Mandarin orange (Citrus reticulata Blanco), an important citrus fruit in India, is grown on a large scale in Nagpur and adjoining districts of the Vidarbha region in Maharashtra. With a rich source of vitamin C and mineral salts, this fruit (popularly known as ‘Nagpur santra’) has a high consumer preference both as fresh fruit as well as refreshing juice. The area under Nagpur orange crop presently in the Vidarbha region is about 80,000 ha, with an annual production of about 5 lakh tonnes. It is claimed to be one of the most remunerative potential foreign exchange-earning crop of not only this region, but also as one of the major horticultural crops after banana and mango at the national stage.

Nonetheless, at present this crop is experiencing the ominous problem of decline, predominantly incited by various diseases caused by different etiological agents such as fungi, bacteria, viruses and phytoplasmas. The fungal pathogen, Phytophthora spp. is well established in this area and is attributed as one of the factors for the Nagpur mandarin decline. However, the role of citrus greening disease (CGD) in Nagpur orange decline has long been questionable and contentious.

Among all the diseases of citrus described to date, CGD (also called ‘Huanglongbing’, a Chinese word, meaning ‘yellow shoot disease’) is considered probably the most destructive and lethal. The disease infects citrus trees of all almost all cultivars and causes substantial economic losses to the citrus industry by shortening the life span of infected trees. The causal pathogen of CGD is a fastidious phloem-limited bacterium, a member of the α-subdivision of the phylum Proteobacteria. Currently three species of the causal organism are recognized: Candidatus Liberibacter asiaticus, Ca. L. africanus and Ca. L. americanus. These bacteria have not been cultured yet. Two psyllids Diaphorina citri and Triozerythreac vector the disease. The former is the principal vector in Asia, Brazil and Florida, while the latter transmits the disease in Africa.

It has been difficult to consistently detect the Liberibacters using traditional biological assays, detection of fluorescent compounds, light or electron microscopy, or ELISA. This is presumably because of the low concentration and the uneven distribution of the pathogens in host plants and vector insects. In addition, the non-specific nature of foliar symptoms makes the disease difficult to distinguish from nutrient deficiencies or other diseases. In recent times, several PCR-based molecular approaches are being developed to detect and differentiate Ca. Liberibacter species using species-specific primers based on sequences of the 16S rDNA and other regions of the bacterial genome.

The present paper reports the molecular identification and characterization of the CGD bacterium associated with Nagpur orange decline through PCR and also deduces its phylogenetic relationship with the members of the phylum Proteobacteria.

Extensive surveys were undertaken across the Nagpur orange belt of Vidarbha during 2006–07. The presence of CGD symptoms was examined as described earlier. Young leaf and twig samples showing characteristic symptoms of CGD in declining trees were collected from private Nagpur orange orchards situated at Katol, Kondhali and Kelmeshwar areas of Nagpur District. Each candidate sample contained 20–40 leaves attached to the branches and was kept in plastic bags at −80°C before being used for DNA extraction. Total DNA was extracted from 200 mg tissue of midrib and petiole of symptomatic leaves as well as from healthy leaves using DNeasy™ Plant Mini Kit (Qiagen, Gmbh, Germany), according to the manufacturer’s protocol.

Specific primers (OH/OL2c) were used for the amplification of 16S rDNA of the CGD bacterium. Primers were synthesized from Integrated DNA Technologies Inc., Coralville, USA. PCR was conducted in 25 μl reaction mixtures (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP, 2.5 units of Taq DNA polymerase and 0.2 μM of each primer) in a MasterCycler (Eppendorf, Germany) with the following set-up: first by incubation at 94°C for 3 min, followed by 35 cycles of a denaturation step at 94°C for 1 min, an annealing step with 58°C for 1 min, extension at 72°C for 1 min and final extension of 10 min at 72°C. The amplified DNA (PCR products) were analysed on 1% agarose gel stained with ethidium bromide. A restriction enzyme digest, using XhoI, of the PCR product (amplified fragment using primers OH/OL2c) was conducted to determine the Liberibacter species present. The mastermix for XhoI was prepared in a 20 μl reaction volume and incubated overnight at 37°C and the digested product was analysed on a 2% agarose gel. The gel was observed under UV transilluminator and photographed in a gel Doc system (Biovis, Mumbai).

To ascertain the nature of the amplification, the amplified DNA fragments were purified from the agarose gel using QIAquick gel extraction kit (Qiagen, Gmbh, Germany). The purified PCR products were cloned into the pGEM- T™ easy vector (Promega Biosciences, California) and sequenced at the commercially available automated DNA sequencing facility (Genei, Bangalore). Search for homologies in the GenBank databases (http://www.ncbi.nlm.nih.gov/blast) was carried out using the BLAST program. Closely related sequences were aligned by using CLUSTAL W version 1.82 and comparison of aligned sequences was done with Bio Edit sequence alignment Editor.

Phylogenetic and evolutionary analysis was conducted using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. The bacterial 16S rRNA gene sequences used for comparisons were obtained from the GenBank databases and were those of Ca. L. asiaticus isolate Malaysia (EU224393.1), Ca. L. asiaticus isolate Florida, USA (EU130553.1), Ca. L. asiaticus isolate China (DQ157274.1), Ca. L. asiaticus isolate Japan (AB008366.1), Ca. L. asiaticus isolate Taiwan (DQ302750.1), Ca. L. asiaticus isolate Poona, India (L22532.1), Ca. L. asiaticus isolate SP-Brazil, (AY919311.1), Ca. L. africanus isolate Nelspruit, South Africa (L22533.1), Ca.
Figure 1. PCR detection of CGD bacterium, Candidatus Liberibacter asiaticus in Nagpur orange samples from Vidarbha region, Maharashtra, India. Agarose gel electrophoresis of DNA extracted from citrus leaf midribs and bark tissues amplified with the O11/O12c primers (a) and digestion of the amplified product with the restriction enzyme XbaI (b). Lane M, 1 kb ladder (MBI Fermentas, MD, USA); lane 1, DNA extracts from healthy citrus; lanes 2-4, DNA extracts from greening-infected Nagpur mandarin plants from different localities of Nagpur District: Kondhali (lane 2), Kalmeshwar (lane 3) and Katol (lane 4).

Figure 2. Phylogenetic tree constructed from alignment of 16S rRNA gene sequences from Ca. Liberibacter and other α-Proteobacteria. GenBank accession numbers are given in parenthesis. Bootstrap values (based on 1000 replications) are indicated at the nodes.

L. africanus subsp. capensis isolate, South Africa (AF137368.1), Ca. L. africanus isolate SP-Brazil (AY742824.1), Sinorhizobium sp. (EF100523.1), Phyllobacterium sp. (EU170548.1) and Rhizobium sp. (DQ337581.1).

PCR using primers O11/O12c produced an amplified fragment of about 1160 bp size, which was observed in all the three plant samples collected from different localities of Nagpur District infected by the CGD bacterium. No amplification was obtained from DNA extracted from healthy citrus (Figure 1a). The result also indicates that the amplified DNA fragment of 16S rDNA was digested by XbaI into two fragments of about 640 and 520 bp (Figure 1b), as reported only for Ca. L. asiaticus (since it has only one XbaI restriction site unlike that of Ca. L. africanus, which has two XbaI restriction sites).

The determined sequences (through cloning and sequencing) of all the three amplified fragments of 16S rDNA were found to be similar (1167 bp long).

When these sequences were compared with the known Liberibacter species available in the GenBank, homology was observed higher with Ca. Liberibacter asiaticus (98.37–99.14%) than with Ca. Liberibacter africanus (94.11–96.4%) or Ca. Liberibacter americanus (93.68%). The sequence homology was found to be even higher when compared with the other members of α-Proteobacteria.

The evolutionary relationship of 14 taxa (Figure 2) was inferred using the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted using MEGA software version 4.0. Evolutionary divergence between the sequences was also estimated, and the results are based on the pairwise analysis of all the 14 sequences. Analyses were conducted using the maximum composite likelihood method in MEGA, version 4.0.

The phylogenetic analysis (Figure 2) revealed that the Nagpur CGD bacterium clustered together with the Ca. L. asiaticus isolates. Ca. L. africanus isolates and Ca. L. americanus isolates formed a different cluster. The genetic distance from other members of α-Proteobacteria (Sinorhizobium sp., Phyllobacterium sp. and Rhizobium sp.) was found to be even higher (Figure 2). Interestingly, within the Ca. L. asiaticus group, genetic distance of Nagpur isolate from the Poona strain was found to be higher.

In summary, PCR detection, sequencing and subsequent sequence analysis of three Nagpur CGD bacterium isolates indicated that all the isolates are the same (1167 bp long) and are closer to Ca. Liberibacter asiaticus than Ca. L. africanus or Ca. L. americanus. The results also indicate that Ca. Liberibacter asiaticus can induce chlorosis and decline symptoms on the mandarin tree.
SCIENTIFIC CORRESPONDENCE

Our studies revealed that the 16S rRNA gene of CGD bacterium is highly conserved and can be used for quick PCR-based detection of CGD bacterium of Nagpur orange plant for the phytosanitary and certification programme. This study provides molecular evidence for the association of *Ca. Liberibacter asiaticus* with Nagpur orange decline in the Vidarbha region. For requirement of certified planting material, citrus growers of this region use locally produced non-certified planting material by private nurseries. Mother plants from which buds were taken for large-scale propagation were often infected with CGD bacterium, which resulted in the spread of the disease on a wider scale rendering the plantation with poor yield potential. Moreover, presence of psyllid vector made the situation even worse. Under these circumstances, it is vital to ensure that growers have access to CGD-free planting material for establishing a profitable citrus industry in this part of India.

Nucleotide sequence accession number: The representative DNA nucleotide sequence of *Ca. Liberibacter asiaticus* Nagpur isolate has been deposited in the GenBank database under accession no. EU 939452.


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Gut content analysis of spiders in coffee ecosystem

Spiders are gaining favour in ecological studies as indicators of environmental quality and as biological control agents in agricultural ecosystems. They are the abundant natural enemies in any agroecosystem and are found in most terrestrial habitats, often present in large numbers. All spiders are predaceous and insects constitute their primary prey. They are generalist predators that may be of importance in reducing and even preventing outbreaks of insect pests in agriculture. Spiders tend to be small, cryptic feeders, have extra-oral digestion and sucking mouthparts, and exhibit amorphous gut contents. All these attributes make it difficult to obtain data on predation rates. Some information can be gathered by direct observation, but gut analysis of field-collected spiders is the least disruptive and the most efficient means to acquire data on predation.

Coffee, a major commercial crop of India harbours rich spider diversity. The crop is also attacked by a variety of pests, including, the coffee green scale, *Coccus viridis* (Green), a major sucking pest of coffee. This correspondence discusses the importance of gut content analysis of spiders in coffee ecosystem and provides suggestions to overcome the difficulties faced in the present techniques. The analysis was carried out with a hypothetically assumed that *C. viridis* is a prey for foliage-dwelling spiders, and *Aularches* sp. could be the prey of spiders inhabiting field margins. This study will lead to better assessment of the predation of *C. viridis* by spiders and their biocontrol potential in coffee plantations. Observations on the analysis of the prey remnants in the gut of spiders are discussed.

Gut content analysis of freshly collected spiders from the coffee plantations of Horticultural Research Station, Yercaud, Tamil Nadu, was carried out by electrophoresis, which is based on the detection of prey enzymes in homogenates of the predator after PAGE and staining for esterase activity to know their preferential feeding habit under field conditions. Two different preyes (*C. viridis* and an acidid, *Aularches* sp.) and eight species of spiders (*Leucauge decorata* (Blackwall), *Oxyopes sp.*, *Dieta vires* (Thorell), *Olias milleti* Pocock, *Telomania dimida* (Simon), *Clubionia sp.*, *Hippusa sp.* and *Plexipus sp.* were used in the experiment. Extraction was carried out according to the method described by Amalin et al., with slight modifications. The samples were macerated in phosphate buffer at the rate of 1 mg of the substrate. Electrophoretic separation of isozymes was achieved with 10% native PAGE, as described by Laemml, with slight modifications as required. Polyacrylamide slab gels with a total gradient concentration of 10% and a cross-link gradient of 4% were prepared between two glass plates using a gel gradient maker. Casing of the gel was done similar to that of SDS–PAGE. The whole electrophoresis set-up was kept in the refrigerator for cooling during the run. Sample solutions diluted with sample buffer were loaded onto the sample wells carefully with the help of a microsyringe. The run was continued until the dye front reached the bottom of the gel.