Prevalence and Diversity of Endosymbionts in Cassava Whiteflies (Hemiptera: Aleyrodidae) From Colombia

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Abstract

Whiteflies cause huge economic losses for cassava (Manihot esculenta Crantz) cultivation. Damage can be caused directly when the insects feed on the phloem and/or indirectly by the transmission of viruses. It has been found that whiteflies maintain a close relationship with some endosymbiotic bacteria and that this interaction produces different effects on host biology and can also facilitate viral transmission. This study aimed to characterize the diversity of secondary endosymbionts (SE) present in whiteflies associated with cassava. Whitefly adults and nymphs were collected from cassava crops at nine locations in Southwestern Colombia. Molecular identification of insects and endosymbionts was carried out using specific mtCOI, wsp, 23s rRNA, and 16s rRNA primers. Phylogenetic trees were constructed from these sequences, both for whitefly species and the endosymbionts found. In addition, morphological identification of whitefly species was made using last instar nymphs. Molecular and morphological evaluation revealed that the most abundant whitefly species was Trialeurodes variabilis (Quaintance) followed by Aleurotrachelus socialis (Bondar) and Bemisia tuberculata (Bondar). One hundred percent of the individuals contained the primary endosymbiont Portiera. The SE Rickettsia, Hamiltonella, Wolbachia, and Fritschea were not detected in the samples tested. Prevalence of Cardinium and Arsenophonus were variable at each locality, Cardinium being most prevalent in A. socialis adults. This study is the first report on the presence of Cardinium and Arsenophonus in A. socialis and T. variabilis. It is also the first report of endosymbiotic diversity in whiteflies associated with cassava in Colombia.

Key words: diversity, whitefly, cytochrome oxidase I, phylogenetics, secondary endosymbionts

After rice and corn, cassava (Manihot esculenta) is the most important source of calories for humans in the tropics and is essential for food security in many countries in the Americas and Africa (FAO 2015). Worldwide, one of the biggest problems for its production is the presence of whiteflies (Hemiptera: Aleyrodidae), a complex of species that causes direct damage by feeding on the phloem and, indirectly, through the transmission of more than 100 different virus species (Legg and Fauquet 2004, Carabali et al. 2008, Navas-Castillo et al. 2011).

Of the 1,556 species of whitefly described (Forero 2008), only 15 are associated with cassava cultivation (Vásquez-Ordóñez et al. 2015), and only Bemisia and Trialeurodes genera are vectors of disease-causing viruses (Bellotti and Arias 2001, Navas-Castillo et al. 2011). In Africa, e.g., Bemisia tabaci (Gennadius) transmits the viruses causing cassava brown streak disease (CBSD) and cassava mosaic disease (CMD), the latter also transmitted by this species in Southern Asia. Both CBSD and CMD annually cause losses of millions of dollars of cassava crops in both Africa and Indian subcontinent (Legg and Fauquet 2004, Ghosh et al. 2015, Karthikeyan et al. 2016).

In Latin America, cassava is mainly colonized by whitefly species that cause losses as a consequence of direct feeding on the phloem (Carabali et al. 2005). Specifically, two species of whitefly, Aleurotrachelus socialis (Bondar) and Trialeurodes variabilis (Quaintance), can cause losses of up to 79% in crop yield depending on the period of infestation and the variety cultivated in the north of South America (Bellotti and Arias 2001, Carabali et al. 2010, Vásquez-Ordóñez et al. 2015). However, there are no reports of virus transmission in cassava by B. tabaci. This is possibly due to the limited capacity of B. tabaci MEAM1 to colonize cassava crops in the Americas (Carabali et al. 2008).

As in other insects, whiteflies harbor intracellular bacteria known as endosymbionts (Baumann 2005, Baumann et al. 2006) that provide their host with essential nutrients that they lack or have little access to (Fedhaar et al. 2007, Santos-Garcia et al. 2012). In some cases, they also participate in the detoxification of secondary metabolites present in their diet (Fedhaar 2011) or are associated with increased resistance to pathogens (Su et al. 2013b) and predators (Oliver et al. 2003), contributing to the evolutionary and
ecological success of the host (Moya et al. 2008, Feldhaar 2011). Endosymbionts are classified into two categories, mainly according to degree of host association. Primary endosymbionts (PE) are usually mutualistic and host-specific, and their transmission is strictly vertical. They exhibit long-term co-evolution with their host and are, therefore, fundamental for survival and/or reproduction (Wernegreen 2002, Su et al. 2013a). Examples of this are Buchnera in aphids, Blochmannia in Camponotus ants, Carsonella ruddi in psyllids, and Portiera aleyrodarum in whiteflies (Wernegreen 2002, Kikuchi 2009, Su et al. 2013a). In the latter case, Portiera participates in the synthesis of essential aminoacids for its host since the phloem that these insects feed on contains low quantities of these aminoacids (Baumann 2005, Santos-Garcia et al. 2012, Upadhyay et al. 2015).

Conversely, secondary endosymbionts (SE) found in a wide variety of hosts can be parasites or mutualists, and their transmission can occur both vertically and horizontally (Gottlieb et al. 2008, Clark et al. 2010, Su et al. 2013a). Also, compared to PEs, SEs are not restricted to bacteriocytes as they do not have an obligate symbiotic relationship with their host and can inhabit a wide variety of tissues including bacteriocytes, salivary glands, Malpighi tubes, and reproductive organs. Additionally, they can be eliminated from their host without affecting the viability of their populations (Ruan et al. 2006, Ahmed et al. 2010, Clark et al. 2010, Xue et al. 2012, Su et al. 2014). However, in some hemipterans where SE complement the functions of PE, the relationship between host and SE has become closer, turning into a co-obligated association (McCutcheon et al. 2009, Manzano-Marín and Latorre 2014).

SE such as Hamiltonella, Wolbachia, Cardinium (Chiel et al. 2007), Rickettsia, Arsenophonus (Thao and Baumann 2004), and Fritschea (Everett et al. 2005) have been detected in whiteflies. Ghanim and Kentsadalo (2009) and Kentsadalo et al. (2008) proposed that some of these endosymbionts may be related to the insecticide resistance exhibited by certain species of whiteflies. Likewise, an association between the presence of SE in these insects and the efficiency of viral transmission has been detected (Morin et al. 2000). Gottlieb et al. (2010) reported the presence of Hamiltonella in B. tabaci MEAM1 which could facilitate transmission of the tomato yellow leaf curl virus; while Rana et al. (2012) detected Arsenophonus in B. tabaci Asia II which may be involved in the transmission of the cotton leaf curl virus. Similarly, it has been observed that the interaction between SE and its host not only enhances its ability to transmit viruses, but also provides several advantages at the fitness level. For example, a significant increase in both the number of eggs and nymph survival, shorter development time and larger body size (Su et al. 2013a), an increase in fertility, tolerance to insecticides and a greater number of female offspring (Brummin et al. 2011). Additionally, SE such as Wolbachia in B. tabaci MED (Xue et al. 2012) can also have important effects on processes such as resistance to parasitoids.

Currently, most of the studies that report endosymbiotic diversity in whiteflies have been carried out in Africa, Asia, and Europe (Ahmed et al. 2010, Gueguen et al. 2010, Thierry et al. 2011, Bing et al. 2013, Škaljic et al. 2013, Tajebe et al. 2014, Ghosh et al. 2015) and mainly focus on B. tabaci, leaving aside other species of this complex. However, in Latin America, specifically in Brazil, Marubayashi et al. (2014) identified the diversity of endosymbionts in different aleurodid species associated with crops such as tomato, cassava, eggplant, and others, reporting the presence of Arsenophonus in Trialeurodes vaporariorum (Westwood); Wolbachia, Arsenophonus, Cardinium, and Fritschea in Bemisia tabesculata (Bondar) and Wolbachia, Cardinium, and Fritschea in Tetraleurodes acacia (Quaintance). In Colombia, despite the importance of endosymbionts, there are no studies, to date, that report on the diversity of SE present in whiteflies. Identification of these endosymbionts is important given the ability of some SE to modify, and even increase, the fitness of their hosts. Their role in facilitating viral transmission by the insect could be influencing the recorded losses in cassava crops. Therefore, this study aimed to identify the SE in whitefly species associated with cassava crops from different localities in southwestern Colombia.

Materials and Methods

Whitefly Collection and DNA Extraction

Whitefly adults were collected from cassava crops at nine locations in the Departments of Cauca, Valle del Cauca, and Quindio in Colombia between August and November 2015 (Fig. 1; Supp Table 1 [online only]). Adults were captured using oral aspirators and preserved in 90% ethanol at −20°C previous to molecular analysis. In addition, the last instar nymphs were collected to corroborate the species present at each locality through morphological identification. Total DNA was extracted following the method proposed by Frohlich et al. (1999), individually macerating 24 to 30 adult females for each location.

Whitefly Identification and SE Screening

For the molecular identification of the Aleyrodidae species, PCRs were performed using specific primers for A. socialis (Lundgren et al. 2014) and universal mitochondrial cytochrome oxidase I (mtCOI) (Folmer et al. 1994) (Table 1). For the detection of SE, specific primers available in the literature were used for Portiera, Rickettsia, Arsenophonus, Hamiltonella, Wolbachia, Fritschea, and Cardinium (Table 1).

PCR reactions were carried out using 2 μl of template DNA, 2 mM dNTPs, 50 mM MgCl 2, 10 μM of each primer, 1 U of Taq BIOLASE DNA polymerase (Bioline, United Kingdom), 10× buffer solution (NH 4). Cycling conditions used for mtCOI (Folmer et al. 1994) were those proposed by Ovall et al. (2014). For the amplification of the endosymbionts, the thermal profile comprised an initial phase at 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, Tm for 45 s (Table 1) and 72°C for 1 min, with a final extension at 72°C for 5 min. All PCRs were carried out in a Veriti thermocycler (ThermoFisher, United States) and negative and positive controls were included. Due to the small sizes of the fragments, the PCR products from A. socialis were visualized on 2% agarose gels while the other products were visualized in gels of 1% agarose with ethidium bromide. For each locality, between 5 and 10 positive amplicons (five amplicons for SE, and 10 for mtCOI gene) were randomly selected and sent to the Macrogen Corporation, United States for sequencing. Sequences were identified by comparison with those deposited in GenBank using BLASTn (NCBI, available online) and were deposited in the NCBI GenBank (GenBank accession numbers: MG812215-MG812280 and MG840316-MG840339).

With minor modifications, for each locality, five to eight nymphs from the last nymph instar were mounted on slides following, the method described by Hodges and Evans (2005). The nymphs were rinsed in 10% potassium hydroxide (KOH) over a period of 24 h (whitish nymphs) to 48 h (dark nymphs), and acid fuchsin was then added to facilitate visualization. Aleyrodidae whitefly species were morphologically determined using keys and descriptions by Caballero (1994), Dooley (2006), Hodges and Evans (2005), Martin (2005), and Ovall et al. (2014) (Supp Table 2 [online only]).
Phylogenetic Analysis

The sequences from both mtCOI and the 16s rDNA gene (Supp Table 3 [online only]) were aligned with sequences available in GenBank using ClustalW (Hall 1999). Subsequently, jModelTest 2.1.6 (Darriba et al. 2012) was used to determine the nucleotide substitution model with best fit according to the AIC criterion. Phylogenetic trees were constructed using 1) the Bayesian inference method in Mr Bayes (Ronquist and Huelsenbeck 2003) with 30,000,000 generations and 2) the maximum likelihood (ML) method in PAUP 4.0 (Swofford 2002) with 1,000 bootstraps. The GTR + I + G nucleotide substitution model was used for mtCOI and Portiera, the TPM2 + G model for Arsenophonus, and the TPM1 + G model for Cardinium. All phylogenetic analyses were performed on the CIPRES platform (Miller et al. 2015).

Results

Whitefly Identification

A total of 264 whitefly adults were analyzed using primers for mtCOI (Table 1). One hundred sixty-three were found to be T. variabilis (62%), 96 A. socialis (36%), and 5 B. tuberculata (2%). Trialeurodes variabilis was dominant in the localities of Armenia (100%), Sevilla (100%), CORPOICA (100%), and Caldono (79%) while A. socialis predominated in the localities of La Tebaida, Santander de Quilichao I and II with percentages ranging from 53% (SQ I) to 83% (La Tebaida). Bemisia tuberculata was detected with percentages lower than 10% in the localities of Cali and SQ I and II. Likewise, a tendency in relation to the dominance of whiteflies in the different localities was observed whereby an increased presence of A. socialis was associated with a decrease in T. variabilis (Fig. 1).
The morphological analysis of the last instar nymphs confirmed the presence of the three species of whitefly associated with cassava crops. All nymphs identified from the Sevilla, CORPOICA, and Armenia locations were *T. variabilis* while both *A. socialis* and *T. variabilis* nymphs were found from the La Tebaida, CIAT, Cali, and SQ I locations. While *B. tuberculata* was only detected in SQ II (Fig. 2; Supp Table 2 [online only]). The above is congruent with *B. tuberculata* and SQ I locations. While nymphs were found from the La Tebaida, CIAT, Cali, and Armenia locations were *A. socialis* and *T. variabilis* and while both species were separated into three different groups: A, B, and C, respectively (Fig. 5).

In the phylogenetic reconstruction based on the 16s rDNA gene for SE *Arsenophonus*, the sequences from this study were grouped into two different clades (Fig. 6). In Clade A were grouped *Arsenophonus* sequences from *A. socialis* and *T. variabilis* collected at the same locality (La Tebaida); whereas in Clade B, two *Arsenophonus* sequences from *B. tuberculata* were grouped together. Similarly, for the phylogenetic reconstruction carried out with the 16s rDNA gene sequences for SE *Cardinium*, the sequences from this study were grouped into two clades (Fig. 7). The *A. socialis* and *T. variabilis* sequences are found in Clade A and those from *B. tuberculata*, as well as one from *T. variabilis* from Santander de Quilichao II, were grouped in Clade B. These results may be demonstrating the presence of different strains for both *Arsenophonus* and *Cardinium* associated with their host.

**Discussion**

Both morphological and molecular results for the identification of whitefly species were congruent, confirming the presence of *T. variabilis*, *A. socialis*, and *B. tuberculata* in Colombian cassava crops. According to Gold et al. (1990), Carabalí et al. (2008, 2010), Vásquez-Ordóñez et al. (2015), cassava crops in Colombia are mostly colonized by these three species of whitefly, being *A. socialis* the predominant species with percentages of prevalence reported ranging from 66 to 88%. However, *T. variabilis* was the dominant species in our study. A possible explanation could be the behavior of *A. socialis* population which increases its size during the rainy season (Bellotti 2008). Nevertheless, in this study sampling was carried out between August and November 2015, a period coinciding with the El Niño phenomenon (IDEAM 2016) characterized by high temperatures and severe droughts. Another factor that could explain these differences is the altitude at which the crops were sampled. *Aleurotrachelus socialis* is generally restricted to altitudes below 1,200 meters above sea level (m.a.s.l) while *T. variabilis* increases at altitudes above 1,000 m.a.s.l. (Bellotti 2008), and six of the nine sampled localities are found above this elevation (Supp Table 1 [online only]).
On the other hand, that the PE *Portiera* was found in 100% of the individuals analyzed is consistent with that reported in studies of endosymbiotic diversity in whiteflies (Bing et al. 2013, Tajebe et al. 2014, Ghosh et al. 2015), and it is expected because this PE is responsible for synthesizing essential amino acids that complement the diet of aleurodids (Santos-Garcia et al. 2012, Rao et al. 2015, Fig. 3. State of individual infection in adults of the three species of whitefly collected in this study. The panels represent the whitefly species detected in each locality. The columns correspond to the endosymbionts evaluated: *P. Portiera*; *H. Hamiltonella*; *R. Rickettsia*; *A. Arsenophonus*; *W. Wolbachia*; *C. Cardinium*. The rows represent the individuals evaluated for each species at each location. The gray boxes indicate positive infection by the corresponding endosymbiont. The location and the number of individuals analyzed (n) are found at the top of each panel.
In the case of SE, our study constitutes the first report of the presence of *Arsenophonus* in *A. socialis* and *T. variabilis*. Typically, this symbiont is prevalent in *T. vaporariorum* (Škaljac et al. 2013, Kapantaidaki et al. 2015). However, in South America, *Arsenophonus* has been reported in populations of *T. vaporariorum*, *B. tabaci* MED, and *B. tuberculata* whiteflies from Brazil (Marubayashi et al. 2014, de Moraes et al. 2017). In this study, *Arsenophonus* was only found to be very prevalent in the locality of La Tebaida, however its role or functionality still unknown. It is possible that the presence of this SE in La Tebaida population is related to host fitness, as occurs with other endosymbionts (Brumin et al. 2011, Xue et al. 2012, Ghosh et al. 2018), or it may be a transient infection propitiated by momentary abiotic factors. More in-depth studies are required in both cases.

Nonetheless, *Cardinium* was detected in most of the localities sampled, prevalence varying according to the locality (8–80%). For South America, particularly in Brazil, Marubayashi et al. (2014) and de Moraes et al. (2017, 2018) reported the presence of this endosymbiont in populations of *B. tabaci* (MEAM 1, MED, and NW), *B. tuberculata* and *Tetraleurodes acaciae*. However, it is not known to host fitness, as occurs with other endosymbionts (Brumin et al. 2011, Xue et al. 2012, Ghosh et al. 2018), or it may be a transient infection propitiated by momentary abiotic factors. More in-depth studies are required in both cases.
whether this bacterium plays any role in the fitness of these populations or if it manipulates host reproduction through cytoplasmic incompatibility or feminization of males, as has been found in some species of parasitoid wasps of the genus Encarsia (Zchori-Fein et al. 2001, Hunter et al. 2003, Engelstädter and Hurst 2009). This is a possibility because Cardinium strains from B. tabaci and Encarsia are highly related: these two insect species exhibit a host (whitefly) parasitoid relationship, making it possible for them to share and transmit bacterial symbionts to each other (Santos-Garcia et al. 2014). It is noteworthy that only in the localities of La Tebaida and Cali was there evidence of double infection of Cardinium and Arsenophonus with high prevalence (>75%). This may be suggesting a possible complementary role for these two endosymbionts within their host and/or low competition for space and resources by both endosymbionts (Vautrin and Vavre 2009, Skaljac et al. 2010). The latter could be related to the location of these symbionts within their host since Arsenophonus is generally located inside the bacteriocytes while Cardinium can be found outside of them (Skaljac et al. 2013, Marubayashi et al. 2014), possibly reducing competition for space.

On the other hand, the low diversity of SE detected in this study could be explained by biological or technical processes. First, it is possible that the PE supplies all host metabolic functions

![Bayesian inference tree constructed from sequences of the 16S rDNA gene for PE Portiera (780 bp). The nodes in parentheses show the Bayesian posterior probability values (>0.6) and the bootstrap values (>70), respectively. The sequences of this study are highlighted in bold print. GenBank accession numbers appear in square brackets.](image-url)
In which case the maintenance of other guests could generate a load on the host, negatively affecting fitness in terms of survival and viability (Gottlieb et al. 2008, Vautrin and Vavre 2009). Secondly, it is likely that the prevalence of endosymbionts inside the insects may be higher than that detected due to the use of inadequate primers, or

(Sloan and Moran 2013, Santos-Garcia et al. 2015) in which case the maintenance of other guests could generate a load on the host, negatively affecting fitness in terms of survival and viability (Gottlieb et al. 2008, Vautrin and Vavre 2009). Secondly, it is likely that the prevalence of endosymbionts inside the insects may be higher than that detected due to the use of inadequate primers, or
to low sensibility of the PCR compared to metagenomics methods (Ghosh et al. 2015), which would permit broad-spectrum detection of the endosymbiotic bacteria present in these insects (Bansal et al. 2014, Gauthier et al. 2015, Coon et al. 2016).

The phylogenetic reconstruction obtained from the mtDNA sequences, in which the division between the Aleurodicinae and Aleyrodinae whitefly subfamilies can be observed, confirm that all sequences from this study were grouped within the Aleyrodinae clade (Fig. 4, clades A, B, and C). It should be noted that sequences from T. variabilis showed several subgroups within clade A (Fig. 4), suggesting a high degree of diversity in mitochondrial DNA within this species of whitefly. However, these differences are not linked to geographical separation, individuals from different subgroups being observed within the same locality.

With respect to the phylogenetic reconstructions for both PE and SE, the formation of two main clades was observed. In the phylogeny of PE Portiera (Fig. 5), the sequences of three whitefly species were grouped within the Aleyrodinae clade, separated into three distinct groups, suggesting the presence of several Portiera strains related to each species of whitefly. This is consistent with reports by Sloan and Moran (2013) and Santos-Garcia et al. (2015) who suggest that the PE strains of Bemisia and Trialeurodes have recently diverged (90 Ma), demonstrating a strong association and a long co-evolutionary process between Portiera and the different whitefly species.

For the phylogeny of SE Arsenophonus, the sequences of this study were grouped into two clades (Fig. 6), which may be evidence of the existence of two genetic groups of Arsenophonus in aleurodids associated with cassava, whereby T. variabilis and A. socialis share the same strain for this SE but B. tuberculata is infected by a different strain. As in our study, Thao and Baumann (2004) also found two different groups of Arsenophonus from sequences of 16-23S rDNA. One group corresponding to Arsenophonus sequences from different species of whitefly, and the other to sequences of this endosymbiont from Bemisia.
Likewise, the sequences from our study obtained for SE Cardinium also were grouped into two well-defined clades. Where sequences from different whitefly species that shared the same locality were found within the same clade. This suggests a possible association of strains of this endosymbiont with the locality in which its host is found rather than with the species of whitefly. Furthermore, the presence of the same strain of both SE Cardinium and Arsenophonus in two different whitefly species suggests the occurrence of horizontal transmission of these endosymbionts. Similarly, de Moraes et al. (2018) found genetically close strains of Hamiltonella and Rickettsia in B. tabaci MED and MEAM 1. Evidencing that it is probable that horizontal transmission is being mediated by the insect host plant since, in this case, these species are in sympathy in the cassava crops studied. The crop may serve as a reservoir and facilitate transmission of the endosymbiont. For example, Caspi-Fluger et al. (2012) and Li et al. (2017) conducted experiments using cotton plants to mediate transmission from insects infected with Rickettsia and Wolbachia, respectively. These authors observed the occurrence of SE transfer from infected insects to the phloem of the plant, where SE could be maintained and subsequently infect SE-free insects that would feed on these plants.

Finally, this is the first study in Colombia demonstrating the endosymbiotic diversity of cassava whiteflies. An association was found between SE and the different species of whitefly analyzed with a higher prevalence of Cardinium in A. socialis than in T. variabilis. Whereas, B. tuberculata was found associated with different strains of Cardinium and Arsenophonus. Likewise, the presence of both SEs was recorded for the first time in A. socialis and T. variabilis. And, it is also worth mentioning the high degree of mitochondrial diversity detected in T. variabilis and a possible increase of this whitefly species in cassava crops in contrast to that reported in previous studies, possibly due to the altitudinal differences of the localities sampled and climatic factors.

**Supplementary Data**

Supplementary data are available at *Journal of Insect Science* online.

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