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Research Article

$(GTG)_5$-rep-PCR demonstrates a clear cut genetic differentiation of *Candidatus* Liberibacter asiaticus isolates infecting mandarin trees in Hormozgan and Sistan and Baluchestan provinces of Iran

Siavash Samavi[1], Heshmatollah Rahimian[1*], Abdoolnabi Bagheri[2] and Valiollah Babaeizad[1]

1. Department of Plant Pathology, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.
2. Plant Protection Research Department, Hormozgan Agricultural and Natural Resources Research and Education Center, Agricultural Research Education and Extension Organization (AREEO), Bandar Abbas, Iran.

Abstract: Huanglongbing (HLB) is one of the most destructive diseases of citrus worldwide. The disease keeps spreading in several citrus-growing areas of southern Iran. The potential of $(GTG)_5$-rep marker in revealing the genetic diversity of geographic isolates of *Candidatus* Liberibacter asiaticus was evaluated in the present study. Twenty-one HLB-infected samples collected from the Hormozgan and Sistan and Baluchestan provinces were used in the trial. PCR with the $(GTG)_5$ primer produced 16 scorable bands, of which nearly 100% were polymorphic among or within the populations. The most observed variation resided within (80.56, $P < 0.10$) and a substantially less (19.44, $P < 0.10$) between the populations. The isolates were distributed in two main (A and B) clusters, each consisting of several subgroups. Group A included Sistan and Baluchestan and Hormozgan isolates and the group B embraced Hormozgan isolates. Based on pairwise genetic differences, the Haji Abad and Hashtbandi populations exhibited the highest between-population variation, and the Siahoo, Hashtbandi, Nikshahr, Haji Abad, and Sarbaz showed the greatest within-population variation. The first three coordinates of the principal coordinate analysis explained more than 72.39% of the variation among or within the populations. The first two coordinates explained 58.20% of band variation in plotting, and the first and third coordinates explained 55.54% of band variation. These results may be inferred that the Hormozgan populations might have diverged from the Sistan populations, or both might have originated separately from an initial or parental population possessing a high genetic diversity.

Keywords: Huanglongbing, *C*Las, $(GTG)_5$, marker, diversity

Introduction

Citrus huanglongbing (HLB), caused by *Candidatus* Liberibacter asiaticus (*C*Las), *Ca* L. africanus (*C*Laf), and *Ca*. L. americanus (*C*Lam) is the most devastating disease affecting the citrus industry worldwide (Bové, 2006; Gottwald, 2010). The disease has been reported from the major citrus

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growing areas in southern Iran (Hormozgan, Kerman, Sistan and Baluchestan and Fars provinces) (Salehi *et al*., 2012; Moslemkhani *et al*., 2015; Salehi and Rasoulipour, 2016). *C*Las is transmitted by grafting and the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayarna (Hemiptera: Sternorrhyncha: Liviidae) (Aubert, 1987; Gottwald *et al.,* 1989; Hung *et al.,* 1999). Faghihi *et al*. (2009) reported detection of *C*Las in psyllid body and Valencia and local orange cultivars (*Citrus sinensis* (L) Osb) using PCR assay in Hormozgan and Sistan and Baluchestan provinces. HLB affects all commercial citrus species and causes substantial economic losses by reducing fruit production, lowering fruit marketability, shortening the lifespan of the affected trees, and their eventual decline (Bové, 2006). HLB symptoms are similar to some nutrient deficiencies and a few other disorders. Hence they may be misdiagnosed in the field without PCR assays (Weiner *et al.,* 2004). The main symptoms of HLB include blotchy mottled leaves, vein chlorosis, yellowing of shoots, and dieback. As the disease advances, the fruits become asymmetrical, remain green in color and smaller in size, and lose marketability (Bové, 2006). HLB-associated phytoplasma was reported as a member of peanut witches'broom (16SrII) phytoplasma group. This was the first of association of a phytoplasma with HLB in sweet lime in the world and first record of association of *C*Las with sour orange (*Citrus aurantium* L.) and sweet lime in Iran (Saberi *et al*., 2017).

Molecular techniques, including amplification and sequencing of the16S rDNA and 16S/23S intergenic spacer regions, outer membrane protein (*omp*) gene, hypervariable effector genes, the *trm*U-*tuf*B-*sec*E-*nus*G-*rpl*KAJL-*rpo*B region (gene cluster region), and microsatellite-based profiling are among the widely used approaches for evaluating the genetic diversity of *C*Las strains (Kashi and King, 2006; Zhou *et al*., 2007; Adkar-Purushothama *et al*., 2009; Puttamuk *et al*., 2014). Repetitive element polymerase chain reaction (rep-PCR) is a powerful molecular tool for investigating the heterogeneity of bacterial populations and differentiation of strains or variants of a species (Gomez-Gil *et al*., 2004; De Vuyst and Vancanneyt, 2007). $(GTG)_5$-PCR amplifies the five GTG repetitive elements distributed throughout the bacterial genome (Cilliers *et al*., 1997; Gevers *et al.,* 2001; De Vuyst *et al*., 2008; Papalexandratou *et al*., 2009). It has been successfully used for the differentiation of bacterial strains (Rademaker *et al*., 2000), including probiotic bacteria (Gevers *et al.,* 2001), and the pathogenic plant species like *Ralstonia solanacearum* strains (Stevens and Van Elsas, 2010; Izadian and Taghavi, 2013).

The goals of the present study were to evaluate the applicability of $(GTG)_5$-PCR in the differentiation of strains of *C*Las associated with HLB-infected mandarin trees and assess the genetic diversity of the prevalent strains in Hormozgan and Sistan and Baluchestan provinces of Iran.

Materials and Methods

Sampling

Between 2019 and 2020, surveys in mandarin orchards in two southern Iranian provinces, including Hormozgan (Hashtbandi, Siahoo and Haji Abad), and Sistan & Baluchestan (Nikshahr and Sarbaz) were conducted, and 21 plant samples (fruit-bearing shoots) with symptoms resembling the HLB disease were collected. The sampling sites and the codes given to samples are listed in Table 1. The samples were carried in cold boxes to the laboratory and kept in a freezer at -40 °C.

DNA extraction and PCR amplification

Total genomic DNA was extracted from 0.5 g of the peduncle of the mandarin fruits by the method outlined by Hung *et al*. (1999). The quality and concentration of the extracted DNAs were checked using a nucleic acid analyzer (Nano-200, Allsheng, China) and agarose gel electrophoresis. The extracted DNA samples were stored at-20 °C.

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The identity of the HLB-associated bacterium as *Candidatus* Liberibacter asiaticus (*C*Las) was affirmed by PCR amplification of the specific fragment of the ribosomal protein *rplKAJL rpoBC* gene of *C*Las using the primer pair A2/J5 (Hocquellet *et al*., 1999; Table 1). Negative controls included the DNA isolated from healthy-looking fruits of uninfected trees.

Table 1 Sampling sites and the specificities of mandarin (*Citrus reticulata* L.) samples used in this study.

No	Code	Province	City	Region	Sample parts used
1	HBS12	Hormozgan	Bandar Abbas	Siahoo, Zakin	Peduncle
2	HBS14	Hormozgan	Bandar Abbas	Siahoo, Zakin	Peduncle
3	HBS19	Hormozgan	Bandar Abbas	Siahoo, Zakin	Peduncle
4	HBS29	Hormozgan	Bandar Abbas	Siahoo, Zakin	Peduncle
5	HBS34	Hormozgan	Bandar Abbas	Siahoo, Zakin	Peduncle
6	HBS35	Hormozgan	Bandar Abbas	Siahoo, Segh	Peduncle
7	HBS36	Hormozgan	Bandar Abbas	Siahoo, Zakin	Peduncle
8	HBS41	Hormozgan	Bandar Abbas	Siahoo, Talgerdoo	Peduncle
9	HBS44	Hormozgan	Bandar Abbas	Siahoo, Khersin	Peduncle
10	HBS3	Hormozgan	Bandar Abbas	Siahoo, Dargaz	Peduncle
11	HHA30	Hormozgan	Bandar Abbas	Siahoo, Talsooroo	Peduncle
12	HHA33	Hormozgan	Haji Abad	Ahmadi	Peduncle
13	HHB1	Hormozgan	Haji Abad	Ahmadi	Peduncle
14	HHF2	Hormozgan	Haji Abad	Baghan	Peduncle
15	HHR20	Hormozgan	Haji Abad	Fareghan	Peduncle
16	HHS14	Hormozgan	Haji Abad	Raeiz	Peduncle
17	HHS7	Hormozgan	Haji Abad	Raeiz	Peduncle
18	HMH17	Hormozgan	Minab	Hashtbandi	Peduncle
19	SN23	Sistan & Baluchestan	Nikshahr	Nikshahr	Peduncle
20	SN26	Sistan & Baluchestan	Nikshahr	Takhtemalek	Peduncle
21	SS15	Sistan & Baluchestan	Sarbaz	Sarbaz	Peduncle

Table 2 Characteristics of the primers used in this study.

Primer name	Sequence (5′–3′)	Target	Amplicon size (bp)	Annealing (°C)	Reference
A2	TATAAAGGTTGACCTTT CGAGTTT	*rplKAJL-rpoBC* operon (β-operon)	669 (*C*Laf)	58	Hocquellet *et al.*, 1999
J5	ACAAAAGCAGAAATAG CACGAACAA		703 (*C*Las)		
GTG	GTGGTGGTGGTGGTG	-		51	Versalovic *et al.*, 1994

PCR was performed using primer pair A2/J5 (Table 2) in 20 µl of reaction mixture containing 10 µl 2X PCR Master Mix (Ampliqon, Denmark), 1 µl of each primer (10 µM), 2 µl of template DNA (ca. 100 ng) and 6 µl sterile distilled water. The thermocycling program consisted of an initial denaturation step at 95 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 40 s and extension at 72 °C for 60 s, with a final extension step at 72 °C for 10 min. Two generated PCR amplicons were sequenced in both directions (Macrogen, South Korea). The sequences were compared with GenBank sequences using BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify and confirm the identity of *C*Las.

(GTG)5-PCR amplifications were carried out with a PeqStar 96 Universal Gradient™ thermal cycler (PeqLab©, Germany) using the $(GTG)_5$ primer (5´-GTGGTGGTGGTGGTG-3´) (Synthesized by

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Methabion, Germany) (Versalovic *et al.*, 1994). The reaction mixture (25 µl) contained 1.5 µM of the $(GTG)_5$ primer, 200 µM of each dNTPs, 2.5 µl of 10X reaction buffer (10 mM Tris-HCl, 2 mM MgCl2 and 1 U of *Taq* polymerase, pH: 8.4) (Cinagen, Iran). A 2 µl aliquot of the extracted DNA was added, as a template, to each PCR mixture. Amplification was carried out with the following cycling: initial denaturation at 95 ºC for 3 min followed by 37 cycles of denaturation (95 ºC for 30 s), annealing (51 ºC for 40 s), and extension (72 ºC for 2 min). A final extension step at 72 ºC for 5 min was also included. The PCR products were electrophoresed on 1.5% gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH:8), stained with FluoroDye™ Nucleic Acid Gel Stain (SMOBIA©, Taiwan), and viewed and photographed on a UV transilluminator (Sambrook *et al.*, 1989).

Data analysis

The DNA fingerprints were transformed into numerical data as bands 1 (presence) or 0 (absence). Using Jaccard and simple matching (SM) similarity coefficients, a pairwise similarity matrix was constructed. A dendrogram was created employing the UPGMA (Unweighted Pair Group Method using Arithmetic Mean) clustering algorithm using the NTSYS software version 2.2 (Rohlf, 2005). Principal coordinate analysis (PCA) was performed on $(GTG)_5$ data using NTSYS software v.2.2. In addition, the SPSS 20 software was used to estimate the correlation between the geographic locations and genetic distances. Combined binary data of the scored bands and the geographic locations were used in statistical analyses. Analysis of molecular variance and estimation of F_{ST} was performed using Arlequin software version 3.5.2.2 (Excoffier and Lischer, 2010). Statistical calculations and graphics for F_{ST} (Weir and Clark Cockerham, 1984) were carried out employing R version 4.0.5 (cran.r-project.org).

Results

PCR detection of *C*Las

Fruits with their attached peduncles showing misshapen and color inversion symptoms were collected from 21 affected local mandarin trees in various locations in Hormozgan and Sistan & Baluchistan provinces. DNA samples extracted from the fruit peduncles were tested for the presence of *C*Las by PCR using primer pair A2/J5. PCR amplifies an approximately 700 bp fragment from all symptomatic fruits but not symptomless ones (Fig. 1). The PCR amplicons were directly sequenced. Analysis using the BLAST corroborated that the nucleotide sequence of the 700 bp fragment was 99.7-100% identical to the corresponding sequences of several strains of *C*Las present in the NCBI (GenBank accession numbers CP010804, JQ973891, MH559117). These sequences were deposited in GenBank under the accession numbers MZ065298 and MZ065320.

(GTG)5-rep-PCR

PCR with $(GTG)_5$ primer produced 19 DNA bands ranging from 400 to 1500 bp; 16 were polymorphic among or within the populations. Most of the observed diversities (80.56%, $P < 0.10$) resided within and considerably less (19.44%, $P < 0.10$) in between the *C*Las populations existing in different areas. The isolates formed two main groups, with group A consisting of the isolates SN23, SS15, SN26, HMH17, HHR20, HHA31, and HBS44 and group B comprising isolates HBS12, HBS35, HBS3, HBS36, HBS41, HBS14, HBS29, HHS14, HHS7, HHF2, HBS34, HBS19, HHA30, and HHB1. The majority of the isolates of group A exist in the Sistan & Baluchestan province. In contrast, all isolates of group B consisted of those existing in the Hormozgan province. Group A consisted of several subgroups, including A1 (HBS12, HBS35, HBS3, HBS36 and HBS41), A2 (HBS14, HBS29, HHS14, HHS7, HHF2, HBS34 and HBS19), A3 (HHA30), and A4 (HHB1). Group B was comprised of subgroups B1

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(HBS44, HHA31, HHR20, and HMH7), B2 (SN26 and SS15), and B3 (SN23) (Fig. 2).

Based on pairwise genetic differences, the Haji Abad and Hashtbandi populations exhibited the highest between-population diversity; these two populations significantly differed from the other populations studied (Figs. 3 and 4). The populations distributed in Siahoo, Minab, Nikshahr, Haji Abad and Sarbaz displayed the greatest within-population variations (Fig. 4). F_{ST} values imply that the populations prevalent in Haji Abad and Hashtbandi regions, with the highest population diversity, have diverged considerably from the other populations studied.

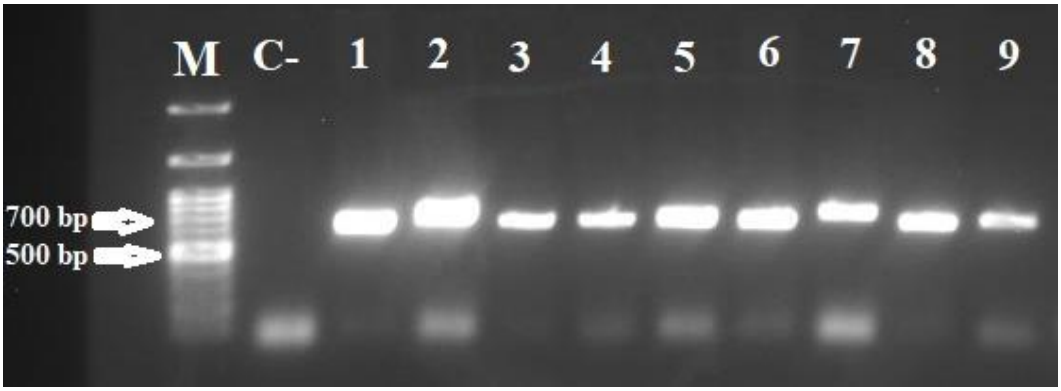

Figure 1 HLB detection from the peduncles of the fruits of *Citrus retculata* by PCR in 1% agarose gel electrophoresis. Lane M: 100 bp DNA ladder; lane C-: Asymptomatic fruit from a healthy mandarin tree; Lanes 1-9: PCR products of approximately 700 bp were amplified using primer pair A2/J5 from symptomatic fruits of HLB-infected mandarin trees.

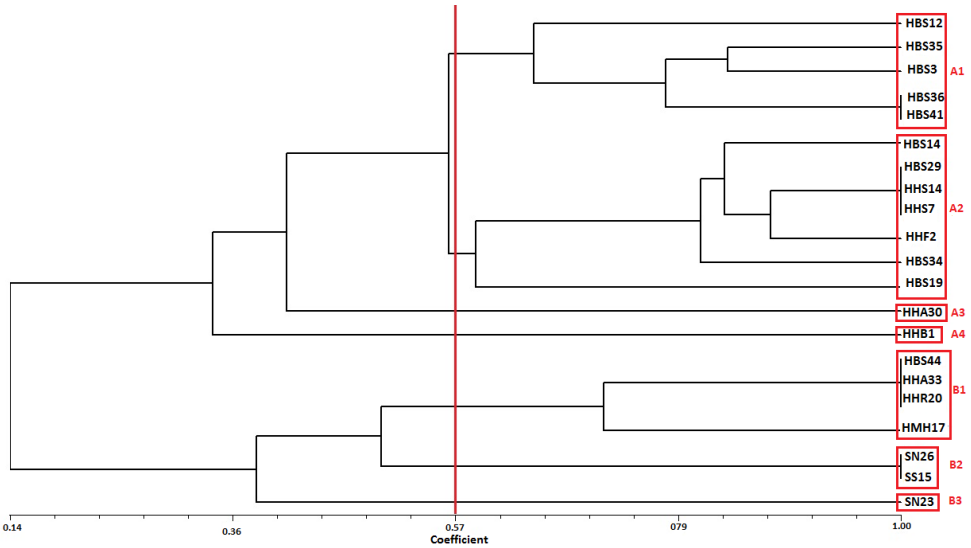

Figure 2 Dendrogram constructed based on the (GTG)-PCR fingerprints of the populations of *Candidatus* Liberibacter asiaticus existing in Hormozgan (H) and Sistan & Bluchestan (S) provinces, southern Iran, using the Jaccard coefficient and UPGMA clustering method.

[DOR: 20.1001.1.22519041.2022.11.1.7.8]

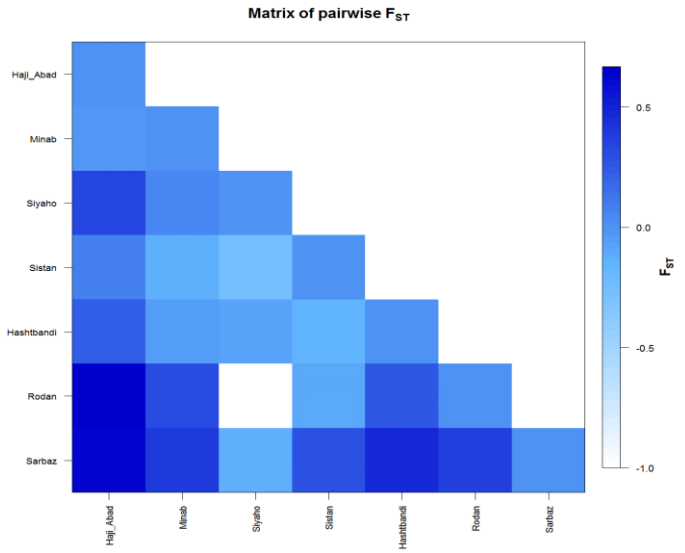

Figure 3 Pairwise F_{ST} of the seven populations existing in the main geographic areas in southern Iran based on their (GTG)5-PCR fingerprints. As shown, the Haji Abad and Hashtbandi populations had the highest between-population diversity.

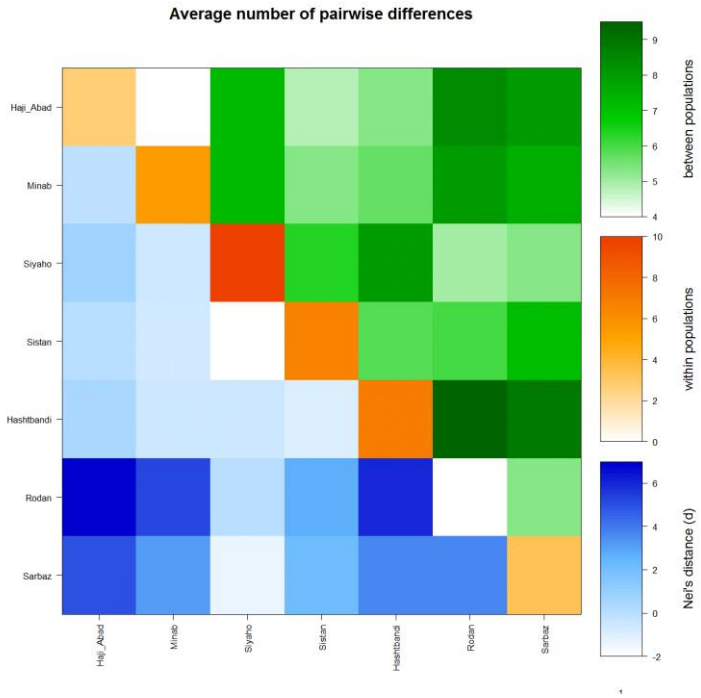

Figure 4 Population pairwise F_{ST} of the seven populations existing in the main geographic areas in southern Iran based on their $(GTG)_{5}$- PCR fingerprints. The populations distributed in Siahoo, Minab (Hashtbandi), Nikshahr, Haji Abad and Sarbaz displayed the greatest within-population variations.

[DOR: 20.1001.1.22519041.2022.11.1.7.8]

Principle components analysis (PCA)

The first three coordinates of the principal coordinate analysis explained more than 72.39% of the variation among or within populations. The first two coordinates explained 58.20% of band variation in plotting, and the first and third coordinates explained 55.54% of band variation. Except for HBS12, HHA30, HBS41, HHB1, and HMH17, most Hormozgan populations were found apart from the Nikshahr populations (Fig. 5A-B). In the first and second biplot options of these three coordinates, *i.e.*, 1 versus 2 and 1 versus 3 (Fig. 5 A, B, C), the three populations of Nikshahr including SN23, SN26 and SS15 were found relatively close to each other and apart from the other populations.

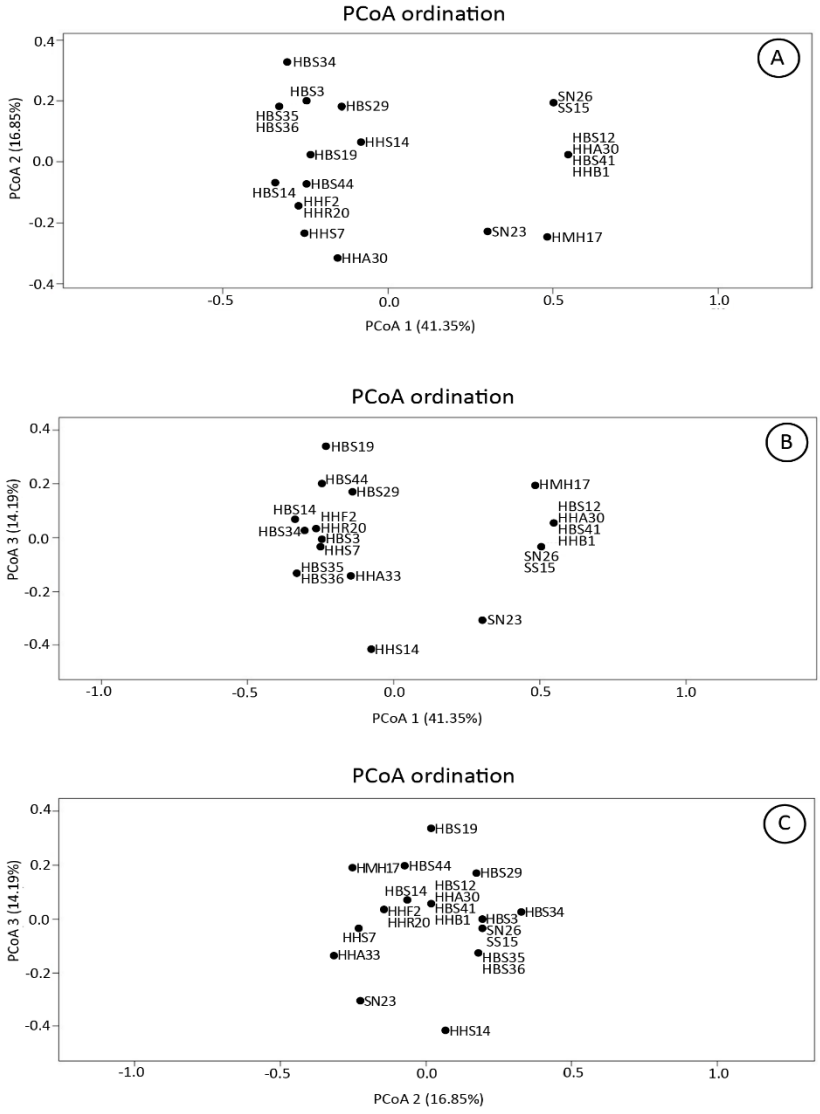

Figure 5 Separation of populations by the first three coordinates in Principal Coordinates Analysis.

[DOR: 20.1001.1.22519041.2022.11.1.7.8]

Discussion

The study of genetic diversity in geographically isolated populations of *Ca.* L. asiaticus', extracted from HLB-affected plants, can shed some light on the genetic diversity of the *C*Las in southern provinces of Iran and help decipher the introduction pattern of the disease. This finding could also assist in finding the relationships among HLB-associated Liberibacters existing in different citrus-growing regions worldwide.

PCR and non-PCR-based molecular techniques have been applied successfully to reveal genetic diversity in different plant microbial pathogens. Random Amplified Polymorphic DNA (RAPD) (Chelossi *et al*., 2004), Amplified Fragment Length polymorphism (AFLP) (PCR-based markers) (Wittwer *et al*., 2005) and Restriction Fragment Length Polymorphism (RFLP) (Non-PCR-based marker) (Mushegian *et al*., 2011) are among the most widely used molecular markers for studying genetic diversity. However, these molecular techniques are challenging to apply for fastidious bacteria such as *C*Las because they require the total genomic DNA of the pathogen. In addition to *C*Las DNA, plant genomic, organellar DNAs, DNA of endophytic bacteria, and phloem-limited prokaryotic pathogens may present in the extracted DNA preparations. $(GTG)_5$-PCR is among the PCR-based markers that have been successfully applied in the differentiation of species and strains of different groups of bacteria (Rademaker *et al*., 2000).

A rather considerable body of information is available on the genetic diversity of *C*Las in some of the affected areas in the world. Katoh *et al.* (2011) and Islam *et al*. (2012) used the SSR markers to study the genetic diversity of the host- and geographic-based populations of *C*Las. Izadian and Taghavi (2013) studied the ability of $(GTG)_5$ and RAPD to reveal the genetic diversity of Iranian isolates of *Ralstonia solanacearum* in potato and tomato and found that both markers were able to separate different biovars of *R. solanacearum*. $(GTG)_5$-PCR has been able to differentiate several Lactobacillus isolates at the species, subspecies, and even at the strain levels (Gevers *et al*., 2001).

In the present study $(GTG)_5$-PCR was found to be of adequate power for demonstrating genetic diversity in the populations of *C*Las. The approach enabled unraveling the substantial heterogeneity of the agent associated with the huanglongbing disease in southern Iran.

In recent years, contrary to the substantial decline in the population of Asian citrus psyllid in the citrus orchards of southern Iran, the outbreak of citrus decline due to HLB in these areas has kept increasing dramatically. In the Siahoo district in Hormozgan province, the prevalence of citrus decline leads to the gradual extinction of its highly favored mandarin variety, an item of utmost importance in the district's economy.

In Iran, the citrus HLB disease was first reported in Sistan and Baluchestan province (Faghihi *et al*., 2009). The disease has spread from there to other citrus-growing areas of southern Iran. Results of the present study, indicating that the *C*Las isolates from Hormozgan are more closely similar to each other than those of Sistan and Baluchestan, are not determinative enough to suppose that the disease in the Homozgan province lends its origin to that in the Sistan and Baluchestan province.

In conclusion, $(GTG)_5$-PCR fingerprinting could successfully determine the phylogenetic relationships among *C*Las strains existing in different citrus-growing areas of the Hormozgan and Sitan and Baluchestan provinces of Iran. Genetically distinct isolates of *C*Las may also vary in pathogenicity and virulence, an issue needing further investigation.

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Author statement

All authors compiled, wrote, and approved this research article.

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روش (GTG)$_5$-rep-PCR تنوع ژنتیکی جدایه‌های *Candidatus* Liberibacter asiaticus را در درختان نارنگی در استان‌های هرمزگان و سیستان و بلوچستان نشان می‌دهد

سیاوش سماوی[1]، حشمت‌الله رحیمیان[1*]، عبدالنبی باقری[2] و ولی‌الله بابایی‌زاد[1]

۱- گروه گیاه‌پزشکی، دانشکده علوم زراعی، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ساری، ایران.
۲- بخش تحقیقات گیاه‌پزشکی، مرکز تحقیقات و آموزش کشاورزی و منابع طبیعی هرمزگان، سازمان تحقیقات، آموزش و ترویج کشاورزی، بندرعباس، ایران.
پست الکترونیکی نویسنده مسئول مکاتبه: rahimian.h@gmail.com
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چکیده: بیماری هوانگ لونگ بینگ (HLB) مرکبات یکی از مخرب‌ترین بیماری‌های مرکبات در دنیا می‌باشد. این بیماری در مناطق مرکبات کاری جنوب ایران در حال گسترش است. در این مطالعه، کارآیی نشانگر (GTG)$_5$ در تنوع ژنتیکی جدایه‌های جغرافیایی *Candidatus* Liberibacter asiaticus بررسی شد. در مجموع ۲۱ نمونه آلوده HLB از استان‌های هرمزگان و سیستان و بلوچستان جمع‌آوری شد. آزمون PCR با آغازگر (GTG)$_5$ ۱۶ باند قابل شمارش ایجاد کرد که تقریباً اغلب آن‌ها دارای چندشکلی (بین یا درون‌جمعیتی) بودند. بیشترین تنوع مشاهده شده مربوط به تنوع درون جمعیتی (۸۰/۵۶، $P < 0.10$) و کم‌ترین آن مربوط به تنوع بین جمعیتی (۱۹/۴۴، $P < 0.10$) بود. جدایه‌ها در دو گروه اصلی (A و B) قرار گرفتند که هر یک، خود از چند زیر گروه تشکیل شدند. گروه اصلی A شامل جدایه‌های سیستان و بلوچستان و هرمزگان و گروه اصلی B شامل جدایه‌های هرمزگان بودند. براساس مقایسه تفاوت‌های ژنتیکی دو به دو، جمعیت‌های حاجی‌آباد و هشتبندی بیشترین تنوع بین‌جمعیتی و جمعیت‌های سیاهو، میناب، نیکشهر، حاجی‌آباد و سرباز بیشترین تنوع درون‌جمعیتی را در بین جمعیت‌های مورد مطالعه نشان دادند. گروه‌بندی جدایه‌ها براساس تجزیه به مختصات اصلی (PCoA) نشان داد که سه بای‌پلات اول بیش از ۷۲/۳۹% از تنوع موجود را به خود اختصاص دادند. بای‌پلات اول (یک در برابر دو)، ۵۸/۲۰% و بای‌پلات دوم (یک در برابر سه)، ۵۵/۵۴% از تنوع را نشان دادند. با نتایج به‌دست آمده از این مطالعه، می‌توان گفت که ممکن است جمعیت‌های هرمزگان از جمعیت سیستان جدا شده باشند یا این‌که این جمعیت‌ها، از یک جمعیت دیگر با تنوع ژنتیکی بالا نشأت گرفته باشند.

واژگان کلیدی: هوانگلونگبینگ، *C*Las، GTG$_5$، نشانگر، تنوع

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