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First analysis of genetic diversity and population structure among different geographic populations of the vector of the Corn stunt disease, *Dalbulus maidis* (Hemiptera: Cicadellidae), in subtropical Argentina

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Primer análisis de la diversidad genética y estructura poblacional entre diferentes poblaciones geográficas del vector de la enfermedad del achaparramiento del maíz, *Dalbulus maidis* (Hemiptera: Cicadellidae), en Argentina subtropical

RESUMEN. El saltahojas del maíz, *Dalbulus maidis* (DeLong & Wolcott) (Hemiptera: Cicadellidae), es el vector del complejo de patógenos que causan la enfermedad conocida como *"Corn stunt"* o achaparramiento del maíz la cual constituye una seria amenaza para la producción de maíz en las regiones tropicales y subtropicales del continente americano. El objetivo de este estudio fue realizar un análisis exploratorio para estimar la estructura y diferenciación genética de poblaciones de *D. maidis* de tres regiones geográficas provenientes de dos campañas agrícolas de cultivo de maíz en el noroeste de Argentina utilizando marcadores moleculares ISSR. Las poblaciones provenientes del Alto Valle y de la Llanura Seca, a pesar de estar separadas geográficamente por montañas de gran elevación, fueron genéticamente similares, mientras que las poblaciones más cercanas provenientes de la Llanura Seca y el Piedemonte Húmedo fueron genéticamente las más diferentes. Los resultados sugirieron que las poblaciones de *D. maidis* en zonas maiceras subtropicales se estructuran de acuerdo con las condiciones climáticas, principalmente humedad y precipitación. Además, esta estructura podría estar influenciada por migrantes anuales de áreas tropicales del norte, así como por individuos que sobreviven ocasionalmente de una estación a otra, como ocurre en la región del Piedemonte Húmedo. La coloración más oscura de las hembras colectadas durante el segundo año en la Llanura Seca y el Piedemonte Húmedo, podrían asociarse a temperaturas más frías durante el invierno anterior a la implantación del cultivo de maíz. Este estudio es el primero realizado en Argentina sobre la diversidad genética de las poblaciones de *D maidis*. Los resultados presentados destacan la necesidad de realizar más investigaciones sobre el origen y el movimiento de las poblaciones de *D. maidis* lo cual permitirá conocer cómo se coloniza el cultivo y desarrollar modelos adecuados para prevenir la enfermedad causada por los patógenos transmitidos por este insecto.

PALABRAS CLAVE. Chicharrita del maíz. Marcadores ISSR. Tucumán. *Zea mays*.

ABSTRACT. The corn leafhopper, *Dalbulus maidis* (DeLong & Wolcott) (Hemiptera: Cicadellidae) is the vector of the Corn stunt disease, caused by a complex of pathogens, a serious threat to corn production in tropical and subtropical regions of the American continent. The aim of this study was to conduct an exploratory analysis to estimate the structure and genetic differentiation of *D. maidis* populations from three geographical regions collected during two maize growing seasons in northwestern Argentina using inter-simple sequence repeats (ISSR) markers. Populations in the High-elevation Valley and Dry Plain sites, despite being geographically separated by mountains of high elevations, were genetically similar, while the nearest populations located in Dry Plain and Humid Piedmont were the most genetically different. The results suggested that populations of *D. maidis* in subtropical maize-growing areas are structured according to the climatic conditions, mainly humidity and precipitation. Additionally, this structure might be influenced by annual migrants from northern tropical areas, as well as by individuals that occasionally survive from one season to the next as occurs in the Humid Piedmont. Darker body coloration of females sampled during the second year in Dry Plain and Humid Piedmont might be associated with colder temperatures during the winter before maize cultivation. This study is the first conducted in Argentina on the genetic diversity of populations of *D. maidis*. The results presented here highlight the need for further investigation into the origin and potential movement of *D. maidis* populations. Knowing how the crop is colonized will aid in the development of well-fitting models, for preventing the disease caused by the pathogens transmitted by this insect.

KEYWORDS. Corn leafhopper. ISSR markers. Tucumán. *Zea mays*.

INTRODUCTION

The corn leafhopper, *Dalbulus maidis* (DeLong & Wolcott) (Hemiptera: Cicadellidae), is a major pest of maize, *Zea mays* L., in the Americas. It is widely distributed in tropical and subtropical areas from southeastern and southwestern USA to central areas of Argentina (Carloni et al., 2013; Virla et al., 2013). It is a monophagous species that only can complete its life cycle on plants belonging to the genus *Zea*, which includes maize and teosintes (Triplehorn & Nault, 1985). Although it prefers to live in areas of low elevations, it is found in a wide range of altitudes from sea level to 3,200 m a.s.l. in the Peruvian Andes (Nault, 1990). At high densities *D. maidis* might seriously affect maize crops (Virla et al., 2021) because it is a vector of four pathogens: the Corn stunt spiroplasma (CSS - *Spiroplasma kunkelii*), the Maize bushy stunt phytoplasma (MBSP), the Maize rayado fino virus (MRFV), and the recently discovered Mastrevirus that causes Maize striate mosaic (Jones & Medina, 2020; Vilanova et al., 2022; Ruiz Posse et al., 2023). These pathogens, either individually or in combination, are responsible for the "Corn stunt", a disease that limits maize production in some regions of the United States and Latin America (Virla et al., 2004; Oliveira et al., 2018). A critical strategy for managing this disease in maize crops is the population reduction of *D. maidis* (Oliveira et al., 2023).

In Argentina, *D. maidis* was reported for the first time in 1948 in Tucumán (Oman, 1948) and was later recorded in 1990, living on maize in different provinces (Virla et al., 1991; Remes Lenicov & Virla, 1999; Giménez Pecci et al., 2002; Paradell et al., 2001, 2005; Saluso, 2006). This vector is distributed mostly to the north of parallel 30° SL, where the Corn stunt spiroplasma (CSS) is the prevalent pathogen (Carloni et al., 2013; Virla et al., 2013). Conversely, south of the 30° parallel, vector populations are less significant, and a strong correlation was found between the prevalence and incidence of CSS and the presence of *D. maidis* (Carloni et al., 2013). The distribution pattern of the vector in subtropical areas is primarily influenced by climatic conditions as well as by the availability of host plants throughout the year (Medina et al., 2012; Oliveira et al., 2013; Virla et al., 2013; Van Nieuwenhove et al., 2016). Populations of the corn leafhopper are important during the summer months, and as maize plants senesce and eventually die off at the end of the growing season, only a few adults may survive the cold winters, especially if there are volunteer plants, or if they take shelter in winter crops or weeds (Virla et al., 2003).

Several biological aspects of *D. maidis* are known like its ability to migrate long distances (Taylor et al., 1993), seasonal polyphenisms (Larsen & Nault, 1994; Oliveira et al., 2004; Moya Raygoza et al., 2005), its level of damage on maize plants (Virla et al., 2021), the population abundance (Oliveira et al., 2013; Meneses et al., 2016; Sánchez Reinoso et al., 2021; Canale et al., 2023) and the spatial and temporal distribution in Brazil (Foresti et al., 2022; da Cunha et al., 2023). The genetic structure of *D. maidis* populations has been studied by means of different molecular markers like RAPD-PCR in populations from Brazil (Oliveira et al., 2007), the cytochrome oxidase subunit I (mtCOI) and the ribosomal internal transcribed spacer (ITS2) in Mexican and Argentinean populations (Palomera et al., 2012), and AFLP in populations from different *Zea* taxa host plants in Mexico (Medina et al., 2012).

Inter-simple sequence repeat (ISSR) are reliable markers that do not require prior knowledge of the target sequences and are highly reproducible due to the length of the primers and the high annealing temperature. Additionally, these markers were found to be highly polymorphic (Zietkiewicz et al., 1994; Kojima et al., 1998;

Bornet & Branchard, 2001). ISSR markers were widely used to study the genetic diversity of insects including species of leafhoppers (Hemiptera: Cicadellidae) in the USA (de León et al., 2004) and rice planthopper (Hemiptera: Delphacidae) pests in Asia (Liu et al., 2010; Xie et al., 2014; Nam et al., 2019; Babu et al., 2023).

Despite the importance of *D. maidis* for maize production in Argentina, the knowledge regarding the population dynamic and the genetic and phenotypic diversity of this leafhopper is limited. Since the incidence of the corn stunt disease is directly linked to vector presence, understanding the genetic diversity and dispersion of *D. maidis* populations is crucial to know further the implications of this leafhopper on the epidemiology of the disease. In this study, we typed the populations of *D. maidis* by means of ISSR markers and studied the genetic and phenotypic diversity as well as the population structure across three distinct geographical regions located within a subtropical area of Argentina. Considering the dependence of the vector on corn as a food source, and the fact that the study was conducted in a subtropical region where the crop is absent during winter months, we hypothesize that populations of *D. maidis* in areas with milder winters, such as the humid Piedmont, which experiences the lowest average number of frost days, may exhibit higher levels of relationship among their members, due to a greater survival rate of the vectors from one season to the next. Secondly, we suggest that the populations inhabiting the dry Chaco plains and humid Piedmont, which are separated by approximately 50 km and are not isolated by orographic barriers, are sympatric. In contrast, the populations residing in the Monte High-altitude Valley, which is geographically isolated from the other regions, are allopatric to the two aforementioned populations.

MATERIAL AND METHODS

Sampling zones

Populations of *D. maidis* were sampled from three distinct geographic regions located in Argentina's subtropical province of Tucumán. The specific regions chosen for this study are representative of the environmental conditions in which maize is typically grown in most subtropical areas of South America and where Corn stunt spiroplasma (CSS) can result in yield losses of up to 70% (Virla et al., 2004). These regions also differ in their maize cultivation practices. The selected regions were: 1) the dry Chaco Plain (Cd), 2) the humid Piedmont (Ch), an ecotone situated between the southern Yungas Forest and Chaco regions, and 3) the Monte high-elevation valleys, Tafí (Ta), as determined by Morrone's (2014) biogeographic classification. The dry Chaco region is located approximately 45 km from the humid Piedmont, without any orographic barriers between them. The high valley, located to the west of the other locations, is 48 km away from the humid Piedmont and is separated by geographic barriers in the form of surrounding mountains with elevations ranging between 2,300 and 2,700 m a.s.l, with an NW-S orientation (Fig. 1).

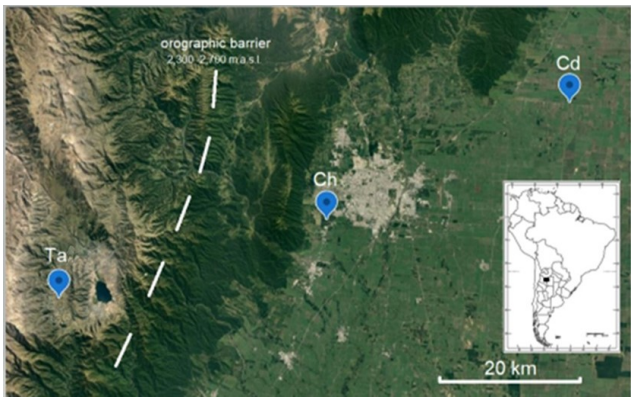

Fig. 1. Geographical locations where *Dalbulus maidis* populations were sampled in Tucumán province. References= Cd: Dry Chaco Plain (dry plain), Ch: Ecotone Yungas-Chaco (humid Piedmont), Ta: Monte high elevation valleys (Tafí).

The ecotone Yungas-Chaco (humid Piedmont) (Ch) is located on the western slopes of the North-South pre-Andean mountains in northwestern Argentina, at altitudes ranging from 500 to 700 m a.s.l. The mesoclimate of this region is characterized by high humidity and warm weather with annual rainfall exceeding 1,000 mm and potential evapotranspiration of around 900 mm. Soil water deficiency is null and the average annual temperature is 20.7 °C, with rare frost events (Zuccardi & Fadda, 1985). In this area, maize is sown in small plots for feeding families or animal subsistence from middle October to the end of January and is harvested from January to June. Crop management lacks technological practices and hardly uses agrochemicals. Volunteer plants are commonly found after harvest. The Dry Chaco Plain (dry plain) (Cd) is about 250-400 m a.s.l. elevation and is characterized by a warm sub-humid dry mesoclimate. Precipitation varies from 650 to 750 mm annually, while the potential evapotranspiration is between 950 and 1000 mm, resulting in a water hydric deficit but a positive water balance in summer. The average annual temperature is 19 °C, and frosts are frequent between June and August, with approximately 12-15 frosts per year (Zuccardi & Fadda, 1985). Unlike the other area here, maize crops often occupy large tracts of land for grain production and their management is highly technological. Maize is mostly seeded in mid-December at the beginning of the wet season. Maize plants dry out in March and are harvested in May. Volunteer plants are rare during winter months, as plots are seeded with winter cereals such as wheat or barley. Monte high elevation valley (Ta) occurs up to 1,500 m a.s.l. and has a temperate semiarid mesoclimate. The annual precipitation is about 400 mm, and the annual evapotranspiration is 500 to 600 mm, with 9 months of water deficiency from April to December. The average annual temperature is 13.2 °C. Frosts occur between March and September (frequency: 44 days/year) (Zuccardi

& Fadda, 1985). Plots are seeded in December when the wet season starts. Maize plants dry out in March and are harvested during the winter. Like the humid Piedmont, maize is cultivated in small plots mostly to family subsistence and agrochemicals are hardly used. No volunteer maize plants occur. Table I provides a summary of the climatic characteristics, geographic data, and location of the sampled sites.

Table I. Summary of the climatic characteristics (*) and geographic data of the sites where populations of *Dalbulus maidis* were sampled in Tucumán Province, Argentina.

Geographic regions	Locality	Latitude (S)	Longitude (W)	Elevation (m a.s.l.)	Average temperature (ºC) hottest month (January)	Average temperature (ºC) coldest month (July)	Average annual rainfall (mm)
Cd	La Virginia	26º45'07,8	64º47'43,7	398	24.2	11.9	710
Ch	El Manantial	26º50'03,41	65º16'30,62	435	26.3	11.9	1084.8
Ta	Las Carreras	26º55'40,7	65º46'19,9	2283	18.6	8.1	393.7

(*) data taken from De Fina (1992). Cd: Dry Chaco Plain; Ch: Ecotone Yungas-Chaco (humid piedmont); Ta: Monte high elevation valley (Tafí)

Experimental design and insect sampling

Maize plots (25 x 20 m) were sown with the landrace named "Maizón" (sweet white corn) during the late October planting window in two growing seasons (2009-2010 and 2011-2012) at three localities belonged to the studied geographic regions: La Virginia, El Manantial, and Las Carreras (Table I). *Dalbulus maidis* specimens were collected using a sweep net during the vegetative stage of maize development in two time periods: December 2009 to January 2010, which we refer to as the first year (Y1), and December 2011 to February 2012, which we refer to as the second year (Y2). The insects were transported alive to the laboratory and stored in absolute ethyl alcohol at -20 °C for further analysis, according to Oliveira et al. (2002). The analysis of morphological variables was performed on live individuals. The taxonomic identification of *D. maidis* was performed according to Triplehorn & Nault (1985). Voucher specimens of *D. maidis* were deposited in the entomological collection at the Museo de Ciencias Naturales, Universidad Nacional de La Plata, Argentina. To perform ISSR and study morphological variables, we randomly selected 10 adult females from each of the three geographic regions sampled in Y1 and Y2. This resulted in six sampled populations named as follows: Y1Ch, Y1Cd, Y1Ta, Y2Ch, Y2Cd, and Y2Ta.

DNA extraction

DNA of each population was extracted from 10 females using the protocol described by Doyle & Doyle (1990). Insects were individually placed in a 1.5 mL Eppendorf tube with 500 µl of extraction buffer (2% CTBA; 0.2% 2-mercaptoethanol; 1.4 M NaCl; 20 mM EDTA; 100 mM Tris- HCL pH 8), homogenized with a micro-pestle, and incubated for 30 min at 65 ºC. The supernatant was transferred to another Eppendorf tube with 500 µl of chloroform-isoamyl alcohol (24:1) and mixed and vortexed. After 2 min. of centrifugation at 13,000 g at room temperature, the aqueous phase containing the DNA was transferred to an Eppendorf tube. The DNA was precipitated by adding 1 volume of cold isopropanol (-20 °C) and centrifuged for 15 min. at 12,000 g. The pellet was washed with 70% cold ethanol, dried, and re-suspended in 50 µl of distilled water.

ISSR-PCR amplification

Nine oligonucleotides were initially used to run PCR with the genomic DNA of *D. maidis*. All oligonucleotides tested resulted in *D. maidis* DNA fragment amplification. Among them, four primers that provided a high number of reproducible fragments were selected. All the reactions included a negative control lacking genomic DNA. Each PCR mixture contained 12 ng of template DNA, 2.5 mM $MgCl_2$, 1 µM primer, 0.2 mM dNTPs, 1X Buffer (500 mM KCl, 100 mM Tris-HCl, pH 9 at 25 °C, 1% Triton X-100) and 1.25 U *Taq*-plus DNA polymerase (Inbio Highway Inc., Tandil, Argentina) in a total volume of 25 µl. PCR was performed in a thermocycler MandJ Research programmed as follows: 1 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 48 °C and 3 min extension at 72 °C, ending with 5 min at 72 °C for a final extension. The optimal annealing temperature for some primers was 53 °C (Table II). Electrophoresis was used to separate PCR amplicons in 1.5% agarose gels stained with ethidium bromide (10 g ml-1). DNA bands were observed with UV light irradiation. Gels were photographed with GeneSnap, and the banding patterns were analysed with GeneTools (SynGene). PCR reactions were conducted with two replications for each specimen and each oligonucleotide to ensure the reproduction of the bands.

Table II. Oligonucleotide primers tested in amplifications of ISRR markers from *Dalbulus maidis* individuals from three regions in the subtropics of Argentina.

Oligonucleotide	Sequence (5´-3´)	Annealing temperature (ºC)
AA5	GAG(AAG)5	48
AA3	(AG)8TA	48
CA5	CAT(ACA)5	48
JA5	TA(AG)8	48
IA5	ACA(CAA)5	48
EN	(GAC)5	48
AN	(CAA)5	48
846	(CA)8RT	53
BA3	(AC)8CT	53

Note: R= A, G.

Molecular data analysis

Based on photographs of the gels, only reproducible fragments were used as markers (Roderick, 1996). Amplified fragments were scored as 1= presence of a

band and 0= absence of a band. Bands with differing intensities were treated equally. Four primers amplified polymorphic regions of the DNA, and these were named M1, M2, M3, and M4. Since ISSR markers are dominant markers, each band represents a single diallelic locus (Williams et al., 1990). Genetic diversity indices for each population were estimated through the Percentage of polymorphic loci (P); Dice's similarity coefficient (DSC); Genetic diversity (GD), Nei's heterozygosity (NeiHe), Effective number of alleles (ENA), and Number of alleles Average (NA) by using Info-gen software (Balzarini & Di Rienzo, 2004). Interpopulation genetic diversity was estimated using binary molecular information (presence and absence of band) of individuals within populations (n= 60). The dissimilarity coefficient between two individuals was calculated using the binary Sokal distance (DSo), where: a: number of matches of type 1-0, for each pair of accesses; b: number of discordances of type 1-1, for each pair of accesses; c: number of discordances of type 0-1, for each pair of accesses; and d: number of matches of type 0-0, for each pair of accesses. For each population pair, the mean and variance were estimated, and an approximate Student's t-test was used to test for equality between population variances (Cruz, 2008). The ISSR molecular data were analysed by Numerical Taxonomy using NTSYS-pc (Numerical Taxonomy System) version 2.10 software program (Rohlf, 1992). A similarity matrix was generated per year or growing season by applying Dice's similarity coefficient. The similarity values were utilized to group individuals via the Unweighted Pair Group Method (UPGMA) and two corresponding dendrograms were constructed. The correlation between similarity and cophenetic coefficients for the clusters (CCC) was computed. Principal coordinate analysis (PCoA) was performed to classify *D. maidis* females from the six sampled populations in tri-dimensional graphics and to estimate the variability explained by each component (Sokal & Rohlf, 1983). This allowed us to improve the cluster identification and evaluate the discriminatory power of each molecular marker (eigenvectors). An analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed from a distance matrix between pairs of individuals. Two hierarchical AMOVA models were used to investigate the partition of total phenotypic variance (Schneider et al., 2000). One model, referred to as model A, divided the total phenotypic variance within and among populations. A second model, referred to as model B, partitioned the total sum of squares among years, among populations within years, and among individuals within populations. A permutational procedure (2,000 random permutations) was used to provide tests of significance for each of the hierarchical variance components (Excoffier et al., 1992). Wright diversity index (Fst) and Nm, number of "migrant" individuals (Wright, 1969), which represents the degree of genetic subdivision and the gene flow between populations, respectively were estimated. Nm was calculated by the formula:

$$Nm = \frac{\left(\frac{1}{FST} - 1\right)}{4}$$

Indices and AMOVAS were calculated using the ARLEQUIN program, version 2000 (Schneider et al., 2000). The STRUCTURE software (Evanno et al., 2005), based on a Bayesian approach, was used to identify clusters of genetically similar populations. The initial burn-in period was set to 10,000 with 1,000,000 Markov chain Monte Carlo iterations. For each K (number of clusters) and each run, 10 independent runs were set as parameters to estimate the population structure. The ΔK of the Evanno method (Evanno et al., 2005) was performed to estimate the K value that best fitted the data, using the STRUCTURE HARVESTER V.0.693 software (Earl & Von Holdt, 2012). The graphical visualization of the population structure was produced with Excel software.

Environmental *versus* genetic classifications

In order to evaluate the congruence between genetic and environmental (climatic and geographic data) classifications, three distance matrixes, using the Euclidean distance coefficient were built from the following data basic matrix: a geographic matrix, with latitude, longitude and altitude data (Table I); a Summer climatic matrix, averaging climatic data of the corn growing period (from December to March) (Table IIIa) and, a Winter climatic matrix, averaging climatic data from the winter period prior to maize sowing season (May-October), given the importance of climatic variables in determining the overwintering survival frequency of *D. maidis* adults (Virla et al., 2003; Van Nieuwenhove et al., 2016) (Table IIIb). The matrix calculated with the binary distance of Sokal (DSo values) was used as the genetic distance matrix. The Mantel test, which assumes a linear correlation between pairs of matrixes, using 1,000 random permutations (NTSYS-pc, Numerical Taxonomy System) version 2.10 software program was used (Mantel, 1967). Furthermore, aimed at the identification of climatic variables that might be relevant for grouping of populations, simple correlations were calculated between means scores of the six populations projected on three principal coordinates axes (PCoA 's tri-dimensional graphic (Fig. 3) and winter and summer climatic means. The Pearson's product-moment coefficient (r) was used (Rohlf et al., 1983).

Morphological variables

The variables evaluated were: total body length (BL) in mm, measured from the apex of the head to the apex of the forewing in repose; relative forewing length (WL) in mm, measured from the base to the apex in repose; and the pattern of body coloration (PCOL), which was categorized in two classes: 1-light (uniformly yellow) and 2-dark (parts of the thorax and abdomen partially darkened). We applied a split-plot design, with the populations as the fixed effect and nested females within

each population as the random effect. For the PCOL variable, we applied a Kruskal-Wallis non-parametric test (Kruskal & Wallis, 1952). For BL and WL quantitative traits the variance analysis was performed. InfoStat software was used (Di Rienzo et al., 2017).

Table III. a) Summer means of climatic characteristics considering the maize crop growing months (from December to March). b) Winter means of climatic characteristics considering the winter before maize crop growing months (from May to October) from the sites where populations of *Dalbulus maidis* were sampled in Tucumán Province, Argentina.

a) Summer means	Tmin[1] ºC	Tmax[2] ºC	Tm[3] ºC	MinRH[4] %	MaxRH[5] %	RHm[6] %	MP[7] mm
Y1Ch	16.50	30.46	22.49	47.11	89.37	70.50	7.10
Y1Cd	19.84	32.16	25.85	39.25	79.84	59.53	9.82
Y1Ta	8.68	18.74	14.27	56.07	91.34	77.23	4.13
Y2Ch	15.18	24.44	19.53	67.46	96.40	85.49	15.9
Y2Cd	20.87	32.51	26.97	36.58	78.77	57.01	3.92
Y2Ta	8.58	18.72	13.27	63.95	96.48	83.83	6.15
b) Winter means	**Tmin ºC**	**Tmax ºC**	**Tm ºC**	**MinRH %**	**MaxRH %**	**RHm %**	**MP mm**
Y1Ch	7.9	24	15.9	35	86	60	67.2
Y1Cd	7	24.9	16	32	82	57	14.8
Y1Ta	1.1	17.7	9.4	34	86	60	14
Y2Ch	10.4	23.6	17	36	80	58	151.8
Y2Cd	7.8	24.2	16	3	87	63	70.7
Y2Ta	1.1	17.6	9.3	35	88	62	3.9

Data were taken during Y1 and Y2 from Estación Experimental Agroindustrial Obispo Colombres, Tucumán Province, Argentina.
[1]Average minimum temperature; [2]Average maximum temperature; [3]Average temperature; [4]Minimum relative humidity; [5]Maximum relative humidity; [6]Average relative humidity; [7]Mean precipitation.

Comparison between morphological and genetic groups

To evaluate the congruence among genetic and morphological groups, three different clusters of populations, selected by PCoA and structural Bayesian approach, were used as classificatory variables. We estimated a Kruskal-Wallis non-parametric test to probe differences between morphological groups (Kruskal & Wallis, 1952).

RESULTS

ISSR profile

The four selected ISSR primers generated informative and reproducible DNA banding patterns. They amplified 130 clear bands. Each fragment was identified by a code as follows: M1 1-32; M2 1-50; M3 1-24; M4 1-24. Among the amplified bands, only 43.07% (56 bands) were polymorphic. The number of scored polymorphic bands and the percentage of polymorphism by each ISSR marker are shown in Table IV.

Table IV. ISSR primers and the polymorphism of their PCR products for *Dalbulus maidis* from three regions in the subtropics of Argentina.

Oligonucleotide	Sequence (5´-3´)	N° of scored bands	N° of polymorphic bands	Polymorphism (%)
AA5	GAG(AAG)5	32	18	56.25
AA3	(AG)8TA	50	18	36
CA5	CAT(ACA)5	24	9	37.5
JA5	TA(AG)8	24	11	45.83
TOTAL		**130**	**56**	

Genetic diversity

The genetic diversity indexes (Table V) showed that the population of the humid Piedmont of the first growing season (Y1Ch) and the population of the high elevation valley of the second growing season (Y2Ta) were the most diverse. On the other hand, insects isolated from Y1Ta and Y2Ch exhibited lower genetic variability.

Table V. Genetic diversity indexes of *Dalbulus maidis* collected from the Argentinean subtropics.

Population	P%[1]	DSC[2]	GD[3]	NeiHe[4]	ENA[5]	NA[6]
Y1Ch	60.7	0.68	0.21	0.22	1.60	90
Y1Cd	46.4	0.74	0.15	0.16	1.46	82
Y1Ta	42.9	0.81	0.12	0.13	1.42	80
Y2Ch	39.3	0.80	0.13	0.14	1.39	78
Y2Cd	48.2	0.76	0.14	0.15	1.48	83
Y2Ta	57.1	0.73	0.17	0.18	1.57	88
MEAN	**49.1**	**0.75**	**0.15**	**0.16**	**1.49**	**83.5**

[1]Percentage of polymorphic loci; [2] Dice's similarity coefficient; [3]Genetic diversity (GD); [4]Nei's heterozygosity; [5]Effective number of alleles; [6]Number of alleles Average.

The Sokal's binary distances and their variances (Table VI) indicated that Y1Ch was significantly different from the other populations ($p < 0.01$) except Y2Ta. Remarkably, Y1Ta showed a significant difference with Y2Ta ($p < 0.01$) and with Y2Ch ($p < 0.05$).

Table VI. Inter-populations distance and variances (V). Sokal's binary distance (DSo) coefficients and the *t*-Student test.

		Y1Ch	Y1Chd	Y1Taf	Y2Ch	Y2Cd	Y2Taf
Y1Ch	D_{SO}	0	0.57**	0.55**	0.61**	0.59**	0.6ns
	V		±0.002	±0.002	±0.002	±0.02	±0.002
Y1Cd	D_{SO}		0	0.47ns	0.56ns	0.55ns	0.47ns
	V			±0.002	±0.002	±0.002	±0.003
Y1Taf	D_{SO}			0	0.59*	0.57ns	0.52**
	V				±0.013	±0.001	±0.002
Y2Ch	D_{SO}				0	0.45ns	0.51
	V					±0.002	±0.004
Y2Cd	D_{SO}					0	0.48ns
	V						±0.003
Y2Taf	D_{SO}						0

*, **: Significant at 0.05, 0.01 respectively; ns: Non significant.

The dendrograms generated using the Dice similarity coefficient (DSC) and UPGMA (Fig. 2) had high CCC values (Cophenetic Correlation Coefficient), indicating low levels of distortion between the similarity matrix and cluster analysis. The percentage of similarity among samples ranged from 0.47 to 0.92, indicating that all sampled individuals, regardless of the region or date sampled, shared at least 50% of their loci. In the first sowing season, individuals collected from each sampling place did not overlap and were clustered into three well defined groups, being the Ch population the most divergent (DSC: 0.54). In the second sowing season, some individuals from Ch (1, 3, 10, 5, and 4) were associated with a few individuals from the Monte high-elevation valley (Ta 7 and 8). This cluster is separated from the rest at a distance of 47% and it is more similar to the cluster formed by the rest of 8 Ta's specimens (DSC: 0.56). Five other individuals from Ch were included in a separate cluster from that formed by all the individuals collected in Cd (DSC: 0.64).

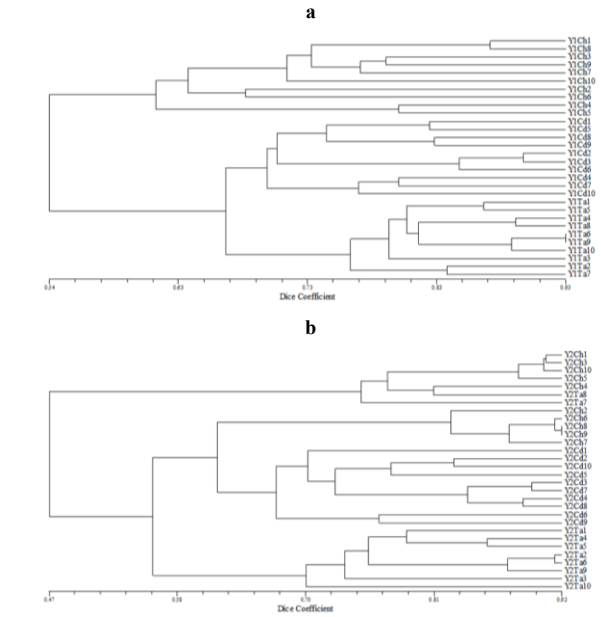

Fig. 2. Dendrograms built using ISSR-PCR markers from the Dice Coefficient and UPGMA method, based on *Dalbulus maidis* females from the six sampled populations. a. First maize growing season (Y1), 48 genetic ISSR polymorphic markers (CCC: 0.833). b. Second maize growing season (Y2), 44 polymorphic markers (CCC: 0.855). References= Ch: Ecotone Yungas-Chaco (humid Piedmont), Cd: Dry Chaco Plains (dry plain), Ta: Monte high elevation valleys (Tafí), CCC: Cophenetic Correlation Coefficient.

Principal coordinate analysis (PCoA) allowed the evaluation of the discriminatory power of each molecular marker, clustering the six sampled populations in four divergent clusters (Fig. 3). The first three axes explained 37.19% of the total variability. Axis 1 (20.2%) separated individuals into 3 groups: Y1Cd and Y1Ta located on the lower left quadrant, Y2Ch and Y2Cd located at the bottom of the right quadrant, and Y1Ch and Y2Ta clustered around the center of axis 1. Thus, axis 2 (9.12%) and axis 3 (7.85%) efficiently separate the population Y1Ch on the upper right quadrant and the one from Y2Ta along the upper right toward the lower left quadrants, on the center of the graphic. This showed that some Y2Ta individuals share some banding patterns with individuals of other population groups, during the same or different planting years.

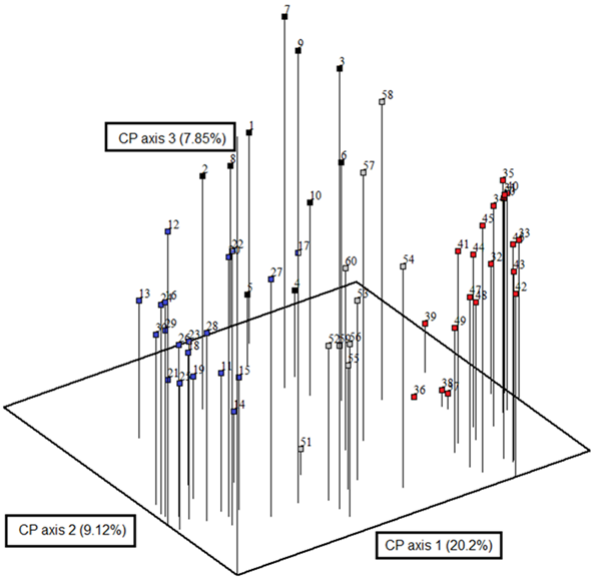

Fig. 3. Principal Coordinate Analysis (PCoA) classifying 60 females of *Dalbulus maidis* from the three geographic regions studied in two growing seasons across the 56 polymorphic ISSRs. Three-dimensional graph built with Dice's genetic distance coefficient, explaining each axis 20.21%, 9.12%, and 7.85% of the variability, respectively. References= Ch: Ecotone Yungas-Chaco (humid piedmont), Cd: Dry Chaco Plains (dry plain), Ta: Monte high elevation valley (Tafí valley). Y1 and Y2: first and second maize growing seasons, respectively. 1-10: Y1Ch, 11-20:Y1Cd, 21-30:Y1Ta, 31-40:Y2Ch, 41-50: Y2Cd, 51-60: Y2Ta.

Molecular markers M1-17, M2-33, and M1-27 were only present in individuals of Y2Ch while M1-1 and M2-33 markers were only present in individuals of Y2Ta indicating the presence of unique private alleles (Kalinowski, 2004). Furthermore, some populations grouped in the same cluster shared markers in an exclusive manner like Y1Cd-Y1Taf that shared M1-15 markers, Y2Ch-Y2Cd that shared M2-16 and M2-21 markers and Y1Cd, Y1Taf, and Y2Taf that shared M1-20 markers.

The Analysis of Molecular Variance (AMOVA) in Model A (Table VIIa) indicated that variation within populations (62.08%) was higher than among populations (37.92%), raising the issue that genetic variability is mostly related to differences among sampled insects, although differences among populations turned out to be significant at 5%. Model B (Table VIIb) showed non-significant differences among years, but significant differences among populations within years ($p < 1\%$), and among individuals within populations ($p < 1\%$).

Table VII. AMOVA for the extraction of ISSR variance components in six populations of *Dalbulus maidis* using 130 ISSR markers.

MODEL A:				
Source of variation	Df[1]	SS[2]	VC[3]	PV[4]
Among populations	5	175.68	3.01	37.92*
Within populations	54	266.90	4.94	62.08
Total	**59**	**442.58**	**7.96**	**100**
MODEL B:				
Source of variation	Df	SS	VC	PV
Among years	1	58.55	0.97	11.68
Among populations within years	4	117.13	2.43	29.14**
Among individuals within populations	54	266.90	4.94	59.18**
Total	**59**	**442.58**	**8.35**	

[1]Degree of freedoms; [2]SS: Squares sum; [3]VC: Variance components; [4]PV: Percentage of Variation; *, **: Significant at 0.05, 0.01.

The Wright's coefficient of co-ancestry or fixation index (Fst) (above diagonal) and the number of "migrants" Nm (below diagonal) are shown in Table VIII. The Fst values fluctuated between 0.037 ≤ Fst ≤ 0.115 and the number of "migrants" ranged between 1.92 ≤ Nm ≤ 6.581, which indicates a low to moderate genetic differentiation.

Table VIII. Wright's diversity measured between pairs of populations collected in the first maize growing season (Y1) and in the second one (Y2) in the Subhumid Chaco (Ch), Dry Chaco (Cd), and high elevation valley (Ta) regions.

	Y1Ch	Y1Cd	Y1Taf	Y2Ch	Y2Cd	Y2Taf
Y1Ch	0	0.064	0.084	0.059	0.062	0.051
Y1Cd	3.626	0	0.045	0.094	0.080	0.037
Y1Taf	2.708	5.257	0	0.115	0.097	0.064
Y2Ch	3.987	2.396	1.920	0	0.038	0.059
Y2Cd	3.769	2.871	2.335	6.399	0	0.041
Y2Taf	4.681	6.581	3.626	4.295	5.833	0

Wright's coefficient or fixation index (Fst) above the diagonal and the number of "migrants" (Nm) below the diagonal.

The population pairs Y1Cd and Y2Ta (Fst= 0.037; Nm= 6.58), Y2Cd and Y2Ch (Fst= 0.038; Nm= 6.39), Y2Cd and Y2Ta (Fst= 0.041; Nm= 5.83), and Y1Cd and Y1Ta (Fst= 0.045; Nm= 5.25) exhibit low values of the coefficient of coancestry (Fst) and a high number of "migrants" (Nm), indicating a lower genetic divergence and higher gene flow between them. Conversely, the population pairs with higher Fst values and lower Nm values, were Y1Ta and Y2Ch (Fst= 0.115; Nm= 1.92), Y1Ta and Y2Cd (Fst= 0.097; Nm= 2.33), and Y1Cd and Y2Cd (Fst= 0.08; Nm= 2.87), pointing higher genetic differences and lower gene flow between them.

Population structure

The Bayesian approach test divided the six sampled populations (K) into two or three major groups. Indeed, the Evanno method identified Δk= 2 as the best value of K that fitted our data (Fig. 4).

Three groups with different genetic structures were Y1Cd-Y1Ta, Y2Cd-Y2Ch, and Y1Ch-Y2Ta. These groups were consistent with the three clusters grouped on the PCoA axis 1 and dendrograms.

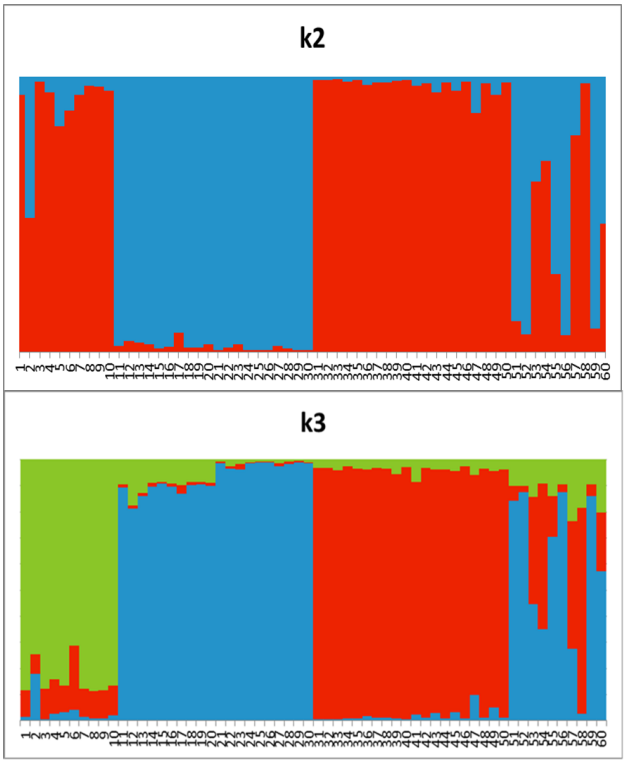

Fig. 4. Cluster analysis using STRUCTURE software (K= 2 and 3). Different colors represent different genetic groups. Each column represents an individual. References= 1-10: Y1Ch, 11-20:Y1Cd, 21-30:Y1Ta, 31-40:Y2Ch, 41-50:Y2Cd, 51-60: Y2Ta.

Congruence among genetic clusters and environmental variables

The Mantel test detected a low and not significant correlation between genetic distances vs. geographic distances matrixes (r= -0.06, t= -0.25; p= 0.41), neither between genetic distances versus climatic distances matrixes, when winter means (from May to October) were used (r= -0.3056, t=-1.1563, p= 0.1238). However, a significant correlation was found (r= -0.49, t=-1.9319, p= 0.03) comparing matrixes of genetic distances vs. climatic distance built with data during the maize growing period (December to March).

Aimed at identifying the climatic variables that might play a relevant role in the population grouping, simple correlations between pairs were calculated. Only significant r values were found between climatic variables and population scores projected along PCoA axes 1 and 3. The first axis was significantly correlated with winter variables: Maximun precipitation (MaxP mm, r= 0.94, p= 0.004), mean precipitation (MP mm, r= 0.89, p= 0.02), and mean relative humidity (MRH %, r= 0.80, p= 0.05). No significant correlation was found for the PCoA axe 2. The third axis showed significant r's with the winter variable: Maximum relative humidity (MaxRH %, r= 0.80, p= 0.05) and with the summer variable: Mean precipitation (MP, r= -0.84, p= 0.03).

Congruence between genetic clusters and morphological variables

Females collected from Y2Cd and Y2Ch had darker bodies with a median of 2 (H= 7.45, p= 0.002). Total body length (BL) and relative wing length (WL) were not significantly different among groups, with median values of H= 1.03 (p= 0.59) and H= 1.62 (p= 0.43), respectively.

DISCUSSION

This is the first study aimed at elucidating the genetic and phenotypic variation of *D. maidis* populations in subtropical regions of Argentina. The number of alleles in a population is a measure of genetic variability which can be shown by different genetic markers (Ferreira & Grattpaglia, 1998). ISSR molecular markers are highly polymorphic and have been useful in detecting genetic variability in insects (Roux et al., 2007; Palma et al., 2015; Kurd et al., 2020; Tabikha & Adss, 2021; Abdolahadi et al., 2022; Nurhasanah et al., 2023). In our study, due to logistic and economic reasons, the estimation of allelic richness might be limited by the sample size (N= 10), which might explain the low number of variable bands detected. Still, such a low number of markers allowed us to identify some level of genetic structuring and subpopulations providing us with new insights into the genetic diversity of *D. maidis.*

Dendrograms generated for samples of each year explained 54% and 47% of the genetic variability for the first and second year, respectively. During the first year, the populations of the three studied regions appeared to be genetically isolated since samples were clustered in three distinct groups each associated with the site of the collection being the Ch population the most divergent. This was consistent with the genetic diversity indexes and the dissimilarity coefficients (DSo) that indicated that the Y1Ch population was the most diverse. In the second year, while some individuals collected in Ch region were closely associated with a few individuals from the Monte high-elevation valley (Ta), others were included in a separate cluster together with individuals collected in the Cd region. Furthermore, the PCoA analysis separated the populations into three groups on the first coordinate: Y1Cd-Y1Ta, Y2Cd-Y2Ch, and Y1Ch-Y2Ta. Furthermore, the existence of unique alleles (Kalinowski, 2004) and cluster-sharing markers in an exclusive manner also denoted certain differentiation in subpopulations. Although the Bayesian approach separated the six populations sampled in the different regions and years into two main groups associated with different genetic patterns, three groups were also identified that were consistent with the results obtained by numerical taxonomy and diversity indexes. The analysis showed that group Y1Ch-Y2Ta was formed by a mixture of alleles, which can justify the higher diversity and the separation from the rest of the populations. Furthermore, both AMOVAS´s models showed that a higher percentage of variability occurred between individuals within populations. The structural parameters Fst and Nm indicated a tendency towards lower genetic divergence and higher migrant number between those populations collected during the same seasons, particularly those from the Cd region and Ta and Ch regions. In our study system, Cd and Ch are separated from the high-altitude crops (Ta) by a high orographic barrier that runs from north to south, the Aconquija Mountains, so the explanation of the high genetic similarity and the higher flow of genes between Ta and Cd is a challenge. We hypothesize that individuals from tropical zones, located to the north of the study area and where vector populations persist throughout the year, might migrate and share their haplotypes year after year with populations from more temperate zones. It is known that the distances travelled by insects whose migrations are seasonally vary from a few meters to hundreds of kilometers, which is additionally under the influence of atmospheric conditions (Otuka et al., 2010; Chapman et al., 2015; Satterfield et al., 2020). The phenomenon of wind-borne migration, in which migrants ascend to altitudes at which they are transported down-wind has been intensively studied in insects and particularly in Hemiptera (Gatehouse, 1997; Reynolds et al., 2017). In general, nocturnal migrants have a greater potential for displacement compared to diurnal migrants (Drake & Farrow, 1988), and species that need to follow changes in habitat availability across regions or geographic areas typically fly at night (Gatehouse & Zhang, 1995). Leafhoppers (Cicadellidae) include nocturnal migrants that can fly on more than one successive night. Previous studies gave evidence of the capability of *D. maidis* for long-distance migration (Taylor et al., 1993; Oliveira et al., 2013; Foresti et al., 2021). It is widely recognized that the climate in tropical South America is of a monsoonal-type nature, characterized by a distinct rainy warm season (Campetella & Vera, 2002). In Tucumán province, the wet winds and rainy season typically occur between the end of November and April, which is spring and summer, when maize is planted. During this period, the region receives mostly nocturnal winds from the South American low-level jet (SALLJ), a phenomenon that occurs over southeastern South America, which is responsible of bringing heat and moisture from the tropical Amazon basin southward into the central plains of southeastern South America (Vera et al., 2006; Salio et al., 2007). In our study system, the three sampled regions are located almost at the same latitude and are influenced by north winds generated by the SALLJ. Our results suggest that annual migrants from the north of Argentina or even from Bolivia (e.g. zones of influence of Santa Cruz de la Sierra, Orán, and Tartagal, where maize crops are grown year-round, might be a permanent source of *D. maidis* that are likely transported by the SALLJ convective system, and such insects might be contributing to the genetic structure of populations of the subtropical corn fields.

Additionally, stochastic environmental factors, which vary from year to year, like winter climatic factors, such as the number of days with frost and total days with temperatures below 17 °C, may impact on the population structure and genetic flow within *D. maidis* populations (Virla et al., 2003; Van Nieuwenhove et al., 2016). Also, volunteer plants are important resources that allow the survival of individuals allowing to colonize the crops the next growing season (Oliveira et al., 2023). Here, we found a significant association between climatic variables and the values of coordinates along the PCoA axes 1 and 3. This suggested that the subdivision division in three groups might be due to a gradient of humidity and precipitation indicating that these two factors are critical in structuring populations of *D. maidis* in subtropical regions of Northwestern Argentina. In line with this, the Humid Piedmont (Ch) region has a higher diversity population, probably because more favorable environmental conditions allow some *D. maidis* individuals to survive between crop seasons. Additionally, Ch populations might increase their diversity due to the arrival of migrant individuals from the north. Previous studies with different molecular markers showed low level of genetic variability in populations of *D. maidis* even if they were separated by long distances. By means of RAPD markers, Oliveira et al. (2007) detected some degree of genetic subdivision in geographically distant locations across north-eastern and central-southern regions in Brazil. Similarly, genetic similarity between experimental populations of *D. maidis* located in areas of central Brazil (located at > 20km) was found by Oliveira et al. (2013). However, Palomera et al. (2012), using the mitochondrial gene cytochrome oxidase subunit I (mtCOI) and the ribosomal internal transcribed spacer (ITS2), found that native Mexican and Argentinean populations of *D. maidis* were genetically similar. By means of amplified fragment length polymorphisms (AFLP) analysis, Medina et al. (2012) found that population structuring of *D. maidis* in western-central and northern Mexico, was mainly due to ecological as well as antrophogenic forces but not to geographical isolation.

According to Larsen & Nault (1994), leafhoppers of the genus *Dalbulus* vary in body size and coloration depending on the season. The increase in body size most likely is associated to energy storage for survival in harsh conditions and migration, while a change in color might be an adaptation with thermoregulatory and cryptic benefits (Tauber et al., 1986). Environmental factors such as temperature, elevation, and latitude might provoke morphological variations observed in *D. maidis* populations (Oliveira et al., 2004; Moya-Raigoza et al., 2005; Moya Raygoza & Garcia Medina, 2010). In this work, we found that females from the examined populations exhibited a similar coloration, total body length or relative wing length. However, we observed that females sampled during the second year in Cd and Ch, were darker than those sampled in the first year. This might be due to colder temperatures during the winter before maize cultivation in the second year suggesting that changes in coloration might be an adaptation to conserve heat. On the contrary, in the high-altitude Tafí valley most of the collected individuals in both years were clear. Since temperatures at Tafí valley are extremely low at this high altitude most probably individuals are unable to survive from one year to the next, so the individuals collected most probably came from new lineages that colonized the crop at seeding.

In this study, we demonstrated that populations in the Monte High-altitude Valley and Dry Plain sites, despite being geographically separated by mountains were genetically similar, while the nearest populations located in Humid Piedmont and Dry Plain were the most genetically different. This structure seems to be influenced by the climatic conditions mainly humidity and precipitation. Additionally, annual migrants from northern tropical areas, as well as individuals that occasionally survive from one season to the next have a high impact on population structure. To confirm the hypothesis that individuals from maize crops in subtropical producing regions receive every year migrant individuals from northern Argentina, studies including a greater number of locations where corn is extensively grown and is severely affected by *D. maidis* should be conducted. Such studies should include tropical populations including possible sources of Bolivia and tropical localities of Argentina. Also, a high number of codominant markers should be used to identify polymorphisms which might help to identify more precise gene flow between populations and a major genetic variability.

This study highlights the need for further investigation into the origin and potential movement of *D. maidis* populations to know how the crop is colonized. Such research will aid in the development of well-fitting models, for preventing diseases transmitted by this insect.

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