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ENTOMOLOGY | ORIGINAL ARTICLE

Molecular Phylogenetic Characterization and Optimized Rearing of *Bactrocera cucurbitae* in Dhaka

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ABSTRACT

Bactrocera (Zeugodacus) cucurbitae, commonly known as the melon fly, is a highly destructive and polyphagous insect pest that poses a significant threat to agriculture in Bangladesh. So, we aimed to accurately identify this species through a combination of morphological and molecular analyses and establish a cost-effective rearing system for lab-based research. More than ±300 fly stocks were produced from the initial wild-collected ±50 larvae in the early three months. We found the applied sawdust as a stable, consistent, aerated, sterilizable, and cost-effective medium; replacing the previous use of moist soil to facilitate pupation and ensure healthy fly populations. A phylogenetic tree was constructed based on the 16S gene of the founding sequence of *B. cucurbitae* and different species of fruit flies available in the GenBank database. The examined family revealed that the mentioned *Bactrocera* species formed a monophyletic group with a 100% bootstrap value. The morphological identification revealed that *Bactrocera cucurbitae* shares most of its morphological features with other species of *Bactrocera*. Meanwhile, the molecular identification method involved the amplification of mitochondrial 16S rRNA gene fragment using universal primers, and sequencing. The resulting 16S rRNA gene sequence, which contains 538 bp (GeneBank Acc Number MW714258), showed a 99% similarity to *B. cucurbitae* from India, China, and Australia. In this study, we established a cost-effective and highly efficient rearing system for *B. cucurbitae*, ensuring optimal resource utilization and successful cultivation of the target species and investigated its life cycle on the natural host media *Momordica charantia* (bitter ground) as part of rearing management. These findings advance our understanding of *B. cucurbitae* evolution and may inform improved pest management strategies. This study highlights the importance of precise species identification, enhanced rearing conditions, and molecular techniques for pest study and control.

1. Introduction

Bangladesh provides a favorable environment for cultivating various vegetables, including cucurbits, due to its conducive soil and agro-climatic conditions (Mondal et al., 2023). However, cucurbit crops are highly vulnerable to insect pests, which pose significant economic challenges to production (Butani & Jotwani, 1984). Among these pests, the cucurbit fruit fly (Bactrocera cucurbitae) is a major concern, causing up to 50% crop damage (York, 1994), with some reports indicating 100% infestation rates (Atwal & Dhaliwal, 2005).

The polyphagous nature, multivoltine reproductive cycle, high mobility, and fecundity of B. cucurbitae make its management particularly challenging (Kamala Jayanthi & Verghese, 2011; S. Singh & Kaur, 2016; Sandeep Singh, 2020). Additionally, morphological similarities in immature stages across Tephritidae species further complicate accurate species identification (Blacket et al., 2012). Effective control strategies require in-depth knowledge of the insect's biology, genetics, and propagation (Cladera et al., 2014), which is crucial for pest control programs and genetic studies (Jayanthi & Abraham, 2002). The development of mass-rearing techniques is essential for large-scale pest control, but it presents significant technical and financial challenges (Parker, 2005). One major limitation is the difficulty in acclimating fruit flies to laboratory conditions while maintaining their reproductive fitness and biological traits (Parker, 2005). Furthermore, B. cucurbitae exhibits high adaptability, dispersal capacity, and reproductive potential, making it a serious invasive pest (Duyck et al., 2004; Hadapad et al., 2019).

Given these challenges, molecular techniques such as PCR-RFLP and DNA barcoding of the 16S rRNA gene

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have emerged as reliable tools for species identification at any developmental stage (Blacket et al., 2012). This study aims to bridge the knowledge gap by developing an efficient laboratory rearing system for B. cucurbitae and utilizing molecular sequencing for precise species identification to support future pest control efforts.

2. Materials and Methods

2.1. Rearing management of *B. cucurbitae*

The *Bactrocera cucurbitae* (melon fly) culture was obtained from infested bitter gourd in Dhaka, with larvae from rural areas, and reared at 25–33°C and 65–80% humidity at Curzon Hall, University of Dhaka, for developmental observation. The test insect melon fly reared at optimal room temperature (25°c to 33°c) in the garden area, Curzon Hall, University of Dhaka, at 65-80% relative humidity to observe the continuous process of the developmental stages. A large plastic bowl (40X40X15 cm,10lt) was wrapped with cloth in which 2/3 plastic bowls (14X10X6.8 cm) containing affected bitter ground were placed on sawdust. In the previous study the moist sand was used to facilitate pupation (Mir et al., 2014). We used sawdust in our study. First, second, and third instar larvae were observed from this. The third instar larvae dropped, and pupation took place in sawdust. After every 3- 4-days, sawdust was sieved and newly formed pupae were collected. Barrel-shaped pupae were found. Then pupae were taken in a petri dish (9 cm diameter), and placed in the adult cage (90×30×50 cm) for emerging adults where both male and female were found. Each rearing cage had Nylon netting mesh on all sides that effectively keeps adult flies from escaping or entering the cage. To collect adult flies for experiments and provide them with food and water, a cage with a round trap entrance was used. Males and females were kept together, with males distinguished by the absence of an ovipositor. A 12% glucose solution was provided in a 250 mL conical flask, with a cotton swab partially immersed to keep it accessible for the flies. A piece of bitter ground was placed on a petri dish in the adult cage for egg collection. The adult females were laying eggs on the parts of fresh bitter ground placed inside the rearing frame cage after successful copulation. A sticky substance secreted by the female helped the laid eggs to adhere to fresh vegetables on which it was deposited. The eggs on the vegetables hatched as 1st instar larvae, and they turned into 2nd instar larvae after a while. The entire process, from egg laying to adult emergence, spanned approximately 28 days, covering one full generation of *Bactrocera cucurbitae*. These pieces were replaced by fresh ones daily to avoid decay. This procedure was repeated until the death of the ovipositing females. Natural feeds were supplied as bitter ground and sugar water. For developing maturation, artificial diet was also provided to the adults, which contained the mixture of 180g sugar and 60g yeast powder. sugar and yeast were mixed in a petri dish and then incubated in 60°c to 65°c for 1 hour. After cooling at the room temperature, the petri dish (9 cm diameter) was placed in the adult cage. A morphometric study was conducted on various life stages of *B. cucurbitae*, which included eggs, freshly hatched first instars, second instars, third instars, pupae, and adults. The study was conducted using two replicates (n = 30 insects per stage). Ideally, scientific studies require at least three replicates for accuracy; however, due to time constraints and limited availability of insects, we had to limit our study to two replicates while ensuring data reliability. Furthermore, in addition to measuring the morphometric parameters, the number of the egg, pupal, and adult stages was also recorded. The study was conducted over a period from March to October 2018, covering approximately three generations of Bactrocera cucurbitae within this timeframe.

2.2. DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from the muscle tissue (0.05 gm) of the fly abdomen, following a method of (Doyle and Doyle 1990) with modification. 500 µl CTAB extraction buffer and 5 µl of proteinase k were added. A total of 500 l CTAB extraction buffer and 5 l proteinase k were added. The mixture was incubated overnight at 56°C. After digestion, an equivalent volume of phenol chloroform was added to the supernatant and centrifuged for 5 minutes at 13,000 rpm. Ethanol was used to precipitate DNA, which was then dissolved in distilled water. Standard PCR technique was utilized for amplifying the 16S rRNA gene, using universal forward and reverse primers. The forward primer, 16S rRNA-F (20-mer), had the sequence 5'-CGCCTGTTTACCAAAAACAT-3', while the reverse primer, 16S rRNA-R (20-mer), had the sequence 5'-CCGGTCTGAACTCAGATCACGT-3'. The amplified PCR products were analyzed through electrophoresis on a 1.0% agarose gel. The purified PCR products were obtained using the GeneJET purification kit and after purification samples were sent to Macrogen, Korea for sequencing and the sequencing was carried out by Sanger Sequencing method.

2.3. Bioinformatics analysis

The GenBank database (NCBI) was used to retrieve the 16S rRNA gene sequence of the experimental fly species as well as other previously identified fly sequences. The serial cloner program 2.6.1 was used to aid in the interspecies alignment. The MEGA 7 program was used to do the multiple sequence alignment, which was followed by the analysis of polymorphism sites and the creation of a phylogenetic tree. Sequence alignment and pairwise distance calculation were used to perform research on two or more biological sequences of equal length.

3. Results

3.1. Development and optimization of a laboratory rearing system for *Bactrocera cucurbitae*

A laboratory-based cost-effective and high-producing rearing system for *B. cucurbitae* has been established in our study (Figure 1), which employs inexpensive equipment such as mesh cages, plastic bowls, and conical flasks, along with a thumb-sized cotton for feeding and natural diet media for breeding. The system utilizes natural diets, resulting in a higher quality and more productive insect population. From the first day of rearing, ±50 larvae were introduced and grew using natural host bitter ground. After four weeks, ±65 newly hatched larvae

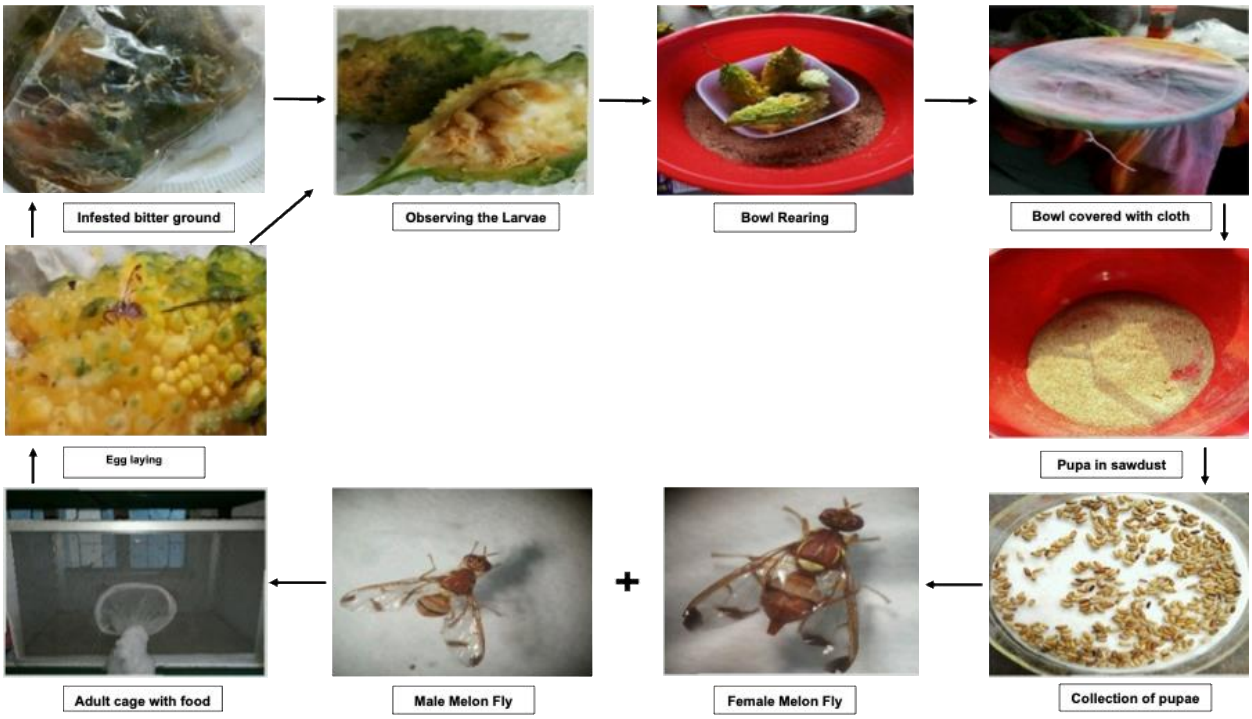


Figure 1. Rearing stages of melon fly

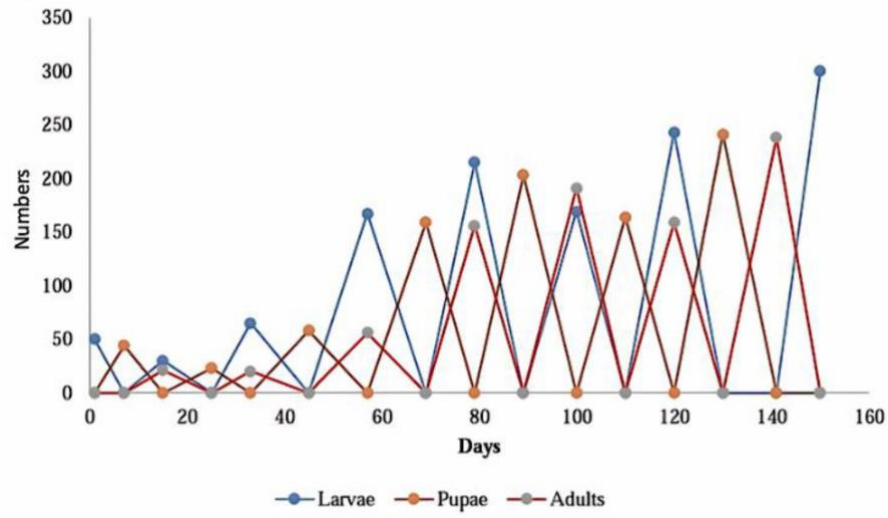


Figure 2. Cumulative number of larvae, pupae and adult collected from the laboratory

were found, and the number of adults grew on average. The number of larvae and pupae increased to ±165 and ±56 respectively after one month, and to ±243 larvae and ±159 pupae after two months. After almost three months, the numbers further increased to ±300 larvae and ±238 pupae. The experiment results are summarized in the graph (Figure 2). The rearing management was effective in producing and growing an adequate number of melon flies in the laboratory.

3.2. Morphological identification and molecular phylogenetic characterization

The identification of Tephritid fruit fly adults in this study was conducted based on their distinctive morphological features. The presence, shape, and color of thoracic vittae, as well as the presence or absence of different setae and relative setal size, were considered as key morphological characteristics.

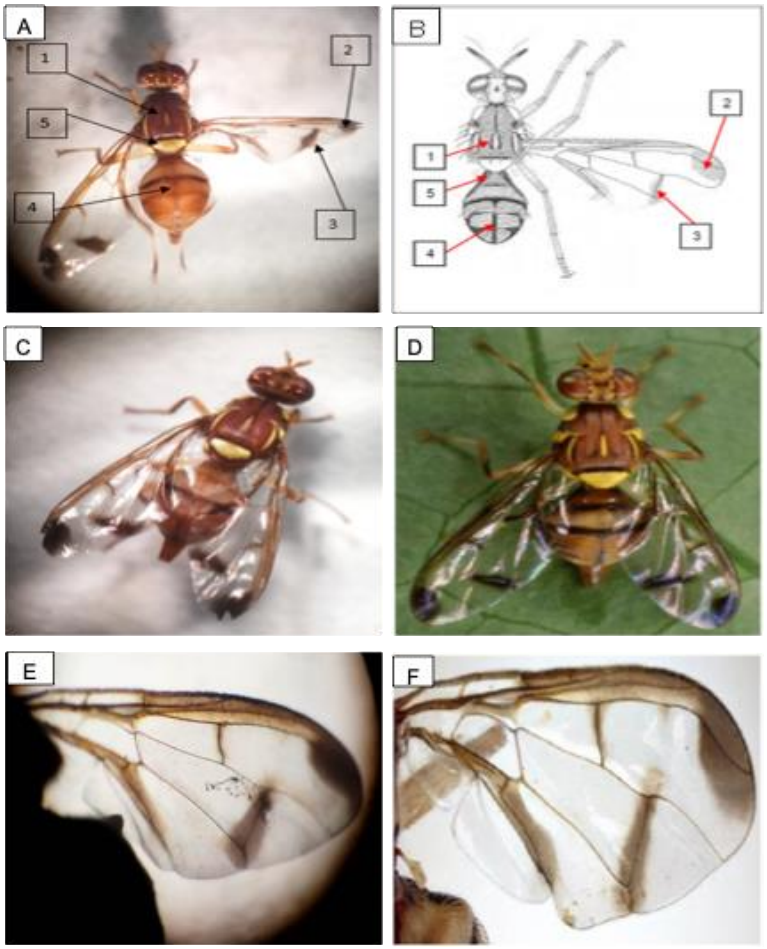


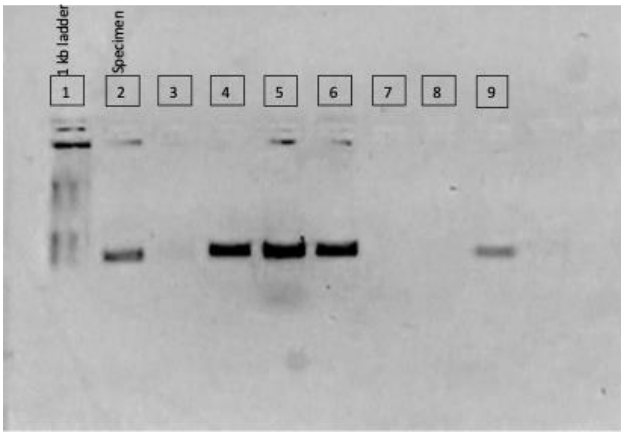
Figure 3. Morphology of B. cucurbitae. Pictures A, C, E were taken from study and B, D, F were from hand book (Plant Health Australia, 2018) respectively. Five key morphological point shown in A and B. They are 1- medial vita, 2-Semi-circle on end of wing, 3 - Infuscation dm-cu vein, 4-T shape, 5-2 sc bristles.

Microscopic examination was carried out using "The Australian handbook for the identification of fruit flies (version 2.1)" as a reference. The application of these morphological diagnostic keys resulted in a 100% similarity rate in species identification, as shown in Figure 3.

In this study, molecular identification of *B. cucurbitae* was conducted using 16S rRNA gene sequencing. Morphological identification of this species can be challenging due to its similarity to closely related species. The DNA was extracted from laboratory-reared specimens, and the 16S rRNA gene was sequenced (Figure 4). The obtained sequences were compared to a database of known 16S rRNA sequences from the gene bank of various *Bactrocera* species, enabling accurate identification of *B. cucurbitae*. The amplification of the 16S rRNA gene was successful, as visualized on a 1.0% Agarose gel (Figure 4), with a fragment length of approximately 600bp. To further confirm the species status, a phylogenetic tree was constructed (Figure 5), utilizing 16S rRNA data from the gene bank DNA database. The phylogenetic analysis demonstrated a monophyletic relationship within the *Bactrocera* species group and a polyphyletic relationship with the outgroup (*Rattus rattus*). The 16S rRNA-based phylogenetic tree revealed that all the *Bactrocera* species formed distinct clades according to their taxonomy, with a 100% bootstrap value. The analysis indicated that the 16S rRNA gene can accurately identify *B. cucurbitae* from other fruit fly species. Comparative analysis with sequences from different continents, such as India, confirmed the monophyletic relationship of the studied *B. cucurbitae* with the collected specimens. The alignments provided strong evidence that the obtained sequence corresponds to a partial sequence of the 16S rRNA gene of *B. cucurbitae*.

4. Discussion

The establishment of a cost-effective rearing system for *B. cucurbitae* involves considering various factors such as environmental conditions, diet, and rearing equipment. Our rearing system utilized bitter gourd, a natural food source for *B. cucurbitae*, ensuring a cost-effective and optimal diet for the flies during their development. The use of natural diets simplified the rearing process by eliminating the need for expensive and complex diet formulations. This approach has significant implications for research and pest management programs, providing a reliable source of insects for experimentation and sterile insect release programs. The successful rearing system offers an efficient and economical means of rearing *B. cucurbitae*, contributing to a better understanding of this significant pest species. Additionally, the life history is mainly egg-larvae-pupae-adult, and in this study, the life cycle is completed in ±26 days (Plant Health Australia 2016). Founding larvae from the egg takes ± 4 day and are continued by the period of three instars.



TTACCAAAAACATGTCTTTTTGAGTTATTTTTAAAGTCTGACCTGCCCACTGAAATTTTTAAATG
GCCGCAGTATTCTAACTGTGCAAAGGTAGCATAATCATTAGTCTTTTAATTGAAGGCTGGTATG
AACGGTTGGACGAGGTATAAGCTGTTTCATATAAATTTATAATAGAATTTTATATTTTAGTCAA
AAAGCTAAAATATTATTAAAAGACGAGAAGACCCTATAAATCTTTATATTTTTTTTTATTTAAAT
TTTTTGGATTTATTTTATTTTTATAATGGAGAATATTTTGTTGGGGTGATGTTAAAATTTAATGA
ACTTTTAATTATAAAAATCATTAATTTATGAGTTATTGATCCATTAGTAGTGATTATAAGATTAA
GTTACTTTAGGGATAACAGCGTAATTTTTTTGGAGAGTTCATATCGATAAAAAAGTTTGCGACC
TCGATGTTGGATTAAGATATAATTTTAGGTGTAGCCGCTTAAATGCTAAGTCTGTTCGACTTTTA
AATTCTTACGTGATCTGAGTT

Figure 4. 1.0% Agarose gel showing the amplified bands of the isolated DNA of B. cucurbitae after PCR. Lane 1 represent DNA marker, Lane 2 showed PCR product. Nucleotide sequence of the partial region of 16S rRna of *B. cucurbitae* and sequence contained 538bp.

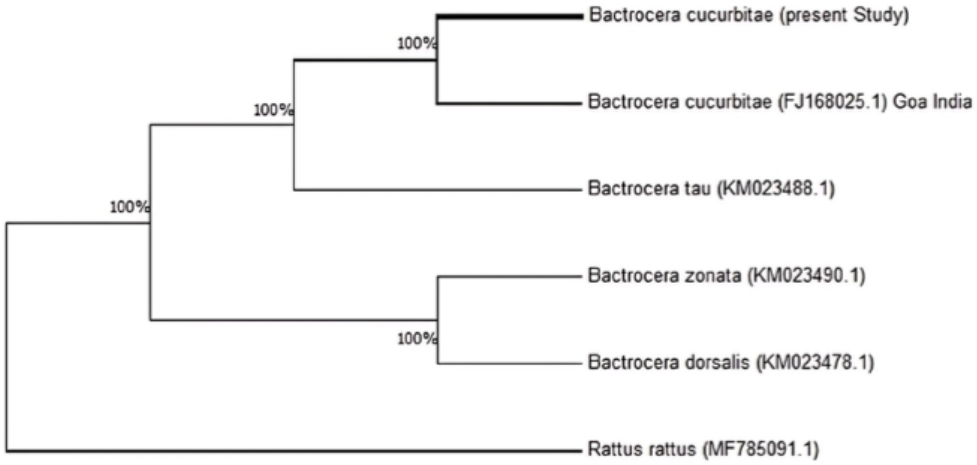


Figure 5. . Phylogenetic tree was constructed with founding sequence of B. cucurbitae and other different kinds of fruit flies based on 16S gene. The 100% bootstrap values indicate strong confidence in the evolutionary relationships among the Bactrocera species, while Rattus rattus serves as an outgroup, confirming that the fruit flies form a distinct and well-supported clade separate from non-related species.

The third instar becomes pupae within ± 10 day, and pupae become adults and require ± 11 day. Monitoring various factors such as egg viability, density and yield of embryonic forms, survival and size of adults, and environmental effects on adults ensured the quality of the rearing process and the continuity of production facilities. The accurate identification of Tephritid fruit fly species is crucial for various purposes, including pest management and quarantine measures. Visual inspection alone can be challenging, as distinguishing between different fly life phases is difficult, with only third instar larvae of some species and possibly pupae being recognizable (White and Elson-Harris 1992).

In this study, the identification of *Bactrocera cucurbitae* was achieved through both morphological and molecular approaches, highlighting the strengths and limitations of each method in fruit fly identification. *B. cucurbitae* is a highly destructive pest species, and accurate identification is crucial for pest management, quarantine, and control efforts. The morphological identification of *B. cucurbitae* was based on established diagnostic features, including the presence and shape of thoracic vittae, as well as setae and their relative sizes, as described in "The Australian Handbook for the Identification of Fruit Flies (Version 2.1)" (Carson, 2012). These characteristics are reliable for identifying fruit fly species, and our study confirmed the species identification with a 100% similarity rate using these keys (Stephens et al., 2015). This approach is widely used in routine pest monitoring and surveillance, particularly for identifying fruit flies in the field (Fletcher, 1987). However, despite its effectiveness, morphological identification can sometimes be challenging due to the high morphological similarity between closely related species, particularly within the *Bactrocera genus* (White and Elson-Harris, 1992).

Molecular tools provide a valuable alternative for precise species identification, especially in cases where morphological features are insufficient or ambiguous. In this study, the 16S rRNA gene sequencing was used for molecular identification. The 16S rRNA gene has been widely employed in molecular phylogenetic studies to

differentiate species within the *Bactrocera* genus, as it offers relatively high resolution and is conserved enough to allow for interspecies comparison (Sivaramakrishnan et al., 2010). Successful DNA extraction from laboratory-reared *B. cucurbitae* specimens, followed by sequencing and subsequent comparison with known sequences in the GenBank database, allowed for accurate species identification, confirming the findings of the morphological analysis. The obtained 16S rRNA sequence showed a high degree of similarity with sequences of *B. cucurbitae* from other regions such as India and China, supporting the notion that *B. cucurbitae* exhibits relatively low genetic divergence across its distribution (Wang et al., 2016). The amplification of the 16S rRNA gene, confirmed by gel electrophoresis (Fig. 4), produced a fragment approximately 600 bp in length, which aligns with expected sizes for this gene region in *Bactrocera* species (Hebert et al., 2003).

The phylogenetic analysis based on 16S rRNA data further validated the species identity. The constructed phylogenetic tree (Fig. 5) revealed a monophyletic relationship within the *Bactrocera* genus, with a distinct separation from the outgroup species *Rattus rattus*. The strong bootstrap support (100%) indicated high confidence in the phylogenetic clustering, which is consistent with previous studies demonstrating the monophyly of the *Bactrocera* genus (Miller et al., 2019). These results are in agreement with previous molecular studies using 16S rRNA for fruit fly identification, which have shown that molecular markers like 16S rRNA are effective in resolving taxonomic ambiguities among closely related species (Miller et al., 2019; Wang et al., 2016).

By comparing the sequence of *B. cucurbitae* obtained from Bangladesh with other sequences from India, we observed a high level of similarity, reinforcing the idea that *B. cucurbitae* populations across these regions share a common genetic lineage. The results from this study support the use of 16S rRNA sequencing as a robust and reliable method for identifying *B. cucurbitae*, complementing traditional morphological methods, especially in cases of taxonomic uncertainty or when distinguishing between similar species (Sivaramakrishnan et al., 2010). Previous studies on *B. cucurbitae* have highlighted the challenges of morphological identification due to similarities with other *Bactrocera* species. For instance, *B. cucurbitae* shares many morphological traits with *Bactrocera dorsalis* and other species in the *Bactrocera* genus, making molecular techniques essential for accurate identification (Fletcher, 1987). Molecular methods, particularly DNA barcoding and 16S rRNA sequencing, have been successfully employed in other studies to clarify the taxonomy of *Bactrocera* species (Hebert et al., 2003; Wang et al., 2016). These findings are consistent with our results, where molecular phylogeny provided a clear differentiation between *B. cucurbitae* and closely related species, emphasizing the superiority of molecular approaches for taxonomic resolution.

5. Conclusion

Diptera is one of the largest insect assemblages and still consists of many species under represented by complete mitochondrial sequences and partial sequences. Analysis of phylogenic relation of *B. cucurbitae* with other tephritid flies and the molecular analysis of 16S rRNA revealed usefulness for observing variation and relation among them based on phylogenetic position; every taxon showed monophyletic relation among the interspecific species in our study. Moreover, mass-rearing of insects is the best of the principal steps to run many processes. In our study, rearing management for the *B. cucurbitae* presents both problems and opportunities due to the environment, diet feeding, temperature, and other facilities. Improvement of growing, raising fecundity, the high mating rate of mass-reared melon flies were observed in our study that may influence the effectiveness of massive production. Future strategies will focus on enhancing knowledge and developing effective control measures to manage melon fly populations through improved biological and ecological approaches.

List of Abbreviations

CPF-Chlorpyrifos, OPs–organophosphorus, DTF-Daily Temperature Fluctuation, CITS-climate-induced toxicant sensitivity, LC_{50}- Lethal Concentration, LD_{50}-Lethal Dose, RAC- Recommended field Application Concentration, CI-Confidence Interval, EC-Effective Concentration, Hb - hemoglobin, PCV -packed cell volume, DAT -day after treatment, AWCD-Average Well-Colour Development, NA-nuclear abnormalities, NGF-nerve growth factor, mAChR-mRNA for the muscarinic acetylcholine receptor, MAG -myelin-associated glycoprotein, GFAP- glial fibrillary acidic protein.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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