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Molecular Identification of Ten Economically Important Fruit Flies (Diptera: Tephritidae) of Bangladesh by Using PCR-RFLP

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Abstract. Proper identification of insect pests at species level is considered as the first step towards implementing successful control strategies against their dispersal. In this context, molecular techniques are best used to support morphological identification. PCR-RFLP is a rapid tool for differentiating tephritid fruit fly pests. In the present study, an initiative was taken to identify ten economically important dacine fruit flies using a PCR amplification technique to successfully establish RFLP patterns of the Internal Transcribed Spacer 1 (ITS1) region. The specific primer pair baITS1f/ baITS1r was used to amplify ~520-840 base pair long fragment of the ITS1 region. The amplified fragments were varied in size among species and able to differentiate at genus level from *Bactrocera* to *Zeugodacus.* But interspecies variations are not clearly distinguished by ITS1 banding profile alone. Due to the ambiguity in band size, they were then subjected to RFLP digestion with two restriction endonuclease enzymes (HhaI and Sau3aI). The restriction enzymes have different cutting sites and thus displayed different banding patterns among species. Analysis of restriction fragments of the ITS1 is able to distinguish six of the ten species successfully. In addition, two morphologically similar species *Bactrocera dorsalis* (Hendel) and *Bactrocera carambolae* (Drew & Hancock) were differentiated by restriction fragments of ITS1. The outcome of this study will enhance early detection and easy monitoring of these quarantine pest species at the port of entry.

Key words: PCR-RFLP, ITS1, fruit fly, *Bactrocera*, *Zeugodacus*

Introduction

Fruit flies in the family Tephritidae include some of the world's most destructive agricultural pests and several of these species share similar, overlapping, or identical morphology, thereby impeding or precluding reliable morphological identification (DeMeyer et al., 2015). Species that appear nearly identical still may exhibit different behaviors, host ranges, tolerances, and physiologies (Gilchrist & Ling 2006; Condon et al., 2008; Gomez-Cendra et al., 2016; Virgilio et al., 2019). Immature stages of different genera are morphologically indistinguishable and are the most likely life stages to be intercepted in food produce (Blacket, 2012). The rejection of fruit cargos due to the presence of maggots is a substantial threat for agricultural and horticultural industries in any fruit-producing country (Stonehouse et al., 2002). Direct damage is associated with fruit drops and rendering fruits inedible. Besides the direct damage to fruits, indirect losses are associated with strict quarantine restrictions that are imposed by importing countries to prevent the entry and establishment of exotic fruit fly species (Ekesi, 2012). Despite severe quarantine procedures, tephritids, especially *Bactrocera* spp. continue to expand their global range, establishing in previously pest-free regions (Koohkanzade et al., 2018). Genus *Bactrocera* and *Zeugodacus* are found mostly in tropical Asia, Australia and the South Pacific regions and cause heavy losses in fruits, flowers and vegetable cultivation.

In Bangladesh, 13 pest and 21 non-pest species of dacine fruit flies have been recorded, the most destructive of which are the oriental fruit fly (*B. dorsalis*), melon fly (*Z. cucurbitae*), pumpkin fruit fly (*Z. tau*), peach fruit fly (*B. zonata*), and the recently reported carambola fruit fly (*B. carambolae*) (Leblanc et al., 2013, 2014, 2019, 2021). Most of these species have been detected repeatedly in survey traps in all the districts of Bangladesh, although *B. carambolae*,

a recent introduction to Bangladesh as evidenced by the genetic profile of the invasive population is still restricted to the southeastern portion of the country (Leblanc et al., 2019). Therefore, simultaneous survey program and accurate identification are required to identify the fruit flies and detect the actual abundance of these harmful tephritid fruit flies in Bangladesh.

Molecular techniques are best used to support or augment morphological identification. In recent years, molecular techniques have taken center stage, as rapid and accurate diagnostics become indispensable for border biosecurity. Several researchers have developed molecular markers for tephritid species diagnosis (Douglas & Haymer, 2001; Kakouli-Durante et al., 2001; Baliraine et al., 2003; Naelole & Haymer, 2003; Ochando et al., 2003). PCR based methods such as DNA barcoding, RFLP are being used for the identification of various pests around the world (Chua et al., 2009). A technique based on RFLP analysis of PCR amplified ribosomal DNA (rDNA) is a quick and sensitive method for generating useful species diagnostic markers (Armstrong et al., 1997). The characteristics of nuclear rDNA have made it of considerable value in systematic studies (Hillis & Dixon, 1991; Brower & DeSalle, 1994) and have been exploited to distinguish closely related species when other traditional markers have failed or are ambiguous (Avise, 1994). Internal Transcribed Spacer (ITS1) of ribosomal DNA genes is used as a molecular marker to identify different economically important fruit fly species. For the ITS1, the size of the PCR amplicon is useful for identification of a few species. However, restriction digestion of the ITS1 PCR amplicon, which denotes the actual sequence in defined regions of the amplicon, is recommended for all analyses as a more robust method of identification (Drew, 2016).

The main objective of this study is to establish PCR-RFLP based quick identification system for fruit flies. To determine the specificity of detection, ITS1 region of a total of ten species of fruit flies were amplified, digested with two restriction endonuclease enzymes (HhaI and Sau3aI) and identified subsequently.

Materials and Methods

Specimen Collection and Morphological Identification

All the ten fruit fly specimens (Table 1) were collected from various locations of the Atomic Energy Research Establishment (AERE) Campus (N 23.954 E 90.280), Savar, Dhaka, a 263.5-acre experimental area comprised of agricultural land, dendrarium, and concrete buildings. Major types of cultivated fruit and vegetable crops grown in this area with a higher abundance and variety of fruit flies. The specimens were collected by traps (Hossain et al., 2019) baited with male lures (cue-lure, methyl eugenol, and zingerone) were maintained in trees, 1.8 m above the ground and about 100 m apart, at each of 10 sites throughout the AERE campus. Alcohol (96%) preserved fruit flies were identified to species level by using the different keys described by Drew and Romig (2016).

Table 1. List of lure types, GPS coordinates, collection date of fruit fly specimens

Scientific Name	Lure Types	GPS Coordinates		Collection Date
		Latitude	Longitude	
Bactrocera dorsalis (Hendel)	ME	23^{0}57$'$17.443”N	90^{0}16$'$50.918”E	15.02.2021
Bactrocera zonata (Saunders)	ME	23^{0}57$'$16.861”N	90^{0}16$'$50.918”E	25.08.2021
Zeugodacus cucurbitae (Coquillett)	CL, Zn	23^{0}57$'$4.421”N	90^{0}16$'$41.333”E	25.08.2021
Zeugodacus diversus (Coquillett)	ME, Zn	23^{0}57$'$16.316”N	90^{0}16$'$36.984”E	13.08.2021
Bactrocera rubigina (Wang & Zhao)	CL, Zn	23^{0}57$'$17.443”N	90^{0}16$'$50.918”E	18.10.2021
Zeugodacus tau (Walker)	CL, Zn	23^{0}57$'$4.421”N	90^{0}16$'$41.333”E	17.02.2021
Bactrocera correcta (Bezzi)	ME	23^{0}57$'$16.861”N	90^{0}16$'$50.918”E	01.02.2021

Bactrocera carambolae (Drew & Hancock)	ME	23^{0}57$^{'}$4.421"N	90^{0}16$^{'}$41.333"E	11.08.2021
Bactrocera syzygii (White & Tsuruta)	Zn	23^{0}57$^{'}$16.316"N	90^{0}16$^{'}$36.984"E	11.08.2021
Bactrocera digressa (Radhakrishnan)	CL, Zn	23^{0}57$^{'}$4.421"N	90^{0}16$^{'}$41.333"E	11.08.2021

Extraction and PCR Amplification

Whole insect (30 gm) was used for extraction of genomic DNA using Qiagen DNeasy® Blood and Tissue Kit. The Primers specific to ITS1; baITS1f 5' GGA AGG ATC ATT ATT GTG TTC C 3' and baITS1r 5' ATG AGC CGA GTG ATC CAC C 3' (McKenzie et al., 1999) resulted in the amplification (Figure 1). PCR was performed in 30 µL of PCR Master Mix (Promega, Madison, WI, USA) consisting 5 µL of extracted template DNA. The PCR cycle conditions were as follows: initial denaturation (94°C for 5 min), 39 cycles of denaturation (94°C for 1 min), primer annealing (60°C for 1 min), and an extension (72°C for 1 min), and a final extension (72°C for 4 min). The success of amplification was evaluated by 1% agarose gel electrophoresis under ultraviolet light. Different banding pattern depicted distinction among species.

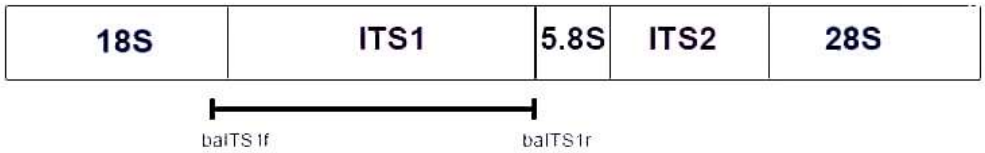

Figure 1: Part of the Ribosomal RNA operon with the location of primer position for ITS1 (Source: Drew, 2016)

Digestion with Enzyme and Restriction Analysis

Without further purification, PCR products from each species were used directly for RFLP digestion using two restriction enzymes HhaI and Sau3AI (New Zealand biolabs) separately. Each digestion consists following components in 20 µl volume.

i) Restriction enzyme (HhaI /Sau3aI) 5 µL
ii) Enzyme specific buffer 2 µL
iii) Bovine serum albumin 0.2 µL (10 ug μL^{-1})
iv) Nuclease free water 12.3 µL &
v) PCR product 5 µL

These digested products were further analyzed by 2% agarose gel electrophoresis under ultraviolet light.

Results and Discussion

The PCR with the primers baITS1f/ baITS1r were successfully resulted in amplification of ITS1 region of 10 fruit fly species (Table 2) (Figure 2). Among ten species ITS1 fragment length fall into two classes and distinctly separate two different genus *Bactrocera* (800-840bp) and *Zeugodacus* (520-550 bp) (Figure 3). But ITS1 banding profiles cannot differentiate at interspecies level accurately. For further clarification restriction digestion was done with two restriction enzymes namely HhaI and Sau3AI.

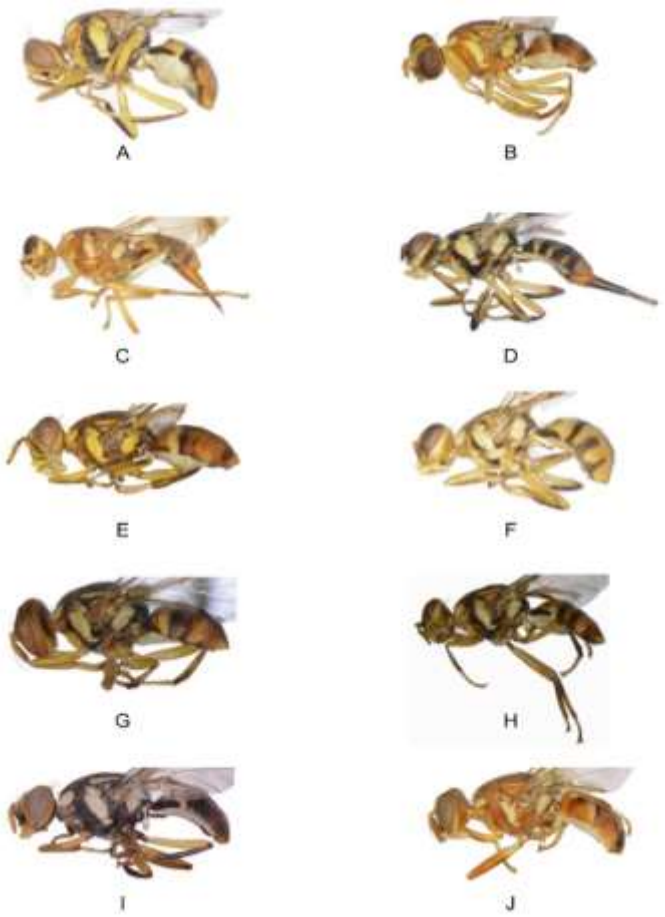

Figure 2. Fruit fly specimens
(A- *Bactrocera dorsalis.* B- *Bactrocera zonata*, C- *Zeugodacus cucurbitae*, D- *Zeugodacus diversus*, E- *Bactrocera rubigina*, F- *Zeugodacus tau*, G- *Bactrocera correcta*, H- *Bactrocera carambolae*, I- *Bactrocera syzygii*, J- *Bactrocera digressa*)

In total seven *Bactrocera* and three *Zeugodacus* species were digested. Distinct banding profiles found for each species by digesting PCR product with either enzyme HhaI or Sau3AI thus enabling their identification at species level (Table 2) (Figure 4, 5). All the ten species have cutting sites for enzyme HhaI and five species for Sau3AI. Within genus *Bacrtocera* five species were easily differentiated by restriction digestion except *B. dorsalis* and *B. zonata* which displayed almost similar banding pattern for both the enzymes were found difficult to clearly separate (Table 2). Similarly, two *Zeugodacus* species (*Z. cucurbitae* and *Z. diversus*) produced same size bands and created uncertainty to identify. This result is consistent with the findings of Drew (2016). He employed PCR-RFLP methods for identification of fruit flies using seven different endonuclease restriction enzymes and described restriction cutting sites for 27 species of fruit flies using these enzymes. Four species (*B. dorsalis, B. zonata, B. carambolae and Z. cucurbitae*) of the present study were showed similar banding profile with Drew's study. Armstrong et al. (1997) developed a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) diagnostic tool to distinguish among species within the genera *Anastrepha* Schiner, *Bactrocera* Macquart and *Ceratitis* that are important to quarantine authorities in New Zealand. They used 18S+ITS for identification of 19 species of fruitflies from four genera by digesting with 4 different restriction enzymes. But they could not able to differentiate closely related species and found almost similar banding patterns. This problem can be solved by nucleotide sequencing. Barr et al. (2021) done DNA sequencing of ITS-1 to a collection of 513 adult flies trapped in California, USA, in the year 2008 to 2018

and showed Internal transcribed spacer1 sequences were successfully recovered from 504 (98%) of these flies.

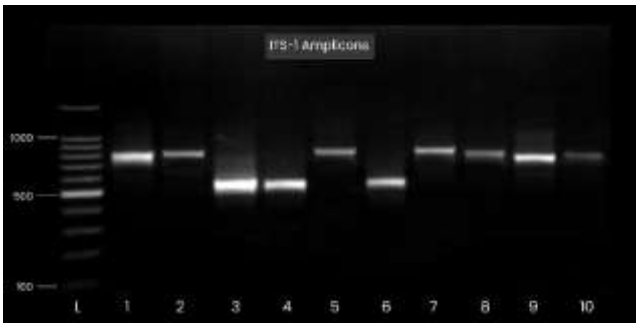

Figure 3. Fragment length pattern of ITS1 (Undigested)
(L= 100bp DNA Ladder; Lanes 1- *Bactrocera dorsalis.* 2- *Bactrocera zonata*, 3- *Zeugodacus cucurbitae*, 4- *Zeugodacus diversus*, 5- *Bactrocera rubigina*, 6- *Zeugodacus tau*, 7- *Bactrocera correcta*, 8- *Bactrocera carambolae*, 9- *Bactrocera syzygii*, 10- *Bactrocera digressa*)

Table 2. PCR-RFLP fragments of ITS1 of fruit flies

Fruit fly species	ITS banding pattern (bp)	Restriction enzymes cutting site (bp)	
		HhaI	Sau3AI
B. dorsalis	800	650,150	×
B. zonata	820	680,140	×
Z. cucurbitae	550	400,150	×
Z. diversus	520	380,140	×
B. rubigina	840	700,140	500,230
Z. tau	600	400,200	×
B. correcta	840	690,150	440,400
B. carambolae	830	680,140	450,380
B. syzygii	810	600,210	420,390
B. digressa	820	620,200	430,390

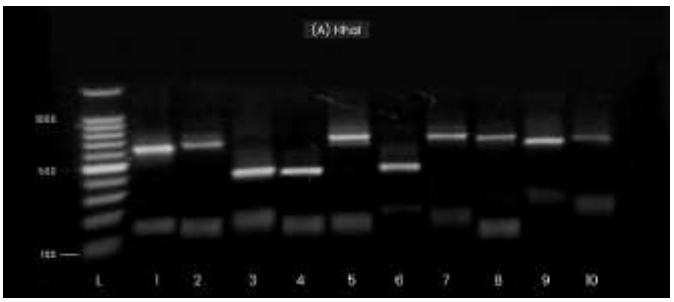

Figure 4. Fragment length pattern of ITS1 digested with enzyme HhaI
(L= 100bp DNA Ladder; Lanes 1- *Bactrocera dorsalis.* 2- *Bactrocera zonata*, 3- *Zeugodacus cucurbitae*, 4- *Zeugodacus diversus*, 5- *Bactrocera rubigina*, 6- *Zeugodacus tau*, 7- *Bactrocera correcta*, 8- *Bactrocera carambolae*, 9- *Bactrocera syzygii*, 10- *Bactrocera digressa*)

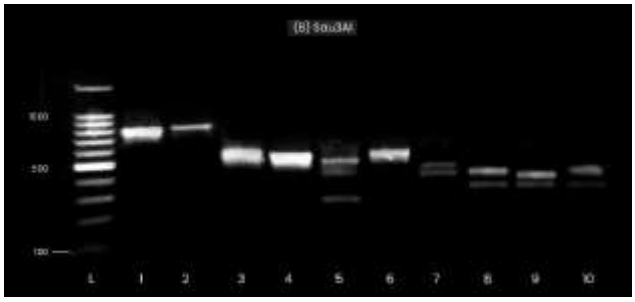

Figure 5. Fragment length pattern of ITS1 digested with enzyme Sau3AI
(L= 100bp DNA Ladder; Lanes 1- *Bactrocera dorsalis.* 2- *Bactrocera zonata*, 3- *Zeugodacus cucurbitae*, 4- *Zeugodacus diversus*, 5- *Bactrocera rubigina*, 6- *Zeugodacus tau*, 7- *Bactrocera correcta*, 8- *Bactrocera carambolae*, 9- *Bactrocera syzygii*, 10- *Bactrocera digressa*)

Restriction fragments shorter than 100 bp were ignored in this analysis, as they could not be observed in 100 bp DNA ladder on 2% agarose gel electrophoresis.

B. dorsalis and *B. carambolae* two major pest species which share almost similar morphological characteristics and cannot be differentiated by COI gene sequencing. In the current study, these two species have been distinguished by their different banding patterns for ITS1 restriction digestion. *Bactrocera dorsalis* had no cutting site for Sau3AI, in contrast, *Bactrocera carambolae* showed to have two cutting sites resulted in producing two bands of 450,380 bp (Table 2) (Figure 4, 5). Notably, DNA sequencing of the nuclear ribosomal internal transcribed spacer 1 (ITS-1) has been adopted by the International Plant Protection Convention (IPPC, 2019) as an internationally accepted method to distinguish between the 2 pestiferous fruit fly species *B. dorsalis* and *B. carambolae* and differentiate successfully. Moreover, Barr et al. (2006) applied polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method to analyses three mitochondrial genes (12S ribosomal RNA, 16S ribosomal RNA, and NADH-dehydrogenase subunit 6) one nuclear locus (the ribosomal internal transcribed spacer region 1, ITS-1) and found it sufficient to diagnose 25 species of *Ceratitis*. However, in future study nucleotide sequencing of ITS1 and mitochondrial COI gene will also be done for accurate identification of fruit fly pests.

Lewter and Szalanski (2007) who employed PCR-RFLP to identify and differentiate fall armyworm (*Spodoptera frugiperda*) noted that the technique offers a very affordable and accurate method for the identification of insect species. The technique is quick and reliable. Chua et al., (2009) showed that this molecular method is effective even when only body parts or immature stages of tephritid fruit flies are present. As continuation of the present study in future, this method will be tested on immature stages of the tephritid fruit flies.

This study had a major contribution in quick identification of tephritid flies under available laboratory facilities prior to further nucleotide sequencing.

Conclusions

PCR- RFLP based identification of fruit flies is first time standardized in Bangladesh at the laboratory of Insect Biotechnology Division, IFRB, AERE. This study demonstrates the utility of PCR-RFLP analysis of ribosomal ITS1 region to identify ten dacine fruit flies. It is a rapid and cost effective tool of identification. The application of this method to identify the species is indispensable in surveillance of fruit fly for successful phytosanitary irradiation program under Bangladesh Atomic Energy Commission. The result of this experiment will also contribute to enhance implementation of SIT based insect management programmes. In further studies, nucleotide sequencing of ITS-1 and mtDNA (COI gene) based identification will be done.

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