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SHORT COMMUNICATION

Identification and differentiation of the cryptic cucurbit fruit fly species *Dacus frontalis*, *Dacus ciliatus* and *Bactrocera cucurbitae* (Diptera: Tephritidae) using PCR–RFLP for quarantine applications

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Abstract
Tephritid fruit flies attacking cucurbits are major agricultural pests that cause severe damage to their host plants, and are listed as quarantine pests in many countries. The classical morphological identification of Tephritid species is an arduous task due to presence of cryptic species complexes, as in those attacking cucurbits, and due to the reduced number of species-specific larval morphological characters. A quick and reliable species differentiation method is required to identify and prevent the establishment of invasive species in new territories. For this purpose, in this study, we developed a PCR–RFLP methodology to differentiate between three cucurbits Tephritid fruit fly species, namely *Dacus frontalis, Dacus ciliatus* and *Bactrocera cucurbitae,* which are hard to differentiate by morphological characters. The PCR–RFLP method is based on the mitochondrial COI gene barcoding region, which was successfully amplified in specimens from the three species. Sequence analysis revealed that the restriction enzyme *Rsa*I clearly separated -among the three target species either at the larval or adult stage. Hence, this method can be used to improve decision-making procedures at quarantine checkpoints, especially when only immature stages are present in each quarantined commodity, avoiding time-consuming rearing until adult emergence for morphological identification.

Keywords Tephritidae · Cucurbits · Invasive species · Species identification · mtCOI · PCR–RFLP

Background

Cucurbits are important agricultural products worldwide, generating high commercial value for agro-industry, and for small hold growers as cucurbits are considered as cash crops (Baloglu 2018). In the Mediterranean region, cucurbits are cultivated the year-round, in greenhouses and open fields given the moderate temperatures and mild winters becoming essential ingredients of the Mediterranean diet (FAO stat 2018; Mnari-Hattab et al. 2008, 2015).

Tephritid fruit flies (Diptera: Tephritidae) are key insect pests of several crops including cucurbits, causing direct (fruit damage and crop loss) and indirect (quarantine measures) economic losses. Many of these Tephritid key species are under area-wide control measures, including integrated pest management strategies to reduce the direct damages, and to comply with quarantine treatments that allow their international trade.

Tephritid fruit flies associated with cucurbits are particularly known for their high damage becoming all of them of quarantine status. The melon fly *Bactrocera* (*Zeugodacus*) *cucurbitae*, which is listed as quarantine pest in many countries, has a great invasive status (CABI 2020). This species is well distributed over whole India, which is considered its native region. Currently, it has been reported in several countries in East and West Africa. It has also been reported from Mauritius, La Réunion and the Seychelles islands (White et al. 2001; De Meyer and Ekesi 2016). The pumpkin Tephritid fruit fly *Dacus frontalis* (Becker) is widely distributed in the Middle East and in North Africa, where it was recorded

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in Algeria, Egypt and more recently in Tunisia (White 2000; EPPO 2003; Ekesi and Billah 2007; Hafsi et al. 2015). The Ethiopian fruit fly or lesser pumpkin fly, *Dacus ciliatus* (Loew), is originated from Africa and has a large distribution within the African continent, where it can be found in all climatic zones (De Meyer and Ekesi 2016). *Dacus ciliatus* is also well established in the Middle East and southern Asia and was also introduced in Mauritius and La Réunion islands (De Meyer and Ekesi 2016). More recently, it was recorded in Turkey (Çalişkan Keçe et al. 2019).

In many cases, *D. ciliatus* has been misidentified as *D. frontalis*, due to reduced number of adult morphological characters. These two species differ only in the coloration of the thoracic katatergite and anatergite, and in the coloration of the femur (White 2006; De Meyer and Ekesi 2016). Moreover, studies on cucurbit pests have shown that *D. ciliatus* is one of the major competitors of the invasive species, *B. cucurbitae,* in Africa (Vayssières et al. 2008; Mwatawala et al. 2010). In areas where *B. cucurbitae* has not been yet established, *D. ciliatus* can be a serious problem (De Meyer and Ekesi 2016). Although the classical morphological discrimination between adults of *B. cucurbitae*, *D. frontalis*, *D. ciliatus* is possible at the adult stage, the differentiation between them at larval stage is potentially error-prone and not reliable (White and Elson-Harris 1992; EPPO 2018). Phytosanitary control measures are rigorously applied when importing cucurbits to prevent the entrance of these invasive pests to new territories. Usually, fruit flies are intercepted at the larval stage in infested fruits, and due to the lack of reliable taxonomic larval characters, species assignments are delayed till adult complete development after rearing, which would consequently delay quarantine decisions. To avoid these delays in species identification, which will increase the invasiveness risk, it is required to develop accurate (high specificity and sensitivity) and rapid detection techniques.

In this context, molecular tools allow the precise and reliable identification to the species level of immature-life-stage specimens, due to the robustness of DNA characters (Darling and Blum 2007). DNA barcoding based on mitochondrial cytochrome oxidase I (COI) gene sequencing has become an effective tool for species identification (Barcoding of Life initiative), including many insect species (Raquin et al. 2018; Anjali et al. 2019; Marullo et al. 2020; Thompson et al. 2020; Lopez-Vaamonde et al. 2021). The polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) analysis has been used to discriminate several Tephritid fly species based on mitochondrial DNA (Chua et al. 2010; Mezghani-Khemakhem et al. 2013).

In this work, we aimed to develop a PCR–RFLP method based on mitochondrial COI barcoding region to discriminate between *B. cucurbitae*, *D. frontalis* and *D. ciliatus* species. This method will allow the reliable discrimination of immature stages of these species becoming of great application in the field of quarantine and phytosanitary control measures. Which would improve quarantine decisions and limit the establishment of these exotic pests in new countries.

Materials and methods

Collection of tephritid fruit fly specimens

Used specimens are listed in Table 1. Adults and larvae of *D. frontalis* were obtained from rearing colonies maintained in the rearing facilities of the High Agronomic Institute of Chott Mariem, Tunisia. Colonies of *D. frontalis* were started in 2014, from larvae collected from infested cucumbers of Kairouan region (Tunisia). Specimens of *D. ciliatus* were provided by the International Center of Insect Physiology and Ecology (ICIPE), Nairobi (Kenya), and those of *B. cucurbitae* were provided by the French Agricultural Research Centre for International Development CIRAD, Réunion (France).

Genomic DNA extraction and PCR amplification of COI gene fragments

DNA was extracted from whole individuals using the CTAB protocol as described by Doyle and Doyle (1987), verified by gel electrophoresis and quantified. The Folmer fragment of the mitochondrial COI gene (COI mtDNA or mtCOI) was amplified using the universal barcoding primer pairs LCO1490 (5'GGT CAACAA ATC ATA AAG ATA TTG G 3') and HCO2198 (5' TAA ACT TCA GGG TGA CCA AAA AAT CA3') (Folmer et al. 1994). The PCR reaction was performed in a total volume of 25 µl containing 50 ng of DNA, 1 unit of Go Taq DNA Polymerase (Promega), 5X of the reaction buffer, 25 mM of $MgCl_2$, 10 mM of dNTP and 10 µM of each primer. The amplification was performed

Table 1 Origin of the three sampled tephritid fruit flies *D. frontalis*, *D. ciliatus* and *B. cucurbitae*

Fruit flies	Countries	Localities	GPS Coordinates
Dacus frontalis	Tunisia	Rakada, Kairouan	35° 35′ 46″ N, 10° 03′ 25″E
Dacus ciliatus	Kenya	Nguruman Escarpment	1°45′42″S, 36°01′32″E
Bactrocera cucurbitae	Réunion	Saint-Pierre	21° 20′ 31″S, 55° 28′ 40″E

in a applied biosystems thermal cycler 2720 programmed with an initial denaturation step for 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 48 °C and 1 min at 72 °C, with a final step of 10 min at 72 °C. The mtCOI amplification was verified by agarose gel electrophoresis.

mtCOI sequencing

Successful COI PCR products were purified using the Wizard PCR purification kit (PROMEGA Inc.) following manufacturer's instructions. Purified PCR products were bi-directionally sequenced on an ABI 3500 Genetic Analyser (Applied Biosystems) using BigDye Direct Cycle Sequencing Kit v.3.1 (Applied Biosystems) and amplification primers. The raw sequencing raw data were analysed and edited manually using BioEdit software, obtaining consensus sequence for each PCR fragment (Hall 1999). Consensus sequences were aligned using MUSCLE program (https://www.ebi.ac.uk/Tools/msa/muscle/) and represented using GeneDoc Editor (V 2.7.000) (Nicholas and Nicholas 1997).

BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Barcode of Life Data System (BOLD) were used to confirm sequence identity and similarity.

The consensus sequences were deposited in GenBank under the following accession numbers: larvae of *D. frontalis*: MZ433293, adult of *D. frontalis*: MZ433292; larvae of *D. ciliatus* MZ433295; adult of *D. ciliatus*: MZ433294; larvae of *B. cucurbitae* MZ433291 and adult of *B. cucurbitae* MZ433290.

Polymerase chain reaction and Restriction fragment length polymorphism (PCR–RFLP) analysis

The obtained sequences of *D. frontalis*, *D. ciliatus* and *B. cucurbitae* were scrutinised using NEBcutter V2.0 program (http://nc2.neb.com/NEBcutter2/) to identify putative discriminatory restriction sites. Finding that the *Rsa*I restriction enzyme (GT↓AC), was able to reflect sequence differences among the three species, as differential fragment sizes.

To verify these results, restriction fragment assay was conducted with mtCOI amplification fragments from each species and developmental stage (larvae and adults). For restriction fragment assays, 15µL of purified PCR products of each sample (one larva and one adult per species) were digested at 37 °C overnight in a 25µL reaction mixture containing 0.2 µl of *Rsa*I enzyme (10U/µl; Eurogentec) and 2.5µL of the corresponding restriction buffer. The digested products were fractionated on a 2% low melting point agarose gel (Cleaver Scientific Ltd), and visualized under ultraviolet light after ethidium bromide staining.

Results and discussion

Mitochondrial DNA (mtDNA) has several advantages for species identification purposes due to the availabilty of universal PCR primers, to its relatively fast mutation rate, and to the international Barcoding of Life initiative that increases the number of available sequences (Hebert et al. 2003; review in https://ibol.org).

In this work, mtCOI gene fragment was successfully amplified from DNA samples of three Tephritid fly species attacking cucurbits, *D. frontalis*, *D. ciliatus* and *B. cucurbitae*., using the Folmer universal COI primers LCO1490 and HCO2198, yielding a single PCR product of ≈ 658 bp. Obtained sequences from *D. frontalis*, *D. ciliatus* and *B. cucurbitae* were deposited at GenBank database, showing 100% BLAST similarity with database specimens, and among tested developmental stages (larvae and adults) (Fig. 1). Based on these nucleotide sequences, the NEBcutter analyses indicated that *Rsa*I enzyme was the best candidate for a PCR–RFLP diagnostic method (Table 2). This new procedure yielded three bands in *B. cucurbitae* (336, 201 and 121 bp) and in *D. frontalis* (354, 201 and 103 bp), and four bands in *D. ciliatus* (217, 201, 137 and 103 bp), clearly distinguishing each species by the PCR–RFLP profile (Fig. 2).

This study presents for the first time, a PCR–RFLP method to asign larvae and adults DNA samples to the corresponding taxonomic species, in these three Tephritid species attacking cucurbits. This methodology can be complementary to classical morphological identification of Tephritid fruit flies (PHA 2011; Hendrichs et al. 2015; Onah et al. 2015) as the second approach can be time consuming, and technically difficult for cryptic Tephritid flies species. In addition, this method, as relying in DNA polymorphisms, allow the assignation to species level independently of the developmental stage tested, either larva or adult, increasing its value as taxonomical character for quarantine purposes (White and Elson-Harris 1992; Hendrichs et al. 2015; Onah et al. 2017; EPPO 2018).

The same technique, PCR–RFLP of mtCOI, was effective in identifying and differentiating among the quarantine species *B. cucurbitae*, *B. zonata* and *Ceratitis capitata,* using *Dde*I and/or *Xmn*I restriction enzymes (Mezghani-Khemakhem et al. 2013); and by separating *B. invadens* from *Ceratitis* spp. with *Rsa*I and *Hsp*92II restriction enzymes (Onah et al. 2015). Similarly, Muraji and Nakahara (2002) used PCR–RFLP on 16S and 12S mitochondrial ribosomal RNA genes for the identification of eighteen *Bactrocera* spp. not including *B. cucurbitae* or *B. zonata* species within the study.

◂**Fig. 1** Nucleotide sequence alignement of *D. frontalis*, *D. ciliatus* and *B. cucurbitae* D.c-L: *D. ciliatus* larvae (MZ433295); D.c-A: *D. ciliatus* adult (MZ433294); B.c-L: *B. cucurbitae* larvae (MZ433291); B.c-A: *B. cucurbitae* adult (MZ433290); D.f-L: *D. frontalis* larvae (MZ433293); D.f-A: *D. frontalis* adult (MZ433292)

Our results are in agreement with the study of Chua et al. (2010), finding the same banding profiles for adults, adult body parts and immature life stages, despite this authors worked with *B. carambolae, B. papayae, B. latifrons* and *B. cucurbitae*.. Indicating that as expected adult, and immature life stages share the same DNA, rendering the developed PCR–RFLP method reliable independently of the source of DNA.

In conclusion, the developed PCR–RFLP method based on mtCOI restriction with *RsaI* allows the differentiation and species assignment of samples from *D. frontalis, D. ciliatus* and *B. cucurbitae*, improving quarantine decision-making at quarantine checkpoints for distinguishing exotic species from native ones, especially at the immature stages when these species are morphologically indistinguishable.

Material used in the study consisted of only one population from each species. At least 2–3 populations from each species should be used. Further, negative controls from other closely-related fruit flies species should be used in future research to exclude false-positive results.

Table 2 Predicted restriction number, position, fragment lengths and number of obtained fragments after digestion of COI PCR products of *D. frontalis*, *D. ciliatus* and *B. cucurbitae* by RsaI

Recognition sequence	Enzyme	species	Restriction site's number	Number of obtained bands	Position	Fragment lengths
5'GT↓AC3' **3'CA↓TG5'**	RsaI	*Dacus frontalis*	2	3	1-201	201
					202-555	354
					556-658	103
		Dacus ciliatus	3	4	1-201	201
					202-338	137
					339-555	217
					556-658	103
		Bactrocera cucurbitae	2	3	1-201	201
					202-322	121
					323-658	336

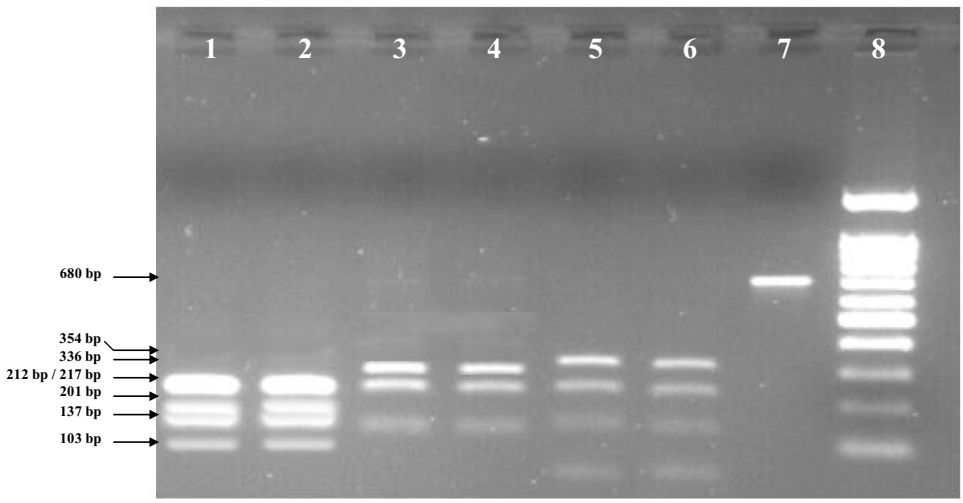

Fig. 2 Fragment length patterns of the three fruit flies digested with the enzyme *Rsa*I. 1: *D. ciliatus* larvae (MZ433295); 2: *D. ciliatus* adult (MZ433294); 3: *B. cucurbitae* larvae (MZ433291); 4: *B. cucurbitae* adult (MZ433290); 5: *D. frontalis* larvae (MZ433293); 6: *D. frontalis* adult (MZ433292); 7: *D. frontalis* undigested and 8: 100 bp ladder (GeneOn)

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Authors' contributions BC, MMK and KA designed the experiment, AH collected insect samples, extracted DNA samples and amplified DNA; CN conducted the restriction enzyme digestion of amplified DNA and sequenced PCR products, SD analysed sequences and selected the suitable restriction enzyme for distinguishing the three

Tephritid insects and AH, SD and MMK wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

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