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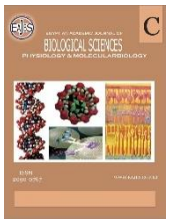

First Report of *Biscogniauxis mediterranae* As Endophytic Fungi Associated with *Quercus infectoria* from A Mountainous Area In Kurdistan Region, Iraq

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ABSTRACT

Endophytic fungi develop symbiotic connections with their hosts in which they live within plant tissues without causing harm Between 2019 and 2020, thirty samples of *Quercus infectoria* were collected from various locations in the Duhok province, Kurdistan Regional Government (KRG), Iraq to investigate the presence of endophytic fungus. Different parts of the oak tree (*Quercus infectoria*), such as the leaf, bud, seed, cork, and lateral branches, were used to isolate the fungal endophytes. Endophytic fungi form symbiotic relationships with their hosts, living within plant tissues without causing harm. A type of xylariaceous fungus called *Biscogniauxia mediterranea* (*B. mediterranea*) was isolated from *Quercus infectoria* (*Q. infectoria*) in the Duhok area. Molecular and morphological methods were used for identification. Our study represents the first report of *B.mediterranea* in Iraq and the first isolation as an endophyte from the *Q.infectoria* plant in the region. Phylogenetic analysis demonstrated the genetic relationship of the Iraqi isolates with strains from different geographic locations. Macrogen Business, a South Korean corporation, conducted bidirectional sequencing on the polymerase chain reaction test (PCR) data. Using BioEdit software, the acquired sequences were edited and assembled, and a consensus sequence was generated for each sample. The sequences underwent Basic Local Alignment Search Tool (BLAST) analysis for the identification of endophytic fungus. The homology of the sequences with reported sequences from other countries ranged from 97.55% to 99.89% as per the National Center for Biotechnology Information's (NCBI) record.

INTRODUCTION

Oak (*Quercus* genus) is among the most common groups northern hemisphere's supply of woody angiosperms, and it is highly regarded for its economic significance and species richness. There are more than 600 species of trees and shrubs in the *Quercus* genus, most of which are deciduous and found in different environments. Because they support the composition and efficiency of diverse forest ecosystems, these *Quercus* species are essential to ecology (Tantray *et al*., 2017). Studies have indicated that a high concentration of compounds with antioxidant activity, like tannins, can be found in leaves and bark. Consequently, these drugs are used in traditional medicine to treat a wide range of illnesses, including hemorrhages, dysentery, and chronic (Taib *et al*., 2020). Endophytic fungi" are fungi that live inside different plant structures, such as stems, nodes, leaves, roots, and other similar parts. During their whole life cycle or just some phases of it.

These fungi establish a symbiotic, mutually beneficial relationship with their host plants devoid of negative effects or pathogenic activities. (Patchett and Newman, 2021; Dasauni *et al*., 2023). The appearance of endophytes has been well-known for over a century. The word endophyte comes from the Greek "endo" or "endon" (meaning within) and "phyte" or "phyton" (meaning plant), interpreted precisely. It is known that every plant hosts one or more endophytes. (Kharwar *et al*., 2008).According to Verma et al. (2007), fungi known as endophytes invade a portion of a plant's internal tissue at some point in its life cycle without exhibiting any outward signs of disease. Certain tree species may be affected by endophytes that live in their roots and affect how diseases develop in those species. (Rigerte *et al*., 2019). Endophytic fungus inhabits nearly every type of plant. These fungi successfully colonize the plant tissues, and no overt symptoms of disease are seen. After that, a mutually advantageous connection develops in which the endophytic fungi living inside the host plant profit from the relationship while the host plant itself benefits (Baron and Rigobelo, 2021).

Charcoal canker disease is mostly caused by *B. mediterranea*, one of the most prevalent fungal diseases in cork oak woods. For a long time, it has been a major issue in Portugal. This fungus is becoming more common in Portuguese forests, particularly in young cork oak trees (Henriques *et al.*, 2014). In *Quercus cerris*, *Quercus frainetto*, and *Quercus pubescens*, charcoal canker disease is also found in Central and Southern Italy (Ragazzi *et al*., 1989).

B. mediterranea is a xylariaceous ascomycete that is typically associated with stressed trees that have been subjected to drought, fires, and mechanical injuries; Nugent *et al*., (2005) reported that water stress was the primary factor in trees' susceptibility to *B. mediterranea* attacks. The fungus can exist in the host tissues as an endophyte (Mazzaglia et al., 2001); however, under stress, it quickly invades bark and woody tissues, manifesting as fungal stroma on branches and trunks (Collado *et al*., 2001). According to Henriques *et al*., (2014), the stroma is made up of clusters of perithecia that carry ascospores, which naturally spread to infect healthy trees by insects. Certain endophytic secondary plant pathogenic fungi are especially susceptible to changes in host physiology brought on by stress (Paoletti *et al*., 2001; Desprez-Loustau *et al*., 2006; Capretti and Battisti, 2007).

Among these, *Biscogniauxia mediterranea*, the causative agent of the oak charcoal disease, lives in host tissues such as twigs, bark, leaves, and, to a lesser extent, wood as an endophyte for a portion of its life cycle (Collado *et al*., 2001; Mazzaglia *et al*., 2001).

When exposed to environmental stress, *B. mediterranea* can quickly invade the bark and xylem tissues of its hosts, causing canker formation and necrosis, which can hasten the decline and final mortality of the tree (Desprez-Loustau *et al*., 2006; Capretti and Battisti, 2007).

The isolation of endophytic fungi from Iraqi forest trees has not been documented in any prior research. However, there were few published works on the endophytic fungi isolated from various vegetable crops (AL-Rifaie and Mohammed-Ameen, 2023; AL-Rifaie and Mohammed-Ameen, 2024) as well as, from leaves or stems of different plants (Hassan *et al*., 2019,2020,2021; Hawar *et al.*,2023; Mohammed and Hawar, 2024; Nuaimy and Hawar 2024).

The aim of the present study was to identify an endophytic species of *Biscogniauxia* isolated from *Q. infectoria* tissues using the LSU genetic marker.

MATERIALS AND METHODS

1. Study Site:

The study was conducted from September to November 2020 and the sample was collected from several locations within the Duhok province, which is located in the northwest of the Kurdistan Region of Iraq. At a height of 433 to 1512 meters above sea

level, it is located between latitudes 36°18' and 37°20' N and longitudes 42°20' and 44°17' E. (Aziz *et al*., 2022)

The rainy season extends from November to March, although there is little to no precipitation throughout the summer months of June through September. The weather in Duhok is influenced by the Mediterranean climate; Winters are typically cold and wet, whereas summers are typically hot and dry. There is 500–1000 millimeters of rain on average every year. The summer temperature range is 20°C to 37°C, and the winter temperature range is 0°C to 15°C, according to Mzuri *et al*., (2022).

2. Samples Collection and Isolation:

Thirty samples were taken from Buds, lateral branches, and seeds of *Q. infectoria*. The samples were stored in sterile paper envelopes and dispatched to the University of Zakho's Department of Biology's mycology lab, where they underwent processing in under 48 hours. Small slices were taken off the buds and the leaves were chopped into tiny pieces in the lab using a paper puncher that had a 5 mm diameter. The prepared samples for each plant were treated separately, undergoing sterilization with (75 %) ethanol for a minute, followed by sterilization with a 3 % sodium hypochlorite solution for (3) minutes. Subsequently, another round of sterilization was conducted using (75 %) ethanol, and a final wash with distilled sterile water (Liu *et al*., 2007).

Samples that had been surface-sterilized were put on sterile paper towels and left to dry on a bench with laminar airflow. Samples were moved to Petri dishes (five segments per plate) and incubated at 25 °C until visible fungal growth. To stop bacterial growth, 50 µg/mL ampicillin and streptomycin were added to the Malt Extract Agar (MEA) medium (Himedia Labs, India) that was placed inside the Petri dishes (Martins *et al*., 2016). Using a sterile needle, the hyphae that emerged from the tissue sections were cut to form pure cultures. These were then subcultured into fresh Malt Extract Agar (MEA) plates and incubated at 25±2◦C. Following a fortnight of incubation, the progression of fungal colonies and sporulation inside cultures were noted.

3. Morphological Identification of *Biscogniauxis mediterranae*:

To identify *Biscogniauxis* isolates from pure cultures on an MEA medium, certain cultural and morphological characteristics were employed, such as conidial size, shape, and color by using a Light microscope (40x). The identification of the fungal isolates was done using several relevant taxonomic references, including Zhang and Zhang, 2006; Simmons, 1986; 2007; Wanasinghe *et al*., 2018; Li *et al*., 2023.

4. Molecular Identification of *B. mediterranae:*

4.1. DNA Extraction:

Pure colonies of isolates of *B.mediterranae* were transferred to a ceramic mortar and ground into a powder using liquid nitrogen. The powder was then placed into sterile tubes and frozen until needed for DNA extraction. The DNA of the isolated fungus was extracted using the Add Prep Genomic DNA Extraction Kit (Korea ®) according to the manufacturer's instructions. Following the instructions in the user's handbook, the purity of the extracted DNA was quantified and evaluated using Thermo Scientific's Nano drop 2000c spectrophotometer. The extracted fungal DNA was amplified using LROR (5′ ACCCGCTGAACTTAAGC–3′) and LR5 (5′ ATCCTGAGGGAAACTTC–3′) (White *et al*., 1990; Vilgalys and Hester, 1990).

4.2. DNA Amplification Using Polymerase Chain Reaction:

Singleplex PCR was utilized to target the LSU genes. Using a 45µl reaction tube, the amplification was carried out using 15µl of Crystal Hot Start DNA Master Mix (0.2 mM of dNTP, 1× Ex Taq Buffer, and 2.0 mM of MgCl2), 1.5µl of each primer's forward and reverse primers (10 pmol), 3µl of template DNA samples, and 21µl of nuclease-free water.

The following thermocycler

parameters were used to amplify the LROR and LR5 genes. Applying the single-plex PCR method: 95°C for 3 minutes was followed by 35 cycles of 94°C for 40 seconds, 67°C for 1 minute, and 72°C for 1 minute. Ten further minutes at 72°C were then dedicated to the final strand elongation (White *et al*., 1990). Red Safe Dye with green fluorescence (GeNet Bio, Korea) was used to dye the PCR products before they were put through 1.5% Agarose gel electrophoresis. An 80-volt electrophoresis runs for 45 minutes.

To assess the band size, Verkley *et al*., (2014) included a DNA ladder with a molecular weight of 100–1000 bps. To sequence the positive PCR results, the forward and reverse primers from the single plex PCR were shipped to Macrogen Company in South Korea. Every sample's consensus sequence was produced by forward and reverse DNA sequencing using BioEdite. After editing, the sequences were put together. The sequences were submitted to the NCBI's BLAST search, which helped determine the species of *Biscogniauxis*.

4.3. Phylogenetic Analysis:

Using neighbor-joining (NJ) techniques, phylogenetic analysis was performed based on the data of the 877 bp LSU gene (Large subunit ribosomal rRNA). The phylogenetic tree was aligned using the Muscle technique and substitution models (K2: Kimura 2 parameter). Alignment gaps were filled in with missing data. Neighbor-joining (NJ) trees were constructed based on the overall character differences, and bootstrap values were calculated from 1,000 replications. MEGA X software 10.1.6 was used to conduct the evolutionary study (Kumar *et al*., 2018).

RESULTS AND DISCUSSION

1. Morphological Identification of *B. mediterranea*:

B. mediterranea has been identified based on its morphological characteristics. These consist of one species of *B. mediterranea*. The culture collection of Zakho University's mycology laboratory has isolates made from representative strains of the designated species safely stored.

Biscogniauxis mediterranae (De Not.). Kountze, 1891.
Kingdom: Fungi
Phylum: Ascomycota
Class: Sordariomycetes
Order: Xylariales
Family: Graphostromataceae
Genus: *Biscogniauxia* Kuntze (1891)

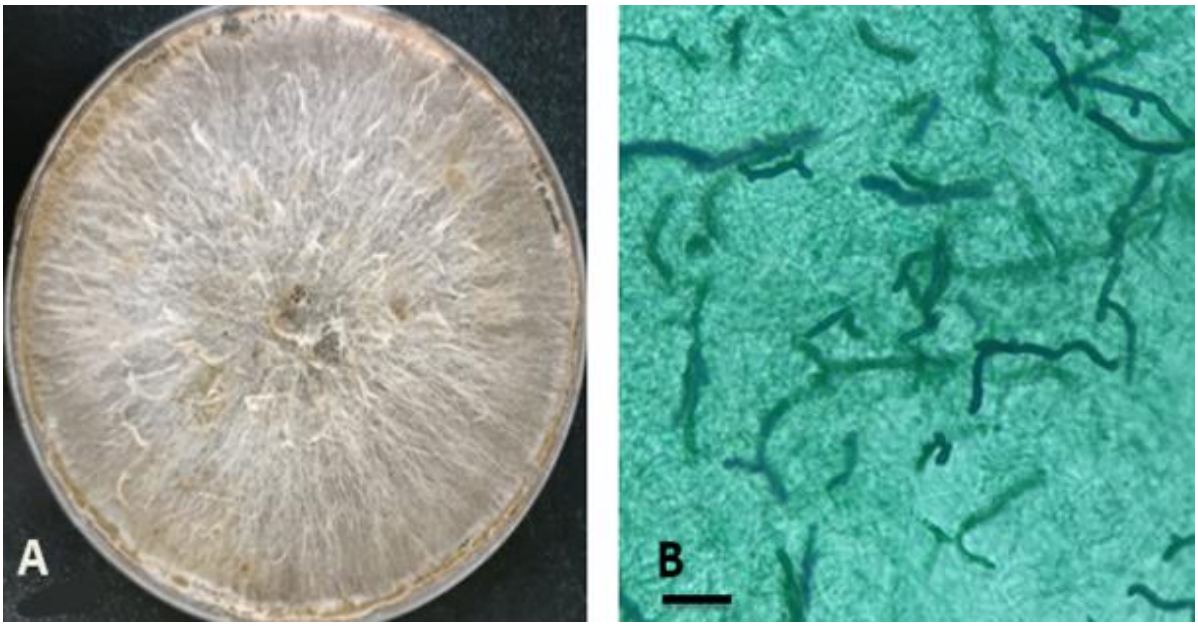
Q, infactoria leaves, buds, lateral branches, and seeds were used to isolate *Biscogniauxia*. The colony grows cottony quickly and is pale white to beige, with an olive patch measuring 8 cm in diameter in the middle of the MEA. The dark, discrete pigments in mycelium characteristics are tiny (Fig. 1).

The genus frequencies among 30 isolates—four isolates from each of the plant tissues were 33.3%, 23.3%, 16.6%, and 13.3%, respectively. The isolates were from buds, lateral branches, seeds, and leaves.

The ascomycete genus *Biscogniauxia*, which belongs to the Xylariaceae family, is distributed worldwide and comprises more than 50 identified taxa. Of particular concern in Portugal is *Biscogniauxia mediterranea*, which is known to cause charcoal canker in cork oak. Other species in this genus can cause canker under certain circumstances. According to strong data, these species serve as endophytes in healthy trees at first and become invasive during periods of water scarcity (Nugent *et al*., 2005; Yangui *et al*., 2019).

Utilizing morphological assessments, cultural characteristics, and DNA sequence data (ITS region), isolates were identified as *B. mediterranea*. The fungus's endophytic existence was confirmed and observed across all surveyed forests, displaying notable variations in frequencies. This occurrence was found to be linked to the densitometric structure of cork oak trees. Conversely, the identification of *B. mediterranea* as a pathogen was limited to Ain Beya and Ain. Sarouia forests in Tunisia (Yangui *et al*., 2021).

Our study represents the first report of *B.mediterranea* in Iraq as well as the first isolation from the *Q.infectoria* plant in the region. *B. mediterranea* was reported as a necrosis pathogen on stems and branches of *Q.castaneifolia*, *Q. brantii*, and *Zelkova carpinifolia* in Iran (Mirabolphathi, 2013). The fungus was also known as a causal pathogen of charcoal disease in *Q.brantii* in Zagros Mountain of Iran (Safaee *et al*., 2017).



Fig; 3.1. *B. mediterranae*: A. colony morphology on MEA, B. mycelium with dark short fragment, Scale bar= 10 µm

2. Phylogenetic Analysis Using Large Subunit Ribosomal RNA Gene (LSU rRNA) for the Identification of *B. mediterranea:*

PCR products of LSU for *B. mediterranea* were sent to Macrogen company in South Korea for DNA sequencing, the obtained sequences were trimmed and aligned using the BioEdite program and submitted to NCBI. The amplified sequence of the PCR product of LSU has been deposited in GenBank under the accession numbers (OP117437, OP117438, and OP117439). The phylogenic tree was constructed by comparing sequences of the current study with other sequences deposited in the NCBI by using MEGA 11 software and applying the neighbor-joining algorithm for the construction of the phylogenic tree. The phylogenic analysis that was conducted among the genus of *Biscogniauxia* species showed that all sequences were in one clade.

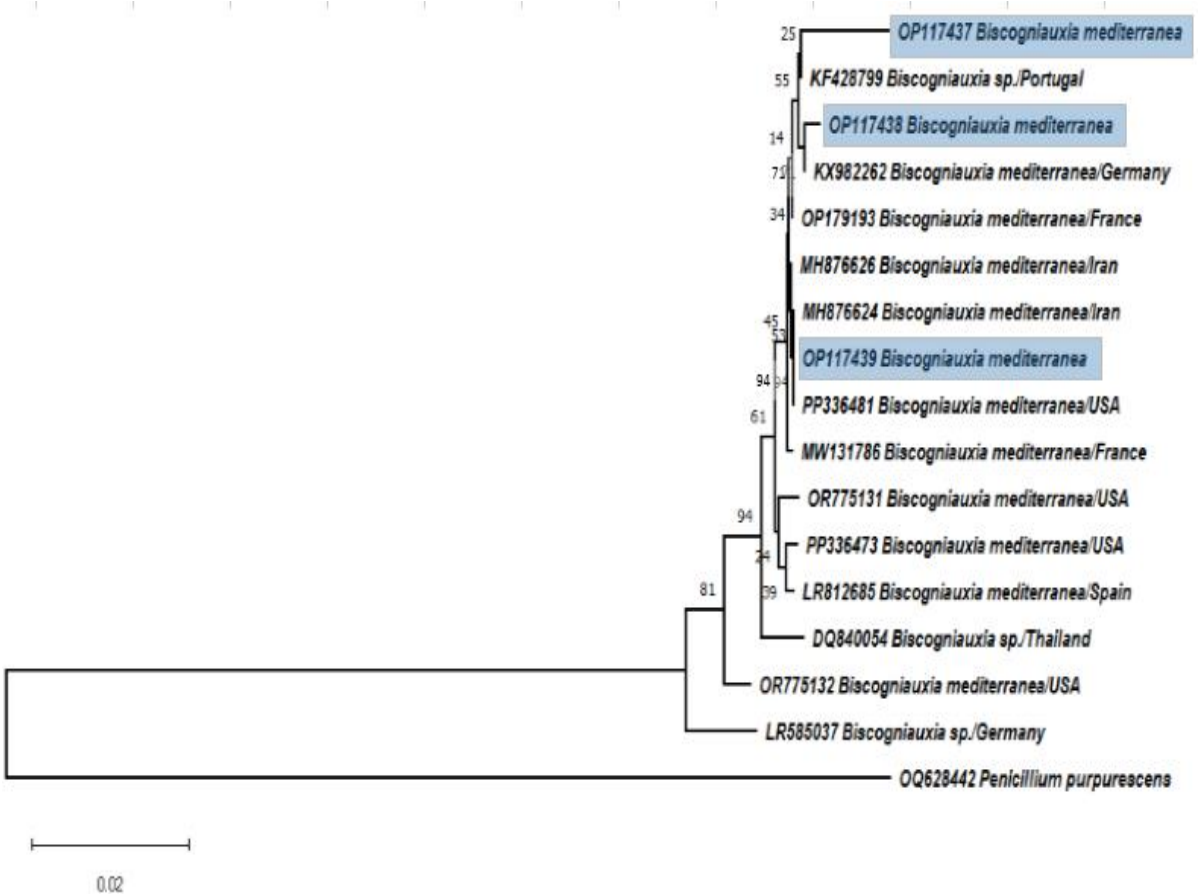
The obtained sequences OP117437, OP117438, and OP117439 belong to *B.mediterranea*. The sequence with accession number (OP117439) for sister taxa with sequence from Iran (MH876624) with 94% bootstrap supports. Our sequence with accession number (OP117438) forms sister taxa with sequences from Germany (KX982262) with 75% bootstrap support. Our sequence with accession number (OP117437) forms an outgroup with different genetic differences as shown in Figure (2).

The phylogenetic tree was generated from 17 sequences including 13 *B. mediterranea* and 3 *Biscogniauxia* sp. sequences in GenBank in addition to the outgroup sequence *Penicillium purpurescens*.

The first group comprised 1 obtained strain of *B. mediterranea* (OP117437) and *Biscogniauxia* sp. (KF428799) from Portugal cluster together with bootstrap (25 %). The second group comprised 1 obtained strain of *B. mediterranea* (OP117438) cluster with a strain of *B. mediterranea* (KX982262) from

Germany obtained from Genbank with bootstrap (14 %). The third group comprised 1 obtained strain of *B. mediterranea* (OP117439) clustered with 2 strains of *B. mediterranea* from Iran (MH876624) and *B. mediterranea* from the USA (PP336473) obtained from GenBank with bootstrap (45% and 53 %) respectively.

All sequences of *B. mediterranea* used the LSU gene, were deposited in GenBank under the accession number (OP117437, OP117438, and OP117439) were 97.55 %-99.76% identical to other available *B. mediterranea* sequences in GenBank. from Iran, France, Spain, the USA, Germany, Thailand, and Portugal. (Table 1).



Fig; 2: Phylogenetic tree of *B. mediterranea* based on Neighbor-Joining analysis with 1000 bootstrap replicates of LSU rRNA sequences on the strain from Iraq (highlighted with blue color) and related *B. mediterranea* from GenBank.

Table 1: Accession numbers of *Biscogniauxia mediterranea* isolates from buds of *Quercus infectoria* and the similarities percentages with other references

Species	Primer	Accession No.	Source / Host	Region	Similarity %
B. mediterranea	LSU	OP117437	Bud/ *Q. infectoria*	Kurdistan/ Iraq	
B. mediterranea		MH876626	CBS 129074	Iran	98.71%
B. mediterranea		OP179193	*Haliclona fulva*	France	98.71%
B. mediterranea		MW131786	leaves /Lycium hybrid cultivar	France	98.59%
B. mediterranea	LSU	OP117438	Bud/ *Q. infectoria*	Kurdistan/ Iraq	
B. mediterranea		MH876624	CBS 129072	Iran	99.33%
B. mediterranea		LR812685	FMR:17639	Spain	99.31%
B. mediterranea		OR775131	Acer saccharum	USA	98.85%
B. mediterranea		PP336473	AZ0048	USA	98.88%
Biscogniauxia sp.		LR585037	1..860	Germany	97.55%
B. mediterranea	LSU	OP117439	Bud/ *Q. infectoria*	Kurdistan/ Iraq	
B. mediterranea		KX982262	strain="LF657"	Germany	99.76%
Biscogniauxia sp.		DQ840054	voucher="JF 06-05	Thailand	98.81%
B. mediterranea		OR775132	Acer rubrum	USA	98.24%
Biscogniauxia sp.		KF428799	untreated drinking water sources	Portugal	99.88%
B. mediterranea		PP336481	CBS 129072	USA	99.89%
Penicillium purpurescens		OQ628442	Seed /*Quercus aegilops*	Kurdistan/ Iraq	Out group

Conclusion

In this study, we conducted a comprehensive investigation to isolate and characterize *B. mediterranea* from *Q. infectoria* in Iraq's Duhok province. Through a combination of morphological and molecular techniques, we confirmed the presence of *B. mediterranea* in *Q. infectoria*, marking its first report in Iraq and its isolation from this particular plant species in the region. Our findings contribute significantly to the understanding of the distribution and genetic diversity of *B. mediterranea*, which is crucial for devising effective strategies to manage charcoal canker disease in oak forests.

Morphological identification revealed characteristic features of *B. mediterranea*, including colony morphology and mycelium characteristics. Molecular identification using LSU gene sequencing further supported the identification of the isolated fungi as *B. mediterranea*. Phylogenetic analysis confirmed the genetic relatedness of Iraqi isolates with strains from different geographic locations, providing insights into the evolutionary relationships within the *Biscogniauxia* genus.

Our study underscores the importance of understanding the ecological dynamics and genetic diversity of fungal pathogens like *B. mediterranea* for effective disease management. By elucidating the presence and genetic characteristics of *B. mediterranea* in Iraq, we contribute valuable information to the global knowledge base on charcoal canker disease. This knowledge can inform the development of targeted management strategies tailored to the unique ecological context of oak forests in Iraq and other regions affected by this devastating disease. Further research focusing on the epidemiology, host-pathogen interactions, and environmental factors influencing disease dynamics will be essential for implementing

sustainable disease management practices and preserving the health of oak ecosystems

Declarations:

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest: The authors declare that they have no competing interests.

Authors Contributions: I hereby verify that all authors mentioned on the title page have made substantial contributions to the conception and design of the study, have thoroughly reviewed the manuscript, confirm the accuracy and authenticity of the data and its interpretation, and consent to its submission.

Availability of Data And Materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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