Sequence Analysis and Comparison of 16S rRNA, 23S rRNA and 16S/23S Intergenic Spacer Region of Greening Bacterium Associated with Yellowing Disease (Huanglongbing) of Kinnow Mandarin

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Abstract High incidence (up to 40%) of symptoms of yellowing and yellow mottling was observed in 5–8 years old orchards of kinnow mandarin (*Citrus reticulata* Balanco ('King' × 'Willow mandarin')) in the Punjab state of India during a survey in January 2007. These symptoms are often confused with nutrient deficiency and other stress related disorders. However, a greening bacterium has been attributed to cause the disease. The disease was graft transmissible and sequencing of 16S rRNA, 16S/23S intergenic spacer region and 23S rRNA of the greening bacterium associated with yellowing disease in kinnow mandarin confirmed it to be *Candidatus Liberibacter asiaticus* ('Ca L. asiaticus') showing maximum identity of 95.9% with 'Ca L. asiaticus' from USA and Brazil in 16S rRNA. The study indicates definite association of 'Ca L. asiaticus' with yellowing/chlorotic mottling symptoms of greening disease of kinnow mandarin in Punjab state of India.

Keywords Citrus greening · Huanglongbing · *Candidatus Liberibacter asiaticus* · Kinnow mandarin · 16S rRNA · 16S/23S intergenic spacer region · 23S rRNA

Introduction

Citrus greening disease is a threatening disease of citrus in India and other citrus growing countries. Kinnow mandarin (*Citrus reticulata* Balanco ('King' × 'Willow mandarin')) is a major horticultural crop of Punjab and it is also grown in Rajasthan and Haryana states of India. During a survey in Punjab, symptoms of yellowing and mottling of leaves were observed up to 40% in kinnow mandarin orchard. These symptoms in different citrus species are generally associated with greening disease caused by greening/huanglongbing bacterium [1]. There are three greening bacterium species which have been associated with greening disease in citrus world over. These are *Candidatus Liberibacter asiaticus* (Ca L. asiaticus); *Candidatus Liberibacter africanus* [2, 3] and *Candidatus Liberibacter americanus* (GenBank Acc. No. AY859542). The bacterium was non-cultivable until recently when Sechler et al. [4] could demonstrate the cultivation of all the three *Candidatus Liberibacter* spp. in a specifically designed Liber A medium. The bacterium is spread by inadvertent vegetative propagation and by insect vector (*Diaphorina citri*) [5, 6] and *Trioza erytreae* [7]. It causes crop losses of 30–100% and citrus production may be eliminated in some production areas [8]. Greening disease has severely affected kinnow mandarin production in Punjab [9] and other northern parts of India and is a major concern to farming community. The greening/huanglongbing disease in citrus has been reported in different citrus species in several parts of India [10–12] and several Asian [2] and African countries in the Arabic Peninsula and Madagascar [13, 14]. As there is no conclusive report available whether symptoms of yellowing or chlorotic mottling in kinnow mandarin is nutritional disorder or caused by a greening bacterium, a study was undertaken on graft transmissibility of the

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disorder and characterization of greening bacterium by cloning, sequencing and phylogenetic analysis of 16S rRNA, 16S/23S intergenic spacer regions and 23S ribosomal RNA.

Materials and Methods

Samples Collection and Maintenance of Citrus Greening Pathogen

Survey of kinnow mandarin was done in the Hoshiarpur district of Punjab during January 2007. We surveyed three orchards each having 100–130 plants of kinnow mandarin. Symptomatic plant materials of kinnow mandarin were collected (Fig. 1) and wedge grafted on one year old healthy sweet orange seedling. The grafted plants were maintained in the glasshouse. Samples of midrib and petiole of symptomatic leaves of kinnow mandarin were also stored at -80°C . Midrib and petiole of stored samples or six months old graft inoculated plants were used for detection and characterization of greening bacterium. Citrus greening bacterium maintained on sweet orange in the

glass house was used as positive control. Healthy seedlings of sweet orange were used as negative control.

Isolation of Total DNA from Plant Leaf Midrib

Leaves of symptomatic kinnow mandarin/graft inoculated sweet orange and healthy leaves of sweet orange were thoroughly washed and blotted dried. They were further cleaned with 70% alcohol. Midrib and petiole were cut with sterilized scissors and ground with liquid nitrogen. Total DNA was isolated from 100 mg midrib and petiole of symptomatic kinnow mandarin/sweet orange and healthy leaves of sweet orange using DNeasy plant mini kit (Qiagen, Germany) as per manufacturer's protocol.

Primers Designing and Synthesis

Sequences of greening bacterium were obtained from NCBI GenBank and Clustal W was used for multiple alignments of these sequences. Three sets of overlapping primers were designed manually to amplify 16S rRNA, 16S/23S rRNA intergenic region and 23S rRNA of citrus greening bacterium. Primer sets were synthesized at Bio-basic, Canada. Primer sets A & B covered the 16S rRNA while primer set C covered the 16S/23S spacer region and 23S rRNA (Table 1).

PCR Amplification

PCR amplification was performed in a thermal cycler (Eppendorf, Germany) in 50 μl reaction mixture using 1 μM of forward and reverse primer of each set (Table 1), 200 μM of each dNTPs, 0.05 unit/ μl of Taq DNA polymerase (Fermentas, Lithuania), $1\times$ reaction buffer, 1.5 mM of MgCl_2 and 5 μl (0.8 μg) of DNA template isolated from healthy and symptomatic citrus plants. The temperature regimes were 94°C for 5 min, followed by 30 cycles of 94°C for 60 s, $58^{\circ}\text{C}/50^{\circ}\text{C}/62^{\circ}\text{C}$ for 60 s, 72°C 60 s, followed by a final extension of 10 min at 72°C . PCR amplification of samples was repeated 2–3 times. PCR products were analyzed on 1% agarose gel in Tris Acetate

Fig. 1 Yellowing symptoms of citrus greening disease in kinnow mandarin

Table 1 Primer sets using for PCR amplification of 16S rRNA, 23S rRNA and 16S/23S intergenic spacer regions of citrus greening bacterium on kinnow mandarin

Primer sets	Primer sequence	T_m $^{\circ}\text{C}$	Annealing temp. $^{\circ}\text{C}$	Expected size	Obtained size
A	F 5'GCG CGT ATG CGA ATA CGA GCG GCA 3'	68.5	62	~ 1168	1167
	R 5'GCC TCG CGA CTT CGC AAC CCA T3'	67.8			
B	F 5'ATG GGT TGC GAA GTC GCG AG3'	64	60	~ 290	289
	R 5'AAG GAG GTG ATC CAG CCG CA 3'	64			
C	F 5'TGC GGC TGG ATC ACC TCC TT 3'	61.9	50	~ 867	866
	R 5'CTT AGA TGT TTC AGT TCC C 3'	53.2			

Table 2 Nucleotide sequence identity of pair wise combinations of 16S rRNA of citrus greening bacterium (1417 bp)

Isolates*	FJ196314	DQ778016	EU644449	L22532	L22533	AY742824	DQ471901	DQ471900	EU812559	EU834130
FJ196314	100.0	95.4	94.8	94.7	93.0	90.4	95.9	95.9	93.1	93.1
DQ778016		100.0	98.3	97.6	96.3	93.9	99.4	99.4	96.3	96.3
EU644449			100.0	97.2	96.2	93.0	98.8	98.8	95.7	95.7
L22532				100.0	96.2	93.0	98.2	98.2	95.1	95.1
L22533					100.0	92.6	96.9	96.9	95.4	95.4
AY742824						100.0	94.0	94.0	93.6	93.6
DQ471901							100.0	100.0	96.7	96.7
DQ471900								100.0	96.7	96.7
EU812559									100.0	100.0
EU834130										100.0

Table 3 Nucleotide sequence identity of pair wise combinations of 16S/23S rRNA intergenic spacer regions of citrus greening bacterium (620 bp)

Isolates*	FJ196314	AB019793	DQ778016	DQ207841	DQ462244	DQ462257	EU644449	U61359	U61360	AY859542	DQ471903	EU812559	EU834130
FJ196314 (This study)	100	99.5	99.5	99.5	99.5	99.5	96.1	98.7	69.5	75.5	99.1	80.4	80.4
AB019793		100	100	100	100	100	96.6	99.1	69.6	75.8	99.6	80.9	80.9
DQ778016			100	100	100	100	96.6	99.1	69.6	75.8	99.6	80.9	80.9
DQ207841				100	100	100	96.6	99.1	69.6	75.8	99.6	80.9	80.9
DQ462244					100	100	96.6	99.1	69.6	75.8	99.6	80.9	80.9
DQ462257						100	96.6	99.1	69.6	75.8	99.6	80.9	80.9
EU644449							100	95.8	66.9	72.7	96.3	77.9	77.9
U61359								100	69.7	75.2	98.8	80.4	80.4
U61360									100	61.3	69.3	65.0	65.0
AY859542										100	75.5	77.1	77.1
DQ471903											100	81.1	80.8
EU812559												100	99.3
EU834130													100

Fig. 2 Gel electrophoresis of PCR products amplified with 3 sets of primers. (I) Primer pair A (II) Primers pair B (III) Primer pair C. Lanes: M 1 kb DNA ladder, H Healthy control sample of kinnow mandarin, I Infected sample kinnow mandarin

Fig. 3 Schematic diagrams of 16S rRNA, 16S-23S rRNA Intergenic spacer regions and 23S rRNA of greening bacterium infecting Kinnow mandarin

Table 4 Nucleotide sequence identity of pair wise combinations of 23S rRNA of citrus greening bacterium (247 bp)

Isolates*	FJ196314	DQ778016	EU644449	EF438153	EU812559	EU834130
FJ196314 (This study)	100	99.5	99.1	99.5	91.1	91.1
DQ778016		100	99.5	100	91.5	91.5
EU644449			100	99.5	91.1	91.1
EF438153				100	91.5	91.5
EU812559					100	100
EU834130						100

*The sequences of greening bacterium used for identity analysis are: AB019793, '*Ca. L. asiaticus*' (Japan); AY859542, '*Ca. L. americanus*' (Brazil); AY742824, '*Ca. L. americanus*' (Brazil); DQ471901, '*Ca. L. asiaticus*' (Brazil); DQ471900, '*Ca. L. asiaticus*' (USA); DQ778016, '*Ca. L. asiaticus*' (China); DQ207841, '*Ca. L. asiaticus*' (China); DQ462244, '*Ca. L. asiaticus*' (China); DQ462257, '*Ca. L. asiaticus*' (China); DQ471903, '*Ca. L. asiaticus*' (Brazil); EF438153, '*Ca. L. asiaticus*' (China); EU644449 '*Ca. L. asiaticus*' (China); EU812559, '*Ca. L. psyllaureus*' (USA); EU834130, '*Ca. L. sp. nz 082226*' (New Zealand) EU644449, '*Ca. L. asiaticus*' (China); EF438153, '*Ca. L. psyllaureus*', FJ196314, '*Ca. L. asiaticus*' (Isolate in this Study); L22532, '*Ca. L. asiaticus*' (Poona); L22533, '*Ca. L. africanus*', (Nelspruit); U61359, '*Ca. L. asiaticus*' (Poona); U61360 '*Ca. L. africanus*' (Nelspruit)

EDTA buffer containing ethidium bromide and the gel was visualized and photographed using a gel documentation apparatus (Bio-Red XR documentation system).

Cloning and Sequencing

The PCR products were excised and eluted from the gel using QIA quick gel extraction kit (Qiagen GmbH, Hilden, Germany). The gel eluted DNA was ligated into pGEM-T easy vector (Promega, Madison, USA) and competent *Escherichia coli* strain DH5 α were transformed by standard molecular biology protocol [15]. Recombinant clones were identified by colony PCR. Selected recombinant clones were sequenced at automated DNA sequencing facility at Bioserve, Hyderabad. BLAST programme was used to compare the homology with sequences available in the NCBI GenBank. Sequences of

16S rRNA, 23S rRNA and 16S/23S rRNA spacer region of greening bacterium available in GenBank were aligned using Clustal X [16]. Sequence identity matrix for pair wise combination of aligned sequences was calculated with Bio Edit sequence alignment editor version 5.09.04 [17]. Sequence phylogram were constructed using the PHYLIP package (boot-strap) analysis with 1000 replicates and rooted trees were generated using NJ TREEVIEW software [18]. A large number of 16S rRNA sequences are available in GenBank and representative sequences of different greening bacterium (10 Sequences) associated with citrus and solanaceous crops from different countries viz., Brazil, China, India, Japan, USA, New Zealand and Africa (Table 2). For analysis of 23S rRNA, a total of 6 sequences were used (Table 4) while 13 sequences were used for analysis of 16S/23S rRNA spacer region (Table 3).

Fig. 4 Phylogenetic tree of 16S rRNA nucleotide sequence of citrus greening isolates. The tree was constructed using Clustal X (Thompson et al. [16]) with bootstrapping (1000 replicates) using

TREEVIEW Software from the alignment of the *Candidatus* species of 16S rRNA. The number at nodes refers to number of times branching was supported

Results and Discussion

Incidence and Losses

Survey in the Hoshiarpur district of Punjab state of India revealed that 144 plants out of 360 plants in three 5–8 years old kinnow mandarin orchards had symptoms of yellowing and mottling typical of greening disease indicating that the incidence of greening disease was up to 40%. Bioassay study revealed that out of six symptomatic plants of kinnow mandarin, 3 plants induced vein yellowing in sweet orange plants.

Amplification, Sequencing and Sequence Analysis of 16 rRNA, 16S/23S rRNA Intergenic Spacer Region and the 23S rRNA

The three sets of over overlapping primer used for PCR amplification of 16S rRNA, 16S/23S rRNA produced PCR products of approximately ~1.1 kb, ~0.3 kb and ~0.8 kb (Fig. 2) and no amplification was obtained from the healthy plant. Sequences of the cloned amplicons obtained by primer sets A, B and C were 1167, 289 and 866 bp in size respectively. Sequences of these clones matched with the corresponding sequence of a Chinese

Fig. 5 Phylogenetic tree of 16S/23S rRNA nucleotide sequence of citrus greening isolates. The tree was constructed using Clustal X (Thompson et al. [16]) with bootstrapping (1000 replicates) using

TREEVIEW Software from the alignment of the *Candidatus* species of 16S/23S rRNA. The number at nodes refers to number of times branching was supported

isolate of '*Candidatus Liberibacter asiaticus*' (Accession number DQ778016). These sequences were aligned together and the total sequence length was 2284 bp in size showing maximum identity of 95.5% with the corresponding sequence of the Chinese isolate of '*Ca. L. asiaticus*' (GenBank Accession No DQ778016). BLAST analysis of the sequence indicated that the sequences of 2284 bp contained 16S rRNA of 1417 bp, 16S/23S spacer region of 620 bp and 23S rRNA of 247 bp (Fig. 3). The 16S rRNA gene sequence of the greening bacterium under study showed an identity of 95.9 with '*Ca. L. asiaticus*' isolate of USA and Brazil (Accession No. DQ471900,

DQ471901), 95.4% with '*Ca. L. asiaticus*' isolate of China (Accession No. DQ778016), 94.7% with '*Ca. L. asiaticus*' Poona isolate (Accession No. L22532), 93% with '*Ca. L. africanus*' isolate Nelspruit (L22533); '*Ca. L. psyllauros*' (Accession No. EU812559); and 90.4% with '*Ca. L. americanus*' (AY742824) (Table 2). Comparison of 5 sequences of 23S rRNA of *Ca. L.* species available in the GenBank and the present isolate of greening bacterium under study showed that 23S rRNA was highly conserved (99–100%) in all the 4 isolates of '*Ca. L. asiaticus*' and two isolates of '*Ca. L. psyllauros*'. However, 23S rRNA of '*Ca. L. asiaticus*' shared 91.5% identity with

Fig. 6 Phylogenetic tree of 23S rRNA nucleotide sequence of citrus greening isolates. The tree was constructed using Clustal X (Thompson et al. [16]) with bootstrapping (1000 replicates) using

TREEVIEW Software from the alignment of the *Candidatus* species 23S rRNA. The number at nodes refer to number of times branching was supported

'*Ca. L. psyllaous*' (Accession No. EU644449)) and '*Ca. L. sp.NZ 082226*' (Accession No. EU834130) (Table 4). A comparison of 16S/23S spacer sequence greening bacterium under study and isolates of '*C. Liberibacter asiaticus*' from India (Poona isolate), China, USA, Brazil showed that the intergenic regions was almost identical (96% to 99.5%). However, it was very distinct in other 3 species of greening bacterium viz., '*Ca. L. americanus*' (Accession No. AY859542), '*Ca. L. africanus*' (Accession No. U61360) and '*Ca. L. psyllaous*' (Accession No. EU644449). 16S/23S spacer sequence of '*Ca. L. asiaticus*', shared only 75.5, 69.5 and 80.5% identity with that of '*Ca. L. americanus*', '*Ca. L. africanus*' and '*Ca. L. psyllaous*' respectively (Table 3). 16S rRNA, 16S/23S intergenic spacer region and the 23S rRNA sequences of greening bacterium under study shared maximum identity with isolates of '*Ca. L. asiaticus*' from China, Brazil and USA. The sequence of the 16S/23S

Intergenic spacer region consisted of 620 bp of nucleotides in '*Ca. L. asiaticus*' and '*Ca. L. psyllaous*' but it was 606 bp in '*Ca. L. americanus*' and 498 bp in '*Ca. L. africanus*'.

The phylogenetic analysis of 16S rRNA of greening bacterium revealed that '*Ca. L. asiaticus*' isolates from different countries grouped together while '*Ca. L. americanus*', '*Ca. L. africanus*' and '*Ca. L. psyllaous*' formed a separate and distinct group (Fig. 4). The Phylogenetic analysis of 16S/23S rRNA Intergenic spacer region showed that 9 isolates of '*Ca. L. asiaticus*' clustered together within the liberibacter group (Fig. 5) and they in turn grouped with '*Ca. L. africanus*', '*Ca. L. asiaticus*', '*Ca. L. americanus*', '*Ca. L. psyllaous*' and '*Ca. L. sp. NZ082226*'. The phylogenetic analysis of 23S rRNA showed that 4 isolates of '*Ca. L. asiaticus*' clustered together within the liberibacter and '*Ca. L. psyllaous*' and '*Ca. L. sp. NZ082226*' clustered together separately (Fig. 6).

The presence of greening disease in citrus species in India was suggested as early as 1965 and the disease could be transmitted by psyllid vector (*Diaphorina citri*) [5]. The disease is graft transmissible as also observed in our study. The association of greening bacterium in citrus species from different parts of India excluding Punjab has been confirmed by electron microscopy and dot blot hybridization [12]. Subsequently the PCR technique was used for detection of greening bacterium in citrus [19]. However, this PCR technique was used in India for the first time to detect greening bacterium associated with mandarin in Bhutan [20] and different citrus species grown in different parts India [10, 11]. Greening disease in kinnow mandarin in Punjab is quite prevalent [9] but there was no information available on definite association and characterization of greening bacterium associated with it. Sequencing of 16S rRNA of greening bacterium associated with greening disease of kinnow mandarin demonstrated that it was very identical (~95%) to sequences of *Ca. L. asiaticus* including a Poona isolate of *Ca. L. asiaticus* (GenBank Accession No. L22532) previously submitted in 1994 from France. The sequence identity of 16S rRNA with other greening bacterium species of African and American continent was in the range of 90–93.1%. Though the sequences of 16S rRNA and 23S rRNA, were comparatively conserved in the three species of greening/huanglongbing bacterium but sequences of 16S/23S spacer region in different species of greening/huanglongbing bacterium viz., '*Ca. L. asiaticus*', '*Ca. L. americanus*', '*Ca. L. africanus*' and '*Ca. L. psyllauros*' were highly variable and can be used to distinguish the different greening bacterium species. Phylogenetic tree based on 16S/23S intergenic spacer region indicated that '*Ca. L. africanus*' Nelspruit had common origin with '*Ca. L. asiaticus*' and both were distinct from '*Ca. L. americanus*', '*Ca. L. psyllauros*' and '*Ca. L. sp. NZ082226*'. It is interesting to observe that '*Ca. L. psyllauros*' was found to infect tomato and potato in USA [21] and '*Ca. L. sp. NZ082226*' (Accession No. EU834130) was found associated with solanaceous crops and a study on the transmission of greening bacterium from citrus to solanaceous crops and vice versa may provide new insight on greening/huanglongbing bacterium.

The nucleotide sequence of '*Ca. L. asiaticus*' associated with kinnow mandarin has been deposited in the Genbank database (Accession No. FJ196314).

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