Genetic diversity of *Candidatus* Liberibacter asiaticus strains from the Central Pacific citrus region in Mexico

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Abstract. Huanglongbing (HLB) is associated with *Candidatus* Liberibacter asiaticus (*C*Las). It is a destructive disease of citrus. The objective of this study was to determine the genetic diversity of 90 strains of CLas infecting eight citrus species trees from the Central Pacific in Mexico. Genetic diversity among *C*Las was estimated by fourteen variable numbers of tandem repeat (VNTRs) loci. Three loci were polymorphic, SSR00 and SSR077 amplified four alleles each, while the locus SSR-A amplified two alleles, and the other loci only one allele per locus, resulting in a total of 21 alleles. Dendrogram analysis showed two clusters. No clear genetic structure was found in relation to geographical origin or host. The cluster I was mostly constituted by the majority of *C*Las strains (82%), but the cluster II comprised twelve strains of *C*Las collected in Tecoman location, State of Colima, and were obtained from different citrus hosts species. The frequency of 17 haplotypes among strains of *C*Las from the states of Nayarit, Jalisco, Colima and Michoacán was analyzed; in Colima 14 haplotypes were determinated, while in Michoacan all strains were identified in one haplotype. These results indicate a large genetic diversity among the strains of *C*Las present in the Central Pacific region in Mexico.

Key words: Huanglongbing, Mexican lime, SSR markers.

Huanglongbing (HLB) is a highly destructive citrus disease associated with *Candidatus* Liberibacter spp., a phloem-limited, gram-negative and noncultured alpha-proteobacterium (Jagoueix *et al*., 1994, Ghosh *et al.,* 2018, Dai *et al.,* 2019). Based on geographic area of their origin and temperature tolerance, they are classified into three species: *Ca*. L. asiaticus (*C*Las), *Ca*. L. africanus (*C*Laf), and *Ca*. L. americanus (*C*Lam). These

bacteria are transmitted by two vectors, *Diaphorina citri* (Hemiptera: Liviidae) that transmits both *C*Las and *C*Lam and *Trioza eryitreae* (Hemiptera: Psyllidae) that is responsible for *C*Laf transmission (Aubert, 1987; Bové, 2006).

The disease can be spread by contaminated plant material grafts that propagate at long distances (Hartung *et al.*, 2010; Wang and Trivedi, 2013). The detection of genetic diversity within pathogen populations is fundamental for ecological and epidemiological studies of a disease (Wang and Triveli, 2013). Knowledge of the genetic structure of a population of pathogens is useful to know the source or origin of a pathogen and the management of the disease (Islam *et al.,* 2012). The first studies for genetic differentiation of *C*Las populations were made using conserved genes as genetic markers, for example: 16S rDNA and 16S/23S regions, *omp*, the *rlp* gene cluster and the bacteriophage-type DNA polymerase region (Bastianel *et al*., 2005; Adkar *et al*., 2009; Tomimura *et al*., 2009; Miyata 2011).

However, genetic diversity into the conserved genes has limited discriminatory power to differentiate close-related isolates in a population (Islam *et al.,* 2012) and most consisted of single-nucleotide polymorphisms (SNPs). In addition, the completion of genome sequence of *C*Las strain Psy 62 is now available (Duan *et al*., 2009), which facilitated the identification of a variable number of tandem repeats (VNTRs), also known as microsatellite or short sequence repeat, which demonstrated the potential for examination of *C*Las genetic variability (Zhou *et al*., 2008; Chen *et al.,* 2010; Katoh *et al.,* 2011; Katoh *et al*., 2012; Islam *et al*., 2012; Wang *et al.,* 2012; Matos *et al*., 2013). The VNTRs can provide sufficient resolution to differentiate closely related isolates and for tracking genotypes of interest; additionally, these markers may help to identify the source of invasive strains (Islam *et al*., 2012; Singh *et al*., 2019).

In Mexico, HLB was first reported in July 2009 in the municipality of Tizimin, state of Yucatan, and in November, of the same year, a new focus of the disease was reported in the Mexican Pacific region, in the states of Jalisco and Nayarit and by 2010 in Sinaloa and Colima also was reported the presence of the disease (SENASICA, 2010). Nowadays, around 80% of the citrus producing areas are infected. Knowledge about the genetic diversity of Mexican *C*Las from different geographical areas and citrus cultivars is indispensable for the management of disease risk. Limited studies are available on the characterization and discrimination of Mexican strains of *C*Las.

The objective of this study was to characterize strains of *C*Las from the Central Pacific region in Mexico in order to identify possible relationship relationships between the geographic origin and the host in relation with the genetic diversity of the pathogen.

MATERIALS AND METHODS

Sampling strategy. Leaf samples were collected from different infected citrus species, including Mexican lime (*Citrus aurantifolia*), Persian lime (*C. latifolia*), sweet orange (*C. siniensi*s), grapefruit (*C. aurantium*), lime (*C. limetta*), alemow (*C. macrophylla*), Volkamer lemon (*C. volkameriana*) and mandarin (*C. reticulata*) that showed blotchy mottle and yellowing, the classical symptoms of HLB disease, from different groves of the states of Colima, Jalisco, Michoacan and Nayarit, all located in the Central Pacific region in Mexico (Table 1).

Grafting of infected plant material. We performed a collection of citrus budwood from plants showing typical symptoms of HLB in major citrus producing areas of Colima, Jalisco, Michoacan,

Table 1. Information of the *Candidatus* Liberibacter asiaticus strains collected from different host and geographic locations in Mexico.

Dendrogram ID	Location ID	Host (genre/specie)	Host (common name)	Location	Year of collection	Cluster
			Strains from Colima			
Colima1	ColCol1	*Citrus macrophylla*	Alemow	Colima		I
Colima2	ColCol2	*Citrus aurantifolia*	Mexican lime	Colima		I
Colima3	ColCol3	*Citrus aurantifolia*	Mexican lime	Colima		I
Colima4	ColCol4	*Citrus aurantifolia*	Mexican lime	Colima		I
Colima5	ColCol5	*Citrus aurantifolia*	Mexican lime	Colima		I
Colima6	ColCol6	*Citrus aurantifolia*	Mexican lime	Colima		I
Colima7	ColCol7	*Citrus aurantifolia*	Mexican lime	Colima		I
Colima8	ColCol8	*Citrus* x *sinensis*	Sweet orange	Colima	2012	I
Colima9	ColCol9	*Citrus* x *sinensis*	Sweet orange	Colima	2012	I
Colima10	ColCol10	*Citrus aurantifolia*	Mexican lime	Colima	2012	I
Colima11	CuCol1	*Citrus aurantifolia*	Mexican lime	Cuauhtemoc	2012	I
Colima12	CuCol2	*Citrus aurantifolia*	Mexican lime	Cuauhtemoc	2012	I
Colima13	CuCol3	*Citrus aurantifolia*	Mexican lime	Cuauhtemoc	2012	I
Colima14	CuCol4	*Citrus aurantifolia*	Mexican lime	Cuauhtemoc	2012	I
Colima15	CuCol5	*Citrus* x *sinensis*	Sweet orange	Cuauhtemoc	2012	I
Colima16	CuCol6	*Citrus* x *sinensis*	Sweet orange	Cuauhtemoc	2012	I
Colima17	TecCol1	*Citrus aurantifolia*	Mexican lime	Tecoman	2010	II
Colima18	TecCol2	*Citrus* x *sinensis*	Sweet orange	Tecoman	2011	I
Colima19	TecCol3	*Citrus aurantium*	Grapefruit	Tecoman	2011	II
Colima20	TecCol4	*Citrus aurantifolia*	Lime	Tecoman	2011	I
Colima21	TecCol5	*Citrus reticulata*	Mandarin	Tecoman	2011	II
Colima22	TecCol6	*Citrus macrophylla*	Macrophylla	Tecoman	2011	II
Colima23	TecCol7	*Citrus macrophylla*	Macrophylla	Tecoman	2011	II
Colima24	TecCol8	*Citrus macrophylla*	Macrophylla	Tecoman	2011	II
Colima25	TecCol9	*Citrus macrophylla*	Macrophylla	Tecoman	2011	II
Colima26	TecCol10	*Citrus macrophylla*	Macrophylla	Tecoman	2011	II
Colima27	TecCol11	*Citrus aurantifolia*	Mexican lime	Tecoman	2011	II
Colima28	TecCol12	*Citrus aurantifolia*	Mexican lime	Tecoman	2011	II
Colima29	TecCol13	*Citrus aurantifolia*	Mexican lime	Tecoman	2011	II
Colima30	TecCol14	*Citrus aurantifolia*	Mexican lime	Tecoman	2011	II
Colima31	CoqCol	*Citrus aurantifolia*	Mexican lime	Coquimatlan	2011	I
			Strains from Michoacan			
Michoacan1	CoaMich1	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan2	CoaMich2	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan3	CoaMich3	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan4	CoaMich4	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan5	CoaMich5	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan6	CoaMich6	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan7	CoaMich7	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan8	CoaMich8	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan9	CoaMich9	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan10	CoaMich10	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan11	CoaMich11	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan12	CoaMich12	*Citrus* x *sinensis*	Sweet orange	Coahuayana	2012	I
Michoacan13	CoaMich13	*Citrus* x *sinensis*	Sweet orange	Coahuayana	2012	I

Table 1. Continue.

Dendrogram ID	Location ID	Host (genre/specie)	Host (common name)	Location	Year of collection	Cluster
Michoacan14	CoaMich14	*Citrus sinensis*	Sweet orange	Coahuayana	2012	I
Michoacan15	CoaMich15	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan16	CoaMich16	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan17	CoaMich17	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan18	CoaMich18	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan19	CoaMich19	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan20	CoaMich20	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan21	CoaMich21	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan22	CoaMich22	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan23	CoaMich23	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan24	CoaMich24	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan25	CoaMich25	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan26	CoaMich26	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan27	CoaMich27	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
Michoacan28	CoaMich28	*Citrus aurantifolia*	Mexican lime	Coahuayana	2012	I
			Strains from Jalisco			
Jalisco1	CiJal1	*Citrus reticulata*	Mandarin	Cihuatlan	2012	I
Jalisco2	CiJal2	*Citrus aurantifolia*	Mexican lime	Cihuatlan	2012	I
Jalisco3	CiJal3	*Citrus aurantifolia*	Lime	Cihuatlan	2012	I
Jalisco4	BNJal1	*Citrus aurantifolia*	Lime	Barra de Navidad	2012	I
Jalisco5	BNJal2	*Citrus aurantifolia*	Lime	Barra de Navidad	2012	I
Jalisco6	BNJal3	*Citrus sinensis*	Sweet orange	Barra de Navidad	2012	I
Jalisco7	BNJal4	*Citrus sinensis*	Sweet orange	Barra de Navidad	2012	I
Jalisco8	BNJal5	*Citrus sinensis*	Sweet orange	Barra de Navidad	2012	I
Jalisco9	BNJal6	*Citrus aurantifolia*	Mexican lime	Barra de Navidad	2012	I
Jalisco10	BNJal7	*Citrus aurantifolia*	Mexican lime	Barra de Navidad	2012	I
Jalisco11	BNJal8	*Citrus aurantifolia*	Mexican lime	Barra de Navidad	2012	I
Jalisco12	BNJal9	*Citrus aurantifolia*	Mexican lime	Barra de Navidad	2012	I
Jalisco13	BNJal10	*Citrus aurantifolia*	Mexican lime	Barra de Navidad	2012	I
Jalisco14	MeJal1	*Citrus aurantifolia*	Mexican lime	Melaque	2012	I
Jalisco15	MeJal2	*Citrus aurantifolia*	Mexican lime	Melaque	2012	I
Jalisco16	SMHJal1	*Citrus latifolia*	Persian lime	San Martin de Hidalgo	2012	II
Jalisco17	CasCJal	*Citrus latifolia*	Persian lime	Casimiro Castillo	2012	I
			Strains from Nayarit			
Nayarit1	TepNay1	*Citrus aurantifolia*	Mexican lime	Tepic	2011	I
Nayarit2	TepNay2	*Citrus latifolia*	Persian lime	Tepic	2012	I
Nayarit3	TepNay3	*Citrus latifolia*	Persian lime	Tepic	2012	I
Nayarit4	TepNay4	*Citrus latifolia*	Persian lime	Tepic	2012	I
Nayarit5	NvoVall1	*Citrus reticulata*	Mandarin	Nuevo Vallarta	2012	I
Nayarit6	NvoVall2	*Citrus reticulata*	Mandarin	Nuevo Vallarta	2012	I
Nayarit7	NvoVall3	*Citrus sinensis*	Sweet orange	Nuevo Vallarta	2012	I
Nayarit8	NvoVall4	*Citrus aurantifolia*	Mexican lime	Nuevo Vallarta	2012	I
Nayarit9	NvoVall5	*Citrus aurantifolia*	Lime	Nuevo Vallarta	2012	I

Table 1. Continue.

Dendrogram ID	Location ID	Host (genre/specie)	Host (common name)	Location	Year of collection	Cluster
			Strains from Yucatan			
Yucatan1	ProYuc1	*Citrus aurantifolia*	Mexican lime	Progreso	2011	I
Yucatan2	ProYuc2	*Citrus latifolia*	Persian lime	Progreso	2011	I
Yucatan3	MocYuc2	*Citrus volkameriana*	Volkameriana	Moctezuma	2011	I
Yucatan4	MocYuc1	*Citrus latifolia*	Persian lime	Moctezuma	2011	I
			Strains from Sinaloa			
Sinaloa	IslaPiedra	*Citrus aurantifolia*	Mexican lime	Isla de Piedra	2011	I

Nayarit and Yucatan. Samples were placed in paper bags, labeled, and transported further processing. All samples were side-grafted on *C. volkameriana* or *C. macrophylla* rootstock and were kept under greenhouse covered with antiaphid mesh at 34/25°C (day/ night) and a photoperiod 14 h light and 10 h darkness for 3 weeks. Weekly observations were made for symptoms appearance (Garnier *et al.* 2000).

DNA extraction. Basically, leaf samples were collected from citrus trees with blotchy mottle and blotchy mottle-like symptoms. Leaves were washed under running tap water and blotted dry with paper towels. The midribs were then excised from the leaf blade. Total genomic DNA was extracted from 4–5 midribs per sample. Samples were ground in liquid nitrogen and DNA was extracted using the CTAB method. Precipitated DNA was dissolved in 100 µL of TE buffer. The quality of DNA samples was checked by spectrophotometry using Nanodrop 2000 (Thermo Scientific; U.S.A.) and electrophoresis in 1.2% agarose gels. All DNA samples were subjected to PCR amplification with primers set OI1/OI2c (Jagouerix *et al*., 1994) to confirm infection of the corresponding trees from which tissue was collected.

VNTRs analysis. The VNTRs analysis was performed with 14 pairs of oligonucleotides (Table 2), each pair for different locus in a total of 90 strains of *C*Las. PCR reaction was carried out in GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Carlsbard, CA, USA) in a total volume of 20 µL which contained 50 ng of template DNA, 0.1 µM of each oligonucleotide (Table 2), 200 mM dNTP's mixture, 20 mL of $MgCl_2$ (30 mM), 1X PCR buffer and 2.5 U of *Taq* Polymerase. The thermal cycles were as follows: initial denaturation at 92 °C for 2 min, 35 cycles of amplification at 92 °C each 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 1 min and a final cycle at 72 °C for 5 min (Katoh *et al.,* 2011; Islam *et al*., 2012). Subsequently, the PCR products were separated by gel electrophoresis in 1.5% agarose, stained in ethidium bromide solution and visualized in a ChemiBis MF-2 phtotodocumenting (Bio-Imaging Systems, Neve Yamin, Israel).

Confirmation of *C*Las by sequencing. To corroborate the presence of *C*Las, only two PCR-amplified products were purified from agarose gels using a commercial kit method (QIAGEN, Hilden, Germany). The sequencing was performed at LANGEBIO (CINVESTAV,

Table 2. Characteristics of the oligonucleotides primers and number of alleles generated in strains of *Candidatus* Liberibacter asiaticus from Mexico.

Locus	Foward primer sequence	Reverse primer sequence	Type of repeat motifs	T (º C)	Size range (bp)	No. of alleles
SSR001	TGAAGTAGCTCTGCAATATCTGA	GGTGAATTAGGATGGAAATGC	$(TACAGAA)_8$	54	400-450	1
SSR002	TTGATAATATAGAAAGAGGCGAAGC	TCCATACCCAAAAGAAAAGCA	$(CAGT)_8$	54	500-650	1
SSR005	ATTGAAGGACGAAACCGATG	TCCCAAGGTTTTCAAATTGC	$(CAGT)_8$	54	500-650	4
SSR006	TCATGTTGATCAGACGCTTTTT	CACTTAATAACGCCCCGAAA	$(TCTTTACA)_3$	54	500-650	1
SSR007	TGGATAGCATGCTCATTTGAA	AAGGCAAATTTCCCCATACG	$(TCAGTA)_3$	54	500-650	1
SSR010	CGTCAGAATAATCAGCGCATA	TGGATTCGAAAGAACCGTCT	$(CAAT)_3$	54	500-650	1
SSR013	AGATTGATGGGCGATAGCTG	TGTCGCATTGTAGACCCTGA	$(TAACTTG)_2$	54	500-650	1
SSR014	AATCCCTTGCTCGTAGGTGA	AAAGATAAGCGACCCGGATT	$(TAAAGAG)_2$	54	400-500	1
SSR022	AATCCCTTGCTCGTA GGTGA	ATTTGAGCCGTGAAACTTCG	$(AAAC)_3$	54	500-650	1
SSR024	GTGGGGAGAGAAGTCGGTTT	ACCGTACCGCTCCAATATGA	$(TTGG)_3$	54	500-650	1
SSR077	TGACTGATGGCAAAAGATGG	AGACACGCCAAACAAGGAAT	$(TTTG)_{14}$	54	500-600	4
SSR-A	CGCCTACAGGAATTTCGTTACG	TCTCATCTTGTTCGTTTATCC	$(TATTCTG)_8$	50	241-434	2
SSR-D	CGGTGTCGGTATCGGTATCATTC	CGAAGAAGAGACGGAGGTTAAGC	$(TTC)_5$	55	158-174	1
SSR-E	GATCAGTAGTCTATCACCAC	TACTGGAAACAAATGGAATAC	$(CTTGTGT)_5$	50	173-290	1

Irapuato, Mexico). DNA sequences were compared by using the Blast Sequence Analysis Tool (Madden, 2013) and T-Coffee (Notredame *et al*., 2000) obtained DNA sequences were compared to those from other *Candidatus* species such as '*Candidatus* Liberibacter solanacearum' (*C*Lso) (AN. CP002371.1), '*Candidatus* Liberibacter americanus' (*C*Lam) (AN. CP006604.1) and '*Candidatus* Liberibacter africanus' (*C*Laf) (AN. CP004021.1) and '*Candidatus* Liberibacter asiaticus' (AN. KJ885230.1, AN. CP029348.1).

Data analysis. The analysis began with the direct observation of the bands obtained in the agarose gel, which were numbered according to their molecular weight and it was assumed that such bands or amplified fragments in different isolates were identical if they had the same molecular weight. Data were considered haploid. We conducted a binary data matrix (assigning a numerical value of 1 to assume the presence and 0 for absence) and genetic variability was estimated by the similarity index developed by Dice (1945) and adapted by Nei (1973) for data molecular ($H = 1 - \Sigma Xi^2$, where H = population genetic variability and ΣXi is the frequency of different alleles at a particular locus, which ranges from zero to one). Then, the matrix was made with a dendrogram using the arithmetic method of unweighted pair group (UPGMA) NTSYS-pc program version 2.0 (Rohlf, 1997).

RESULTS

Grafting of infected plant material. Of the 90 samples collected in the states of Colima, Michoacan, Jalisco, Nayarit, Yucatan and Sinaloa, all induced severe vein yellowing, leaf mottle and nutritional-like deficiency symptoms in Alemow and Volkamer lemon within six months from grafting thus were retained as affected by HLB, which will be kept as an *in vivo* collection.

Genetic diversity. Fourteen VNTRs loci were used to determinate the genetic diversity of 90 Mexican strains of *C*Las collected from different citrus species and geographical regions, the results showed that the loci SSR005 and SSR077 amplified four alleles each, while SSR-A amplified two alleles, and the other loci (SSR001; SSR002; SSR006; SSR007; SSR010; SSR013; SSR014; SSR022; SSR024; SSR-D and SSR-E) only one allele per loci, resulting a total of 21 alleles (Table 2).

Dendrogram generated indicated two clusters among *C*Las strains analyzed in this study (Figure 1). In relationto the geographical origin of the strains of *C*Las, those from Colima were found in both clusters; however, it was not possible to demonstrate the relationship with their origin. In comparison with the host, cluster I was formed by *C*Las strains obtained from Mexican lime, sweet orange, lime, Persian lime and Volkamer lemon; while the cluster II was constituted by Mexican lime, grapefruit, alemow and mandarin. Only, the samples of Mexican lime were present in both clusters.

Based on the combination of the allelic data obtained from 14 VNTRs loci, 17 haplotypes were identified in a total of 90 strains of *C*Las from Colima, Nayarit, Jalisco and Michoacan, for which only two haplotypes were found to be unique at Nayarit (Figure 2). An overlapping of identical haplotype was found in different geographical locations, such as haplotype 1, which was found in strains from all locations. While haplotypes 2, 3 and 13 were found in only two or three states. However, majority of the haplotypes (4, 5, 6, 7, 8, 9, 10, 11, 12, 14, and 15) were found in Colima only. When haplotypes distribution was compared with respect to host, no identical haplotypes appeared to be restricted to a particular type of the citrus species.

Confirmation of *C*Las by sequencing. The sequencing results were expressed as a dendrogram. In the phylogenetic analysis, two sequences obtained in this study were compared to those from other *Candidatus* Liberibacter spp. (Figure 3). These two sequences were clustered with *C*Las (AN. CP029348.1) sequence from Anaheim, USA being genetically similar (0.03621). This cluster was associated with the origin and closeness of this strain. The *C*Lam (AN. CP006604.1), *C*Laf (AN. CP004021.1), *C*Lso (AN. CP002371.1) species of *Candidatus*, were not genetically similar, only they present some conserved regions of DNA (Figure 4). The *C*Las sequence from Maharashtra, India (AN. KJ885230.1) was also genetically different from the sequences in this study, probably from different geographical origin of this strain (Asia).

DISCUSSION

TThe study on genetic diversity of *C*Las is limited, initially were employed to certain genes, such as RNA 16S/23S, *omp* region, sequence-rpoBC rplKAJL operon, closely-related effector genes *lasAI* and *lasAII* and miniature inverted-repeat transposable elements, *MITEs* (Villechanoux *et al*., 1993; Planet *et al*. 1995; Jagoueix *et al*., 1997; Subandiyah *et al.*, 2000; Bastianel *et al*., 2005; Zhou *et al*., 2008; Adkar *et al*., 2009; Ding *et al.,* 2009; Furuya *et al.,* 2010; Puttamuk *et al.,* 2014; Wang *et al.,* 2015), where some sequencing has been unsuccessful (Deng *et al*., 2008) or limited to a few SNPs (Bastianel *et al*., 2005) and may not be sufficient for population analyses.

However, with the publication of the complete sequence of *C*Las genome (Duan *et al*., 2009), the availability of this information, has facilitated the implementation of methodologies such as VNTRs,

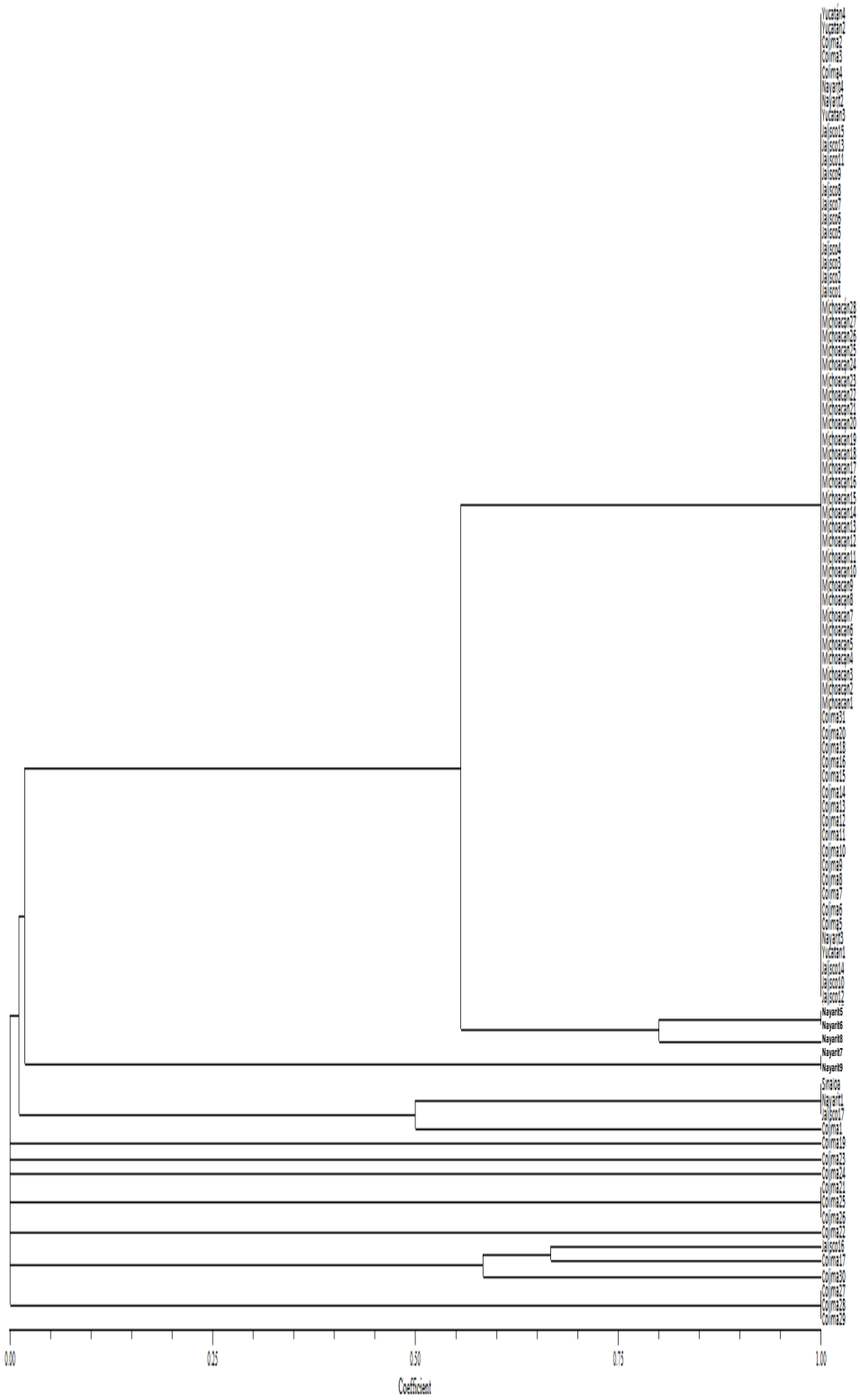

Figure 1. UPGMA dendrogram showing the genetic relationships of 90 *Candidatus* Liberibacter asiaticus strains from different locations from Mexico [Colima (2-16, 18, 20, 31), Jalisco (1-15), Michoacan (1-28), Nayarit (2-4) and Yucatan (1-4)].

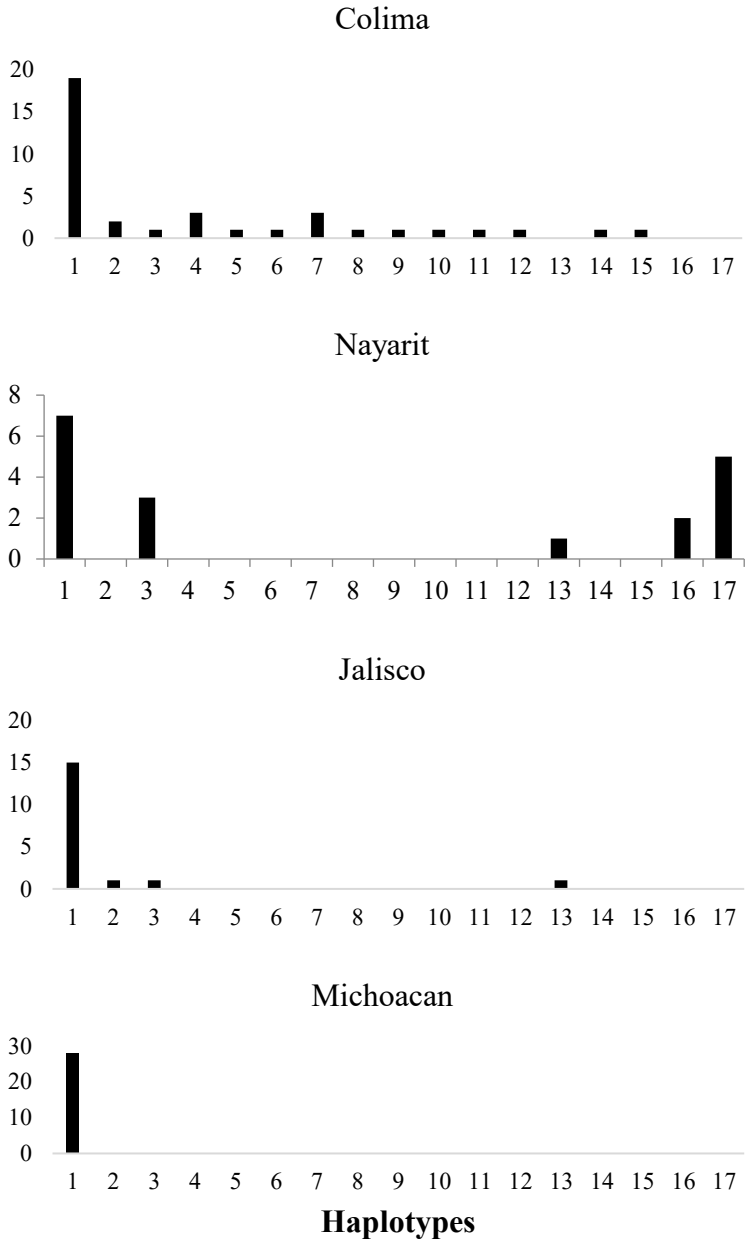

Figure 2. Frequency of each haplotype microsatellite among 90 strains of *Candidatus* Liberibacter asiaticus from the states of Nayarit, Jalisco, Colima and Michoacan. A total of 17 haplotypes were identified.

Figure 3. Dendrogram based on the sequence comparison of some *Candidatus* species. DNA sequence alignment shows that there is a 99% homology between the DNA sequences from the strains described in the present work and that from *C*Las obtained in the genebank from a strain in the USA (Figura 4). Therefore, the sequencing confirms the presence of *C*Las in this study.

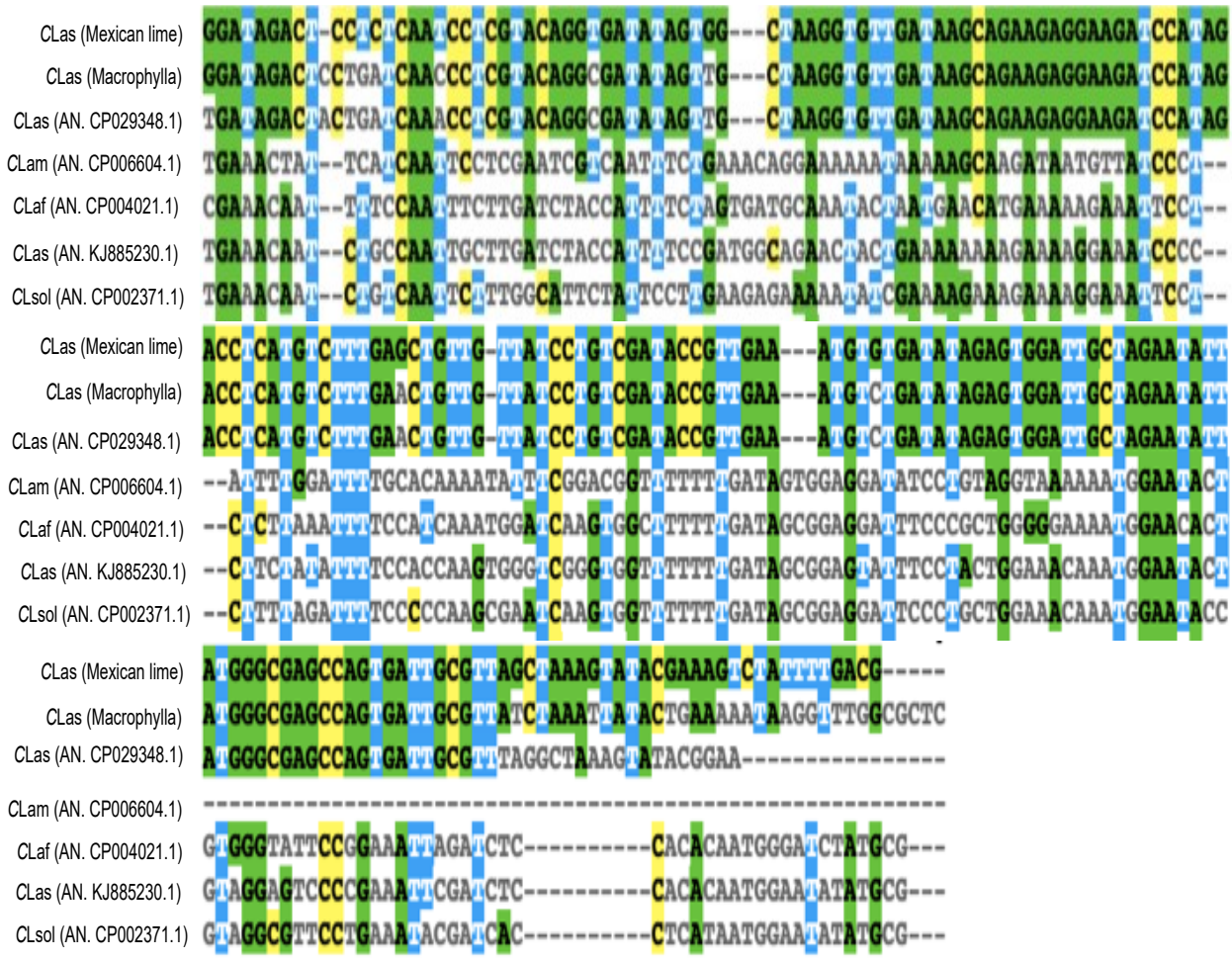

Figure 4. Nucleotide polymorphisms founded in SSR005 locus and compared with others *Candidatus* species. The two sequences analyzed are highly similar with *C*Las (AN. CP029348.1) from United States and low similar with other species (*C*Lam, *C*Laf and *C*Lsol).

which demonstrated that can be used to estimate the genetic diversity and populations structure of strains of *C*Las outside of Asia continent (Katoh *et al*., 2012; Islam *et al.,* 2012; Matos *et al.,* 2013). The power of VNTRs facilitates the analysis of regional *C*Las populations from HLB-affected plants and to prove the identification of introduction patterns and predict the possible relationship of HLB-associated Liberibacter distribution amongst growing regions (Islam *et al.*, 2012).

Similar studies were previously carried out to a regional or country scale to better understand *C*Las genetic variation, for example Meneguim *et al*. (2011), employed PCR-RFLP analyses and sequencing of the β-operon ribosomal protein genes for conducted studies of the genetic diversity of strains of *C*Las present in the state of Paraná, Brazil, showing a phylogenetic tree where all strains were genetically identical, regardless of geographical origins. Similarly, to investigate the diversity of 23 strains of *C*Las from seven provinces in China, Hu *et al.* (2011), used the *omp* genes and showed that the isolates under study shared 99% identity with *C*Las (AY642159), and within the phylogenetic tree the isolates from China were grouped with the Asian strains.

In this study 90 strains of *C*Las from different states of Mexico and different citrus host were examined by VNTRs-based analysis, which demonstrated the capacity for determining the genetic diversity of Mexican strains of *C*Las, being not possible to related the geographic origin with the molecular analysis. Initially, Katoh *et al.* (2011), developed 27 loci and were used to identify the genetic diversity of 84 *C*Las strains from Japan, in which only four loci were highly polymorphic. The locus SSR001 detected nine alleles, the locus SSR002 three, the SSR005 seven alleles and three alleles the locus SSR077. Nevertheless, in this study using Mexican *C*Las strains, the loci SSR005 and SSR077 detected four alleles, while the loci SSR001 and SSR002 detected one allele. For subsequent studies on the genetic variability among Mexican strains of *C*Las from several states and among the same state could be analyze the loci SSR005 and SSR077 because they were the most informative, both had the highest number of alleles per locus, while other loci may not be useful to distinguish among strains from other states because they were uninformative among *C*Las strains from the states analyzed in this study.

In 2012, Islam *et al*. showed that samples from Mexico, which exhibit the HA and HB haplotypes, were similarly to samples from Florida. These results showed that the origin of the strains of *C*Las from Mexico is Florida. Neverless, in our study other haplotypes were identified. In another study a dendrogram analysis performed by Katoh *et al*. (2012), *C*Las strains from India, East Timor, Papua New Guinea and Florida, showed that the cluster was mostly consistent with the geographic origin of the isolate. Furthermore, the differences in the nucleotide sequence were not associated with the citrus species. This was also shown by an analysis of the 16S rDNA, 16S/23S intergenic spacer, *omp*, *trm*U-*tuf*B-*sec*E-*nus*G-rplKAJL-rpoB, and bacteriophage-type DNA polymerase regions; where Tomimura *et al*. (2009) proved that all Indonesian *C*Las isolates clustered in one group. These authors also showed that other clusters were not correlated with geographic distribution neither related to the citrus host.

Singh *et al*. (2019) analyzed the genetic diversity using tandem repeat numbers (TRN) in variable CLIBASIA_01645 loci in 55 different citrus plants from India. The TRN showed single amplicons (~650 bp) in most of the 55 samples, except for a few amplicons up to 700 bp. Their results are similar to those obtained in the present work. Therefore, this technique is useful to estimate the genetic diversity of *C*Las.

In the present study, we show the use of VNTRs markers can provide enough information about genetic diversity among closely related strains and that might be correlated to a specific host species. This was observed since cluster I was formed by *C*Las strains collected from Mexican lime, sweet orange, lime, Persian lime, and Volkamer lemon; while the cluster II was constituted by Mexican lime, grapefruit, alemow, and mandarin (Figure 1). Only, strains from Mexican lime were assigned on both clusters. In general, the genetic diversity was higher among *C*Las strains across the different regions sampled. However, the large number of unique haplotypes identified, and the high level of genetic diversity observed in this study suggest that this pathogen has much higher diversity in Colima, this is probably due to the presence of different hosts, large areas of crop in a large valley, and frequent use of chemicals.

The characterization of citrus populations affected by HLB can help identify patterns and predict the possible introduction of distribution relationships associated with different citrus producers. The VNTRs loci can be useful for monitoring the genotypes of interest, in addition, these markers can help identify the origins of invasive *C*Las strains also that genetic differences are not just found in samples from different geographic areas, but also in a single area and a single tree (Islam *et al*., 2012). In addition to improving our understanding of the spatial and dynamics of the HLB disease, these insights are helpful in designing effective integrated pest and disease management for the HLB problem.

CONCLUSIONES

A broad genetic diversity was determined by VNTRs analysis of 90 strains of *C*Las infecting eight citrus species from the Central Pacific in Mexico. A no clear genetic structure was found in relation to the geographical origin or host of the strains of *C*Las analyzed.

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