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# MOLECULAR CHARACTERIZATION OF POLLINATORS IN COTTON ECOSYSTEM

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**ABSTRACT**

**DNA barcoding using cytochrome *c* oxidase I (mtCO1) for molecular characterization is a taxonomic method that uses a short genetic marker in an insect DNA to identify a species, including an unknown species. The coming-to-light of this identification tool is timely when we are facing perhaps the greatest rate of species loss in recent millennia. This study contributes to increasing the number of published accounts of DNA barcoding and accurately distinguishing the pollinator fauna of cotton in Punjab, India. The mtCOI region of all the samples was amplified, cloned and the nucleotide sequences were determined and analyzed. This study reveals that specimens collected in cotton belong to order Hymenoptera and Diptera. Hymenopterans include *Apis dorsata*, *Apis florea*, *Xylocopa fenestrata*, *Sceliphron madraspatanum and Polistes wattii*. Dipterns include *Eristalinus quinquelineatus* and *Musca convexifrons.***

**Key words:** Cotton, pollinator fauna, *Apis dorsata*, *Apis florea*, *Xylocopa fenestrata*, *Sceliphron madraspatanum*, *Polistes wattii*, *Eristalinus quinquelineatus*, *Musca convexifrons*, DNA barcode, mtCO1, phylogeny

Pollination is a prime case of a supporting service that is being adversely influenced by modern farming practices, as well as by other factors such as pollution, global warming, urbanization, and industrialization (Klein et al., 2007). Pollination is essential to most plants including commercial crops for reproduction. This ecosystem function is provided by many animals. Without this service, many plant species would be driven to extinction, and cultivation of many modern crops would be impossible (FAO, 2009; Abrol, 2011). Many crops such as cucurbits are wholly dependent on crosspollination by pollinator insects while many other crops show significant yield increases when cross-pollinated (Ollerton et al., 2011; Marini et al., 2015). It has been estimated that pollination is responsible for as much as 75% of agricultural food production (Klein et al., 2007).

Cotton, is an important cash crop in India as well as in the Punjab state (Anonymous, 2019). It is also known as 'white gold' or 'queen of fibre'. Cotton flower is large, axillary, terminal and solitary and the plant have sympodial development of fruiting branches. The inner most bud of the lowest and oldest branch is the first to open while the outermost bud of the highest and youngest branch is the last one to do so. When the flower opens, it is white or creamy white in colour, changing to pink towards the end of the day and becoming red the following morning. On the second day, the petals wither and fall. Though self-pollination is the general rule, cross pollination also occurs in cotton ranging from 5 to 50% or more (Stephen and Finker, 1953; Muhammad et al., 2020). Pollen grains of cotton are relatively heavy and sticky in nature; therefore, anemophily does not play any significant role. So, array of pollinators plays a vital role in the pollination of cotton (Free, 1970; Rhodes, 2002).

Genetic-based systems, primarily DNA barcoding using cytochrome c oxidase I (cox1) as per (Hebert et al., 2003a; b) is now advocated as an accurate approach for identification of the world's biota (Waugh, 2007). To facilitate DNA barcoding at the larger scale, the Barcode of Life Data System (BOLD) has been developed (Ratnasingham and Hebert, 2007). Standardization of a universal and sequence able locus present in most of the taxa of interest for DNA barcoding that can be amplified with universal PCR primers, is the best method to assess a large variation among species and a relatively small amount of variation within the species (Hebert et al., 2003a). This has become a solution for accurate identification of pollinators in agriculture (FAO, 2009). The present study was, thus, aimed to identify pollinators of cotton in the state of Punjab (India) using DNA barcoding.

## MATERIALS AND METHODS

The insects examined were collected from various cultivars of cotton (*Gossypium arboreum* L.; transgenic and non-transgenic *Gossypium hirsutum* L.) at the Regional Research Station of the Punjab Agricultural University, at Faridkot, Punjab, India (30° 40' 41.4696"

N, 74° 44' 22.3980'' E, 196 masl) during 2018. The pollinators were collected at three-hour intervals starting from 0600 to 1800 hr by net sweeping, at three randomly selected places once every week on each cotton cultivar throughout the flowering period. For DNA barcoding, the specimens were collected and preserved in 95% ethanol and kept at -20°C till further use. Genomic DNA was extracted from thorax and legs tissue by standardized CTAB method. A nearly 700 base pair (bp) fragment of the mitochondrial cytochrome oxidase subunit I (mtCOI) barcode region was amplified with the universal primers COI-LepF (5′-ATTCAACCAATCATAAAGATATTGG-3′) and COI-LepR (5′-TAAACTTCTGGATGTCC AAAAAATCA-3′). All PCR amplifications were carried out in a programmable DNA thermocycler (Eppendorf ™). The optimized PCR conditions (per 50 µL) using Taq DNA polymerase (Promega Corporation, USA) were 5 µL of 10 X PCR buffer with 4.5 µL of 25 mM $MgCl_2$, 10.0 µL of 1mM dNTPs, 2.0 µL (10 µM) each of forward and reverse primer, 2.5 U of Taq DNA polymerase, 17 µL of ultra pure water (Invitrogen). Thermocycler conditions were as follows: initial denaturation for 5 min at 94°C followed by 35 cycles of denaturing for 1 min at 94°C, annealing for 1.0 min at 52°C and extension time of 2.0 min at 72°C, with a final extension for 5 min at 72 °C. PCR products were visualized on 0.75% agarose gel after electrophoresis. The size of the amplified bands was confirmed by co-running a molecular weight standard (100 bp DNA ladder plus, Fermentas, LifeSciences) along with the samples in the gel.

Single bands were purified using a NucleoSpin gel and PCR clean-up (TaKaRa Bio). The purified DNA fragments were ligated into a 'PCR product cloning plasmid vector pGEM®-T easy vector'. The ligation reaction product was transformed into JM 109 competent host cells using 'pGEM®-T Easy Vector System II by Promega Corporation' following manufacturer's protocol. 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 representing the different pollinators were edited using DNA software Chromaslite 201 and CLC Sequence Viewer 7.8.1 (CLC bio A/S). Accession numbers were generated by submitting the sequences to the GenBank database. The sequences were also submitted to BOLD database which is a freely available online workbench for collection and management of DNA barcodes from all over the world enriching barcode reference libraries installed at University of Guelph, Canada.The sequences obtained were blasted in BLASTn programme of NCBI and all the samples were identified based on maximum homology. The mtCOI sequences of ~658bp of hymenopteran and dipteran pollinators available in NCBI database were downloaded to find out the genetic differences and analyze the similarities between the samples (Saitou and Nei, 1987; Kumar et al., 2016). Tree robustness was evaluated by bootstrapping with 1000 replicates. The sequences were aligned, and phylogenetic tree was developed using Neighbor-Joining (NJ) statistical method based on Tamura3 model with the MEGA7 programme. Data deposition is made in the GenBank repository found at https://www. ncbi.nlm.nih.gov/ and BOLD repository found at https://www.boldsystems.org/. Other data is available from the authors for noncommercial purposes on reasonable request.

## RESULTS AND DISCUSSION

DNA sequences from a 658 bp mtCOI region were analyzed from various pollinators of cotton in Punjab. PCR amplification with mtCOI primers to amplify the 5' end of COI gene resulted in approx. 700bp product. The PCR product was purified using gel extraction kit (TaKaRa Bio) and cloned in sequencing vector pGEM®-T Easy Vector. The nucleotide sequences of cloned mtCO1 gene were determined and the sequences obtained were blasted in BLASTn programme of NCBI. All the pollinator samples collected from cotton grown in Faridkot (Punjab) were identified to be *Apis dorsata*, *Apis florea*, *Xylocopa fenestrata*, *Sceliphron madraspatanum*, *Polistes wattii*, *Eristalinus quinquelineatus* and *Musca convexifrons,* based on the maximum homology (99-100%) (Table 1). The CO1 region in almost all the samples was in the range of 653-658 bp. Out of seven, five belonged to Hymenoptera representing the major families Apidae (3), Sphecidae (1) and Vespidae (1) and two to order Diptera - one each from Syrphidae and Muscidae families. The sequences were submitted to NCBI-GenBank and Barcode of Life Database (www.barcodeoflife.org) and were assigned respective BOLD IDs.

Sequence data of COI barcode region of the pollinators collected from cotton cultivars were aligned and phylogenetic relation was determined using neighbour joining statistical method to differentiate the result of alignment of nucleotide sequence of pollinators with same homologous sequences from different countries which were selected from GenBank

Table 1. Samples collected and details

| Sample No. | Order | Family | Identification | GenBank accession No. | BOLD ID | Query coverage (%) | Identity (%) |
|---|---|---|---|---|---|---|---|
| 1 | Hymenoptera | Apidae | *Apis dorsata* | MN163112 | AAA2328 | 99 | 93.98 |
| 2 | | Apidae | *Apis florea* | MN163113 | ADL8684 | 100 | 99.85 |
| 3 | | Apidae | *Xylocopa fenestrata* | MN163114 | AAE4670 | 100 | 100 |
| 4 | | Sphecidae | *Sceliphron madraspatanum* | MN148443 | AAP9891 | 100 | 98.94 |
| 5 | | Vespidae | *Polistes wattii* | MN163110 | AAE1384 | 100 | 99.85 |
| 6 | Diptera | Syrphidae | *Eristalinus quinquelineatus* | MN163109 | AAF3600 | 100 | 92.56 |
| 7 | | Muscidae | *Musca convexifrons* | MN163115 | AAX3117 | 100 | 100 |

as references using MEGA 7 software (Fig. 1). Phylogenetic analysis of Hymenoptera formed two distinct clades which included family Apidae in one group and Sphecidae and Vespidae in another. The Phylogenetic tree had a total branch length of 0.65 base substitutions per site. Also, in Diptera, two distinct groups were formed during Phylogenetic analysis which included the families Syrphidae and Muscidae; the phylogenetic tree has a total branch length of 0.18 base substitutions per site (Fig. 2).

Wind is an important source of pollination process for 18% of species among the Angiosperms (Culley et al., 2002). Free (1970) noted that pollen grains of cotton are relatively heavy and sticky in nature; therefore, winds cannot carry pollen moving away. So, array of pollinators plays a vital role in the pollination of cotton. Being polytrophic in nature and more flower-constant, bees effectively pollinate many crops (Calderone, 2012; Hung et al., 2018). Tanda and Goyal (1979) from Indian Punjab reported that the most important insect pollinators on *Gossypium arboreum* were apoids among which the most abundant were *Apis mellifera* L., *Apis cerana* Fabricius, *Apis dorsata* Fabricius and *Apis florea* Fabricius in decreasing order of their intensity. They also opined that intensive bee pollination increased the yield of this species of cotton by 14.2 to 15.8%. There was also a great improvement in the quality of the cotton.

Khan et al. (2016) reported from Pakistan that cotton crop was rich in hymenopteran pollinators with genera including *Apis, Xylocopa*, *Sceliphron*, *Polistes*, *Vespa, Campsomeriella, Andrena, Megachila, Halictus* and *Nomia.* El-Sarrag et al. (1993) reported that in Sudan, among the different Hymenopteran pollinators visiting on cotton, the most dominant species were *A. mellifera* and *Bombus* sp. Nachappa (2004) reported that in Dharwad (Karnataka state, India), Bt cotton flowers were visited by *A. mellifera*, *A. cerana* and *A. dorsata* and these collectively constituted about 75% of total pollinators' population on the crop. Naik et al. (2014) recorded that eight species of pollinators were foraging on BG-I and BG-II cotton in Karnataka, and seven of them belonged to Hymenoptera and one to Diptera.

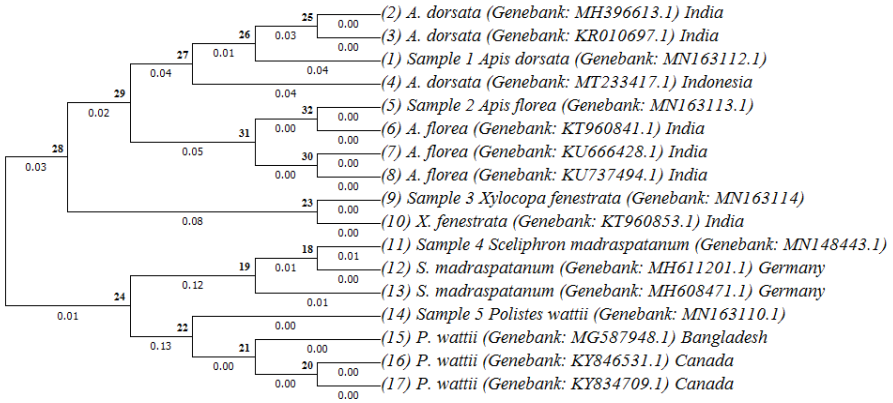


Fig. 1. Phylogenetic sequence based on mt COI gene for Hymenopteran pollinators in cotton. The tree was constructed using the Neighbor-Joining method and evolutionary distances were computed using the Tamura 3-parameter model. There were a total of 574 positions in the final dataset. Numbers at branch points indicate 1000 bootstrap value

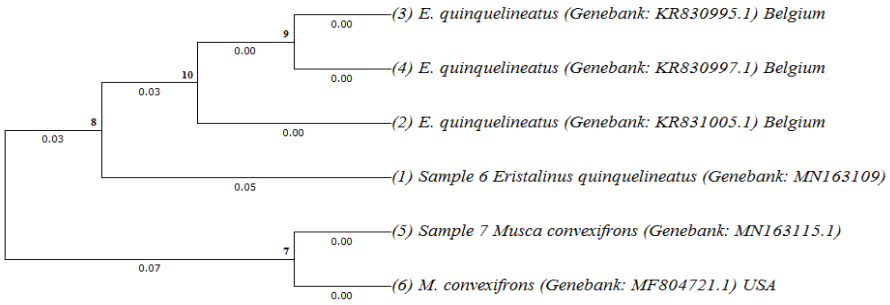


Fig. 2. Phylogenetic sequence based on mt COI gene for Dipteran pollinators in cotton. The tree was constructed using the Neighbor-Joining method and evolutionary distances were computed using the Tamura 3-parameter model. There were a total of 634 positions in the final dataset. Numbers at branch points indicate 1000 bootstrap value

Among honey bees, *A. cerana* was the most dominant followed by *A. florea* and *A. dorsata*. Non-Apis bees were *Xylocopa* sp., *Pithitis* sp., *Sceliphron* sp., *Polistes* sp., and dipteran pollinator was *Eristalins obliqus* L. These findings are also supported by the earlier findings of Naik et al. (2011).

Pise and Viraktamath (2015) reported that 18 species of pollinators were found foraging on Bt and non-Bt cotton blossoms in Karnataka; of these, nine belonged to the order Hymenoptera, eight to Lepidoptera and one to Diptera. Among Hymenoptera, *A. dorsata* was the most dominant followed by *A. cerana* and *A. florea*. Other hymenopteran pollinators were *Xylocopa* sp., *Ceratina* sp., *Thyreus* sp., *Megachile* sp. and *Vespa* sp.; *E. obliqus* was dipteran pollinator. Sinduja et al. (2016) reported from Tamil Nadu (India) that dominant group of pollinators in cotton was Hymenoptera which included *A. cerana*, *A. dorsata*, *A. florea*, *Tetragonula iridipennis* Smith, *Megachile* sp. and *Xylocopa* sp. According to Viraktamath and Nachappa (2004), insect pollinators *viz., A. mellifera*, *A. cerana*, *A. dorsata*, *Xylocopa* sp., *Megachile* sp., *Hemaris* sp., *Telicota* sp. and *Catopsile pyrantha* L. revealed higher abundance in Bt cotton compared to the non-Bt cotton.

Previous works on using mtCOI sequences to identify the pollinators in cotton are scanty. Present study confirms that DNA barcoding based on COI sequences can be applied for taxonomic identification of pollinating insects. Sometimes, the morphological markers used in insect identification depict similarities making the precise identification either difficult or ambiguous. Molecular marker helps to facilitate the identification in shorter period with even scanty knowledge of taxonomy and provides the higher degree of the precision. However, the molecular taxonomy is helpful where the species has already been identified and described and sequence of the gene for the species with authenticity is known and available in public domain. For the new species or where sequence of the species is not already available, the identity can be established only with classical tools. DNA barcode can facilitate integrative approaches in species identification. Mitochondrial cytochrome oxidase subunit I (COI) has been used extensively by molecular biologists around the world to identify insect species (Jalali et al.,2015). COI barcoding sequences can be used to identify insect species at all stages of development (Armstrong and Ball., 2005; Ball and Armstrong., 2006). Another added advantage is that DNA barcoding has been applied to identify the Hymenoptera (Makkar et al., 2016; 2018; Sheffield et al. 2017; Taye et al. 2018) Diptera (Rotty et al., 2018) and Orthoptera (Trewick, 2008).

In the present study, COI sequence analysis showed that there was a variety of pollinating insects in cotton. Insect pollinators of cotton plants have been identified using mtCOI DNA markers, viz, *Apis dorsata*, *Apis florea*, *Xylocopa fenestrata*, *Sceliphron madraspatanum*, *Polistes wattii*, *Eristalinus quinquelineatus* and *Musca convexifrons*. The present data indicate that COI barcoding provides a useful identification tool for pollinators. Studying more populations of pollinators from cotton blooms grown in different agro-climatic zones of India can facilitate cataloguing the genetic variations existing in different geographical regions. The precise identification of pollinating insects is the key in formulating strategies for their conservation and augmentation programmes in cotton towards yield enhancement.

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## AUTHORS CONTRIBUTION STATEMENT

Conceptualization of research work and designing of experiments (PKC, JS); Execution of field/lab experiments and data collection (KB, BM); Analysis of data and interpretation (KB, BM); Preparations of manuscript (KB, BM, PKC, JS).

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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