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ORIGINAL ARTICLE

Facultative symbionts associated with aphid populations in citrus orchards in northern Tunisia

François RENOZ[1], Inès PONS[1], Christine NOËL[1], Kaouthar LEBDI-GRISSA[2], Vincent FORAY[3] and Thierry HANCE[1]

[1] Earth and Life Institute, Biodiversity Research Centre, UC Louvain, ELIB – Croix du sud 4–5 bte L7.07.04, 1348 Louvain-la-Neuve, Belgium; e-mails: francois.renoz@uclouvain.be, ines.pons@uclouvain.be, christine.noel56@hotmail.com, Thierry.hance@uclouvain.be

[2] Laboratory of Entomology and Acarology, National Agronomic Institute of Tunisia (INAT) – University of Carthage, 43 Avenue Charles Nicolle, Cité Mahrajène, 1082 Tunis, Tunisia, e-mail: grissak@yahoo.fr

[3] Institut de Recherche sur la Biologie de l'Insecte (IRBI), UMR CNRS 7261, 37200 Tours, France; e-mail: vincent.foray@univ-tours.fr

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Abstract. Like many insects, aphids (Hemiptera: Aphididae) can host a wide diversity of symbiotic bacteria that can be important drivers of their evolutionary ecology. In addition to the nutritional obligate symbiont *Buchnera aphidicola*, these phloem-sap feeding insects can host various facultative symbionts whose functional diversity depends on complex interactions with the host genotype and environmental factors. During sampling in citrus orchards in northern Tunisia, we collected aphids on citrus plants. The specimens belonged either to the cotton-melon aphid *Aphis gossypii* or the green citrus aphid *Aphis spiraecola*. We analysed the prevalence of *Arsenophonus*, *Serratia symbiotica*, *Hamiltonella defensa* and *Regiella insecticola*, four facultative symbionts frequently found in the genus *Aphis* and potentially associated with phenotypic effects related to nutrition, protection against parasites and tolerance of high temperatures. We found that the diversity and prevalence of facultative symbionts differed between these two aphid species that exploit similar ecological niches. In particular, we found a high prevalence of *Arsenophonus* in *A. gossypii* populations and that the defensive symbiont *H. defensa* was only present in *A. spiraecola* populations. These results are discussed in light of the ecology and life cycles of each of the species of aphid studied.

INTRODUCTION

Like most insects that feed on plant-phloem, aphids (Hemiptera: Aphididae) host symbiotic bacteria in their tissues that can have significant consequences on their evolutionary ecology (Oliver et al., 2010; Ferrari & Vavre, 2011). The unbalanced diet of these sap-feeding insects renders them dependent on an obligate symbiont, *B. aphidicola*, which provides them with essential amino acids and vitamins (Douglas, 1998; Russell et al., 2017). Aphids can also carry an array of heritable facultative symbionts (e.g. *H. defensa*, *S. symbiotica*, *R. insecticola*, etc.) that occur more sporadically in host insect populations and are maintained in these by vertical transmission and intra- and interspecific horizontal transfers (Sandström et al., 2001; Caspi-Fluger et al., 2011; Gehrer & Vorburger, 2012). These facultative bacterial partners can impart dramatic phenotypic effects to their host, sometimes beneficial and sometimes harmful depending on specific environmental conditions (Oliver et al., 2006, 2010). The beneficial phenotypic effects include protection against parasitoid wasps and other parasites (Oliver et al., 2003; Scarborough et al., 2005), tolerance of high temperatures (Montllor et al., 2002; Burke et al., 2010), body colour modification (Tsuchida et al., 2010), host plant use (Tsuchida et al., 2004; Wagner et al., 2015) and reproductive manipulation (Simon et al., 2011).

Since facultative symbionts may have beneficial effects (e.g. defence against parasites, improvement of nutrition, etc.) or detrimental effects on their host (e.g. fitness cost), there is an interest in determining the bacterial partners present in these insects, in particular in species that are crop pests. The presence of these bacteria in aphid populations may interfere with control programs, whether biological (e.g. influence on host resistance to parasitoid wasps) or chemicals (e.g. influence on host susceptibility to insecticides) (Skaljac et al., 2018; Vorburger, 2018). In this study, we examined the prevalence and co-occurrence of *Arsenophonus*, *H. defensa*, *S. symbiotica* and *R. insecticola* in *A. gossypii* and *A. spiraecola* populations infesting citrus plants in northern Tunisia. These two species of aphid are among the most destructive pests of citrus crops in the Mediterranean basin (Halima-Kamel & Hamouda, 2004; Jacas et al., 2010). The four targeted symbionts are frequently found in aphids of the genus Aphis (Najar-Rodríguez et al., 2009; Brady & White 2013; Brady et al., 2014; Wulff & White, 2015) and are known to be involved in a variety of effects including resistance to parasitoids, tolerance of high temperatures and host nutrition (Oliver et al., 2003, 2006; Tsuchida et al., 2004; Burke et al., 2010; Duron, 2014; Wulff & White, 2015). It is hypothesized that aphids exploiting similar ecological niches, although belonging to different species or to geographically distant populations, tend to exhibit similar combinations of symbionts (McLean et al., 2011; Henry et al., 2013, 2015; Brady et al., 2014). In citrus orchards where the sampling was carried out, *A. gossypii* and *A. spiraecola* populations exploit similar niches (e.g. they feed on the same host-plant species, are attacked by the same natural enemies and are subject to similar climactic conditions) (Boukhris-Bouhachem, 2011; Limem Sellami et al., 2013; Elhaddad et al., 2016; Boukhris-Bouhachem et al., 2017). Thus, determining the presence of these facultative symbionts in *A. gossypii* and *A. spiraecola* also offer the possibility of testing the hypothesis that populations of these two aphid species are likely to host similar combinations of symbionts.

MATERIALS AND METHODS

Aphid collection

During sampling in the North of Tunisia [on the Cap Bon peninsula and around the capital Tunis where citrus crops are concentrated (Metoui et al., 2014)], we collected specimens of *A. gossypii* and *A. spiraecola* (Table S1). The samples consisted of three wingless parthenogenetic adult females from the same colony (i.e. from the same leaf). A total of 68 colonies at 9 locations were sampled. Samples collected at the same location were at least 500 m apart and came from different orchards. Aphids were stored in 95% ethanol at 4°C until used and then identified based on morphological criteria (Blackman & Eastop, 2000).

Species-specific screening for facultative symbionts

DNA of the collected aphids was extracted using the DNeasy Blood & Tissue Kit (QIAGEN) following the instructions of the manufacturer. Each DNA extraction was performed on a pool of three individuals from the same colony to reduce the risk of missing infection when facultative symbionts are present. We screened each specimen for the facultative symbiont species *Arsenophonus*, *S. symbiotica*, *R. insecticola* and *H. defensa*. We amplified a partial region of the 16S rRNA gene using specific primers (Table 1). The PCR assays were performed in a final volume of 15 µl containing 1 µl of the template DNA lysate, 0.5 µM of each primer, 200 µM dNTP's, 1 × buffer and 0.625 unit of Taq DNA polymerase (Roche). The PCR reaction conditions consisted of 40 cycles of 95°C for 1 min, 55°C for 1 min 30 s and 72°C for 1 min 30 s. DNA samples found positive for the different symbiont species in the context of a previous deep 16S rRNA sequencing analysis were used as positive controls (Fakhour et al., 2018). Negative controls consisted of sterilized water instead of genomic DNA. The PCR products were stained with ethidium bromide and visualized on a 1% agarose gel. Half of the amplified samples were sequenced and the resulting sequences were validated using BLAST on GenBank.

Statistical analyses

To evaluate potential factors contributing to the facultative symbiont infections, generalized linear models (GLMs) were examined on the basis of the presence/absence of the respective facultative symbionts in the two species of aphid (binomial error structure, logit-link functions). For each facultative symbiont, the model considered the species of aphid and site sampled as fixed factors. We also carried out Fisher's exact tests to compare the global distribution of facultative symbionts between aphid species. To test the levels of co-occurrence of facultative symbionts, we performed a multiple regression analysis of the presence/absence of each symbiont against that of the other symbionts using generalized linear mixed models (binomial-error and logit-link functions). Finally, the graphical representation of facultative symbiont communities was done using the Mondrian function implemented in R (Gueguen et al., 2010). All statistical analyses were performed using the software R version 3.6.1 (R Development Core team, 2014).

RESULTS

Of the 68 citrus aphid colonies sampled at 9 locations in northern Tunisia, we identified 19 colonies of the cotton-melon aphid, *A. gossypii*, and 49 colonies of the green citrus aphid, *A. spiraecola*. For *A. gossypii*, 89% (17/19) of the colonies were infected, whereas only 45% (22/49) of the *A. spiraecola* colonies were positive for facultative symbionts (Fig. 1). In general, these two aphids differed in terms of the species composition of their symbionts (Fisher's exact test, $p < 0.0001$). More precisely, for *Arsenophonus*, its prevalence was significantly higher in *A. gossypii* than in *A. spiraecola* ($\chi^2 = 18.38$, df = 1, $p < 0.001$, Fig. 2A). *Arsenophonus* was clearly the facultative symbiont that was most frequently recorded in the citrus aphids studied with a prevalence reaching 73% (14/19) in *A. gossypii*, either as a single infection (47%) or in combination with other symbionts (26%) (Fig. 1). In *A. spiraecola* colonies, the prevalence of this symbiont was 18% (9/49). In terms of infection with the symbionts *S. symbiotica* and

Table 1. Primers used in this study.

Gene	Product	Primers (5'–3')		Tm	Fragment size (bp)	References
Arsenophonus sp. 16S rRNA	Ribosomal RNA	16SA1F Ars16SR	AGAGTTTGATCMTGGCTCAG GCAATGTCTTATTAACACAT	55°C	960	Fukatsu & Nikoh, 1998 Tsuchida et al., 2002
Serratia symbiotica 16S rRNA	Ribosomal RNA	16SA1F PASScmpR	AGAGTTTGATCMTGGCTCAG GCAATGTCTTATTAACACAT	55°C	480	Fukatsu & Nikoh, 1998 Fukatsu et al., 2000
Regiella insecticola 16S rRNA	Ribosomal RNA	U99F 16SB4	ATCGGGGAGTAGCTTGCTAC CTAGAGATCGTCGCCTAGGTA	55°C	200	Sandström et al., 2001 Tsuchida et al., 2002
Hamiltonella defensa 16S rRNA	Ribosomal RNA	PABSF 16SB1	AGCACAGTTTACTGAGTTCA TACGGYTACCTTGTTACGACTT	55°C	1660	Darby et al., 2002 Fukatsu & Nikoh, 1998

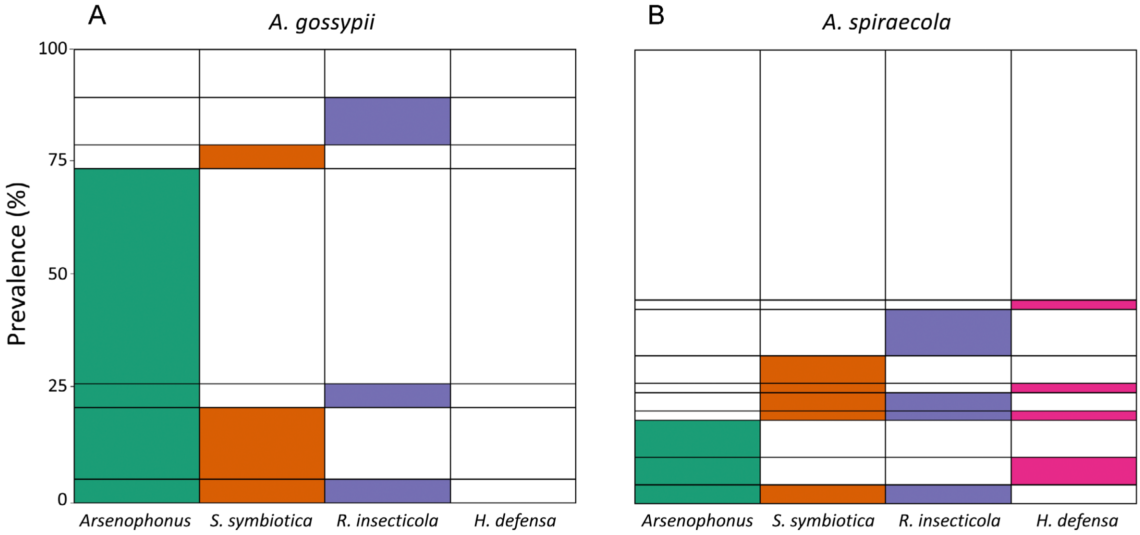

Fig. 1. Structure of facultative bacterial symbiont communities recorded in (A) *A. gossypii* (N = 19) and (B) *A. spiraecola* (N = 49). Uninfected individuals are depicted in white. For each column, the coloured area corresponds to the total prevalence of this symbiont in a particular species of aphid (corresponding to different sampled populations). Rows consisting of several coloured areas indicate multiple infections of the same sample, but do not necessarily reflect co-infection of the same aphid, because each sample consisted of three aphids from the same colony.

R. insecticola there was no significant difference between the two species of aphid (*S. symbiotica*: $\chi^2 = 0.51$, df = 1, p = 0.47; *R. insecticola*: $\chi^2 = 0.0035$, df = 1, p = 0.95, Fig. 2B, C). *S. symbiotica* occurred either as a single infection or in combination with other facultative symbionts in both species with a prevalence of 26% (5/19) in *A. gossypii* and 16% (8/49) in *A. spiraecola*. The prevalence of *R. insecticola* was about 20% in both aphids. Finally, for *H. defensa* the prevalence of infection was significantly higher in *A. spiraecola* than in *A. gossypii* ($\chi^2 = 4.15$, df = 1, p = 0.042, Fig. 2D) as it was not detected in *A. gossypii* but the prevalence of infection of *A. spiraecola* colonies was 12% (6/49). In terms of the sites sampled, there was no significant difference between sites for *Arsenophonus* ($\chi^2 = 11.62$, df = 8, p = 0.17) and *R. insecticola* symbionts ($\chi^2 = 12.29$, df = 8, p = 0.14), but for *S. symbiotica* and *H. defensa*, the prevalence of infection was significantly dependent on the site sampled (*S. symbiotica*: $\chi^2 = 21.48$, df = 8, p = 0.006; *H. defensa*: $\chi^2 = 22.61$, df = 8, p = 0.0039).

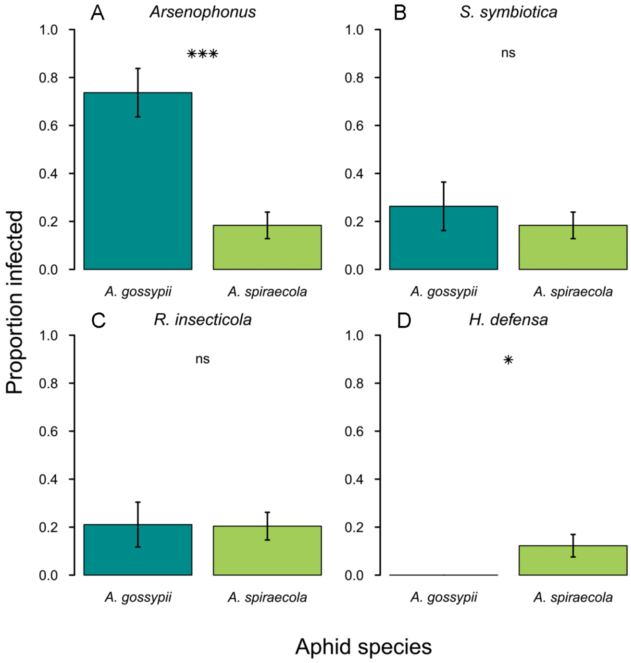

Fig. 2. Proportion of aphids infected with facultative symbionts targeted in natural populations of *A. gossypii* (N = 19) and *A. spiraecola* (N = 49). A – *Arsenophonus*, B – *S. symbiotica*, C – *R. insecticola*, D – *H. defensa*. Error bars depict the standard error. Significant differences are shown (ns – not significant; * – $p < 0.05$; *** – $p < 0.001$).

We also determined the prevalence of multiple infections, that is, the presence of more than one facultative symbiont species in the same sample. Twenty-one percent (14/68) of the samples were infected by at least two facultative symbionts. For *A. gossypii*, all possible combinations of *Arsenophonus*, *S. symbiotica* and *R. insecticola* were recorded other than a dual infection with *S. symbiotica* and *R. insecticola* (Fig. 1A). For *A. spiraecola*, we recorded single infections with each symbiont, but samples with two or three symbionts were uncommon and followed no discernible pattern (Fig. 1B). If we only consider those samples infected with at least one facultative symbiont, the prevalence of multiple infections is 29% (5/17) in *A. gossypii* and 41% (9/22) in *A. spiraecola* with no significant difference between the two aphids (Fisher's exact test, p = 0.52). However, none of the combinations were recorded significantly more or less frequently than expected by chance. Even the combination *Arsenophonus*-*S. symbiotica* of 16% (3/19) for the *A. gossypii* samples did not deviate significantly from a random association ($\chi^2 = 0.14512$, df = 1, p = 0.70).

DISCUSSION

In this study, we collected citrus aphids from orchards in northern Tunisia. We identified the individuals collected and found that they all belonged to either the species *A.*

gossypii or *A. spiraecola*. We determined whether these two species were infected with facultative symbionts. Four symbionts, namely *Arsenophonus*, *S. symbiotica*, *R. insecticola* and *H. defensa*, were recorded either in *A. gossypii* or *A. spiraecola*. These symbionts are widely recorded occurring in the genus *Aphis* (Carletto et al., 2008; Jones et al., 2011; Brady & White, 2013; Jousselin et al., 2013; Arneodo & Ortego, 2014; Brady et al., 2014; Wulff & White, 2015; Zhao et al., 2016; Zytynska & Weisser, 2016; Desneux et al., 2018) and are known to have associated effects that may contribute to the ecological success of their host. The majority of the *A. spiraecola* samples were not infected with facultative symbionts, although all the targeted symbionts were detected in this species. However, in *A. gossypii*, the presence of at least one facultative symbiont is almost systematic. *H. defensa* was recorded in *A. spiraecola* but not in *A. gossypii* even though previous studies have reported the presence of *H. defensa* in this aphid (Zhao et al., 2016; Ayoubi et al., 2020). We recorded similar percentage infections of *A. gossypii* and *A. spiraecola* with *S. symbiotica* and *R. insecticola*, but the percentage infection of these aphids with *Arsenophonus* differed. In *A. gossypii* populations, the prevalence of *Arsenophonus* was around 75%, which is similar to that recorded in other studies (Jones et al., 2011; Zhao et al., 2016; Ayoubi et al., 2018; Zhang et al., 2018).

Although the effects associated with the symbionts identified in *A. gossypii* and *A. spiraecola* are unknown, inferences can be drawn based on their functions in other aphid species. *H. defensa* is well known for protecting the pea aphid *A. pisum* (Oliver et al., 2003, 2009; Weldon et al., 2013) and black bean aphid *Aphis fabae* (Schmid et al., 2012; Rouchet & Vorburger, 2014) against parasitoids. In *A. pisum*, *S. symbiotica* has some ability to protect its host against parasitoids (Oliver et al., 2006; Pons et al., 2019) and high temperatures (Montllor et al., 2002; Burke et al., 2010). *R. insecticola* is reported to affect the performance of *A. pisum* on host plants (Tsuchida et al., 2004) and protect *A. fabae* against parasitoids (Vorburger et al., 2010). The effects associated with *Arsenophonus* are unclear. Bacteriophages required for protective symbiosis are recorded in various strains of this symbiont (Duron, 2014), but no defensive properties are reported for *Aphis glycines* infected with *Arsenophonus* (Wulff et al., 2013). Due to the high prevalence of *Arsenophonus* in populations of *A. gossypii* it is suggested that this facultative symbiont may be involved in host nutrition by mediating host plant range (Wagner et al., 2015; Zhao et al., 2016; Tian et al., 2019; Ayoubi et al., 2020).

Previous large-scale surveys indicate that aphids exploiting similar ecological niches, although belonging to different species or geographically distant populations, tend to have similar combinations of facultative symbionts (Ferrari et al., 2012; Henry et al., 2013, 2015; Brady et al., 2014; Wagner et al., 2015). In our study, *A. gossypii* and *A. spiraecola* were collected from similar food resources (i.e. citrus plants) distributed over a small geographical area and therefore subject to similar climatic conditions. In addition, the two species share many natural enemies (Boukhris-Bouhachem, 2011; Limem Sellami et al., 2013), are frequently tended by ants (Kaneko, 2018; Karami-Jamour et al., 2018) and transmit the same viruses, such as citrus tristeza virus (CTV) (Elhaddad et al., 2016; Boukhris-Bouhachem et al., 2017). In our study, the two aphids differ in terms of symbiont composition, while they occupy very similar ecological niches. The factors that may determine the presence of facultative symbionts in aphid populations remain poorly understood. A recent study indicates that the bacterial flora associated with *A. gossypii* is strongly affected by its host plants (Xu et al., 2019). However, other studies indicate that the composition of microbial communities hosted by aphids may largely depend on host genotype (Fakhour et al., 2018; McLean et al., 2019). In addition, it is possible that microclimatic conditions and other local variables (natural enemies, surrounding vegetation, etc.) have a major influence on the structure of symbiotic communities (Zytynska et al., 2019). *A. gossypii* and *A. spiraecola* are both highly polyphagous species and can readily switch from one host plant species to another. Therefore, it is also possible that their feeding behaviour and the food environment they encountered previously determines the composition of the symbiont community associated with them.

There was a high incidence of multiple infections, of up to 42%, in the *A. spiraecola* samples, which did not differ from that recorded for *A. gossypii*. Further, there was no evidence for preferential associations or the exclusion of particular symbionts. Multiple infections by facultative symbionts are mostly reported for the pea aphid *A. pisum* (Leonardo & Muiru, 2003; Oliver et al., 2006; Nyabuga et al., 2010; Ferrari et al., 2012; Henry et al., 2013; Russell et al., 2013). The reports for the genus *Aphis* are conflicting as *A. fabae*, *A. craccivora* and *A. gossypii* are rarely reported hosting more than one facultative symbiont (Chandler et al., 2008; Najar-Rodríguez et al., 2009; Brady et al., 2014; Henry et al., 2015; Zhao et al., 2016; Zhang et al., 2018) whereas one individual of the black bean aphid *A. fabae* can harbour up to four different symbionts (Zytynska et al., 2015). Our results are consistent with the latter study and indicate that multiple infections by different symbionts are probably less rare in the genus *Aphis* than previously thought. However, in the context of this study, artefacts due to the methodology cannot be excluded. Indeed, we assessed the presence of facultative symbionts using three individuals per colony and contamination from other colonies cannot be completely excluded. Host infection by several facultative symbionts is known to affect host fitness in different ways dependent on the symbionts involved (Oliver et al., 2006; Łukasik et al., 2013; Tsuchida et al., 2014; Leclair et al., 2016). Specific multiple infection patterns could either result in an amplification of the beneficial effects associated with each symbiont (Oliver et al., 2006) or in severe costs for host aphids (Oliver et al., 2006). However, apparent preferential associations or exclusion of symbionts might be explained by drift in certain cases (Mathé-Hubert et al., 2019). Patterns of multiple infections in species of aphids may also depend on specific features related to their respective lifecycle, such as feeding behaviour (i.e. the degree of polyphagy), alternation between a primary and a secondary host plants (i.e. heteroecious cycle) or a sexual reproductive phase that promotes the exchange of facultative symbionts between males and females (Moran & Dunbar, 2006).

In conclusion, the prevalence of several facultative symbionts in populations of two species of aphids was quantified. In the citrus orchards in northern Tunisia, *A. gossypii* and *A. spiraecola* exploit similar niches and are major pests. It is important to keep in mind that, despite the fact that the four targeted symbiont species are frequently found in aphids of the genus Aphis, other facultative symbionts can infect these aphids. For example, several studies report the presence of *Wolbachia* in *A. gossypii* (Jones et al., 2011; Zhao et al., 2016). In addition, like other field studies, this study is a snapshot as the prevalence of symbionts in insect populations can change depending on environmental pressures. However, it is relevant to evaluate the presence of facultative bacteria in insect pests, as in addition to being beneficial for their hosts they may make them more vulnerable to certain stresses. Due to their associated effects, bacterial symbionts can interfere with control programs, be it biological control or chemical control.

DECLARATION OF INTEREST. None.

AUTHOR CONTRIBUTIONS. FR conceived and designed the study; VF and KLG sampled the aphids; FR, IP and CN did the research; FR, VF and IP analysed the data; FR wrote the first draft of the manuscript; IP, VF and TH revised the manuscript. All authors approved the manuscript for publication.

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Table S1. Detection of the targeted facultative symbionts in the different colonies of *A. spiraecola* and *A. gossypii* in different regions of the North of Tunisia.

Colony ID	Sampling date	Collection site	Aphid species	Host plant species	*Arsenophonus*	*S. symbiotica*	*R. insecticola*	*H. defensa*
1	31/04/2013	Béni Khalled	*A. gossypii*	*Citrus sinensis*	+	+		
2	31/04/2013	Béni Khalled	*A. gossypii*	*Citrus sinensis*	+	+		
3	31/04/2013	Menzel Bouzelfa	*A. gossypii*	*Citrus sinensis* 'Thomson Navel'	+	+	+	
4	31/04/2013	Menzel Bouzelfa	*A. gossypii*	*Citrus sinensis* 'Thomson Navel'	+	+		
5	31/04/2013	Menzel Bouzelfa	*A. gossypii*	*Citrus* × limon	+			
6	31/04/2013	Takilsa	*A. gossypii*	*Citrus sinensis* 'Washington Navel'	+			
7	31/04/2013	Takilsa	*A. gossypii*	*Citrus sinensis* 'Washington Navel'	+			
8	31/04/2013	Takilsa	*A. gossypii*	*Citrus sinensis* 'Washington Navel'	+			
9	31/04/2013	Takilsa	*A. gossypii*	*Citrus sinensis* 'Washington Navel'				
10	31/04/2013	Takilsa	*A. gossypii*	*Citrus sinensis*	+			
11	31/04/2013	Takilsa	*A. gossypii*	*Citrus sinensis*	+			
12	31/04/2013	Takilsa	*A. gossypii*	*Citrus sinensis*	+			
13	3/05/2013	Mornag	*A. gossypii*	*Citrus clementina*		+		
14	3/05/2013	Mornag	*A. gossypii*	*Citrus clementina*	+		+	
15	3/05/2013	Mornag	*A. gossypii*	*Citrus clementina*	+			
16	3/05/2013	Mornag	*A. gossypii*	*Citrus clementina*			+	
17	3/05/2013	Mornag	*A. gossypii*	*Citrus clementina*				
18	3/05/2013	Khalidia	*A. gossypii*	*Citrus sinensis* 'Thomson Navel'	+			
19	3/05/2013	Khalidia	*A. gossypii*	*Citrus sinensis* 'Thomson Navel'			+	
20	31/04/2013	Grombalia	*A. spiraecola*	*Citrus clementina*	+			+
21	31/04/2013	Grombalia	*A. spiraecola*	*Citrus clementina*	+			+
22	31/04/2013	Grombalia	*A. spiraecola*	*Citrus clementina*	+			+
23	31/04/2013	Grombalia	*A. spiraecola*	*Citrus clementina*				
24	31/04/2013	Grombalia	*A. spiraecola*	*Citrus clementina*		+		+
25	31/04/2013	Béni Khalled	*A. spiraecola*	*Citrus* × limon				+

Table S1 (continued).

Colony ID	Sampling date	Collection site	Aphid species	Host plant species	*Arsenophonus*	*S. symbiotica*	*R. insecticola*	*H. defensa*
26	31/04/2013	Béni Khalled	*A. spiraecola*	*Citrus sinensis*				
27	31/04/2013	Béni Khalled	*A. spiraecola*	*Citrus sinensis*				
28	31/04/2013	Béni Khalled	*A. spiraecola*	*Citrus sinensis*				
29	31/04/2013	Béni Khalled	*A. spiraecola*	*Citrus sinensis*				
30	31/04/2013	Menzel Bouzelfa	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'		+	+	+
31	31/04/2013	Menzel Bouzelfa	*A. spiraecola*	*Citrus* × limon				
32	31/04/2013	Menzel Bouzelfa	*A. spiraecola*	*Citrus* × limon		+		
33	31/04/2013	Selinem	*A. spiraecola*	*Citrus sinensis*	+			
34	31/04/2013	Selinem	*A. spiraecola*	*Citrus sinensis*				
35	31/04/2013	Takilsa	*A. spiraecola*	*Citrus sinensis* 'Washington Navel'			+	
36	31/04/2013	Takilsa	*A. spiraecola*	*Citrus sinensis* 'Washington Navel'				
37	31/04/2013	Takilsa	*A. spiraecola*	*Citrus sinensis*				
38	31/04/2013	Takilsa	*A. spiraecola*	*Citrus sinensis*				
39	2/05/2013	Bou Salem	*A. spiraecola*	*Citrus clementina*				
40	2/05/2013	Bou Salem	*A. spiraecola*	*Citrus clementina*				
41	2/05/2013	Bou Salem	*A. spiraecola*	*Citrus clementina*			+	
42	2/05/2013	Bou Salem	*A. spiraecola*	*Citrus sinensis* 'Washington Navel'			+	
43	2/05/2013	Bou Salem	*A. spiraecola*	*Citrus sinensis* 'Washington Navel'				
44	2/05/2013	Jendouba	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'			+	
45	2/05/2013	Jendouba	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'				
46	2/05/2013	Jendouba	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'				
47	2/05/2013	Jendouba	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'				
48	2/05/2013	Jendouba	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'				
49	2/05/2013	Jendouba	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'				
50	2/05/2013	Jendouba	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'		+	+	
51	2/05/2013	Jendouba	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'		+	+	
52	2/05/2013	Jendouba	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'		+		
53	2/05/2013	Jendouba	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'		+		
54	3/05/2013	Mornag	*A. spiraecola*	*Citrus clementina*	+	+	+	
55	3/05/2013	Mornag	*A. spiraecola*	*Citrus clementina*	+			
56	3/05/2013	Mornag	*A. spiraecola*	*Citrus clementina*	+	+	+	
57	3/05/2013	Mornag	*A. spiraecola*	*Citrus clementina*				
58	3/05/2013	Mornag	*A. spiraecola*	*Citrus* × limon				
59	3/05/2013	Mornag	*A. spiraecola*	*Citrus* × limon				
60	3/05/2013	Mornag	*A. spiraecola*	*Citrus* × limon			+	
61	3/05/2013	Mornag	*A. spiraecola*	*Citrus* × limon				
62	3/05/2013	Khalidia	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'	+			
63	3/05/2013	Khalidia	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'				
64	3/05/2013	Khalidia	*A. spiraecola*	*Citrus sinensis* 'Thomson Navel'				
65	3/05/2013	Khalidia	*A. spiraecola*	*Citrus clementina*				
66	3/05/2013	Khalidia	*A. spiraecola*	*Citrus clementina*				
67	3/05/2013	Khalidia	*A. spiraecola*	*Citrus clementina*	+			
68	3/05/2013	Khalidia	*A. spiraecola*	*Citrus sinensis* 'Maltaise demi-sanguine'				