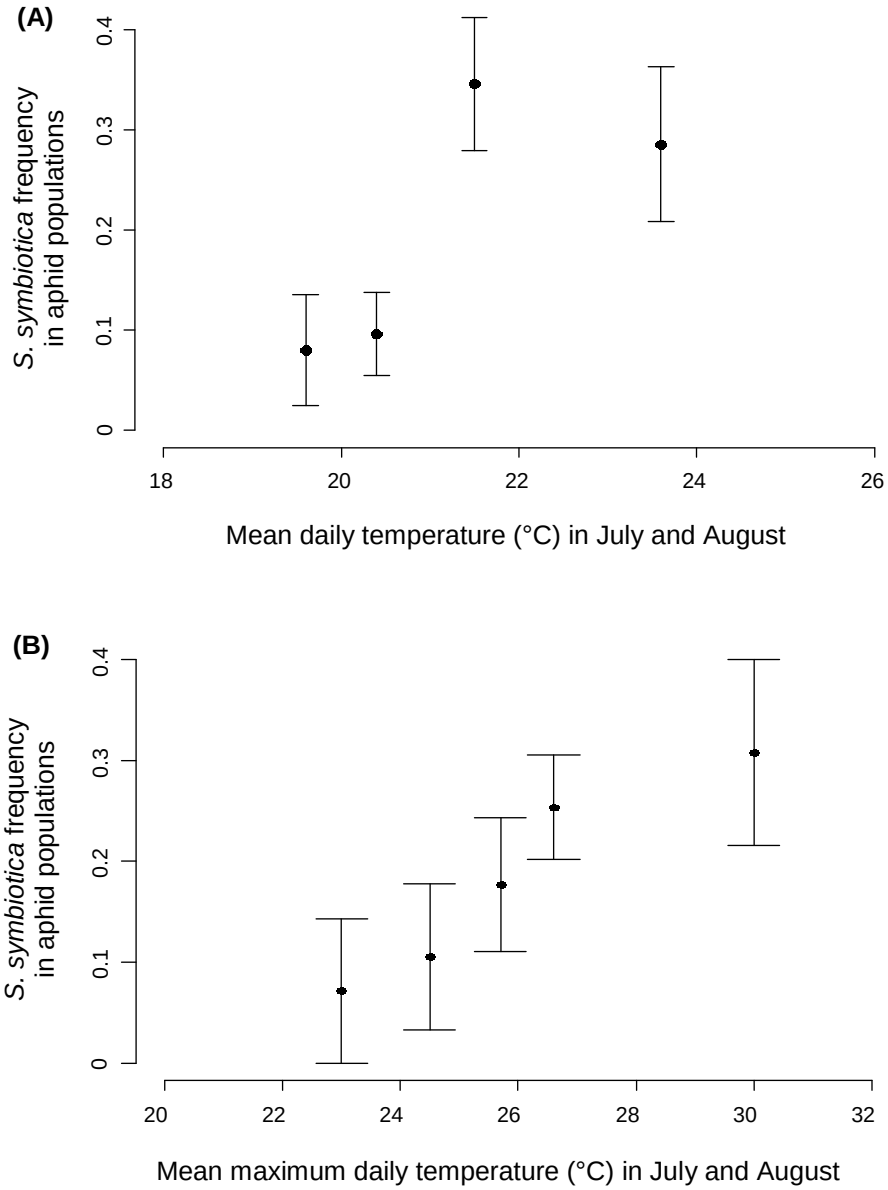


Figure 1. Relationships between *S. symbiotica* frequency across Belgian aphid populations and the mean daily temperature (**A**) or the maximum daily temperature (**B**) during sampling.

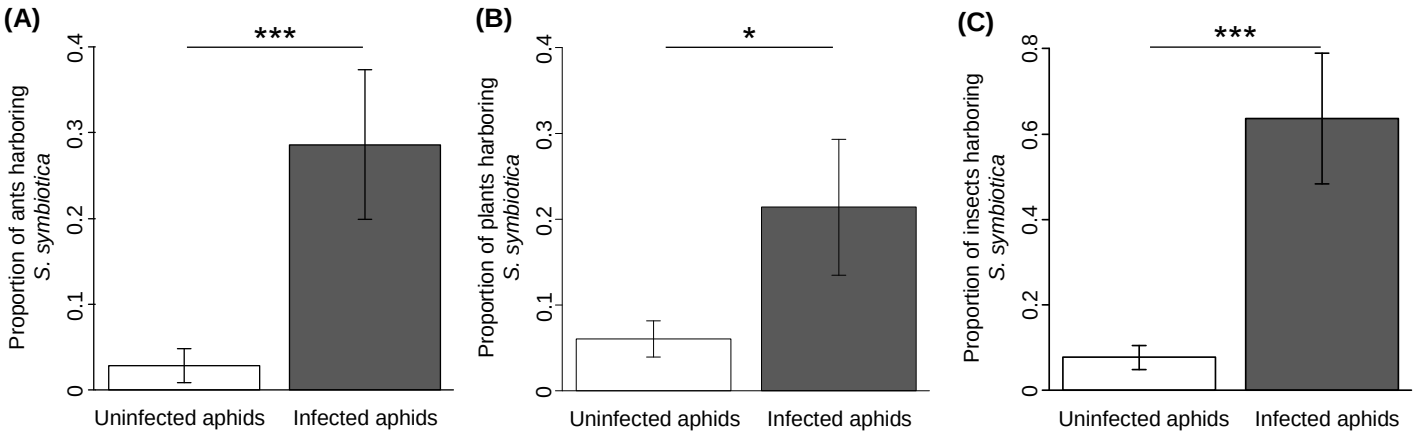


Figure 2. Prevalence of *S. symbiotica* in tending ants (A), plant samples (B) and associated insects (C) differ depending on the infection status of the aphid colonies. Columns represent the proportion of samples infected with *S. symbiotica* according to uninfected aphid colonies (white) or infected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

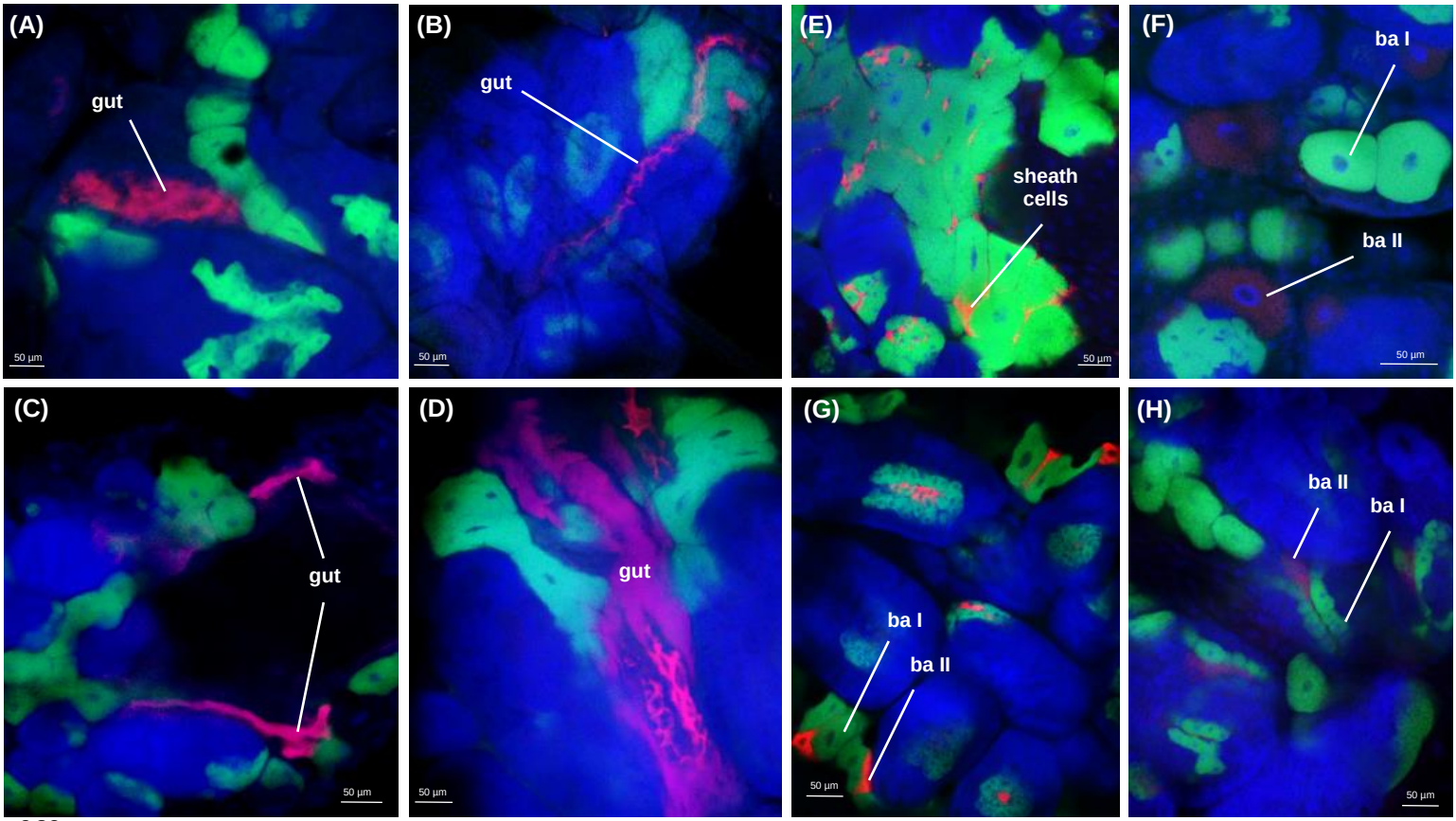


Figure 3. Whole-mount FISH of *S. symbiotica* in naturally infected aphids (ventral views). Red Cy3 signals are *S. symbiotica*, green Cy5 signals are *B. aphidicola*, and blue SYTOX Green signals are aphid tissues. **(A-D)** *S. symbiotica* found residing at the gut level of aphids. **(E)** *S. symbiotica* found residing at the sheath cells level of aphids. **(F-H)** *S. 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 mordvilkoi* (ID 135), **(G)** *Periphyllus testudinaceus* (ID 1), and **(H)** *Aphis fabae* (ID 380).

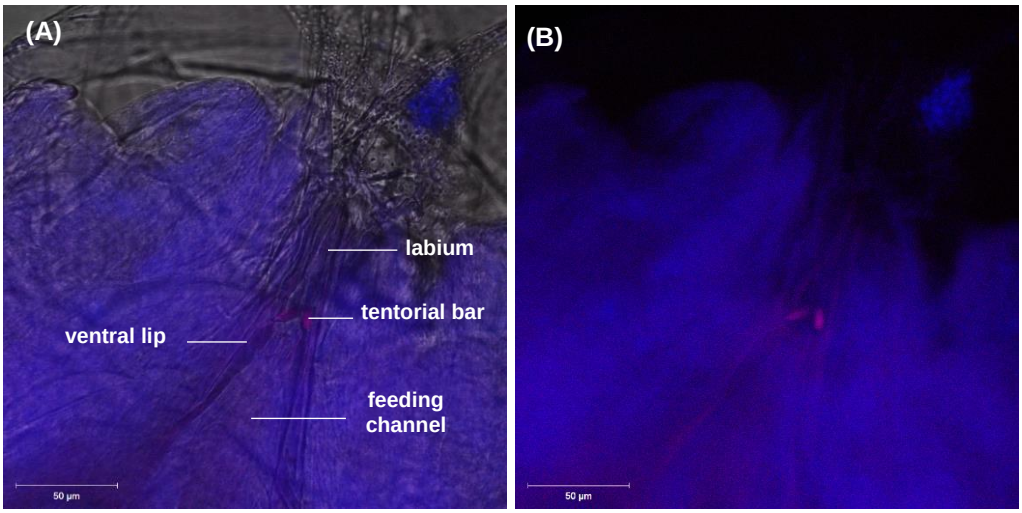
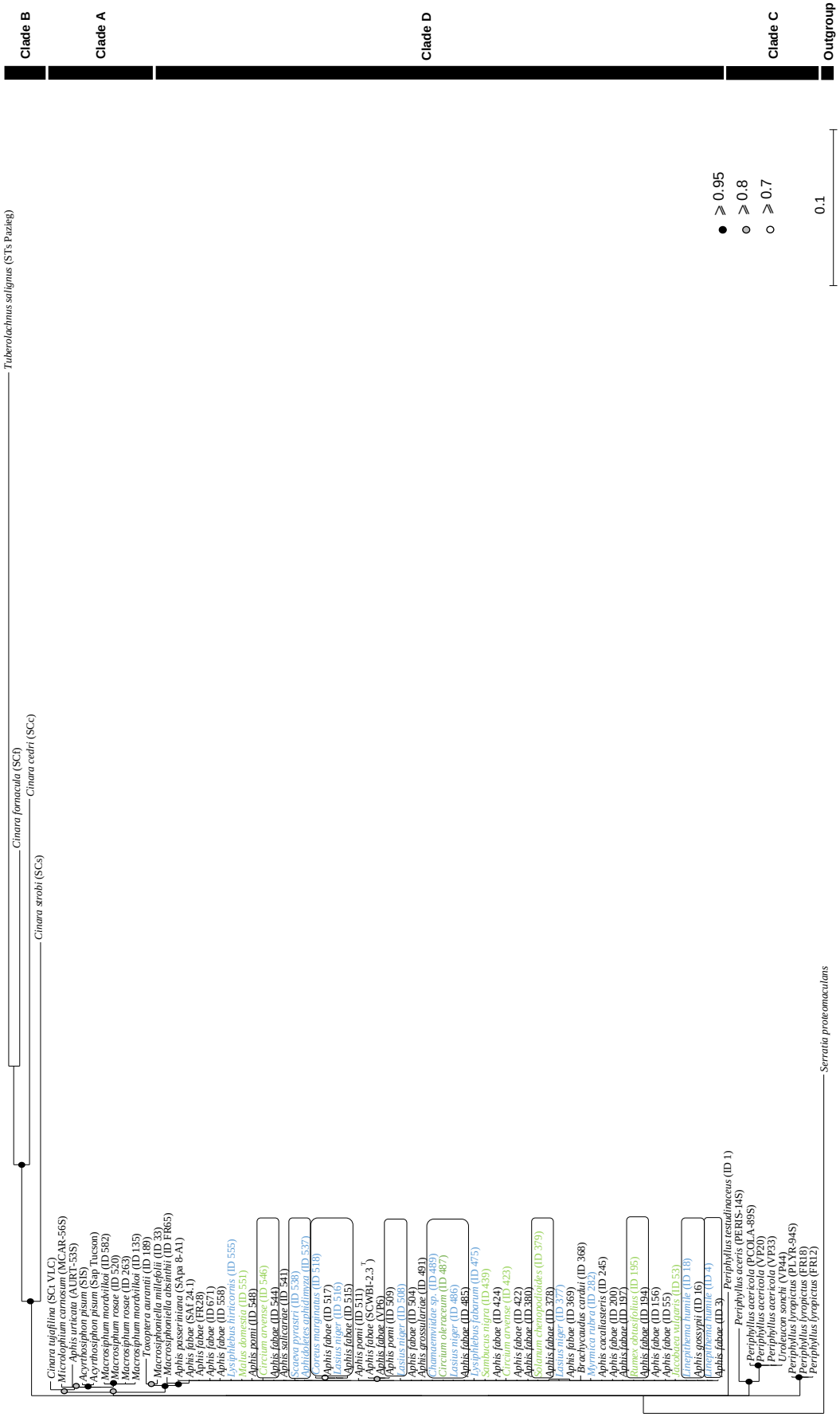


Figure 4. Whole-mount FISH of *S. symbiotica* in a naturally infected hoverfly larva (Prothorax, ventral view). Red Cy3 signals are *S. symbiotica*, and blue SYTOX Green signals are insect tissues. A is with bright field and B is without bright field. *S. symbiotica* is located at the level of the tentorial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



990

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, grey 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 aphid host plant samples and the blue names the insect samples associated to the aphid colonies. Rectangles correspond to individuals belonging to the same colony. Clade D correspond to the clade D of the previous 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.

Table 1. Summary of the natural occurrence of *S. symbiotica* within 250 aphid colonies, across the different aphid genera. Aphids positive to specific primers correspond to aphids positive to the three primers designed to detect *S. symbiotica* strains with a potential gut localization.

Aphid genus		Number of colonies	Positive to *S. symbiotica*
Aphidinae			
Macrosophini			
	Acyrthosiphon	3	0
	Brachycaudus	3	**1**
	Capitophorus	1	**1**
	Cavariella	5	**2**
	Corylobium	1	0
	Dysaphis	1	0
	Hyadaphis	1	0
	Hyalopteroides	4	**1**
	Hyperomyzus	10	0
	Macrosiphoniella	8	**1**
	Macrosiphum	5	**4**
	Megoura	1	0
	Metopeurum	2	0
	Metopolophium	4	0
	Myzus	1	0
	Sitobion	8	**1**
	Straticobium	1	0
	Uroleucon	49	0
Aphidini			
	Aphis	121	**35**
	Hyalopterus	3	0
	Rhopalosiphum	1	0
	Schizaphis	3	**2**
	Toxoptera	6	**2**
Chaitophorinae			
Chaitophorini			
	Chaitophorus	1	0
	Periphyllus	1	**1**
Calaphidinae			
Panaphidini			
	Pterocallis	1	0
	Tinocallis	1	0
	Not identified	4	0
Total		**250**	**51**

Table 2. Summary of the natural occurrence of *S. symbiotica* within 161 host plants. Plants positive to specific primers correspond to plants positive to the three primers designed to detect *S. symbiotica* strains with a potential localization in aphid gut and free-living capacity. The number of plants associated with infected aphids, as well as the number of plants infected with *S. symbiotica* associated with infected aphids were considered.

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

Spirea	2	0	0	-	0	-
Symphytum	1	0	0	-	0	-
Tanacetum	6	**1**	0	-	0	-
Verbena	2	0	0	-	0	-
Vesca	1	0	0	-	0	-
Vicia	1	0	0	-	0	-
Total	**161**	**14**	**37**	**-**	**6**	**-**

Table 3. Summary of the natural occurrence of *S. symbiotica* within 203 insects associated with aphid colonies. Insects positive to specific primers correspond to insects positive to the three primers designed to detect *S. symbiotica* strains with a potential localization in aphid gut and free-living capacity. The number of insects infected with *S. symbiotica* associated with infected aphids was considered.

Insect genus		**Number of samples**	**Positive to *S. symbiotica***	**Infected insect associated with infected aphids**	
				Number	Infected Aphid genus
Ants					
	Camponotus	3	0	-	-
	Formica	1	0	-	-
	Lasius	78	**7**	6	Capitophorus, Aphis
	Linepithema	2	**2**	2	*Aphis*
	Myrmica	7	**1**	0	-
	Not identified	7	0	-	-
Aphid Midge Larvae					
	Aphidoletes	7	**2**	1	*Aphis*
Bugs					
	Coreus	2	**1**	1	*Aphis*
	Deraeocoris	1	0	-	-
	Dictyla	2	**1**	0	-
	Dicyphus	1	0	-	-
	Dolycoris	3	0	-	-
	Graphosoma	1	0	-	-
	Malacocoris	1	0	-	-
	Orius	1	0	-	-
	Orthops	1	0	-	-
	Pinalitus	1	0	-	-
	Not identified	1	0	-	-
Fly Larvae					
	Chamaemyiidae	1	**1**	1	*Aphis*
Hoverfly Larvae					
	Episyrphus	6	**1**	1	*Aphis*
	Eupeodes	3	0	-	-
	Paragus	7	0	-	-
	Platycheirus	2	0	-	-
	Scaeva	11	**3**	1	*Capitophorus*
	Syrphus	5	0	-	-
Lacewing Larvae					
	Chrysoperla	1	0	-	-

Ladybugs					
	Coccinella	8	**1**	0	-
	Harmonia	7	**1**	1	*Aphis*
	Hippodamia	2	0	-	-
	Propylea	2	0	-	-
	Rhyzobius	1	0	-	-
	Scymnus	1	0	-	-
	Not identified	1	0	-	-
Moth Larvae					
	Eupithecia	3	**1**	1	*Aphis*
	Hecatera	1	0	-	-
	Mompha	1	0	-	-
	Pieris	1	0	-	-
Parasitoids					
	Aphelinus	1	0	-	-
	Aphidius	2	**1**	0	-
	Binodoxys	2	0	-	-
	Diglyphus	1	0	-	-
	Eulophinae	1	0	-	-
	Lysiphlebus	7	**2**	0	-
	Pachyneuron	1	0	-	-
	Pnigalio	1	0	-	-
	Tetrastichinae	3	0	-	-
Total		**203**	**25**	**15**	**-**

1029