



## Research Article

# Morphological and Molecular Identification of Pathogenic Fungi Associated with the Red Palm Weevil *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae)

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**Abstract** | This research study was conducted by collecting red palm weevil samples known as one of the most serious pests from infested date palm trees across several provinces in central Iraq during 2025. The survey results indicated the presence of pathogenic fungi is the most important natural enemies associated with the red palm weevil and most widespread. The entomopathogenic fungi were isolated from the samples and cultured on artificial media. The fungi were morphologically identified based on the most prominent diagnostic morphological characteristics distinguishing among species. To confirm the morphological identification, molecular identification was performed using PCR amplification with ITS primers. The results showed the presence of four different species of pathogenic fungi infecting the red palm weevil. Upon analysis of nucleotide sequences and comparison with the data available in the global gene bank NCBI (GenBank), the registered local isolate (PX556663.1) showed 99% similarity with *Trichoderma harzianum* registered under accession number (OP810430.1). The local isolate of *Beauveria bassiana* (PX556664.1) also showed 99% similarity with the reference isolate (MG813215.1). The isolate (PX556665.1) showed 99% similarity with the reference sequence FJ545316.1 isolated from *Metarhizium anisopliae*. Regarding the isolate (PX556666.1), it showed 99% similarity with the sequence EF513009.1 isolated from *Lecanicillium lecanii*. This confirmed the accuracy of the morphological and molecular identification of these entomopathogenic fungi. This study indicates the possibility of using entomopathogenic fungi as a biological control agent for managing this important pest.

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## Introduction

The red palm weevil *Rhynchophorus ferrugineus* (Alderawii *et al.*, 2020), which belongs to the family Curculionidae and the order Coleoptera, is

also known as the Asian palm weevil or the Indian red palm weevil. Its original habitat to the countries of Southeast Asia. It is one of the major invasive pest species in the world, as it destroys about 40 species of palm trees worldwide, and date palm is considered

one of the most important host species for the red palm weevil (Augul, 2019; Morici, 1998; Faleiro, 2006). In addition to its high capacity for oviposition, which may reach between 200–300 eggs, its tolerance to different climatic conditions, and its ability to fly (Al-Jubouri, 2025). The red palm weevil was detected for the first time in Iraq during October 2015 in the Safwan area, southwest of Basra Governorate (Aletby, 2016), and the number of infested palms increased from 12 palms in 2015 to 111 palms in 16 orchards during the year 2016 (Alderawii *et al.*, 2020).

Entomopathogenic fungi are used in integrated management programs among different control methods with the aim of reducing the use of pesticides and chemical materials that have major harmful effects on the agricultural ecosystem, and of finding alternatives for managing this rapidly spreading and dangerous pest. It has been found that entomopathogenic fungi possess the ability to manage this pest and reduce its damage, as confirmed by Alwaneen *et al.* (2024). It is known that more than 50 species of natural enemies (fungi, viruses, bacteria, nematodes, animals, and predatory and parasitic insects) attack the red palm weevil in its native areas and in invaded regions (Mazza *et al.*, 2014). *Beauveria bassiana* and *Metarhizium anisopliae* are among the most important insect disease-causing fungi and are currently used against a wide range of arthropods, especially insects (Vega and Kaya, 2012). Alwaneen *et al.* (2024) indicated the testing of the virulence and pathogenicity of 15 different fungal isolates belonging to the genera *Beauveria*, *Metarhizium*, and *Purpureocillium* as entomopathogenic fungi through laboratory rearing of stages of the red palm weevil *R. ferrugineus*. The results of the study showed that all fungal isolates were highly virulent against larvae, and the biological tests for horizontal transmission indicated that infected individuals transmitted the disease to healthy individuals.

In Iraq, the first record of the entomopathogenic fungus *Trichoderma harzianum* and the confirmation of its pathogenicity against the red palm weevil in Basra Governorate were reported in 2024, and the fungus was identified morphologically and molecularly (Hasan *et al.*, 2025). Given that the red palm weevil is a recently invasive pest to Iraq, the study aimed to conduct a survey and morphological and molecular identification of the entomopathogenic fungi associated with the red palm weevil, and consequently

to assess the possibility of using entomopathogenic fungi as a biological control agent for managing this pest.

## Materials and Methods

### *Sample collection*

Larvae, pupae, and adults of the red palm weevil were collected by manual and direct collection from infested palm trees from four provinces in central Iraq (Diyala, Baghdad, Wasit, Babil) by excavating the infestation site using an iron crowbar. The stages infected with fungal causative agents were isolated based on the abnormal appearance and color or the growth of fungal mycelium on the insect body, and they were placed in 60 mL tubes, each one separately. The collection location and date were recorded on the tubes, then they were transferred to the laboratory, where the following steps were performed to conduct the isolation and identification process.

### *Isolation and identification of pathogenic fungi from red palm weevil stages*

The insect stages of the red palm weevil (larvae, pupae, adults) were sterilized; the fungal infection was clear and distinctive, especially in the pupal stage. They were surface-sterilized using sodium hypochlorite (bleach) diluted to 1%.

The samples were washed with sterile distilled water twice, each time for 1–2 minutes, then dried using sterile filter paper.

PDA medium was prepared at 40 g per liter of water and sterilized in the autoclave at 121°C and a pressure of 1.5 bar for 15–20 minutes.

The previously sterilized insects were cultured in 9 cm Petri dishes containing PDA medium, and the dishes were placed in the incubator at 25°C, with continuous monitoring; after 48–72 hours, the fungus began to emerge.

The insect stages were cultured, each one separately, in 9 cm Petri dishes containing culture medium sterilized in the autoclave at 121°C and a pressure of 1.5 bar for 15–20 minutes.

Dishes were incubated at 25 ± 2°C for 3 days, followed by fungal purification through transfer of a portion of fungal mycelium to new dishes; this procedure

was repeated and incubated for an additional 5 days (López-Luján *et al.*, 2022).

The fungi were morphologically identified based on colony characteristics and the microscopic characteristics of the structures and spores formed by the fungi, using the approved taxonomic keys. The primary identification was conducted by Prof. Dr. Huria Hussein Al-Jubouri / University of Baghdad / College of Agricultural Engineering Sciences, specialized in fungal identification.

Percentage of fungal occurrence = (number of plates in which the fungus appeared / total number of plates) × 100.

#### *Pathogenicity test of the isolated entomopathogenic fungi from red palm weevil stages*

To confirm the pathogenicity of the fungi isolated from the insect stages, larvae of different ages were isolated from infested palms. Three larvae were placed in each replicate after being treated with a fungal suspension produced by scraping the fungus from the plates in an amount estimated at 1 g and substituting it into an appropriate amount of distilled water, then spraying it directly onto the larvae before placing them in plastic containers with dimensions of 30 cm length and 15 cm width containing sawdust from the infested palm from which the larvae were isolated, with three replicates per treatment using a completely randomized design (CRD). Containers were placed in an incubator at 25°C and examined periodically. When larvae died, they were isolated, sterilized, and re-cultured on the culture medium as mentioned previously to confirm the presence of the same fungus used for treatment (Hasan *et al.*, 2025).

#### *Molecular identification of fungi isolated from red palm weevil stages*

Molecular identification was performed for four fungal isolates, including (*T. harzianum*, *B. bassiana*, *M. anisopliae*, *L. lecanii*), which had been previously identified morphologically, using the polymerase chain reaction (PCR) technique in the laboratories of Al-Masdar Al-Ilmi Company for Training, Development, and General Services, located in Baghdad / Al-Qadisiyah district, according to the following steps:

#### *DNA extraction (DNA Extraction)*

Deoxyribonucleic acid (DNA) was extracted

from pure fungal cultures (mycelium) using the extraction kit (FavorPrep Fungi/Yeast Genomic DNA Extraction Mini Kit) supplied by the Korean company FAVROGEN, following the manufacturer's instructions.

**Table 1:** *ITS primer sequences used for PCR amplification and their melting temperature (T<sub>m</sub>), GC content, and expected product size (bp).*

| Primer | Sequence | Primer sequence                | T <sub>m</sub> (°C) | GC%  | Size of product (bp) |
|--------|----------|--------------------------------|---------------------|------|----------------------|
| ITS    | F        | 5'- TCCG-TAGGT-GAACCTG-CGG -3' | 60.3                | 50 % | 550-650              |
|        | R        | 5' TCCTC-CGCTTATT-GATATGC-3'   | 57.8                | 41 % |                      |

**Table 2:** *Thermal cycling conditions for PCR amplification of the ITS region.*

| No. | Phase                | T <sub>m</sub> (°C) | Time  | No. of cycle |
|-----|----------------------|---------------------|-------|--------------|
| 1-  | Initial Denaturation | 95°C                | 5 min | 1 cycle      |
| 2-  | Denaturation -2      | 95°C                | sec45 | 35 cycle     |
| 3-  | Annealing            | 52°C                | 1 min |              |
| 4-  | Extension-1          | 72°C                | 1 min |              |
| 5-  | Extension -2         | 72°C                | min.  | 1 cycle      |

#### *Primer preparation (Primers):*

Specialized primers for the (ITS) region produced by the Korean company Macrogen were used to amplify the DNA of the three studied isolates; these are general fungal primers as shown in Table 1.

Primers were frozen and dissolved in nuclease-free ddH<sub>2</sub>O solution to obtain a final concentration of 100 pmol/μL as a stock solution, which was stored frozen at -20°C for subsequent use. Then, 10 picomoles per microliter (picomole/μL) were extracted from the stock solution and mixed with 90 microliters (μL) of nuclease-free double-distilled water (ddH<sub>2</sub>O) to achieve a final volume of 100 μL.

#### *PCR amplification (PCR amplification)*

The reaction mixture was prepared by mixing 12.5 μL of Master mix with 10 picomole/μL of F primer and 10 picomole/μL of R primer, along with 1.5 μL of DNA and 9 μL of distilled water. Then, the thermal cycler (PCR) was set according to the instructions in Table 2 below

**Table 3:** *Fungi associated with red palm weevil stages and their locations.*

| Governorate / Area    | Fungal occurrence (%)<br><i>Beauveria bassiana</i> | Fungal occurrence (%)<br><i>Metarhizium anisopliae</i> | Fungal occurrence (%)<br><i>Trichoderma harzianum</i> | Fungal occurrence (%)<br><i>Lecanicillium lecanii</i> |
|-----------------------|----------------------------------------------------|--------------------------------------------------------|-------------------------------------------------------|-------------------------------------------------------|
| Wasit / Al-Suwaira    | 20                                                 | 16                                                     | 0                                                     | 0                                                     |
| Wasit / Taj Al-Din    | 14                                                 | 18                                                     | 0                                                     | 0                                                     |
| Baghdad / Abu Ghraib  | 0                                                  | 12                                                     | 26                                                    | 0                                                     |
| Diyala / Al-Muqdadiah | 8                                                  | 0                                                      | 0                                                     | 4                                                     |
| Babil / Al-Markaz     | 12                                                 | 0                                                      | 4                                                     | 0                                                     |
| Total                 | 54                                                 | 46                                                     | 28                                                    | 4                                                     |

### *Agarose gel electrophoresis*

To verify the success of the amplification process and determine the size of the products, the PCR products were electrophoresed.

1- An agarose gel was prepared at a concentration of 1.5% by dissolving 1 gram of agarose powder in 100 ml of 1X TAE buffer, then the mixture was dissolved by heating. The solution was left to cool to 50–55°C with continuous stirring in order to distribute the heat evenly.

2- Three microliters of RedSafe dye were added, then the ends of the tray into which the gel would be poured were sealed with two layers of adhesive tape.

3- The combs were inserted into the gel casting tray and followed by pouring the solution into the tray.

4- The solution was allowed to harden at room temperature, after which the combs were gently taken out and the adhesive tape was peeled off from the tray. Subsequently, the gel was transferred into the electrophoresis chamber that was filled with 1x WAE buffer.

5- Five microliters of DNA samples were mixed with 3 microliters of DNA loading dye and placed into the designated wells on the agarose gel.

6- The agarose gel was run by applying an electric current at 80 volts and 65 amperes for one hour. Then, the results were examined using a UV transilluminator.

### *Purification of PCR products and determination of nucleotide sequence (DNA sequencing)*

After confirming the appearance of single and clear bands for each of the studied fungal isolates at the required size (approximately 550–650 base pairs)

using electrophoresis, the PCR products were sent to Macrogen Company in South Korea. The product underwent Purification, then nucleotide sequencing of the ITS region was conducted using an ABI3730XL Automated DNA Sequencer. After obtaining the results, the data were processed and the sequences were corrected using BioEdit software, then compared with globally registered sequences in GenBank using the BLASTn search tool of NCBI to accurately determine the fungal species.

## **Results and Discussion**

The survey results showed the recording of four pathogenic fungi of the red palm weevil during the period from February to October 2025 in central Iraq, occurring naturally without any intervention or any artificial inoculation procedures specifically for controlling the red palm weevil. It is suggested that the infection occurred naturally through the presence of fungal inoculum used in the control of other insects in the near previous periods.

The results of Table 3 showed that the fungus *B. bassiana* was the most present and widespread entomopathogenic fungus, as it was found in four areas of different provinces during the sample collection operations, and the percentage of its occurrence reached 54% of the total number of samples. It was followed by the fungus *M. anisopliae*, as it appeared in three different areas and at different times of sample collection as well, and its occurrence percentage reached 46%. As for the fungus *T. harzianum*, it appeared in two areas and several times during the sample collection period, with an occurrence percentage of 28%. The fungus *L. lecanii* was the rarest fungus during the survey operations, as it appeared in one area and only once during the sample collection period, with an occurrence percentage of 4%.



**Figure 1:** Agarose gel electrophoresis (1.5%) of PCR amplification products of the ITS region. 1 = *T. harzianum*, 2 = *L. lecanii*, 3 = *M. anisopliae*, 4 = *B. bassiana*

#### Morphological characterization of fungi

The morphological characteristics of all isolates were observed at the age of 7 days on culture media, and examination after mounting on glass slides showed that the fungus *T. harzianum* had spores of a semi-spherical shape with a size range of 1.2–1.7  $\mu\text{m}$ , the conidium length was 8.70–9.85  $\mu\text{m}$ , and the conidiophores were distributed in a pyramidal pattern with the presence of dense branching, and it was found that the color of the mycelium ranged between yellow and green, as shown in many studies that agree with these results (Hiba *et al.*, 2019; Asis *et al.*, 2021; Inglis *et al.*, 2020).

It was found that *L. lecanii* produced white colonies that turned to creamy and pale yellow color, with radial growth and a cottony appearance, and the fungal hyphae were septate with thin walls. The conidiophores were erect and circular in shape. As for the spores, they were hyaline, unicellular, with smooth walls. Conidial sizes were small, ranging between 2.9–8.8  $\mu\text{m}$ . The conidial shape was either cylindrical with rounded ends, or crescent-shaped with two sharp ends, or crescent-shaped with one end more pronounced, or lanceolate, or ovoid to ellipsoidal, and this is consistent with what was reported by (Cortez-Madrugal *et al.*, 2003; Subramaniam *et al.*, 2021; Neware *et al.*, 2025; Liu *et al.*, 2025).

The fungus *B. bassiana* formed white colonies with a radial and dense appearance and a soft velvety texture resembling cotton during the conidial production stage. The hyphae were smooth, fine, and septate with clear septa. The conidiophores were short and took a zigzag arrangement to form spores. The conidia were ovoid, unicellular, and their length reached 2.23  $\mu\text{m}$  and their width reached 1.23  $\mu\text{m}$ , and this is consistent with (Neware *et al.*, 2025; Safavi, 2010).

As for the fungus *M. anisopliae*, it formed white

colonies with radial growth that gradually turned green with the progress of conidial formation. The colonies were characterized by a powdery texture and produced very large numbers of spores. The hyphae were hyaline, branched, and septate. The spores were carried on the conidiophores in chains, which gives the colonies the powdery texture. Conidial sizes ranged between 5–5.5  $\mu\text{m}$ , and this is consistent with what was reported by (Neware *et al.*, 2025; Moslim and Kamarudin, 2014).

#### Results of molecular identification and nucleotide sequencing:

The appearance of clear and distinct bands for all four studied isolates: *Beauveria bassiana*, *Metarhizium anisopliae*, *T. harzianum*, and *Lecanicillium lecanii* with a molecular size between 550–650 base pairs (bp) was visualized by the results from agarose gel electrophoresis (1.5%). Figure 1 Genomic Sequencing of ITS Amplification by PCR. These bands were generated through the amplification of a genetic region, specifically ITS, via PCR.

Upon analysis of nucleotide sequences and comparison with the data available in the global gene bank NCBI (GenBank) using the search tool (BLASTn), the registered local isolate (PX556663.1) recorded 99% similarity with the species *T. harzianum* registered under accession number (OP810430.1) for the reference Indian isolate, and this result agrees with the studies of Hasan *et al.*, 2025, Kaushik *et al.*, 2020, Mukherjee *et al.*, 2020; and Islam *et al.*, 2022. The local isolate of the fungus *B. bassiana* (PX556664.1) also showed 99% similarity with the Italian reference isolate (MG813215.1), and this result was consistent with the findings of Bovio *et al.*, 2018. As for the local isolate of the fungus *M. anisopliae* (PX556665.1), it matched by 99% with the reference sequence of the Chinese isolate FJ545316.1, which is in agreement with what was mentioned by Fred *et al.*, 2011. Regarding the local isolate of the fungus *L. lecanii* (PX556666.1), it showed 99% similarity with the sequence EF513009.1 for the Greek isolate, and this agrees with what was reported by Kouvelis *et al.*, 2008. The accuracy of identifying these entomopathogenic fungi morphologically and molecularly has been verified.

Following the analysis of the nucleotide sequences and their comparison with global data, the fungal

**Table 4:** Local fungal isolates registered in NCBI GenBank and their similarity to reference isolates based on the ITS region.

| Fungal isolate         | Accession number of registered local isolates in NCBI GenBank | Accession number of registered reference isolates in NCBI GenBank | Similarity (%) | Genetic region |
|------------------------|---------------------------------------------------------------|-------------------------------------------------------------------|----------------|----------------|
| Trichoderma harzianum  | PX556663.1                                                    | OP810430.1                                                        | 99%            | ITS            |
| Beauveria bassiana     | PX556664.1                                                    | MG813215.1                                                        | 99%            | ITS            |
| Metarhizium anisopliae | PX556665.1                                                    | FJ545316.1                                                        | 99%            | ITS            |
| Lecanicillium lecanii  | PX556666.1                                                    | EF513009.1                                                        | 99%            | ITS            |

isolates were registered and documented in the NCBI GenBank. The results demonstrated high genetic identity (99%) with reference strains, and specific Accession Numbers were obtained for each isolate, as detailed in Table 4.

#### Pathogenicity test

The results showed the effectiveness of the four entomopathogenic fungi in the study in killing red palm weevil larvae, as the onset of lethargy and a change in larval color were recorded after 3 days of treatment, and the mortality percentage reached 100% after 7 days of treatment in all treatments. It is likely that the effect of entomopathogenic fungi is due to multiple mechanisms such as direct parasitism on the insect and the production of secondary metabolites that kill the insect, as indicated by the mechanism of Bihal *et al.* (2023).

## Conclusions and Recommendations

The survey results showed the presence of four different species of entomopathogenic fungi of the red palm weevil in four different provinces in central Iraq, and this confirms that entomopathogenic fungi are used in integrated management programs among different control methods with the aim of reducing the use of pesticides and chemical materials that have major harmful effects on the agricultural ecosystem and finding alternatives for managing this rapidly spreading and dangerous pest.

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## Novelty Statement

Morphological and molecular identification of the entomopathogenic fungi associated with the red palm weevil was examined and find-out entomopathogenic fungi as a biological control agent for managing this pest.

## Authors Contribution

**Shehab Ahmed Abbas:** Conducted the experiment both in field and laboratory, recorded the data, analyzed it and wrote the manuscript.

**Feryal Bahjat Hermize:** Proposed the research idea, provided the materials and supervised the project.

#### Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

#### Conflict of interest

The authors have declared no conflict of interest.

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