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Morphological and molecular identification of some indigenous fruit fly species in Sennar State

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ABSTRACT

Fruit flies (Diptera:Tephritidae) are among the major constrains in commercial horticultural production in many African countries. DNA barcoding was used in this study to obtain quick and precise identification and to confirm the morphological identification of fruit fly specimens. Yellow sticky and Dome traps were used to collect some specimens from Sennar State whereas other specimens were reared under laboratory conditions at University of Gezira, from infested fruits. DNA from different specimens was extracted using the NucleoSpin® Tissue method. Three DNA fragments with sizes of 340, 220 and 280 bp. (base pairs) were recognized using specific primers and amplified from the 5' region of the cox1 gene from the mitochondrial DNA to give a full barcode of 660 bp. Then, purified PCR products were subjected to sequencing reactions using the BigDye Cycle Sequencing Kit. The nucleotide sequences were aligned using the cluster W algorithm included in the Bioedit 7.0 software package. Sequence divergences were determined using similarity index (P-distance model). Morphologically, six fruit fly species, namely *B. invadens*, *B. cucurbitae*, *C. cosyra*, *C. quinaria*, *D. ciliatus* and *D. longistylus* were identified. The molecular phylo-genetic tree of the Sudanese fruit flies was established by sequencing of 12 specimens representing five fruit fly species at the mitochondrial cytochrome oxidase cox1 gene fragments. The morphological identification of the fruit fly specimens was confirmed by molecular identification. Although *B. invadens* is a highly variable species, specimens of the same species collected using yellow sticky traps, methyl eugenol and those reared from magad fruits (wild plant belonging to the family *Cucurbitaceae*) appeared to have the same or with a little variation (0.7%) in the DNA sequences cox1 fragments. *D. ciliatus* and *B. cucurbitae* appeared in separate clusters, with no variation among sequences of their specimens. The three common fruit fly genera: *Bactrocera*, *Ceratitis* and *Dacus* exhibited low intra-specific variation compared with that between inter-species in the amplified sequences.

INTRODUCTION

The family Tephritidae includes about 4257 species arranged in 500 genera, among these 1400 species are known to develop in fruits. Equatorial Africa is the original home of 915 fruit fly (FF) species belonging to 148 genera, out of which 299 species develop in either wild or cultivated fruits (Thompson, 1998). The taxonomic characters available are restricted to at least the late larval or adult stages of development (Norrbon and Foote, 1989). When eggs or early larval stages are found, considerable time and effort must be devoted to rearing them to a stage in which morphological identifications can be made. The most common basis for species identification is using the morphological characters; these are: the thorax features such as the presence or absence of lateral or medial stripes in the scutum, their color and color of the scutellum, wing pattern such as venation, color, the presence of bands and spots; abdominal feature: Tergites overlapped, bands, pectin, color and ovipositor length, legs color and leathering; and the major features of the head (White and Elson-Harris, 1992).

Species identification based on morphological features has limitations, which sometimes lead to incorrect identification. Therefore, there is a need for a more precise and quick approach for taxon recognition such as the DNA-based identification system. This approach can be especially useful for identification of larval forms or incomplete specimens lacking diagnostic morphological characters. Identification based on certain genetic criteria theoretically can be made at any phase of the life cycle (Hebert *et al.,* 2003 a). DNA barcoding in combination with the molecular systematic methods of DNA taxonomy provide powerful tools to aid in more comprehensive species discoveries and deeper understanding of evolutionary relationships and speciation (Hebert *et al.,* 2003 a and b). There is a growing literature demonstrating that cox1 will reliably discriminate a diverse range of taxa at the species level (Hebert *et al.,* 2003 a and b). Correct identification of a species causing infestation is a critical step in a commodity treatment program (Armstrong and Couey, 1989). An accurate species identification requires a comprehensive molecular database against which unknowns can be compared. However, it is clear that most of the biological diversity in the world is undocumented. Therefore, another goal of DNA barcoding is to facilitate the species-discovery process. Large scale and standardized sequencing, when integrated with existing taxonomic practice, could contribute significantly to the challenges of identifying individuals and increasing the rate of discovering biological diversity. The objective of this research was to identify the prevailing fruit fly species which attack the different types of host fruits grown in Sennar State, Sudan, using morphological and molecular techniques.

MATERIALS AND METHODS

Infested fruit with wriggling larvae were collected from mango, guava and cucurbits including magad (wild plant which belongs to the family *Cucurbitaceae*) from Sennar State, Sudan. Larvae were reared under laboratory conditions (temperature 25^0C, relative humidity 70%) to obtain the adult stage. Emerged adults were kept in cadges and fed in a nourishing media, composed of sugar and yeast in a ratio of 4:1 (Lux *et al.,* 2003), for few days to complete their development and coloration. In addition, many other fruit fly specimens were collected using yellow sticky traps and Dome traps. All the collected fruit flies (FF) specimens were morphologically identified using a simplified key to the common FF species (Maxwell, 2005), and another key prepared by White (2006) at the Royal Museum for Central Africa, Belgium.

Nineteen fruit fly specimens were selected according to the host, type of trap and variation which exists among specimens belonging to the same species to be used for DNA barcoding analysis. Analysis was carried out at the Molecular Biology Laboratory, Royal Belgium Institute for Natural Sciences, Brussels, Belgium.

Information concerning locality and details of the specimens is presented in Table 1. As a general principle, barcode analysis should follow collection as soon as possible, since all specimens give good quality and little amount of DNA (Mehrdad *et al.,* 2005).

Genomic DNA extraction was done using only the abdomen to limit damage to specimens. The NucleoSpin® tissue method (Alex *et al.,* 2005) was used for DNA extraction. Tissues were incubated for 48 hr, and DNA was extracted following the standard protocol for animal tissues.

Three fragments with sizes of 220, 280 and 340 bp were recognized and amplified from the 5’ region of the cox1 gene from the mitochondrial DNA to give a full barcode of 660 bp, using different combinations of six newly designed primers. Table 2 shows primers, their sequence and the length of the fragments. Primers were developed by the Joint Experimental Units (JEMU) of Royal Belgium Institute for Natural Sciences (RBINS) and Royal Museum for Central African (RMCA). The process of amplification was conducted in a thermocycler (Biometra). The amplification products were separated electrophoretically in a 1.2% agarose gel. The running procedure was done according to the manufacturer’s instructions for 15 min. The gel was visualized and photographed on a UV trans-illuminator equipped with a digital camera. The positive PCR products were purified using a vacuum pressure device, a sample of 20 µl of each PCR product were added to 30 µl Nano water (Nano pure +++/Deionized+) and then the plate was put under vacuum (400-600 atmosphere) for 15 min.

Purified PCR products were subjected to sequencing reactions. The process of sequencing was conducted in Biometra TP professional thermocycler using the Big Dye Cycle Sequencing Kit. A forward and reverse reaction was performed for each sample using M13 Fa and M13 Re primers (Massimilliano *et al.,* 2009). The sequenced products were purified following the clean-up protocol and sequenced in both directions with an ABI 3130 genetic analyzer (applied biosystem), following manufacturer’s instructions.

Phylogenetic analysis, nucleotide sequences were aligned using the cluster W algorithm included in the Bioedit 7.0 software package (Hall, 1999). Sequence divergences were calculated using similarity index (P-distance model) (Eucledean distance), (Romesburg, 1984) to study the pattern of divergence in and among species.

RESULTS AND DISCUSSION

Morphological identification

The variability existing among the collected adults indicated the presence of different fruit fly species.

Group 1

Adult: Face, antennal furrow with dark spot, scutum brown to black, but with high degree of variation from dark brown to complete black. Scutellum: Yellow in color, except for a narrow black band at the base; only lateral yellow stripes were found without medium stripes, anatergite and katatergite were both yellow (Plate 1A). Wing: Costal band complete; shallow, anal streak present (Plate 1B). Abdominal tergites: 3 to 5 with a black T-shaped mark. Adults belong to this group were identified as the invading FF *Bactrocera invadens* (Drew, Tsuruta and White).

Group 2

Adult: Showed the following characters: Scutum: With medial and lateral setae (Plate 2A), prescutellar acrostical setae present (Plate 2C). Wing : With stripes and a large black spot at the wing tip (Plate 2B). Abdomen: usually brown with a gold to brown horizontal band and a faint black “T” (Plate 2A); these are the characteristics of the melon FF, *B. cucurbitae* **(**Coquillet).

Group 3

Adult: Orbital bristle not modified; Wing: Yellowish; costal band and distal cross band were joined (Plate 3B); one anepisternal bristle; Scutellum: With three separated apical spots (Plate 3A). Abdomen: without black bands or spots , Adults belong to this group were identified as mango FF, *C. cosyra* (Walker).

Group 4

Adult: Adult were recognized by the following features: Head: Two pairs of frontal bristles. Two pairs of orbital setae. Thorax: Postpronotal seta present; Scutum: Yellowish; number of pale whitish to yellow postsutural stripes are two (sublateral). Scutum has no blackish dorsocentral stripe. Mediotergite: Uniformly yellowish to orange-brown. Scutum: Entirely microtrichose scutellum with a dark and pale pattern; with five isolated dark spots (3 apical, 2 distal; Plate 4A). Legs: Femora is slender. Fore-femur: with regular bristles. Mid-femur and hind-femur: without spine-like bristles. Femora is entirely of one color. Wing: *Ceratitis*-like. Wing pattern is mostly yellowish, or mostly brownish. Dark longitudinal streaks through basal cells present. Crossvein r-m covered by a major crossband. Crossvein dm-cu covered by a major crossband which reaches posterior margin of the wing. Crossveins r-m and dm-cu are not covered by a single crossband. Subbasal cross band is present (Plate 4B). Abdomen: Ovate. Abdominal tergites: They are separate. Abdominal tergite 1: Broader at apex than at base. Abdominal tergite 5 is normal. Abdominal tergites are 3–5, predominantly yellow to orange brown. Abdominal tergites are without medial dark stripe. This group was identified as the five spotted FF, *Ceratitis quinaria* (Bezzi.).

Group 5

Adult: This group possessed the following features: Face, antennal furrow with dark spot. Wing with narrow costal band, with small apical spot at tip (Plate 5B), two dark spots on scutum. Abdominal tergites 3 with 2 dark spots on either side (Plate 5A), identified as the lesser melon FF, *Dacus ciliatus* (Loew).

Group 6

Adult: Medium in size. Face, antennal furrow with dark spot. Frons, frontal setae 2, orbital seta 1 (Plate 6A). Thorax: Postpronotal lobe yellow; notopleural callus yellow; notopleural xanthine wedge-shaped, connected to notopleural callust; medial vitta present. Scutellum: without dark patterning, except for basal dark margin (Plate 6A); Wing: Basal cells bc and c are without an almost complete covering of microtrichia; cell bm is without microtrichia. Crossvein R-M beyond middle of cell dm. Costal band absent. Cells bc and c hyaline, without any cross banding (Plate 6B). Abdomen: Tergites I–V all fused. Male: Tergite III without pecten (Plate 6B). Female: Ovi-scape is about as long as the abdomen (Plate 6A); this species has one of the longest ovipositors known in the genus. Those were identified as *D. longistylus* (Wiedemann). The most common morphological features used to differentiate between *Dacus* and *Bactrocera* species are presented in Table 4A, while those presented in Table 4B differentiate between *C. cosyra* and *C. quinaria*.

Molecular identification

All the nucleotide sequences obtained were compared to the molecular data to identify the indigenous fruit fly species. Seven of the samples used were not included in the final results, because some of them gave no bands after being amplified. Others gave false sequence reading. The molecular identification was compatible with morphological classification patterns. The molecular analysis confirmed the morphological identification and investigated the molecular phylogeny of the Sudanese fruit flies by sequencing 12 specimens representative of five species at the mitochondrial cox1 gene fragments. Massimilliani *et al.* (2009) tested the performance of DNA barcoding in three Tephritid genera by considering 493 DNA barcodes involving 86 species belonging to the genera *Bactrocera*, *Ceratitis* and *Dacus* and he found that DNA barcoding could be effective under well-defined conditions, where only a limited number of well-known Tephritid taxa, with well characterised intraspecific variation, are to be distinguished.

Although *B. invadens* is a species with significant variation in terms of the scutum color, specimens of the same species collected using yellow sticky traps, ME and those reared from the wild magad fruits appeared with the same DNA sequences (variation=zero). However, those reared from guava appeared in a separate cluster (Fig. 1) with a variation of 0.7% (Table 3) of the previous group.

Specimens belonging to species *D. ciliatus* and *B. cucurbitae* appeared in separate clusters (Fig. 1) with no variation among sequences of their specimen (Table 3). Results showed that the three common FF genera *Ceratitis, Bactrocera* and *Dacus* have low intraspecific variation in the sequenced region of DNA compared with that between species (interspecific; Fig. 2), which is necessary for accurate diagnosis.

CONCLUSIONS

The DNA barcoding technique proved to be an effective method for identification of FF species. The three common FF genera: *Ceratitis*, *Bactrocera* and *Dacus* exhibited low intra-specific variation in the sequenced cox1 region compared to that between species (interspecific), which is necessary for accurate diagnosis.

Proposed future work

Efforts should be directed towards exploring the utility of the DNA-based diagnostic tools at the level of immature stages (the available stages inside the fruits during inspection), to facilitate identification of these pests at ports of entry.

ACKNOWLEDGEMENTS

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Table 1. Specimens used in the study, their locality, details of collection and molecular analysis.

Specimen Code	Genus	Species	Notes	Fragment 1	Fragment 2	Fragment 3
AB31545710	*Dacus*	*longistylus*	Attracted to ME	+	+	+
AB31509612	*Bactrocera*	*cucurbitae*	Reared from magad	+	+	+
AB31509583	*Bactrocera*	*cucurbitae*	Attracted to ME	+	+	+
AB31517438	*Bactrocera*	*cucurbitae*	Caught using yellow sticky trap	+	+	+
AB31509466	*Bactrocera*	*invadens*	Reared from magad	+	+	+
AB31545683	*Bactrocera*	*invadens*	Reared from mango	+	+	+
AB31545659	*Bactrocera*	*invadens*	Reared from mango	+	+	+
AB31509535	*Bactrocera*	*invadens*	Reared from guava	+	+	+
AB31497875	*Bactrocera*	*invadens*	Caught using yellow sticky trap	+	+	+
AB31517449	*Bactrocera*	*invadens*	Caught using yellow sticky trap	-	-	-
AB31545721	*Bactrocera*	*invadens*	Attracted to ME	+	+	+
AB31509453	*Dacus*	*longistylus*	Reared from mango	+	+	+
AB31545667	*Bactrocera*	*invadens*	Reared from guava	+	+	+
AB31545681	*Dacus*	*ciliatus*	Reared from pumpkin	-	+	+
AB31517435	*Dacus*	*ciliatus*	Reared from watermelon	+	+	+
AB31517461	*Dacus*	*ciliatus*	Reared from watermelon	+	+	+
AB31545672	*Ceratitis*	*quinaria*	Reared from guava	+	+	+
AB31509602	*Ceratitis*	*cosyra*	Reared from mango	+	+	+
AB31545720	*Ceratitis*	*cosyra*	Reared from mango	+	+	+

(-): No bands appear.
(+): developed bands.

Table 2. Primers and their sequence and length of the amplified fragments.

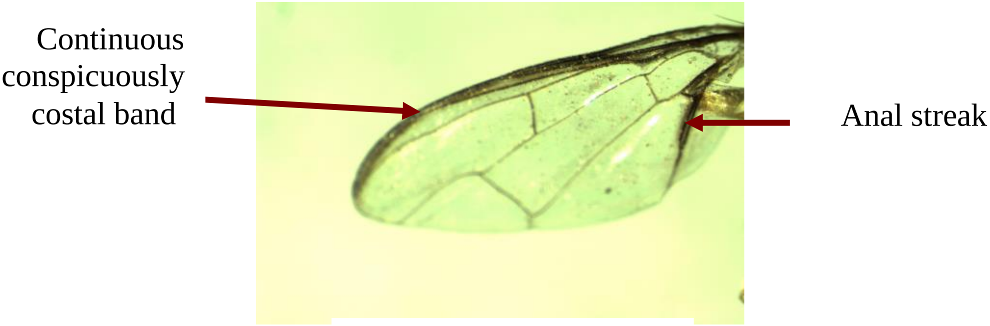
Fragment	Length	Primers	Sequence of the primers
1	340bp	Fa CO1L1440_1464dt	TGTAAAACGACGGCCAGTTTCAACAATCATAAGATATTGG
		Re Teph_H343_362t (Rc)	ATAGTAGAAAACGGAGCTGGGTCATAGCTGTTTCCTG-
2	220 bp	Fa Teph_L280_306t	ATGTAAAACGACGGCCAGTCGAATAAATAATATAAGATTTTGATTA
		Re Teph_H526_548t (Rc)	TTTGACCGAATACCTTTATTTGTGTCATAGCTGTTTCCTG
3	280bp	Fa Teph_L499_521t	TGTAAAACGACGGCCAGTATTAATATACGATCAACAGGAAT
		Re CO1H2123_2148dt(Rc)	CAGGAAACAGCTATGACTAACTTCGGRTGCCAAAAATCA

Table 3. Similarly matrix pattern (expressed as P-distance) of DNA sequences of different fruit fly specimens.

	1	2	3	4	5	6	7	8	9	10	11
1											
2	0										
3	0.131	0.131									
4	0.128	0.128	0.007								
5	0.131	0.131	0.00	0.007							
6	0.131	0.131	0.00	0.007	0.00						
7	0.128	0.128	0.007	0.00	0.007	0.007					
8	0.152	0.152	0.128	0.125	0.128	0.128	0.125				
9	0.158	0.158	0.135	0.131	0.135	0.135	0.131	0.007			
10	0.168	0.168	0.135	0.131	0.135	0.135	0.131	0.064	0.064		
11	0.135	0.135	0.138	0.131	0.138	0.138	0.131	0.118	0.125	0.152	
12	0.135	0.135	0.138	0.131	0.138	0.138	0.131	0.118	0.125	0.152	0.00

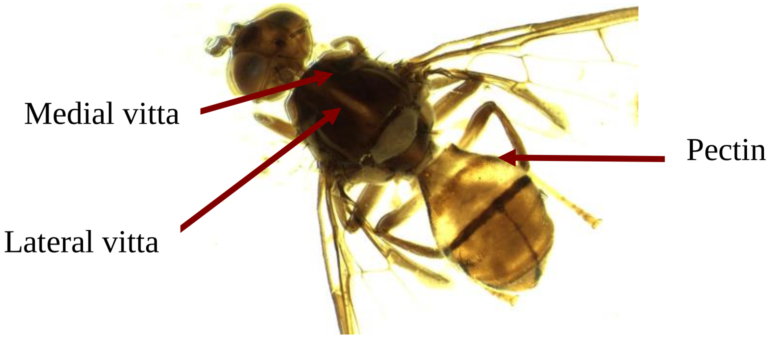
1 *B. cucrbitae* (reared from Magad)
2 *B. cucurbitae* (caught using yellow sticky traps)
3 *B. invadens* (reared from Magad)
4 *B. invadens* (reared from guava)
5 *B. invadens* (caught using yellow sticky traps)
6 *B. invadens* (caught using methyl eugenoul)
7 *B. invadens* (reared from guava)
8 *C. cosyra* (reared from mango)
9 *C. cosyra* (reared from mango)
10 *C. quinaria* (reared from guava)
11 *D. cilitatus* (reared from pumpkin)
12 *D. cilitatus* (reared from water melon)

(A) Lateral view

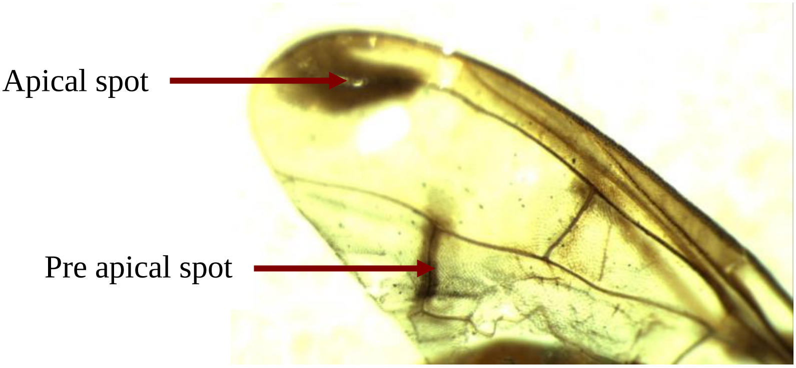


(B) Wing

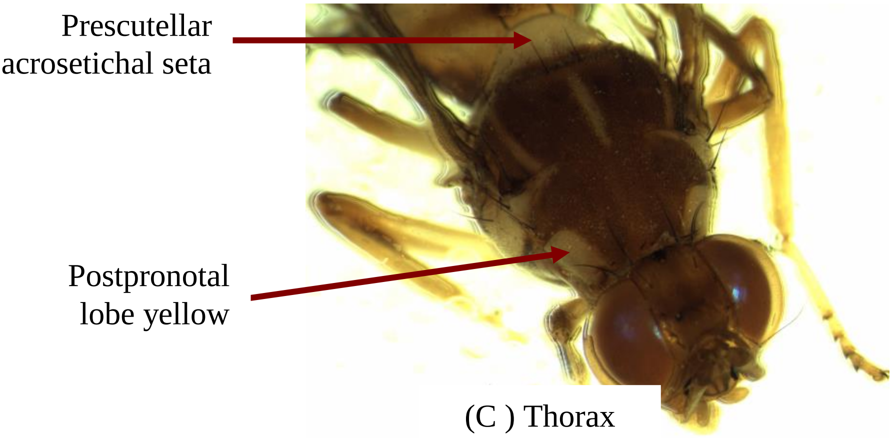
Plate 1. Morphological features of *B. invadens*.



(A) Thorax and abdomen

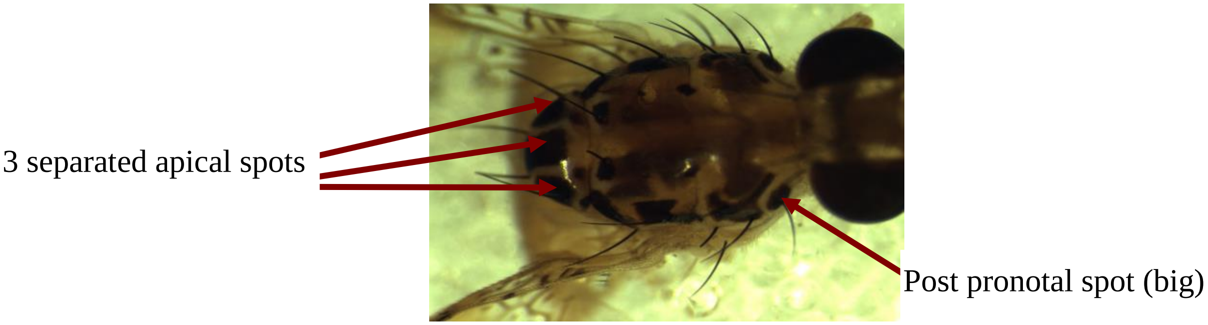


(B) Wing



(C) Thorax

Plate 2. Morphological feature of *B. cucurbitae*.



(A) Thorax

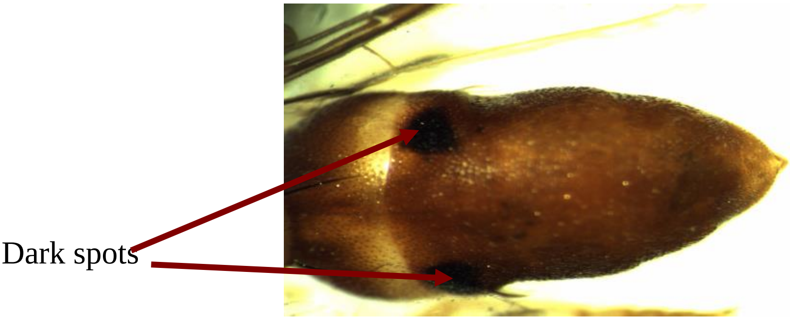
(B) Wing venation

Plate 3. Morphological features of *C. cosyra*.

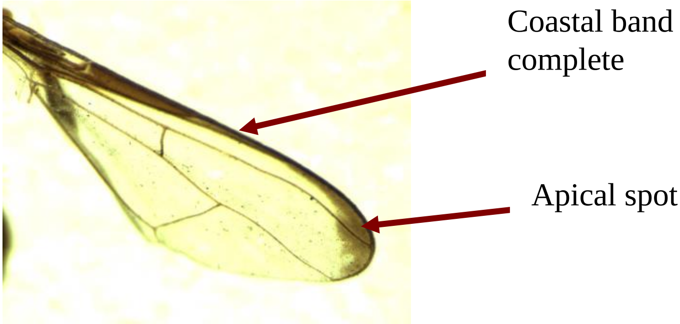
(A) Thorax

(B) Wing venation

Plate 4. Morphological features of *C. quinaria*.



(A) abdomen



(B) Wing

Plate 5. Morphological features of *D. ciliatus*.

(A) Scutum

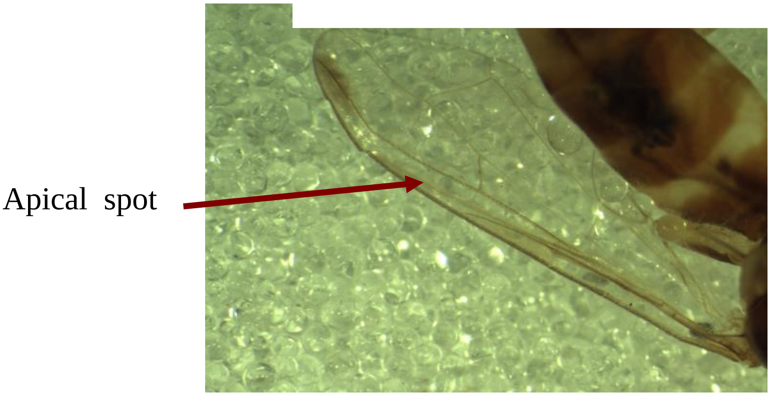
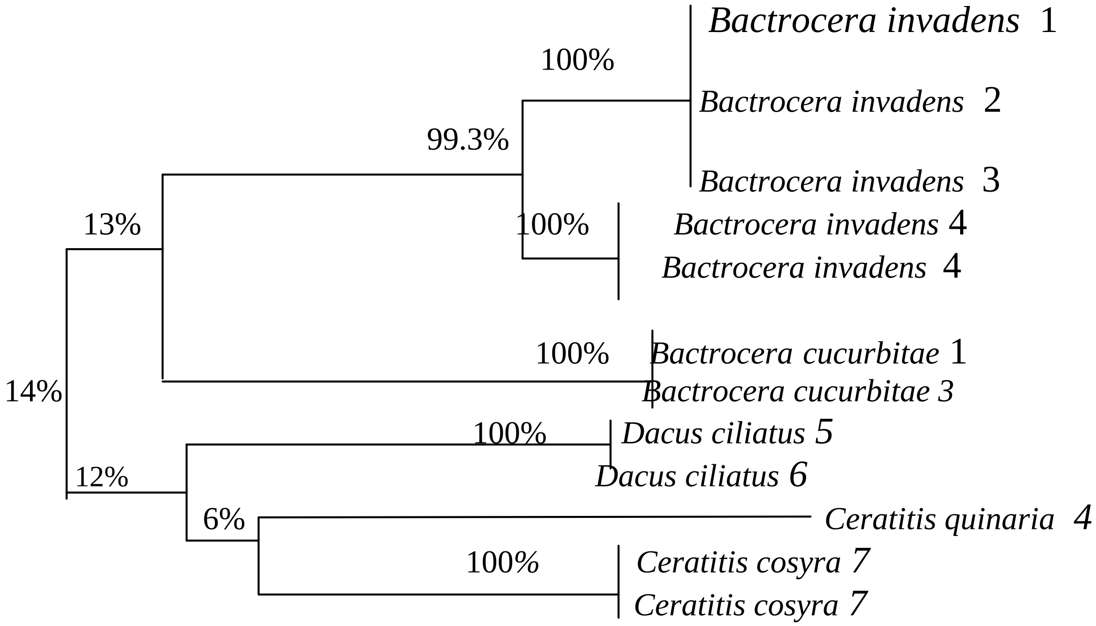


Plate 6. Morphological features of *D. longistylus*.



Source of specimens:

1- Yellow sticky traps.
2- ME.
3- Reared from magad.
4- Reared from guava.
5- Reared from pumpkin.
6- Reared from water melon.
7- Reared from mango.

Fig.1. Dendrogram showing relationship between different FF species based on their DNA sequences similarity (%).

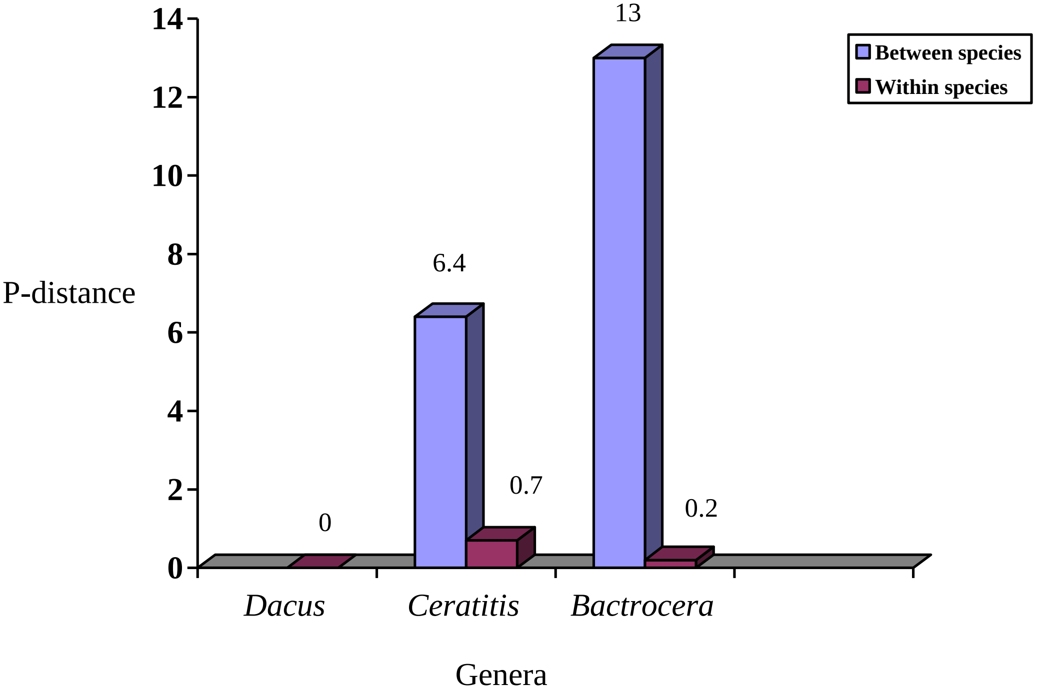


Fig. 2. Intra and inter- specific genetic variation in three genera of Tephritid fruit flies.

التصنيف عن طريق الشكل الظاهري والأحياء الجزيئية لبعض الأنواع المحلية من ذبابة الفاكهة في ولاية سنار

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الخلاصة

تعتبر ذبابة الفاكهة (Diptera : Tephritidae) من أهم الآفات التي تعيق الإنتاج البستاني التجارى في عدد من الدول الأفريقية. أستخدمت تقنية التحليل الجزيئي (DNA barcoding) للحصول على تصنيف سريع ودقيق لأنواع ذبابة الفاكهة ولتأكيد التصنيف عن طريق الشكل الظاهري (مورفولوجي) لهذه الانواع. أستخدمت المصائد الصفراء اللاصقة والمصائد المقبية لجمع الحشرات الكاملة لذبابة الفاكهة من ولاية سنار، السودان، إضافة إلى تلك التي رُبيت من اليرقات تحت الظروف المعملية بجامعة الجزيرة. أستخلص الـDNA من الحشرات الكاملة لذبابة الفاكهة باستخدام طريقة NucleoSpin® Tissue. ثلاثة قطع بحجم 340، 220 و 280 زوج قاعدي قد تم التعرف عليها وإكثارها من المنطقة الخامسة للجين *Cox1* من DNA الميتوكوندريا لتعطي هذه القطع شريطاً من الـDNA بحجم 660 زوج قاعدي، استخدم في هذه العملية ثلاثة أزواج من البادئات المصممة خصيصاً لهذا الغرض. اجريت تفاعلات التسلسل النيوكليوتيدى لنواتج الـPCR المنقاة باستخدام طريقة الـ Big Dye Cycle sequencing kit. اجرى التسلسل الخطى للنيوكليوتيدات باستخدامcluster W algorithm المضمن فى ال Bioedit 7.0 software package. خلال هذه الدراسة ستة أنواع من ذبابة الفاكهة هي *B. invadens, B. cucurbitae, C. cosyra, C. quinaria, D. ciliatus* and *D. longistylus* تم تصنيفها عن طريق الشكل الظاهري. تم استخدام نتائج التحليل الجزيئ للأنواع الحشرية في تصميم شجرة التشابه (Phylogenetic tree) والتي وضحت درجة التقارب بين أنواع ذبابة الفاكهة الموجودة في السودان، أيضاً تم تحديد التسلسل النيوكلوتيدى لـ12 عينة حشرية تمثل خمسة أنواع من ذبابة الفاكهة، هذا التسلسل تمت مقارنته مع قواعد للبيانات وأعطى نتائج مماثلة للتصنيف عن طريق الشكل الظاهري. رغماً أن النوع *B. invadens* أظهر تبايناً في الشكل الظاهري إلا أن عينات النوع التي جمعت بإستخدام المصائد الصفراء اللاصقة والمصائد المقبية وتلك التي رُبيت من نبات المقد (نبات بري يتبع لعائلة القرعيات) قد أظهر إختلافاً صغيراً تراوح بين 0– 0.7% على مستوى تسلسل القواعد النتروجينية. عينات النوعين *D. ciliatus* و*B.cucurbitae* ظهرا في عنقودين (two clusters) مع عدم وجود اختلافات في تسلسل القواعد النتروجينة لعيناتهما. أجناس ذبابة الفاكهة الثلاثة الأكثر شيوعاً *Bactrocera, Ceratitis* و *Dacus* أظهرت تبايناً أقل في تسلسل القواعد النتروجينية داخل النوع عن ذلك الذي بين الأنواع.