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New endophytic fungal species of Chaetomiaceae (Ascomycota) in Iraq

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Abstract. *Al-Rifaie AA, Ameen MKM. 2023. New endophytic fungal species of Chaetomiaceae (Ascomycota) in Iraq. Biodiversitas 24: 5270-5277.* The present study was conducted to isolate and identify some endophytic fungi from vegetable crops in Iraq. Samples of eight vegetable plants, namely *Abelmoschus esculentus*, *Mentha piperita*, *Vicia faba*, *Petroselinum sativum*, *Ocimum basilicum*, *Lawsonia inermis*, *Beta vulgaris and Apium graveolens* were collected from three regions in Basrah (Abu Al-Khaseeb, Karmat Ali and the Centre of Basrah). Isolation was done by solid culture method and moist culture method. The recovered endophytic fungi were purified and axenic cultures of each isolated species were then identified on the basis of their macro and micro-morphological features. Morphological identification was further confirmed by molecular analysis through DNA extraction and sequencing by PCR amplification of the ITS4 and ITS5 gene primers. Phylogenetic examination revealed that five novel endophytic fungal species related to the family Chaetomiaceae were isolated from vegetable plants, including *Chaetomium cucumericola*, *C. madrasense*, *Amesia atrobrunnea*, *A. cymbiformis* and *Botryotichum verrucosum.* Three species, including *C. cucumericola*, *A. cymbiformis* and *B. verrucosum*, were documented for the first time in the Iraqi mycobiota. To the best of our acknowledgment, this study is the first to investigating endophytic fungi from vegetables in Iraq.

Keywords: Ascomycota, *Chaetomium*, endophyte fungi, Iraq, vegetables

INTRODUCTION

Endophytic fungi are organisms that inhabit all healthy plant tissues throughout at least a portion or the entire life cycle of the plant without causing disease or noticeable morphological alterations (Wen et al. 2022). The presence of fungi within plant tissues has been recognized since the late 19th century, and the term "endophyte" was introduced by de Bary in 1866. Generally, endophytic fungi can colonize within intracellular and/or extracellular spaces of the plant tissues as a host. Some of these fungi are considered a serious plant pathogen such as *Fusarium* and *Aspergillus* species (Al-Rifaie and Al-Maqtoofi 2018; Minati and Mohammed-Ameen 2019a; Minati and Mohammed-Amee 2019b). Endophytic fungi typically can transmit into inside plant's hosts through two main modes of transmission: vertical transmission via host seeds and horizontal transmission through air and soil microorganisms for colonization (Tian et al. 2022). However, these microorganisms have a significant ability to promote the host plants growth via capability of producing secondary metabolites resulting in enhancing the plant's resistance to biotic and abiotic stresses (Rashmi et al. 2019). For this reason, the research community over the last three decades has expanded the efforts to get the advantages of endophytic fungi considering them valuable putative sources of bioactive compounds that have medicinal properties for plants, animals and humans that have antibacterial, antifungal, antiviral, anti-inflammatory, antitumor, antimalarial activity (Singh and Kumar 2023). Meanwhile, investigation wide resources for isolation and characterization of endophytic fungi can enrich providing fungal species with a putative ability for production valuable metabolites and discovery the biodiversity of those microorganisms is needed (Gupta et al. 2023). One of the most common endophytic fungi is the genus *Chaetomium* that have a wide application in the field of healthcare and agriculture (Hashem et al. 2023).

The Chaetomiaceae is a large family that was identified by Winter (1885). This family is exemplified by *Chaetomium* Kunze. It belongs to the order Sordariales (Ascomycota) and currently comprises 25 genera (Alidadi et al. 2020). The Chaetomiaceae family is widely recognized for its saprophytic nature and showcases a remarkable diversity, encompassing more than 400 documented species (Rao et al. 2023). Members of the genus *Chaetomium* occupy various ecological niches, frequently being isolated from sources such as soil, seeds, decomposing plants, herbal drugs, sugarcane, and dung (Moya et al. 2020). Species of the genus *Chaetomium* are distinguished morphologically by its superficial ostiolate ascomata, which are adorned with terminal and terminal hairs and connected to the substrate via rhizoidal hyphae (Fortes and Vitoria 2023). The asci within this genus are typically clavate to fusiform, occasionally cylindrical, and possess thin walls without apical structures, ultimately fading away. Ascospores found in *Chaetomium* are aseptate and feature one or two germ pores, displaying hues ranging from brown to grey-brown (Asgari and Zare 2011). Morphological identification of the genus *Chaetomium* is highly limited due to extreme similarities with closely related genera, such as *Thielavia* and *Humicola* (Wang et al. 2022). For this reason, identification on the base of molecular and sequence analysis is needed as some species

may lack reproductive structures production over time (Wang et al. 2016; 'Aini et al. 2022). Over the past few years, many studies have been conducted to group, classify, and accurately identify different species of *Chaetomium*, utilizing DNA sequencing as a key tool (Sekhar et al. 2018). Very limited studies have been conducted in Arabic countries and Iraq for the genetic characterization of *Chaetomium* species (Abdullah et al. 2015; Abdel-Azeem 2020; Al-Dossary et al. 2021; Alfartosy et al. 2021).

Due to the lack studies about isolation and identification of *Chaetomium* species as endophytic fungi from Iraqi vegetable plants, the aim of present work was to investigate the endophytic fungi community and biodiversity of the genus *Chaetomium* that exist within Iraqi environments from vegetables resources as hosts based on morphological characterization. Furthermore, molecular sequencing was performed to confirm morphological identification to understand the existence and diversity of *Chaetomium* species as an endophyte.

MATERIALS AND METHODS

Samples collection

A total of 50 cultivated vegetable plant samples were collected from three different regions in Basrah province, Iraq (Abu Al-Khaseeb, Karmat Ali and the Centre of Basrah) as shown in (Figure 1) in the period from 1st January to 1st September 2022. Plant samples, including mature leaves, stems, roots, flowers, and fruits, were collected and kept in a separate clean plastic bag. After collection, all samples were brought to the Postgraduate Fungi Research Laboratory at the University of Basrah, College of Science, Department of Biology, for isolation process.

Isolation and purification of endophytic fungi

Isolation of fungal endophytes was performed by following two methods:

Solid culture media

This method was done according to Ibrahim et al. (2021). Briefly, after sterilizing the surface layer of each sample, they were thoroughly washed under running tap water to eliminate dust and debris. Next, the samples were washed with autoclaved distilled water. Using sterile razor blades, vegetable samples were cut into small pieces with dimensions 2 and 4 mm in length.

The vegetable segments were surface disinfected with 70% ethanol for 60 seconds, followed by 0.5% sodium hypochlorite for 5 minutes, 70% ethanol for 30 seconds, and finally with sterilized distilled water for 5 minutes, and then air-dried using sterilized filter paper. Finally, vegetable segments were placed on a Petri dish containing Potato Dextrose Agar (PDA) supplemented with 0.01% Chloramphenicol antibiotic, and 3 replicates were made per plant part.

The plates were carefully sealed and incubated at 25°C for 5-7 days. Samples were monitored daily for detection the growth of endophytic fungi. Recovered fungi were selected and transferred into a new PDA plate for preservation and 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.

Moist chamber method

Vegetable samples were cut, measuring approximately 4-5 mm, then washed twice with tap water, and followed by the same sterilization process described above. The dried plant pieces were arranged in large Petri dishes (15 cm in diameter) containing sterile filter paper that was moistened with sterile distilled water to provide moisture. Subsequently, the Petri dishes were placed in an incubator at 25 ± 2°C for 15 to 20 days (Sarsaiya et al. 2020). Plates were monitored daily for detection 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.

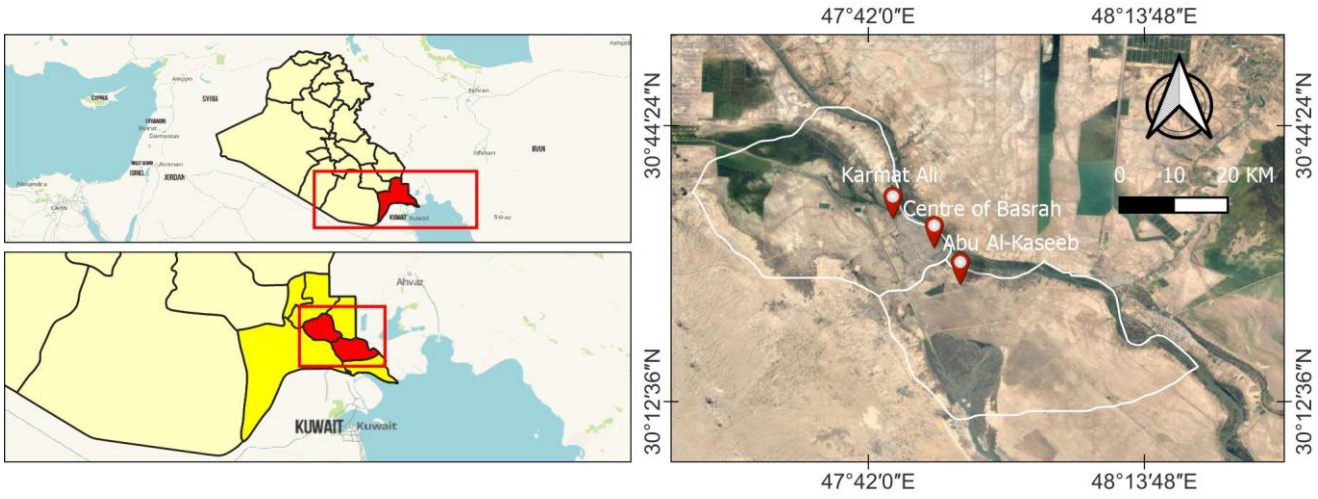

Figure 1. Location of vegetable samples collection for endophytic fungi isolation in Basrah, Iraq

Molecular identification of isolated endophytic fungi

Deoxyribonucleic Acid (DNA) of isolated endophytic fungi was extracted using (Yeast Genomic DNA Kit Cat. No. GBYB100, Geneaid, Taiwan). Briefly, axenic culture of each endophytic fungi isolates on PDA was freshly sub-cultured on PDA and incubated for 7 days at 25 ± 2°C. Pure fungal biomass of 50-200 mg was transferred to a clean 1.5 mL microcentrifuge tube for DNA extraction following the manufacture instructions. Extracted DNA of endophytic fungi was amplified by the ITS1-5.8S-ITS2 region sequencing of the rRNA encoding gene unit using the primers ITS5ext (5'-GTAACAAGGTTTCCGTAGGTG-3') and ITS4 ext (5'TTCTTTTCCTCCGCTTATTGATATGC3') (White et al. 1990). PCR amplification condition of the ITS region was conducted according to procedures described by Al-Maqtoofi and Thornton (2016). The amplified DNA bands of the fungal species were sent to Macrogen company in South Korea for additional sequencing analysis.

The species identity was determined based on a sequence identity of over 95% (Altschul et al. 1997) of the ITS region of recovered species using 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 made in (MEGA11) (Tamura et al. 2013). Sequence phylogeny was generated using Maximum Likelihood (ML) with the best nucleotide substitution model, Jukes-Cantor 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.

RESULTS AND DISCUSSION

Taxonomy of isolated species

There is a complex relationship between endophytes and their host plants. Host-endophyte interactions can range from mutualism through commensalism to parasitism. The mutual relationship has benefits for both fungi and plant by which the endophytic fungi receive nutrition and protection from host plant while the plant getting advantage from fungal metabolites production to enhance competitive ability and increase resistance to herbivores, insect, pathogens and various biotic and abiotic stresses (Jia et al. 2016). In addition, endophytic fungi have been reported to promote the host plant growth and development as well as plant physiology by producing special substances mainly secondary metabolites and enzymes (Poveda et al. 2022). However, in cryptic existence, endophytes play a role in ecosystems by becoming decomposers among the primary colonizers of dead plant tissues (Batzer and Mueller 2020; Fontana et al. 2021).

The results of the phenotypic and molecular analysis showed that five endophytic fungi isolated from vegetables belonged to the Ascomycota group. These identified species were related to the family of Chaetomiaceae. Two species belonged to the genus *Chaetomium* (*Chaetomium cucumericola* and *C. madrasense*) and others belonged to other genera of the Chaetomiaceae family, namely, *Amesia atrobrunnea*, *Amesia cymbiforms* and *Botryotichum verrucosum.* Among them three species, including *C. cucumericola*, *A. cymbiformis* and *B. verrucosum* were isolated in the Iraqi Mycobiota, Basrah, for the first time (Figure 2). Details of collection areas in Basrah, Iraq and recovered endophytic fungi related to the genus *Chaetomium* and their substrate of isolation are shown in Table 1. Special morphological characteristics, such as superficial ascomata and hairs extension that surrounded or the presence of stalked ascospores, are the most common identifying phenotypic feature of *Chaetomium* species (Abdel-Azeem 2020). Due to slight morphological diversity and variation among *Chaetomium* species, identification based on morphological features alone is insufficient for the identification of *Chaetomium* species (Abdel-Azeem et al. 2021). Therefore, molecular techniques would be considered a vital tool for discrimination between *Chaetomium* species particularly for isolates that lack production reproductive structures (Hanin and Fitriasari 2019).

Table 1. Details of isolated *Chaetomium* species area, vegetable sources and ITS accession numbers

Isolates code	Species	Substrates	Collection area	ITS accession numbers
25	*Amesia atrobrunnea*	*Abelmoschus esculentus* *Mentha piperita* *Vicia faba*	Centre of Basrah Karmat Ali	OP168751
37	*Amesia cymbiformis*	*Petroselinum sativum* *Ocimum basilicum*	Centre of Basrah Karmat Ali	OR352900
13	*Chaetomium madrasense*	*Mentha piperita* *Lawsonia inermis*	Centre of Basrah	OP168757
B1	*Chaetomium cucumericola*	*Beta vulgaris*	Abu Al-Khaseeb	OP168755
A3	*Botryotrichum verrucosum*	*Apium graveolens*	Abu Al-Khaseeb	OP168726

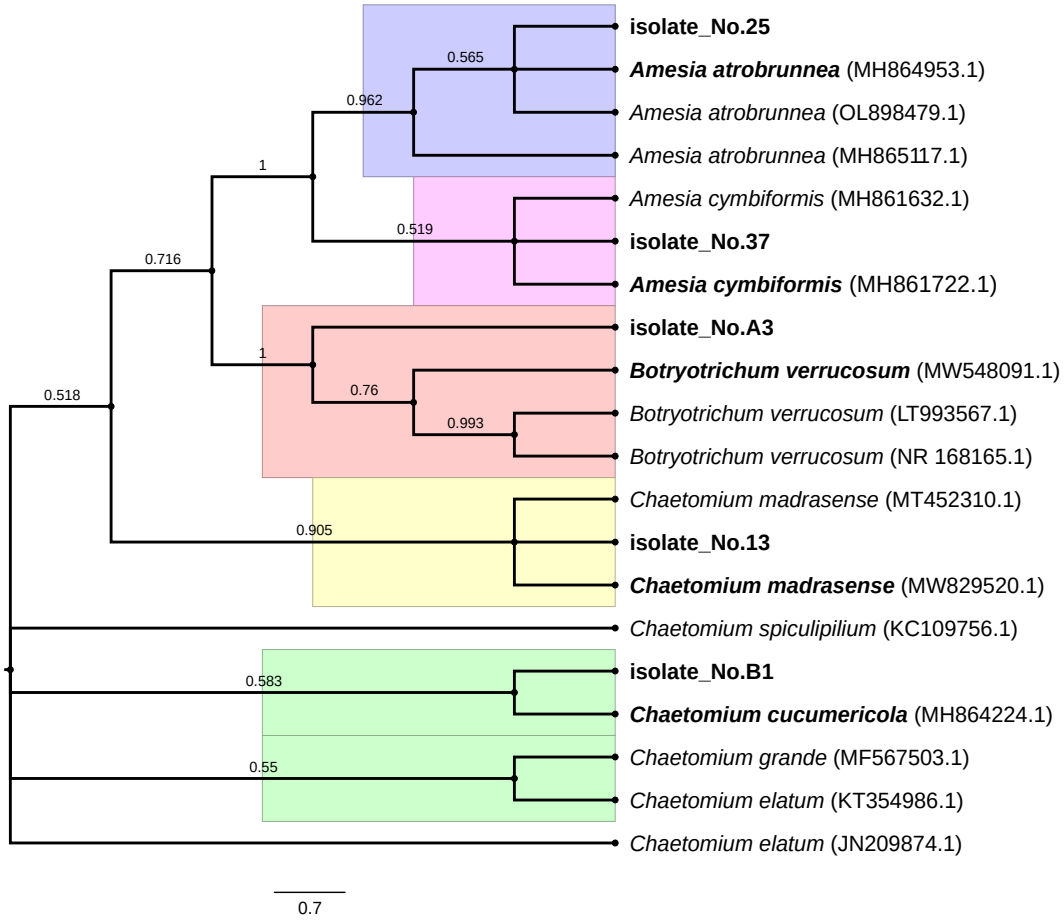

Figure 2. Phylogenetic tree represents neighbor-joining analysis of ITS domain sequences depicting the relationships of five isolated endophytic fungi (isolate_No.25 to isolate No B1) with closely related reference sequences of *Chaetomium* species retrieved from NCBI. Each numerical value represents the percentage of bootstrap samples, a total of 1000 samples, that support the internal branches with a confidence level of 50% or higher

Amesia atrobrunnea *(Ames) Wang and Samson, Stud. Mycol. 84: 158. (2016) (Figure 3)*

Basionym: *Chaetomium atrobrunneum* Ames, Mycologia 41: 641. 1949.

Culture characteristics: On PDA media, the colonies displayed a greyish-white appearance, characterized by floccose mycelium. The central part of the colonies appeared looser, with a texture ranging from white to pale olivaceous buff due to the presence of ascomata mixed with aerial hyphae. After 7 days at 25°C, the colony diameter reached approximately 7 cm. The reverse side color was greyish to black, and underneath the fruiting bodies, a dark color was observed. On PCA, the colony exhibited a white color and diameter of colony was about 4-5 cm within seven days. The reverse side of the colony remained uncolored.

Morphological Description: Ascomata was superficial, ostiolate ovate, or subglobose ascomata 75-165 µm high, 70-140 µm diameter, black in reflected light. Ascomata wall exhibited texture angular in surface view and was black to dark brown in color. The hairs were flexuous, smooth, and septate and were 2.3-3.5 µm in diameter near the base flexuous, sometimes branched, and smooth, the lateral hairs are similar and shorter in length. The asci were clustered, with a club-shaped or spindle-shaped spore-bearing part measuring 9-25 µm and stalks that are 9.5-21 µm long. Each ascus contains 8 ascospores irregularly arranged. The ascospores mature into an olivaceous-brown color and are fusiform or elongated, measuring 8-10 × 4.5-5 µm, featuring an apical or slightly subapical germ pore located at the more attenuated end. The asexual form of this organism remained unknown.

Material examined: The fungus was isolated from three plant samples collected from two areas in Basrah province (The Centre of Basrah and Karmat Ali). GenBank accession number (OP168751) of the isolate was confirmed by NCBI. Wang et al. (2016) previously named this species as *Cheatomium atrobrunneum.* After extensive phylogenetic analysis was transferred to closely related a new genus named *Amesia.* The main features of this genus were the variety of ascomatal hair extensions that covered the ascoma, and ascospore shape. Also, phylogenetic analysis revealed that this species clustered with another isolated species in the current study, *A. cymbiformis* in the same clade (Figure 4). The findings were consistent with Wang et al. (2016) showing that these two species are

associated with each other and can be distinguished on the basis of ascospore size and shape.

Amesia cymbiformis *(Lodha) Wang and Samson, comb. nov.2016. (Figure 4)*

Basionym: *Chaetomium cymbiforme* Lodha, J. Indian Bot. Soc. 43:127. 1963.

Culture and morphological characteristics: Colonies grown on PDA exhibited a yellowish-pale color with a white edge, reaching a diameter approximately 7 cm after seven days at 25°C. Sparse white aerial hyphae were visible, and the reverse side of colony was pale and uncolored. On PCA, the edge of colonies was smooth and complete, with the diameter of colonies reaching around 3-4 cm within seven days at 25°C. The ascomata were superficial and ostiolate, displaying an olivaceous-grey hue under reflected light due to the presence of ascomatal hairs. They were subglobose to ovate in shape, measuring 140-250 µm in height and 100-255 µm in diameter. The ascomatal wall appeared brown, and the surface exhibited a textura angularis pattern in view. The terminal hairs were flexuous, sometimes curled, smooth, brown, and approximately 2-3.5 µm in diameter near the base. The lateral hairs were also flexuous but shorter in comparison. The asci were clustered and clavate or fusiform in shape, with a spore-bearing part measuring 16-26 µm and stalks about 6-15 µm long. Each ascus contained 8 ascospores arranged irregularly and are evanescent. The ascospores mature into an olivaceous-brown color, appearing ovate with dimensions of (7-9 × 5-8) µm and featuring an apical germ pore at the attenuated end. The asexual form of this organism remained unknown.

Material examined: The fungus was isolated from two plant samples taken from two places in Basrah province (Centre of Basrah and Karmat Ali) GenBank accession number (OR352900).

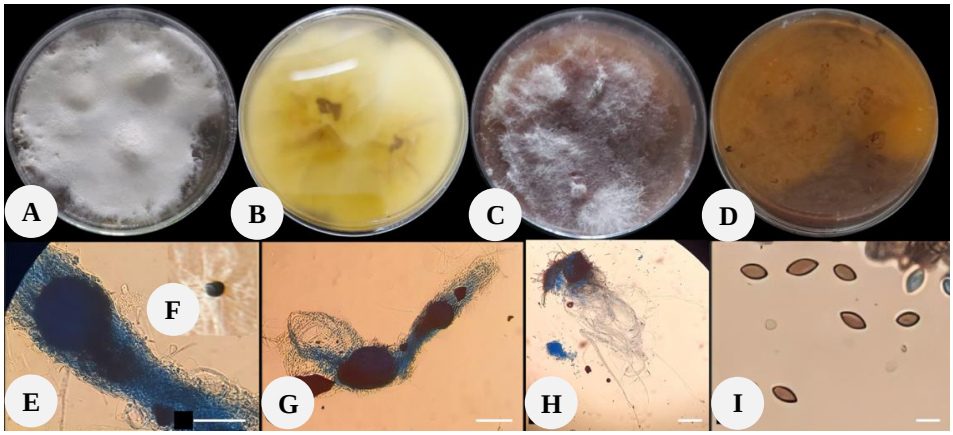

Figure 3. *Amnesia atrobrunnea*. A: *A. atrobrunnea* on PDA front view; B: *A. atrobrunnea* on PDA reverse view; C: *A. atrobrunnea* on PCA front view; D: *A. atrobrunnea* on PCA reverse view; E, G, H: Ascoma; F: Fruiting body; I: Ascospores. Bars: E, G, H = 100 µm; I = 8 µm

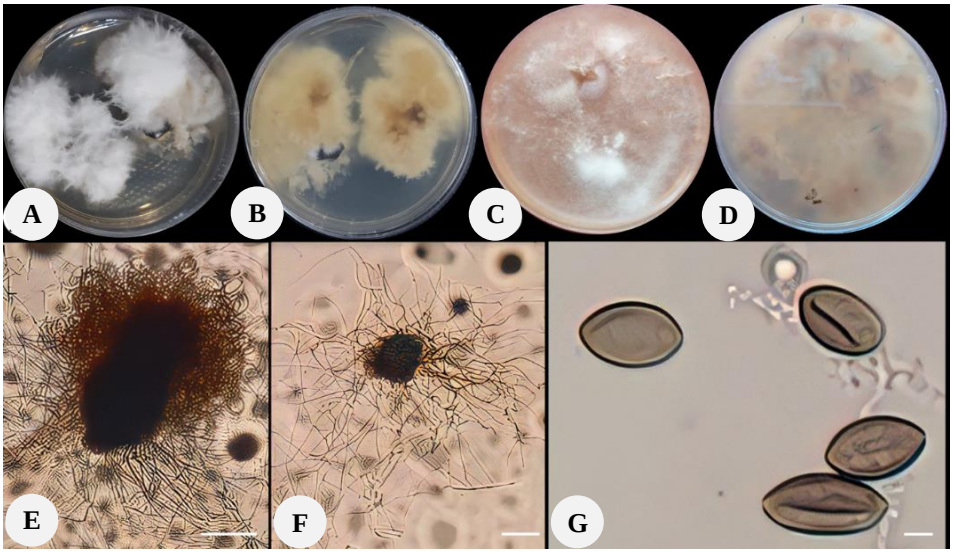

Figure 4. *Amnesia cymbiformis*. A: *A. cymbiformis* on PDA front view; B: *A. cymbiformis* on PDA reverse view; C: *A. cymbiformis* on PCA front view; D: *A. cymbiformis* on PCA reverse view; E and F: Ascoma; G: Ascospores. Bars: E = 90 µm; F= 110 µm; G = 6 µm

Chaetomium madrasense *Natarajan, Proc. Indian Acad. Sci., B. 74: 255. 1971. (Figure 5)*

Culture characteristics: On PDA, colonies of (*Chaetomium madrasense*) had cottony white aerial hyphae, yellow exudates found in the center, and diameter reached 8 cm at 25°C in seven days. Reverse side was white with little orange, however, the area beneath the fruiting bodies appeared dark.

Morphological characteristics: The fruiting bodies were found on the surface and possess ostioles. They varied in color from olive-green to somewhat dark olive-orange or grey. Under reflected light, they exhibited ascomatal hairs and had a spherical to ovoidal shape, measuring 130-300 µm in height and 100-260 µm in diameter. Terminal hairs were fairly abundant, brown, the surface exhibited fine verrucose texture and may appear coiled, undulated, or sometimes, showed simple branches. Lateral hairs were similar in appearance to the terminal hairs. Asci were clustered together and appeared fusiform or clavate. The spore-bearing part of the ascus measured 28-38 × 13-20 µm, with stalks about 16-30 µm in length. Each ascus contained eight biseriate ascospores. The ascospores matured into an olivaceous-brown color and had a broad limoniform to nearly globose shape, often slightly apiculate at both ends. They were bilaterally flattened. The asexual form of this organism was not found.

Material examined: The fungus was isolated from one plant sample taken from one place in Basra province (Centre of Basrah) GenBank accession number (OP168757).

Chaetomium cucumericola *Wang, Crous and L. Lombard 2015 (Figure 6)*

Cucumericola is a term used to name a fungus that isolated from a plant host, cucumber (*Cucumis sativus*).

Culture characteristics: Colonies on PDA were cottony, floccose, with thick white aerial hyphae, yellow to orange color pigmentation appeared on the reverse side. Diameter was 8 cm at 25°C in seven days. On PCA, white aerial hyphae appeared, reverse side was pale orange to sometimes uncolored.

Cultures sterile: Efforts to induce sporulation on PDA, PCA, and OA media were unsuccessful. The result was consistent with Wang's group (Wang et al. 2016a; Wang et al. 2016b).

Material examined: The fungus was isolated from one plant sample (root and stem) taken from two areas in Basrah province (Abu Al-Khaseeb) GenBank accession number (OP168755).

Botryotricum verrucosum *(Pugh, Blakeman and Morgan-Jones) Wang and Houbraken (2018) (Figure 7)*

Culture characteristics: Colonies on PDA were white-yellowish in the center with a black edge, reverse side was yellow but dark beneath the fruiting bodies diameter about 8 cm at 25°C in seven days. On PCA, the colony displayed complete or slightly white aerial hyphae, while the area beneath the fruiting bodies appeared dark. Fruiting bodies were superficial with ostioles, olive dark brown or colonies somewhat dark olive-orange to grey, spherical to ovoidal, 300-400 µm in height, 200-350 µm in diameter. The terminal hairs were abundant and exhibited a flexuous, undulated, coiled, or arcuate structure, with their apices showing an incurved to coiled appearance. The asci were clavate and contain 8 biseriate or irregularly arranged ascospores. Ascospores have a limoniform to quadrangular shape that was bilaterally flattened, and feature an apical germ pore tapering to the ends with an apical germ spore, 8-13 × 5-8 µm.

Material examined: The fungus was isolated from one plant samples taken from one place in Basrah province (Abu Al-Kaseeb) GenBank accession number (OP168726).

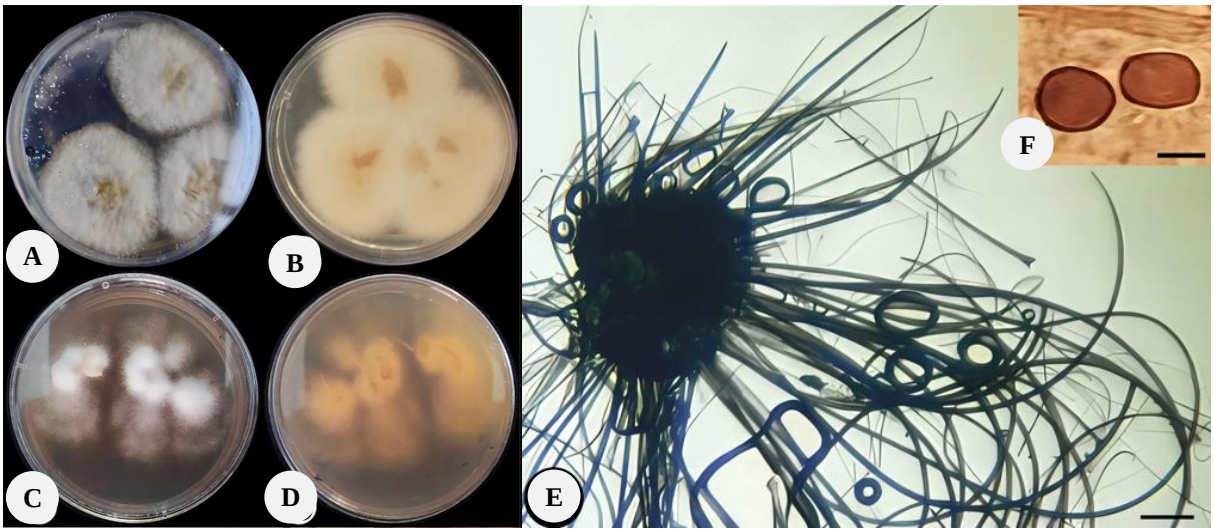

Figure 5. *Chaetomium madrasense*. A: *C. madrasense* on PDA front view; B: *C. madrasense* on PDA reverse view; C: *C. madrasense* on PCA front view; D: *C. madrasense* on PCA reverse view; E: Ascoma; F: Ascospores. Bars: E = 100 µm; F = 10 µm

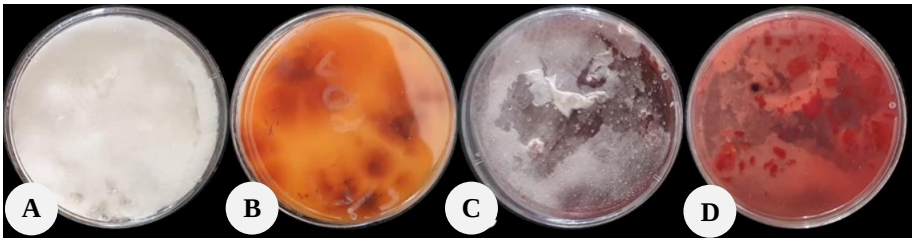

Figure 6. *Chaetomium cucumericola*. A: *C. cucumericola* on PDA front view; B: *C. cucumericola* on PDA reverse view; C: *C. cucumericola* on PCA front view; D: *C. cucumericola* on PCA reverse view

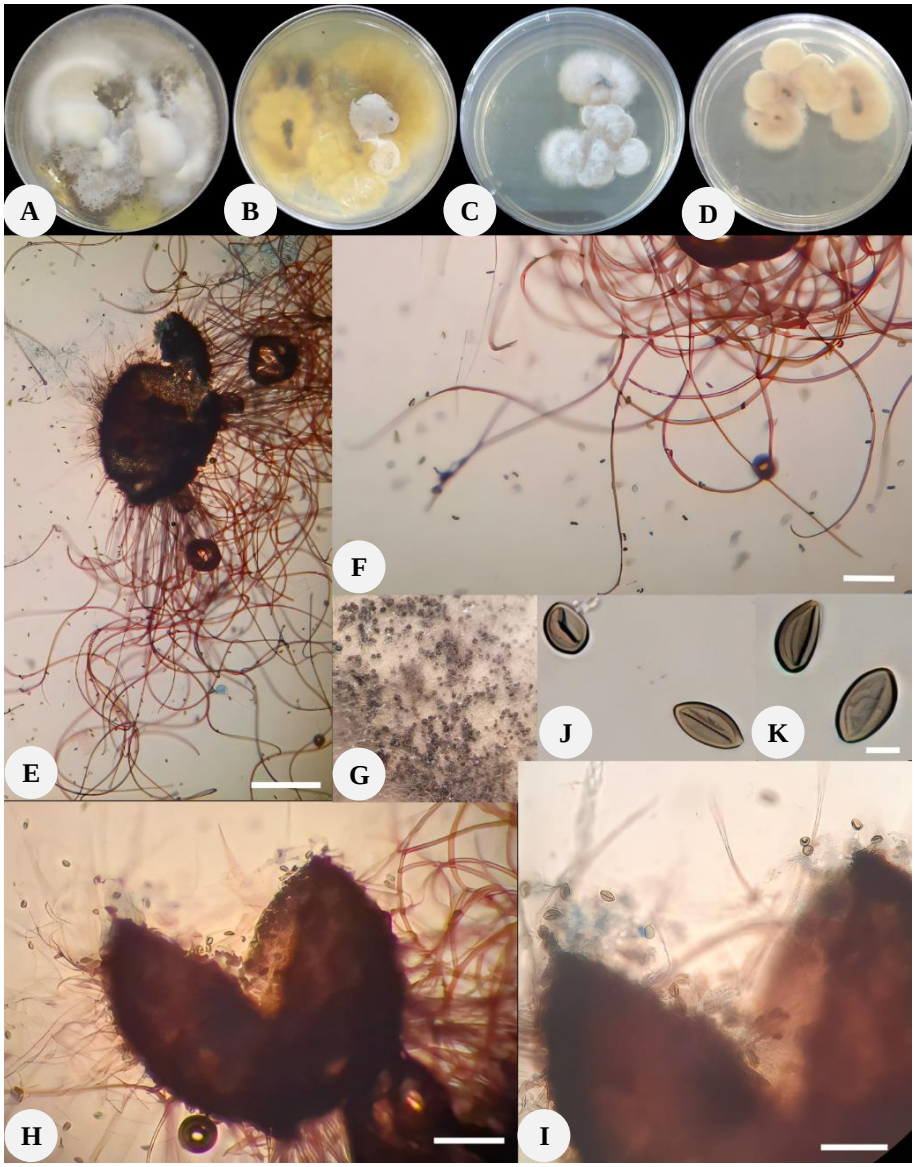

Figure 7. *Botryotricum verrucosum*. A: *B. verrucosum* on PDA front view ; B: *B. verrucosum* on PDA reverse side; C: *B. verrucosum* on PCA front view; D: *B. verrucosum* on PCA reverse side; E, H, I: Ascoma. F: Ascomatal hairs. G: Fruiting body. J, K: Ascospore. Bars: E, H = 100 µm; I = 20 µm; F = 10 µm; J, K = 6 µm

In conclusion, the present study marked the initial investigation of the family Chaetomiaceae as endophytic fungi in Basrah, Iraq, particularly vegetable samples. The isolated species exhibited a wide range of morphological features, indicating that reliance only on morphological characteristics for identification may not be sufficient and should be supplemented with modern phylogenetic techniques. Among the five identified species, three species, namely *Chaetomium cucumericola*, *Amesia cymbiformis*, and *Botryotichum verrucosum* were documented for the first time in the Iraqi mycobiota.

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