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Research

Spatial Distribution of Whitefly Species (Hemiptera: Aleyrodidae) and Identification of Secondary Bacterial Endosymbionts in Tomato Fields in Costa Rica

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Abstract

In Costa Rica, tomato (*Solanum lycopersicum* Linnaeus) Linnaeus (Solanales: Solanaceae) is one of the crops most severely affected by the whiteflies (Hemiptera: Aleyrodidae) *Trialeurodes vaporariorum* (Westwood) and the *Bemisia tabaci* (Gennadius) species complex. The objective of this study was to monitor the spatial distribution and diversity of these species and to detect the presence of secondary bacterial endosymbionts in individuals collected in areas of intensive tomato production. In total, 628 whitefly individuals were identified to the species level using restriction analysis (PCR-RFLP) of a fragment of the mitochondrial cytochrome C oxidase I gene (mtCOI). *Trialeurodes vaporariorum* was the predominant species, followed by *B. tabaci* Mediterranean (MED). *Bemisia tabaci* New World (NW) and *B. tabaci* Middle East-Asia Minor 1 (MEAM1) were present in lower numbers. The mtCOI fragment was sequenced for 89 individuals and a single haplotype was found for each whitefly species. Using molecular markers, the 628 individuals were analyzed for the presence of four endosymbionts. *Arsenophonus* Gherna et al. (Enterobacterales: Morganellaceae) was most frequently associated with *T. vaporariorum*, whereas *Wolbachia* Hertig (Rickettsiales: Anaplasmataceae) and *Rickettsia* da Rocha-Lima (Rickettsiales: Rickettsiaceae) were associated with *B. tabaci* MED. This study confirmed that *B. tabaci* NW has not been completely displaced by the invasive species *B. tabaci* MED and *B. tabaci* MEAM1 present in the country. An association was found between whitefly species present in tomato and certain secondary endosymbionts, elevation was the most likely environmental factor to affect their frequency.

Key words: *Trialeurodes vaporariorum*, *Bemisia tabaci*, *Rickettsia*, *Wolbachia*, *Arsenophonus*

Whiteflies (Hemiptera: Aleyrodidae) are a group of insects with a worldwide distribution. There are at least 1,550 described species, of which 50 are pests causing important losses in agriculture (Liu et al. 2015). The most economically important species are *Trialeurodes vaporariorum* Westwood and the species complex traditionally grouped within *Bemisia tabaci* Gennadius (Jones 2003, De Barro et al. 2011, Brown 2016). Both species can be vectors of different genera of plant viruses (Navas-Castillo et al. 2011). The identification of these species is particularly important in tropical and subtropical regions where increased whitefly populations and associated crop losses have been reported over the past decades in bean, tomato, pepper, and other crops (Varma and Malathi 2003, Navas-Castillo et al. 2011, Barboza et al. 2019b). *Bemisia tabaci* is a complex of morphologically indistinguishable species (Boykin and De Barro 2014, Boykin et al. 2017) that can be identified using molecular techniques. Identification of whitefly species is relevant for agriculture because species have been observed to differ in resistance to insecticides and efficiency of pathogen transmission (Perring et al. 1993).

In Costa Rica, *B. tabaci* New World (NW), *B. tabaci* Middle East-Asia Minor 1 (MEAM1) (both species formerly known as biotype A and B, respectively), and *T. vaporariorum* were present in different regions of the country prior to 2006 (Morales et al. 2005, Hilje and Morales 2008). In 2009, *B. tabaci* Mediterranean (MED) or the species commonly known as biotype Q was reported in greenhouses in the western Central Region (Guevara-Coto et al. 2011), and soon after (2011–2012), it was found in field and greenhouse-grown tomato and sweet pepper (Barboza et al. 2019a). Other recent reports have also informed of the presence of *B. tabaci* MED in

field-grown crops in America (Alemandri et al. 2015, McKenzie and Osborne 2017).

One interaction that has been shown to have a wide impact on the ecology of whiteflies is bacterial endosymbiosis (Baumann 2005). To date, one primary and at least seven secondary endosymbionts have been described in these hemipterans (Bing et al. 2013a, b; Wang et al. 2019). In addition to providing nutrients to the insect, secondary endosymbiosis with *Hamiltonella* Candidatus Hamiltonella (Enterobacterales: Enterobacteriaceae), *Rickettsia*, or *Wolbachia* confers resistance to parasitic wasps and viral and fungal pathogens and improves tolerance to heat stress (Scarborough et al. 2005, Guay et al. 2009, Łukasik et al. 2013). The secondary endosymbionts *Arsenophonus*, *Rickettsia*, and *Wolbachia* are capable of altering insect reproduction with the apparent effect of self-dispersal in the host population, although this may require that the symbiont be located in the reproductive organs (Gottlieb et al. 2008, Engelstädter and Hurst 2009). Secondary endosymbionts can also participate in whitefly-virus pathosystems, as in the case of *Hamiltonella* and *Rickettsia*, which can facilitate virus transmission through direct or indirect interaction with the virus (Gottlieb et al. 2010, Kliot et al. 2014, Czosnek et al. 2017, Bello et al. 2019). In addition to the individual effects produced by each endosymbiont, the presence of endosymbiont species in different combinations can affect the susceptibility of whiteflies to various insecticidal compounds (Chu et al. 2011). The association between whiteflies and specific secondary bacterial endosymbionts can vary with geographical location, host plant, whitefly species or genetic group, and even within individuals of the same population, and it is important to characterize these associations (Gueguen et al. 2010, Pan et al. 2012, de Moraes et al. 2018). The objective of the present study was to monitor the distribution and diversity of whitefly species and detect their secondary bacterial endosymbionts in the principal zones of tomato production in Costa Rica.

Materials and Methods

Whitefly Collection

Adult whiteflies were collected in 81 commercial tomato fields, 32 of which were visited in 2015 and 49 in 2016 (Fig. 1, Table 1). All of the insects collected at a single site constituted a sample. At least five individuals were analyzed per sample, except for 15 samples that contained fewer than five individuals (Table 1). Costa Rica is a tropical country with two seasons: the dry season, which extends from January to April, and the rainy season, from July to November. The country is divided into seven provinces, six of which were included in the surveys (Alajuela, Cartago, Guanacaste, Heredia, Puntarenas, and San José). The Limón province was not included because it is not an area of tomato production. The sampling sites were located in tomato fields at elevations of 800–1,600 m above sea level (m a.s.l.), with temperatures ranging from 18 to 30°C. Mean annual precipitation was from 950 to 4,000 mm (Ortiz-Malavasi 2009). Individual whiteflies were collected during the dry season using a manual aspirator. Whiteflies were placed in 70% alcohol in vials and stored at −20°C until analyzed at the Centro de Investigación en Biología Celular y Molecular (CIBCM), Universidad de Costa Rica.

Extraction of Whitefly DNA

DNA was extracted from 628 adult whiteflies using a modification of the Chelex 100 (Sigma, Saint Louis, MO) method described by Walsh et al. (1991). Each individual was crushed without liquid in a 1.5-ml tube and then 30 µl of distilled water were added. Chelex 100 was added (30 µl) to 50% and the suspension was homogenized. Suspensions were incubated at 56°C for 15 min and at 99°C for 3 min, then centrifuged at 14,000 rpm for 5 min. Total DNA was obtained by extracting 30 µl of supernatant from each tube.

Identification of Whitefly Species by PCR-RFLP

An ~800-bp fragment of the mtCOI gene was amplified by PCR using the primers C1-J-2915 (Simon et al. 1994) and 801c (Dalmon et al. 2008). The PCR mix included DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific, Carlsbad, CA) at a final concentration of 1×, 3 µl of nucleic acids, and 0.3 µM of primers in a total reaction volume of 25 µl. A variation of the thermal profile described by Frohlich et al. (1999) was used with an initial denaturing at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 37°C for 45 s, and 70°C for 30 s, and a final extension at 72°C for 5 min.

The PCR product was digested in independent reactions with the restriction enzymes *Tru*9I (Promega, Madison, WI) and TaqI (Thermo Fisher Scientific) at 65°C for 3 h. Each reaction consisted of 1 U of enzyme, buffer solution and water in a final volume of 15 µl. DNA fragments were visualized in a 2.5% agarose gel stained with GelRed (Biotium, Fremont, CA). The diagnostic band patterns described by Bosco et al. (2006) were used to differentiate *B. tabaci* MEAM1, *B. tabaci* MED, *B. tabaci* Asia I, *B. tabaci* Sub-Sahara Africa 2, *B. tabaci* Italy, and *T. vaporariorum*.

Sequence Analysis of the mtCOI Gene

The ~800 bp mtCOI gene fragments from 89 individuals were sequenced (Macrogen Inc., Seoul, Korea) (Table 1). Sequences were assembled using the Staden Package (Bonfield et al. 1995) and aligned using the MUSCLE algorithm (MEGA 7) (Kumar et al. 2016). Sequences were compared with those available in the databases using the similarity percentage obtained with the BlastN tool (Altschul et al. 1990).

The number of haplotypes for each species was estimated using DnaSP v5.10.1. Haplotypes were compared with those described by Barboza et al. (2019a). The fragments analyzed were 532 bp for *T. vaporariorum* (nt 77–608 of mtCOI gene, KF991608), and 657 bp for *B. tabaci* MED, *B. tabaci* MEAM1, and *B. tabaci* NW (nt 1–657 of mtCOI gene, KJ606633). The seven sequences obtained for *B. tabaci* NW were compared with 21 accessions of this species available in GenBank (Supp Table 1 [online only]). A phylogenetic tree was constructed using Mr. Bayes 3.2.6 (Huelsenbeck et al. 2001), considering 10 million generations, eight Markov chains, sampling each 2,000 generations and a mixed nucleotide substitution method (Ronquist and Huelsenbeck 2003). One sequence for each haplotype was considered for phylogeny construction using the *B. tabaci* NW2 sequence JN689353 as an external group. Aligned haplotypes of *B. tabaci* NW (Table 2) were analyzed for single nucleotide polymorphisms (SNPs) using AF342770 from Honduras as a reference sequence.

Detection of Secondary Bacterial Endosymbionts

Each of the 628 whiteflies was analyzed and individually considered for the presence of secondary bacterial endosymbionts. Although at least seven secondary endosymbionts have been described in whiteflies (*Wolbachia, Arsenophonus, Hamiltonella, Rickettsia, Cardinium, Fritschea*, and *Hemipteriphilus*), in this study, it was included the ones possibly involved in the whiteflies fitness according to literature. Independent PCRs were performed to amplify the 23S rDNA genes of *Arsenophonus* and the 16S of *Wolbachia* and

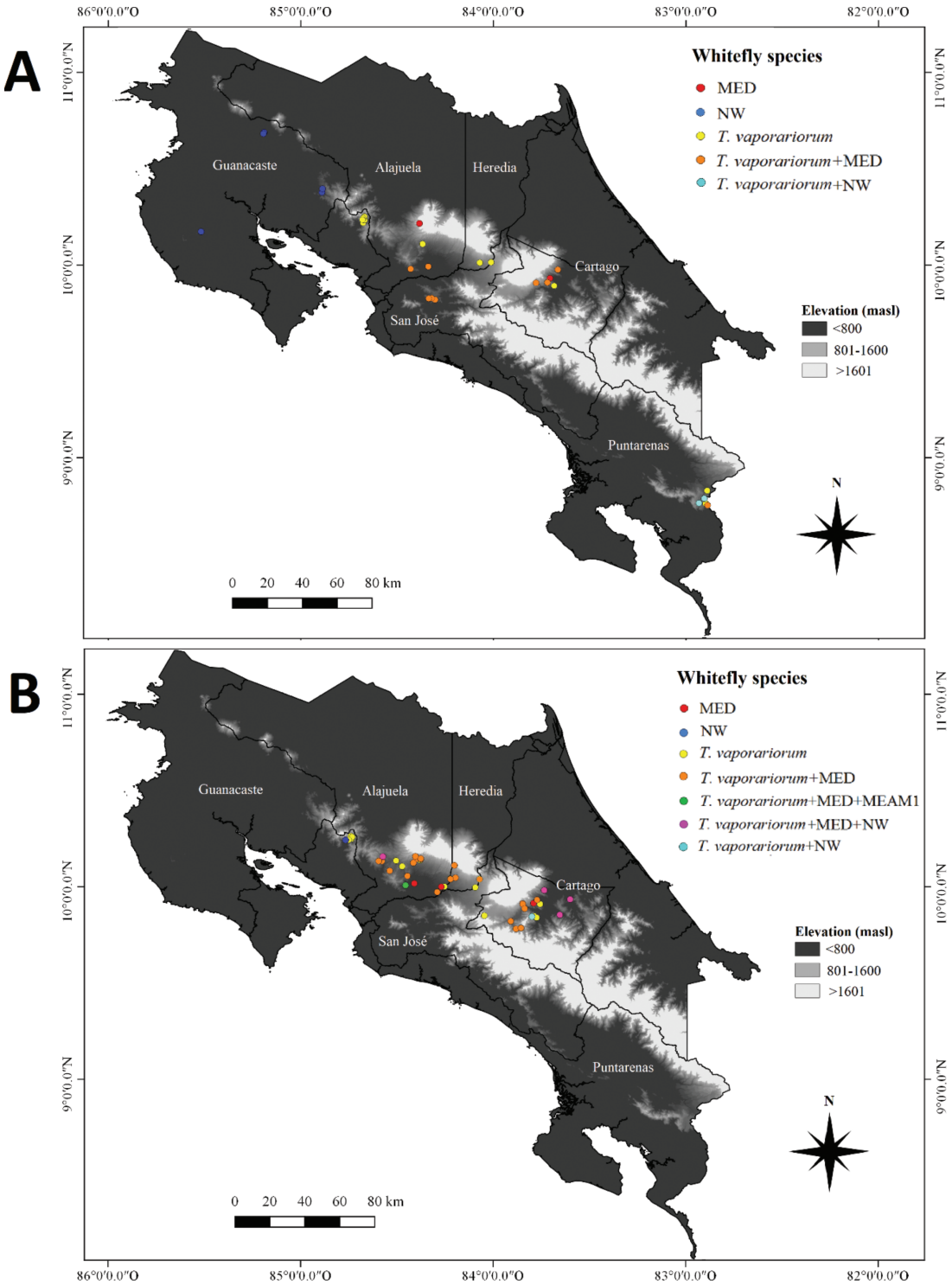

Fig. 1. Distribution of whitefly species identified in surveys of field-grown tomato in 2015 (A) and 2016 (B).

Rickettsia (Thao and Baumann 2004, Heddi et al. 1999, Gottlieb 2006). The primers Hb-F/Hb-R and Ham-F/Ham-R (Zchori-Fein and Brown 2002) were tested for detection of *Hamiltonella*, but due to a cross-reaction with *Arsenophonus*, which was confirmed by sequencing, this species was excluded from the study.

Each PCR contained DreamTaq PCR Master Mix (2×; Thermo Fisher Scientific) at a final concentration of 1×, 2.5 µl of DNA, and 0.3 µM of each primer in a reaction volume of 12.5 µl. PCR specificity was confirmed by sequencing randomly selected PCR products for each endosymbiont. A χ^2-test was performed between the frequency of hosts and the tested secondary endosymbionts to determine whether any association exists between the host and endosymbionts, using the statistical package R, version 3.4.2 (http://cran.r-project/org).

Environmental Factors Analysis

For each sampling site, latitude, longitude, and elevation were recorded. Average yearly precipitation and temperatures were also determined for each site based on the Holdridge life zones system. To determine how environmental variables affected host and endosymbiont frequencies, spatial autocorrelation on host frequency was initially tested by means of a Mantel correlation test (excluding *B. tabaci* NW and *B. tabaci* MEAM1 hosts due to small frequencies) as implemented by Sepúlveda et al. (2017).

A generalized linear mixed model (*glmm*) was used to estimate the probability of finding *T. vaporariorum* over *B. tabaci* MED host as a binomially distributed response variable dependent on latitude, longitude, elevation, precipitation, and temperature, with year as a random factor. Separate *glmm* analysis were also run to model each endosymbiont frequency on the same explanatory variables. However, in these separate analysis, the proportion of *T. vaporariorum* hosts (*B. tabaci* MED = 1 – *T. vaporariorum*) was included as a covariate. *Glmm*'s were fit using a binomial distribution and a logit link function, using the lme4 library in the R statistical language version 3.6.2.

Results

Identification of Whitefly Species by PCR-RFLP

In total, 81 samples were collected in the main areas of tomato production in Costa Rica. The mtCOI fragment was analyzed by PCR-RFLP for 628 individual whiteflies, 178 of which were collected in 2015 and 450 in 2016. The species were identified as *T. vaporariorum, B. tabaci* MED, *B. tabaci* MEAM1, and *B. tabaci* NW. *Bemisia tabaci* NW was not identified by the diagnostic bands described by Bosco et al. (2006); however, it could be distinguished from the other species based on the band pattern generated with *Tru*9I (184, 138, 107, and 84 bp) and by the lack of digestion with TaqI (Supp Fig. 1 [online only]).

Trialeurodes vaporariorum was the most frequent species in tomato fields in both sampling years. In total, 430 whiteflies individuals were identified, while *B. tabaci* results showed that *B. tabaci* MED was the most frequent species in comparison with *B. tabaci* NW and *B. tabaci* MEAM1 (Table 1). Species representativeness varied among the provinces; in Puntarenas in 2015, *T. vaporariorum* individuals were 27% points higher than *B. tabaci* MED. In Heredia, *T. vaporariorum* was the only species detected. *Bemisia tabaci* MED was not present in samples from Guanacaste or Heredia, and *B. tabaci* NW was found in low numbers in Guanacaste and Puntarenas (6% or less). In 2016, *T. vaporariorum* was more frequent than *B. tabaci* MED (4–17% points higher) in Alajuela, Cartago, and Heredia. *Bemisia tabaci* MED was not present in samples from Puntarenas or San José, whereas *B. tabaci* NW individuals were found in low numbers in Alajuela, Cartago and Puntarenas (2% or less). It is important to note that the only two *B. tabaci* MEAM1 individuals identified in this study were found in Alajuela (Table 1).

Two or more whitefly species were found in sympatry in several sampling sites. In 2015, *T. vaporariorum* and *B. tabaci* MED were found to coexist in nine of the sampling sites, and *T. vaporariorum* and *B. tabaci* NW were found together in two sites. In the remaining 21 samples, only one species was present (*T. vaporariorum, B. tabaci* MED, or *B. tabaci* NW; Fig. 1A). In 2016, *T. vaporariorum* and *B. tabaci* MED were found together in 22 sites; these two species occurred most frequently together and were distributed together throughout the Central Region (Alajuela, San José, Heredia, and Cartago). *Trialeurodes vaporariorum* and *B. tabaci* NW were found together in one site. Note that two new combinations of coexisting species were found in 2016: *T. vaporariorum*, *B. tabaci* MED, and *B. tabaci* NW in four sites and *T. vaporariorum*, *B. tabaci* MED, and *B. tabaci* MEAM1 in one site. In the remaining 21 sites sampled, only one species was identified: *T. vaporariorum*, *B. tabaci* MED, or *B. tabaci* NW (Fig. 1B).

Elevation was another variable considered in this study. All of the whitefly species identified were found between 800 and 1,600 m a.s.l. *Bemisia tabaci* NW was the most abundant species at sites below 800 m a.s.l., whereas *B. tabaci* MED could be found at elevations above 1,600 m a.s.l. *Trialeurodes vaporariorum* was found at all elevations. The two *B. tabaci* MEAM1 individuals were found at ~800 m a.s.l.

Table 1. Species frequency of whitefly species *Trialeurodes vaporariorum*, *Bemisia tabaci* Mediterranean (MED), *B. tabaci* New World (NW), and *B. tabaci* Middle East-Asia Minor 1 (MEAM1) collected in areas of tomato production

		% Whitefly species[a] (number of individuals)					
			Bemisia tabaci				
Year	Province	*Trialeurodes vaporariorum*	MED	NW	MEAM1	No. of individuals	Whitefly species (number of sequences)
2015	Alajuela	11 (19)	10 (18)	0	0	37	
2016		25 (114)	14 (64)	0.4 (2)	0.4 (2)	142	Tv (18), MED (12), NW (1), MEAM1 (1)
2015	Cartago[b]	8 (14)	13 (23)	0	0	37	
2016		26 (115)	9 (41)	2 (8)	0	204	Tv (21), MED (13), NW (3)
2015	Guanacaste[b]	0	0	6 (10)	0	10	
2015	Heredia	11 (20)	0	0	0	20	
2016		7 (32)	3 (12)	0	0	44	Tv (4), MED (2)
2015	Puntarenas[b]	28 (50)	1 (2)	2 (3)	0	55	Tv (2), MED (1), NW (1)
2016		11 (50)	0	1 (4)	0	54	Tv (5), NW (2)
2015	San José	6 (10)	5 (9)	0	0	19	Tv (1), MED (1)
2016		1 (6)	0	0	0	6	Tv (1)
Total number of individuals		68.5 (430)	26.9 (169)	4.3 (27)	0.3 (2)	628	

[a]Percent calculated with respect to total number of individuals sampled per year.

[b]Provinces with less than five individuals identified.

Table 2. Single nucleotide polymorphisms (SNPs) detected in American *Bemisia tabaci* NW haplotypes from the 657 nucleotide sequence of the mtCOI gene fragment considered in this study[a]

Haplotipe	Number of SNP in the amplified mtCOI fragment																															
	1	9	13	23	27	29	33	86	100	109	114	155	159	163	166	167	178	180	182	233	269	361	386	422	443	454	482	497	521	545	558	632
NW-i	G	G	T	C	G	C	A	C	G	T	A	T	A	G	T	T	G	T	G	C	T	C	A	T	C	A	C	A	A	T	T	T
NW-ii	-	-	**A**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NW-iii	A	**A**	-	T	-	-	-	T	-	-	-	C	**G**	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	C	C	-
NW-iv	-	-	-	T	-	-	-	T	-	-	-	-	**G**	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	C	C	-
NW-v	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-
NW-vi	-	-	-	T	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	**T**	-	-	-	-	-	-	-	C	C	-
NW-vii	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	**T**	-	-	T	-	T	-	-	C	C	-
NW-viii	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
NW-ix	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	T	-	T	-	-	C	C	-
NW-x	-	**A**	-	T	-	-	-	T	**A**	-	-	-	**G**	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	C	C	-
NW-xi	-	-	-	T	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	**C**	-	-	-	C	C	-
NW-xii	-	-	-	G	**A**	-	**T**	-	-	**C**	**C**	-	-	**A**	**C**	C	**C**	**C**	**C**	-	-	-	-	-	-	-	-	-	G	C	C	C
NW-xiii	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	C	G	C	-	C
NW-xiv	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	C	C	-
NW-xv	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	C	C	C

Nonsynonymous (nsSNP) are boxed and highlighted in bold to indicate where the amino acid could have been altered.

[a]Nucleotide positions refer to positions on the mtCOI gene of *B. tabaci* NW GenBank AF342770.

Analysis of Partial mtCOI Gene Sequences

The mtCOI gene was partially sequenced for 89 whitefly individuals previously identified by PCR–RFLP. Included were 52 sequences from *T. vaporariorum,* 29 from *B. tabaci* MED, 7 from *B. tabaci* NW, and 1 from *B. tabaci* MEAM1 (Table 1). A single haplotype was found for each of the four whitefly species identified. These were deposited in GenBank with the accession numbers MH510175 (*T. vaporariorum*), MH510176 (*B. tabaci* MED), MH510178 (*B. tabaci* NW), and MH510177 (*B. tabaci* MEAM1). Haplotypes found in the *T. vaporariorum*, *B. tabaci* MED, and *B. tabaci* MEAM1 sequences were compared with those described in the Western Hemisphere by Barboza et al. (2019a) and were found to correspond to Tv-i, MED-i and MEAM1-i. Whitefly individuals with the Tv-i haplotype sequence were found in all of the provinces where *T. vaporariorum* was present. MED-i haplotype sequence was found in the Alajuela, Heredia, Cartago, and San José provinces, whereas MEAM1-i haplotype was present in the Alajuela province. Analysis of the *B. tabaci* NW sequences in the present study allowed the identification of 15 haplotypes of this species. In Fig. 2, the phylogenetic tree showed that sequences from Costa Rica (MH510178) were close to whitefly individuals described in Panama (DQ130060, DQ130061) but did not coincide with any other haplotypes reported for this species in America. SNP analysis detected the presence of 32 SNPs, of which 18 caused nonsynonymous changes (nsSNPs) in the amino acid coding sequence (Table 2).

Detection of Secondary Bacterial Endosymbionts

The 628 identified whitefly individuals were screened for the presence of secondary bacterial endosymbionts. Sequences of PCR products from each endosymbiont were analyzed and used as positive controls in subsequent amplifications. The frequency of hosts and their symbionts were not independent ($\chi^2 = 544.05$, df = 2, $P < 0.01$). The data show that *Wolbachia* and *Rickettsia* are more common in *B. tabaci* MED species, whereas *Arsenophonus* is more frequent in *T. vaporariorum*. In the *B. tabaci* NW and *B. tabaci* MEAM1 species, *Rickettsia* was the only secondary endosymbiont detected.

Bemisia tabaci MED showed a higher number of endosymbionts than *T. vaporariorum* and *B. tabaci* NW. The most frequent combination of endosymbionts found together in this species, *Wolbachia* and *Rickettsia*, was present in 65.3% of the individuals in 2015 and 68.4% in 2016 (Fig. 3). *Arsenophonus* was detected in over 80% of the *T. vaporariorum* individuals analyzed in both sampling periods, whereas *Rickettsia* was found in over 90% of the *B. tabaci* MED individuals in both years. *Wolbachia* was also found in *B. tabaci* MED in 69.2% of the individuals collected in 2015 and 76% in 2016 (Fig. 3). No secondary endosymbionts were detected in *B. tabaci* NW individuals collected in 2015, but in 2016, *Rickettsia* was found in 35.7% of the *B. tabaci* NW individuals and in the two *B. tabaci* MEAM1 individuals (figure not shown).

Analysis of abundance data by whitefly species, endosymbiont and province showed that *Arsenophonus* was present in the majority of the *T. vaporariorum* individuals analyzed in Alajuela, Cartago, Heredia, Puntarenas, and San José (Table 3). *Arsenophonus* was also associated with *B. tabaci* MED from Alajuela, Cartago, Heredia, and San José in lesser amounts (17% or less). *Rickettsia* was the predominant species in *B. tabaci* MED individuals; abundance of *B. tabaci* MED individuals harboring *Rickettsia* ranged from 89% in Alajuela to 100% in Heredia, Puntarenas, and San José. *Rickettsia* was also present in *T. vaporariorum* individuals in Alajuela, Cartago, and Heredia (≤21%). *Bemisia tabaci* NW individuals harboring *Rickettsia* were detected only in Alajuela and Cartago, whereas

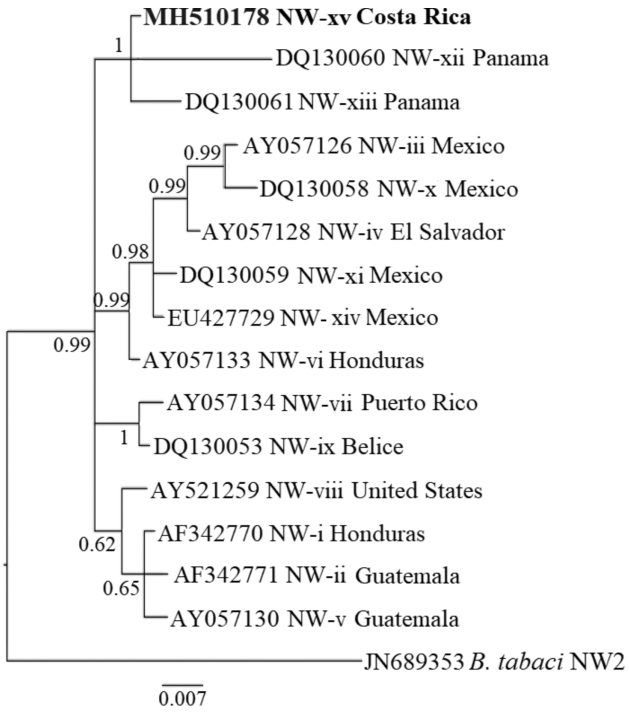

Fig. 2. Phylogenetic tree of *Bemisia tabaci* NW considering 657 nucleotides of the mtCOI gene sequences reported for the western hemisphere and the *B. tabaci* NW2 (GenBank JN689353) sequence as an external group. Sequences were aligned using MUSCLE and the tree was built by Bayesian inference. Numbers close to the branches represent Bayesian posterior probabilities; bars indicate the number of substitutions per site. The haplotype from Costa Rica is in bold.

B. tabaci MED individuals harboring *Wolbachia* ranged from 44% in San José to 100% in Puntarenas. *Wolbachia* was also present in *T. vaporariorum* individuals in Alajuela and Cartago in low numbers (2% or less).

Environmental Factors Analysis

Neither evidence of spatial autocorrelation (Mantel r_M = 0.025; P = 0.252) was found nor statistical support for the effect of latitude, longitude, precipitation, or temperature on host or endosymbiont frequencies (P > 0.05). The frequency of *T. vaporariorum* was marginally affected by elevation (β = 0.111, P = 0.091). Secondary endosymbiont frequencies were always affected by the frequency of *T. vaporariorum* hosts; the likelihood of *Arsenophonus* increased with each increase in *T. vaporariorum* frequency (β = 1.986, P < 0.001). Since *T. vaporariorum* tends to increase with elevation, so does the frequency of *Arsenophonus* endosymbionts. *Wolbachia* and *Rickettsia* frequencies both decreased significantly with an increase in *T. vaporariorum* frequency (i.e., meaning they increased with *B. tabaci* MED frequency; β_W = –3.316, P < 0.001; β_R = –2.816, P < 0.001 for *Wolbachia* and *Rickettsia*, respectively); and both their frequencies increased with elevation (β_W = 0.2094, P = 0.082; β_R = 0.2135, P = 0.032). Also, a quadratic term of elevation to see if frequencies increase at intermediate elevations was tested; however, quadratic terms were not significant, and based on AIC including a polynomial term did not improve the fit of the models.

These previous results suggest that *Arsenophonus* is more likely found in *T. vaporariorum* hosts at higher elevations, whereas *Wolbachia* and *Rickettsia* are more likely found in *B. tabaci* MED hosts. These patterns are independent of geographical location and are unlikely to be affected by temperature or precipitation. The most likely environmental factor shaping the frequency of hosts and symbionts is elevation. In our data set, elevation and precipitation were not significantly correlated (r = 0.151; P > 0.05), nor elevation and temperature (r = –0.314; P > 0.05).

Discussion

In this study, whitefly individuals were collected in 81 tomato fields in Costa Rica in 2015 and 2016. *Trialeurodes vaporariorum* was the most abundant species. Previous reports indicated the presence of this species in the country associated with different crops, especially tomato (Morales et al. 2005, Guevara-Coto et al. 2011, Vargas-Asencio et al. 2013). The present study confirms the distribution of *B. tabaci* MED in field-grown tomato, especially in the Central Region of the country where the majority of tomato production is located. In 2009, Guevara-Coto et al. (2011) made the first report of this species in greenhouse production. Later surveys identified this species in open fields (Barboza et al. 2019a).

Bemisia tabaci NW was also detected. This species was last reported in the country over 10 yr ago (Morales et al. 2005) and was believed to be absent due to the presence of the invasive whiteflies *B. tabaci* MED and *B. tabaci* MEAM1. In the present study, *B. tabaci* NW was more abundant in the outer limits of the Central Region where tomato production is less intensive. This finding could indicate that this species is being displaced by invasive species; however, *B. tabaci* NW, *B. tabaci* MED, and *T. vaporariorum* could still be found in sympatry in tomato. The invasive *B. tabaci* MED may be displacing the native species in Costa Rica, due to its lower susceptibility to several insecticides such as piriproxyfen and the neonicotinoids (Horowitz and Ishaaya 2014). These are among the most commonly applied compounds in Costa Rica; piriproxyfen is the most widely used insecticide in important crops in terms of production and consumption, including tomato (Morera, 2015). Similar reports have been published from Venezuela and Brazil (Romay et al. 2011, Marubayashi et al. 2012).

Bemisia tabaci MEAM1 was the least abundant species in this study. This result is similar to that of a 2011–2012 survey in which this species was found only in sweet pepper and bean (Barboza et al. 2019a). Recent reports in Western Hemisphere indicate that *B. tabaci* MEAM1 is the predominant species in the *B. tabaci* complex in important agricultural regions (Marubayashi et al. 2012, McKenzie et al. 2012, Rodríguez et al. 2012, Romay et al. 2016), although Alemandri et al. (2015) reported a similar frequency for *B. tabaci* MEAM1 and *B. tabaci* NW2 in bean in Argentina. Despite its predominance in these agricultural regions, studies in controlled environments show that application of the most common insecticides causes the artificial selection of *B. tabaci* MED over *B. tabaci* MEAM1 in tomato crops (Sun et al. 2013); this may be one of the reasons for the low abundance of *B. tabaci* MEAM1 reported in this study.

Twenty-two haplotypes for the mtCOI region have been described for *T. vaporariorum* in the Western Hemisphere; however, only individuals with the Tv-i haplotype were identified in this study. This is the same haplotype found in most of *T. vaporariorum* individuals previously reported by Barboza et al. (2019a) and also matched with whitefly sequences available from Spain and France. Out of the seven *B. tabaci* MED haplotypes previously reported in America (Barboza et al. 2019a), a single haplotype was detected in the current study (MED-i). Previously, the MED-i and MED-ii haplotypes were identified in Costa Rica, which suggests that individuals with

the MED-ii haplotype may be present in low abundance. Individuals with the *B. tabaci* MEAM1-i haplotype were found in the Alajuela province. The sequences were identical to previously reported from individuals collected in sweet pepper and common bean in the Cartago province (GenBank KY441488). Our findings confirm that this haplotype is present in Costa Rica, although in low numbers. In this study, a *B. tabaci* NW haplotype is described for the first time and compared with sequences available for this species in the online databases (www.GenBank.com). With the exception of one sequence from Sudan (Gueguen et al. 2010), this species has not been found in other continents. *B. tabaci* NW sequences taken from the databases and included in the phylogenetic tree corresponded to samples from North and Central America ranging from the United States to Panama (Fig. 2). In contrast, the NW2 species has been reported in South America (Marubayashi et al. 2012, Alemandri et al. 2015). The high frequency of nsSNPs present in *B. tabaci* NW may be explained by the fact that this species is endemic to America and may have adapted to different agroclimatic conditions in the countries where it is present.

The presence of three endosymbionts was confirmed in whiteflies collected in tomato fields in the main areas of tomato production in the country. Regarding the endosymbionts not considered in this

Fig. 3. Abundance of endosymbionts *Arsenophonus, Rickettsia* and *Wolbachia* in whitefly species *Trialeurodes vaporariorum, Bemisia tabaci* MED and *B. tabaci* NW collected in tomato fields in 2015 and 2016.

Table 3. Bacterial endosymbionts frequency identified in individual whiteflies collected in the main areas of tomato production in Costa Rica

Province	Whitefly species (number of individuals)	% Endosymbiont		
		Arsenophonus	*Rickettsia*	*Wolbachia*
Alajuela	*Trialeurodes vaporariorum* (133)	100	8	2
	Bemisia tabaci MED (82)	4	89	76
	Bemisia tabaci NW (2)	0	100	0
	Bemisia tabaci MEAM1 (2)	0	100	0
Cartago	*Trialeurodes vaporariorum* (129)	81	9	1
	Bemisia tabaci MED (64)	2	95	80
	Bemisia tabaci NW (8)	0	38	0
Guanacaste	*Bemisia tabaci* NW (10)	0	0	0
Heredia	*Trialeurodes vaporariorum* (52)	83	21	0
	Bemisia tabaci MED (12)	17	100	50
Puntarenas	*Trialeurodes vaporariorum* (100)	84	0	0
	Bemisia tabaci MED (2)	0	100	100
	Bemisia tabaci NW (7)	0	0	0
San José	*Trialeurodes vaporariorum* (16)	56	0	0
	Bemisia tabaci MED (9)	11	100	44

study, to our knowledge, it is not clear if *Cardinium* and *Fritschea* play a role in whiteflies population fitness or in an ecological interaction relevant for agricultural purposes in the studied species. In America, *Cardinium* has been found in low numbers in *B. tabaci* MEAM1 and *B. tabaci* MED from Brazil (De Moraes et al. 2018). *Fritschea* has been identified in low frequencies in *B. tabaci* MEAM1 and *B. tabaci* NW2 (Marubayashi et al. 2014) and has been absent in many other populations (Skaljac et al. 2010, Chu et al. 2011, Ghosh et al. 2015). The secondary endosymbiont *Hemipteriphilus* has been reported in *B. tabaci* group of species named China 1 or formerly known as biotype ZHJ3 (Bing et al. 2013a, b).

In this study, an association between *Arsenophonus* and *T. vaporariorum* was found, similar to that described by Kapantaidaki et al. (2014) and Skaljac et al. (2013). The composition of endosymbiont populations associated with *B. tabaci* MED varies among countries in Latin America, and for the most part, *Arsenophonus* is absent (Barbosa et al. 2014). In Costa Rica, *Rickettsia* was found in more than 90% of the *B. tabaci* MED individuals. It is important to note that this endosymbiont is characteristically found outside of the bacteriocyte in the hemolymph (Caspi-Fluger et al. 2011) and can interact with organs in the body cavity of the insect and with circulative viruses such as *Tomato yellow leaf curl virus* (TYLCV) (Kliot et al. 2014). Further studies of this endosymbiont in *B. tabaci* MED populations may provide more information about its impact on the ecology of this species in Costa Rica.

Rickettsia was present in *B. tabaci* NW individuals in the Central Region of the country, but not in the Puntarenas or Guanacaste provinces. This may be evidence of a horizontal interspecific transmission event (Chrostek et al. 2017, Li et al. 2017a,b). The whiteflies collected in the Central Region were found coexisting in tomato fields with high numbers of *B. tabaci* MED, which is frequently associated with *Rickettsia* (Table 3). The frequent association of *B. tabaci* MED and *Rickettsia* in different sampling sites (Table 3) may be due to the genetic homogeneity found in *B. tabaci* MED (a single haplotype). Previous studies have shown a relationship between whitefly genetic heterogeneity and the heterogeneity of their endosymbionts (Gnankiné et al. 2012, de Moraes et al. 2018). The association of *Wolbachia* with *B. tabaci* MED also requires attention since it has been associated with increased adaptation and number of female progeny in *B. tabaci* MED (Xue et al. 2012).

A number of studies have suggested that environmental factors can affect the frequency of the endosymbiont on the host (Doremus et al. 2017, Sepúlveda et al. 2017, Zhu et al. 2018). In this study, precipitation, temperature, and elevation were considered. Secondary endosymbionts have been reported to help hosts cope with extreme environmental conditions (Oliver et al. 2010), nevertheless, in temperatures that vary from 18 to 30°C and annual precipitation that ranges from 950 to 4,000 mm, the secondary endosymbiont presence in whiteflies was more likely to be affected by elevation (800–1,600 m a.s.l.). It is known that *B. tabaci* MED does not adapt well to elevations exceeding 1,000 m a.s.l. (Morales and Jones 2004), so it was expected to find a lower frequency of this species relative to *T. vaporariorum* at higher elevations. The fact that *Wolbachia* and *Rickettsia* frequencies in *B. tabaci* MED did not increase significantly with elevation is probably related to the introduction of this species. Because there are a few introduced genetic groups of *B. tabaci* in the country (MED-i and MED-ii) and secondary endosymbionts are mainly vertically transmitted, if few environmental factors are involved, these whiteflies will tend to harbor the same endosymbionts. Studies in aphids show that the vertical transmission efficiency can be affected by temperature (Liu et al. 2019), but according to our results, temperature was not correlated with elevation. In the case of *T. vaporariorum*, it was more likely to be found harboring *Arsenophonus* at higher elevations. Although *Arsenophonus* is highly associated with *T. vaporariorum* around the world, the increase of their presence in the host at higher elevations has not been mentioned before. Because it is an endemic species, the pattern could be related to adaptation, but more data are needed to support this claim.

In conclusion, through surveys conducted in 2015 and 2016, whitefly diversity in commercial tomato fields in Costa Rica was monitored. *Trialeurodes vaporariorum* was the predominant species; however, the presence of *B. tabaci* MED has increased in the last 10 yr in some areas such as Cartago, where previously only *T. vaporariorum* was detected. The identification of *B. tabaci* NW individuals confirmed that this species has not been completely displaced by the invasive species *B. tabaci* MED and *B. tabaci* MEAM1. Haplotype analysis confirmed the presence of the most frequent groups of *T. vaporariorum*, *B. tabaci* MED, and *B. tabaci* MEAM1 previously reported in the country; however, the haplotype found for

B. tabaci NW did not coincide with any other reported in America. An association between whitefly species and secondary bacterial endosymbionts was observed and elevation was an environmental factor most likely to affect their presence. *Arsenophonus* was found most frequently in *T. vaporariorum* at higher elevations and the endosymbionts *Wolbachia* and *Rickettsia* were most frequently associated with *B. tabaci* MED.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.Table S1. Sequences for the mtCOI region of *B. tabaci* NW from America and their respective haplotypes obtained from GenBank and used in this study.Figure S1. Electrophoretic analysis in a 1 % agarose gel of the restriction of ~800 bp mtCOI gene fragments of PCR products digested with *Tru9*I and *TaqI*. Gel order: 1=*B. tabaci* Mediterranean, 2=*Bemisia tabaci* Middle East-Asia Minor 1, 3=*Trialeurodes vaporariorum*, 4=*B. tabaci* New World.

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