Article

New haplotypes of *Diaphorina citri*, vector of *Candidatus* Liberibacter in citrus areas of Mexico

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Abstract

New haplotypes of *Diaphorina citri*, also known as the Asian citrus psyllid, were identified, called DcitACC-1, DcitACC-2, and DcitACC-3. The studies were based on the amplification of DNA of the mitochondrial COI gene and individuals from different citrus areas of the country were used, in some producing areas of the country no samplings have been carried out before, specifically in the municipalities of Acatlán de Pérez, Oaxaca, Misantla and Tantoyuca, Veracruz, and Huejutla, Hidalgo , so adult insects were collected without distinction of gender. The number of individuals collected from each site depended on the availability of insects at the site. A total of 60 individuals were collected. DNA amplification was performed with the specific primers DCITRI COI-L and DCITRI COI-R, the product of the PCR reaction was sequenced at the Institute for Scientific and Technological Research of San Luis Potosí (IPICYT, for its acronym in Spanish). The sequences obtained were compared with those reported in Genbank and it was determined that there is a matrix line corresponding to haplotypes Dcit-01 and Dcit-04 with identification numbers FJ190300 and FJ190 306 (Boykin, 2007). Twenty-two sequences were obtained, which were analyzed with the Oligo analyzer and Clustal Omega programs and 11 sequences equal to haplotypes Dcit-01 and Dcit-04 were identified. The results showed 13 sequences with differences in three specific nucleotides: 61, 253 and 636, which are reported in this work as new haplotypes.

Keywords: *Diaphorina citri*, haplotypes, psyllid.

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Introduction

There are different pests that affect the production of citrus, some are vectors of causative agents of diseases, so they are considered the most important due to the impact they generate in the spread of pathogens such as viruses, bacteria, and fungi. The most important pest in citrus production is *Diaphorina citri*, also known as Asian citrus psyllid (ACP) (Martín *et al*., 2014). The psyllid causes significant damage, as it feeds on tender shoots; it introduces its piercing-sucking mouthparts to feed on the sap that flows through the sieve tubes (Bové, 2006). It is capable of staying in citrus-associated weeds (Clarke and Brown, 2018).

ACP is a pest native to Asia that has been reported in different countries of America, among them are Brazil, Argentina, the United States of America, and Mexico, where it was first reported in the state of Campeche in 2002 (López-Arroyo *et al*., 2005). Because it is an exotic pet, a process known as speciation is likely to occur during dispersion (Prentis *et al*., 2008). One of the factors that influences this process is the different climatic conditions to which individuals are exposed; that is, the need to adapt to the environment generates evolutionary changes at the genetic level and that can manifest themselves in different ways (Boykin *et al*., 2012).

Some of these changes can be size, color, gestation time, reproduction times, alternative hosts, and resistance to control strategies, etc. (Moncayo-Donoso *et al*., 2014). Currently, studies related to the ACP are aimed at evolutionary, ecological, and biological issues. That is why studies that help understand the behavior between the insect vector and the pathogen that causes HLB have been prioritized in order to find elements to develop management strategies that help reduce losses in citrus production (Flores-Aguilar *et al*., 2020).

Molecular studies are tools that allow identifying the genetic, phylogenetic and population dynamics variants of ACP. The techniques used are based on the study of mitochondrial DNA and with it the structure of its population genetics that deduce and explain the population behavior of the ACP of different regions (Moncayo *et al*., 2014). Currently, molecular studies for the identification and description of genetic variants are related to the cytochrome oxidase (COI) gene (Fuentes *et al*., 2018).

In COI, ascending genetic characteristics are inherited, which is why the COI gene turns out to be the best object of study, since it allows tracing the ancestors of any individual (Paternina *et al*., 2016). De León *et al*. (2011) report that there is a high gene flow between ACP populations when comparing population records from different countries such as South Africa, the United States of America, Mexico, Argentina and Brazil. Using the mitochondrial gene (COI), they found that there are 23 global haplotypes and that, in Mexico, the dominant haplotype is H9. This is found in the states of Yucatán, Nuevo León, Tamaulipas, and San Luis Potosí.

In the Caribbean, 12 haplotypes from a sample of 46 individuals were described by using the mitochondrial COI gene, of which H2 and H7 are dominant, these haplotypes are not reported for Mexico, but they are reported for Texas and Florida. Genetic mutations are related to different factors, in this case, ACP individuals were found in weeds near citrus groves, so it is important to

verify if there is presence of individuals in vegetation near crops (Clarke and Brown, 2018). Boykin *et al*. (2012) analyzed 212 sequences obtained from 52 populations in different parts of the world, where they found that the sequences reported by each country are closely similar and only have differences in one of their nucleotides.

In said work, they were grouped according to their similarities and were reduced to eight global haplotypes that are distributed in the world. These haplotypes are in countries where there are various biotic and abiotic factors of regions with broad national and international gene flow. The relevance of studies of the genetic diversity of populations is reflected in the mutations of individuals, some are expressed physically, such as size and elongation of the body, eyes, wings and even mouthparts, there are also ecological expressions in which individuals modify their life and reproduction cycles, as well as mating times and adaptation to climates and altitudes, even with limited samples of collections or collections preserved for their morphological study and which have also shown variations in the distribution of populations (Lotta-Arevalo *et al*., 2020).

There are cases in which new unknown genetic variants have been identified in isolated populations; that is, populations where gene flow is limited and generates evolutionary pressure on groups of individuals, which causes genetically manifested alterations (Bonal *et al*., 2019). Evolutionary changes can be generated by different factors, one of them is the use of chemical agents for the control of pest insects, in which genetic differences have been found in situations where intensive control strategies are used in order to minimize populations; however, this generates a selection pressure on individuals who in turn mutate and become resistant (Atencia *et al*., 2018).

Materials and methods

The present work aimed to study the genetic variants of *Diaphorina citri* through the use of the endpoint PCR technique in order to know the distribution of populations in citrus areas of the country. Likewise, to describe and know if there are new ACP haplotypes in citrus areas where no previous studies have been carried out. The criteria for the selection of groves were: plants up to 10 years old, groves that are next to bodies of water and citrus collection centers such as packers and waxers, groves in which any type of citrus is produced.

With the help of a mouth aspirator, adult females and males were collected in the spring-summer period of 2020 in six states of the republic: Ciudad Mante, Tamaulipas, Montemorelos, Nuevo León; Tecomán, Colima, Misantla (Misantla is a municipality and belongs to Veracruz), (Veracruz is the state, not the municipality); Tantoyuca, Veracruz; Huejutla, Hidalgo and Acatlán de Pérez Figueroa, Oaxaca. A total of 60 adult individuals were used, which were processed to obtain DNA, then 55 were subjected to PCR to amplify their DNA.

Due to the low availability of individuals in the groves at each sampling site, there were different numbers of individuals (Table 1). The psyllids were extracted from commercial orange, lemon and tangerine plantations that showed no apparent symptoms of the presence of HLB. The criteria for

the selection of groves were plants up to 10 years old, groves that are next to bodies of water and collection centers. The insects were collected with the help of a manual aspirator and transported in vials with 96% alcohol. The extraction was carried out following the CTAB method modified by Almeyda and Rocha, (2001). One psyllid was used for each extraction to make a total of 55 extractions.

Table1. Codes and number of individuals for each collection site.

Site	Code	Site	Code
Ciudad Mante, Tamaupas (17 individuals)	A1	Monte Morelos, Nuevo León (10 individuals)	A7
	A2		A8
	A11		A9
	A12		A10
	A13		A39
	A14		A40
	A15		A41
	A16		A42
	A17		A43
	A29		A44
	A30	Huejutla, Hidalgo (6 individuals)	A17
	A50		A18
	A51		A19
	A52		A31
	A53		A32
	A54		A33
	A55	Acatlán de Pérez, Oaxaca (5 individuals)	A20
	A56		A21
Misantla, Veracruz (4 individuals)	A3		A22
	A4		A23
	A5		A24
	A6	Tecomán, Colima (8 individuals)	A25
Tantoyuca, Veracruz (5 individuals)	A34		A26
	A35		A27
	A36		A28
	A37		A47
	A38		A48
			A49
			A56

A Select Bioproducts endpoint thermal cycler was used. DNA from one insect was processed separately, for a total of five individuals for each collection site. The primers used were: DCITRI COI-L (AGGAGGTGGAGACCCAATCT) and DCITRI COI-R (TCAATTGGGGGAGAG TTTTG) (Clarke and Brown, 2018). The amplification conditions were: predenaturation temperature 94 °C for 4 min, 40 cycles with denaturation temperatures at 94 °C for 40 s, alignment at 58 °C for 40 s, with an extension of 1 min at 72 °C.

The molecular weight marker used was one of 100 bp of the invitrogen brand for a fragment of 812 bp, which were visualized in a 1.5% agarose gel using a transilluminator (Figure 1). The DNA obtained from the insects for the description of haplotypes was sent to the Institute for Scientific and Technological Research of San Luis Potosí (IPICYT, for its acronym in Spanish). The sequences obtained, product of the PCR tests, were compared with those reported in Genbank using the OligoAnalyzer and Clustal Omega programs; likewise, with the help of the latter, a dendrogram of said sequences was made.

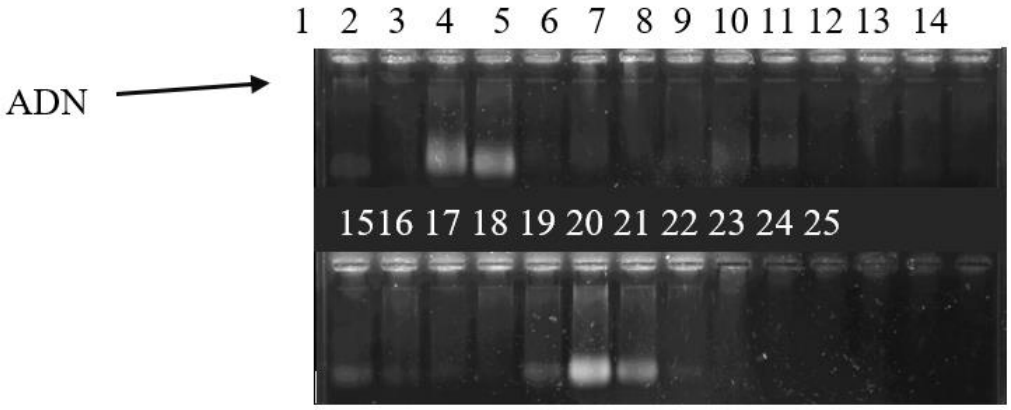

Figure 1. 2% agarose gel showing DNA extraction by psyllid. Wells: 1-5 Misantla, Ver.; 6-15 Cd. Mante, Tamps.; and 16-25 Montemorelos, N. L.

Results

The DNA that resulted as a product of PCR, in some individuals for the identification of ACP haplotypes, did amplify, so it was visualized in the agarose gel. In the gel it was observed that the DNA amplification was at molecular weight 683bp (Figure 2). In total, 23 final amplifications were obtained from a total of 60 insects collected and 55 processed, which were sent for sequencing.

Three new haplotypes were found and were named DcitACC-1, DcitACC-2, and DcitACC-3. The result of the comparisons between sequences obtained and between sampling sites presents differences in some nucleotides between individuals, even when they are part of the same population. Once the sequences obtained were compared with those reported in Genbank, it was found that there is a common ancestor with the haplotypes Dcit-01 and Dcit-04 with identification numbers FJ190300 and FJ190306 (Boykin *et al*., 2007).

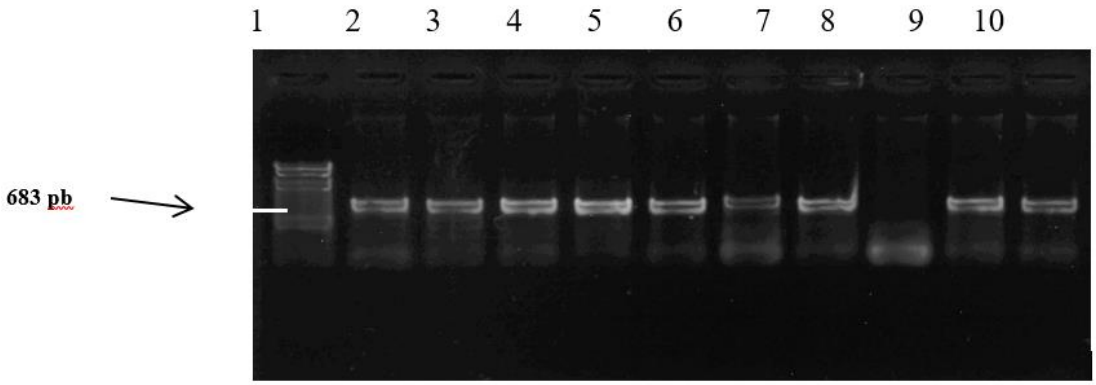

Figure 2. DNA amplification with the primers DCITRI COI-L and DCITRI COI-R for mitochondrial gen of *Diaphorina citri*. Wells: 1) molecular marker; 2) Ciudad Mante, Tamaulipas; 3-6) Misantla, Veracruz; 7) Huejutla, Hidalgo; 8, 10 and 11) Acatlán de Pérez, Oaxaca. Well 9 without reaction.

Of the 23 individuals selected from the five states, 11 are equal to haplotypes Dcit-01 and Decit-04. It was found that 13 sequences corresponding to: Misantla, Veracruz; Huejutla, Hidalgo; Ciudad Mante, Tamaulipas; Acatlán de Pérez, Oaxaca; and General Terán, Nuevo León, have differences in three specific nucleotides; that is, haplotype Dcit-01 has a 'T' base, while the 12 samples mentioned above have a 'G' base. Another difference is shown in nucleotide 253, where there is normally a 'T' base, 7 of the 12 samples have a 'C' base (Table 2).

Table 2. Differences between nucleotides of *D. citri* haplotypes found using the mitochondrial COI gene. (1) Common ancestor reported in Genbank, (2-14) New haplotypes.

Site	Sample	Nucleotides			Haplotypes
		61	253	636	
1. Genbank	Dcit-01	T	T	T	FJ190300, FJ190306
	Dcit-04	T	T	T	
2. Tecomán, Colima	A25	G	C	T	DcitACC-1 (New)
3. Tantoyuca, Veracruz	A34	G	C	T	
4. Huejutla, Hidalgo	A33	G	C	T	
5. C. D. Mante. Tamaulipas	A51	G	C	T	
6. Misantla, Veracruz	A5	G	C	T	
7. Misantla, Veracruz	A6	G	C	T	
8. Acatlán de Pérez, Oaxaca	A23	G	C	T	
9. Tantoyuca, Veracruz	A36	G	T	T	DcitACC-2 (New)
10. Tantoyuca, Veracruz	A35	G	T	T	
11. Montemorelos, Nuevo León	A43	G	T	T	
12. Misantla, Veracruz	A3	G	T	T	
13. Tecomán, Colima	A49	G	T	T	
14. Acatlán de Pérez, Oaxaca	A22	G	T	A	DcitACC-3 (New)

Regarding the haplotype Dcit-04, the differences are in the same nucleotides as Dcit-01. The dendrogram shows the genetic distances that exist between groups of individuals and the haplotypes Dcit-01 and Decir-04 reported in Genbank. It can be seen that the haplotypes called DcitACC-1, DcitACC-2 and DcitACC-3 are genetically different from haplotypes FJ190300 and FJ190306 (Boykin *et al*., 2007). The haplotypes DcitACC-1 and DcitACC-3 are genetically closer, while DcitACC-2 is more distant. The dendrogram shows that the group DcitACC-2 is different from the haplotypes reported for Mexico and in turn is different from the groups DcitACC-1 and DcitACC-3 (Figure 3).

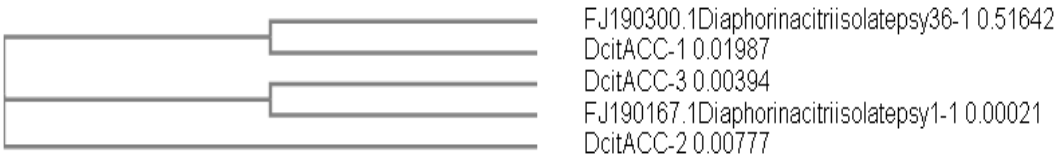

Figure 3. Dendrogram generated from the sequences obtained for each of the groups of individuals processed.

Discussion

In this work, a matrix line recorded in Genbank with the access number FJ190346, and which is named as Dcit-01 (Boykin *et al*., 2007), was identified; that is, it is a common ancestor that is reported in different parts of the world including Mexico and that, in addition, in our country is the one that predominates. The record FJ190346 served as a reference to detect the differences that exist specifically in three nucleotides (Table 2).

The genetic variations are related to new reports in which the presence of ACP where it was not previously found has been documented (Monzó *et al*., 2015). According to what was reported by Luo and Agnarsson (2018), there are other matrix lines identified due to the distribution of the sampled sites that correspond to countries with factors that influence ecological habits in the development of ACP, as well as conditions different from those of Mexico, for example: climate, relief, predators, host varieties, etc.

Another important point is that the sequences reported in Mexico correspond only to a geographical region of the country, where the mobility of populations may be limited by factors such as: traceability of citrus, climatic conditions that are not found in other parts of the country and little availability of ACP individuals due to the intensity of the applications for their control, this suggests that sampling is limited and therefore the number of existing haplotypes has not been identified.

ACP populations are related to different climatic conditions and others such as the intensive use of pesticides and other control strategies to which they are exposed (Lashkari *et al*., 2014). The present work identified new genetic variants of ACP taken from different parts of the country and which, in turn, is an indicator of a wider diversity in the populations. This point can be contrasted with the work of Guidolin *et al*. (2014), where they report a wide distribution of genetic variants and relate them to the location of sampling points.

There are some coincidences with what was reported by Clarke and Brown (2018), where they relate the distribution and variety of ACP populations with climate change due to the deterioration in the usual temperature ranges of the regions where individuals historically develop and reproduce.

The temperature ranges for the optimal development of ACP range from 25 °C to 28 °C; nevertheless, there are reports that it survives at temperatures of -7 °C to 48 °C (Aurambout *et al*., 2009). The variants found in this work may be related to the climatic conditions in which they live, where the average maximum temperature is 26 °C to 30 °C and the minimum are 18 °C to 21 °C, while 22 °C to 25 °C is the average temperature range that predominates, indicating that this temperature range is not the optimum in which the ACP develops (Díaz-Padilla *et al*., 2018).

Another aspect is the control strategies, the use and abuse of chemical control that generates degrees of resistance that are manifested in the genetic expression and modification (Mora-Aguilera *et al*., 2016). This point coincides with the results obtained in this work, the low populations found in citrus areas are the result of the control strategies used since in some groves the sampling was carried out at the time where they were making applications.

In this work, new haplotypes were found in some of the main citrus-producing states of the country, due to the importance that these sites represent, large-scale applications are made, and this exerts selection pressure on individuals. This coincides with what was mentioned by Atencia *et al*.(2018), where in areas of high pest impact, constant applications have been made, the collections suggest mutations of known haplotypes in new haplotypes with variations in two or three nucleotides originated due to selection pressure, even in limited quantities of collections of individuals.

It has been reported that, in the Caribbean, there are haplotypes that are not found in Mexico, weeds near citrus plantations have been sampled, this may be an indicator of the short-distance mobility that ACP individuals may have to take refuge when conditions do not allow them to remain in their usual hosts (Clarke and Brown, 2018). In Mexico, there are few reports about the genetic variants of ACP, works such as that of Grafton-Cardwell *et al*. (2013) refer to populations and their genetic variants found in Brazil and Florida; that is, they consider samples only from some citrus areas of the region.

Restricting sampling only to the main citrus-producing areas or to regions with high gene flow where populations are not isolated is one of the factors limiting the identification and description of ACP haplotypes (Moncayo-Donoso *et al*., 2014). While this work had collections from areas that have not been sampled or reported previously, for this reason there could be significant differences in the sequences obtained. The individuals collected in this work are from some states where citrus activity is not the main one, and it is also important to mention that, in some groves, the ACP populations were very low due to the control that is carried out because it is a pest of great importance. This coincides with what was reported by Bonal *et al*. (2019), where genetic variations were identified in isolated populations of *Curculio* spp. Where genetic exchange is limited by the distance between populations, this causes individuals to mutate. This could be reflected in the modification of reproduction times or even morphometric changes, although studies to support it are lacking.

The collection sites may be the main reason that differences have been identified in the sequences obtained with the works since, taking into account what was reported by Ajene *et al*. (2022), they are directly obtained from the gene bank, suggesting that the processed individuals were obtained from the main citrus-producing areas of various countries or from places where there is a wide genetic plasticity, while the samplings of this work were carried out in isolated geographical areas in places where citrus crops do not predominate despite being commercial groves.

Conclusions

The sample used in this work was sufficient for the identification of new haplotypes, in the same way, it was possible to identify their genetic distribution and geographical location. The new haplotypes identified coincide with the geographical regions of the country where no research regarding genetic variants has been carried out, this confirms that it is necessary to implement samplings in groves in areas where citrus cultivation is not dominant. The distribution of haplotypes in Mexico is wider and is not restricted only to those reported in Genbank.

The identification of three new haplotypes suggests that, in future works, the collections should be concentrated on the largest number of individuals present in the crops, since due to the applications and control strategies, there is no abundance of individuals. The limited availability of ACP in some groves hinders the expansion and feedback of studies. It is important to highlight that there are groves where the flow of populations is restricted due to the isolation of the place where they appear, in this research new haplotypes were found in places where the groves were surrounded by other crops or livestock.

The contribution in the identification of new haplotypes contributes to generate ACP control strategies and complements other identification works related to the habits of the psyllid. It is suggested to carry out morphological and ecological studies in ACP populations to identify how the different haplotypes expressed themselves between sites. In future works it is important to expand the collection sites to regions of the country where no sampling has been carried out, consider all citrus and even collect in backyard gardens and weeds near groves; likewise, to collect again in the main producing areas of the country considering the aforementioned criteria to monitor mutations and record new genetic variants.

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