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LETÍCIA APARECIDA DE MORAES

LEVANTAMENTO DE MOSCA-BRANCA ASSOCIADA ÀS PLANTAS ORNAMENTAIS E HORTALIÇAS E CARACTERIZAÇÃO DE SEUS ENDOSSIMBIONTES

BOTUCATU
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Tese apresentada à Faculdade de Ciências Agronômicas da UNESP - campus de Botucatu, para obtenção do título de Doutora em Agronomia (Proteção de Plantas).

Orientador: Prof. Dr. Marcelo Agenor Pavan
Coorientador: Dr. Julio Massaharu Marubayashi

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Botucatu, 23 de janeiro de 2017.

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Jussara e Dinorah, por sempre me apoiarem.
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RESUMO

Bemisia tabaci (Hemiptera: Aleyrodidae), é um complexo composto por pelo menos 37 espécies crípticas e representa uma das mais importantes pragas agrícolas do mundo, já que é um inseto altamente polifago e considerado um supervector de vírus, uma vez que sozinho é capaz de transmitir mais de 300 espécies, como os begomovírus (gênero *Begomovirus*, família *Geminiviridae*) e crinivírus (gênero *Crinivirus*, família *Closteroviridae*). Mais de duas décadas depois que a espécie *B. tabaci* Middle East Asia Menor 1 (MEAM1, biótipo B) invadiu e se estabeleceu no Brasil através de plantas ornamentais, a presença da *B. tabaci* especie Mediterranean (MED, biótipo Q) foi relatada pela primeira vez no Rio Grande do Sul em 2014, e, recentemente, nos estados de São Paulo e Paraná. Em 2015, espécimes de moscas-brancas coletadas em cultivos comerciais protegidos de begônias, hortênsias, petúnias e poinsettias em São Paulo, bem como de begônias e poinsetias de floriculturas e *Capsicum* spp. associado a *Emilia fosbergii* em estufas usadas anteriormente para poinsettia no Paraná, foram todos identificados como pertencentes a espécie MED. Adicionalmente, os endosimbiontes secundários identificados foram *Arsenophonus, Hamiltonella* e *Rickettsia* foram detectados por PCR e confirmados por sequenciamento e análise de FISH, divergindo dos encontrados nas moscas MED do Rio Grande do Sul, as quais abrigavam *Hamiltonella* e *Cardinium*. Em 2015, portanto, a primeira pesquisa no Estado de São Paulo revelou que a espécie MED estava presente apenas em cultivos protegidos de ornamentais e floriculturas, ou seja, associadas a ornamentais. Em 2016, no entanto, uma segunda e mais extensa pesquisa realizada em São Paulo e Paraná mostraram que MED se espalhou por várias e importantes hortaliças, não somente em estufas, mas também para campos abertos localizados próximos de onde MED foi detectada em plantas ornamentais previamente. Os conjuntos de endossimbiontes, cujos sets foram compostos por *Arsenophonus, Hamiltonella*, *Rickettsia* e *Wolbachia* são diferentes também tanto da MED de São Paulo e Paraná de 2015, como da MED detectada no Rio Grande do Sul em 2014. Através da análise filogenética do gene mtCOI usando o banco de dados global de mosca-branca, os espécimes representam diferentes haplótipos divididos em dois grupos dentro da espécie MED. Além disso, neste trabalho

houve o primeiro relato da presença do endossimbionte *Arsenophonus* infectando *B. tabaci* MEAM1.

Palavras-chave: *Bemisia tabaci*, mtCOI, MED, MEAM1, dinamica populacional.

ABSTRACT

Bemisia tabaci (Hemiptera: Aleyrodidae), it is a complex consisting of at least 37 cryptic species and is one of the most important agricultural pests worldwide, since it is a highly polyphagous insect and considered a virus supervector once it alone transmits more than 300 species, such as begomovirus (genus *Begomovirus*, Geminiviridae family) and crinivirus (genus *Crinivirus*, Closteroviridae family). More than two decades after the species *B. tabaci* Middle East Asia Minor 1 (MEAM1, biotype B) invaded and settled in Brazil through ornamental plants, the presence of *B. tabaci* Mediterraneann species (MED, biotype Q) was first reported in Rio Grande do Sul in 2014, and recently in São Paulo and Paraná States. In 2015, specimens of whiteflies collected in commercial greenhouses of begonias, hydrangeas, petunias and poinsettias in São Paulo, as well as begonias and poinsettias from flower shops and *Capsicum* spp. associated with *Emilia fosbergii* in greenhouses used previously for poinsettia in Paraná, they were all identified as belonging to MED species. In addition, the identified secondary endosymbionts were *Arsenophonus, Hamiltonella* and *Rickettsia* were detected by PCR and confirmed by sequencing and FISH analysis, diverging from the set of MED from Rio Grande do Sul, which harbored *Hamiltonella* and *Cardinium*. In 2015, therefore, the first research in São Paulo revealed that the MED species was present only in greenhouses of ornamentals and flower shops, associated with ornamental. In 2016, however, a second and more extensive research conducted in São Paulo and Paraná showed that MED has spread to several important vegetables, not only in greenhouses, but also to open fields located close to where MED was detected in ornamental plants previously. The endosymbionts, whose sets were composed of *Arsenophonus*, *Hamiltonella*, *Rickettsia* and *Wolbachia* are also different from both the MED of São Paulo and Paraná in 2015, and the MED of Rio Grande do Sul in 2014. Through phylogenetic analysis of gene mtCOI using the whitefly global database, specimens has shown to represent different haplotypes divided into two groups within the species MED. In addition, this study was the first report of *Arsenophonus* endosymbiont present infecting *B. tabaci* MEAM1.

Key words: *Bemisia tabaci*, mtCOI, MED, MEAM1, population dynamics

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1 INTRODUÇÃO

Nas últimas duas décadas, a emergencia e a evolução dos vírus causou mudanças na predominância daqueles transmitidos por insetos em ecossistemas agrícolas. Uma dos casos mais destacados atualmente foi o surgimento da *Bemisia tabaci* (Hemiptera: Aleyrodidae) como um sugador de floema extremamente polífago capaz de hospedar-se em mais de 600 plantas hospedeiras (POLSTON et al., 2014), bem como considerado um supervetor que sozinho é capaz de transmitir mais de 300 espécies virais, representando 5 gêneros, incluindo virus de DNA e RNA de diversos formatos (GILBERTSON et al., 2015).

B. tabaci é considerado um complexo de espécies crípticas, uma vez que engloba grupos morfologicamente indistinguíveis, mas ecologicamente e geneticamente distintos (DINSDALE et al., 2010;. XU et al., 2010; DE BARRO et al., 2011; KANAKALA; GHANIM, 2015), composto de p
elo menos 37 espécies putativas com base na sequência genética da subunidade citocromo-oxidase mitocondrial I (mtCOI) (FIRDAUS et al., 2013; BOYKIN; DE BARRO, 2014; KANAKALA; GHANIM, 2015). Dois destes, conhecidos atualmente como Middle East Asia Menor 1 (MEAM1, biotipo B) e Mediterranean (MED, biotipo Q) são consideradas as pragas mais invasivas de uma ampla variedade de culturas em todo o mundo. Infere-se que se espalharam globalmente através do comércio de ornamentais e hortaliças, surgindo assim como nova praga agrícola em várias regiões (CHEEK; MACDONALD, 1994; DALTON, 2006; Global Invasive Species Database, http://www.issg.org/database).

A variabilidade de *B. tabaci* também é relatada atraves dos endossimbiontes secundários que abriga (GUEGUEN et al., 2010;. GNANKINE et al., 2013). *B. tabaci* carrega o simbionte obrigatório ou primário *Portiera aleyrodidarum*, que é essencial para a sua sobrevivência e desenvolvimento (BAUMANN, 2005). Além disso, o inseto pode abrigar simbiontes facultativos previamente descritos como o *Arsenophonus*, *Hamiltonella*, *Wolbachia*, *Cardinium*, *Fritschea*, *Rickettsia* e o mais recentemente descoberto, organismo Orientia-like OLO (BING et al., 2013), cujos papéis estão relacionados com a ecologia e evolução do sistema

hospedeiro-inseto (BAUMANN, 2005; GOTTLIEB et al., 2006; KANAKALA; GHANIM, 2015).

No Brasil, *B. tabaci* foi relatada pela primeira vez em 1928 na Bahia (BONDAR,1928) e a população manteve-se baixa até o início dos anos 1990 com a introdução de MEAM1 no Estado de São Paulo (LOURENÇÃO; NAGAI, 1994). Altas perdas foram relatadas na agricultura brasileira desde então, principalmente em Solanaceae (ZERBINI et al., 1996, RIBEIRO et al., 1998; FARIA et al., 2000). Pesquisas anteriores realizadas no Estado de São Paulo indicaram apenas a presença de espécies MEAM1 (ROCHA et al., 2011; MARUBAYASHI et al., 2013). No Paraná, por sua vez, em diversas culturas, incluindo plantas ornamentais, predominavam as as espécies MEAM1 e New World até então (MARTINEZ et al., 2000).

No entanto, cerca de 25 anos após a invasão de espécies MEAM1 (LOURENÇÃO; NAGAI, 1994), a introdução da espécie MED de *B. tabaci* nessas regiões foi identificada. Em 2015, uma pesquisa no Estado de São Paulo, Brasil, revelou que a espécie MED foi introduzida e é predominante em plantas ornamentais de estufas comerciais, mas não em campo aberto ou em culturas hortícolas, onde MEAM1 prevalecia até então. A presença de espécies MED no Brasil foi previamente relatada pela primeira vez em 2014 na região sul, cerca de 1500 km de distância de São Paulo em *Capsicum annuum* e *Ipomoea batatas* (BARBOSA et al., 2014), muito provavelmente devido à proximidade com a Argentina e o Uruguai, onde MED já foi identificada (GRILLE et al., 2011). Estas moscas também abrigavam um conjunto diferente de endossimbiontes das populações de MED presentes em São Paulo e Paraná.

Estudos recentes, utilizando análises filogenéticas moleculares do gene mtCOI mostraram que as espécies MED têm altos níveis de diversidade genética e podem ser divididos em grupos genéticos (GUEGUEN et al., 2010), alguns destes são restritos às áreas geográficas (TSAGKARAKOU et al., 2007; AHMED et al., 2009; MCKENZIE et al., 2012; MOUTON et al., 2012; PARRELLA et al., 2014), outros distribuídos em todo o mundo tendo particularmente elevada resistência à neonicotinóides que são eficazes contra MEAM1 (ELBERT; NAUEN 2000; HOROWITZ et al., 2005; NAUEN; DENHOLM 2005) e alta eficiência de transmissão de *Tomato yellow leaf curl virus* (TYLCV) (PARK et al., 2012).

Portanto, a espécie MED é considerada uma praga agrícola extremamente importante.

Introduções de pragas invasivas e seus impactos ecológicos e economicos negativos aumentaram particularmente com o comércio internacional globalizado (WESTPHAL et al., 2008), e assim vem causando muitos danos à agricultura, florestas e plantas ornamentais. O complexo *B. tabaci* é classificado como uma dos principais organismos invasores pela União Internacional para a Conservação da Natureza e dos Recursos Naturais (IUCN, http://www.issg.org).

É apresentada aqui a dinâmica populacional da espécie Mediterranean nos Estados de São Paulo e Paraná desde o seu primeiro relatório nessas áreas, nos anos de 2015 e 2016, bem como a constituição de endossimbiontes e o estudo filogenético dessas moscas, uma vez que o acompanhamento regular da dinâmica *B. tabaci* é fundamental para a detecção precoce do estabelecimento desta nova espécie para o cenário brasileiro.

2 REVISÃO BIBLIOGRÁFICA

2.1 Hemiptera: Aleyrodidae: *Bemisia tabaci* (Gennadius)

A ordem Hemiptera, descrita por Linnaeus (1758), e cujo significado vem do latim “Hemi” = metade e “ptera” = asas, está dentre as 29 ordens pertencentes à classe insecta (GALLO, 2002). A subordem Sternorrhyncha esta dentro desta ordem e compreende em torno de 16.000 espécies organizadas em quatro superfamílias: Aleyrodoidea (mosca-branca); Psylloidea (psilídeos); Aphidoidea (afídeos) e Coccoidea (cochonilhas) (GULLAN; MARTIN, 2009).

A família Aleyrodidae é dividida em duas subfamílias: subfamília Aleurodicinae e Aleyrodinae. A subfamília Aleurodicinae compreende cerca de 130 espécies sem grande importância econômica, como por exemplo, *Aleurodicus dugesii* e *Aleurodicus dipersus*, descritas principalmente na América do Sul, América Central e Caribe. A subfamília Aleyrodinae, por sua vez, apresenta cerca de 1560 espécies distribuídas em 161 gêneros, e compreende os insetos conhecidos como mosca-branca, cujas espécies de destaque são: *Trialeurodes vaporariorum* (Westwood), *Bemisia afer* e as espécies pertencentes ao complexo *Bemisia tabaci* (Gennadius) (NOLDUS; VAN LENTEREN, 1990; GAMARRA et al., 2010; DE BARRO et al., 2011).

Quanto à morfologia desses insetos, medem de 1 a 2 mm, e as fêmeas são ligeiramente maiores que os machos (NATESHAN et al., 1996). Os adultos têm o dorso de coloração amarelada e as asas brancas. Suas asas cobrem quase todo o corpo, e portanto a cor predominante é o branco, assim originando seu nome popular de mosca-branca (TOSCANO et al., 2002). O ciclo de vida desses insetos é composto por três fases (ovo, estádio de ninfa e adulto) e sofre influência das condições climáticas e ambientais, principalmente temperatura, umidade relativa do ar e planta hospedeira (GILL, 1990).

As fêmeas chegam a ovipositar de 130 a 400 ovos durante o ciclo, dependendo de diversos fatores como a planta hospedeira e a temperatura (TOSCANO et al., 2002). A reprodução é sexuada ou por partenogênese arrenótoca, na qual fêmeas não fecundadas resultam em ovos que darão origem à machos estéreis (GILL, 1990). A mosca-branca é encontrada principalmente nos trópicos e subtrópicos, porém sua presença já foi descrita em todos

continentes (BROWN et al., 2000). O aumento populacional está relacionado à expansão da monocultura da maioria das espécies cultivadas, às condições dos sistemas agrícolas modernos, ao aumento da utilização de agrotóxicos, selecionando populações resistentes e, principalmente, à facil adaptação da *B. tabaci* aos diversos hospedeiros (BROWN et al., 1995).

2.2 *Bemisia tabaci*: complexo de espécies crípticas

O primeiro relato de *B. tabaci* foi em 1889 por Gennadius, quem a nomeou incialmente de *Aleurodes tabaci*, presente em plantas de tabaco na Grécia. Uma nova identificação foi realizada no sudoeste dos Estados Unidos da América (EUA) em plantas de *Physalis alkenkekgi* L e descrita como *Aleyrodes inconspicua* (PERRING, 2001), onze anos depois da primeira descrição. No Brasil, sua primeira aparição foi em 1928 no Estado da Bahia, em plantas de *Euphorbia hirtella* e foi denominada como *Bemisia costalimai* (BONDAR, 1928).

Após eventos de surtos populacionais nas décadas de 70 e 80 no mundo e consequentes prejuízos agrícolas, *B. tabaci* passou a ficar conhecida como uma praga de cultivos agrícolas tanto de campo aberto quanto de estufas, ganhando status de praga global (Global Invasive Species Database, http://www.issg.org/database). No Brasil, *B. tabaci* passou a fazer parte do cenário agrícola nacional em meados da década de 90, a partir da introdução do biótipo B, hoje denominada de espécie MEAM1 no Estado de São Paulo, através do comércio internacional de plantas ornamentais, e associação com a transmissão de vírus, principalmente do gênero *Begomovirus* (LOURENÇÃO; NAGAI, 1994; ZERBINI et al., 2002) e mais recentemente do gênero *Crinivirus*, com o vírus *Tomato chlorosis virus* (ToCV) (BARBOSA et al., 2011).

Estudos mais recentes utilizando a análise do gene mitocondrial cytochroma oxidase I (mtCOI) demonstraram que *B. tabaci* é considerado um complexo de espécies cripticas (DINSDALE et al., 2010; DE BARRO et al., 2011) constituído de indivíduos morfologicamente indistinguíveis, porém que exibem variabilidade genética e biológica quanto a gama de hospedeiros, grau de fecundidade, composição de endossimbiontes e capacidade de transmissão das diferentes espécies de vírus (BROWN et al., 1995; FROHLICH et al., 1999; GUEGUEN et al., 2010).

Atualmente são reconhecidas pelo menos 37 espécies distintas de *B. tabaci* divididas em 11 grupos, conforme apresentado na Figura 1. Dentre todas as espécies, MEAM1 e MED se detacam pelo carater altamente invasivo mundialmente.

A espécie MEAM1, previamente denominada como biótipo B, é a de maior distribuição mundial. Essa espécie foi inicialmente encontrada presente em *Euphorbia pulcherrima*, popularmente conhecida como poinsétia ou bico de papagaio, na Flórida, Estados Unidos (COSTA; BROWN, 1990). No Brasil, MEAM1 entrou de modo abrupto e desencadeou o primeiro grande evento de invasão da espécie no Estado de São Paulo, no começo de 1990, provavelmente pelo comércio internacional de plantas ornamentais (LOURENÇÃO; NAGAI, 1994) e está relacionada com o aumento vertiginoso da incidência de begomovírus, principalmente em solanáceas, a partir desse período (RIBEIRO et al., 2003). Essa espécie tem sido predominante no Estado de São Paulo desde então (ROCHA et al., 2011; MARUBAYASHI et al., 2013).

A espécie MED (biótipo Q) por sua vez, é predominante nos países da Europa e Ásia. Nauen & Denholm (2005) observaram na Espanha que essa espécie tem alta capacidade de se tornar resistente a inseticidas Tendo particularmente elevada resistência a neonicotinóides que são eficazes contra MEAM1 (ELBERT; NAUEN 2000, HOROWITZ et al., 2005; NAUEN; DENHOLM, 2005) e elevada eficiência de transmissão do *Tomato yellow chlorosis virus* (LI et al., 2010; PARK et al., 2012). Portanto, a espécie MED é considerada uma praga agrícola extremamente importante.

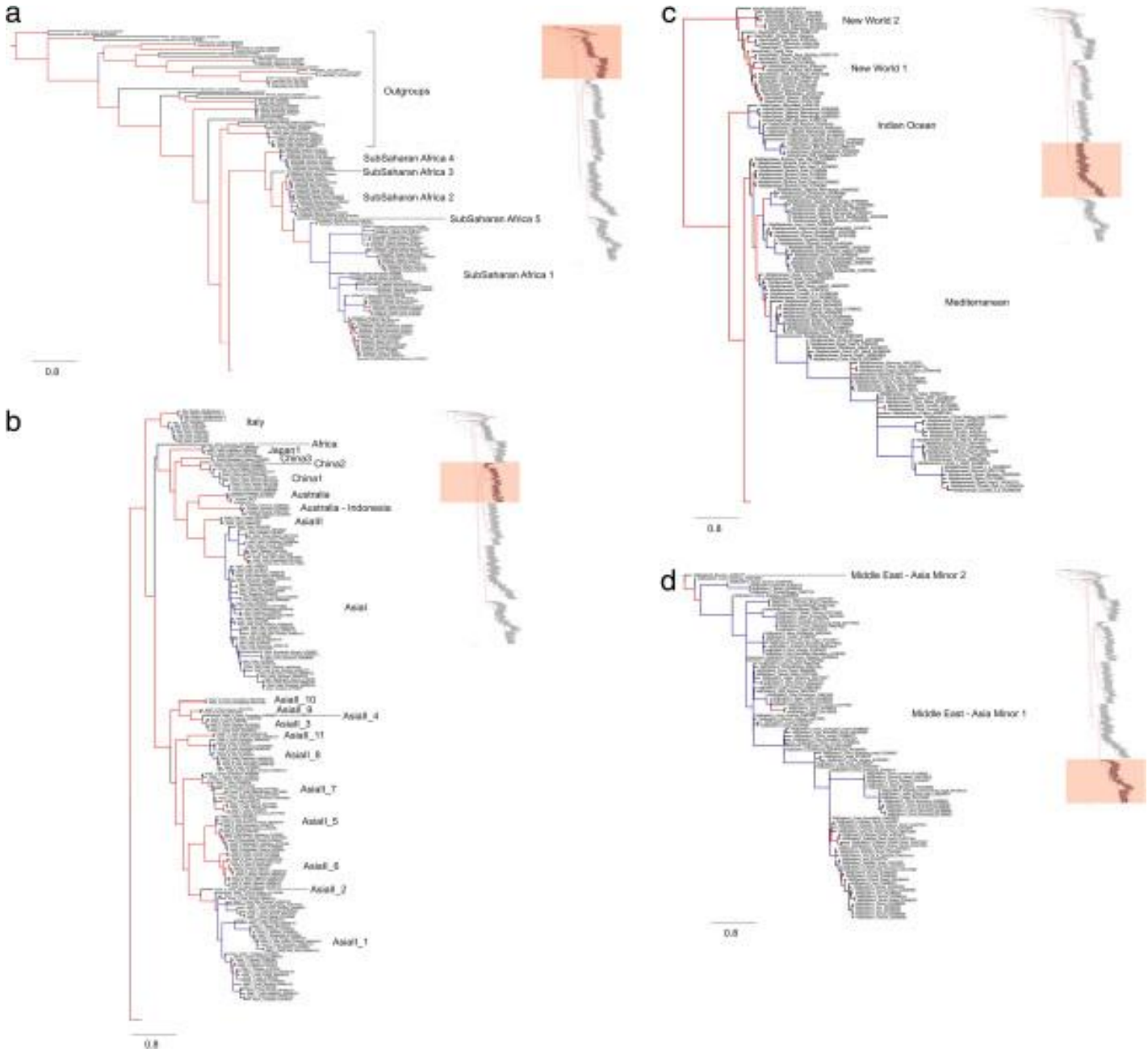
Nas Américas, as espécies MED tem uma presença mais limitada sendo relatada pela primeira vez nos Estados Unidos em 2004 (DALTON, 2006), posteriormente no México, em 2007 (MARTINEZ-CARRILLO; BROWN, 2007), Guatemala em 2009 (BETHKE et al., 2009) e mais recentemente na Argentina, Uruguai (GRILLE et al., 2011) e Brasil (BARBOSA et al., 2015). Nos últimos anos, a expansão global da espécie MED desde a sua origem na bacia do Mediterrâneo para pelo menos 12 países (Canadá, China, Costa Rica, Guatemala, Japão, México, Holanda, Nova Zelândia, Coreia do Sul, Uruguai, Estados Unidos) (DE BARRO et al., 2011) e mais recentemente Brasil (BARBOSA et al., 2015) impõe uma série de preocupações.

A titulo de curiosidade, o primeiro exemplar de mosca-branca relatada e

denominada de *Aleyordes tabaci* em 1889 por Panayotis Gennadius, na Grécia, foi preservada no museu de história natural de Smithsonian (Smithsonian National Museum of Natural History), e teve o seu gene mtCOI recentemente analisado, cujo resultado foi que este pertencia à espécie MED (TAY et al., 2012).

Outro membro do complexo de espécies de *B. tabaci*, New World (NW), também referida na literatura como biótipo A, foi identificado pela primeira vez nos Estados Unidos em batata-doce. No Brasil, New World teve seu primeiro relato provavelmente na Bahia em 1928 em *E. pulcherrima*, *Nicotiana glauca* e *N. tabacum* , coincidindo em ser o primeiro relato de mosca-branca no país (BONDAR, 1928). Esta espécie ainda ocorre no Estado de São Paulo, restrita ao litoral sul paulista (MARUBAYASHI et al., 2013). A espécie New World 2 foi descrita primeiramente por Alemandri et al., (2012). Na Argentina NW2 é a espécie predominante em soja (ALEMANDRI et al., 2012), enquanto que no Brasil é encontrada com frequência em *Euphorbia heterophylla* e em soja, bem como há a ocorrência da espécie New World 2 no Estado de São Paulo (MARUBAYASHI et al., 2013).

Figura 1 - Filogenia gerada pelo programa MrBayes v. 3.2.2 no super computador Magnus para Aleyrodidae usando 30 milhões de gerações, árvores amostradas a cada 1000 gerações e 25% descartado como “burnin”. A árvore foi dividida em quatro painéis (a-d). O sombreado vermelho na árvore de resumo (canto superior direito de cada painel) indica onde a seção se encaixa na árvore geral. A filogenia contendo todas as amostras é mostrada à direita ea seção destacada no



salmão é ampliada à esquerda (BOYKIN et al., 2013).

2.3 Importância econômica como supervetor de fitovirus

Nos últimos tempos, *B. tabaci* vem recebendo o status de super vetor viral,

devido a carterísticas determinantes, tais como sua alta taxa de fecundidade; sua facilidade de dispersão por longas distancias pelo vento e por transporte de material vegetal; ser cosmopolita e portanto presente no mundo todo; altamente polifaga, com uma vasta gama de hospedeiras vegetais; e ser capaz de transmitir ao redor de 300 fitoviroses, correspondentes a 5 generos virais distintos, que diferente quanto à forma dos vírions e modo de transmissão. Muitos destes vírus são emergentes no mundo e incluem vírus pertencentes aos gêneros *Begomovirus, Carlavirus, Crinivirus, Ipomovirus e Torradovirus* (GILBERTSON et al., 2015), conforme apresentado na Tabela 1.

Tabela 1. Resumo dos gêneros de vírus transmitidos por mosca-branca do complexo *Bemisia tabaci.*

Genero	Tamanho e forma dos virions	Material Genético	Modo de Transmissão	Número de espécies
Begomovirus	Geminado 18-30 nm	ssDNA	Persistente circulativo	288
Ipomovirus	Flexuoso Filamentoso 12-15x800-950 nm	ssRNA	Semi persistente	6
Crinivirus	Flexuoso Filamentoso 10-13x650-900 nm	ssRNA	Semi persistente	13
Carlavirus	Flexuoso Filamentoso 12-13x470-1000 nm	ssRNA	Semi persistente	2
Torradovirus	Esférico 28-30 nm	ssRNA	Semi persistente	2

Fonte: Adaptado de Gilbertson et al., (2015).

A transmissão de vírus por *B. tabaci* pode ser do tipo não persistente, persistente e semi-persistente. Para vírus que são transmitidos de maneira não persistente e semipersistente, o aparelho bucal do vetor, em geral, são os locais de retenção de vírus. A transmissão dos crinivírus é do tipo semipersistente e para a maioria dos begomovírus é do tipo persistente circulativo (NG; FALK, 2006; HOGENHOUT et al., 2008; POLSTON et al., 2014). No entanto, há um relato na literatura do *Tomato yellow leaf curl virus* em Israel (TYLCV) como sendo transmitido da forma persistente propagativa (GHANIM et al., 1998). Na transmissão não persistente, o inseto adquire o vírus por picadas de prova e o tempo de retenção é de minutos ou horas. Na transmissão semipersistente são necessários minutos para aquisição e o tempo de retenção pode ser de horas a dias. A transmissão persistente necessita que o inseto se alimente por horas na planta para adquiri-lo e o tempo de retenção do vírus na hemolinfa pode durar

dias ou até mesmo toda a vida do inseto. Neste tipo de interação o vírus pode replicar no inseto (propagativo) ou não replicar (NG & FALK, 2006; HOGENHOUT et al., 2008).

Dentre os grupos de vírus transmitidos por *B. tabaci*, os pertencentes ao gênero *Begomovirus* tem maior quantidade de espécies descritas, apresentam maior importância econômica e provocam doenças consideradas graves e de grande importância agroeconômica em cultivos de campo aberto e de estufas (STANLEY et al., 2005; BRIDDON; MARKHAN, 2001; MORALES; ANDERSON, 2001). Na década de 60, Costa (1965) realizou o primeiro relato brasileiro de uma doença causada por begomovirus em feijão, *Phaseolus vulgaris* L. O vírus foi denominado *Bean golden mosaic virus* (BGMV). O aumento populacional de *B. tabaci* durante a década de 70 foi determinante para aumento da incidência do BGMV e foi atribuída ao acréscimo em área cultivada com soja, uma excelente hospedeira do inseto (COSTA, 1975).

No Brasil, foram descritas até o momento trinta e seis espécies de begomovírus de maior importância infectando feijão, quiabo, pimentão, batata, soja e batata doce, das quais quatorze espécies foram descritas infectando tomateiro no campo (INOUE-NAGATA et al., 2016): *Tomato golden mosaic virus* (TGMV) (MATYIS et al., 1975), *Tomato rugose mosaic virus* (ToRMV) (FERNANDES et al., 2006), *Tomato chlorotic mottle virus* (ToCMoV) (RIBEIRO et al., 2003), *Tomato yellow spot virus* (ToYSV) (CALEGARIO et al., 2007), *Tomato severe rugose virus* (ToSRV) (FERNANDES et al., 2008), *Tomato common mosaic virus*(ToCmMV), *Tomato mild mosaic virus* (ToMlMV) (CASTILLO-URQUIZA et al., 2008), *Tomato yellow vein streak virus* (ToYVSV) (ALBUQUERQUE et al., 2010), *Tomato interveinal chlorosis virus* (ToICV), *Tomato mottle leaf curl virus* (TMoLCV), e *Tomato golden vein virus* (TGVV) (ALBUQUERQUE et al., 2012), *Sida micrantha mosaic virus, Sida mottle virus* (INOUE-NAGATA et al., 2016)

A introdução e a excelente adaptação às condições brasileiras de *B. tabaci*, MEAM 1 (biótipo B) está diretamente relacionada com a disseminação dessas espécies virais. A partir da introdução desta espécie no início dos anos 90 surtos de begomovirus foram observados em solanáceas em diferentes Estados brasileiros como São Paulo, Bahia, Pernambuco e Distrito Federal, acarretando perdas que podem chegar a 100% na produção de tomate (RIBEIRO et al., 1998;

ZERBINI et al., 1996; FARIA et al., 2000).

2.4 *Bemisia tabaci* e seus endossimbiontes

Endossimbiontes bacterianos são comuns na natureza e predominantes em artrópodes (BAUMANN, 2005). *B. tabaci* abriga um endossimbionte primário ou obrigatório, denominado *Portiera aleyrodidarum* (THAO; BAUMANN, 2004). Os endossimbiontes obrigatórios são transmitidos de forma vertical entre as gerações e coevoluíram com os insetos por um longo período e tem formado uma relação íntima com seus hospedeiros (BAUMANN, 2005). Além dos obrigatórios, *B. tabaci* pode ser infectada por endossimbiontes secundários que por sua vez são facultativos e a transmissão pode ocorrer tanto verticalmente, como horizontalmente (SINTUPACHEE et al., 2006; CHIEL et al., 2009; ROSELL et al., 2010).

Atualmente, são conhecidos sete endossimbiontes secundários infectando *B.tabaci*: *Arsenophonus*, *Hamiltonella* (THAO; BAUMANN, 2004; MORAN et al., 2005), *Fritschea* (EVERETT et al., 2005), *Cardinium* (WEEKS; BREEUWER, 2003), *Rickettsia* (GOTTLIEB et al., 2006), *Wolbachia* (ZCHORI-FEIN & BROWN, 2002) e *Orientia* like organism (OLO) (BING et al., 2013). A presença destes podem ter efeitos diferentes no inseto hospedeiro. *Rickettsia*, *Hamiltonella* e Wolbachia, por exemplo, podem fornecer nutrientes como aminoácidos essênciais a sobrevivência do inseto (BROWNLIE et al., 2009; KOGA et al., 2003), conferir resistência a vespas parasitas e fungos ou vírus patogênicos (FERRARI et al., 2004; HAINE, 2008; HEDGES et al., 2008; OLIVER et al., 2003; SCARBOROUGH et al., 2005; TEIXEIRA et al., 2008; VORBURGER et al., 2010), além de conferir tolerância ao estresse térmico (MONTLLOR et al., 2002). Em contrapartida, endossimbiontes como *Wolbachia*, *Arsenophonus*, *Cardinium* e *Rickettsia* podem causar alterações na reprodução dos insetos, forçando a assexualidade, matando machos e provocando alterações genéticas, além de induzir incompatibilidade citoplasmática em conjunto com a partenogênese, com efeito aparente de auxiliar a disseminação dos endossimbiontes em populações de insetos hospedeiros (ENGELSTADTER; HURST, 2009; WERREN, 1997; WERREN et al., 2008).

Em relação à presença destes endossimbiontes nas espécies pertencentes

ao complexo *B. tabaci*, observou-se que *P. aleyrodidarum* está presente em todas as espécies do complexo, sendo essencial para sua sobrevivência (THAO; BAUMANN, 2004; BAUMANN, 2005; SKALJAC et al., 2010), mas composição de endossimbiontes secundários entre as diferentes espécies e mesmo dentro da mesma espécie de *B. tabaci*, por sua vez, pode variar. *Rickettsia* e *Hamiltonella* são características de *B. tabaci* MEAM 1, entretanto outros endossimbiontes secundários podem ser encontrados nestas espécies, como: *Cardinium* e *Fritschea* (CHIEL et al., 2007; MARUBAYASHI et al., 2014). Nas espécies do grupo New World (NW e NW 2) *Fritschea* é o endossimbionte mais comumente encontrado (THAO et al., 2003; MARUBAYASHI et al., 2014), porém também podem estar presentes *Hamiltonella* e *Cardinium* infectando sobretudo NW 2 (MARUBAYASHI et al., 2014).

Na espécie MED presente em Israel são encontrados *Wolbachia***,** *Rickettsia* e *Arsenophonus* (CHIEL et al., 2007), enquanto que na Croácia, *Hamiltonella*, *Wolbachia*, *Cardinium* e *Rickettsia* (SKALJAC et al., 2010). Na França, Uruguai e Coreia do Sul apresentam os endossimbiontes *Hamiltonella* e *Cardinium*, sendo que algumas populações de MED da França, além destes, podem ser infectadas também por *Wolbachia* (GUEGUEN et al., 2010)**.**

Himler et al., (2011) verificaram que em seis anos *Rickettsia* passou a predominar em uma população de *B. tabaci* encontrada em batata-doce no sudoeste dos Estados Unidos. *B. tabaci* infectada com *Rickettsia*, em condições laboratorias controladas, produz muitos adultos que sobrevivem melhor e produzem mais moscas-brancas do sexo feminino, o que poderia explicar a rápida predominância deste endossimbionte, uma vez que proporciona melhor ‘fitness’ à mosca-branca.

Quanto à transmissão de fitovirus, sabe-se que a transmissão de TYLCV depende da proteína de 63-kDa GroEL produzida por *Hamiltonella*, porém independe da proteína GroEL produzida pelos demais endossimbiontes. A GroEL de *Hamiltonella* é capaz de interagir com a proteína capsidial do TYLCV, enquanto que a de outros endossimbiontes não, protegendo o vírus (GOTTLIEB et al., 2010). Isto explicaria o fato do MEAM1de Israel (que possui *Hamiltonella*) transmitir eficientemente o vírus, enquanto que MED, que não possui este endossimbionte secundário, transmite o vírus com baixa eficiência.

Recentemente Kliot et al., (2014) observaram que *Rickettsia* tem um papel

importante na transmissão do TYLCV. Trabalhando com linhas isogênicas de *B. tabaci* MEAM1, porém uma com *Rickettsia* (R^+) e outra não (R^-), verificaram que a linha R^+ transmitia melhor o TYLCV (79% de transmissão) quando a comparada a linha R^- (41%) e que esta transmissão estava ligada ao fato da presença de *Rickettsia* proporcionar maior aquisição do vírus.

Todos os endossimbiontes, com excessão de *Rickettsia*, se localizam em uma célula especializada chamada “bacteriocytes”. *Rickettsia* está presente nas células do sistema digestivo, salivar e reprodutivo da *B. tabaci*, atingindo altas concentrações no inseto, sendo assim encontrada em toda a cavidade do corpo da *B. tabaci*. Por *Rickettsia* infectar órgãos imprescindíveis ao inseto, esta poderia estar influenciando mais a biologia do inseto comparada aos demais endossimbiontes localizados somente nos ‘bacteriocytes’ (BRUMIN et al., 2012).

A técnical confocal de “Flourescense *in situ* Hybridization” (FISH) permite visualizar as bactérias dentro do corpo do inseto, e ajuda a elucidar as interações entre esses organismos, bem como compreender os efeitos que abrigando conjuntos específicos podem trazer para a biologia de insetos e ecologia.

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CAPÍTULO 01

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New invasion of *Bemisia tabaci* Mediterraneann species in Brazil

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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, which commenced in the 1990s mainly by ornamentals plants in São Paulo State. More than two decades after this invasion, the presence of the Mediterraneann (MED) invasive species of *B. tabaci* 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 and *Capsicum* spp. associated with *Emilia fosbergii* in greenhouses previously used for poinsettia in Paraná, were all identified as belonging to MED species. Through phylogenetic analysis of the mtCOI gene using the whitefly database, the specimens collected were divided in two different groups among the MED species. Furthermore, the secondary endosymbionts *Arsenophonus*, *Hamiltonella* and *Rickettsia* were detected by PCR and confirmed by sequencing and FISH analysis for MED from São Paulo and Paraná, differing from MED detected in Rio Grande do Sul that harbored *Hamiltonella* and *Cardinium.* The phylogenetic analysis showed the presence of high levels of genetic variation among the MED populations in Brazil and the different sets of secondary endosymbionts between them indicating that different invasions of MED may have occurred in Brazil.

Key words: whiteflies, MED groups, mtCOI gene, endosymbionts.

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, once encompasses morphologically indistinguishable, but ecologically and genetically distinct groups (Dinsdale *et al.,* 2010; Xu *et al.,* 2010; De Barro *et al.,* 2011; Kanakala & Ghanim, 2015), composed of at least 37 putative species based on mitochondrial

cytochrome oxidase subunit I (mtCOI) gene sequences (Dinsdale *et al.,* 2010; De Barro *et al.,* 2011; Esterhuizen *et al.,* 2013; Firdaus *et al.,* 2013; Boykin & De Barro, 2014; Parrella *et al.,* 2014). Two of these, known currently as Middle East-Asia Minor 1 (MEAM1) and Mediterraneann (MED) are considered the most invasive pests of a wide variety of field crops worldwide, and cause direct plant damage and transmit more than over 300 species, representing 5 genera, including DNA and RNA viruses of several shapes. (Jones, 2003; Navas-Castillo *et al.,* 2011; Parrella *et al.,* 2014, Gilbertson *et al.,* 2015). As well, *B. tabaci* have been spread globally through trade of ornamental and vegetable plant material, emerging as new agricultural pests in several regions (Cheek & Macdonald, 1994; Dalton, 2006; Global Invasive Species Database, http://www.issg.org/database).

In Brazil, *B. tabaci* was first reported in 1928 in Bahia (Bondar 1928) and the population remained low until the early 1990s with the introduction of MEAM1 in São Paulo State (Lourenção & Nagai, 1994). High losses have been reported on Brazilian agriculture ever since, mainly in Solanaceae (Zerbini *et al.,* 1996; Ribeiro *et al.,* 1998; Faria & Mirtes, 2000).

In recent years the global spread of MED from its origin in the countries bordering the Mediterraneann Basin to at least 11 countries (Canada, China, Costa Rica, Guatemala, Japan, Mexico, the Netherlands, New Zealand, South Korea, Uruguay, United States) (De Barro *et al.,* 2011) and more recently Brazil (Barbosa *et al.,* 2015) has imposed a lot of concerns. MED species has a more limited presence in the Americas, being first reported in the United States in 2004 (Dalton, 2006) and subsequently in Mexico in 2007 (Martinez-Carrillo & Brown, 2007), Guatemala in 2009 (Bethke et al., 2009) and more recently in Argentina, Uruguay (Grille *et al.,* 2011) and Brazil (Barbosa *et al.,* 2015).

Recent studies using molecular phylogenetic analyses of the mtCOI gene have showed that MED species have high levels of genetic diversity and can be divided into genetic groups (Gueguen *et al.,* 2010), some of these are restricted to geographic areas (Tsagkarakou *et al.,* 2007; Ahmed *et al.,* 2009; McKenzie *et al.,* 2012; Mouton *et al.,* 2012; Parrella *et al.,* 2014), others distributed throughout the world having particularly high resistance to neonicotinoids that are effective against MEAM1 (Elbert & Nauen 2000; Horowitz *et al.,* 2005; Nauen & Denholm 2005) and high transmission efficiency of *Tomato yellow leaf curl virus* (TYLCV)

(Li *et al.,* 2010; Park *et al.,* 2012). Therefore, the MED species is considered an extremely important agricultural pest.

The variability of *B. tabaci* is also reported by the secondary endosymbionts that it harbors (Gueguen *et al.,* 2010; Gnankine *et al.,* 2013). *B. tabaci* has the primary symbiont *Portiera aleyrodidarum*, which is essential for their survival and development (Baumann, 2005) and is located in specialized cells called "bacteriocytes". Furthermore, the insect may harbor secondary symbionts previously described as *Arsenophonus*, *Hamiltonella*, *Wolbachia, Cardinium*, *Fritschea*, *Rickettsia* and later discovered, *Orientia*-like organism OLO (Bing *et al.,* 2013). These secondary symbionts are associated with their hosts and can play a very important role in the ecology and evolution of these (Baumann, 2005; Gottlieb et al., 2006; Kanakala & Ghanim, 2015). The MED species has a frequent association with *Arsenophonus* and *Wolbachia*, and in Israel MED is not associate with *Hamiltonella* (Chiel et al., 2007). On the other hand, populations of MED from other regions of the world harbor *Hamiltonella* and *Cardinium* (Baumann, 2005).

Here we present evidence that *B. tabaci* MED species was introduced in São Paulo and Paraná States by ornamental plants as well as different MED mitochondrial variants could be identified, with MED populations containing different sets of secondary endosymbionts, suggesting different invasions of this species into Brazil and a new concern to Brazilian agriculture.

EXPERIMENTAL METHODS

Whitefly sampling

Adults, nymphs and eggs of *B. tabaci* were collected from ornamental plants produced in commercial greenhouses and weeds associated to the ornamental cultures, or floricultures, between March and October 2015 in several São Paulo and Paraná States locations (Table 1). The specimens of each sample were collected using a hand-held aspirator, preserved immediately in 95% ethanol and stored at 4ºC until further processing for species genotyping, endosymbiont and for FISH analysis. Seventeen populations represented by 10 individuals of each were analyzed.

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'-CGGAGCTTGCGCCTTAGTC-3') and Bem23R (5'-CGGCTTTATCATAGCTCTCGT-3') witch amplifies a microsatellite locus of about 200 bp and 400 bp for MEAM1 and MED, respectively (De Barro et al., 2003). 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) analysis of the amplicons (Bosco et al., 2006), 5 µl of each PCR (880 bp) was digested with one unit of TaqI at 65ºC 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 compared with those present in GenBank database from National Center for Biotechnology Information (NCBI) by using BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis

Phylogenetic analysis were carried out using the 22 unique *B. tabaci* mtCOI sequences obtained in this study (Figure 1) added to the global *B. tabaci* mtCOI dataset (Boykin & De Barro 2014) and also 15 Mediterraneann sequences from GenBank (Access numbers: KF991610, KJ606613, KJ606633, JF304725, EU760723, JF304724, EU881713, EU427725, FJ88480, AB908058, AB908065, EU760751, DQ365877, EF080821, EU760756) totaling 595 sequences. Multiple sequence alignment was prepared using MAFFT (Katoh and Toh, 2008), and manually adjusted using Geneious 9.1.5. MtCOI sequences ranged from 613 to 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 & Brown, 2002), *Rickettsia* (Gottlieb et al., 2006), *Wolbachia* (Heddi et al., 1999), *Arsenophonus* (Thao & Baumann, 2004), *Cardinium* (Weeks & Breeuwer, 2003) and *Fritschea* (Everett et al., 2005) using genus-specific primers targeting the 16 S or 23 S 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 (Gottlieb et al., 2008; Skaljac et al., 2010; Skaljac et al., 2013). 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 hours 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). At least 10 specimens were analyzed for each endosymbiont for each developmental stage.

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 floricultures from Paraná were identified as MED species. MED was also identified colonizing *Capsicum* spp and *Emilia fosbergii* grown in greenhouses where previously poinsettia had been cultivated. The species present in *Myrtus communis* and *Ficus sp.* from Arujá was identified as *Bemisia berbericola* and 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 work showed the high variability among the MED populations collected in Brazil that were divided into two different groups (as indicated by “A” and “B”) (Figure 1).

Table 1. Samples and localities of adult, nymph and eggs of whitefly specimens collected in ornamental greenhouses in São Paulo and floricultures and greenhouses from Paraná State cities.

Sample	Plant	City	Coordinates	Species
1	*Myrtus communis / Ficus spp.*	Arujá-SP	S23°23'20,07" W46°19'13,27"	*B. berbericola*
2	*Begonia spp.*	Mogi das Cruzes-SP	S23°22'19,95" W46°10'35,02"	MED Group B
3	*Hydrangea macrophylla*	Mogi das Cruzes-SP	S23°22'19,95" W46°10'35,02"	MED Group A
4	*Begonia spp.*	Mogi das Cruzes-SP	S23°22'19,95" W46°10'35,02"	MED Group B
5	*Begonia spp.*	Mogi das Cruzes-SP	S23°22'19,95" W46°10'35,02"	MED Group B
6	*Hydrangea macrophylla*	Mogi das Cruzes-SP	S23°22'19,95" W46°10'35,02"	MED Group B
7	*Begonia spp.*	Guarulhos-SP	S23°23'31,13" W46°21'37,43"	MED Group B
8	*Begonia spp.*	Guarulhos-SP	S23°23'31,13" W46°21'37,43"	MED Group B
9	*Euphorbia pulcherrima*	Atibaia-SP	S23°2'23,87" W46°35'1,54"	MED Group A
10	*Conyza bonariensis*	Atibaia-SP	S23°23'31,13" W46°21'37,43"	MEAM1
11	*Ruta graveolens L.*	Atibaia-SP	S23°23'31,13" W46°21'37,43"	MEAM1
12	*Euphorbia pulcherrima*	São Pedro-SP	S22°32'55" W47°54’50"	MED Group B
13	*Petunia spp.*	Mogi Mirim-SP	S22°25'55" 46°57'30"	MED Group B
14*	*Begonia spp.*	Londrina-PR	S23°26'48" W51°11'22"	MED Group B
15*	*Euphorbia pulcherrima*	Londrina-PR	S23°26'48" W51°11'22"	MED Group B
16	*Emilia fosbergii*	Marialva-PR	S23°47'9" W51°83'6"	MED Group B
17	*Capsicum spp.*	Marialva-PR	S23°47'9" W51°83'6"	MED Group B

- Colected from Flowershops

Figure 1- Phylogenetic tree of the partial mtCOI gene from *Bemisia tabaci* totalizing 595 sequences conducted using MrBayes v. 3.2.2 on the Magnus supercomputer. The Mediterraneann clade is shown highlighted and expanded. Sequences obtained in this work are highlighted in green. The host, city of collection, population number and the GenBank access number are indicated.

The primary endosymbiont *P. aleyrodidarum*, which is essential for *B. tabaci* persistence, was detected in all whiteflies tested, providing proof that DNA extractions were sufficient for the rest of the PCRs. The most prevalent co-infection observed in the majority of samples tested was *Rickettsia*, *Hamiltonella* and *Arsenophonus*, although it could be observed that those bacterial populations are not well established yet once they were not present in 100% of the individuals tested (Figure 2).

We confirmed the presence of all symbionts through PCR, sequencing (data not shown) and FISH analysis (Figure 3).

Figure 2 -. Individual and mixed infections with endosymbionts in MED *Bemisia tabaci* populations collected in São Paulo and Paraná State cities. The population number id, species name, and symbionts sets are indicated**.**

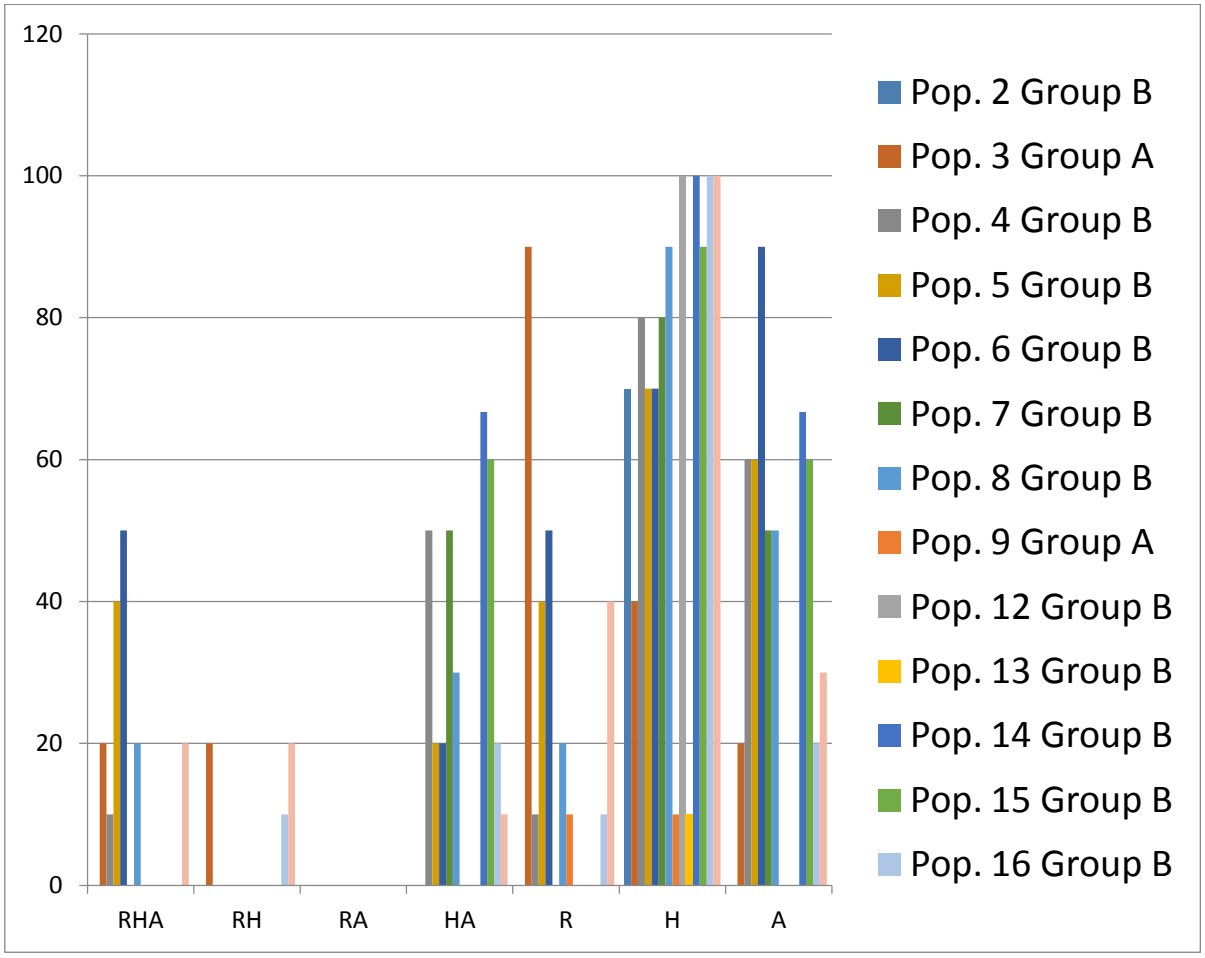
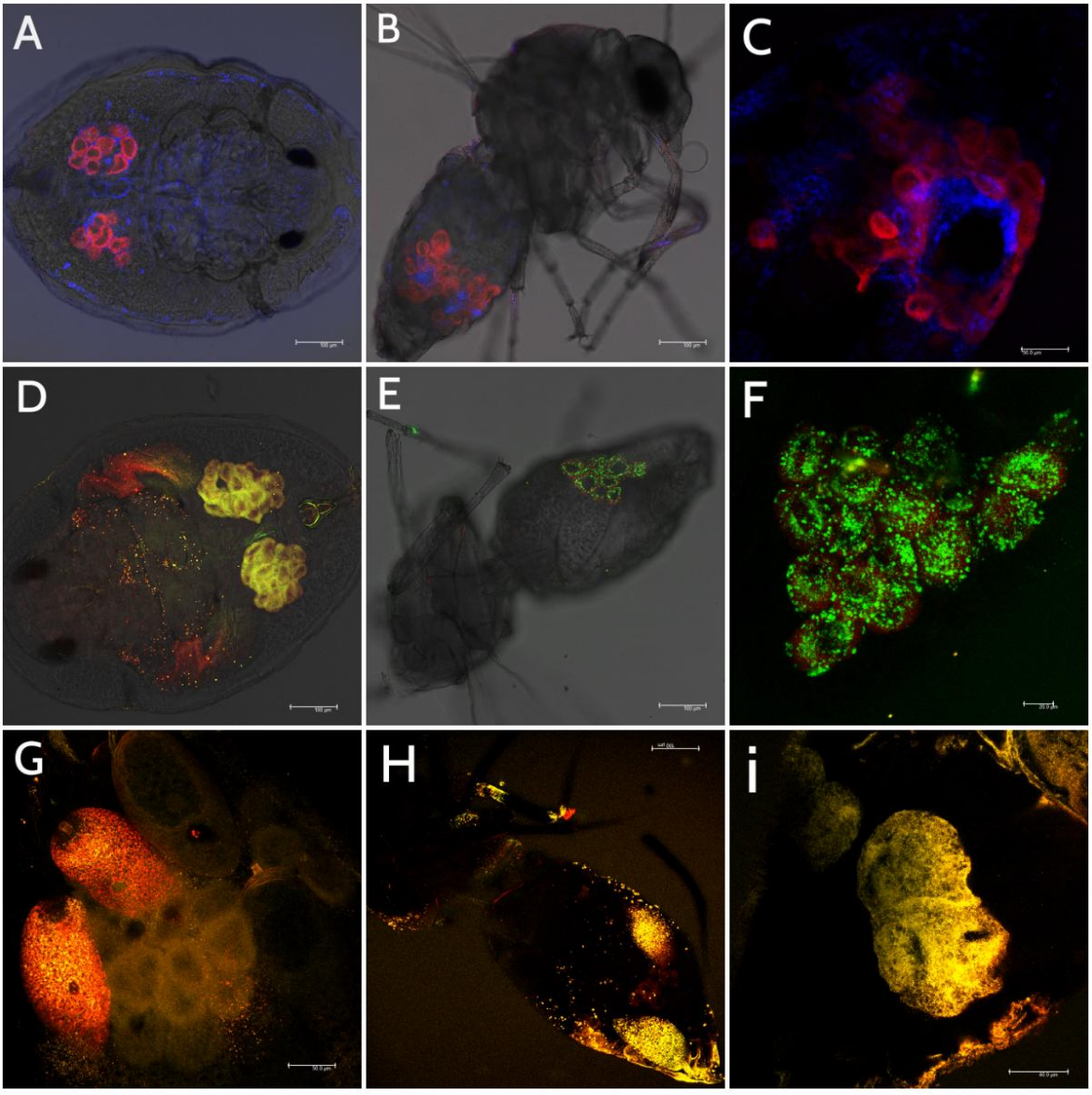


Figure 3. *Bemisia tabaci* MED species adults and nymphs visualized using confocal microscope after FISH analysis, in dark field. Figure 2A: *Portiera* (Red) *Hamiltonella* (Green) from population 2 nymph. Figures 2B and 2C: *Portiera* (Red) and *Rickettsia* (Blue) from population 6 adults. Figure 2D: *Portiera* (Red) and *Rickettsia* (Blue) from population 5 nymph. Figures 2E and 2F: *Portiera* (Red) and *Hamiltonella* (Green) from population 2(2E) and 12(2F) adults. Figures 2G, 2H and 2i: *Portiera* (Red) and *Arsenophonus* (Yellow) from population 7 (2G, 2i) and 16 (2H). Figure 2G and 2i: Close look at eggs (2G) and bacteriocytes (2i) at the bacteriome region.



DISCUSSION

In the present work, the phylogenetic analysis showed the presence of high levels of genetic variation among the MED populations in Brazil and the different sets of secondary endosymbionts between them indicating that different invasions of MED may have occurred in this country. Previous surveys were conducted in São Paulo State and indicated only the presence of MEAM1 species (Rocha *et al.,* 2011; Marubayashi *et al.,* 2013). On the other hand, a previous survey in several crops in Paraná State, including ornamentals, showed that the prevalent species of *B. tabaci* in that area were MEAM1 and New World species (Martinez et. al., 2000).

However, about 25 years after the invasion of MEAM1 species (Lourenção & Nagai, 1994), we identified a new invasion in these regions, the MED species of *B. tabaci*. This is the first report of MED species associated with ornamental plants in Brazil. 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 has already been identified (Grille *et al.,* 2011). However, those MED species were collected from *Capsicum annuum* and *Ipomoeae batatas,* and harbor *Hamiltonella* and *Cardinium* secondary endosymbionts. Here, we have verified the presence of two different groups of the MED *B. tabaci* associated with ornamental plants in Brazil, most of the specimens collected in São Paulo being placed on group “B” that harbors MED from different regions of the world (Figure 1). MED species collected in Paraná from poinsettias, begonias, *Capsicum* spp. and *Emilia fosbergii* and the previously collected in Rio Grande do Sul State (“MED_Brasil_KF991610) were also placed on group “B”. Populations of MED collected in Atibaia and Mogi das Cruzes from poinsettia and hydrangea, respectively, were classified as belonging to the group A composed by populations from Egypt, Syria, Turkey, Japan and Argentina.

Associated with the mtCOI genetic variability found for MED populations collected from ornamental plants in Brazil, the secondary endosymbiont analysis reveals that they are not homogenous, suggesting a recent introduction of the MED species in those regions, in settlement process of endosymbionts in these. The secondary endosymbiont composition differs between the MED groups (Gueguen *et al.,* 2010; Gnankine *et al.,* 2013). 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 work, we noted that group “A” populations harbors *Hamiltonella*, although it was not fixed in all individuals. In Population “3” 40% of the insects were infected and in Population “9” only 10% were infected with *Hamiltonella* (Figure 2). Over all, there was no specific pattern regarding infection with secondary symbionts among the populations of groups “A” or “B” tested in this work. According to Ahmed *et al.,* (2013), endosymbionts of *B. tabaci* species show evidence for variable degrees of horizontal transmission. Primary endosymbionts are almost entirely vertically transmitted whereas the secondary endosymbionts show evidence of horizontal transfers between different species within the *B. tabaci* species complex.

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 that harboring specific sets may bring to the insect biology and ecology. In this study we provide results of the co-localization of *Hamiltonella* with *Portiera,* as well as *Arsenophonus* with *Portiera* in bacteriocytes and eggs. *Rickettsia* seems to be spread all over the insect body, mainly at the circumference of the bacteriocyte, and do not co-localize with *Portiera*. In nymphs, though, the symbionts localization behaviors are similar; *Portiera* and *Hamiltonella* seem to co-localize, while *Rickettsia* is spread outside the bacteriome. The localizations of the bacteria in nymphs and adults are similar to the ones previously reported (Skaljac *et al.,* 2010; Caspi-Fluger *et al.,* 2011; Skaljac *et al.,* 2012; Marubayashi *et al.,* 2014). This also suggests that secondary endosymbiont genetic variation may not reflect host genetic variation and should not be used to infer taxonomic relationships within the *B. tabaci* species complex. This is not surprising as secondary endosymbionts are facultative in their association with the host and so not always present within all individuals within a species (Ahmed *et al.,* 2013).

The secondary endosymbionts play important roles in ecological and evolutionary aspects of their hosts, for example enhancing insecticide susceptibility (Kontsedalov *et al.,* 2008; Kontsedalov *et al.,* 2009), facilitating virus transmission (Gottlieb *et al.,* 2010; Rana *et al.,* 2012; Su *et al.,* 2013), conferring high-temperature tolerance (Brumin *et al.,* 2011), female bias, fitness benefits

(Himler *et al.,* 2011) and resistance to a parasitic wasp (Mahadav *et al.,* 2008). *Hamiltonella* was suggested to increase TYLCV transmission capacity (Gottlieb *et al.,* 2010; Su *et al.,* 2013), which was likely related to differences between the subgroups in virus transmission capacity. TYLCV occurs in the Old World (Czosnek *et al.,* 2001) and has already been detected in the USA, Mexico, Dominican Republic, Jamaica, Puerto Rico (Duffy & Holmes, 2007), Cuba (Quiñones *et al.,* 2002, Duffy & Holmes 2007) and Venezuela (Zambrano *et al.,* 2007). It has yet not been reported in Brazil, but there are suitable conditions for its fast spread if introduced in this country (Inoue-Nagata *et al.,* 2004), 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 & Ghanim, 2015).

The genetic differences found for MED populations from species identified in Argentina, Uruguay and Rio Grande do Sul, as well as the different set of secondary endosymbionts suggests that the MED introductions in São Paulo and Paraná States were independent from the one occurred on Rio Grande do Sul, and may be associated with ornamental plants brought to Brazil from different countries. In addition, MEAM1 species were not identified in the collection areas, which is an indication that MED may be taking place, probably due to their advantage over high resistance to neonicotinoids reported previously (Elbert & Nauen 2000; Horowitz *et al.,* 2005; Nauen & Denholm, 2005), or the ability to take off MEAM1.

Just as occurred in 1990 when MEAM1 was introduced by ornamental plants in Brazil (Lourenção & Nagai, 1994), our work demonstrated once more the importance of ornamental plants in the introduction of exotic pest as in our case the invasive MED species in Brazil. 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. 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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CAPÍTULO 02

Redigido conforme as normas da Revista Bulletin of Entomological Reasearcht (Fator de Impacto: 1.761) no formato de Full Research Paper.

POPULATION EVOLVEMENT OF *Bemisia tabaci* MEDITERRANEANN SPECIES (BIOTYPE Q) IN BRAZIL.

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ABSTRACT

Bemisia tabaci (Hemiptera: Aleyrodidae) is a highly polyphagous insect and considered a supervector of viruses that alone transmits over 300 species. More than three decades after the *B. tabaci* Middle East-Asia Minor 1 species (MEAM1, biotype B) invasion in Brazil, the presence of the *B. tabaci* Mediterraneann species (MED, biotype Q) was first reported in Rio Grande do Sul, and recently in São Paulo and Paraná States. In 2015, a first survey in São Paulo State revealed that MED species was present only in commercial ornamentals greenhouses and flower shops. In 2016, however, a second and more extensive survey performed in São Paulo and Paraná showed that MED has spread through several important vegetable cultures, either in greenhouses and open fields located near where MED was detected in ornamental plants. The endosymbiont sets are different as well. *Arsenophonus*, *Hamiltonella* and *Rickettsia* were detected by PCR and confirmed by sequencing for MED from São Paulo and Paraná in 2015, differing from MED detected in Rio Grande do Sul and from the MED collected in 2016, whose sets were composed by *Arsenophonus*, *Hamiltonella*, *Rickettsia* and *Wolbachia.* The phylogenetic analysis of the MED populations of Brazil showed the presence of different haplotypes divided in two groups.

Key words: whiteflies, MED groups, mtCOI gene, endosymbionts.

INTRODUCTION

Over the past 2 decades, virus emergence and evolution has caused changes in the predominance of insect-transmitted viruses in agricultural ecosystems. One of the most highlighted trends has been the emergence of *Bemisia tabaci*

(Hemiptera: Aleyrodidae) as an extreme polyphagous phloem-feeder sucking on more than 600 host plants (Polston et al., 2014) and a supervector that alone transmits over 300 species, representing 5 genera, including DNA and RNA viruses of several shapes (Gilbertson et al., 2015).

B. tabaci is a cryptic species complex, once encompasses morphologically indistinguishable, but ecologically and genetically distinct groups (Dinsdale *et al.,* 2010; Xu *et al.,* 2010; De Barro *et al.,* 2011; Kanakala & Ghanim, 2015), composed of at least 37 putative species based on mitochondrial cytochrome oxidase subunit I (mtCOI) gene sequences (Firdaus *et al.,* 2013; Boykin & De Barro, 2014; Muhad & Kanakala, 2015). Two of these, known currently as Middle East-Asia Minor 1 (MEAM1) and Mediterraneann (MED) are considered the most invasive pests of a wide variety of field crops worldwide. It may has been spread globally through trade of ornamental and vegetable plant material, emerging as new agricultural pest in several regions (Cheek & Macdonald, 1994; Dalton*,* 2006; Global Invasive Species Database, http://www.issg.org/database).

B. tabaci variability is also reported by the secondary endosymbionts that it harbors (Gueguen *et al.,* 2010; Gnankine *et al.,* 2013). *B. tabaci* has the primary symbiont *Portiera aleyrodidarum*, which is essential for their survival and development (Baumann, 2005). Furthermore, the insect may harbor facultative symbionts previously described as *Arsenophonus*, *Hamiltonella*, *Wolbachia, Cardinium*, *Fritschea*, *Rickettsia* and later discovered, *Orientia*-like organism OLO (Bing *et al.,* 2013), whose roles are related to the host-insect ecology and evolution (Baumann, 2005; Gottlieb et al., 2006; Kanakala & Ghanim, 2015).

In Brazil, *B. tabaci* was first reported in 1928 in Bahia (Bondar 1928) and the population remained low until the early 1990s with the introduction of MEAM1 in São Paulo State (Lourenção & Nagai, 1994). High losses have been reported on Brazilian agriculture ever since, mainly in Solanaceae (Zerbini *et al.,* 1996; Ribeiro *et al.,* 1998; Faria & Mirtes, 2000).

Previous surveys were conducted in São Paulo State and indicated only the presence of MEAM1 species (Rocha *et al.,* 2011; Marubayashi *et al.,* 2013). On the other hand, a previous survey in several crops in Paraná State, including ornamentals, showed that the prevalent species of *B. tabaci* in that area were MEAM1 and New World species (Martinez et. al., 2000).

However, about 25 years after the invasion of MEAM1 species (Lourenção & Nagai, 1994), a new invasion of the MED species of *B. tabaci* in these regions were identified (Moraes et al., 2016 in press). In 2015, a survey in São Paulo State, Brazil, revealed that MED species was introduced and is predominating in commercial ornamentals greenhouses, but not in open field neither in vegetable cultures, where MEAM1 prevailed ever since (Moraes et al., 2016 in press). The presence of MED species in Brazil was previously first reported in 2014 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 has already been identified (Grille *et al.,* 2011). However, those MED were collected from *Capsicum annuum* and *Ipomoeae batatas,* and harbor a different set of endosymbionts from the São Paulo and Paraná MED populations.

In the Americas, MED species has a more limited presence being first reported in the United States in 2004 (Dalton, 2006) and subsequently in Mexico in 2007 (Martinez-Carrillo & Brown, 2007), Guatemala in 2009 (Bethke et al., 2009) and more recently in Argentina, Uruguay (Grille *et al.,* 2011) and Brazil (Barbosa *et al.,* 2015, Moraes et al., 2016 in press). In recent years, the global spread of MED from its origin, bordering the Mediterraneann Basin, to at least 12 countries (Canada, China, Costa Rica, Guatemala, Japan, Mexico, the Netherlands, New Zealand, South Korea, Uruguay, United States) (De Barro *et al.,* 2011) and more recently Brazil (Barbosa *et al.,* 2015, Moraes et al., 2016 in press) has imposed a lot of concerns.

Recent studies using molecular phylogenetic analyses of the mtCOI gene have showed that MED species have high levels of genetic diversity and can be divided into genetic groups (Gueguen *et al.,* 2010), some of these are restricted to geographic areas (Tsagkarakou *et al.,* 2007; Ahmed *et al.,* 2009; McKenzie *et al.,* 2012; Mouton *et al.,* 2012; Parrella *et al.,* 2014), others distributed throughout the world having particularly high resistance to neonicotinoids that are effective against MEAM1 (Elbert & Nauen 2000; Horowitz *et al.,* 2005; Nauen & Denholm 2005) and high transmission efficiency of *Tomato yellow leaf curl virus* (TYLCV) (Li *et al.,* 2010; Park *et al.,* 2012). Therefore, the MED species is considered an extremely important agricultural pest.

Introductions of invasive insect pests and their negative ecological and economic impacts have particularly increased with the international trade and

globalization (Westphal et al., 2008), and thus causing much damage to agricultural, forest and ornamental plants. The whitefly *B. tabaci* species complex is classified as one of the major invasive organism in the world by the International Union for the Conservation of nature and Natural Resources (IUCN, http://www.issg.org).

Population dynamics studies are important because they provide useful information for the development of models involving pest management (Gilbert et al., 1976), as it is possible to obtain an image of the population over a given period of time (Odum, 1988). Such studies may indicate the distribution and abundance of insects, as well as elucidate ecological interactions of pests and natural enemies (Silveira Neto et al., 1976) and abiotic factors, which are important means of natural mortality (Trnkaa et al., 2007 ; Batalden et al., 2007); allowing to predict the occurrence of outbreaks and, the correct use of control measures.

Here we present the population dynamics of Mediterraneann species in São Paulo and Paraná States since its first report in those areas, in the years of 2015 and 2016, once regular monitoring of *B. tabaci* dynamics is critical for early detection of the establishment of this new species for the Brazilian scenario. MED populations have shown to be containing different sets of secondary endosymbionts, and besides, the phylogenetic analysis showed the presence of high levels of genetic variation among the MED populations in Brazil. Also, this is the first report of *Arsenophonus* infecting MEAM1. Our work suggests different invasions and the spread of this species in this country and a new concern to Brazilian agriculture.

MATERIALS AND METHODS

Whitefly sampling

Adults of *B. tabaci* were collected from ornamental plants produced in commercial greenhouses and weeds associated to the ornamental cultures, floricultures, and vegetables between March 2015 and October 2016 in several São Paulo, Paraná States and Minas Gerais locations (Table 1). The specimens of each sample were collected using a hand-held aspirator, preserved immediately

in 95% ethanol and stored at 4ºC until further processing for species genotyping and endosymbiont set. 56 populations represented by 10 individuals of each were analyzed.

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'-CGGAGCTTGCGCCTTAGTC-3') and Bem23R (5'-CGGCTTTATCATAGCTCTCGT-3') witch amplifies a microsatellite locus of about 200 bp and 400 bp for MEAM1 and MED, respectively (De Barro et al., 2003). 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) analysis of the amplicons (Bosco et al., 2006), 5 µl of each PCR (880 bp) was digested with one unit of TaqI at 65ºC 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 compared with those present in GenBank database from National Center for Biotechnology Information (NCBI) by using BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis

Phylogenetic analysis were carried out using the 31 unique *B. tabaci* mtCOI sequences obtained in this study (Figure 1) added to the global *B. tabaci* mtCOI dataset (Boykin & De Barro 2014) totaling 595 sequences. Multiple sequence alignment was prepared using MAFFT (Katoh and Toh, 2008), and manually adjusted using Geneious 9.1.5. MtCOI sequences ranged from 613 to 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 & Brown, 2002), *Rickettsia* (Gottlieb et al., 2006), *Wolbachia* (Heddi et al., 1999), *Arsenophonus* (Thao & Baumann, 2004), *Cardinium* (Weeks & Breeuwer, 2003) and *Fritschea* (Everett et al., 2005) using genus-specific primers targeting the 16 S or 23 S 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.

RESULTS

The species identification results indicated that MED species has spread from the ornamental inside greenhouses to vegetables either in greenhouses and open field (Table 1).

Furthermore, the phylogenetic analysis of *B. tabaci* mtCOI sequences conducted in this work showed the high variability among the MED populations collected in Brazil, that were divided into two different groups (as indicated by “A” and “B”), as previously reported in ornamental plants (Moraes et al., 2016 in press) (Figure 1).

The primary endosymbiont *P. aleyrodidarum*, which is essential for *B. tabaci* persistence, was detected in all whiteflies tested, providing proof that DNA extractions were reliable for the other endosymbionts PCRs. The co-infection

observed in the samples tested was *Rickettsia*, *Hamiltonella*, *Arsenophonus* and *Wolbachia,* although it could be observed that those bacterial populations are not well established yet once they were not present in 100% of the individuals tested (Table 1).

We confirmed the presence of all symbionts through PCR and sequencing (data not shown).

Figure 1- Phylogenetic tree of the partial mtCOI gene from *Bemisia tabaci* totalizing 595 sequences conducted using MrBayes v. 3.2.2 on the Magnus supercomputer. The Mediterraneann clade is shown highlighted and expanded. Sequences obtained in this work are highlighted in green. The host and city of collection are indicated.

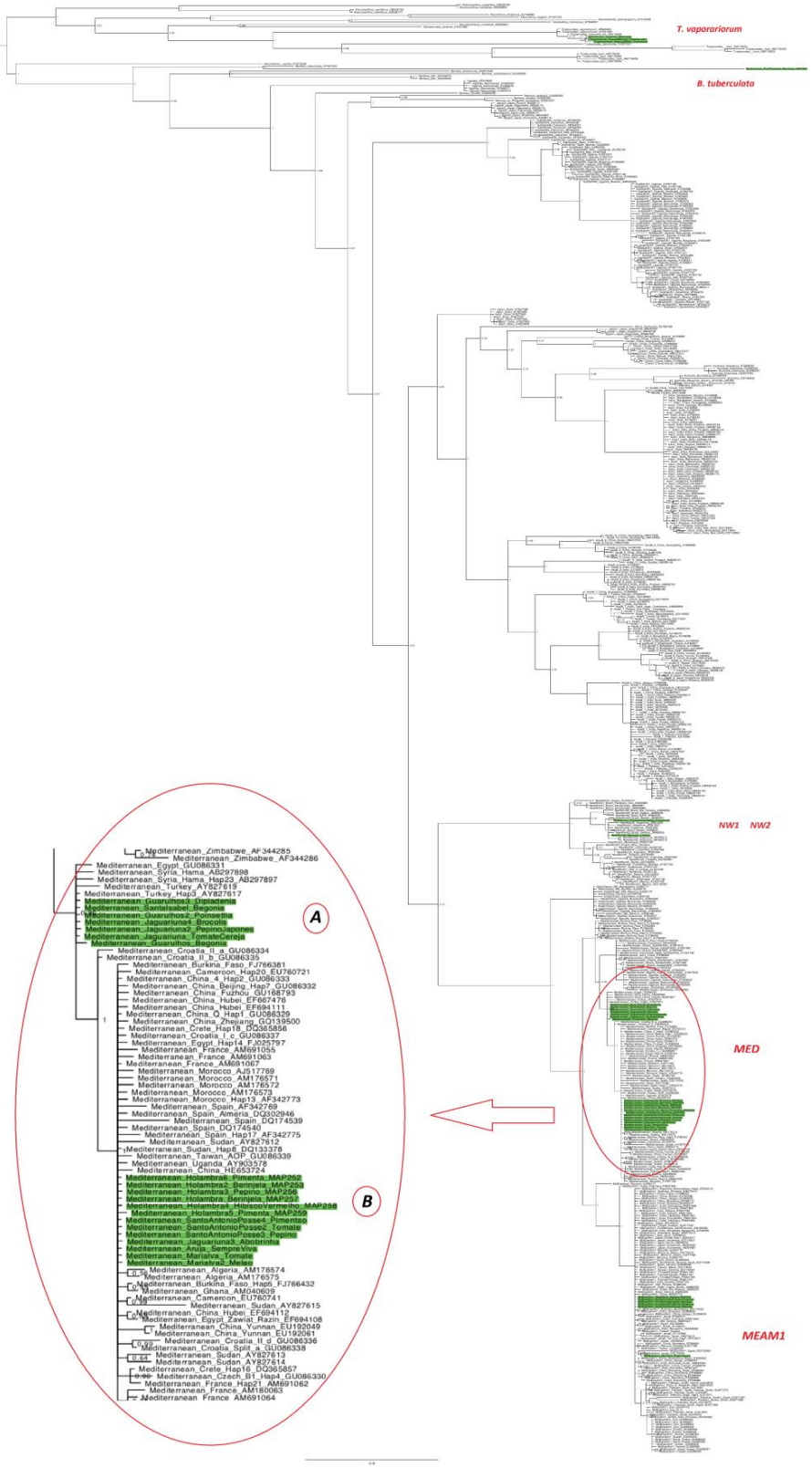

Table 1. Samples and localities of adult, nymph and eggs of whitefly specimens collected in 2016 in ornamental and vegetable greenhouses, open fields and flowershops in São Paulo, Paraná and Minas Gerais States cities, and individual endosymbiont infection status observed in MED *B. tabaci* populations. O. F.= Open Field, G.= Grenhouse, F.= Flowershop, H= *Hamiltonella*; R= *Rickettsia*; W= *Wolbachia*; A= *Arsenophonus*.

					Endosymbionts %			
Plant	**City**	**Coordinates**	**Species**	**Condition**	**H**	**R**	**W**	**A**
Manihot esculenta	Presidnte Prudente-SP	S23°23'20,07'' W46°19'13,27''	*B. tuberculata*	O.F.	100	90	-	-
Begonia spp.	Mogi das Cruzes-SP	S23°22'19,95'' W46°10'35,02''	MED Group B	G.	80	90	-	-
Hydrangea macrophylla	Mogi das Cruzes-SP	S23°22'19,95'' W46°10'35,02''	MED Group A	G.	90	90	-	-
Begonia spp.	Mogi das Cruzes-SP	S23°22'19,95'' W46°10'35,02''	MED Group B	G.	100	70	-	-
Begonia spp.	Mogi das Cruzes-SP	S23°22'19,95'' W46°10'35,02''	MED Group B	G.	70	80	-	10
Hydrangea macrophylla	Mogi das Cruzes-SP	S23°22'19,95'' W46°10'35,02''	MED Group B	G.	70	70	-	20
Begonia spp.	Guarulhos-SP	S23°23'31,13'' W46°21'37,43''	MED Group B	G.	-	90	-	-
Begonia spp.	Guarulhos-SP	S23°23'31,13'' W46°21'37,43''	MED Group B	G.	100	-	-	-
Mentha rotundifolia	Londrina-PR	S23°23'23'' W52°11'32''	*T. vaporariorum*	O. F.	-	-	-	40
Emilia sonchifolia	Sapucaí Mirim -MG	S22°74'55'' W45°7994'22''	*T. vaporariorum*	O. F.	-	-	-	80
Sida spp.	Sapucaí Mirim -MG	S22°74'55'' W45°7994'22''	*T. vaporariorum*	O. F.	-	-	-	100
Petunia spp.	Mogi Mirim-SP	S22°25'55'' W46°57'30''	MED Group B	G.	80	50	-	-
Begonia spp.	Londrina-PR	S23°26'48'' W51°11'22''	MED Group B	F.	30	-	-	20
Euphorbia pulcherrima	Londrina-PR	S23°26'48'' W51°11'22''	MED Group B	F.	90	-	-	50
Emilia fosbergii	Marialva-PR	S23°47'9'' W51°83'6''	MED Group B	O. F.	40	90	-	-
Capsicum spp.	Marialva-PR	S23°47'9'' W51°83'6''	MED Group B	O. F.	100	10	-	-
Euphorbia heterophylla	Botucatu-SP	S22°50'46'' W48°26'6''	New World 2	O. F.	100	-	100	100
Sida spp.	Pindamonhangaba-SP	S22°95'0'' W45°45'0''	New World 1	O. F.	70	-	90	80
Solanum tuberosum	Mogi Mirim-SP	S22°23'25'' W46°51'30''	MEAM1	O. F.	*	*	*	*
Capsicum annum	Santo Antonio de Posse-SP	S22°39'21'' W46°56'21''	MEAM1	O. F.	100	100	-	-
Solanum melongena	Pindamonhangaba-SP	S22°93'50'' W45°45'08''	MEAM1	O. F.	100	100	-	-
Citrullus lanatus	Presidente Prudente-SP	S22°19'42'' W51°23'57''	MEAM1	O. F.	100	100	-	-
Brassica oleracea var. capitata	Santo Antonio de Posse-SP	S22°39'45'' W46°56'87''	MEAM1	O. F.	100	100	-	-
Phaseolus	Marialva-PR	S23°47'59''	MEAM1	O. F.	100	90	-	-

vulgaris		W51°81'16"						
Solanum tuberosum	Mogi Mirim-SP	S22°25'55" W46°57'30"	MEAM1	O. F.	*	*	*	*
Cucumis sativus	Jaguariúna-SP	S22°67'34" W47°06'71"	MED Group A	G.	40	80	20	50
Solanum lycopersicum	Jaguariúna-SP	S22°67'04" W47°06'21"	MED Group A	O. F.	20	100	60	50
Begonia spp.	Guarulhos-SP	S23°23'31,13" W46°21'37,43"	MED Group A	G.	-	100	70	-
Mandevilla spp.	Guarulhos-SP	S23°23'31,13" W46°21'37,43"	MED Group A	G.	-	80	30	-
Begonia spp.	Santa Isabel-SP	S23°37'18" W46°17'61"	MED Group A	G.	-	100	-	90
Euphorbia pulcherrima	Guarulhos-SP	S23°23'31,13" W46°21'37,43"	MED Group A	G.	40	30	-	10
Brassica oleracea var. italica	Jaguariuna-SP	S22°67'04" W47°06'21"	MED Group A	O. F.	60	80	20	30
Capsicum spp.	Holambra-SP	S22°62'60" W47°22'66"	MED Group B	F.	*	*	*	*
Solanum melongena	Holambra-SP	S22°62'60" W47°22'66"	MED Group B	F.	*	*	*	*
Cucumis sativus	Holambra-SP	S22°61'34" W47°22'21"	MED Group B	O. F.	*	*	*	*
Solanum melongena	Holambra-SP	S22°62'60" W47°22'66"	MED Group B	F.	*	*	*	*
Hibiscus spp.	Holambra-SP	S22°61'34" W47°22'21"	MED Group B	G.	*	*	*	*
Capsicum spp.	Holambra-SP	S22°61'34" W47°22'21"	MED Group B	G.	*	*	*	*
Capsicum annum	Santo Antonio de Posse-SP	S22°39'45" W46°56'87"	MED Group B	O. F.	_	80	-	-
Solanum lycopercicum	Santo Antonio de Posse-SP	S22°39'45" W46°56'83"	MED Group B	O. F.	100	-	-	-
Cucumis sativus	Santo Antonio de Posse-SP	S22°39'45" W46°56'12"	MED Group B	O. F.	50	90	-	40
Cucubita pepo	Jaguariuna-SP	S22°67'04" W47°06'21"	MED Group A	O. F.	40	50	30	50
Helichrysum bracteatum	Arujá-SP	S23°34'87" W46°31'59"	MED Group B	G.	40	10	-	-
Solanum lycopercicum	Marialva-PR	S23°23'54" W51°82'16"	MED Group B	O. F.	80	-	-	-
Cucumis melo	Marialva-PR	S23°47'60" W51°82'16"	MED Group B	O. F.	80	-	-	-
Citrullus lanatus	Anhamas-SP	S22°19'42" W51°23'57"	MEAM1	O. F.	100	100	-	-
Cucubita pepo	Presidente Venceslau-SP	S22°19'42" W51°23’	MEAM1	O. F.	100	100	-	-
Manihot esculenta	Montalvão-SP	S22°19'44" W51°23'23"	*B. tuberculata*	O. F.	-	-	100	100
Cucubita pepo	Montalvão-SP	S22°19'44" W51°23'23"	MEAM1	O. F.	100	90	-	-
Solanum lycopersicum	Arujá-SP	S23°40'18" W46°35'73"	MED	G.	30	-	-	-
Capsicum spp.	Arujá-SP	S23°40'18" W46°35'73"	MED	G.	30	30	-	-
Euphorbia pulcherrima	Santa Isabel-SP	S23°37'18" W46°17'61"	MED Group A	G.	40	30	-	10
Manihot	Holambra-SP	S22°95'60"	MED	O. F.	*	*	*	*

esculenta		W45°43'75"						
Brassica oleracea	Pindamonhangaba-SP	S22°95'40" W45°45'45"	MEAM1	O. F.	100	100	-	-
Euphorbia pulcherrima	Campinas-SP	S22°54'20" W47°03'39"	MED	F.	10	20	10	
Euphorbia pulcherrima	Tatui-SP	S23°21'24" W47°53'32"	MED	F.	-	-	-	-

*Not tested

DISCUSSION

In 2015, a first survey in São Paulo State revealed that MED species had been introduced and was present only in commercial ornamentals greenhouses and not in open field neither in vegetable cultures, where MEAM1 prevailed ever since (Moraes et al., 2016, in press). In 2016, however, a second and more extensive survey performed in São Paulo, Paraná and Minas Gerais showed that MED has spread through several important vegetable cultures, either in greenhouses and open fields located near where MED was detected only in ornamental plants previously.

The phylogenetic analysis of *B. tabaci* mtCOI sequences conducted in this work emphasizes the high variability among the MED populations collected in Brazil, divided into two different groups (as indicated by “A” and “B”), as already reported previously (Moraes et al., 2016, in press) (Figure 1). The genetic differences found for MED populations between themselves and from species identified in Rio Grande do Sul (Barbosa et al., 2014), as well as the different set of secondary endosymbionts suggests that the MED introductions in São Paulo and Paraná States in 2015 were independent from the one occurred on Rio Grande do Sul in 2014, and may be associated with ornamental plants brought to Brazil from different countries. In addition, MEAM1 species were still not identified in the collection areas, as the same scenario found in 2015, which is an indication that MED may be taking place, probably due to their advantage over high resistance to neonicotinoids reported previously (Elbert & Nauen 2000; Horowitz *et al.,* 2005; Nauen & Denholm, 2005), or the ability to take off MEAM1, depending on the host plant.

Associated with the mtCOI genetic variability found for MED populations collected from ornamental plants in Brazil, the secondary endosymbiont analysis reveals that they are not homogenous, suggesting a recent introduction of the

MED species in those regions, in settlement process of endosymbionts in these. It is known that secondary endosymbiont composition differs between the MED groups (Gueguen *et al.,* 2010; Gnankine *et al.,* 2013). 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 work, we noted that group "A" and "B" populations harbors *Hamiltonella*, although it was not fixed in all individuals, and MED Group "B" was slightly more infected with this bacteria than MED Group "A" (Table 1). Over all, there was no specific pattern regarding infection with secondary symbionts among the populations of groups "A" or "B" tested in this work, except for the fact that MED Group "A" populations harbors *Wolbachia*. Interestinly, MED Group A reported in 2015 didn't harbor this endosymbiont (Moraes et al., 2016, in press). In 2016, Group "A" harbored in a higher rate: *Rickettsia*, *Arsenophonus* and *Wolbachia*, and in a lower rate *Hamiltonella*. On the oher hand, Group "B" was highly infected with *Hamiltonella* and *Rickettsia*, and slightly with *Arsenophonus*. According to Ahmed *et al.,* (2013), endosymbionts of *B. tabaci* species show evidence for variable degrees of horizontal transmission. Primary endosymbionts are almost entirely vertically transmitted whereas the secondary endosymbionts show evidence of horizontal transfers between different species within the *B. tabaci* species complex.

The secondary endosymbionts play important roles in ecological and evolutionary aspects of their hosts, for example enhancing insecticide susceptibility (Kontsedalov *et al.,* 2008; Kontsedalov *et al.,* 2009), facilitating virus transmission (Gottlieb *et al.,* 2010; Rana *et al.,* 2012; Su *et al.,* 2013), conferring high-temperature tolerance (Brumin *et al.,* 2011), female bias, fitness benefits (Himler *et al.,* 2011) and resistance to a parasitic wasp (Mahadav *et al.,* 2008). *Hamiltonella* was suggested to increase TYLCV transmission capacity (Gottlieb *et al.,* 2010; Su *et al.,* 2013), which was likely related to differences between the subgroups in virus transmission capacity. TYLCV occurs in the Old World (Czosnek *et al.,* 2001) and has already been detected in the USA, Mexico, Dominican Republic, Jamaica, Puerto Rico (Duffy & Holmes, 2007), Cuba (Quiñones *et al.,* 2002, Duffy & Holmes 2007) and Venezuela (Zambrano *et al.,* 2007). It has yet not been reported in Brazil, but there are suitable conditions for its fast spread if introduced in this country (Inoue-Nagata *et al.,* 2004), since

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 & Ghanim, 2015).

In 2016, the phylogenetic analysis showed the persistence of high levels of genetic variation among the MED populations in Brazil. Different sets of secondary endosymbionts were also noticed, and *Wolbachaia* became present in MED Group “A” populations. Both facts emphasize the hypothesis that different invasions of MED may have occurred in this country.

Our work demonstrated the spread of MED species from ornamental greenhouses to vegetables in open fields in Brazil, and that this new species harbors genetic and endosymbionts divergences. This study opens new doors and raises questions that must be further clarified over the different groups of MED *B. tabaci* behavior. Biological aspects must be verifyed, such as the insecticide resistance under Brazilian conditions, the competition with the MEAM1 species, and its ability to transmit begomoviruses and criniviruses.

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3 CONSIDERAÇÕES FINAIS

1. Plantas ornamentais são importantes hospedeiras de *Bemisia tabaci* espécie Mediterranean no Brasil;

2. A variabilidade genética do mtCOI evidencia eventos distintos de introdução de *Bemisia tabaci* Mediterranean no Brasil.