

**MOLECULAR DIAGNOSIS AND COMPARISON STUDY TO THE RED PALM WEEVIL *RHYNCHOPHOROUS FERRUGINEUS* (OLIVIER, 1790) IN BASRAH PROVINCE - IRAQ**

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**ABSTRACT**

The Red Palm Weevil (RPW) *Rhynchophorus ferrugineus* (Olivier) is well-known as serious insect pest of date palm and other palm species. The pest was firstly recorded in Iraq in 2015. This study was confirmed the identification of this pest using molecular diagnostic (PCR and sequence analysis) and investigate the genetic relationship by comparison of the sequences with other recorded copies worldwide. The results confirmed the identity of the *R. ferrugineus* (Olivier) molecularly. Ten copies of mitochondrial COI gene of Iraqi samples (MF092880.1-MF092889.1) were firstly registered in National Center of Biotechnology Information (NCBI). The sequences comparison of Iraqi sample genes with worldwide genes showed high similarity with Chinese and Mediterranean COI copies especially the Tunisian one, which may assumes that they were descended from the same origin. The study concluded that the invention of this insect to Iraq could be occurred directly from farms in border area between Iraq and Kuwait or by infested offshoot that imported from other countries.

**Keywords:** *Rhynchophorus ferrugineus*, Date Palm, distribution, Identification.

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دراسة تشخيصية ومقارنة لحشرة سوسة النخيل الحمراء (*Rhynchophorus ferrugineus* (Olivier, 1790) في محافظة

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المستخلص

تعد حشرة سوسة النخيل الحمراء (*Rhynchophorus ferrugineus* (Olivier) من الآفات الحشرية الشرهة التي تهاجم نخيل التمر والعديد من أنواع النخيل الأخرى. سجلت هذه الآفة لأول مرة في العراق عام 2015. شملت هذه الدراسة تأكيد تشخيص النماذج الحشرية التي صنفت مظهرها في دراسة سابقة باستخدام الطرق الجزيئية كما استخدمت طرق المعلوماتية الحيوية في تقصي مصدر الإصابة عن طريق دراسة العلاقة الجينية بين النسخ العراقية للجين cytochrome c oxidase subunit I (COI) و النسخ المسجلة عالمياً في المركز الوطني لمعلومات التقنية الحيوية الأمريكي NCBI. أوضحت النتائج تأكيد تشخيص نماذج سوسة النخيل الحمراء (*Rhynchophorus ferrugineus* (Olivier) جزيئياً اعتماداً على الجين COI والذي سجلت 10 نسخ عراقية منه في الـ NCBI (MF092880.1 – MF092889.1). أوضحت نتائج المقارنة لتتابعات القواعد النروجينية للجين COI للنماذج العراقية المسجلة مع نسخ الـ COI الأخرى المودعة في الـ NCBI تشابه عالي مع النسخ الصينية ونسخ حوض البحر الأبيض المتوسط وخصوصاً النسخة التونسية Tunisia – KX228866.1 والذي قد يوحي بالاعتقاد أنها انحدرت من نفس الأصول. استنتجت الدراسة بأن مصدر الإصابة بالآفة قد يكون مباشراً من المزارع الكويتية القريبة من الحدود العراقية أو غير مباشر من الفسائل المصابة المستوردة من الدول الأخرى.

كلمات مفتاحية: سوسة النخيل الحمراء، نخلة التمر، الانتشار، التشخيص

## INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is one of the major fruit crops that grown anciently in Iraq, which was expected to be the origin area of the date palm, which is then distributed to the Arab Peninsula, Middle East, and North Africa since a long historical periods (6). Recently the plant growing in about 30 countries distributed in four continents included Asia, Africa, America, and Australia (7). Date palm trees usually infested by a wide range of insect pests of different orders involves Coleoptera, Lepidoptera, and Hemiptera that mostly causes severe damages to the plant which leads to yield losses (7). Red palm weevil (RPW) *Rhynchophorus ferrugineus* (Olivier) is a serious pest of palms, which is reported to attack 27 palm species worldwide but the major hosts are *Cocos nucifera*, *Phoenix canariensis*, and *Phoenix dactylifera* (9). The pest currently present in Africa: Egypt, Libya, Morocco, and Tunisia; America: Netherlands Antilles; Asia: Bahrain, Bangladesh, Cambodia, China, India, Indonesia, Iran, Iraq, Israel, Japan, Jordan, Kuwait, Lao, Lebanon, Malaysia, Myanmar, Oman, Pakistan, Philippines, Qatar, Saudi Arabia, Singapore, Sri Lanka, Syria, Taiwan, Thailand, United Arab Emirates, Viet Nam, and Yemen; Europe: Albania, Cyprus, France, Georgia, Greece, Italy, Malta, Portugal, Slovenia, Spain, Turkey, and United Kingdom; Oceania: Samoa, Solomon Islands, and Vanuatu (9). The red palm weevil was firstly mentioned by (11) in Basrah province, Iraq but the subsequent researchers denied the existence of the pest. On October 2015, Agricultural Directorate of Basra and in collaboration with the Plant Protection Directorate in Baghdad were recorded the appearance of red palm weevil in orchard located in Safwan border area near Kuwait (4). The RPW usually attacks the top of plant then transfer to the trunk of the young palm trees *P.dactylifera* about one meter from the ground (12). The wounds and injured tissues following date palm service operations like offshoot removal and frond shaving considered as assistant factors to RPW infestation (7). The females of RPW usually lays about 300 eggs individually in the separate holes in the wounded or injured areas.

The eggs, which is mostly, sized  $2.62 \times 1.12$  mm, creamy white, shiny and oblong, will hatch within 2-5 days into larva. Larvae body are pyriform comprised of 13 segments, about 50 mm long and 20 mm wide, legless, creamy white with brown-russet-red to brown-black head capsule with strongly chitinized mouthparts. The larvae starts boring and feeding on the soft succulent tissues around apical meristems at the early stages then move to the crown or trunk edges to form cocoon. The larval stage may range from one to three months depending on temperature and host species. The pupation occurs in cylindrical fibrous cocoons for two to three weeks ended with adult weevil emergence, so the life cycle will completed in about 4 months (5, 7). Adults are large, about 35 mm long and 10 mm wide, although they can be up to 42 mm and 16 mm wide with a long rostrum contains short brownish seta (hairs) on the anterior dorsal half in meals and disappearance of this structure in females. They are reddish-brown in colour with variable dark markings on the pronotum. The RPW adults have the ability to fly for distances ranging from 100 to 800 m because of well-developed wings that they have. The adults usually stay in the same palm until complete consumption of the meristem of the palm or its offshoots then the weevil left the dead palm to a new one (5, 7). The detection of the infestation is very difficult unless the Palm been severely damaged as larvae and adults mostly destroy the interior parts of the palm without showing any detectable signs. The early symptoms are eggs lying notches, cocoons in the base of the palm, irregular crown growing, wilting, desiccation and necrosis of the fronds as a result of lacking water, and tunnelling in the trunk and stem which leads to hollowing out it that resulting in reduce the mechanical resistance of the trunk which exposing palm to collapse. The injured tissues could be infected with opportunistic bacteria and/or fungi to speed up the deterioration of the palm (1, 5, 7). The objective of this study was to confirm the diagnostic of RPW (*R. ferrugineus*) molecularly and compare the COI gene sequences with other worldwide copies to investigate the source of infestation in Basrah province.

## MATERIALS AND METHODS

**Samples :** The samples were gotten from previous study (2), which were collected from a farm in Safwan border region about less than 1 km from Iraq-Kuwait border line (Figure 1) in 1<sup>st</sup> of October 2015. The farm included



**Fig. 1. Samples collection site at Safwan border area**

### Molecular Identification

**Genomic DNA Extraction :** The total genomic DNA (G-DNA) was obtained from thorax area as it is rich with muscle tissues. The tissue was flash frozen with liquid nitrogen then grinding to fine powder with mortar and pestle. Up to 25 mg of ground tissue was transferred to 1.5 ml Eppendorf tube to extract total G-DNA using Geneaid DNA extracting kit (gSYNC™, GS100, Geneaid Biotech Ltd.) according to the manufacturer instruction. The quantity and quality of G-DNA was confirmed using Nanodrop (Termo Scientific™, NanoDrop 2000) under 260/280 nm wavelength.

### Primer Design

Primers designed to amplify 453 bp of cytochrome c oxidase subunit I (COI) region of RPW mitochondrion genome using primer-3-plus software (16). The sequences of *Rhynchophorus ferrugineus* mitochondrion, complete genome copies: NC\_028535.1 and KT\_428893.1 were aligned using BLAST software (3) to select the targeted amplification area. The forward primer was COI-LAB-F:

5'CCCCCTCTCTCACTCTTCTCT 3' which positioned from 1654-1674 of *R.ferrugineus* mitochondrial genome, where the reverse primer was COI-LAB-R: 5'TATGGGAAATTATTCCAAATCCTG 3'

several cultivars of date palm involved Berhi, Hylawy, Sayer and Boraim but the severe injury was in Berhi and Hylawy cultivars. The collected samples placed in sealed plastic bags then saved in 70% ethanol for next experiments.

which positioned from 2016-1993 of *R.ferrugineus* mitochondrial genome

### PCR Reaction

The PCR reaction performed using 50µl Eppendorf tube contained 25µl of total reaction component which included 12.5 µl of master mix (Taq DNA Polymerase Master Mix RED, Ampliqon, Denmark), 12.5 µl of each primer, 100 ng of template DNA, the volume completed to 25 µl with DNase free DD-water. The PCR amplification performed in thermal cycler (MyGenie™ 96/384 thermal Block, BioNEER, Inc.) and the reaction conditions involved 90°C for 5 min followed by 35 cycles of 94°C for 1 min, 60 °C for 1 min and 72 °C for 2 min then final extension on 72 °C for 5 min. The quality and quantity of PCR product was confirmed using nano drop device (Termo Scientific™, NanoDrop 2000). The PCR product samples send to Macrogen Inc. (Macrogen Korea: 10F, 254 Beotkkot-ro, Geumcheon-qu, Seoul, 08511, Rep. of Korea) for sequencing.

### Sequence Analysis

The sequences were processed using Chromas version 2.6.2 (14) and the assembly of sequences (forward and reverse) was performed using CAP3-PRABI-Doua software (13). The assembled sequences were submitted to the National center of biotechnology information (NCBI) for registration. The molecular identity of the samples performed

by multiple alignment of each sample sequence with NCBI database using Basic local alignment search tool (BLAST) software (3). A comparison among all our identified sequences with all NCBI copies was carried out using BLAST and the best compatible sequences of BLAST throughput were processed by performing new multiple alignment to build neighbor joining tree using MEGA 5 software (15) to determine the similarity relationship among related COI copies.

## RESULTS AND DISCUSSION

**Molecular Identification:** After deposition of the sequences and their protein translation in the NCBI, the molecular identification of all collected samples was confirmed as: Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Holometabola; Coleoptera; Polyphaga; Cucujiformia; Curculionidae; Dryophthorinae; *Rhynchophorus ferrugineus* (Table 1).

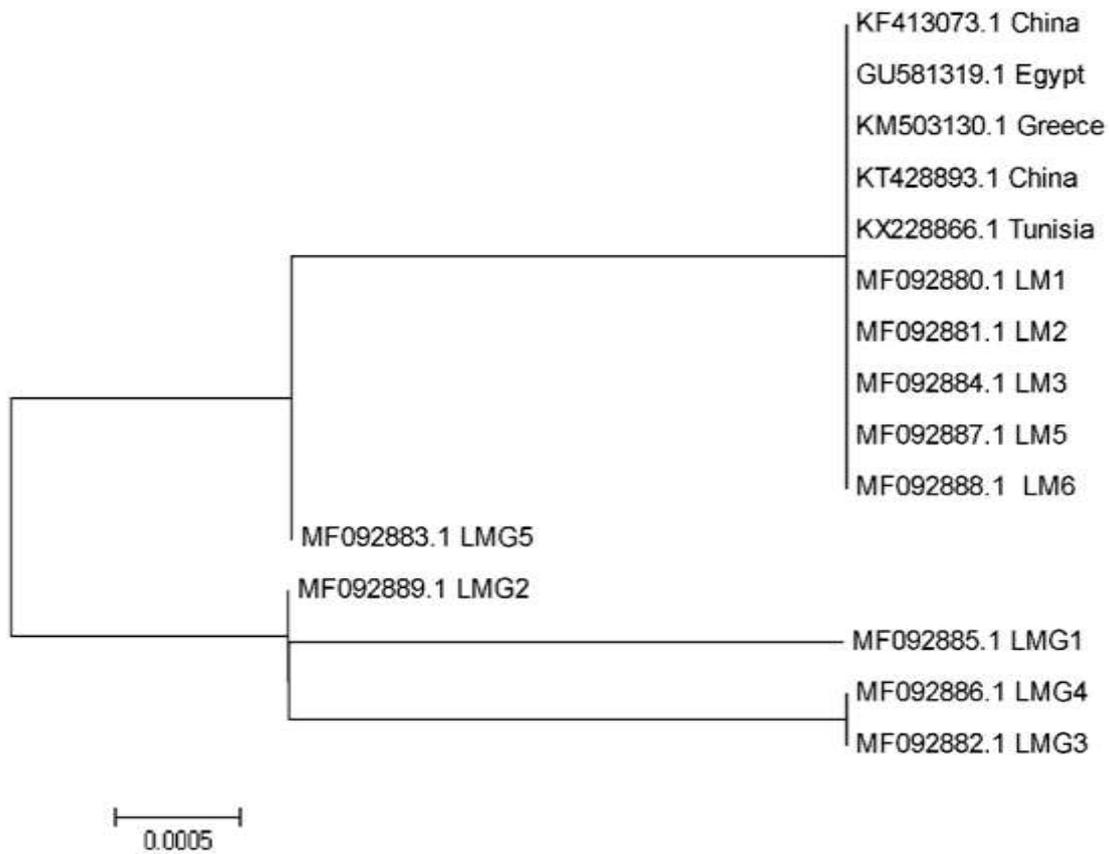
**Table 1. *Rhynchophorus ferrugineus* mitochondrial cytochrome c oxidase subunit I (COI) gene copies of collected registered samples and most similar samples worldwide**

Iraqi recorded samples		Most similar COI copy*			
Sample name	Accession #	Accession # - location	Score	Query cover %	Identity
LM1	MF092880.1	KX228866.1 - Tunisia	837	100	100
LM2	MF092881.1	KX228866.1 - Tunisia	837	100	100
LM3	MF092884.1	KX228866.1 - Tunisia	837	100	100
LM5	MF092887.1	KX228866.1 - Tunisia	837	100	100
LM6	MF092888.1	KX228866.1 - Tunisia	837	100	100
LMG1	MF092885.1	KX228866.1 - Tunisia	821	100	99
LMG2	MF092889.1	KX228866.1 - Tunisia	826	100	99
LMG3	MF092882.1	KX228866.1 - Tunisia	821	100	99
LMG4	MF092886.1	KX228866.1 - Tunisia	821	100	99
LMG5	MF092883.1	KX228866.1 - Tunisia	832	100	99

\*The similarity results obtained from NCBI-BLAST alignment comparison results in 5 Aug 2017.

**Bioinformatics:** The comparison of the studied sequence samples with all deposited cytochrome c oxidase subunit I (COI) gene copies of *R.ferrugineus* in the NCBI database presented high similarity (99-100% identity) with five non-Iraqi *R.ferrugineus* COI gene copies (KX228866.1 - Tunisia, KT428893.1 - China, KM503130.1 - Greece, GU581319.1 - Egypt and KF413073.1 - China) respectively. Furthermore, among the mentioned five copies, the Tunisian one was the most similar to all Iraqi samples (Table 1). A phylogenetic neighbor joining tree results (Figure 2) showed grouping of examined samples to two main related groups within differences ranged from (0 – 0.66%) among examined COI copies. The first group involved (KF413073.1- China,

LM6, GU581319.1- Egypt, KM503130.1- Greece, KT428893.1-China, KX228866.1-Tunisia, MF092880.1-LM1, MF092881.1 - LM2, MF092884.1-LM3, MF092887.1-LM5, MF092888.1-LM6, and MF092887.1-LMG5) respectively where the second group involved (MF092889.1-LMG2, MF092885.1-LMG1, MF092886.1-LMG4, MF092884.1-LMG3) respectively. Anyway, the differences were very low to distinguish among aligned sequences ( $P=1.0$ ).



**Fig. 2. Neighbour joining tree to determine the relationship among Iraqi samples of *Rhynchophorus ferrugineus* mitochondrial cytochrome c oxidase subunit I (COI) gene copies and the most similar worldwide copies**

Red palm weevil was not recorded among the usual and/or local pests on palms in Iraq until 2015 when it was first recorded in Safwan, Basrah by the Agricultural Directorate of Basra (4). Previously, RPW considered as a major palm pest in all Iraq surrounding countries that share the same borders especially Saudi Arabia (since 1987) and Kuwait (since 1988), which were the oldest invented countries in this area followed by Iran (since 1990) (9, 10). The molecular identification confirmed the pest identification; furthermore, it connected the collected samples with the most related worldwide samples, which gave us preliminary perception about the source of this infestation. There are two possible scenarios may investigates the appearance of this weevil in Iraq, that were either through direct invention by flying adults from the nearby farms of Kuwait or by infested offshoots or tissue culture date palm clones that imported to Iraq. The first scenario, which is the most accepted, is the direct transfer of the pest from nearby farms of Kuwait, which are distributed beneath the Iraq

– Kuwait borders especially that the infested Iraqi farm located in less than 1km away from borders and less than 2 km from some Kuwaiti farms in Al-Abdily border area. The second scenario proposed that RPW entered Iraq through uncontrolled importing of infested palm plants especially in the last few years when a huge number of UAE, Iranian and even Saudi tissue culture plants (clones) were imported and grown in Iraqi orchards especially in Basrah province. The comparison results showed high genetic similarity with RPW samples that collected from Mediterranean area especially the Tunisian one. According to (9), there is no available details about RPW in Kuwait, UAE and Iran adding to that, there is no registered (COI) copies in the NCBI related to these regions, so we think that could explain why the Mediterranean and chines samples were the most related to Iraqi ones. Furthermore, when we tried to inspect the source of the first infestation of RPW in all mentioned countries in the literature, we'll found that it related nearly to the same source, "the ornamental

palm offshoots imported from Middle East” (9, 10). Similar study reported that the haplotypes of COI gene showed significant relatedness to their geographic regions and suggested that the invasion history of RPW for last thirty years followed three main routes; the east haplotype and two of west haplotypes, which were the Middle East and Mediterranean ones (8). The mentioned study also reported that the Middle East and Mediterranean haplotypes were descended from different geographical populations. The results of this study could be support the possibility that the related samples in our neighbor-joining tree returned to the same origin, which is may clearly explain the high genetic similarity among them.

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