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MOLECULAR IDENTIFICATION AND PRELIMINARY REGISTRATION OF TWO *RHYNCHOPHORUS FERRUGINEUS* STRAINS IN IRAQ

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ABSTRACT

The study was conducted during the 2023-2024 agricultural season on the *Rhynchophorus ferrugineus* in infested orchards in Diyala Governorate, from the beginning of March to the end of November 2024. The aim was to conduct a molecular diagnosis of *Rhynchophorus ferrugineus*. The results of the study confirmed the initial recording of two strains of the insect in Diyala Governorate. Polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS) region, one of the most conserved regions in many organisms. The gene sequences of the PCR products were analyzed using the Sanger method. They matched to global strains and registered in the National Center for Biotechnology Information's Global Gen Bank. The evolutionary tree was drawn, and the genetic divergence values between them and the global strains were also evaluated using MEGA11. For the first time in Iraq, the study recorded two strains of the insect, which were deposited under serial numbers PQ496644 and PQ522231, respectively. The results also confirmed the existence of varying matching rates between local strains and many strains recorded in the Kingdom of Saudi Arabia and Greece, as indicated by genetic divergence values. The strain with serial number PQ496644 was most similar to the Greek strain, and both strains belonged to the species *Rhynchophorus ferrugineus*.

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INTRODUCTION

Date palm (Phoenix dactyliferaL.) is a cross-pollinating fruit tree, due to its dioecious nature (Munir *et al*., 2016). Date palm Phoenix dactyliferaL is one of the oldest fruit trees in the subtropical regions date palm management (Alkenani and Alshehri 2025) and (Jubeir and Ahmed 2019). Date palms (Phoenix dactylifera L.) belonging to the family Aracaceae are the most important fruit trees in Iraq (Alpresem *et al*., 2019). *Rhynchophorus ferrugineus* is one of the most important insect pests attacking date palms in most regions of the world (Cousin *et al., 2019)*. The first occurrence of the insect in Iraq was recorded in 2015 in Basra Governorate (Alsaad and Aletby,2018), affecting more than 40 cultivated date palm varieties. This pest is considered a complex insect, as all stages are found inside the palm

trunk (Al-Dosary *et al.,* 2016). The eggs are typically 1.12–2.62 mm in size, creamy white, shiny, and oblong, and hatch into larvae within 2–5 days. The larval body is pear-shaped, consisting of 13 segments, about 50 mm long and 20 mm wide, legless, creamy white in colour with a reddish-black cap capsule with strong mouthparts (Ivan *et al.,* 2018). An accurate identification of an insect is essential for an effective pest management program (Taher and Alyousuf 2023). Molecular techniques are used to accurately distinguish between species (Sinjare, 2024; Faraj *et al.,* 2019).

The larvae begin by burrowing and feeding on the soft, tender tissue around the apical meristem in the early stages, then migrate to the edges of the crown or trunk to form a cocoon. The larval stage may last from one to three months, depending on temperature and host species. The larval stage is fibrous and cylindrical, lasting two to three weeks, and the adult weevil emerges, completing the life cycle in about four months (Cristofaro *et al.,* 2023).

Adult larvae are large, measuring approximately 35 mm long and 10 mm wide, although they can reach 42 mm and 16 mm wide. They have a long proboscis containing short brown hairs on the anterior dorsal half of the male, but no such hairs are present in the female. They are reddish-brown in color with dark spots on their body. Adult red-palm weevil insects can fly distances ranging from 100 to 800 meters due to their well-developed wings. Adult insects typically remain in the same palm tree until the entire palm tissue or branches are consumed due to overcrowding. Then it leaves the dead palm tree for a new one (Bardan, 2020).

Molecular diagnosis is a powerful and modern tool, as it is highly accurate and enables the detection of subtle genetic changes and mutations that may not be apparent with traditional methods such as phenotypic diagnosis. Molecular diagnosis also offers rapid turnaround, which helps accelerate treatment initiation and inform appropriate control decisions (Ibrahim *et al*. 2023).

The red palm weevil *(Rhynchophorus ferrugineus*) is one of the most destructive pests affecting date palm plantations worldwide. Despite its economic significance, molecular studies on this species remain limited, with most identification efforts relying on morphological characteristics, which can be ambiguous and insufficient for distinguishing closely related strains. Therefore, in this study, we employed molecular diagnostic techniques based on the nuclear genome, which serves as the primary and most reliable reference for accurate strain-level identification and genetic differentiation.

MATERIALS AND METHODS

After conducting a field survey and confirming the presence of the insect in Diyala Governorate, samples of the entire insect were taken for molecular diagnosis from the areas of (Al-Abara, Al-Wajhiyah, and Al-Hadid). One insect was taken

from each area for molecular diagnosis. The samples were placed in an alcoholic solution (ethanol) at 70% concentration and transferred to a laboratory for molecular diagnosis according to the method described by Sabit *et al*. (2021).

Molecular Identification of Rhynchophorus ferrugineus

DNA Extraction

DNA was extracted from R. ferrugineus using the Addprep Genomyc Extraction KIT, supplied by ADD INC, following standard DNA extraction procedures and according to the method described by Asghar et al. (2015).

Primers

Primers for the diagnosis of Rhynchophorus ferrugineus were designed based on the ITS gene sequence located at the National Center for Biotechnology Information (NCBI) using the primer design software from Macrogen Inc. (South Korea), accession number HC01102850, and the nitrogenous base sequence as shown in Table 1.

Table (1): shows the genetic sequence of the ITS gene and the sequence of primers

output gross	Primer sequence	Primer	No
418	TGGCGCTCGTAATCATCATA	ITS F	1
	CTGGCTGAGGGTCGTATCTC	ITS R	2

The concentration and purity of DNA were measured using a nano drop device

Polymerase chain reaction (PCR)

To identify R. ferrugineus in this study, a polymerase chain reaction (PCR) test was performed using Maxime PCR premix kits prepared by the Korean company. The PCR was performed in a volume of 20 microliters, containing 1 microliter of forward primer, 1 microliter of reverse primer, and 1 microliter of extracted DNA. All components were then placed in a tube prepared by the Korean company, and the volume was brought to 20 microliters with nuclease-free water. To amplify the DNA of Rhynchophorus ferrugineus, follow the steps and PCR reaction conditions shown in Table 2.

Electrical deportation

1% acarose gel was prepared in a glass flask by dissolving 1 g of acarose in 100 ml of TAE solution and heating in a microwave until the gel was completely dissolved. The mixture was then cooled to 50-55°C. 5 ml of Red Saf nucleic acid staining solution, prepared by Intron Korea, was added to the plate and gently poured to avoid air bubbles. The gel was then left at room temperature to set. After the gel solidified, the plate was placed in a horizontal gel electrophoresis tank and immersed in TAE solution. The solution rises to the surface of the gel (0.5-1 mm) and then the comb is gradually raised, the samples are placed inside the holes and the size indicator is placed inside the hole on one side of the gel. The electrodes are

connected to carry the current from the negative pole to the positive pole at 80 volts for 45 minutes. After that, the plate is lifted from inside the transfer tank and placed in the UV Transilluminator, and the gel is photographed at a magnification of 2X so that the sections are clear and the molecular sizes are estimated by comparing with the size indicator bands on the side of the gel.

Table (2): shows the steps and conditions of the PCR reaction

Number of cycles	Required period	Celsius	Steps
1	3 minutes	95	Primary denaturation
35	30 seconds	95	denaturation
30	30 seconds	56	Annealing
30	1 minute	72	Extension
1	5 minutes	72	Final Extension
-	∞	4	Cooling

Phuphisut *et al* 2021 and Taha, 2022 and Kadhim *et al*, 2019

Genetic Sequencing and Matching with the World Gene Bank's Documented Strains

PCR amplification products, along with the designated primers, were sent to Macrogen (South Korea) for genetic sequencing using the Sanger method (Crossley *et al*., 2020). The genetic sequence files were then received in text files and matched with the gene results of the documented strains in the World Gene Bank using the Blast program to determine the degree of genetic divergence and closeness to these strains, as well as to register them in the bank.

Drawing the Evolutionary Tree of the Registered Strains

To draw the evolutionary tree of the strains under current study, their gene sequences were aligned with the corresponding sequences documented in the Global Gen Bank for the Rhynchophorus ferrugineus using Mega 11 (Tamura *et al.,* 2021). According to the methodology demonstrated in Table 3, the genetic divergence values between the registered strains and the global strains were estimated using the same program.

Table (3): shows the paragraphs and methods used in drawing the evolutionary tree.

Items	Method
Alignment Method	Clustal W
Test Used	Maximum likelihood tree
Model Used	Kimura 2 parameter model
Bootstrap P Values	Estimated using 100 replicates of the dataset.

RESULTS AND DISCUSSION

Molecular Diagnosis of the Rhynchophorus ferrugineus

The complete genome of the samples under study was extracted from the thorax of the insect, a region rich in muscle tissue. The genome concentration reached 1.8 Lu/ng and purity reached 1.9 ~. This was measured using the Nano drope device, a highly efficient device for estimating DNA concentration and purity. It is also easy to use, inexpensive, and provides results simultaneously.

Phuphisut *et al* (2021) confirmed that in a study that evaluated several targets within the insect genome, the current work utilizes sequence regions within the ribosomal DNA (rDNA) gene complex. This section of the genome contains the 18S, 5S, 8S, and 28S rRNA genes, which share a relatively conserved nucleotide sequence across fungi. It also includes the variable DNA sequence regions of the overlapping internal transcribed spacer (ITS) regions, called ITS1 and ITS2.

Although not translated into proteins, ITS-coding regions play a critical role in the development of functional RNA, with sequence variations between species showing promise as signature regions for molecular assays (Sadder *et al*., 2015). The discriminating power of ITS1 was significantly higher than that of ITS2, as the prevalence of dorsal markers varied from region to region. A novel ITS1 sequence from Rhynchophorus frida was found in the GenBank database.

Electrophoresis results of the PCR reaction showing the binding of the primer used in the study to the 418 pb band of the ITS2 gene region, Figure 1.

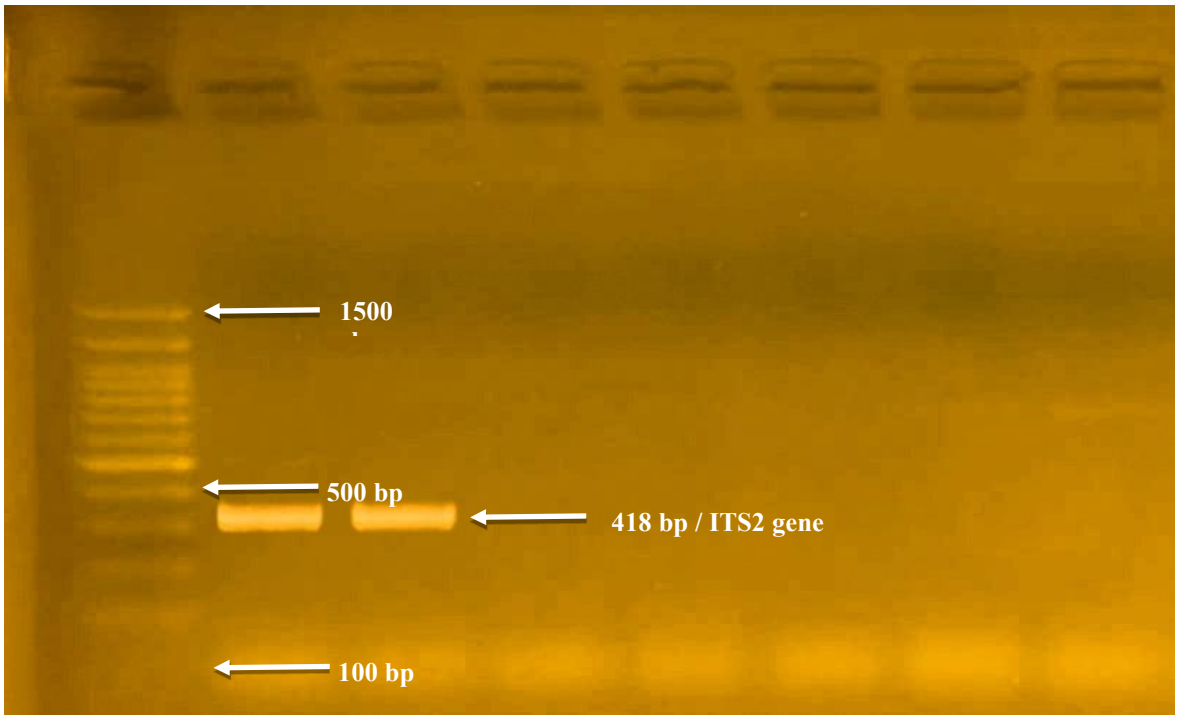


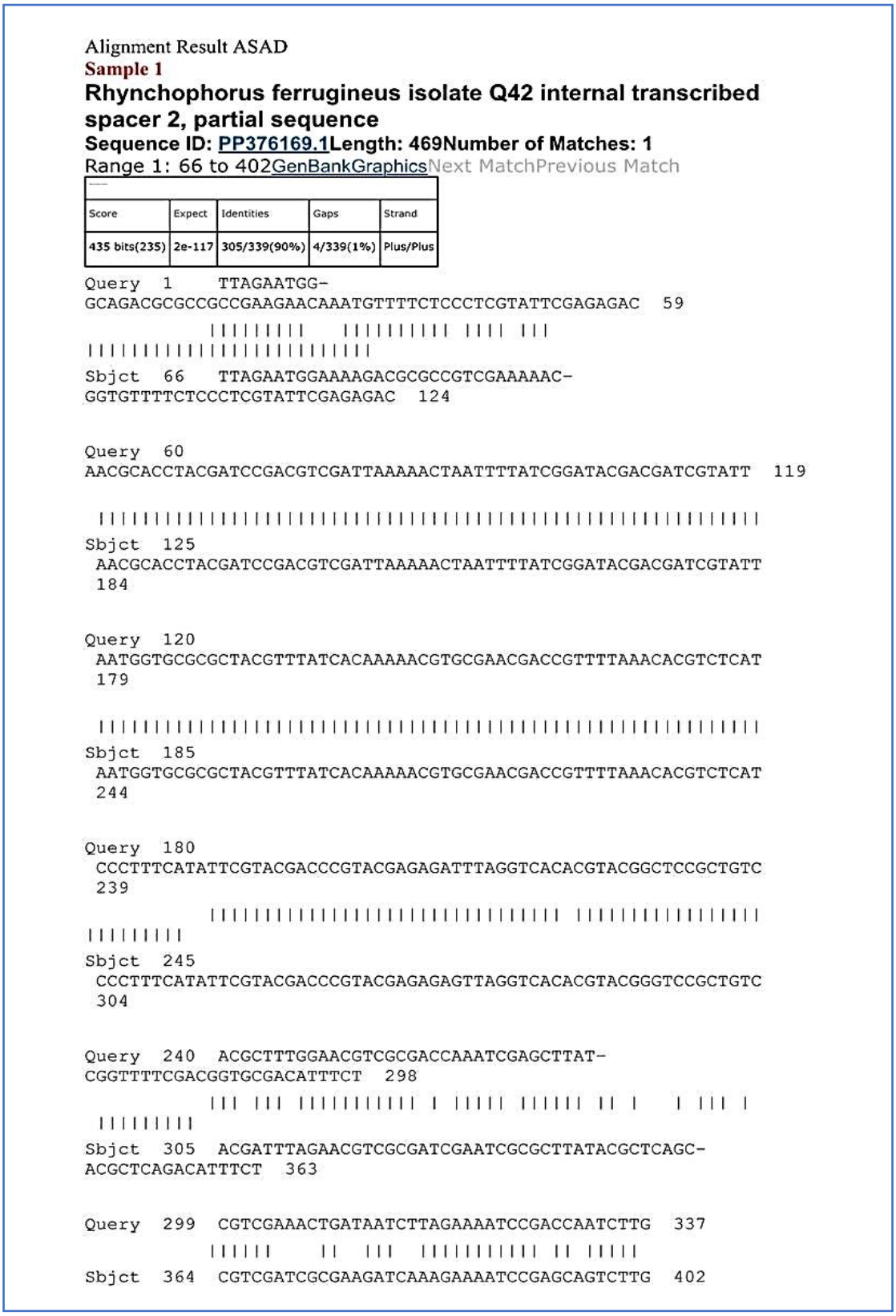
Figure (1): shows the electrophoresis results of the PCR reaction, showing the binding of the primer used in the study at the 418 pb band of the ITS2 gene region. The

genetic sequences under study matched and were registered in the National Gene Bank (NCBI).

Figure (1) shows the electrophoresis results of the PCR reaction, showing the binding of the primer used in the study at the 418 pb band of the ITS2 gene region.

The main objective of genetic sequencing and matching with strains registered in the Global Gen Bank is to diagnose the individuals under study accurately and to identify genetic variations and discrepancies relative to global strains. In general, the samples under study for genetic analysis were compatible with the global samples (91.5% and 86.85%). The results also led to the registration of two strains in the Global Gen Bank, named AM01 and AMo2, referring to the first letter of the name of both the student and the supervisor (Figure 2, 3).

By comparing the results, it was found that the strain AMo2 belonging to the species Rhynchophorus ferrugineus has a lower percentage of identity with the global strains documented in the international gene bank than the strain AM01. This indicates the presence of genetic variation, and the reason for this is attributed to genetic changes, as well as the geographical area, with the presence of one strain from Greece, which was shown by the results of identity with the strains present in the global gene bank.



Alignment Result ASAD

Sample 1

Rhynchophorus ferrugineus isolate Q42 internal transcribed spacer 2, partial sequence

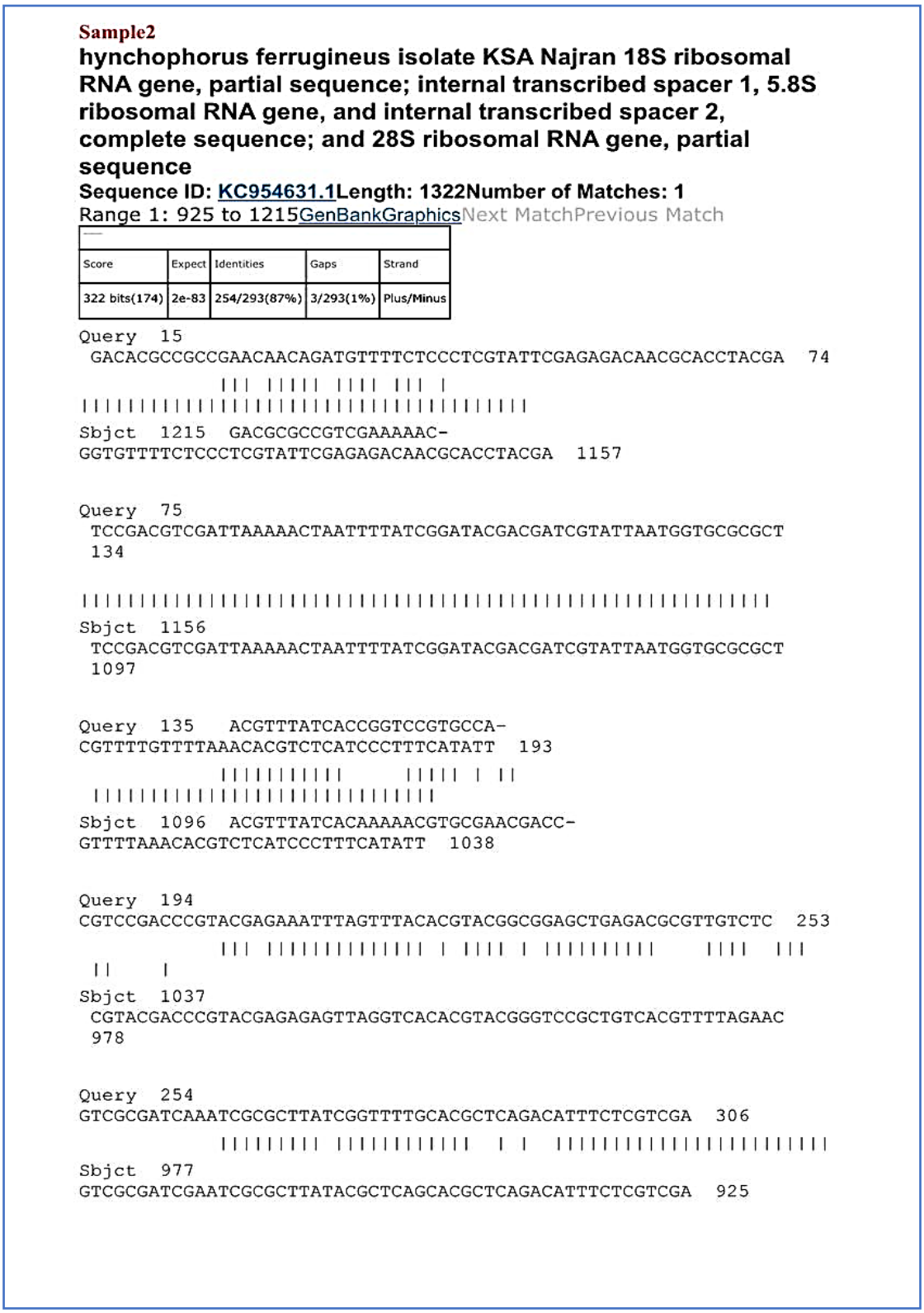
Sequence ID: PP376169.1Length: 469Number of Matches: 1

Range 1: 66 to 402GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
435 bits(235)	2e-117	305/339(90%)	4/339(1%)	Plus/Plus

```
Query  1    TTAGAATGG-
GCAGACGCGCCGCCGAAGAACAAATGTTTTCTCCCTCGTATTCGAGAGAC  59
           |||||||||   |||||||||| |||| |||
|||||||||||||||||||||||||
Sbjct  66   TTAGAATGGAAAAGACGCGCCGTCGAAAAAC-
GGTGTTTTCTCCCTCGTATTCGAGAGAC  124


Query  60
AACGCACCTACGATCCGACGTCGATTAAAAACTAATTTTATCGGATACGACGATCGTATT  119

 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  125
 AACGCACCTACGATCCGACGTCGATTAAAAACTAATTTTATCGGATACGACGATCGTATT
 184


Query  120
 AATGGTGCGCGCTACGTTTATCACAAAAACGTGCGAACGACCGTTTTAAACACGTCTCAT
 179

 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  185
 AATGGTGCGCGCTACGTTTATCACAAAAACGTGCGAACGACCGTTTTAAACACGTCTCAT
 244


Query  180
 CCCTTTCATATTCGTACGACCCGTACGAGAGATTTAGGTCACACGTACGGCTCCGCTGTC
 239
           |||||||||||||||||||||||||||||| ||||||||||||||||
|||||||||
Sbjct  245
 CCCTTTCATATTCGTACGACCCGTACGAGAGAGTTAGGTCACACGTACGGGTCCGCTGTC
 304


Query  240  ACGCTTTGGAACGTCGCGACCAAATCGAGCTTAT-
CGGTTTTCGACGGTGCGACATTTCT  298
           ||| ||| |||||||||||| | ||||| |||||| || |   | ||| |
 |||||||||
Sbjct  305  ACGATTTAGAACGTCGCGATCGAATCGCGCTTATACGCTCAGC-
ACGCTCAGACATTTCT  363


Query  299  CGTCGAAACTGATAATCTTAGAAAATCCGACCAATCTTG  337
           ||||||    ||  |||  |||||||||||| || |||||
Sbjct  364  CGTCGATCGCGAAGATCAAAGAAAATCCGAGCAGTCTTG  402
```

Figure (2): shows a model of matching the gene sequences of Rhynchophorus ferrugineus with the strains documented in the NCBI Gen Bank. The matching ratios of the studied strains to the global strains documented in the World Gen Bank



Sample2

hynchophorus ferrugineus isolate KSA Najran 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: KC954631.1Length: 1322Number of Matches: 1

Range 1: 925 to 1215GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
322 bits(174)	2e-83	254/293(87%)	3/293(1%)	Plus/Minus

```
Query  15
 GACACGCCGCCGAACAACAGATGTTTTCTCCCTCGTATTCGAGAGACAACGCACCTACGA  74
            ||| ||||| |||| ||| |
||||||||||||||||||||||||||||||||||||||
Sbjct  1215  GACGCGCCGTCGAAAAAC-
GGTGTTTTCTCCCTCGTATTCGAGAGACAACGCACCTACGA  1157


Query  75
 TCCGACGTCGATTAAAAACTAATTTTATCGGATACGACGATCGTATTAATGGTGCGCGCT
 134

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1156
 TCCGACGTCGATTAAAAACTAATTTTATCGGATACGACGATCGTATTAATGGTGCGCGCT
 1097


Query  135   ACGTTTATCACCGGTCCGTGCCA-
CGTTTTGTTTTAAACACGTCTCATCCCTTTCATATT  193
            ||||||||||     ||||| | ||
 ||||||||||||||||||||||||||||
Sbjct  1096  ACGTTTATCACAAAAACGTGCGAACGACC-
GTTTTAAACACGTCTCATCCCTTTCATATT  1038


Query  194
CGTCCGACCCGTACGAGAAATTTAGTTTACACGTACGGCGGAGCTGAGACGCGTTGTCTC  253
            ||| |||||||||||||| | |||| | ||||||||||    ||||  |||
 ||    |
Sbjct  1037
 CGTACGACCCGTACGAGAGAGTTAGGTCACACGTACGGGTCCGCTGTCACGTTTTAGAAC
 978


Query  254
GTCGCGATCAAATCGCGCTTATCGGTTTTGCACGCTCAGACATTTCTCGTCGA  306
            ||||||||| ||||||||||||  | |  ||||||||||||||||||||||||
Sbjct  977
GTCGCGATCGAATCGCGCTTATACGCTCAGCACGCTCAGACATTTCTCGTCGA  925
```

Figure (3): shows a model of matching the gene sequences of Rhynchophorus ferrugineus with the strains documented in the NCBI Gen Bank. The matching ratios of the studied strains to the global strains documented in the World Gen Bank

This genetic diversity is evidence of the biological diversity in ecosystems, which ensures the development of species and their adaptation to new ecosystems as well as maintaining their evolutionary potential in light of global change, as shown in Table (4).

Table (4): shows the percentage of conformity of the studied sample strains with global strains

No	Isolates	Accession Numbers	Release Date	Matching with NCBI			
				Query Cover	Identity %	Acc. Numbers	Country
1	*Rhynchophorus ferrugineus strain AM01*	PQ496644	22-10-2024	100%	89.97	PP376169	Saudi Arabia
				100%	89.97	PP376159	Saudi Arabia
				100%	89.97	PP376171	Saudi Arabia
2	*Rhynchophorus ferrugineus strain AM02*	PQ522231	26-10-2024	91%	84.50	PP376129	Saudi Arabia
				91%	84.50	PP376150	Saudi Arabia
				91%	84.50	KM503122	Greece

At the level of Iraq, studies related to molecular diagnosis of Rhynchophorus ferrugineus strains were very few and were limited to a diagnostic study in Basra Governorate, the 2016 study (Aletby and Al-Saad 2018), where they were able to molecularly diagnose the Rhynchophorus ferrugineus strain by targeting the COI gene region.

Evolutionary tree and genetic divergence:

To determine the degrees of genetic proximity and distance between the strains registered in the Global Gen Bank and to confirm the results of the molecular diagnosis of the ITS gene, a phylogenetic tree was drawn with several global strains belonging to both Saudi Arabia and Greece (Figure 4) The phylogenetic tree was designed using the maximum likelihood method and the Kimura 2-parameter model, where the clades represent the association of strains with each other. In general, closely related strains are clustered and supported by high bootstrap values.

Bootstrap values, expressed as percentages of connected clades, indicate the level of confidence or stability of the observed nodes in the evolutionary tree. A bootstrap value of 100 indicates a high level of confidence in the assembly. This is evident with the AM01 and Greek clades, which may indicate a common ancestor. The remaining values, from 34 to 47, indicate a divergent phylogenetic relationship when compared to the clades under study. This indicates that they are not very

similar, which in turn is attributed to environmental differences or the nature of the geographical area.

Table (5) shows the genetic divergence values between the gene sequences of the species under study and their global counterparts registered in World Gen Bank.

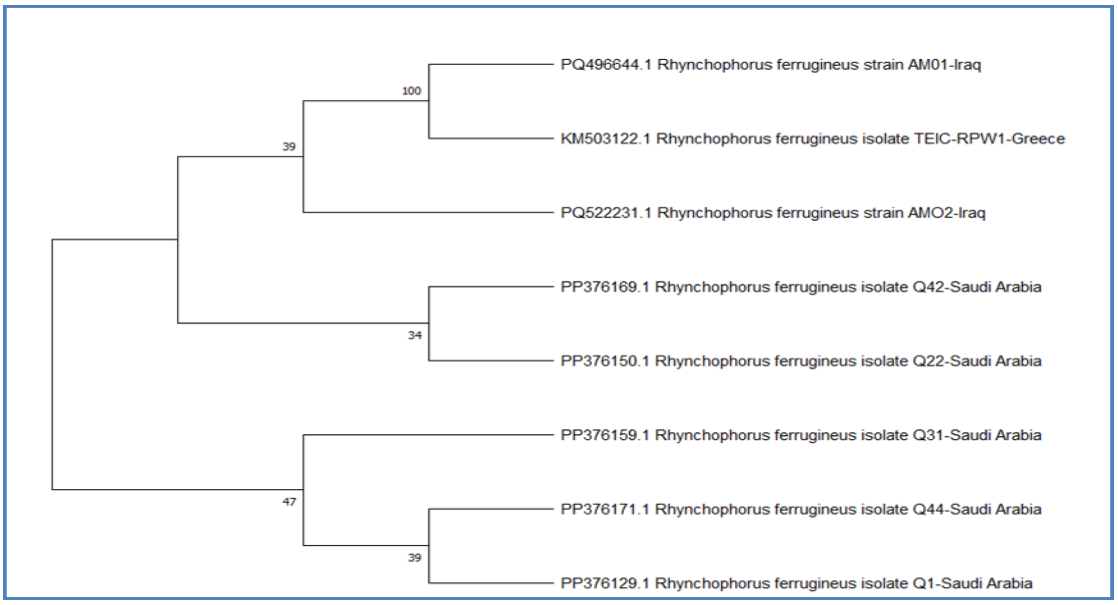


Figure (4): shows the evolutionary tree between the local red palm weevil strain and the global strains documented in the global gene bank

Table (5): shows the genetic divergence values between the genetic lineages of the species under study and registered in the World Gene Bank

N0.	Strains	1	2	3	4	5	6	7	8
1	*R.ferrugineus* Strain AM01-Iraq								
2	*Rh.ferrugineus* Isolate Q42-SaudiArabia	1.111							
3	*R. ferrugineus* Isolate Q31-SaudiArabia	1.099	0.008						
4	*R. ferrugineus* Isolate Q44-Saudi Arabia	1.099	0.008	0.004					
5	*R.ferrugineus* Strain AMO2-Iraq	1.503	0.198	0.198	0.198				
6	*R.ferrugineus* Isolate Q1-Saudi Arabia	1.099	0.006	0.002	0.002	0.198			
7	*R.ferrugineus* Isolate Q22-SaudiArabia	1.129	0.006	0.006	0.006	0.194	0.004		
8	*R.ferrugineus* Isolate TEIC-RPW1-Greece	0.105	1.209	1.224	1.224	1.881	1.214	1.209	

The values were extracted using the Kimura 2-parameter model and the MEGA11 program (Tamura *et al*., 2021). Zero values indicate no genetic

divergence between strains. Genetic divergence increases with increasing values. It is worth noting that the values ranged between (0.00 and 1.50) for the strains registered in the World Gen Bank for the strains under study, El-Mergawy *et al*. (2011) reported that genetic differences were detected among Rhynchophorus, with genetic distances between the five species ranging from 0.058 to 0.095. Sabit *et al* (2021) found that the genetic distance between Rhynchophorus species ranged from 0.0% to 3.0%. One sequence was randomly selected from the samples collected from each site and deposited in Gene bank as shown in Table (5).

CONCLUSIONS

The results of the study, using polymerase chain reaction (PCR) technology, confirmed the registration of two strains of the red palm weevil (Rhynchophorus ferrugineus) in Iraq for the first time, specifically in the infected area of Diyala Governorate. The sequences of these two strains were deposited in the global GenBank database under the numbers PQ496644 and PQ522231, respectively. Genetic sequence analyses revealed differences in the similarity rates between the local strains and other strains previously recorded in Saudi Arabia and Greece, as indicated by genetic divergence values. It was found that the strain registered under the number PQ496644 is almost identical to the Greek strain, confirming their belonging to the same species. The study used the internal transcribed spacer (ITS), one of the most conserved regions in many organisms. PCR technology demonstrated high efficiency in amplifying this region and accurately identifying the strains. The PCR products were analyzed using Sanger sequencing and compared with global strains registered in the GenBank database—Gen Bank of the US National Center for Biotechnology Information (NCBI).

ACKNOWLEDGMENT

The authors thank the University Mosul and Agriculture and Forestry College for their kindly aid in conducting the undertaken study.

CONFLICT OF INTEREST

The authors state that there are no conflicts of interest with the publication of this work.

التشخيص الجزيئي والتسجيل الأولي لسلالتين من سوسة النخيل الحمراء *Rhynchophorus ferrugineus* في العراق

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قسم وقاية النبات / كلية الزراعة والغابات / جامعة الموصل / الموصل / العراق[2]

الخلاصة

أجريت الدراسة خلال الموسم الزراعي 2023 – 2024 على سوسة النخيل في بساتين محافظة ديالى المصابة بالحشرة للفترة من بداية شهر اذار ولغاية نهاية شهر تشرين الثاني 2024 بهدف اجراء التشخيص الجزيئي لحشرة سوسة النخيل *Rhynchophorus* sp واثبتت نتائج الدراسة وجود تسجيل اولي لسلالتين من الحشرة في محافظة ديالى اذ استخدم لهذا الغرض تقنية تفاعل البلمرة المتسلسل Polymerase Chain Reaction (PCR) وذلك لتضخيم منطقة Internal transcribed spacer (ITS) التي تعد من أكثر المناطق محافظة في العديد من الكائنات الحية. كما أجري تحليل التسلسلات الجينية لنواتج تفاعل PCR باستخدام طريقة Sanger ثم مطابقتها مع السلالات العالمية وتسجيلها في بنك الجينات العالمي National Center for Biotechnology Information ورسم الشجرة التطورية وتقييم قيم التباعد الوراثي بينها وبين السلالات العالمية، باستخدام برنامج MEGA11. تمكنت الدراسة ولأول مرة في العراق من تسجيل سلالتين للحشرة واودعت بالارقام التسلسلية PQ496644 و PQ522231 على التوالي. كما أكدت النتائج وجود نسب تطابق متباينة بين السلالات المحلية وعدد من السلالات المسجلة في المملكة العربية السعودية واليونان وأكدت ذلك قيم التباعد الوراثي وكانت السلالة ذات الرقم التسلسلي PQ496644 أكثر تطابقا مع السلالة اليونانية وكانت السلالتين عائدة للنوع *Rhynchophorus ferrugineus*.

الكلمات المفتاحية: سوسة النخيل الحمراء، التشخيص الجزيئي، بنك الجينات، الشجرة التطورية، التباعد الوراثي.

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