Detection of Maize Bushy Stunt Phytoplasma in Leafhoppers Collected in Native Corn Crops Grown at High Elevations in Southeast Mexico

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Detection of maize bushy stunt phytoplasma in leafhoppers collected in native corn crops grown at high elevations in southeast Mexico

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Abstract

Phytoplasmas are wall-less bacteria, unculturable in vitro, and transmitted primarily by leafhoppers (Cicadellidae). Maize bushy stunt disease has been linked to phytoplasmas belonging to the16SrI-B subgroup and vectored by leafhoppers in the genus *Dalbulus* spp. (Hemiptera: Cicadellidae). The recent detection of maize bushy stunt affecting native corn, maize, in the southeast highlands of Mexico motivated the survey to determine which leafhoppers were associated with this crop during the 2013-2014 growing season. We detected 7 leafhopper genera in native corn cultivated 2,400 meters above sea level (masl), with 4 of these genera reported for the first time in corn. Based on external morphology and male genitalia, we identified *Idiodonus wickhami* (Ball) (Hemiptera: Cicadellidae), *Amblysellus grex* (Oman) (Hemiptera: Cicadellidae), *Empoasca fabae* (Harris) (Hemiptera: Cicadellidae), *Macrosteles quadrilineatus* (Forbes) (Hemiptera: Cicadellidae), and *Dalbulus elimatus* (Ball) (Hemiptera: Cicadellidae). We were not able to identify the leafhopper genera *Graphocephala* (Hemiptera: Cicadellidae) and *Erythridula* (Hemiptera: Cicadellidae) to species because of a lack of male leafhoppers. Nymphal stages of *I. wickhami* also were identified using taxonomic and molecular tools. The presence of adults and nymphs of *I. wickhami* in the crop suggest that native corn grown in the southeast highlands of Mexico is a feeding and reproductive host for *I. wickhami.* Moreover, *I. wickhami* was found infected with 16SrI-B strain maize bushy stunt-Ver while *D. elimatus*, a well-known maize bushy stunt phytoplasma vector, was found infected with the 16SrI-B strain maize bushy stunt-Pueb.

Key Words: Maize, phytoplasma, MBS, *Idiodonus* spp., *Dalbulus* spp.

Resumen

Los fitoplasmas son bacterias sin pared celular, no cultivables in vitro, y transmitidos principalmente por saltahojas (Cicadellidae). La enfermedad del enanismo arbustivo de maiz (enanismo arbustivo del maíz, por sus siglas en inglés) se ha relacionado con fitoplasmas pertenecientes al subgrupo 16SrI-B y transmitidas por saltahojas dentr del género *Dalbulus* spp. (Hemiptera: Cicadellidae). La detección reciente de maize bushy stunt que afecta el maíz nativo en el altiplano sureste de México, motivó el sondeo para determinar cuales saltahojas estan asociadas con este cultivo durante la temporada de crecimiento del 2013-2014. Detectamos 7e géneros de saltahojas en el maíz nativo cultivado a 2,400 msnm, con 4 de estos géneros reportados por primera vez en maíz. En base a la morfología externa y los genitales masculinos identificamos a *Idiodonus wickhami* (Bola) (Hemiptera: Cicadellidae), *Amblysellus grex* (Omán) (Hemiptera: Cicadellidae), *Empoasca fabae* (Harris) (Hemiptera: Cicadellidae), *Macrosteles quadrilineatus* (Forbes) (Hemiptera: Cicadellidae) y *Dalbulus elimatus* (Bola) (Hemiptera: Cicadellidae). No pudimos identificar los géneros de saltahojas *Graphocephala* (Hemiptera: Cicadellidae) y *Erythridula* (Hemiptera: Cicadellidae) al nivel de especie debido a la falta de cicadélidos machos. También, se identificaron los estadios de ninfas de *I. wickhami* utilizando herramientas taxonómicas y moleculares. La presencia de adultos y ninfas de *I. wickhami* en el cultivo sugiere que el maíz nativo cultivado en las tierras altas del sureste de México es un hospedero sobre el cual *I. wickhami* se alimenta y reproduce. Además, *I. wickhami* se encontró infectada con la cepa 16SrI-B enanismo arbustivo del maíz-Ver, mientras que *D. elimatus*, un conocido vector de fitoplasma del enanismo arbustivo del maíz, se encontró infectado con la cepa 16SrI-B enanismo arbustivo del maíz-Pueb.

Palabras Clave: Maiz, fitoplasma, MBS, *Idiodonus* spp., *Dalbulus* spp.

Maize bushy stunt is the most serious disease affecting corn in the Americas (Alvarez et al. 2014; Pérez-López et al. 2016). Maize bushy stunt has been associated in previous studies with phytoplasma strains related to '*Candidatus* Phytoplasma asteris', which belongs to the 16SrI-B subgroup. Phytoplasmas are vectored by phloem-feeding insects, primarily leafhoppers (Hemiptera: Cicadellidae) and members of the genus *Dalbulus* have been identified as the vector of maize bushy stunt phytoplasma in maize. *Dalbulus maidis* (DeLong & Wolcott),

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D. elimatus (Ball), *D. guevari* (DeLong), *D. quinquenotatus* (DeLong & Nault), *D. gelbus* (DeLong) (Hemiptera: Cicadellidae), and *D. tripsacoides* (DeLong and Nault) (Hemiptera: Cicadellidae) transmit maize bushy stunt phytoplasma, with the corn leafhopper, *D. maidis* Delong, and the Mexican corn leafhopper, *D. elimatus* Ball, being the most efficient vectors (Madden & Nault 1983; Moya-Raygoza & Nault 1998; Weintraub & Beanland 2006). *Dalbulus maidis* can be found at low altitudes while *D. elimatus* is distributed at altitudes higher than 1,000 meters above sea level (masl) (Pinedo-Escatel & Moya-Raygoza 2015).

Along with maize bushy stunt phytoplasma, other pathogens are efficiently transmitted by leafhoppers from the genus *Dalbulus*, such as maize rayado fino virus and corn stunt spiroplasma *Spiroplasma kunkeii* (Whitcomb et al. 1986) (Entomoplasmatales: Spiroplasmataceae) (Moya-Raygoza et al. 2012). Together, the 3 pathogens form the corn stunt complex, which is a well-known cause of yield loss in corn production in Central and South America. The overlap in symptom expression caused by these pathogens has led to confusion and inaccurate disease diagnosis (Nault 1983).

Corn (*Zea mays* L. ssp. *mays*) (Poaceae) was first domesticated in Mexico around 10,000 years ago (Doebley 2004). Centuries of local selection and seed interchanges have led to the development of native corn varieties with unique genotypes (Serratos 2009). In the highlands of the state of Puebla, Mexico (> 2,400 masl), small agricultural communities use indigenous corn varieties that are adapted to the specific environmental conditions present at high altitudes (Perales & Golicher 2014). The recent detection of maize bushy stunt phytoplasma in native corn in the highlands of southeast Mexico could become a serious economic problem for the local subsistence farmers (Pérez-López et al. 2016). Phytoplasma diseases can be controlled with chemicals or cultural practices that target the insect vectors (Weintraub 2007). However, the maize bushy stunt phytoplasma vector(s) identity and biodiversity in crops of native corn grown at high elevations is not known.

This study is a preliminary survey of leafhoppers associated with native corn grown in "Sierra Norte de Puebla," at the high-altitude community of Las Trancas located in southeast Mexico. The objective of this research was to identify the leafhopper species present in native corn crops and to determine their maize bushy stunt phytoplasma infection status.

Materials and Methods

STUDY AREA AND LEAFHOPPER COLLECTION

The field survey was conducted in 2014 in native corn fields, located in the municipality of Ejido Las Trancas in the region of Zaragoza of Puebla in Mexico (19.7293°N, 97.8634°W; 2,400 masl). Fields were cropped mainly with white and blue varieties (Pérez-López et al. 2016). Leafhoppers were sampled in 2 corn fields 5 kilometers apart, once in Jul 2014 and once in Nov 2014 (4 and 8 mo after seeding). The 2 cornfields and their direct surroundings were never treated with insecticide. Insects were collected with a sweep net (diam 38 cm) and 10 sweeps per field were taken along a short transect of 10 footsteps, starting 10 m from the border of the field.

LEAFHOPPER IDENTIFICATION

Leafhopper adults were counted and identified in the laboratory using a binocular microscope. Species were keyed according to several features such as length, morphology, color, and genitalia, using figures and data referenced in DeLong (1946, 1931), Forbes (1885), Oman (1949), Hepner (1978) and Young (1977).

Molecular tools were used to identify the leafhopper nymphs. Total DNA was extracted from 5 insects using a modified CTAB method (Pérez-López et al. 2016). DNA extracts were amplified using the mitochondrial cytochrome c oxidase 1 (CO1, *cox*1) specific primers Uni-MinibarR1/Uni-MinibarF1, following the protocol previously described (Meusnier et al. 2008). PCR products were examined using 1% agarose gel electrophoresis and ethidium bromide-stained products were visualized using a GelDoc (BioRad, Mississauga, Ontario, Canada). CO1 amplification generated an approximately 150bp DNA fragment when observed under an ultraviolet (UV) transiluminator at 365 nm (AIML 26, Alpha Innotech Corp., San Leandro, California, USA).

PHYTOPLASMA DETECTION

Leafhoppers were grouped by genus or species and DNA was extracted from 2 or more individuals randomly selected, using a modified CTAB method (Pérez-López et al. 2016). DNA extracts were diluted 1:10 with 10mM Tris-Cl, pH 8.5, and used as a template in PCR to amplify the 16S rRNA-encoding gene F2nR2 fragment with primers R16F2n/R16R2 and *cpn60* Universal Target (*cpn60* UT) sequence with primers H279p/H280p: D0317/D0318 (1:7 ratio) (Gundersen & Lee 1996; Dumonceaux et al. 2014). PCR products were examined using 1% agarose gel electrophoresis with ethidium bromide staining and visualized using a GelDoc. F2nR2 and *cpn60* UT amplifications produced 1.2kb and 604 bp DNA fragments, respectively.

DNA SEQUENCING AND PHYLOGENETIC ANALYSES

The 16S, *cpn60* UT, and *cox*1 mini-barcode amplicons were purified using a QIAquick® PCR Purification Kit (QUIAGEN, Mississauga, Ontario, Canada), and directly sequenced using the corresponding primers.

Table 1. Leafhoppers collected in this study and their phytoplasma infection status.

				No. collected		
Collection date	Genus	Species	Phytoplasma status	Male	Female	Nymph
Jul 2014	*Idiodonus*	*wickhami*	+	6	5	
Jul 2014			-			20 'Red speckled'
Jul 2014	*Macrosteles*	*quadrilineatus*	*	0	1	
Jul 2014	*Amblysellus*	*grex*	-	1	2	
Jul 2014	*Empoasca*	*fabae*	-	3	4	
Jul 2014	*Graphocephala*	na	*	0	1	
Jul 2014	*Erythridula*	na	*	0	1	
Nov 2014	*Dalbulus*	*elimatus*	+	19	14	

*Samples were not analyzed due to the collection of only 1 leafhopper.

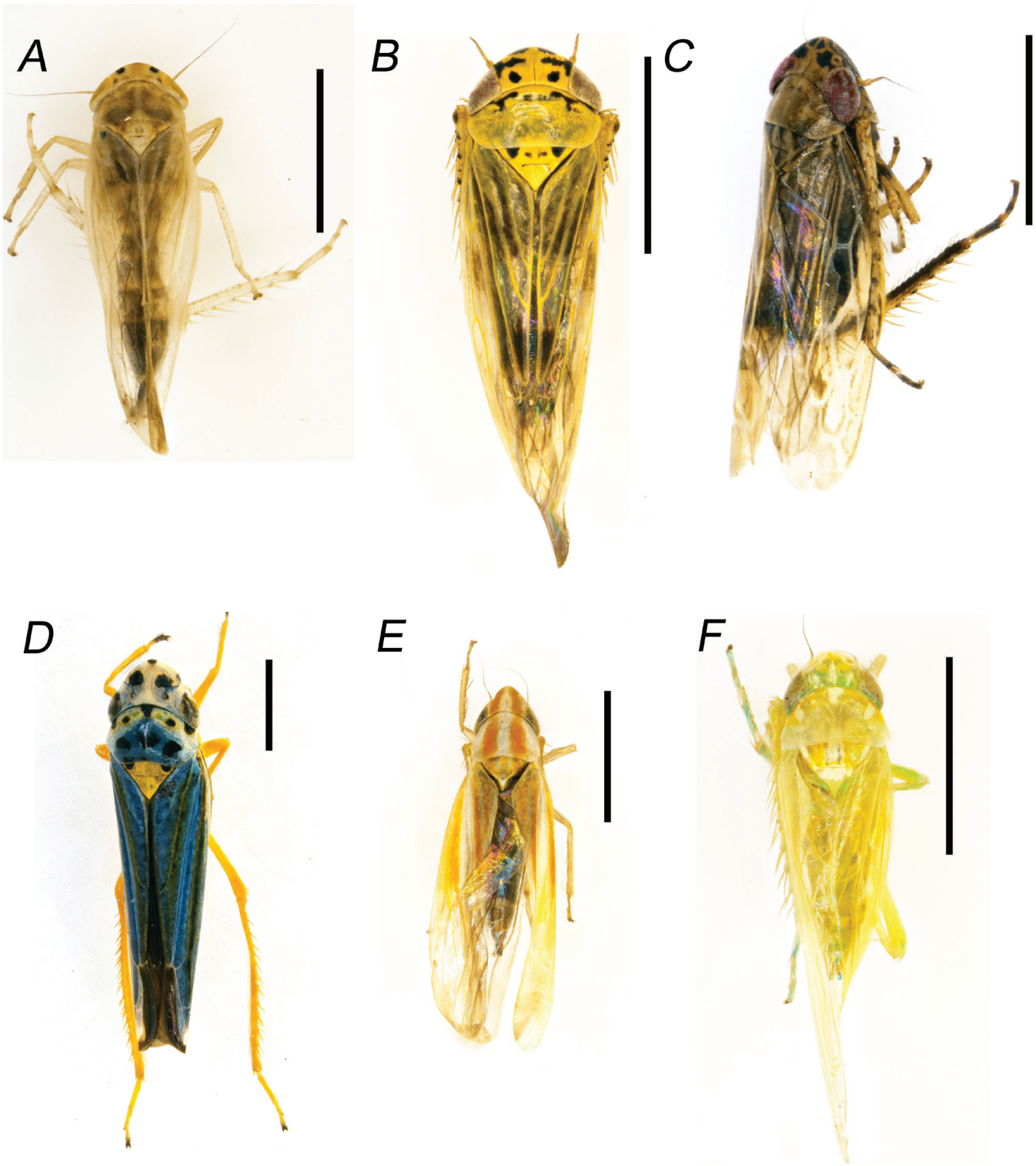

Fig. 1. Six of the 7 leafhopper genera (Hemiptera: Cicadellidae) detected in this study. Dorsal view of: (A) *Dalbulus*, (B) *Macrosteles*, (C) *Amblysellus*, (D) *Graphocephala*, (E) *Erythridula*, (F) *Empoasca*.

Phylogenetic analyses were conducted using the neighbor-joining method with MEGA v6.0 (Tamura et al. 2013), with 1,000 bootstrap replicates. All the sequences obtained were assembled using the Staden package (Bonfield & Whitwham 2010), and compared with reference sequences from GenBank through the BLAST program (http://www.ncbi.nlm.nih.gov). *Acholeplasma laidlawii* (Edward and Freund 1970) (Acholeplasmatales: Acholeplasmataceae) strain PG-8A (U14905) was used as outgroup to root the tree generated for F2nR2 and *cpn60* UT, and a member of the family Membracidae (GU013584) was used as outgroup to root the tree generated with *cox*1 mini-barcode.

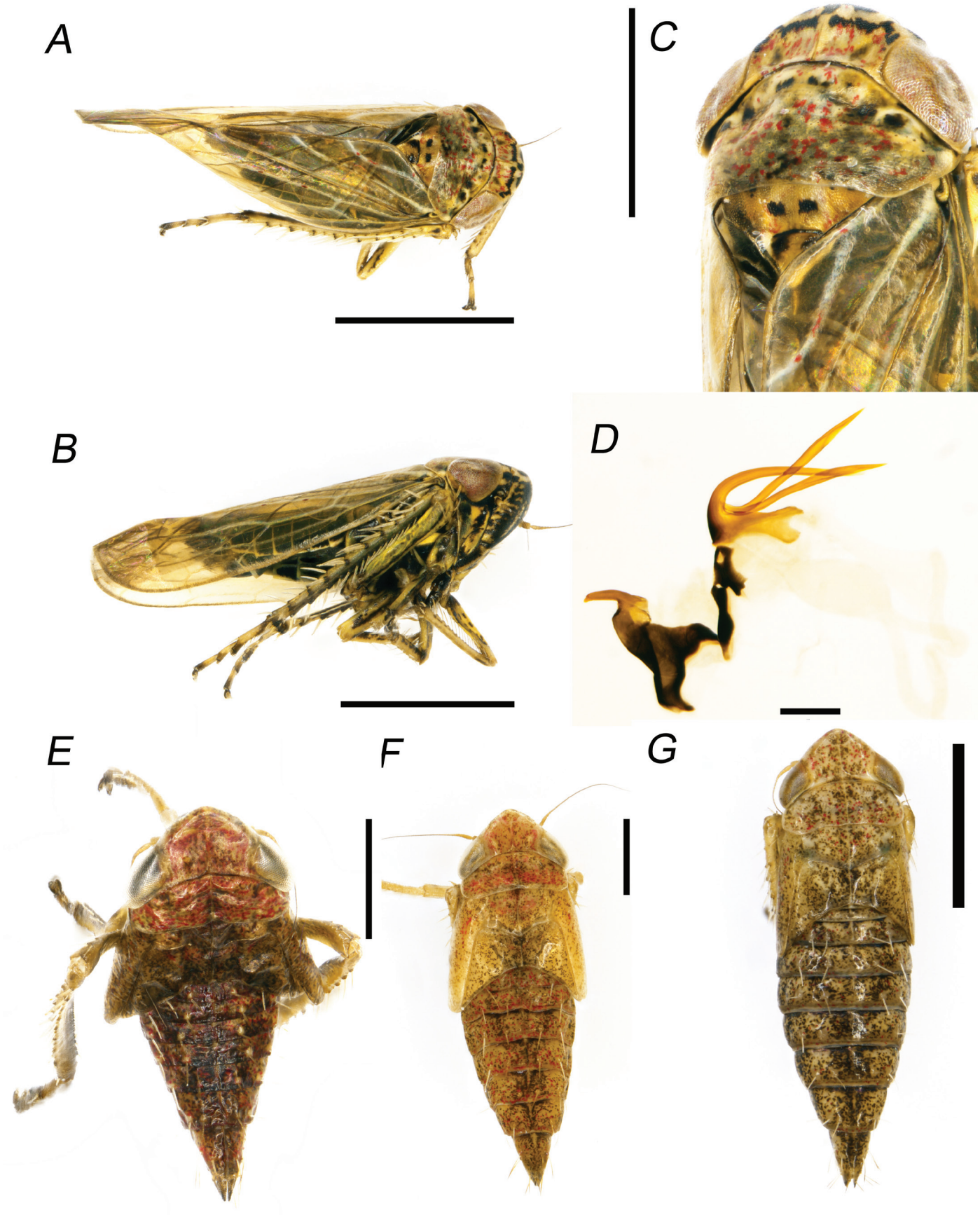

Fig. 2. *Idiodonus wickhami* Ball. (Hemiptera: Cicadellidae). (A) Dorsal view, (B) ventral view, (C) vertex, pronotum and scutellum, (D) Male genitalia, (E-G) *I. wickhami* nymphs.

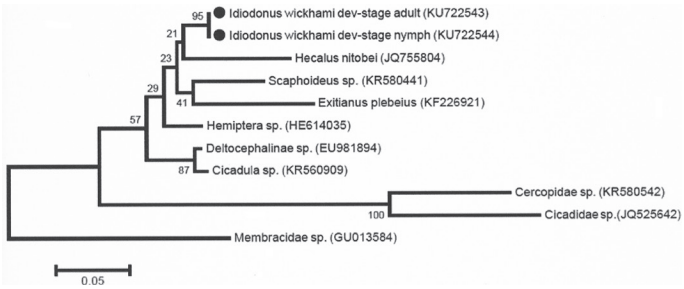

Fig. 3. Evolutionary analysis conducted through a neighbor-joining phylogenetic tree between the *cox*1 mini-barcode sequences obtained for the red speckled nymphs and *Idiodonus wickhami* (Hemiptera: Cicadellidae) (both marked with a circle) with reference sequences from GenBank. Bar 5 substitution in 100 positions.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSES

The 1.2 kb of F2nR2 sequence obtained from phytoplasma-positive leafhoppers were digested with endonucleases *Alu*I, *Bst*UI, *Hae*III, *Hinf*I and *Tsp509*I (Thermo Scientific, Mississauga, Ontario, Canada), and the restriction fragment length polymorphism (RFLP) pattern compared between them and with the previously recorded RFLP pattern (Lee et al. 2004). Reactions with *Alu*I, *Bst*UI, *Hae*III, and *Hinf*I were incubated at 37 °C, while reaction with *Tsp509*I was incubated at 65 °C according to the manufacturer's recommendations (Thermo Scientific, Mississauga, Ontario, Canada). Once digested, the samples were observed through electrophoresis using 4% UltraPure™ Agarose 1000 gel (Invitrogen, Mississauga, Ontario, Canada) stained with ethidium bromide (Pérez-López et al. 2016).

SINGLE NUCLEOTIDE POLYMORPHISM ANALYSIS

The *cpn60* UT sequences obtained from the phytoplasma-positive leafhoppers were aligned using ClustalW (Thompson et al. 1994) with 20 publicly available *cpn60*-encoding genes and *cpn60* UT sequences from '*Ca.* P. asteris'-related strains. Sequences were then trimmed to the 552 bp *cpn60* UT for phytoplasmas, using the Staden package (Bonfield & Whitwham 2010; Dumonceaux et al. 2014). The single nucleotide polymorphisms (SNP) were noted as previously described (Pérez-López et al. 2016).

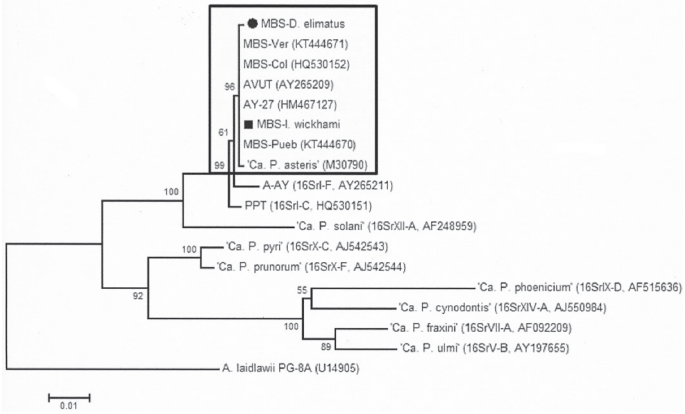

Fig. 4. Evolutionary analysis conducted through a neighbor-joining phylogenetic tree between the 16S rRNA sequences amplified in this study from phytoplasma DNA, bar 1 substitution in 100 positions. Sequences in the grey square belong to the subgroup 16SrI-B. Sequences amplified from leafhoppers (Hemiptera: Cicadellidae) *Dalbulus elimatus* marked with a circle and from *Idiodonus wickhami* marked with a square.

Results

LEAFHOPPER COLLECTION AND IDENTIFICATION

A total of 80 leafhopper (Hemiptera: Cicadellidae) specimens in different developmental stages was collected during the 2 surveys (Table 1). Based on their external morphology and male genitalia characteristics, the following leafhopper species were identified: *Idiodonus wickhami* (Ball), *Amblysellus grex* (Oman), *Empoasca fabae* (Harris), and *Dalbulus elimatus* (Ball) (Figs. 1, 2). Female specimens only were found in the genera *Macrosteles*, *Graphocephala*, and *Erythridula.* Specimens belonging to the genus *Macrosteles* were classified as *Macrosteles quadrilineatus* (Forbes) (Hemiptera: Cicadellidae) through the measurement of the wing ratio (Saguez et al. 2015). No species identification could be conducted for *Graphocephala* and *Erythridula*.

One type of nymph, with a 'red speckled' pattern, was collected (Table 1). Red speckled nymphs showed red spots similar to the spots observed on the body of *I. wickhami*. The phylogenetic tree generated for *cox*1 from *I. wickhami* (GenBank accession no. KU722543) and from the red speckled nymphs (GenBank accession no. KU722544) showed that both sequences formed a well-supported independent phylogenetic group (bootstrap value 95 %) (Fig. 3). Both sequences showed a 93% or higher nucleotide sequence identity with *Cicadellidae* sp. (GenBank accession no. HF968661), and 100 % between them, suggesting that the red speckled nymphs are the nymphal stages of *I. wickhami*.

PHYTOPLASMA DETECTION AND IDENTIFICATION

Leafhoppers in 2 of the 7 genera collected tested positive for the presence of phytoplasma DNA. The sequence fragments of about 1.2 kb F2nR2 and about 605 bp of *cpn60* UT, were amplified from DNA extracts obtained from *I. wickhami* and *D. elimatus*. The F2nR2 sequences obtained from both leafhopper species (GenBank accession no. KU722546 and KU722545 for *I. wickhami* and *D. elimatus*, respectively) through direct sequencing showed 99% nucleotide identity with maize bushy stunt phytoplasma strain Puebla and Veracruz (maize bushy stunt-Pueb and maize bushy stunt-Ver) (GenBank accession no. KT444670 and KT444671, respectively). The *cpn60* UT sequence obtained from *I. wickhami* (GenBank accession no. KU722542) showed 100% nucleotide identity with the strain maize bushy stunt-Pueb (Gen-

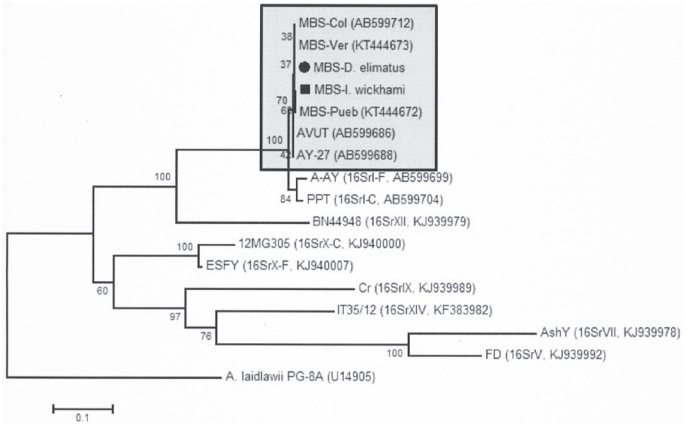

Fig. 5. Phylogenetic relationship inferred from analysis of *cpn60* UT sequences, bar 1 substitution in 10 positions. Sequences in the pink square belong to the subgroup 16SrI-B. Sequences amplified from leafhoppers (Hemiptera: Cicadellidae) *Dalbulus elimatus* marked with a circle and from *Idiodonus wickhami* marked with a square.

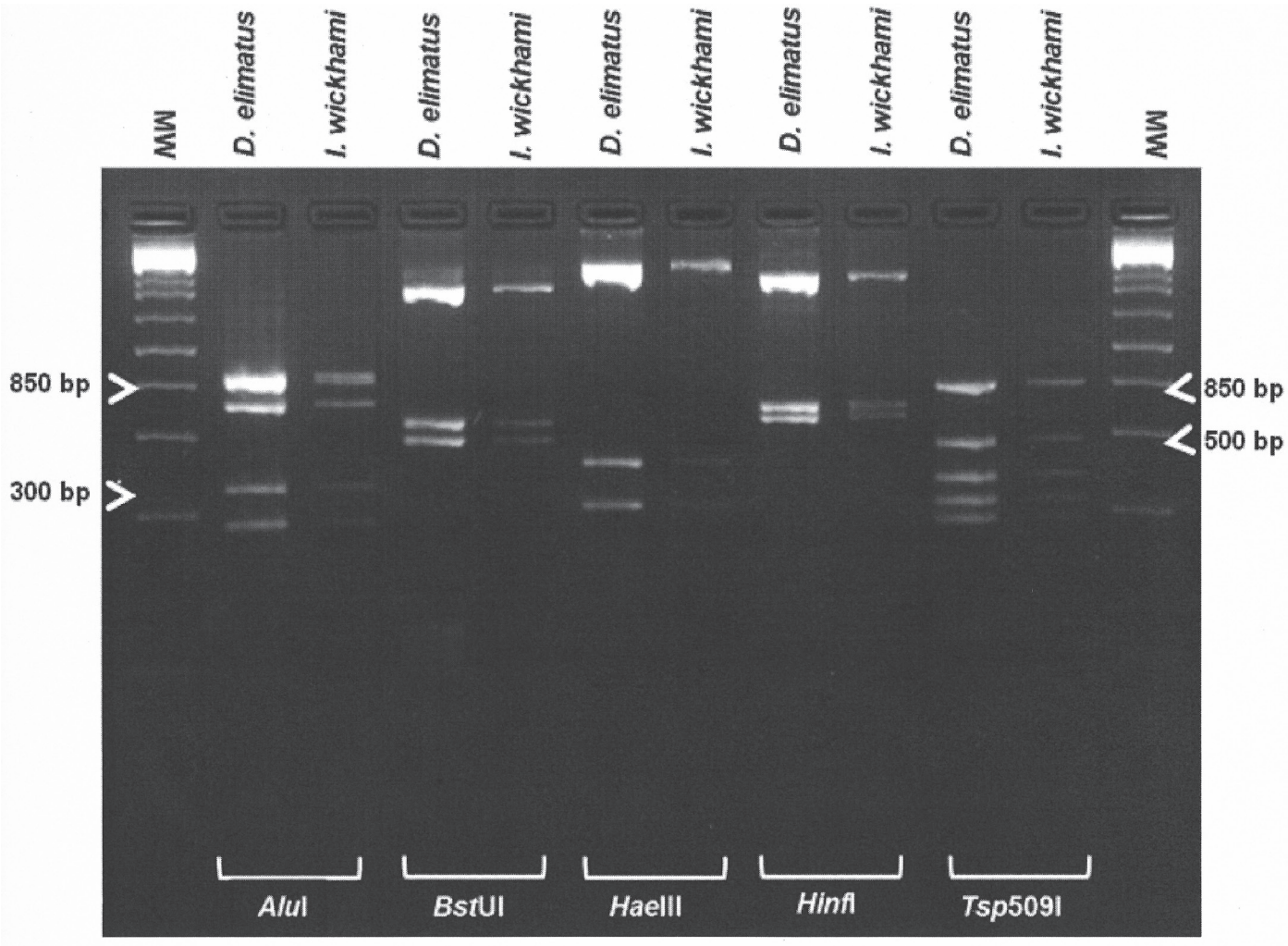

Fig. 6. Electrophoresis agarose gel showing RFLP pattern comparison between the F2nR2 sequences amplified from *Dalbulus elimatus* and *Idiodonus wickhami* digested with *Alu*I, *Bst*UI, *Hae*III, *Hinf*I, and *Tsp509*I. Molecular weight (MW) marker, 1 kb plus.

Bank accession no. KT444672), and the *cpn60* UT sequence obtained from *D. elimatus* (GenBank accession no. KU722541) showed 100% nucleotide identity with the strain maize bushy stunt-Ver (GenBank accession no. KT444673). The F2nR2 sequences obtained from *I. wickhami* and *D. elimatus* showed 99% nucleotide identity between them. Similarly, the *cpn60* UT sequences obtained from *I. wickhami* and *D. elimatus* also showed 99% nucleotide identity between them. Maize bushy stunt-Pueb and maize bushy stunt-Ver are members of 16SrI-B subgroup, '*Ca*. P. asteris'-related strains. The phylogenetic tree derived from the analysis of F2nR2 sequences and *cpn60* UT sequences obtained from the leafhoppers were consistent between them and showed that the sequences clustered with strains within the 16SrI-B subgroup (Figs. 4, 5).

The RFLP profiles obtained after the digestion of the F2nR2 sequences amplified from DNA extracts of *I. wickhami* and *D. elimatus* were identical between them (Fig. 6). The pattern observed was identical to the RFLP pattern described for 16SrI-B strains (Lee et al. 2004). The SNP analysis of *cpn60* UT sequences confirmed the previous results showing that the *cpn60* UT sequence obtained from *D. elimatus* is identical to the strain maize bushy stunt-Ver and maize bushy stunt-Col (GenBank accession no. AB599712) while the sequence amplified from *I. wickhami* is identical to the strain maize bushy stunt-Pueb (Fig. 7).

Discussion

Maize bushy stunt disease has been detected throughout Latin America and the southern United States, with *D. maidis* and *D. elimatus* as vectors. All genera found in this study have been described as Nearctic leafhoppers with a distribution in the southern USA and throughout Mexico (Dmitriev & Dietrich 2009; Feil et al. 2000).

Species *D. elimatus*, *A. grex*, *E. fabae*, and *M. quadrilineatus* have been reported in corn previously, although maize has been described as a non-preferred host for *M. quadrilineatus* (Kunkel 1946; Madden & Nault 1983; Hammond & Stinner 1987; Zhou et al. 2003). However, the presence of *I. wickhami*, *Graphocephala* sp., and *Erythridula* sp. in corn crops has not been reported. Native corn is usually grown in mixed cultures with other crops such as potato, amaranth or common beans (Waddington et al. 1990. In the Sierra Norte de Puebla community, corn crops also are weedy because most crops are grown without herbicide treatments. The study area was surrounded by potato plants, and we caught more female *E. fabae* than male (Table 1), which suggests that the potato leafhopper may have immigrated into corn from the neighboring potato crop. This same explanation also can apply to *M. quadrilineatus*, *A. grex*, *Graphocephala* sp., and *Erythridula* sp. because more female leafhoppers were caught than males (Table 1). An excess of female leafhoppers as evidence of immigration previously

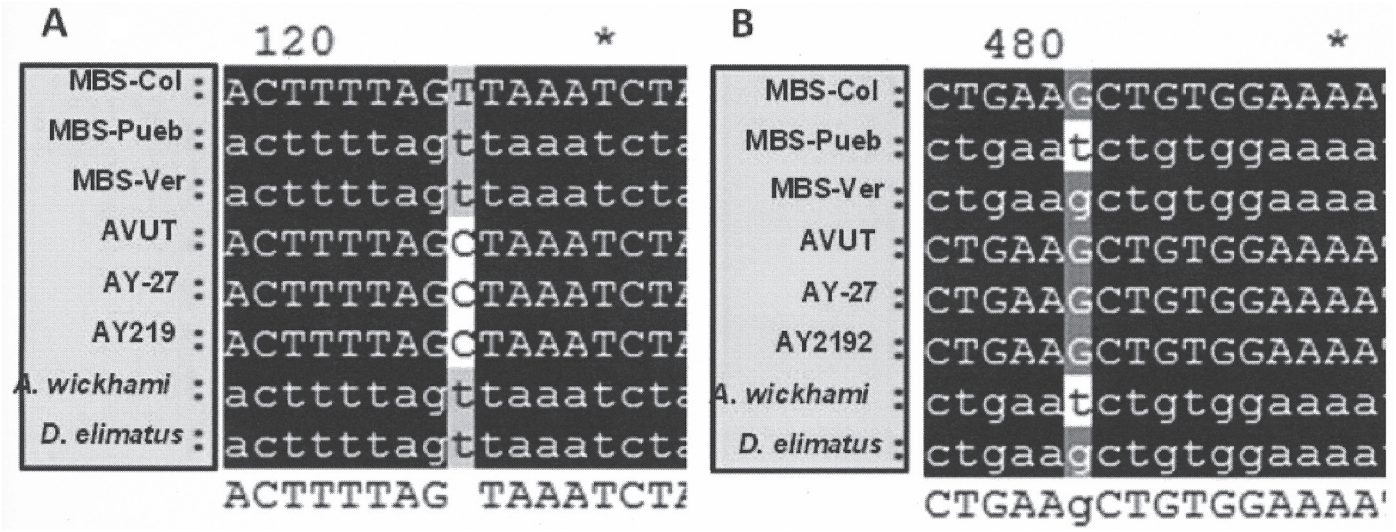

Fig. 7. Single nucleotide polymorphism of *cpn60* UT sequences amplified from *Dalbulus elimatus* and *Idiodonus wickhami* compared with the 16SrI-B strains maize bushy stunt-Col (AB599712), maize bushy stunt-Pueb (KT444672), maize bushy stunt-Ver (KT444673), AVUT (AB599686), AY-27 (AB599688), and AY2192 (AB599687). (A) Similarities between the maize bushy stunt strains and the sequences obtained from the leafhoppers. (B) Similarity between maize bushy stunt-Pueb and the phytoplasma detected associated with *Idiodonus wickhami* and similarity between maize bushy stunt-Col and maize bushy stunt-Ver with the phytoplasma associated with *Dalbulus elimatus*, based on SNP in *cpn60* UT sequences.

is described for *M. quadrilineatus*, *E. fabae*, and *D. elimatus* (Drake & Chapman 1965; Emmen et al. 2004; Moya-Raygoza et al. 2012). The collection dates also may influence the differences between the number of females and males (Pinedo-Escatel & Moya-Raygoza 2015). To our knowledge, this is the first report of *I. wickhami*, *Graphocephala* sp., and *Erythridula* sp. in corn fields, and this is the first report of the association of *A. grex*, *E. fabae*, *I. wickhami*, *Graphocephala* sp., and *Erythridula* sp. in native corn fields grown at altitudes of 2,400 masl.

Based on nucleotide identity, phylogenetic analysis, and morphological similarities, we suggested that the red speckled nymphs are the progeny of *I. wickhami*. This morphological characteristic is a well-known feature of the species *I. wickhami* (DeLong 1946). The presence of a high number of red speckled nymphs in the samples suggests that this leafhopper species is reproducing on native corn, but further experiments must be performed in order to confirm these results.

Maize bushy stunt phytoplasmas DNA was detected in *D. elimatus* and adult *I. wickhami*. The detection of maize bushy stunt phytoplasma in *D. elimatus* has been reported previously (Esau et al. 1976; Nault 1980, 1983). Interestingly, maize bushy stunt phytoplasma also was detected in DNA extracts of *I. wickhami*, a leafhopper not previously identified as a corn feeder or a phytoplasma vector. The most intriguing finding was the identification through the SNP of *cpn60* UT sequences of strain maize bushy stunt-Ver from *D. elimatus* and strain maize bushy stunt-Pueb from *I. wickhami*. The strain maize bushy stunt-Ver is closely related to the strain maize bushy stunt Colombia, while maize bushy stunt-Pueb is different from previously identified maize bushy stunt phytoplasmas strains, based on *cpn60* UT sequences (Pérez-López et al. 2016).

In conclusion, in this study maize bushy stunt phytoplasma was detected in 2 abundant leafhoppers, *D. elimatus* and *I. wickhami*. Also, native corn was identified as a probable new host for *I. wickhami*. This study is the first step towards identifying the vectors of maize bushy stunt phytoplasma in indigenous corn varieties produced in small agricultural communities in southern Mexico. Further surveys to characterize leafhopper populations and transmission bioassays are necessary in order to develop management strategies that are sustainable for those rural communities.

Acknowledgments

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