

Molecular identification of a novel endophytic fungus *Botryotrichum murorum* associated with tomato in Iraq

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Abstract. *Al-Rifaie AA, Al-Maqtoofi MY. 2025. Molecular identification of a novel endophytic fungus Botryotrichum murorum associated with tomato in Iraq. Biodiversitas 26: 2970-2977.* Due to the economic importance of tomato crops in Iraq, this study was designed to investigate new endophytic fungal species related to Sordariomycetes (Ascomycota), a class known for its diverse ecological roles. From Al-Zubair region, which includes well-known farms of tomato and located in the South of Basrah Province in Iraq, a total of 35 cultivated vegetable tomato (*Solanum lycopersicum*) samples were collected. These samples were processed by cutting them into small pieces sized (5-6 mm) using sterile blades. Two isolation protocols, including the Solid Culture Method (SCM) and the Moist Culture Method (MCM), were employed for endophytic fungal isolation. The recovered endophytic fungi were purified, and axenic cultures of each isolated fungal species were then identified initially based on macro and micro-morphological features. For accurate taxonomy, molecular technique was utilized by genomic DNA extraction and PCR amplification of multigene sequences such as ITS (Internal Transcribed Spacers 1 and 2 including the 5.8S nrDNA), LSU (D1/D2 domains of the 28S nrDNA), RPB2 (partial RNA polymerase II second largest subunit gene), TEF-1 α (translation elongation factor 1-alpha gene) and β -tubulin (TUB2) gene were used for identification. Phylogenetic analysis showed the isolation of *Botryotrichum murorum* as a dominant endophytic fungal species. This species was reported for the first time as a novel isolation in the Iraqi mycobiota.

Keywords: Ascomycota, *Botryotrichum*, endophytic fungi, Iraq, Sordariomycetes

INTRODUCTION

The growing human population has led to a rising demand for food, particularly tomatoes, to support various life forms while ensuring global food security. For this reason, losing crops can seriously impact food availability. It is necessary to develop sustainable strategies to help manage abnormal conditions affecting economic crops such as tomato (*Solanum lycopersicum* L.). Many studies have suggested that fungal endophytes have the potential ability to protect host plants against the damage caused by severe biotic and abiotic stress such as drought. These fungal endophytes hold promising potential as protective agents to enhance the resilience and productivity of traditional agricultural crops (Miranda et al. 2023). *Ampelomyces* sp. and *Penicillium chrysogenum* isolated from tomato plant associated with enhancing root biomass in comparison to non-symbiotic plants and yield under optimal, drought, and salt stress conditions, with potential as biofertilizers for improving future agricultural production (Morsy et al. 2020).

Endophytic fungi offer unique and sustainable approaches that could reduce crop losses while limiting the detrimental impact of conventional chemical treatments in addressing biotic and abiotic challenges (Najjar 2025). The immunity of host plants could compromise, leading to some endophytic fungi converted to serious plant pathogens such as *Fusarium* and *Aspergillus* species resulting in losing crops due to spoilage and this can affect human health through mycotoxins contamination (Al-Maqtoofi and Thornton 2016;

Al-Rifaie and Al-Maqtoofi 2018, 2019; Al-Maqtoofi and Al-Rifaie 2019). Endophytic fungi, residing within plant tissues, orchestrate an intricate interplay that significantly influences plant health and resilience (Usman et al. 2024). The ability of endophytic fungi to produce diverse chemical entities, including plant growth hormones, highlights their potential to modulate host gene expression, influencing defense mechanisms and secondary metabolic pathways (Usman et al. 2024; Zhao et al. 2024).

Screening of fungal species associated with tomato plant tissues revealed that the most frequently isolated classes were Sordariomycetes, Dothideomycetes, and Eurotiomycetes, all from the phylum Ascomycota, including identified species such as *Hanseniaspora uvarum*, *Dothideomycetes* sp., *Trichosporon cutaneum*, *Phialophora verrucosa*, *Erysiphe pisi*, *Sodiomyces alkalinus*, *Bipolaris sorokiniana*, and *Coelomomyces lativittatus* (Dong et al. 2021). Sordariomycetes class has approximately more than 3,000 genera and more than 15,000 species, making it one of the most taxonomically rich groupings of fungus (Wang et al. 2023). Within Sordariomycetes, the family of Chaetomiaceae is the most prominent family (Al-Rifaie and Ameen 2023).

Chaetomium species are a unique group of endophytic fungi characterized by several distinct biological and ecological features. *Chaetomium* species have a particular ability to produce extensive secondary metabolites, which enables the production of a diverse range of bioactive compounds that could be valuable in medicine, agriculture,

and industry (Tspinana et al. 2023). Recent molecular phylogenetic studies have revealed the polyphyletic nature of several genera traditionally classified based on morphology, including *Botryotrichum*, *Canariomyces*, *Chaetomium*, *Chaetomidium*, and *Thielavia* (Wang et al. 2022). *Botryotrichum murorum*, synonym for *Chaetomium murorum* related to the family Chaetomiaceae, is common in soil and endophytes. *B. murorum* resembles members of the genus *Chaetomium* by its superficial ostiolate ascomata and terminal hairs and is connected to the substrate via rhizoidal hyphae. The asci within this genus are typically clavate to fusiform, occasionally cylindrical (Fortes and Vitória 2023).

B. murorum showed unbranched ascomatal hairs with circinate tips and ellipsoid ascospores, which are the characteristics of *C. murorum*. A recent study showed that *C. murorum*, *Emilmuelleria spirotricha*, and *Thermomyces verrucosus* were related to *Botryotrichum* species, and new combinations, such as *B. murorum*, *B. spirotrichum*, and *B. verrucosum*, based on phylogenetic analyses (Wang et al. 2016a, 2019). *B. murorum* did not produce an asexual state and, in contrast to many closely related fungi, it is sensitive to high temperatures, showing restricted growth when incubated above 35°C. (Ryu et al. 2023). *Botryotrichum* species tend to produce a diverse range of secondary metabolites, which have significant advantages on plant host growth and defenses such as growing hormones, antimicrobial and antifungal (Ebrahim and Ebada 2021). This symbiotic relationship can be harnessed in agriculture to improve crop resilience and productivity (Fite et al. 2023). Due to the importance of tomato crops and the lack of studies about the isolation and identification of endophytic fungi from Iraqi tomato plants, the current study aimed to

investigate the endophytic fungi diversity in Iraqi tomato plants.

MATERIALS AND METHODS

Samples collection

A total of 35 cultivated tomato plant samples were collected from different regions within Al-Zubair farms, which are very well-known for their extensive tomato cultivation in Basrah Province, which is located in the South of Iraq, as shown in (Figure 1). The latitude of Al-Zubair, Iraq, is 30.389462, and the longitude is 47.705727. Al-Zubair is situated in the southern part of Iraq, within Basrah Province. The region is geographically positioned at the GPS coordinates of 30° 23' 22.0632" N latitude and 47° 42' 20.6172" E longitude.

The sampling collection was conducted between the 1st of February 2023 and to 1st of April 2023, ensuring a comprehensive representation of the region's tomato plants. Samples included roots, leaves, and stems sourced exclusively from disease-free greenhouse-grown tomato plants. Each sample was carefully collected in clean and sterile plastic bags to prevent contamination and preserve sample integrity during transportation. The collected materials were then promptly transported to the Postgraduate Fungi Research Laboratory (PFRL) at the Department of Biology, College of Science, University of Basrah. The samples were systematically processed for the isolation of endophytic fungi, adhering to standardized research laboratory approaches, ensuring optimal conditions for isolating and identifying endophytic fungal species associated with tomato plants.

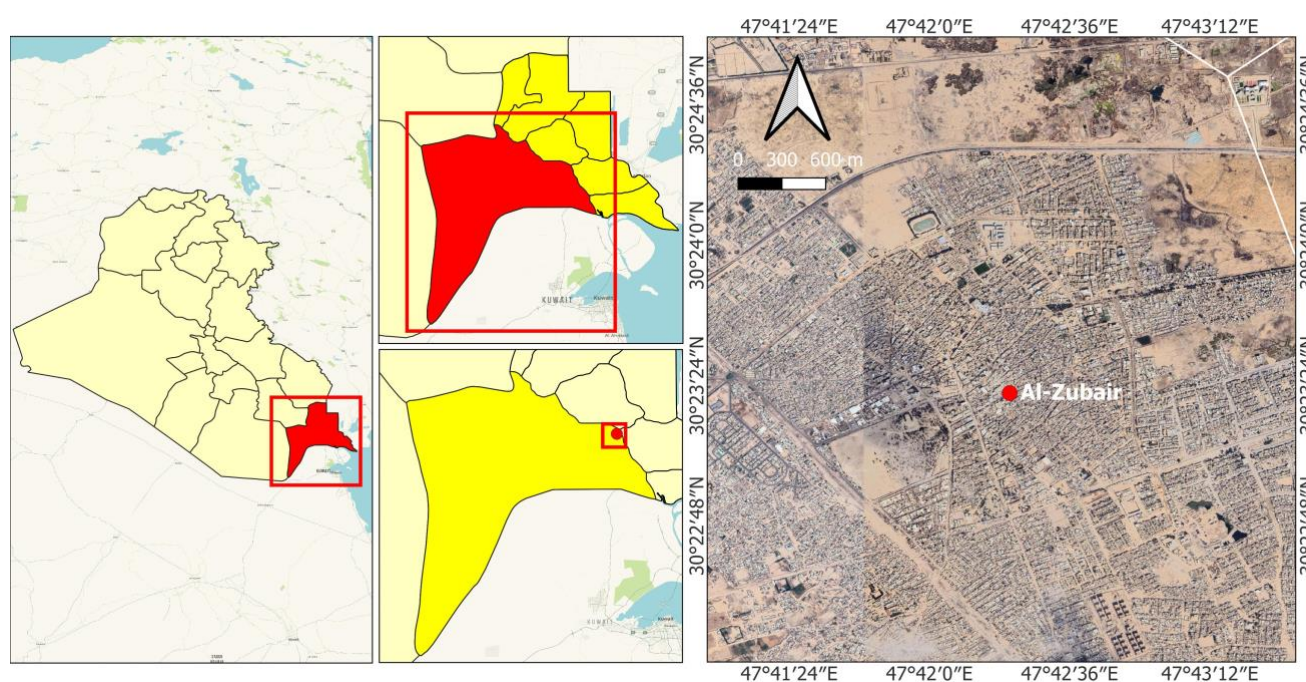


Figure 1. Location of vegetable samples collection for endophytic fungi isolation in Al-Zubair, Basrah Province, Iraq

Isolation of endophytic fungi from tomato tissues

Two distinct methods were conducted for endophytic fungi isolation: the Solid Culture Media (SCM) and the Moist Chamber Method (MCM). Prior to isolation, all tomato plant samples were thoroughly washed under running tap water to eliminate dust and debris, followed by rinsing with sterilized distilled water to ensure surface cleaning. For the SCM method, sterile razor blades were used to cut tomato plant samples into small segments measuring approximately (4-5) mm in length. Surface sterilization was then performed to eliminate epiphytic microorganisms. This process involved immersing the samples in 70% ethanol for 60 seconds, followed by treatment with 0.5% sodium hypochlorite for 5 minutes, then a second immersion in 70% ethanol for 30 seconds, and a final rinse with sterilized distilled water for 5 minutes. The sterilized plant samples were air-dried using sterilized filter paper to remove excess moisture at room temperature. Finally, three replicates for each tomato plant segment to ensure reliability were placed on a Petri dish containing Potato Dextrose Agar (PDA) supplemented with 0.01% Chloramphenicol antibiotic to inhibit bacterial growth. The plates were incubated at 25°C for 5-7 days with daily monitoring for emerging endophytic fungal growth from inside plant segments. Once fungal growth was observed, individual endophytic fungal colonies were transferred to new PDA plates to obtain axenic cultures for preservation and further identification. The pure isolates of endophytic fungi were then separately transferred to PDA slants and a sterilized glycerol solution at a 50% (v/v) concentration for long-term preservation.

The MCM protocol was performed following Sarsaiya et al. (2020). Briefly, tomato plant samples were cut into small pieces, approximately (0.5-1) cm in size, and then washed twice with tap water, followed by the same sterilization process described above. The sterilized and dried plant pieces were placed in large glass Petri dishes (15 cm in diameter) containing sterile filter paper moistened with sterile distilled water to maintain humidity. This method allowed for the natural emergence of endophytic fungi under controlled moisture conditions. The plates were incubated at 25±2°C for 15 to 20 days. Plates were monitored daily for detection of the growth of endophytic fungi.

Axenic cultures of isolated endophytic fungi were preserved in The Postgraduate Fungal Research Laboratory, Department of Biology, College of Science, University of Basrah, Iraq, for identification and analysis.

Morphological characterization

The cultural and morphological characteristics of the isolate X7 were thoroughly investigated using various growth media, including Potato Dextrose Agar (PDA) and Potato Carrot Agar (PCA). The colony characteristics, such as color, texture, and growth patterns, were meticulously examined under a light microscope (Olympus, Tokyo, Japan) and a dissecting microscope (Leica M80, Germany). The documented observations provided insights into the isolate's structural details, such as hyphal morphology, spore formation, and other reproductive structures, contributing to a comprehensive understanding of its taxonomic and functional traits.

Molecular identification of isolated endophytic fungi

After 7 days of incubation, the biomass of axenic endophytic fungi was harvested to extract genomic DNA (gDNA) at room temperature by using the kit provided by Geneaid Company (Yeast Genomic DNA Kit Cat. No. GBYB100, Geneaid, Taiwan) following carefully the manufacturer's instructions to ensure optimal results. Initially, approximately (50-100) mg of fungal biomass was carefully transferred into a clean 1.5 mL microcentrifuge tube. After gDNA was successfully extracted, Polymerase Chain Reaction (PCR) amplifications were conducted using four sets of primers to target specific genetic regions. The primers included ITS4/ITS5 for the complete ITS1/5.8S/ITS2 regions as described by White et al. (1990), LSU-F/LSU-R for Large Subunit (LSU, 28S) of the ribosomal RNA (rRNA), RPB2AM-1bF/RPB2AM-7R for RNA polymerase II subunit gene (Liu et al. 1999), and EF1-1018F/ EF1-1620R for partial translation elongation factor 1 α (TEF1- α) gene, in addition to Bt2a/ Bt2b for tubulin gene following the methodology was described by Wang et al. (2014). The used primers were described in detail as shown in Table (1).

Table 1. Primers used for *Botryotrichum* species identification

Gene	Primer	Sequence (5'→3')	Length (bp)	Reference
Internal Transcribed Spacer (ITS)	ITS5	CTTGGTCATTTAGAGGAAGTAA	~ 600	(Raudabaugh and Aime 2023)
	ITS4	TCCTCCGCTTATTGATATGC		
Large Subunit (LSU, 28S) of the rRNA	LSU-F	ACCCGCTGAACTTAAGC	~ 1200	(Raudabaugh and Aime 2023)
	LSU-R	CGCCAGTTCCTGCTTACC		
RNA polymerase II subunit (RPB2)	RPB2AM-1bF	GAYGAYMGWGATCAYTTYGG	~ 1200	(Wang et al. 2022)
	RPB2-7R	ACCCTCAGTGTAGTGACCCTTGCC		
Translation Elongation Factor 1 α (TEF1- α)	EF1-1018F	GAYTTCATCAAGAACATGAT	~ 700	(Meyer et al. 2019)
	EF1-1620R	GACGTTGAADCCRACRTTGTC		
β -tubulin (TUB2) genes	Bt2a	GGTAACCAATCGGTGCTGCTTTC	~ 500	(Wang et al. 2022)
	Bt2b	ACCCTCAGTGTAGTGACCCTTGCC		

The PCR amplification conditions were tailored for each primer pair to enhance the specificity and yield of the reactions. For each primer pair as following (ITS region and LSU) amplifications, the PCR conditions began with an initial denaturation step for 5 minutes at 95°C; 35 cycles of denaturation at 94°C for 30 seconds; followed by annealing step at 55°C; and 1.5 minutes at 72°C; followed by a final 10 minutes extension step at 72°C (Al-Maqtoufi and Thornton 2016). For (RBP2; TEF1- α ; TUB2) genes, a gradient PCR was employed. The final PCR amplification conditions were followed: an initial denaturation of 5 minutes at 94°C followed by 40 cycles of denaturation at 94°C for 30 seconds and an annealing step at 57°C for 30 seconds followed by 90 seconds at 72°C and then 7 minutes at 72°C for final TEF1- α extension; while for RBP2 same conditions but with annealing at 51.8°C for 30 seconds and 35 cycles. Unfortunately, attempts to amplify the TUB2 gene through gradient PCR were unsuccessful across the range of temperatures tested, from 48°C to 68°C. After gDNA amplification by PCR, the bands of the fungal species were sent to the Macrogen Company in South Korea for additional sequencing analysis.

Phylogenetic analysis

Molecular identification of the isolated endophytic fungus was conducted using a robust, multi-step approach. ITS barcoding was used for primary identification. The RBP2 and TEF1- α gene sequences were used for secondary and precise molecular identification. Sequences generated from these primers were analyzed by comparing them with other fungal sequences obtained from GenBank, ensuring accurate taxonomic placement. The sequences were subsequently deposited in GenBank, and their corresponding accession numbers were documented in Table 2. The species identity was determined based on a sequence identity of over 95% (Altschul et al. 1997) utilizing the Basic Local Alignment Search Tool (BLAST) and was compared with the GenBank sequence database at the National Center for Biotechnology Information (NCBI). Alignments for each data set were conducted using (MEGA11) (Tamura et al. 2013). All positions containing gaps and missing data were eliminated. Sequence phylogeny was generated using Maximum Likelihood (ML) with the best nucleotide substitution model and the Kimura 2-parameter model with the lowest BIC scores (Bayesian Information Criterion). The bootstrap consensus tree was inferred from 1000 replicates. After genomic analysis, the sequence data of isolated endophytic fungi were submitted to GenBank for accession number verification.

Table 2. Details of isolated *Botryotrichum murorum* from tomato (*Solanum lycopersicum*) source in Al-Zubair region and gene accession numbers

Isolates code	Species	Accession numbers
X7	<i>Botryotrichum</i> sp.	(ITS) OP168745
X7	<i>Botryotrichum</i> sp.	(TEF) OP974691
X7	<i>Botryotrichum murorum</i>	(LSU) PV147361
X7	<i>Botryotrichum murorum</i>	(RBP2) PV172615

RESULTS AND DISCUSSION

Identification of isolated species

Endophytic fungi have a significant positive impact on plant hosts by promoting crop production and improving tolerance to biotic and abiotic stresses, such as pests, diseases, drought, and salinity. In recent years, studies highlighted the role of endophytic fungi as the main microbial sources for secondary metabolites with high potential application for both human health and environmental sustainability. This has shifted focus toward exploring more endophytic fungi, particularly those associated with plants. These fungi are increasingly recognized for their ability to produce novel bioactive compounds, offering promising opportunities for diverse applications in biotechnology, medicine, and agriculture (Li et al. 2022). The primary function of bioactive metabolites synthesized by endophytic fungi is to increase the plant host resistance to microbial pathogens and enhance plant growth and productivity, such as flavonoids, carotenoids, melatonin, terpenoids, phenolics, alkaloids, peptides (Watts et al. 2023). Due to endophytic fungi's ability to provide a wide range of secondary metabolites to their plant hosts, there is a high chance of finding endophytic fungi that inhabit tomato plants. This relation can be mutualism through commensalism to parasitism. Through mutualism, the endophytic fungi receive nutrition from the host plant while the plant gets the advantages of fungal metabolites (Jha et al. 2023). This study is a part of extensive program of screening endophytic fungi in Iraq. In this particular study, our primary objective was to focus on the identification and characterization of a unique fungal isolate, *B. murorum*, which demonstrated distinct morphological and molecular features. While a total of 35 tomato plant samples were collected and various sections were examined, the comprehensive analysis and diversity profiling of all fungal endophytes recovered from each plant part have been intentionally reserved for a future, more detailed study. This upcoming work will address the comparative diversity, ecological roles, and potential applications of these isolates. During this study, the aim was to investigate the endophytic fungi that have mutualism and commensalism relationships with tomato hosts.

Tomato (*Solanum lycopersicum*) is one of the essential nutritional economic crops for daily use all over the world and produced by many countries particularly South of Iraq, and is the second most important crop after potato. Tomato crops are enriched with incredible nutritional elements such as vitamins A and C, minerals, and lycopene (antioxidants) that are essential for human health (Ali et al. 2020). During this work, the results revealed a notable dominance of a specific species of endophytic fungi colonizing tomato plants cultivated in Al-Zubair farms located in the Basrah province of Iraq.

The results of the phenotypic identification and phylogenetic analysis showed that the isolated endophytic fungus from tomato plant tissues belonged to the Ascomycota group, the class Sordariomycetes, the family Chaetomiaceae. This fungus was identified as *B. murorum* and reported as a novel record of isolation from tomato as a

host for the first time in Iraqi Mycobiota (Figure 2). The dominance of *B. murorum* suggests a potentially strong association between the fungus and tomato plant that enables the plant host to tolerate the local environmental conditions, as well as a possible mutualistic relationship with the host plant. This relation could play a crucial role in enhancing the resilience and productivity of tomato crops in the South of Iraq in Al-Zubair farms.

The selection of appropriate primers is crucial for the accurate amplification, sequencing, and identification of *Chaetomium* species. The ITS region is used widely as a fungal barcode for primary identification as it has limitations in species detection. The same outcome was noticed when TEF1-a primers were used. Based on these markers, isolated

endophytic fungus, *B. murorum*, was identified as *Botryotrichum* sp., as shown in (Figures 2.A and 2.C). In the current study, the amplification of the β -tubulin gene failed for detection of the isolated *Botryotrichum* species using gradient PCR with a wide range of annealing temperatures; this might be due to difficulty in aligning TUB2 primers with isolated *Botryotrichum* species gDNA. However, many studies used TUB2 primers for the identification of *Botryotrichum* at the species level (Alidadi et al. 2020; Wang et al. 2022; Qian et al. 2024). In addition, phylogenetic analysis of LSU and RPB2 sequencing revealed the accurate identification at the species level of *B. murorum* (Figures 2.B and 2.D).

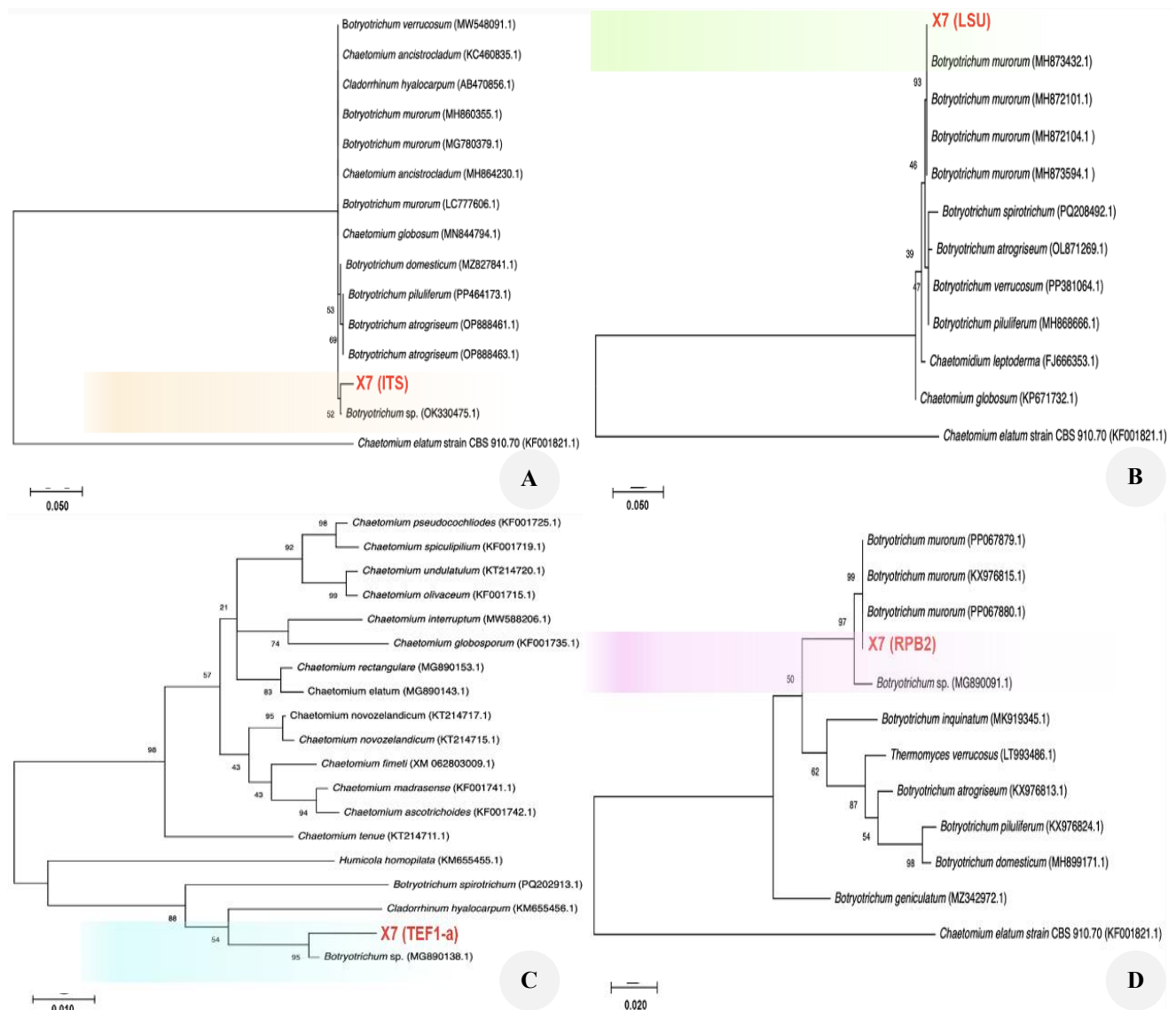


Figure 2. Phylogenetic analysis of *Botryotrichum murorum* using multiple genetic markers. Phylogenetic trees were constructed using: A. ITS, B. LSU, C. TEF1- α , and D. RPB2 markers based on neighbor-joining analysis. The trees depict the relationship of the isolated endophytic fungus (isolate_No.X7, highlighted in red) with closely related reference sequences from the family Chaetomiaceae (retrieved from NCBI). Bootstrap values (based on 1000 replicates) are shown at nodes, with values $\geq 50\%$ indicating confidence levels in branch support

These sets of primers are essential for reliable identification. For this reason, LSU and RPB2 primers are particularly recommended due to ease of amplification and taxonomic clarity. Many studies recommended relay on using LSU and RPB2 for specific and accurate identification of *Botryotrichum* species (Wang et al. 2016b; Wang et al. 2022; Ryu et al. 2023). For the description of new *Botryotrichum* species, we recommend generating at least making primary identification of isolated endophytic fungi using ITS and TEF1-a and then conducting the secondary identification using LSU and RPB2. The relationships of the new species will be confidently determined using these four sets of primer approaches to recognize new species more easily.

Studies have reported that *B. piluliferum* and *B. murorum* isolated from interior substrates of housed such as plaster walls, ceiling tiles, and anthropogenic environments. The species *B. murorum* was identified in the liquor cerebrospinalis of *Homo sapiens* as well (Wang et al. 2016b) and cover crops, including *Astragalus sinicus* and *Vicia villosa* (Qian et al. 2024). Globally, there were no studies indicating the isolation of *B. murorum* as endophytic fungi from tomatoes.

Chaetomium species could serve as a source of multifarious bioactive natural compounds for controlling plant diseases and developing bioorganic fertilizers that can help, support, and protect plant hosts from biotic and abiotic conditions (Yang et al. 2023). The positive relation between *B. murorum* and tomato can explain the dense productivity of tomatoes in Al-Zubair farms in Basrah, Iraq. However, the mechanisms by which *B. murorum* functions in tomatoes are completely unknown, as this is a new plant host report. The mechanism that enhances growth promotion or stress tolerance is not within the scope of this current work. For this reason, further studies are required to determine the stress tolerance and growth promotion mechanisms in response to *B. murorum* colonization.

Morphological characteristics are unique for *Botryotrichum*, such as superficial ascomata and hair extensions that surround or the presence of stalked ascospores that are considered the common identifying phenotypic of the species (Abdel-Azeem 2020; Wang et al. 2022). However, the identification of *Botryotrichum* species based on morphological features only is insufficient due to the identification of slight morphological diversity and variation among this genus (Abdel-Azeem et al. 2021). Therefore, molecular techniques would be considered a vital tool for discrimination between these genera, particularly for isolates that lack production reproductive structures (Dos Reis et al. 2022).

***Botryotrichum murorum* (Corda) X. Wei Wang & Samson (2016)**

Culture characteristics

Colonies on PDA sparse smoke grey-olive with aerial hyphae mixed with pale olivaceous grey ascomata, about 8 cm in diameter at 25°C in seven days, reverse pale purplish grey to fuscous black (Figure 3).

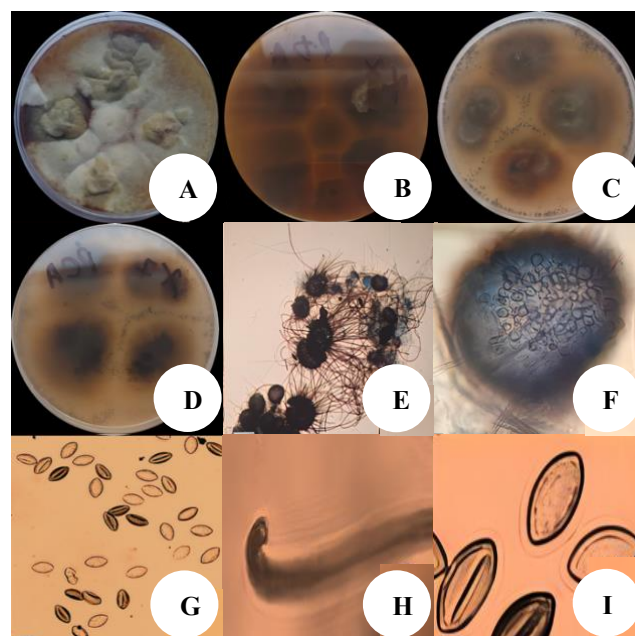


Figure 3. *Botryotrichum murorum*. A. *Botryotrichum murorum* on PDA front, B. *Botryotrichum murorum* on PDA reverse side, C. *Botryotrichum murorum* on PCA front, D. *Botryotrichum murorum* on PCA reverse side, E. Fruiting body, F. Ascoma with asci, G and I. Ascospore, H. Ascomatal hairs. Note: E. 100 μ m, F. 50 μ m, G. 20 μ m, H and I. 8 μ m

Morphological characteristics

Fruiting bodies were superficial with ostioles, olive dark brown or colonies somewhat dark olive to grey, spherical to ovoidal, 100-500 μ m in height, 200-350 μ m in diameter. Terminal hairs, usually over four times longer than ascomata, flexuous or undulate, often circinate at the apex, or undulate, occasionally recurved or circinate at the apex olivaceous brown, up to about 3 mm long. Lateral hairs are shorter. Ascospores olivaceous brown when mature, ellipsoidal-fusiform, (7.5-18 \times 7.5-10) μ m, with an apical germ pore. The asexual morph is unknown.

Material examined

The fungus was isolated from tomato (*S. lycopersicum*) samples that were taken from Al-Zubair farms in Basrah Province. This fungus was isolated for the first time in the world by (Wang et al. 2016a) from indoor environments.

In conclusion, this study is considered the first exploration of *B. murorum* as an endophytic fungus isolated from a novel plant host, tomato, in Basrah, Iraq. This fungus belongs to the Chaetomiaceae family and Sordariomycetes class. This fungus exhibits diverse morphological traits, highlighting the limitations of relying on morphological characteristics that make reliance only on morphological identification insufficient. Phylogenetic analyses of ITS and TEF1-a alongside LSU and RPB2 sequences are recommended for *Botryotrichum* species identification. Further studies focusing on the bioactive metabolites of *B. murorum* are essential. In addition, the bioactive metabolites produced by *B. murorum* require further investigation, as they hold potential applications in agriculture and medicine.

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