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DNA barcoding and phylogenetic analysis of major fruit fly pests occurring in New Delhi

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ABSTRACT: Accurate and rapid species identification of tephritid fruit flies is critical for effective pest management, particularly where morphologically similar species coexist. The present study aimed to document economically important fruit fly pests in Delhi and to establish DNA barcode-based identification using mitochondrial cytochrome c oxidase subunit I (mtCOI) gene. Adult flies were collected using cue lure and methyl eugenol traps and also through laboratory rearing of infested fruit and vegetables. All samples were morphologically identified and representative samples were subjected to COI gene amplification and Sanger sequencing. A total of 264 specimens representing five species, *Zeugodacus cucurbitae* (Coquillett, 1899); *Z. tau* Walker, 1849; *Bactrocera dorsalis* (Hendel, 1912); *B. zonata* (Saunders, 1842) and *B. divenderi* Maneesh, Hancock & Prabhakar, 2022 were recorded from cucurbitaceous vegetables and fruit crops including mango, guava and pomegranate. Twelve barcodes, showing high sequence similarity (98.06-100%) were generated using the universal primer pair (LCO1490 and HCO2198) and deposited in GenBank. Phylogenetic analyses using the Neighbor-Joining (NJ) method recovered distinct clusters corresponding to the morphologically identified species and clearly separated the genera *Zeugodacus* and *Bactrocera*. The generated DNA barcodes provide a valuable molecular reference for fruit fly identification in Delhi and demonstrate the usefulness of COI barcoding as a tool for surveillance, diagnostics and integrated management.

Keywords: COI, Dacinae, Fruit fly surveillance, Molecular identification, Tephritidae

INTRODUCTION

Tephritidae, commonly known as fruit flies, constitute one of the largest and most diverse families within the order Diptera. Globally, this family encompasses more than 5000 species (Bragard *et al.,* 2020), of which nearly 200 are economically important (Papadopoulos *et al.,* 2024). In the Indian subcontinent, 335 species of fruit flies have been recorded, belonging to 97 genera, 22 tribes and 5 subfamilies (Singh *et al*., 2025). Fruit flies cause major losses in fruit and vegetable production by laying eggs in ripening fruits, leading to spoilage and reducing their market value (Dias *et al.,* 2022; He *et al.,* 2023). These pests pose serious challenges to the agricultural sector by inflicting direct damage, making fruits unmarketable and contributing to considerable post-harvest losses (Ekesi *et al.,* 2016). Their high reproductive capacity, broad larval host range, rapid dispersal, extreme polyphagy and wide climatic tolerance enable them to multiply quickly under favourable conditions, further intensifying their destructive impact on fruits and vegetables (Vasudha and Agarwal, 2019; He *et al.,* 2023). Rapid and fast identification of fruit fliesis crucial in effective pest management and decision making (Armstrong and Ball, 2005; Kunprom and Pramual, 2016). The taxonomy of the fruit flies mostly relies on the morphological characters of the adults (Frias *et al.,* 2008); however, it becomes challenging when there is high variation within a species or when closely related species exhibit strong morphological similarity and the immature stages (Armstrong *et al.,* 1997; Mitra *et al.,* 2025). Accurate identification is particularly challenging within the *Bactrocera dorsalis* species complex, which comprises approximately 90 closely related species, including nearly 30 species attracted to methyl eugenol (ME). Morphological identification of *B. dorsalis* is often complicated by overlapping diagnostic characters with closely related species, particularly *B. carambolae* and *B. occipitalis*, resulting in potential misidentification (Leblanc *et al.,* 2015; Pieterse *et al.,* 2017; Taddei *et al.,* 2023). Compared to traditional taxonomic identification molecular detection methods are rapid, highly specific and also useful in identifying immature stages, cryptic

taxa as well as degraded and specimens with lost body parts (Avise, 1994; Hebert *et al.,* 2003; Hillary and Ceasar, 2023; Kumar *et al*., 2025; Barman *et al*., 2025). Several molecular approaches have been developed to support fruit fly identification, including PCR-based techniques such as restriction length polymorphism (RFLP) (Armstrong *et al*., 1997; Barr *et al*., 2006), amplified fragment length polymorphism (AFLP) (Kakouli-Duarte *et al*., 2001), and oligonucleotide array-based assays (Naeole and Haymer 2003).Among these, DNA barcoding based on mtCOI has become one of the most widely used methods for tephritid identification because of its high discriminatory power and ability to complement morphological identification. (Armstrong and Ball, 2005; Black *et al.,* 2012).

This study aimed to document the economically important tephritid pests occurring in Delhi and establish a DNA barcode reference library based on mtCOI gene. Despite the economic importance of fruit flies, comprehensive information on molecular characterization of tephritid species is lacking.Fruit fly specimens were collected from various agricultural fields and orchards and identified morphologically using standard taxonomic keys and validated through COI DNA barcoding. Molecular phylogenetic analysis was subsequently carried out to assess genetic relationships among the recorded species.

MATERIALS AND METHODS

Insect collection and rearing

Adult male fruit flies were collected at weekly intervals from October 2024 to June 2025 covering the winter and summer seasons using pheromone traps developed by the Division of Entomology, ICAR - Indian Agricultural Research Institute (ICAR-IARI), New Delhi. Traps provided with cuelure and methyl eugenol were installed at a density of one trap per acre in experimental vegetable fields and orchards following developer's recommendations. In addition to trap collections, infested fruits and vegetables were collected from the same field and orchards for laboratory rearing. Each infested fruit or vegetable sample was placed individually in a 3 litre glass jar half filled with sterile sands to allow pupation followed by adult emergence, enabling the host association of each emerged adult to be recorded. Emerged adults were reared with honey solution and sugar cube at Division of Entomology. The culture was maintained at 28±2°C and 75% relative humidity. Adult specimens collected from traps were stored in absolute alcohol for further molecular study.

Morphological identification

Adult fruit flies collected from pheromone traps and those reared from infested fruits and vegetables were identified morphologically using the taxonomic keys of White and Elson-Harris (1992), Ramani (1997), David and Ramani (2011), Leblanc *et al.* (2021), Leblanc (2022), and Singh *et al*. (2022). Species identification was based on diagnostic morphological characters, including facial markings, scutal vittae, wing banding pattern, leg coloration, abdominal markings, and the presence or absence of a male pecten. The principal diagnostic characters used were as follows: *Zeugodacus cucurbitae* – orange-brown body with a fulvous face bearing two black spots, yellow medial and lateral scutal vittae, and a broad costal band expanded into an apical spot with radial-medial and subapical bands; *Z. tau* – dark brown scutum with yellow vittae, oval facial spots, an apically expanded costal band, and a male pecten on tergite III; *Bactrocera dorsalis* – yellowish face with two black facial spots, black to reddish-brown scutum with yellow lateral vittae, entirely yellow fore and mid femora, a continuous costal band, and an orange-red abdomen with a distinct T-shaped marking; *B. zonata* – pale orange-brown body with oval facial spots, a discontinuous costal band forming an apical spot, reddish-brown abdomen, and a male pecten on tergite III; and *B. divenderi* – entirely black face without facial spots, narrow postsutural lateral yellow vittae extending to the intra-alar seta, predominantly black fore and mid femora, a narrow non-expanded costal band and a predominantly black abdomen with a narrow pale transverse band on tergite II. Representative specimens were photographed dorsally using a Leica EC4 digital camera mounted on a Leica M205FA stereo zoom auto-montage microscope (Leica Microsystems, Germany), and the images were processed using Adobe Photoshop (Version 23.1.0). All voucher specimens were deposited in the National Pusa Collection (NPC), Division of Entomology, ICAR–Indian Agricultural Research Institute, New Delhi (INPC: http://grbio.org/cool/nwky-2b63).

DNA extraction

DNA was extracted from three legs of one side from adults and half body parts of maggots using CTAB

protocol. The lysis buffer of total 10 ml was prepared by 3.5 ml of 10% CTAB detergent, 2.8 ml of 5M NaCl, 1.0 ml of 1M Tris-HCl, 0.4 ml of 0.5M EDTA (pH 8.0), 20 µl of β-mercaptoethanol and 2.3 ml of sterile distilled water. Each insect tissue was put in a 1.5 ml microcentrifuge tube and homogenized in 500 µl of the extraction buffer with the help of a micro-pestle. The homogenate was incubated at 65°C for 2 hrs with gentle mixing every 10 mins. Following incubation, an equal volume (500µl) of chloroform:isoamyl alcohol (24:1) was added to each tube, mixed thoroughly and centrifuged at 6000 rpm for 15 min. The aqueous phase was carefully transferred to a new tube and 400µlof chilled isopropanol were added to precipitate the DNA. The mixture was kept at -20°C for 2 hrs. DNA was pelleted by centrifugation at 12000 rpm for 20 mins at 4°C. The supernatant was gently discarded and the pellet was washed with 70% ethanol. After removing residual ethanol by air drying at room temperature the DNA pellet was dissolved in 50 µl of TE buffer. The quality of the isolated DNA was assessed through 1% agarose gel electrophoresis and the purity and concentration were assessed using NanoDrop™ One Spectrophotometer (Thermo Scientific™). All the extracted DNA was stored at - 20°C for further molecular study.

PCR amplification of COI gene

The standard barcoding region of COI gene was amplified using universal barcoding primers LCO 1490 (Forward): GGTCAAATCATAAAGATATTGG and HCO2198 (Reverse): TAAACTTCAGGGTGACCAAAAAATCA (Folmer *et al.,* 1994). The PCR reaction of total 25 µl, consist of 12.5 µl of EmeraldAmp® MAX PCR Master Mix, 1 µl of forward and reverse primers, 8.5 µl of nuclease free water and 2 µl of template DNA. The PCR parameters for *Z. cucurbitae* is as follows: Initial denaturing at 95°C for 4 min followed by 35 cycles of 1 min denaturing at 95°C, 1 min annealing at 52°C and 1.30 min extension at 72°C followed by final extension for 7 min at 72°C. The PCR parameters for other species was as follows: initial denaturation at 94°C for 1 min, followed by 4 cycles of 94°C for 30 sec, 45°C for 40 sec and 72°C for 1 min, followed by 34 cycles of 94°C for 30 sec 51°C for 40 sec and 72°C for 1 min, followed by final extension at 72°C for 5 min. The reaction was stopped by an indefinite hold at 4 °C. The resulting products of PCR were separated using 1.6% agarose gel electrophoresis stained with ethidium bromide and visualized with a gel documentation system (BioRad Lab.).

Sequencing and characterization

The PCR amplified region of the COI gene from different fruit fly species was sequenced using Sanger dideoxy sequencing from Barcode Bioscience (Bengaluru, India). The sequences were then manually assembled using two or more forward and reverse sequence with the help of BioEdit software Version 7.2.5. The noisy sequences were trimmed from both ends of forward and reverse sequences. Subsequently, the sequences were aligned and consensus sequences were created for each species. The resulting consensus sequences were then compared in NCBI through BLASTN software to assess the quality and percentage similarity of the sequences with already submitted sequences. The assembled sequence was submitted to NCBI GenBank and accession number was obtained.

Phylogenetic analysis:

For phylogenetic analysis,COI gene sequence of respective fruit fly species generated in this study, along with sequences retrieved from NCBI database, were used.COI gene sequences submitted from different geographic locations worldwide (Supplementary Table 1) were collected from NCBI. For species lacking global representation, the available sequence entries in the database were used for the evaluation. A total of ten sequences per species were retrieved for phylogenetic analysis (Supplementary Table 1). Additionally, three sequences of *Culex quinquefasciatus* from three different geographic locations were retrieved from NCBI to serve as the out-group (Supplementary Table 1). The phylogenetic tree was constructed following the Neighbor-Joining (NJ) (Saitou and Nei, 1987) and Maximum Composite Likelihood methods (Tamura *et al.,* 2004) with 1000 bootstrap replicates in MEGA12 (Kumar *et al.,* 2024). The NJ tree was further refined and visually improved using the web-based iTOL tool (https://itol.embl.de/).

RESULTS AND DISCUSSION

A total number of 264 fruit fly specimens representing five species of fruit flies (*Zeugodacus cucurbitae, Z. tau, Bactrocera dorsalis, B. zonata* and *B. divenderi*) from the subfamily Dacinae were found in the selected vegetable fields and orchards of the already known hosts. The details of recorded species and the host plant from which they were collected are given in Table 1. The photographic illustrations of the representatives from the collected species were shown in Fig. 1.

Table 1: Details of the fruit fly species along with their recorded host plant and attractants

Sl. No	Insect Species	Host Plant (Collected from)	Attractants	Total no. of specimens
1.	*Zeugodacus cucurbitae* (Coquillett, 1899)	*Luffa aegyptiaca* (Sponge gourd), *Cucurbita pepo* (Summer squash) and *Cucumis sativus* (Cucumber)	Cue lure	66
2.	*Zeugodacus tau* Walker, 1849	*Luffa aegyptiaca* (Sponge gourd), *Cucurbita pepo* (Summer squash) and *Cucumis sativus* (Cucumber)	Cue lure	52
3.	*Bactrocera dorsalis* (Hendel, 1912)	*Mangifera indica* (Mango), *Punica granatum* (Pomegranate) and *Psidium guajava* (Guava)	Methyl eugenol	56
4.	*Bactrocera zonata* (Saunders, 1842)	*Mangifera indica* (Mango), and *Psidium guajava* (Guava)	Methyl eugenol	58
5.	*Bactrocera divenderi* Maneesh, Hancock & Prabhakar, 2022	*Luffa aegyptiaca* (Sponge gourd)	Cue lure	32

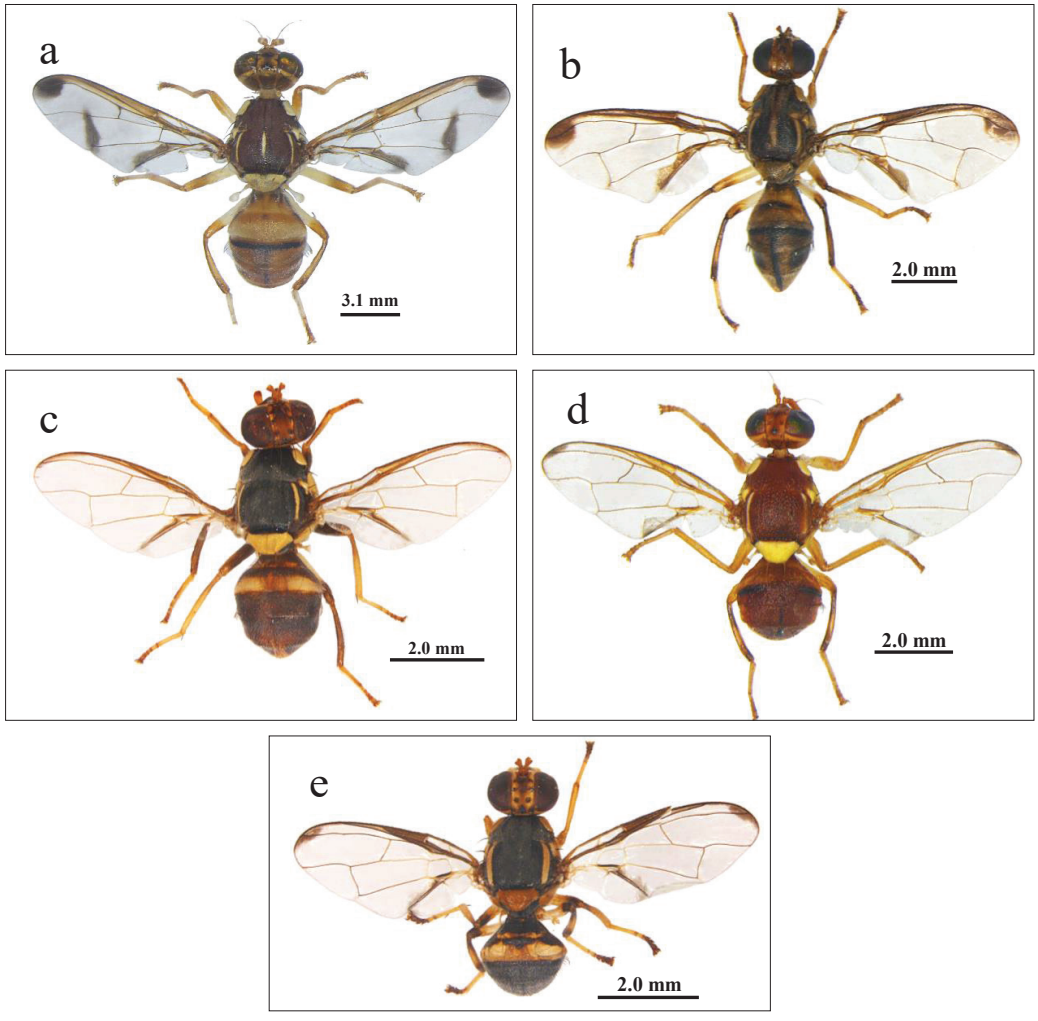

Fig. 1. Fruit fly species recorded in the present study (a) *Zeugodacus cucurbitae*, (b) *Z. tau*, (c) *Bactrocera dorsalis*, (d) *B. zonata*, (e) *B. divenderi*.

Table 2: List of accession numbers along with their % similarity with already submitted sequences in NCBI database, compared through BLASTN software.

Sl. No	Insect Species	Accession No	% Similarity
1.	*Zeugodacus cucurbitae*	PX672281, PX672755, PX699579	99.82-100.00%
2.	*Zeugodacus tau*	PX671287, PX701743	99.27-99.47%
3.	*Bactrocera dorsalis*	PX672976, PX699589	99.79-100.00%
4.	*Bactrocera zonata*	PX671429, PX672856, PX699593	99.50-100.00%
5.	*Bactrocera divenderi*	PX671250, PX699590	98.06-100.00%

Barcode generations

The consensus COI gene sequences made through bioinformatic software using forward and reverse sequences obtained from sanger sequencing of the PCR amplified product, assessed through NCBI BLASTN software. The BLASTN comparison of the submitted sequences showed very high degree of similarity with the NCBI database sequence of the same species (Table 2). A total number of twelve barcodes were generated in this study and accession numbers were obtained for all the species studied (Table 2). Two new barcodes were generated for *Z. tau*, *B. dorsalis* and *B. divenderi* and three new barcodes were generated for *Z. cucurbitae* and *B. zonata*, collected from various locations of ICAR-IARI, New Delhi. The relatively low sequence similarity (98.06%) observed for one *B. divenderi* sequence may be attributed to the limited molecular data currently available for this recently described species. *B. divenderi* was previously misidentified as *B. nigrofemoralis* White & Tsuruta and its economic importance as a pest remained unrecognizeduntil its formal description (Singh *et al*., 2022). Consequently, the limited representation of *B. divenderi* sequences in public databases may have contributed to the lower BLAST similarity observed in the present study.

Phylogenetic analysis

The results of phylogenetic analyses are summarized in Fig. 2. and reveal clear and consistent evolutionary relationships among the studied species. The topology of tree indicates that the positions of most species are highly stable in the Neighbor-Joining (NJ) analyses, reflecting strong phylogenetic signals in the dataset. *B. zonata* clusters closely with *B. divenderi* and both species are grouped under a single well-supported node, indicating a close evolutionary relationship. *B. dorsalis* also shows close affinity with these two species and together *B. zonata*, *B. divenderi* and *B. dorsalis* form a single monophyletic clade, suggesting a shared common ancestor. In contrast, *Z. cucurbitae* and *Z. tau* cluster together to form a distinct monophyletic group. This group is clearly separated from *Bactrocera* clade, highlighting the genetic divergence between the two genera and confirming their distinct evolutionary lineages.

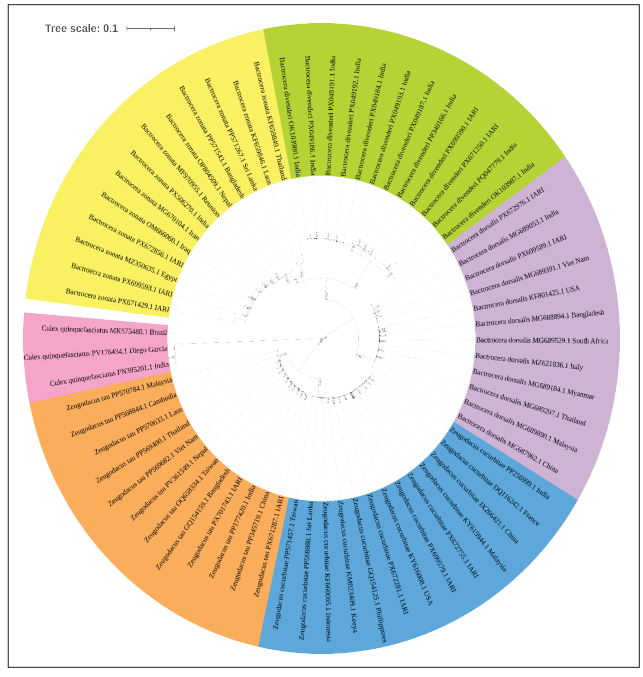

Fig. 2. Phylogenetic tree of recorded fruit fly species, constructed using the Neighbor-Joining (NJ) method taking *Culex quinquefasciatusas* an outgroup. Bootstrap values were calculated from 1000 replicates and are shown on the branches. Accession numbers of the sequences along with the country names from where they were submitted, shown in the tree and the de novo sequences were mentioned with the names of IARI after their individual accession numbers.

DNA barcoding based on the mitochondrial cytochrome c oxidase subunit I (COI) gene has become a widely accepted tool for the identification of tephritid

fruit flies and complements conventional morphology, particularly for closely related species and immature stages (Armstrong and Ball, 2005; Black *et al.*, 2012). A comprehensive COI reference library developed for 265 species of the tribe Dacini demonstrated that COI barcodes provide reliable species identification for most fruit flies, although species complexes such as the *Bactrocera dorsalis* complex may require additional molecular markers because approximately 11.3% of species were found to be non-monophyletic (Doorenweerd *et al*., 2022). In the present study, five economically important tephritid species infesting fruit and vegetable crops in Delhi were identified using both morphological characters and COI barcodes. A total of 264 specimens were collected, and representative specimens generated 12 COI barcode sequences showing 98.06–100% similarity with existing GenBank sequences. Neighbor-Joining phylogenetic analysis recovered distinct genetic clusters corresponding to the morphologically identified species and clearly separated the genera *Zeugodacus* and *Bactrocera*, consistent with previous studies (Kunprom and Pramual, 2016; Shashank *et al*., 2022). The relatively lower similarity (98.06%) observed for one *Bactrocera divenderi* sequence is likely due to the limited availability of reference sequences for this recently described species, which was previously misidentified as *B. nigrofemoralis* (Singh *et al*., 2022). The COI barcode sequences generated in this study provide valuable molecular reference data for the fruit fly fauna of Delhi and will support future surveillance, molecular diagnostics, and integrated pest management programs.

CONCLUSION

Accurate identification of fruit fly species infesting fruit and vegetable crops is essential for effective pest management and quarantine enforcement. In the present study, five fruit fly species were recorded from the New Delhi. Emergence studies from infested fruits confirmed that *Z. cucurbitae*, *Z. tau* and *B. divenderi* are the recorded species infesting cucurbits, whereas *B. dorsalis* and *B. zonata* are the major species infesting mango and guava. In pomegranate, *B. dorsalis* was identified as the sole recorded fruit fly species at the study location. DNA barcoding proved to be a reliable and powerful tool for the rapid and precise identification of fruit fly species occurring in New Delhi.

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AUTHOR CONTRIBUTION

Suman Barman: Writing – original draft, Visualization, Validation, Investigation, Formal analysis.

Pathour Rajendra Shashank: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Susheel Kumar Sharma: Supervision, resources, editing & review.

Godavari H: Data collection and identification.

Mukesh Kumar Dhillon: Supervision, resources, editing & review

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DATA AVAILABILITY

The sequence data used in this study are submitted to NCBI, and details of the data is provided within the manuscript.

DECLARATIONS

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests: The authors declare no competing interests.

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