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 the 16SrI-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), Amblyseius grex (Oman) (Hemiptera: Cicadellidae), Empoasca fabae (Harris) (Hemiptera: Cicadellidae), Macrosteles quadrilineatus (Forbes) (Hemiptera: Cicadellidae), and Dalbulus elimatus (Bola) (Hemiptera: Cicadellidae). We were not able to identify the leafhopper genera Graphocephala (Hemiptera: Cicadellidae) and Erythrina (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 en vitro, y transmitidos principalmente por saltahojas (Cicadellidae). La enfermedad del enanismo arbustivo del maíz (enanismo arbustivo del maíz, por sus siglas en inglés) se ha relacionado con fitoplasmas pertenecientes al subgrupo 16SrI-B y transmitidas por saltahojas durante el género Dalbulus spp. (Hemiptera: Cicadellidae). La detección reciente de maíz 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 7 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), Amblyseius grex (Oman) (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 Erythrina (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),
Idiodonus wickhami

tive corn crops and to determine their maize bushy stunt phytoplasma
community of Las Trancas located in southeast Mexico. The objective
vector(s) identity and biodiversity in crops of native corn grown at
farmers (Pérez-López et al. 2016). Phytoplasma diseases can be con-
controlled with chemicals or cultural practices that target the insect vec-
tors (Weintraub & Beanland 2006).

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: Spiroplasmata-
ceae) (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 ex-
pression caused by these pathogens has led to confusion and inaccu-
rate disease diagnosis (Nault 1983).

Corn (Zea mays L. ssp. mays) (Poaceae) was first domesticated
in Mexico around 10,000 years ago (Doebely 2004). Centuries of lo-
cal 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 agricul-
tural communities use indigenous corn varieties that are adapted
starting 10 m from the border of the field.

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 na-
tive 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, lo-
cated 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 in-
secticide. 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 meth-
od (Pérez-López et al. 2016). DNA extracts were amplified using the
mitochondrial cytochrome c oxidase 1 (CO1, cox1) specific primers
Uni-MinibarR1/Uni-MinibarF1, following the protocol previously de-
scribed (Meusnier et al. 2008). PCR products were examined using
1% agarose gel electrophoresis and ethidium bromide-stained prod-
cuts were visualized using a GelDoc (BioRad, Mississauga, Ontario,
Canada). CO1 amplification generated an approximately 150bp DNA
fragment when observed under an ultraviolet (UV) transilluminator
at 365 nm (AIML 26, Alpha Innotech Corp., San Leandro, California,
USA).

PHYTOPLASMA DETECTION

Leafhoppers were grouped by genus or species and DNA was ex-
tracted 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 electro-
phoresis with ethidium bromide staining and visualized using a GelDoc
BioDoc (BioRad, Mississauga, Ontario, Canada). 16S CO1 and data referenced in DeLong (1946, 1931), Forbes (1885), Oman
(1949), Hepner (1978) and Young (1977).

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fragment when observed under an ultraviolet (UV) transilluminator
at 365 nm (AIML 26, Alpha Innotech Corp., San Leandro, California,
USA).

DNA SEQUENCING AND PHYLOGENETIC ANALYSES

The 16S cpn60 UT, and cox1 mini-barcode amplicons were purified
using a QiAquick® PCR Purification Kit (QIAGEN, Mississauga, Ontario,
Canada), and directly sequenced using the corresponding primers.

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

| Collection date | Genus          | Species            | Phytoplasma status | Male | Female | Nymph |
|-----------------|----------------|--------------------|--------------------|------|--------|-------|
| Jul 2014        | Idiodonus      | wickhami           | +                  | 6    | 5      | 20 ‘Red speckled’ |
| Jul 2014        |                |                    |                    |      |        |       |
| Jul 2014        | Macrosteles    | quadrilineatus      |                    | 0   | 1      |       |
| Jul 2014        | Amblysellus    | grex               |                    | 1   | 2      |       |
| Jul 2014        | Empoasca       | fabeae             |                    | 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.
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 cox1 mini-barcode.

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*.
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.
SINGLE NUCLEOTIDE POLYMORPHISM ANALYSIS

The 1.2 kb of F2nR2 sequence obtained from phytoplasma-positive leafhoppers were digested with endonucleases AluI, BstUI, HaeIII, HinfI and Tsp509I (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 AluI, BstUI, HaeIII, and HinfI were incubated at 37 °C, while reaction with Tsp509I 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, 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). The single nucleotide polymorphisms (SNP) were noted as previously described (Pérez-López et al. 2016).

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), Ambyllus grex (Oman), Erythrina fabae (Harris), and Dalbulus eliminatus (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 cox1 from I. wickhami (GenBank accession no. KT722543) and from the red speckled nymphs (GenBank accession no. KT722544) 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.

RESULTS

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. eliminatus. The F2nR2 sequences obtained from both leafhopper species (GenBank accession no. KT722546 and KT722545 for I. wickhami and D. eliminatus, 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. KT722544) 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.

"**Fig. 3.** Evolutionary analysis conducted through a neighbor-joining phylogenetic tree between the cox1 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.""
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

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**Fig. 6.** Electrophoresis agarose gel showing RFLP pattern comparison between the F2nR2 sequences amplified from *Dalbulus elimatus* and *Idiodonus wickhami* digested with *Alul*, *BstUI*, *HaeIII*, *HinfI*, and *Tsp509I*. Molecular weight (MW) marker, 1 kb plus.
is described for *M. quadrilineatus*, *E. fabae*, and *D. elima-
tus* (Drake & Chapman 1965; Emmen et al. 2004; Moya-Raygoza et al. 2012). The collection dates also may influence the differences between the num-
er of females and males (Pinedo-Escatel & Moya-Raygoza 2015). To
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ber of females and males (Pinedo-Escatel & Moya-Raygoza 2015). To

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