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Molecular Identification and Characterization of Fruit Flies of Genus *Bactrocera* (Diptera: Tephritidae) in Pakistan

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JNA, TS and SJNA collected samples, performed experiments and wrote the article. JNA analysis the data. SM, FZ, TS and SJNA reviewed the data.

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ABSTRACT

Molecular identification and characterization of tephritid fruit flies (Diptera: Tephritidae), *Bactrocera zonata, Bactrocera dorsalis* and *Bactrocera cucurbitae* was done in Punjab, Pakistan through PCR analysis using mitochondrial cytochrome oxidase I (COI) gene based primers. Phylogenetic analysis was also complemented to differentiate fruit flies identified from other countries of the world. The sequencing results and phylogenetic analysis of collected specimens indicated that sequences of *B. dorsalis, B. cucurbitae* and *B. zonata* have 99-100% similarity with fruit flies reported from other countries. This is the first report of molecular identification and characterization of Tephritid fruit flies infesting fruits in Punjab, Pakistan.

INTRODUCTION

The fruit flies belonging to the family Tephritidae are almost present worldwide in all major fruit and vegetable growing area (Clarke *et al*., 2005) causing a high potential damage to vegetable and fruit crop production (White and Elson Haris, 1992). In Indian subcontinent, nearly 325 species of fruit flies are present, of which 205 are from India (Kapoor, 2005). The fruit fly (*Bactrocera* spp.) is the most dangerous insect pest and a major yield limiting factor that causes 13.40 to 46.60% weight loss and 16 - 40% loss (Hasseb, 2011; Ukey *et al*., 2012). The most common fruit flies, *Bactrocera dorsalis, B. zonata* and *B. cucurbitae* belonging to genus *Bactocera* are the major widely occurring insect pests while *B. correcta*, *B. diversa* and *B. latifrons* are still confined to their place of origin (Kapoor, 2005). In Pakistan, fruit flies are pests of vegetable and fruit crops particularly for guava where *B. zonata* alone causes damage up to 5 to 100% (Syed *et al*., 1970). The main damage is caused during the development of larvae inside the fruit (Stonehouse *et al*., 2005) andtheir control is difficult by adopting a single control measure methodology (Dhilon *et al*., 2005). The morphological identification and separation of immature stages, adults or sibling species of fruit flies using diagnostic taxonomic keys require tedious procedure of microscopic observation of male genitalia of adults and preparation of samples (White and Hancock, 1987; Pogue, 2002). Further, for females or immature stages, unambiguous keys are not frequently available and substantial host overlapping and attraction to specific pheromone confined the use of this technique (Meagher *et al*., 2008). The identification and differentiation of *Bactrocera* complexes is of utmost importance, therefore, DNA barcoding has been proposed to identify known species from single specimens (Hebert *et al*., 2003) based on cytochrome oxidase I (COI) gene (Nagoshi and Meagher, 2003; Manzoor *et al*., 2018). The objective of this research was to confirm the identification of fruit fly by DNA barcoding using mitochondrial COI gene.

MATERIALS AND METHODS

Methyl eugenol and cure lure treated cotton wicks were placed in fruit fly traps installed during 2017-2018 in a guava orchards surrounding with mango fields at Post Graduate Agriculture Research Centre (PARS), University of Agriculture Faisalabad. The adult specimens of three *Bactrocera* spp. were collected from infested fruits of guava in the same experimental farm. Before proceeding with the molecular identification, adult fruit flies were observed under light microscope (Micros, Austria) connected with camera (Canon EOS 750 D, USA). In addition, CD-ROM computer based tool from Lawson *et al*. (2003) was compared with those described in taxonomic keys of Drew

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and Hancock (1994) and Lawson *et al*. (2003). Some of the collected specimens were preserved in 75% alcohol or either stored at −20 °C in a freezer for molecular study. The *Bactrocera zonata* (Saunders), *Bactrocera dorsalis* (Hendel) and *Bactrocera cucurbitae* (Coquillett) species of fruit flies collected from orchards were investigated.

Total genomic DNA from adult body (legs) of fruit fly was extracted with slight modifications to increase DNA yield (Ahmad *et al*., 2017). Mitochondrial cytochrome oxidase I (mtCOI) based primers were employed for polymerase chain reaction (PCR) in PCR machine (PeqSTAR, Germany) with primers LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′)andHCO -2198(5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) according to described conditions (Fleming *et al*., 2000; Ahmad *et al*., 2018). PCR amplified products were stored at -20 °C till gel electrophoresis and sequencing.

The amplified PCR products were tested for the confirmation of genomic DNA presence using gel electrophoresis in 1.5.0% agarose gel stained with ethidium bromide. The size of DNA fragments from fruit fly samples was estimated by comparing with 1 kb DNA marker (GeneMark-Korea). The amplified band corresponding to the target PCR product was documented using SYNGENE Gel documentation system under UV light.

The 710bp PCR products were sequenced directly in both directions using COI forward and reverse primers and analyzed using Lasergene v. 7.1 software package (DNASTAR, USA). The sequences were further aligned with CLUSTAL OMEGA multiple sequence alignment tool. The comparison of obtained sequences with sequences available in GenBank was accomplished using BLAST service available at http://www.ncbi.nlm.nih.gov:80/BLAST. Phylogenetic studies were performed with MEGA6 software employing the "Maximum Likelihood method" (Nei and Kumar, 2000; Tamura *et al*., 2013). The reference accession numbers of NCBI database sequences for *B. dorsalis* (KF998634.1, KF801366.1, KF801377.1, KF998789.1, KF318566.1), *B. zonata* (KP296150.1, KP851002.1, KT151121.1, KX758098.1, JX965418.1, KJ753909.1, KF801374.1) and *B. cucurbitae* (KJ753953.1, KJ753952.1, KT151115.1, JX129494.1, JX129494.1, KY113315.1, KY113291.1, KY113283.1) were used in construction of ML tree to differentiate Pak fruit flies samples (*B. cucurbitae* (MK296116)*; B. zonata (*MK296117 (C) *B. dorsalis* (MK296118).

RESULTS AND DISCUSSION

Total of the 1,738 specimens collected from guava and mango orchards in Punjab, the morphological studies of the species using the light microscope revealed that 1,085 samples belonged to *B. zonata,* 446 to *B. cucurbitae* and 207 to *B. dorsalis* (Supplementary Fig. 1). The melon fly, B. cucurbitae, is larger than housefly and is principally an Asian species that can be distinguished morphologically by the presence of midial vita on scutum and apical spot, complete costal band and marking on R-M and Dm-Cu cross veins on wings (Supplementary Fig. 1). *B. zonata* is differentiable from the other species by the absence of central vita on scutum and by only narrow yellow lateral vita on each side of thorax. It is usually about the size of housefly and on wings; the costal band area is reduced to an apical spot and has no microtrichia in the narrowed basal area of cell br. There is usually a presence of one pair of dark marks on tergite III with no medial dark line except on tergite V (Supplementary Fig. 1B). *B. dorsalis* has a clear T shaped dark mark on the abdomen and continuous apical costal bands on the wings with two yellow stripes on thorax (Supplementary Fig. 1A). Supplementary Figure 2 shows the amplified products (710 bp) of COI of 9 DNAs of fruit flies.

The PCR products (710bp) were sequenced and aligned with a multiple sequence alignment tool (Supplementary Fig. 2). The result showed the highest percentage of nucleotides identity (99-100%) of the current studied NCBI submitted nucleotides sequences of *B. cucurbitae* (MK296116) *B. zonata* (MK296117) and *B. dorsalis* (MK296118) with NCBI available 3 *Bactrocera* sequences KJ753953.1, KP296150.1 and KF998634.1 respectively (Fig. 1). The99-100% identity of studied *B. dorsalis* DNA sequences with maximum score coverage (98-99) for *B. dorsalis* submitted sequences *(*KF998634.1, KF801366.1, KF801377.1, KF318566.1) was obtained (Table 1). The results also showed that identity of *B. zonata and B. cucurbitae* was 99% with the submitted sequences of *B. zonatas* (KP296150.1, KP851002.1, KT151121.1, KX758098.1, JX965418.1) and *B. cucurbitae* (KJ753953.1, KJ753952.1, KT151115.1, JX129494.1, JX129494.1, KY113315.1) whereas 94-93% similarity was observed for *B. correcta* (KJ753909.1), and *B. dorsalis* (KF801374.1), respectively (Table 1). The DNA sequences of studied *Bactrocera* species exhibited the same clade in phylogenetic tree whereas different clades were observed by different species of this genus (Fig. 2). The genetic evolutionary divergence among the studied species of Pakistan showed little genetic variation with closely related species whereas it exhibited increasing pattern of divergence for other species of genus *Bactrocera* as indicated in phylogenetic tree branches (Fig. 2). Molecular identification and differentiation of fruit flies have been reported, using mitochondrial DNA, 18 *Bactrocera* spp. except 2 (*B. carambolae* and *B. papaya*) and 3 (B. *dorsalis, B. correcta* and *B. zonata*) have

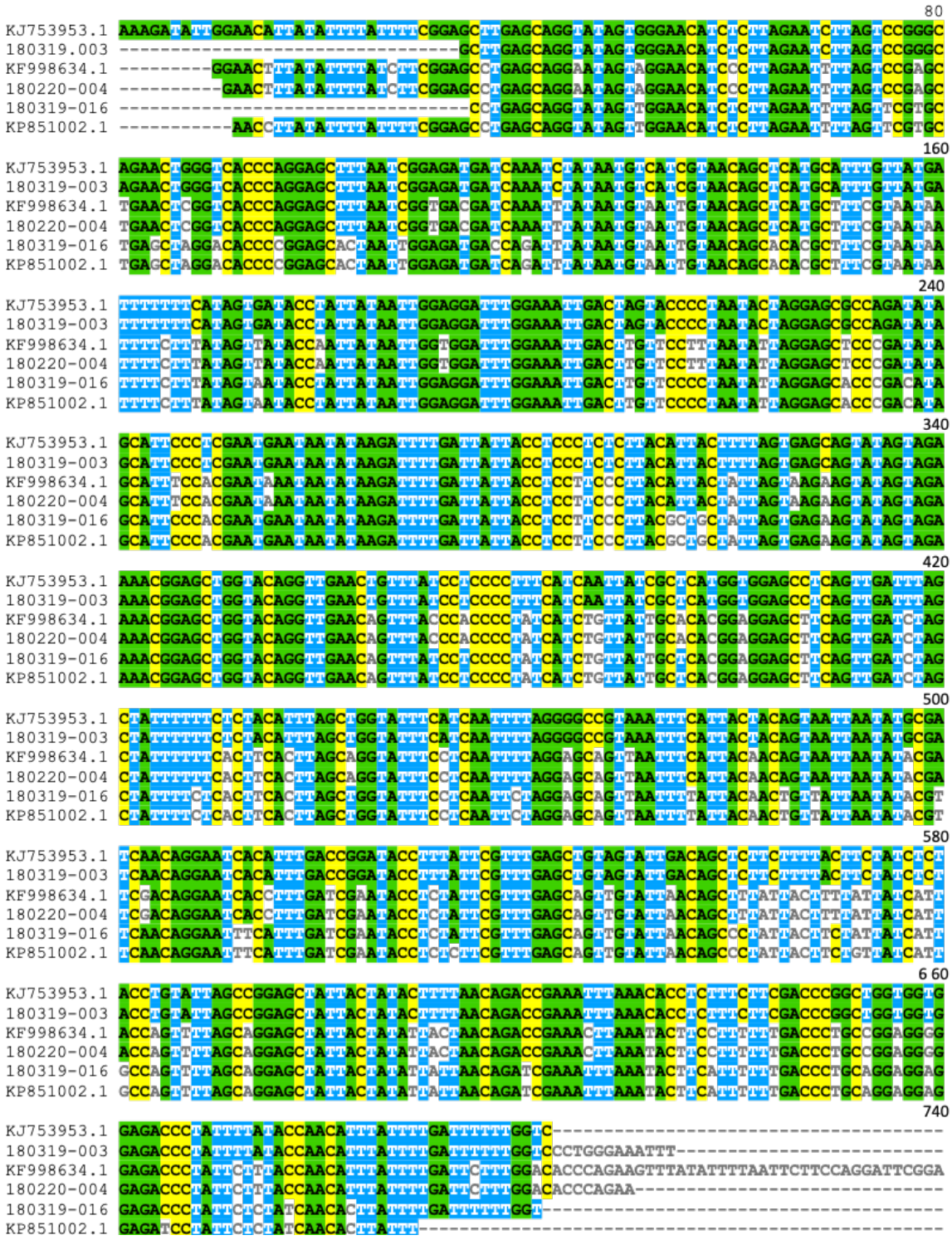

Fig. 1. Multiple alignments of DNA sequences through CLUSTAL OMEGA from DNA amplification of 3 Pakistani *Bactrocera* species, *B. cucurbitae 180319-003 (*MK296116) isolate FSD-03, *B. dorsalis 180319-004 (*MK296118)isolate FSD-18 and *B. zonata 180319-016 (*MK296117) isolate FSD-16 with NCBI GenBank sequences, *B. Cucurbitae* (KJ753953.1), *B. dorsalis* (KF998634.1) and *B. zonata* (KP851002.1)

Table 1.- Comparison of the fragments of the cytochrome oxidase I (COI) gene of *Bactrocera* species collected from guava and mango orchards of Punjab, Pakistan with those available in the GenBank.

Pakistani strains	*Bactrocera* species	Identity	Accessions numbers
MK296116 *B. cucurbitae* isolate FSD-03	*Bactrocera cucurbitae* voucher GSY2	99%	KJ753953.1
	Bactrocera cucurbitae voucher GSY1	99%	KJ753952.1
	Bactrocera cucurbitae voucher GB245247	99%	KT151115.1
	Bactrocera carambolae isolate FF13_	99%	JX129494.1
	Bactrocera cucurbitae isolate UKM000163	99%	JX129494.1
	Zeugodacus cucurbitae isolate KD708	99%	KY113315.1
	Zeugodacus cucurbitae isolate TL2815	99%	KY113291.1
MK296117 *B. zonata* isolate FSD-16	*Bactrocera zonata* mitochondrion	99%	KP296150.1
	Bactrocera zonata mitochondrion	99%	KP851002.1
	Bactrocera zonata voucher GB170	99%	KT151121.1
	Bactrocera zonata isolate 32 hasa1	99%	KX758098.1
	Bactrocera zonata isolate tao	94%	JX965418.1
	Bactrocera correcta voucher FSL5	93%	KJ753909.1
	Bactrocera dorsalis isolate BX120711-056	91%	KF801374.1
MK296118 *B. zonata* isolate FSD-18	*Bactrocera dorsalis* voucher DUHA1	100%	KF998634.1
	Bactrocera dorsalis isolate BX120426-003	99%	KF801366.1
	Bactrocera dorsalis isolate BX120711-138	99%	KF801377.1
	Bactrocera dorsalis strain SH2	99%	KF318566.1

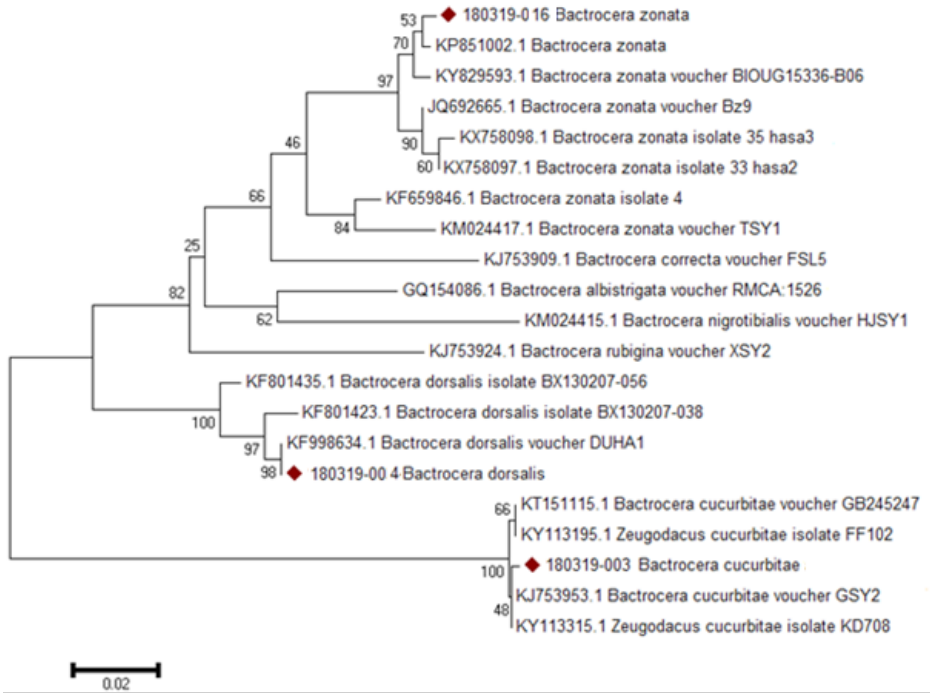

Fig. 2. Phylogenetic analysis by Maximum Likelihood Method produced from DNA sequences of 3 *Bactrocera* species using mitochondrial Cytochrome oxidase I (mCOI) gene based primers. (A) *B. cucurbitae 180319-003* (MK296116) (B) *B. zonata 180319-016 (*MK296117) (C) *B. dorsalis 180319-004 (*MK296118)

also been identified from other countries (Muraji and Nakahara, 2002; Asokan *et al*., 2007). Alignment of sequences of fruit fly species showed 92% similarity between *B. dorsalis, B. correcta* and *B. zonata.* Similarly, Chua *et al*. (2009) studied species identification of *Bactrocera* spp. using COI (F/R) and UEA (F/R) based primers and RFLP analysis. In Pakistan, only 3 *Bactrocera* species have been found in guava and mango orchards of Punjab, Pakistan as compared to many species reported from India.

CONCLUSION

The only three species belonging to the family Tephritidae (*B. dorsalis, B. correcta* and *B. zonata)* was found and collected from guava and mango orchards in Punjab. COI gene was successfully used for their identification and characterization of *Bactrocera* spps.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2019.51.6.2275.2280

Statement of conflict of interest

The authors declare no conflict of interest.

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