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New invasion of *Bemisia tabaci* Mediterranean species in Brazil associated to ornamental plants

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Abstract In Brazil, the first major invasion event of *Bemisia tabaci* was that of Middle East–Asia Minor 1 (MEAM1) species, formerly termed as B biotype, which commenced in the 1990s mainly by ornamental plants in São Paulo State. More than two decades after this invasion, the presence of the Mediterranean (MED) species of *B. tabaci*, formerly Q biotype, was reported in Rio Grande do Sul, the southernmost State of Brazil, and now in São Paulo and Paraná States, in southeastern Brazil. Specimens of whiteflies collected from commercial begonia, hydrangea, petunia and poinsettia greenhouses in São Paulo, and also from begonias and poinsettias collected in flower shops in Paraná, were all identified as belonging to MED species. Furthermore, the secondary endosymbionts *Arsenophonus*, *Hamiltonella* and *Rickettsia* of MED from São Paulo and Paraná were detected by PCR and their presence confirmed by sequencing and FISH analysis, and those results differed from MED detected in Rio Grande do Sul that harbored only *Hamiltonella* and *Cardinium.* Our results suggest a new MED invasion into Brazil and is associated with ornamental plants. The two MED populations are genetically different and suggest that they are separate invasions.

Keywords Whiteflies · MEAM1 · mtCOI · Endosymbionts

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Introduction

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is one of the most invasive and damaging pests of a wide variety of horticultural, ornamental and field crops worldwide, causing heavy economic losses (De Barro et al. 2011). *B. tabaci* is a cryptic species complex encompassing morphologically indistinguishable but ecologically and genetically distinct groups (Xu et al. 2010; De Barro et al. 2011; Kanakala and Ghanim 2015), composed of at least 43 cryptic biological species based on mitochondrial cytochrome oxidase subunit I (mtCOI) gene sequences (De Barro et al. 2011; Tay et al. 2017). Two of these cryptic species, Middle East-Asia Minor 1 - MEAM1 (commonly known as biotype B) and Mediterranean – MED (biotype Q) are considered

the most invasive whitefly pests, causing direct plant damage and transmitting more than over 300 viruses, representing 5 genera, including DNA and RNA viruses (Gilbertson et al. 2015).

B. tabaci species from the New World are native for the Americas and were reported for the first time in Brazil in 1928 in the State of Bahia (Barbosa et al. 2015; Bondar 1928). The populations of whiteflies in Brazil remained low until the early 1990s with the introduction of MEAM1 in São Paulo State (Lourenção and Nagai 1994). High losses have been reported on Brazilian agriculture ever since, mainly in the Solanaceae (Ribeiro et al. 1998).

In recent years the global spread of MED from its origin in the countries bordering the Mediterranean Basin has imposed a lot of concerns (De Barro et al. 2011). MED species has particularly developed resistance to neonicotinoids (Horowitz et al. 2005; Nauen and Denholm 2005) and in some countries was associated with high transmission efficiencies of *Tomato yellow leaf curl virus* (TYLCV), one of the most devastating viruses in the world (Li et al. 2010). MED species has a limited presence in the Americas, being first reported in the United States in 2004 (Dalton 2006) and subsequently in Mexico in 2007 (Martinez-Carrillo and Brown 2007), Guatemala in 2009 (Bethke et al. 2009) and more recently in Argentina and Uruguay (Grille et al. 2011) and in the South of Brazil (Barbosa et al. 2015).

The variability of *B. tabaci* is also reported by the secondary endosymbionts that it harbors (Gueguen et al. 2010; Bing et al. 2013). *B. tabaci* harbors the primary symbiont *Portiera aleyrodidarum*, which is essential for its survival and development and is located within specialized cells called "bacteriocytes" (Kanakala and Ghanim 2015). Furthermore, the insect may be infected with secondary symbionts including *Arsenophonus*, *Hamiltonella*, *Wolbachia, Cardinium*, *Fritschea* and *Rickettsia*. These secondary symbionts are associated with their insect hosts and can play a very important role in their ecology and evolution (Gottlieb et al. 2006; Kanakala and Ghanim 2015). The MED species has a frequent association with *Arsenophonus* and *Wolbachia*, and in Israel this species was not associated with *Hamiltonella* (Chiel et al. 2007).

Here we present evidence for a second introduction of *B. tabaci* MED species in Brazil, which was found on ornamental plants in São Paulo and Paraná States. The newly introduced population harbors a different set of secondary endosymbionts compared to the first introduction and the analysis of the mtCOI gene suggests a genetic variability of this species in Brazil, representing a new concern to agriculture in this country.

Experimental methods

Whitefly sampling

Adults, nymphs and eggs of *B. tabaci* were collected in different ornamental plants and weeds associated to these ornamental plants. The sampling was carried out both in commercial greenhouses and in flower shops between March and October 2015 in several São Paulo and Paraná States locations (Table 1). Adults were collected using a hand-held aspirator; nymphs and eggs were collected with the aid of a brush. The specimens were immediately transferred to a tube containing 95% ethanol and stored at 4 °C until further processing. Each population had 10 individuals used for mtCOI and microsatellite analysis and 10 more individuals used for Fluorescent in situ hybridization (FISH) to confirm the presence of the endosymbionts from representative samples.

DNA extraction, mtCOI gene amplification and enzyme restriction

Total nucleic acids were extracted from each individual following a modified Chelex method. *B. tabaci* adults were crushed and homogenized in 20 μl of Chelex 5% solution in a 0.2 ml Eppendorf tube. The tube was agitated for few seconds, and then incubated at 56 °C for 15 min and at 99 °C for 3 min. After centrifugation at 14,000 rpm for 5 min, the supernatant was then collected and used as a template for the PCR amplification.

All DNA samples were first subjected to PCR analysis to differentiate MEAM1 from MED using the primers pair Bem23F (5′-CGGAGCTTGCGCCT TAGTC-3′) and Bem23R (5′-CGGCTTTATCATAG CTCTCGT-3′) described by (De Barro et al. 2003), which amplifies a microsatellite locus of about 200 bp and 400 bp for MEAM1 and MED, respectively (Skaljac et al. 2010; Kontsedalov et al. 2012). The samples were then screened with the generic insect primers C1-J-2195 and TL2-N-3014 that amplify a fragment of the mtCOI (Frohlich et al. 1999). For Restriction fragment length polymorphism (RFLP)

Table 1 Samples, localities, species identification and set of secondary endosymbionts of adults and nymphs of whitefly specimens collected in ornamental greenhouses and flower shops from São Paulo and Paraná State

City	Coordinates	Date of Collections	Hosts	Species	RHA	RH	RA	HA	R	H	A
Arujá-SP	S23°23′20,07″ W46°19′13,27”	April 2015	*Myrtus communis / Ficus* spp.	*B. berbericola*	-	-	-	-	-	-	-
Mogi das Cruzes-SP	S23°22′19,95″ W46°10′35,02”	April 2015	*Begonia* spp.	MED	0	0	0	0	0	70	0
Mogi das Cruzes-SP	S23°22′19,95″ W46°10′35,02”	August 2015	*Hydrangea macrophylla*	MED	20	20	0	0	90	40	20
Mogi das Cruzes-SP	S23°22′19,95″ W46°10′35,02”	November 2015	*Begonia* spp.	MED	10	0	0	50	10	80	60
Mogi das Cruzes-SP	S23°22′19,95″ W46°10′35,02”	December 2015	*Begonia* spp.	MED	40	0	0	20	40	70	60
Mogi das Cruzes-SP	S23°22′19,95″ W46°10′35,02”	January 2016	*Hydrangea macrophylla*	MED	50	0	0	20	50	70	90
Guarulhos-SP	S23°23′31,13″ W46°21′37,43”	April 2015	*Begonia* spp.	MED	0	0	0	50	0	80	50
Guarulhos-SP	S23°23′31,13″ W46°21′37,43”	May 2015	*Begonia* spp.	MED	20	0	0	30	20	90	50
Atibaia-SP	S23°2′23,87″ W46°35′1,54”	February 2015	*Euphorbia pulcherrima*	MED	0	0	0	0	10	10	0
Atibaia-SP	S23°23′31,13″ W46°21′37,43”	February 2015	*Conyza bonariensis*	MEAM1	-	-	-	-	-	-	-
Atibaia-SP	S23°23′31,13″ W46°21′37,43”	February 2015	*Ruta graveolens*	MEAM1	-	-	-	-	-	-	-
São Pedro-SP	S22°32′55″ W47°54′50”	September 2015	*Euphorbia pulcherrima*	MED	0	0	0	0	0	100	0
Mogi Mirim-SP	S22°25′55″ W46°57′30”	September 2015	*Petunia* spp.	MED	0	0	0	0	0	10	0
Londrina-PR	S23°26′48″ W51°11′22”	December 2015	*Begonia* spp.	MED	0	0	0	66,7	0	100	66,7
Londrina-PR	S23°26′48″ W51°11′22”	December 2015	*Euphorbia pulcherrima*	MED	0	0	0	60	0	90	60
Marialva-PR	S23°47′9″ W51°83′6”	December 2015	*Emilia fosbergii*	MED	0	10	0	20	10	100	20
Marialva-PR	S23°47′9″ W51°83′6”	December 2015	*Capsicum* spp.	MED	20	20	0	10	40	100	30

analysis of the amplicons (Bosco et al. 2006), 5 μl of each PCR (880 bp) was digested with one unit of TaqI at 65 °C for 2 h in a final volume of 15 μl. The restricted DNA was visualized by electrophoresis in 2% agarose gel stained with Gel Red ®.

PCR products amplified from mtCOI of *B. tabaci* were purified (QIAquick Gel Extraction Kit Qiagen) and sequenced (Macrogen, South Korea) in both directions using C1 -J-2195/TL2-N-3014 and sequence analyzed and comparedwith those present in GenBank using BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis

Phylogenetic analysis were carried out using the 23 unique *B. tabaci* mtCOI sequences obtained in this study (Fig. 1) added to the new global *B. tabaci* mtCOI dataset (Boykin and De Barro 2014) downloaded straight from GenBank on June 15, 2017 totaling 567 sequences. Multiple sequence alignment was prepared using MAFFT (Katoh and Toh 2008) within the Geneious v9.1.5 software. The mtCOI sequences were trimmed and the fragment obtained was 663 bp in length. Bayesian analyses were conducted using Mr. Bayes v. 3.2.2 (Ronquist et al. 2012) and were run in parallel across 384 nodes on the Magnus supercomputer (located at the Pawsey Centre, Western Australia). Analyses were run for 3 million generations with sampling every 1000 generations. Each analysis consisted of four independent runs, each utilizing four coupled Markov chains. The run convergence was monitored by finding the plateau in the likelihood scores (standard deviation of split frequencies <0.0015). The first 25% of each run was discarded as burn-in for the estimation of a majority rule consensus topology and posterior probability for each node. Trees were visualized, edited and rooted using FigTree (Rambaut 2012).

Molecular detection of endosymbionts

The same DNA originated from each individual was used for the screening of *Portiera aleyrodidarum* (Muyzer et al. 1996), and the six secondary endosymbionts *Hamiltonella* (Zchori-Fein and Brown 2002), *Rickettsia* (Gottlieb et al. 2006), *Wolbachia* (Heddi et al. 1999), *Arsenophonus* (Thao and Baumann 2004), *Cardinium* (Weeks et al. 2003) and *Fritschea* (Everett et al. 2005) using genus-specific primers targeting the 16S or 23S rDNA genes. PCR cycling was performed as described by Marubayashi et al. (2014). The endosymbiont presence confirmation was performed by sequencing the amplified sequences from representative individuals.

Localization of endosymbionts using fluorescence in situ hybridization (FISH)

For endosymbiont localization in whiteflies, FISH analysis was performed for 10 more specimens from each population (Gottlieb et al. 2008; Skaljac et al. 2010). After collecting the insect samples, they were immediately transferred to Carnoy's fixative (Chloroform:Ethanol:Acetic acid = 6:3:1) for overnight fixation. The samples were then bleached with 6% H_2O_2 in ethanol for 2 h and hybridized overnight in hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% SDS, 30% Formamide) containing 10 pmol/ml of fluorescent probes specifically targeting the different symbionts as described by Marubayashi et al. (2014). The specimens were then mounted in microscope slides and viewed under an Olympus IX81 Fluoview 500 confocal microscope (Olympus, Tokyo, Japan).

Results

The species identification results indicated that all the whiteflies specimens collected from the ornamentals cultivated in greenhouses from Mogi das Cruzes, Guarulhos, Atibaia, São Pedro and Mogi Mirim, as well as from poinsettia and begonias sold by flower shops from Paraná were identified as MED species. MED was also identified colonizing ornamental pepper (*Capsicum* spp.) and *Emilia fosbergii* grown in greenhouses where previously poinsettia had been cultivated. The whiteflies present in the ornamental bushes from Arujá, such as *Myrtus communis* and *Ficus* sp. were identified as *Bemisia berbericola*. MEAM1 was present in *Conyza bonariensis* and *Ruta graveolens* L. from Atibaia (Table 1).

Furthermore, the phylogenetic analysis of *B. tabaci* mtCOI sequences conducted in this study showed high variability among the MED populations collected in Brazil (Fig. 1).

The primary endosymbiont *P. aleyrodidarum*, which is essential for *B. tabaci* survival, was detected in all whiteflies tested and the most prevalent co-infection

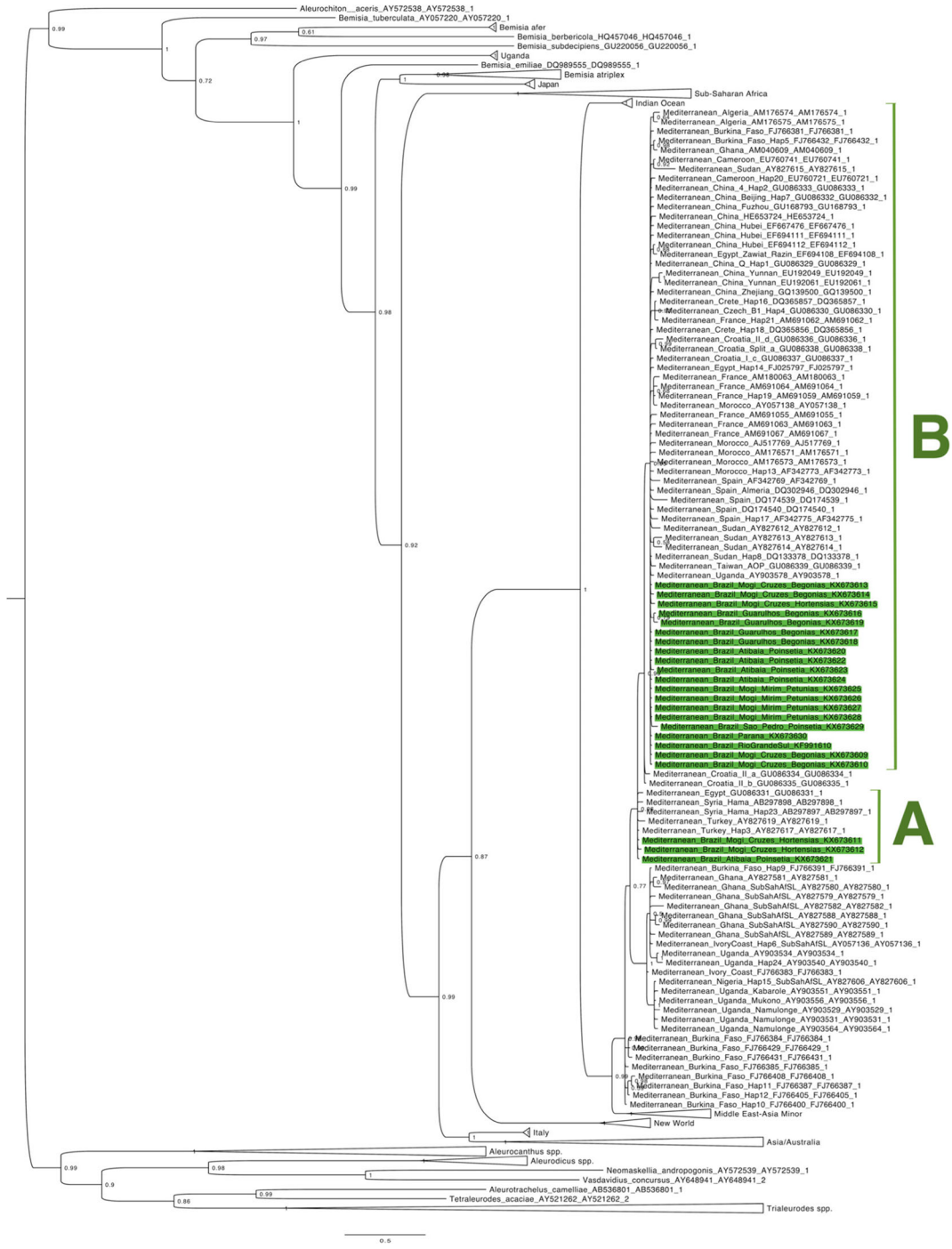

Fig. 1 Phylogenetic tree of the partial mtCOI gene from *Bemisia tabaci* conducted using MrBayes v. 3.2.2 on the Magnus supercomputer. The alignment consisted of 567 sequences but only the Mediterranean clade is shown expanded, other clades are collapsed. Sequences obtained in this study are highlighted in green. The host, city of collection, population number and the GenBank access number are indicated

observed in the majority of samples tested was *Rickettsia*, *Hamiltonella* and *Arsenophonus* (Table 1).

We confirmed the presence of all symbionts through PCR, sequencing (data not shown) and FISH analysis (Fig. 2).

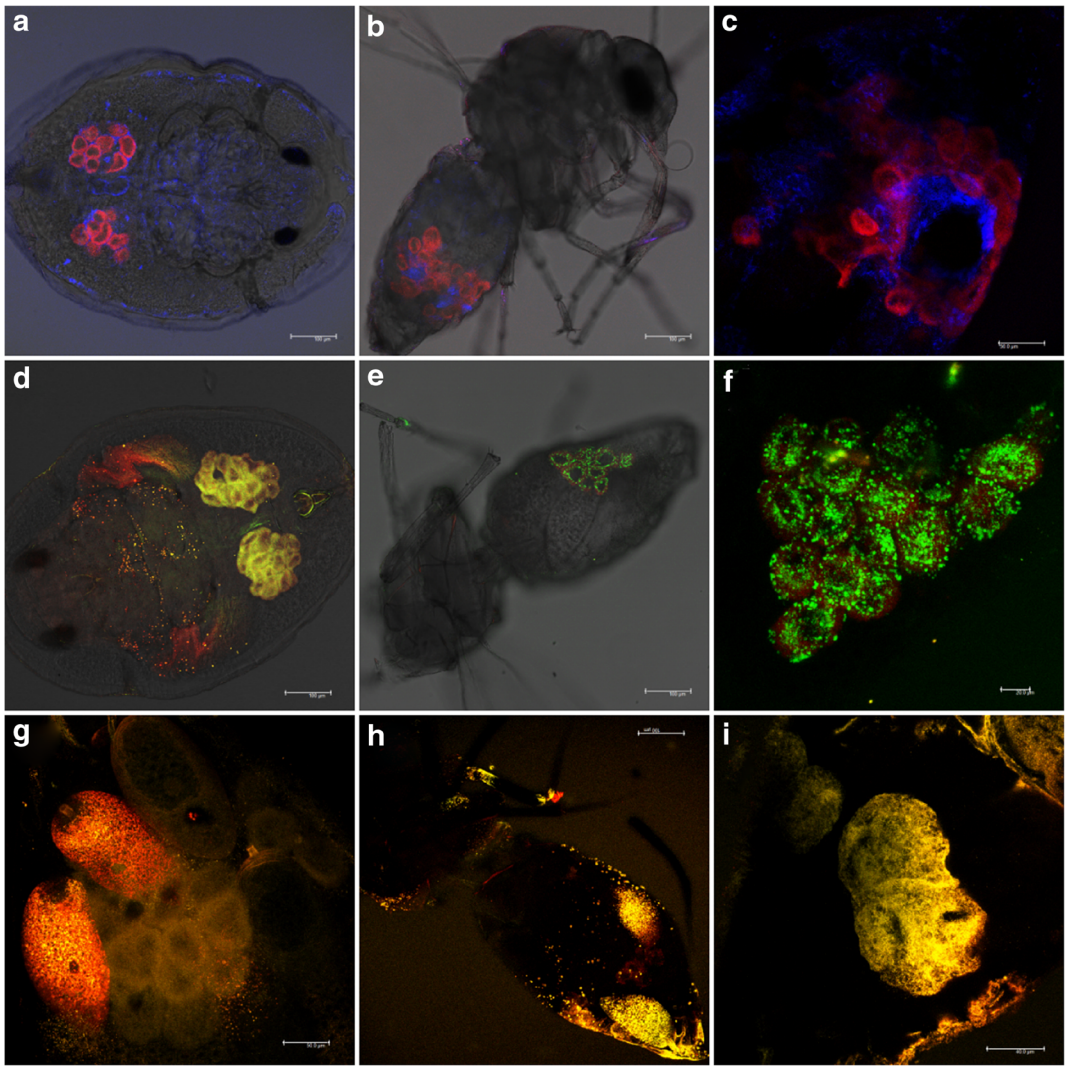

Fig. 2 *Bemisia tabaci* MED species adults and nymphs visualized using confocal microscope after FISH analysis, in dark field. **a**: *Portiera* (Red) *Hamiltonella* (Green) from population 2 nymph. **b** and **c**: *Portiera* (Red) and *Rickettsia* (Blue) from population 6 adults. **d:** *Portiera* (Red) and *Rickettsia* (Blue) from population 5 nymph. **e** and **f**: *Portiera* (Red) and *Hamiltonella* (Green) from population 2(2E) and 12(2F) adults. **g**, **h** and **i**: *Portiera* (Red) and *Arsenophonus* (Yellow) from population 7 (2G, 2i) and 16 (2H). **g** and **i**: Close look at eggs (**g**) and bacteriocytes (**i**) at the bacteriome region

Discussion

In the present study, we identified a new invasion of MED species in Brazil associated with ornamental plants. The phylogenetic analysis also provided evidence for different mtCOI haplotypes among these populations and different sets of secondary endosymbionts, suggesting distinct invasions that occurred recently in São Paulo, since surveys conducted previously indicated the occurrence of only MEAM1 species (Marubayashi et al. 2013).

The presence of MED species in Brazil was recently reported in the southern region, about 1500 km distant from São Paulo (Barbosa et al. 2015) most probably due to the proximity to Argentina and Uruguay, where MED had previously been identified (Grille et al. 2011). However, those MED individuals were collected from *Capsicum annuum* and *Ipomoea batatas,* and harbor *Hamiltonella* and *Cardinium* secondary endosymbionts (Barbosa et al. 2015), different from the MED found in ornamental plants that may have *Arsenophonus*, *Hamiltonella* and *Rickettsia*. The phylogenetic analysis

separated MED populations of Brazil in two different subclades. Most of the specimens of MED collected in São Paulo were placed in subclade "B" that harbors MED from different regions of the world (Fig. 1). MED species collected in Paraná from poinsettias, begonias, *Capsicum* spp. and *Emilia fosbergii* and the previously collected in Rio Grande do Sul State (KF991610) were also placed in subclade "B". Populations of MED collected in Atibaia and Mogi das Cruzes from poinsettia and hydrangea, respectively, were placed in subclade "A", composed by populations from Egypt, Syria and Turkey.

Recent studies using molecular phylogenetic analyses of the mtCOI gene and microsatellite analysis have showed that MED and MEAM1 species exhibit moderate to high levels of genetic diversity suggesting that they stemmed from large founding populations that have maintained ancestral variation (Hadjistylli et al. 2016). Gueguen et al. (2010) using mtCOI phylogenies observed that MED populations could be divided into genetic groups, some of these are restricted to geographic areas (Ahmed et al. 2009; Parrella et al. 2014), others distributed throughout the world (Horowitz et al. 2005; Nauen and Denholm 2005).

The genetic variability found for MED populations collected from ornamental plants in Brazil based on mtCOI analysis, and the high heterogeneity of the secondary endosymbionts detected in the MED populations suggest that multiple MED populations were introduced into different regions. Additionally, the secondary endosymbiont composition we obtained is different from the MED groups reported earlier (Gueguen et al. 2010). Notably, regarding to the infection status with *Hamiltonella*, Gueguen et al. (2010) observed that almost all individuals of one clade (called as MED "Q1") were infected with this bacterium, but the other two (former MED "Q2" and MED "Q3") were not. In our study, we noticed that subclade "A" populations harbors *Hamiltonella*, although it was not fixed in all individuals (Table 1).

The FISH analysis allows visualizing the bacteria inside the insect body, and helps to elucidate the interactions between those organisms, as well to understand the effects of these symbionts on the biology and ecology of the host insects. The localizations of the bacteria in nymphs and adults obtained in this study are similar to the ones previously reported (Skaljac et al. 2010; Škaljac et al. 2012; Marubayashi et al. 2014). *Hamiltonella* was suggested to increase TYLCV transmission capacity (Gottlieb et al. 2010). TYLCV occurs in the Old World (Czosnek et al. 2001) and has already been detected in the USA, Mexico, Dominican Republic, Jamaica, Puerto Rico, Cuba (Duffy and Holmes 2007) and Venezuela (Zambrano et al. 2007). It has not yet been reported in Brazil, but there are suitable conditions for its fast spread if introduced in this country, since we verified that Brazilian MED populations harbor *Hamiltonella*, whose GroEL protein was associated with higher TYLCV transmission efficiency (Gottlieb et al. 2010), and *Rickettsia,* which is also associated with enhanced TYLCV transmission (Kliot et al. 2014; Kanakala and Ghanim 2015).

The genetic differences between the newly reported MED population and the populations previously reported in Argentina, Uruguay and Rio Grande do Sul, as well as the different set of secondary endosymbionts among these populations suggests that the MED introductions in São Paulo and Paraná States were independent from the one that occurred on Rio Grande do Sul, and might be associated with ornamental plants brought to Brazil from different countries.

In 1990, ornamental plants were responsible for the introduction of MEAM1 in Brazil (Lourenção and Nagai 1994). In our study, we suggest a newly introduced population of MED in Brazil reported in ornamental plants, demonstrating the importance of these crops to host exotic pests. This study opens new research on *B. tabaci* in this country and its wide areas where *B. tabaci* populations may exist. Further surveys must be conducted to verify whether the MED species found in São Paulo and Paraná is present in other States and in other cropping systems. It is very well documented that MED species has lower susceptibility to several insecticidal compounds and under repeated applications of either pyriproxyfen or a neonicotinoid, the MED species is more competitive than MEAM1 (Horowitz et al. 2005; Horowitz and Ishaaya 2014). There is also a risk that *B. tabaci* MED could move from ornamental greenhouse production to open agriculture. In the United States, the MED species, that for dozen years was restricted to greenhouse-grown ornamental plants, was recently found in residential landscapes and open field agricultural production, raising concerns about the increase of pesticide use and the risk of moving to crops like cotton (McKenzie and Lance S. Osborne 2017). So, we can conclude that the expansion of MED from protected ornamental crops to open field crops is a real threat to Brazilian agriculture. Moreover, biological

aspects of MED species, such as its ability to transmit begomoviruses and the insecticide resistance under Brazilian conditions, compared to the MEAM1 species, must be further clarified.

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