Status bakterijskih simbionata u tri vrste štitastih moljaca (Hemiptera: Aleyrodidae): Bemisia tabaci, Trialeurodes vaporariorum i Siphoninus phillyreae u Hrvatskoj, Crnoj Gori i odabranim područjim ...

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University of Zagreb

FACULTY OF SCIENCE
DIVISION OF BIOLOGY

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DOKTORSKI RAD

Zagreb, 2012

This doctoral thesis was prepared at the Institute for Adriatic Crops and the Department of Entomology, Volcani Center in Israel, led by Dr. Katja Zanic, Prof. Murad Ghanim and Prof. Gordana Rusak, as a part of postgraduate studies at the Division of Biology, Faculty of Science, University of Zagreb.

University of Zagreb
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Doctoral Thesis

STATUS OF BACTERIAL SYMBIONTS OF THREE WHITEFLY SPECIES (HEMIPTERA: ALEYRODIDAE): *Bemisia tabaci*, *Trialeurodes vaporariorum* AND *Siphoninus phillyreae* IN CROATIA, MONTENEGRO AND SELECTED AREAS OF BOSNIA AND HERZEGOVINA

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Whiteflies (Hemiptera: Aleyrodidae) are cosmopolitan phloem-feeders that cause serious damage in numerous agricultural crops. They harbor a primary bacterial symbiont, and a diverse array of secondary symbionts (SS) which may influence several aspects of the insect's biology.

The aim of this study was to identify the status of *Bemisia tabaci* (Gennadius), *Trialeurodes vaporariorum* (Westwood) and *Siphoninus phillyreae* (Haliday) in Croatia, selected locations of Bosnia and Herzegovina, and Montenegro, in terms of: geographic distribution; infections by SS and their spatial localization in all developmental stages (from egg to adult); monitoring geminiviruses presence in surveyed areas, and ability of the studied *B. tabaci* to transmit geminiviruses. Our studies based on molecular methodology revealed: unique co-infection and localization patterns by SS in three whitefly species, which differed from previously reported studies; absence of geminiviruses in the examined areas, but efficient geminivirus transmission by *B. tabaci* from infected to healthy plant in experimental conditions.

Appearance of devastating geminiviruses can be expected in examined area due to specific SS composition and their presence in neighboring countries. Finally, the conducted studies contribute to investigating SS function in whiteflies, and can be used in designing programs to control these important agricultural pests.

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Štitasti moljci (Hemiptera: Aleyrodidae) su kozmopoliti koji se hrane sadržajem floema, a štete uzrokuju na brojnim poljoprivrednim kulturama. Sadrže primarnog bakterijskog simbionta, te niz sekundarnih simbionata (SS) koji mogu utjecati na različite aspekte biologije kukca.

Istraživanje je započeto, kako bi se utvrdio status *Bemisia tabaci* (Gennadius), *Trialeurodes vaporariorum* (Westwood) i *Siphoninus phillyreae* (Haliday) u Hrvatskoj, odabranim područjima Bosne i Hercegovine, te Crnoj Gori, u smislu: geografske rasprostranjenosti; infekcije i lokalizacije SS u razvojnim stadijima (od jaja do odraslog oblika); monitoringa pojave geminivirusa na području istraživanja i sposobnost prijenosa istih pomoću vektora *B. tabaci*. Istraživanje temeljeno na molekularnoj metodologiji je pokazalo:

jedinstvene koinfekcije i lokalizacije SS kod tri vrste štitastih moljaca, koje se razlikuju od prijašnjih istraživanja; nepostojanje geminivirusa u istraženom području, ali efikasnost prijenosa geminivirusa u eksperimentalnim uvjetima, pomoću *B. tabaci*, od inficirane na zdravu biljku.

Pojavnost devastirajućih geminivirusa je moguća na području istraživanja zbog specifičnog sastava SS i prisutnosti ovih virusa u susjednim zemljama. Provedena studija doprinosi istraživanju funkcije SS, te može biti korištena u osmišljavanju programa kontrole ovih važnih štetnika u poljoprivredi.

(150 stranica; 44 slika; 9 tablica; 213 literaturnih navoda; jezik izvornika-engleski)

Ključne riječi: Bakterijski simbionti, FISH, geminivirusi, hortikultura, štitasti moljci

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Content

1. INTRODUCTION

Whiteflies (Hemiptera: Aleyrodidae) are cosmopolitan phloem-feeding pests that cause serious problems in numerous agricultural crops. They 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). The economically most important, sweet potato whitefly Bemisia tabaci (Gennadius) can feed on more than 600 different plant species (Secker et al., 1998) and transmits 111 economically important plant viruses (Jones, 2003).

Since, 1990s there has been ongoing debate of whether B. tabaci was a complex species, composed of biotypes or a complex of multiple cryptic species. Today, it is known on the basis of molecular data, primarly mitochondrial oxidase I (mtCOI), that B. tabaci represents a species complex consisting of 24 morphologically indistinguishable species, placed in 11 well-defined high-level groups (De Barro, 2011). The world's two most widespread members of the B. tabaci species complex are: Middle East-Asia Minor 1 (MEAM1, commonly known as B biotype) and Mediterranean (MED, commonly known as Q biotype). MEAM1 and MED became global invaders and the most damaging due to ornamental plants trade (De Barro, 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; Perring, 2001).

The greenhouse whitefly Trialeurodes vaporariorum (Westwood) and the ash whitefly Siphoninus phillyreae (Haliday) are common pests in many agrosystems worldwide that 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 and Hamon, 1990). Whereas T. vaporariorum and S. phillyreae can be identified based on external morphology (Figure 3), B. tabaci genetic groups (species) 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 *et al* 2008). All whitefly species are known to harbor the primary endosymbiont *Portiera aleyrodidarum* (Thao and Baumann, 2004b), which supplements their unbalanced phloem diet. *P*. *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 Gammaproteobacteria *Arsenophonus* (Enterobacteriales), *Hamiltonella* (Enterobacteriales) (Thao and Baumann, 2004b; Moran *et al* 2005), *Fritschea* (Chlamydiales) (Everett *et al*., 2005) and *Cardinium* (Bacteroidetes) (Weeks and Breeuwer, 2003) and the Alphaproteobacteria *Rickettsia* (Rickettsiales) (Gottlieb *et al*., 2006) and *Wolbachia* (Rickettsiales) (Zchori-Fein and Brown, 2002), whose function is unknown. In other insects, *Wolbachia* and *Rickettsia* have been reported to manipulate their host's reproduction via parthenogenesis induction, male killing, feminization and cytoplasmic incompatibility (Dale and Moran, 2006). *Hamiltonella defensa*, a secondary symbiont from the pea aphid *Acyrthosiphon pisum* (Harris), has been shown to confer resistance against the parasitoids *Aphidius ervi* (Haliday) and *Aphidius eadyi* Stary, González & Hall (Oliver *et al*., 2003; Ferarri *et al*., 2004; Bensadia *et al*., 2005). The secondary symbiont *Arsenophonus nasoniae* has been shown to induce a female-biased sex ratio distortion in the parasitoid wasp *Nasonia vitripennis* (Walker), similar to the phenotype observed with some strains of *Wolbachia* spp. (Werren *et al*., 1986; Gherna *et al*., 1991). *Cardinium* has been implicated in several cases of reproductive manipulation in arthropods (Zchori-Fein and Perlman, 2004). A clear association between common *B. tabaci* genetic groups (species) and secondary symbionts has been observed in Israeli populations: *Hamiltonella* is detected only in the MEAM1, *Wolbachia* and *Arsenophonus* only in the MED and *Rickettsia* in both genetic groups (Chiel *et al*., 2007). *Fritschea* has only been detected in the New World (commonly known as A biotype) group, from the United States (Thao *et al*., 2003), and only *Arsenophonus* has been associated with *T. vaporariorum* (Thao and Baumann, 2004a). Recent studies have shown that different single and mixed infections with

secondary symbionts in *B. tabaci* can affect the insects' ability to tolerate synthetic pesticides (Kontsedalov *et al*., 2008; Ghanim and Kontsedalov, 2009).

Among *B*. *tabci* transmited viruses, *Tomato yellow leaf curl virus* (TYLCV) (*Begomovirus, Geminiviridae*) is one of the most economically important viruses. It is the type member and representative of the complex of viruses associated with the tomato yellow leaf curl disease (TYLCD) with ssDNA genome, a plant-infecting group of viruses that have single or double genomic components enveloped by an icosahedral coat protein. These viruses infect tomatoes and other vegetable and ornamental crops and cause severe losses estimated by billions of dollars each year. TYLCV affects the plant phloem and it is exclusively transmitted by *B. tabaci* (Czosnek and Laterrot*,* 1997; Czosnek *et al.,* 2001). Differences in the bacterial symbiont complements in the two *B. tabaci* genetic groups (MEAM1 and MED) raised the hypothesis that the efficiency of TYLCV transmission by the MEAM1 and MED depends on a secondary symbionts composition. TYLCV transmission depends upon a 63-kDa GroEL protein produced by the vector's symbiotic bacteria. Taking into account all symbionts, the GroEL protein produced by *Hamiltonella* (present in the MEAM1, but absent in the MED) enables TYLCV transmission, while the other symbionts from both biotypes do not seem to be involved in transmission of this virus (Gottlieb *et al.*, 2010).

1.1. The aim, specific objectives and scope of the work, methods and hypothesis

In Croatia, Bosnia and Herzegovina and Montenegro, whiteflies (*B. tabaci*, *T. vaporariorum* and *S. phillyreae*) were incompletely explored. The aim of this doctoral disertation was to define the status of these important agricultural pests, using an arsenal of biological and molecular analyses.

Specific objectives and scope of the work were:

1) To describe the geographic distribution of three whitefly species in Croatia, Montenegro and selected areas of Bosnia and Herzegovina;

2) To identify species of *B.tabaci* and infection status and co-infections by secondary symbionts (*Rickettsia*, *Hamiltonella*, *Arsenophonus*, *Wolbachia*, *Cardinium* and *Fritschea*) in sampled populations of three whitefly species;

3) To record spatial localization of secondary symbionts within the all developmental stages of *B. tabaci*, *T. vaporariorum* and *S. phillyreae*;

4) To monitor the appearance of plant viruses with emphasis on the occurrence of whitefly transmitted, geminiviruses (in particular TYLCV), in vegetables, in Croatia, Montenegro and selected areas of Bosnia and Herzegovina;

5) To test TYLCV transmission efficiency of Croatian MED *B. tabaci*.

Methods used to accomplish the mentioned objectives:

1) Morphological identification of the whiteflies (Martin *et al.*, 2000);
2) Whitefly sampling with Pasteur pipette attached to a hand-held aspirator and their rearing in the insect proof cages placed in the growth chamber;
3) DNA extraction (from single insect individual (Shahjahan *et al*.,1995), multiple insect individuals and plant samples);
4) Polymerase Chain Reaction (PCR) detection of *B. tabaci* species, secondary bacterial symbionts and geminiviruses;
5) Cloning, colony PCR and sequencing of the insert of interest (secondary symbiont, phytovirus);
6) Fluorescence *in situ* hybridization analysis (FISH) of symbionts in developmental stages of *B. tabaci* and *T. vaporariorum*, and TYLCV in tomato stems (Sakurai *et al*., 2005);
7) Transmission Electron Microscopic (TEM) analysis of *T. vaporariorum*;
8) Acquisition and transmission of TYLCV by *B. tabaci* species.

Hypothesis:

Whitefly populations, collected in the surveyed countries harbor different symbiontic contents, based on their phylogenetic distance. The different symbiotic contents are expected to have different effects on TYLCV transmission, especially within the *Bemisia* group. Understanding these insect-symbiont-virus interactions are expected to contribute to our understanding these multitrophic interactions, for designing approaches for better whitefly control.

2. LITERATURE REVIEW

2.1. The whiteflies (Hemiptera: Aleyrodidae)

2.1.1. Systematics, morphology and life cycle

Order: Hemiptera

Suborder: Sternorrhyncha

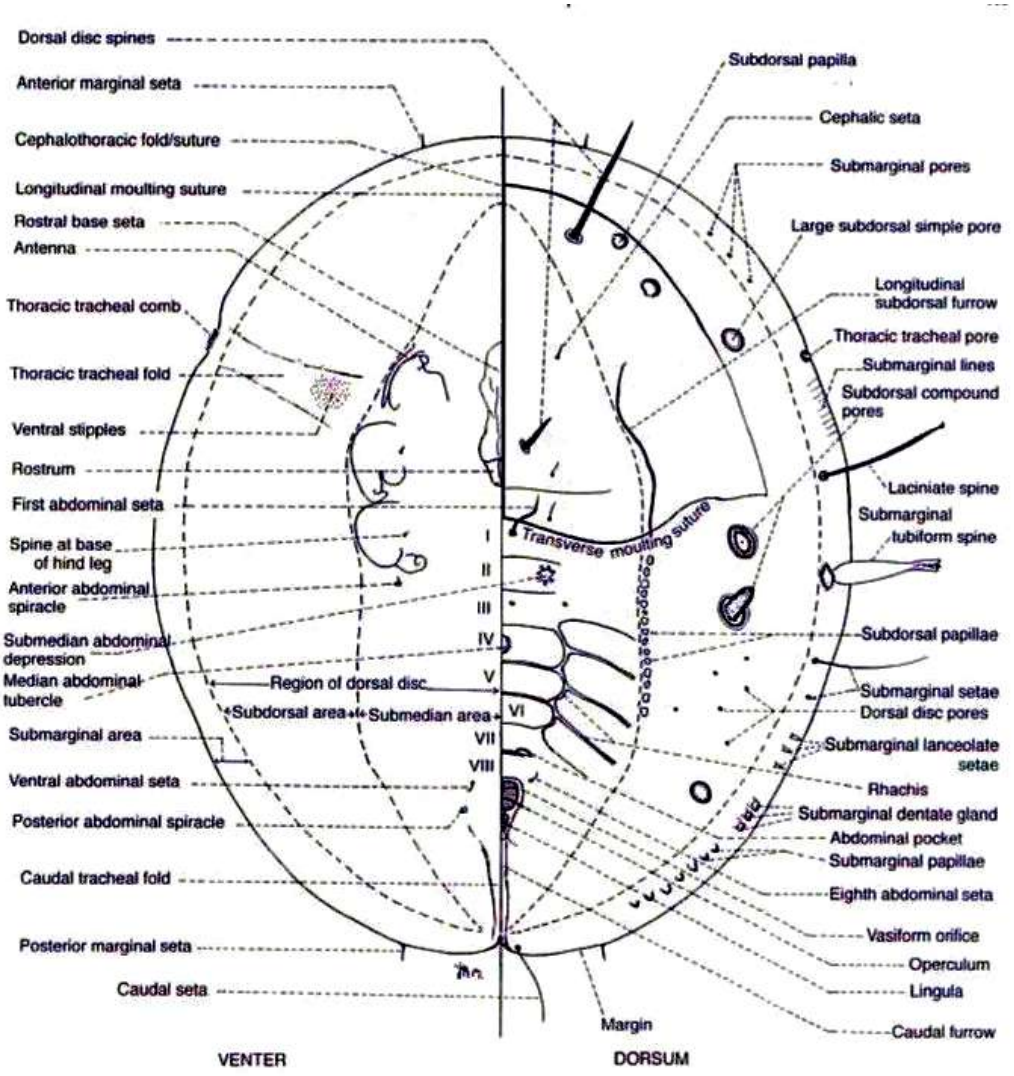
Superfamily: Aleyrodoidea

Family: Aleyrodidae

Whiteflies are small phloem sap-sucking insects that belong to the order Hemiptera and comprise a single superfamily, Aleyrodoidea, within the suborder Sternorrhyncha. They are all placed in a single family, Aleyrodidae (Martin *et al*., 2000). The common name “whiteflies” was obtained due to waxy wings and bodies of the adults. There are approximately 1500 known species of whiteflies (Martin, 2004). Of these, 33 species from 20 genera are considered common and economically important. Adult whiteflies are 1-3 mm in body length, where females are bigger in body size than males. Other features of sexual dimorphism manifest through the differences in genitalia, the numbers of ventral abdominal wax plates and antennae. Both males and females are winged, having 4 membranous wings without cross veins. Reproduction in whiteflies is sexual and occasionally arrhenotokyous partenogenesis. Many species lay their eggs in one or more semicircular or circular concentric rows, but others scatter their eggs over the leaf. Each female whitefly can produce approximately 200 eggs in a lifetime (http://www.issg.org/database/). The number of larval stages (instars) is four, and the fourth stage is usually termed a puparium (Hodges and Evans, 2005). This stage with red eye spots is often incorrectly called pupal stage, because insects in the order Hemiptera have paurometabolus metamorphosis (Byrne and Bellows, 1991). The first instar whitefly larvae, usually called ‘crawlers’, are mobile and can walk a short distance to locate suitable feeding sites. The remaining three larval instars are sessile and individuals are unable to relocate themselves if feeding conditions deteriorate (Figure 1).

Figure 1. Developmental stages of whiteflies (e.g. *Bemisia tabaci*). (a, b, c and d): adult (a), eggs (b), nymphal stages (c) and pupa (d). Arrow indicates developmental direction.

The adults leave the pupal case through an inverted T-slit in the dorsum. Immediately after emergence, the adults of many species begin to feed, even before the wings are unfolded. The systematics of the whiteflies is currently based almost entirely on the puparial stage (Martin *et al.*, 2000) (Figure 2). Duration of the life cycle in whiteflies depends on the species, temperature, relative humidity (RH) and host plant (Lopez-Avila, 1986).



(Martin *et al*., 2000)

Figure 2. General morphology of a whitefly puparium.

External morphology of the puparial stage alterate, due to physical characteristics of leaf surfaces. Some species of Aleyrodidae have more than one generation per year, and in tropical to subtropical climates, continuous generations may occur with slowed development during short, cool periods. In contrast, several species on non-herbaceous hosts have only one or two generations. Amongst the Sternorrhyncha, whiteflies appear to be a recently evolved group, with the oldest known fossil remains 135 million years ago (Schlee, 1970). Regarding the host plants, majority of whiteflies colonize only dicotyledonous angiosperms, while small number feed on monocots.

This doctoral dissertation involves three economically important whitefly species in Croatia and two neighboring countries. These are: ***Bemisia tabaci* (Gennadius, 1889)**, ***Trialeurodes vaporariorum* (Westwood, 1856)** and ***Siphoninus phillyreae* (Haliday, 1835)** (Figure 3).

Figure 3. Phenotypical differences between three whitefly species. (a, c and e): adults of *Bemisia tabaci* (a), *Trialeurodes vaporariorum* (c) and *Siphoninus phillyreae* (e). (b, d and f): pupal stages of *B. tabaci* (b), *T. vaporariorum* (d) and *S. phillyreae* (f).

2.2. Species, ecology and pest status of Bemisia tabaci, Trialeurodes vaporariorum and Siphoninus phillyreae

2.2.1. General remarks on *Bemisia tabaci, Trialeurodes vaporariorum* and *Siphoninus phillyreae*

a) *Bemisia tabaci* (common names: cotton/tobacco/cassava/sweet potato whitefly) is an extremely polyphagous cosmopolitan insect adapted to arid desert, tropical, subtropical and Mediterranean conditions, where prolonged freezing temperatures are rare or nonexistent (Brown, 2007). *B. tabaci* has been spread throughout the world through the transport of infested plant products and ornamentals. Small size and big reproductive potential enabled *B. tabaci* to spread fast and to have explosive population growth. According to 'Global Invasive Species Database' *B. tabaci* is placed in a 100 of the world's worst invasive alien species (http://www.issg.org). It also represents an insect with the highest diversity of bacterial symbionts (Gottlieb *et al.,* 2008). *B. tabaci* feeds on more than 600 plant species (Secker *et al.*, 1998), transmits 111 phytoviruses (Jones, 2003) and easily becomes resistant to wide array of insecticides (Palumbo *et al.*, 2001). In the sweet potato whitefly, development from egg to adult on 25°C (65% RH, host plant: *Lycopersicon esculentum* Mill.) is 22.3 days (Salas and Mendoza, 1995). *B. tabaci* is a complex of 11 major clades containing at least 24 morphologically indistinguishable species. Phylogenetic analysis based on mitochondrial cytochrome oxidase I (mtCOI) molecular clocks revealed that origin of *B. tabaci* stretches back approximately 4 million years (De Barro *et al.*, 2011).

b) *Trialeurodes vaporariorum* (common names: greenhouse or glasshouse whitefly) is alike *B. tabaci*, very polyphagous and cosmopolitan, although, less common in tropical Asia. Duration of the life cycle (egg to adult) in greenhouse whitefly, on the *L. esculentum* Mill. and the temperature of 22°C, lasts 28 days (Smith, 2009). *T. vaporariorum* is known to share habitat with *B. tabaci* (pers. observation) (Figure 8 a) and transmits three phytoviruses of no economic importance (Jones, 2003). Excessive

use of insecticides has resulted in *T. vaporariorum* resistance to different insecticide classes (Omer *et al.,* 1992, 1993).

c) *Siphoninus phillyreae* (common name: ash whitefly) is widely distributed (Europe and Mediterranean countries, except Scandinavia, Middle East, Ethiopian region; Oriental region (India, Pakistan); Australia, New Zeland, Mexico, USA (California, Florida). *Siphoninus phillyreae* is an oligophagous insect, preferring woody hosts such as Oleaceae, Punicaceae, Rosaceae, particularly from genus *Crataegus*, *Fraxinus*, *Olea*, *Phillyrea*, *Pyrus* and *Citrus*. Life cycle duration (egg to adult) on 25°C in ash whitefly lasts 27.7 days on pomegranate (*Punica granatum* L.) (Leddy *et al*., 1995). *S. phillyreae* is not known as a virus vector (Jones, 2003).

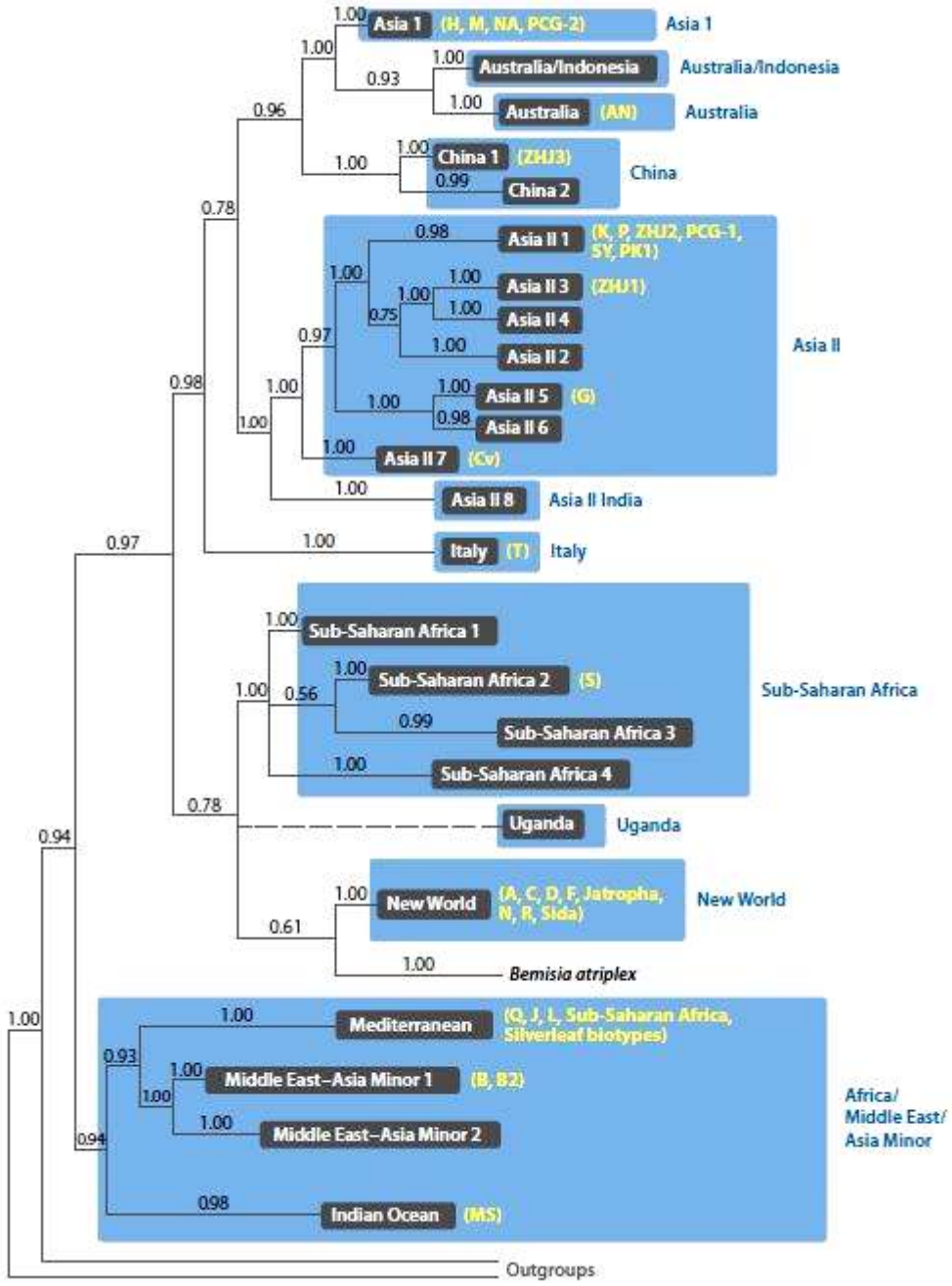
2.2.2. Species complex of *Bemisia tabaci*

The genetic complexity within *B. tabaci* was first recognized in the 1950s when morphologically indistinguishable populations were reported to differ in host range, host plant adaptability, and plant virus-transmission capabilities (Bird, 1957; Mound, 1963; Costa and Russell, 1975; Bird and Maramorosch, 1978). This led to the development of the concept that *B. tabaci* was composed of a series of biotypes (Costa and Brown, 1991; Bedford *et al*., 1994). Bellows *et al*. (1994), initiated species debate by raising the commonly named B biotype to a species status of *Bemisia argentifolii* Bellows and Perring, due to specific phytotoxic silverleafing symptoms it caused. At least 36 biotypes have been reported based on differences in biological characteristics (De Barro *et al*., 2011).

The allozymes, especially esterase and *randomly amplified polymorphic DNA – polymerase chain reaction* (RAPD-PCR), were used to separate different *B. tabaci* groups (Wool *et al*., 1989; Gawel and Bartlett, 1993) and this provided the first insights into genetic variability of *B. tabaci*. Whereas, ribosomal genes (16S rDNA), nuclear ribosomal integenic transcribed spacer 1 (ITS1), mitochondrial cytochrome oxidase I (mtCOI) and microsatellites uncovered phylogenetic structure of *B. tabaci*.

In general, biotypes in *B. tabaci* are primarily distinguished based on genetic markers and not on the base of biological characteristics; therefore the term biotype is inadequate and misleading. Finally, the biotype terminology was needed to be changed. Based on mtCOI phylogenetic analysis Dinsdale *et al*. (2010) postulate that *B. tabaci* is a complex of 11 groups (Asia 1, Australia/Indonesia, Australia, China, Asia II, Asia II India, Italy, Sub-Saharan Africa, Uganda, New World and Africa/Middle East/Asia Minor), consisting of 24 species (Figure 4). Hu *et al*. (2011) used the mentioned phylogenetic analysis and increased the number of *B. tabaci* species to 28. Gueguen *et al*. (2010) based on COI phylogeny differentiate six genetic groups: B, Q1 (western Mediterranean populations), Q2 (Middle Eastern populations), Q3 (Burkina Faso), ASL and MS).

Parasitoid diversity led to conclusion that the centre of *B. tabaci* evolution was Indian subcontinent (Mound and Halsey, 1978), while today, based on mtCOI phylogenetic analysis, it is considered that sub-Saharan Africa may be the origin of *B. tabaci*.



(De Barro *et al*., 2011)

Figure 4. The summary of the evolutionary relationships of the 11 groups (blue boxes) and 24 species (black boxes) of *Bemisia tabaci* based on mitochondrial cytochrome oxidase I (mtCOI) phylogenetic analysis. Commonly known biotypes are indicated in the brackets. *Bemisia atriplex* is insufficiently explored group, due to lack of mtCOI information.

2.2.2.1. Most common and invasive *Bemisia tabaci* species; asymmetric mating interactions and thermotolerance

The two best-known *B. tabaci* species are: MEAM1 and MED, which together have proven to be extremely invasive. MED is thought to have a native range extending from the western Mediterranean Basin through to Egypt while MEAM1 extends through the Middle East into Asia Minor (Frohlich *et al*., 1999; De Barro *et al*., 2000).

The MEAM1 and MED now predominate in agricultural systems in tropics, subtropics and temperate areas (Brown, 2007) and they have overlapping host range in vegetable crops and ornamentals. Frohlich *et al*. (1999) concluded that MEAM1 group was introduced from Old World to New World (e.g. USA, Puerto Rico) via the international plant trade. This statement was initially supported by biological differences between MEAM1 and indigenous New World group, whereas this was later supported by phylogenetical analysis. Comparing to New World group, MEAM1 has broader range of host plants, as well as the ability to disperse across long distances, in search of new host plants (Brown *et al*., 1995). New World genetic group of *B. tabaci* do not exhibit this behavior. Arid climate, where food sources are not plentiful through the whole year, forced MEAM1 to adapt to a wide range of host plants (Frohlich *et al*., 1999).

Circumstantial evidence indicates that in many regions, introduction of the MEAM1 has led to the displacement of the indigenous *B. tabaci* population, belonging to different genetic groups (Brown *et al*., 1995; Perring, 1995; Lima *et al*., 2002; Zang *et al*., 2006). In China (Zhejiang) and Australia (Queensland), indigenous groups Asia II 3 and Australia, respectively, were completely displaced by MEAM1 group of *B. tabaci* in a period less than 15 years (Liu *et al*., 2007). Liu *et al*. (2007) conducted a study to investigate the process of replacement of indigenous genetic group of *B. tabaci* by the invading MEAM1. The competitive ability of MEAM1 results partly from its capacity to adjust sex ratio in favor of its population increase, and partly from its capacity to interfere with the mating of indigenous individuals. When the proportion of males is increased in mixed (invading and indigenous group) population, MEAM1 adults respond by increasing the frequency of copulation and consequently increasing the proportion of female progeny. Interestingly, MEAM1 females respond independently of whether the

males are all MEAM1 or a mix. This interaction is extraordinary because the indigenous males actually help to promote copulation among the invaders and consequently increase the invaders' competitive capacity. In contrast, the indigenous females do not respond to increased numbers of adult males. Moreover, copulation by indigenous individuals is partly blocked by MEAM1 males that readily attempt to court with females of either genetic group. These asymmetric mating interactions have obvious population-level implications because the increase in the proportion of MEAM1 females and the concomitant decrease in the proportion of indigenous females results in an immediate higher population growth rate for MEAM1 and a lower growth rate for the indigenous population. Identification of the progeny using DNA markers detected no hybrids between MEAM1 and Asia II 3 or Australia genetic groups, which confirmed reproductive isolation between these tested groups.

In many countries it was reported that MED is replacing MEAM1, possibly, due to climate changes (e.g. global warming) or continuous and repeated insecticide spraying. MED is known to develop resistance to most of the insecticide groups (Horowitz *et al*., 2005) and their continuous use can lead to MED dominance.

Mahadav *et al*. (2009) conducted an experiment to compare gene expression and thermotolerance in the MED and MEAM1 before and after exposure to heat stress. This experiment monitored expression of heat stress, cytoskeleton and mitochondria genes, which may be induced or repressed in abiotic stress conditions, such as high temperature. Mortality rate at 40°C was in MEAM1 twice of those in MED group, while differences in thermotolerance were linked mainly to genetic background, rather than biological characteristics (e.g. bacterial symbionts). Majority of the monitored genes were higher expressed only in the MED group during the heat-shock, while some mitochondrial genes showed decreased levels of expression in both genetic groups of *B. tabaci*, or induction in one group and repression in the other. Genes such as *ATP synthase* and *cytochrome oxidase subunit 1* were induced in MEAM1 and repressed in MED, while *NADH-ubiquinone oxidoreductase* was repressed in MEAM1 and induced in MED. It seems that MED group down-regulates the expression of the most of the mitochondrial genes related to mitochondrial respiration and energy production. Many genes involved in the mentioned study, which are related to adaptation to stress

conditions, are already up-regulated under normal 25°C conditions which may suggest that the MED group can better and rapidly adapt to changing climates while the MEAM1 may less tolerate these changes.

2.2.3. Bacterial symbionts of the whiteflies

Insects host a wide diversity of symbiotic bacteria, which have important ecological phenotypes ranging from parasitism to mutualism (Buchner, 1965; Moran *et al*., 2008). It is estimated that average 15% of all insect species harbor bacterial symbionts (Douglas, 1998). Symbiotic bacteria might affect: development (Braendle *et al*., 2003; Koropatnick *et al*., 2004), nutrition (Baumann, 2005; Backhed *et al*., 2005), reproduction and speciation (Stouthamer *et al*., 1999; Hurst and Jiggins, 2000; Bandi *et al*., 2001; Hurst and Werren, 2001), defense against natural enemies (Piel, 2002; Oliver *et al.,* 2003; Scarborough *et al.,* 2005), and immunity (Macdonald and Monteleone, 2005).

Primary or obligatory bacterial symbionts, or obligate mutualist, live in host organs (specialized for housing symbiotic associates) called bacteriosomes which consist of bacteriocytes (Figure 5). All whiteflies have a small, paired, and roundish or oval orange bacteriosome (Buchner, 1965). Depending on the host group, the bacteriosome may consist of fat body cells, gut wall cells, or highly specialized cells developed in embryos (Buchner, 1965; Braendle *et al*., 2003).

Studies using electron microscope have indicated that whitefly bacteriocytes contain a pleomorphic bacterium called *Portiera aleyrodidarum* (Thao and Baumann, 2004b). These bacteria are essential for the host and required for supplementing unbalanced phloem sap diet, which is rich in carbohydrates and deficient in essential amino acids (Sandström and Moran, 1999). *P. aleyrodidarum* is AT-rich member of the gamma subdivision of the Proteobacteria (Baumann, 2005) and is exclusively vertically transmitted through migration of the bacteriocytes to the ovaries and the eggs (Costa *et al*., 1996). Primary symbionts, have extremely reduced genome and they lack mobile genetic elements, bacteriophage and repetitive DNA, while secondary or facultative

bacterial symbionts, have larger genome with many repetitive regions and unusually high numbers of mobile elements (Moran *et al*., 2008).

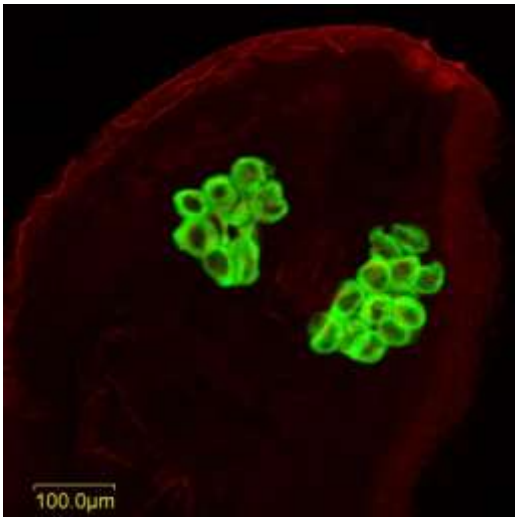


Figure 5. Fluorescent *in situ* hybridization (FISH) of the pair bacteriosomes consisted of bacteriocytes (green) in *Bemisia tabaci* nymph.

Secondary symbionts, usually do not reside in specialized organs and are not necessary for host survival. They can invade various cell types, including reproductive organs, and may reside extracellularly in the body cavity (hemolymph) (Dobson *et al*., 1999; Fukatsu *et al*., 2000; Brumin *et al*., 2012). In many cases, secondary symbionts experimentally introduced to previously uninfected hosts, establish stable, maternally inherited infection (Pontes and Dale, 2006; Chen *et al*., 2000) indicating that the persistence of the symbiosis is largely achieved through symbiont capabilities rather than host adaptations for maintaining symbiosis. In hosts with bacteriosomes, secondary symbiont may invade bacteriocytes where they co-reside, or even exclude primary symbiont (Buchner, 1965; Fukatsu *et al*., 2000; Gottlieb *et al*., 2008). Secondary symbionts can be divided into two groups: 1) Facultative mutualists: are beneficial in terms to provide their hosts to live longer and to reproduce more. These benefits include protection against natural enemies, heat, or other mortality factors. 2) Reproductive manipulators: are parasites that spread by increasing host reproduction through daughters at the expense of reproduction through sons (Stouthamer *et al*., 1999; Himler *et al*., 2011). They often provide incompatibility between infected and uninfected strain; son-killing (which

potentially increases investment in daughters); feminization of genetic males; and parthenogenesis. The best studied reproductive manipulator is *Wolbachia pipientis*, which is widely distributed in arthropods (Stouthamer *et al*., 1999).

Secondary symbionts often occur at low titers in hosts and show irregular distribution among host tissues and species. In general, they are typically vertically transmitted from mother to offspring and often transmission rates are near 100% (Ferrari and Vavre, 2011). Aditionally, secondary symbionts can be transferred horizontally between individuals and between species (Sandström *et al*., 2001; Russell *et al*., 2003; Caspi-Fluger *et al*., 2012). It is suggested that primary symbiont evolve from secondary symbiont that lose horizontal transmission capabilities. For example, genus *Arsenophonus* comprises secondary symbionts that are sometimes reproductive manipulators and harmful to hosts (Gherna *et al*., 1991) and sometimes beneficial, but facultative (Russell *et al*., 2003; Baumann, 2005; Hansen *et al*., 2007), whereas *Wolbachia* and *Rickettsia* show a spectrum of interactions with hosts, ranging from pathogenic to obligatory for host survival and development (Perlman *et al*., 2006; Perotti *et al*., 2006; Darby *et al*., 2007). In general, symbionts that are strictly vertically transmitted must either increase the fitness of their host or manipulate host reproduction in ways that benefit their own transmission (Bull 1983; O'Neill *et al*., 1997). *B. tabaci* populations from around the world have been reported to harbor several secondary symbionts, including *Hamiltonella*, *Arsenophonus*, *Cardinium*, *Wolbachia*, *Rickettsia* and *Fritschea* (Nirgianaki *et al*., 2003; Baumann, 2005; Gottlieb *et al*., 2006; Li *et al*., 2007), while only *Arsenophonus* was reported from populations of *T. vaporariorum* and *S. phillyreae* (Thao and Baumann, 2004a).

Secondary symbionts in *B. tabaci* significantly influence its ability to tolerate insecticides. It was reported that single but mainly double infections with secondary symbionts significantly influence susceptibility of *B. tabaci* (MED) to insecticides (Ghanim and Kontsedalov, 2009) and to heat stress (Brumin *et al*., 2011).

The same host individual can be multiple infected with several symbionts, most often in the form of one or two primary symbionts and one or two secondary symbionts (Dale and Moran, 2006).

2.2.3.1. *Rickettsia*

Domain: Bacteria
Phylum: Proteobacteria
Class: Alphaproteobacteria
Order: Rickettsiales
Family: Rickettsiaceae
Genus: Rickettsia

Bacteria in the genus *Rickettsia* are intracellular and best known as arthropod-vectored pathogens of vertebrate hosts (Raoult and Roux 1997). *Rickettsia* species are the causative agents of numerous diseases of humans, including epidemic typhus (Zinsser, 1963).

Members of the genus *Rickettsia* were traditionally classified into: 1) the spotted fever group (*Rickettsia conorii* and *Rickettsia rickettsii* vectored by ticks); and 2) the typhus group, including *Rickettsia typhi*, the cause of murine typhus, transmitted by fleas and *Rickettsia prowazekii*, the agent of epidemic typhus, transmitted by lice. Surveys of microbial diversity of many hosts, particularly arthropods, are continually uncovering new *Rickettsia*, including many that do not fall into the previously recognized subgroups. These have received much less attention than their relatives in blood-feeding hosts because they are not clearly pathogenic to humans, and in most cases, their effects on their hosts have not been discovered (Perlman *et al*., 2006). Most *Rickettsia* species appear to be facultative symbionts of invertebrates.

The *Rickettsia* involved in reproductive manipulation was the first found in a host that is not a blood feeder. The *Rickettsia* in the ladybird beetle *Adalia bipunctata* is associated with male embryo mortality (Werren *et al*., 1994). Recently, the first strong evidence for an association between *Rickettsia* and parthenogenetic reproduction was reported. In parthenogenetic populations of the parasitoid *Neochrysocharis formosa* (Hymenoptera: Eulophidae), over 99.5% of individuals are females and all appear infected with *Rickettsia*, but not *Wolbachia*, *Cardinium* or other symbionts (Hagimori *et al*., 2006).

A number of *Rickettsia* that appear closely related to *R. bellii* are symbionts of herbivorous arthropods, with the best-studied, being the *Rickettsia* infecting the pea aphid *Acyrthosiphon pisum*. The effect of *Rickettsia* on pea aphids is not clear, but negative effects such as reduced body weight, lower fecundity and suppressed *Buchnera* (primary symbiont of aphids) densities have been reported (Chen *et al*., 2000; Sakurai *et al*., 2005).

Rickettsia-infected whiteflies compared with uninfected whiteflies, produced more offspring, had higher survival to adulthood, developed faster, and produced a higher proportion of daughters. The symbiont thus functions as both mutualist and reproductive manipulator (Himler *et al*., 2011). Brumin *et al*. (2011) revealed that exposing a *Rickettsia*-infected whitefly population to increasing temperatures significantly increases its tolerance to heat shock (40°C), compared to a *Rickettsia*-free population.

Furthermore, parthenogenetic booklouse *Liposcelis bostrychophila* (Psocoptera) is infected with *Rickettsia* that is a strict obligatory symbiont, localized in the bacteriocytes. Essential role of this *Rickettsia* is the early development of the oocyte (Perotti *et al*., 2006).

The diversity of transmission modes of *Rickettsia* is striking, and includes horizontal, vertical and mixed transmission (Perlman *et al*., 2006). In glossiphoniid leeches, eggs are all infected with *Rickettsia*, suggesting a high rate of vertical transmission (Kikuchi *et al*., 2002; Kikuchi and Fukatsu, 2005). Gottlieb *et al*. (2006) postulate that the *Rickettsia* enters the oocyte together with the infected bacteriocytes, then multiplies and spreads throughout the egg during embryogenesis.

It is suggested that most of *Rickettsia* travel in the arthropod host from the gut to the haemocoel and then to the salivary glands where they may be horizontally transmitted to the host (Perlman *et al*., 2006).

Interestingly, it has been shown that *Empoasca* planthoppers (*Rickettsia*-infected) infect papaya with mentioned symbiont, causing ‘papaya bunchy top disease' (Davis *et al*., 1998). So far, this is the only *Rickettsia* identified as a plant pathogen.

Caspi-Fluger *et al*. (2011a) performed a combination of experiments where it was found that *Rickettsia* were transferred from *B. tabaci* to a plant (cotton, basil, black

nightshade), moved inside the phloem, and could be acquired by other whiteflies. This experiment also confirmed that plants can serve as a reservoir for horizontal transmission of *Rickettsia*, a mechanism which may explain the occurrence of phylogenetically similar symbionts among unrelated phytophagous insect species.

The most unusual form of transmission is in *R. prowazekii*, which appears to be better adapted to its vertebrate host than its louse host (Azad and Beard, 1998). *R. prowazekii* is pathogenic to the louse, generally killing it within two weeks, and is not transovarially transmitted.

In Israel, *Rickettsia* in *B. tabaci* showed two localization patterns. In the ‘‘scattered’’ pattern, *Rickettsia* is localized throughout the whitefly hemocoel and different tissues, excluding the bacteriocytes, while in the ‘‘confined’’ pattern, *Rickettsia* is restricted to the bacteriocytes (Gottlieb *et al*., 2008; Caspi-Fluger *et al*., 2011b). Kontsedalov *et al*. (2008) revealed that, in the presence of *Rickettsia*, the whitefly’s susceptibility to five out of the six insecticides tested (thiametoxam, acetamiprid, imidacloprid, diafenthiuron, pyriproxyfen, spiromesifen) was increased, in spite of their variable mode of action and target stages.

Co-infections of multiple *Rickettsia* strains, and of *Rickettsia* and other symbionts, are common and we would expect strong competition among symbionts for access to host cells, resulting in trade-offs between transmission modes and tissue specificities (Perlman *et al*., 2006).

2.2.3.2. *Wolbachia*

Domain: Bacteria
Phylum: Proteobacteria
Class: Alphaproteobacteria
Order: Rickettsiales
Family: Rickettsiaceae
Genus: Wolbachia

Wolbachia is a group of bacteria, associated with reproductive alterations in their hosts (Werren, 1997; Stouthamer *et al*., 1999; Stevens *et al*., 2001). Many studies place *Wolbachia* among the most common intracellular bacteria known, with estimates of several million infected species (Werren *et al.*, 1995; Jayaprakash and Hoy, 2000; Werren and Windsor, 2000; Hilgenboecker *et al*. 2008; Werren *et al*., 2008). Jeyaprakash and Hoy (2000), recorded that 76% of 63 arthropod species were *Wolbachia* infected. The widespread distribution of *Wolbachia* as well as the manipulation of host's reproductive system places this symbiont among the most promising targets for disease/pest control. A significant amount of *Wolbachia* genomic information is available since the genome of four *Wolbachia* strains (*w*Mel, *w*Ri, *w*Pip and *w*Bm) has been completed (Wu *et al*., 2004; Foster *et al*., 2005; Klasson *et al*., 2008; Klasson *et al*., 2009).

Wolbachia is mainly localized in the reproductive tissues of arthropods and it has developed a number of strategies to ensure transmission by manipulating the host reproductive system (Werren *et al.*, 2008). These strategies include: a) feminization, the conversion of genetic males into females, b) parthenogenesis, the production of offspring in the absence of sexual reproduction, c) male killing, the killing of infected males to the benefit of infected female siblings and d) cytoplasmic incompatibility (CI), the inability of infected males to successfully fertilize eggs from either uninfected females or from females infected with different *Wolbachia* types.

Feminization - The conversion of genetic male offspring into females doubles the potential *Wolbachia* transmission to the following generation. In terrestrial isopod hosts,

Wolbachia within genetic males inhibits the development of the androgenic gland and the production of the androgenic hormone (Azzouna *et al*., 2004). **Parthenogenesis**, the production of female offspring in the absence of sperm fertilization offers an obvious advantage to a maternally inherited microorganism. Currently documented cases of *Wolbachia* induced parthenogenesis are found within haplodiploid species belonging to Thysanoptera (Arakaki *et al.*, 2001), Acari (Weeks and Breeuwer, 2001) and Hymenoptera (Stouthamer *et al.*, 1993; Zchori-Fein *et al.,* 1995).

The killing of genetic males by *Wolbachia* has been described in four different Arthropod orders: Diptera (Hurst *et al.*, 2000; Dyer and Jaenike, 2004), Coleoptera (Fialho and Stevens 2000; Majerus *et al*., 2000), Lepidoptera (Jiggins *et al.*, 2000) and Arachnida (Zeh *et al.*, 2005).

Despite the fact that **cytoplasmic incompatibility (CI)** is the most commonly described reproductive abnormality induced by *Wolbachia*, the underlying mechanism still remains under investigation. CI can be *unidirectional* or *bidirectional*, depending on the number of *Wolbachia* strains involved (Breeuwer and Werren, 1990; O'Neill and Karr, 1990; Bourtzis *et al.*, 1998; Bourtzis *et al.,* 2008). *Unidirectional* CI describes the embryonic lethality observed when a *Wolbachia*-infected male mates with an uninfected female. All the other possible crosses are fully compatible, favoring the relative fitness of infected females and the spread of *Wolbachia*. *Bidirectional* CI occurs between populations infected with different *Wolbachia* strains, when an infected male mates with a female lacking the same *Wolbachia* strain (Werren, 1998; Bordenstein, 2003; Telschow *et al*., 2005).

In addition to the above mentioned reproductive abnormalities, *Wolbachia* can positively or negatively influence other aspects of host fitness. In *Aedes albopictus* (Diptera: Culicidae), fitness benefits resulting from *Wolbachia* infection affect both fecundity and longevity (Dobson *et al.*, 2002). Infected females produce more eggs and live longer than uninfected females while no effect on males has been observed.

Which type of manipulation is expressed depends on bacterial properties as well as host genomic background (Boyle *et al*., 1993; Poinsot *et al*., 1998).

Molecular phylogeny of *Wolbachia* is not parallel to that of their hosts. This strongly suggests horizontal transmission between species (Vavre *et al*., 1999) which could be a reason for its widespread distribution in arthropods. Although mechanisms of horizontal transmission are not completely known, some defined routes of *Wolbachia* transmission, between close or distant related arthropods, include predators, prey and associated competitors. To date, examples of natural or experimental horizontal transmission of *Wolbachia* between distant related insects were noted at following relations: hymenopteran parasitoids-*Drosophila* species (Vavre *et al*., 1999), Asian tiger mosquito (*A. albopictus*)-*Drosophila simulans* (Braig *et al*., 1994), intertidal amphipods *Talorchestia deshayesii* and *Orchestia gammarellus* -semiaquatic isopods (*Sphaeroma rugicauda*, *S. hookeri* and the sea slater *Ligia oceanica*) (Cordaux *et al*., 2001).

Gottlieb *et al*. (2008) localized *Wolbachia* in the eggs and nymphs of the *B. tabaci*, where it was detected mostly at the circumference of and inside the bacteriocytes. The same localization was also seen in adult females, but in some individuals *Wolbachia* concentrations could also be observed in the abdomen, outside of the bacteriocytes. The localization of *Wolbachia* in *Drosophila* reproductive organs (ovaries and testes) has been studied to understand its mode of vertical transmission and its influence on host reproduction (Clark *et al*., 2002; Veneti *et al*., 2004; Ferree *et al*., 2005). In several insect hosts, this symbiont has been found in organs such as the salivary glands, gut structures, malpighian tubes, fat bodies, and brain (Min and Benzer, 1997; Ljichi *et al*., 2002; Mitsuhashi *et al*., 2002). Coexistence of *Wolbachia* with other bacterial genera has been shown in the aphid *Cinara cedri,* where it is found in the bacteriocytes together with *Serratia symbiotica*, and in the weevil *Sitophilus oryzae*, where it coexists with the primary symbiont (Heddi *et al*., 1999; Gomez-Valero *et al*., 2004).

2.2.3.3. *Hamiltonella*

Domain: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: Hamiltonella

Hamiltonella is a maternally transmitted defensive secondary symbiont found sporadically in sap-feeding insects, including aphids, psyllids, and whiteflies (Sandström *et al*., 2001; Russell *et al*., 2003).

In pea aphids (*A. pisum*), *Hamiltonella* can block larval development of the solitary endoparasitoid wasps, *Aphidius ervi* and *Aphidius eadyi*, rescuing the aphid host (Oliver *et al*., 2003; Ferrari *et al*., 2004; Bensadia *et al*., 2005). It occurs sporadically in *A. pisum* and is beneficial only when parasitoids are present (Oliver *et al*., 2008). Infection frequencies increase under strong parasitoid attack and decrease when parasitoids are absent. In aphids, *Hamiltonella* can also be transmitted horizontally, either intraspecifically (e.g., sexually (Moran and Dunbar, 2006) or interspecifically (Russell *et al*., 2003; Degnan and Moran, 2008). Moreover, its protection has been shown to be transferable between distantly related aphid species (Oliver *et al*., 2005). *Hamiltonella* and the aphid host rely on *Buchnera* that synthesize essential amino acids from this limited carbon and nitrogen source (van Ham *et al*., 2003; Nakabachi *et al*., 2006).

Chiel *et al*. (2009) investigated transmission of *Hamiltonella* from the host *B. tabaci*, to three species of whitefly parasitoids: *Eretmocerus emiratus*, *Eretmocerus eremicus* and *Encarsia pergandiella*. *Hamiltonella* did not establish in any of the parasitoids tested, and none of the parasitoids acquired *Hamiltonella* by host feeding. The possible reason may be the localization of the symbiont within the bacteriosomes (Gottlieb *et al*., 2008). There is a lack of information about the mobility of *Hamiltonella* in whiteflies, yet the fact that *Hamiltonella* reside in *B. tabaci* bacteriocytes where they are vertically transmitted

along with the *Portiera*, suggests that *Hamiltonella* may have more limited mobility, compared to other secondary symbionts.

Hamiltonella produces 63 kDa GroEL protein (chaperonin) that binds the coat protein of *B. tabaci* transmitted *Tomato yellow leaf curl virus* (TYLCV) (Begomovirus, Geminiviridae) and therefore enables efficient transmission of the virus by its vector (Gottlieb *et al*., 2010).

2.2.3.4. *Arsenophonus*

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: Arsenophonus

Members of the genus *Arsenophonus* comprise a large group of intracellular, bacterial endosymbionts that are maternally inherited and widely distributed in arthropods of medical, veterinary, and agricultural importance (Dale *et al*., 2006).

Arsenophonus-host relationships range from parasitism to mutualism, with the various phenotypes such as reproductive manipulation (male-killing) (Gherna *et al*., 1991), phytopathogenicity (Zreik *et al*., 1998) or obligatory mutualism (Allen *et al*., 2007; Perotti *et al*., 2007). However, in most reported symbiotic associations, the impact of this symbiont on the host phenotype remains unknown (Mouton *et al*., 2012).

The same host species sometimes harbors several *Arsenophonus* strains, a pattern that is probably due to the *Arsenophonus* ability to be horizontally transferred, as recently demonstrated in the hymenopteran parasitoids of the family Pteromalidae (Duron *et al*., 2010). Previous studies have shown that whitefly species can host different strains of several bacteria (Zchori-Fein and Brown, 2002; Novakova *et al*., 2009). A limited

number of whitefly species such as: *B. tabaci*, *T. vaporariorum*, *S. phillyreae*, etc., are reported to be infected with *Arsenophonus* (Thao and Baumann, 2004a).

Localization of *Arsenophonus* was found restricted to the bacteriocytes in all *Arsenophonus*-harboring populations at all *B. tabaci* developmental stages. The highest detectable signal for this symbiont, observed as string-shaped, was found near the nuclei of the bacteriocytes (Gottlieb *et al*., 2008).

2.2.3.5. *Cardinium*

Domain: Bacteria

Phylum: Cytophaga-Flavobacterium-Bacteroides

Class: Bacteroidetes

Order: Bacteroidales

Family: Bacteroidaceae

Genus: Cardinium

Cardinium genus includes intracellularly inherited symbionts of arthropods (Zchori-Fein *et al*., 2004). They have been detected in 6–7% of arthropods screened, and has been found in the Hymenoptera, Hemiptera and Acari (Weeks *et al*., 2003; Zchori-Fein and Perlman, 2004). Recently, a high incidence (22%) has been detected in spiders (Araneae) (Duron *et al*., 2008). *Cardinium* may cause reproductive manipulation, including feminization (Weeks *et al.,* 2001), cytoplasmic incompatibility (Hunter *et al*., 2003; Gotoh *et al*., 2007) and thelytokous parthenogenesis (females produce only daughters from unfertilized eggs) (Zchori-Fein *et al*., 2001, 2004; Provencher *et al*., 2005; Groot and Breeuwer, 2006; Matalon *et al*., 2007). *Cardinium* also increases the fecundity of a mite host (Weeks and Stouthamer, 2004).

In *Encarsia hispida*, Giorgini *et al*. (2009) confirmed *Cardinium* as the causal agent of asexual reproduction, or thelytoky. Antibiotic treatment of the infected adult females results in uninfected diploid, which is very unusual. *Cardinium* is required to feminize

diploid male embryos and thus must interact with elements of the host sex determination system.

Very similar *Cardinium* strains infect distantly related insect species, which could lead to conclusion about horizontal transmission of this symbiont among insects (Zchori-Fein and Perlman, 2004).

In *B. tabaci*, Gottlieb *et al*. (2008) localized *Cardinium* in the bacteriocytes, although it was also found outside these cells in a random distribution. In nymphs, higher concentrations of this bacterium were found in the abdomen and upper part of the head, whereas, in females, the symbionts occupied abdomen tissue, as well as the bacteriocytes. In *B. tabaci* males, *Cardinium* appeared to be randomly spread. No *Cardinium* signal was detected in the thorax or head of adults.

2.2.3.6. *Fritschea*

Domain: Bacteria

Phylum: Chlamydiae

Class: Chlamydiae

Order: Chlamydiales

Family: Simkaniaceae

Genus: Fritschea

Candidatus Fritschea bemisiae is a chlamydial endosymbiont found in the bacteriocytes of *B. tabaci*, which are transmitted vertically to oocytes (Costa *et al*., 1996). However, nothing is known about the function of *F. bemisiae* in *B. tabaci* (Everett *et al*., 2005). The prevalence of chlamydiae in insects is not known.

2.2.4. World distribution of *Bemisia tabaci* secondary symbionts and species

Chiel *et al*. (2007) monitored species dependent composition of secondary symbionts in Israeli *B. tabaci* populations. They found MED and MEAM1 species, where *Hamiltonella* infected MEAM1; *Arsenophonus* and *Wolbachia* infected MED, while *Rickettsia* infected both species.

According to Gorsane *et al*. (2011), both MED and MEAM1 *B. tabaci* species were found in Tunisia, where *Hamiltonella* and *Cardinium* infected both species, *Rickettsia* infected only MEAM1, while *Wolbachia* and *Fritschea* were detected in MED species. Furthermore, MED species were only found in Moroccan and France populations (Gueguen *et al*., 2010). They were infected with *Hamiltonella* and *Cardinium*, while *Wolbachia* appeared sporadically.

Perring (2001) reviewed summarized information on *B. tabaci* species. Therefore, in Egypt, Denmark, Netherlands, Cyprus and Italy, MEAM1 groups were identified, while in Spain, MEAM1, MED and 'Sub Saharan Africa 2' species were affirmed. Žanić *et al*. (2005) recorded predominance of MED *B. tabaci* species in coastal Croatia.

Invasive MED and MEAM1 *B. tabaci* species appeared in China together with indigenous Cv (China) species (Ahmed *et al*., 2010; Chu *et al*., 2006). Both MED and MEAM1 populations were infected with *Hamiltonella*, *Rickettsia*, *Arsenophonus*, *Wolbachia* and *Cardinium*, while indigenous populations were infected with *Wolbachia* and *Arsenophonus*.

2.2.5. Plant viruses transmitted by whiteflies

Whiteflies in the genera Bemisia and Trialeurodes transmit plant viruses. In the genus Bemisia, only *B. tabaci* has been shown to be a vector, whereas in the Trialeurodes genus, *T. vaporariorum*, *T. abutilonea* Haldeman and *T. ricini* (Misra) transmit viruses.

Bemisia tabaci is a confirmed vector of 111 plant viruses in the genera Begomovirus (Geminiviridae), Crinivirus (Closteroviridae), Carlavirus (Betaflexiviridae) and Ipomovirus (Potyviridae) (Jones, 2003). Begomoviruses are the most numerous and exclusively *B.*

tabaci-transmitted viruses that cause crop yield losses of between 20% and 100% (Brown and Bird, 1992). Symptoms include yellow mosaics, yellow veining, leaf curling, stunting and vein thickening. According to Virus Taxonomy List of 2011, there have been generally determined 192 species of begomoviruses (http://www.ictvonline.org/). Economically important plant viruses transmitted by *T. vaporariorum* belong to the genus Closterovirus and Crinivirus. Both are members of Closteroviridae.

In September 2011, European and Mediterranean Plant Protection Organization (EPPO) (http://www.eppo.org/) listed following whitefly transmitted plant viruses considered as quarantine pests:

<u>- on the A1 list (a pest not present in the specific area):</u>

- *Bean golden mosaic virus* (Begomovirus) – transmitted by *B. tabaci*
- *Tomato mottle virus* (Begomovirus) – transmitted by *B. tabaci*
- *Lettuce infectious yellows virus* (Crinivirus) – transmitted by *B. tabaci*
- *Potato yellow vein virus* (Crinivirus) – transmitted by *T. vaporariorum*

<u>- on the A2 list (a pest present in the specific area, but not widely distributed and being officially controlled):</u>

- *Tomato yellow leaf curl virus* (TYLCV) and related viruses (Begomovirus) – transmitted by *B. tabaci*
- *Squash leaf curl virus* (Begomovirus) – transmitted by *B. tabaci*
- *Cucurbit yellow stunting disorder virus* (Crinivirus) – transmitted by *B. tabaci*
- *Tomato chlorosis virus* (Crinivirus) – transmitted by *T. vaporariorum*
- *Tomato infectious chlorosis virus* (Crinivirus) – transmitted by *T. vaporariorum*

In this doctoral dissertation, the special emphasis is on *B. tabaci* transmitted, TYLCV, due to its economic importance and comprehensive researches conducted in past few decades.

2.2.5.1. Tomato yellow leaf curl disease

During the 1960s a new disease reported in the Jordan valley in Israel caused severe damages to a newly introduced tomato variety to the market. This disease was later called tomato yellow leaf curl disease (TYLCD) (Cohen and Nitzany, 1966). TYLCV was found to be the causative agent of this disease and was associated with outbreaks of the whitefly *B. tabaci* populations nearby cotton fields, which were newly grown in this area. These cotton fields helped *B. tabaci* populations to build up to high levels, and outbreaks of the disease were seen afterwards. Although symptoms of TYLCD on plants were observed as early as the 1930s, outbreaks of the disease were not observed until *B. tabaci* populations greatly increased. TYLCV was observed to have geminate shape in 1980 (Russo *et al*., 1980) and a few years later the single-stranded DNA (ssDNA) genome was fully cloned and sequenced, and the virus was shown to be a monopartite geminivirus (Navot *et al*., 1991). Since the late 1990s research regarding TYLCV was focused on understanding the interactions between TYLCV, plants that it infects, and its only vector, *B. tabaci*. Research on virus–plant interactions included understanding virus movement, symptoms induction, replication, and plant response to the virus, while on the virus-vector front research was aimed to understand mechanisms of acquisition, retention, and transmission of TYLCV by *B. tabaci*.

TYLCV is known today to occur in almost all continents around the globe, including Asia, Africa, Europe, and North America (Czosnek and Latterot, 1997). In the European–Mediterranean region TYLCV has caused significant crop losses within the tomato industries of southern Spain, the Canary Islands, Portugal and parts of Italy and has been shown to infect the common weed *Solanum nigrum* L., which can act as a reservoir of infection (Bedford *et al*., 1998). TYLCV is the most widespread virus in Algeria, Canary Islands, Cyprus, Egypt, Greece, Israel, Jordan, Morocco, Portugal, Spain, Tunisia and Turkey. A recent outbreak of TYLCV in the Rhone delta area of France was believed to have been eradicated (Bertaux *et al*., 2001), but there has since been a second outbreak. Both outbreaks have been attributed to the movement of infected tomato plants from Spain. The only vector in all countries is *B. tabaci* and epidemics are often associated with a rise in populations of this vector.

2.2.5.2. Interactions of *Bemisia tabaci* and TYLCV

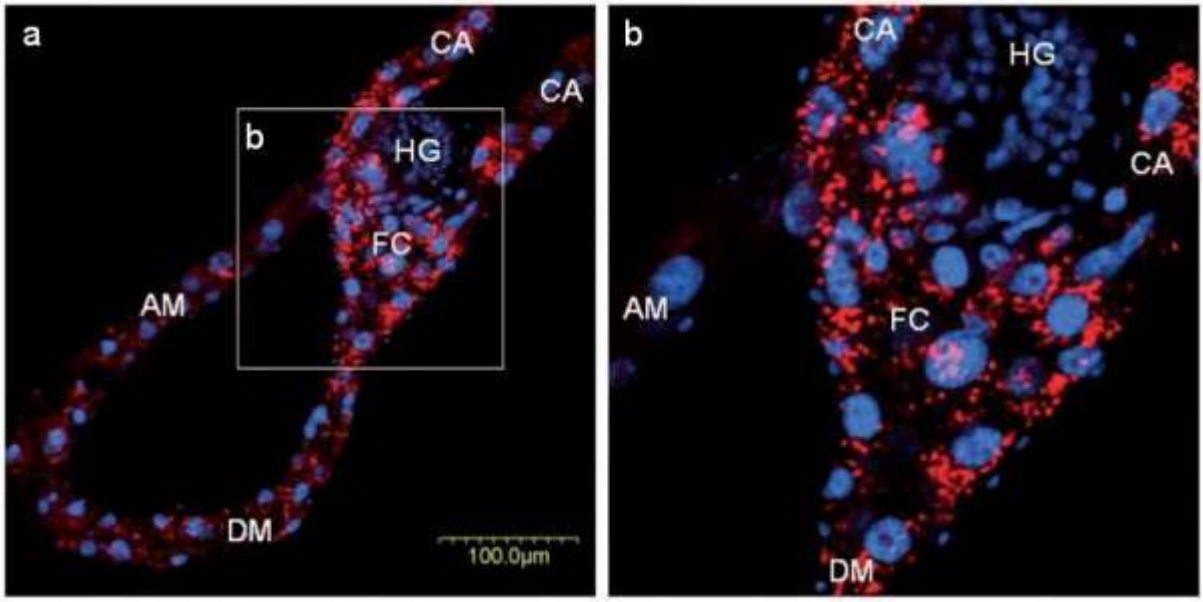
TYLCV is vectored by *B. tabaci* in a persistent circulative manner (Ghanim *et al*., 2001a). The latent period of the virus in the vector lasts between 8–24 h (Ghanim *et al*., 2001a), and once the virus circulates and passes the latent period, it can be transmitted to a new plant after 5 min of inoculation access period (IAP) (Atzmon *et al*., 1998). The virus can also be acquired from an infected plant after 5 min of acquisition access period (AAP) (Atzmon *et al*., 1998).

It is acquired as a virion from the plant phloem as a result of different pressure values between the plant cell and the opening–closing cibarial pump in the insect mouth apparatus (Ghanim and Medina, 2007). The virions pass along the food canal in the stylet with sugars and other substances form the phloem and reach the esophagus. The esophagus is a chitin-lined tissue that does not allow food/virion penetration to the haemolymph (Ghanim *et al*., 2001b). The first tissue through which virions can cross to the haemolymph is a modification of the digestive system called the filter chamber (Ghanim *et al*., 2001b). The filter chamber is a complicated structure that combines tissue from the midgut, hindgut, and the caeca. Membranes from these organs interdigitate to form this complicated structure that insures direct absorption of “pure” useful substances for the insect into the haemolymph, while more “complicated” food is pushed into the descending midgut by the muscular caeca. It is hypothesized that the majority of the TYLCV virions are absorbed from the filter chamber into the haemolymph, while a minority of the virions circulate into the descending then the ascending midgut, and cross the midgut epithelial cells to the haemolymph (Ghanim and Medina, 2007). Recent studies using localization methods have shown extensive location of TYLCV virions in the filter chamber area, and their concentration decreases toward the descending and the ascending midguts (Ghanim *et al*., 2009) (Figure 6). In the haemolymph, TYLCV virions interact with a 63 KDa GroEL protein produced by the endosymbiotic bacteria of *B. tabaci*, which protects the virions from proteolysis by the insect’s immune system (Morin *et al*., 1999, 2000). Virions cross the digestive system into the haemolymph within 1 h, while reaching the digestive system from the stylet lasts 40 min (Ghanim *et al*., 2001a). The virions circulate in the haemolymph and are de-

tected in the primary salivary glands after 7 h from the AAP. A second recognition barrier is thought to reside on the apical membrane of the primary salivary gland of *B. tabaci* (Brown and Czosnek, 2002). Once the virions reach the secretory salivary cells in the primary salivary glands, they are secreted with the saliva into the salivary duct, and then to the salivary canal in the stylet, from where they are injected into the plant. Not much is known about the molecular interactions between TYLCV and *B. tabaci*. Different studies were aimed to address replication of TYLCV in *B. tabaci*, and the actual view is that TYLCV and geminiviruses do not replicate in their vectors.

Unlike other geminiviruses, TYLCV is transovarially transmitted from females to their offspring through the egg (Ghanim *et al*., 1998), and it can be transmitted between males and females during sexual intercourse (Ghanim and Czosnek, 2000).

In the experiment with *Squash leaf curl virus* (SLCV), Rosell *et al*. (1999) described the pathway of the virus in *B. tabaci* while in the same study, the *T. vaporariorum* showed ability to acquire, but not transmit the virus. After acquisition SLCV was not detected in the hemolymph and in the saliva of *T. vaporariorum*.



(Skaljac and Ghanim, 2010)

Figure 6. Fluorescent in situ hybridization (FISH) of the midgut with Tomato yellow leaf curl virus-specific probe (red) from viruliferous *Bemisia tabaci* adult females. (a and b): Specific localization of the virus transcripts in the filter chamber (FC) and caeca (CA). The signal decreases in the descending midgut (DM) and disappears in the ascending midgut (AM). HG, hindgut (a). Magnified FC (b). DAPI stained nuclei (blue).

2.2.5.3. TYLCV – Plant interactions

TYLCV is released into the plant phloem by *B. tabaci* as virions or ssDNA to the nucleus where replication is followed using a polymerase machinery in host cells. Once in the nucleus of a permissive cell, the ssDNA is converted in a double-stranded replicative intermediate DNA (dsDNA). The dsDNA associates with histones and can be visualized as 'minichromosomes' that serve as templates for transcription and subsequent rolling circle replication yielding multiple copies of plus stranded ssDNA. After replication, it invades most of the plant tissues including roots, and symptoms become visible within 2 weeks or less (Figure 7), depending on the environment conditions and the vigor of the infected host. It is not clear whether TYLCV whole virions or DNA genomes enter the sieve element cell nucleus (Kunik *et al*., 1998), and in which cell types in the phloem they replicate (sieve cells, companion cells, or phloem parenchyma cells). After

replication and encapsidation, a process which may take several days and reach a peak at 11–13 days post inoculation, virions are transported via sieve cells in long-distance movement supported by TYLCV coat protein (CP), and symptoms are visible after another 2–4 days (Michelson *et al*., 1997). Although it is accepted that in the infected plant, TYLCV remains confined to the phloem, Michelson *et al*. (1997) showed that viral DNA could be detected in mesophyll tissues and parenchyma cells, maybe as a result of tissue collapse or ageing. Several recent studies also showed the presence of begomoviral DNA in phloem, cambium, and xylem tissues (Rasheed *et al*., 2006).

Figure 7. Symptoms of Tomato yellow leaf curl virus (TYLCV) on tomato plants in Israel. (a) Chlorosis and curling of young tomato leaves. (b) Infected tomato plant stunting in compared to healthy plants.

2.2.5.4. Efficiency of TYLCV transmission in correlation with *Bemisia tabaci* species and secondary bacterial symbiont composition

Ghanim *et al*. (2001a) confirmed that MEAM1 genetic group of *B. tabaci* from Israel has the higher transmission efficiency of TYLCV than MED group. Israeli MEAM1 group tested positive for the presence of *Hamiltonella* and *Rickettsia*, while Israeli MED group harbored *Arsenophonus*, *Wolbachia*, and *Rickettsia* (Chiel *et al.,* 2007). Gottlieb *et al*. (2010) conducted an experiment to elucidate possible differences in TYLCV transmission efficiency between the MEAM1 and MED in correlation to symbiotic

content. An experiment with MEAM1 *B. tabaci* populations harboring either *Hamiltonella* alone or *Hamiltonella* with *Rickettsia*, TYLCV was transmitted with an 80% efficacy. These results suggested that *Hamiltonella*, the only symbiont present in Israeli *B. tabaci* MEAM1 group and absent from the Israeli MED group, is most probably a secondary symbiont necessary for TYLCV transmission (Gottlieb *et al*., 2010). Later, it was confirmed that GroEL proteins produced by *Wolbachia*, *Arsenophonus*, *Rickettsia*, and *Portiera* do not interact with TYLCV CP in the Israeli MED group and only the GroEL produced by *Hamiltonella* interacts with TYLCV CP in the Israeli *B. tabaci* MEAM1. Therefore, reduced transmissibility of TYLCV by the mentioned MED group is a result of the lack of interaction between the virus and the appropriate *Hamiltonella* GroEL protein in the hemolymph. Israeli population of *B. tabaci* from the MED group with no *Hamiltonella* presence was artificially fed with *in vitro*-synthesized *Hamiltonella* GroEL protein, however, the transmission of TYLCV did not increase.

In *B. tabaci* populations from Spain, TYLCV was efficiently transmitted with both genetic groups. Spanish MED group harbored *Hamiltonella* and TYLCV transmission was even more efficient than with Spanish MEAM1 genetic group (Sanchez-Campos *et al*., 1999; Jiang *et al*., 2004).

2.2.6. Whitefly pest status

Whiteflies pierce plant tissues and ingest phloem sap with stylet mouthparts. *B. tabaci* and *T*. *vaporariorum* are notorious and extremely polyphagous pests of annual plants (vegetables and ornamentals). We consider them as pests of crops belonging to Compositae, Cucurbitaceae, Euphorbiaceae and Solanaceae. *S*. *phillyreae* prefers perennial plants from Oleaceae, Punicaceae and Rosaceae.

Direct feeding of whitefly adults and nymphs, results in indirect damage through numerous chlorotic spots on the leaves, honeydew (highly concentrated carbohydrate secreted solution) and sooty moulds (e.g. Cladosporium; Alternaria) contamination of the leaves and fruits, physiological disorders, and plant virus infection (Figure 9 c,d,e). Honeydew and belonging sooty moulds reduce plant photosynthetic activity and

commercial value of the plant products. In heavy infestations, close observation of the underside of the leaves will show numerous adults and the tiny yellow/white nymphal individuals of *B. tabaci* and *T. vaporariourum* (Figure 8 a,b), or nymphs with variable number of dorsal siphons in the case of *S. phillyreae* (Figure 3 f).

Whitefly developmental stages are vertically distributed in the infested plant with respect to leaf age. Adults, eggs, and first instar nymphs are predominantly found on young fully developed leaves, second and third instar nymphs on middle aged leaves, and fourth instars, pupae, and pupal cases on middle aged and old leaves.

In greenhouses, *B. tabaci* shows invasiveness and may displace *T. vaporariorum* within two generations when these species co-exist simultaneously over the temperature range 20°C to 30°C. Displacement of *T. vaporariorum* by *B. tabaci* may be related to host plant suitability. Aggregations of adult *B. tabaci* on leaves initiates' avoidance behavior in adult *T. vaporariorum* which rarely settle in close proximity to *B. tabaci* (Hoddle, http://biocontrol.ucr.edu).

Generally, reliable estimation of the economic impact of the whiteflies on worldwide agriculture have been difficult to obtain due to extensive areas affected, the numbers of plant hosts involved, and different monetary systems (Oliviera *et al*., 2001).

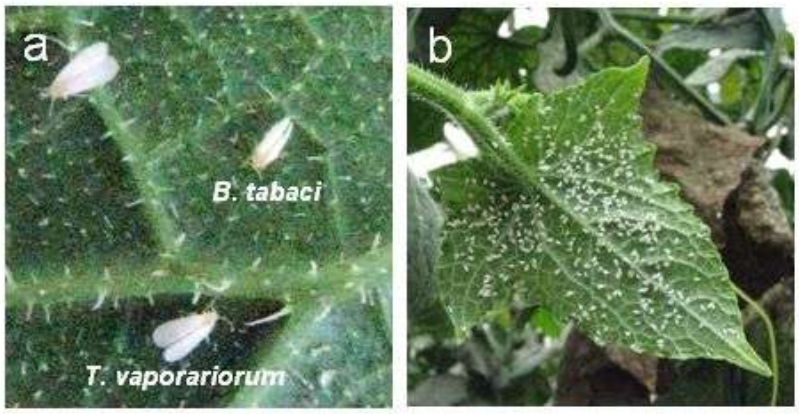


Figure 8. Whitefly infestation (a and b): *Bemisia tabaci* and *Trialeurodes vaporariorum* share habitat (a), heavy whitefly infestation on young cucumber leaf (b).

2.2.6.1. Whitefly control

Whiteflies are mainly controled with chemical substances, such as, insecticides. Due to developed resistance to a wide range of insecticides from different chemical groups and high reproductive potential, the effectiveness of chemical control is greatly reduced.
There is no specific treatment that could be used for a long period in the fight against these pests, therefore, integrated pest management (IPM) could be the most effective program to regulate the number and reproduction of the whiteflies.

IPM includes a combination of:

- physical and mechanical control, and cultural practice: visual traps (yellow sticky traps) (Figure 9 f); 'insect-proof' mesh and UV absorbing plastic films in the greenhouses and in the field; not over-fertilizing and implementation of hygiene measures (to remove the old plants and the weed which could serve as a source of infection) (Figure 9 a,b).

- biological control: natural enemies (predators, pathogens, and parasitoids) weaken organisms either by prematurely killing the pest through predation, disease or parasitism, or by reducing the reproductive output or competitiveness of the target. Biological control is the intentional use of natural enemies by crop producers to control pest populations. Predators, pathogens, and parasitoids are commercially available for use against whiteflies in greenhouses.

  Predators are organisms that obtain energy by consuming more than one individual prey over the course of their lifetime. Often, both immature and adult stages are predatory.

  B. tabaci and *T*. *vaporariorum* is attacked by predatory species representing eight arthropod orders, including members of the families: Phytoseiidae (Acari), Coccinellidae (Coleoptera), Syrphidae (Diptera), Anthocoridae, Nabidae, and Miridae (all Hemiptera), Chrysopidae and Coniopterygidae (both Neuroptera). At least four species of predators that are commercially available have been

evaluated for their ability to control *B. tabaci* and *T*. *vaporariorum* on greenhouse grown crops: beetle *Delphastus catalinae* (Horn), Myrid bug *Macrolophus caliginosus* (Wagner); mites: *Amblyseius swirskii* (Athias-Henriot) and *Amblydromalus limonicus* (Garman & McGregor) (http://www.koppert.com/pests/whitefly).

Entomopathogens are disease-causing organisms which either kill or weaken the host and they include: bacteria, viruses, protozoa, and fungi. The most commonly observed fungal pathogens of the whiteflies are: *Paecilomyces fumosoroseus* (Wize) Brown and Smith, *Aschersonia aleyrodes* Webber, *Verticillium lecanii* (Zimmerman) Viegas and *Beauveria bassiana* (Balsamo) Vuillemin. The fungus *Verticillium lecanii* is a base of several commercial products against *B. tabaci* and *T*. *vaporariorum* (http://www.koppert.com/pests/whitefly).

Immature stages of the **parasitoids** develop at the expense of a single whitefly individual which is referred to as the host. Parasitoids kill their host upon completing development. Whitefly parasitoids belong to three hymenopterous families: Platygasteridae (e.g., *Amitus* spp.), Aphelinidae (e.g., *Eretmocerus* and *Encarsia* spp.), and the Eulophidae (e.g., *Euderomphale* spp.). The best studied whitefly parasitoids are *Encarsia Formosa* Gahan, and *Eretmocerus eremicus* Rose and Zolnerowich, both of which are commercially available and suitable in IPM of *B. tabaci* and *T*. *vaporariorum* (http://www.koppert.com/pests/whitefly). Furthermore, according to the laboratory and the field study of Gerling *et al*. (2004), mortality of *S. phillyreae* nymphs caused by *Encarsia inaron* ranged between 24 and 78%. This parasitoid wasp was introduced from Israel into the US and New Zeland and led to successful biological control of the whitefly *S. phillyreae*.

- <u>chemical control</u>: Insecticides are still important tools in integrated management of the whiteflies. However, excessive use of insecticides has resulted in resistance to different insecticide groups (organophosphates, pyrethroids, insect growth regulators, neonicotinoids). Due to the toxic effects on human health and

other organisms, insecticide manufacturers are compelled looking into less aggressive substances, which are also effective in combating pests.

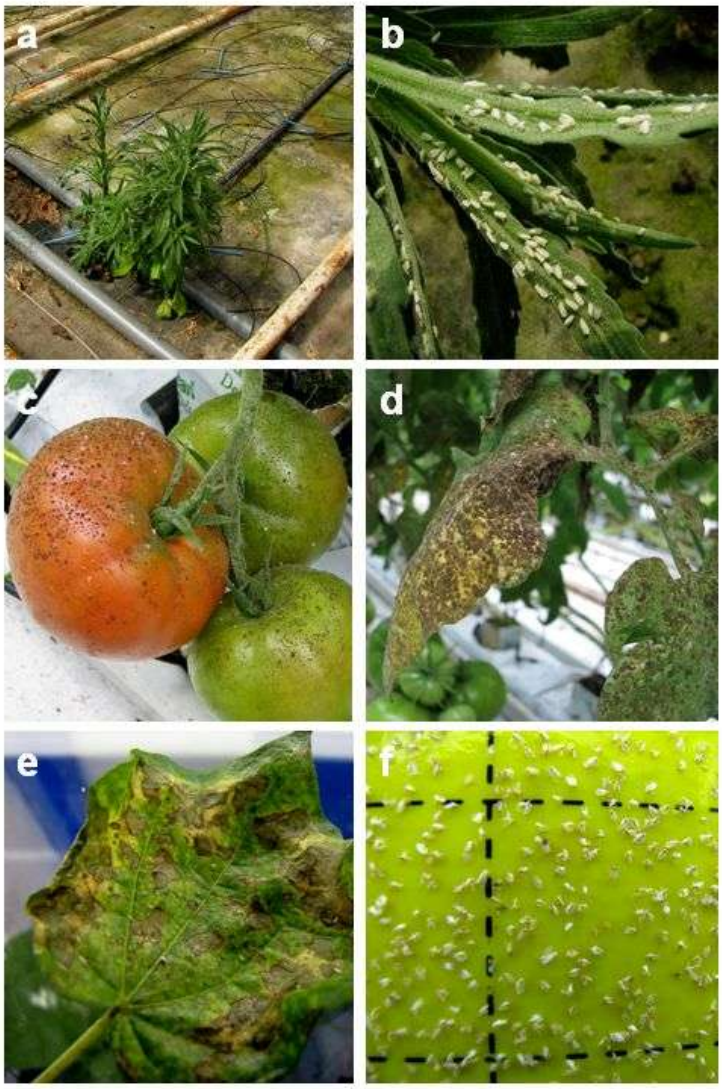


Figure 9. Whitefly damage and control. (a-f): Weed infested with whiteflies in the greenhouse (a and b); sooty mould fungi developed on honeydew on tomato fruit and leaf (c and d); physiological disorders on the cotton leaf as a result of whitefly infestation (e); yellow sticky trap with captured whiteflies (f).

3. MATERIALS AND METHODS

3.1. Whitefly populations

The populations of sweet potato whitefly *B. tabaci* and greenhouse whitefly *T. vaporariorum* were collected from open fields and/or greenhouses during the period 2008-2011 across Croatia, Montenegro and selected locations of Bosnia and Herzegovina, while the ash whitefly (*S. phillyreae*) populations were collected from pomegranate orchards in coastal Croatia and Montenegro during the vegetation period of 2011 (Figure 10, Table 1).

Figure 10. Locations of whitefly populations in Croatia, Bosnia and Herzegovina and Montenegro according to population numbering in Table 1.

Details on collecting locations and host plants are shown in Table 1. Although sampling were arranged to include the populations from all parts of the country, in some areas no whiteflies were found. *Bemisia tabaci* and *T. vaporariorum* were collected from different host plants while *S. phillyreae* was collected only from pomegranate (*P. granatum* L.). Adult individuals were collected using a Pasteur pipette attached to a hand-held aspirator, and were immediately preserved in absolute ethanol and kept at room temperature until processing. Each collected population was collected from different leafs, on different plants. Two populations imported via the trade in ornamentals, one population of *B. tabaci* originated from Italy, and one population of *T. vaporariorum* originated from Slovenia, were found in Croatia, at the nymphal stage on host plants *Hibiscus* sp. and *Euphorbia pulcherrima* Willd., respectively. These infested plants were maintained in insect-proof cages (designed by Dr. M. Ghanim, Volcani center, Israel) in the growth chamber under standard conditions (26 ± 2°C, 60% RH, 14/10 h of light/dark) until adult emergence.

Table 1. The list of whitefly populations (*Bemisia tabaci, Trialeurode vaporariorum* and *Siphoninus phillyreae*) tested in this study.

Population No.	Location	Coordinates	Country	Genetic group or Species	Host plant	Date
1	Pula	44°53'03'' N, 13°54'53'' E	Croatia	MED	*Euphorbia pulcherrima*	11. 2008
2	Zadar	44°05'50'' N, 15°15'46'' E	Croatia	MED	*Hibiscus* sp.	10. 2008
3	Zadar	44°05'50'' N, 15°15'46'' E	Croatia*	MED	*Hibiscus* sp.	04. 2011
4	Turanj	43°58'14'' N, 15°24'59'' E	Croatia	MED	*Lycopersicon esculentum*	11. 2008
5	Turanj	43°58'14'' N, 15°24'59'' E	Croatia	MED	*Euphorbia pulcherrima*	11. 2008
6	Kaštela	43°32'53'' N, 16°17'38'' E	Croatia	MED	*Hibiscus* sp.	11. 2008
7	Brač-Supetar	43°22'48'' N, 16°32'50'' E	Croatia	MED	*Cucumis sativus*	08. 2009
8	Opuzen	43°00'54'' N, 17°33'40'' E	Croatia	MED	*Cucumis melo*	07. 2010
9	Cavtat	42°34'52'' N, 18°13'12'' E	Croatia	MED	*Ipomea* spp.	07. 2009
10	Veljaci	43°11'58'' N, 17°29'33'' E	Bosnia and Herzegovina	MED	*Cucurbita pepo*	08. 2009
11	Višići	43°05'05'' N, 17°42'32'' E	Bosnia and Herzegovina	MED	*Datura stramonium*	07. 2009
12	Ulcinj	41°55'26'' N, 19°14'08'' E	Montenegro	MED	*Dipladenia sanderi*	06. 2011
13	Bar	42°05'08'' N, 19°08'11'' E	Montenegro	MED	*Dipladenia sanderi*	06. 2011
14	Podgorica	42°27'43'' N, 19°16'40'' E	Montenegro	MEAM1	*Hibiscus* sp.	05. 2008
15	Podgorica	42°27'43'' N, 19°16'40'' E	Montenegro	MEAM1	*Sonchus oleraceus*	06. 2011
16	Čepin	45°31'23'' N, 18°34'08'' E	Croatia	*T. vaporariorum*	*Gerbera* sp.	05. 2009
17	Velika Ludina	45°36'19'' N, 16°34'45'' E	Croatia	*T. vaporariorum*	*Datura stramonium*	07. 2009
18	Zabok	46°01'41'' N, 15°54'24'' E	Croatia	*T. vaporariorum*	*Cucurbita pepo*	07. 2009
19	Donja Lomnica	45°42'43'' N, 16°01'08'' E	Croatia	*T. vaporariorum*	*Fragaria* sp.	07. 2009
20	Karlovac	45°29'17'' N, 15°32'29'' E	Croatia	*T. vaporariorum*	*Cucurbita pepo*	07. 2009
21	Novigrad	45°19'12'' N, 13°34'11'' E	Croatia	*T. vaporariorum*	*Lycopersicon esculentum*	05. 2009
22	Pula	44°53'03'' N, 13°54'53'' E	Croatia	*T. vaporariorum*	*Petunia* sp.	05. 2009
23	Turanj	43°58'14'' N, 15°24'59'' E	Croatia	*T. vaporariorum*	*Lycopersicon esculentum*	07. 2009
24	Split	43°30'20'' N, 16°30'24'' E	Croatia	*T. vaporariorum*	*Nicotiana megalosiphon*	07. 2009
25	Split	43°30'20'' N, 16°30'24'' E	Croatia**	*T. vaporariorum*	*Euphorbia pulcherrima*	12. 2010
26	Tugare	43°28'22'' N, 16°39'01'' E	Croatia	*T. vaporariorum*	*Cucumis sativus*	07. 2009
27	Brač-Supetar	43°22'48'' N, 16°32'50'' E	Croatia	*T. vaporariorum*	*Cucumis sativus*	07. 2009
28	Metković	43°02'57'' N, 17°38'45'' E	Croatia	*T. vaporariorum*	*Lycopersicon esculentum*	07. 2009
29	Dubrovnik	42°38'43'' N, 18°07'13'' E	Croatia	*T. vaporariorum*	*Gerbera* sp.	07. 2009
30	Veljaci	43°11'58'' N, 17°29'33'' E	Bosnia and Herzegovina	*T. vaporariorum*	*Cucumis sativus*	07. 2009
31	Gabela polje	43°04'14'' N, 17°41'37'' E	Bosnia and Herzegovina	*T. vaporariorum*	*Lycopersicon esculentum*	07. 2010
32	Ulcinj	41°55'26'' N, 19°14'08'' E	Montenegro	*T. vaporariorum*	*Salvia* sp.	06. 2011
33	Bar	42°05'08'' N, 19°08'11'' E	Montenegro	*T. vaporariorum*	*Cucumis sativus*	06. 2011
34	Radanovići	42°21'39'' N, 18°45'37'' E	Montenegro	*T. vaporariorum*	*Geranium* sp.	06. 2011

35	Podgorica	42°27'43'' N, 19°16'40'' E	Montenegro	*T. vaporariorum*	*Sonchus oleraceus*	06. 2011
36	Sutorina	42°28'13'' N, 18°28'51'' E	Montenegro	*T. vaporariorum*	*Sonchus oleraceus*	06. 2011
37	Ljuta	42°32'03'' N, 18°22'43'' E	Croatia	*S. phillyreae*	*Punica granatum*	06. 2011
38	Trsteno	42°42'45'' N, 17°58'41'' E	Croatia	*S. phillyreae*	*Punica granatum*	06. 2011
39	Opuzen	43°00'45'' N, 17°32'13'' E	Croatia	*S. phillyreae*	*Punica granatum*	06. 2011
40	Pozla Gora	43°08'07'' N, 17°30'56'' E	Croatia	*S. phillyreae*	*Punica granatum*	06. 2011
41	Brač-Pučišća	43°20'58'' N, 16°44'03'' E	Croatia	*S. phillyreae*	*Punica granatum*	05. 2011
42	Brač-Supetar	43°22'50'' N, 16°32'50'' E	Croatia	*S. phillyreae*	*Punica granatum*	05. 2011
43	Ulcinj	41°55'26'' N, 19°14'08'' E	Montenegro	*S. phillyreae*	*Punica granatum*	06. 2011
44	Bar	42°05'08'' N, 19°08'11'' E	Montenegro	*S. phillyreae*	*Punica granatum*	06. 2011
45	Podgorica	42°27'43'' N, 19°16'40'' E	Montenegro	*S. phillyreae*	*Punica granatum*	06. 2011

***** Population imported from Slovenia (Čatež ob Savi, 45°53'28'' N, 15°36'09'' E).
** Population imported from Italy (Rome, region Lazio, 41°39'14'' N, 12°59'14'' E).

3.2. Whitefly rearing

After field collections, three *B. tabaci* (Zadar, Podgorica (2008) and Turanj) and one *T. vaporariorum* (Split) population were directly transferred as adults to insect-proof cages containing plant seedlings. *B. tabaci* was released on cotton cv. Acala (Zeraim Gedera, Israel) while bean cv. Berggold (Impex s.r.l. Sementi, Italy) was infested with *T. vaporariorum*. These adults were set to lay eggs and to establish a colony. The established colonies were maintained in cages in the growth chamber under standard conditions (26 ± 2°C, 60% RH, 14/10 h of light/dark) (Figure 11).

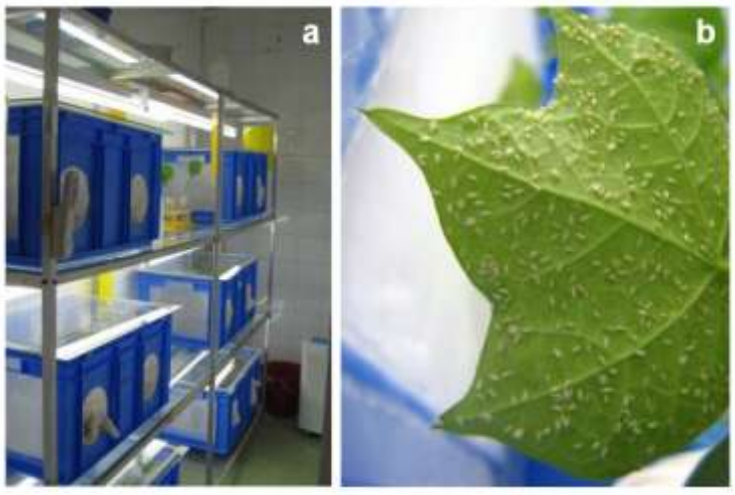


Figure 11. Insects growing chamber. (a) Insect-proof cages, (b) Whitefly, adults, reared on the cotton plant.

3.3. *DNA analysis*

3.3.1. Individual whitefly DNA extraction

Individual whitefly DNA extraction was done according to the protocols of Frohlich et *al.* (1999) and Chiel et *al.* (2007). Genomic DNA of each individual was extracted in lysis buffer (1M Tris, 0.5M EDTA, Igepal, Proteinase K (20 mg/mL), and double distilled water). Each individual was macerated separately in a lysis buffer. DNA lysate was heated up to 65˚C for 15 minutes in a thermo block (BIOSAN, Bio TDM-100), then

heated up to 96˚C for 10 minutes, transferred on ice for few minutes, vortexed and stored on 4˚C until processing.

3.3.2. Plant and insect DNA extraction

a) CTAB plant DNA extraction

Genomic plant DNA was extracted as follows (pers. comm. Dr. M. Ghanim, Volcani center, Israel):

- plant sample was macerated with micro pestle in a 1.5 mL tube containing CTAB buffer (99% Hexadecyltrimethyl-ammonium bromide, 1M Tris, 5M NaCl, 0.5M EDTA, β-mercaptoethanol, double distilled water);
- macerate was incubated on 65˚C for 60 minutes;
- after incubation, it was centrifuged (13500 rpm) for 10 minutes at room temperature (upper phase was transferred into a new tube);
- equal volume of chloroform was added and tube was inverted for the few times to get a cloudy solution;
- solution was centrifuged (13500 rpm) for 15 minutes at room temperature (upper phase was transferred into a new tube);
- 0.6 volumes of cold isopropanol and 0.08 volumes of 7.5M ammonium acetate was added and a tube was placed on -20˚C for 30 minutes to overnight;
- afterwards, solution was centrifuged for 30 minutes (13500 rpm) at 4˚C;
- supernatant was decanted and 200 µL of cold 70% ethanol was added to wash the DNA pellet;
- ethanol with the pellet were centrifuged for five minutes (7000 rpm) at room temperature;
- supernatant was decanted and washing step was repeated;
- DNA pellet was left to dry and afterwards resuspended in 50 µL double distilled water.

b) CTAB insect DNA extraction

According to protocol of Shahjahan et *al.* (1995), insect genomic DNA was extracted as follows:

- insect sample (n=10-100) was macerated with micro pestle in a 1.5 mL tube contaninig CTAB buffer (99% Hexadecyltrimethyl-ammonium bromide, 1M Tris, 5M NaCl, 0.5M EDTA, β-mercaptoethanol, double distilled water);
- macerate was incubated on 37˚C for 45 minutes;
- centrifuged (13500 rpm) for 5 minutes at room temperature (upper phase was transferred into a new tube);
- equal volume of phenol/chlorophorm/isoamyl alcohol (25:24:1) was added and the tube was inverted for the few times;
- solution was centrifuged (13500 rpm) for 5 minutes at room temperature (aquatic upper fraction was transferred into a new tube);
- equal volume of chlorophorm was added and the tube was inverted for the few times;
- solution was centrifuged (13500 rpm) for 5 minutes at room temperature (aquatic fraction was transferred into a new tube);
- 0.2 volumes of 5M NaCl and 1 volume of isopropanol (room temperature) were added and the tube was inverted for the few times;
- solution was centrifuged for 10 minutes (13500 rpm) at room temperature;
- supernatant was decanted and 200 μL of cold 70% ethanol was added to wash the DNA pellet;
- ethanol with the pellet were centrifuged for 5 minutes (13500 rpm) at room temperature;
- supernatant was decanted and washing step was repeated;
- DNA pellet was left to dry and afterwards resuspended in 50 μL double distilled water.

Concentrations of plant and insect DNA were measured using NanoDrop 2000 UV spectrophotometer (Thermo Scientific).

3.3.3. Polymerase Chain Reaction (PCR)

PCR was carried out in 20 µl volume containing 4 µl DNA lysate, 20 pmole of each primer, 10 mM dNTP mix, 10X Dream Taq buffer (+$MgCl_2$) and 5 units/µl of Dream Taq polymerase (Fermentas). The following conditions were applied: initial denaturation at 95°C, 3 min; subsequent denaturations, 30 s; annealing temperature depended on set of primers used in the reaction (Table 2), 30 s; elongation at 72°C, 30 s; final incubation at 70°C, 5 min; total of 35 cycles (Applied Biosystems, 2720 Thermal Cycler). PCR products were visualized and photographed under UV on a 1.5% agarose gel containing ethidium bromide (BIO RAD PowerPac Basic, Sub-Cell GT, UVITEC Cambridge, Canon PowerShot SX10IS).

3.3.4. Cloning, colony PCR and sequencing

In order to verify the PCR products, selected bands were excised, eluted (QIAquick Gel Extraction Kit, QIAGEN) and DNA was cloned into *Escherichia coli* using pGEM T-Easy plasmid vector (pGEM-T Vector System II, Promega), according to protocol of Promega (Revised 6/09, Part TM042) as follows:

a) **Ligation**

- Single ligation reaction was prepared by 0.5 µL of pGEM T-Easy plasmid vector; 5 µL T_4 buffer (2X Ligation Buffer); 1 µL T_4 ligase; 7.5 µL PCR product (50 ng/ µL) and 1 µL double distilled water;
- Reaction was mixed by pipetting and incubated overnight at 4˚C.

b) **Transformation**

Preparation of LB plates with ampicillin:

- agar was added to 1L of liquid LB medium (Bacto-Tryptone, Bacto-Yeast extract, NaCl, pH 7);
- mixture was autoclaved (on 120˚C for 30 minutes), cooled to 50˚C and ampicilin was added to a final concentration of 100 μg/ml;
- medium (30-35 mL) was poured to a petri dish and left to harden;
- LB plates with ampicillin were stored at 4˚C until use.

Tube with competent *E. coli* cells (stored previously at -80˚C) was kept on ice for 5 minutes to thaw; afterwards ligation product was added to *E. coli* cells, slightly mixed by flicking the tube and placed on ice for 30 seconds. Mixture was placed on 42˚C for 1 minute and 30 seconds and afterwards placed on ice for 5 minutes. Furthermore, 600 μL of LB medium was added to a tube with transformed competent cells and the mixture was placed in the shaker for 1 hour and 30 minutes at 37˚C (150 rpm). Finally, 200 μL of transformed *E. coli* was plated on LB/ampicilin plates and incubated for 16-24 hours at 37˚C.

c) **Colony PCR**

In order to check whether *E. coli* colony has a cloned insert, colony PCR was employed. Several colonies were randomly marked and picked up from the LB (ampicillin) plates, with a pipette tip and placed individually in the PCR mix (instead of DNA lysate) with a set of primers adequate for the cloned DNA. PCR procedure was performed as described before, while bands on agarose gel indicated ‘insert-positive’ colonies and their absence indicated ‘insert-negative’ colonies.

d) **Sequencing**

'Insert – positive' colonies were picked up from LB (ampicillin) plates and placed in 5 mL of liquid LB medium with 5 µL of ampicillin (100 mg/ µL) overnight at 37˚C.
Isolation of cloned plasmid (HiYield Plasmid Mini Kit) was conducted from overnight multiplied 'insert – positive' *E. coli* and sent for sequencing (ABI 3700 DNA analyzer, Hylabs, Rehovot, Israel; 3730xl DNA analyzer, Macrogen Europe, Amsterdam, Netherlands). The sequences were compared with those in the databases using BLAST algorithm in NCBI (http://www.ncbi.nlm.nih.gov/).

3.4. Identification of Bemisia tabaci genetic groups

Bemisia tabaci species were identified using microsatellite markers with the primer pair Bem23 which distinguishes MED from MEAM1 genetic group. based on the amplified fragment size (De Barro *et al*., 2003). Another method was used to verify the MED and MEAM1 which consisted of sequencing a fragment of the mitochondrial (mt) COI gene after amplification by PCR (Frohlich *et al.*, 1999; Gueguen *et al*., 2010). The PCR conditions for amplifying mtCOI and the microsatellite markers together with the primer sequences are given in Table 2.

3.5. Screening of secondary bacterial symbionts

Adult females (n=10–20 per population) of the three whitefly species were individually tested for the presence of secondary bacterial symbionts and for genetic group determination in the case of *B*. *tabaci*. The presence of the following secondary symbionts: *Rickettsia* (R), *Hamiltonella* (H), *Arsenophonus* (A), *Wolbachia* (W), *Cardinium* (C) and *Fritschea* (F), was tested by PCR using genus-specific primers (16S or 23S rDNA) shown in Table 2.

Table 2. The list of primers used in the study.

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
C1-J-2195 L2-N-3014	TTGATTTTT TGGTCATCCAGAAGT TCCAATGCACTAATCTGCCATATTA	51/850	Cytochrome oxidase I (mtCOI)	Frohlich *et al*., 1999
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	59/600	*Arsenophonus* 23S rDNA	Thao & Baumann, 2004a
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
P1V P5C	ATACTTGGAC ACCTAATGGC AGTCACGGGCCCTTACAA	51/500	TYLCV	Gottlieb *et al*. 2010
SPG1 SPG2	CCCCKGTGCGWRAATCCAT ATCCVAAYWTYCAGGGAGCTAA	45/900	*Geminiviridae* regulatory proteins	Li *et al*., 2004

3.6. Fluorescent in situ hybridization analysis (FISH)

3.6.1. Insect and plant sample preparation for in situ hybridization

Whitefly specimens from Croatian populations (adults, nymphs and eggs) or hand-cut longitudinal stem tomato (*L. esculentum)* sections were fixed in Carnoy's fixative (chloroform:ethanol:glacial acetic acid, 6:3:1) overnight, at room temperature. Fixed samples were then transferred to 6% hydrogen peroxide (H_2O_2) for two hours at room temperature. After H_2O_2 treatment, insect or plant material were stored in absolute ethanol until processing (Sakurai *et al*., 2005).

3.6.2. Fluorescent *in situ* hybridization

In order to localize bacterial symbionts in all developmental stages of *B. tabaci*, *T. vaporariorum* and *S. phillyreae*, as well as to detect and localize TYLCV in tomato stem tissue, FISH was employed.

Previously fixed insect specimens (0.7-1.0 μm thick) or plant sections were washed once for one minute in hybridization buffer (HB) (20mM Tris pH 8.0, 0.9M NaCl, 0.01% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) formamide). Short symbiont-specific (16S/23S rRNA) or short oligonucleotide (complementary to TYLCV coat protein) DNA probes harboring a fluorescent Cy3/Cy5 molecule on their 5' end (10 pmol fluorescent probe/ml in HB) (Table 3) were hybridized to specimen overnight. Afterwards specimens were washed once for one minute, in HB, mounted whole and viewed under an IX81 Olympus FluoView500 confocal microscope (Olympus, Tokyo, Japan) (Gottllieb *et al*., 2006). Specificity of detection was confirmed using the following controls: no probe, RNase digested and healthy plants.

For each developmental whitefly stage, at least 50 specimens were viewed under the microscope to confirm reproducibility. In addition, each population was tested with all of the probes listed in Table 3 as controls. Thus, staining of a population known not to have a particular symbiont but harboring others was performed.

Table 3. The list of probes used for fluorescent *in situ* hybridization (FISH) in this study.

Target	Probe name and dye	Sequence (5´→ 3´)	Reference
Portiera	BTP1-Cy3	TGTCAGTGTCAGCCCAGAAG	Gottlieb *et al*., 2006
Rickettsia	Rb1-Cy5	TCCACGTCGCCGTCTTGC	Gottlieb *et al*., 2006
Hamiltonella	BTH-Cy5/Cy3	CCAGATTCCCAGACTTTACTCA	Gottlieb *et al*., 2008
Cardinium	Card-Cy5	TATCAATTGCAGTTCTAGCG	Matalon *et al*., 2007
Arsenophonus	Ars2-Cy5	TCATGACCACAACCTCCAAA	Gottlieb *et al*., 2008
Wolbachia	W1-Cy5	CTTCTGTGAGTACCGTCATTATC	Ferree *et al*., 2005
TYLCV	TYLCV-Cy3	GGAACATCAGGGCTTCGATA	Ghanim *et al*., 2009

3.7. Transmission electron microscopic (TEM) analysis of Trialeurodes vaporariorum

TEM analysis was applied in order to visualize secondary symbionts on sub-cellular level.

Freshly collected females of *T. vaporariorum* were anesthetized with acetone vapour and their abdomens were detached from the body in the fixative (4% paraformaldehyde in 1XPBS buffer (NaCl, KCl, Na_2HPO_4, KH_2PO_4, pH 7.4)) for 1-4 hours at room temperature.

Fixated abdomens were rinsed three times in 1XPBS buffer for 20 minutes and then dehydrated in series of ethanol as follows:

- 15 minutes in 50% ethanol,
- 15 minutes in 70% ethanol,
- 15 minutes in 90% ethanol,
- 15 minutes in 100% ethanol.

Dehydrated specimens were placed in 100% ethanol: acrylic resin (LR White Embedding Medium, Pelco, Technical notes), 1:1, for two hours, then transferred twice to 100% acrylic resin for one hour. Finally, specimens were placed in fresh 100% acrylic resin overnight at 4˚C.

Afterwards, specimens were rinsed twice in 100% acrylic resin for 30 minutes and processed as follows:

- abdomens were placed in the centres of the beem embed capsules bottom; capsules were filled with acrylic resin and lids were closed;
- capsules were placed in the incubator for 48 hours at 65°C for acrylic resin to polymerize.

Acrylic blocs were sectioned with ultramicrotome on 60-90 nm thick sections. Those sections were stained with aqueous uranyl acetate and lead citrate (Ghanim *et al*.,

2001b), and after examined in a Tecnai G2; FEI Philips electron microscope (Eindhoven, The Netherlands).

3.8. Monitoring of geminiviruses (Geminiviridae) in Croatia, Montenegro and selected locations of Bosnia and Herzegovina

Leaf samples of vegetables, showing geminiviruses (*Geminiviridae*) like symptoms (mosaic, chlorosis, discoloration, curling, stunting and asymmetry) were collected from open fields or greenhouses during the years 2009-2011, across Croatia, Montenegro and selected locations of Bosnia and Herzegovina. Plant samples with geminiviruses like symptoms were collected regardless of whiteflies infestation. In some cases, prior to sampling, whiteflies were sprayed with insecticides, therefore, no whiteflies could be found. Details on sampling locations and inspected plant species are shown in Table 4.
In order to test the geminiviruses infection, plant samples were processed by:
- DNA extraction;
- PCR by using specific TYLCV and geminiviruses primers (Table 2);
- cloning and sequencing, as described before.

Table 4. The list of vegetable samples tested for the presence of geminiviruses.

Sample No.	Origin of plant sample	Coordinates	Country	Whitefly species	Plant with viruse symptoms/ Year of sampling
1	Zabok	46°01'41" N, 15°54'24" E	Croatia	*T. vaporariorum*	*Cucurbita pepo*/2009
2	Donja Bistra	45°54'07" N, 15°51'37" E	Croatia	*T. vaporariorum*	*Citrullus lanatus*/2009
3	Karlovac	45°29'17" N, 15°32'29" E	Croatia	*T. vaporariorum*	*Lycopersicon esculentum*/2009
4	Brlog	44°56'31" N, 15°07'47" E	Croatia	/	*Cucurbita pepo*/2009
5	Kompolje	44°54'32" N, 15°10'17" E	Croatia	/	*Capsicum sp.*/2009
6	Brač-Supetar	43°22'48" N, 16°32'50" E	Croatia	MED	*Cucumis sativus*/2009
7	Opuzen	43°00'54" N, 17°33'40" E	Croatia	/	*Lycopersicon esculentum*/2009
8	Opuzen	43°00'54" N, 17°33'40" E	Croatia	/	*Lycopersicon esculentum*/2009
9	Opuzen	43°00'54" N, 17°33'40" E	Croatia	/	*Lycopersicon esculentum*/2009
10	Opuzen	43°00'54" N, 17°33'40" E	Croatia	MED	*Cucumis melo*/2010
11	Opuzen	43°00'54" N, 17°33'40" E	Croatia	MED	*Cucumis melo*/2010
12	Opuzen	43°00'54" N, 17°33'40" E	Croatia	MED	*Cucumis melo*/2010
13	Čibača	42°37'36" N, 18°10'49" E	Croatia	MED	*Lycopersicon esculentum*/2009
14	Veljaci	43°11'58" N, 17°29'33" E	Bosnia and Herzegovina	MED	*Cucurbita pepo*/2009
15	Veljaci	43°11'58" N, 17°29'33" E	Bosnia and Herzegovina	*T. vaporariorum*	*Cucurbita pepo*/2009
16	Darza	41°57'00" N, 19°19'12" E	Montenegro	MED	*Cucumis melo*/2011
17	Darza	41°57'00" N, 19°19'12" E	Montenegro	MED	*Cucumis melo*/2011
18	Darza	41°57'00" N, 19°19'12" E	Montenegro	MED	*Cucumis melo*/2011
19	Darza	41°57'00" N, 19°19'12" E	Montenegro	MED	*Cucumis melo*/2011

/ - absence of whiteflies infestation

3.9. Acquisition and transmission of TYLCV by Bemisia tabaci associated to MEAM1 and MED genetic groups

All experiments were conducted in controlled conditions (26 ± 2°C, 60% RH, 14/10 h of light/dark) of a growth chamber. Five to 8 days after emergence, 100 adults of *B. tabaci* belonged to two genetic groups (Israel MEAM1 and Croatia (Zadar) MED) were separately caged with a virus-infected tomato plant (TYLCV isolate from Israel, maintained in tomato plants (*L. esculentum* cv. Daniela) by whitefly-mediated transmission) for 48 hours. Adults were then removed from the infected plant, and five individuals were placed in a leaf cage on a virus-free tomato plant for 24 hours, in 12 replicates for each genetic group (Figure 12). After inoculation, whiteflies were collected from groups of five and tested for the presence of TYLCV. Ten days after virus transmission, the plants were tested with PCR and prepared for FISH analysis to detect the presence of the virus according to Ghanim *et al*. (2001a).

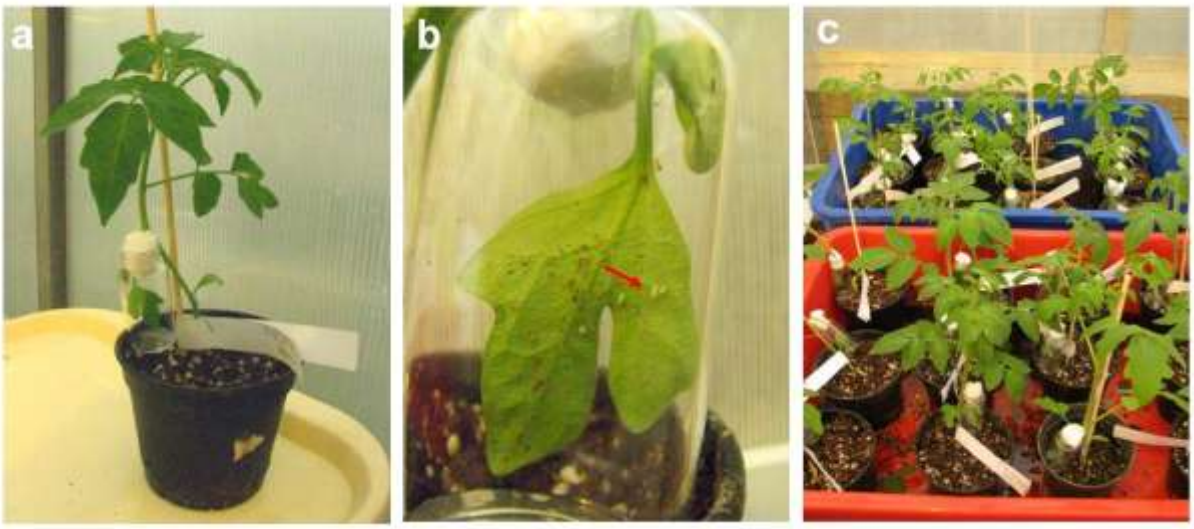


Figure 12. Transmission of Tomato yellow leaf curl virus (TYLCV) by adults of *Bemisia tabaci*. (a) Healthy tomato plant with leaf cage. (b) Leaf cage with TYLCV infected *B. tabaci* adults (arrow). (c) Experiment of TYLCV transmission in 12 replicates.

4. RESULTS

4.1. Bemisia tabaci distribution and infection by secondary symbionts in Croatia

Bemisia tabaci collections in Croatia were conducted in the period 2008-2011. Nine populations, described in Table 1 and Figure 10, were found only in the coastal part of the country. Interestingly, testing the collected populations revealed only the MED genetic group belonging to Q1 subgroup as assessed by sequencing COI gene based on the work conducted by Gueguen *et al*. (2010). Twenty individuals from each population were tested for the presence of the different symbionts known from whiteflies using genus-specific primers for each symbiont (Table 2). *Portiera aleyrodidarum*, the primary symbiont, was detected in all individuals tested and provided as a control for the quality of the extracted DNA. Each diagram in Figure 13 shows single and mixed infections by secondary symbionts detected in all of the individuals in each *B*. *tabaci* population. For example, the population collected from Turanj on *E. pulcherrima* (Table 1) contained only two individuals that were singly infected with *Rickettsia*, two individuals that harbored only *Hamiltonella*, one individual that harbored only *Wolbachia* and three individuals that harbored only *Cardinium*. This population also contained two individuals that were doubly infected with *Rickettsia* and *Hamiltonella*, one individual that was doubly infected with *Wolbachia* and *Cardinium*, one individual that was infected with three symbionts: *Rickettsia, Wolbachia* and *Cardinium*, and one individual that showed the highest level of mixed infection with four symbionts: *Rickettsia, Hamiltonella, Wolbachia* and *Cardinium*. Among the 20 individuals tested from that location, seven did not contain any of the tested secondary symbionts. *Fritschea* was not detected in any of the Croatian *B. tabaci* population tested in this study. Although the described population from Turanj showed a high level of mixed infection, containing one individual harboring four different symbionts, mixed infections with four symbionts were not common in many of the tested populations.

All populations harbored at least one symbiont or more in some of the individuals tested, and overall, secondary symbionts were highly prevalent with 82% (145/176)

of the individuals having at least one symbiont, 18% did not contain any of the tested secondary symbionts (Table 5).

Between all symbionts, *Hamiltonella* showed the highest prevalence and was detected in 59% of the individuals tested (Figure 14); sometimes it was the only symbiont detected in a particular population (Pula, Cavtat) and it was fixed or close to fixation in some populations, for example in those collected in Brač-Supetar, Opuzen and Cavtat.

The presence of each symbiont varied considerably between populations. For example *Hamiltonella* was fixed in the population from Brač-Supetar, and this population did not harbor *Rickettsia*. However, in the population from Zadar, *Hamiltonella* was found in only one individual while *Rickettsia* was almost fixed. All symbionts tested were found in at least one or more cases in which they were co-infecting the same individual.

The *B. tabaci* population imported from Italy via the trade in ornamentals to Croatia (Zadar) in the nymphal stage (Table 1) was identified as MED member of species complex. In that population, *Hamiltonella* was close to fixation and four individuals were singly infected with this symbiont. Seven individuals showed double infections: three with *Rickettsia* and *Hamiltonella*, three with *Arsenophonus* and *Hamiltonella*, and one with *Wolbachia* and *Hamiltonella*. Six individuals were infected with three types of symbionts and three individuals showed infection with four secondary symbionts (Figure 13).

In general, 53% of all tested Croatian *B. tabaci* individuals showed single infection with secondary symbionts, 23% showed double infection, 5% showed triple infection and 2% of all tested individuals showed infection with four symbionts (Figure 15).

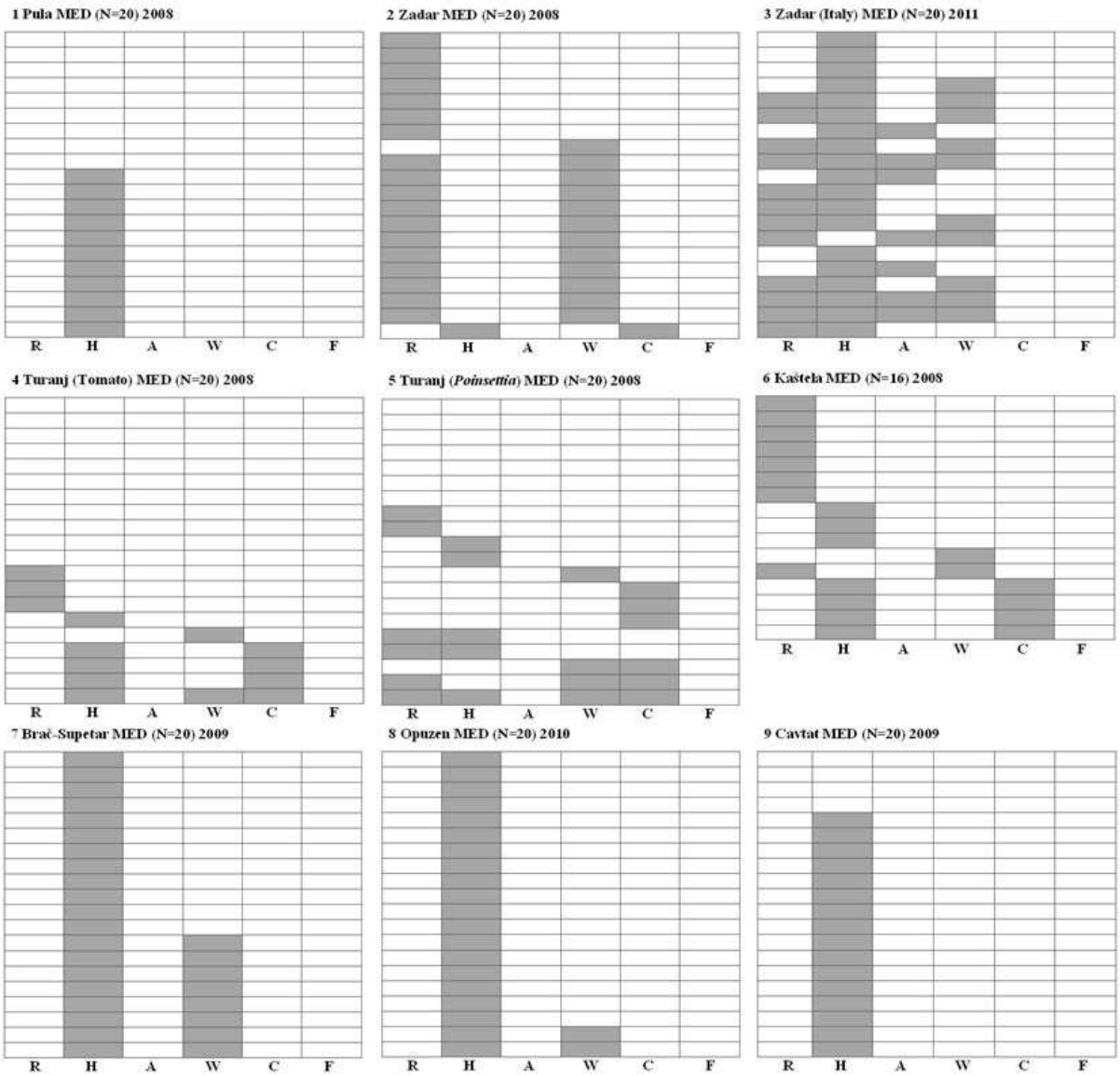


Figure 13. Individual and multiple infections by secondary bacterial symbionts in the Croatian *Bemisia tabaci* populations included in the survey and described in Table 1. Eight populations collected across coastal Croatia and one population imported from Italy were tested for the presence of secondary bacterial symbionts. Each table represents one population and each column represents one type of symbiont; the number of rows per table represents the number of individuals tested per population. Gray fields indicate positive infection for the tested symbiont. Population number, number of tested individuals, genetic group, and geographical location are indicated at the top of each table. Symbionts: R - *Rickettsia,* H - *Hamiltonella*, A - *Arsenophonus*, W - *Wolbachia*, C - *Cardinium*, F – *Fritschea*.

Table 5. Summary inspection of *Bemisia tabaci* populations from Croatia (2008 – 2011).

a) Infection type in Croatian *B. tabaci* populations with secondary symbionts (SS)

b) The portion of secondary symbionts (SS) in tested Croatian *B. tabaci* populations

a) The type of infection with SS	**Number and percentage of infected individuals** (Total No. of tested individuals, **N=176**)
Non infected individuals	31 (18%)
Single infected individuals	93 (53%)
Double infected individuals	40 (23%)
Triple infected individuals	8 (5%)
Fourfold infected individuals	4 (2%)
b) The portion of SS	**Number and percentage of infected individuals** (Total No. of tested individuals, **N=176**)
Rickettsia infected individuals	47 (27%)
Hamiltonella infected individuals	104 (59%)
Arsenophonus infected individuals	7 (4%)
Wolbachia infected individuals	40 (23%)
Cardinium infected individuals	15 (9%)
Fritschea infected individuals	0 (0%)

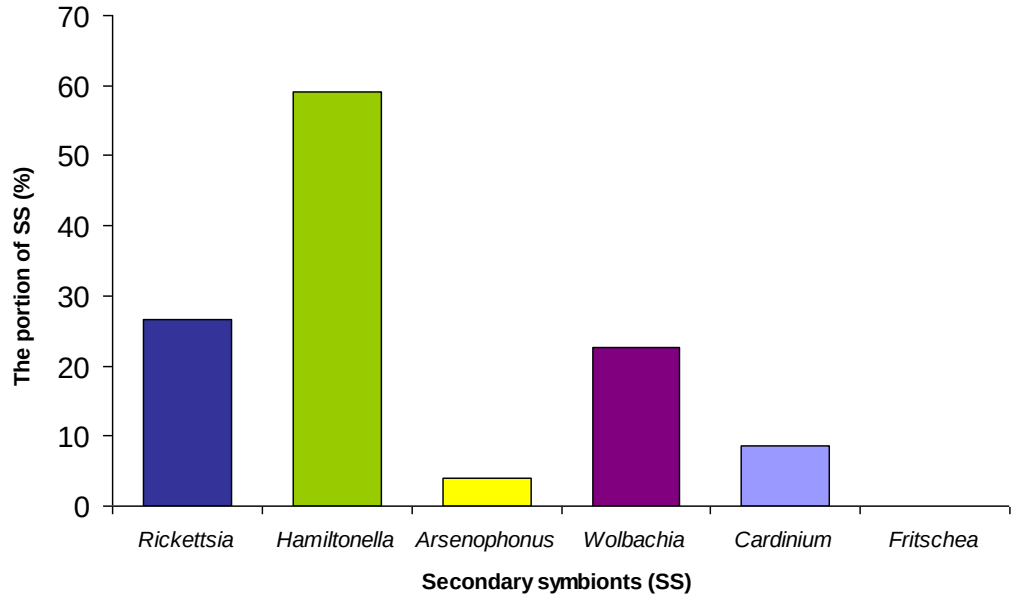


Figure 14. The portion of secondary symbionts (SS) in tested *Bemisia tabaci* populations from Croatia (2008-2011).

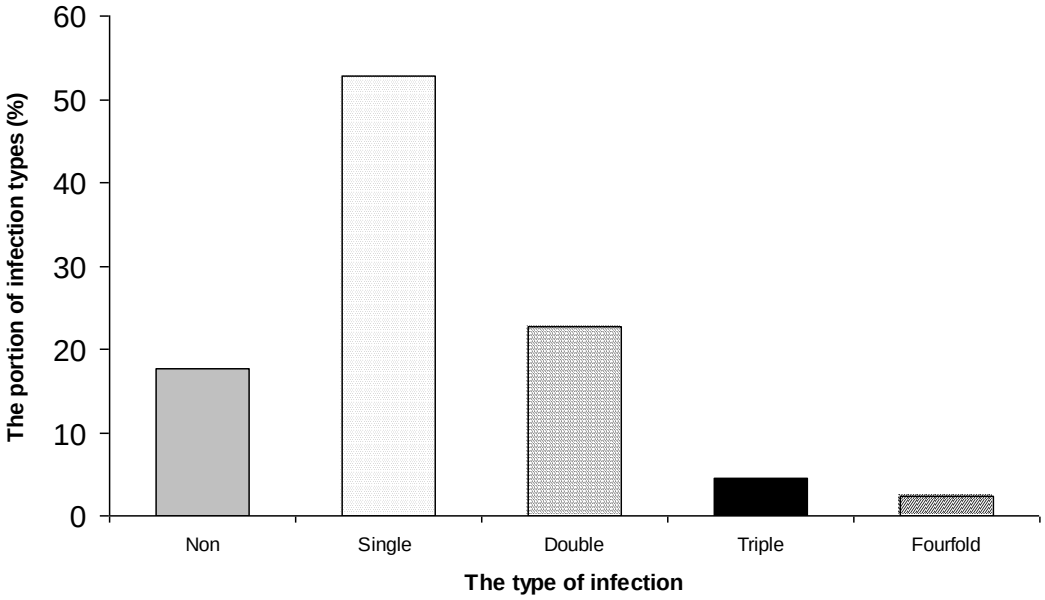


Figure 15. Infection types by secondary symbionts (SS), recorded in *Bemisia tabaci* populations from Croatia (2008-2011).

4.2. Bemisia tabaci distribution and infection by secondary symbionts in Bosnia and Herzegovina and Montenegro

The first *B. tabaci* population was collected in May 2008 at Podgorica (Montenegro); two populations were collected from Bosnia and Herzegovina in the summer 2009, while three *B. tabaci* populations were collected in June 2011 in greenhouses across Montenegro (Table 1). The *B. tabaci* populations sampled in Podgorica (2008, 2011) were identified as MEAM1, and the two others (from Bar and Ulcinj) were identified as MED, as well as *B. tabaci* populations from Bosnia and Herzegovina (Veljaci, Višići). All MED *B. tabaci* from neighbouring countries of Croatia belonbged to Q1 subgroup as previously described. Twenty adult females from each population were tested for the presence of the six secondary symbionts, while detection of *P. aleyrodidarum*, served as a control for the quality of the extracted DNA.

The MEAM1 population collected in Podgorica in 2008, was infected with *Hamiltonella* and *Wolbachia*. *Hamiltonella* was detected in 70% of individuals, while *Wolbachia* infected 30% of individuals. Nine individuals were single infected with *Hamiltonella*, one was single infected with *Wolbachia*, while five individuals were double infected with *Hamiltonella* and *Wolbachia.* *Bemisia tabaci* population of the same genetic group, collected on the same location in 2011, was infected with *Rickettsia* and *Hamiltonella*. In this population *Hamiltonella* was fixed while *Rickettsia* was close to fixation. Seventeen individuals of that population showed double infections with *Rickettsia* and *Hamiltonella*, while three individuals were infected only with *Hamiltonella*.

The *B. tabaci* MED populations collected in Bar and Ulcinj on *Dipladenia sanderi* Hemsl. showed a high level of multiple infections with *Rickettsia*, *Hamiltonella, Arsenophonus* and *Wolbachia*. The population collected in Bar showed fixed infection with *Rickettsia*, while *Arsenophonus* was close to fixation in both of them (Bar and Ulcinj). *Hamiltonella* was present in 65% and 30% of the tested individuals in the Bar and Ulcinj populations. Finally, 40% of individuals of the Bar population, and 30% of Ulcinj population were infected with *Wolbachia* (Figure 16).

Bemisia tabaci population collected from Veljaci (Bosnia and Herzegovina) showed similar infection status with *Hamiltonella* and *Wolbachia*, as mentioned population from Podgorica (collection, 2008). Only *Hamiltonella* was detected in population

collected from Višići (Bosnia and Herzegovina) where it was close to fixation, infecting 90% of individuals.

Hamiltonella showed the highest prevalence in tested populations from Montenegro as well as from Bosnia and Herzegovina, detected in 72% of the tested individuals (Figure 17). Within all *B. tabaci* populations from Montenegro and Bosnia and Herzegovina, 43% of all individuals showed single infection with secondary symbionts, 26% showed double infection, 14% were infected with three symbionts, and 5% showed infection with four symbionts (Figure 18). Overall, 88% (106/120) of all *B. tabaci* individuals from Montenegro and Bosnia and Herzegovina were infected with at least one secondary symbiont; 12% of individuals did not contain any of the tested secondary symbionts (Table 6).

Neither *Fritschea* nor *Cardinium* were detected in *B. tabaci* populations tested. *Bemisia tabaci* populations, principally from Montenegro, showed high variability and heterogeneity in their secondary symbionts.

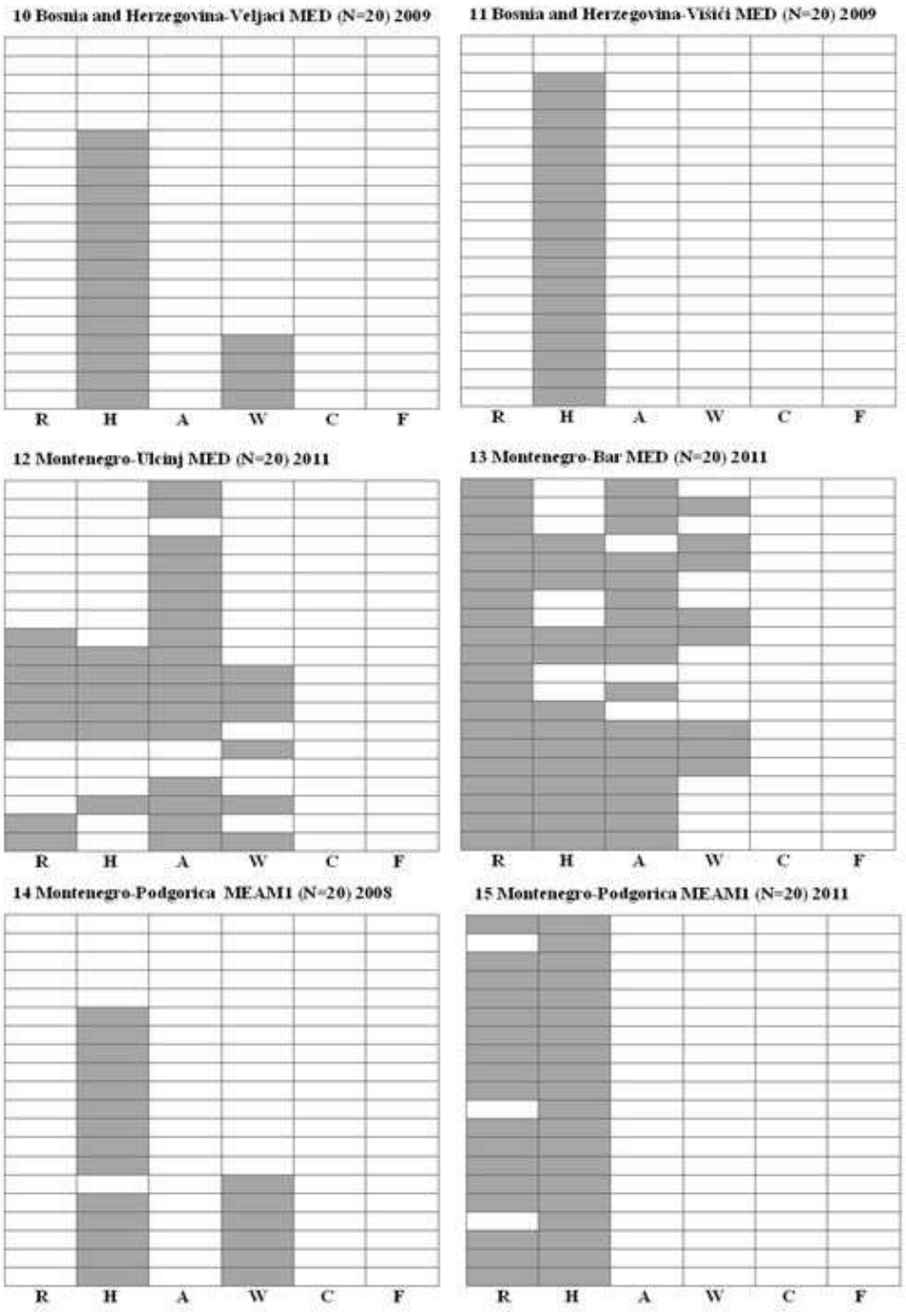


Figure 16. Individual and multiple infections by secondary bacterial symbionts in Bosnia and Herzegovina, and Montenegro *Bemisia tabaci* populations included in the survey, and described in Table 1. Two populations collected from Bosnia and Herzegovina and four populations collected across Montenegro were tested for the presence of secondary bacterial symbionts. Each table represents one population and each column represents one type of symbiont; the 20 rows per table represent the 20 individuals tested per population. Gray fields indicate positive infection for the tested symbiont. Population number, number of tested individuals, genetic group, geographical location and country of origin are indicated at the top of each table. Symbionts: R - *Rickettsia,* H - *Hamiltonella*, A - *Arsenophonus*, W - *Wolbachia*, C - *Cardinium*, F – *Fritschea*.

Table 6. Summary inspection of *B*emisia *tabaci* populations from Bosnia and Herzegovina, and Montenegro (2008 – 2011).

a) Infection type in tested Bosnia and Herzegovina, and Montenegro *B. tabaci* populations with secondary symbionts (SS)
b) The portion of secondary symbionts (SS) in tested Bosnia and Herzegovina, and Montenegro *B. tabaci* populations

a) The type of infection with SS	**Number and percentage of infected individuals** (Total No. of tested individuals, **N=120**)
Non infected individuals	14 (12%)
Single infected individuals	52 (43%)
Double infected individuals	31 (26%)
Triple infected individuals	17 (14%)
Fourfold infected individuals	6 (5%)
b) The portion of SS	**Number and percentage of infected individuals** (Total No. of tested individuals, **N=120**)
Rickettsia infected individuals	45 (38%)
Hamiltonella infected individuals	86 (72%)
Arsenophonus infected individuals	34 (28%)
Wolbachia infected individuals	24 (20%)
Cardinium infected individuals	0 (0%)
Fritschea infected individuals	0 (0%)

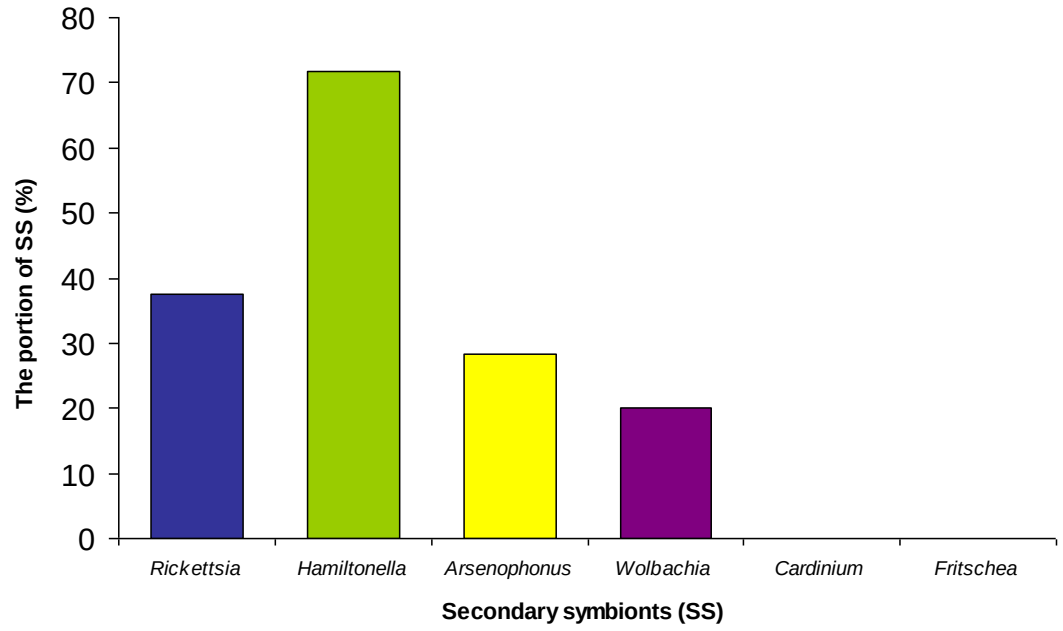


Figure 17. The portion of secondary symbionts (SS) in tested *Bemisia tabaci* populations from Bosnia and Herzegovina, and Montenegro (2008-2011).

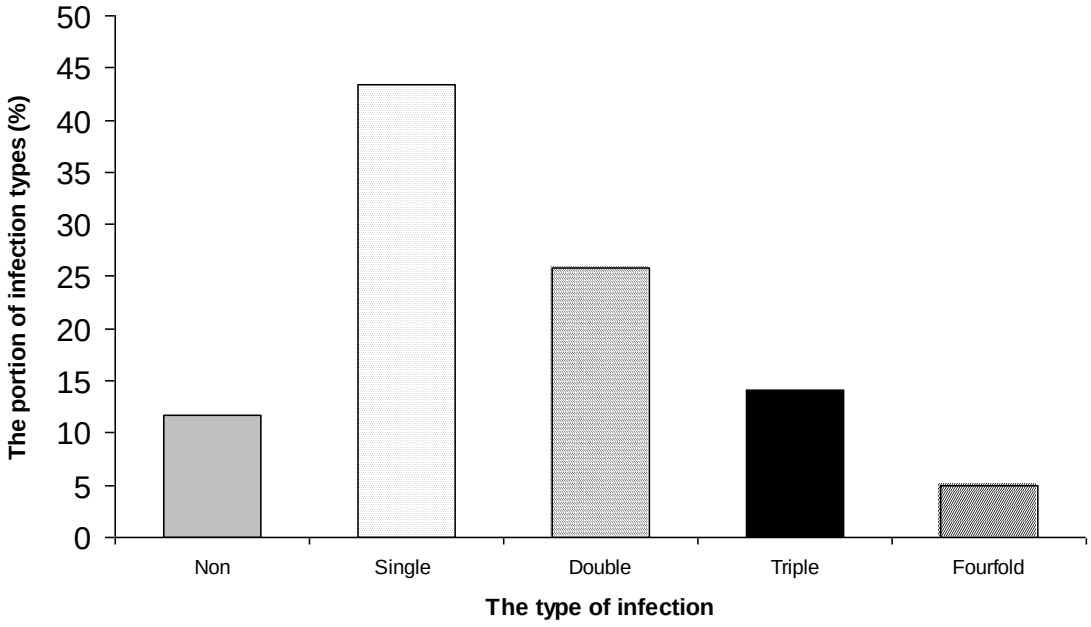


Figure 18. Infection types by secondary symbionts (SS), recorded in *Bemisia tabaci* populations from Bosnia and Herzegovina, and Montenegro (2008-2011).

4.3. Trialeurodes vaporariorum distribution and infection by secondary symbionts in Croatia

Fourteen *T. vaporariorum* populations were collected across Croatian coastal and inland regions in the period 2009-2010 (Figure 10), and tested for the presence of secondary symbionts. *Trialeurodes vaporariorum* was much more prevalent than *B. tabaci* in most of the regions, usually with heavy infestations in agricultural crops. *Portiera aleyrodidarum*, the primary symbiont, was detected in all individuals tested. Out of the six secondary symbionts tested, in the collected *T. vaporariorum* populations, only *Arsenophonus* and *Hamiltonella* were detected (Figure 19). *Arsenophonus* was more prevalent than *Hamiltonella*: it appeared in 72% of all individuals tested (101/140), as a single infection in 35% of all individuals, while *Hamiltonella* was detected in 44% of all individuals, and appeared as a single infection in 6% of all individuals (Figure 20). The prevalence of *Arsenophonus* was always higher or equal to that of *Hamiltonella* in all populations tested except for population from the island Brač, location Supetar. Two of tested populations (Pula and Turanj) were not infected with *Hamiltonella* and one population (Metković) showed fixation of both symbionts.

Trialeurodes vaporariorum population imported via the trade in ornamentals from Čatež ob Savi (Slovenia) to Split (Croatia), also showed infection with *Arsenophonus* and *Hamiltonella*. *Arsenophonus* was prevalent with 80% infected individuals, while *Hamiltonella* infected 50% of the tested individuals. Three individuals were singly infected with *Arsenophonus* and five individuals were double-infected with *Arsenophonus* and *Hamiltonella*. Two individuals did not contain any of the tested secondary symbionts.

In total, 79% (110/140) of all *T. vaporariorum* individuals collected from Croatia were infected with at least one secondary symbiont; 21% of individuals did not contain any of the tested secondary symbionts (Table 7). Single infections were scored in 41%, while 37% of tested individuals were doubly infected with *Arsenophonus* and *Hamiltonella* (Figure 21).

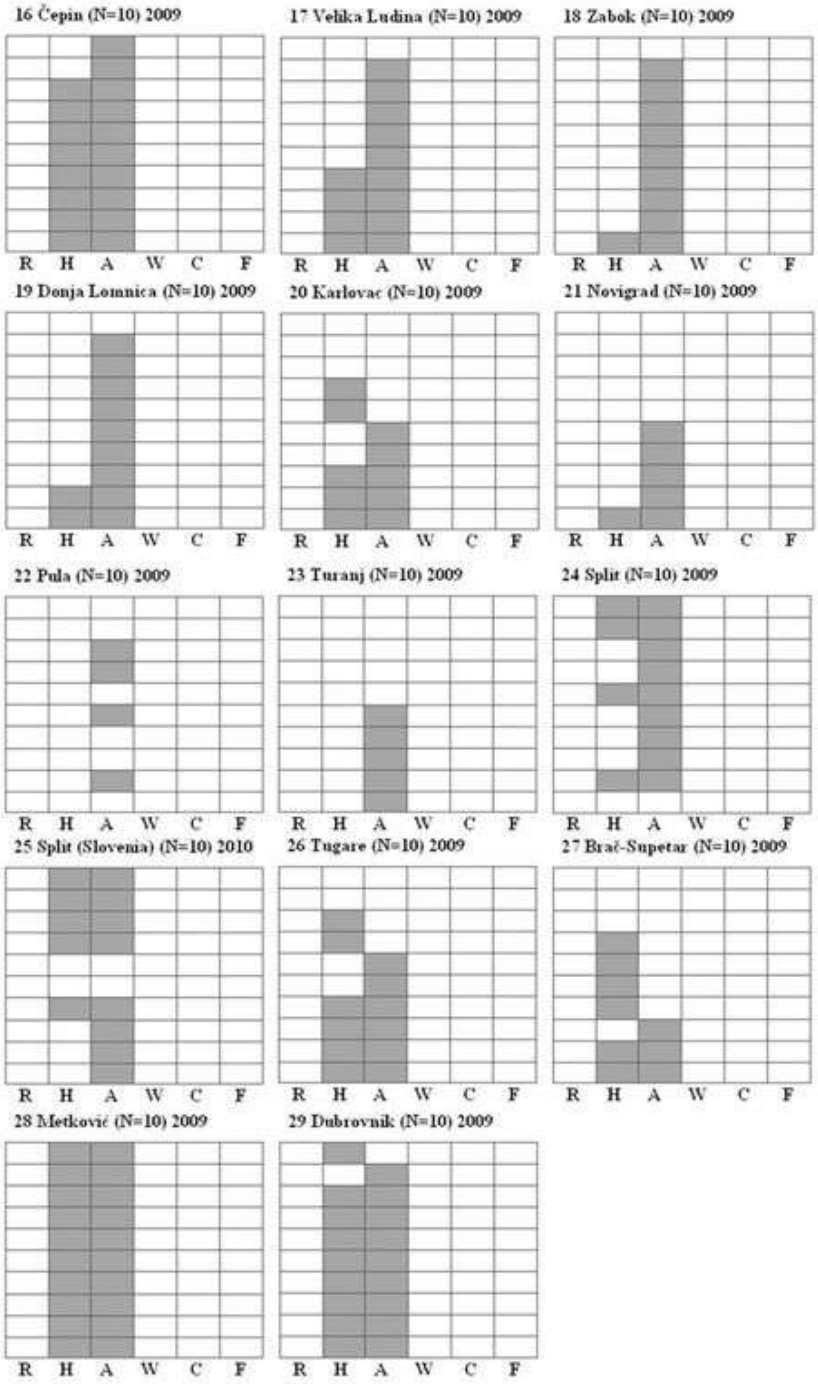


Figure 19. Individual and multiple infections by secondary bacterial symbionts in Croatian *Trialeurodes vaporariorum* populations included in the survey, and described in Table 1. Thirteen populations collected across Croatia and one population introduced from Slovenia were tested for the presence of secondary bacterial symbionts. Each table represents one population and each column represents one type of symbiont; the 10 rows per table represent the 10 individuals tested per population. Gray fields indicate positive infection for the tested symbiont. Population number, number of tested individuals and geographical location are indicated at the top of each table. Symbionts: R - *Rickettsia,* H - *Hamiltonella*, A - *Arsenophonus*, W - *Wolbachia*, C - *Cardinium*, F – *Fritschea*.

Table 7. Summary inspection of *Trialeurodes vaporariorum* populations from Croatia (2009-2010).

a) Infection type in Croatian *T. vaporariorum* populations with secondary symbionts (SS)

b) The portion of secondary symbionts (SS) in tested Croatian *T. vaporariorum* populations

a) The type of infection with SS	**Number and percentage of infected individuals** (Total No. of tested individuals, **N=140**)
Non infected individuals	30 (21%)
Single infected individuals	58 (41%)
Double infected individuals	52 (37%)
Triple infected individuals	0 (0%)
Fourfold infected individuals	0 (0%)
b) The portion of SS	**Number and percentage of infected individuals** (Total No. of tested individuals, **N=140**)
Rickettsia infected individuals	0 (0%)
Hamiltonella infected individuals	61 (44%)
Arsenophonus infected individuals	101 (72%)
Wolbachia infected individuals	0 (0%)
Cardinium infected individuals	0 (0%)
Fritschea infected individuals	0 (0%)

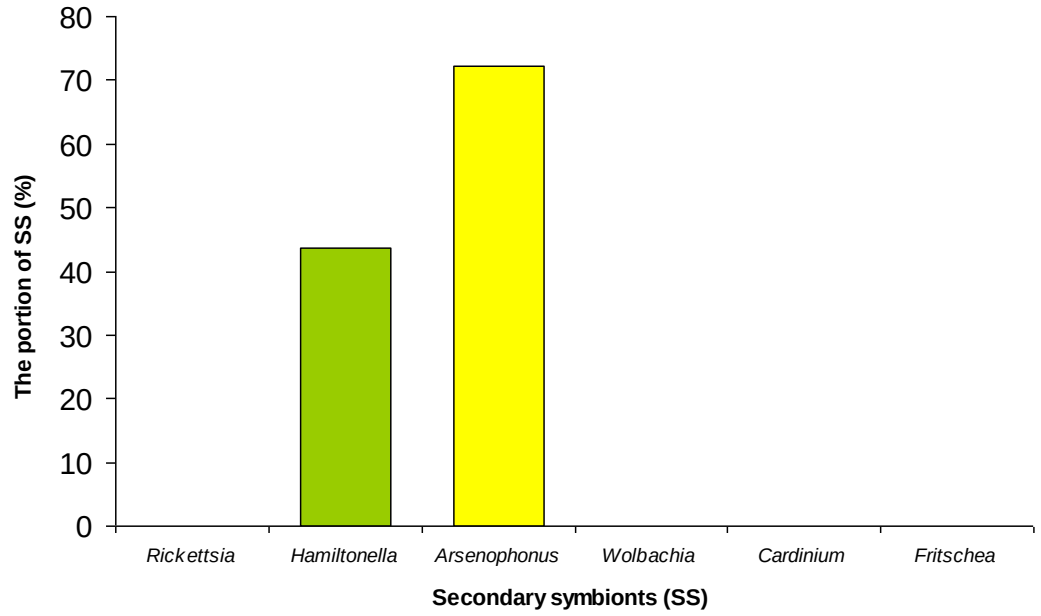


Figure 20. The portion of secondary symbionts (SS) in tested *Trialeurodes vaporariorum* populations from Croatia (2009-2010).

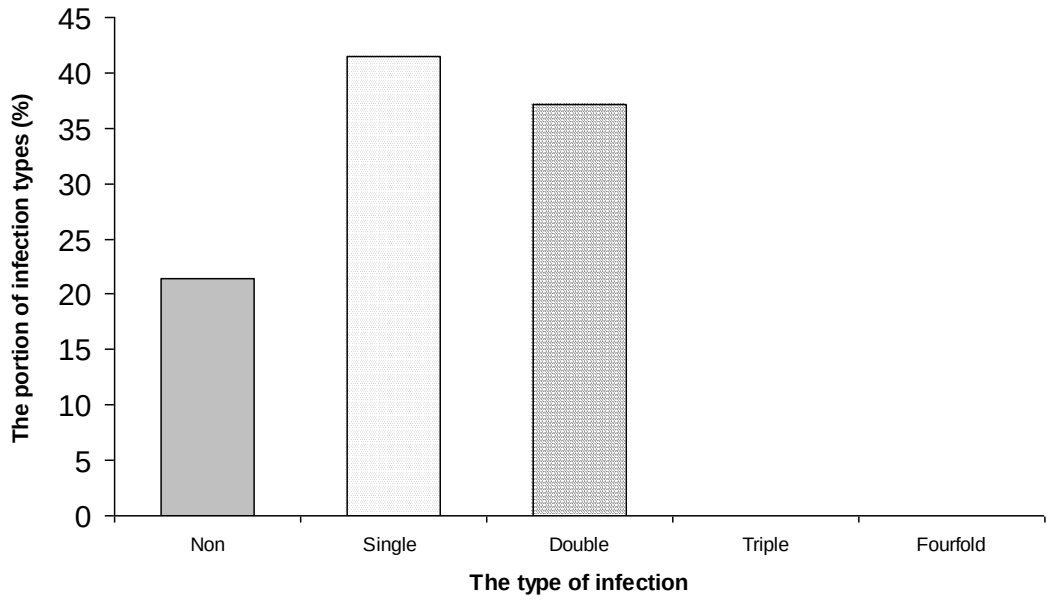


Figure 21. Infection types by secondary symbionts (SS), recorded in *Trialeurodes vaporariorum* populations from Croatia (2009-2010).

4.4. Trialeurodes vaporariorum distribution and infection by secondary symbionts in Bosnia and Herzegovina and Montenegro

In 2009-2010, two *T. vaporariorum* populations were collected from selected locations of Bosnia and Herzegovina, while five populations were collected in 2011 across Montenegro (Figure 10; Table 1). *Trialeurodes vaporariorum* was more frequent than *B. tabaci* in all examined locations of Bosnia and Herzegovina, and Montenegro. Ten to twenty adult females from each population were tested for the presence of the six secondary symbionts (Figure 22), while *P. aleyrodidarum*, was detected in all individuals.

Trialeurodes vaporariorum collected from two locations in Bosnia and Herzegovina (Veljaci, Gabela Polje) showed similar infection status like the populations collected in Croatia. *Arsenophonus* was found in populations collected from Veljaci and Gabela Polje, in 90 and 80% of infected individuals, respectively. *Hamiltonella* infected 70 and 60% of the mentioned populations, respectively. No other secondary symbionts were found in *T. vaporariorum* populations from Bosnia and Herzegovina.

Interestingly, an inspection of secondary symbiont infections of *T. vaporariorum* collected from Montenegro, showed high degree of heterogeneity. *Arsenophonus* was fixed in populations collected in Ulcinj, Bar and Radanovići, while it was close to fixation in populations collected in Podgorica and Sutorina. In addition, 20 to 45% of all individuals from Montenegro were infected by *Hamiltonella*, whereas *Wolbachia* was present in only 10% of the individuals, in two of the tested *T. vaporariorum* populations (Podgorica, Ulcinj). One individual belonging to the population collected in Podgorica had a single infection with *Wolbachia*, while another individual from the same population had a double infection with *Arsenophonus* and *Wolbachia*. In *T. vaporariorum* population from Ulcinj, one individual was triple-infected with *Wolbachia*, *Arsenophonus* and *Rickettsia*. *Cardinium* was found in populations collected in Bar, Sutorina, Radanovići and Podgorica in 5, 15, 25 and 35% of infected individuals, respectively. No individuals in the populations from Montenegro showed single infections with *Cardinium*, whereas most of them showed double infections with *Cardinium* and *Arsenophonus*. Two individuals, from Bar and Radanovići, were triple-infected with *Cardinium*, *Arsenophonus* and *Hamiltonella*. *Rickettsia* was found in populations collected in Ulcinj and Radanovići, with 5 and 10% of the individuals infected, respectively. In these two populations, one individual was infected with

Rickettsia, *Arsenophonus* and *Wolbachia* while another individual was infected with *Rickettsia* and *Arsenophonus*. *Arsenophonus* showed the highest prevalence in populations from Montenegro, detected in 97% of the tested individuals.

Single infection with secondary symbionts was recorded in 46% of all tested individuals from Bosnia and Herzegovina, and Montenegro, 47% of all individuals showed double infection and 3% of all individuals showed infection with three symbionts (Figure 24; Table 8). In general, *Arsenophonus* showed the highest prevalence in tested populations from Croatian neighbouring countries, detected in 96% of the tested individuals (Figure 23; Table 8). Overall, 96% (106/110) of all *T. vaporariorum* individuals from Bosnia and Herzegovina, and Montenegro were infected with at least one secondary symbiont, while only four individuals (4%) did not contain any of the tested secondary symbionts.
Fritschea was not detected in any of the *T. vaporariorum* population tested from Croatian neighbouring countries.

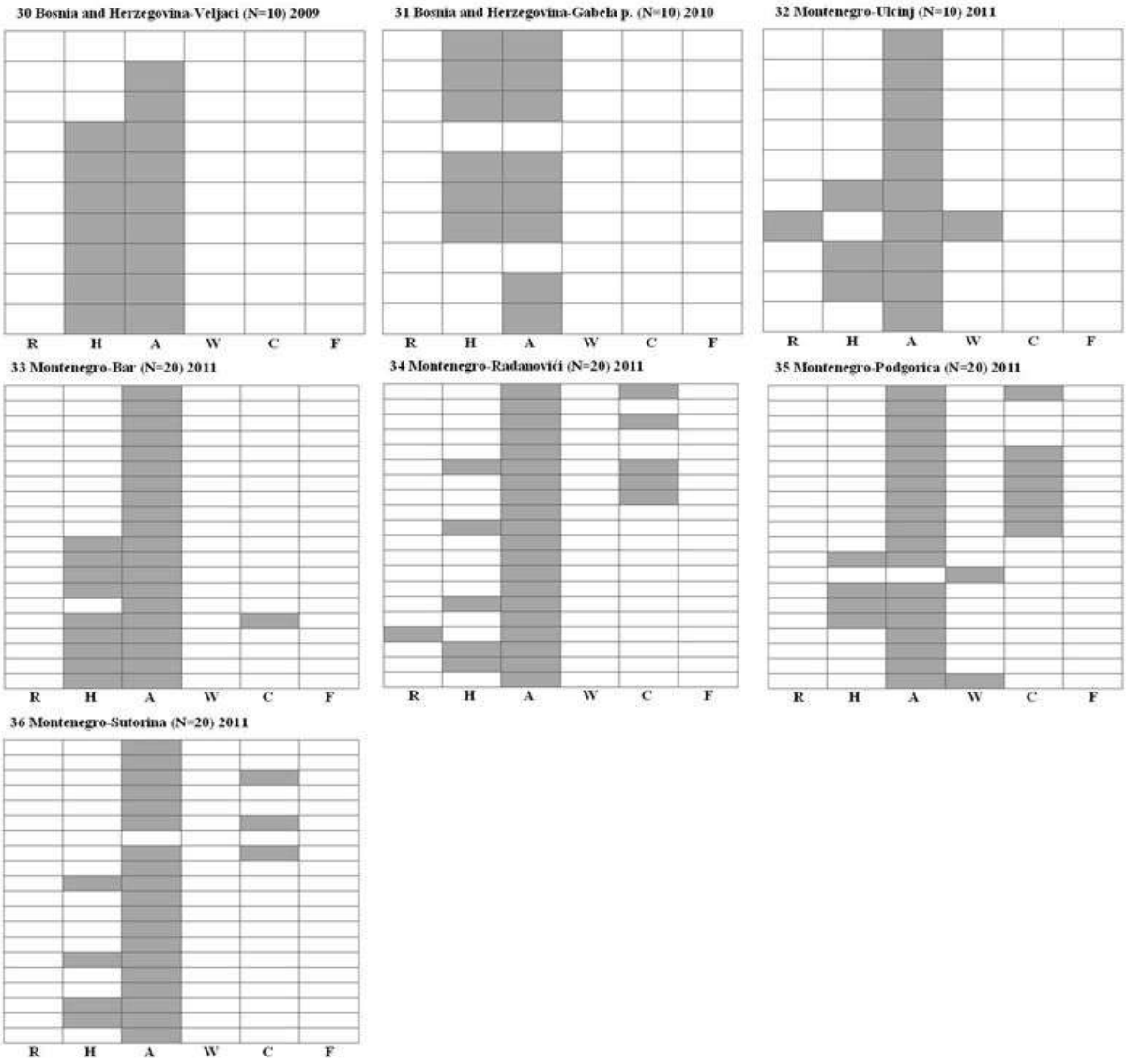


Figure 22. Individual and multiple infections by secondary bacterial symbionts in Bosnia and Herzegovina, and Montenegro *Trialeurodes vaporariorum* populations included in the survey and described in Table 1. Two populations collected from Bosnia and Herzegovina and five populations collected across Montenegro were tested for the presence of secondary bacterial symbionts. Each table represents one population and each column represents one type of symbiont; the 10 or 20 rows per table represent the 10 or 20 individuals tested per population. Gray fields indicate positive infection for the tested symbiont. Population number, number of tested individuals, geographical location and country of origin are indicated at the top of each table. Symbionts: R - *Rickettsia,* H - *Hamiltonella*, A - *Arsenophonus*, W - *Wolbachia*, C - *Cardinium*, F – *Fritschea*.

Table 8. Summary inspection of *Trialeurodes vaporariorum* populations from Bosnia and Herzegovina, and Montenegro (2009-2010).

a) Infection type in Bosnia and Herzegovina, and Montenegro *T. vaporariorum* populations with secondary symbionts (SS)
b) The portion of secondary symbionts (SS) in tested Bosnia and Herzegovina and Montenegro *T. vaporariorum* populations

a) The type of infection with SS	**Number and percentage of infected individuals** (Total No. of tested individuals, **N=110**)
Non infected individuals	4 (4%)
Single infected individuals	51 (46%)
Double infected individuals	52 (47%)
Triple infected individuals	3 (3%)
Fourfold infected individuals	0 (0%)
b) The portion of SS	**Number and percentage of infected individuals** (Total No. of tested individuals, **N=110**)
Rickettsia infected individuals	2 (2%)
Hamiltonella infected individuals	38 (35%)
Arsenophonus infected individuals	105 (96%)
Wolbachia infected individuals	3 (3%)
Cardinium infected individuals	16 (15%)
Fritschea infected individuals	0 (0%)

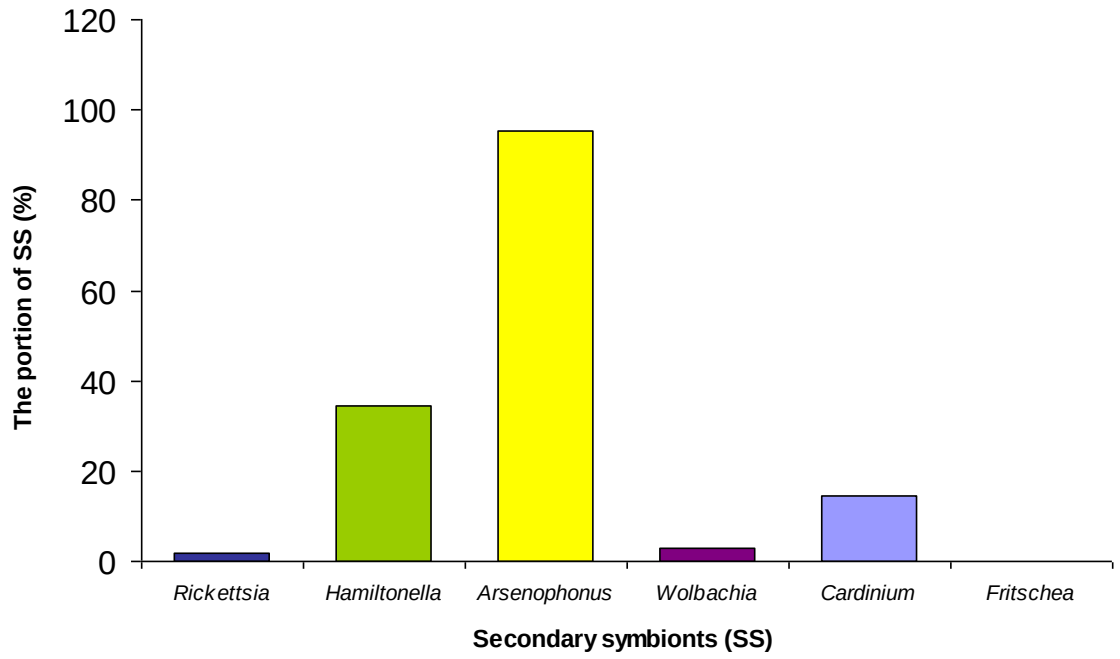


Figure 23. The portion of secondary symbionts (SS) in tested *Trialeurodes vaporariorum* populations from Bosnia and Herzegovina, and Montenegro (2009-2010).

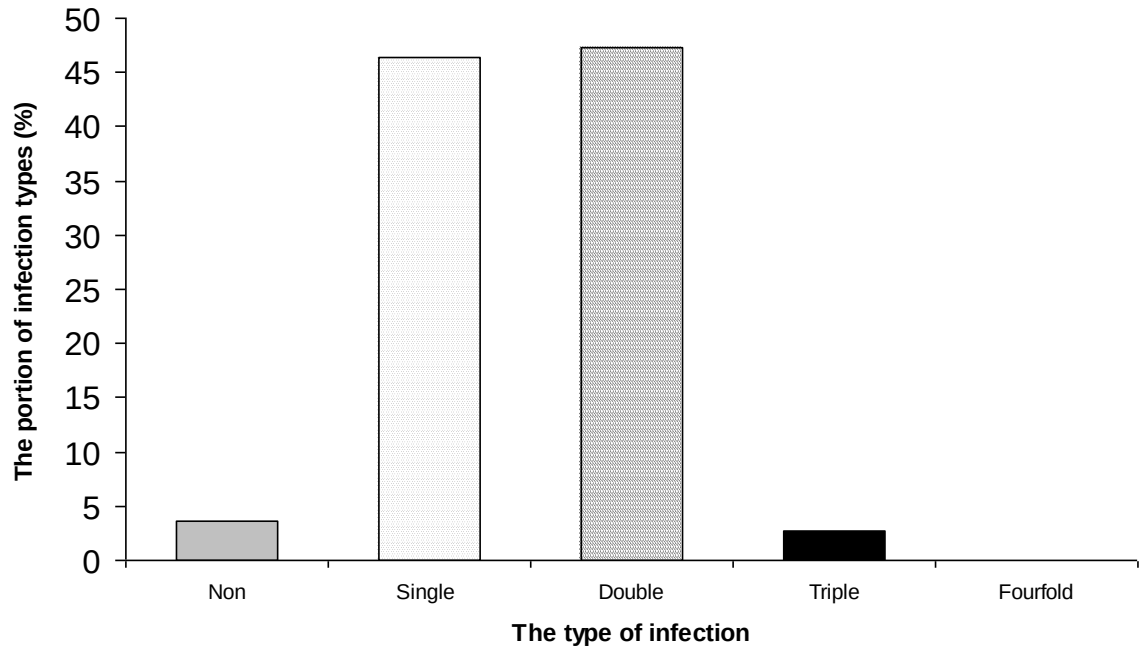


Figure 24. Infection types by secondary symbionts (SS), recorded in *Trialeurodes vaporariorum* populations from Bosnia and Herzegovina, and Montenegro (2009-2010).

4.5. Siphoninus phillyreae distribution and infection by secondary symbionts in Croatia and Montenegro

In spring 2011, heavy ash whitefly infestation of pomegranate was observed in Croatia and Montenegro. We therefore initiated a screening for secondary symbionts in its nine populations collected across coastal Croatia and Montenegro (Figure 10). *Portiera aleyrodidarum* was detected in all individuals tested and served as a control for DNA quality. Twenty adult females from each population were tested for the presence of six symbionts known from whiteflies. Figure 25 shows individual and multiple infections by secondary bacterial symbionts in the assessed *S. phillyreae* populations. Interestingly, all tested populations showed high heterogeneity in secondary symbiont composition. *Hamiltonella* showed the highest prevalence in assessed populations, detected in 87% of all tested individuals (Figure 26). It was fixed in the Croatian population collected on island Brač (location Pučišća), while it was close to fixation in all other tested populations, except the Croatian population collected on Brač (location Supetar) and the Montenegro population (location Ulcinj), in which 70 and 75% of individuals, respectively, were infected. All tested populations were positive for *Wolbachia*, 15 to 70% infected individuals belonged to Croatian populations, while 20 to 50% individuals from Montenegro populations being infected with this symbiont. *Cardinium* presence was variable among the different populations: three of six Croatian *S. phillyreae* populations were infected with *Cardinium*, with infection ranging from 5 to 70% of all individuals, while it was not detected in any *S. phillyreae* population from Montenegro. *Arsenophonus* was present in all populations tested. It was close to fixation in Croatian populations collected in Opuzen and Ljuta, whereas 20 to 65% of individuals from other Croatian locations were infected with *Arsenophonus*. *Siphoninus phillyreae* population collected in Bar showed fixed infection with *Arsenophonus*, while it was close to fixation in two other populations from Montenegro.

Interestingly, in most of the *S. phillyreae* populations tested, *Arsenophonus* appeared as two phenotypes or more likely, two strains. PCR products of *Arsenophonus* appeared at ~580 bp (*Arsenophonus* 580) and ~760 bp (*Arsenophonus* 760) (Figure 28). BLASTn analysis showed 97% similarity of the 580-bp large product to 23S rDNA of the *Arsenophonus* symbiont of *B. tabaci,* whereas the 760-bp large product showed 100% similarity to 23S rDNA of the *Arsenophonus* symbiont of *S. phillyreae*.

The strain *Arsenophonus* 760 did not appear in populations from Brač (Pučišća) or Ljuta which were generally positive for *Arsenophonus* 580. *Arsenophonus* 760 appeared in the seven other *S. phillyreae* populations tested in this study, in 5 to 50% of the total number of *Arsenophonus*-positive individuals.

In general, 24% of all tested *S. phillyreae* individuals showed single infection with secondary symbionts, 47% showed double infection, 26% showed triple infection and 2% of all tested individuals showed infection with four symbionts (Table 9; Figure 27). Overall, ~ 98% (177/180) of all *S. phillyreae* individuals were infected with at least one secondary symbiont and from all of the populations, three individuals did not contain any of the tested secondary symbionts.

Rickettsia and *Fritschea* were not detected in any *S. phillyreae* population tested in this study.

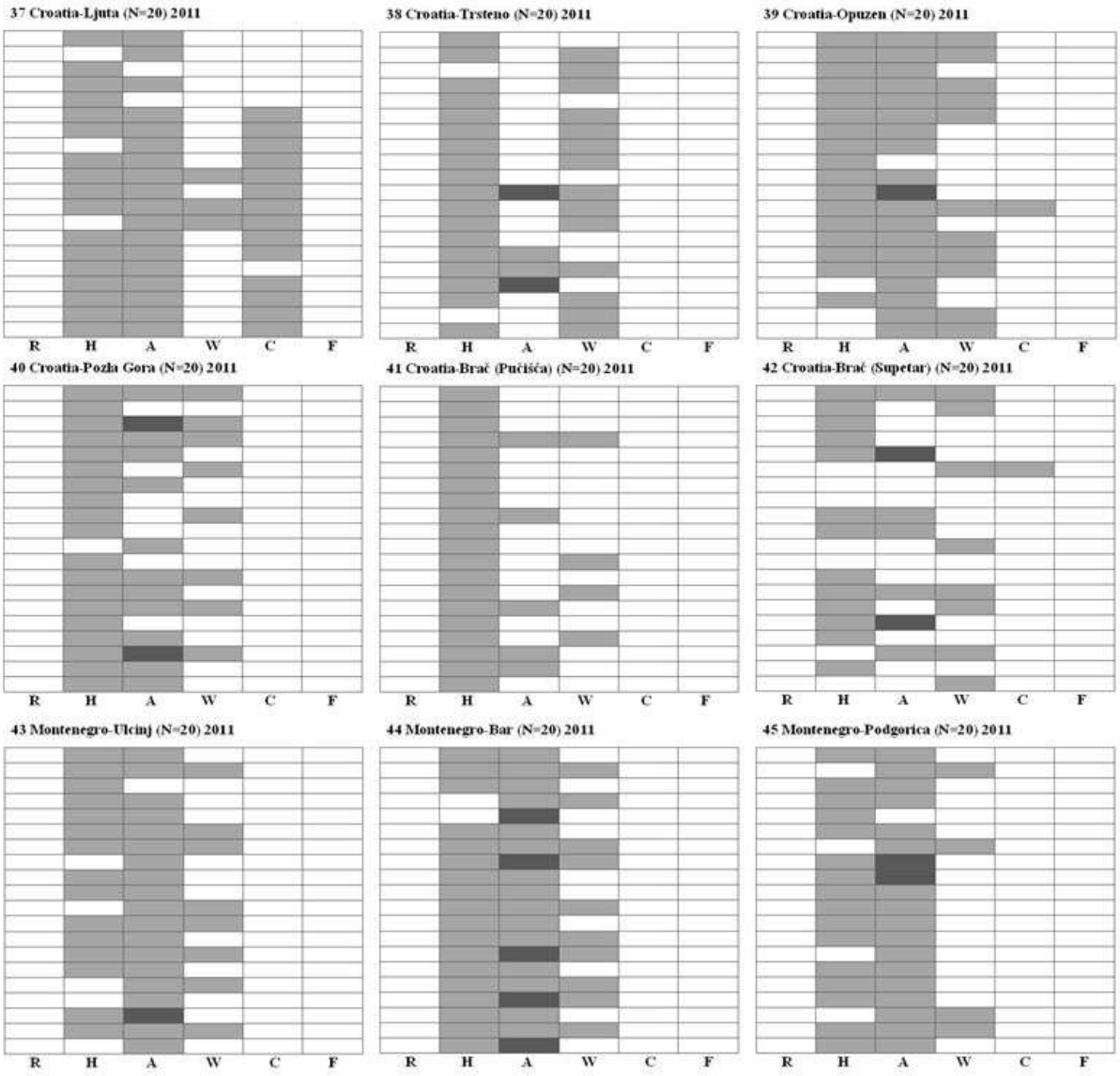


Figure 25. Individual and multiple infections by secondary bacterial symbionts in nine *Siphoninus phillyreae* populations included in the survey, and described in Table 1. Six populations collected across coastal Croatia and three populations collected across Montenegro were tested for the presence of secondary bacterial symbionts. Each table represents one population and each column represents one type of symbiont; the 20 rows per table represent the 20 individuals tested per population. Gray fields indicate positive infection for the tested symbionts (R, H, W, C, F) and *Arsenophonus* (A) recorded as *Arsenophonus* 580; dark gray fields indicate positive infection with *Arsenophonus* (A) recorded as *Arsenophonus* 760. Population number, number of tested individuals, geographical location and country of origin are indicated at the top of each table. Symbionts: R - *Rickettsia,* H - *Hamiltonella*, A - *Arsenophonus*, W - *Wolbachia*, C - *Cardinium*, F – *Fritschea*.

Table 9. Summary inspection of *Siphoninus phillyreae* populations included in the study (Croatia and Montenegro, 2011).

c) Infection type in *S. phillyreae* populations with secondary symbionts (SS)

d) The portion of secondary symbionts (SS) in *S. phillyreae* analysed populations

a) The type of infection with SS	**Number and percentage of infected individuals** (Total No. of tested individuals, **N=180**)
Non infected individuals	3 (2%)
Single infected individuals	43 (24%)
Double infected individuals	85 (47%)
Triple infected individuals	46 (26%)
Fourfold infected individuals	3 (2%)
b) The portion of SS	**Number and percentage of infected individuals** (Total No. of tested individuals, **N=180**)
Rickettsia infected individuals	0 (0%)
Hamiltonella infected individuals	156 (87%)
Arsenophonus 580 infected individuals	108 (60%)
Arsenophonus 760 infected individuals	15 (8%)
Wolbachia infected individuals	70 (39 %)
Cardinium infected individuals	16 (9%)
Fritschea infected individuals	0 (0%)

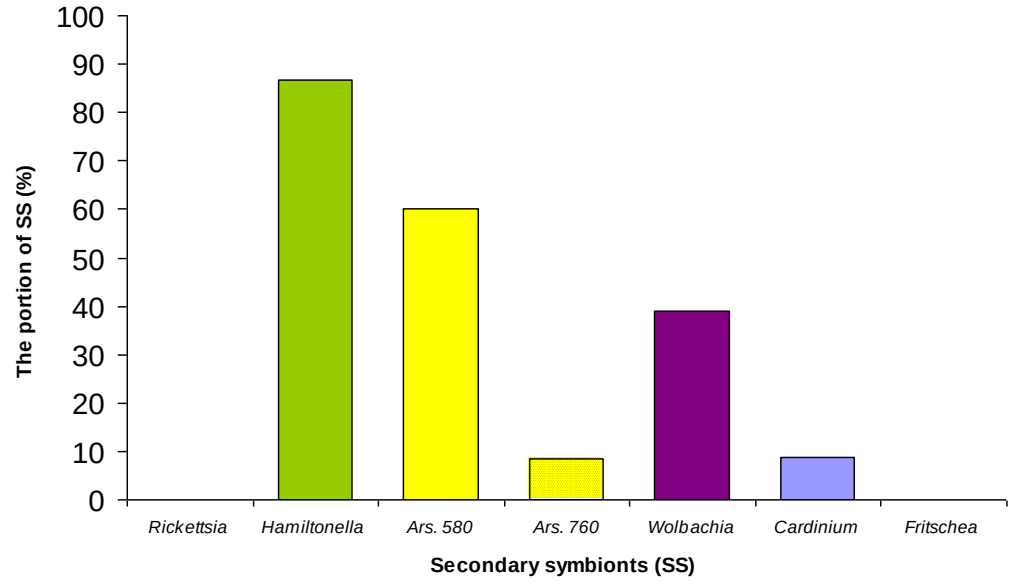


Figure 26. The portion of secondary symbionts (SS) in tested in *Siphoninus phillyreae* populations from Croatia and Montenegro (2011).

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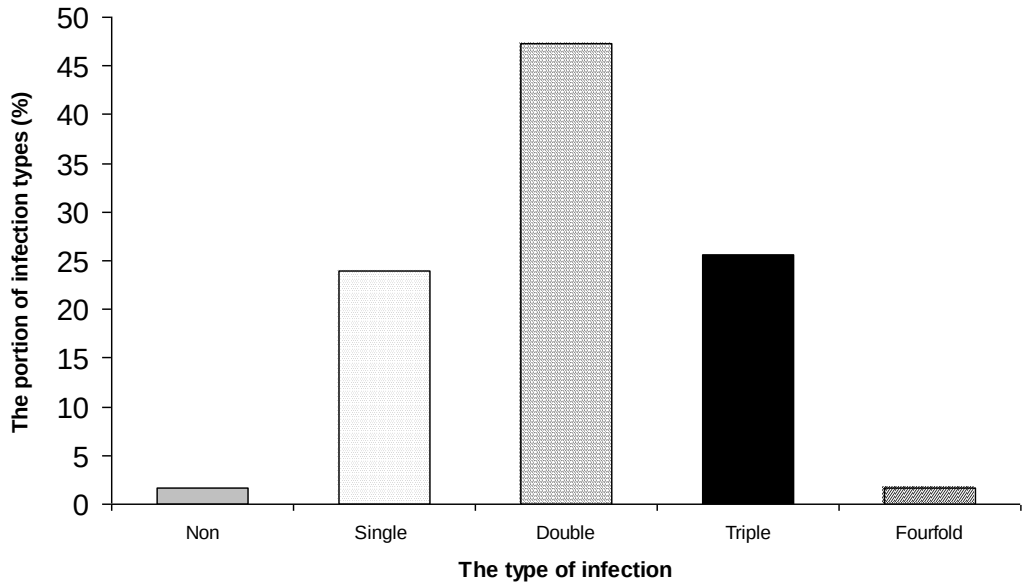

Figure 27. Infection types by secondary symbionts (SS), recorded in *Siphoninus phillyreae* populations from Croatia and Montenegro (2011).

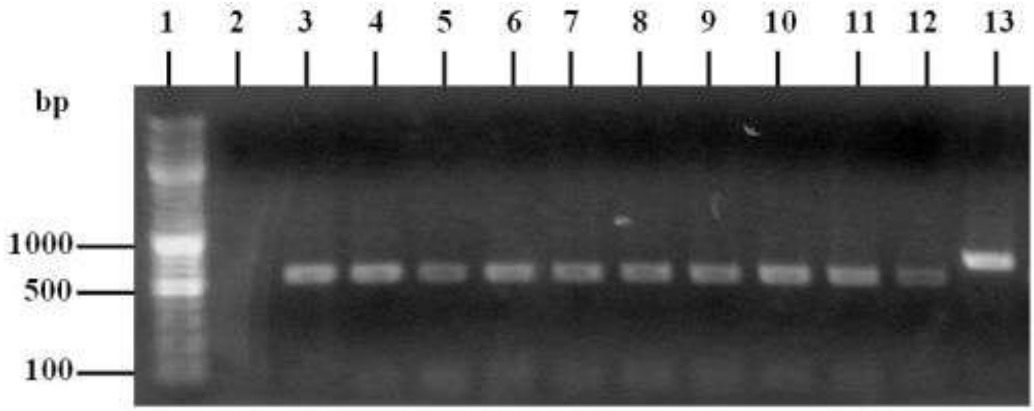

Figure 28. Agarose gel electrophoresis of polymerase chain reaction (PCR) products obtained with genus-specific primers Ars23S-1 and Ars23S-2 for *Arsenophonus* in whiteflies. Lane 1, DNA molecular marker (100-10,000 bp); lane 2, negative control; lanes 3–12, 10 *Arsenophonus* PCR products from *Siphoninus phillyreae*, product size 580 bp; lane 13, *Arsenophonus* PCR product from *Siphoninus phllyreae* individual, product size 760 bp.

4.6. Secondary symbionts sequences of Bemisia tabaci, Trialeurodes vaporariorum and Siphoninus phillyreae, recorded in present study

Cloned PCR products of six secondary symbionts known from whiteflies were sequenced and run against the non-redundant nucleotide database using the BLAST algorithm (NCBI). The percentage of similarity was scored between sequences from the present study and sequences present in the NCBI database. BLAST results confirmed presence of specific secondary sybmionts known from whiteflies.

4.6.1. Sequences of *Bemisia tabaci* secondary symbionts in this study

- ***Rickettsia*** **sequence**

>TGGGGCTTGCCCTTAGTTAGTGGCAGACGGGTGAGTAACACGTGGGAATCTGCCCATCAGTACGGAATAACTTT
TAGAAATAAAAGCTAATACCGTATATTCTCTACGGAGGAAAGATTTATCGCTGATGGATGAGCCCGCGTCAGATT
AGGTAGTTGGTGAGGTAATGGCTTACCAAGCCTACGATCTGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGC
AATACCGAGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTTAGCAAGGAAGATAATGACGTTACTTGCAGA
AAAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAAGACGGAGGGGGCTAGCGTTGTTCGGAATTACTGGGCG
TAAAGAGTGCGTAGGCGGTTTAGTAAGTTGGAAGTGAAAGCCTGGGGCTTAACCTCGGAATTGCTTTCAAAACTA
CTAATCTAGAGTGTAGTAGGGGATGATGGAATTCCTAGTGTAGAGGTGAAATTCTTAGATATTAGGAGGAACACC
AGTGGCGAAGCGGTCATCTGGGCTACAACTGACGCTGATGCACGAAAGCGTGGGGAGCAAACAGGATGATACCCT
GGTAGTCCACGCCGTAAACGATGAGTGCTGATATTGGGATATTTTCTCTCGGTTTCGCAGCTACGCATTAAGCAC
TCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCTCGCACAAGCGGTGGAGCATGC
GGTTTAATTCATGTTCCGAAAAACCTTACCACCCTTGACATGGTGGTCGCGGATCGCA

Sequence size: 882 bp

Similarity: 99% to sequence, GenBank access number: GU563842.1.

- ***Hamiltonella*** **sequence**

>GACGAAGGGTACTGCTGGAAACGGCAGCTAATACCGCATGAAGTCGTGAGACCAAAGTGGGGGACCTTCGGGCC
TCACGCCGTCGGATGAGCCCAGATGAGATTAGCTGGTAGGTAGGGTAAAGGCTTACCTAGGCGACGATCTCTAGC
GGGTCTGAGAGGATAGCCCGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
ATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCACGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTC
AGCGAGGAGGAAGCGATAAATGCCAATACCATTTATTTTTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCG
TGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATAACTGGGCGTAAAGGGCATGTAGGCGGTG
AGTTAAGTCAGATGTGAAATCCCCGAGCTCAACTTGGGAATGGCATTTGAAACTGGGTCGCTAGAGTTTTCTAGA
GGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGATATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCT
GGAGAAAGACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAA
ACGATGTCGATTTGGAGGTTCGGCCTTGTAAACTAAAAAAAAACGTCTATGAGGTAC

Sequence size: 731 bp

Similarity: 99% to sequence, GenBank access number: JF795506.1.

- ***Arsenophonus*** **sequence**

>TTTGTTAAACAGTTCATGATCGAGATGATGTCACCGAGATTCCCCCAGTAGCGGGGAGCGAACGGGGAGCAGCC
CAGAGTCAACATCAATATTTACCGCATGAGAAGGGTCTGGAAAGGCCGGCAACAAAGGGTGATAGCCCCCCGTAT
CTGAAACGGTAAGTGTTGTGAACTCGAAGAGTAGGGCGGGACACGTGTTATCCTGTTTGAATATGGGGGGACCAT
CCTCCAAGGCTAAATACTCCTGACTGACCGATAGTGAACCAGTACCGTGAGGAAAAGGTAAAAAAACCCCGGCGA
GGGGAGTAAAATAGAACCTGAAACTCTGTACGTACAAGCAGTGGGAGCACCCGAAAGGGTGTGACTGCGTACCTT
TTTTATATGCGTACCTTTTGTATAAAAGGTCAGCGACTTATATTCTGTAGCACGGTTAACCGGATAGAGAAGCCG
TATGGAAACCGACTCTTAACTGGGCGTTAAGTTCCAGGGTATAGACCCGAAACCCGGTGATCTAGCCATGGGCAG
GTTGAAGGTTGGGTAACACTAACTGGAGGACC

Sequence size: 556 bp

Similarity: 99% to sequence, GenBank access number: FJ766369.1.

- ***Wolbachia*** **sequence**

>TGGTGGAGTAATAGCCTACCAGGCAATGATCTATAGCTGATCTGAAGGATGATAGCCCACTGGAACTGAGATAC
GGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCACTGCCGCATGAG
TGAAGAAGGCCTTTGGGTTGTAAAGCTCTTTTAGTGAGGAAGATAATGACGGTACTCACAGAAGAAGTCCTGGCT
AACTCCGTGCCAGCAGCCGCGGTAATACGGAGAGGGCTAGCGTTATTCGGAATTATTGGGCGTAAAGGGCGCGTA
GGCTGATTAATAAGTTAAAAGTGAAATCCCGAGGCTTAACCTTGGAACTGCTTTTAAAACTATTAATCTAGAGAT
TGAAAGAGGATAGAGGAATTCCTGATGTAGAGGTAAAATTCGTAAATATTAGGAGGAACACCAGTGGCGAAGGCG
TCTATCTGGTTCAAATCTGACGCTGAGCGCGAAGGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CTTAAACGATGAATGTTAAATATGGGAAGTTTACTTTCTGTATCAGCTA

Sequence size: 573 bp

Similarity: 98% to sequence, GenBank access number: JF795503.1.

- ***Cardinium*** **sequence**

>GCTACATGGGTAGGGGGTTCTTAGTGGAAGATCCCCCACACTGGCACTGAGATACGGGCCAGACTCCTACGGGA
GGCAGCAGTAGGGAATATTGGTCAATGGGCGAAAGCCTGAACCAGCCATGCCGCGTGCAGGATGACAGCTCTCTG
AGTTGTAAACTGCTTTTGTACAGGAGCAAAACAATCCCTGCGGGGGTTCTTGAGAGTACTGTAAGAATAAGCACC
GGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGAAGGTGCAAGCGTTATCCGGTTTTATTGGGTTTAAAGGGTG
CGTAGGCGGCTTATTAAGTCAGTTGTGAAATCCTAGTGCTTAACGCTAGAACTGCAATTGATACTATTAGGCTTG
AGTTAAGATAGGTA

Sequence size: 388 bp

Similarity: 98% to sequence, GenBank access number: HM452326.1.

- **mtCOI (MED) sequence**

>TAACTGCTAATTGACTATTGGTATCTTAGGGTTTATTGTTTGAGGACATCATATATTTACAGTTGGAATAGATG
TAGATACTCGAGCTTATTTCACTTCAGCTACTATGATTATTGCCGTTCCTACAGGAATTAAAATTTTTAGTTGGC
TTGCTACTTTGGGTGGAATAAAGTCCAATAAATTCAGGCCCCTTGGCCTTTGATTTACAGGATTTTTATTTTTAT
TTACTATAGGTGGATTAACTGGAATTATTCTTGGTAACTCTTCTGTAGATGTGTGTTTGCATGACACTTATTTTG
TTGTTGCGCATTTTCATTATGTCTTATCAATAGGAATTATTTTTGCTATTGTAGGAGGAGTTATCTATTGATTTC
CATTAATCTTGGGCTTAACCTTAAATAATTATAGCTTGGTGTCTCAATTTTATATCATGTTCATTGGAGTAAATT
TAACTTTTTTTCCTCAGCATTTTCTTGGTTTGGGGGGAATGCCTCGCCGATATTCAGATTATGCTGATTGTTATC
TAGTATGGAACAAAATTTCTTCTGCGGAAATTTTTTAAAAAAAACCCCGATGGGGGAGGGGAGGGCATGATCCTG
GGGTCGGGTTCTTAGTGCTCCCCCGACGAAAAATCCATCATTTGATT

Sequence size: 646 bp

Similarity: 99% to sequence, GenBank access number: HM488325.1.

- **mtCOI (MEAM1) sequence**

>CCATATGCTATTGACTATTGGTATCTTAGGGTTTATTGTTTGAGGACATCATATATTTACAGTTGGAATAGATG
TAGATACTCGAGCTTATTTCACTTCAGCTACTATGATTATTGCTGTTCCTACAGGAATTAAAATTTTTAGTTGGC
TTGCTACTTTGGGTGGAATAAAGTCCAATAAATTCAGGCCCCTTGGCCTTTGATTTACAGGATTTTTATTTTTAT
TTACTATAGGTGGATTAACTGGAATTATTCTTGGTAACTCTTCTGTAGATGTGTGTTTGCATGACACTTATTTTG
TTGTTGCGCATTTTCATTATGTCTTATCAATAGGAATTATTTTTGCTATTGTAGGAGGAGTTATCTATTGATTTC
CATTAATCTTGGGTTTAACCTTAAATAATTATAGTTTGGTGTCTCAATTTTATATCATGTTCATTGGAGTAAACT
TAACTTTTTTTCCTCAGCATTTTCTTGGTTTGGGGGGAATGCCTCGCCGATATTCAGATTATGCTGATTGTTATC
TAGTATGGAACAAAATTTCTTCTGCGGGAAGGTTTTTTTGGAGTACACCCAACCTCCCCCTATACAAATTCCCCC
AAAATGTACCCATCGAGCCAGTGTCCCCCCCTCCTTAAGACG

Sequence size: 641 bp

Similarity: 99% to sequence, GenBank access number: HQ877591.1.

4.6.2. Sequences of *Trialeurodes vaporariorum* secondary symbionts in this study

- ***Rickettsia* sequence**

>AGTCACGGACTAATTGGGGCTTGCTCCAATTAGTTAGTGGCAGACGGGTGAGTAACACGTGGGAATCTGCCCAT
CAGTACGGAATAACTTTTAGAAATAAAAGCTAATACCGTATATTCTCTACGGAGGAAAGATTTATCGCTGATGGA
TGAGCCCGCGTCAGATTAGGTAGTTGGTGAGGTAATGGCTTACCAAGCCTACGATCTGTAGCTGGTCTGAGAGGA
TGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG
CGAAAGCCTGATCCAGCAATACCGAGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTTAGCAAGGAAGATA
ATGACGTTACTTGCAGAAAAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAAGACGGAGGGGGCTAGCGTTG
TTCGGAATTACTGGGCGTAAAGAGTGCGTAGGCGGTTTAGTAAGTTGGAAGTGAAAGCCTGGGGCTTAACCTCGG
AATTGCTTTCAAAACTACTAATCTAGAGTGTAGTAGGGGATGATGGAATTCCTAGTGTAGAGGTGAAATTCTTAG
ATATTAGGAGGAACACCAGTGGCGAAGGCGGTCATCTGGGCTACAACTGACGCTGATGCACGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGATATTGGGATATTTTCTCTCGGTT
TCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGC
TCGCACAAGCGGTGGAGCATGCGGTTTAATTCGATGTTACGCGAAAAACCTTACCAACCCTTGACATGGTGGTCG
CGGATCGCATCCTCTCTCATACTAAAAAAAAAAAA

Sequence size: 934 bp

Similarity: 100% to sequence, GenBank access number: GU563842.1.

- ***Hamiltonella* sequence**

>ATGAAGTCGTGAGACCAAGTGGGGGACCTTCGGGCCTCACGCCGTCGGTGAGCCCAGATGAGATTAGCTGGTAG
GTAGGGTAAAGGCTTACCTAGGCGACGATCTCTAGCGGGTCTGAGAGGATAGCCCGCCACACTGGAACTGAGACA
CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCACG
TGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGCGATAAATGCCAATACCATTTATTTT
TGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAA
TCGGAATAACTGGGCGTAAAGGGCATGTAGGCGGTGAGTTAAGTCAGATGTGAAATCCCCGAGCTCAACTTGGGA
ATGGCATTTGAAACTGGGTCGCTAGAGTTTTCTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGA
TATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGAGAAAGACTGACGCTGAGGTGCGAAAGCGTGGGGAGC
AAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGCGGTCTTGAAACTA

Sequence size: 670 bp

Similarity: 99% to sequence, GenBank access number: HM452317.1.

- ***Arsenophonus* sequence**

>TACCCCGAGGAAAAGAAATCAACCGAGATTCCCCCAGTAGCGGCGAGCGGACGGGGAGCAGCCCAGAGTCAGCA
TCAATATTTACCGCAGGAGAAGGGTCTGGAAAGCCGGCAATAAAGGGTGATAAGCCCCGTATCTGAAGCGGTAAG
TGTTGTGAACTCGAAGAGTAGGGCGGGACACGTGTTATCCTGTCTGAATATGGGGGGACCGCTACTGCTAGACCG
GGGAAAGGACCCGAGGAAAACCTGAACCGTCCAGCCAAAGGGACTGCGTCCTTGGGTCAACTTATTGGCTAGGAG
GAAACGAGTCTTGGGTTTGGGTATACCAAACCTCGGAGTAGGTACTGCC

Sequence size: 348 bp

Similarity: 99% to sequence, GenBank access number: EU559190.1.

- ***Wolbachia* sequence**

>CTTGTGTATAGCTAGTTGGTGGAGTATAGCCTACCAAGGCAATGATCTATAGCTGATCTGAGAGGATGATCAGC
CACACTGGAACTGAGATACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCC
TGATCCAGCCATGCCGCATGAGTGAAGAAGGCCTTTGGGTTGTAAAGCTCTTTTAGTGAGGAAGATAATGACGGT
ACTCACAGAAGAAGTCCTGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGAGGGCTAGCGTTATTCGGAAT
TATTGGGCGTAAAGGGCGCGTAGGCTGATTAATAAGTTAAAAGTGAAATCCCGAGGCTTTAACCTTGGAATTTGC
TTTTTAAAACTAATTAATCTAGAGATTGGAAAGGAGGAATAGAAGGAAATTTCCTGGATGGTAAGGGAAAAATTC
GTTAAAATAATTTAGGGAGGGAACCACCCATGGGCGAAAGGGCGTTCTATTCTTGGTCAAAATCTTGACGCTTGA
ACCCCAAAGGCGTGGGGAAAACAGGATTAGTTCCCCTGGTATCCAACTTGAAAACAAGAAATGTAAAAATGGGAT
TTTTTCGGTTTACCGAA

Sequence size: 616 bp

Similarity: 91% to sequence, GenBank access number: JF795503.1.

- ***Cardinium* sequence**

>CCGTTATGAAGCATGGCTCACCAAGGCTACGATGGGTAGGGGTTCTTAGTGGAAGATCCCCCACACTGGCACTG
AGATACGGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGGTCAATGGGCGAAAGCCTGAACCAGCCATG
CCGCGTGCAGGATGACAGCTCTCTGAGTTGTAAACTGCTTTTGTACAGGAGCAAAACAATCCCTGCGGGGGTTCT
TGAGAGTACTGTAAGAATAAGCACCGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGAAGGTGCAAGCGTTAT
CCGGTTTTATTGGGTTTAAAGGGTGCGTAGGCGGCTTATTAAGTCAGTTGTGAAATCCTAGTGCTTAACGCTAGA
ACTGCAATTGATACTATTAGGCTTGAGACCAAAATAGGTAACGCCCTCCCCCCCAATTCGAAATTACAATGAAAA
ACAATCCTGGCAGAAACTTTCTCATTGGATTACATAAGCCTCACAAACGCGTAAGCGTGTGGAGCAGACAGGATT
TGATACTCTGGTAGTCCTTTCTGTAAGGGATGAATGTTAAATATAGGGAGTTTGCTTTTGTTTTTTTGTTAAGGT
GGCCGGTGGTGTATG

Sequence size: 614 bp

Similarity: 91% to sequence, GenBank access number: HM452326.1.

4.6.3. Sequences of *Siphoninus phillyreae* secondary symbionts in this study

- ***Arsenophonus* 580 sequence**

>TTCAAAGTGTCACGTACGGAATACCGACCAACGAGATTCCCCCAGTACGGCGAGCAACGGGGAGCCCCAGAGTC
AACATCAATATTTACCGCAGGAGAAGAGTCTGAAAAGACCGGCAATAAAGGGTGATAGCCCCGTATCTAAAACGG
TAAGTGTTGTGAACTCAAAGAGTAGGGCGGGACACGTGTTATCCTGTCTGAATATGGGGGGACCATCCTCCAAAG
CTAAATACTCCTGACTGACCGATAGTGAACCAGTACCGTGAGGGAAAGGCGAAAAGAACCCCGGCGAGGGGAGTG
AAATAGAACCTGAAACCGTGTACGTACAAGCAGTGGGAGCACCCAAAAAAGTGTGACTGCGTACCTTTTGTATAA
TGGGTCAGCGACTTATATTCTGTAGCAAGGTTAACCAGATAGGGGAGCCGTAGGGAAACCGAGTCTTAACTGGGC
GTTAAGTTGCAGGGTATAGACCCGAAACCCGGTGATCTAGCCATGGGCAGGTTGAAGGTTGGGTAACACAATGGA
GGACCA

Sequence size: 530 bp

Similarity: 96% to sequence, GenBank access number: JF795493.1.

- ***Arsenophonus* 760 sequence**

>TTACAACTTGTGCACGTCTTTTGTACAGACCACCGAGATTCCCCTAGTAGCGGCGAGCGAACGGGGAGTAGCCC
AGAGTCAGCATCGATATTAGCATCAGGAGAAGGGTCTGGAAAGGCCAGCCATAAAGGGTGATAGCCCCGTATCCG
AAGGKGTTAGTATCGTGAGCTCGAAGAGTAGGGCGGGACACGTGTTATCCTGTCTGAATAAGGGGGGACCATCCT
CCAAGGCTAAATACTCCTGACTGACCGATAGTGAACCAGTACCGTGAGGGAAAGGCGAAAAAACCCCGGCGAGGG
GAGTGAAAGAGAACCTGAAACCGTGTACGTACAAGCAGTGGGAGCCCCATCACTCAAGTGCCTCGGCAGTTGAGT
GATGGTATACCATCACAATAACAGCGCAGGCTTTGCCAACAAGGTTGTTCGGTTTTTTCAAGCCGCAGCGCATGT
AGCTGAAGTACACGAGCAGCGGAAGTTAAAAAAGCGGGCAAGATTGGCCGCAAAGACAAGCGGGACATTTGAGGA
TGGGGTGACTGCGTACCTTTTGTATAATGGGTCAGCGACTTATATTCTGTACGGTTAACCGTATAGGGGAGCCGT
AGGGAAACCGAGTCTTAACTGGGCGTCAAGTTGCAGGGTATAGACCCGAAACCCGGTGATCTAGCCATGGGCAGG
TTTGAAGGTTGGGTAACACTAACGGAGGACA

Sequence size: 705 bp

Similarity: 99% to sequence, GenBank access number: AY264669.1.

- ***Cardinium* sequence**

>CAGTGTACGTATGACGAGTTCGTATAGGGTAAGGGTTCTTAGTGGAAGATCACCCACACTGGCACTGAGATACG
GGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGGTCAATGGGCGAAAGCCTGAACCAGCCATGCCGCGTG
CAGGATGACAGCTCTCTGAGTTGTAAACTGCTTTTGTACAGGAGCAAAACAATCCCTGCGGGGGTTCTTGAGAGT
ACTGTAAGAATAAGCACCGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGAAGGTGCAAGCGTTATCCGGTTT
TATTGGGTTTAAAGGGTGCGTAGGCGGCTTATTAAGTCAGTTGTGAAATCCTAGTGCTTAACGCTAGAACTGCAA
TTGATACTATTAGGCTTGAGTTAAGAATAGGTCAGCCGCGCCGCCCGGCCTGTTGGGAATTTTTTCGACCGGCAG
GGCAGGGTAGCTCCAATTTCGCCAGCCGCGGATGTTAAGAATGCGGGCCACATT

Sequence size: 514 bp

Similarity: 99% to sequence, GenBank access number: HM452328.1.

- ***Hamiltonella* sequence**

>ATCGGCTGCGTGTCATCGTGCTAATACCGTCATGAAGTCGCGAGACCAAAGTGGGGGACCTTCGGGCCTCACGC
CTTCGGATGAGCCCAGATGAGATTAGCTGGTAGGTAAGGTAAAGGCTTACCTAGGCGACGATCTCTAGCGGGTCT
GAGAGGATAGCCCGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA
CAATGGGCGAAAGCCTGATGCAGCCATGCCACGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAG
GAGGAAGCGATAAATGCGAATACCATTTATTTTTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATAACTGGGCGTAAAGGGCATGTAGGCGGTGAGTTAA
GTCAGATGTGAAATCCCCGAGCTCAACTTGGGAATGGCATTTGAAACTGGGTCGCTAGAGTTTTCTAGAGGGGGG
TAGAATTCCAGGTGTAGCGGTGAAATGCGTAGATATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGAGAA
AGACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATG
TCGATTTGGAGGTTGCTCTTTGAACATAAAAAA

Sequence size: 614 bp

Similarity: 98% to sequence, GenBank access number: HM452317.1.

- ***Wolbachia*** **sequence**

>TATCGATTGCGAGTTGGTGGGGTAATAGCCTACCAAGGTAATGATCTATAGCTGATCTGAGAGGATGATCAGCC
ACACTGGAACTGAGATACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCT
GATCCAGCCATGCCGCATGAGTGAAGAAGGCCTTTGGGTTGTAAAGCTCTTTTAGTGAGGAAGATAATGACGGTA
CTCACAGAAGAAGTCCTGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGAGGGCTAGTGTTATTCGGAATT
ATTGGGCGTAAAGGGCGCGTAGGCTGGTTAATAAGTTAAAAGTGAAATCCCGAGGCTTAACCTTGGAATTGCTTT
TAAAACTATTAATCTAGAGATTGAAAGAGGATAGAGGAATTCCTGATGTAGAGGTAAAATTCGTAAATATTAGGA
GGAACACCAGTGGCGAAGGCGTCTATCTGGTTCAAATCTGACGCTGAAGCGCGAAGGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCTGTAAACGATGAATGTTAAATATAGGGAGTTTACTTTCTTTTTTTACAGCCC

Sequence size: 614 bp

Similarity: 98% to sequence, GenBank access number: JF795503.1.

4.7. Localization of secondary symbionts in Croatian Bemisia tabaci, Trialeurodes vaporariorum and Siphoninus phillyreae populations

None of the controls used with the samples submitted to fluorescence *in situ* hybridization (FISH) showed any signal (Figure 29). All tested symbionts varied in their localization pattern, which could be divided into two types: total confinement to the bacteriocyte during all developmental stages, and confinement combined with scattered localization outside the bacteriosome during some of the developmental stages.

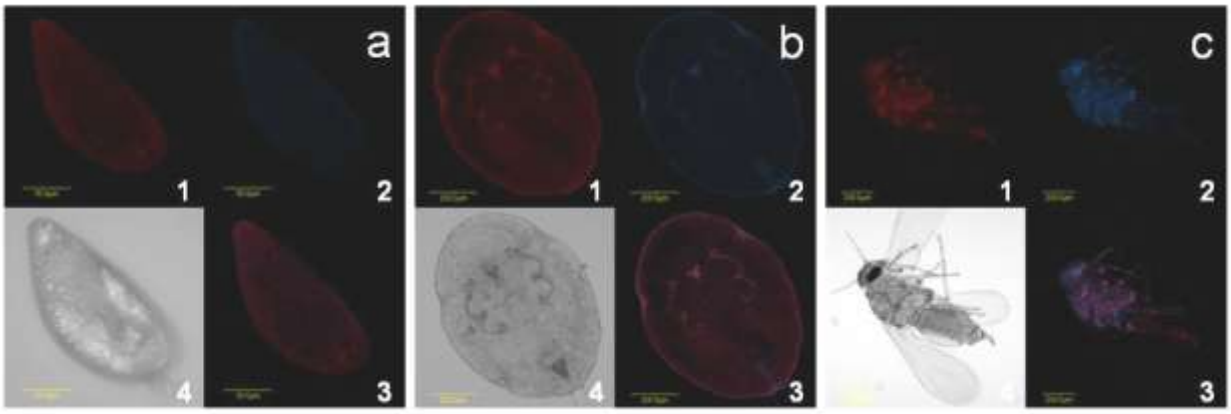

Figure 29. Negative control fluorescent *in situ* hybridization (FISH) of whitefly eggs, nymphs and adults under confocal microscope. (a, b and c): egg (a), nymph (b) and adult (c). (1, 2, 3 and 4): Cy3 channel (dark field) (1); Cy5 channel (dark field) (2); overlapped Cy3 and Cy5 channel (dark field) (3); bright field (4).

The localization of *Portiera* in *S. phillyreae* revealed and interesting structure of the bacterisome, which was not previously seen in other whitefly species. The two separate bacteriosomes that were generally seen in the nymphal stages of *B. tabaci* and *T. vaporariorum* (for example Figures 30 d-f and 32 a-f), in *S. phillyreae* were always connected during all nymphal developmental stages (Figures 37 a, c and 40 b, c).

Some bacterial symbionts, like *Cardinium* in *S. phillyreae* and *T. vaporariorum* or *Rickettsia* in the latter species, were not detected in any of developmental stages, possibly due to low concentration of bacterium, necessary for detection.

4.7.1. Localization of *Hamiltonella* and *Arsenophonus* in *Bemisia tabaci*, *Trialeurodes vaporariorum and Siphoninus phillyreae*

Hamiltonella was localized to small areas inside the bacteriocyte: these areas appeared sometimes as independent and homogenous small patches as in *T. vaporariorum* (Figure 33 a-i) and sometimes continuous and irregular as in *B. tabaci* (Figure 30 a-l). These patterns of localization were observed in eggs, nymphs and adults of both *T. vaporariorum* and *B. tabaci*. The pattern of localization of *Arsenophonus* in *T. vaporariorum* was similar to that of *Hamiltonella* (Figure 32 a-f).
Both symbionts always co-localized with *Portiera* which occupied most of the bacteriocyte. *Hamiltonella* and *Arsenophonus* were never observed outside the bacteriocyte in *B. tabaci* and *T. vaporariorum*. Double FISH of *Arsenophonus* and *Hamiltonella* detected their presence to exactly the same areas, in egg, nymph and adult stages of *T. vaporariorum* (Figure 34 a-i).
Since FISH does not allow sub-cellular localization, TEM analysis on *T. vaporariorum* bacteriosome sections was employed. Interestingly, *Arsenophonus*-like and *Hamiltonella*-like structures were observed in pleomorphic *Portiera*-like cells (Figure 35 a, b). This result could perhaps partially indicate and is in agreement with the FISH results of co-localization of *Arsenophonus* and *Hamiltonella* in *T. vaporariorum*.

In *S*. *phillyreae*, *Hamiltonella* was localized outside the bacteriosome and showed a random and scattered localization pattern in adults (Figure 37 d-f) and nymphs (Figure 37 a-c). *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 thorax (Figure 37 d-f) of the *S*. *phillyreae*.
Arsenophonus co-localized with *Portiera,* inside the bacteriosome in *S. phillyreae* eggs (Figure 36 a-c) and adults (Figure 36 d-f), as it was shown for *Arsenophonus* from *B. tabaci* MED (Figure 31 a-c) and *T. vaporariorum* (Figure 32 a-f).

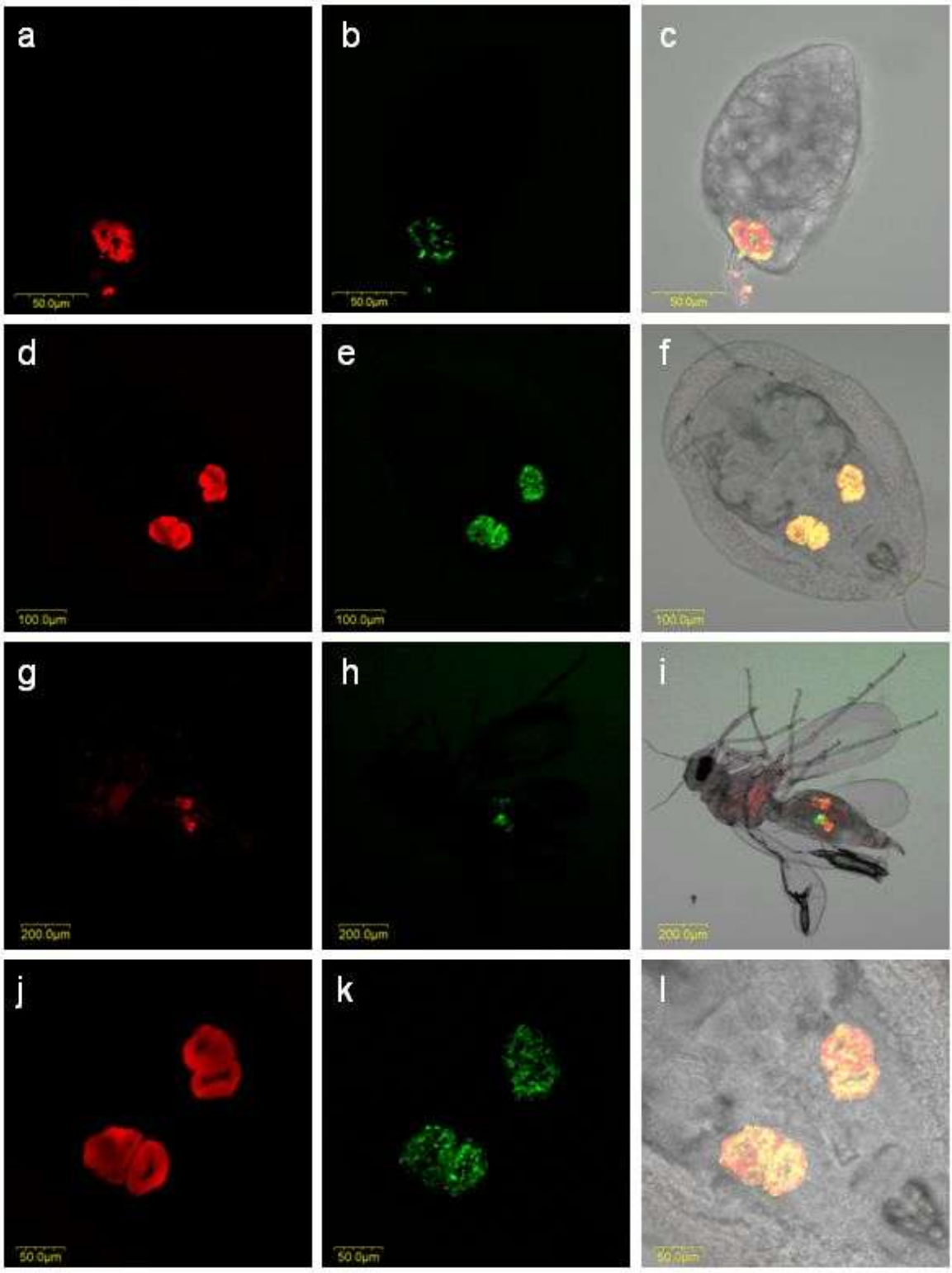

Figure 30. *Portiera* and *Hamiltonella* fluorescent *in situ* hybridization (FISH) of *Bemisia tabaci* eggs, nymphs and adults. *Portiera*-specific probe (red) and *Hamiltonella*-specific probe (green) were used. (a, b, d, e, g and h): double FISH of *Portiera* and *Hamiltonella* in eggs (a, b), nymphs (d, e) and adults (g, h) under dark field. (c, f and i): double FISH of *Portiera* and *Hamiltonella* in eggs (c), nymphs (f) and adults (i) under bright field. (j, k and l): double FISH of *Portiera* and *Hamiltonella* of the focused bacteriosome of the nymph, under dark (j, k) and bright field (l).

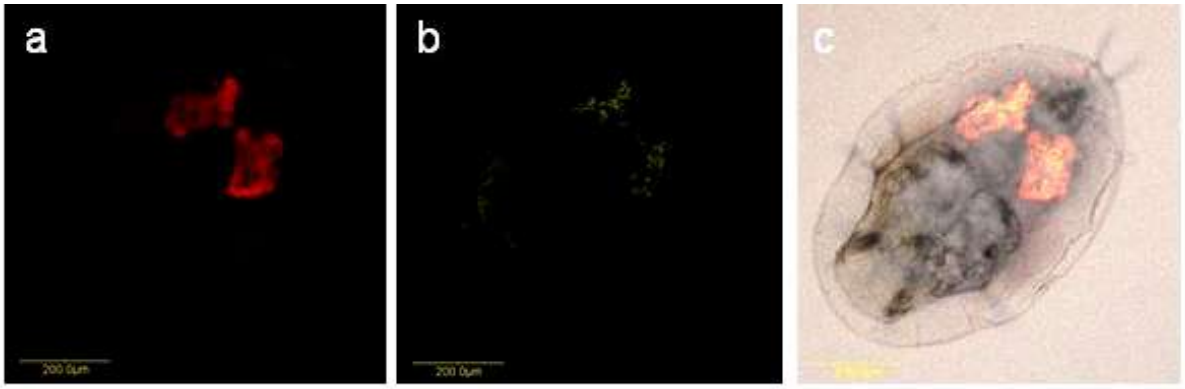

Figure 31. *Portiera* and *Arsenophonus* fluorescent *in situ* hybridization (FISH) of *Bemisia tabaci* nymphs. *Portiera*-specific probe (red) and *Arsenophonus*-specific probe (yellow) were used. (a, b and c): double FISH of *Portiera* and *Arsenophonus* in nymphs, under dark (a, b) and bright field (c).

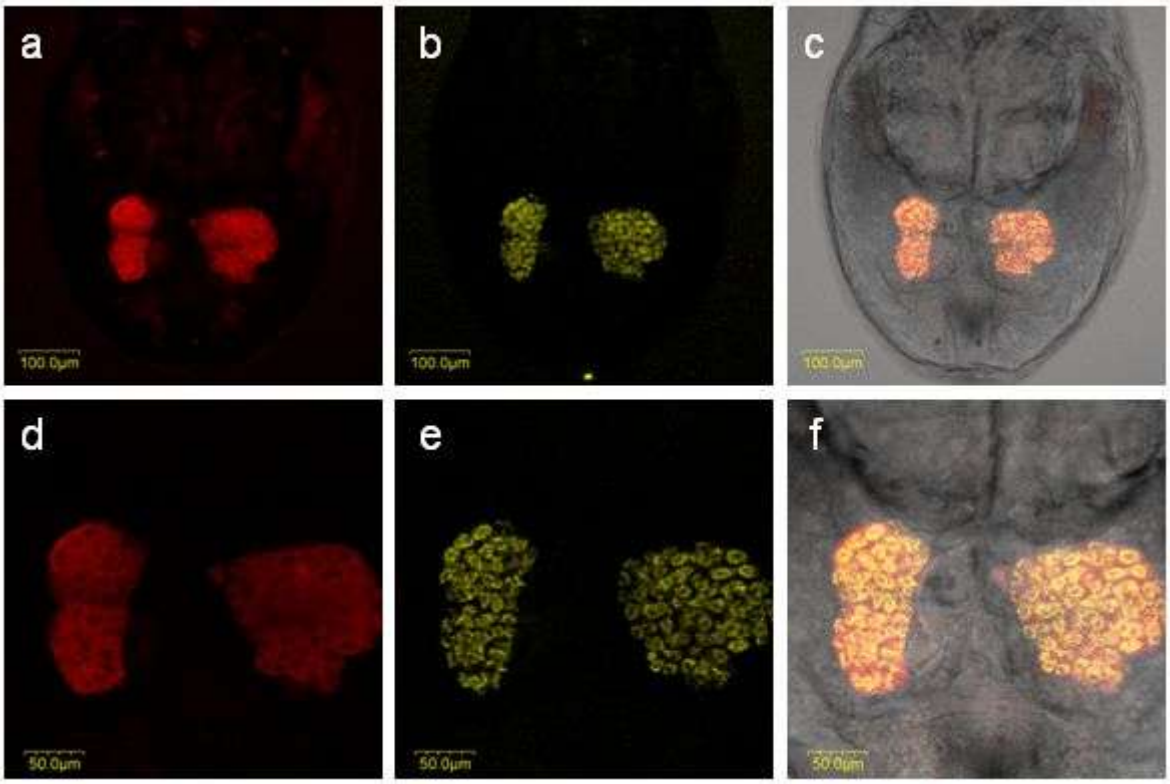

Figure 32. *Portiera* and *Arsenophonus* fluorescent *in situ* hybridization (FISH) of *Trialeurodes vaporariorum* nymphs. *Portiera*-specific probe (red) and *Arsenophonus*-specific probe (yellow) were used. (a, b and c): double FISH of *Portiera* and *Arsenophonus* in nymphs, under dark (a, b) and bright field (c). (d, e and f): double FISH of *Portiera* and *Arsenophonus* of the focused bacteriosome of the nymph, under dark (d, e) and bright field (f).

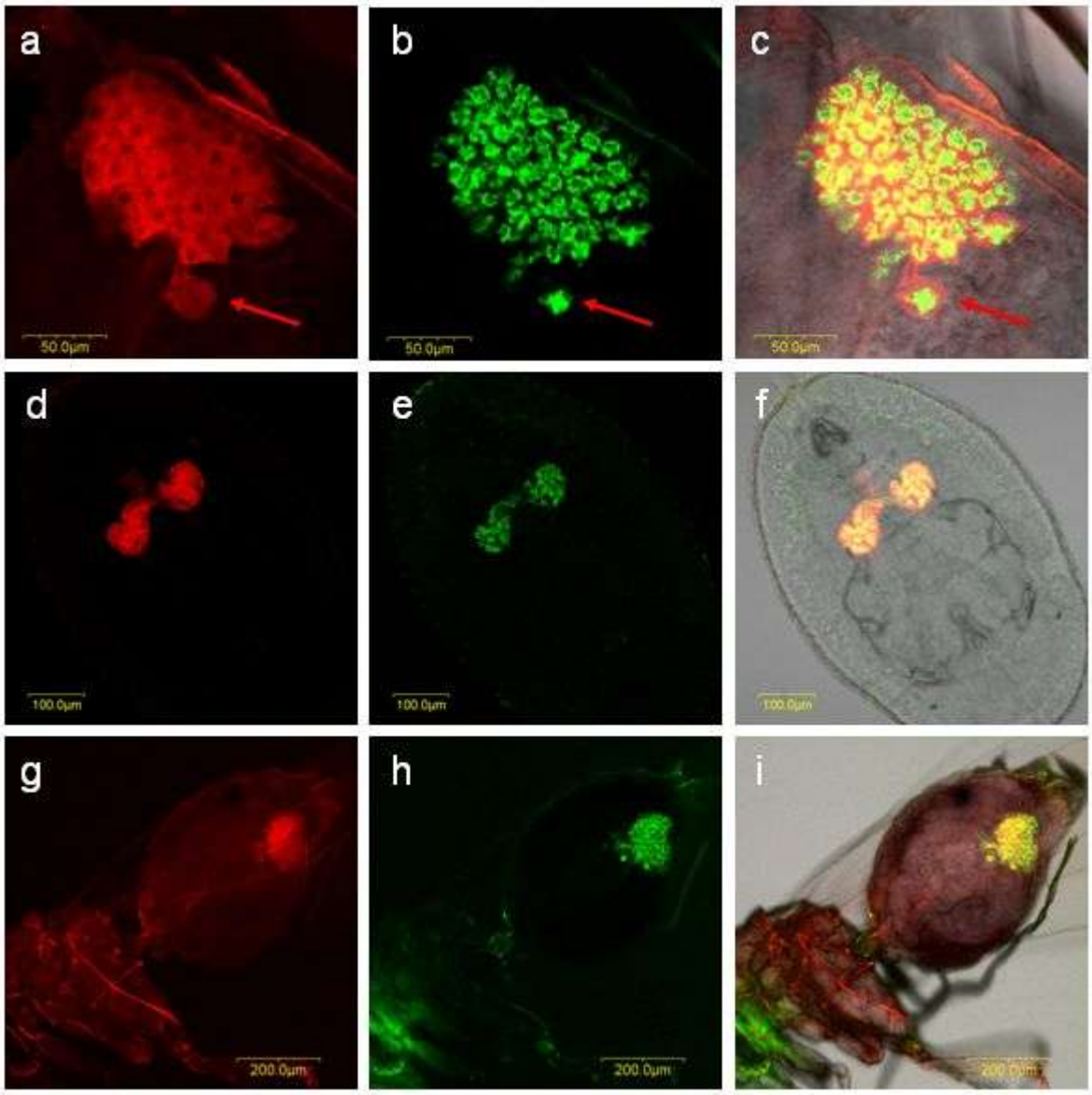

Figure 33. *Portiera* and *Hamiltonella* fluorescent *in situ* hybridization (FISH) of *Trialeurodes vaporariorum* eggs, nymphs and adults. *Portiera*-specific probe (red) and *Hamiltonella*-specific probe (green) were used. Red arrow shows the position of the egg (bacteriosome) inside the female abdomen. (a, b, d, e, g and h): double FISH of *Portiera* and *Hamiltonella* in eggs (a, b), nymphs (d, e) and adults (g, h) under dark field. (c, f and i): double FISH of *Portiera* and *Hamiltonella* in eggs (c), nymphs (f) and adults (i) under bright field.

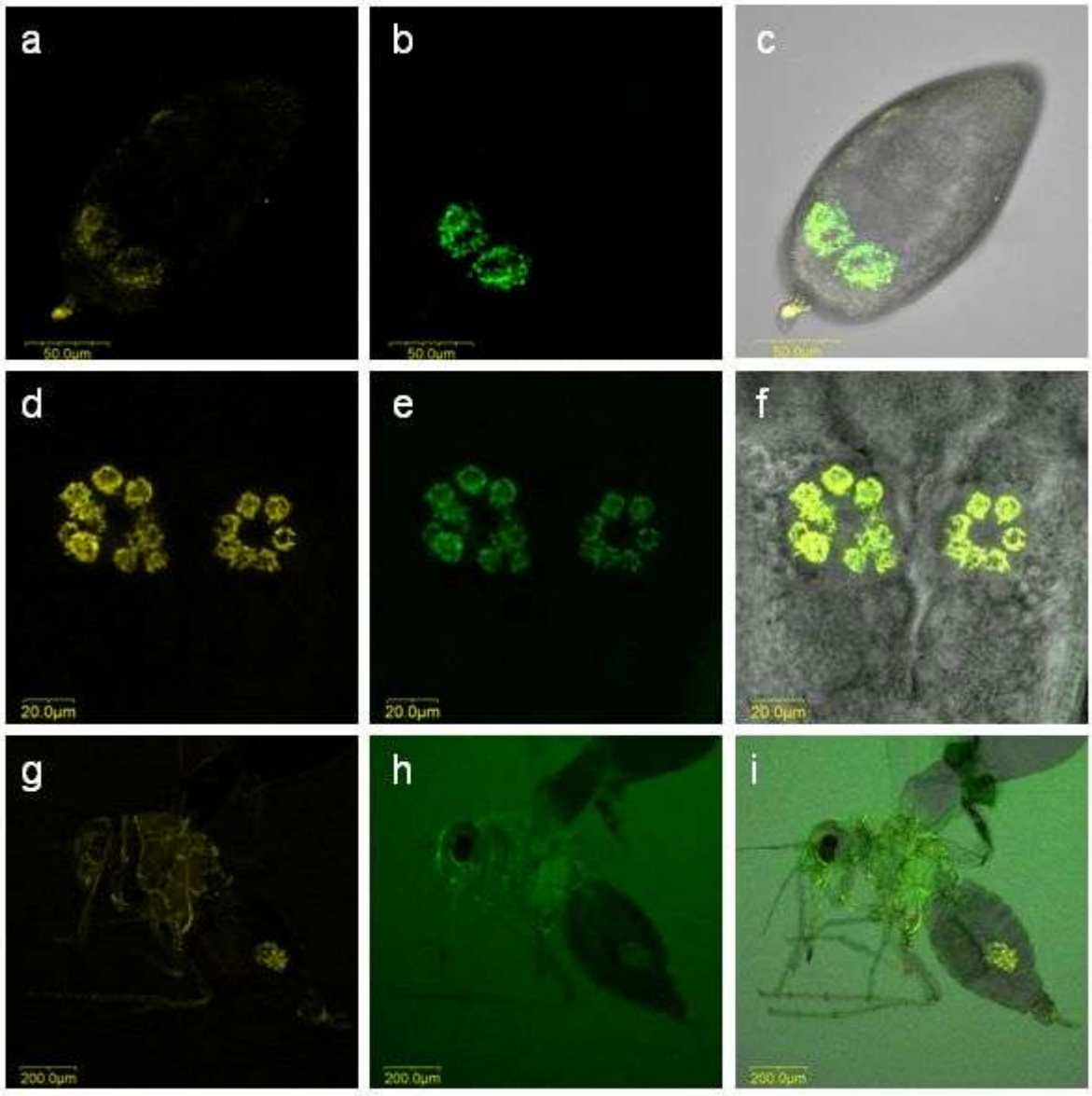

Figure 34. *Arsenophonus* and *Hamiltonella* fluorescent *in situ* hybridization (FISH) of *Trialeurodes vaporariorum* eggs, nymphs and adults. *Arsenophonus*-specific probe (yellow) and *Hamiltonella*-specific probe (green) were used. (a, b, d, e, g and h): double FISH of *Arsenophonus* and *Hamiltonella* in eggs (a, b), nymphs (d, e) and adults (g, h) under dark field. (c, f and i): double FISH of *Arsenophonus* and *Hamiltonella* in eggs (c), nymphs (f) and adults (i) under bright field.

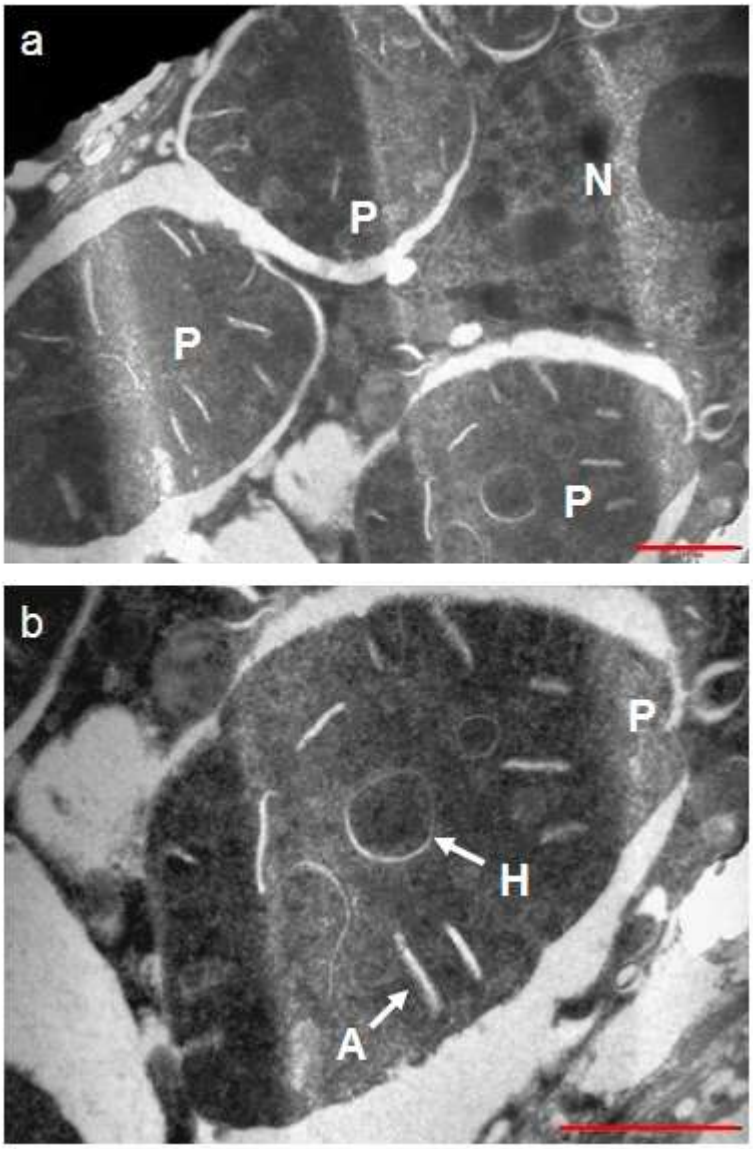

Figure 35. Transmission electron microscopic (TEM) analysis of *Trialeurodes vaporariorum* showing a cross section of the bacteriosome. (a) Three pleomorphic *Portiera*-like cells (P) in the bacteriocyte. (b) Portiera-like structure contains *Hamiltonella*-like (H) *Arsenophonus*-like (A) bacteria, indicated with arrows. Scale bar=1000 nm. N-nucleus.

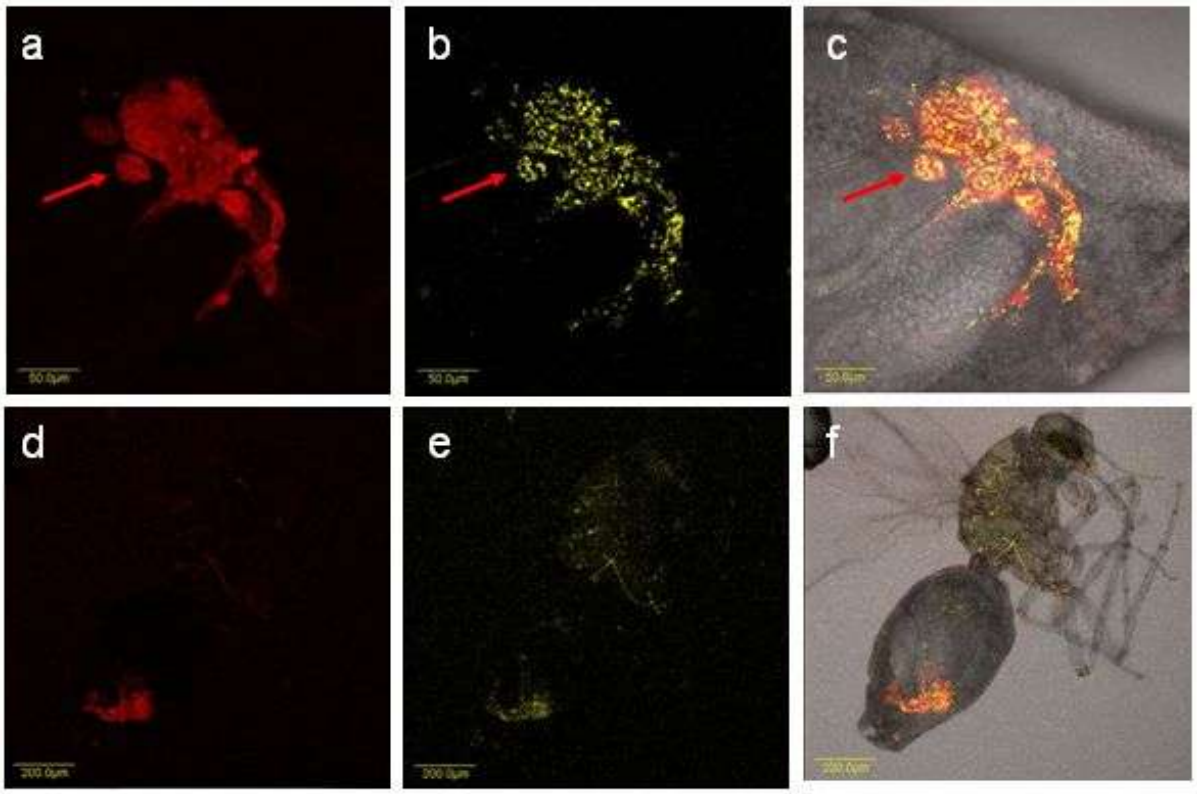

Figure 36. *Portiera* and *Arsenophonus* fluorescent *in situ* hybridization (FISH) of *Siphoninus phillyreae* eggs and adults. *Portiera*-specific probe (red) and *Arsenophonus*-specific probe (yellow) were used. Red arrow shows the position of the egg (bacteriosome) inside the female abdomen. (a, b, d and e): double FISH of *Portiera* and *Arsenophonus* in eggs (a, b) and adults (d, e) under dark field. (c and f): double FISH of *Portiera* and *Arsenophonus* in eggs (c) and adults (f) under bright field.

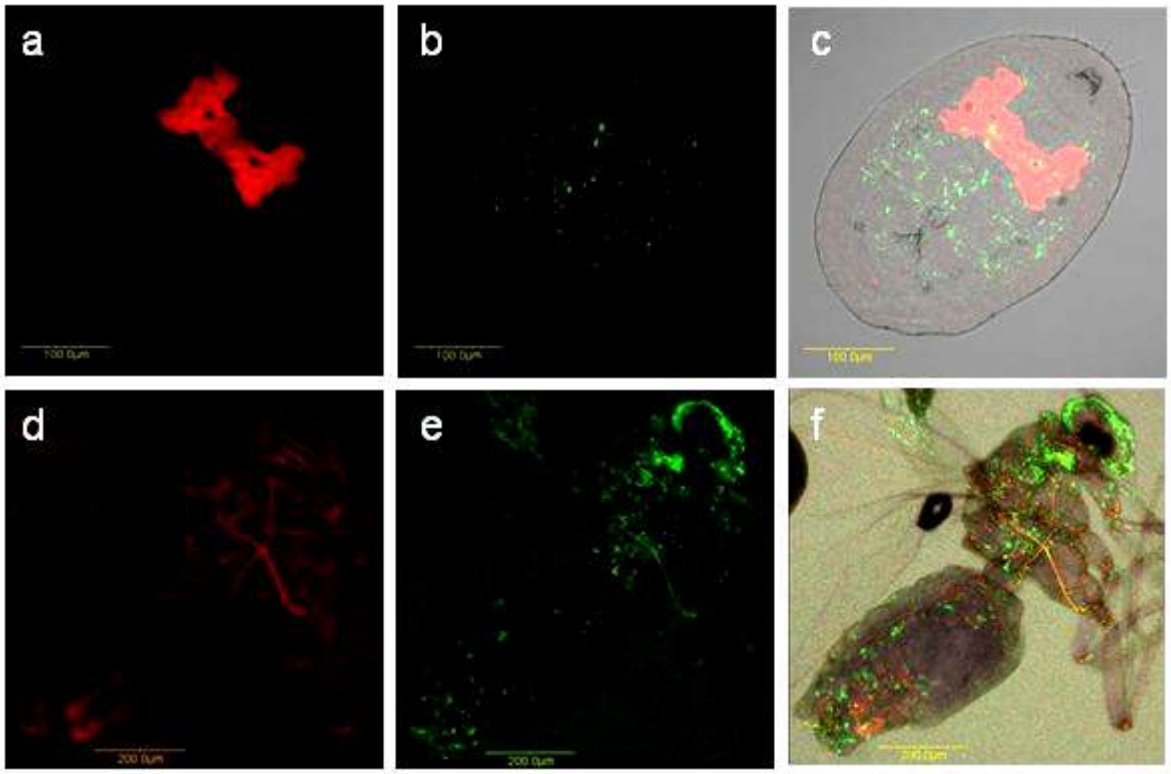

Figure 37. *Portiera* and *Hamiltonella* fluorescent *in situ* hybridization (FISH) of *Siphoninus phillyreae* eggs, nymphs and adults. *Portiera*-specific probe (red) and *Hamiltonella*-specific probe (green) were used. (a, b, d and e): double FISH of *Portiera* and *Hamiltonella* in nymphs (a, b) and adults (d, e) under dark field. (c and f): double FISH of *Portiera* and *Hamiltonella* in nymphs (c) and adults (f) under bright field.

4.7.2. Localization of *Cardinium* in *Bemisia tabaci*

Cardinium showed a dual localization pattern, outside and inside the bacteriocyte, with *Portiera* in the same *B. tabaci* individuals (Figure 38). *Cardinium*, like all symbionts that are confined to the bacteriocyte, is transovarially transferred from the mother to the offspring through the egg. In the egg's early developmental stages, it was confined to the bacteriocyte (Figure 38 a-c); however, in older eggs (5-7 days), it was also observed outside the bacteriocyte, while in later nymphal (Figure 35 d-f) and adult stages (Figure 38 g-i), it occupied most of the body tissues, including the bacteriocyte.

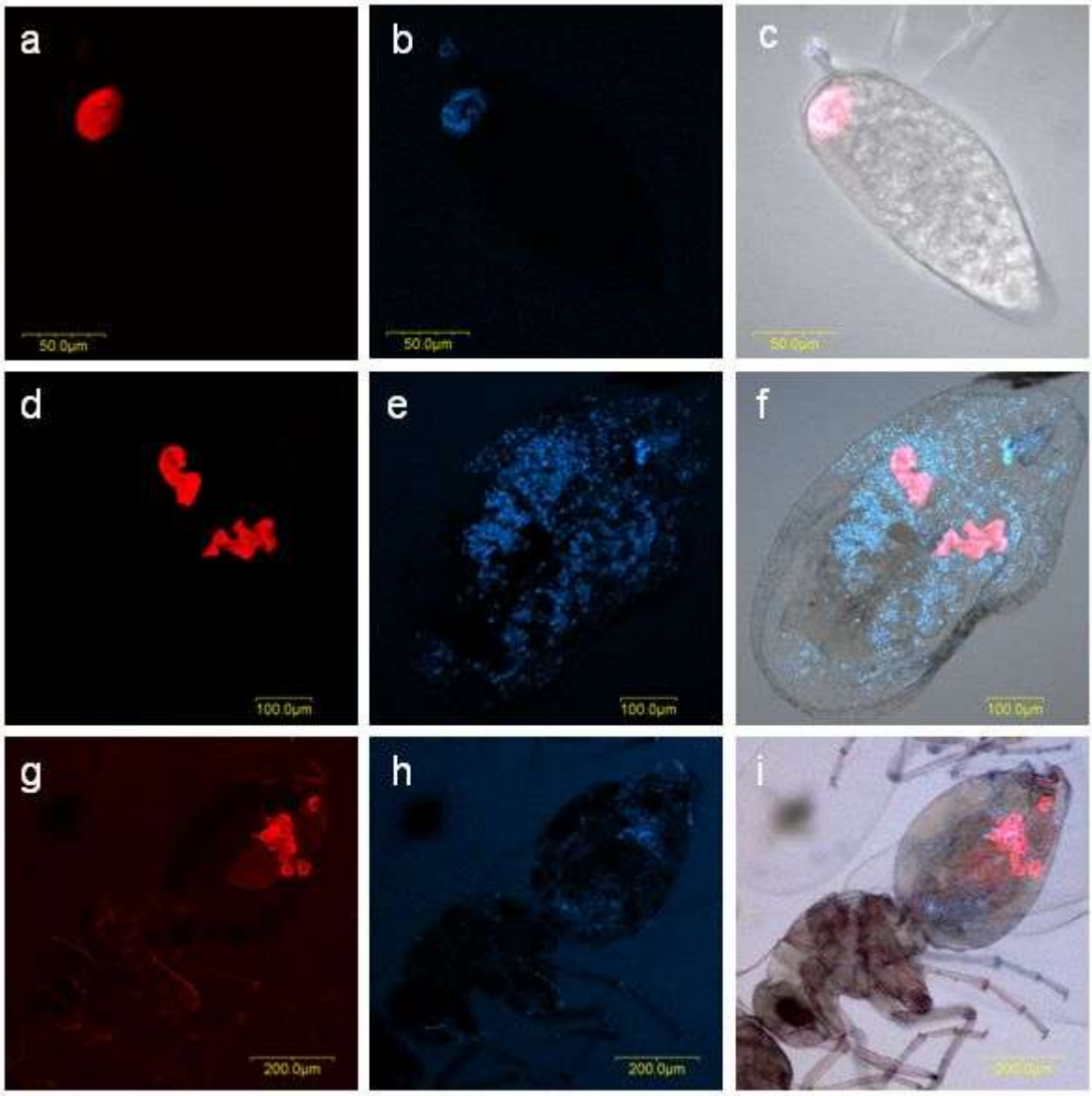

Figure 38. *Portiera* and *Cardinium* fluorescent *in situ* hybridization (FISH) of *Bemisia tabaci* eggs, nymphs and adults. *Portiera*-specific probe (red) and *Cardinium*-specific probe (blue) were used. (a, b, d, e, g and h): double FISH of *Portiera* and *Cardinium* in eggs (a, b), nymphs (d, e) and adults (g, h) under dark field. (c, f and i): double FISH of *Portiera* and *Cardinium* in eggs (c), nymphs (f) and adults (i) under bright field.

4.7.3. Localization of *Wolbachia* in *Bemisia tabaci* and *Siphoninus phillyreae*

Wolbachia could only be detected inside the bacteriocytes with the primary symbiont, therefore signal was not detected in any other organ at any developmental stage of *B. tabaci* (Figure 39 a-f) or *S. phillyreae* (Figure 40 d-f). The localization signal was evenly distributed in the bacteriocyte cells, but it was stronger at the cell's circumference. This different localization pattern than previously described (Gottlieb *et al*., 2008) suggests the presence of a different strain of *Wolbachia* in whitefly populations included in this study. *Wolbachia* was not detected in the nymphal stage of *S. phillyreae* (Figure 40 a-c) possibly due to low amounts of the bacterium.

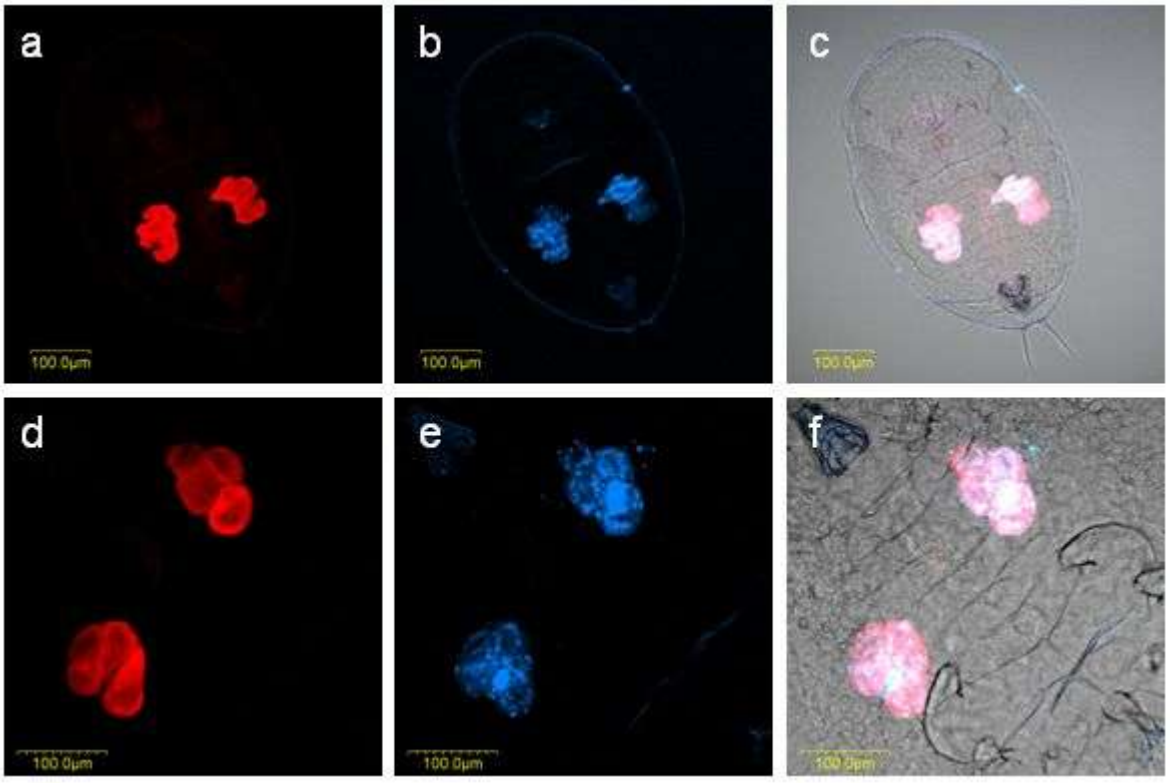

Figure 39. *Portiera* and *Wolbachia* fluorescent *in situ* hybridization (FISH) of *Bemisia tabaci* nymphs. *Portiera*-specific probe (red) and *Wolbachia*-specific probe (blue) were used. (a, b and c): double FISH of *Portiera* and *Wolbachia* in nymphs, under dark (a, b) and bright field (c). (d, e and f): double FISH of *Portiera* and *Wolbachia* of the focused bacteriosome of the nymph, under dark (d, e) and bright field (f).

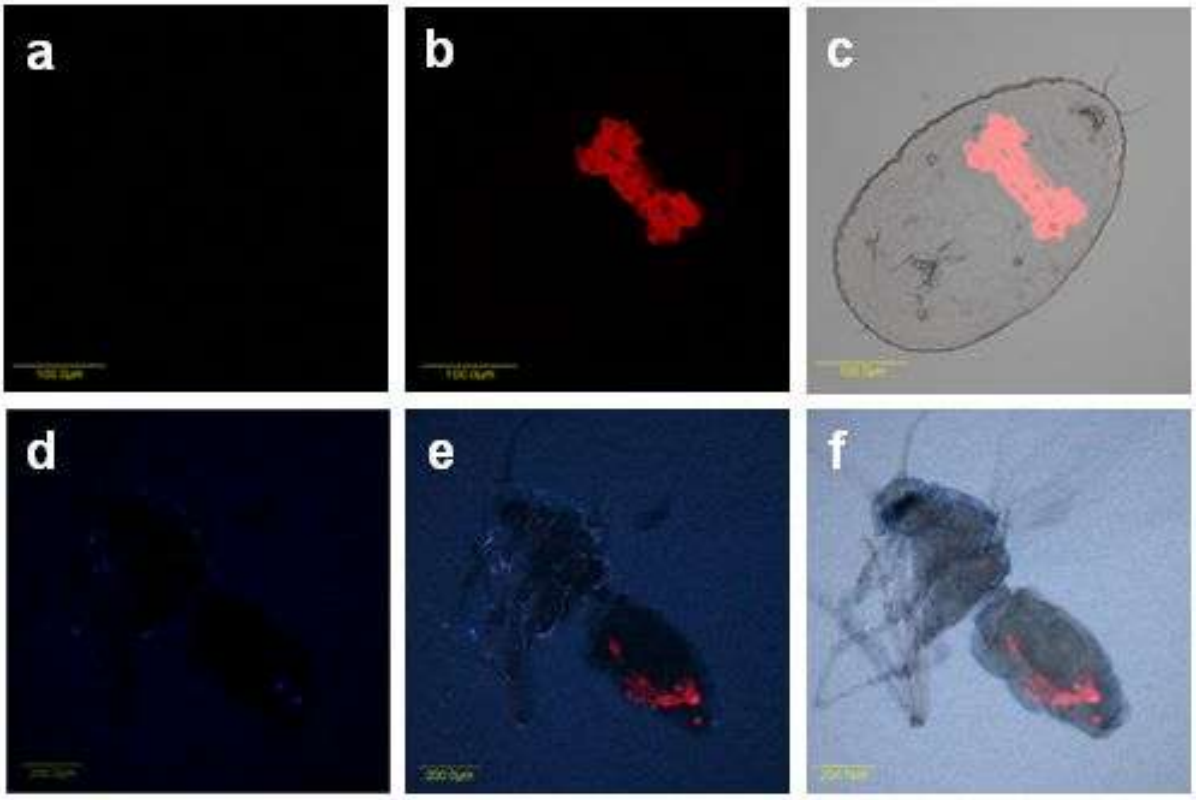

Figure 40. *Portiera* and *Wolbachia* fluorescent *in situ* hybridization (FISH) of *Siphoninus phillyreae* nymphs and adults. *Portiera*-specific probe (red) and *Wolbachia*-specific probe (blue) were used. (a, b and c): double FISH of *Portiera* and *Wolbachia* in nymphs, under dark (a, b) and bright field (c). (d, e and f): double FISH of *Portiera* and *Wolbachia* in adults, under dark (d, e) and bright field (f).

4.7.4. Localization of *Rickettsia* in *Bemisia tabaci*

Rickettsia occupied the most of the egg cavity including the bacteriocyte (Figure 41 a-c). In nymphal stages, *Rickettsia* appeared to be localized inside and outside the bacteriocytes (Figure 41 d-f). In this phenotype, *Rickettsia* cells were mostly concentrated at the circumference of the bacteriocyte cells with some sort of adhesion. Furthermore, in adults, a much higher concentration of *Rickettsia*-associated signal was consistently observed near and around the bacteriocytes relative to the rest of the body (Figure 41 g-l). *Rickettsia* was also observed in the head, thorax and abdomen.

Observed localization of *Rickettsia* in digestive system (stylet, midgut) of *B. tabaci* adults as well as other tissues and organs, such as salivary glands and haemolymph, suggests potential circulative pathway (Figure 42) of this secondary symbiont, which could be plant-mediated.

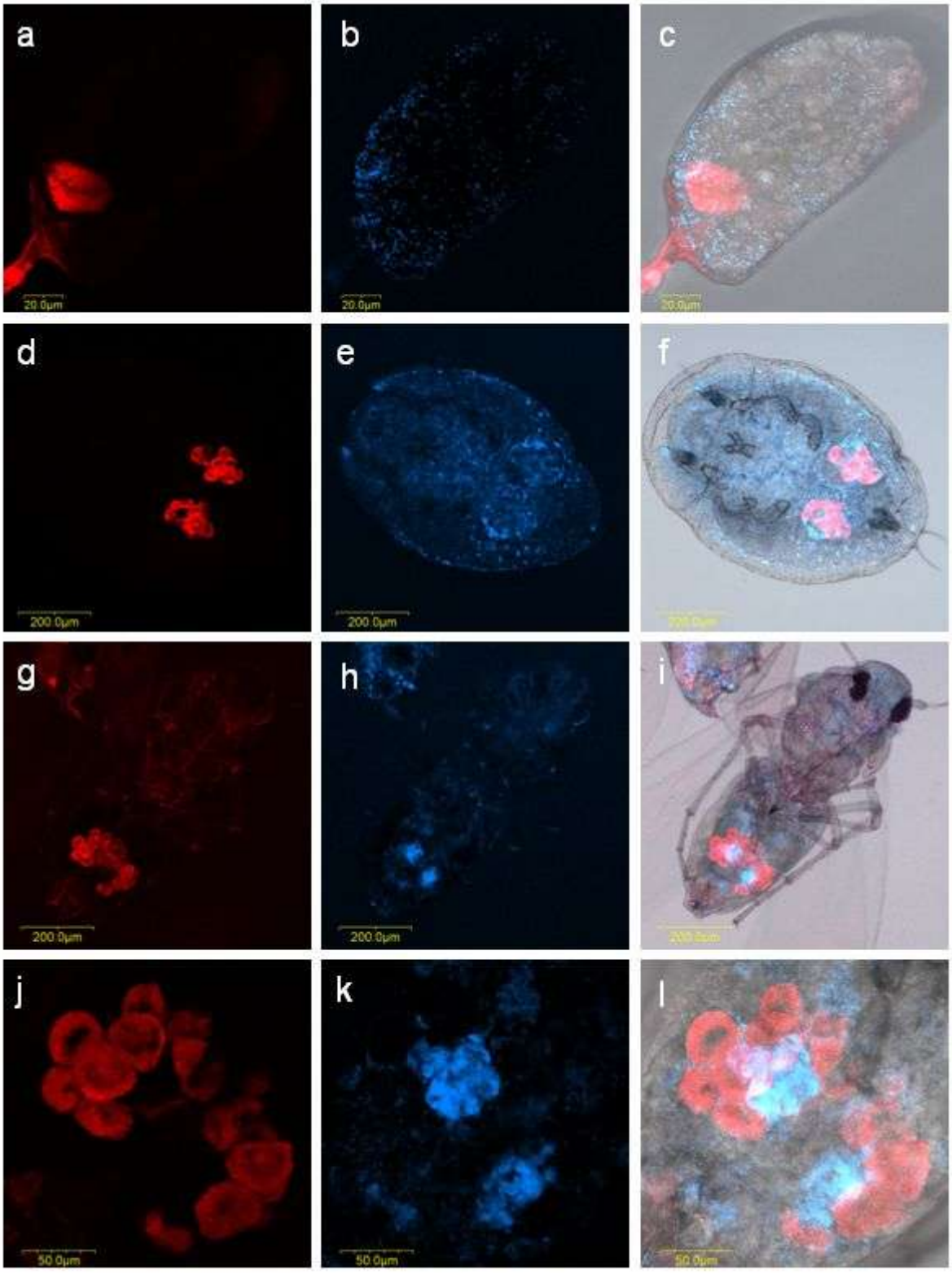

Figure 41. *Portiera* and *Rickettsia* fluorescent *in situ* hybridization (FISH) of *Bemisia tabaci* eggs, nymphs and adults. *Portiera*-specific probe (red) and *Rickettsia*-specific probe (blue) were used. (a, b, d, e, g and h): double FISH of *Portiera* and *Rickettsia* in eggs (a, b), nymphs (d, e) and adults (g, h) under dark field. (c, f and i): double FISH of *Portiera* and *Rickettsia* in eggs (c), nymphs (f) and adults

(i) under bright field. (j, k and l): double FISH of *Portiera* and *Rickettsia* of the focused bacteriosome of an adult, under dark (j, k) and bright field (l).

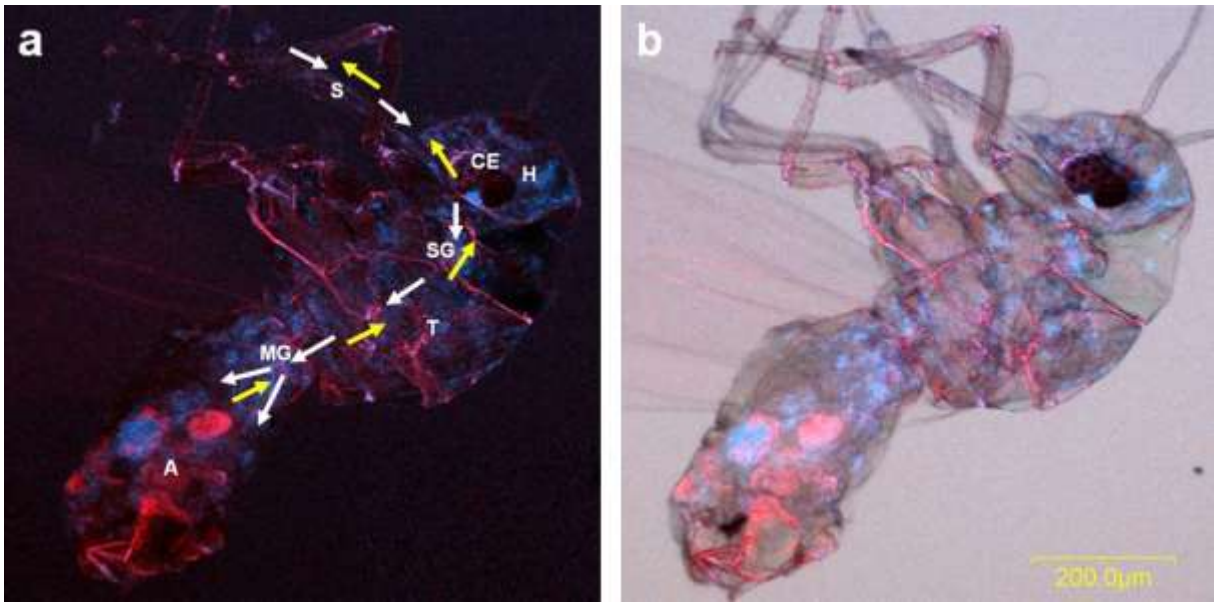

Figure 42. Potential circulative pathway of *Rickettsia* in adults of *Bemisia tabaci*. *Portiera*-specific probe (red) and *Rickettsia*-specific probe (blue) were used. (a and b): double fluorescent *in situ* hybridization (FISH) of *Portiera* and *Rickettsia* in adult (a) under dark field and (b) under bright field. White arrows show possible *Rickettsia* pathway in digestive system, from stylet to midgut, while yellow arrows indicate possible reverse *Rickettsia* pathway in the haemolymph to salivary glands and stylet. CE, compound eye; H, head; MG, midgut; S, stylet; SG, salivary gland; T, torax.

4.8. Geminiviruses monitoring, Bemisia tabaci transmission efficiency and TYLCV localization in tomato stem tissue

4.8.1. Geminiviruses monitoring in Croatia, Montenegro and selected locations of Bosnia and Herzegovina

Simultaneously with whitefly sampling, geminiviruses like symptoms (mosaic, chlorosis, discolorations, curling, stunting and asymmetry) were monitored on different plant hosts and collected if found (Table 4). Sometimes, plant samples were collected regardless of whitefly infestation.

Thirteen vegetable leaf samples that were showing geminiviruses like symptoms were collected across Croatia in 2009-2010. Two melon leaf samples were collected in Bosnia and Herzegovina, while four melon leaf samples were collected in Montenegro in 2011. Collected plant samples in Montenegro were infested with *B. tabaci* MED and this was the first recorded appearance of *B. tabaci* in their outdoor conditions (Table 4).

Plant samples were processed with DNA extraction, PCR amplification by use of geminivirus and TYLCV primers and sequencing, as previously described. All plant samples from Croatia, Bosnia and Herzegovina, and Montenegro resulted negative for the presence of TYLCV and other geminiviruses.

4.8.2. *Bemisia tabaci* transmission efficiency and localization of TYLCV in tomato stem tissue

Bemisia tabaci population of MEAM1 genetic group from Israel (Figure 43) and Croatian *B. tabaci* MED population (Zadar, collected in 2008) (described in Table 1; Figure 13) were tested for TYLCV transmission efficiency.

Tested Israeli *B. tabaci* population hosted two secondary symbionts, *Rickettsia* and *Hamiltonella*. *Hamiltonella* showed fixation, while *Rickettsia* infected 70% of individuals. Six individuals were single infected with *Hamiltonella* and 14 individuals showed double infection with *Rickettsia* and *Hamiltonella*. *Arsenophonus*, *Wolbachia*, *Cardinium* and *Fritshchea* were not detected.

To perceive whether Croatian *B. tabaci* MED population is capable in TYLCV transmission, an experiment which included both populations was conducted. Israeli *B. tabaci* MEAM1 population, served as a positive control.

Twelve replication groups of *B. tabaci* per population, including five individuals per each, were set for 24 hours of TYLCV acquisition and afterwards 48 hours of TYLCV inoculation. PCR analysis of *B. tabaci* showed that all 12 Israeli groups were positive for TYLCV, whereas 10 Croatian groups resulted positive for TYLCV. Ten days after inoculation tomato plants were tested for the presence of TYLCV using PCR and FISH (Figure 44). Eight out of 12 plants inoculated with Israeli *B. tabaci* were positive for TYLCV, whereas six out of 12 plants inoculated with Croatian *B. tabaci* were positive for the same geminivirus. FISH analysis showed TYLCV transcripts in sieve elements of the phloem (Figure 44 c), while surrounding tissues were virus free.

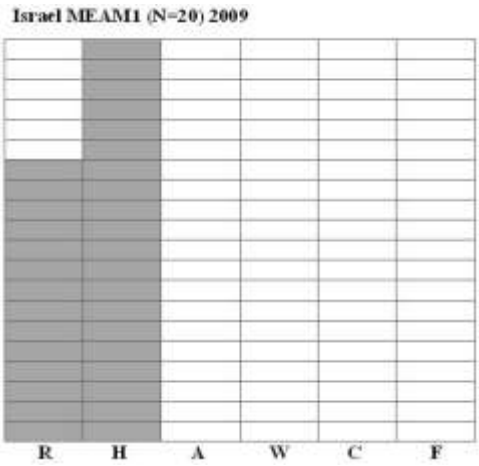

Figure 43. Individual and multiple infections by secondary bacterial symbionts in the Israeli *Bemisia tabaci* Middle East Asia Minor 1 (MEAM1) population. The table represents one population and each column represents one type of symbiont; 20 rows per table represent 20 individuals tested in the population. Gray fields indicate positive infection for the tested symbiont. Number of tested individuals, genetic group, and country of origin are indicated at the top of each table. Symbionts: R - *Rickettsia,* H - *Hamiltonella*, A - *Arsenophonus*, W - *Wolbachia*, C - *Cardinium*, F – *Fritschea*.

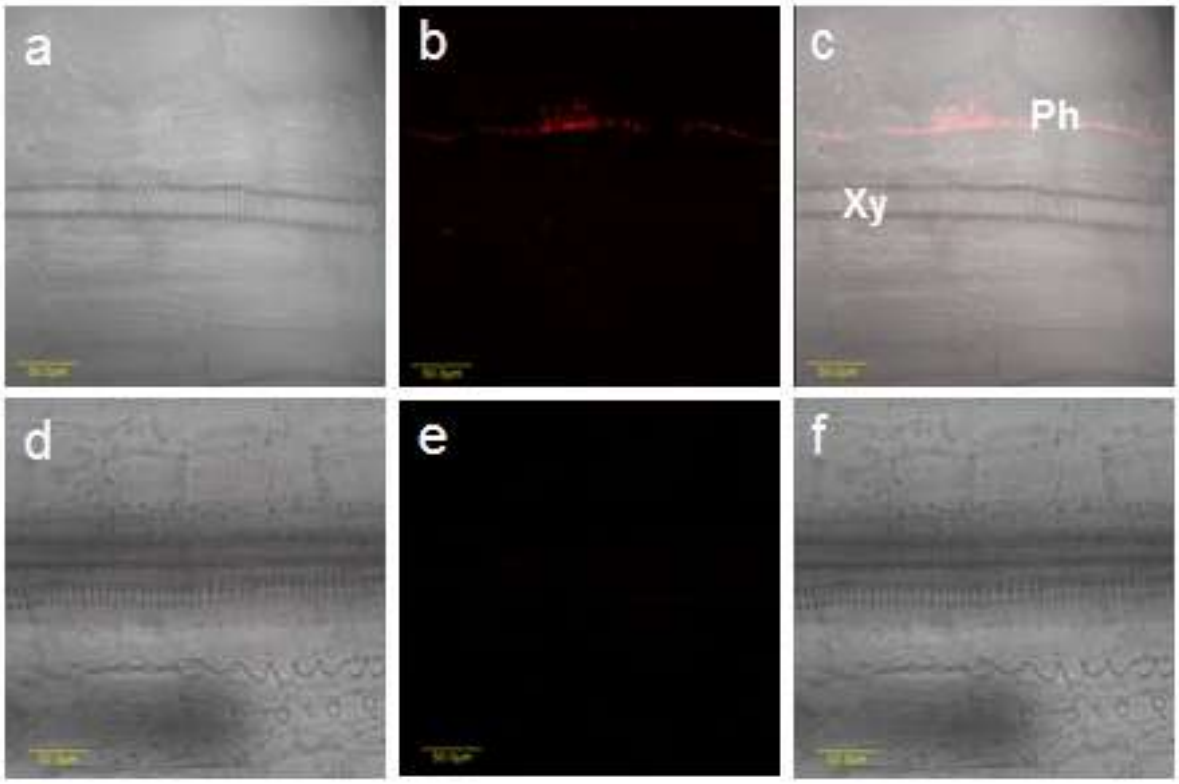

Figure 44. Fluorescent *in situ* hybridization (FISH) of tomato hand-cut stem sections infected with Tomato yellow leaf curl virus (TYLCV). (a, b and c): Localization of TYLCV (red) using TYLCV-specific probe in the phloem (Ph), under bright (a, c) and dark (b) field. (d, e and f): Control section from non infected plant under bright (d, f) and dark field (f). Xy, xylem.

5. DISCUSSION

This doctoral dissertation presents a comprehensive study on economically important whitefly species (*B. tabaci*, *T. vaporariorum* and *S. phillyreae*), their secondary bacterial symbionts and whitefly transmitted phytoviruses, in Croatia, Montenegro and selected locations of Bosnia and Herzegovina, in the period of 2008-2011.

T. vaporariorum was observed as predominant species in all examined areas, while in Croatia, *B. tabaci* was present only in the coastal region (Figure 10). Due to climate differences between the coastal and inland regions of Croatia, *B. tabaci* is not able to maintain throughout the year. In 2011, heavy *S. phillyreae* infestation of pomegranate, led us to initiate a first survey of its secondary symbionts in Croatia and Montenegro within areas of pomegranate growing. Predominance of *T. vaporariorum* is most likely a result of an earlier introduction and lower ecological requirements in compare to other two whiteflies.

In Croatia, only MED group of *B. tabaci* was found, and it harbored *Rickettsia, Wolbachia, Cardinium* and *Hamiltonella*. In spring 2011, in Croatia (location Zadar), the *B. tabaci* population was found on *Hibiscus* sp. imported from Italy (Table 1). Infection of this *B. tabaci* MED population with *Arsenophonus* represented the first finding of this secondary symbiont in Croatia since 2008. In selected locations of Bosnia and Herzegovina MED group of *B. tabaci* was only identified and it harboured *Hamiltonella* and *Wolbachia*, while in Montenegro the presence of both MED and MEAM1 genetic groups of *B. tabaci* were found. MED group of *B. tabaci* in Montenegro showed high degree of heterogeneity and it harbored *Rickettsia*, *Hamiltonella*, *Arsenophonus* and *Wolbachia*, whereas MEAM1 group was less heterogeneous and it was infected with *Rickettsia*, *Hamiltonella* and *Wolbachia.* The MED populations found in Croatia and Montenegro were confirmed to belong to the Q1 group as assessed by sequencing 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 and the Q1 populations described by Gueguen *et al*. (2010) harbor the same similar secondary symbiont loads. These results confirm the Q1 identity of the populations tested in the current study.

B. tabaci MEAM1 (location Podgorica) collected in Montenegro in 2008 was similar in symbiont composition to a MED *B. tabaci* population collected in Croatia (location

Brač) and another MED population collected in Bosnia and Herzegovina (location Veljaci), carrying only *Hamiltonella* and *Wolbachia*. The MEAM1 *B. tabaci* population collected in the same location in Montenegro (Podgorica) three years later (in 2011) showed infection status similar to that of a MEAM1 *B. tabaci* from Israel (Figure 43), harboring only *Rickettsia* and *Hamiltonella* (Chiel *et al*., 2007).

Two MED *B. tabaci* populations from Montenegro and Croatian MED population imported from Italy contained *Arsenophonus* and *Hamiltonella*, among other symbionts, unlike the MED *B. tabaci* in Israel (belonged to Q2 subgroup) which has never been infected with *Hamiltonella*, but harbored *Rickettsia*, *Arsenophonus* and *Wolbachia* (Chiel *et al*., 2007). In general, the composition of secondary symbionts in *B. tabaci* populations from three screened countries reveals high diversity and heterogeneity among the different species (genetic groups) and populations.

The first comprehensive study on secondary symbionts of *T. vaporariorum* was initiated in Croatia and extended to Bosnia and Herzegovina and Montenegro. In Croatia and Bosnia and Herzegovina, *T. vaporariorum* harbored *Arsenophonus* and *Hamiltonella*. Co-infections of these two symbionts were frequent, while *Arsenophonus* was only secondary symbiont present in two *T. vaporariorum* populations from Croatia (locations Pula and Turanj). Similar infection status with *Arsenophonus* and *Hamiltonella* showed population found in Croatia but imported from Slovenia.

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 highly prevalent in all of them, showing a pattern of fixation or near fixation. These *Arsenophonus* infection results are similar to those reported on *T. vaporariorum* populations from Croatia and from Bosnia and Herzegovina. Previously, Thao and Baumann (2004a) have reported the presence of *Arsenophonus* in *T. vaporariorum*. According to Gottlieb *et al*. (2008), fixation of the secondary symbiont in the population, indicates a mutualistic or obligatory association with the host insect and indicates a functional advantage for the host, rather than manipulation of reproduction. *T. vaporariorum* sometimes shared habitat with *B. tabaci* on collecting locations (Figure 8).

In Croatia and Montenegro, the first study on secondary symbionts of *S. phillyreae* was also initiated. The presence of *Hamiltonella*, *Arsenophonus*, *Wolbachia* and

Cardinium was determined in the screened populations. *Hamiltonella* showed the highest prevalence, infecting 87% 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 (Opuzen and Ljuta). Interestingly, *Arsenophonus* appeared as two phenotypes, as reflected by different PCR product sizes (580 and 760 bp) of the amplified 23S rDNA, in most of the *S. phillyreae* populations tested (Figure 28). Close *Arsenophonus* relatives such as *Proteus*, *Yersinia*, *Providencia* and *Salmonella* have been suggested to have an intervening sequence (IVS) inserted in their 23S rDNA (Miller *et al*., 2000) which results in increased PCR product size. Three whitefly species (*Aleurodicus disperses* Russell, *Aleuroplatus gelatinosus* (Cockerell) and *S. phillyreae* have been shown to carry *Arsenophonus* strains with IVSs (Thao and Baumann, 2004a). The most intriguing aspect of the IVS is its uneven and sporadic distribution among bacteria, suggesting its inheritance by horizontal transfer. It appears that possession of an IVS is not a distinct species characteristic and is unevenly allocated within 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. The IVS is an example of the dynamic nature of secondary symbiont genomes which are frequently invaded by mobile elements such as insertion sequences and bacteriophages (Gottlieb *et al*., 2008).

In general, infections with only one secondary symbiont were the most common in *B. tabaci* populations from all regions in the study and in *T. vaporariorum* populations from Croatia. *Trialeurodes vaporariorum* populations from Montenegro and Bosnia and Herzegovina shared almost equal percentage of single and double infections, while in the *S. phillyreae* the most common were infections with two secondary symbionts. *Hamiltonela* was the most prevalent symbiont from all *B. tabaci* and *S. phillyreae* populations in this study, while from all *T. vaporariorum* populations, *Arsenophonus* was the most prevalent secondary symbiont. In compare to Israeli populations of *B. tabaci*, double infections with *Rickettsia* and *Hamiltonella* were prevalent in MEAM1 group, whereas *Rickettsia* and *Arsenophonus* infections were prevalent in MED group (Chiel *et al*., 2007).

All of the symbionts detected in *B. tabaci*, *T. vaporariorum* and *S. phillyreae* were located together with the primary symbiont *Portiera* in the bacteriocytes at one or

more developmental stages. However, some were strictly localized to the bacteriocytes during all developmental stages, such as *Hamiltonella* in *B. tabaci* and *T. vaporariorum*, *Wolbachia* in *B. tabaci* and *S. phillyreae* or *Arsenophonus* in all three species. Others were located inside and outside the bacteriocyte, like *Rickettsia* and *Cardinium* in *B. tabaci* or *Hamiltonella* in *S. phillyreae.*

Previously described (Chiel *et al*., 2007), Israeli *B. tabaci* (MED populations have never been reported to harbor *Hamiltonella* and those populations were infected with *Arsenophonus* at high rates, therefore the two symbionts could not be observed in the same individual. Gottlieb *et al*. (2008) observed *Hamiltonella* in Israeli *B. tabaci* (MED and MEAM1) by FISH and TEM, where it showed continuous and irregular localization phenotype which is in agreement with results of this study.

Cardinium has been shown by TEM to localize to the bacteriocytes of the New world group of *B. tabaci* (Costa *et al*., 1995). In some cases, multiple infections of *Cardinium* with two (*Wolbachia* and *Rickettsia*) or three (*Rickettsia, Wolbachia* and *Hamiltonella*) symbionts were observed in tested Croatian *B. tabaci* populations. The localization pattern of *Cardinium* as seen by FISH was different from *Wolbachia* and *Hamiltonella* and similar to that of *Rickettsia* that co-localized with it. TEM has revealed the presence of *Cardinium* in the spermatid cytoplasm, residual bodies, and cyst cell cytoplasm of *B. tabaci* males (Bao *et al*., 1996). Studies on other hosts have reported the presence of *Cardinium* in a diverse array of tissues, including the reproductive tract (Zchori-Fein *et al.,* 2001), fat bodies, and salivary glands (Bigliardi *et al*., 2006; Marzorati *et al*., 2006), as well as inside bacteriocytes surrounded by oogonia in the apical region of the ovary (Sacchi *et al*., 2008) as presented in this study.

In this study, *Wolbachia* has been shown only to localize at the circumference of and inside the bacteriocytes in nymphs and adults of *B. tabaci* and *S. phillyreae*. In *B. tabaci*, Gottlieb *et al*. (2008) localized *Wolbachia* in some individuals only in the abdomen outside the bacteriocyte, while in others it was only localized inside the bacteriocytes. Different spatial localization patterns of *Wolbachia* may suggest the presence of different strains.

Rickettsia is vertically transferred with the primary symbiont into the newly developing egg. Once the new bacteriocyte cell enters the mature developing egg, it moves towards the center of the egg, and *Rickettsia* leaves it and occupies most of the egg cavity (Figure 41 a-c). At later stages (nymphs and adults), it is found throughout the

body, except in the bacteriocytes (Ghanim *et al*., 2001b; Gottlieb *et al*., 2006; Brumin *et al*., 2012). In the confined phenotype, *Rickettsia* is always associated with the bacteriocyte and never observed outside it. In this study, confined phenotype was never observed, and *Rickettsia* distribution in the eggs was similar to previously published results (Gottlieb *et al*., 2006), although its localization differed in the later stages where it was localized inside and outside of the bacteriocytes.

Symbionts that are strictly localized to the bacteriocytes are vertically transmitted and thus they may contribute to their host's fitness (Wernegreen, 2004). However, they are less likely to be able to manipulate their host's reproduction since this requires invading reproductive organs outside the bacteriocyte. Thus, the restricted localization of *Hamiltonella* in both *B. tabaci* and *T. vaporariorum*, *Wolbachia* in *B. tabaci* and *S. phillyreae* or *Arsenophonus* in all three species, suggests their involvement in providing the host with a functional advantage rather than in manipulating its reproduction. Interestingly, in this study, *Wolbachia* was localized to the bacteriocyte and was not observed outside it, invading other organs. *Wolbachia* can be found in all major insect orders at various different frequencies, and it has been associated with reproductive disorders (Stouthamer *et al*., 1999). However, the localization pattern in *B. tabaci* and *S. phillyreae* observed here suggests that *Wolbachia* does not manipulate reproduction in these whiteflies, but maybe performs other unknown functions. It cannot be excluded that at some stages of the adult development, *Wolbachia* may invade the reproductive system and causes known reproductive manipulations, however, discovering this requires more investigations.

One major advantage of the confined localization of some symbionts with the primary symbiont in the bacteriocyte is that the host immune system is thus avoided, representing a bidirectional advantage for the host which invests fewer resources in maintaining the symbiont levels and for the symbiont, which is not recognized by the immune system of the host. This confined localization ensures low cell numbers of the bacterium because of the limited space in the bacteriosome, and thus for the host, a lower fitness cost is associated with maintaining the symbiont. An additional advantage for the symbiont is the ease of vertical transmission from one generation to the next. "Hitching a ride" with the primary symbiont in the bacteriocyte exempts the secondary symbiont from invading and entering the egg alone during oogenesis, and ensures its transmission during the transfer of the bacteriocyte to the egg (Stouthamer *et al*., 1999). The localization pattern of the secondary symbionts

confined to the bacteriocyte in three whitefly species showed some specific localization to patches. This localization pattern was consistent in all of the individuals tested, and suggests specific sharing inside the bacteriocyte, with each symbiont, primary and secondary, occupying its own niche. Interestingly, all of the symbionts detected in *B. tabaci* were found to co-exist in the same individual, in varying percentages, suggesting little or no competition for space, with the exception of *Arsenophonus* and *Hamiltonella* which were not found together in *B. tabaci* (except population imported from Italy, Figure 13), although they were found together in *T. vaporariorum* and *S. phillyreae*. Interestingly, using FISH, in *T. vaporariorum*, their localization pattern in the bacteriocyte looked exactly the same, suggesting localization in exactly the same places or one inside the other. Afterwards, TEM localization suggested the presence of *Arsenophonus*-like and *Hamiltonella*-like inside the pleomorphic *Portiera*-like cells (Figure 35), which could possibly explain FISH localization in *T. vaporariorum*. Similar localization was presented by von Dohlen *et al*. (2001), where they found in bacteriosome of citrus mealybug, *Planococcus citri* (Risso) a kind of symbiosis in which gamma proteobacteria lived inside beta proteobacteria.

In contrast to the symbionts that were restricted to the bacteriocytes, *Rickettsia* and *Cardinium* in *B. tabaci* and *Hamiltonella* in *S. phillyreae* showed a scattered localization pattern and were seen outside the bacteriocyte. Rickettsia *and Cardinium* are known to manipulate host reproduction in many arthropods (Perlman *et al*., 2006; Hunter and Zchori-Fein, 2006), and this fits well with their localization pattern in *B. tabaci*. Previously, *Rickettsia* has been shown to exhibit two different localization phenotypes: scattered throughout the body and confined to the bacteriocyte (Gottlieb *et al*., 2008; Caspi-Fluger *et al*., 2011b). These two phenotypes were never observed together in the same individuals. It is not clear whether these localization phenotypes are characteristic of the host or if they are due to different bacteria localizing differently in the host's body. In this study, FISH results showed the presence of both scattered and confined phenotypes in the same individuals for *Rickettsia* (Figures 41, 42), and *Cardinium* (Figure 38). These phenotypes are similar to the obligatory *Rickettsia* in booklice, in which it was found to appear with both phenotypes in the same individual (Perotti *et al*., 2006). We further observed concentration of *Rickettsia* at the circumference of the bacteriocyte, suggesting a stage in which *Rickettsia*

concentrates around the developing oocytes for entry, for transferral to the next generation (Figure 41 g-l).

Localization of *Rickettsia* in *B. tabaci* within this study suggested its presence in the stylet, salivary glands, midgut and hemolymph (Figure 42) as was recently confirmed (Brumin *et al*., 2012). These organs are involved in microorganisms circulative transmission routes, like that of TYLCV (Ghanim *et al*., 2001a; Ghanim *et al*., 2001b). Previous studies detected *Rickettsia* in the salivary glands and midgut of cat fleas (Bouyer *et al*., 2001; Macaluso *et al*., 2008) and hemolymph of aphids (Chen *et al*., 1997), while Caspi-Fluger *et al*. (2011a) found that *Rickettsia* was transferred from *B. tabaci* to a plant, moved inside the phloem, and could be acquired by uninfected whiteflies. Therefore, it is possible that *Rickettsia* use the same circulative pathway in the insect as geminiviruses and finally, plants can serve as a reservoir for horizontal transmission of *Rickettsia*, which may explain the occurrence of phylogenetically similar symbionts among unrelated phytophagous insect species (Caspi-Fluger *et al*., 2011a).

Hamiltonella was the only endosymbiont common in high percentage to all whitefly species included in the study. Sequences of the 16S rRNA gene of *Hamiltonella* from the different *B. tabaci* populations tested in this study were identical as was the case with sequences of the same gene from all *T. vaporariorum* and *S. phillyreae* populations. Comparing the sequences of the 16S rRNA gene from *Hamiltonella* of all whitefly species revealed 95% similarity. This high similarity suggests different strains of *Hamiltonella* that colonize whitefly species, however, ancient occurrence of horizontal transfer between the whitefly species, after which *Hamiltonella* became localized to the bacteriocyte, cannot be excluded. *B. tabaci* and *T. vaporariorum* feed through the plant phloem and share host plants (Figure 8a), and horizontal transmission can therefore occur through the host (Ferree *et al*., 2005; Sintupachee *et al*., 2006). For the first time in *S. phillyrae, Hamiltonella* was observed in the circumference of the bacteriocytes and in many other tissues as well. The presence of *Hamiltonella* in the stylet, head and other body tissues of *S. phillyreae* could be another hint for a possible horizontal transmission of this symbiont as previously seen in *Rickettsia* (Caspi-Fluger *et al*., 2011a). Furthermore, whiteflies share host plants with other phloem-feeders such as aphids, planthoppers and leafhoppers, which are also known to harbor endosymbionts (Chen *et al*., 1997; Ferree *et al*., 2005; Sintupachee *et al*., 2006). These insects can inject endosymbionts into the

vascular system which then follow the circulative pathway of transmission, reaching the salivary glands of the insect which might be involved in transmitting these symbionts (Gray *et al*., 1999; Caspi-Fluger *et al*., 2011a). A recent study has shown that salivary glands can indeed be infected by endosymbionts, as in the case of *Cardinium* in *Scaphoideus titanus* (Zchori-Fein *et al*., 2001; Bigliardi *et al*., 2006).

It is difficult to hypothesize how infections with symbionts occurred among whiteflies on an evolutionary scale: it might have been the result of horizontal transmission, loss or new acquisition of symbionts, which would partially explain the mixed infections and heterogeneity among some of the collected populations. Some populations showed very low infection rates or lacked some of the symbionts, suggesting the recent introduction of those symbionts into the populations, possibly through horizontal transfer or introduction of new whitefly populations (e.g. imported Croatian *B. tabaci* MED population with *Arsenophonus*, location Zadar) with new symbiotic complements into investigated area of three countries, via regular trade of plants. For example, among the 20 individuals tested in the Zadar population of *B. tabaci*, only one individual showed infection with *Hamiltonella* and *Cardinium*. The multiple infections observed among some of the populations, such as those from Turanj and Kaštela, can also be explained by efficient horizontal transfers, which allowed the appearance of maximum symbionts in one population. However, some other internal factors may influence maximum horizontal transfers and maximum infection rates in the same individuals. These factors include competition for space and resources among two or more symbionts (Gottlieb *et al*., 2008; Vautrin and Vavre, 2009), or on the contrary, positive interaction between the symbionts may contribute to maximum infection in one individual (Vautrin *et al*., 2008). Another important factor is the host response to the presence of these symbionts which in most cases will influence the bacterial community residing within the host. The occurrence of mixed infections in both species also suggests that these secondary symbionts are non-essential for these whiteflies, allowing their presence to be variable. In report of Chen *et al*. (2000) *Hamiltonella* was found in 40% of *B. tabaci* populations, while 0 to 40% of pea aphid populations have been found to harbor *Rickettsia* (Chen *et al*., 2000; Darby *et al*., 2001; Tsuchida *et al*., 2002; Darby *et al*., 2003; Haynes *et al*., 2003; Ferrari *et al*., 2004). Only *Hamiltonella* was highly prevalent in *B. tabaci* populations and sometimes reached fixation, an indication of a mutualistic or obligatory interaction with the insect. Such interactions can occur via

complementation of the primary symbiont's function with regard to completing the host's dietary needs or enhancing host fitness.

In this study, the presence of whitefly transmitted viruses was monitored with emphasis on economically important geminiviruses. Total 19 vegetable samples collected in three countries during 2009-2011, were negative for the presence of TYLCV and other geminiviruses. Gottlieb *et al*. (2010) investigated transmission efficiency of TYLCV in relation to secondary symbionts of *B. tabaci.* TYLCV was transmitted with Israeli MEAM1 *B. tabaci* infected with *Hamiltonella* alone or with *Hamiltonella* and *Rickettsia* with 80% efficacy, due to GroEL protein produced by *Hamiltonella* which facilitates virus transmission, whereas transmission efficacy was 9% with *Hamiltonella* free Israeli MED *B. tabaci*.
Since, 59% of individuals from Croatian MED *B. tabaci* populations were infected with *Hamiltonella* we investigated TYLCV transmission efficacy of one *Hamiltonella* positive MED *B. tabaci* (location Zadar). TYLCV transmission efficiency of Croatian MED *B. tabaci* population was 50% in compare to 67% transmission efficiency of Israeli MEAM1 *B. tabaci* population included in this study. Previous comparisons of transmission capabilities of MEAM1 and MED populations from Spain showed that both biotypes were able to transmit TYLCV, the MED biotype was even more efficient than the MEAM1 in this respect (Sanchez-Campos *et al*., 1999; Jiang *et al*., 2004).

This 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 (Moran and Baumann, 1994; Aksoy *et al*., 1997). Herein, three different whitefly genera and species were found to share similar secondary symbionts, which also suggest that symbionts can survive, reproduce, and undergo efficient colonization in novel hosts. Co-infections revealed a unique pattern of co-sharing the bacteriocyte by the primary and different secondary symbionts. Co-sharing of the same cell by multiple symbionts while maintaining infections over time by vertical transmission through the egg is unique in whiteflies. This sharing provides a unique system to study interactions among bacteria that co-inhabit the same cell. Positive and/or negative interactions among these symbionts-cooperation and antagonism are part of the multiple interactions that one can expect within their small niche. Competition between symbionts for space and resources may affect their

small environment and their host. The host can be affected through competition between the primary and secondary symbionts within the bacteriocyte.

Recorded compositions of secondary symbionts contribute to a better understanding of their function in the assessed whiteflies, which can then be used in artificial interference and manipulation to disrupt this diverse community as a better means of designing programs to control these important agricultural pests.

6. CONCLUSIONS

This doctoral dissertation is the first comprehensive study on status of three economically important whitefly species (*B. tabaci*, *T. vaporariorum* and *S. phillyreae*), their distribution, secondary bacterial symbionts and viruses that they transmit, in Croatia, Montenegro and selected locations in Bosnia and Herzegovina.

Based on the conducted study, following conclusions were made:

- *T. vaporariorum* was observed as the predominant species in all examined areas, whereas in Croatia *B. tabaci* was only found in the coastal region. Sometimes, *T. vaporariorum* and *B. tabaci* were observed to share habitat. *S. phillyreae* was found in the area of pomegranate growing.

- MED (Q, subgroup Q1) species of *B. tabaci* was only found to be present in Croatia and Bosnia and Herzegovina, while in Montenegro both MED (Q, subgroup Q1) and MEAM1 species of *B. tabaci* were found.

- In Croatia, MED *B. tabaci* was found to harbor *Rickettsia, Wolbachia, Cardinium* and *Hamiltonella*, while in 2011 *Arsenophonus* was found for the first time to infect MED population imported from Italy to Croatia (Zadar). MED *B. tabaci* from Bosnia and Herzegovina harbored *Hamiltonella* and *Wolbachia*, whereas MED *B. tabaci* from Montenegro showed infection with *Rickettsia*, *Hamiltonella*, *Arsenophonus* and *Wolbachia*. MEAM1 group found only in Montenegro harbored *Rickettsia*, *Hamiltonella* and *Wolbachia.*

- Secondary symbiont co-infection in *B. tabaci* suggests that we cannot associate specific genetic groups (species) with secondary symbiont composition as previously postulated.

- Secondary symbionts localization in *B. tabaci* revealed presence of *Rickettsia* and *Cardinium* inside and outside of bacteriosome, while *Wolbachia* was only restricted to bacteriocyte. These localization patterns could suggest new and unknown functions

of these symbionts as well as the presence of new bacterial strains. Localization of *Rickettsia* in *B. tabaci* suggested that *Rickettsia* use the same circulative pathway in the insect as geminiviruses, since it was observed in stylet, salivary glands, midgut and hemolymph of an insect.

- For the first time, monitoring of secondary symbionts composition and their localization in developmental stages of *T. vaporario*rum and *S. phillyreae* was conducted. Unique co-infection patterns were revealed in the investigated whitefly species.

- In Croatia and Bosnia and Herzegovina, *T. vaporariorum* was found to harbor *Arsenophonus* and *Hamiltonella*, while in Montenegro, *T. vaporariorum* harbored *Rickettsia*, *Hamiltonella*, *Arsenophonus*, *Wolbachia* and *Cardinium*.

- FISH localization of *Arsenophonus* and *Hamiltonella* in *T. vaporariorum* populations from Croatia revealed their presence at exactly same places in the bacteriocytes. Afterwards TEM analysis suggested localization of both *Arsenophonus* and *Hamiltonella* inside of *Portiera* cell, which would explain FISH results.

- *Hamiltonella* in *S. phillyreae* populations from Croatia was localized in the bacteriocytes and in many other body tissues of an insect. This was the first report of *Hamiltonella* detected outside the bacteriosome.

- The observation of *Rickettsia*, *Wolbachia* and *Cardinium* in *T. vaporariorum* populations from Montenegro, as well as the high diversity of secondary symbionts in *S. phyillireae* suggests horizontal transfer of secondary symbionts between whitefly species.

- To date, geminiviruses (TYLCV) were not detected in Croatia and neighboring countries. The results show that Croatian *B. tabaci* populations are capable in TYLCV transmission, due to presence of *Hamiltonella*, which facilitates virus transmission. Appearance of TYLCV in Croatia can be expected in the near future because of it’s presence in neighboring countries (Italy, Spain, Portugal) and intensive international plant trading.

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8. APPENDIX

8.1. Curriculum vitae

Basic information

Name and surname: **Marisa Škaljac**
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Personal web page: **http://www.krs.hr/en/about/staff/personal/marisa/**
Nationality: **Croatian**
Date and place of birth: **24.11.1982, Koblenz (Germany)**

Work experience (chronologically)

March 2008 till present; Institute for Adriatic crops, Split

- research asistant
- field: molecular biology, microbiology

Education

2008; Faculty of Science, University of Split, Split

- B.Sc. in biology and chemistry
- Thesis title: Application of FISH in visualization of 18S – 5.8S – 26S rDNA in *Allium ampeloprasum* L. – supervisor: Professor Jasna Puizina

2008; Faculty of Science, University of Zagreb, Zagreb

- Ph.D. student

Training

2009, 2010 (total 7 months), Volcani center (Department of entomology), Israel

Funding:

- Fellowship (for doctoral students) for project ´Exploring *Bemisia tabaci* biotypes, their status as virus vectors and the diversity of their secondary symbionts in Croatia´, funded by The National Foundation for Science, Higher Education and Technological Development of the Republic of Croatia (NZZ).
  Supervisor: Prof. Murad Ghanim
- COST FA0701 fellowship (Short Term Scientific Mission) for the project 'Localization of secondary symbionts in two whitefly species from Croatia'.
  Supervisor: Prof. Murad Ghanim

Languages

Croatian–mother tongue
English–(speaking, writing, reading)

Research and other projects

- 2007-2010 Abiotic and biotic stressors in vegetable growing systems in karst (Support: Ministry of Science, Education and Sports, Republic of Croatia; Position: PhD student)

- 2007-2009 The role of nitrogen on vegetable in close hydroponics system (Support: Support: Ministry of Agriculture, Fisheries and Rural Development, Republic of Croatia; Position: Collaborating investigator)

- 2009 Exploring *Bemisia tabaci* biotypes, their status as virus vectors and the diversity of their secondary symbionts in Croatia (Support: The National Foundation

for Science, Higher Education and Technological Development of the Republic of Croatia, Position: Fellowship candidate)

- 2008-2012 COST FA0701 ‘Ahropod symbioses from fundamental studies to pest and disease management’; working group: WG 1: Arthropod Symbiont Diversity, WG 3: Host-Symbiont Interactions (Position: member of the Management Committee)

- 2011-2013 Whiteflies (Aleyrodidae), viruses that they transmit and Mediterranean fruit fly (Tephritidae) in horticulture of Croatia and Montenegro (Bilateral project: Croatia–Monte Negro, Support: Ministry of Science, Education and Sports, Republic of Croatia; Position: Collaborating investigator)

- 2012-2015 New introduced invasive pests in plant production of Montenegro; Ministry of Science, Montenegro (Position: Collaborating investigator)

Membership in science organizations

2008 till present; Croatian Biological Society

Committees and working groups

COST FA0701 (Athropod symbiosis from fundamental studies to pest and disease management). Action duration: 2008-2012, 27 countries participating

- Member of the working group:
  WG 1: Arthropod Symbiont Diversity
  WG 3: Host-Symbiont Interactions

- Member of the Management Committee

Publications

Journal articles and review articles in CC journals

- **Škaljac M**, Žanić K, Hrnčić S, Radonjić S, Perović T, Ghanim M (2012) Diversity and localization of bacterial symbionts in three whitefly species (Hemiptera: Aleyrodidae) from the east coast of the Adriatic Sea. Bulletin of entomological research 15: 1-12

- **Škaljac M**, Kostanjšek R, Žanić K (2012) The presence of *Wolbachia* in *Tuta absoluta* (Lepidoptera: Gelechiidae) populations from coastal Croatia and Montenegro. African entomology 20(1): 191–194

- Ercisli S, Radunić M, Gadže J, Ipek A, **Škaljac M**, Čmelik Z (2012) S-Rnase based S-genotyping of Croatian sweet cherry (*Prunus avium* L.) genotypes. Scientia horticulturae 139: 21-24

- Žanić K, Dumičić G, **Škaljac M**, Goreta Ban S, Urlić B (2011) The effects of nitrogen rate and the ratio of NO_3^-:NH_4^+ on *Bemisia tabac*i populations in hydroponic tomato crops. Crop protection 30: 228-233

- Gottlieb Y, Zchori-Fein E, Mozes-Daube N, Kontsedalov S, **Škaljac M**, Brumin M, Sobol I, Czosnek H, Vavre F, Fleury F, Ghanim M (2010) The transmission efficiency of *Tomato Yellow Leaf Curl Virus* by the whitefly *Bemisia tabaci* is correlated with the presence of a specific symbiotic bacterium species. Journal of Virology 84 (18): 9310-9317

- **Škaljac M**, Ghanim M (2010) Tomato yellow leaf curl disease and the plant-virus-vector interactions. Israel Journal of Plant Sciences 58: 103-111

- **Škaljac M**, Žanić K, Goreta Ban S, Konstedalov S, Ghanim M (2010) Co-infection and localization of secondary symbionts in two whitefly species. BMC Microbiology 10: 142-1-142-15

Scientific papers in other journals (cited in CAB Abstracts)

- **Škaljac M**, Žanić K, Ghanim M (2011) *Squash leaf curl virus* (SLCV) i *Watermelon chlorotic stunt virus* (WmCSV) - potencijalna opasnost uzgoju dinje i lubenice u Hrvatskoj. Glasilo biljne zaštite 11 (6): 407-413

- **Škaljac M**, Žanić K, Ghanim M (2011) Tomato yellow curl virus: vector-simptomi-prevencija. Glasilo biljne zaštite 11 (4): 289-296

- Žanić K, Ban D, **Škaljac M**, Dumičić G, Goreta Ban S, Žnidarčić D (2009) Aphid population in watermelon (Citrullus lanatus Thunb.) production. Acta agriculturae Slovenica 93 (2):189-192

Abstracts in Book of abstracts

- **Škaljac M**, Žanić K, Hrnčić S, Ghanim M (2012) Composition and localization of bacterial symbionts in three whitefly species (Hemiptera: Aleyrodidae) from Croatia, Montenegro and Bosnia and Herzegovina // 11. HRVATSKI BIOLOŠKI KONGRES s medunarodnim sudjelovanjem 11th CROATIAN BIOLOGICAL CONGRESS with International Participation / Sven D. Jelaska, Göran I.V. Klobučar, Lucija Šerić, Jelaska Dunja, Leljak Levanić, Žaklin Lukša (ur.). Zagreb: Hrvatsko biološko društvo 1885, 129-130 (lecture,international peer-review,abstract, scientific)

- **Škaljac M**, Žanić K, Goreta Ban S, Hrnčić S, Radonjić S, Perović T, Ghanim, M (2011) Rasprostranjenost i sastav bakterijskih simbionata dviju vrsta štitastih moljaca u Crnoj Gori. Društvo za zaštitu bilja Srbije, XI savjetovanje o zaštiti bilja, November 28-December 03, Zlatibor, Srbija (poster,abstract,scientific)

- **Škaljac M**, Žanić K, Bućan L, Ghanim M (2010) Bacterial symbionts of sweetpotato and greenhouse whitefly in Croatia. 54th Plant Protection symposium, 9-12 February, Opatija, Croatia (lecture, abstract, scientific)

- **Škaljac M**, Žanić K, Ghanim M (2010) Distribution and secondary symbiont infection status of two whitefly species in Croatia. Workshop Genomics and Metagenomics, Funchal, Portugal (poster, international peer-review, abstract, scientific)

- **Škaljac M**, Žanić K, Goreta Ban S, Ghanim M (2009) *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) Pest Status and Association with Secondary Symbionts in Croatia. 5th International Bemisia Workshop. Book of abstracts. Yu-Yuan Guo, editor(s). Guangzhou : South China Agricultural University 13-13 (lecture, international peer-review, abstract, scientific)

- **Škaljac M,** Ghanim M, Žanić K (2009) Status infekcije i raspodjela sekundarnih simbionata štitastog moljca *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) u obalnom području Hrvatske. Zbornik sažetaka / Besendorfer, Višnja ; Kopjar, Nevenka ; Vidaković-Cifrek, Zeljka ; Tkalec, Mirta ; Bauer, Nataša ; Lukša, Žaklin, editor(s). Zagreb: Nacionalna i sveučilišna knjižnica 90-91 (lecture, international peer-review, abstract, scientific)

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- Žanić K, Dumičić G, Bućan L, **Škaljac M** (2009) Utjecaj gnojidbe dušikom na populaciju duhanovog štitastog moljca (*Bemisia tabaci)* na rajčici. 44. Hrvatski i 4. međunarodni simpozij agronoma-Zbornik sažetaka. Marić, Sonja; Lončarić, Zdenko, editor(s). Osijek: Poljoprivredni fakultet Sveučilišta Josipa Jurja Strossmayera u Osijeku 137-138 (lecture, international peer-review, abstract, scientific)