

# Fluorescence in situ hybridization analysis of endosymbiont genera reveals novel infection patterns in a tomato-infesting *Bemisia tabaci* population from Brazil

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**Abstract** The *Bemisia tabaci* cryptic species complex harbors a diversified flora of primary and secondary endosymbionts, which plays crucial roles in many aspects of the insect biology. The endosymbiont infection pattern is dependent upon many factors, including host plant and geographic origin. In Brazil, the invasion of *B. tabaci* Middle East-Asia Minor-1 (MEAM 1) populations was observed in tomato (*Solanum lycopersicum* L.) fields in the 1990s, which was followed by severe begomovirus epidemics. Here we confirmed the vertical transmission of “*Candidatus* Portiera” and the localization of secondary endosymbionts in distinct *B. tabaci* developmental stages. *Hamiltonella defensa* was detected in bacteriocytes but also scattered in the leg muscles as well as in the male heads. *Wolbachia*-specific signals were found in eggs, nymphs, male adults, and female gonads in predominant association with bacteriocytes. These results were somewhat surprising since *Wolbachia* infection is rarely found in MEAM 1 populations from the Old World. Our results reinforce the notion that endosymbiont infection pattern may vary according to the insect population, gender, developmental stage, and geographic origin. This characterization will provide tools to study the endosymbiont function in the transmission

ability/efficiency of a complex of tomato-infecting bipartite begomoviruses by *B. tabaci* under Neotropical conditions.

**Keywords** Endosymbionts · *Bemisia tabaci* MEAM 1 · *Wolbachia*

## Introduction

The whitefly [*Bemisia tabaci* (Genn.) (Hemiptera: *Aleyrodidae*)] cryptic species complex is among the most important pests in a wide range of economically important plants such as tomatoes (*Solanum lycopersicum* L.), leaf vegetables, cucurbits, legumes, cassava, cotton, and ornamental plants (De Barro et al. 2011). More than one thousand of whitefly species have been described (Brown 2007), but only three are recognized as plant virus vectors (Thompson 2011). *Bemisia tabaci* is a very efficient vector of viral species from the genera *Begomovirus* (family *Geminiviridae*), *Torradovirus* (*Secoviridae*), *Ipomovirus* (*Potyviridae*), and *Crinivirus* (*Closteroviridae*) (Navas-Castillo et al. 2011). Nymphs and adults of *B. tabaci* can also induce physiological disorders in many host plants, including the silver-leaf syndrome in cucurbits, tomato irregular ripening, and brocoli light stalk (Thompson 2011).

The diversity of *B. tabaci* was initially categorized as biotypes, which are populations that share similar morphological characteristics as well as biological features such as virus-transmission ability/efficiency, host range, host-specific adaptation, life history traits, tolerance to adverse climatic conditions, induction of specific plant physiological disorders and tolerance to insecticides (Berry et al. 2004; De Barro et al. 2011; Navas-Castillo et al. 2011). Currently, *B. tabaci* is recognized as a genetically diverse complex of distinct cryptic species (De Barro et al. 2011). Studies based upon the mitochondrial *cytochrome oxidase I (COI)* gene sequence phylogeny

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indicated the worldwide occurrence of at least 31 cryptic species in 11 high-level genetic groups (Dinsdale et al. 2010; Lee et al. 2013). The cosmopolitan *B. tabaci* species from Middle East-Asia Minor 1 (MEAM 1) is a major pest and an important vector of viruses in many tropical and subtropical areas (De Barro et al. 2011; Navas-Castillo et al. 2011).

Distinct *B. tabaci* cryptic species and even geographically isolated populations may differ in relation to the taxonomic diversity and relative frequency of the endosymbionts that they may harbor (Thompson 2011; De Barro et al. 2011). Endosymbionts are organisms that live within other organisms establishing distinct types of interactions with their hosts (Buchner 1965; Moran and Baumann 1994). In fact, whiteflies and other groups of insects house a diversified bacterial community (Baumann et al. 1993; Chiel et al. 2007; Zchori-Fein and Brown 2002; Zchori-Fein et al. 2014), which includes the widespread obligate (primary) endosymbiont “*Candidatus* Portiera aleyrodidarum” (here referred to as “*Ca. Portiera*”) and so far seven secondary (facultative) endosymbiont genera viz. *Arsenophonus*, *Cardinium*, *Fritschea*, *Hamiltonella defensa*, *Rickettsia*, and *Wolbachia* (Clark et al. 1992; Nirgianaki et al. 2003; Thao and Baumann 2004; Everett et al. 2005; Gottlieb et al. 2006; Chiel et al. 2009; Ahmed et al. 2013). More recently, an *Orientia*-like organism (Bing et al. 2013) was also included in the list as *Candidatus Hemipteriphilus asiaticus*.

The endosymbiont flora plays important roles in many aspects of the host biology in a wide array of insects (Chiel et al. 2009). There are evidences that these interactions are crucial for insect fitness, survival, and nutrition (Moran et al. 2003). For example, the presence of *H. defensa* may confer host resistance against parasitoids (Ferrari et al. 2004; Bensadia et al. 2005) and plays a role in accelerating *B. tabaci* rate of growth, especially during periods of host nutritional stress (Su et al. 2014). “*Ca. Portiera*” was found to be a major supplier to its insect host diet of essential amino acids (Jiang et al. 2013) and carotenoids (Santos-Garcia et al. 2012). Endosymbiotic associations may also be involved in modulating host immunocompetence variation (Braquart-Varnier et al. 2008). On the other hand, negative impacts of endosymbiont flora have also been reported. For example, the presence of *Rickettsia* is associated with increased susceptibility of *B. tabaci* to insecticides (Kontsedalov et al. 2008). In extreme cases, the symbiotic nature may move towards highly virulent parasitism (e.g., *Wolbachia-Drosophila*), causing degeneration and early death of the insect host (Min and Benzer 1997). Insect endosymbionts can also manipulate the reproduction rate of their hosts and enhance their own rate of transmission via distinct sex-ratio-distorting mechanisms such as male-killing activities, cytoplasmic incompatibility, and feminization (Weeks et al. 2003).

The primary endosymbiont “*Ca. Portiera*” has been found across distinct *B. tabaci* populations, plant hosts, and insect gender (Pan et al. 2012b). Complete genome assembly was

carried out using genomic DNA from two “*Ca. Portiera*” strains obtained in association with MEAM 1 and *B. tabaci* Mediterranean subgroup Q (MED Q) species. These analyses indicated that these strains have a 351-kb circular molecule and the genomes of the two groups share around 99.8 % identity (Jiang et al. 2013). Even though displaying almost identical gene repertoire and order, some species-specific genetic differences were identified (Santos-Garcia et al. 2012; Jiang et al. 2013).

There is a growing amount of evidences that some secondary endosymbionts might have group-specific incidence. For instance, *H. defensa* has been found associated with populations of *B. tabaci* from the MEAM 1 group, whereas *Arsenophonus* and *Rickettsia* were found indistinctly in MEAM 1 and MED Q species. On the other hand, *Wolbachia* infection was either not reported or found at very low frequency in *B. tabaci* MEAM 1 populations from the Old World (Chiel et al. 2007; Chu et al. 2011; Pan et al. 2012a).

Frequency and infection dynamics of secondary endosymbionts in whiteflies are variable according to host species, geographic area, insect species, and the prevalent gender in the field populations (Chu et al. 2011; Pan et al. 2012b). Percentage of *Wolbachia* infection was very low in MEAM 1 species and MED Q field populations in China and co-infection with others secondary endosymbionts was never found (Pan et al. 2012a). In Israel, *Wolbachia* associations with bacteriocytes were found in individuals from the MED Q group, but it was not found in samples from the MEAM 1 (Gottlieb et al. 2008). On the other hand, MED Q populations from Croatia showed localization of *Wolbachia* only inside the bacteriocytes and not in other organ at any developmental stage (Škaljac et al. 2010). The co-infection levels of secondary endosymbionts in *B. tabaci* populations may also change according to the species (Škaljac et al. 2010; Škaljac et al. 2013). Populations of MEAM 1 from China showed a significantly higher frequency of double (*Rickettsia-Hamiltonella* and *Rickettsia-Cardinium*) and triple (*Rickettsia-Hamiltonella-Cardinium*) co-infections than that found on MED Q populations (Pan et al. 2012a).

In Brazil, *B. tabaci* (former biotype A—New World species, Dinsdale et al. 2010) was first reported in the late 1960s colonizing cotton and legume crops. At that time, the begomovirus-induced damages were restricted to the “golden mosaic disease” of field beans (Costa et al. 1973). The invasion of *B. tabaci* MEAM 1 populations was confirmed in tomato fields in the 1990s, which was followed by severe begomovirus epidemics (Ribeiro et al. 1994). The rapid spread of the MEAM 1 into the country in association with its wide host range allowed for the natural transfer of new begomoviruses from weeds and/or native *Solanaceae* species to cultivated tomato, intensifying the selection pressure towards a higher number of novel begomoviruses in this crop (Ribeiro et al. 2003; Fernandes et al. 2008; Fernandes 2010). Studies on diversity of *B. tabaci* populations associated with the tomato crop are now under way in Brazil (Ribeiro et al.

2003; Fernandes 2010; Marubayashi et al. 2013). These studies are indicating that populations closely related to *B. tabaci* MEAM 1 are predominant in the Brazilian tomato fields.

Although whitefly endosymbionts have been object of many recent studies worldwide, there is an overall few works documenting the endosymbiont diversity in whiteflies populations colonizing the tomato crop in Brazil (Marubayashi et al. 2013, 2014). Currently, the secondary endosymbiont *Wolbachia* is being a subject of extensive studies in many different organisms, mainly because it can manipulate the reproduction rate of their hosts and enhance their own rate of transmission and therefore might serve as a biological control agent (Mitsuhashi et al. 2002). During preliminary surveys, we faced a surprising fact that *Wolbachia* could be detected at high frequency in nymphs and eggs of our experimental tomato-infesting *B. tabaci* population. In this context, considering the increase of interest in localizing and detecting endosymbionts in whiteflies populations around the world, we decided to study the presence of three genera (“*Ca. Portiera*”, *Wolbachia*, and *H. defensa*) in a representative tomato-infesting *B. tabaci* MEAM 1 population in distinct developmental stages using whole-mount fluorescence in situ hybridization analysis (FISH) analysis. “*Ca. Portiera*” (a primary endosymbiont with well-established localization in the bacteriocytes) was employed as an internal control in these FISH assays.

## Materials and methods

### *Bemisia tabaci* MEAM 1 population and colony maintenance

Begomovirus-free *B. tabaci* MEAM 1 colony was originally obtained from infested tomato plants under field conditions in Brasília-DF (Central Brazil). This population was then reared on virus-free cabbage (*Brassica oleracea* var. *capitata*) plants during approximately 6 months. This insect population was kept into isolated cages at the greenhouse system of the Experimental Biology Station (EBS) of UnB in Brasília-DF, Brazil. These host plants were cultivated in 2 L-plastic pots filled with commercial solid substrate (Bioplant®, Nova Ponte-MG, Brazil). The average temperature in the greenhouse was of  $28 \pm 2$  °C and the relative air humidity was  $69.7 \pm 5$  %.

### DNA extraction and PCR assay for *B. tabaci* identification

Total genomic DNA was purified from a sample of ten whitefly adults using a CTAB 2X buffer and organic solvents essentially as described by Boiteux et al. (1999). Sequence analyses were carried out using a partial *cytochrome oxidase subunit I (COI)* mitochondrial gene segment, which is rich in

genetically informative single nucleotide polymorphisms. The 700 bp target PCR product was amplified using the prime pair: C1-J-2195 (5'-TTG ATT TTT TGG TCA TCC AGA AGT-3') and L2-N-3014 (5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3') (Simon et al. 1994). PCR amplification of individual DNA samples was carried and amplicons were purified from the gel using the PureLink (Invitrogen) purification Kit prior to direct sequencing. Sequencing was done at an ABI 3100 (Applied Biosystems) sequencer from the Genomic Analysis Lab (at Centro Nacional de Pesquisa em Hortaliças, CNPH) located in Brasília-DF, Brazil. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al. 2013). Multiple sequence alignments were constructed using Clustal W (Thompson et al. 1994). Sequences reported by Dinsdale et al. (2010) were downloaded in addition to our sequenced amplicon (accession number KM013848). Adjustments were performed manually and when necessary, ends of the sequences were trimmed. Phylogenetic reconstruction was performed using the Maximum Likelihood method, with 1000 Bootstrap replications, defined by the substitution model of Hasegawa et al. (1985), (HKY85) and Gamma distributed with Invariant sites (G + I). MrBayes 3.1 was used for Bayesian phylogenetic inference (Ronquist and Huelsenbeck 2003). Analyses were run for 10 million generations by using four Markov chains and sampling every thousand generations. At the end of each run the average standard variation of split frequencies was below 0.01. Runs converged with the potential scale reduction factor (PSRF) at 1.0 for all parameters. Tree was visualized with FigTree (FigTree, 2006–2009, A. Rambaut).

### Endosymbiont genomic DNA extraction, PCR detection, and sequence characterization

Total genomic DNA extraction was carried out essentially as described by Aljanabi and Martinez (1997) and Santos et al. (2003), but with some minor modifications. Successful genomic DNA extractions of a single to up ten whitefly adults were obtained after increasing the Proteinase K (50 µL/mL) volume in the extraction buffer from 1 µL to 3.5 µL. Amplification was performed in 12.5 µL as described previously by Zchori-Fein and Brown (2002) and Zhou et al. (1998), using the specific primer pairs listed in Table 1. Sequencing was done at an ABI 3100 (Applied Biosystems) sequencer apparatus from the Genomic Analysis Lab (at CNPH) located in Brasília-DF, Brazil.

### Whole-mount fluorescence in situ hybridization (FISH) analyses

Whole-mount FISH of the samples (obtained from distinct *B. tabaci* MEAM 1 developmental stages) was performed

**Table 1** PCR primers employed for detection of the obligate “*Ca. Portiera*” and for two facultative endosymbionts (*Wolbachia* and *H. defensa*) in the representative *Bemisia tabaci* MEAM 1 population from tomatoes in Brazil

Endosymbiont	Primer code and genomic regions	Primer pairs and their 5'-3' sequence	Amplicon size (bp)	PCR Annealing T(°C)
“ <i>Ca. Portiera</i> ” <sup>1</sup>	Forward 28 Reverse 1098 (16S rDNA)	TGCAAGTCGAGCGGCATCAT AAAGTTCCCGCCTTATGCGT	1000–1100	54 °C
<i>Wolbachia</i> <sup>2</sup>	Forward wsp81 Reverse wsp691 ( <i>wsp</i> gene)	TGGTCCAATAAGTGATGAAGAAAC AAAAATTAACGCTACTCCA	590–625	55 °C
<i>Hamiltonella defensa</i> <sup>1</sup>	Forward Ham Reverse Ham (16SrDNA)	TGAGTAAAGTCTGGAATCTGG AGTTCAAGACCGCAACCTC	700	60 °C

<sup>1</sup> Zchori-Fein & Brown (2002)<sup>2</sup> Zhou et al. (1998), Holden et al. (1993)

essentially as described by Gottlieb et al. (2006) using endosymbiont-specific probes (Table 2) and analyzed under a confocal laser scanning microscope (Leica model TCS SP5). Briefly, eggs, adults, and nymphs of *B. tabaci* were collected from the abaxial leaf surface of cabbage plants with the aid of a stereomicroscope (20X magnification) and then transferred to microcentrifuge tubes containing Carnoy's fixative (chloroform: ethanol: glacial acetic acid, 6:3:1) and left overnight (12 h) at a refrigerator (4 °C) (Gottlieb et al. 2006). Carnoy's fixative was removed while keeping the insect samples within the microcentrifuge tubes. After that, ethanol-diluted H<sub>2</sub>O<sub>2</sub> (6 %) was added (for at least 2 h) to the tubes aiming to de-stain the insect tissues. This step is crucial to inactive autofluorescent pigments as well as endogenous enzymes (e.g., RNAses) present in the insect bodies. Next, the H<sub>2</sub>O<sub>2</sub> was completely removed and the insect samples were washed with absolute ethanol. The samples were kept on ethanol up to the hybridization procedure. In the next step, the ethanol was removed and the insect samples were washed with the hybridization solution (20 mM TRIS-HCL pH 8.0; 0.9 M NaCl; 0.01 % SDS; 30 % formamide) for 30 min. The endosymbiont-specific probes (Table 2) were added (10 pmol/mL) to the hybridization buffer. The samples were allowed to hybridize for at least 12 h under room temperature and dark conditions. The insect samples were removed from the microcentrifuge tubes and then washed for 5 min with the hybridization solution (but without SDS). Stained samples were carefully transferred slides and analyzed under a confocal laser scanning microscope (Leica model TCS SP5, single sequential scan images for Cy3 and Cy5 probes). Cy3 was

excited with 543 nm (emission 560–590 nm) and Cy5 with 633 nm (emission 670–700 nm). Signal quantification was performed using ImageJ (National Institutes of Health, Bethesda, MD). Specificity of stained samples was confirmed by using the following internal controls: RNase digested samples, samples containing only hybridization solution without probe, and samples without any treatment. Antibiotic treatments cannot eliminate secondary endosymbionts in a specific manner and they also induce adverse effects on the insect host fitness (Ahmed et al. 2010; Ruan et al. 2006). Therefore, controls with antibiotic treatments were not employed in our experimental whitefly population. Negative controls were carried out according to previous FISH analyses as recommended by Gottlieb et al. (2006, 2008). “*Ca. Portiera*” (a primary endosymbiont with well-known localization in the bacteriocytes) was employed as an internal control in these FISH assays. Quantitative analysis of co-localization of the single sequential scan images was performed using ImageJ (National Institutes of Health, Bethesda, MD) and the Coloc-2 plugin with a sample of 50 individuals of each *B. tabaci* developmental stage. Five samples were taken in each image of each *B. tabaci* developmental stage the “*Ca. Portiera*” and *Wolbachia* combination. These values were used to calculate the means and the Pearson's correlation coefficient values. Student's one-sample *t*-test was used to test the significance of Pearson's correlation coefficient measurements (McDonald and Dunn 2013) of “*Ca. Portiera*” and *Wolbachia* co-localization. All statistical analyses were performed using the software package Statistica v. 7.0.61.0 (StatSoft, Inc.).

**Table 2** Probes employed in the fluorescence in situ hybridization assays for detection of “*Ca. Portiera*” and two secondary endosymbionts (*Wolbachia* and *H. defensa*) in the bodies of alldevelopmental stages of a representative *Bemisia tabaci* MEAM 1 population from tomatoes in Brazil. Probes were labeled with either the fluorophore Cy3 or the fluorophore Cy5

Probe/fluorophor code	Target endosymbiont	Probe sequence 5'-3'	Reference
BTP1-Cy3	“ <i>Ca. Portiera</i> ”	TGTCAGTGTGACCCAGAAG	Gottlieb et al. (2006; 2008)
BTH-Cy5	<i>H. defensa</i>	CCAGATTCAGACTTTACTCA	Gottlieb et al. (2008)
W2-Cy5	<i>Wolbachia</i>	CTTCTGTGAGTACCGTCATTATC	Mitsuhashi et al. (2002)

## Results and discussion

### *Bemisia tabaci* species identification via sequence analysis of the cytochrome oxidase subunit I (COI) mitochondrial gene

A single amplicon of expected size ( $\approx 700$  bp) was purified and sequenced in both directions. Using maximum likelihood analysis of the COI gene segment, which is rich in genetically informative single nucleotide polymorphisms, allow us to unequivocally classify our population as belonging to the cosmopolitan *B. tabaci* MEAM 1 group (Supporting Information S1), which is so far the predominant tomato-infesting whitefly species in Brazil (Marubayashi et al. 2013). BLAST analysis showed 100 % identity with one *B. tabaci* MEAM 1 population (named as population Jo-122) reported on tomato plants in Jordan (GenBank accession KC789961.1). Our results are in agreement with the phylogenetic tree generated by Dinsdale et al. (2010) in which our sequence (GenBank accession KM013848) clustered to the same clade within the MEAM 1 group. This Brazilian *B. tabaci* MEAM 1 population was employed in all subsequent assays.

### PCR detection and amplicon sequence analysis of *Wolbachia* and *H. defensa* associated with the tomato *B. tabaci* MEAM 1 population

Single PCR amplicons of the expected size were obtained for each of the three endosymbiont-specific primer pairs. Table 3 shows the results obtained from the PCR procedures. Sequence analysis of the *Wolbachia*, and *H. defensa*-specific amplicons displayed identity values ranging from 97–100 % with sequences available at GenBank. *H. defensa* amplicons were visualized in all 12 female and 12 male individual

**Table 3** Relative frequency of endosymbiont-specific fluorescence in situ hybridization probe signals visualized in the bodies of distinct developmental stages of a representative *Bemisia tabaci* MEAM 1 population from tomatoes in Brazil and confirmed via PCR. Specific probes and hybridization assays were carried out to detect the one obligate “*Ca. Portiera*” and two facultative (secondary) endosymbionts (*Wolbachia* and *H. defensa*)

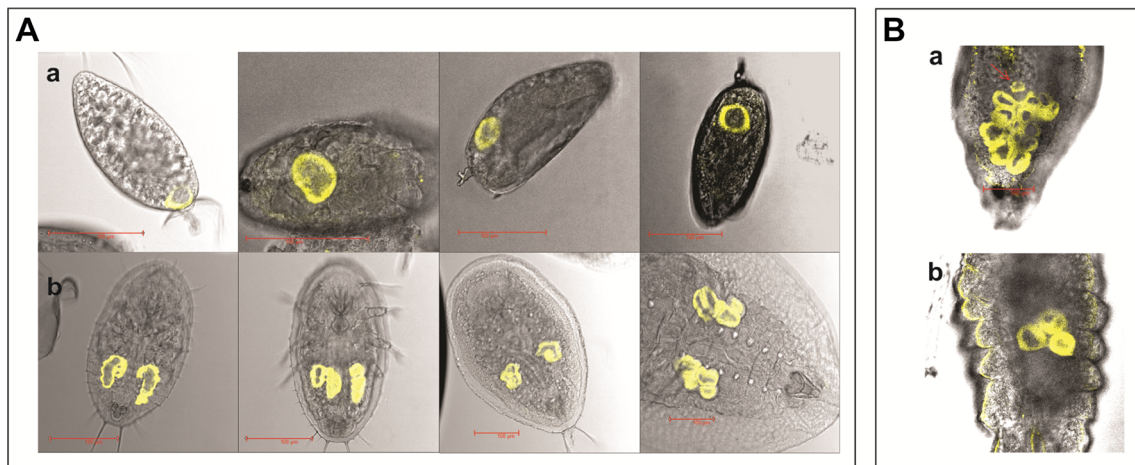
Endosymbionts	Adult Female	Adult Male	Nymphs	Eggs
<i>Wolbachia</i> (FISH)	20/10 <sup>a</sup>	20/04	20/16	20/17
<i>Wolbachia</i> (PCR)	12/10*	12/05	n.d	n.d
<i>Ca. Portiera</i> (FISH)	20/10	20/03	20/19	20/18
<i>Ca. Portiera</i> (PCR)	12/12	12/11	n.d	n.d
<i>Hamiltonella</i> (FISH)	20/06	20/02	10/05	10/05
<i>Hamiltonella</i> (PCR)	12/12	12/12	n.d	n.d

<sup>a</sup>Number of evaluated samples in each *B. tabaci* developmental stage / number of positive samples (= detection of probe-specific signals). n.d not determined

samples (100 %). *Wolbachia* genomic segments were amplified from ten out of 12 female samples (83.3 %) and from five out of 12 male adult samples (41.6 %), indicating higher frequencies of *Wolbachia* in female than in male adults. These results are in agreement with that obtained with the FISH analysis. Also, we were able to detect *Wolbachia* via FISH in 80 to 85 % of nymph and egg samples. Hence, is left for future work the verification if *Wolbachia* does alter sex ratio on this population.

### Vertical transmission of “*Ca. Portiera*” in the Brazilian *B. tabaci* MEAM 1 population

The primary (obligate) endosymbiont “*Ca. Portiera*” was detected in *B. tabaci* eggs and in nymphs in association with bacteriocytes, which are large polyploidy, symbiotic bacteria-bearing host cells that are grouped into organ-like structures, called bacteriomes (Baumann 2005). The Fig. 1 panel A shows the results of the localization of the “*Ca. Portiera*” in distinct egg stages. Endosymbiont probe signal was consistently observed in the distal segment of the egg corresponding to embryo pole (adjacent to the egg short stalk). This localization is suggestive of the potential maternal transmission of the endosymbiont to the embryo. In previous studies with *B. tabaci*, “*Ca. Portiera*” was detected mainly at the circumference of the bacteriocytes, which are located adjacent to the ovarioles (Gottlieb et al. 2006, 2008). “*Ca. Portiera*” is already known to be localized in association with the bacteriocytes in distinct whitefly populations (Guo et al. 2014) as shown at Fig. 1 panel A picture b and Fig. 1 Panel B shows the localization of “*Ca. Portiera*” in female and male adults. Probe signals were observed in association with bacteriocytes. In female adults (Fig. 1 panel B picture a), the endosymbiont signal was found in the ovaries mainly in association with bacteriocytes. This same figure displays an egg within the female abdomen in close vicinity with bacteriocytes that seems to be colonized by “*Ca. Portiera*”. Moreover, a distinct signal quantity of “*Ca. Portiera*” was observed when comparing female and male adult samples (Fig. 1 panel B, pictures a and b). The corrected total cell fluorescence mean values were overall less in the male adult samples when compared with the values obtained from female adult samples under identical hybridization conditions, suggesting that “*Ca. Portiera*” proliferate more efficiently in females (data not shown). This prokaryote is generally passed from host to host via vertical transmission (Feldhaar 2011). This vertical transmission of “*Ca. Portiera*” is speculated to be due to the physical contact of the primordial eggs with bacteriocyte within the female abdomen. The association of this prokaryote with the egg region where the insect embryo is located give support to the hypothesis that this could be a major mechanism associated with its vertical transmission in our MEAM 1 population.



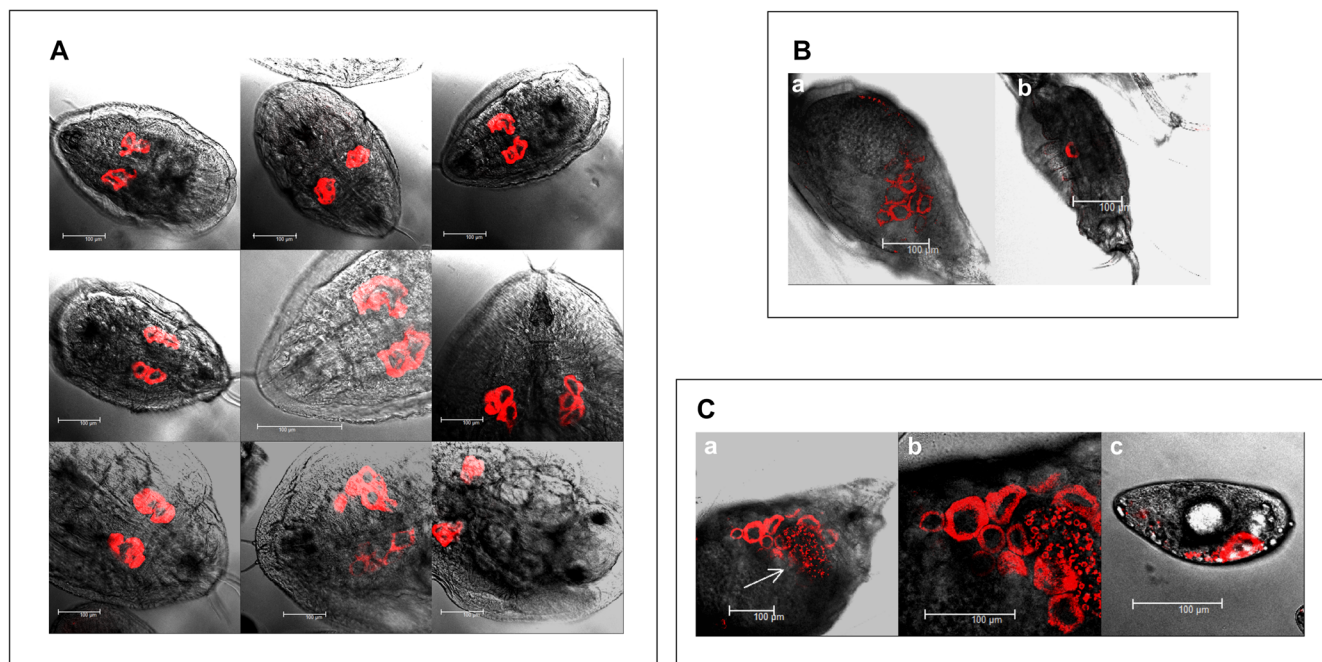
**Fig. 1** Whole-mount fluorescence in situ hybridization of the obligate (primary) endosymbiont “*Candidatus Portiera aleyrodidarum*”. (Panel a, sub-panel a) *Bemisia tabaci* MEAM 1 eggs and (Panel a, sub-panel b) nymphs; (Panel b, picture a) *Bemisia tabaci* MEAM 1 female and (Panel

b, picture b) male adults. The red arrow shows “*Ca. Portiera*” in ovaries in association with bacteriocytes. Fluorescence signals were generated by the specific probe BTP1-Cy3 (pseudocolored yellow). Bars=100 μm

### Localization of *Wolbachia* in the bodies of distinct developmental stages of the *B. tabaci* MEAM 1 population

Signals derived from the *Wolbachia*-specific probe (W2-Cy5) were detected in *B. tabaci* eggs, nymphs, males, and females. *Wolbachia*-specific signals coincided with the female gonads at the tip of abdomen (Fig. 2 panel B, picture a). These signals were also found in close association with bacteriocytes in both

nymphs (Fig. 2 Panel A) and male adults (Fig. 2 panel B, picture b). However, in some eggs, *Wolbachia* signals were not detected in strict association with bacteriocytes. A small fraction of the probe signal was also found scattered and diffuse throughout the egg in regions not clearly associated with bacteriocytes (Fig. 2 panel C, picture c). To our knowledge, this is a novel localization pattern described for *Wolbachia* in the Brazilian MEAM 1 population, since it was not observed



**Fig. 2** Whole-mount fluorescence in situ hybridization using the specific-probe W2-Cy5 (pseudocolored red) of the secondary endosymbiont *Wolbachia*. Distinct nymphal stages of *Bemisia tabaci* MEAM 1 population from Brazil (Panel a); female (Panel b, picture a) and male (Panel b, picture b) samples of *Bemisia tabaci* MEAM 1. Panel

c (picture b) is a magnification of Panel c (picture a), illustrating that *Wolbachia*-specific signals were localized at the female gonads (at the tip of abdomen) in close association with bacteriocytes (white arrows). In some eggs, probe signals were not in strict association with bacteriocytes but also scattered in other areas (Panel c, picture c). Bars=100 μm

in previous studies carried out with *B. tabaci* MED samples from Israel (Gottlieb et al. 2008). Interestingly, Gottlieb et al. (2008) detected *Wolbachia* signals outside the bacteriocytes in some *B. tabaci* MED Q adults. However, in eggs and nymphs the *Wolbachia*-specific signals were located exclusively at the circumference and/or inside bacteriocytes (Gottlieb et al. 2008). Biological differences between *Wolbachia* strains from Brazil and Israel could be an explanation for these variations. Genetic variability among *Wolbachia* populations from *B. tabaci* MED Q from China and Mediterranean regions has been identified (Ahmed et al. 2009). In fact, there are already reports of at least two distinct *Wolbachia* strains coexisting, for example, in closely related mulberry leafhopper species (Mitsuhashi et al. 2002). In our samples, *Wolbachia* was found at high frequency especially in eggs and nymphs, which is in contrast with the scenario described for MEAM 1 and MED Q field populations in China (Pan et al. 2012a). It is important to note that in Israel, *Wolbachia* association with bacteriocytes was found in *B. tabaci* MED Q individuals, but it was not found in MEAM 1 samples (Gottlieb et al. 2008). Moreover, Zchori-Fein et al. (2014) studied the variations in the symbiotic communities of *B. tabaci* over 2000 individuals from independent screenings. In this analysis, *Wolbachia* was again not detected in MEAM 1 individuals. Therefore, our report seems to be the first confirmation of *Wolbachia* in strong association with *B. tabaci* MEAM 1 individuals in the Neotropical areas.

#### **Co-localization of *Wolbachia* and “*Ca. Portiera*” in the bodies of distinct developmental stages of the Brazilian *B. tabaci* MEAM 1 population**

The results from several multiple images (Fig. 3 panels A from D) of each *B. tabaci* developmental stages indicated that “*Ca. Portiera*” and *Wolbachia* are occupying the very same sites within nymphs, male, and female adults of their insect host. “*Ca. Portiera*” and *Wolbachia* signals were associated mainly with bacteriocytes. However, as previously discussed, in some eggs *Wolbachia* signals were also detected in scattered regions not in close association with bacteriocytes. The co-localization analysis using ImageJ (Coloc-2 plugin) estimated the Pearson’s correlation coefficient value of 0.788 for eggs, of 0.802 for first instar nymphs, and of 0.875 for female adults, indicating that the intensity distribution between channels and, therefore, the coexistence of both probe-specific signals in a very close physical location. In addition, all the Pearson’s correlation coefficient values showed a value significantly greater than zero, which was verified by using the Student’s one-sample *t*-test method. To verify the possibility of obtaining an improved double staining result, the concentration of the “*Ca. Portiera*”-specific hybridization probe BTP1-Cy3 was 10-fold increased. However, no significant increase of the fluorescence signal could be observed, possibly indicating that,

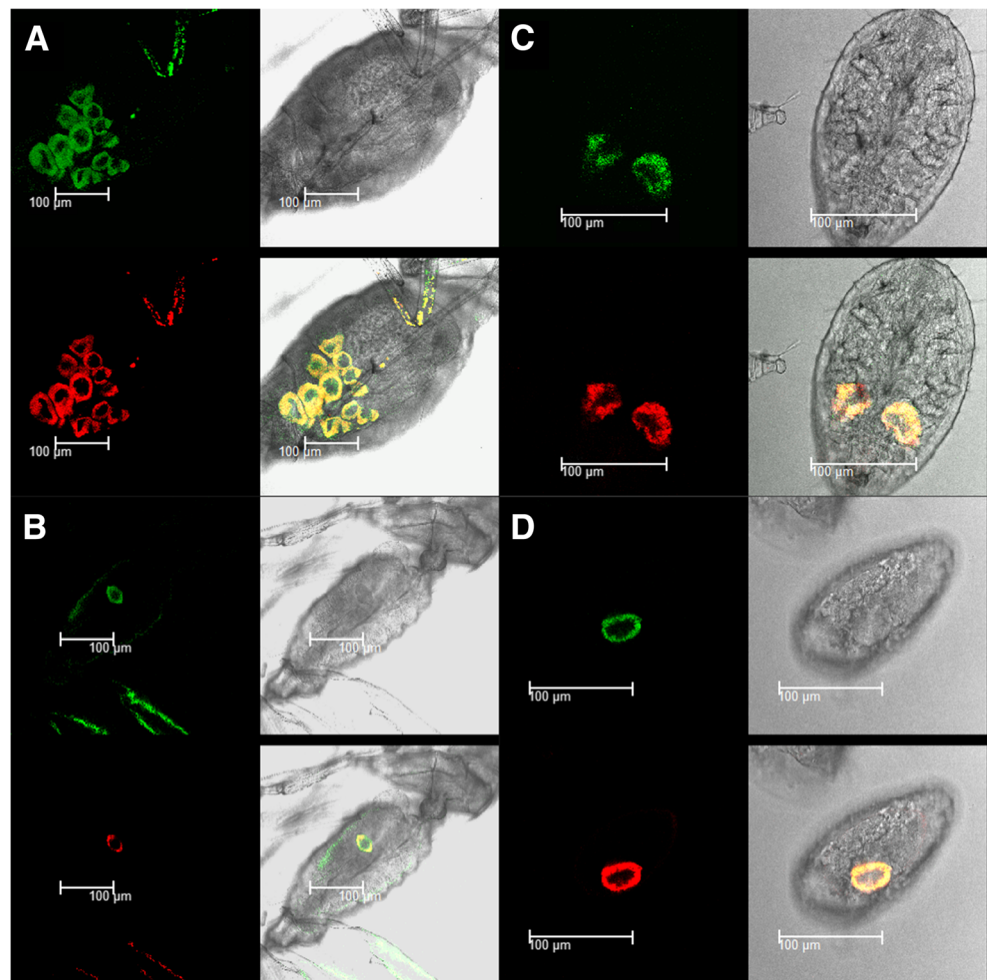
under our experimental conditions, the hybridization of the second probe (the *Wolbachia*-specific W2-Cy5) is somehow favored in relation to that of “*Ca. Portiera*”. Quantitative PCR analysis to obtain a more precise estimation of the amount of each endosymbiont DNA occurring in female and male adults is left for future work.

#### **Localization of *H. defensa* in the bodies of distinct developmental stages of the Brazilian *B. tabaci* MEAM 1 population**

The signals derived from the probe specific for the secondary endosymbiont *H. defensa* in *B. tabaci* were detected in male and female adults, eggs, and nymphs. *H. defensa*-specific signals were quite variable in terms of localization, being detected in the leg muscles (e.g., coxa), in the head (e.g., cibarium) (Fig. 4A) in the female ovaries (Fig. 4B) as well as at the abdomen tip corresponding to the reproductive organs of the males (Fig. 4C). Probe signals were observed at the tip as well as in the proximal abdomen segment (gonads) of the nymphs in association with bacteriocytes (Fig. 4D), but a less intense signal was also observed scattered within the eggs (Fig. 4E), indicating that *Hamiltonella* is not exclusively confined to bacteriocytes in this tomato MEAM 1 population. These results are in sharp contrast with that reported by Gottlieb et al. (2008).

Currently, works aiming to localize distinct endosymbiont genera associated with *B. tabaci* populations have been done mainly with MEAM 1 and MED Q species from the Old World (Gottlieb et al. 2006, 2008, 2010; Brown 2007). There is an overall lack of studies documenting the diversity as well as biological properties of the prokaryotic endosymbiont community associated with recent invasion of exotic *B. tabaci* MEAM 1 populations into Brazil. Gottlieb et al. (2008) investigated the localization of a group of endosymbionts in the MEAM 1 and MED Q species from Israel. These authors subdivided the bacterial flora of *B. tabaci* into two subgroups according to their localization within the host body: (1) endosymbionts with exclusive association with bacteriocytes and (2) endosymbionts not associated with bacteriocytes. “*Ca. Portiera*” and *H. defensa* were found to be members of the first group, whereas *Wolbachia* and *Cardinium* were classified in the second group (Gottlieb et al. 2008). In the Brazilian *B. tabaci* MEAM 1 population employed in the present study, *H. defensa* and *Wolbachia* distribution in some developmental stages was not in complete agreement with the classification proposed by Gottlieb et al. (2008). This set of data reinforces the notion that localization and distribution of endosymbionts might vary according to the species/ecotype of occurrence of a given *B. tabaci* population. Previous work indicated that *B. tabaci* monopartite begomovirus transmission efficiency might be related to the diversity and relative composition of endosymbiont community living in association with the insect host (Gottlieb et al. 2010).

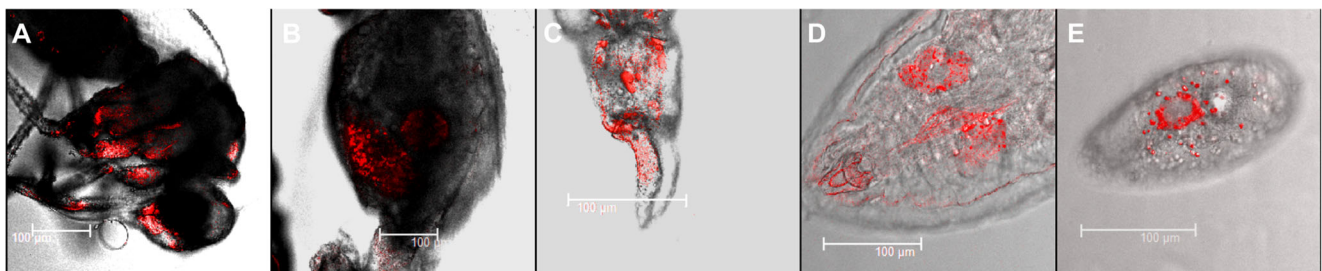
**Fig. 3** Co-localization of the primary endosymbiont “*Candidatus Portiera aleyrodidarum*” and the secondary endosymbiont *Wolbachia* in the tomato-infesting *Bemisia tabaci* MEAM 1 population from Brazil. “*Ca. Portiera*” is shown in areas with green signals (generated by the specific-probe BTP1-Cy3, pseudocolored green) and *Wolbachia* is shown in areas with red signals (generated by the specific-probe W2-Cy5, pseudocolored red). Co-location signals (yellow) were observed mainly in bacteriocytes of female adult (Panel a), male adult (Panel b), nymphs (Panel c), and eggs (Panel d) samples. It was possible to visualize the auto-fluorescence of chitins on legs and wings. Bars=100  $\mu$ m



#### Relative frequency and comparative analysis of endosymbiont-specific fluorescence probe signals in distinct developmental stages and distinct genders of a Brazilian MEAM 1 population

Probe-specific signals of all three endosymbionts visualized in distinct developmental stages were overall more frequently detected in nymphs and eggs (Table 3). Female samples displayed higher infection percentages and higher levels of

colonization when compared with male adult samples. In addition, we observed that the amount of all three bacteria was overall lower in the male adult samples when compared with the female samples (Fig. 1 panel B, 2 panel B). In our study, *Wolbachia* was found with relative frequency and at detectable levels within the body of male and female adults in the *B. tabaci* MEAM 1 population. This somewhat surprising occurrence of *Wolbachia* in the Brazilian *B. tabaci* MEAM 1 population may have significant epidemiological impacts. Is



**Fig. 4** Whole-mount fluorescence in situ hybridization using specific-probe BTH-Cy5 (pseudocolored red) of the facultative (secondary) endosymbiont *H. defensa* in a representative *Bemisia tabaci* MEAM 1 population from tomatoes in Brazil. *H. defensa*-specific signals were

localized in **a** the male leg muscles, in the head mouthparts, in **b** the female ovaries, **c** male reproductive organs at the abdomen tip, **d** distal tips and in the proximal abdomen segment of the nymphs, and **e** in the bacteriocytes but also scattered into the eggs. Bars=100  $\mu$ m



important to point out that other secondary endosymbionts (e.g., *Wolbachia*) were also found playing a key role in increasing rates of monopartite begomovirus transmission by MED Q populations (Gottlieb et al. 2010). In fact, it is quite possible that GroEL proteins might be begomovirus-specific or even virus strain-specific. In Brazil, so far only bipartite begomovirus species have been detected in tomato and other crops (Ribeiro et al. 2003; Fernandes et al. 2008; Fernandes 2010), with exception for sweet-potatoes where solely monopartite species were detected (Paprotka et al. 2010). In this scenario, it would be of interest to carry out additional studies aiming to demonstrate if the localization of the endosymbionts and their gene products might be correlated with the transmission ability/efficiency by the Brazilian MEAM 1 populations of the complex of more than 20 bipartite begomovirus species described infecting tomatoes so far (Ribeiro et al. 2003; Fernandes et al. 2008; Fernandes 2010).

In conclusion, our results represent the first set of analyses about the interactions that are occurring among endosymbiont genera and a representative tomato-infesting *B. tabaci* MEAM 1 population from the Neotropics. All endosymbionts were found in our samples, revealing the occurrence of distinct and novel infection and colonization patterns. “*Ca. Portiera*” was located exclusively at the circumference and/or inside bacteriocytes as expected. However, a peculiar sub-localization pattern was observed for *H. defensa* and for *Wolbachia* in eggs. Strong *H. defensa*-specific probe signals were detected in the head and in the region associated with the male reproductive organs, which is in sharp contrast with previous studies (Marubayashi et al. 2014), which found this bacterium always confined to the bacteriosome in MEAM 1 and New World 2 species from Brazil. In addition, our results reinforced the notion that *H. defensa* is also associated with *B. tabaci* MEAM 1 population. On the other hand, *Wolbachia* infection/colonization (which is associated with MED and rarely found in MEAM 1 populations from the Old World) was present at high frequency in eggs and nymphs. All these features are new and reported for the first time in the Brazilian whitefly population.

There is a clear need for further investigations on the interactions among Brazilian bipartite begomoviruses, viruliferous whiteflies and secondary endosymbionts, with special emphasis on key prokaryotic genera such as *H. defensa* and, according to our results, also *Wolbachia*. These additional studies will be necessary in order to elucidate their function in this complex multi-kingdom (tomato – insect vector species – *Begomovirus* species – prokaryotic endosymbionts) interaction (Brown 2007) under Neotropical conditions.

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