Molecular identification of fruit flies, *Bactrocera* spp. (Diptera: Tephritidae) using mitochondrial cytochrome oxidase I

Identification of fruit flies using conventional taxonomy has certain limitations owing to homoplasmy on most morphological characters and difficulty in identification in egg and larval stages[1]. On the other hand, molecular identification augments conventional taxonomy, in which species identification is not limited by polymorphism, sex and stage of development of the target species. In molecular identification of fruit flies, microsatellites[1], internal transcribed spacer 1 (ITS1)[2], amplified fragment length polymorphism (AFLP)[3], nuclear gene *period*[4], 16S rRNA, 12S rRNA, mitochondrial cytochrome oxidase II (mtCOII) + $tRNA_{Lys}$ + $tRNA_{Asp}$[5], etc. have been employed as markers. Presently, the Consortium for the Barcode of Life (COBL) advocates the use of mitochondrial cytochrome oxidase I (mtCOI)[6] for species identification, as it exhibits reliable inter-species variation[7]. Here we report the mtCOI-based identification of three fruit flies, *Bactrocera dorsalis* (Hendel), *B. correcta* (Bezzi) and *B. zonata* (Saunders), important pests of mango (*Mangifera indica* L.) and guava (*Psidium gujava* L.)[8].

The above three species of fruit flies were collected from the infested fruits of mango and guava in the experimental farm of the Indian Institute of Horticultural Research (IIHR), Bangalore. Morphological identification of the above three species was carried out prior to molecular studies. DNA was isolated from single adult female of each of the above three species using CTAB method[9] and 2 µl was made use of as template for PCR reaction.

PCR was carried out in a thermal cycler (Primus 96; MWG Biotech, Germany) with the following cycles: 94°C for 3 min as initial denaturation followed by 40 cycles of 94°C for 30 s, 53°C for 45 s, 72°C for 1 min and 72°C for 20 min as final extension. Primers specific to mtCOI, viz. mtD7.2F – 5′ATT AGG AGC HCC HGA YAT AGC ATT3′ and mtD9.2R – 5′GAG GCA AGA TTA AAA TAT AAA CTT CTG3′ resulting in the amplification of approximately 500 bp fragment were used in the present study[10]. PCR was performed in a 25-µl total reaction volume containing 20 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP and 0.5 U of *Taq* polymerase (Fermentas, GmBH, Germany). The amplified products were resolved in 1.5% agarose gel, stained with ethidium bromide (10 µg/ml) and visualized in a gel documentation system (UVP, UK).

The PCR-amplified fragments were eluted using Perfect prep® gel clean-up according to the manufacturer's protocol (Eppendorf, Germany) and ligated into the general purpose cloning vector, InsT/Aclone™ (Fermentas, GmBH, Germany). 5 µl of the ligated vector was cloned into 200 µl of competent *Escherichia coli* (DH5α) cells by heat treatment at 42°C for 45 s and the whole content was transferred into a tube containing 800 µl of SOC (tryptone – 2% w/v, yeast extract – 0.5% w/v, NaCl – 8.6 mM, KCl – 2.5 mM, $MgSO_4$ – 2.0 mM, glucose – 20 mM in 1000 ml water, pH 7.0) and rotated at 150 rpm at 37°C for 1 h. 200 µl of the above culture was spread on Luria Bertani agar (LBA) (tryptone – 10 g, yeast extract – 5 g, NaCl – 5 g, agar – 15 g in 1000 ml of water, pH 7.0) containing ampicillin (100 µg/ml), IPTG (4 µg/ml) and X-gal (40 µg/ml) and incubated at 37°C for 16 h. Blue/white selection was carried out and all the white colonies (cells harbouring the insert) were maintained on LBA containing ampicillin (100 µg/ml), incubated at 37°C overnight and stored at 4°C until further use.

Plasmids were prepared from the overnight culture of the positive colonies cultured in LB broth (enzymatic casein – 10 g, yeast extract – 5 g, NaCl – 5 g in 1000 water, pH 7.0) using modified alkali lysis method[11]. Plasmids were resolved in 1.0% agarose gel, stained with ethidium bromide (10 µg/ml) and visualized in a gel documentation system (UVP, UK). For sequencing, plasmids were isolated using plasmid kit mini (Qiagen, Germany) according to the manufacturer's protocol, from overnight cultures of the five selected clones multiplied in LB broth overnight. Sequencing was carried out in an automated sequencer (ABI PRISM 310 Applied Biosystems, USA) using M13 universal primers, both in forward and reverse directions. Homology search was carried out using BLAST[12] and differences in mtCOI sequences of *B. dorsalis*, *B. correcta* and *B. zonata* were determined using the sequence alignment editor 'Bioedit'. Sequences for the above three species have been deposited with the NCBI database and the accession numbers are DQ838978, DQ838979 and DQ838980 respectively, for *B. dorsalis*, *B. correcta* and *B. zonata*. A cladogram was developed using 'Treeview'[13].

A single fragement of approximately 500 bp was amplified for *B. dorsalis*, *B. correcta* and *B. zonata* (Figure 1). Sequencing results showed that the total nucleotide length obtained was 440 bases, for all the three species of fruit flies. Alignment of the above sequences in Bioedit revealed that there was 92% similarity between *B. dorsalis* and *B. correcta* and also between *B. correcta* and *B. zonata*. The number of nucleotides that were different between *B. dorsalis* and *B. correcta* as well as between *B. correcta* and *B. zonata* was 32 and 28, respectively. Highest variation (11%) was observed between *B. dorsalis* and *B. zonata*, where there was difference in 45 nucleotides (Figure 2). BLAST search of the above three sequences showed that maximum similarity was observed for the respective species in the NCBI database. The dendrogram developed for the above three species of fruit flies showed that three of them formed a different clade (Figure 3). Molecular identification has corroborated the morphological identification in the present study. Molecular evolution and phylognetic relationship of tephritid fruit flies could also be eluci-

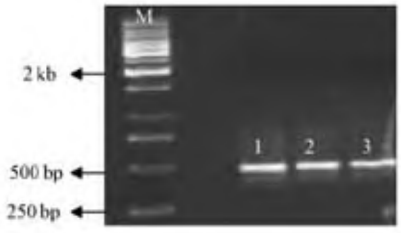

Figure 1. PCR amplification of DNA from *Bactrocera* spp. with primers specific to mtCOI. Lane M, 1 kb ladder; lane 1, *Bactrocera dorsalis*; lane 2, *B. correcta*, and lane 3, *B. zonata*.

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                          10        20        30        40        50        60        70        80
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B. dorsalis (DQ838978)    TCCACGAATGAATAATATAAGATTTTGATTATTACCTCCTTCCCTTACATTACTATTAGTAAGAAGTATAGTAGAAAACG
B. correcta (DQ838979)    .........A.........................C.....................................T.
B. zonata (DQ838980)      C........A.....................................GC.G........G................T.

                          90       100       110       120       130       140       150       160
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B. dorsalis (DQ838978)    GAGCTGGTACAGGTTGAACAGTTTACCCACCCCTATCATCTGTTATCGCACACGGAGGAGCTTCAGTTGACCTAGCTATT
B. correcta (DQ838979)    ............................T.................T..T.............G.....T.........
B. zonata (DQ838980)      ........................T..T.................T..T........G...........T.........

                        170       180       190       200       210       220       230       240
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B. dorsalis (DQ838978)    TTTTCACTTCACTTAGCGGGTATTTCCTCAATTTTAGGAGCAGTAAATTTCATTACAACAGTAATTAATATACGATCGAC
B. correcta (DQ838979)    ..C.....C........T..............................T..C.....C..T.....C..G........
B. zonata (DQ838980)      ..C..............C................C..........T.....T..C.....T..T.....C.....T..A..

                          250       260       270       280       290       300       310       320
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B. dorsalis (DQ838978)    AGGAATCACCTTTGATCGAATACCTCTATTCGTTTGAGCAGTTGTATTAACAGCTTTATTACTTTTATTATCATTACCAG
B. correcta (DQ838979)    ......TT.A.....C.....................................G...C........C......
B. zonata (DQ838980)      ......TT.A.....C...........C..............C...........CC........C..............

                          330       340       350       360       370       380       390       400
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B. dorsalis (DQ838978)    TTTTAGCAGGGGCTATTACTATACTACTAACAGACCGAAACTTAAATACTTCCTTTTTTGACCCTGCCGGAGGAGGAGAT
B. correcta (DQ838979)    ..........A.....C.........T.......T..............................A...........C
B. zonata (DQ838980)      .......T..A............T..T.......T.....T.C.........A..............A............

                          410       420       430       440
                          ....|....|....|....|....|....|....|....|
B. dorsalis (DQ838978)    CCTATTCTTTACCAACATTTATTTTGATTCTTTGGACACC
B. correcta (DQ838979)    ..A.....C........C.....................
B. zonata (DQ838980)      ........C..T.....C.....................
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Figure 2. Consensus sequences of 440 bp fragment from the *mtCOI* gene for *B. dorsalis* (DQ838978), *B. correcta* (DQ838979) and *B. zonata* (DQ838980). Dots indicate nucleotides identical throughout the species compared.

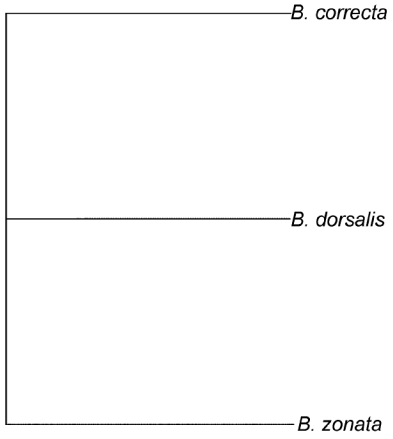

Figure 3. Rectangular cladogram for *B. dorsalis*, *B. correcta* and *B. zonata* based on the mtCOI partial sequence.

dated based on mtCOI[14]. Molecular identification is also a valuable tool when there is a problem of polymorphism, as it has been shown in the case of *Ceratitis capitata* (Wiedemann) and *Anastrepha fraterculus* (Wiedemann)[15]. In addition, molecular identification could also reveal multiple infestations as against the morphological identification, which could be used to separate the species[16].

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