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Genetic diversity among '*Candidatus* Liberibacter asiaticus' isolates based on single nucleotide polymorphisms in 16S rRNA and ribosomal protein genes

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Abstract - Citrus Huanglongbing (HLB) is one of the most destructive and widespread diseases of citrus, caused by '*Candidatus* Liberibacter', a non-cultured, phloem-restricted alpha-proteobacterium. In the present study, '*Ca.* Liberibacter' was detected in symptomatic citrus plants growing in the Karnataka state (South India) by amplification of 16S rRNA and β-operon ribosomal protein (β-rp) genes, using PCR primers specific for African and Asian '*Ca.* Liberibacter'. Sequencing of the 16S rRNA amplified fragment revealed that '*Ca.* Liberibacter', here identified belongs to the species '*Ca.* Liberibacter asiaticus'. The alignments of 16S rRNA and ribosomal protein gene sequences of all known '*Ca.* Liberibacter asiaticus'-related, allowed to recognize Single Nucleotide Polymorphisms (SNPs). Basing on SNP analysis, the strains were grouped in fourteen 16SrRNA SNP genetic lineages (16Sr-I to 16Sr-XIV) and three β-rp SNP genetic lineages (rp-I to rp-III). Only the strains of '*Ca.* Liberibacter asiaticus' from the Karnataka state belonged to the genetic lineages 16Sr-I and rp-I. RFLP diagnostic tests on the discriminative 16S rRNA SNPs were set up to identify this lineage. These results revealed the presence of a new '*Ca.* Liberibacter asiaticus' genetic lineage in the Indian sub-continent, where at least four genetically diverse SNP lineages were found. These findings could open new opportunities for in-depth studies on biological niches and traits of '*Ca*. Liberibacter asiaticus'.

Key words: Huanglongbing (HLB); '*Candidatus* Liberibacter'; single nucleotide polymorphisms; genetic lineage.

INTRODUCTION

Huanglongbing (HLB), whose name in Chinese means "yellow dragon disease", is a destructive disease of citrus, first reported in southern China in 1919 (Reinking, 1919) and now known to occur in next to 40 different Asian, African, Oceania and American countries. The same disease is denoted by different names like "greening" in South Africa (Oberholzer *et al.*, 1965), "die-back" in India (Capoor, 1963), "mottle leaf" in Philippines (Martinez and Wallace, 1967; Salibe and Cortez, 1968), "vein phloem degeneration" in Indonesia (Tirtawidjaja *et al.*, 1965), and so on. HLB represents a major threat to the world citrus industry and is invading new citrus growing areas (Bové, 2006). The disease causes severe losses in the production of sweet orange (*Citrus sinensis*), mandarin (*Citrus reticulata*), lemon (*Citrus limon*), grapefruit (*Citrus paradisi*), and other economically important citrus species (Halbert and Manjunanth, 2004; Coletta-Filho *et al.*, 2005). However, except preventing the trees from becoming infected, no control strategies are known for containing the spreading of HLB. For this reason, HLB is becoming a night-mare for regions still free from the disease, such as western Asia, Mediterranean basin, Australia, and Pacific Ocean islands.

The etiological agent of HLB is a phloem-restricted, non-cultured, Gram-negative bacterium that has been characterized in 1994 basing on its 16S rDNA sequence and shown to be a new genus, named '*Candidatus* Liberibacter', in the α-proteobacteria subdivision (Jagoueix *et al.*, 1994). Presently, three species of '*Ca.* Liberibacter' are known to infect citrus plants: '*Ca.* Liberibacter asiaticus' ('*Ca*. L. asiaticus'), a heat-tolerant bacterial species transmitted by the Asian citrus psyllid *Diaphorina citri* (Capoor *et al.*, 1967) in Asia, Brazil, and Florida (USA)(Halbert, 2005); '*Ca.* Liberibacter africanus' ('*Ca.* L. africanus') with its subspecies 'capensis', a heat-sensitive species vectored by the African citrus psyllid *Trioza erytreae* in the African countries (Aubert, 1987; Garnier *et al.*, 2000; Bové, 2006); '*Ca.* Liberibacter americanus' ('*Ca.* L. americanus'), another heat-tolerant

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species transmitted by *Diaphorina citri* in Brazil (Texeira *et al.*, 2005a, 2005b). '*Ca.* Liberibacter psyllaurous' ('*Ca.* L. psyllaurous'), causing diseases in tomato and potato in western North America, where it is vectored by the psyllid *Bactericera cockerelli* (Sulc) (Hansen *et al*., 2008); and '*Ca.* Liberibacter solanacearum' ('*Ca.* L. solanacearum'), causing diseases in tomato and pepper in New Zealand, where its vector is not still defined (Liefting *et al*., 2009).

A variety of citrus are largely grown in India, where infections by '*Ca*. L. asiaticus' were reported in Poona region (Jagoueix *et al*., 1996), central India. Die-back symptoms in Coorg mandarins were described in Karnataka State, southern India, and the association with HLB was confirmed by positive dot blot hybridization (Varma *et al.*, 1993). HLB disease showed symptoms such as blotchy mottle condition of the leaves (Elizabeth *et al*., 2005), that results in the development of yellow shoots, the early and very characteristic symptom of the diseases; tree stunting and declining; bear a few, small-sized and deformed fruits, that are poorly colored giving greening appearance. Roots start decaying from the rootlets (da Graça, 1991; Batool *et al*., 2007). The objectives of this work were to investigate the genetic diversity of '*Ca.* Liberibacter asiaticus' in Karnataka State in citrus trees with die-back HLB symptoms. PCR and sequence analyses of 16S rRNA and β-ribosomal protein (*rpl*KAJL-*rpo*BC operon) genes revealed the presence of a new '*Ca.* L. asiaticus' SNP genetic lineage within Indian sub-continent. These findings could stimulate future researches on molecular markers describing the diversity among '*Ca*. Liberibacter asiaticus' populations and their epidemics.

MATERIALS AND METHODS

Plant material. Leaf samples were collected from 13 *Citrus reticulata* plants exhibiting typical HLB symptoms in Karnataka state, southern India and leaves of five asymptomatic citrus plants were sampled in different localities away from the HLB-diseased plantations (Table 1). Samples were stored at 4 °C and transported to the laboratory for DNA isolation and further analyses of 16S rRNA and β-operon ribosomal protein gene sequences.

Total DNA extraction. From both asymptomatic and symptomatic leaf samples, midribs were chopped to a fine mince with a razor blade and were subjected to total DNA isolation as previously reported by Adkar-Purushothama *et al.* (2007) with some modifications. Veins weighing approximately 1g were homogenized with 2 mL of pre-heated extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA pH 8.0, 100 mM Tris-Cl pH 8.0) using mortar and pestle. Clear homogenate was transferred to 15 mL tube containing RNaseA (100 mg/mL) using Miracloth-linned funnel and incubated at 65 °C for 20 min. During incubation, mixture was vortexed several times. Nucleic acids were extracted with an equal volume of phenol:chloroform (1:1). DNA was precipitated with an equal volume of ice-cold 99.5% isopropanol. After incubation at -20 °C for 1 h and centrifugation at 15000 rpm for 15 min, the obtained pellet was rinsed with 70% ethanol and air dried. Nucleic acids were re-suspended in 100 µL of nucleic acid-free sterile water.

Detection of '*Candidatus* Liberibacter' by PCR amplification. Two different PCR primer pairs specific for African and Asian '*Ca* Liberibacter' were used to detect the pathogen. Primer pair OI1/OI2c was employed for the amplification of a partial sequence (1167 bp) of 16S rRNA (Jagoueix *et al.*, 1994, 1996; Urasaki *et al.*, 2008); primer pair A2/J5 was utilized for amplifying a partial sequence of β-operon (*rpl*KAJL-*rpo*BC operon) ribosomal protein (β-rp) genes (Hocquellet *et al.*, 1999; Das *et al.*, 2007). The A2/J5 primer pair allows for the discrimination of the species '*Ca.* L. asiaticus' and '*Ca.* L. africanus' basing on the amplicon expected length, of respectively 703 and 669 bp (Hocquellet *et al.*, 1999). Total genomic DNA isolated from HLB-symptomatic and healthy leaf midrib samples were subjected to PCR after diluting to 1:10 before using as

TABLE 1 - PCR-based detection of '*Candidatus* Liberibacter' in leaf samples from citrus-growing regions in Karnataka state, southern India

Sample ID	Specific origin		Observation of HLB symptoms	Liberibacter detection (PCR)	
	District	Area		16S rDNA	rplKAJL-rpoBC
1	Kodagu	Madikeri	+	+	+
2		Gonnikoppa	+	+	+
3		Siddapura	+	+	+
4		Polibetta	+	+	+
5		Thithimati	+	+	+
6		Bagamandala	+	+	+
7		Suntikoppa	+	+	+
8		Amathi	+	+	+
9		Srimangala	–	–	–
10		Galibidu, Madikeri	–	–	–
11		Madapura	–	–	–
12		Sanivarsante	–	–	–
13		Maddenadu	–	–	–
14	Chikmagalur	Balehonnur	+	+	+
15		Chikmagalur	+	+	+
16	Hassan	Sakaleshapura	+	+	+
17		Arehalli	+	+	+
18	Shimoga	Shimoga	+	+	+

TABLE 2 - 16S rDNA sequences of '*Candidatus* Liberibacter asiaticus' isolates used in this study

No.	Isolate	Isolation source	Origin	Accession No.
1	Karnataka - Madikeri	Mandarin	India: Karnataka	FJ827777
2	Karnataka - Polibetta	Mandarin	India: Karnataka	FJ827779
3	Poona	Citrus	India: Poona	L22532
4	n.d.	Mandarin	India	FJ196314
5	CGBNM1	Citrus	India: Nagpur	EU939452
6	Okinawa	Citrus	Japan: Okinawa	AB008366
7	n.d.	Citrus	China	AY192576
8	n.d.	n.d.	China: Guangdong	DQ157273
9	n.d.	Citrus	China: Guangdong	DQ303210
10	Guangdong-PG	n.d.	China: Guangdong	DQ431999
11	Guangdong-HP	n.d.	China: Guangdong	DQ432005
12	n.d.	n.d.	China: Guangxi	DQ157274
13	Guangxi-HJC	n.d.	China: Guangxi	DQ431997
14	Guangxi-STY	n.d.	China: Guangxi	DQ432000
15	GuangXi-GL-1	n.d.	China: Guangxi	DQ778016
16	GuangXi-GL-7-CHN	n.d.	China: Guangxi	EU921614
17	GuangXi-GL-10-CHN	n.d.	China: Guangxi	EU921615
18	GuangXi-GL-19-CHN	n.d.	China: Guangxi	EU921616
19	HN-BJNM	n.d.	China: Guangxi	DQ432001
20	Fujian-NHE	n.d.	China: Fujian	DQ431998
21	n.d.	n.d.	China: Guizhou	DQ157275
22	Hunan-NG	n.d.	China: Hunan	DQ432002
23	Jiangxi-GC	n.d.	China: Jiangxi	DQ432003
24	Chongqing-ZG	n.d.	China: Chongqing	DQ432004
25	Kumquat 1	n.d.	Taiwan	DQ302750
26	GFB-Selangor (P)	Pummelo	Malaysia: Selangor	EU224393
27	GFB-S	Citrus	Malaysia: Selangor	EU371107
28	GFB-PahangKelantan (SO)	Mandarin	Malaysia: Pahang and Kelantan	EU224394
29	GFB-T	Citrus	Malaysia: Terengganu	EU371106
30	LSg1	Plant	Brazil: Sao Paulo	AY919311
31	n.d.	Citrus	Brazil: Sao Paulo	DQ471901
32	Sao Paulo-285	n.d.	Brazil: Sao Paulo	EU921613
33	Sao Paulo-Duatina-53	n.d.	Brazil: Sao Paulo	EU921622
34	n.d.	citrus	USA: Florida	DQ471900
35	F11Dade	*Diaphorina citri*	USA: Florida	EU130552
36	F16Nassau	*Diaphorina citri*	USA: Florida	EU130553
37	F17PalmBeach	*Diaphorina citri*	USA: Florida	EU130554
38	F18Polk	*Diaphorina citri*	USA: Florida	EU130555
39	n.d.	*Diaphorina citri*	USA: Florida	EU130556
40	12166	Citrus	USA: Florida	EU265646
41	Florida-8	n.d.	USA: Florida	EU921617
42	Florida-1808	n.d.	USA: Florida	EU921618
43	LJZ-745	Citrus	USA: Florida	FJ236554
44	LJZ-451	n.d.	USA: Florida	FJ263696
45	LJZ-4620	n.d.	USA: Florida	FJ263697
46	LJZ-4621	n.d.	USA: Florida	FJ263698
47	LJZ-4622	n.d.	USA: Florida	FJ263699
48	LJZ-4730	n.d.	USA: Florida	FJ263700
49	LJZ-5670	n.d.	USA: Florida	FJ263701
50	LJZ-575	n.d.	USA: Florida	FJ263702
51	LJZ-5719	n.d.	USA: Florida	FJ263703
52	LJZ-5818	n.d.	USA: Florida	FJ263704
53	Sihui	n.d.	n.d.	EU644449
54	n.d.	*Diaphorina citri*	n.d.	AB038369

n.d.: not determined.

templates. Reaction mixtures without DNA template were used as negative controls. PCRs were conducted in 25 µL of reaction mixture containing 10 mM Tris-HCl, pH 8.7; 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 µM each primer, 1.25 U of *Taq* DNA polymerase (Promega Corporation, Madison, WI, USA), and 1 µL of template DNA. PCRs were done in a MasterCycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: initial denaturation at 94 °C for 3 min; 40 cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min for OI1/OI2c primers and at 58 °C for 1 min for A2/J5 primers, and extension at 72 °C for 1.5 min. Final extension was carried out for 15 min at 72 °C. Amplified DNA fragments were analyzed by electrophoresis on 1% agarose gel in 1x Tris-Acetate-EDTA buffer, stained with ethidium bromide, and documented using a Biorad UV transilluminator (Bio-Rad, Hercules, CA, USA).

Cloning and sequencing of 16S rDNA and *rpl*KAJL-*rpo*BC operon genes. The obtained amplicons were puri-

TABLE 3 - Ribosomal protein-based SNP genetic lineages among '*Candidatus* Liberibacter asiaticus'

Isolate	Source	Origin	Accession No.	Confirmed SNP in rp[a]					rp SNP lineage
				29	192	261	457	662	
Karnataka - Madikeri	Mandarin	India: Karnataka	FJ827778	T	G	T	A	T	I
Karnataka - Polibetta	Mandarin	India: Karnataka	FJ827780	T	G	T	A	T	
n.d.	n.d.	India	AY266352	G	A	T	A	T	II
n.d.	*Citrus sinesis*	India	FJ177536	G	A	T	A	T	
n.d.	n.d.	China: Guizhon	DQ157276	G	A	T	A	T	
n.d.	n.d.	China: Guangdong	DQ157278	G	A	T	A	T	
n.d.	n.d.	China: Guangxi	DQ157277	G	A	T	A	T	
n.d.	*Citrus grandis*	China	DQ303211	G	A	T	A	T	
Clone ZJ05	n.d.	China	FJ557239	G	A	T	A	T	
n.d.	n.d.	Brazil	EU078703	G	A	T	A	T	
n.d.	n.d.	Brazil	DQ47904	G	A	T	A	T	
n.d.	Citrus	USA: Florida	EU265648	G	A	T	A	T	
n.d.	*Citrus paradix*	Japan	AY342001	G	A	T	A	T	
n.d.	n.d.	n.d.	M94319	G	A	T	A	T	
Cu-c	n.d.	Cuba	FJ394022	G	A	T	A	T	
GC-1	n.d.	Cuba	EU649780	G	A	C	G	C	III
GC-2	n.d.	Cuba	EU649781	G	A	C	G	C	

[a] Bases from start of the A2/J5 fragment of the ribosomal protein gene sequences.
n.d.: not determined.

fied using gel purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified DNA was used for ligation in plasmid vector pCR2.1TOPO (Invitrogen, Carlsbad, CA, USA) and cloned in *Escherichia coli* TOP10 cells, according to the manufacturer's protocol. Both strands of cloned inserts were sequenced to achieve at least 4X coverage per base position. DNA sequencing was performed at Primm S.r.l., Milan, Italy. Sequence results were preliminarily analyzed in BioEdit software version 7.0.9.0 (Hall, 1999), and assembled by using CLC Free Workbench version 4.6 software (http://www.clcbio.com/index.php?id=28). 16S rRNA and β-rp sequences of two representative '*Ca.* Liberibacter' strains, retrieved respectively in the Polibetta and Madikeri regions of the Karnataka state, were deposited in National Center of Biotechnology Information (NCBI) GenBank database under accession numbers from FJ827777 to FJ827780.

Phylogenetic analyses and SNP identification. Sequences retrieved in this work and all the 16S rRNA and β-rp nucleotide sequences of '*Ca.* Liberibacter asiaticus' available in NCBI database (Table 2 and Table 3) were trimmed at primer annealing positions and aligned by using ClustalX (V1.83). For phylogenetic analyses, 16S rRNA gene sequences of all known '*Ca.* Liberibacter asiaticus' together with related representative strains were analyzed by minimum evolution analysis carried out using Neighbor-Joining method and bootstrap replicated 1000 times with MEGA4 software (http://www.megasoftware.net/ index.html) (Tamura *et al.*, 2007).

Presence of SNPs in recognition sites for restriction endonucleases was determined by automated *in silico* restriction digestion assays with the program pDRAW32 (AcaClone Software, http://www.acaclone.com). Virtual RFLP patterns of restriction digestion products were captured according to Wei *et al.* (2007). SNPs detected through virtual-RFLP analyses were validated by experimental enzymatic RFLP analyses of PCR products amplified from citrus plants infected by '*Ca.* Liberibacter asiaticus' in Karnataka state. Restriction reactions were carried out with enzymes *Bpu*10I, *Rsa*I, and *Sac*II (New England Biolabs, Inc. U.K.) in separate tubes containing a total volume of 20 µL consisting of PCR product (40 µg DNA), 10 units of selected enzyme and 2 µL of respective digestion buffer as recommended by the manufacturer. The reaction mixture was incubated overnight at 37 ºC. Restriction fragments were visualized by electrophoresis through 6% polyacrylamide gel. Sizes of the fragments were estimated by comparison with molecular weight marker Φx174 digested by enzyme *Hae*III (Invitrogen).

RESULTS AND DISCUSSION

Detection of '*Ca.* Liberibacter asiaticus'

Amplicons of the expected length were obtained with both primer pairs OI1/OI2c and A2/J5 from templates derived from all HLB-symptomatic samples studied. No amplification was observed in DNA isolated from healthy citrus plants and in DNA-free negative controls (Table 1). These results confirm the presence of '*Ca.* Liberibacter' in HLB affected citrus in different regions of the Karnataka state. All the β-rp amplicons were about 700 bp long, indicating that HLB bacteria in South India belong to the species '*Candidatus* Liberibacter asiaticus' (data not shown).

Phylogenetic analysis

16S rRNA amplicons obtained from two representative HLB affected citrus plants were cloned and sequenced to obtain 4X coverage. Each sample was sequenced using two external primers as well as internal primers, such that each base is checked four times. Thus, obtained sequences were compared and analyzed for eliminating any possible sequence errors. The 1167 bp long sequences obtained shared 100% nucleotide identity, indicating low polymorphism level among strains retrieved from the same geographical region. The sequences had 97-99% sequence identity with previously reported nucleotide sequences of '*Ca.* Liberib-

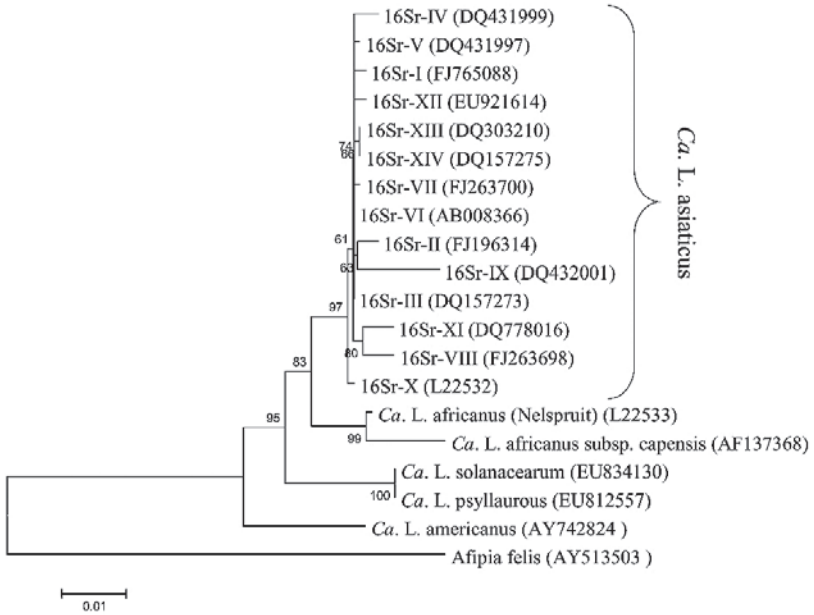

FIG. 1 - Phylogenetic tree inferred from '*Candidatus* Liberibacter' 16S rDNA OI1/OI2c fragments. *Escherichia coli* was used as outgroup to root the tree. Bootstrap values are displayed at tree nodes. GenBank accession numbers of nucleotide sequences are shown along with the names of '*Ca.* Liberibacter' isolates and other bacterial species. Nucleotide sequences determined in this study is indicated in bold characters.

acter asiaticus', confirming the PCR data, and showed 94% sequence identity with '*Ca.* Liberibacter americanus', 94-96% with '*Ca.* Liberibacter africanus'-related isolates, 95-96% with '*Ca.* Liberibacter solanacearum'. Minimum evolution phylogenetic analysis of 16S rRNA gene sequences revealed that '*Ca.* Liberibacter asiaticus' isolates from Karnataka clustered together in a phylogenetic subclade with known '*Ca.* Liberibacter asiaticus' isolates (Fig. 1).

SNP genetic lineages among '*Candidatus* Liberibacter asiaticus'

Alignment of all the known '*Ca.* Liberibacter asiaticus' 16S rDNA nucleotide sequences revealed an unexpected high genetic diversity. Fourteen 16Sr SNP lineages (16Sr-I to 16Sr-XIV) (Table 4) were determined by the presence of 21 SNPs. Each SNP was detected in at least two nucleotide sequences from two independent isolation sources, supporting that they are not due to PCR or sequencing errors (Wei *et al*., 2008; Quaglino *et al*., 2009). Interestingly, the sequences retrieved in this work, obtained from the Karnataka state, belong to a new lineage (16Sr-I) differentiated by three SNPs, two nucleotide transitions at position numbers 414 and 1051, and one transversion at position number 643 (Table 4).

Analysis of the 17 available ribosomal protein genes of the β-rp operon revealed SNPs on five nucleotide positions (Table 3). On the basis of these SNPs, only three ß-rp SNP genetic lineages were revealed among '*Ca*. Liberibacter asiaticus'. Even on this genetic locus the Karnataka strains where different from all the other known strains. Two SNPs (transition at position number 29 and 192) distinguished Karnataka lineage rp-I from the other two '*Ca.* Liberibacter asiaticus' lineages (Table 3). Translation of β-rp genes revealed that Open Reading Frame (ORF) for L10 protein (*rplJ* gene) starts from position at nucleotide 208 from A2 primer initiation site. Consequently, both the diagnostic SNPs were positioned outside the coding region of ribosomal protein genes of the β-operon, probably a region of sequence variability within the β-operon.

Validation of SNPs in 16Sr and rp lineages among '*Candidatus* Liberibacter asiaticus'

Computer simulated RFLP analyses was carried out in order to detect restriction enzymes whose digestion site overlapped with SNP positions previously identified. Three SNPs at nucleotide positions 414, 643, and 1051 in 16S rDNA, which can differentiate Karnataka SNP lineage 16Sr-I, were located within recognition site for restriction enzymes *Sac*II, *Bpu*10I, and *Rsa*I, respectively. In detail, *Bpu*10I and *Sac*II did not digest the amplicons of SNP lineage 16Sr-I (fragment size 1167bp), whereas all the other SNP lineages were digested once by yielding two fragments (*Bpu*10I yields fragments of 635 bp + 532 bp and *Sac*II results in fragment of 759 bp + 408 bp). *Rsa*I digested 16S rDNA PCR products of Liberibacter belonging to SNP lineage 16Sr-I resulting in two fragments of 769 bp + 398 bp, while other SNP lineages were digested at two positions with fragments of 769 bp + 271 bp + 127 bp. In case of β-rp genes, the identified SNPs were not located within restriction site for known endonucleases. *In silico* digestion patterns were validated on the amplicons obtained from the HLB affected Karnataka citrus trees. All the analyzed samples showed the same restriction profiles, indicating that all the individuals contained the same '*Ca.* Liberibacter asiaticus' phylotype (16Sr-I).

*Xba*I-RFLP analysis of 16S rRNA sequences was reported to differentiate between '*Ca*. Liberibacter asiaticus' and '*Ca*. Liberibacter africanus' (Jagoueix *et al.*, 1996). The usefulness of SNP-RFLP analysis for classifying '*Ca.* Liberibacter' species was confirmed also in the present work. *Sac*II, *Bpu*10I, *Rsa*I-RFLP analysis constitute potential diagnostic assays to identify Karnataka phylotypes.

TABLE 4 - 16Sr SNP genetic lineages among '*Candidatus* Liberibacter asiaticus'

16Sr SNP lineage	Isolate[a]	Confirmed SNP in 16S rDNA[b]																				
		9	11	76	180	224	337	360	384	414[c]	643[c]	676	771	772	807	849	909	982	1034	1051[c]	1085	1138
I	1; 2	G	–	T	A	A	T	T	A	T	C	T	C	T	A	T	T	A	T	C	A	T
II	4; 17; 18; 25; 31; 32; 33; 34; 41; 42	G	G	T	A	A	T	T	A	C	G	T	C	T	A	T	T	A	T	T	A	T
III	8; 12	C	–	T	A	A	T	T	A	C	G	T	C	T	A	T	T	A	T	T	A	T
IV	10; 11; 22	G	–	T	A	A	C	A	A	C	G	T	C	T	A	T	T	T	T	T	A	C
V	13; 24	G	–	T	A	A	T	T	G	C	G	T	C	T	A	T	T	A	C	T	A	T
VI	5; 6; 7; 14; 20; 23; 26; 28; 29; 30; 35; 36; 37; 38; 39; 40; 43; 44; 45; 47; 51; 52; 53; 54	G	–	T	A	A	T	T	A	C	G	T	C	T	A	T	T	A	T	T	A	T
VII	48; 49	G	–	T	A	A	T	T	A	C	G	T	C	T	G	T	T	A	T	T	A	T
VIII	46; 50	G	–	C	G	G	T	T	A	C	G	C	T	G	A	T	T	A	T	T	G	T
IX	19; 27	G	–	T	A	A	T	T	A	C	G	T	C	T	A	T	A	A	T	T	A	T
X	3	G	–	T	A	A	T	T	A	C	G	T	C	T	A	T	T	A	T	N	A	T
XI	15	G	G	T	A	A	T	T	A	C	G	T	T	G	A	T	T	A	T	T	A	T
XII	16	G	G	T	A	A	T	T	A	C	G	T	C	T	A	T	T	A	T	G	A	T
XIII	9	G	–	T	A	A	T	T	A	C	G	T	C	T	A	C	T	A	T	T	A	T
XIV	21	C	–	T	A	A	T	T	A	C	G	T	C	T	A	C	T	A	T	T	A	T

[a] See Table 2 for description of '*Ca.* Liberibacter asiaticus' isolates.

[b] Bases from start of the OI1/OI2c fragment of the 16S rRNA gene sequences.

[c] Distinguishing SNPs of '*Ca.* L. asiaticus' Karnataka isolate.

Genetic diversity and ecology of '*Candidatus* Liberibacter asiaticus'

Previously, based on 16S and 16S/23S rDNA intergenic spacer region sequence similarity , '*Ca.* Liberibacter asiaticus' strains from Asia were broadly clustered in only two main groups: Okinawa-related (Japan) and Poona-related (India) isolates. In detail, Subandiyah and colleagues (2000) underscored that Liberibacter isolates detected in Thailand, Nepal, Philippines, Indonesia and Taiwan shared identical 16S rDNA and 16S/23S rDNA sequences with Japanese isolate; liberibacter isolates identified in China shared best sequence homology with Poona isolate. These data are supported by recent work on molecular characterization of 31 Liberibacter isolates from Southeast Asian countries; in other words these isolates shared 100% 16S rDNA sequence identity with Okinawa isolate (Tomimura *et al*., 2009) (these 31 samples are not included in Table 2 as all are identical to Okinawa strain). Furthermore, '*Ca*. Liberibacter asiaticus' isolates reported from São Paulo state, Brazil, grouped with Okinawa isolate (Coletta-Filho *et al.*, 2005). In the present study, presence of repeated SNPs in at least two independent sequences of 16S rRNA and ribosomal protein genes allowed grouping of the '*Ca*. Liberibacter asiaticus' into 14 SNP genetic lineages. These are: 16Sr-II and 16Sr-VI, two widely distributed groups, reported from India, China, Taiwan, Japan (Okinawa isolate), Malaysia, Brazil, and Florida (USA); 16Sr-III, -IV, -V, -XI, -XII, -XIII, and -XIV, reported only from China; 16Sr-VII and -VIII, identified only in Florida; 16Sr-I (Karnataka) and 16Sr-X (Poona isolate), found only in India; 16Sr-IX, described from China and Malaysia. At our knowledge, this is the first study reporting the analysis of 16S rDNA sequence diversity among '*Ca*. Liberibacter asiaticus' isolates from Asian and American continents. As observed from previous studies (Subandiyah *et al*., 2000; Tomimura *et al*., 2009), majority of Liberibacter asiaticus from Southeast Asian countries grouped in the same SNP lineage of Okinawa strain (16Sr-VI) (the 31 isolates described by Tomimura and colleagues (2009) were not included in Table 2 as all are identical to Okinawa strain). On the other hand, diverse SNP lineages were identified in isolates from India, China, and American countries, revealing an unexpected high level of genetic heterogeneity among '*Ca*. Liberibacter asiaticus' isolates. These evidences are in contrast with previous studies by Subandiyah *et al*. (2000) and Bovè (2006) where less number of isolates were analyzed. Interestingly, diverse SNP lineages were detected in different geographic areas, characterized by different climatic conditions (altitude, temperature, humidity, precipitations). In the Indian subcontinent, four genetically diverse SNP lineages, 16Sr-I, 16Sr-II, 16Sr-VI and 16Sr-X, were detected in different geographic regions. It is well known that variations in microclimate can determine radical alterations in phylogenetic diversity of bacterial populations (Zhang *et al*., 2004; Appunu *et al.*, 2008; Cai *et al.*, 2008). Detection of molecular markers in 16S rRNA and ribosomal protein genes, reported in the present study, will help in broad grouping and subgrouping of '*Ca* Liberibacter asiaticus' isolates. In addition, the use of these molecular markers along with those recently reported in the nucleotide sequences of the outer membrane protein gene region (*omp*), of the gene cluster region *trm*U-*tuf*B-*sec*E-*nus*G-*rpl*KAJL-*rpo*B and of the bacteriophage-type DNA polymerase region (Tomimura *et al*., 2009), could provide information for the understand-

ing of geographical distribution and biological traits such as virulence of '*Ca*. Liberibacter asiaticus'-related strains that could be exploited in fighting disease spreading.

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