ZIBELINE INTERNATIONAL™
PUBLISHING
ISSN: 2521-2931 (Print)
ISSN: 2521-293X (Online)
CODEN: MJSAEJ

Malaysian Journal of Sustainable Agriculture
(MJSA)

DOI: http://doi.org/10.26480/mjsa.02.2022.110.116

RESEARCH ARTICLE

SPECIES IDENTIFICATION OF ECONOMIC IMPORTANT ADULT FRUIT FLIES BASED ON DNA BARCODING (MT DNA *COI*) AND LARVAE BASED ON SPECIES SPECIFIC PRIMERS FROM CENTRAL AND SOUTH PARTS OF BANGLADESH

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ARTICLE DETAILS

Article History:

Received 26 April 2022
Accepted 30 May 2022
Available online 02 June 2022

ABSTRACT

Bangladesh is an agro-based country. Several vegetables and fruits contribute greatly to the national economy. Fruit fly species (Diptera: Tephritidae) have been a serious threat to agriculture in Bangladesh as well as worldwide. Morphological identification sometimes creates misidentification in adult stages, while in eggs, larvae and pupal stages are totally difficult. Nowadays, molecular identification based on DNA barcoding is an effective and rapid identification tool. However, this technique is very limited use in Bangladesh. In this study, adult samples were collected (trapping with ME and CUE) from three different geographic locations (Dhaka, Chittagong and Barisal) of Bangladesh. Adult flies were identified based on DNA barcoding (amplify the sequences with *COI* gene), and larvae were identified based on species-specific primers. *Bactrocera dorsalis* (Hendel), *Zeugodacus tau* (Walker) and *Zeugodacus cucurbitae* (Coquillett) were identified adult species found in all the locations, whereas *B. dorsalis* was found in a higher number. In case of host fly identification on the basis of larvae, *B. dorsalis* was identified from guava in three locations, indicating guava fruit is the suitable host in Bangladesh. Proper management should be taken to control these pest species; otherwise, they will become a great threat to the agriculture of Bangladesh.

KEYWORDS

Bactrocera dorsalis, *Zeugodacus tau*, *Zeugodacus cucurbitae*, molecular identification, DNA barcodes, species-specific primer.

1. INTRODUCTION

Bangladesh has fertile land and a conducive climate for the growth of various agricultural products (Hoq et al., 2012). Agriculture is the most dominant sector of the economy of Bangladesh, which contributes more than 15 percent of the GDP (BBS, 2018). Approximately 70% of the total population lives in rural areas and depends directly or indirectly on agriculture for their livelihoods. Bangladesh has been blessed with various fruits and vegetables, including more than 90 vegetables and 60 fruits (Uddin et al., 2005). The main vegetables are tomato, brinjal, potato, aroids, cabbage, cauliflower, pumpkin, bottle gourd, pointed gourd, bitter gourd, yard long bean, cucumber, and hyacinth bean, and fruits include banana, guava, jack fruit, mango, papaya, lemons, pummelo, pineapple, litchi and ber (jujube) are the most important (Hasanuzzaman, 2003).

Fruit flies (family: Tephritidae) are a group of agricultural pests that damage a wide range of fruits and vegetables and pose tremendous threats worldwide, with both quantitative and qualitative losses (FAO/IAEA, 2013). About 4000 described species from 500 genera of tephritids were identified (Qin et al., 2015). Among them, more than 250 species are considered economically important (Li et al., 2013). Most of them belong to six: *Anastrepha, Bactrocera, Ceratitis, Dacus, Zeugodacus*, and *Rhagoletis* (White and Elson-Harris, 1992; Van Houdt et al., 2010). Moreover, 118 species have been known to occur on the Indian subcontinent as cultivation fruits and vegetable pests (David and Ramani, 2011; Drew and Romig, 2013; David et al., 2016, 2017; Leblanc et al., 2018). In Bangladesh, fruit fly also causes massive damage to both vegetables and fruit production (Alim et al., 2012). Recently, 29 fruit fly species (13 pests and 16 non-pest species) were identified from the rural environment and forest areas of Bangladesh by six years surveys (2013-2018), of which *Bactrocera dorsalis* (Hendel), *B. zonata* (Saunders), *Zeugodacus tau* (Walker) and *Zeugodacus cucurbitae* (Coquillett) was found as a major species (Leblanc et al., 2019).

Identifying species is a key part of the recognition and description of biodiversity. Traditional identification (morphological) needs experts like taxonomists and trained technicians who can clearly identify taxa, as it needs special skills and vast experience. But there has been a substantial decrease in the number of taxonomists and other experts. Therefore, alternative and accurate methods of identification are necessary so that non-experts can do it the very easy way. DNA-based identification methods have been the most promising approaches instead of morphological data for identifying the taxa (Busse et al., 1996; Blaxter,

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	Website: www.myjsustainagri.com	**DOI:** 10.26480/mysj.02.2022.110.116

Cite The Article: Sultana Afroz, Md Shibly Noman, Yue Zhang, Md Yousuf Ali, Md Rubel Mahmud, Zhihong Li (2022). Species Identification of Economic Important Adult Fruit Flies Based on DNA Barcoding (*MT DNA COI*) and Larvae Based on Species Specific Primers from Central and South Parts of Bangladesh. *Malaysian Journal of Sustainable Agricultures, 6(2): 110-116.*

2003). Nowadays, DNA barcoding has become a powerful tool for the successful identification of fruit flies. On the basis of the *COI* gene, several rapid diagnostic approaches have been established, such as real-time PCR, PCR-RFLP (restriction fragment length polymorphisms), microfluid dynamic array techniques, and loop-mediated isothermal amplification (LAMP). Such methods were successfully implemented in identifying several economically important fruit fly species.

In some studies, polymerase chain reaction (PCR) with species-specific primers have also been used to resolve the need for post-amplification digestion and DNA sequencing that enables quicker identification (Chua et al., 2010). The two pairs of species-specific primers were successfully developed by based on 1517 bp of the mtDNA *COI* gene that could distinguish B. carambolae and B. papayae under normal PCR testing conditions (Chua et al., 2010). A group researchers developed species primers based on DNA barcode sequences for the identification of *B. dorsalis* and *B. zonata*, and this approach could accurately classify all life stages of the target species (Asokan et al., 2011). Some of researcher developed two pairs of species-specific primers that can easily identify all developmental stages of *B. minax* and *B. tsuneonis*, which is very cost-effective molecular method of identification (Zheng et al., 2019). Nowadays, molecular identification based on DNA barcoding is an effective and rapid identification tool. However, this technique is very limited use in Bangladesh. Hence, the present study was taken to identify the economic important adult fruit fly species using DNA barcoding (mt DNA *COI*) and larvae using species specific primer.

2. MATERIAL AND METHODS

2.1 Experimental Area

Samples were collected from three divisions Dhaka, Barisal and Chittagong of Bangladesh during June and December 2018 (Figure 1). Adult fruit fly samples were collected from the commercial vegetable farm (Dhaka), guava orchard of Horticulture Centre, Madaripur, and vegetable field (Barisal) and Commercial mango and citrus orchard (Chittagong).

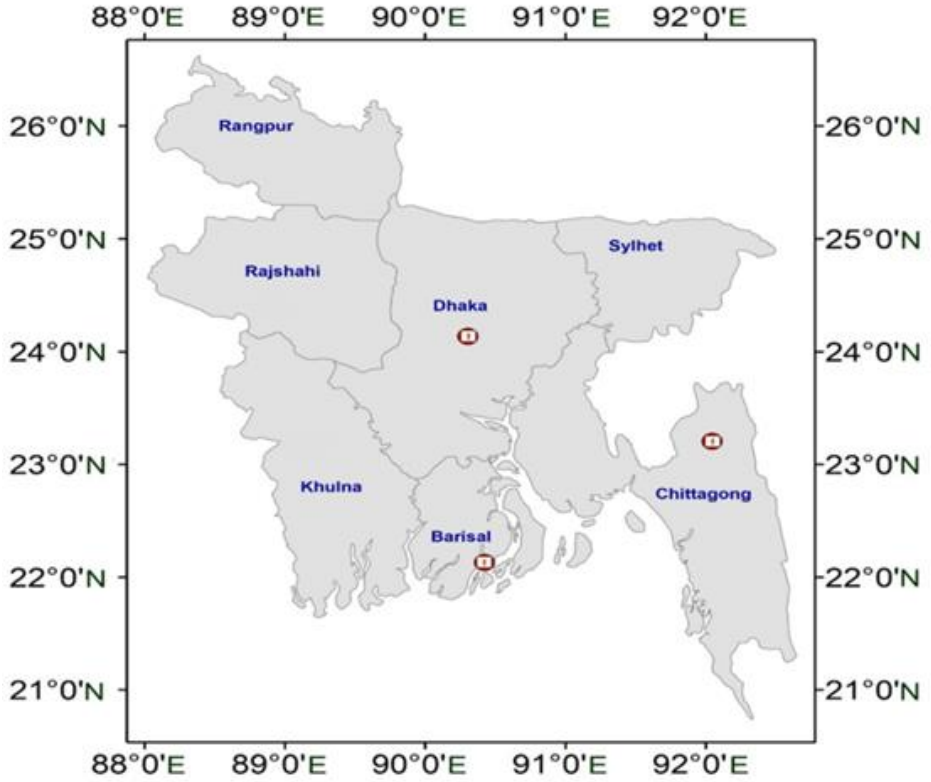

Figure 1: Map showing the different sampling locations of Bangladesh. Map was prepared based on the coordinate of location using ArcGIS 10.0

2.2 Study Species and Collection Design

The areas were surrounded by traps that were hung 10m apart from each other. Methyl Eugenol (ME) and Cue types of lure (CUE) were used for trapping adult fruit flies. The lure was exchanged after five days. Adult fruit flies were removed from traps every day. Larvae from guava were collected from three locations in two divisions. The collected samples were counted and put into small tubes with 100% alcohol.

2.3 DNA Extraction, Barcodes Amplification by PCR and Sequencing of Adult Flies

A total of 12 adult individuals from three locations were used for species identification. DNA was extracted from the leg of adult fruit fly using the commercial DNA mini kit, TIANGEN Total DNA Kit (China) followed by the instructions of manufacturing company. DNA quality and concentration was tested by a Quawell UV-Vis Q5000 spectrophotometer (Quawell Technology Inc., San Jose, CA, USA). Only the good quality of DNA samples was used for sequencing. The universal COI gene primers LCO-1490 (5'-GGTCAACAAATCATAAAGATATTG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') were used to amply the DNA (Former, 1994). Polymerase chain reaction (PCR) was completed in a final volume of 50 µl containing 25 µl of 2×Taq Master Mix, 2 µl of forward and 2 µl of reverse primers, 19 of µl sterilized distilled water (ddH$_2$O) and 2 µl of DNA template. The reaction condition was described as follows:94˚C for 3 minutes, followed by 35cycles of 94˚C for 1 minute, 53˚C for 1 minute, and 72˚C for 1 minute and then a final incubation at 72˚C for 10 minutes. The reaction was performed on Veriti TM 96 –well Thermal Cycler (ABI, USA). The remaining DNA samples of all specimens were preserved at -80˚C in Plant Quarantine and Invasion Biology Lab (CAUPQL) in the Department of Entomology of China Agricultural University. The PCR products were purified and sequenced in both directions (forward and reverse) by BGI, Beijing, China.

2.4 Sequences Analysis

DNAMAN 5.2.2.0 was used for sequence assembly, and further analysis was done for the low-quality sequence. The multiple alignments of sequences were done by MEGA 7. For consistency with the standard sequences for species identification, sequences of *COI* (barcoding region) were submitted to the BOLD system.

2.5 The Neighbour-Joining Tree

The neighbour-joining tree (NJ) was constructed for phylogenetic analysis of the sequence of samples of related species and some sequences derived from the Gene Bank database to verify the above identification. MEGA (version 7) was used to build the neighbor-joining (NJ) tree with NJ method and Kimura 2-Parameter (Hebert, 2003a). Kimura -2 parameter was also determined using MEGA version 7 for computing the pairwise genetic distance. The associations were calculated on the basis of genetic distances.

2.6 Species-Specific Gene Amplification and Gel Electrophoresis for Larvae

Three larvae were used for identification. DNA was extracted from larvae using the commercial tissue/cell DNA mini kit, TIANGEN Total DNA Kit (China). DNA samples of all specimens were deposited at -80°C in Plant Quarantine Lab in the Department of Entomology of China Agricultural University. Species-specific primer of 10 species was used for larvae identification (Table 1), which were collected (Jiang, 2015). Primers were synthesised by Tsingke Biotechnology Co, Ltd, Beijing, China. Polymerase chain reaction (PCR) was completed in a final volume of 50 µl containing 25 µl of 2×Taq Master Mix, 2 µl of forward and 2 µl of reverse primers, 19 of µl sterilized distilled water (ddH2O) and 2 µl of DNA template. Reaction condition was described as follows: 95˚C for 3 minutes, followed by 30 cycles of 95˚C for 15 seconds, 60˚C for 1 minute, and then a final incubation at 60˚C for 1 minute. A total of 6 µl PCR product was used for electrophoretic analysis to get the specific band for species identification. D2000 marker and 6 µl PCR product was poured into 1.5 agarose gel with a dye and was run 1x TAE buffer with 120V for 30 minutes. The result was observed under the UV light of gel imaging system.

Table 1: Species-Specific Primer List of Economically Important Fruit Flies in This Study

SL No	Fruit Fly Species	Code	Primer Sequence (5` -3`)	Length (bp)	Max TM
1	*B. correcta*	BBCOF	TGACTTGTCCCCCTAATACTG	21	65.6
		BBCOR	GTCGATCGCATGTTAATAACG	21	
2	*B. dorsalis*	BBDF	GCTATTTTTTCACTTCACTTAACG	24	60.2
		BBDR	AGTATTTAAGTTTCGGTCTGTTAG	24	
3	*B. latifrons*	BBLF	CGAATAAACAATATAAGATTTTGG	24	61.4
		BBLR	GTGATGAAGTTAACTGCTCCTAAG	24	
4	*B. tryoni*	BBTF	ATTAATCGGAGACGATCAG	19	61.7
		BBTR	AGCTAAATCAACTGAAACC	19	
5	*B. zonata*	BBZR	ACTTGTTCCCCTAATATTAGGAACC	25	64.1
		BBZF	TGTTAATACAACTGCTCAGACGAAG	25	
6	*B(Z) bezziana*	BZBF	CTCCTGATATAGCATTCACC	20	59.0
		BZBR	AAGTATAGTGATAGCTCCAACC	22	
7	*B(Z) cilifera*	BZCIF	GGCTGTAAATTTTATCACTACAGTC	25	64.4
		BZCIR	CGGTCTGTCAAAAGTATAGTAATG	24	
8	*B(Z) cucurbitae*	BZCUF	GGAGATGATCTAATCTATAATGTC	24	63.1
		BZCUR	GCTCAAACGAATAAAGGTAAC	21	
9	*B(Z) scutellata*	BZSF	CTCGGAGCCCCAGATATAACC	21	65.3
		BZSR	GGGCTGTTAATACTACTGCTCAG	23	
10	*B(Z) tau*	BZTF	GGAGCACCAGATATAGCG	18	63.0
		BZTR	GGTATTCGGTCAAATGTAATC	21	

3. RESULTS

3.1 Collection Information of Adult Samples in Different Locations of Bangladesh

From the three different locations of Bangladesh, a total of 1897 fruit fly individuals were collected (Table 2). Based on morphology, 1291 individuals belonged to *Bactrocera,* and606 individuals were *Zeugodacus.* The genus *Bactrocera* was found more in all regions (Table 2).

Table 2: Collection Information of Adult Samples From Bangladesh In This Study

Date Of Collection	Locations			Lure Type	Caught Genus	Number of Specimens
	Name	Latitude	Longitude			
December/18	Dhaka	24.134	90.3035	ME	*Bactrocera*	287
				CUE	*Zeugodacus*	133
June/2018	Barisal	22.128	90.423	ME	*Bactrocera*	452
				CUE	*Zeugodacus*	161
June/2018	Chittagong	23.203	92.0443	ME	*Bactrocera*	552
				CUE	*Zeugodacus*	312

Table 3: Collection Information of Larvae Samples from Bangladesh in This Study

Date of Collection	Location			Host	Number of Specimens
	Location	Latitude	Longitude		
June/2019	Madaripur	22.128	90.423	Guava	110
	Bakerjong	--	--	Guava	91
June/2019	Chittagong	23.203	92.0443	Guava	96

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3.2 DNA Sequences and Analysis of DNA Sequences

Each individual provides a high level of DNA quality and a fragment of ~700bp produced by PCR amplification with universal COI gene primers. PCR amplification was accomplished successfully, and 700bp COI gene sequence was obtained (Figure 2).

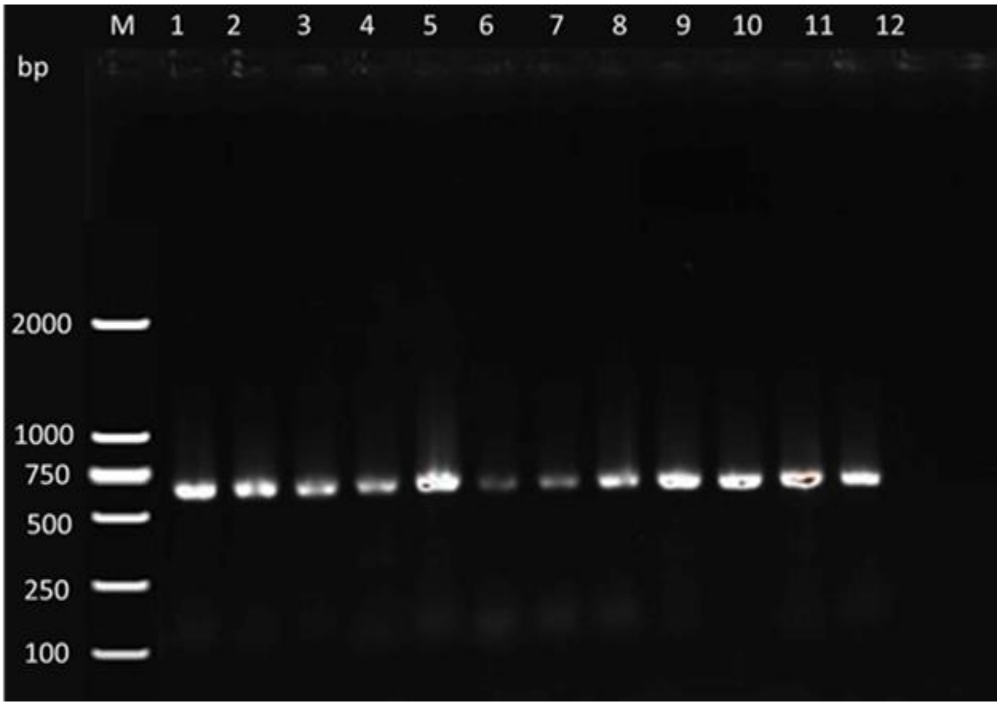

Figure 2: Test of PCR result on *Bactrocera* and *Zeugodacus* by gel electrophoresis. Line 1-4, *Bactrocera* spp.; line 5-12, *Zeugodacus* and line M: D2000 Marker.

3.3 Analysis of DNA Sequences

Sequences of 9 individuals (1 from each location) were obtained from the three locations (Dhaka, Chittagong and Barisal) of Bangladesh, and their similarity was compared with standard sequences of the Life Data System Barcode (BOLD) for species identification. Three identified species/location was presented in Table 4. Compared with all the COI sequences in BOLD, sequence similarity between species of *B. dorsalis*100%, *Z. cucurbitae* 100% *and Z. tau* 100%.

Table 4: Identified adult fruit fly species from different location of Bangladesh

	Samples Id	Location	Identified Species	Similarity (%)
1	BDK1	Chittagong	*Bactrocera dorsalis*	100
2	BDK2	Chittagong	*Zeugodacus tau*	100
3	BDK3	Chittagong	*Zeugodacus cucurbitae*	100
4	BDD1	Dhaka	*Bactrocera dorsalis*	100
5	BDD2	Dhaka	*Zeugodacus tau*	100
6	BDD3	Dhaka	*Zeugodacus cucurbitae*	100
7	BDB1	Barisal	*Bactrocera dorsalis*	100
8	BDB2	Barisal	*Zeugodacus tau*	100
9	BDB3	Barisal	*Zeugodacus cucurbitae*	100

BDK= Chittagong (East part), BDD= Dhaka (Centre part), BDB= Barisal (South part),

3.4 Neighbor-Joining (NJ) Tree

The data were presented in the neighbour-joining (NJ) tree only for the sole purpose of clustering species on a distance basis based on sequence similarity rather than character-based sequence clustering. From the NJ tree, it was calculated that the obtained species was similar to the species obtained from NCBI of the same species (Figure 3).

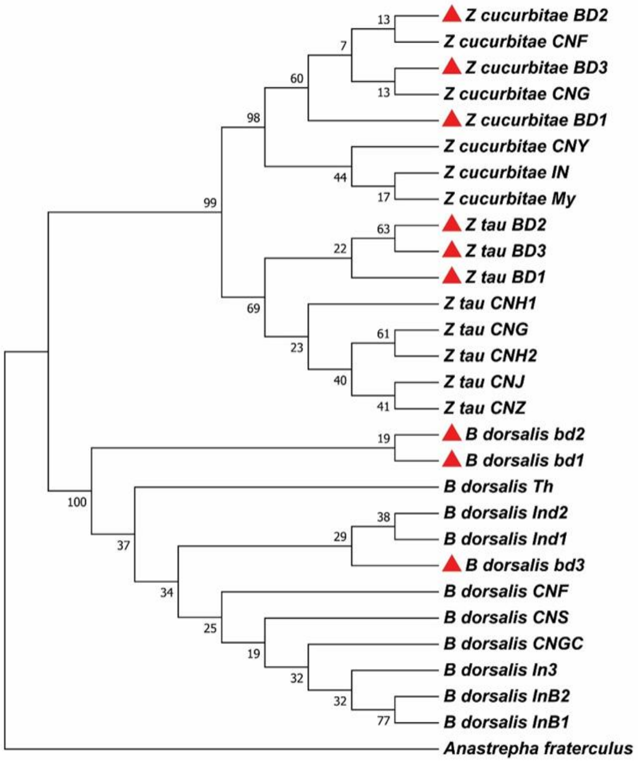

Figure 3: Neighbor Joining (NJ) tree developed from COI barcoding analysis showing the phylogenetic position of 9 fruit fly species *B. dorsalis*, *Z. cucurbitae*, *Z. tau* and Anastrepha *frateculus. Anastrepha frateculus* was out of the group. Percent bootstrap value of 1000 replicates.

3.5 Larvae Identification by Species-Specific Primers

According to the presence or absence of a band in the gel profile, the specificity and sensitivity of particular primer were tested. A total of 10 species-specific primers were used to identify the larvae from guava. 250 bp fragment was amplified inline - 2 indicated that *B. dorsalis* was found in the larvae, collected from guava in different locations (Figure 4. A-C).

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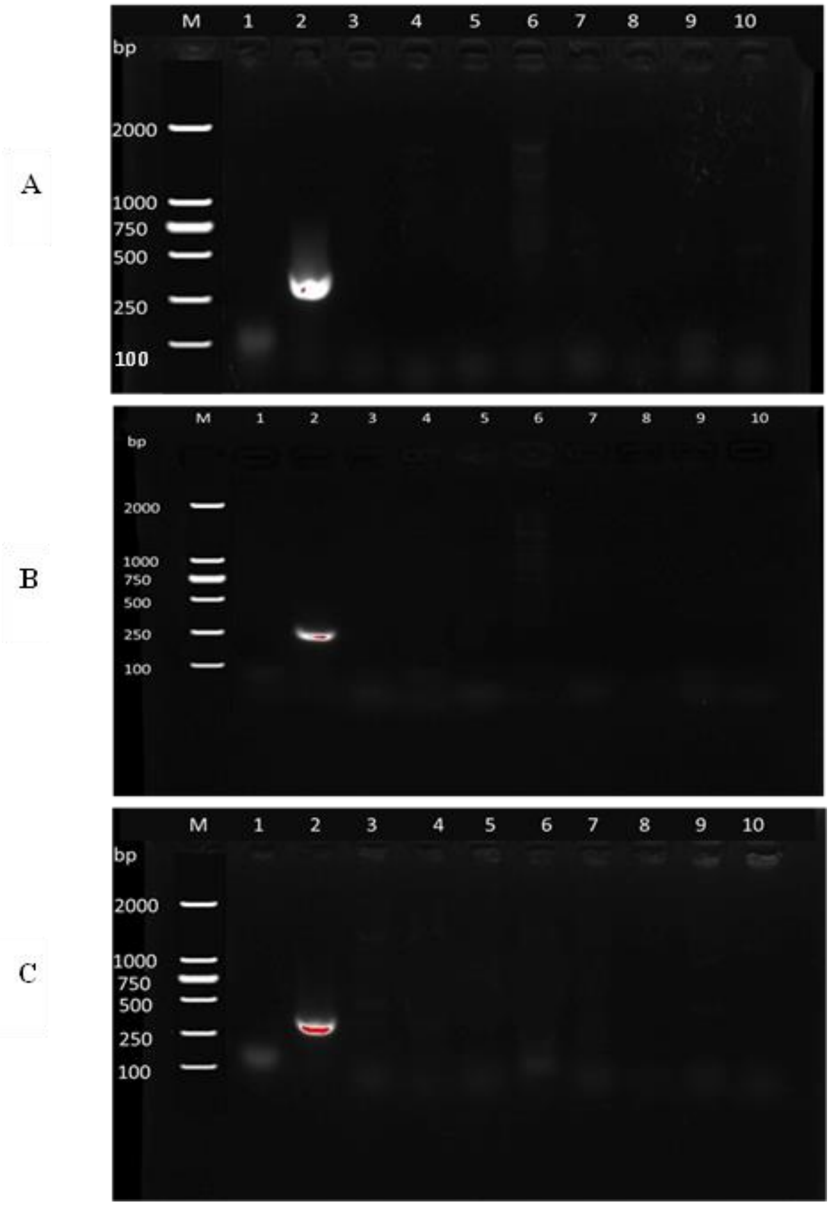

Figure 4: Diagnosis of larvaebased on species specific primer from guava fruit. A. Chittagong sample, B. Madaripur sample, 3. Bakergonj sample. Line 1 is B. correcta, Line 2 is B. dorsalis, Line 3 is B. latifrons, Line 4 is B. tryoni, Line 5 is B. zonata , Line 6 is Z. bezziana, Line 7 is Z.cilifera, Line 8 is Z.cucurbitae, Line 9 is Z.scutellata, Line 10 is Z.tau and line M:D2000 Marker

4. DISCUSSION

The morphological characteristics of adult insects may be difficult to separate *Bactrocera* sibling species or the identification of arrogant stages (White et al., 1997). Molecular dedication of fruit flies would have been an advantage in such instances (Greenstone, 2006). A group researcher proposed a technique for amplifying a 648 bp region of the mitochondrial cytochrome-c oxidase subunit 1 (*COI)* gene to ensure rapid and accurate identification of a wide range of biological specimens (Hebert et al., 2003 a, b). The Life Barcode project was then introduced to encourage DNA barcoding as a global standard for sequence-based eukaryote identification. In the present study for the identification of adult fruit fly species, the DNA barcoding technique (mt DNA *COI*) was used. Several studies have been reported. A studied the *COI* sequences of fruit fly samples collected from New Zealand ports, and the findings were compatible with the previous results of the constraint fragment duration polymorphisms (RFLPs) (Armstrong et al., 2005).

However, identification using DNA barcodes detected species recognized RFLP analysis could not remember (Armstrong et al., 2005). The DNA barcoding (amplification of the sequence by COI gene) method was applied to identify the larvae collected from guava fruit of Thailand, resulting in the identification of *B. correct* (Buahom et al., 2011). Some researchers only differentiated four species of *Anastrepha* (*Anastrepha grandis*, A. *serpentine*, *A. ludens*, and *A. striata*) based on barcode data, though they used 539 DNA sequence from 74 species (Barr et al., 2017). Out of 10 species of *Bactrocera,* eight species were easily distinguished by based on the standard DNA barcoding region of the *COI* gene. Bangladesh is divided into eight divisions (Manger et al., 2018). The south part is comprising of Khulna, Barisal and Chittagong division. Khulna division is famous for mainly vegetables and some parts have guava orchard. The Barisal division is famous for guava production.

The Chittagong division is the only hilly area of Bangladesh, famous for mango and citrus production. Vegetables also have been grown in some parts of this region. In the central part (Dhaka division), some commercial mixed fruits orchards and vegetable farms are also present. During the sample collection period of this study, only guava fruits and some summer vegetables were presented in the field. Only two genera of fruit fly were collected, of which *Bactrocera* was found to bethe higher in number in all locations. Alam reported 10 tephritids fruit flies from Bangladesh (East Pakistan) (Alam, 1967). A group researchers recorded 11 tephritids fruit flies from Bangladesh (Kapoor et al., 1980). In some study, they identified 15 species of fruit fly belonging to *Bactrocera* (6), *Daculus* (1), *Hemigymnodacus* (1), *Parasinodacus* (1), *Sinodacus* (1), *Dacus* (3)and *Zeugodacus* (2) during one-year survey of different locations (field, orchard, forest and rural areas) of Bangladesh.

Leblanc and his colleagues again did a six-year survey, including most of Bangladesh and found 29 fruit fly species, of which 13 species were considered pests and 16 were non-pest species (Leblanc et al., 2019). In this study three species, *vizB. dorsalis, Z. tau,* and *Z. cucurbitae* were identified from different areas of Bangladesh in a short survey (June and December 2018). The report of the molecular identification of fruit flies in

Bangladesh is limited. Recently, a six-year survey (2013-2018) in the different locations of Bangladesh found 13 pest species of fruit flies, of which *B. dorsalis* found to be the dominant species followed by *Z. tau*, *Z. cucurbitae* and *B. zonata* (Leblanc et al., 2019). But in this study, *B. zonata* did not collect. The lower number of identified species may be due to the short period of the survey, seasonal variation, host unavailability in the crop field or orchard and the locations.

Some researchers conducted a two-year survey (November 2017 to October 2019) in the different agricultural areas of Bangladesh and found 13 pests and non-pests' species, of which three pest species, *viz. B. dorsalis* (58%), *Z. tau* (13.5%) and *Z. cucurbitae* (23.6%) were found to be dominant (Hossain et al., 2019). They also noted that *B. dorsalis* was higher in wet summer months (May to August), whereas *Z. cucurbitae* was higher in March and May. In this study, found only two genera may be due to the seasonal variation and host availability in the field and maybe the selection of farmers' field or commercial and research orchard and the short time of the survey. For insect's host identification, the identification of larvae is the best option.

Being the higher production and demand of guava in Bangladesh, larvae from this fruit of three different areas were examined. *B. dorsalis* was identified from the guava of all three locations, seems that guava is one of the hosts of *B. dorsalis* in Bangladesh. Our finding is disagreed with the study (Leblanc et al., 2013). They found that the peach fruit fly, *B. zonata* is a serious pest of a variety of fruits especially in mango, carambola and guava in Bangladesh. But in our neighbor country India, the pest status of *B. zonata* is considered equal to or greater than that of the Oriental fruit fly (*B. dorsalis*) and they may overlap in the same crop (Kapoor, 1993). So, in near future *B. dorsalis* may be severe problem in our agriculture.

5. CONCLUSION

Bangladesh is very small but agro-based country in South Asia. Fruit fly causes serious damage to several vegetables and fruit crops every year. Survey is very important for identification of insects. Long time, cover the most of locations and season wise survey is effective for studying insects in a country that will be included in future research. Molecular identification is an effective method, as it can diagnosis very accurately and rapidly. Identification is very important to take proper and timely control measures; however, molecular identification is limited in Bangladesh. Therefore, this study could be the foundation of future insect identification research in Bangladesh. In this study, B. *dorsalis, Z. tau*and *Z. cucurbitae* were found in the three locations. *B. dorsalis* was identified from guava in all three locations. So it needs to pay more attention to managing these harmful pest species and saving the crops; results will increase the production.

FUNDING

This work was supported by the Natural Science Foundation Project of China (No. 31972341).

ACKNOWLEDGEMENTS

We thank, S.M. Kamrul Hasan Chowdhury, Bangladesh Agricultural Research Institute, Bangladesh. We also thank the other members of Plant Quarantine and Invasion Biology Laboratory, China Agricultural University (CAUPQL).

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Cite The Article: Sultana Afroz, Md Shibly Noman, Yue Zhang, Md Yousuf Ali, Md Rubel Mahmud, Zhihong Li (2022). Species Identification of Economic Important Adult Fruit Flies Based on DNA Barcoding (*MT DNA COI*) and Larvae Based on Species Specific Primers from Central and South Parts of Bangladesh. *Malaysian Journal of Sustainable Agricultures, 6(2): 110-116.*