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Molecular Profiling of Selected *Aspergillus* Species Parasitizing Stored Wheat Seeds in the Silos of Tikrit City, Iraq

Aya Fadhil Noori*[1] , Rafea Zeidan Mukhlif[2]
[1,2]Department of Biology, College of Science, University of Tikrit, Iraq
*e-mail:aya.f.noori.bio449@st.tu.edu.iq

ABSTRACT: This study aimed to isolate and identify fungal species.*Aspergillus*From wheat seeds stored in silos in Tikrit city, Salah al-Din Governorate, to assess the risks of fungal contamination on the safety of stored crops. Samples were collected from two local wheat seed varieties (with and without sorghum), and fungi were isolated from them using culture techniques on medium.PDA. Fungal isolates were identified based on morphological characteristics (colony color, texture, conidiophore and conidia shape), in addition to molecular identification using DNA sequencing of the ITS region of rRNA, after amplification using primers ITS1 and ITS4. BLAST analysis results showed a high match between the local isolates and the isolates registered in the GenBank database, where the following accession numbers were registered: *A. fumigatus* (PV363322), *A. terreus (PV363323), A. niger (PV363324),* and *A. flavus* (PV363325). Genetic analysis results also showed relative differences among some isolates, indicating potential genetic diversity within the local environment. These results demonstrate that stored wheat seeds are susceptible to contamination by fungal species known to produce dangerous mycotoxins, posing an environmental and health threat. Together, the results of molecular and phenotypic identification confirm the accuracy of isolate classification and its role in assessing fungal risks in storage silos.

keywords
Aspergillus,wheat,ITS, Genbank, PCR, *A.niger, A.flavus, A.fumigatus, A.terreus*

INTRODUCTION

Wheat is one of the most important agricultural crops in Iraq, and the most cultivated and consumed.Wheat grains are considered a complete food because they contain a range of important nutrients, such as vitamin B and some minerals such as copper and magnesium. They are also a rich source of protein, containing more than 10% protein and more than 9% vitamins, and constitute approximately 20% of the total calories in the diet [1]. Wheat seeds are exposed to various types of fungal infections during the planting, harvesting, and storage stages. Fungi are among the most prominent causes of damage to stored seeds, due to their ability to form microscopic spores capable of surviving in storage conditions for long periods [2]. Many fungal species are common on stored wheat seeds, and the genus *Aspergillus* is considered one of the most dangerous due to its rapid spread and high capacity to produce mycotoxins. Its species grow rapidly under moderate to high temperatures [3]. The genus Aspergillus is one of the most widespread fungal genera worldwide and belongs to the filamentous fungi (molds). This genus includes more than 300 known species, many of which are pathogenic to humans and animals [4]. This is attributed to its ability to produce multiple types of mycotoxins such as aflatoxins, ochratoxins, gliotoxin, and citrinin, which cause liver and kidney diseases [5], and may affect genetic material and lead to mutations [6]. These fungi live in soil, air, organic matter, and agricultural crops, and have the ability to adapt to different environments, as they can live as saprophytes, obligate or facultative parasites [7]. Aspergillus is a fungus belonging to the phylum Ascomycetes. It was previously classified as a Deuteromycete due to the lack of discovery of its sexual phase. However, after the identification of sexual reproduction through the production of ascospores, it was classified as a sac fungus [8]. A study [9] showed that Aspergillus grows on stored wheat grains under conditions of moisture and moderate to high temperatures, and can produce toxins at temperatures up to 40°C. Most of these toxins are heat-resistant [10]. The growth rates of different Aspergillus species vary depending on their ability to adapt and survive in environmental conditions [11]. Studies have shown that the most common Aspergillus species in wheat seeds stored in silos in Salah al-Din Governorate are: *A. flavus, A. niger, A. fumigatus, and A. terreus,* all of

which are known for their ability to produce mycotoxins. Given the great similarity between different species within the same genus, relying solely on phenotypic characteristics may not be sufficient for accurate diagnosis, as growth and medium characteristics are factors that influence colony morphology. Therefore, molecular diagnosis based on DNA sequencing is an accurate and crucial tool for species identification [12], using the polymerase chain reaction (PCR) technique to amplify DNA [13].

MATERIALS AND METHODS

1_Sample collection

Twenty wheat seed samples were collected from the silos of Tikrit city, Salah al-Din Governorate, Iraq, during November 2024. The samples included two local wheat varieties (Bakhdam and Badun Khadam). The samples were transferred to the Mycology Laboratory in the Department of Life Sciences, College of Science, Tikrit University, to study fungal contamination associated with the two varieties under local storage conditions.

2_ Sample culture

Use the mediumPDA for fungal isolation, where wheat seeds were sterilized with 1% sodium hypochlorite for one minute, after which the seeds were rinsed with sterile water, then the sterilized seeds were planted on PDA medium at 25°C for 5-7 days, and the antibiotic tetracycline was added to the medium to prevent bacterial growth. More than one colony of *Aspergillus spp* appeared in both varieties, where 4 types of Aspergillus spp were isolated, then the pure growing colonies were transferred to new plates to complete the diagnosis and molecular study.

3_Morphological and microscopic diagnosis

Fungal isolates were identified morphologically and microscopically based on morphological and histological characteristics..The morphological characteristics of the isolates, such as colony color and texture, were observed. Microscopic slides were prepared using lactophenol cotton blue staining to examine the microscopic characteristics, such as conidia and conidia morphology, based on the taxonomic keys for fungi according to [14].

4- Molecular diagnosis

1-4: DNA extractionDNA

DNA was extracted.DNA from fungal isolates was isolated using a Fungal DNA Isolation Kit (ZYMO, USA) according to the manufacturer's recommendations for fungal DNA. DNA purity and concentration were verified using a NanoDrop device as described in [15].

2-4: Enlarge the areaITS using polymerase chain reaction (PCR)

The interior area has been enlarged.ITS using primers ITS1 and ITS4 as described by [13]. The PCR reaction was prepared in a total volume of 25 µL, by adding 5 µL of template DNA, 10 µL of Taq PCR Pri Mix, and 1 µL each of primers ITS1 and ITS4 to amplify the ITS region of fungal DNA as shown in Table (1), and the volume was completed with deionized water. The reaction consisted of three main cycles, repeated 35 times as follows: denaturation at 95°C for 30 seconds, annealing at 65°C for 40 seconds, and extension at 72°C for 60 seconds. The cycle was concluded with a final stage at 72°C for 10 minutes to ensure the formation of the new DNA strand.

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- TCCGTAGGTGAACCTGCGG -3'	60.3	50	500-650 base pair
Reverse	5' TCCTCCGCTTATTGATATGC-3'	57.8	41	

Table (1): Sequence of primers used to amplify the regionITS of fungal DNA.

3-4: Analysis of resultsPCR
The reaction products were separated.PCR was performed by electrophoresis on a 0.75% agarose gel using 50 ml of distilled water and 2 µl of red dye. Bands were detected using UV light at 302 nm. The bands were compared with a standard DNA ladder [16].
4-4: Sequencing and AnalysisBLAST
To accurately analyze the molecular data and confirm the molecular diagnosis of the studied fungal species and determine the closest sequence matches of these species with the isolates recorded inGenBank. Samples were sequenced by Biomeer Biotechnology, Korea. ITS region sequence analysis was performed using BLAST. Sequences of each isolate were also deposited in GenBank. Local isolates were matched with global isolates registered in GenBank.

5-4: Sequence analysis and phylogenetic tree construction
Sequences were analyzed.ITS analysis of fungal isolates was performed using MEGA X [17]. Multiple sequence alignments were performed, and the genetic similarity ratios between each two isolates were calculated based on the pairwise distances matrix using the Kimura-2 parameter. The results were presented in a table for pairwise comparison.

RESULTS

1- Results of collecting isolates
Four pure isolates of the fungus were obtained.*Aspergillus spo.*They all showed good growth on medium.PDA After incubation for 5-7 days at 25°C, a significant variation in phenotypic characteristics was observed among the isolates, indicating the diversity of the isolated fungal species.

2- Results of morphological and microscopic diagnosis
The fungal isolates showed morphological and microscopic characteristics that helped in their diagnosis, both morphologically and microscopically. All the isolates studied belong to the genus*Aspergillus*This genus belongs to the kingdom *Fungi, subkingdom Dikarya, phylum Ascomycota, subphylum Pezizomycotina, class Ascomycetes, subclass Eurotiomycetidae, order Eurotiales, and family Trichocomaceae.*
The isolated species appeared as follows:
*Aspergillus flavus*It forms yellowish-green colonies with a velvety surface and spherical conidia.*Aspergillus niger:*Black colonies with a velvety surface and rough conidia appearing in chains. *Aspergillus terreus:*Light brown, smooth colonies, velvety, spherical conidia. *Aspergillus fumigatus:*Grey or bluish-green colonies, with velvety, spherical conidia that appear as chains[14]. As shown in Figure 1.

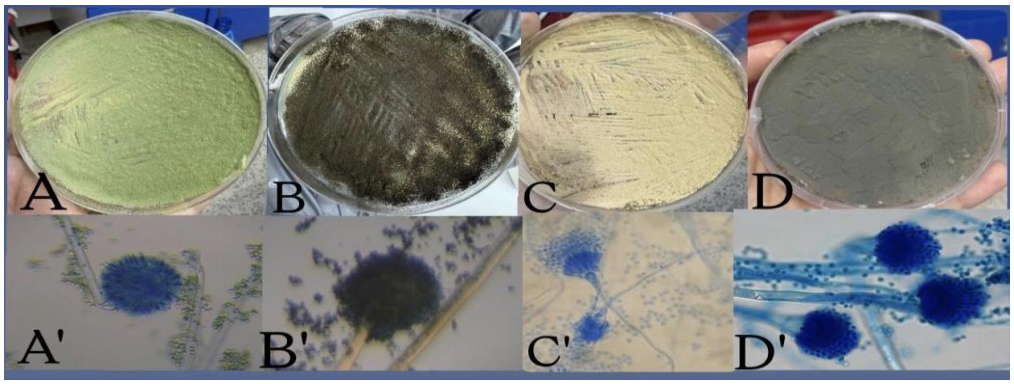

Figure 1: Morphological and microscopic diagnosis of four types of*Aspergillus*spp. isolated from wheat seeds.
(A) External appearance of *A.flavus* isolate on PDA medium,
(A') Micrograph of the same isolate showing a conidial head and spherical conidia.
(B) External appearance of an *A.niger* isolate
(B') Micrograph showing black conidia arranged as rough chains.

(C) External appearance of an *A.terreus* isolate
(C') Photomicrograph showing a conidial head and spherical conidia.
(D) External appearance of *A.fumigatus* isolate
(D') Micrograph showing a gray head with small, smooth conidia.

3- Molecular diagnosis results

1-3: DNA extraction resultsDNA
ExtractedHigh purity DNA was obtained from all isolates, with purity ranging from 1.7 to 1.9 (A260/A280), and quantities were sufficient for successful PCR.

2-3: Area amplification resultsITS and PCR
A reaction was made.PCR using primers ITS1 and ITS4, all isolates showed clear bands on agarose gel with a molecular weight of approximately 600 base pairs, indicating successful amplification of the ITS region, as shown in Figure (2).

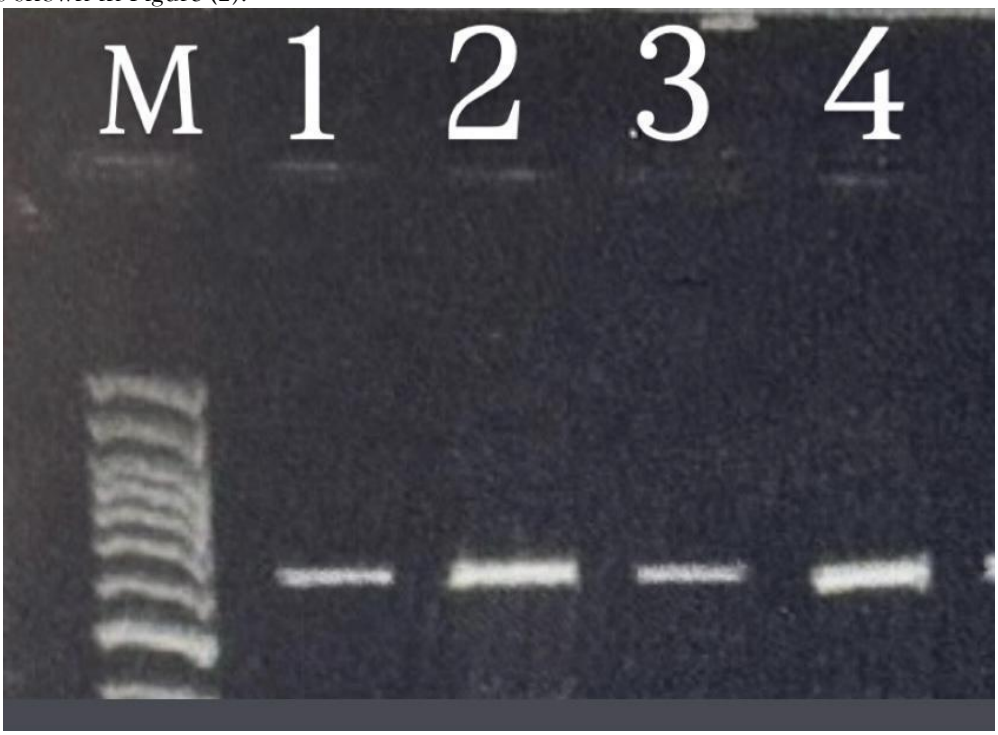

Image of the transfer of productsPCR on agarose gel showing amplification bands of the ITS region of the studied isolates. M: molecular ladder, 1–4: amplification products of the isolates (*A. flavus, A. niger, A. terreus, A. fumigatus).*

3-3: Sequencing and analysis resultsBLAST
Outputs sentThe purified PCR was sent to Biomeer Biotechnology, Korea, for sequencing of the ITS region. After receiving the sequences, they were analyzed using the BLAST program available in the NCBI database. The researcher compared the sequences with isolates registered in the GenBank database. The 5.8 rDNA gene sequences of the studied isolates have been deposited in the GenBank database. Accession numbers:

A.fumigatus: PV363322
A.terreus:PV363323
A.niger:PV363324
A.flavus:PV363325

Results of comparing local isolates with global isolates recorded inGenbank:

A. fumigatus showed 100% homology to the isolate recorded in Germany (accession number: LR536678.1), confirming that the local isolate belongs to this species.
*A.terreus*It showed 99.6% similarity to the isolate recorded in France (accession number:KY624794.1). This confirms that the local isolate belongs to this species.
*A.niger*It showed 99.9% similarity to the isolate recorded in the Netherlands (accession number:MN308513.1). This confirms that the local isolate belongs to this species.
*A.flavus*It showed 99.8% similarity to the isolate recorded in Italy (accession number:ONO75443.1). This confirms that the local isolate belongs to this species.

The quality of the sequence was also checked using sequence maps (chromatograms), which showed good clarity in the peaks of nitrogenous bases A, T, C, and G, reflecting the quality of the results and the purity of the sequencing process for all isolates, as in Figures (3) to (6):

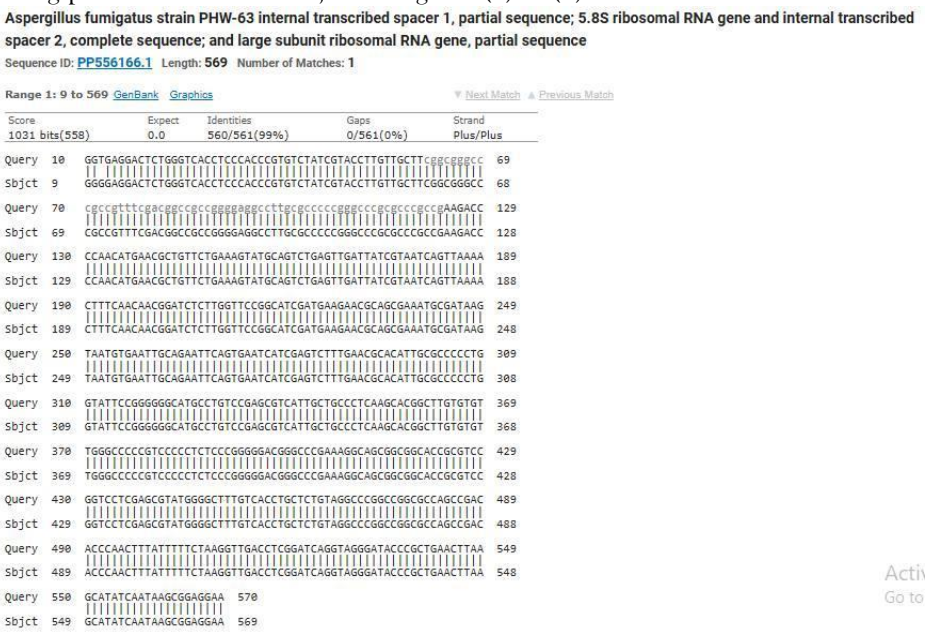

Figure (3): Sequence mapITS for *A.fumigatus* isolation

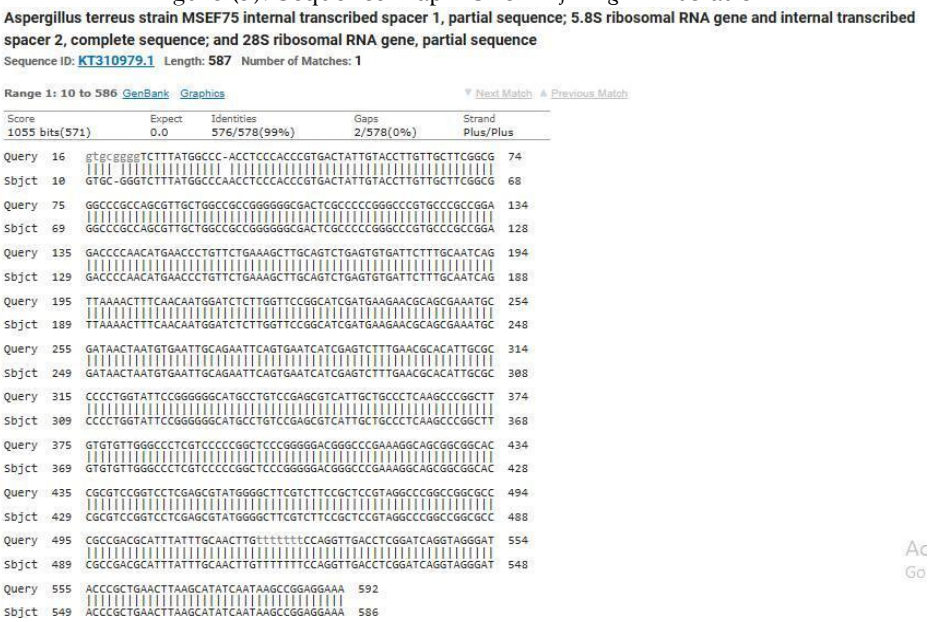

Figure (4): Sequence mapITS for *A.terreus* isolation

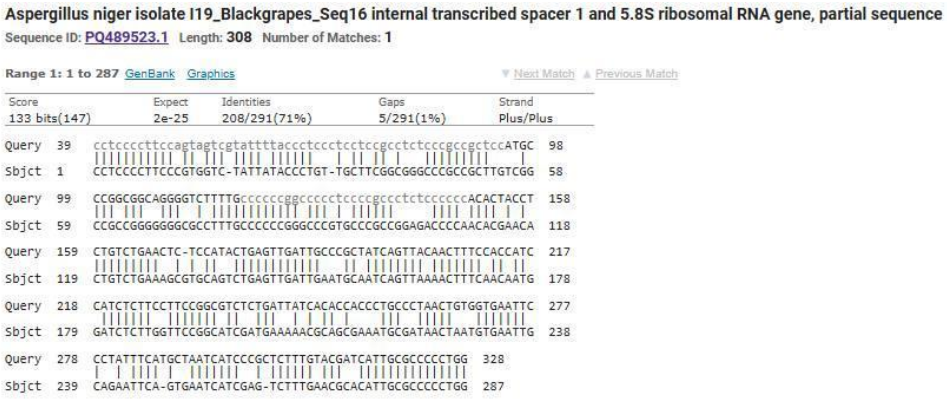

Figure (5): Sequence mapITS for *A.niger* isolation

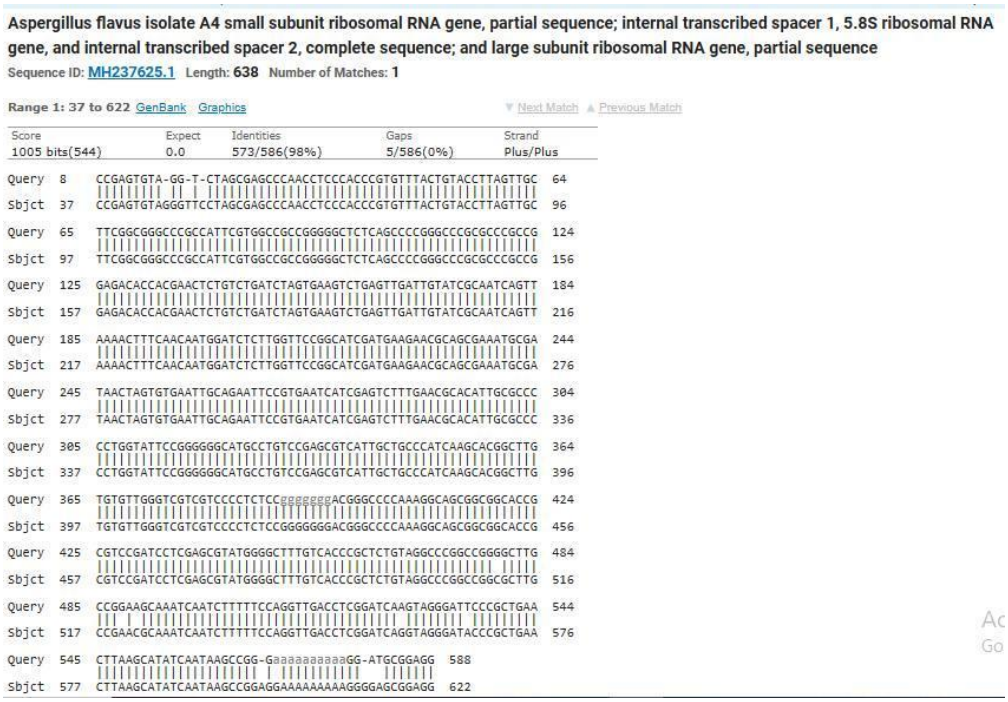

Figure (6): Sequence mapITS for *A.flavus* isolate

3-4: Results of sequence analysis and phylogenetic tree construction

The genetic sequences of the region were analyzed.ITS using the nearest neighbor-joining method to construct a phylogenetic tree. The results showed that the samples were divided into two main groups. The first group included Sample1 (*Aspergillus fumigatus)* and Sample4 (*Aspergillus flavus*) with a convergence rate of 85%, and they shared a common ancestor with Sample5 (*Aspergillus niger*). The second group included Sample2 (*Aspergillus terreus*), in addition to Sample3 (*Aspergillus flavus) and Sample6 (Rhizopus arrhizus*), which are two samples included in the analysis for comparison purposes only and are not part of the main study focus, as shown in Figure (7).

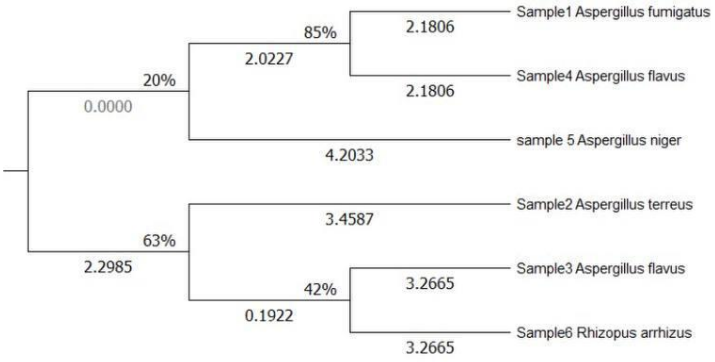

Figure (7): Genetic differences tree of isolates*Aspergillus spp*Local based on region sequence.ITS and compared with other isolates using the Neighbor-Joining method. The tree shows the bootstrap values for genetic associations. Other species: *Rhizopus arrhizus and Aspergillus flavus* were included in Sapmle3, taken from other isolates, for comparison purposes only, and were not analyzed in the current study.

DISCUSSION

1- Physical and microscopic diagnosis

The studied fungal isolates showed a clear diversity in morphological characters such as colony color, texture, and shape of conidiophores and conidia, which are characteristic of the genus.*Aspergillus*,It is considered one of the approved classification characteristics as stated in the guide [14]. The reliance on morphological traits remains important in fungal studies, especially in environments lacking molecular capabilities, as noted by [18].

2- Enlarge the areaITS using PCR technology

Polymerase chain reaction results showed thatPCR) was successful in amplifying the ITS region, with the measured band sizes being approximately 600 base pairs. This is consistent with what [13]. reported using ITS primers to identify fungi. Selecting the ITS region is effective in fungal classification to distinguish closely related species, and the results showed good clarity and purity, indicating the quality of the extracted DNA.

3- Sequencing and analysisBLAST

When analyzing the sequence of a regionITS showed that isolates of *A. flavus, A. niger, A. terreus, and A. fumigatus* showed high similarity to globally recorded isolates, with a matching percentage ranging from 100% to 99.6%, which accurately supports molecular diagnosis, as confirmed by studies such as [4]. However, some minor differences in the base sequence appeared between some local isolates and their counterparts, which may indicate the presence of local genetic mutations.

4- Sequence analysis and phylogenetic tree construction

The phylogenetic tree based on the analysis of the region's sequences reflectsThe ITS gene segment is effective in distinguishing between fungal species, especially those belonging to the genus Aspergillus. Several studies have indicated that the ITS region is one of the most widely used and reliable molecular regions in fungal classification, given its sufficient variability to enable species discrimination [19].

The results of the current tree showed a genetic closeness between the sample.*Aspergillus*fumigatus and the A. flavus specimen, indicating a possible evolutionary similarity, which is in line with what was stated by [20]. about the possibility of some Aspergillus species converging in genetic traits despite the difference in their environmental or physiological manifestations.

As for the two samples*A. flavus*and Rhizopus arrhizus, were included in the analysis for phylogenetic comparison purposes (Outgroup), and their presence helped clarify the limits of genetic differentiation between the studied specimens.
These results are consistent with other studies that have used genetic analysis to elucidate the evolutionary relationships between fungal species, and emphasize the importance of programs such asMEGA X in building accurate trees that reflect the true genetic structure [17].

5- The importance of molecular analysis in diagnosis

The combination of phenotypic and molecular analysis has contributed to enhancing the reliability of the diagnosis, which is confirmed by recent literature that recommends the use of two complementary approaches as in (The use of sequencing and BLAST analysis is also an accurate tool for revealing genetic relationships among fungi, especially when comparing sequences with the GenBank database.

Conclusion
The results of the study indicate that wheat seeds stored in silos in Tikrit city, Salah al-Din Governorate, contain different types of fungi.*Aspergillus,*which were identified using phenotypic and molecular diagnostic techniques. The results confirm that these isolates match globally known species, enhancing the accuracy of molecular diagnosis and confirming that the local isolates belong to the same species studied in previous research. The study also showed the presence of fungal contamination in post-harvest grains, reflecting their sensitivity to surrounding environmental conditions. This study highlights the importance of identifying the health and environmental risks associated with the contamination of stored grains and emphasizes the need to take preventive measures in storage silos to limit the growth of fungi.*Aspergillus,*which may pose a threat to crop quality and safety.

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