Presence of citrus greening (Huanglingbing) disease and its psyllid vector in the North-Eastern region of India confirmed by PCR technique

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Citrus greening or Huanglingbing disease caused by a nonculturable, phloem-limited bacterium, Candidatus Liberibacter asiaticus, is one of the most serious and destructive citrus diseases in the world. The presence of this disease in citrus orchards in the North-Eastern region of India has been confirmed through polymerase chain reaction (PCR)-based molecular detection. Samples of DNA extracted from leaves of putatively infected different citrus cultivars and from the vector of the disease, Diaphorina citri, were subjected to analysis using PCR and produced DNA amplicons characteristic of Ca. L. asiaticus. This is the first molecular evidence confirming the presence of greening disease and its psyllid vector in NE India.

Keywords: Candidatus Liberibacter asiaticus, citrus, greening disease, PCR detection, psyllid vector.

The nutritional value of citrus fruits is well known in our dietary requirements. Presently, it is the third largest fruit industry after mango and banana in India. The North-Eastern (NE) region of India offers favourable climatic conditions for cultivation of various citrus species. Submountain and hilly tracts of states like Meghalaya, Assam, Manipur, Arunachal Pradesh, Mizoram, Nagaland, Tripura, Sikkim and Darjeeling District, West Bengal grow excellent quality citrus fruits. Different citrus species, viz. mandarin, sweet orange, lemon and other limes are cultivated in all the states of the NE region covering 57.2 thousand hectares, with a total production of 306 thousand tonnes. The entire citrus orchards in NE India are of seedling origin, with few budded or grafted plants at some experimental research stations. Many other crops, citrus in this region is plagued with a host of diseases caused by different etiological agents such as fungi, bacteria, viruses and phytoplasmas. Among all the diseases of citrus described to date, citrus greening disease (CGD) is considered to be probably the most destructive and lethal. The disease infects citrus trees of almost all cultivars and causes substantial economic losses to the citrus industry by shortening the lifespan of infected trees. It is estimated that globally more than 60 million trees had been destroyed by the disease. The name of the disease has been

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recently changed from ‘greening’ to the Chinese word ‘huanglongbing’ (since the disease was first discovered in China)\textsuperscript{3}. Huanglongbing means ‘yellow dragon disease’, a description used because infected trees turn yellow, usually starting from one branch or one part of the tree, before they die (as if attacked by a yellow dragon). The term ‘greening’ on the other hand, originates from the colour of the infected fruits. Instead of becoming orange when ripe, fruits of affected trees remain green. The disease is graft- and vector-transmissible\textsuperscript{4,5}.

The causal pathogen of CGD is a fastidious bacterium, a member of the \( \alpha \)-subdivision of the phylum Proteobacteria\textsuperscript{6}. The bacterium resides in the sieve tube elements in the phloem tissue of infected plants and is systemic in insect vectors. CGD exists in nature in three forms that differ by a combination of environmental conditions and insect vectors. CGD caused by \textit{Candidatus Liberibacter asiaticus}\textsuperscript{7}, a heat-tolerant form found in Asia, is vectored by \textit{Diaphorina citri}, the Asian citrus psyllid\textsuperscript{8}. CGD caused by \textit{Ca. L. africanus}\textsuperscript{9}, a heat-sensitive form found in Southern Africa, is vectored by \textit{Trioza erytreae}, the African citrus psyllid\textsuperscript{10}. CGD caused by \textit{Ca. L. americanus}, another heat-tolerant form found in Brazil\textsuperscript{11,12}, is vectored by \textit{D. citri}. \textit{Ca. L. asiaticus} has been recently reported from south Miami-Dade county, Florida, USA\textsuperscript{13,14}. However, Koch’s postulates have not been fulfilled because the bacteria have not been cultured yet. (The term \textit{Candidatus} is used for bacterial species that cannot be cultured. If \textit{Candidatus} is used, the actual genus and species are not italicized.) The Asian citrus psyllid (\textit{D. citri}), which transmits Asian greening is a member of Sternorrhyncha: Psyllidae. It is considered a serious pest of citrus in the world due to its ability to efficiently transmit the greening agent. The bacteria reproduce in the hemolymph and salivary glands of the insects after they feed on infected plants\textsuperscript{9,14,15}. Once the citrus psyllids acquire the bacteria, they transmit it to new hosts for the remainder of their life cycle in a persistent, propagative manner\textsuperscript{14,15}. Only adult psyllids are known to transmit the bacteria under natural conditions\textsuperscript{16}.

In recent years several accurate diagnostic techniques have been developed. PCR is a powerful method which has greatly facilitated detection of citrus greening agents both in infected plants as well as in psyllid vectors\textsuperscript{17-20}. Here we report the presence of \textit{Ca. L. asiaticus} in different citrus varieties grown in the NE region based on PCR detection using specific primers. We could also detect the greening pathogen from the psyllid vectors caught from these regions.

Surveys were conducted during February 2006 in the NE region of India covering some parts of the Sikkim, North Bengal, Meghalaya and Assam (Figure 1) for recording the incidence and distribution of CGD. Nearly 50 orchards of various citrus cultivars were surveyed. Due to differences in the size and condition of the orchards, flushing density and time available at each locality for inspection of the trees, it was not possible to standardize the sampling methods. However, about 50–60\% of total trees in an orchard was inspected depending on the size of the orchard. The presence of greening symptoms was noted at most of the localities surveyed (Table 1). Different kinds of symptoms were observed, viz. motting (M), severe chlorosis with green veins (SCGV), pale green colour in young leaves (PG), zinc deficiency-like symptoms (Zn), vein yellowing (Yv) and general yellowing (Y). In severe cases leaves become almost chlorotic with scattered green spots (Green Island, GI). Symptoms were often seen on a part of the canopy (sectorial chlorosis, SC). While M-, Y- and Yv-type of symptoms were found in older leaves, the PG- and SCGV-type of symptoms were noticed in relatively younger leaves. Infected trees were sparsely foliated, affected by extensive twig dieback (Figure 2 a–c).

Collection of citrus leaf and budstick samples showing greening-like symptoms was carried out from different locations surveyed. Adult psyllids (Figure 2 d–e) were collected from different orchards with the help of an aspirator for PCR diagnosis of greening bacterium. As citrus psyllids (\textit{D. citri}) develop only on the new flush and as the flushing period varies considerably in different parts of the NE region, the psyllid population could not be observed in some orchards (Table 1). Obviously, the absence of psyllids during the inspection of a particular orchard does not mean that they have never previously been present. The samples were brought back to National Research Centre for Citrus, Nagpur for PCR testing. Total DNA was extracted and purified from 200 mg leaf mid-ribs and bark tissues of symptomatic as well as healthy citrus plants using DNeasy\textsuperscript{TM} Plant Mini Kit (Qiagen)

![Figure 1. The surveyed area.](image)

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Table 1. Incidence of citrus greening disease and its psyllid vector in different parts of NE India

<table>
<thead>
<tr>
<th>State Location, District</th>
<th>Cultivar</th>
<th>Symptoms observed</th>
<th>Per cent infection</th>
<th>Presence (+) or absence (–) of psyllid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sikkim</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nazitam, East Sikkim</td>
<td>Sikkim mandarin</td>
<td>SCGV, Zn, Yv</td>
<td>16.6</td>
<td>+</td>
</tr>
<tr>
<td>Beyong, East Sikkim</td>
<td>Sikkim mandarin</td>
<td>SC, Y, PG</td>
<td>13.3</td>
<td>–</td>
</tr>
<tr>
<td>Lower Sripatum, South Sikkim</td>
<td>Sikkim mandarin</td>
<td>Zn, M</td>
<td>12.5</td>
<td>+</td>
</tr>
<tr>
<td>Namrek Zoom, South Sikkim</td>
<td>Sikkim mandarin</td>
<td>SCGV, Y, M,</td>
<td>30.0</td>
<td>+</td>
</tr>
<tr>
<td>West Bengal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower Dongra, Kalimpong</td>
<td>Darjeeling mandarin</td>
<td>SCGV, GI, Yv</td>
<td>20.0</td>
<td>+</td>
</tr>
<tr>
<td>Ichhey busti, Warlingaan</td>
<td>Darjeeling mandarin</td>
<td>SCGV, M, Y</td>
<td>17.8</td>
<td>+</td>
</tr>
<tr>
<td>Takling busti, Karseeng</td>
<td>Darjeeling mandarin</td>
<td>Zn, Y</td>
<td>10.0</td>
<td>–</td>
</tr>
<tr>
<td>Meghalaya</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Umsning, Ribhoo</td>
<td>Khasi mandarin</td>
<td>Y, Zn</td>
<td>6.6</td>
<td>–</td>
</tr>
<tr>
<td>Umsning, Ribhoo</td>
<td>Sweet orange</td>
<td>Zn, M, GI</td>
<td>12.5</td>
<td>–</td>
</tr>
<tr>
<td>Ponshutia, East Khasi hill</td>
<td>Khasi mandarin</td>
<td>SCGV</td>
<td>5.0</td>
<td>–</td>
</tr>
<tr>
<td>Pynursla, East Khasi hill</td>
<td>Khasi mandarin</td>
<td>Y</td>
<td>8.3</td>
<td>–</td>
</tr>
<tr>
<td>Riahjolong, Dowki, Jaintia hill</td>
<td>Khasi mandarin</td>
<td>SCGV, Y</td>
<td>7.3</td>
<td>+</td>
</tr>
<tr>
<td>Tamubhill, Dowki, Jaintia hill</td>
<td>Khasi mandarin</td>
<td>Zn, Y</td>
<td>8.3</td>
<td>+</td>
</tr>
<tr>
<td>Assam</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kahikuchi, Kamrup District</td>
<td>Assam lemon</td>
<td>M, Yv</td>
<td>43.7</td>
<td>+</td>
</tr>
<tr>
<td>Kahikuchi, Kamrup District</td>
<td>Acid lime</td>
<td>SCGV</td>
<td>8.0</td>
<td>+</td>
</tr>
</tbody>
</table>

Y = yellowing, SCGV = severe chlorosis with green main veins, SC = sectorial chlorosis, Zn = Zn-deficiency like, Yv = vein yellowing, M = Mottling, GZ = green island, PG = pale green young leaves.

Table 2. Primer sequences used for PCR amplification of citrus greening bacterium from greening-infected plants and psyllid vectors

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequences (5’–3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O11</td>
<td>GCCGCGTATGCAATTACGAGCCGGCA</td>
<td></td>
</tr>
<tr>
<td>O12c</td>
<td>GCCCTCGCGAATTCCGAACCCAT</td>
<td>1160</td>
</tr>
<tr>
<td>A2</td>
<td>TATAAGGTTGACCCTTCGAGTTT</td>
<td></td>
</tr>
<tr>
<td>J5</td>
<td>ACCAAAAGCAGAAAATAGCAGAACA</td>
<td>703</td>
</tr>
</tbody>
</table>

Figure 2. Citrus greening disease and its psyllid vector. a. Symptoms of greening disease in Darjeeling mandarin plants at Kalimpong, West Bengal. b. Close-up of SCGV-type leaf symptoms. c. Typical mottling symptom of Assam lemon leaves due to greening disease at Kahikuchi, Assam. d. Citrus psyllids collection in test tubes. e. Close-up of citrus psyllid adult.

According to the manufacturer’s instructions, for detection of greening bacterium in psyllids, a simple protocol was followed to extract DNA for the PCR test. A pool of ten numbers of adult psyllids was placed in a 1.5 ml microcentrifuge tube; 180 μl PBS was added and the sample was homogenized using a disposable microtube pestle. DNA was extracted and subsequently purified using DNeasy™ Tissue Kit (Qiagen) according to the manufacturer’s instructions. The primers used were designed on the basis of the sequence information reported, which is conserved among Asian strains of the greening bacterium. Two sets of greening-specific primers were used for the amplification of 16S rDNA (O11/ O12c) and ribosomal protein genes (A2/J5) (Table 2). Primers were synthesized from Integrated DNA Technologies Inc., Coralville, USA.

PCR was conducted in 25 μl reaction mixtures (10 mM Tris-HCl, pH 8.7; 50 mM KCl, 2 mM MgCl2, 0.2 mM each dNTP; 1.25 units of Taq DNA polymerase and 0.2 μM each primer and 2 μl of extracted DNA). All the PCR reagents were procured from Qiagen, GmbH, Germany. The PCR reaction was carried out in a MasterCy-
observed in citrus orchards at Kamrup District, Assam; Jaintia District, Meghalaya; Warlinggaon and Kalimpong Districts, West Bengal, and South Sikkim District, Sikkim (Table 1).PCR using primers OI1/OI2c and A2/J5 produced an amplified fragment of expected size (1160 and 703 bp respectively), which was observed in plant samples infected with different isolates of Ca. L. asiaticus, collected from different locations. No amplification was obtained from water or DNA extracted from healthy citrus (Figure 3). A PCR product of correct size was also amplified from the psyllids collected on symptomatic citrus trees from different orchards, whereas no fragment was obtained from psyllids collected from healthy citrus orchard. A band of about 703 bp was obtained with the primer pair A2/J5, indicating the presence of Ca. L. asiaticus within the psyllids collected from different places (Figure 4).

Molecular analysis of samples collected during this survey has shown that greening is prevalent in these regions. As the disease was found in all places visited, its geographical distribution must be large and probably includes areas that could not be surveyed. Our study confirms the earlier report of prevalence of CGD in this region on the basis of visual observation of typical symptoms and limited biological indexing using indicator hosts22,23. However, to our knowledge, there has been no report of the presence of greening disease and its psyllid vector in these parts of India derived from PCR-based molecular tests. The PCR technique can be used for early diagnosis of the disease prior to symptom appearance, particularly at seedling stage and in young citrus plants, so as to prevent a widespread outbreak of this disease. By the distribution of greening bacteria across orchards of NE India that we verified in this study and due to the presence of the vector, D. citri, an integrated management programme against CGD needs to be implemented urgently to avoid any further spread of the disease.


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Occurrence of monazite in the auriferous zones of Gadag gold field, Karnataka

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Monazite (Ce, La, Nd)PO4 occurs as an accessory mineral in the auriferous zones of Gadag gold field, Karnataka. It is characterized by uniform LREE (CeLa + Nd) content and Ce/La ratio, and higher concentration of P2O5 (av. 36.06 wt%). Occurrence of monazite in the auriferous zones could be attributed to the mobilization and concentration of LREE during hydrothermal alteration, related with gold mineralization in the area. Near-consistent association of monazite with auriferous zones suggests a possible genetic link between monazite and gold mineralization.

Keywords: Auriferous zones, Gadag gold field, gold mineralization, monazite.

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