# Molecular identification of Chinese citrus fly, *Bactrocera minax* (Diptera: Tephritidae) in Nepal

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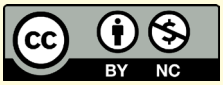

## ABSTRACT

An accurate identification at the species level is often the first step in successfully controlling, mitigating and managing of insect pests. Species identification utilizing molecular approaches can complement morphological identification, often resulting more accurate result. Tephritid fruit fly insects can be identified quickly using DNA barcoding technology. In this study, Chinese fruit fly *(Bactrocera minax)*, a destructive citrus pest collected in Nepal, was identified using barcoding method with the sequence of mitochondrial cytochrome c oxidase I (COI) gene.

**Keywords:** *Bactrocera minax*, Chinese citrus fly, DNA barcode

## 1. Introduction

Fruit flies of the Tephritidae family in Diptera order are the most damaging agricultural pests, especially in horticultural crops (tree fruits and fruit vegetables) (Vargas *et al.* 2015). Numerous fruit fly species have similar, overlapping, or identical features, making definitive morphological identification difficult or impossible (DeMeyer *et al.* 2015). Even though some fruit fly species appear to be morphologically identical, they may have distinct characteristics, host plants preferences, and genetic make-ups (Virgilio *et al.* 2019; Gomez-Cendra *et al.* 2016). Fruit fly damage is often associated with fruit drops, quality deterioration and inedible products. Apart from direct fruit loss, severe quarantine requirements to prevent exotic fruit fly species from entering also use a lot of resources of importing countries (Ekesi 2012). However, despite all the strict quarantine efforts, tephritids, particularly *Bactrocera* spp., continues to spread globally even to strategic pest-free areas (Koohkanzade *et al.* 2018).

Fruit flies of *Bactrocera, Zeugodacus,* and *Dacus* are mainly found inflicting significant damages in tree fruits and vegetable

fruits productions in Nepal. Among different fruit fly species reported from Nepal, the Chinese citrus fly has found the most serious damage in citrus orchards of Nepal (Adhikari *et al.* 2020; Adhikari & Joshi 2018). Rapid and reliable insect pest identification and diagnosis is often the most important information for the containment and mitigation of pest damages, and, in this respect, molecular techniques have shown some promising results. There are several molecular markers that can be utilized to identify tephritid species (Ochando *et al.* 2003; Douglas & Haymer 2001), and various techniques such as DNA barcoding and RFLP are often the preferred diagnostic tools in the present context (Chua *et al.* 2009).

*Bactrocera minax* was first collected in December 1984 from a sweet orange in Helambu, Sindhupalchok district, Nepal (Joshi & Manandhar 2001). The identified fruit flies specimens as *B. tsuneonis* are displayed in the Reference Museum of National Entomology Research Center (NERC), Nepal Agricultural Research Council at Khumaltar, Lalitpur. Later, on September 26, 2007, Dr. Gary J. Steck, Curator of Diptera, Florida State Collection of Arthropods, Florida, USA corrected the identity of *B. tsuneonis* of NERC to *Bactrocera minax* (Paudyal *et al.* 2016; Joshi 2019). The Chinese citrus fly (*B. minax*) is morphologically similar to the Japanese fruit fly (*B. tsuneonis*) (Drew & Romig 2013), but it lacks anterior supra-alar setae (EPPO 2021). Because of that, the prior specimen (EPPO/CABI 1996) was misidentified as *B. tsuneonis* rather than *B. minax*. In Nepal, characterizing studies of fruit fly species in molecular level are uttermost limited. In this study, the morphologically identified *B. minax* specimens are verified in the light of DNA barcoding analysis.

## 2. Materials And Methods

### 2.1 Collection of Specimens and Morphological Identification

Maggots infested sweet orange fruits (variety: Sindhuli Local) were collected in early November 2020 in Golanjor-5, Khaniyakharka (latitude: 27°17.145' N, longitude: 85°58.675' E, altitude: 1341 masl), Sindhuli (Fig. 1). Infested fruits were cut opened to expose maggots. Mature larvae (n = 200) were then collected and placed in 10 plastic containers (dimension: 15 cm height and 10 cm circumference) filled with garden soil (loamy soil of 20.7% average moisture content) 20 prepupae in each container. Pupation of these collected larvae took place at the experiment site. Jar's opening was closed with the help of a muslin piece and a rubber band to avoid larval escape. Soil in the jar was stirred after two months; pupae were examined, counted and recorded. Pupae placed in the same containers, fastened with a piece of nylon mesh. All the emerged adult fruit flies (n = 180) were morphologically identified as Chinese citrus fly in May (2021). Morphological characteristics of these fruit fly species has been described by Adhikari & Joshi (2018). Five dry fruit fly specimens (Fig. 2) out of the identified specimens were sent to the Center for Molecular Dynamics Nepal laboratory for DNA barcoding analysis. Since all the five fruit fly specimens were morphologically identical, only one representative sample was taken for molecular study.

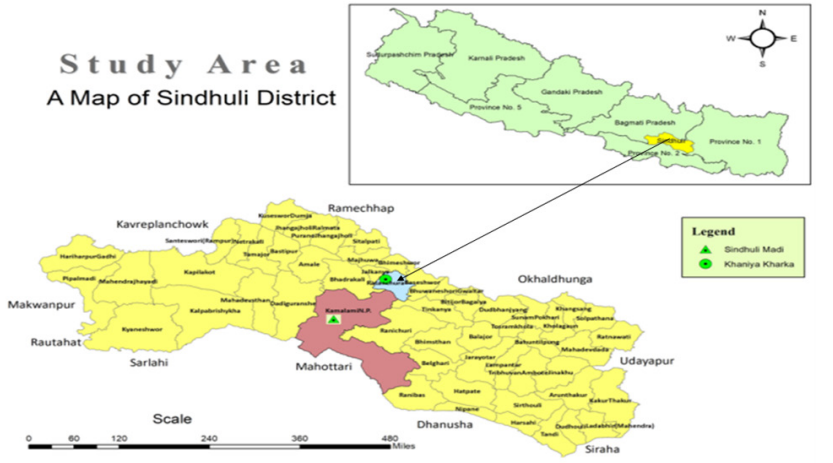


*Fig. 1: Map of study site: Golanjor-5, Khaniyakharka, Sindhuli, Nepal.*


*Fig. 2: Fruit fly specimen, Bactrocera minax (a: male and b: female).*

**2.2 DNA Barcoding of Fruit Fly:** For DNA barcoding of Chinese citrus fly the partial mitochondrial cytochrome c oxidase subunit I (COI) gene was used (Hebert *et al.* 2013).

**2.3 DNA Extraction:** DNA was extracted from the sample obtained by chopping specimens into small pieces and lysed with Lysis buffer and Proteinase K (56 °C for 10 hours). DNA extraction was conducted using GeneAll Exgene™ Tissue SV kit following the manufacturer's protocol (GeneAll® Exgene TM Protocol) with slight modification in lysis step using an additional CTAB lysis buffer to dissolve the exoskeleton. Final DNA was eluted in 50 µL TE buffer in order to obtain the concentrated DNA.

**2.4 PCR of COI Mitochondrial Gene Marker:** PCR was carried out in a 25 µL reaction volume-consisting 12.5 µL multiplex master mix (Qiagen, Germany), 5.0 µL Q solution (Qiagen, Germany), 4.50 µL of RNAse free water (Qiagen, Germany), 1.0µL of each forward and reverse primer and 1 µL of undiluted extracted DNA. Water based negative control to rule out any contamination was also used. Thermo cycling condition was slightly modified for this PCR (Table 1) (Hebert *et al.* 2013). Fig. 3 shows the Agarose Gel Electrophoresis of COI PCR Product from Chinese citrus fruit fly.

The Primer Pair used were:

Forward: LCO1490:
5'-GGTCAACAAATCATAAAGATATTGG-3'

Reverse HCO2198:
5'-TAAACTTCAGGGTGACCAAAAAATCA-3'

**Table 1.** Thermo cycling condition for PCR

| Steps | Temp. (°C) | Time (min) | Cycles |
|---|---|---|---|
| Initial denaturation | 95 | 15 | x1 |
| Denaturation | 94 | 1.0 | x5 |
| Annealing | 45 | 1.5 | |
| Extension | 72 | 1.5 | |
| Denaturation | 94 | 1.0 | x35 |
| Annealing | 50 | 1.5 | |
| Extension | 72 | 1.0 | |
| Final extension | 72 | 5.0 | x1 |
| Cool down | 04 | forever | x1 |

**2.5 Agarose Gel Electrophoresis:** PCR products were visualized in 1.5% agarose gel electrophoresis. A 100 bp DNA ladder from Solis Biodyne was used as reference. The expected PCR amplicon was nearly 650 bp. (Source: Hebert *et al.* 2013)

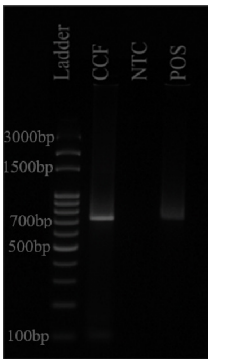


*Fig. 3: Agarose Gel Electrophoresis of COI PCR Product from citrus fruit fly DNA.*

### Production of Barcode

The amplified PCR product (amplicon) was purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermofisher, Catalog No. 78200.200.UL) for 30 minutes at 55°C to digest the unused primers, followed by deactivation step at 85°C for 10 minutes. The purified PCR product was then sequenced on ABI thermocycler using BigDye™ Terminator V3.1 Cycle Sequencing Kit (Catalog No. 4337455) as per the manufacturer's protocol. Excess salts and dye terminators were removed using BigDye® XTerminator™ Purification Kit (Catalog No.4376486) following the manufacturer's instruction. The sample was then loaded on ABI 310 Genetic Analyzer for sequencing. The sequence was annotated using BLAST programme in NCBI database. Tt was then submitted at NCBI GenBank and a unique accession number was obtained for the sequence. Using Barcode of Life Data (BOLD) System v4 (boldsystems.org), a barcode from the DNA sequence has been produced (Fig. 5).

## 3. RESULTS AND DISCUSSION

### 3.1 Sequence Electropherogram

The portion of sequence electropherogram as produced by Sanger Sequencing for the COI marker in fruit fly sample is presented in Fig. 4.

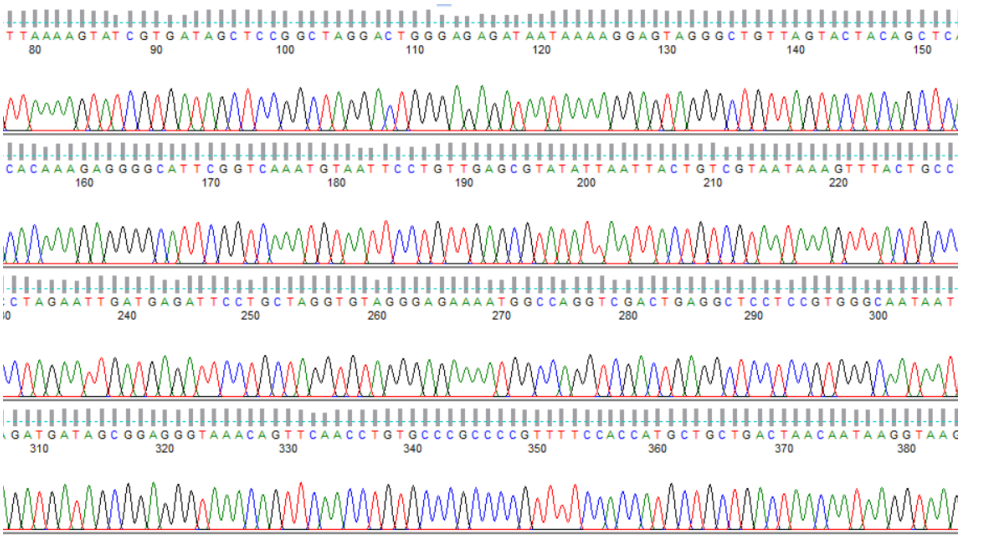

*Fig. 4: Portion of sequence electropherogram as produced by Sanger Sequencing for the COI marker in fruit fly sample.*

### 3.2 Sequence Fasta and Barcode

The electropherogram represented the sequence of COI marker sequenced in Sanger that was further converted to FASTA format for analysis. Fig. 5 shows the barcode.

>ON619567.1 *Bactrocera minax* isolate CCF1/INPL/NPL cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial


*Fig. 5: DNA Barcode of Bactrocera minax generated by BOLD System v4.*

The sequence can also be accessed in NCBI Genbank with accession no. ON619567.1. Basic Local Alignment Search Tool (BLAST) was used to perform the homologous nucleotide search for taxonomic identification. The highly similar sequences search for the obtained sequence showed the sequence to be most likely *Bactrocera minax* as shown in Table 2.

**Table 2:** NCBI BLAST result of the sequence with most significant alignment

| Query length (bp) | BLAST matched Accession | Scientific Name | Common Name | QueryCoverage | E value | Percent identity |
|---|---|---|---|---|---|---|
| 620 | KU985287.1 | *Bactrocera minax* | Chinese citrus fly | 98% | 0 | 99.67% |

Sanger sequencing has been employed in this study to perform the molecular confirmation of the suspected citrus fruit fly with a remarkable 99.67% identity. Various other approaches have also been used for the molecular characterization of citrus fly. Lin *et al.* (2007) applied the PCR-RFLP analysis of mitochondrial and ribosomal DNA to develop a quick molecular diagnostic approach for the identification of *Bactrocera (Tetradacus) tsuneonis* and *Bactrocera (Tetradacus) minax*. These two species were separated based on their restrictive patterns using four primer pairs and five restriction endonucleases. They designed 4 primer pairs targeting the 4 different regions of cry1 genes and following the amplification of cry1 genes, they performed restriction digestion using 5 different restriction endonuclease to obtain variable number of bands. Observing the pattern of bands in electrophoresis they differentiated the *Bactrocera* species. In this study COI gene was specifically targeted due to its extensive research history across wide range of organisms making it suitable for taxonomic identification across broad spectrum of insect species (Zenker *et al.* 2020). The COI gene has a vast amount of reference sequence data available in databases like the Barcode of Life Data Systems (BOLD) and GenBank, which aids in the identification process (López *et al.* 2023). These databases provide a comprehensive collection of COI sequences for numerous insect species, making it easier to compare and match unknown sequences to known species. Other genes like cry1 gene may have limited reference sequence data available, particularly for non-target insect species. In addition, Sanger sequencing provides a high level of resolution and accuracy in determining DNA sequence of target gene, enabling single base pair differences, insertions and deletions, allowing for precise identification and differentiation of closely related species (Cheng *et al.* 2023). Hence, Sanger sequencing using COI gene provides a robust and standardized approach for DNA barcoding and species discrimination. This rapid and cost effective tool for identification of an organism can be very critical in the surveillance and management of fruit files.

## 4. Conclusion

Molecular identification of Chinese citrus fly is performed for the first time in Nepal. It is a rapid and a cost effective tool of identification of an organism. For effective surveillance and management techniques, especially in the case of fruit flies, species identification is crucial. By correct diagnosis of the Chinese citrus fly, specific control strategies can be developed to prevent its expansion and reduce damage to citrus crops. Additionally, this knowledge can help with quarantine operations and protect the agriculture sector.

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