Molecular identification and diversity of Asian Citrus Psyllids using mtCoI gene sequences

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ABSTRACT: The current study involves identification of 10 specimens of Asian citrus psylla infesting citrus in different geographical regions of India through DNA barcoding. Psyllids are phloem feeding insects that cause a serious devastation to citrus. Considering the invasiveness of the pest, it is necessary to identify them rapidly and accurately. The classical taxonomy is associated with certain limitations while, COI based identification comes handy as it is applicable on developmental stages. The technique uses \(^1\) region of mitochondrial cytochrome oxidase-I for reliable and accurate species identification. Further, our study revealed that among 794 bp there were 23 variable nucleotides with 7 nucleotides being Parsimony informative, also overall Transition (ti) / Transversion (tv) bias was found to be R=4.95. In addition, the Neighbour-Joining (NJ) tree showed clear clades with an out group Pachypsylla venusta (NCBI accession number:AY278317). Hence our study showed that DNA barcoding will assist in rapid and accurate identification of species which will be a valuable tool in pest management.

Keywords: Asian citrus psylla, DNA barcoding, Huanglongbing, novel COI primer

INTRODUCTION

Diaphorina citri Kuwayama (Hemiptera: Psyllidae), commonly known as Asian Citrus Psyllid (ACP) or Asiatic psylla is a phloem feeding pest on citrus. It is widely distributed in many parts of Asia viz. India, China, Burma, Malaysia, Indonesia, Taiwan, Mauritius, Arabia (Hoy and Nguyen, 1996). In India, as compared to southern parts it is distributed in northern part because of high cultivation of citrus (Randhawa and Srivastava, 1986). Apart from Asian continent, it is observed in several parts of Africa (Bové, 2006). The damage caused by psyllid leads to deformation in plant shoots because of toxic substance present in their saliva. Also, the insect excretes copious amounts of honey dew which forms sooty mould on the leaf surface which hinders the photosynthesis their by leads to eventual death of plant (Chien and Chu, 1996). The ACP is most devastating when accompanied by pathogens causing yellow shoot disease known as huang long bing (HLB) often called as yellow dragon disease in China (Anonymous, 1996). When the vectored pathogen i.e., phloem-inhabiting bacterium, Candidatus liberibacterasiaticus ( Lallem and et al., 1986; Pluke et al., 2008) causes the disease, the affected plant shoots turn to characteristic yellow. In addition, it causes mottling, chlorosis which resembles zinc deficiency (Caoop et al., 1974). Eventually brings down the marketability and economical blow-off to the growers as well as the citrus processing industries. In view of the invasiveness of this pest, an effective, rapid and timely identification has to be carried out so that the epidemic of the pest can be managed. Though morphological identification is useful and has limiting factors like availability of adult specimens for identification, in experienced specialists, unavailability of identification ‘keys’ at all developmental stages or damaged specimens, where not only a trained person but even the specialist may face difficulty in identification. In this regard, the current study adopts molecular species identification based on mitochondrial cytochrome oxidase I (COI), obviate since the technique not only the necessities associated with the morphological identification but expedite in quarantine at the ports of entry (Asokan et al., 2015).
Use of molecular markers envisioned the versatility associated with mitochondrial DNA, in species identification, phylogenetic (Caterino et al., 2000) and population genetic studies (Behura, 2006). Over past three decades, molecular markers are extensively used in species identification which augmented conventional taxonomy. The uniqueness like quick, precise and ease to use properties, maternal inheritance, high copy number and less interspecific variations as compared with other markers allied with mitochondrial gene made them popular in molecular systematic (Savolainen et al., 2005). The concept of DNA barcoding was put forward by Hebert et al., 2003a. The aim of DNA barcoding is to facilitate a proficient and reliable method in species identification across the taxa. The barcoding is carried out from a short segment of DNA sequence from the mitochondrial cytochrome oxidase-1 gene, which is termed as a standard region or barcode region. At present, iBOL (International consortium for barcode of life) upholds the use of universal standard-COI for species level identification since it exhibits reliable interspecific variations (Savolainen et al., 2005). Currently, our study employed COI specific primer for molecular species identification of ten Asian citrus psylla from different regions of India (Table 1).

**MATERIALS AND METHODS**

Samples of Asian citrus psylla, were collected from different geographical locations of India and preserved in ethyl alcohol (70%). Samples were stored in -20°C until DNA was extracted.

**Amplification using novel COI primers**

An amplification of 794bp fragment of COI region was successful using novel primers [generated from EST library generated USDA (Hunter et al., 2008)]. The primer used in the present study was DCITRI COI-L 5' - AGGAGGTGGAGACCCAATCT and DCITRI COI-R 5' - TCAATTGGGGGAGAGTTTTG.

**DNA Isolation, In-vitro Amplification and Sequencing**

Total DNA was isolated from a small incised portion of specimen abdomen. Rest of the specimen was deposited as specimen voucher at IARI, New Delhi in the division of Entomology. Total DNA was extracted using cetyltrimethyl ammonium Bromide method (Rugman-Jones et al., 2006) which involved grinding of tissue in a sterile mortar and pestle with liquid nitrogen. Later, it was homogenized with 300µl of STE buffer [100mMNaCl, 10mM TrisHcl (pH-8.0) and 1m MEDTA (pH-8.0)]. The homogenized tissue was taken in sterile Eppendorf tube and incubated for 1 hour at 65°C with frequent tapping of the tube for every 15-20 min for proper homogenization. After completion of incubation, the homogenate was centrifuged at 8000rpm for 15 min at room temperature followed by ethanol precipitation. The precipitated DNA was dissolved in 20.0 µl of nuclease free water (Eppendorf, Germany). Quantification of the DNA was carried out using Nanodrop (Thermo scientific, Germany) as per the standard operational procedures. Further dilution is carried out, such that the working solution reaches 20-25ng/µl concentration. Glycerol stock (10%) of total DNA was made and preserved at -80°C for future reference purpose. Polymerase Chain Reaction (PCR) was carried out in a thermal cycler (AB-Applied Biosystems, Veriti 96 wells, USA) with following PCR cocktail for 25μl of reaction volume: two micro litres of DNA template, 20 Pico mol of both forward and reverse primers (DCITRI COI-L and DCITRI COI-R respectively), 2.5mM of Taq buffer, 0.25mM of dNTP mix, 2.5 mM of MgCl₂, 0.5U of Taq DNA polymerase (Fermentas Life Sciences) and with temperature conditions set as 94°C for 4 minutes as initial denaturation, followed by 35 cycles of 94°C for 40 seconds, annealing 48°C for 40seconds extension at 72°C for 45 seconds, 72°C for 10 minutes as final extension and 10°C as hold. The amplicons were resolved in 1.0% agarose gel with ethidium bromide (10µg/mL) and documented under UVP (Gel documentation system).

**Molecular Cloning and Sequencing**

The amplicons were eluted using Nucleospin Extract-II kit (Macherey Nagel, Germany) according to manufacturer protocol. Using PTZ57R/T a general purpose cloning vector (Fermentas GMBH, Germany), the PCR product was ligated as per the manufacturer procedures. Transformation is carried out using *Escherichia coli* (DH₅- strain) cells according to standard protocol (Fermentas GMBH, St. Leon-Rot, Germany; Thermo scientific). Further, the cells were incubated overnight at 37°C on LB-Agar comprising ampicillin (100mg/mL) to develop colonies. Blue/White screening was carried out and white colonies harbouring positive transformants were inoculated into LB broth and was incubated overnight in a shaker incubator at 37°C and 220 rpm for proliferation of clones. Plasmids were isolated using Gene JET Plasmid MiniPrep Kit (Fermentas, Germany) as per manufacturer protocol. Sequencing was carried out in an automated sequencer.
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Fig. 1. Consensus sequence of 794 bp from the mitochondrial cytochrome oxidase I (COI) gene for Citrus psylla species collected from different locations in India

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**RESULTS AND DISCUSSION**

The PCR amplified 10 distinct specimens, using COI specific novel primer was successfully sequenced. It revealed a length of 794bp and showed maximum hits for the respective species when BLAST search was carried out. To rule out the possibility associated with the sequencing error, triplicates sequencing for individual psylla specimen was carried out. Also, all the triplicate sequences were devoid of nuclear copies amplification since there were no stop codons within the region and there were no report of indels. The sequences generated in this study were deposited in NCBI-Gen Bank under the following accessions KF702297, KF702298, KF702299, KF702300, KF702301, KF702302, KF702303, KF702304, KF702305 and KF702306.

Pairwise alignment of all the psylla species under study showed 23 variations out of 794bp accounting to 2.89 % difference (Fig. 1). Sequence analysis showed 7 parsimony informative (Pi) sites. A bootstrap test for resolution of clustering patterns in the tree was conducted using MEGA 5.0 with 1,000 replications (Fig. 2) (Tamura et al., 2011). The nucleotide frequencies of all the psylla specimens were 32.39% (A), 36.79% (T/U), 16.94% (C) and 13.88% (G). The overall Transition (ti)/Transversion (tv) bias was found to be (R=) 4.95.

Reliable, accurate and timely identification of notorious insect pests like *D. citri* is vital and challenging worldwide, since they occupy predominantly to all other insects both in diversity as well as in population (Whitcomb *et al.*, 1988). More clarity is required to

![Fig. 2. Phylogenetic tree of citrus psylla based on nucleotide sequences using MEGA 7.0 (NJ method with 1000 bootstrap replicates). Out group used is *Pachypsylla venusta* (NCBI accession number AY78317).](image)
distinguish and to identify these pests which are of quarantine significance (Armstrong and Ball, 2005). In such an occasion, molecular species identification using COI marker has added advantages like, it is independent of life stages, polymorphism and gender (Asokan et al., 2011). Mitochondrial cytochrome oxidase marker is versatile because of its prevalence in cells by having a high copy number in comparison with nuclear genes. Mitochondrial cytochrome oxidase I gene also possess unique properties like maternal inheritance, highly conserved with short intragenic regions and absence of introns (Simon et al., 1994). Apart from routine molecular identifications, COI is also employed in discovery of new species (Foottit, 1997; Sallum et al., 2008, 2010; Bourke et al., 2013; Foster et al., 2013) and to interpret the prevalence of biotypes (Shufran et al., 2000). Recently, Boykin et al., (2012), studied the existence of relationship between different collections of D. citri around the world employing mitochondrial cytochrome oxidase-I.

Moreover, none of the citrus growing country in the world has successfully managed in eradication of HLB (Citrus greening disease) completely. This can be achieved only on successful and timely identification of the invasive pest. In this context, more research is required in priority on a timely and reliable diagnosis irrespective of the life stages and gender. Data procured in current study provides information which can be used in identification, management and quarantine purposes.

### Table 1. Specimens collected from different locations in India and NCBI accessions

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>NCBI Accession</th>
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<tbody>
<tr>
<td>Citrus psylla</td>
<td>Sriganganagar (Rajasthan)</td>
<td>KF702297</td>
</tr>
<tr>
<td>Citrus psylla</td>
<td>Ludhiana (Punjab)</td>
<td>KF702298</td>
</tr>
<tr>
<td>Citrus psylla</td>
<td>Harayana</td>
<td>KF702299</td>
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<td>Uttarkhand</td>
<td>KF702300</td>
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<tr>
<td>Citrus psylla</td>
<td>Kinkhede (Nagpur)</td>
<td>KF702301</td>
</tr>
<tr>
<td>Citrus psylla</td>
<td>Warud (Amravati)</td>
<td>KF702302</td>
</tr>
<tr>
<td>Citrus psylla</td>
<td>NRCC, Nagpur</td>
<td>KF702303</td>
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<td>Delhi</td>
<td>KF702306</td>
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### REFERENCES


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