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Diversity and localization of bacterial symbionts in three whitefly species (Hemiptera: Aleyrodidae) from the east coast of the Adriatic Sea

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Abstract

Several whitefly species (Hemiptera: Aleyrodidae) are cosmopolitan phloem-feeders that cause serious damage in numerous agricultural crops. All whitefly species harbor a primary bacterial symbiont and a diverse array of secondary symbionts which may influence several aspects of the insect's biology. We surveyed infections by secondary symbionts in *Bemisia tabaci* (Gennadius), *Trialeurodes vaporariorum* (Westwood) and *Siphoninus phillyreae* (Haliday) from areas in the east cost of the Adriatic Sea. Both the Middle East-Asia Minor 1 (MEAM1) and Mediterranean (MED) *B. tabaci* genetic groups were detected in Montenegro, whereas only the MED was confirmed in Croatia. *Trialeurodes vaporariorum* and *S. phillyreae* were found in all areas surveyed. MEAM1 and MED exhibited similarity to previously reported infections, while populations of *T. vaporariorum* from Montenegro harbored *Rickettsia, Wolbachia* and *Cardinium* in addition to previously reported *Hamiltonella* and *Arsenopnohus*. *Siphoninus phillyreae* harbored *Hamiltonella, Wolbachia, Cardinium* and *Arsenophonus*, with the latter appearing in two alleles. Multiple infections of all symbionts were common in the three insect species tested, with some reaching near fixation. Florescent *in situ* hybridization showed new localization patterns for *Hamiltonella* in *S. phillyreae*, and the morphology of the bacteriosome differed from that observed in other whitefly species. Our results show new infections with bacterial symbionts in the whitefly species studied. Infections with the same symbionts in reproductively isolated whitefly species confirm complex relationships between whiteflies and bacterial symbionts, and suggest possible horizontal transfer of some of these bacteria.

Keywords: *Bemisia tabaci*, *Trialeurodes vaporariorum*, *Siphoninus phillyreae*, secondary symbionts

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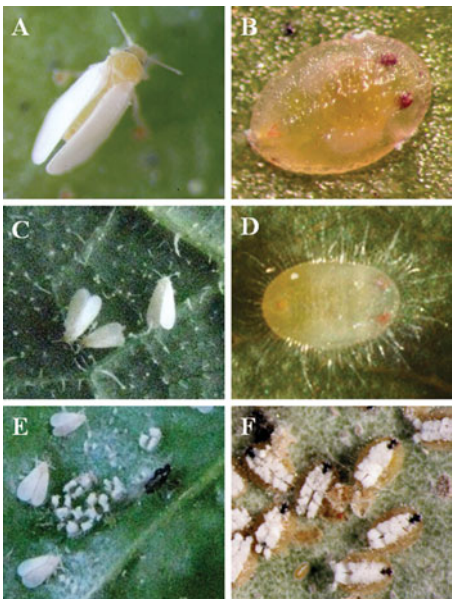

Fig. 1. Phenotypical differences between three whitefly species: adults of (A) *B. tabaci*, (C) *T. vaporariorum* and (E) *S. phillyreae*; pupal stages of (B) *B. tabaci*, (D) *T. vaporariorum* and (F) *S. phillyreae*.

Introduction

Several whitefly species (Hemiptera: Aleyrodidae) are cosmopolitan phloem-feeding pests that cause serious problems in numerous agricultural crops. Few of these species are known to heavily damage plants through direct feeding, honeydew secretion and virus transmission. Among the many whitefly species known to date, only genus *Bemisia* and *Trialeurodes* are known to serve as virus vectors (Jones, 2003). Whiteflies can adapt to a wide range of climates, from arid desert to tropical, subtropical and Mediterranean conditions, where prolonged freezing temperatures are rare or nonexistent (Brown, 2007). *Bemisia tabaci* (Gennadius) represents a species complex consisting of at least 30 morphologically indistinguishable species, placed in 11 well-defined high-level groups, based on biochemical and molecular markers (Dinsdale *et al*., 2010; Hu *et al*., 2011; Alemandri *et al*., 2012). The world's two most widespread members of the *B. tabaci* species complex are the Middle East-Asia Minor 1 (MEAM1, known as B biotype) and Mediterranean (MED, known as Q biotype). MEAM1 and MED became global invaders and the most damaging, due to the ornamental plants trade (De Barro & Ahmed, 2011). They are known for their wide host range, high fecundity, insecticide resistance and ability to transmit plant viruses and induce plant disorders (Brown *et al*., 1995; Secker *et al*., 1998; Perring, 2001). The greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) and the ash whitefly *Siphoninus phillyreae* (Haliday) are common pests in many agrosystems worldwide and cause serious damage by direct feeding on plants and secretion of large amounts of honeydew. Although *S. phillyreae* is less known in agricultural crops, it is known in horticultural crops such as pomegranate, pear, olive and citrus (Nguyen & Hamon, 1990).

In Croatia, *T. vaporariorum* has been a serious pest in greenhouse crops since the 1970s, and it is still the predominant species, while *B. tabaci* MED was first recorded in 2000 (Zanic *et al*., 2005). These two pests cause significant losses on numerous horticultural crops. Similar damage is caused by both pests in the south eastern part of Bosnia and Herzegovina. The first report of *B. tabaci* on ornamentals from Montenegro was confirmed in 2008 (Skaljac *et al*., 2010). The control of all whitefly species that cause agricultural damage in all East Adriatic countries relies on adopting integrated pest management practices. *Siphoninus phillyreae* is a major pest of pomegranate in this area, and new infestations of *Phillyrea* spp. originate from shrubs, where the pest overwinters. Whereas *T. vaporariorum* and *S. phillyreae* can be identified based on external morphology (fig. 1), *B. tabaci* genetic groups can only be identified using DNA markers (Boykin *et al*., 2007).

Many insects are known to host a diverse array of bacterial symbionts which appear to interact with their hosts on several levels, ranging from parasitism to mutualism (Buchner, 1965; Moran & Baumann, 1994). All whitefly species are known to harbor the primary symbiont *Portiera aleyrodidarum*, which supplements their deficient phloem diet with some essential nutrients (Thao & Baumann, 2004a). *Portiera aleyrodidarum* is confined to specialized cells (bacteriocytes) and is vertically transmitted (Baumann, 2005). In addition, *B. tabaci* populations from around the world have been reported to harbor several secondary symbionts, including *Hamiltonella*, *Arsenophonus*, *Cardinium*, *Wolbachia*, *Rickettsia* and *Fritschea*, whose functions are mostly unknown in this species (Nirgianaki *et al*., 2003; Baumann, 2005; Gottlieb *et al*., 2006; Li *et al*., 2007). In other insects, *Wolbachia*, *Rickettsia*, *Arsenophonus* and *Cardinium* have been implicated in manipulating their host's reproduction via several mechanisms (Werren *et al*., 1986; Gherna *et al*., 1991; Zchori-Fein & Perlman, 2004; Dale & Moran, 2006). *Hamiltonella* has been shown to confer resistance against parasitoids in the pea aphid *Acyrthosiphon pisum* (Oliver *et al*., 2003; Ferrari *et al*., 2004; Bensadia *et al*., 2005) and to increase the ability of *B. tabaci* to be an efficient virus vector (Gottlieb *et al*., 2010). *Rickettsia* in *B. tabaci* has been shown to confer resistance to heat stress (Brumin *et al*., 2011) to increase its susceptibility to chemical insecticides (Kontsedalov *et al*., 2008) and to provide the whitefly with fitness benefits (Himler *et al*., 2011).

In 2008, we conducted a survey on the distribution of *B. tabaci* and *T. vaporariorum* populations and their infection status with bacterial symbionts in Croatia, and Bosnia and Herzegovina (Skaljac *et al*., 2010). In the same study, the localization of the different endosymbionts within both whitefly species was investigated using fluorescent *in situ* hybridizations (FISH), and new localization patterns were discovered (Skaljac *et al*., 2010). The infection status of *B. tabaci* differed from previously published results (Chiel *et al*., 2007; Gottlieb *et al*., 2008; Gueguen *et al*., 2010). Here, we surveyed more populations of *T. vaporariorum*, *B. tabaci* and an additional whitefly species, *S. phillyreae*, and focused on populations imported in Croatia and Montenegro. Unexpectedly, the infection statuses of the different whitefly populations differed from those obtained in 2008, and new infections were discovered. These results may suggest movement of whitefly populations within and between Eastern Adriatic neighboring countries over time. The results further confirm recent findings obtained from China which showed that, over time, infection levels in secondary symbionts of *B. tabaci* populations change markedly for some endosymbionts (Chu *et al*., 2011).

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Table 1. Presence (+) or absence (−) of secondary symbionts (SS) in *B. tabaci*, *T. vaporariorum* and *S. phillyreae* populations tested in this study.

Population number	Country/Location	Host plant	Species	SS presence/absence					
				R	H	Ars760	Ars580	W	C
1	Montenegro/ Ulcinj	*Dipladenia sanderi*	MED	+	+	−	+	+	−
2	Montenegro/Bar	*Dipladenia sanderi*	MED	+	+	−	+	+	−
3	Montenegro/Podgorica	*Sonchus oleraceus*	MEAM1	+	+	−	−	−	−
4	Croatia*/Zadar	*Hibiscus* sp.	MED	+	+	−	+	+	−
5	Montenegro/Ulcinj	*Salvia* sp.	*T. vaporariorum*	+	+	−	+	+	−
6	Montenegro/Bar	*Cucumis sativus*	*T. vaporariorum*	−	+	−	+	−	+
7	Montenegro/ Radanovići	*Geranium* sp.	*T. vaporariorum*	+	+	−	+	−	+
8	Montenegro/Podgorica	*Sonchus oleraceus*	*T. vaporariorum*	−	+	−	+	+	+
9	Montenegro/Sutorina	*Sonchus oleraceus*	*T. vaporariorum*	−	+	−	+	−	+
10	Croatia**/Split	*Euphorbia pulcherrima*	*T. vaporariorum*	−	+	−	+	−	−
11	Montenegro/Ulcinj	*Punica granatum*	*S. phillyreae*	−	+	+	+	+	−
12	Montenegro/Bar	*Punica granatum*	*S. phillyreae*	−	+	+	+	+	−
13	Montenegro/Podgorica	*Punica granatum*	*S. phillyreae*	−	+	+	+	+	−
14	Croatia/Ljuta	*Punica granatum*	*S. phillyreae*	−	+	−	+	+	+
15	Croatia/Trsteno	*Punica granatum*	*S. phillyreae*	−	+	+	+	+	−
16	Croatia/Opuzen	*Punica granatum*	*S. phillyreae*	−	+	+	+	+	+
17	Croatia/Pozla Gora	*Punica granatum*	*S. phillyreae*	−	+	+	+	+	−
18	Croatia/Brač-Pučišća	*Punica granatum*	*S. phillyreae*	−	+	−	+	+	−
19	Croatia/Brač-Supetar	*Punica granatum*	*S. phillyreae*	−	+	+	+	+	+

* Population imported by trade from Italy (Rome, region Lazio).
** Population imported by trade from Slovenia (Čatež ob Savi).
R, *Rickettsia*; H, *Hamiltonella*; Ars580, *Arsenophonus* 580 bp; Ars760, *Arsenophonus* 760 bp; W, *Wolbachia*; C, *Cardinium*

Materials and methods

Whitefly populations

Three whitefly species were collected in summer 2011 from open fields or greenhouses in coastal Croatia and Montenegro (fig. 2). Sampling locations and host plants are shown in table 1. The sampling of *B. tabaci* and *T. vaporariorum* in Croatia included only new populations, imported on ornamental plants, and that from Montenegro contained new *B. tabaci* and *T. vaporariorum* populations collected from different locations. Collections were made in the summer when high population densities were available. *Bemisia tabaci* and *T. vaporariorum* nymphs were collected in the laboratory from leaves of different host plant samples in the field and moved into ethanol. *Siphoninus phillyreae* nymphs were similarly collected from leaves of pomegranate trees (*Punica granatum* L.) in gardens and orchards. Adults of the three whitefly species were collected using a Pasteur pipette attached to a hand-held aspirator and moved into ethanol. Scarcity in the egg stage was observed for all studied species, possibly due the very fast generation turnover in the summer season, thus eggs were not used for subsequent analysis. The collected insects were kept at −20°C, until processing. One imported population of *B. tabaci* from Italy and one *T. vaporariorum* population from Slovenia were found in Croatia at the nymphal stage on host plants *Hibiscus* sp. and *Euphorbia pulcherrima* Willd., respectively. Imported infested plants were maintained in insect-proof cages in the laboratory under standard conditions (26 ± 2°C, 60% RH, 14:10 h of light:dark) until adult emergence.

Screening of secondary bacterial symbionts and B. tabaci *species*

Adult females (n = 10–20 per population) from the studied whitefly species were tested for the presence of secondary bacterial symbionts, as were species (MED or MEAM1) of four *B. tabaci* populations. Genomic DNA of each individual was extracted in lysis buffer as previously described and used for species and secondary symbiont screening (Chiel *et al.*, 2007). *Bemisia tabaci* MED or MEAM1 groups were identified using microsattelite markers by polymerase chain reaction (PCR) amplification using Bem 23 primers and fragment size. The product size obtained from B biotype (MEAM1) was 200 bp and from the Q biotype (MED) was 400 bp. Three *B. tabaci* MED populations that were collected in this study were confirmed to belong to the Q1 group based on Mitochondrial Cytochrome Oxidase I (COI) sequences that were obtained following the protocol described in Gueguen *et al.* (2010). The sequences showed 99.5–99.8% similarity (0.2–0.5% divergence) to the Q1 group, while representative Q2 and Q3 group sequences generated in Gueguen *et al.* (2010) diverged by 1.5–2.0%. Numerous studies around the world using different molecular methods have identified six secondary symbionts in whiteflies: *Rickettsia* (R), *Hamiltonella* (H), *Arsenophonus* (A), *Wolbachia* (W), *Cardinium* (C) and *Fritschea* (F). The presence of these secondary symbionts was tested by PCR using genus-specific primers (16S or 23S rDNA) (table 2). PCR was carried out in 20 µl volume containing 4 µl DNA lysate, 20 pmol of each primer, 10 mM dNTP mix, 10 Dream Taq buffer (+$MgCl_2$) and 5 units μl^{-1} of Dream Taq polymerase (Fermentas). PCR products were visualized on a 1.5% agarose gel containing ethidium bromide. To verify the PCR products, bands were eluted and DNA was sent for sequencing (3730 xl DNA analyzer, Macrogen Europe, Amsterdam, Netherlands). The sequences were compared with those in the databases using BLAST algorithm in NCBI; 10–20 samples per set of primers were sequenced.

Fluorescent in situ *hybridization analysis*

FISH analysis of *S. phillyreae* adults and nymphs and *B. tabaci* nymphs (for reference) was performed as previously

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Table 2. List of primers used in the research.

Primer name	Sequence (5′ → 3′)	Anealing (°C)/Size (bp)	Gene	Reference
Bem 23 F Bem 23 R	CGGAGCTTGCGCCTTAGTC CGGCTTTATCATAGCTCTCGT	55/MEAM1 = 200 MED = 400	Microsatellite	De Barro *et al*., 2003
Rb F Rb R	GCTCAGAACGAACGCTATC GAAGGAAAGCATCTCTGC	59/900	*Rickettsia* 16S rDNA	Gottlieb *et al*., 2006
92 F Hb R	TGAGTAAAGTCTGGGAATCTGG AGTTCAAGACCGCAACCTC	62/700	*Hamiltonella* 16S rDNA	Zchori-Fein & Brown, 2002
Ars23S-1 Ars23S-2	CGTTTGATGAATTCATAGTCAAA GGTCCTCCAGTTAGTGTTACCCAAC	59/580	*Arsenophonus* 23S rDNA	Thao & Baumann, 2004b
Wol16S F Wol16S R	CGG GGGAAAAATTTATTGCT AGCTGTAATACAGAAAGTAAA	55/650	*Wolbachia* 16S rDNA	Chiel *et al*., 2007
CFB F CFB R	GCGGTGTAAAATGAGCGTG ACCTMTTCTTAACTCAAGCCT	59/500	*Cardinium* 16S rDNA	Weeks & Breeuwer, 2003
U23 F 23SIG R	GATGCCTTGGCATTGATAGGCGATGAAGGA TGGCTCATCATGCAAAAGGCA	55/600	*Chlamydia* 23S rDNA	Everett *et al*., 2005

Fig. 2. Locations of collected whitefly populations in coastal Croatia and Montenegro according to population numbers in table 1.

described (Skaljac *et al*., 2010), using short symbiont-specific 16S/23S rRNA DNA probes harboring a fluorescent Cy3/Cy5 molecule on their 5′ end (table 3 in Skaljac *et al*., 2010). Stained samples were mounted whole and viewed using an IX81 Olympus FluoView 500 confocal microscope (Olympus, Tokyo, Japan). For each developmental stage, at least 30 specimens were viewed under the microscope to confirm reproducibility. Optical sections (0.7–1.0 μm thick) were

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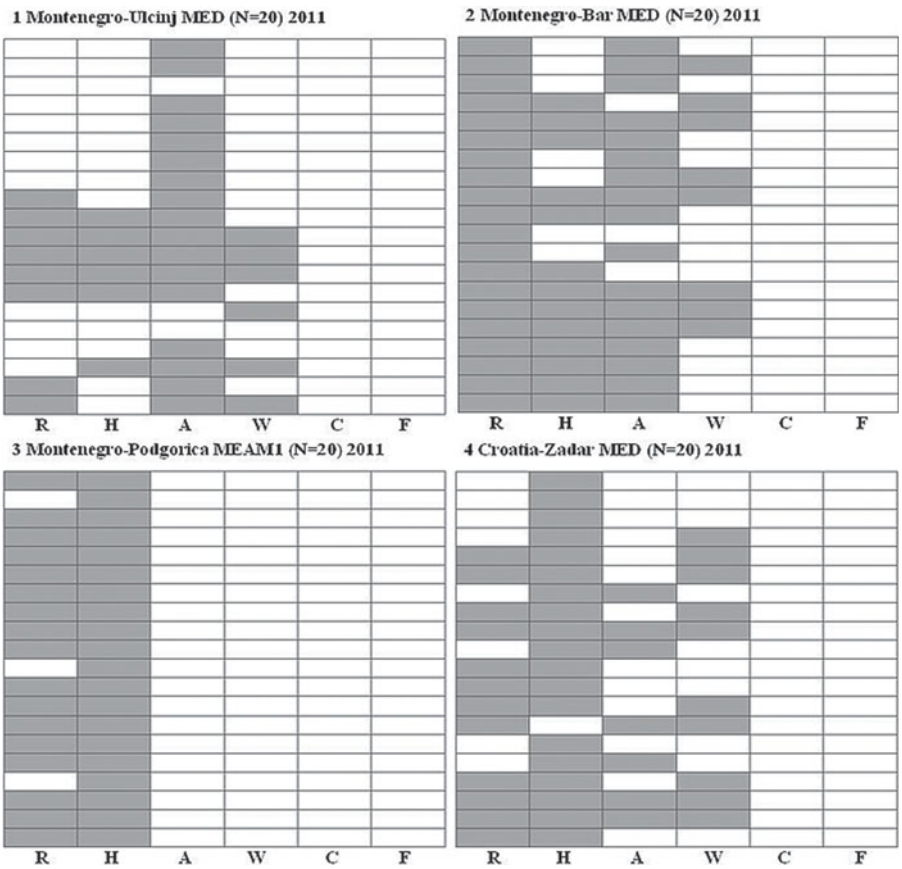

Fig. 3. Individual and multiple infections by secondary bacterial symbionts in four *B. tabaci* populations from Montenegro and Croatia (introduced from Italy). Each square represents one population, and each column represents one type of symbiont; the 10–20 rows per table represent the 10–20 individuals tested per population. Gray fields indicate positive infection for the tested symbiont. Population number, country and geographical location, species, number of tested individuals and year of sampling are indicated at the top of each table. Symbionts: R, *Rickettsia*; H, *Hamiltonella*; A, *Arsenophonus*; W, *Wolbachia*; C, *Cardinium*; F, *Fritschea*.

prepared from each specimen. Specificity of detection was confirmed using no probe staining and RNase digested specimen staining. In addition, each population was tested with all of the probes as controls. Thus, staining of a population known not to have a particular symbiont but harboring others was performed.

Results

General results for all whitefly species tested

Twenty individuals from each collected population were tested for the presence of the different secondary symbionts in each individual using genus-specific primers, except for two *T. vaporariorum* populations where only ten individuals were analyzed. *Portiera aleyrodidarum*, the primary symbiont of whiteflies, was detected in all individuals tested and served as a control for the quality of the extracted DNA. *Fritschea* was not detected in any of the whitefly individuals tested.

Bemisia tabaci *infection by secondary symbionts*

Bemisia tabaci populations were collected in Montenegro and Croatia (table 1 and fig. 3). Three populations were collected in Montenegro and were identified as MEAM1 and MED groups. The MEAM1 population was infected with *Hamiltonella* and *Rickettsia*. *Hamiltonella* was fixed in the population, while *Rickettsia* was highly prevalent. The MED populations collected in Bar and Ulcinj showed high levels of multiple infections with *Rickettsia*, *Hamiltonella*, *Arsenophonus* and *Wolbachia*, with some of these symbionts being fixed or close to fixation such as *Rickettsia* and *Arsenophonus*. *Hamiltonella* was present in 30–95% of the tested populations, *Wolbachia* in 30–40% and *Rickettsia* in about 65% of the tested individuals. Within all *B. tabaci* populations from Montenegro, 22% of all individuals showed single infection with secondary symbionts, 40% showed double infection, 22% were infected with three symbionts, and 13% showed infection with four symbionts. Overall, 97% (58/60) of all *B. tabaci* individuals from Montenegro were infected with at least one secondary symbiont; only two individuals did not contain any of the tested secondary symbionts. The *B. tabaci* MED population imported from Italy to Croatia (table 1) was infected with *Hamiltonella*, *Rickettsia*, *Arsenophonus* and *Wolbachia* (fig. 3), with high prevalence of mixed infections.

Trialeurodes vaporariorum *infection by secondary symbionts*

Five *T. vaporariorum* populations were collected in Montenegro and one in Croatia (fig. 4). *Trialeurodes*

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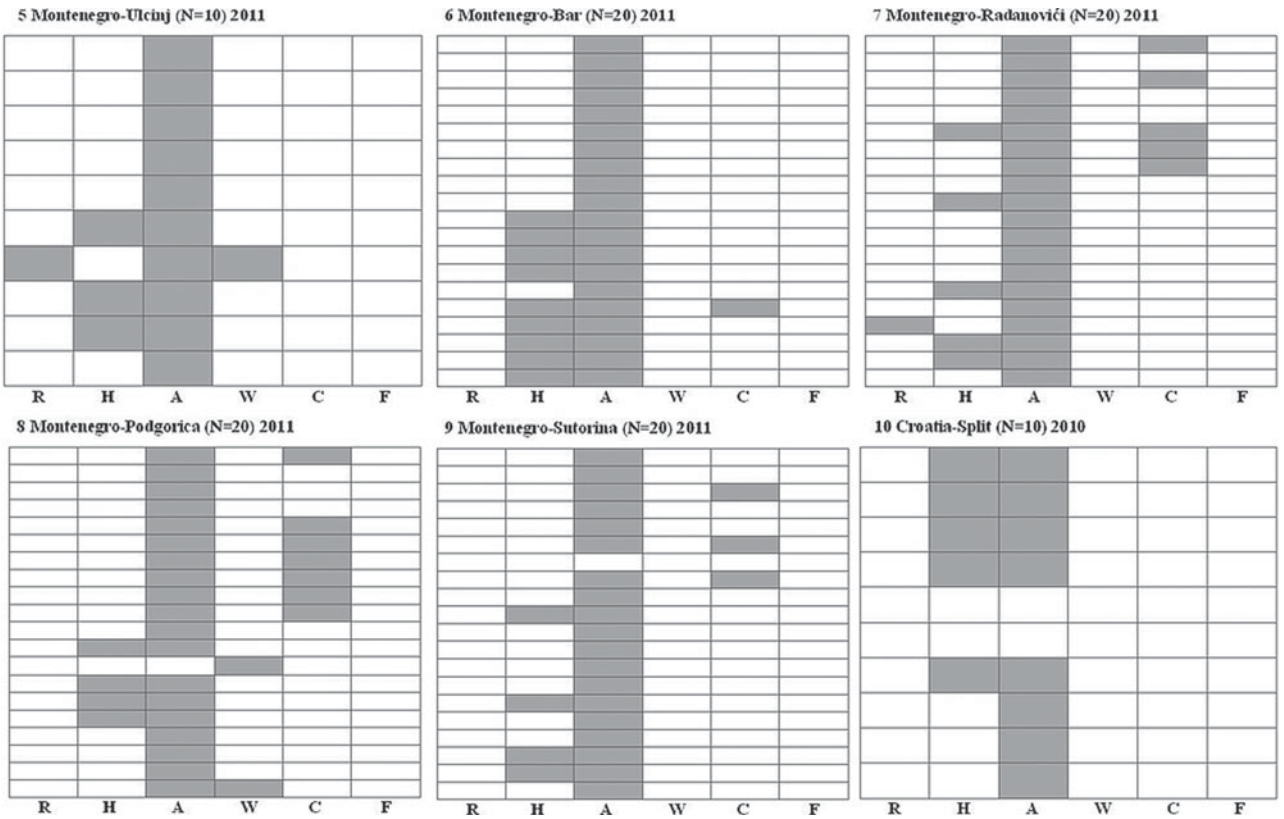

Fig. 4. Individual and multiple infections by secondary bacterial symbionts in the six *T. vaporariorum* populations collected from Montenegro and Croatia (introduced from Slovenia). For more information see the legend of fig. 3.

vaporariorum was more prevalent than *B. tabaci* in all examined locations of Montenegro, as is the case in Croatia. *Arsenophonus* was the most prevalent symbiont and was fixed or close to fixation in the populations collected in Montenegro (fig. 4). In addition, 20–45% infection rates with *Hamiltonella* were detected, whereas *Cardinium* infected about 30% of the individuals. *Wolbachia* and *Rickettsia* were present in only 3% of all individuals tested. Since almost all *T. vaporariorum* individuals harbored *Arsenophonus*, double infections were prevalent and included, besides *Arsenophonus*, one of the other detected symbionts, such as *Cardinium*, *Wolbachia* and *Hamiltonella*. Only three cases of triple infections were found. Two of the cases included *Arsenophonus*, *Cardinium* and *Hamiltonella*, and the third case included *Arsenophonus*, *Wolbachia* and *Rickettsia*. Overall, 99% (89/90) of all *T. vaporariorum* individuals from Montenegro were infected with at least one secondary symbiont, while only one individual did not contain any of the tested secondary symbionts. One *T. vaporariorum* population imported from Slovenia to Croatia (Split) showed infection with *Arsenophonus* and *Hamiltonella*. *Arsenophonus* was more prevalent with an 80% infection rate in all individuals examined, while *Hamiltonella* infected 50% of the tested individuals. Double infections with both symbionts, as well as individuals that did not contain any secondary symbiont, were also detected.

Siphoninus phillyreae *infection by secondary symbionts*

Nine populations of *S. phillyreae* were collected across coastal Croatia and Montenegro (fig. 2) in summer 2011, when heavy ash whitefly infestations of pomegranate were observed in both countries. These populations were tested for individual and multiple infections by secondary symbionts (fig. 5). All populations showed high heterogeneity in secondary symbiont composition. *Hamiltonella* showed the highest prevalence in tested populations, detected in 85% of the tested individuals and was fixed or close to fixation in the populations collected in Croatia. *Wolbachia* appeared with infection rate ranging from 15% to 70% of individuals from the populations collected both in Croatia and Montenegro. Infection rates with *Cardinium* ranged from 5% to 70% of all individuals in the Croatian populations but were not detected in any *S. phillyreae* population collected in Montenegro. *Arsenophonus* was present in all populations tested, and infection rates ranged from 20% to about 100%. In most of the *S. phillyreae* populations tested, *Arsenophonus* exhibited two alleles, a 580 bp (Ars580) and a 760 bp (Ars760) PCR products. Both products showed close to 100% similarity to the 23S rDNA of the *Arsenophonus* symbiont of *S. phillyreae*. Overall, about 98% (177/180) of all *S. phillyreae* individuals were infected with at least one secondary symbiont.

Localization of secondary symbionts in S. phillyreae

Since localization of secondary symbionts in *B. tabaci* and *T. vaporariorum* was previously studied (Gottlieb *et al.*, 2008; Skaljac *et al.*, 2010), we focused our FISH experiments on *S. phillyreae*. Only adults and nymphs were tested due to the

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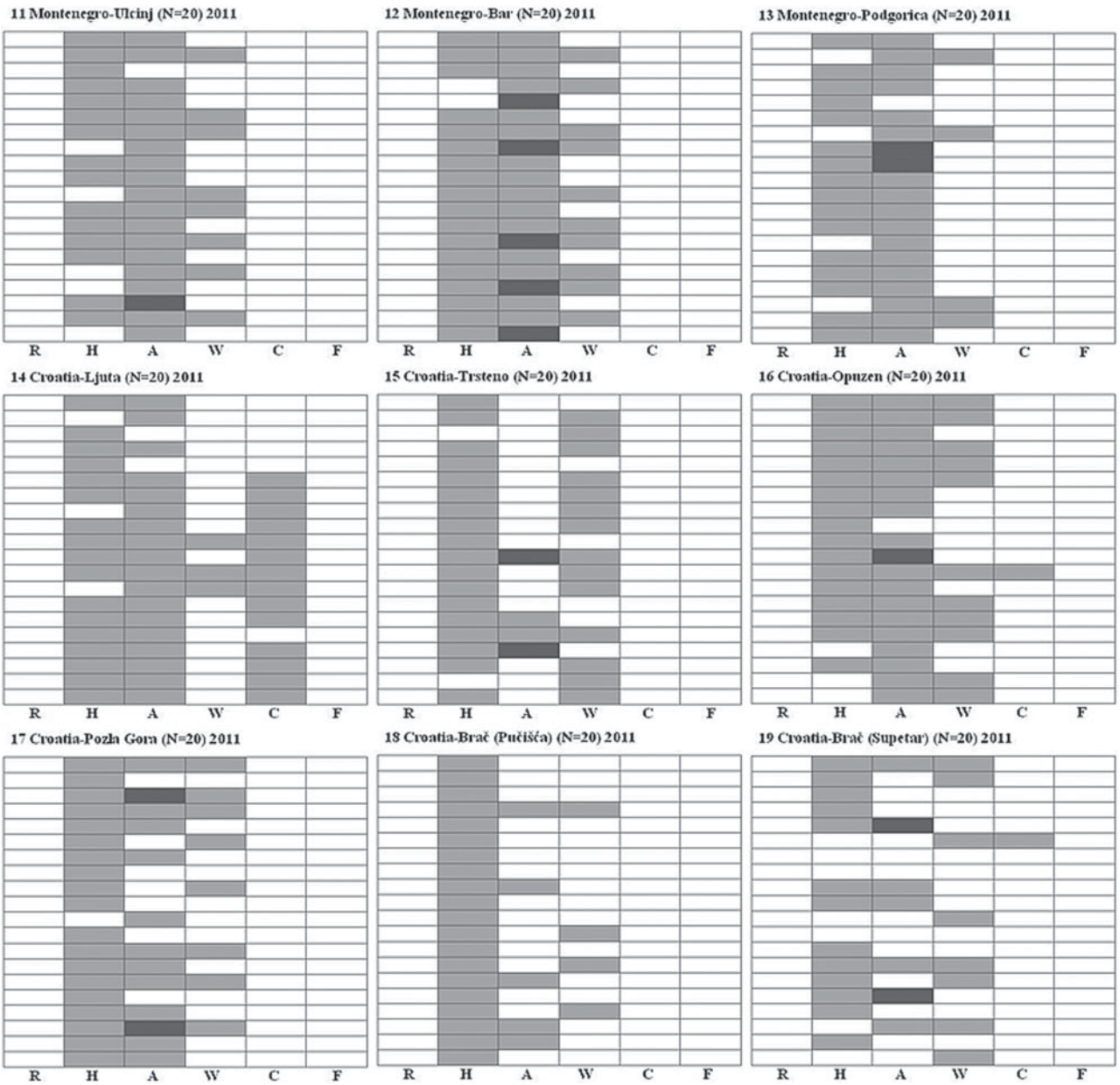

Fig. 5. Individual and multiple infections by secondary bacterial symbionts in the nine *S. phillyreae* populations from coastal Croatia and Montenegro. For more information, see the legend of fig. 3. Gray fields for (A) *Arsenophonus* represent the Ars580 allele and dark gray fields represent *Arsenophonus* with the Ars760 allele.

scarcity of eggs in the collected locations and difficulties in collecting enough eggs for the analyses. *Bemisia tabaci* nymphs were used in the FISH experiments for reference. In the developmental stages tested, symbionts were either randomly scattered outside the bacteriocyte or confined to the bacteriocyte with the primary symbiont. Interestingly, localization patterns of some of the symbionts differed from the previously reported ones. The localization of *Portiera*, the primary symbiont, revealed an interesting structure of the bacterisome, which had not been seen previously in other whitefly species. In the nymphal stages of *B. tabaci* and *T. vaporariorum*, the bacteriosomes always appear as two separate structures (for example figs 6H–I, 7H–I and 8H–I); whereas, in *S. phillyreae*, they are always connected (figs 6E–F and 7E–F). Surprisingly, *Hamiltonella* was localized outside the bacteriosome and showed a random and scattered localization pattern in adults (fig. 6A–C) and nymphs (fig. 6D–F), compared with the confined localization of *Hamiltonella* in *B. tabaci* (fig. 6G–I). *Hamiltonella* was observed in the circumference of bacteriocytes in adults or nymphs, where co-localized with *Portiera*. It was localized in other tissues as well, mainly in the abdomen and adult head, and was sometimes observed in the thorax (fig. 6A–C). This is the first time such a localization pattern has been observed in a whitefly species. Previously, only confined

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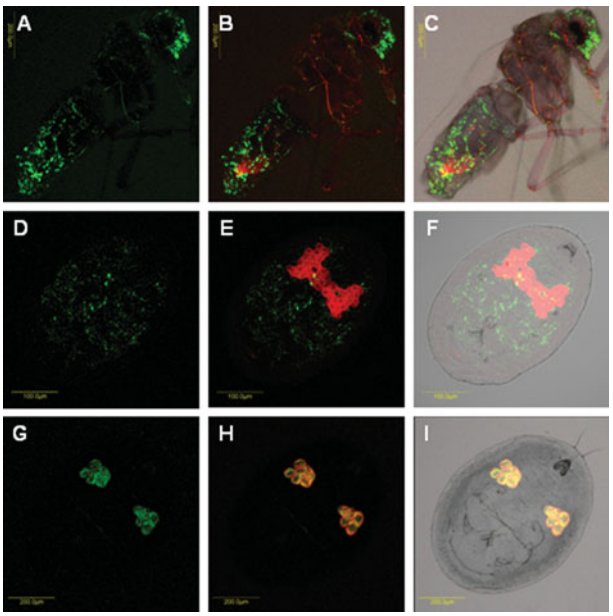

Fig. 6. *Portiera* and *Hamiltonella* FISH of *S. phillyreae* (A–C) adults and (D–F) nymphs and *B. tabaci* (G–I) nymphs. *Portiera*-specific probe (red) and *Hamiltonella*-specific probe (green) were used. (A, D and G) FISH of *Hamiltonella* alone, (B, E and H) double FISH of *Hamiltonella* and *Portiera* under dark field, and (C, F and I) double FISH of *Hamiltonella* and *Portiera* under bright field are shown.

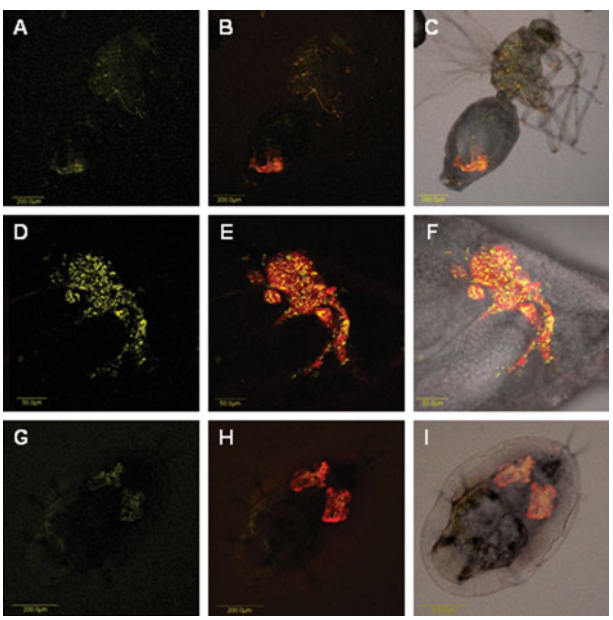

Fig. 7. (A–F) *Portiera* and *Arsenophonus* FISH of *S. phillyreae* adults and (G–I) *B. tabaci* nymphs. *Portiera*-specific probe (red) and *Arsenophonus*-specific probe (yellow) were used. (A, D and G) FISH of *Arsenophonus* alone, (B, E and H) double FISH of *Arsenophonus* and *Portiera* under dark field, and (C, F and I) double FISH of *Arsenophonus* and *Portiera* under bright field. D, E and F are zoom-in images of the FISH signals in A, B and C, respectively.

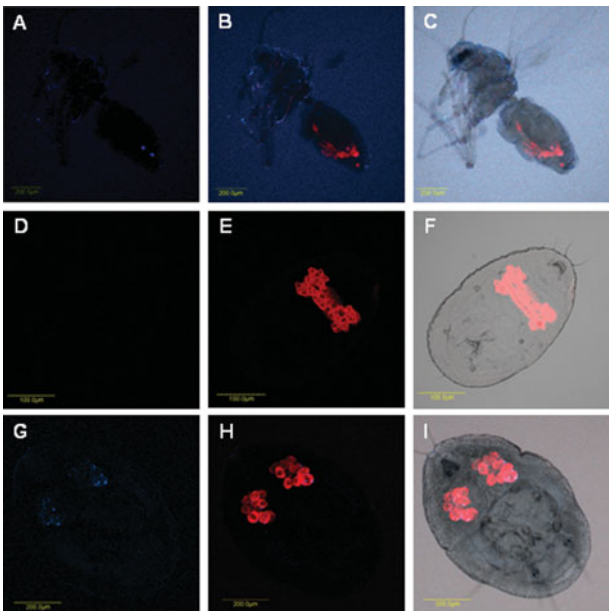

Fig. 8. *Portiera* and *Wolbachia* FISH of *S. phillyreae* adults (A–C) and nymphs (D–F) and *B. tabaci* nymphs (G–I). *Portiera*-specific probe (red) and *Wolbachia*-specific probe (blue) were used. FISH of *Wolbachia* alone (A, D and G), double FISH of *Hamiltonella* and *Portiera* under dark field (B, E and H), and double FISH of *Hamiltonella* and *Portiera* under bright field (C, F and I) are shown.

localization had been observed in *B. tabaci* and *T. vaporariorum* (Gottlieb *et al*., 2008; Skaljac *et al*., 2010).

Arsenophonus co-localized with *Portiera*, inside the bacteriosome in *S. phillyreae* adults (fig. 7A–F), as was previously shown for *Arsenophonus* from *B. tabaci* MED and *T. vaporariorum* (Gottlieb *et al*., 2008; Skaljac *et al*., 2010), and was also confirmed here (fig. 7G–I). *Arsenophonus* was observed to be rod-shaped in TEM and light microscopy results of cell lines infected with this bacterium (Szklarzewicz & Moskal, 2001) and appears to have a similar localization pattern in *S. phillyreae*.

Wolbachia has been previously shown to localize at the circumference of and inside the bacteriocytes of *B. tabaci* and was also seen in the abdomen outside the bacteriosome (Gottlieb *et al*., 2008). Further FISH analysis on *B. tabaci* populations from Croatia have shown that *Wolbachia* could only be detected inside the bacteriocytes with the primary symbiont, and was not detected in any other organ at any developmental stage (Skaljac *et al*., 2010). In the current study, *Wolbachia* was confined to the bacteriosome in the adult stage of *S. phillyreae* (fig. 8A–C) but was not detected in the nymphal stage (fig. 8D–F), possibly due to low amounts of the bacterium. This localization pattern is similar to the confined pattern that was previously observed in *B. tabaci* and confirmed in the current study (fig. 8G–I). The localization of *Wolbachia* in other insects is diverse and was shown to localize to several organs, including the salivary glands, gut, Malpighian tubules, fat body and brain (Min & Benzer, 1997; Ijichi *et al*., 2002; Mitsuhashi *et al*., 2002).

Cardinium was the only endosymbionts not detected in any of the developmental stages of *S. phillyreae* using FISH, possibly due to low amounts of the bacterium which are below detection levels. Previous research has shown that

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Cardinium localizes both inside and outside the bacteriosome of *B. tabaci*; however, it was not detected in *T. vaporariorum* (Gottlieb *et al.*, 2008; Skaljac *et al.*, 2010). *Rickettsia* was not detected by PCR, suggesting that this bacterium does not infect *S. phillyreae*.

Discussion

This study reports on a detailed screening of secondary symbionts of *B. tabaci* and *T. vaporariorum* in Montenegro, extending a previous screening of these whiteflies in Croatia, and provides a first such screen of *S. phillyreae* populations in the two countries. A first report of *Arsenophonus* in a Croatian *B. tabaci* MED population, introduced via the plant trade, further clarifies the previously observed diversity in secondary symbionts of Croatian whitefly populations. The MED populations found in Croatia and Montenegro were confirmed to belong to the Q1 group, as assessed by sequencing the COI gene based on the work conducted by Gueguen *et al.* (2010). This fact is particularly important and relevant to the current study because the Q1 group reported here, the populations that were previously described (Skaljac *et al.*, 2010) from Croatia and the Q1 populations described by Gueguen *et al.* (2010) harbor similar secondary symbiont loads. These results confirm the Q1 identity of the populations tested in the current study. A *B. tabaci* population from Montenegro, identified as MEAM1, was found to harbor *Rickettsia* and *Hamiltonella*, while the MED group from Montenegro harbored *Rickettsia, Hamiltonella, Arsenophonus* and *Wolbachia*. According to our previous data, a MEAM1 *B. tabaci* population collected in Montenegro (Podgorica) in 2008 was similar in symbiont composition to a MED *B. tabaci* population collected in Croatia and another MED population collected in Bosnia and Herzegovina, carrying only *Hamiltonella* and *Wolbachia* (Skaljac *et al.*, 2010). The *B. tabaci* MEAM1 population collected in the same location in Montenegro, three years later (in 2011) showed infection status similar to that of a *B. tabaci* MEAM1 from Israel, harboring only *Rickettsia* and *Hamiltonella* (Chiel *et al.*, 2007). In the present survey, two *B. tabaci* populations from Montenegro, identified as the MED group, contained *Arsenophonus* and *Hamiltonella*, among other symbionts. Interestingly, in neighboring Croatia and in Bosnia and Herzegovina, only the MED Q1 was found among the tested *B. tabaci* populations and none of them carried *Arsenophonus*, unlike the MED group in Israel that belongs to the Q2 group, which has never been infected with *Hamiltonella* (Chiel *et al.*, 2007; Gueguen *et al.*, 2010; Skaljac *et al.*, 2010). In *B. tabaci* MED populations from Montenegro, *Arsenophonus* and *Hamiltonella* were found together in 43% of the tested individuals, similar to Q1 populations described from Burkina, while this was never recorded in the Q2 or Q3 *B. tabaci* groups described from several countries (Gueguen *et al.*, 2010). Co-infection of *Arsenophonus* and *Hamiltonella* was also frequent in *T. vaporariorum* populations from Croatia and from Bosnia and Herzegovina, whereas in the present survey, these two symbionts were found together in 28% of the individuals in *T. vaporariorum* populations from Montenegro (Skaljac *et al.*, 2010). Based on our previous and current study, and the study of Gueguen *et al.* (2010), the composition of secondary symbionts in *B. tabaci* populations from Croatia, Bosnia and Herzegovina, and Montenegro reveals high diversity and heterogeneity among the different species and populations (Skaljac *et al.*, 2010). This can be attributed to the existence of the MED and MEAM1 groups, and the Q1, Q2 and Q3 subgroups of MED.

In summer 2011, a *B. tabaci* population was found on *Hibiscus* sp. imported from Italy into Croatia. Infection of this *B. tabaci* MED population with *Arsenophonus* represented the first finding of this secondary symbiont in Croatia since 2008. This further clarifies the unique co-infection pattern in recently tested Croatian *B. tabaci* populations, which is suggested to be due to horizontal symbiont transfer, introduction of new whitefly populations via the plant trade or whitefly populations with new infections that exist in some niches and were not sampled in the survey conducted in 2010. Chu *et al.* (2011) showed that the symbiotic composition in the MED and MEAM1 groups tested in China markedly changed over time, suggesting that other unknown factors may influence this composition.

Trialeurodes vaporariorum is much more prevalent than *B. tabaci* in Croatia and Montenegro, most likely due to climate conditions. In a previous survey, *Arsenophonus* and *Hamiltonella* were the only two symbionts detected in *T. vaporariorum* populations collected in Croatia, and Bosnia and Herzegovina (Skaljac *et al.*, 2010). In the current study, a more diverse composition of secondary symbionts was recorded in *T. vaporariorum* populations from Montenegro, where five tested populations harbored *Rickettsia, Hamiltonella, Arsenophonus, Wolbachia* and *Cardinium*. *Arsenophonus* was prevalent in all of them, showing a pattern of fixation or near fixation. The present *Arsenophonus* infections are similar to those reported from *T. vaporariorum* populations collected in Croatia, and Bosnia and Herzegovina. Such infection rates may indicate a mutualistic or obligatory association with the insect host and indicate either a functional advantage for the host or manipulation of its reproduction (Gottlieb *et al.*, 2008). Indeed, *Arsenophonus* was shown to be a reproductive manipulator in other insects (Werren *et al.*, 1986), and further investigation is needed to confirm or refute this hypothesis in *T. vaporariorum*.

Heavy *S. phillyreae* infestation of pomegranate led us to initiate a first survey of its secondary symbionts in Croatia and Montenegro. We found *S. phillyreae* to harbor *Hamiltonella, Arsenophonus, Wolbachia* and *Cardinium*. *Hamiltonella* showed the highest prevalence, infecting 85% of all tested individuals. In most of the populations tested, *Hamiltonella* was fixed or close to fixation, whereas *Arsenophonus* showed similar infection status in all *S. phillyreae* populations from Montenegro and two Croatian populations. Interestingly, *Arsenophonus* appeared in two alleles of the 23S rDNA amplification. Close *Arsenophonus* relatives, such as *Proteus, Yersinia, Providencia* and *Salmonella*, and other whitefly species such as *Aleurodicus disperses* and *Aleuroplantus gelatinosus* besides *S. phillyreae* have been suggested to have an intervening sequence (IVS) inserted in their 23S rDNA which results in a longer PCR (Miller *et al.*, 2000; Thao & Baumann, 2004b). It appears that possession of an IVS is not a distinct species characteristic and is unevenly allocated within bacterial genera (Miller *et al.*, 2000). This would clearly explain why several individuals in the tested *S. phillyreae* populations appear to have an IVS while others do not.

Our study supports the hypothesis that closely related heritable bacteria are often distributed across distantly related insect hosts, due to possible horizontal transfer or host switching (Aksoy *et al.*, 1997; Moran *et al.*, 2008). In this report, three different whitefly genera and species were found to share similar secondary symbionts, which also suggest that

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symbionts can survive, reproduce and undergo efficient colonization in new arthropod hosts. *Arsenophonus* and *Hamiltonella* have been shown to share and persist in new insect hosts, *A. pisum*, after transfer via microinjection from their natural aphid hosts (Russell & Moran, 2005). The butterfly *Acraea encedon* (L.) from Tanzania is distantly related to the butterfly *Hypolimnas bolina* (L.) from the Fiji Islands, but they share an identical male-killing *Wolbachia*, which strongly implicates horizontal transmission of the male killing element (Dyson *et al*., 2002). Recently, *Rickettsia* was shown to be horizontally transferred between *B. tabaci* individuals via the plant host, explaining the presence of this secondary symbiont in distantly related *B. tabaci* species (Caspi-Fluger *et al*., 2011). Moran *et al*. (2008) showed another example for the existence of closely related symbionts in evolutionary distant hosts, which may suggest inter- and intra-specific mechanisms for horizontal transmission.

A few of the populations surveyed here, particularly of *T. vaporariorum* from Montenegro, showed low infection rates with some secondary symbionts (*Rickettsia, Wolbachia* and *Cardinium*), suggesting recent introduction through horizontal transfer, the aforementioned plant trade or unknown factors that may influence the symbiotic composition over time, as was recently shown in Chinese populations of *B. tabaci* (Chu *et al*., 2011). Zchori-Fein & Perlman (2004) presented a phylogenetic analysis of 16S rDNA of *Cardinium*, which revealed that distantly related arthropods can harbor closely related symbionts, and closely related *Cardinium* were found to cluster among closely related hosts. This pattern suggests host specialization and horizontal transmission, which is particularly likely between *B. tabaci* and *T. vaporariorum*, since they are known to share plant hosts (Skaljac *et al*., 2010).

Although the populations of the different whitefly species tested in this study were collected from different host plants, we did not detect significant correlation between host plant and specific symbiotic content in any of the three species. The domination of *S. phillyreae* on pomegranate may indicate the adaptation of this specific species to the plant; however, other factors may affect this adaptation, such as the ability of the insect to manipulate plant secondary metabolites and toxic materials, and climate conditions. Further investigations are required to obtain conclusions regarding the host-insect adaptation, and whether secondary symbionts play a role in these interactions. Such investigations under controlled conditions may shed light on the contribution of the host plant to the composition of secondary symbionts in each whitefly species.

Finally, our study revealed unique co-infection patterns in *B. tabaci, T. vaporariorum* and *S. phillyreae* from Croatia and Montenegro. Our previous and present study of secondary symbiont co-infection in *B. tabaci* suggest that it is difficult to associate genetic groups (species) with secondary symbiont composition. The observation of *Rickettsia, Wolbachia* and *Cardinium* in *T. vaporariorum*, as well as the high diversity of secondary symbionts in *S. phillyreae*, suggest horizontal transfer of secondary symbionts between whitefly species. This study and recent studies from China (Chu *et al*., 2011) indicate that the symbiotic composition within whitefly populations is subject to change over time and space. These changes are possibly influences by a diversity of factors which are not fully known, but might be related to climate conditions, host plants, genetic background and other factors that are yet to be discovered. Recorded compositions of secondary symbionts contribute to a better understanding of their ecology and evolution within the assessed whitefly species and, subsequently, to designing research for discovering the functional role of secondary symbionts in their insect hosts.

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