IS THERE ANY GENETIC VARIATION AMONG NATIVE MEXICAN AND ARGENTINIAN POPULATIONS OF *DALBULUS MAIDIS* (HEMIPTERA: CICADELLIDAE)?

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Abstract

The corn leafhopper *Dalbulus maidis* (Delong & Wolcott) (Hemiptera: Cicadellidae) originated in Mexico, but is found from southeastern and southwestern USA to Argentina. Differences in reproductive and phenotypic traits between Mexican (native) and Argentinian (adventive) populations have been previously reported, but information on their genetic variation is currently unavailable. The objective was to investigate possible genetic variability among *D. maidis* populations collected in Mexico on maize and maize relatives (annual and perennial teosintes) and on maize in Argentina. A region of the mitochondrial gene coding for the cytochrome oxidase subunit I (mtCOI) and the ribosomal internal transcribed spacer (ITS2) were sequenced and analyzed. We developed the forward and reverse primers for the DNA amplification of COI in *D. maidis* (dalCOI). Twenty two and 17 sequences for dalCOI and ITS2, respectively, were generated and analyzed. No genetic variation among Mexican and Argentinian populations was found in the ribosomal region and low genetic variation was found in the mitochondrial region. These results could be explained by the short evolutionary time scale, since both maize and the corn leafhopper moved throughout the Americas only in the most recent millenia, or in part to the limited host range, and thus a limited change in the corn leafhopper associated bacteria.

Key Words: Genetic variation, COI, ITS2, adventive, maize, teosintes

Resumen

La chicharrita del maíz *Dalbulus maidis* (Delong & Wolcott) (Hemiptera: Cicadellidae) se originó en México pero se le encuentra desde el sur de los Estados Unidos de América hasta Argentina. Diferencias en la reproducción y caracteres fenotípicos entre poblaciones de *D. maidis* de México (nativas) y Argentina se han registrado previamente, pero se desconoce sobre su variación genética. El objetivo de este estudio fue investigar una posible variación genética entre poblaciones de *D. maidis* de México colectadas en maíz y sus parientes silvestres (teosintes anuales y perenes) y en maíz de Argentina. Una porción del gene mitocondrial que codifica para la subunidad 1 del citocromo oxidasa (mtCOI) y el espaciador interno transcrito del ADN ribosomal (ITS2) fueron secuenciados y analizados. En este estudio se desarrolló el cebador para la amplificación de COI en *D. maidis* (dalCOI). Veintidós y 17 secuencias para mtCOI y ITS2, respectivamente, fueron generados y analizados. No se observó variación genética entre poblaciones de México y Argentina en el ITS2 y se encontró poca variación genética en el mtCOI. La falta o poca variación genética podría ser el resultado de un corto tiempo evolutivo, desde que el maíz y la chicharrita del maíz se dispersaron en América, o en parte a un número limitado de plantas hospederas, consecuentemente a un cambio limitado en la asociación bacteria y *D. maidis*.

The corn leafhopper, *Dalbulus maidis* (De-long & Wolcott) (Hemiptera: Cicadellidae), has a wide geographical distribution, and is found from southern USA to Argentina. It is one of the most important pests of maize (*Zea mays* L. ssp. *mays*) throughout Latin America, because it efficiently transmits 3 plant pathogens: maize rayado fino virus (*Marafivirus*), and the bacteria; corn stunt spiroplasma (*Spiroplasma kunkelii*) and maize bushy stunt phytoplasma (*Candidatus* Phytoplasma asteris) (Nault 1980). The corn leafhopper originated in Mexico, probably at the same time as maize (Nault 1990), which evolved from its annual teosinte relative (*Zea mays* ssp. *parviglumis* Iltis & Doebly) about 9,000 yr ago (Matsuoka et al. 2002). Likewise, annual teosintes originated from perennial teosintes (Fukunaga et al. 2005).

Through time, maize and the corn leafhopper have spread together. The reason has to do with their biology and geographical distribution. *Dalbulus maidis* feeds and reproduces only on maize and annual and perennial teosintes. While teosintes grow mostly in Mexico, maize has been dispersed by humans in the Americas. Argentina represents the most distant country away from Mexico where *D. maidis* has been found, at 30 °S (Paradell et al. 2001). Isotopic and macrobotanical data suggest that maize arrived 2,000 years ago to the lowlands and 1,000 years ago to the highlands of west central Argentina (Gil et al. 2006). Most likely the corn leafhopper got there at the same time.

A previous study showed that corn leafhoppers from the native Mexico and the most distant Argentinian regions differ in body size and reproduction rate, the Argentinian individuals being larger and more fertile than the Mexican ones (Moya-Raygoza & Garcia-Medina 2010). Further phenotypic diversity has been observed between samples collected at different elevations in Mexico, i.e., the leafhoppers from high elevations are bigger and darker and have longer wings compared with leafhoppers from low elevations (< 700 m) (Moya-Raygoza et al. 2005). However, it is unknown if *D. maidis* population from both countries show genetic variation. Therefore, the objective of this study was to investigate if *D. maidis* populations from Mexico are genetically different from Argentinian populations. We applied genetic analyses to corn leafhoppers collected at high and low elevations, on maize in Argentina and Mexico and annual and perennial teosintes in Mexico. The genetic variability has been checked at 2 different DNA sequences: the ribosomal internal transcribed spacer region 2 (ITS2) and the mitochondrial gene coding for the subunit I of the cytochrome oxidase (mtCOI). These genomic regions are commonly employed as molecular markers, and they have already proved to be useful for separating distant groups of individuals within an insect species and resolving population genetic structures (DeBarro 2000; Behura 2006).

MATERIALS AND METHODS

Insect Collection

Corn leafhopper adults were collected with a heavy sweep net (38-cm-diam net ring) over the foliage of maize and teosintes. In Mexico, adults were trapped in 2008 from the annual, *Zea mays* ssp. *parviglumis,* and the perennial, *Zea perennis* (Hitch.) Reeves & Manglesdorf teosinte plants. In contrast, the Argentinian adults were collected on maize in 2006 and 2009. In Mexico, 4 different sites from low and high elevations were sampled, and in Argentina 8 sites, including those from frontier region, were sampled. In each site 20 corn leafhopper adults were collected. In addition, we used *Macrosteles* as outgroup (Tables 1A and 1B). Corn leafhopper adults were identified using the keys of Triplehorn & Nault (1985) and adults were stored in 95% ethanol until the DNA extraction.

DNA Isolation

Genomic DNA was extracted using the protocol described by Marzachí et al. (1998). Single adult leafhoppers were ground in 500 μL of CTAB-based buffer (2% w/v cetyl-trimethyl-ammonium-bromide (CTAB); 1.4 M NaCl; 20 mM EDTA, pH8.0; 100 mM Tris-HCl, pH 8.0; 0.2% mercaptoethanol) using sterile carborundum and a micropestle in a 1.5 mL Eppendorf tube. The suspension was vortexed, incubated for 30 min at 60 °C, and centrifuged for 5 min at 12000-13000 rpm at room temperature. The supernatant was transferred into a new 1.5-mL Eppendorf tube, along with 1 volume of chloroform-isoamyl alcohol (24:1) and repeatedly inverted. Next, the suspension was centrifuged for 5 min at 12000-13000 rpm at room temperature and the supernatant transferred in a new 1.5 mL Eppendorf tube. The DNA was precipitated by adding1 volume of cold isopropanol (-20 °C) and centrifuging for 20 min at 12000-13000 rpm at 4 °C. The pellet was washed with cold 70% ethanol, and centrifuged for 8-10 min at 12000-13000 rpm at 4 °C. Finally, the pellet was dried in a vacuum and re-suspended in 30-100 μL of Tris-EDTA (ethylenediaminetetraacetc acid) solution.

DNA Amplification and Sequencing

A fragment of the mitochondrial cytochrome oxidase I gene (mtCOI) was initially amplified with the primers LCO (5´- GCTCAACAAAT-CATAAAGATATTGG-3´) and HCO (5´-TA-AACTTCAGGGTGACCAAAAAATCA-3´), previously described as conserved primers for invertebrate DNA (Folmer et al. 1994). However, the resulting PCR was poorly reproducible, even

TABLE 1A. *DALBULUS MAIDIS* ADULTS COLLECTED AND SEQUENCED ON PERENNIAL TEOSINTE, ANNUAL TEOSINTE, AND MAIZE FROM MEXICO AND ARGENTINA. THE ITS2 AND THE DALCOI SUBUNIT OF THESE SAMPLES WERE SEQUENCED.

Location	Country	Host plant	ITS2 accession number in GenBank	dalCOI accession number in GenBank	Elevation m asl. and latitude	Collection date
Piedra Ancha	Mexico	perennial teosinte (*Z. perennis*)	JN411704	JN411692	2000 H, N19° 38´	Jul 25, 2008
Guachinango	Mexico	annual teosinte (*Z.* ssp. *parviglumis*)	JN411705	JN411693	1420 H, N20° 35´	Jul 25, 2008
El Grullo	Mexico	maize (*Z. mays* ssp. *mays*)	JN411706	JN411694	880 L, N19° 47´	Jul 20, 2008
Zapopan	Mexico	maize (*Z. mays* ssp. *mays*)	JN411707	JN411695	1570 H, N20° 44´	Jul 20,2008
El Manantial	Argentina	maize (*Z. mays* ssp. *mays*)	JN411708	JN411696	435 L, S26° 50´	Feb 5, 2006
Los Nogales	Argentina	maize (*Z. mays* ssp. *mays*)	JN411709	JN411697	630 L, S26° 42´	Feb 5, 2006
Animana	Argentina	maize (*Z. mays* ssp. *mays*)	JN411710	JN411698	1623 H, S25° 57´	Feb 5, 2006
El Mollar	Argentina	maize (*Z. mays* ssp. *mays*)	JN411711	JN411699	2005 H, S26° 56´	Feb 5, 2006
Los Nogales 1	Argentina	maize (*Z. mays* ssp. *mays*) *f*	NS	JN411700	586 L, S26° 42´	Mar 15,2009
El Cerrillo	Argentina	maize (*Z. mays* ssp. *mays*) *f*	JN411712	JN411701	401 L, S29° 58´	Mar19,2009
Los Sauces	Argentina	maize (*Z. mays* ssp. *mays*) *f*	JN411713	JN411702	450 L, S30° 12´	Mar19,2009
Jesus Maria	Argentina	maize (*Z. mays* ssp. *mays*) *f*	NS	JN411703	544 L, S31° 04´	Mar16,2009

Abbreviations: *f* = frontier or southern distribution limit of *D. maidis* in argentina. H = high elevations. L = low elevations. NS = not sequenced.

under different reaction conditions. Therefore, a new primer pair specific for *D. maidis* mtCOI region was designed, i.e., dalCOI fwd (5´TAG CTC AAC CTG GGT CGT TT), and dalCOI rev (5´TGG TAT AGG ATT GGG TCA CCA). PCR reactions were performed in 50 µL final volume containing 50-150 ng of DNA template, 1 X buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.5 µM of each primer and 1 U/µl *Taq*. The mtCOI fragments were amplified under the following conditions: 94 °C for 5 min, followed by 35 cycles each of 30 s at 94 °C, 60 s at 58 °C, and 60 s at 72 °C. Final cycles of 30 s at 94 °C, 60 s at 52 °C and 10 s at 72 °C were executed.

Similarly, the ITS2 PCR fragments were generated using the ITS2 fwd (5´ TGTGAACTGC-GAGACACA GC) and ITS2 rev (5´ATG CTT AAA TTT AGG GGG TA) primers (Collins & Paskewitz 1996). The PCR conditions were the same as described for mtCOI amplification, except the annealing temperature was 54°C.

PCR products were visualized on 1% agarose gels to confirm amplification of samples and non amplification of negative controls. Then, PCR products were purified with the Pure Link PCR Purification Kit (Invitrogen, Carlsbad, California) and sequenced. Sequencing reactions were performed using BigDye Terminator version 3.1 chemistry (Applied Biosystems, Foster City, California) and purified with the AutoSeq G-50 Dye Terminator Removal Kit (GE Healthcare). The samples were run on an ABI 310 automated sequencer and edited using CROMAS Lite Version 2.01 (McCarthy 2005). Finally, the manual alignment of the DNA sequences was done using MacClade (Maddison & Maddison 2000). We sequenced 2 insects per site in most of the cases and the same insects were used to sequence dalCOI and ITS2.

Data Analysis

Genetic diversity among the sampled populations was estimated using haplotype diversity (*h*) and nucleotide diversity (µ), as implemented in DNASP 4.10 (Librado & Rozas 2009).

RESULTS

The dalCOI and ITS2 sequences representative of the different populations are available in GenBank; and their accession numbers are given in Tables 1A and 1B. In total, we obtained 41 sequences. For ITS2, 17 sequences belong to *D. maidis* and two to *Macrosteles* sp. Similarly, 22 sequences represent the dalCOI subunit of *D. maidis*. In this study the primer for dalCOI and the annealing temperature were developed specifically for *D. maidis*, and we designated this primer dalCOI (Table 2). The amplified ITS2 region was 481 bp long, which was reduced to 470

TABLE 1B. OUTGROUP ACCESSIONS ANALYZED.

Specie	Locality	Country	ITS2 accession number in GenBank	COI accession number in GenBank
Macrosteles quadripunctulatus	Grugliasco	Italy	JN411714	NS
Macrosteles sp.	California	USA	NS	&EU 981868
Macrosteles quadrilineatus	California	USA	NS	&EU 981884

&Data from Leroux and Rubinoff (2009) taken from Genbank. NS = not sequenced.

bp after gap exclusion. Polymorphic sites defined only one haplotype, therefore, the nucleotide diversity was zero (Table 3).

Meanwhile, the amplified and aligned region of dalCOI was 352 bp long, which was reduced to 325 bp after gap exclusion. Only one site was polymorphic, 324 were singleton variable sites, and one was parsimony informative. Polymorphic sites defined 3 haplotypes including the outgroup (Table 3). The haplotype diversity was 0.6023 and the nucleotide diversity (μ) was 0.0381. The values of *h* and μ decreased to 0.5303 and 0.00163, respectively, when the outgroup was eliminated from the analysis (Table 3).

DISCUSSION

No significant genetic variation was found in the nuclear Internal Transcribed Spacer Region 2 (ITS2) among *D. maidis* populations, and low genetic variation was found in their mitochondrial dalCOI primer sets. Similarly, no variation was recorded between the teosinte and maize populations evaluated, in spite of the fact that maize radiated from its teosinte ancestors about 9,000 yr ago. It is unknown when *D. maidis* arrived in Argentina, however because *D. maidis* only reproduces and feeds on teosinte and maize, we suggest that the corn leafhopper followed the maize movement from Mexico to Argentina. Studies have reported that maize was domesticated from the annual teosinte, *Zea mays* ssp. *parviglumis*, in central Mexico (Matsuoka et al. 2002). At a later time maize was moved to Central America (Dickau et al. 2007), and finally arrived in Argentina 1,000 or 2,000 yr ago (Gil et al. 2006). Previous studies showed that the distribution of teosintes is limited to Central America (Fukunaga et al. 2005). Since gene flow between Mexican and Argentinian *D. maidis* populations is not possible, the recency of colonization in evolutionary terms of Argentina by *D. maidis* populations from Mexico might explain the paucity of genetic differentiation.

We used markers that were not used previously to test genetic variation among *D. maidis* populations. However, mtCOI and ITS have been used to elucidate relationships among populations of insects, including hemipterans (Frohlich et al. 1999; De Barro et al. 2000; De la Rua et al. 2006). Previously, Oliveira et al. (2007) used RAPD-PCR and they found some variations between northeastern and south central Brazilian populations of *D. maidis*. They concluded that the relatively high rates of gene flow within south central Brazilian populations suggest the occurrence of *D. maidis* migration within that region, whereas the northeastern population seems to be genetically isolated. Also Dietrich et al. (1998) used mitochondrial NADH gene to elucidate the phylogeny of the genus *Dalbulus*, and they found little intraspecific variation among *D. maidis* populations in 3 sites in Mexico: Poza Rica, Tlatizapan, and Texcoco.

Both mtCOI and ITS markers are generally sensitive enough to pick out some within-species genetic variations, especially when the species occur across a large geographical area, such as the case of *D. maidis*. We do not exclude the presence of genetic variations that can be highlighted using a more diverse set of genetic markers. Nevertheless, the conserved sequences of the 2 markers used in this study clearly reveal that the Argentinian and Mexican populations of *D. maidis* are closely related, as would be expected for highly monophagous insect populations.

Interestingly, Carpane (2007) found very high genetic similarity of several isolates of the bacteria corn stunt spiroplasma (CSS), specifically transmitted by *D. maidis*, throughout North and

TABLE 2. PRIMER CHARACTERISTICS USED IN THE STUDY.

Primer name	Primer sequence (5´-3´)	Annealing temperature	Annealing time
dalCOI	fwd (5´TAG CTC AAC CTG GGT CGT TT)	58 °C	60 s
ITS2	fwd (5´TGT GAA CTG CGA GAC ACA GC)	54 °C	60 s

TABLE 3. SUMMARY OF DALCOI AND ITS2 HAPLOTYPE DISTRIBUTIONS, HAPLOTYPE DIVERSITY AND NUCLEOTIDE DIVERSITY INDICES.

Marker	Haplotype 1	Haplotype 2	Haplotype 3	h	µ
COI with outgroup	*Macrosteles*	Mex (Piedra Ancha) Mex (Guachinango) Mex (El Grullo) Mex (Zapopan)	Arg (El Mollar) Arg (Los Nogales) Arg (Animana) Arg (Los Nogales 1) Arg (El Cerrillo) Arg (Los Sauces) Arg (Jesús María)	0.6026	0.0381
COI without outgroup	Mex (Piedra Ancha) Mex (Guachinango) Mex (El Grullo) Mex (Zapopan	Arg (El Mollar) Arg (Los Nogales) Arg (Animana) Arg (Los Nogales 1) Arg (El Cerrillo) Arg (Los Sauces) Arg (Jesús María)		0.5303	0.00163
ITS2 with outgroup	*Macrosteles*	Mex (Piedra Ancha) Mex (Guachinango) Mex (El Grullo) Mex (Zapopan) Arg (El Manantial) Arg (Los Nogales) Arg (Animana) Arg (El Cerrillo) Arg (Los Sauces) Arg (El Mollar)		0	0.0614
ITS2 without outgroup	Mex (Piedra Ancha) Mex (Guachinango) Mex (El Grullo) Mex (Zapopan) Arg (El Manantial) Arg (Los Nogales) Arg (Animana) Arg (El Cerrillo) Arg (Los Sauces) Arg (El Mollar)			0	0

H = haplotype diversity, µ = nucleotide diversity. The accession number of each of these materials is given in tables 1a or 1b. Mex = Mexico, Arg = Argentina.

South America. This study revealed that isolates of CSS from Argentina, Brazil, California, Costa Rica, Florida and Mexico share a strong similarity at the DNA sequence level. The lack of genetic variation in both vector and its spiroplasma seems to suggest the absence of spatial-temporal barriers among the different *D. maidis* and spiroplasma populations.

Absence of genetic variations in *D. maidis* and CSS could be the result of a short evolutionary time of their association. Previous studies have found morphological and reproductive differences between Mexican and Argentinian *D. maidis* populations (Moya-Raygoza & Garcia-Medina 2010), but these are most likely linked to temperature differences between these locations. Also, as noted above, Oliveira et al. (2007) found morphological variation among *D. maidis* populations in Brazil, but they did not find important genetic variation, which, again, suggests an effect of temperature on the biology of *D. maidis*. Insects generally demonstrate high adaptation to environmental conditions and high phenotypic plasticity (Nijhout 2003). For example, *D. maidis* individuals from high elevations in Mexico are darker, larger and have longer wings than individuals from low elevations in Mexico where temperatures are warmer (Moya-Raygoza et al. 2005). The same reaction to temperature changes was found in *Drosophila melanogaster* (Nijhout 2003). When *Drosophila* lines were exposed to low temperatures for five years, heritable and cellular changes in body size were found (Partridge et al. 1994). Recently, the genetic bases of body size variation were described in *D. melanogaster* (Turner et al. 2011), and similar bases may apply to other insects such *D. maidis*.

Finally, a more definitive examination may be needed to focus on differences in the vector's capacity to acquire and transmit CSS, or other pathogens. Biological differences continue to provide clues to the ever-changing genomes of theis important insect pest species.

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