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# Exploring the genetic variability of citrus butterfly (*Papilio demoleus*) using DNA barcode

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ABSTRACT

The present study is first and initial report from India on developing barcodes and molecular identification of citrus swallow tail butterfly (*Papilio demoleus* L.) based on mtCOI region and species composition from Abohar, Jalandhar, Ludhiana, Sangrur areas of Punjab on citrus host plants. The corresponding genomic DNA isolation, PCR amplification, detectable genetic divergence, molecular identification and phylogenetic tree were assessed. Using the specific set of primers the samples yielded specific fragment of 658 bp. The amplified PCR product was sequenced and identified as *Papilio demoleus* and submitted to BOLD database. The 658 bp mtCOI gene sequences from Abohar, Ludhiana, Jalandhar were 100% similar however the sequence from Sangrur region show mutations at three different positions showing a variation of 0.45% from rest of the populations of Punjab. The phylogenetic tree was developed and it was revealed that all the populations of Punjab are in same cluster and when compared with populations from other countries, it form two main clusters which are different from each other by 3.70%. The cluster one includes all the populations of Australia, India, USA and Pakistan and the cluster two include totally different populations from Canada. In cluster one the populations from Australia and USA forms one subgroup while populations from India and Pakistan form second subgroup.

**Key words:** Citrus butterfly, DNA barcode, Genetic variations, mtCOI region, *Papilio demoleus*

Citrus is an important agricultural commodity which is cultivated in the tropical and subtropical regions. Citrus industry is the third largest fruit industry in the world occupying six per cent of the total area under various fruits. In India citrus crop occupies a prominent place covering an area of about 9.76 million ha with an annual production of 11.71 MT (NHB-2017) with an average yield of citrus fruits in India is alarmingly low (8.8 t/ha) compared to other developed countries (india.gov.in).

Pest problem is one of the major constraints in the production of any crop. Citrus in India are attacked by more than 250 insect-pests including all the stages of growth (Bhutani 1979). Out of these, 165 species are important in India causing an estimated loss of 30% in yields (Pruthi and Mani 1945). Among the different pest species infesting citrus, *Papilio* is considered as one of the most prevalent and destructive pest in terms of its foliage damaging ability. *Papilio* species initially have originated in Madagascar and are native to Asia, ranging from Iran in the west and southern Japan and Australia in the east and are extremely prevalent in India due to its easy adaptability to various climatic conditions. Among the various *Papilio* species that attack citrus, the *Papilio demoleus* L has a successful dispersal and has become a major pest of citrus plants throughout Asia. *Papilio demoleus* (Papilionidae: Lepidoptera) is a regular and serious pest on all citrus species in India and ubiquitously found in the plains, on the hills of peninsular India and up to 7000 feet in the Himalayas (Sarada *et al*. 2014). *Papilio demoleus* is known by different names such as citrus butterfly, lime swallowtail, citrus swallowtail or chequered swallowtail butterfly. It is a highly destructive pest whose larval forms feed on tender leaves and also cause serious damage to citrus plant in nurseries, young seedlings, and new flush of full grown up. Six sub species of *P. demoleus* have been recognized (Lewis *et al*. 2015), viz. *P. d. demoleus* Linnaeus, 1758 - Across Asia from China to the Arabian Peninsula; *P. d. libanius* Fruhstorfer, 1908 - Taiwan, Philippines, Sula, Talaud; *P. d. malayanus* Wallace, 1865 - Sumatra and the Malaysian peninsula; *P. d. novoguineensis* Rothschild, 1908 - Papua New Guinea, *P. d. sthenelus* Macleay, 1826 – Australia; *P. d. stenelinus* Rothschild, 1895 - Sumba, Flores and Alor. Morphological characters of the adult butterfly are unable to properly define species and subspecies of *Papilio* and it was reviewed that larval morphology sometimes provides the informative characters for distinguishing species in the genus. In case of high invasiveness of *Papilio* in citrus, rapid and effective

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identification needs to be carried out for further management practices. Now a days, identification of insects collected from the field using morphological traits has limiting factors like availability of adult specimens, lack of taxonomic keys at all stages of insect life cycle, lack of experienced taxonomists, damaged specimens cannot be identified morphologically and above all it is time consuming. Identification of immature or larval specimens is even more challenging.

In the context of reduced taxonomic expertise in morphology, the availability of molecular data makes this issue alternatively accessible (Brown *et al*. 1999, Tautz 2002, 2003). In this regard, the current study adopts molecular identification based on mitochondrial cytochrome oxidase I (COI) gene fragment, which is the most conservative protein-coding genes in the mitochondrial genome of animals (Burns 2008). Studies have been conducted on historical distribution, morphometrics, biology, morphological identification of citrus butterfly (Atwal 1964, Ahad 2004, Haldhar *et al.* 2010, Sarada 2014, Ramakrishna Rao 2015, Patel *et al*. 2017), however, only one report on genetic categorization of six North-Indian species of butterfly from Chandigarh belonging to Pierinae sub family, using the partial 16S rRNA sequence is available. Moreover, no information of mtCOI sequence of *Papilio* population from India is available even though they are so diverse in nature. Keeping in view the severity of the problem and importance of molecular techniques in identification of insects at species level, this study reports use of mitochondrial cytochrome oxidase I (COI) sequence based analysis of *Papilio* population from India for their identification up to species level and genetic variations among the from population from other countries. The DNA barcode data will be a reference for future molecular identification of this pest.

## MATERIALS AND METHODS

### *Insect collection*

The populations of citrus butterfly larvae were collected from citrus growing areas of Punjab, viz. Abohar, Jalandhar, Ludhiana and Sangrur regions. Two or more larvae were immediately preserved in absolute alcohol in glass vials till further used for isolation of genomic DNA.

### *PCR amplification and cloning of mtCOI gene*

Single individual larvae from each population samples were picked with clean forceps, washed with 70% ethanol and then kept in a tube containing sterile water. Two larvae from each population were used as two replications. The genomic DNA was extracted using CTAB method with little modifications. Single larva was taken in a 1.5 ml microcentrifuge tube and suspended in 200 µl CTAB extraction buffer followed by 10 µl proteinase K (20 ug/ml) and homogenized with a micro pestle. The samples were incubated for 2 h at 65°C until completely dissolved. Following the incubation period, centrifuged the homogenate for 5 min at 10000 rpm. The supernatant was transferred to a new eppendorf tube. Equal volume of chloroform/isoamyl alcohol (CIA) was added and mixed by vortexing for 10 seconds then centrifuged the sample for 5 min at 15000 to separate the phases. The upper aqueous layer was transferred into a new 1.5 eppendorf tube, and equal volume of chilled isopropanol was added and kept at −20°C for 20 min for precipitation. After 20 min, the sample was centrifuged at 10000 rpm (4°C) for 10 min. The supernatant was decanted off and the pellet was washed with 500µl of 70% ethanol twice. The pellet was air-dried and eluted in 50 µl TE (Tris EDTA, 100 mM) buffer containing RNAseA (10 µg $ml^{-1}$) and stored at −20 °C. The quality of the isolated DNA was determined using horizontal 0.75 per cent agarose gel electrophoresis. The DNA bands were checked on UV Gel Documentation system (Alfa Innotech). The mitochondrial cytochrome oxidase I (mtCOI) gene region from total DNA of citrus butterfly larvae with specific primers set (F- ATTCAACCAATCATAAAGATATTGG and R- TAAACTTCTGGATGTCCAAAAAATCA) as described by Hajibabaei *et al.* (2006). Each PCR reaction mixture (25 µl total reaction volumes)consisted of insect DNA- 10 ng, primers (10 µM)- 1.0 µl each, 10X reaction buffer- 2.0 µl, *Taq* polymerase 2 U, 5 mM dNTPs mix- 1.0 µl, and distilled water to make- 25 µl. The PCR amplification was accomplished at an annealing temperature of 52°C in a programmable DNA thermocycler (Eppendorf) using the following thermal profile: initial denaturation for 5 min at 94°C, followed by 34 cycles of 0.5 min at 94°C, 0.5 min at 52°C, 1 min at 72°C with a subsequent final extension at 72°C for10 min, and stored at 4°C. The PCR products (10 µl) were checked on horizontal agarose gel electrophoresis on 1 per cent agarose gel by co-running a molecular weight standard (100 bp DNA ladder plus, Fermentas, Life Sciences).

The amplified PCR product was purified from agarose gel using 'Gel and PCR clean-up' kit (Macherey-Nagel Ltd). The purified DNA fragment was cloned vector pGEM-T easy vector (Promega) and transformed into *Escherichia coli* DH5α host cells as per manufacturer's protocol. The white clones were selected from LB plates and tested to confirm the cloning by plasmid isolation and restriction with EcoR1 and Pst1. The inserted DNA in the respective recombinant clones was custom sequenced for both strands, using custom sequencing services of M/S Xcelris (Ahmedabad, India). The final sequences mtCOI gene fragments from the citrus butterfly populations were edited using CLC Sequence Viewer 6.5.4 (CLC bio A/S) and 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 for future molecular identification of this group (Table 1).

### *Phylogenetic analysis*

The sequences obtained were subjected to blast analysis in BLASTn programme of NCBI and all the samples were identified based on maximum homology. The mtCOI sequences of 658 bp of citrus butterfly populations available in NCBI database of Australia, China, Canada, Pakistan,

Table 1 Sample details of citrus butterfly collected from Punjab and blast analysis

| Location | Host | Sample ID | BOLD ID | Query coverage (%) | Identity (%) | Identification |
|---|---|---|---|---|---|---|
| Abohar | Citrus | Pd Ab-1 | LEPIN025-13 | 100 | 100 | *Papilio demolues* |
| Ludhiana | Citrus | Pd L1 | LEPIN030-13 | 100 | 100 | *P. demolues* |
| Hoshiarpur | Citrus | Pd Ho1-83 | LEPIN043-13 | 100 | 100 | *P. demolues* |
| Ludhiana | Citrus | Pd L1-133 | LEPIN059-14 | 100 | 100 | *P. demolues* |
| Sangrur | Citrus | Pd Sr-305 | PBCER001-15 | 100 | 99 | *P. demolues* |

USA were downloaded and compared with populatios from Punjab to find out the genetic differences. The sequences were aligned with ClustwalW programme and phylogenetic tree was developed using neighbor joining method based on Tamura3 model (Tamura 1992) with the MEGA7 program (Kumar *et al.* 2016).

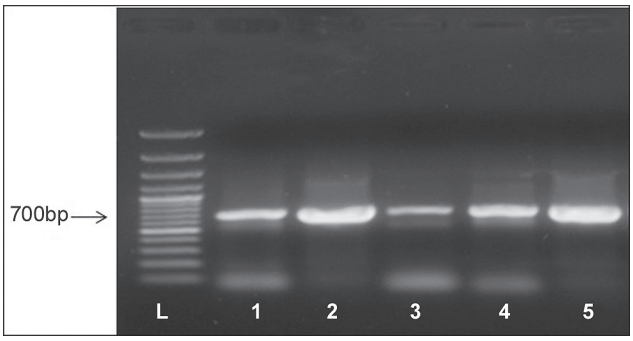


Fig 1 PCR amplification of mtCOI gene from different population of *Papilio demolues* collected from Punjab. L: 100bp ladder, 1-5 different populations of *P. demoleus.*

## RESULTS AND DISCUSSION

PCR amplification of five samples from different regions of Punjab with mtCOI primers yielded specific fragment of 658 bp as observed on 1% agarose gel (Fig 1). The amplified PCR product was purified and was cloned in pGEMT easy vector and was successfully transformed into bacterial *Escherichia coli* DH5α host cells. All the samples from Punjab were identified as *Papilio demoleus* showing maximum homology of nucleotide sequences of mtCOI (Table 1). Sequences were edited for any misread and removal of plasmid sequences using CLC sequencing workbench and submitted to Barcode of Life Database (BOLD) database and BOLD ID were assigned as given in Table 1.


Fig 2 Multiple alignment of *Papilio demoleus* mitochondrial cytochrome oxidase 1 sequences collected from different regions of Punjab.

All five sequences of mtCOI of *P. demoleus* collected from Punjab were aligned to each other on CLC workbench. Multiple sequence analysis helped to analyze the sequence homology and phylogenetic analysis can be conducted to assess the sequences shared evolutionary origins (Phillip *et al.* 2000). The multiple sequence alignment shows only few variations among the sequences (Fig 2). It was observed that the 658 bp sequences from Abohar, Ludhiana, Jalandhar were 100% similar, however, the sequence from Sangrur region show mutation of 'G instead of A' at 433rd position and 'C instead of T' at two positions 238 and 574 (Fig 2). Hebert *et al.* (2003) reported mtCOI region appeared to possess enough sequence divergence to basically show differences between closely related species. The mtCOI nucleotide sequence variability amongst different citrus butterfly *P. demoleus* populations was quite less which indicated that all the local populations of in the present study were almost genetically similar and belonged to same species. DNA barcoding has been successfully used to identify and assess the genetic diversity of many insects, viz. *Bemisia tabaci, Mythimna separata, Helicoverpa armigera, Drosicha mangiferae, Leucinodes orbonalis* (Ashfaq *et al.* 2011, Jindal *et al.* 2015, Jindal *et al*. 2016, Shashank *et al*. 2015, Banta *et al*. 2016). The low level of genetic variations has been reported from population collected from same geographical regions for *Amritodus atkinsonii, Mythimna separata, Drosicha mangiferae* (Jindal *et al*. 2016, Jindal *et al*. 2015, Banta *et al*. 2016). On the other hand, at least 43 cryptic species of *Bemisia tabaci* has been reported through analysis of sequences of mtCOI gene (DeBarro and Ahmed 2011, Tay *et al.* 2017)

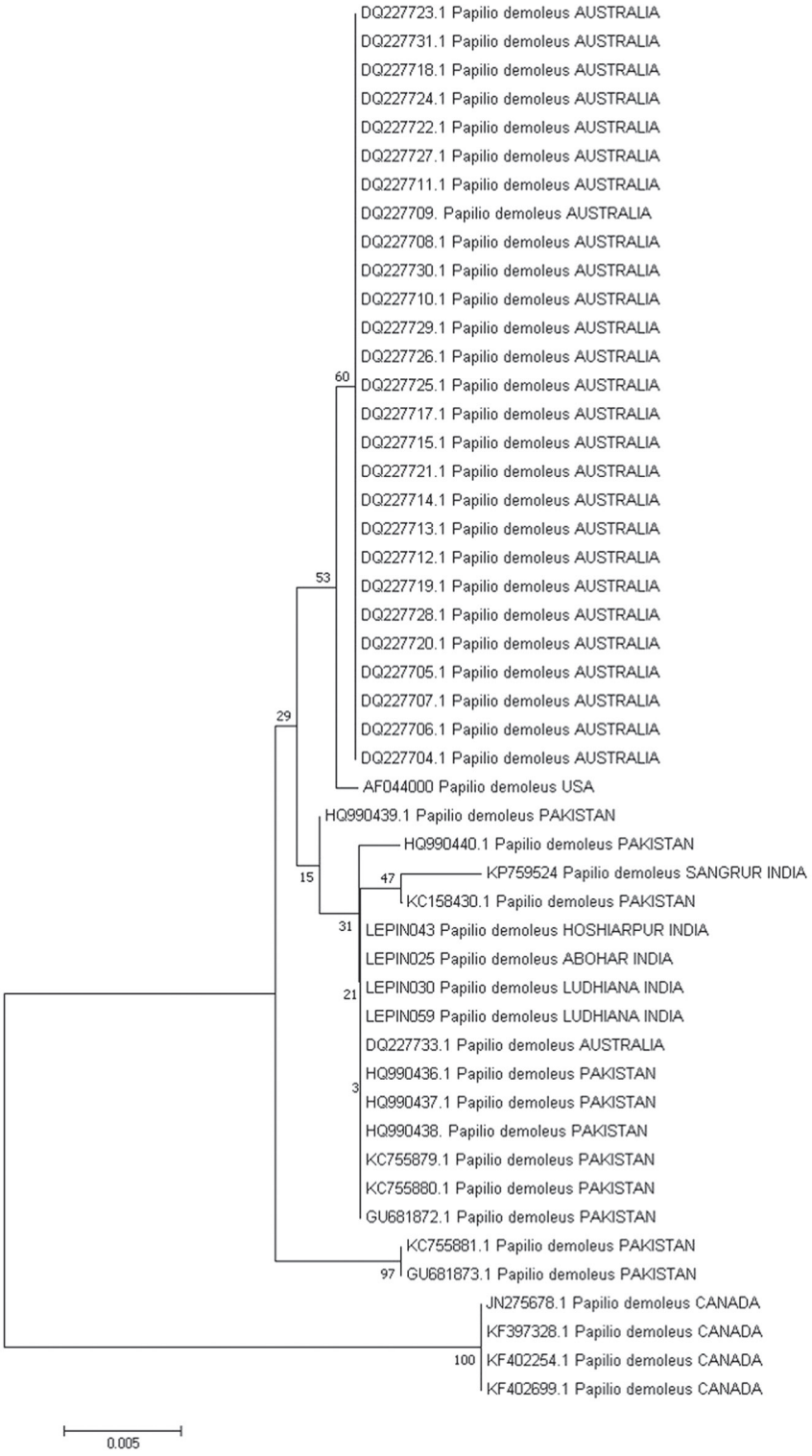


Fig 3 Phylogenetic analysis of *Papilio demoleus* populations from different regions of world. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Tamura 3-parameter method. There were a total of 582 positions in the final dataset.

The genetic factors play a crucial role in influencing the likelihood of species monophyle and the degree of intraspecific variation and the extent of divergence between species (Cantarel *et al.* 2006). Tsao and Yeh (2008) reported very low level DNA based discrimination of subspecies of 89 swallowtail butterfly from Taiwan with phylogenetic analysis grouped together members of same species or genus.

Sequences of mtCOI gene of *P. demoleus* of different regions of world were downloaded from NCBI and were

Table 2 Estimates of evolutionary divergence over sequence pairs between five major groups made after phylogenetic analysis of *Papilio deloleus* populations from different regions of world. Analyses were conducted using the Tamura 3-parameter model. The rate variation among sites was modeled with a gamma distribution

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| USA | | | | | |
| Australia | 0.002 | | | | |
| Indian_and_Pakistan | 0.004 | 0.006 | | | |
| Pakistan-1 | 0.011 | 0.009 | 0.008 | | |
| Canada | 0.037 | 0.035 | 0.037 | 0.037 | |

aligned with the local Punjab to find out the genetic variability using neighbour joining statistical method. The phylogenetic analysis revealed that the sequences are quite region specific and form two main clusters which are significantly different from each other by 3.7% (Fig 3). The cluster one includes all the populations of Australia, India, Pakistan and one from USA and the cluster two formed an entirely different group of populations from Canada and the maximum genetic distance between these two clusters is 0.037 (Table 2). The *P. demoleous* population from Australia (Eastwood *et al.* 2006) and Canada (Hebert *et al.* 2003) form totally different two groups with one sequence of Australia in the cluster of India and Pakistan populations. Such type of increased genetic divergence is associated with geographical distances, but the increase in genetic divergence is very less to prevent the identification and geographical relatedness of species (Lukhtanov *et al*. 2009, Gaikwad *et al*. 2012, Ashfaq *et al*. 2013). The local populations from Punjab (Abohar, Jalandhar and Ludhiana) lie in same cluster except for one population from Sangrur, India. The Sangrur population shows a variation of 0.45% from rest of the populations of Punjab (Fig 3). Our data clearly demonstrate that relatively high intraspecific variation and relatively low genetic variability from the closest species characterize virtually all local species. There is only one sequence (cds) from USA which takes a position in cluster one with Australia population (Fig 3). In cluster one, a subgroup is formed by populations from Pakistan and India as the two countries are on the same continental shelf and share common boundaries, biological attributes and less climatic variations. Although two populations (KC755881.1 and GU681873.1) were placed separately but the genetic distance from the major cluster with Indian and Pakistan population is only 0.008 (Table 2). The differences in Sangrur and two Pakistan population from same geographical regions may be due to existence of intraspecific variation in any population from a region.

This is one of the first reports from India on molecular identification and species composition of citrus butterfly *P. demoleus* from different geographical areas of Punjab on citrus host plants. The genetic variability of population from all over the world indicated that Canada population is genetically different from all other populations. The gene sequences submitted to BOLD database will serve as preliminary data for future research plan. It may also aid pest management programs by allowing implementation of species specific management strategies.

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