Natural Occurrence of Secondary Bacterial Symbionts in Aphids from Tunisia, with a Focus on Genus *Hyalopterus*

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Abstract

Aphids (Hemiptera: Aphididae) can harbor two types of bacterial symbionts. In addition to the obligate endosymbiont *Buchnera aphidicola* Munson, Baumann and Kinsey 1991 (Enterobacteriales: Enterobacteriaceae), several facultative symbiotic bacteria, called secondary (S) symbionts, have been identified among many important pest aphid species. To determine interpopulational diversity of S-symbionts, we carried out a survey in a total of 18 populations of six aphid species collected from six localities in Tunisia, by performing a diagnostic polymerase chain reaction analysis of partial 16S–23S rRNA operon sequences. While 61.7% of individuals contained only *Buchnera*, three S-symbionts were found at different frequencies. *Arsenophonus* sp. Gherna et al. 1991 (Enterobacteriales: Enterobacteriaceae) was found in all species under study except for *Acyrtosiphon pisum* (Harris 1776) (Aphidinae: Macrosiphini); *Serratia symbiotica* Moran et al. 2005 (Enterobacteriales: Enterobacteriaceae) was present in all analyzed individuals of *A. pisum* but only sporadically in *Aphis spiraecola* (Patch 1914) (Aphidinae: Aphidini) and *Hyalopterus amygdali* (Blanchard 1840) (Aphidinae: Aphidini), while *Hamiltonella defensa* Moran et al. 2005 (Enterobacteriales: Enterobacteriaceae) was found in all analyzed individuals of one population of *Aphis gossypii* (Glover 1877) (Aphidinae: Aphidini) and sporadically in two populations of *Hyalopterus*. The lysogenic bacteriophage APSE-1 (*A. pisum* secondary endosymbiont, type 1) was detected in the three populations infected with *H. defensa*. This bacteriophage has been associated with moderate protection against braconid parasitoids in pea aphids. The high prevalence of *Arsenophonus* sp. in our samples is in accordance with previous studies indicating that, among gammaproteobacteria, this genus is one of the most widespread insect facultative symbionts.

Key words: *Hyalopterus*, parasitoid, secondary symbiont, *Arsenophonus*, *Hamiltonella defensa*, APSE

Aphids (Insecta: Hemiptera: Aphididae) maintain a well-characterized obligate mutualistic relationship with the gamma-proteobacterium *Buchnera aphidicola* (Munson et al. 1991). The bacterium lives within specialized aphid cells (bacteriocytes), it is maternally transmitted to the offspring and, like other bacteriocyte-associated endosymbionts, it provides to its host nutrients that are lacking in its unbalanced phloem diet, mainly essential amino acids and some vitamins (Baumann 2005). In addition to its obligate partner *B. aphidicola*, many aphids harbor additional facultative symbionts (Oliver et al. 2010). Although they are normally vertically transmitted from mother to progeny, these facultative symbionts can also undergo horizontal transmission, including transfer between host species (Sandström et al. 2001, Russell et al. 2003).

Aphid facultative symbionts are highly diverse. Among them, *Serratia symbiotica* and *Regiella insecticola* (Moran et al. 2005a) have only been found in aphids, while *Hamiltonella defensa* is also present in other sap-feeding insects such as psyllids and whiteflies

(Clark et al. 1992, Moran et al. 2005a, Rao et al. 2012). Some others, such as *Arsenophonus* Gherna et al. 1991 (Enterobacteriales: Enterobacteriaceae) (Gherna et al. 1991), *Rickettsia* da Rocha-Lima, 1916 (Rickettsiales: Rickettsiaceae), and *Spiroplasma* Saglio, L'Hospital, Laflèche, Dupont, Bové, Tully and Freundt, 1973 (Entomoplasmatales: Spiroplasmataceae), are known to infect a wide variety of insects (Russell et al. 2003). These so-called secondary (S) symbionts are not essential for host survival, but their presence has been related with several positive effects on their hosts, mainly protection against certain environmental stresses, such as heat stress or natural enemies (Feldhaar 2011, Oliver et al. 2014). S-symbionts have not been found in all aphid species, nor in all populations of one species. However, at least in some cases, the lack of detection might be due to sampling problems. In addition to the pea aphid *Acyrtosiphon pisum* (Harris 1776) (Aphidinae: Macrosiphini), up to 156 species have been screened for particular

facultative symbionts (Zytynska and Weisser 2016), mostly in a limited number of individuals, while only a few species from the subfamily Aphidinae have been extensively analyzed (mainly from genus *Aphis* Linnaeus 1758; e.g., *Aphis fabae* Scopoli 1763, *Aphis gossypii* Glover 1877, and *Aphis craccivora* Koch 1854).

Aphids must face the attack of numerous natural enemies, most notably hymenopteran parasitoids (Le Ralec et al. 2010), which are generally host specific. Many studies have shown that the infection of *H. defensa* strains provide defense to pea aphids against the parasitic wasp *Aphidius ervi* (Haliday 1834) (Hymenoptera: Braconidae: Aphidiinae) by killing the developing wasp larva or embryo in the aphid hemocoel (Oliver et al. 2003, 2005, 2006, 2009; Ferrari et al. 2004; Bensadia et al. 2006; Guay et al. 2009) and producing mummies (aphid remains containing developing parasitoids). In addition to the presence of protective symbionts, the resistance of *A. pisum* to the attack of *A. ervi* in natural populations has been linked to the aphid genetic background, the host plant, and temperature (Martinez et al. 2014a, McLean and Godfray 2015).

H. defensa has also been involved in the resistance of other aphid species to the attack of diverse aphidiine braconids, such as the protection of the black bean aphid, *A. fabae*, against *Lysiphlebus fabarum* (Marshall 1896) (Hymenoptera: Braconidae) (Vorburger et al. 2009, 2013; Schmid et al. 2012; Cayetano and Vorburger 2015), and of the cowpea aphid, *A. craccivora*, against *Binodoxys communis* (Gahan 1927) (Hymenoptera: Braconidae) (Desneux et al. 2009). In any case, the protection provided by *H. defensa* is not general toward all parasitoid species (Asplen et al. 2014; McLean et al. 2015; Martínez et al., 2016). Other aphid S-symbionts are also able to improve resistance to parasitoid wasps, albeit to a lesser extent. These include *R. insecticola* providing protection against *Aphidius colemani* (Hymenoptera: Braconidae) in *Myzus persicae* (Sulzer 1776) (Aphidinae, Macrosiphini) and *A. fabae* (Nyabuga et al. 2010, Vorburger et al. 2010), and coinfections of *H. defensa*–*R. insecticola*, *H. defensa*–*S. symbiotica*, *H. defensa*–*Spiroplasma*, and *R. insecticola*–*Spiroplasma* in *A. pisum* (Oliver et al. 2006, Nyabuga et al. 2010). The coinfection of *H. defensa* and the facultative symbiont referred as PAXs (pea aphid X-type symbiont) was suggested to improve aphid resistance to parasitoids after exposure to heat stress conditions (Guay et al. 2009), although a recent study failed to corroborate this synergic effect (Doremus et al. 2017).

Besides the presumed influence of the aphid and parasitoid genotypes, the degree of protection provided by *H. defensa* is highly variable among bacterial strains (Martinez et al. 2014a,b, 2017; Rouchet and Vorburger 2012). Part of this variation must be due to the *H. defensa* chromosomal gene content, but it is also greatly influenced by the presence of a lysogenic lambda-like bacteriophage. Originally identified in strains from *A. pisum*, it was designated as ‘*A. pisum* secondary endosymbiont’ (APSE) phage (van der Wilk et al. 1999). Later on, APSE was found to be common not only in *H. defensa* strains of aphids but also in whiteflies and psyllids (Moran et al. 2005b; Degnan and Moran 2008a,b). Several APSE variants have been identified (Moran et al. 2005b, Degnan and Moran 2008a), each of them harboring a specific virulence cassette region, which encode homologs of toxins that are suspected to target embryonic wasp cells (Li et al. 2002, Nyabuga et al. 2010). The decrease of aphid mortality due to the attack of braconid parasitoids in aphids infected with *H. defensa* correlates to the presence of APSE, and it fluctuates among strains depending on the phage variant (Moran et al. 2005b, Oliver et al. 2005, Weldon et al. 2013). These variants might also be involved in the specificity against different parasitoid species (Degnan and Moran 2008a, Asplen et al. 2014). Strains lacking APSE (Degnan and Moran 2008a, Weldon et al. 2013), or with defective phages that carry inactivated toxin genes (Martinez et al. 2014b),

provide no protection. APSE is not only present in *H. defensa*; Duron (2014) reported a wide variety of APSE phages in *Arsenophonus*, an S-symbiont exceptionally widespread in insects groups (including aphids, white flies, parasitoid wasps, triatomine bugs, louse flies, and bat flies; Nováková et al. 2009). However, the role of APSE in *Arsenophonus* symbioses remains to be characterized.

Aphids are considered a serious pest, causing great economic damages due to the direct removal of plant nutrients or the transmission of viruses causing plant diseases (Leclant 1996, Lozier et al. 2008a). The mealy aphids of the genus *Hyalopterus* (Koch 1854) (Hemiptera: Aphididae: Aphidinae) cause big damages in agricultural fields in Tunisia, where fruit trees represent 30% of agricultural production. They form colonies on the lower leaf surface, on twigs and even on branches, where they feed on phloem, causing premature leaf drop, retarded tree growth, and fewer and smaller fruits. *Hyalopterus* is a cosmopolitan genus, Mediterranean in origin, with only three recognized species *Hyalopterus pruni* (Geoffroy 1762), *Hyalopterus amygdali* (Blanchard 1840), and *Hyalopterus arundiniformis* (Ghulamullah 1941) (Aphidinae: Aphidini: Rhopalosiphina) (Lozier et al. 2007, Rakauskas et al. 2013, Favret et al. 2017). All three species are associated with common reed (*Phragmites communis* Cavanilles (Trinius) EX Steudel 1841, Poales: Poaceae) as summer host, and different *Prunus* species (Linnaeus 1753, Rosales: Rosaceae) as winter/spring hosts: *H. amygdali* is associated with almond trees (*Prunus dulcis* (Mill.) D. A. Webb), the second most abundant fruit tree in Tunisia (194,740 ha, 8.5% of the tree area); *H. pruni* is associated with plum trees (*Prunus domestica* Linnaeus) and blackthorn (*Prunus spinosa* Linnaeus); and *H. arundiniformis* with peach trees (*Prunus persica* (Linnaeus) Stokes 1812 NON Batsch 1801), distributed over 16.5 thousand hectares in Tunisia; all three species can also be found on apricot trees (*Prunus armeniaca* Linnaeus) (Blackman and Eastop 1994, 2000; Lozier et al. 2008a), which occupies 8.5 thousand hectares. Screening of *Hyalopterus* for facultative symbionts rendered negative results (Russell et al. 2003, Tsuchida et al. 2005) except in one study (Henry et al. 2015), where a few individuals were found to be infected by *H. defensa*.

Predators and parasitoids can be used for biological control of invasive aphids as an alternative to chemical pesticides. Because host and parasites coevolve (Rehman and Powell 2010), it is desirable to use natural enemies from the pest's region of origin in order to get the best possible biological control (Rosen 1986). *Aphidius transcaspicus* (Telenga 1958) (Hymenoptera: Braconidae: Aphidiinae) is a specific parasitoid of *Hyalopterus* from the Mediterranean, and has been selected to be used as a biological control agent against *H. pruni* (Latham and Mills 2012). Our laboratory has been studying this braconid for many years in order to use it as a biological control against the *Hyalopterus* pest in orchards. To do so, it is necessary to release high amounts of the wasp in the fields and, therefore, we need a way to mass rearing this parasitoid. The difficulties of rearing *A. transcaspicus* in aphids of the *Hyalopterus* complex under controlled conditions in greenhouse oriented us to try different aphid hosts. In addition to its natural host, at least in laboratory conditions, *A. transcaspicus* has been able to attack successfully several other aphid pests, such as *A. pisum*, *Aphis spiraeicola* (Patch 1914) (unpublished data from our group [SZ]), *A. fabae* (Latham and Mills 2010), *A. gossypii*, *A. craccivora*, and *M. persicae* (Wang and Messing 2006), among others.

To select the best aphid host for mass rearing of *A. transcaspicus*, it is also important to determine the putative presence of protective symbionts in the available aphid populations (Vorburger 2018). As a starting point, and prior to making experimental determinations of susceptibility to parasitoids under controlled conditions, we have performed an analysis for the presence of S-symbionts in aphids from six different species collected in different locations in the North-Western

and central-East of Tunisia, in the Gulf of Hammamet and near the border with Algeria (Fig. 1; Table 1). This is the first time that the presence of S-symbionts has been determined in aphid populations from Tunisia.

Materials and Methods

Aphid Collection

Aphids of different species within subfamily Aphidinae were field collected from natural populations in different geographical locations of Tunisia in 2014, 2015, and 2016 (Table 1; Fig. 1). These include one species of the tribe Macrosiphini (*A. pisum*) and five species from the tribe Aphidini, two of them from the genus *Aphis* (*A. gossypii* and *A. spiraecola*), plus all three known species from genus *Hyalopterus* (*H. amygdali*, *H. arundiniformis*, and *H. pruni*). No specific permissions were required for these collection activities and sampling did not involve endangered or protected species. In addition, aphid populations were established from viviparous individuals randomly collected in spring 2014, and reared in the laboratory under controlled conditions. The rearing was conducted on young shoots of common reed and almond for *Hyalopterus* species

Fig. 1. Map of North Tunisia indicating the areas of aphids sampling.

in Plexiglas's cages (50 × 50 × 60 cm in size) in glass greenhouses, as well as in warm rooms maintained at different temperatures, while *A. pisum* was reared on broad bean and pea at 25°C (Table 1), all of them with a relative humidity of 70%.

DNA Extraction

Aphids were collected and stored in absolute ethanol until DNA extraction. Total DNA (τ -DNA) of single individuals (or four aphids when indicated, see below) was obtained for all populations under study. Aphids were placed in a 1.5-ml microtube, washed with phosphate-buffered saline (PBS) buffer (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH 7.4), and crushed in 180 μl of PBS buffer with a sterilized plastic pestle, using a pellet pestle cordless motor (Kimble Chase, Vineland, NJ). τ -DNA was extracted using DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer instructions.

Diagnostic Screening for Secondary Symbionts and APSE

The detection of the secondary bacteria was based on diagnostic polymerase chain reaction (PCR) analysis of partial 16S–23S rRNA operon sequences (Russell et al. 2003; Guay et al. 2009).

PCR amplifications were performed on insect τ -DNA, with appropriate primer pairs (Table 2), using 50–60 μmol of each primer/50 μl reaction, with KAPATaq DNA Polymerase Kit (Kapa Biosystems, Wilmington, MA). All PCR reactions included a negative control without template DNA. The thermal cycling profile was as follows: an initial denaturation step at 94°C for 4 min, followed by 40 cycles of 94°C for 50 s, the appropriate annealing temperature (see Table 2) for 1 min, and 72°C for 1.5 min, plus a final extension step of 7 min at 72°C. PCR products were run on 1% agarose gels with ethidium bromide and visualized with UV light.

As a positive control of τ -DNA quality, a first PCR reaction (control PCR, C) was performed using universal direct and reverse primers to amplify a fragment of the 16S rRNA gene, which also amplifies *B. aphidicola*. Aphid DNA samples where *Buchnera* could not be

Table 1. Insect samples analyzed in this study

Population ID	Aphid	Host plant	Origin	Collection time
Ap-1	<i>Acyrtosiphon pisum</i>	Broad bean	Chott-Mariem ^a (25°C)	May 2015
		Pea	Chott-Mariem ^a (25°C)	May 2015
Ag-1	<i>Aphis gossypii</i>	Chinese hibiscus	Chott-Mariem	May 2015
Ag-2	<i>A. gossypii</i>	Muskmelon	Chott-Mariem	May 2015
As-1	<i>A. spiraecola</i>	Plum	Kalaa Kebira	May 2015
Ha-1	<i>Hyalopterus amygdali</i>	Common reed	Chott-Mariem ^a (25, 28, 30, and 35°C)	May 2015
Ha-2	<i>H. amygdali</i>	Almond	Akouda ^a (15, 20, 25, and 35°C)	June 2015
Ha-3	<i>H. amygdali</i>	Almond	Chott-Mariem	December 2015
Ha-4	<i>H. amygdali</i>	Almond	Jammel	May 2015, May 2016
Ha-5	<i>H. amygdali</i>	Almond	Kef	June 2014, April 2016
Ha-6	<i>H. amygdali</i>	Almond	Zaghuan	May 2016
Ha-7	<i>H. amygdali</i>	Almond	Akouda	December 2015
Har-1	<i>Hyalopterus arundiniformis</i>	Peach	Chott-Mariem	March 2016
Har-2	<i>H. arundiniformis</i>	Peach	Jammel	April 2016
Har-3	<i>H. arundiniformis</i>	Peach	Kef	April 2016
Har-4	<i>H. arundiniformis</i>	Apricot	Akouda	December 2015, April 2016
Hpr-1	<i>Hyalopterus pruni</i>	Apricot	Kef	June 2014, April 2016
Hpr-2	<i>H. pruni</i>	Blackthorn	Kef	June 2014, May 2016
Hpr-3	<i>H. pruni</i>	Plum	Kef	April 2016

^aNatural origin of populations maintained in greenhouse at the temperatures indicated in brackets.

confirmed must be considered unreliable and were discarded for further analysis. Next, for the detection of S-symbionts (Diagnostic PCR 1, DP1), we used a universal direct primer targeting the 16S rRNA gene, and a universal reverse primer targeting the 23S rRNA gene.

According to the experimental protocol of Sandström et al. (2001), the amplicons obtained from the first quality PCR test and DP1 were digested with the restriction enzymes ClaI, SacI, SalI, and XbaI, and the digestions were run in 1% agarose gels to further confirm infection status. The results were indicative of the presence of one (*B. aphidicola*) or more than one bacterial species in the sample. This was an important step in order to discard false-negative results for the presence of S-symbionts during the following steps. All positive samples were PCR amplified using primers for the 16S–23S operon, which are able to amplify only S-symbionts because the two genes are separated in *B. aphidicola* (DP1; Table 2). If no amplicon was obtained in this analysis, all the procedures were repeated but including four aphids in a single DNA extraction. When the results remained negative, the sample was considered to be free of S-symbionts. Positive DNA samples for S-symbionts were tested for the presence of APSE phages using specific primers (Table 2) to amplify a fragment of the gene P45, encoding the phage DNA polymerase.

For the specific detection of each suspected S-symbiont (Diagnostic PCR 2, DP2), we used a specific forward primer for each symbiont and a universal reverse primer (Table 2). When needed,

amplicons with the expected size were excised from the gel prior to sequencing. If more than one S-symbiont was suspected, purified amplicons were cloned using pGEM-T Easy Vector System I Kit (Promega). At least 10 individual clones were analyzed by direct colony PCR, using T7 and SP6 universal primers to amplify the inserts.

DNA Sequencing and Phylogenetic Analysis

ABI sequencing was performed on successfully amplified products from the DP1, DP2, and APSE analyses at the SCSIE sequencing facility of the Universitat de València. Sequencing reads were quality surveyed and assembled with the Staden Package (<http://staden.sourceforge.net/>; Staden et al. 2000). Sequences were then compared with those in the GenBank nucleotide sequence database using BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), to confirm the identity of the detected symbiont. The DNA sequences determined in this study have been deposited in GenBank with the following accession numbers: *Arsenophonus* sp., MG257468–MG257478; *Erwinia* sp., MG257479; *H. defensa*, MG257480–MG257482; *S. symbiotica*, MG257483–MG257485; and APSE: MG257486.

APSE sequences available in GenBank were used to perform a sequence alignment with MAFFT v7.037b software (Katoh and Standley 2013; Table 3). Uninformative positions were located and

Table 2. Primers used in this study

Target	Primer name	Primer sequence (5'–3')	Gene	Use	T _m (°C)	Reference
Bacteria	1502R	GTTACGACTTCACCCAG	16S rRNA	C, S	52	Russell et al. 2003
Bacteria	10F	AGTTTGATCATGGCTCAGATTG	16S rRNA	C, DP1, S	52	Sandström et al. 2001
S-symbiont	480R	CACGGTACTGGTTCACATATCGGTC	23S rRNA	DP1	52	Sandström et al. 2001
<i>Arsenophonus</i>	Ars16SR	TTAGCTCCGGAGGCCACAGT	16S rRNA	DP2, S	51	Tsuchida et al. 2002
<i>Hamiltonella defensa</i>	PABSF	AGCGCAGTTTACTGAGTTCA	16S rRNA	DP2, S	55	Darby et al. 2001
PAXS	PAXSF	GAAGCAATGCAAAGAGTGTTGC	16S rRNA	DP2	55	Guay et al. 2009
<i>Regiella insecticola</i>	U99F	ATCGGGGAGTAGCTTGCTAC	16S rRNA	DP2	56	Sandström et al. 2001
<i>Serratia symbiotica</i>	PASScmp	GCAATGTCTTATTAACACAT	16S rRNA	DP2, S	55	Fukatsu et al. 2000
APSE	APSE30.1F	ACGGCACTTAAACGCTATCC	P45	P, S	53	Taylor et al. 2011
APSE	APSE31.1R	TGGGATGTGTATGGACGTTG	P45	P, S	53	Degnan and Moran 2008a

C, control PCR reaction; DP1, diagnostic PCR for the presence of S-symbionts; DP2, diagnostic PCR for the presence of a specific S-symbiont; P, phage detection; S, sequencing.

Table 3. APSE sequences included in the phylogenetic analysis

Accession number	Bacteria	Insect host	Reference
HM594699.1	<i>Arsenophonus</i> sp.	<i>Trialeurodes vaporariorum</i>	Taylor et al. (2011)
HM594700.1	<i>Arsenophonus nasoniae</i>	<i>Spalangia cameroni</i>	Taylor et al. (2011)
HM594701.1	<i>A. nasoniae</i>	<i>Eupelmus vesicularis</i>	Taylor et al. (2011)
HM594702.1	<i>A. nasoniae</i>	<i>Nasonia vitripennis</i>	Taylor et al. (2011)
AF157835.1	<i>Hamiltonella defensa</i>	<i>Acyrtosiphon pisum</i>	Van der Wilk et al. (1999)
EU021718.1	<i>H. defensa</i> T5A	<i>A. pisum</i>	Degnan and Moran (2008b)
EU021719.1	<i>H. defensa</i> A1A	<i>A. pisum</i>	Degnan and Moran (2008b)
EU021722.1	<i>H. defensa</i> 5ATac	<i>A. pisum</i>	Degnan and Moran (2008b)
EU021991.1	<i>H. defensa</i> A2F	<i>A. pisum</i>	Degnan and Moran (2008b)
EU021996.1	<i>H. defensa</i> N4	<i>Chaitophorus</i> sp.	Degnan and Moran (2008b)
EU794057.1	<i>H. defensa</i> A1A	<i>A. pisum</i>	Degnan and Moran (2008b)
KT028654.1	<i>H. defensa</i>	<i>A. pisum</i> (clone Medicago_217)	McLean and Godfray (2015)
KT028655.1	<i>H. defensa</i>	<i>A. pisum</i> (clone Medicago_308)	McLean and Godfray (2015)
KT028670.1	<i>H. defensa</i>	<i>A. pisum</i> (clone Ononis_257)	McLean and Godfray (2015)
KT028671.1	<i>H. defensa</i>	<i>A. pisum</i> (clone Ononis_256)	McLean and Godfray (2015)
MG257486	<i>H. defensa</i>	<i>Aphis gossypii</i> , <i>H. amigdali</i> , <i>Hyalopterus pruni</i>	This study
EU039462.1	S-symbiont	<i>Glycaspis brimblecombei</i>	Hansen et al. (2007)

masked with GBLOCKS 0.91b (Castresana 2000). ClustalX (Larkin et al. 2007) was used to change the file format from fasta to nexus, and the obtained nexus file was manually edited to add MrBayes parameters. Finally, a phylogenetic analysis was performed using Bayesian Inference with MrBayes v3.2.5 software (Ronquist et al. 2012). Nodal support was estimated by posterior probabilities using the sumt command in MrBayes. We used FigTree v1.4.0 software (<http://tree.bio.ed.ac.uk/software/figtree/>) to visualize and edit the phylogenetic tree.

Results and Discussion

A. transcaspicus is a specific and indigenous parasitoid of the Mediterranean region that has co-evolved with its hosts, aphids from genus *Hyalopterus* (Lozier et al. 2008b, Latham and Mills 2012), and it has been selected to be used as a biological control agent against these aphids. However, because *Hyalopterus*'s host plants are trees belonging to *Prunus* genus and reeds, it is time consuming and expensive to rear big amounts of *A. transcaspicus* on *Hyalopterus* spp. under controlled conditions. In laboratory conditions, and even in the open field, *A. transcaspicus* can also attack other aphid species (Wang and Messing 2006; Latham and Mills 2010). While trying to find an alternative host for mass rearing of *A. transcaspicus*, we noticed different mummification (<7%) and emergence rates (<20%) among several species, including *A. pisum*, *A. fabae*, *A. gossypii*, and *A. spiraeola* (unpublished data from our group [SZ]). This phenomenon might be caused by the presence of S-symbionts with a protective role against the attack of parasitoids, as described in other aphids. However, there is only scarce information about the presence of S-symbionts in *Hyalopterus* spp., and only a few studies included samples from Africa or Mediterranean coastal regions (Zytynska and Weisser 2016). For this reason, we decided to determine the presence of putatively protective symbionts in *Hyalopterus* spp. and potential alternative hosts, reared in our greenhouse and from

different natural populations of the northwestern and central-east of Tunisia (Fig. 1; Table 1).

We screened a total of 194 aphids from six different species. In a first step, μ DNA was obtained from single individuals and all samples were analyzed by PCR using universal primers for the 16S rRNA gene (Table 2), in order to test for DNA quality. Because *B. aphidicola* must always be present, only the 162 positive samples were further analyzed. Second, the obtained amplicons were digested with four different restriction enzymes that gave a known pattern for *B. aphidicola* and the three most common S-symbionts, *S. symbiotica*, *H. defensa*, and *R. insecticola* (Sandström et al. 2001). All samples that gave positive results were also PCR-amplified using primers that were able to amplify only S-symbionts (DP1; Table 2). Next, samples that gave positive DP1 results were further analyzed with species-specific primers (DP2; Table 2). Finally, the obtained DP2 amplicons were sequenced. For those samples that were positive in the DP1 analysis but did not give any DP2 amplicon, the product of DP1 was sequenced instead, allowing the detection of bacteria for which no species-specific primers were designed. All obtained sequences were analyzed by BLAST, to confirm the identity of the detected symbiont. The sequencing step revealed that the *Arsenophonus*-specific primers gave some unspecific amplification of samples that contained *Hamiltonella*. The final results are summarized in Table 4.

Nearly all screenings for the presence of S-symbiont across insects have used diagnostic PCR to determine the presence of particular bacterial species (Oliver et al. 2014). In fact, Henry et al. (2015) showed that species-specific primers are very reliable in detecting S-symbionts in aphids because the use of NGS technologies gave almost the same results. Therefore, even though the number of aphids analyzed is limited and we cannot discard false-negative results, our results indicate a relatively high incidence of S-symbionts in the analyzed populations.

The most frequently found facultative symbiont was *Arsenophonus* spp. In fact, except for *A. pisum*, we found it in all

Table 4. S-symbionts identified in the aphid populations analyzed

Population ID	Number of individuals analyzed	<i>Arsenophonus</i> sp.	<i>Serratia symbiotica</i>	<i>Hamiltonella defensa</i> ^a	<i>Erwinia</i> sp.
Ap-1 (bean)	4		4		
Ap-1 (pea)	4		4		
Ag-1	5			5	
Ag-2	11	11			
As-1	7	3	1 ^b		
Ha-1 (natural)	9	3	1	1	
Ha-1 (greenhouse)	25	11	2 ^c		
Ha-2	17				
Ha-3	5				
Ha-4	12	4			
Ha-5	10	1			
Ha-6	4				
Ha-7	4	1			
Har-1	4	1			
Har-2	4	3			
Har-3	4	1			
Har-4	10	5			
Hpr-1	4	1			
Hpr-2	8			1	
Hpr-3	11				1

^aAll samples positive for *Hamiltonella* were also positive for APSE-1.

^bThe same individual gave a positive result for *Arsenophonus* and *Serratia*.

^cOne individual positive for *Serratia* was also positive for *Arsenophonus*.

species under study, although not in all populations and, in many cases, it was the only S-symbiont detected. This is the first statement of the presence of *Arsenophonus* in the genus *Hyalopterus*. Enterobacteriaceae of genus *Arsenophonus* have been increasingly discovered as facultative symbionts in insects belonging to multiple taxa, and it has been estimated to infect almost 5% of arthropods (Nováková et al. 2009). This genus presents a high ecological versatility, with switches between entirely different life styles, from male-killers (e.g. *Arsenophonus nasoniae* Gherna et al. 1991 (Enterobacteriales: Enterobacteriaceae) infecting the parasitic wasp *Nasonia vitripennis* Walker 1836, Hymenoptera: Pteromalinae; Taylor et al. 2011) to beneficial symbiont, as it seems to be the case of the soybean aphid, *Aphis glycines* (Matsumura, Aphidinae: Aphidini) (Wulff and White 2015). Nevertheless, despite being found in 7% of all aphid species, and 30% of species within the genus *Aphis* (Jouselin et al. 2013), the function of this bacterium is unknown in most aphids.

Hamiltonella was sporadically found only in three populations analyzed in this work, each one belonging to a different species. We detected it in all individuals analyzed from a natural population of *A. gossypii* feeding on Chinese hibiscus, but not on a second natural population of the same aphid species feeding on melon. It was also found in a single individual in one natural population of *H. pruni* feeding on apricot, and in another single individual of

H. amygdali feeding on the summer host plant common reed, but not in any of the analyzed aphids from colonies established in a greenhouse derived from the later natural population (Table 4). Previous studies on the presence of *H. defensa* in pea aphids indicate that this S-symbiont appears sporadically in *A. pisum* populations, and it is beneficial only when parasitoids are present (Oliver et al. 2008). Therefore, infection frequencies decrease in the absence of parasitoids.

Because the protective effect of *Hamiltonella* has been related with the presence of the APSE prophage, we screened for the incidence of APSE infections using a PCR assay based on the P45 gene, involved in phage DNA synthesis (Degnan and Moran 2008b). The analysis revealed that all samples that gave positive results for *Hamiltonella* also possessed APSE, and that the obtained amplicon was identical in all of them. The subsequent phylogenetic analysis suggests that it belongs to the APSE-1 clade (Fig. 2). In previous studies performed on *A. pisum*, this strain has been associated with moderate-to-strong protection against *A. ervi* (Degnan and Moran 2008a, Oliver et al. 2009, Weldon et al. 2013, McLean 2015). APSE has also been frequently detected in *Arsenophonus* spp. (Duron 2014). For this reason, the same screening analysis was also performed for all samples that gave positive results for other S-symbionts, but all of them gave negative results for the presence of the prophage.

Fig. 2. Phylogenetic analysis of the APSE-P45 gene partial nucleotide sequences obtained from the different populations analyzed (in bold). Bayesian posterior probabilities are indicated as nodal support. Scale bar represents substitutions per site.

S. symbiotica was found in three of the aphid species analyzed. It was present in all analyzed individuals from the *A. pisum* populations maintained in our greenhouse in Chott-Mariem, reared on peas and beans, established from a natural population in 2014. This is not an unexpected result because *S. symbiotica* has been frequently associated with pea aphids, being more abundant in warm conditions (Montllor et al. 2002). *S. symbiotica* in pea aphids has also been suggested to provide mild protection against parasitism by *A. ervi* (Oliver et al. 2003), although in that case no direct fitness benefit was observed (Oliver et al. 2008). In fact, because there is limited evidence for known defensive factors in the *S. symbiotica* genome (Burke and Moran 2011), the above observations might be related to other benefits, such as thermal tolerance (Montllor et al. 2002, Russell and Moran 2006).

Among the different species of tribe Aphidini analyzed, *S. symbiotica* was only sporadically detected in one population of *H. amygdali* from Chott-Mariem. The S-symbiont was detected in one individual collected on spring 2015 from a natural population feeding on common reed, and also in two aphids from a population established from the original one and reared in a greenhouse at 28°C. However, it was not found in aphids from the same population reared at lower or higher temperatures (25, 30, and 35°C). The same natural population of *H. amygdali* gave also one positive result for the presence of *H. defensa*, although not simultaneously with *S. symbiotica*. It is unusual to find such small incidence of *Serratia* infections in aphids from a warm area. It might indicate that the increased thermal tolerance attributed to this bacterium is not a general trait for different aphid species.

Besides the common aphid heritable S-symbionts, the sequencing analysis of the amplicon obtained in the DP1 analysis of one *H. pruni* aphid captured on blackthorn allowed the identification of *Erwinia* spp. Winslow et al., 1920 (Enterobacteriales: Enterobacteriaceae), a plant pathogen that several studies have detected as a gut associate in some aphids (reviewed in Gauthier et al. 2015). Its presence in the sampled aphids might just reflect their acquisition from the host plant during feeding. Although two species of this genus, *E. aphidicola* and *E. iniecta*, have been described to negatively affect the infected aphid (Harada et al. 1997, Campillo et al. 2015), it is not clear whether *Erwinia* acts as an aphid pathogen, commensal, mutualist, it uses aphids as alternative hosts to plants, or it is just acquired during aphid feeding, so that the aphid acts as a vector for the plant pathogen.

Concluding Remarks

Recently, Zytynska and Weisser (2016) performed a comprehensive search of all available information about the presence of aphids S-symbionts, to highlight the geographical areas that should be explored in order to have a better understanding of the global distributions of these facultative bacteria. At the continental scale, Africa is one of the less explored world areas regarding this subject, with only a few studies performed on a limited number of aphid species. Therefore, even though the number of aphids analyzed in this work is limited, this is the first survey on the presence of S-symbionts in Tunisian aphids causing important damage in crops. Our results indicate that S-symbionts, mainly *Arsenophonus* spp., are widely present in the analyzed populations. Further studies need to be conducted in order to determine whether their presence has ecologically important benefits and the impact of temperature and plant hosts in such benefits. In any case, the presence of *Hamiltonella* is sporadic in the analyzed populations, although

all strains detected present APSE. To select for a good candidate for massive rearing of *A. transcaspicus*, it would be interesting to previously screen for the presence of APSE, and select one APSE-free population.

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