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Molecular identification of mango hoppers infesting mango trees in Punjab through DNA barcoding

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ABSTRACT

Hoppers belonging to *Amritodus* spp. and *Idioscopus* spp. have been reported infesting mango trees in India. In this study, the mango hoppers were collected from Patiala (Nabha), Jalandhar and Ludhiana districts of Punjab for their molecular identification using DNA barcoding. The mitochondrial cytochrome oxidase I (*mtCOI*) gene from these samples was PCR amplified, cloned in vector and sequenced. The DNA sequences obtained were subjected to analysis through BLASTn programme of NCBI and all three samples were identified as *Amritodus atkinsoni* with 99% similarity of *mtCOI* gene sequence. Sequence alignment of 624 bp *mtCOI* of *Amritodus* populations showed the changes in nucleotides at 4 different positions. The phylogenetic tree was prepared for *mtCOI* gene sequences of different populations (number) of *A. atkinsoni* (1), *A. brevistylus* (2), *Idioscopus niveosparus* (1), *I. nagpurensis* (3) and *I. clypealis* (1) and populations from Punjab. The tree revealed two Clades, i.e. first corresponding to *A. atkinsonii* and *A. brevistylus*, while, Clade 2 consist of three species of *Idioscopus* with three sub-clusters for each *I. clypealis*, *I. niveosparus* and *I. nagpurensis.* Nucleotide pairwise distances ranged from 0.002 to 0.199. The analysis revealed very low genetic variations among the south and north Indian populations of *A. atkinsoni*.

Key words: *Amritodus* sp., DNA barcoding, *Idioscopus* sp., mango hopper.

INTRODUCTION

The mango (*Mangifera indica* L.) belonging to the family Anacardiaceae, is one of the most important fruits of India. Production and quality of mango are mainly hampered by the incidence of about 400 minor and major insects pests like thrips, scale insects, fruit flies, shoot borers, mealy bugs, leaf blisters and mango hoppers (Pena *et al.*, 12) of which leaf hoppers are major pests (Hati *et al.*, 9). Mango hoppers have been reported as the most noxious insects, which colonize the crop both during flushing, flowering and fruiting stages with maximum incidence at flowering stage. Both adults and nymphs suck the sap from the inflorescence and shoots. They secrete honey dew which leads to the growth of sooty mould on the dorsal surfaces of leaves, branches and fruits, which interferes with the photosynthetic activity of the plant resulting in the non-setting of flowers and cause immature fruit drop. So far, 22 species of mango hoppers have been recognized (Dalvi *et al.*, 6). The most common species of mango hoppers in India are *Amritodus atkinsoni*, *Idioscopus niveosparsus* (*I. nitidulus*), *I. nagpurensis*, *I. clypealis*, *A. splendens*, *Busoniominus manjunathi*, *I. anasnyal*, *I. jayshriae* and *A. brevistylus* (Reddy and Dinesh, 13; Thangam *et al*., 14; Asokan *et al*., 2). Most of these species have been recorded from South India. From plain regions of India, *I. nagpurensis* has been recorded. Chakrabarti (5) stated that *A. atkinsonii* is more common in North India. However, no particular recent report on species of mango hoppers prevailing in Punjab is available. Keeping in view the damaging potential of mango hoppers and their complex species, it is important to identify the correct species/ genera of mango hoppers. Although the classical taxonomy holds its significance, however, identification of mango hoppers to the species level is often challenging due to their small size and lack of taxonomists. With the advent of molecular biology and molecular tools, use of DNA sequences of mitochondrial cytochrome oxidase1 gene (*mtCOI*) has been used to identify the insect species. Several studies have shown that 658 bp fragment of *mtCOI* (known as DNA region) has been used to identify and distinguish different animal species including insects, birds, fishes *etc*. Recently, Asokan *et al.* (2) have developed DNA barcodes for five species of mango leaf hoppers collected from India. This study aimed to identify mango hoppers species occurring in Punjab using DNA barcoding and genetic variations from population occurring in south India.

MATERIALS AND METHODS

The adults of mango hopper were collected from three different mango growing locations of Punjab (Table 1). The adults were immediately preserved in

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Table 1. Mango hopper populations collected from different regions of Punjab.

Location	Genebank Acc. No.	GPS coordinates	Barcode Index No.
Ludhiana, Punjab	-	30°89'N / 75°80'E	PBHOR016-15
Jalandhar, Punjab	-	31°32'N / 75°57'E	PBHOR018-15
Nabha, Patiala, Punjab	KP759542	30°37'N, 76°15'E	HEMP025-14

absolute alcohol in glass vials (15 mm dia and 50 mm height) till further use for isolation of genomic DNA. Individual adult from each population was thoroughly washed with sterile double-distilled water. The genomic DNA was extracted using NucleoSpin Tissue XS kit (Macherey-Nagel GmbH & Co.) as per manufacturer's protocol. Two adults from each population were used as replications. The isolated total genomic DNA was stored at -20°C until used. The quality of the isolated DNA was determined using horizontal agarose (0.75% agarose containing ethidium bromide 1 µg/ ml) gel electrophoresis in 1×Tris-acetate-EDTA buffer at 75 V for 1 h. The DNA bands were visualized and recorded using a UV gel documentation system (Ultra Cam). The mitochondrial cytochrome oxidase I (*mtCOI*) gene region from total DNA of mango hopper was amplified with specific primers set (F- attcaaccaatcataaagatattgg and R- taaacttctggatgtccaaaaaatca) (Hajibabaei *et al*., 8). Each PCR reaction mixture consisted of insect DNA- 10 ng, primers (10 µM)- 1.0 µl each, 10× *Taq* reaction buffer- 2.0 µl, *Taq* polymerase-2 U, 5 mM dNTPs mix- 1.0 µl, and distilled water to make- 20 µl. The PCR amplification was accomplished in a programmable DNA thermolcycler (Mastercycler Gradient, Eppendorf) using a PCR programme: 95°C-5 min. (95°C- 1 min., 52°C-1 min., 72°C-2 min.) × 35 cycles, 72°C-10 min. and stored at 4°C. The PCR product was analyzed by horizontal agarose gel electrophoresis by co-running a molecular weight standard (100 bp DNA ladder plus, Fermentas Life Sciences) along with the samples (Fig. 1).

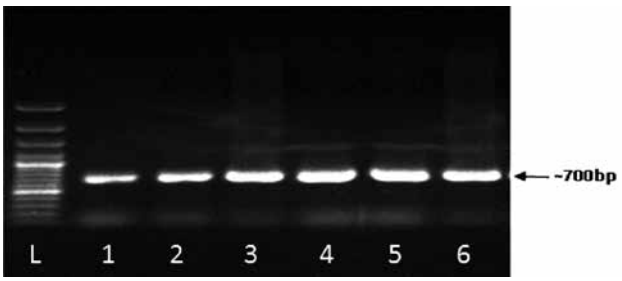

Fig. 1. PCR amplification of *mtCOI* gene from genomic DNA of mango hopper populations collected from different location of Punjab. L = 100 bp DNA ladder, 1-6 represents the mango hopper population from three locations with duplicate samples.

The amplified PCR product was purified from agarose gel block using 'QIAquick Gel Extraction Kit' (Qiagen) as per manufacturer's protocol. The purified DNA fragment was cloned in a sequencing vector pTZ57R/T using 'InsT/A Clone PCR product cloning kit' (Fermentas Life Sciences) and transformed into *Escherichia coli* DH5α host cells as per manufacturer's protocol. The positive clones were confirmed through PCR with specific primers and restriction analysis. The inserted DNA (amplified PCR product) in the respective recombinant clones was custom sequenced for both strands, using custom sequencing services of M/s Xcelris (Ahmedabad, India). The final sequence of all the individual *mtCOI* gene fragments from the mango hopper populations were edited using DNA software Chromaslite 201 and CLC Sequence Viewer 6.5.4 (CLC bio A/S) and submitted to BOLD database and GenBank (Table 1).

All the *mtCOI* sequences representing different populations were aligned using CLC Sequence Viewer 6.5.4 (CLC bio A/S) and the sequence divergence was observed. The comparative analysis of different *mtCOI* sequences from different mango hopper samples was done using MEGA6 programme. The ten *mtCOI* sequences of mango hopper populations of south India, available in GenBank were downloaded from NCBI GenBank database. All the 13 sequences were aligned and processed to homologous region to develop comparative genetic analysis. The sequences phylogenetic tree was created using Neighbor-Joining and BioNJ algorithm with maximum likelihood method with 500 replicates for bootstrap analysis with the MEGA6 programme. The genetic distances were also calculated.

RESULTS AND DISCUSSION

Mitochondrial cytochrome oxidasel is the preferred universal barcode for animal kingdom. Using total genomic DNA from individuals of mango hopper population as template, *mtCOI* specific primers resulted in amplification of a single amplicon of ~700 bp (Fig. 1). PCR and restriction analysis yield the desired fragments on agarose gel, which confirmed the cloning of DNA fragment in vector. The analysis of duplicate sequences for each population revealed no mismatches, hence no sequencing errors. The sequences were submitted

to Barcode of Life Database (www.barcodeoflife.org) and were assigned respective BOLD IDs (Table 1). The sequence obtained were blasted in BLASTn programme of NCBI and all the samples were identified as *Amritodus atkinsoni* with 99% similarity of mtCO1 gene sequence of GenBank Acc. No. HQ268819. The *mtCOI* sequence alignment showed a very low variation between the three sequences.

The *mtCO1* sequence (5' end) of different populations (number) of *A. atkinsoni* (1), *A. brevistylus* (2), *Idioscopus niveosparus* (1), *I. nagpurensis* (3) and *I. clypealis* (1) available in NCBI GenBank database were retrieved. All the 11 sequences including population from Punjab were aligned using CLC sequence viewer programme and processed to 561 bp DNA barcode region. The phylogenetic tree revealed two clades (Fig. 2). The Clade 1 corresponds to *A. atkinsonii* and *A. brevistylus*, while clade 2 had the *Idioscopus nagpurensis*, *I. niveosparsus* and *I. clypealis.* All the three samples from Punjab and one from South India fall in Clade 1. The multiple alignment of 624 bp fragment of *A. atkinsoni* revealed the variations at 19th, 27th, 396th and 544th positions (Fig. 2). It showed a very low level of genetic variations in *A. atkinsoni* population of north and south India. The phylogenetic analysis revealed two clades (Fig. 3). Clade 1 consist of two sub clusters of *A. atkinsoni* and *A. brevistylus.* The clade 2 consist of three separate sub-clusters corresponding to three different species of *Idioscopus.* A pairwise distance matrix was generated using Tamura 3-parameter model (Table 2). Nucleotide pairwise distances ranged from 0.002 to 0.199. The highest nucleotide variations were between *A. atkinsonii* Ludhiana population and *I. clypealis* voucher IIHR-BT-05 HQ268815.1 with genetic distance value of 0.199. Among the genus *Amritodus* and *Idioscopus*, the genetic distance ranged from 0.002 to 0.083 and 0.002 to 0.139, respectively. The overall results showed a very low level of genetic variations among the different populations of south and north India.

The present study confirms the presence of *Amritodus atkinsoni* from three very distinct locations in Punjab using DNA barcoding. Asokan *et al.* (2) has developed DNA barcode for five mango hoppers, *viz*., *Amritodus atkinsoni*, *A. brevistylus*, *Idioscopus niveosparsus*, *I. clypealis* and *I. nagpurensis.* The Punjab population showed maximum similarity with the DNA barcode of *Amritodus atkinsoni*, which led to identification of mango hoppers species. The phylogenetic tree showed that south and north Indian population of mango hoppers are almost similar. These *mtCOI* sequences will act as baseline to monitor development of any biotypes of mango hopper in future. The DNA barcoding is useful for identification of different species of spiders and insects like tussock moth, true bugs, armyworm (*Spodoptera*) *etc.* (Barrett and Hebert, 4; Ball and Armstrong, 3; Park *et al*., 11; Nagoshi *et al*., 10) and invasive species identification (Armstrong and Ball, 1). In addition, it will also help in monitoring the development of biotypes/ cryptic species in insects (Dinsdale *et al*., 7). It will help in the identification and conservation of the evolutionary processes that generate and preserve biodiversity. The present study establishes the prevalence of *Amritodus atkinsoni* in three districts of Punjab. Chakrabarti (5) has stated the existence of different species of mango hoppers in lower Gangetic regions only, and only a few studies were conducted on developing

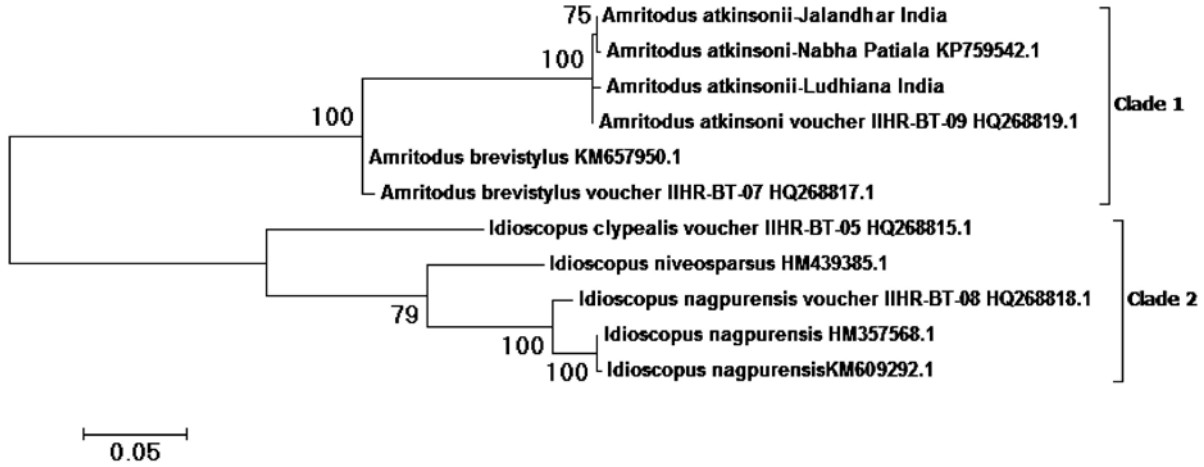

Fig. 2. Phylogenetic analysis of different populations of *Amritodus atkinsoni* of India using Maximum Likelihood method based on the Timura 3-parameter model. All positions containing gaps and missing data were eliminated. There were a total of 621 positions in the final dataset.

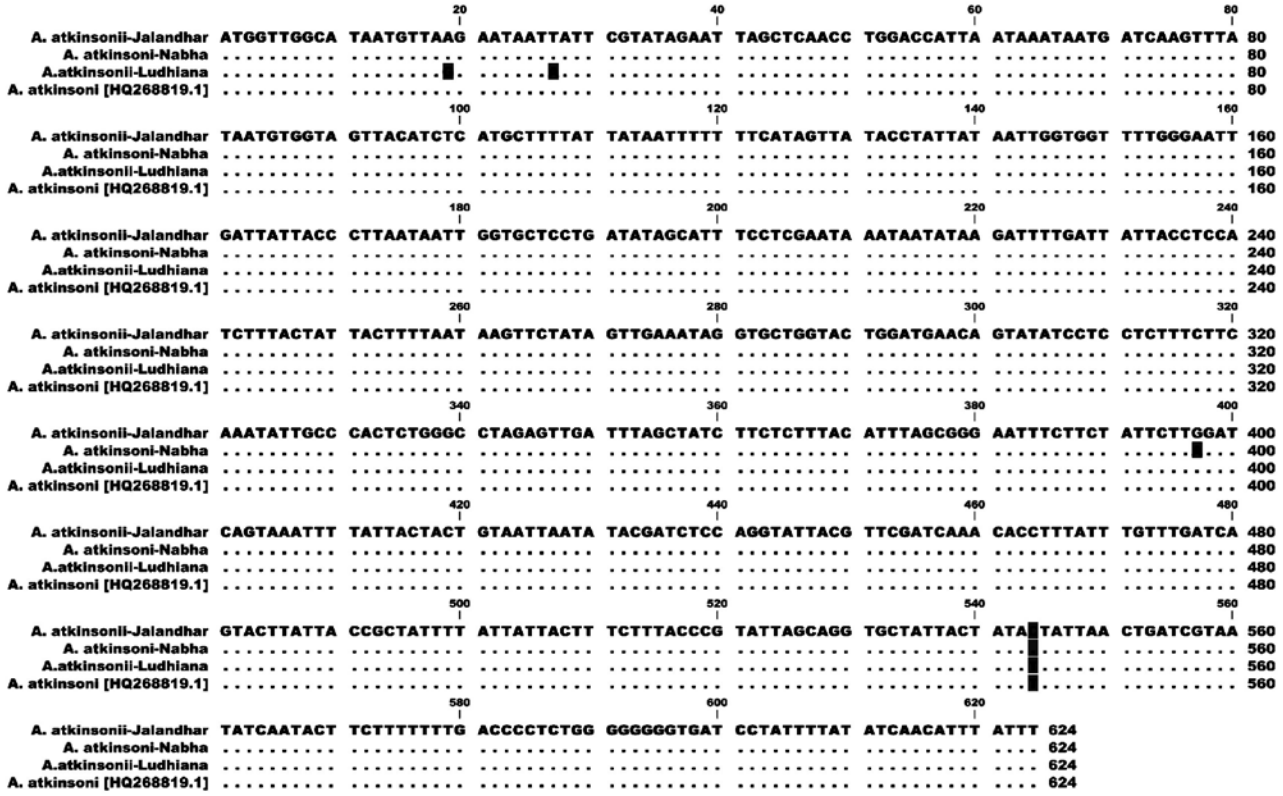

Fig. 3. Multiple alignment of *Amritodus atkinsoni* population of Punjab and South India. The nucleotides indicate changes in sequence are highlighted.

Table 2. Estimates of evolutionary divergence between sequences. Analyses were conducted using the Tamura 3-parameter model. The analysis involved 13 nucleotide sequences. There were a total of 621 positions in the final dataset.

Nucleotide sequence		1	2	3	4	5	6	7	8	9	10	11
A. atkinsoni-Nabha_Patiala [KP759542.1]	1											
A. atkinsoni voucher IIHR-BT-09 [HQ268819.1]	2	0.003										
A. atkinsoni-Jalandhar	3	0.002	0.002									
A. atkinsoni-Ludhiana	4	0.006	0.003	0.005								
A. brevistylus [KM657950.1]	5	0.077	0.074	0.076	0.077							
A. brevistylus voucher IIHR-BT-07 [HQ268817.1]	6	0.083	0.079	0.081	0.083	0.005						
Idioscopus clypealis voucher IIHR-BT-05 [HQ268815.1]	7	0.195	0.195	0.193	0.199	0.168	0.175					
I. nagpurensis [HM357568.1]	8	0.167	0.163	0.165	0.167	0.147	0.149	0.139				
I. nagpurensis voucher IIHR-BT-08 [HQ268818.1]	9	0.159	0.155	0.157	0.157	0.141	0.143	0.135	0.026			
I. nagpurensis [KM609292.1]	10	0.169	0.165	0.167	0.169	0.149	0.151	0.137	0.002	0.028		
I. niveosparsus [HM439385.1]	11	0.165	0.161	0.163	0.165	0.151	0.157	0.132	0.091	0.089	0.093	

DNA barcodes. Therefore, more number of samples from different regions of India needs to be collected and characterized using DNA barcode to catalogue diversity of mango hopper in India.

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