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Full Length Article

Molecular Confirmation of *Dacus ciliatus* from Balochistan, Pakistan, Based on mt-COI Gene

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Abstract

The pumpkin fly, *Dacus ciliatus*, is a destructive pest of *Cucurbitaceae* crops, poses significant challenges to agricultural productivity worldwide. Despite its economic impact, limited molecular research has been conducted on this species, particularly in Pakistan. In this study, we conducted comprehensive investigations on the presence and identification of *D. ciliatus* in Quetta city of Balochistan, Pakistan, using morphological and molecular techniques. Pheromone traps installed in Zucchini and tomato fields yielded adult male fruit flies, predominantly identified as *Zeugdacus cucurbitae* and *Bactrocera zonata.* Additionally, morphological analysis of infested zucchini samples revealed the presence of *D. ciliatus*. Molecular confirmation through mitochondrial cytochrome oxidase I (mt-COI) sequencing further verified these findings, indicating 100% match with *D. ciliatus* reported from Iraq and 99.8% similarity with an Indian specimen. This study is the first molecular confirmation of *D. ciliatus* in Pakistan, highlighting its potential implications for agricultural management strategies in the region.

Keywords: *D. ciliates*; Morphological; mt-COI gene; Pheromone; Pumpkin fly

Introduction

Dacus ciliatus, commonly known as the lesser pumpkin fly, is an invasive specie belonging to the family *Tephritidae* (Weems 2004). It is an oligophagous pest that affects crops in both temperate and tropical regions, particularly targeting plants of the Cucurbitaceae family (Khater 2020; Nair *et al.* 2021). Native to Africa and Asia, it was first identified in India in 1914 and subsequently recorded in Ombo, Upper Egypt, in 1953 (Weems 2004).

D. ciliatus is a major pest of the *Cucurbitaceae*, 23 plants of this plant family are considered the main hosts of *D. ciliatus* with several other crops apparently of less importance (Weems 2004). The spread of this specie ranges from dry coastal areas to damper zones of medium altitude, with heavy infestations reported in Egypt and in South Africa. Its presence is also reported from Pakistan, India, Sri-Lanka, Bangladesh, Mauritius, Reunion Islands, Egypt and other parts of Africa (White and Elson-Harris 1992; Drew and Romig 2013).

The female fly of this specie typically oviposits inside young fruits or in vegetative parts of the plant, such as stems, and within male and female flowers. The larvae develop within the fleshy tissues of stems or fruits. Infestations have been recorded on several fruit crops, including marrow, gourds, squash, cucumber and cantaloupe (Fetoh 2006). It lays approximately 210 eggs over their lifespan, depositing them in clusters of 5-15 eggs (Khater 2020). Upon hatching, the larvae feed on the host plant, causing significant damage to ripe fruits. Under laboratory conditions, the complete life cycle of *D. ciliatus* is observed to last between 19 and 22 days. The egg stage lasts for 2-4 days, the larval stage for 4-6 days, the pupal stage for 8-10 days and the pre-oviposition period is at least 4 days. Typically, 3-4 eggs are deposited in a single puncture made by the female's ovipositor, although up to 8 eggs may occasionally be deposited in a single puncture, often near the fruit stalk. When more than 10 eggs are laid in a single fruit, larval competition for resources may result in undersized adult flies. Pupation occurs in the soil (Weems 2004).

The economic impact arises due to the requirement for expensive insecticides to manage and control the spread of this invasive fruit fly (Khater 2020). In Pakistan *D. ciliatus* was initially identified in 1992 (White and Elson-Harris 1992) but was not reported thereafter. During the course of our study, this specie was identified in the zucchini field of Quetta city, Balochistan, Pakistan. Our research marks a pioneering effort in Pakistan, representing the first molecular confirmation of this specie in the country. The objective of

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this study was to identify fruit fly species affecting vegetable crops in Quetta, Balochistan, Pakistan with a particular focus on investigating the presence and identification of *Dacus ciliatus* specie through morphological and molecular techniques, thereby contributing to enhanced pest management strategies in the region.

Materials and Methods

Fruit fly collection and handling

Adult male fruit flies were collected by installing pheromone traps. Two types of pheromones; cue lure and methyl eugenol were employed. The traps were installed in the Hazara town area of Quetta, Balochistan, Pakistan within a vegetable field comprising zucchini, tomato and capsicum. The geographic coordinates of each sampling site; 30.162664°N latitude and 66.958544°E longitude were precisely recorded using global positioning system (GPS). The pheromone traps consisted of plastic bottles with dimensions of 20 cm in length and 12 cm in diameter featured with two holes on each side for fly entry. The male lures, consisting of a small cotton wick soaked with 2 mL of either methyl eugenol (ME) or cue lure (CL) and a few drops of insecticide (Bifenthrin 10% EC), were suspended at the center of each trap. Male flies were attracted to the lures and swiftly killed by the insecticide on the cotton wick. The traps were positioned two meters above ground level and suspended with a tree. Simultaneously, the vegetables in the field were also inspected for fruit fly infestation. Infested zucchinis were gathered and forwarded to the Molecular Virology Lab (MVL) for rearing.

Laboratory rearing and morphological identification of fruit flies

The trap was installed on June 11, 2023. After a month the adult male fruit flies obtained from the trap were counted and recorded. While, the infested zucchini samples were kept in laboratory rearing facility in molecular virology lab (MVL) with controlled conditions of 25 ± 2°C temperature, 50 ± 10% relative humidity (RH), and 12:12 (light: dark) photoperiod. The infested vegetables were observed daily for pupation and emergence. Once the larvae fully matured, they spontaneously emerged from the fruit and pupated in a 10-15 cm deep layer of 5-8% moist sterilized sand (Susanto *et al.* 2022). The puparium was subsequently separated from the sand substrate by sieving method and shifted on 2-5 cm deep layer of sterilized sand. After the emergence of first-generation, fruit flies and trap captured flies were identified using the morphological key described by David and Ramani (2011) and Zubair *et al.* (2019).

Isolation of DNA from individual fly

The specimens collected from both the pheromone trap and infested zucchini consisted of three species. While successful identification was achieved for two of the species, there was some uncertainty in the identification of the third species. As a result, our study involved DNA extraction for molecular analysis to resolve this ambiguity. The DNA extraction was carried out for the un-identified fly using the Cetyl trimethyl ammonium bromide (CTAB) method as previously described by Amad *et al.* (2019) with minor modifications. The abdomen portion of the fly was separated from the rest of the body and homogenized in 1.5 mL centrifuge tube containing 150 μL of lysis buffer containing 100 m*M* Tris-HCl, 1.4 *M* NaCl, 20 m*M* EDTA, 2% CTAB (Sigma-Aldrich, Darmstadt, Germany) and 7 μL of proteinase K (Fermentas). The resulting lysate was incubated at 55°C for 1 h. Further, 150 μL of chloroform: isoamyl-alcohol (24:1) was added. The emulsion was then separated by centrifugation at 14,000 rpm for 10 min. Supernatant is then shifted to separate tubes. For DNA precipitation, 150 μL of 100% ethanol and 30 μL of sodium acetate were added, followed by -20°C freezing for 2 h. The resulting pellet was collected by centrifugation at 14,000 rpm for 10 min, washed with 150 μL of 70% ethanol and centrifuged again at 14,000 rpm for 10 min. After air drying, the pellet was dissolved in 20 μL of ddH_2O and stored at -20°C.

Amplification and sequencing of mt-COI gene region

Isolated DNA from the fly were subjected to polymerase chain reaction (PCR) using universal primers LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') targeting the 680 bp mitochondrial cytochrome c oxidase subunit I (mt-COI) gene barcode region, as described by Sharma and Kobayashi (2014). The PCR procedure initiated with an initial denaturation step at 94°C for 10 min, proceeding 35 cycles comprising denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 45 s. The process concluded with a final extension step at 72°C for 10 min. The total PCR reaction mixture was 25 μL, containing 2.5 μL of template DNA, 0.25 μL (5 U/μL) Taq polymerase (Fermentas, USA), 2.5 μL (10 X) Taq buffer (Fermentas, USA), 1.5 μL (25 m*M*) $MgCl_2$ (Fermentas, USA), 2.5 μL (2 m*M*) dNTPs (Fermentas, USA), 0.5 μL (10 pMol) of both forward and reverse primers and 14.75 μL ddH_2O. The final amplified products were separated using electrophoresis in a 1% agarose gel, using a 1 Kb ladder, with a gel run time of 30-35 min at 80V. DNA bands were visualized under UV light in an ultraviolet (UV) transilluminator and the results were compared with the ladder to determine the size of the DNA samples. The PCR products were then subjected to Sanger DNA sequencing using the forward primer by Macrogen (South Korea).

Phylogenetic tree and matrix analysis of mt-COI sequences of flies

To identify the sequence, the sequence was refined and aligned using nBLAST tool which is available at National

Center for Biotechnology Information (NCBI) https://blast.ncbi.nlm.nih.gov/Blast.cgi. Subsequently, the sequence was submitted to NCBI for its accession number. After assigning accession number to our sequence, phylogenetic analysis was performed, for that closely related sequences were retrieved from the database in FASTA format and aligned using Muscle executed in MEGA version XI (Edgar 2004). The maximum composite likelihood method was used to construct a phylogenetic tree (Tamura *et al.* 2013) with bootstrap values of 1000, using *Drosophila melanogaster* (KP730938.1) as an outgroup.

For the validation of phylogenetic analysis, matrix analysis of the sequences was also performed using the sequence demarcation tool (SDT v. 1.2) (Muhire *et al.* 2014). The SDT tool classify our sequences by comparing each sequence in pairs. This software takes sequences in FASTA format, aligns every pair of sequences and calculates pairwise similarity scores and finally displays these scores in color-coded matrix form. The identity scores are calculated as 1-(M/N) where M is the number of mismatching nucleotides and N the total number of positions along the alignment.

Results

Morphological identification of fruit flies

The total number of adult male fruit flies collected from pheromone traps were 36 of which 8 were morphologically identified to be *Bactrocera zonata* while 26 were identified to be *Zeugodacus cucurbitae* using the taxonomic key of David and Ramani (2011) and Zubair *et al.* (2019). Additionally, 44 first generation flies were emerged from the infested zucchini samples. These were morphologically identified as *D. ciliatus*. The identification was further confirmed through DNA sequencing of the amplified mt-COI region of the fly sample.

Mt-COI amplification and Phylogenetic analysis

For the identification of first-generation flies obtained from infested zucchini samples, single fly was subjected to DNA extraction for molecular analysis in our study. BLAST searches for the amplified region of mt-COI revealed 99% similarity between our sequence having accession number PP380152 with *D. ciliatus* India (MH733833.1) and 100% match with *D. ciliatus* Iraq (JQ668130.1). For the phylogenetic analysis, we downloaded the most similar four sequences of *D. ciliatus*, two sequences of *D. frontalis* (MZ433293.1) (KJ703664.1), two sequence of *Zeugodacus tau* (MN842264.1) (MW093433.1) and two sequence of *Bactrocera diversa* (KJ753934.1) (KJ753933.1) as reference sequence. The tree clearly illustrates the clustering of our sequence (PP380152) with *D. ciliatus*. In the tree the sequence of *Drosophila melanogaster* (KP730938.1) was used as out group. This marks the first-time molecular report of this specie in the country. The phylogenetic tree in Fig. 1

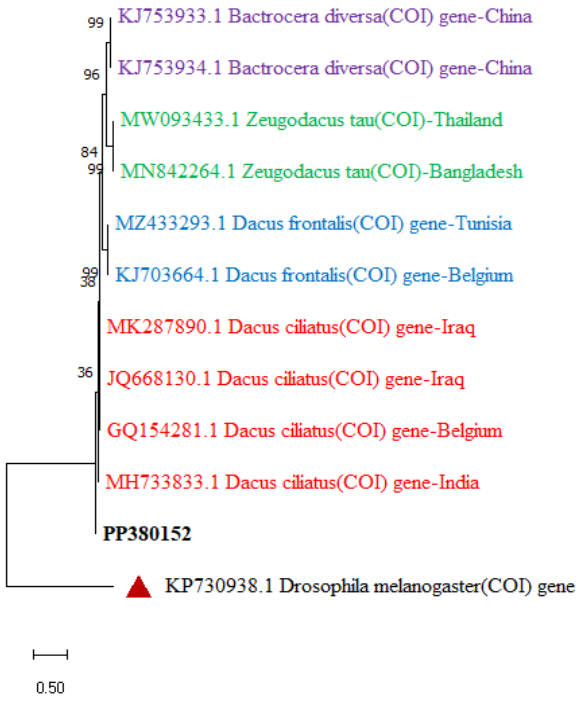

Fig. 1: Phylogenetic tree of mt-COI sequence of *Dacus ciliatus*
The phylogenetic tree was constructed using the amplified sequence of mitochondrial cytochrome oxidase I (mt-COI) gene of our sequence (PP380152) and other sequences downloaded from NCBI as reference sequence. The numbers on tree branches indicate bootstrap value while the bar at the bottom is the scale of branch lengths

was created using the Maximum Composite Likelihood method in MEGA XI (Tamura *et al.* 2021) with bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985).

Matrix analysis of sequences

Our phylogenetic analysis was validated using the sequence demarcation tool (SDT v. 1.2) as depicted in Fig. 2 revealing four distinct clusters. A colored key is also given in the right side of matrix, which indicates the percentage similarity between pairwise identities. In the percentage pairwise identity matrix, the most similar sequences are highlighted in dark brown. The matrix exhibits four triangles of dark brown color, aligning with the clustering observed in our phylogenetic tree (Table 1).

The identification of *D. ciliatus* in zucchini fields in Quetta highlights the potential threat this species poses to agricultural production in Pakistan, particularly crops that belong to Cucurbitaceae family. *D. ciliatus* is known for its oligophagous nature, primarily targeting cucurbit crops but also infesting a variety of other economically significant crops (Khater 2020). In our study, morphological analysis of field-collected samples confirmed the infestation of zucchini with Tephrtidae fruit flies. This finding was consistent with previous reports from Egypt and South Africa, where infestations of cucurbit crops by *D. ciliatus* were documented (Weems 2004).

Table 1: Pairwise sequence identity score of mt-COI gene of fruit flies

PP380152	100.0											
D.ciliatus India	99.8	100.0										
D.ciliatus Iraq	100.0	99.8	100.0									
D.ciliatus Iraq	99.8	99.4	99.8	100.0								
D.ciliatus Belgium	99.2	99.0	99.2	99.1	100.0							
D.ciliatus Tunisia	91.1	90.9	91.1	91.2	91.2	100.0						
D. frontalis Belgium	91.1	90.9	91.1	91.2	91.2	100.0	100.0					
B. diversa China	88.7	88.7	88.4	88.9	87.7	86.6	86.6	100.0				
B. diversa China	88.5	88.5	88.4	88.8	87.7	86.0	86.0	99.4	100.0			
Z. tau Thailand	87.4	87.3	87.4	87.5	87.0	85.4	85.4	88.1	87.7	100.0		
Z. tau Bangladesh	87.4	87.3	87.3	87.6	86.9	85.7	85.7	88.1	87.6	99.2	100.0	
D. melanogaster	61.5	61.0	61.1	58.6	58.3	64.4	64.4	60.4	59.9	60.6	62.0	100.0
	1	2	3	4	5	6	7	8	9	10	11	12

In the Table the pairwise sequence identity analysis of mt-COI gene of our sequence (PP380152) shows 99-100% similarity with *Dacus ciliatus* downloaded from NCBI as reference sequences. This pairwise sequence identity score was measured using sequence demarcation tool version 1.2 (SDT v. 1.2)

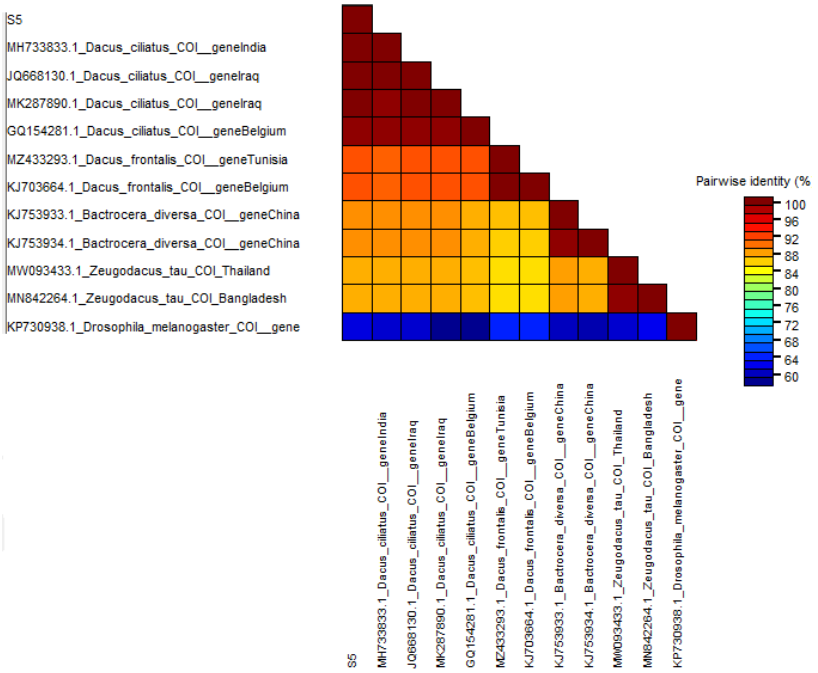

Fig. 2: Color coded pairwise identity matrix of mt-COI gene of Fruit flies
The color coded pairwise identity matrix was generated from 12 mt-COI sequences of fruit flies. Each colored cell represents a share identity score between 2 sequences (one indicated horizontally to the left and other vertically at the bottom)

Discussion

The successful molecular identification of *D. ciliatus* in this study emphasizes the importance of integrating molecular techniques with traditional morphological approaches for pest identification. While pheromone traps captured predominantly *Z. cucurbitae* and *B. zonata* and *D. ciliatus* was only identified through careful rearing of larvae from infested zucchini samples and subsequent DNA sequencing. As the *D. ciliatus* was found only in infested zucchini, this indicates that relying solely on pheromone traps to estimate the diversity of fruit fly species in an area is not sufficient (Kambura 2016). The use of morphological keys (David and Ramani 2011; Zubair *et al.* 2019) remains essential for initial identification, but molecular confirmation is critical for accurate species identification, especially when dealing with closely related species or cryptic species complexes (Gul *et al.* 2024).

The detection of *D. ciliatus* in Pakistan also has significant implications for pest management and highlights the need to re-evaluate the widespread use of insecticide to control fruit fly population. This species causes significant damage to crops through larval feeding. Therefore, use of expensive pest control measure is insufficient and requires Integrated Pest Management (IPM) strategies, including the use of pheromone traps, biological control, and targeted insecticide applications to minimize the economic impact of this invasive pest (Khater 2020).

Moreover, the discovery of *D. ciliatus* in Quetta highlights the need for regular surveillance and monitoring efforts in other parts of Pakistan. Given that this species has

been reported in several neighboring countries, such as India, Sri Lanka, and Bangladesh (Drew and Romig 2013), the probability of the presence of this specie in other parts of Pakistan is also high. The ability of *D. ciliatus* to adapt to both temperate and tropical environments further increases its potential to spread and establish in different ecological zones (Weems 2004).

Phylogenetic analysis conducted in this study revealed that the *D. ciliatus* isolated from Pakistan from our study clustered closely with other *D. ciliatus* sequences from Iraq and India, confirming the genetic relatedness of populations across South Asia and the Middle East. The phylogenetic relationships deduced from mt-COI sequences support the idea of gene flow between these regions that may be due to the movement of infested agricultural products or natural distribution of the flies. This observation is consistent with studies conducted on other fruit fly species, where regional genetic connectivity has been documented (Prabhakar *et al.* 2013; Gul *et al.* 2024).

Conclusion

Our research provides the first molecular confirmation of the presence of *D. ciliatus* in Pakistan, a finding that holds critical implications for agriculture and pest management in the region. The integration of molecular and morphological techniques proved essential for the accurate identification of this pest, and our results highlight the need for surveillance efforts. Further research is required to explore the population dynamics of *D. ciliatus* in Pakistan and to develop pest control strategies that can be used to control the population of this pest in cucurbit crops.

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Authors Contributions

Anbareen Gul: Original drafting, sample processing, data collection, result compilation; Mariam Badam: Data and result compilation; Syed Hamid Jalal Shah: Data and result compilation; Javaria Qazi: Idea of the study, original drafting, result compilation.

Conflict of Interest

It is important to note that there is no competent interest associated with this manuscript.

Data Availability

All data generated or analyzed during this study is present in its full form in this manuscript.

Ethics Approval

This study did not involve any endangered or protected species. Thus, no specific ethical approval was required.

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