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MOLECULAR IDENTIFICATION OF CUCURBIT FLY *Dacus ciliatus* (DIPTERA: TEPHRITIDAE) INFEST CUCURBITACEAE FAMILY BASED ON MITOCHONDRIAL GENE IN KURDISTAN REGION- IRAQ.

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ABSTRACT

- Fruit fly hosts survey was carried out in many villages at three provinces (Duhok, Erbil and Sulaimaniyah /Kurdistan region, Iraq) during period 15/9-1/12/2017 and 15/5-30/9/2018. Ethiopian fruit fly *Dacus ciliates* (Loew) which belongs to the genus Dacus, family Tephritidae, order Diptera were found infesting most vegetables like cucubitaceae and some of fruit trees like fig. For molecular identification, polymerase chain reaction (PCR) amplification technique was used to amplify a single or a few copies of a pieces of DNA to millions of copies of a particular DNA sequence. For rearing this pest, damaged fruits were collected and kept in a round cages galvanized by sieves cloth (35 cm diameter, 40 cm hight), containing a layer of 3 cm soil to facilitate pupation

KEYWORDS: Fruit flies, Tephritidea, Dacini, Molecular, Mitochonderial DNA.
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INTRODUCTION

Tephritid fruit flies are considered an insect group of major economic significance in agriculture. It's attack different types of commercial and wild fruits and vegetables, causing considerable damage to agricultural crops (De Meyer *et al.*2012). There are about 450 genera and about 4,300 described species within Tephritidae family in the worldwide, making it one of the largest families within Diptera (Norrbom *et al.* 1998). The fruit fly species that cause the damage are not well known. Sub-Saharan Africa is the aboriginal home to 915 fruit fly species from 148 genera, of which 299 species develop in either wild or cultivated fruit. Both of the lesser pumpkin fly, *Dacus ciliatus* (Loew) and the greater pumpkin fly, *Dacus frontalis* (Becker) which belong order Diptera family Tephritidae (Typetidae or Trupaneidae) are a group of about 4000 known species and nearly about 80% of their larvae develop in the seeds (flowers or fruits) of higher plants, and therefore known as fruit flies (White, 2000). Both flies are serious pests that cause high loss in yield and cause damage sometimes reached 100%. According EPPO (2009) both species could be arranged as highly serious agricultural pests.

The genus Dacus causes severe damage to fruits and vegetables in Asia. The cucumber fly, *D. ciliatus* was recorded as a serious pest on cucurbitaceae since 1947 by Azab and Kira (1954), continued nearly untill 1980 and disappeared then appeared again after 25 years in Egypt (Fetoh, 2003). *Dacus ciliatus* Loew., is a major pest of the most of eastern, southern, and central Africa, Arabian Peninsula, Pakistan and India. (Azab, *et al*., 1970; Nagappan *et al.*, 1971; White and Elson-Harris, 1994). It is also known as Ethiopian fruit fly. *D. ciliatus* is a pest of the most of eastern, southern, and central Africa, Arabian Peninsula, Pakistan and India. Its color is orange, with facial spots. There are two black spots in abdomen particularly in females (White and Elson-Harris, 1994). The fruits of cucumbers are exposed to being infected with six types of flies in autumnal cultivation in central Iraq, and these species are arranged according to their economic importance as *Dacus ciliatus (Loew), Dacus frontalis(Becker), Atherigona orintalis (Schin.), Atherigona varia(Meigen), Myiopardalis pardalina (Bigot) and Ceratitis capitata (Wiedeman)* Al-Jorany *et.*

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al. (2015). In Iran, (Fars, Khorasan Razavi, Tehran, Khuzestan and Hormozgan) provinces. this pest is a major pest of cucumber, watermelon and cantaloupe. Also milkweed and colocynth are other hosts of this pest in Iran (Arghand, 1983 and Cheraghian, 2012). Many species of these Tephritid fruit flies are morphologically similar but differ in their behavior such as reproductive potentials, competitive abilities and dispersive power (Duyck *et. al*. 2004). Generally, accurate identification of insect species is essential, especially in the sibling species, in order to give right information for ecology, biology and control methods, the molecular biology methods helps to classify and control pests in clear, easy and quick manner. PCR is now a common and often indispensable technique. There are three major steps involved in the PCR technique: denaturation, annealing, and extension.

The main objective of this work is to differentiate the Ethiopian fruit fly, *D. ciliatus* from other species of the same genus by molecular and morphological characters.

MATERIALS AND METHODS

First: Survey

• Fields in three governorates (Duhok, Erbil, and Sulaimaniyah) / Kurdistan region - Iraq were selected. The fields and their locations Y(lat) and X(long) are shown in Table (1) & Fig. (1).

Table (1): Cucurbit fly's specimens collected from locations in Kurdistan region Iraq during 2017-2018

No.	Governorate	Location	* X(long)	** Y(lat)
1	Duhok	Summel	36.51	42.59
		Qadish	37.05	43.21
		Bamarze	36.36	43.34
2	Erbil	Selke	36.41	44.58
		Khalifan	37.15	42.15
		Gardasher	36.17	43.50
3	Sulaymanyiah	Khalakan	36.01	44.50
		Hassan tappa	36.52	43.48
		Basharat upper	35.54	44.58

***** X(long.) = Longitude ****** Y(lat.) = Latitude

Fig. (1): Shows the survey areas of *Dacus ciliatus*
: The sampling location

* Part of Ph.D. Thesis

Second:Samples collection

Samples of *Dacus ciliatus* were collected from cucurbit fields (squash and cucumber fields) weekly during period of pest activity (late of May until the late of October) in the 2017 and 2018 from many villages at Duhok, Erbil and Sulaymaniyah in Kurdistan region, Iraq. The infested small cucumbers and squash with larvae were collected and transferred to laboratory.

Third: Insect sample's preparation (insect stock)

Damaged fruits, that were collected from 9 localities of the three Governorates during the late of May until the late of October, were kept in a round cages galvanized by sieves cloth (35 cm diameter, 40 cm height), containing a layer of 2 cm soil to facilitate pupation. A sugar solution (10%) was used for feeding the adults in the cages. Insect rearing and all bioassay tests were performed at 26 ± 1 °C and 65 ± 5% relative humidity under a 12:12 (L: D) photoperiod in the growth chamber (Vayssie`res *et. al.* 2008) and (Hussein *et.al.* 2006). The adults kept in plastic containers filled with 96% alcohol for maintaining the insect's sample for molecular study. (Jalali *et. al.* 2015).

Fourth: Molecular studies:

Hundred specimens had been selected for the extraction of DNA. Fifty specimens were sent to the sequences (Intergene genetic center/ Ankara / Turkey). The steps of this work were summarized as following:

1. Extract the Mitochondrial DNA (mtDNA) of the *Ducus ciliatus*.
2. Use the specific species primer for amplifying the mitochondrial DNA.
3. A mitochondrial DNA was amplifying by PCR (Polymerase Chain Reaction) technique.
4. Purification of the mtDNA product had done for the step three.
5. Purified mtDNA product had sent to the sequencing.
6. Compare the results of sequencing with the samples of the neighbor regions of Kurdistan.

Fifth: Chemicals and primers that used in this research

A/ Chemicals need in molecular technique were:

1) PCR Buffer
2) MgCl2
3) dNTP
4) TAQ
5) H2O (sterile water)

B/ The Primers:

Mitochondrial Cytochrome Oxidase Subunit I gene (COI): (Folmer *et.al.* 1994)

LCO1490F 5′-
GGTCAACAAATCATAAAGATATTGG-3′
HCO2198R 5′-
TAAACTTCAGGGTGACCAAAAAATA-3′

C/ DNA Extraction Kit

Qiagen DNAeasy Tissue Extraction Kit

*** Extraction of mtDNA.**

The following chemicals were used for mtDNA extraction :

1- Lysis Buffer
2- Phenol
3- Chloroform
4- Ammonium acetate
5- Absolute ethanol
6- TE Buffer

***Polymerase Chain Reaction (PCR) Amplification**

The mitochondrial DNA polymerase chain reaction (mtDNA-PCR) method was used to amplifying the mtDNA of selected samples. All the chemicals that are used in this step shown in Table2

(Table2): The Chemicals used for one sample

Volume	Reagent name
5.0µL	PCR Buffer
8.0 µL	MgCl2
1.25 µL	dNTP
1.25 µL	Primer F
1.25 µL	Primer R
0.25 µL	TAQ
7.5 µL	H2O
24.5 µL	Total master mix
0.5 µL	Template DNA

Table (3): Kit components

Cat.No. / Reagents	K-3000 (50 prep.)	K-3001 (200 prep.)
Spin column	50 ea	50 ea *x* 4
Collection tube	100 ea	100 ea *x* 4
Buffer TL	20 ml	20 ml *x* 4
Buffer GB	12 ml	12 ml *x* 4
Buffer GW1	20 ml	20 ml *x* 4
Buffer GW2	10 ml	10 ml *x* 4
Buffer GE	10 ml	10 ml *x* 4
Proteinase K Sol.(20mg/ml) (should be stored at – 20 °c)	1.2 ml	1.2 ml *x* 4

D/ PCR Program for the COI gene:

1- Hot start temperature 95°c (for 5 minute)
2- Denaturation temperature 95°c (for 1 minute)
3- Annealing temperature 55°c (for 1 minute)
4- Extension temperature 75°c (for 1.45 minute)
5- The above steps are repeated for 35 cycles
6- The final extension temperature 72° c (for 5 minute).

E: Sequencing step:

The amplified products which had been obtained from PCR analysis were sent to commercial sequencing company (Intergene genetic center/ Ankara). Each sample was bi directionally sequenced and checked for quality and frame shifts by using NCBI BLAST. All sequences were uploaded to GenBank.

RESULTS AND DISCUSSION

Morphological identification of** ***Dacus ciliatus**:**

The fruit fly specimens (Fig. 2) were identified according to key of the species *Dacus ciliates.* (Diptera: Tephritidae: Dacinae) by Luc Leblanc, *et.al.* (2013). Adults of Dacus species were identified according to morphological features, accepted with Menon *et.al*.(1968), Malan & Giliomee (1969), and Azab *et.al.* (1971).

Characters of *Dacus ciliatus:*

1- Adult semi-oval peaked toward the posterior part.
2- Length 4-5 mm, width 2-2.5 mm.
3- Yellow head with brown thorax and abdomen.

4- The legs are yellow in color.

Fig. (2): Cucurbit fly adult

***Molecular Identification:**

*** PCR amplification:**

The identification of the species was determined by using the mitochondrial DNA polymerase chain reaction (mtDNA-PCR) method. The arrangement of genes in mitochondrial genomes has been studied in insects. The results of PCR amplification detected one band as shown in Fig.(3) indicated that there is only one species of Dacus namely: *Dacus ciliatus*. The length of the polymerase chain reaction products is 708bp.while Morphological classification used in this study revealed that there are two different Dacus species.

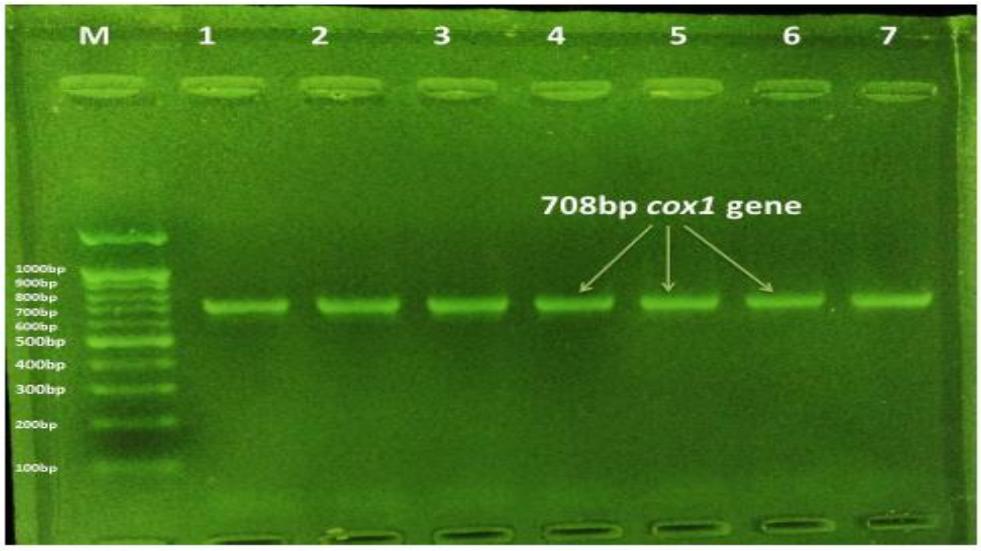

Fig. (3): Agarose gel electrophoresis of species specific PCR amplification of *Dacus ciliatus* genomic DNA. Electrophoresis performed on 1% Agarose gel and run with 3 volt/cm. Lane 1= Marker (Molecular weight marker is a 1000bp ladder; it means that each band with the next one has 100bp difference), lane 1-7 *Dacus ciliatus* (each of them isolated from single adult specimen cucurbit fly), the length of the polymerase chain reaction products is 708bp.

Fifteen species of insects have had their mitochondrial genomes sequenced completely; the mitochondria of insects contain their own double-stranded circular genomes, which range from 14,503 bp (Beckenbach and Joy 2009) to 19,517 bp in size (Lewis *et al.* 1995). The DNA that was extracted from somatic tissue in mitochondria was subjected to PCR amplification of 708 bp region near the 5′ terminus of the COI gene following standard protocols.

The sequence of bases of (MK 287888) Iraq *D. ciliates*

1 atcataaaga tattggaaca ttatatttta ttttcggagc ctgagcaggc atagtaggaa
61 catcccttag aattctagtt cgtgctgaat taggacaccc cggagcttta attggagacg
121 accaaattta taacgtaatt gtaacagctc acgcatttgt aataattttc tttatagtaa
181 tacctattat aattggaggg tttggaaatt gattagtacc attaatatta ggtgctccag
241 atatagcatt cccccgaata aataatataa gtttttgatt actaccccca tctcttacct
301 tacttttagt cagcagtata gtggaaaacg gagctggaac aggttgaaca gtgtatcctc
361 ctctatcatc aatcattgct cacggaggag catctgtaga tttagctatc ttttccttac
421 atttagcagg tatttcttca attttagggg ctgtaaattt tattacaaca gttattaata
481 tacgatctac aggaattagt tttgaccgaa tacctctatt tgtttgagct gttgtattaa
541 ctgcattatt attacttctt tcccttccag tactagctgg agctattact atattattaa
601 cagaccgaaa cttaaacaca tctttcttcg accccgctgg aggaggagac cctattcttt
661 accaacattt attttgattt tttggtcacc ctgaag

The sequence of bases of (MK 287889) Iraq *D. ciliates*

1 aaatcataaa gatattggaa cattatattt tattttcgga gcctgagcag gcatagtagg
61 aacatccctt agaattctag ttcgtgctga attaggacac cccggagctt taattggaga
121 cgaccaaatt tataacgtaa ttgtaacagc tcacgcattt gtaataattt tctttatagt
181 aatacctatt ataattggag ggtttggaaa ttgattagta ccattaatat taggtgctcc
241 agatatagca ttccccccgaa taaataatat aagtttttga ttactacctc catctcttac
301 cttacttta gtcagcagta tagtggaaaa cggagccgga acaggttgaa cagtgtatcc
361 ccctctatca tcagtcattg ctcacggagg agcatctgta gatttagcca tcttttcctt
421 acatttagca ggtatttctt caattttagg ggctgtaaat tttattacaa cagttattaa
481 tatacgatct acaggaatta gctttgaccg aatacctctg tttgtttgag ctgttgtatt
541 aactgcatta ttattacttc tttcccttcc agtactagct ggagctatta ctatattatt
601 aacagaccga aacttaaaca catctttctt cgaccccgct ggaggaggag accctattct
661 ttaccaacat ttattttgat tttttggtca ccctgaag

The sequence of bases of (MK 287890) Iraq *D. ciliates*

1 ctttatgatt tgttgacaaa tcataaagat attggaacat tatattttat tttcggagcc
61 tgagcaggca tagtaggaac atcccttaga attctagttc gtgctgaatt aggacacccc
121 ggagctttaa ttggagacga ccaaatttat aacgtaattg taacagctca cgcatttgta
181 ataattttct ttatagtaat acctattata attggagggt ttggaaattg attagtacca
241 ttaatattag gtgctccaga tatagcattc ccccgaataa ataatataag tttttgatta
301 ctacctccat ctcttacctt acttttagtc agcagtatag tggaaaacgg agccggaaca
361 ggttgaacag tgtatccccc tctatcatca gtcattgctc acggaggagc atctgtagat
421 ttagccatct tttccttaca tttagcaggt atttcttcaa ttttaggggc tgtaaatttt
481 attacaacag ttattaatat acgatctaca ggaattagct ttgaccgaat acctctgttt
541 gtttgagctg ttgtattaac tgcattatta ttacttcttt cccttccagt actagctgga
601 gctattacta tattattaac agaccgaaac ttaaacacat ctttcttcga ccccgctgga
661 ggaggagacc ctattcttta ccaacattta ttttgatttt ttggtcaccc tgaag

Final Remark:

All the specimens of Dacus species that were collected from three sites of Kurdistan region /Iraq were subjected to morphological characterization using different classification keys (David *et al.*, 2011; Drew *et al*., 2002; Drew *et al*., 2007; Drew *et al*., 2013; and Leblanc *et al*., 2013). Results showed that there is only one species of Dacus species populated in this region called *Dacus ciliates*. Molecular techniques are providing the scientists with more mechanistic tools for scientists to confirm the organism, so that the molecular techniques are a

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uniform and practical method for species identification of insects. The mitochondria of insects contain their own double-stranded circular genomes (Fig 4), which range from 14,503 bp (Beckenbech and Joy 2009) to 19,517 bp in size (Lewis *et al.* 1995). The agarose Gel electrophoresis of the PCR products for about 100 specimens, with the species-specific primers indicated that there is only one species of Dacus (*Dacus ciliates*) in Kurdistan region / Iraq. The length of the polymerase chain reaction products was approximately 708 bp. DNA sequencing which is a molecular based technique was used to confirm the above results. All sequencing results of COI-mtDNA were sent to the GenBank (in USA) to be checked. The GenBank firstly submitted a code number for each sequence as: Bankit 2174377, Bankit 2174378, and Bankit 2174379 respectively, and after about one month, the GenBank sent an accession number for each sequence in the mid of December 2019 as: MK 287888, MK 287889, and MK 287890 respectively, as clarified in Table (4). The sequencing map of COI- mtDNA of *Dacus sp.* that collected in Kurdistan region-Iraq, has been registered as a new according to IGB.

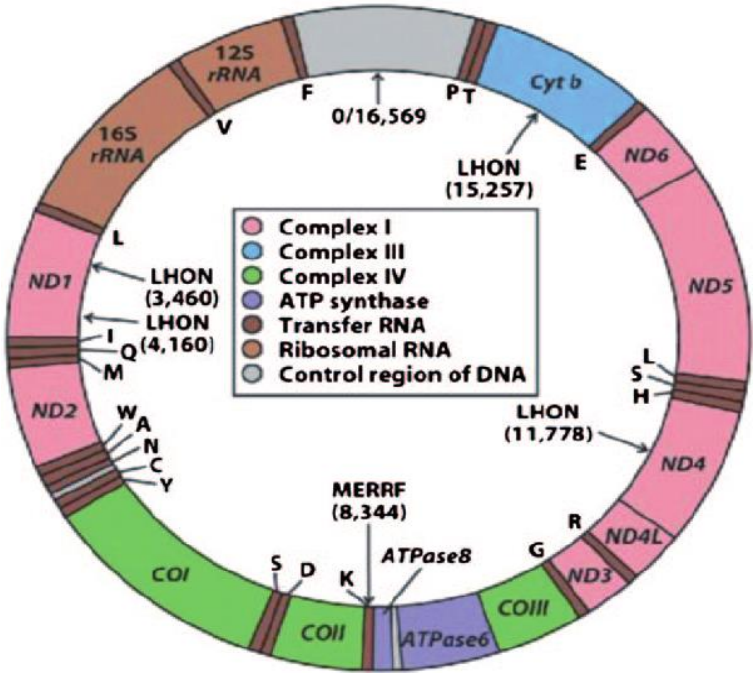

Fig. (4): Organization of insect mitochondrial genome (Source: http://chimerasthebooks.blogspot.in/2011_12_01_archive.html)

Table (4): GenBank code number and accession number of COI-mtDNA sequence

Specimen No.	Sampling location in Iraq	Species identity	Gen Bank	
			Code Number	Accession Number
1c-1	Bamarze/Duhok	*Dacus ciliatus*	Bankit 2174379	MK 287890
2c-9	Gardasher/Erbil	*Dacus ciliatus*	Bankit 2174378	MK 287889
3b-9	HassanTappa/ Sulaymaniyah	*Dacus ciliatus*	Bankit 2174377	MK 287888

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پوختە

نمونێن مێشا کولندی **Dacus ciliatus** زارە ڤییین گە لە ك گوندێن سە ر هە ر سێ پارێزگە هێن هە رێما کوردستانا عیراقێ د ماوێ 9/15 – 12/1/ 2017 تا کو 5/15 – 9/30 / 2018 هاتنە کومکرن. مێشا کولندی کول پرانیا زە رزە واتێن خێزانا کولندی ین بە ر بە لاف هاتنە دیتن. بکارئینانا کە وییا پە لمە را ئێك لدویف ئێك (PCR) کە وییا زانستی یا مولوکولی ژ بو ستویرکرنا کوپییە کی یان چە ند کوپییە کا ژ پارچە کا ترشێ ناووکێ، و بدە ستڤە ئینانا هزاران بو ملیونانا کوپیا ژ زنجیرا (DNA). ئە و فێقییێن زە رە ردار هاتنە کومکرن و پاراستن دناف هند ھ ك ساڤیکێت بازنە یی دا یت وە رپێچای ب پارچە کا تولێ بێژینکا (تیرێ 35 سم و ب درێژاهیا 40 سم)، و تە خە کا ئاخێ دناڤدا دروست کریە ب ستویراتیا 3 سم ژ بو تمامکرنا پروسێسا پوپاینێ.

التحديد الجزيئي لذبابة القرعيات ((*Dacus ciliatus* (Diptera: Tephritidae) التي تصيب العائلة الباذنجانية في إقليم كودستان / العراق ، استناداً إلى جين المايتوكوندريا COI

الخلاصة

تم إجراء مسح لعينات ذبابة القرعيات للعديد من القرى لثلاث محافظات دهوك ، إربيل والسليمانية / إقليم كردستان /العراق خلال الفترة 15 / 9 -1 / 2017/12 و15 / 5-30 / 2018/9. تم العثور على ذبابة القرعيات *Dacus ciliates* Loew التي تنتمي إلى عائلة Tephritide رتبة ثنائية الاجنحة التي تصيب معظم الخضروات مثل العائلة الباذنجانية. استخدمنا تقنية تفاعل البلمرة المتسلسل (PCR) وهو تقنية علمية في البيولوجيا الجزيئية لتضخيم نسخة واحدة أو بضع نسخ من قطعة من الحمض النووي ، وتوليد الآلاف إلى ملايين النسخ من تسلسل DNA معين. تم جمع الفواكه التالفة وحفظها في أقفاص مستديرة مغلفنة بقطعة من قماش المناخل (قطر 35 سم ، بارتفاع 40 سم) ، تحتوي على طبقة من 3 سم تربة لتسهيل عملية التعذير .