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## Morphological and Molecular Identification of Jujube Fruit Fly *Carpomya incompleta* (Becker, 1903) (Diptera: Tephritidae) southern Iraq

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**Abstract:** An accurate identification of an insect is essential for the effective pest management program. However, due to the restrictions of phenotypic identification, molecular process (e.g. DNA barcoding) is used to overcome some of these limitations. The present paper was aimed to identify the Jujube fruit fly by morphological characters of the adults and molecular DNA barcoding of the larval stage in southeast Iraq. The adults was morphologically identified as *Carpomya incompleta* (Becker, 1903) (Diptera: Tephritidae). Results of the molecular method revealed that the mitochondrial COI – COII marker was successfully identified immature stage of the species. The sequence analysis results identified the specimens (AHL1 and AHL2) as *C. incompleta*. Sequences of the specimens were recorded in The US National Center for Biotechnology Information (NCBI) under accession numbers (ON045002.1 and ON045003.1, respectively). These results were consistent with the phenotypic identification of the Jujube fruit flies.

**Keywords:** *Carpomya incompleta*, Fruit flies, Identification, Jujube, *Ziziphus* spp.

### Introduction

Fruit fly species, *Carpomya* spp. (Tephritidae: Diptera) are economically important pests infesting some flowering plants, fruits, some vegetables and trees in different type of the climates in the world (Norrbon, 1997; Abdel-Galil *et al.*, 2014; Ciceoi *et al.*, 2017; Korneyev *et al.*, 2017); the genus *Carpomya* includes several species *C. vesuviana* Costa, 1854 (by monotypy), *C. (Carpomya) incompleta* (Becker, 1903), *C. (Carpomya) schineri* (Loew, 1856), *C. (Goniglossum) liat* (Freidberg, 2016), *C. (Goniglossum) wiedemanni* (Meigen, 1826), *C. (Myopardalis) pardalina* (Bigot, 1891) and *Carpomya tica* (Norrbon, 1997) (Norrbon, 1997; Korneyev

*et al.*, 2017).

Only *C. incompleta* and *C. vesuviana* attack Jujube (*Ziziphus* spp.) fruits (Rhamanaceae) (Aluja & Norrbom, 1999); *C. incompleta* is more common in Europe (France, Italy and Romania) (Ciceoi *et al.*, 2017; Korneyev *et al.*, 2017), Asia (Iraq, Iran, Oman, Saudi Arabia, the United Arab Emirates and Yemen) (Korneyev & Dirlbek, 2000; Ghanim *et al.*, 2014; Parchami-Araghi *et al.*, 2015; Korneyev *et al.*, 2017), and Africa (Egypt, Eritrea, Ethiopia, Libya, Niger, Senegal, Sudan, and Morocco) (Zakari-Moussa *et al.*, 2012; Abdel-Galil *et al.*, 2014; El Harym & Belqat, 2017; Korneyev *et al.*, 2017). However, *C. vesuviana*

was recorded in Italy, Ukraine, Russia, Afghanistan, Azerbaijan, Cyprus, Iran, Iraq, Lebanon, Syria, Tajikistan, Turkey, Turkmenistan, Uzbekistan, Cambodia, India, Pakistan, Thailand and India (Kapoor, 2002; Farrar *et al.*, 2004; Mumford, 2005; Korneyev *et al.*, 2017).

Insect identification at the species level is essential to understanding the biological diversity and evolutionary relationships of species (Mayhew, 2018); the identification of insect pest species is usually done by the traditional method using insect phenotypic/morphological traits (Tahir *et al.*, 2018). However, the process of phenotypic identification is often difficult and time consuming, as well as the difficulty of diagnosing pests in immature stages is common because the most morphotaxonomic keys are according to adults only (Barrett & Hebert, 2005). Also, the phenotypic plasticity complicates the process of morphometric identifications of species, so a high level of expertise is required to use taxonomic keys (Ball & Armstrong, 2006). Depending on the phenotypic identification of *C. incompleta*, this species differs from other congeneric species, as it lacks the dark markings, bare areas and black microtrichose of other species. However, the genus was included *C. incompleta* based on other similarities with the species *C. schineri* and *C. vesuviana* (Norrbom, 1997).

The molecular identification method is now widely used by taxonomists to solve the complexity associated with the traditional morphological method (Navajas & Fenton, 2000; Tahir *et al.*, 2018). DNA barcoding is the easiest and the most widely used method (Nagoshi *et al.*, 2011; Van der Bank *et al.*, 2012); Mitochondrial DNA (mtDNA) is an independent genome found in the mitochondria, and this type of genetic material

is inherited from mothers only. The sequences of nitrogenous bases in certain regions of this independent genome, especially the COXI-COXII region, are important molecular markers used to diagnosis most arthropods to the species level. (De la Rúa *et al.*, 2009).

Annually, Jujube trees are affected by several pests (Alyousuf *et al.*, 2004), such as Jujube fruit fly *C. incompleta* infests the Jujube fruits in Iraq during the growth season from November to April (Jebbar, 1996), causing economic losses to the crop that require management to decline their population and enhancement the fruit quality (Al-Yousuf & Al-Miahy, 2007; Ismael *et al.*, 2022). However, there are no molecular diagnostic studies for this pest species in Iraq. Therefore, the study aimed to provide a pictorial phenotypic identification method for *C. incompleta*, and the molecular diagnosis was conducted in order to better understand the identification of this insect pest.

## Materials & Methods

### Morphological identification

Specimens of the Jujube fruit fly were collected from Jujube orchards by using attractant traps at Shatt Al-Arab and Khor Al-Zubair regions, Basrah province, Iraq. The Specimens were preserved in 70% ethyl alcohol and sent to the Natural History Museum - University of Baghdad for identification. The insect was morphologically identified as *Carpomyia incompleta* (Becker, 1903) (Diptera: Tephritidae) according to the taxonomical key of Korneyev *et al.* (2017).

External features of both males and females in larval and adult stages (12 specimens) were examined using a Novel optical microscope and imaged using a Sony a7R II camera.

**Molecular identification**

**DNA extraction**

The DNA extraction process was carried out in the genetic engineering laboratory of the College of Agriculture, University of Basrah; the extraction was done using a special extraction kit (Geneaid Korea, GS100), according to the manufacturer instructions and the extracted DNA was kept at -20°C until use.

**Estimation of quality and quantity of genomic DNA**

The quantity and quality of mtDNA were estimated for all samples extracted by the Nano drop device (American company Thermo-Scientific), at wave lengths 260-280 nm; the extracted DNA was used in subsequent experiments.

**Polymerase Chain Reaction (PCR)**

A molecular polymerase chain reaction (PCR) was carried out to amplify the COXI-COXII

gene region in the mitochondrial genome. The primers (Macrogen, South Korea) detailed in table (1) was used; this region has high variability among species and high stability within one species of arthropods. The primers were prepared at a concentration of 10 picomoles.microlitre<sup>-1</sup>, according to the manufacturer's instructions.

The PCR reaction was carried out using a reaction mixture of 50 µl of the reaction components, 25 µl of the prepared reaction mixture (RED, Ampliqon, Denmark, Taq DNA Polymerase Master Mix), 2.5 µl of each primer and 100 ng of template DNA, which increased to the volume of 50 microliter using DD-Water. Amplification was done by using a thermal cycler (Techne TC-3000X Thermal Cycler, UK) according to the thermal cycling program mentioned in table (2).

**Table (1) Primers used in the PCR polymerase chain reaction**

Primer	Primer sequence	TM	Expected size (bp)	Reference
LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	54.80	710~	Folmer <i>et al.</i> , 1994
HC02198	5'-TAAACTTAGGGTGAAAAAATCA-3'	59.62		

**Table (2): Thermal cycling program for PCR reactions for the COI-COII genomic region**

Primer	Stages	Time (min)	Cycle no.	Tem. (°C)	reference
LCO1490 / HC02198	Initiation	4	1	94	(Folmer <i>et al.</i> , 1994)
	denaturation	0.5		94	
	Annealing	1	35	57	
	Extension	1		72	
	Final extension	10	1	72	
	Storage			∞	

### Technique Relay for Product (PCR)

An electrophoresis of the PCR reaction product was carried out to confirm the amplification process for the targeted gene segments, the process was conducted on a (2%) agarose gel at a voltage of 95 mV. Solution 1X TAE was used as an electrolyte buffer to conduct the migration process in a migration tank (Cleaver Scientific MultiSUB electrophoresis unit ev222, England).

Diamond Dye (Promega, USA) at X1 concentration was used to stain the samples and DNA molecular weight index (Ladder (100 bp). After migration, the samples were detected using Gel documentation (Cleaver “Clear view UVT254/312, England).

### DNA sequencing PCR

After confirming the success of the amplification of the targeted gene segments by electrophoresis, the samples (PCR product) were sent to Macrogene Company (South Korea) for reading the sequences of nitrogenous bases for each sample.

### Molecular identification of samples

The sequence processing was performed using Chromas Ver. 2.6.6 (Technelysium Pty, Ltd). The processed sequences were compared with the deposited sequences at the US National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) to determine identification, which is based on the Maximum score, the Query cover, and the identity percentage provided by the research tool; the highest rates of the mentioned criteria were adopted to confirm the identification to the species level. After confirming the identification, the confirmed sample sequences were deposited and registered in NCBI GenBank®

([www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/)).

## Results

### Morphological identification

External features of larvae and adults, males and females of Jujube fruit fly *C. incompleta* were measured; the newly hatched larvae were characterized as worm-shaped, translucent white in colour, quickly turning to milky colour (Fig. 1). The average length and width of the three larval instars was (3.23 and 0.74), (5.3 and 1.42), and (8.7 and 1.96) mm, respectively. It is possible to distinguish between the larvae of the three larval ages due to the clear differences in the shape and size of the pharyngeal vertical structure; this structure includes the mouth hooks curved forward; each hook which is dark brown is the Mandible jaw. However, an anther structure is found in the thorax and consists of a triangular structure which is called the hypostomal sclerite, it connects to a hardened structure inside the first thoracic segment, called the pharyngeal sclerite, consisting of two parts, one upper and the other ventral.



**Fig. (1): Jujube fruit fly larvae *C. incompleta*.**

The adults are characterized by their small size, yellow colour, streamlined compressed shape; the average length of females and males reached 4.91 and 4.11 mm, respectively. The average shortest distance between the two ends of the extended wings was 7.13 mm (Figs. 2 and 3). The head is less wide than the thorax

region. The compound eyes are large and oval in shape. The surface of the compound eyes is comprised of the optical units Ommatidium; the three simple eyes are located in the vertex; the face is pale yellow. The forehead is broad and convex bearing a pair of antennae, while the gena is located between the compound eyes and the edge of the head; and compound eyes connects with the forehead by frontal suture (Lunula), which form the shape U upside down. The proboscis is a short and enlarged structure; the lateral ends of the proboscis contain fine hairs for taste.

Thorax region has three lines of fine dark hairs on the dorsal surface of the scutum; there are two small dark spots on the lower side of the scutellum (Figs. 2 and 3).

The coxa of front legs, which are longer than those in the other legs, are free from the hairs. There are two parallel rows of spines distributed along the femur of the front and hind legs, while there is one row of those spines on one side of the femur in the middle legs. Also, there is a tibial spur attached only in the middle leg, which is absent in the other legs (Figs. 2 and 3).



Fig. (2): A. Dorsal side, B. Ventral side of a female Jujube fruit fly *C. incompleta*.

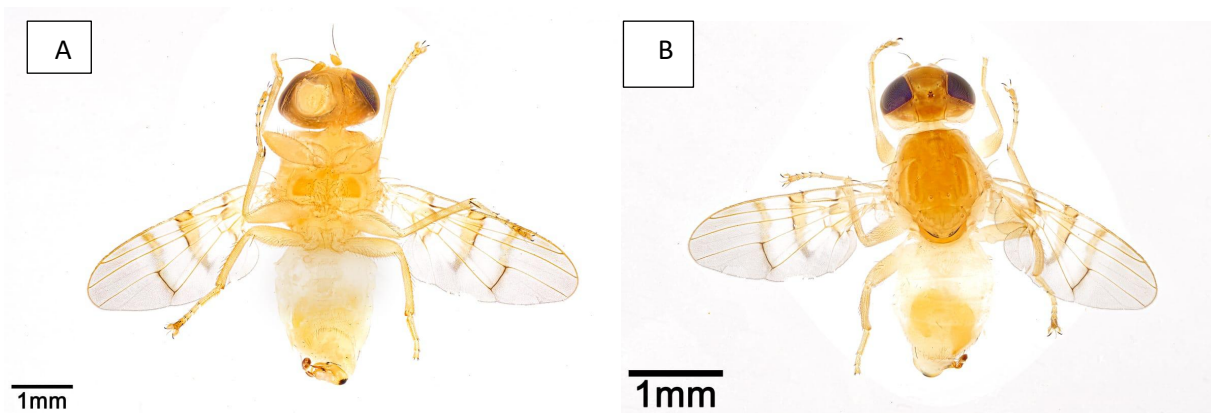


Fig. (3): A. Dorsal side B. Ventral side of a male Jujube fruit fly *C. incompleta*.

The front wings were distinguished by the presence of three pale cross bands. At the wing veins, (third medial + first cubital) veins  $M_3 + Cu_1$  and (second cubital + second Anal) veins  $Cu_2 + A_2$  do not extend to the rear edge of the wing. The costal bristle on the front edge of the wing is also small (Fig. 2 and 3).

The external appearance of the abdomen was also distinguished by the presence of

thick, coarse hairs surrounding the edges of the abdomen; there were no appendages carried by the abdomen except for the external genital appendages. The ovipositor has a tapering end; its dimension is about 0.63 mm in length and 0.131 mm in width (Fig. 2).

### Molecular identification

The results of the electrophoresis (Fig. 4) of the PCR product showed two clear bands, plus ladder, with a size of about 710 base pairs, confirming the amplification of the COXI-COXII target region. The sequence analysis results, using BLAST, also confirmed that the specimens (AHL1 and AHL2) were identified as *Carpomya incompleta*. The specimens were deposited in The US National Center for Biotechnology Information NCBI under accession numbers (ON045002.1 and ON045003.12), respectively (Table 3). These results were consistent with the results of the phenotypic identification of the Jujube fruit flies.

### Phylogenetic analysis of the genus *Carpomya*

The results in fig. (5) confirmed the identity of the species of the current study as they were grouped in one cluster with a 90% similarity, while the genetic distance between them was 0.00. The phylogenetic tree showed that the species grouped into two main groups. The first group included the two clusters *C. vesuvina* and *C. incompleta*, whose spread in the eastern and southeastern parts of the globe (Iraq and Southeast Asia); while the second group, represented by *C. schineri*, which was oriented in the northern and western parts of the globe (Europe and America).

**Table (3): Molecular identification of Jujube fruit fly *Carpomya incompleta* specimens.**

No.	Isolation name	Scientific name	NCBI registration number
1	AHL1	<i>Carpomya incompleta</i>	ON045002.1
2	AHL2	<i>Carpomya incompleta</i>	ON045003.1

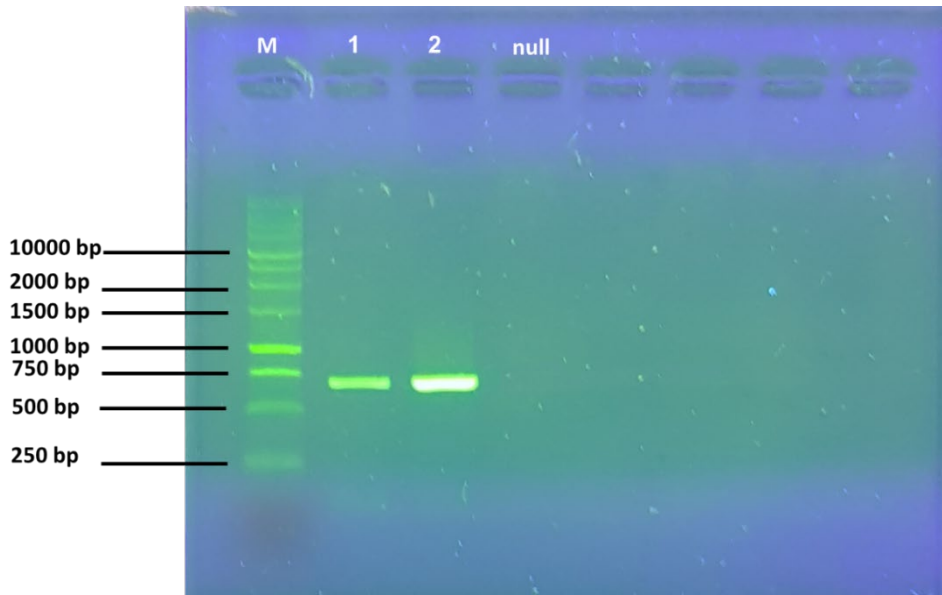


Fig. (4): Results of electrophoresis of PCR product for Jujube fruit fly samples, *Carpomya incompleta*. 1) AHL1 sample and 2) AHL2 sample.

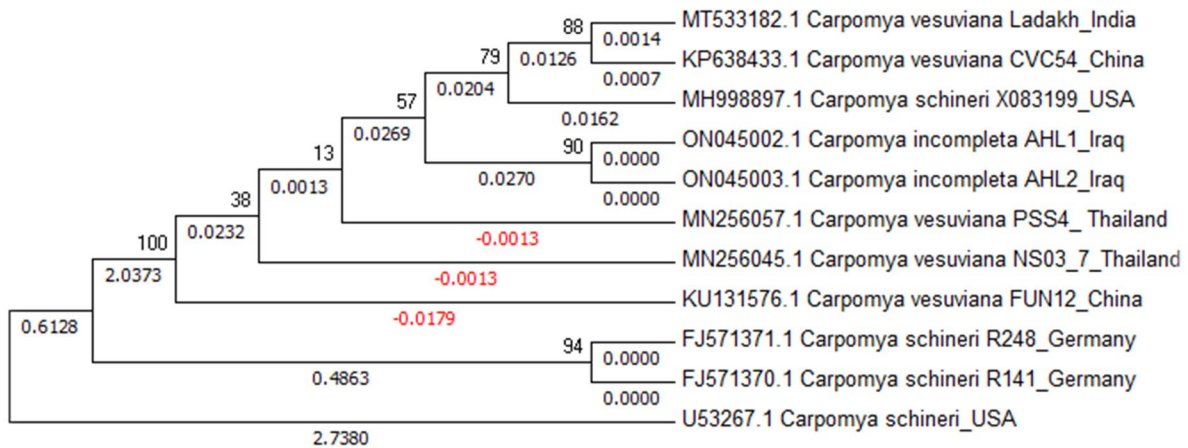


Fig. (5): Phylogenetic tree drawn according to the neighbour joining algorithm to describe the relationship between the species of the genus *Carpomya*, including *C. incompleta*.

## Discussion

In the present study, the key morphological markers, which have been implemented in the identification process were subsequently confirmed by DNA barcoding. The morphological markers of the adults Jujube fruit fly are congruent with the (Jebbar, 1996). However, the morphometric methods failed to identify immature Jujube fruit fly. The

morphological identification of the immature stage of the fly had some restrictions. It is difficult to identify many of the immatures of the economically important pests by morphological taxonomic keys even by taxonomists due to the phenotypically similarities between some of insect pests species (Clarke *et al.*, 2005). The results of molecular identification markers has played an important role in effectively resolving this

complexity and assigning this species to the proper taxon. In this study, mitochondrial COI – COII marker was successfully identified the species by using the immature stage. Several studies have also reported difficulties with morphometric identification of the immatures of different insect species, including larvae of Queensland fruit fly *Bactrocera tryoni* (Blacket *et al.*, 2012).

DNA barcoding provides a fast and reliable methods to distinguish among species (Ide *et al.*, 2016; Menabit *et al.*, 2022). The originality of the DNA barcoding depends on the polymorphism among examined DNA sequences. Barcode sequence polymorphism is mostly quite low among individuals of the same species than in those of narrowly related species; the larger the barcode gap, the more accurate results (Hebert *et al.*, 2004; Dasmahapatra *et al.*, 2010).

Depending on the results of neighbor-joining tree, it is difficult to differentiate between the two species *C. vesuviana* and *C. incompleta*, at the molecular level. Therefore, it has become necessary to rely on phenotypic and genotypic markers of mature and immature stages of *C. incompleta*. Interestingly, a phylogenetic analysis study based on a morphological trait was performed by Norrbom (1997) to determine the relationships among species of *Carpomya* showed an almost identical pattern of clustering that presented in our model, in which the species *C. incompleta* was closely related to *C. vesuviana* and both were close to *C. schineri*, while the rest of the species gathered in clusters relatively far away from them, this is indeed can explain the importance of combining the results of morphological and molecular profiles to insure identity of insect species, especially the closely related ones that is lead to accurate identification of the examined samples.

## Conclusion

The adults fruit flies infesting Jujube fruits was Morphometrically identified as *Carpomyia incompleta* (Becker, 1903) (Tephritidae: Diptera). The mitochondrial COI – COII marker was successfully identified the species using the immature stage. These results were consistent with the phenotypic identification of the Jujube fruit flies.

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## Contributions of authors

**H.M.T:** Writing original draft.

**A. A.:** Formal analysis, visualization, supervision, project administration.

**Both authors:** Methodology, software, investigation resources data and editing of the manuscript.

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## Conflicts of interest

The authors declare that they have no conflict of interests.

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التشخيص المظهري والجزئي لذبابة ثمار السدر (*Carpomyia incompleta* (Becker, 1903)  
(Tephritidae: Diptera)

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**المستخلص:** يعد التشخيص الدقيق للحشرة أمراً ضرورياً لبرامج مكافحة الآفات الفعال، نظراً لقيود التشخيص المظهري ، يتم استخدام العملية الجزيئية (تشفير الحمض النووي الشريطي) للتغلب على بعض تلك القيود. هدفت هذه الدراسة إلى تشخيص على ذبابة ثمار السدر مظهرياً ووراثياً باستخدام الترميز الشريطي للحمض النووي. تم تشخيص الحشرة البالغة مظهرياً على أنهم (*Carpomyia incompleta* (Becker, 1903) (Tephritidae: Diptera). أظهرت نتائج الطريقة الجزيئية أن علامة COI-COII للميتوكوندريا قد شخصت الأنواع بنجاح باستخدام الادوار الحشرية غير البالغة. أكدت نتائج تحليل التسلسل أيضاً أن العينات (AHL1 و AHL2) تم تحديدها على أنها *C. incompleta*. تم تسجيل العينات في المركز الوطني الأمريكي لمعلومات التكنولوجيا الحيوية NCBI تحت أرقام الانضمام (ON045002.1 و ON045003.12) ، على التوالي. كانت هذه النتائج متوافقة مع التشخيص المظهري لذباب ثمار السدر.

الكلمات المفتاحية: *Carpomyia incompleta* ، تشخيص، ذباب الفاكهة، *Zizaphus* spp