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Molecular Diagnosis by Polymerase chain reaction method (PCR) of *Aspergillus* Fungi Isolated from the Air of the area between the two Holy Mosques in Holy Karbala

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Abstract:

The study included molecular diagnosis of some types of *Aspergillus* sp. fungi for specific areas between the two holy mosques of the city of Karbala. With the aim of identifying the types of Aspergillus present in the region and their importance to community health. Samples were taken from the atmospheric air using an air-sampler system. Using the technique of random polymorphic DNA replication (genetic fingerprinting) after the DNA was extracted from each isolate at a final concentration of pb (524) per 2-3 grams of wet mycelium with 100% purity, using primers ITS1 and ITS4, and by electrophoresis through a gel. Agarose with a concentration of 1.5%, dyed with ethidium bromide, and photographed under ultraviolet light. The genetic fingerprint of two isolates isolated from the air was determined by the appearance of DNA duplication bands for each isolate with one or more primers, and they included: Aspergillus quadrilineatus, *Aspergillus terreus*, and they were registered in the bank. The global genome with serial numbers OR492487 and OR497735) respectively, with a similarity rate of 100% with the global fungi.

Key word; Fungi, Molecular diagnosis of fungi, Aspergillus spp.

Introduction:

Fungi are eukaryotic organisms. The number of species documented to date has reached approximately 100,000 fungal species, with an average of 1,200,000 fungal species annually. (Kirk et al., 2008). Many species of fungi pose a major challenge in the contemporary era due to their wide distribution in the natural environment. They are a major factor contributing to bacterial deterioration of food products. (Gizachew et al., 2016) Fungi cause diseases in humans, resulting in more than 1.5-2 million deaths annually, and more than a billion people suffer from acute fungal diseases (Kirk et al., 2008). Previous research indicated that fungi constitute A major threat to both animals and plants, making it one of the most worrisome microorganisms. In fact, it accounts for about 65% of infections when compared to other disease-causing agents. (Fisher et al., 2012) In light of these circumstances, global warming and the climate changes accompanying it have led to an increased incidence of many fungal diseases (Ostrosky-Zeichner et al., 2010). Therefore, there are increasing concerns about the emergence of epidemics, which These are likely to be attributed

to fungal pathogens in the 21st century, which can be attributed to the effects of climate change and weakened immunity.

This, in turn, leads to high levels of resistance and challenges in alleviating health problems caused by the complex relationship between chronic diseases and fungal infections. Hence, the medical importance of fungi is greatly magnified, necessitating a comprehensive approach towards human preparedness in combating fungal infections. Hence, further investigation is needed to explore alternative treatments to synthetic antibiotics. (Albuquerque & Casadevall, 2012). Infections caused by spores or fungal filaments that spread through the atmosphere constitute a global health dilemma for populations around the world. Inhalation of airborne particles laden with fungal spores leads to many afflictions, including bronchopulmonary aspergillosis, sinonasal aspergillosis, and invasive aspergillosis, which represents the most serious and atypical infection affecting the respiratory or other body systems and stands as a primary trigger. to death among individuals with immunodeficiency (Badran et al., 2018). Fungal species present in the air play an important role in respiratory allergies and asthma (Mezzari et al., 2002). Fungi are not limited to allergies, asthma, and respiratory diseases, but rather extend to more than This is like cancer, as well as what is known as Aspergillosis, which is caused by some types of fungi (Aspergillus).

The group of fungi of the genus Aspergillus is considered one of the most widespread species in most environments and is one of the most important producers of aflatoxin toxins (Balina et al., 2018). Epidemiological studies have shown that the incidence of invasive aspergillosis (A. terreus) has increased compared to other species in the genus (Baddley et al. ., 2003). A. terreus infection causes a mortality rate of up to 100% in people who develop invasive aspergillosis. Compared with 20 other Aspergillus species, A. terreus infection is associated with poor prognosis and high mortality. In fact, invasive aspergillosis has been named as the leading cause of death in leukemia and stem cell transplant patients (Birren et al., 2004). Therefore, there are increasing concerns about the possibility of epidemics caused by fungal pathogens in the twenty-first century due to climate change and weakened immune systems. This can lead to increased resistance and challenges in treating health problems resulting from links between chronic diseases and fungal infections.

As a result, the medical importance of fungi is greatly magnified, highlighting the necessity of human preparedness in the fight against fungal infections. This in turn calls for extensive research into exploring alternative solutions to synthetic antibiotics. Many studies conducted in Iraq have proven the presence of fungal contamination in the atmosphere. In particular, the investigation into fungal contamination in the city of Hilla conclusively demonstrated the widespread spread of fungi in the air. And the dominance of the Aspergillus genus (Khairallah & Jawad, 2017). A comparative study was conducted on air fungi in the city of Karbala, which proved the presence and spread of fungi. The study confirmed in its comparison the spread of the Aspergillus genus in the air and its dominance in that region (Muhammad & Muhammad, 2007). It is considered a group of genus *Aspergillus* fungi. *Aspergillus* Given the studies mentioned above and the high temperatures witnessed in the city of Karbala due to the sandy surface soil and the spread of fungi in suspended dust particles, as well as the scarcity of research on airborne fungi in Iraq in general and in Karbala specifically, the study focuses on airborne fungi in specific areas. of the city and its impact on the population. The research was conducted during October 2022 with the aim of mitigating or reducing the spread of pollution through health education, guidance and counselling. The implementation of techniques to sterilize and disinfect the atmosphere was used. The use of traditional methodologies was considered insufficient in most cases due to patterns Asymmetric and polymorphic phenomena, as well as diverse environmental conditions.

Materials and Methods:

1. Prepare the medium (potato-dextrose-acchar (PDA), according to the

manufacturer's instructions (HIMEDIA-India), by dissolving 39 g of the crushed medium in one liter of distilled water to which the antibiotic chloramphenicol has been added at a concentration of 502 mg/l. The medium was sterilized. In an autoclave at a temperature of 121 degrees Celsius and under a pressure of 1.5 atmospheres for 15 minutes, after cooling, pour the medium into plastic Petri dishes.

2. Samples were collected using a steel air-sampler system manufactured by Merck KGaA (KGaA), by placing the dish containing the Potato-Dextrose-Agar (PDA) medium. The lid was removed from the dish, placed inside the nozzle of the device, closed tightly, and turned on. The device is carried out in accordance with the manufacturer's terms and conditions, and the device must be walked at a height of 1.5 meters above ground level for 60 seconds, after which the process of withdrawing microbes from the air is stopped by pressing the stop button and removing the dish from inside the device, and the dishes are covered with their lids that were kept in sterile paper.
3. The dishes were taken to the laboratory for incubation at a temperature of 38°C, and after a week of incubation, the growing fungi were diagnosed according to what was stated in (Ellis et al., 2014). The isolates were purified several times in order to obtain pure fungal isolates.
4. Traditional methods were used for diagnosis, then PCR technology was used, as follows: -

First: DNA extraction method:

The examination was performed according to the manufacturer's instructions (FAVORGEN) to extract the fungal DNA, as follows: 100 µl of fungal culture was taken and 1 ml of FA Buffer was added to the fiber fraction to obtain DNA. TG1 Buffer and TG2 Buffer were added to the samples and mixed, and after some additions and dilutions, DNA was obtained that can be stored or preserved between 4 and -20 °C. Promega's (Green Master MIX) PCR method (KIT) was performed according to the manufacturer's protocol. After DNA isolation, quantification was performed using Macrogen (Korean ITS 4 and ITS1) specific primers were used and PCR analysis was processed. Specific primers (nucleotide primers, Prime) were used to identify

Forward primer TCCGTAGGTGAACCTGCGG.

Reverse primer: TCCTCCGCTTATTGATATGC

Second: Analysis of PCR test results:

The PCR test was performed according to Promega's (KIT) method, where Green Master MIX and two types of special primers (ITS 4 and Microgen (ITS1) were used for all fungi as shown in (Table 1). Agarose gel electrophoresis was performed using 1.5% of agarose gel To read the result of the polymerase reaction, the sequence analysis of the PCR product is as follows: (Mishra et al., 2010) 1.5-2 µg of agarose gel was dissolved in 100 ml of TBE buffer solution at a concentration of 1 Microwave, then leave the gel to cool at a temperature of 50 °C, then 3 µl of ethidium bromide DNA dye was added and mixed well with the gel, after which the agarose gel was poured into a rectangular basin containing a comb to determine the PCR samples, then the gel was allowed to solidify at room temperature. 15 min, then the comb was carefully removed from the gel and transferred to the electrophoresis bath.

Samples for the PCR product were loaded and placed in the holes of the gel, a DNA 100 ladder was used to measure the PCR product and placed in the first hole, and then DNA samples were placed in the remaining holes. The agarose gel was immersed in buffer solution in the concentrate and then the relay was operated at 80 V, 58 A, for 75 min. After migration, the gel containing the PCR product was scanned using UV gel documentation to determine the product per unit of measurement (Sambrook & Russell, 2006).

Table 1. The specific primers ITS 1 and ITS4 MICROGEN KOREA were used.

Primers	Sequence		Amplicon
ITS 1	F	TCCGTAGGTGAACCTGCGG	524bp
ITS 4	R	TCCTCCGCTTATTGATATGC	

After that, the PCR results were sent to Macrogen Company in South Korea for the purpose of determining the sequence of the nitrogenous bases for each fungal sample. The data received from the company was evaluated and analyzed using the Chromas program, and for the purpose of determining the similarity between the studied mushrooms and the fungi recorded internationally, the Basic Local Alignment Search program was used. Tool (BLAST) of the National Center for Biotechnology Information website. Search results: National Center for Biotechnology Information.

Results and Discussion:

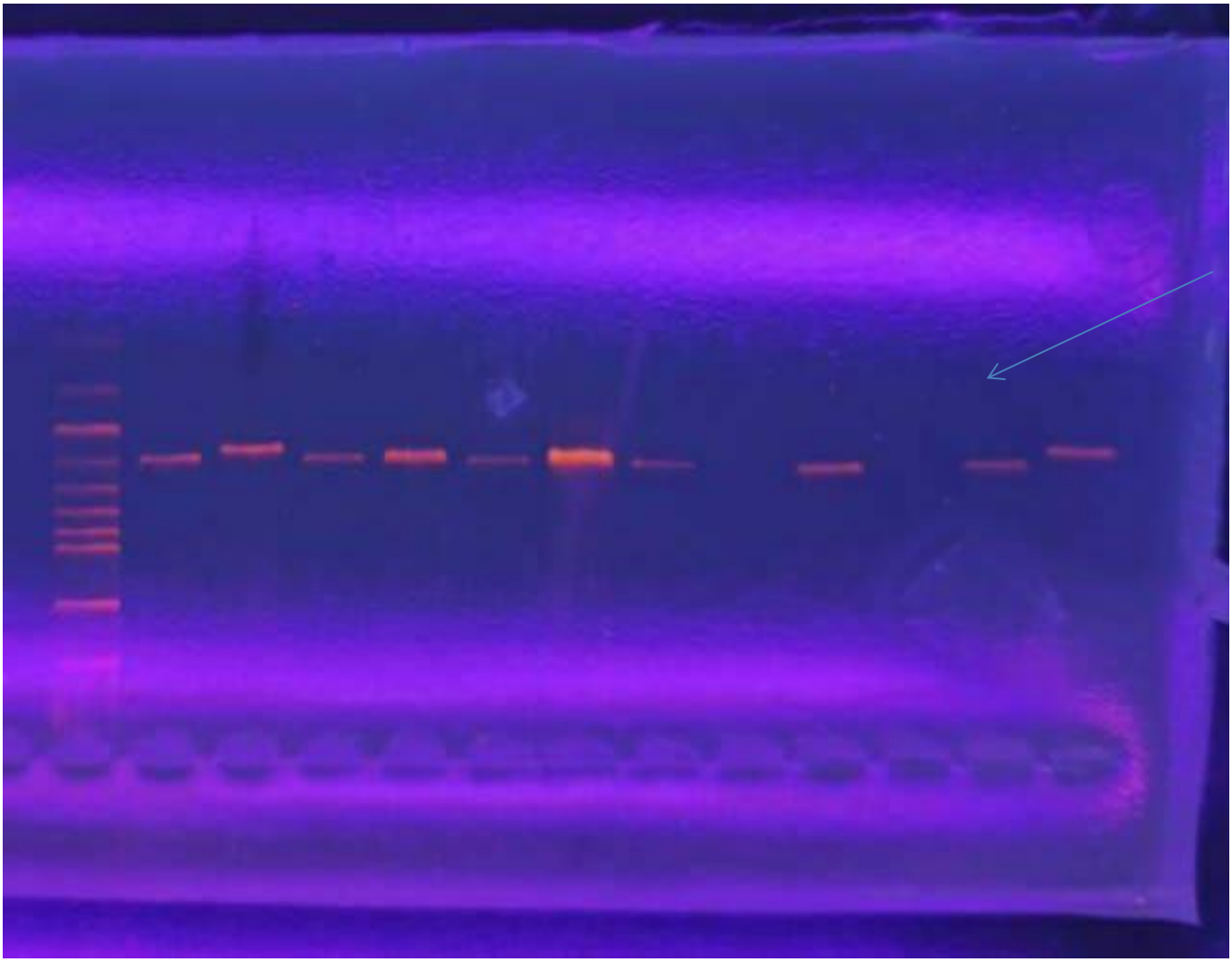

Figure (1). Demonstrates the process of electrophoresis (EIectrophoresis) on a 1.5% agarose gel at a voltage of 100 for an hour for isolates of the genus Aspergillus with a PCR product of size pb524.

Detection using (P.C.R) technology

A- Analysis of the sequences of the amplification products

The amplified gene products were sent to the Korean company Macrogen to determine the sequences of the nitrogenous bases, and these sequences were approved by comparing them with the information available about this gene on the website of the National Center for Biotechnology Information (NCBI) through the website (https://www (ncbi.nlm.nih.gov/nuccore)

According to the BLAST Nucleotide program, in order to identify the type of the selected isolate, the base sequences were recorded at NCBI by filling out the form for registering the ITS18 gene to obtain a special accession number for the local isolate. The phylogenetic tree was also drawn. tree of the local isolate after matching it to closely related strains in NCBI and based on the Blast Tree View program.

The results of the isolates showed a 100% match with global isolates, as shown in Table No. (3). It was considered the first registration of isolates in the GenBank and was given special numbers, as shown in Table No. (2).

Table No. (2) shows the numbers registered in the GenBank for genetically diagnosed fungal isolates.

Fungi type	Isolate number in GenBank
Aspergillus qyadrilineatus	OR492487
Aspergillus terreus	OR497735

The interstitial region ITS1 and ITS4 in the ribosomal gene S18 is considered stable and is used successfully in distinguishing different types of fungi and gives decisive results in diagnosis (Holland, 2010).

B- Determine the sequence of nitrogenous bases and analyze bioinformatics and the genetic tree

The results of the analysis of the nucleotide sequence of the doubled DNA bands were shown using the NCBI program and comparing them with the data available at the National Center for Biotechnology Information (NCBI). The four isolates sent each belong to...

The isolate bearing the number OR492487 and registered in the GenBank belongs to the fungus Aspergillus quadrilineatus, where the match rate with global isolates was 100%, as shown in Figure No. (1-1).

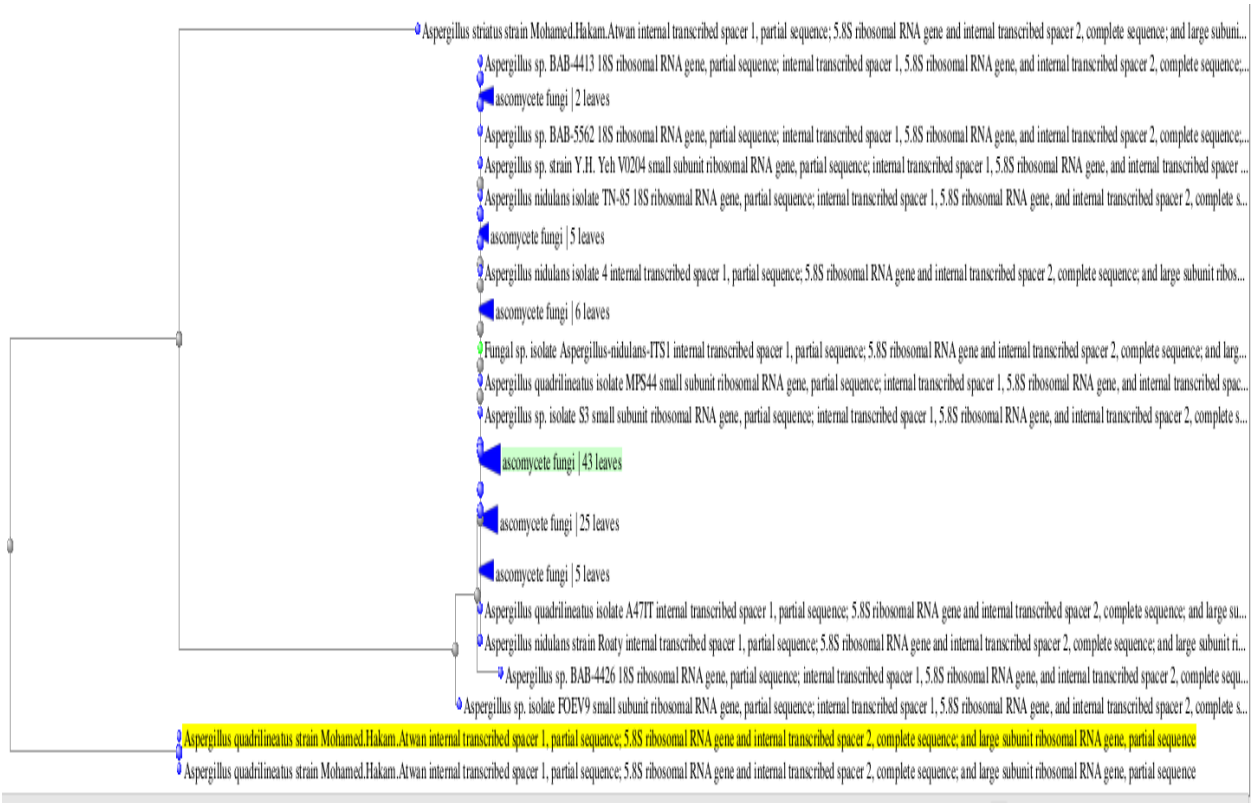

Figure (1-1) shows the conformity of the Aspergillus quadrilineatus isolate with global isolates in GenBank

Figure (2-1): The genetic tree of the fungus Aspergillus quadrilineatus (marked in yellow), which was created based on the sequences of its nitrogenous bases for the ITS-rDNA region, in addition to the sequences of known international strains of the same fungus obtained from the GenBank data repository. Genetic distances were calculated using the neighbor-joining method.

The isolate bearing the number OR497735 and registered in the GenBank belongs to the fungus Aspergillus terreus, and the match rate with global isolates was 100%, as shown in Figure No. (1-3).

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Aspergillus terreus strain Mohamed.Hakam.Atwan internal transcribed spacer 1, partial sequence; 5.8S ribosoma...	Aspergillus terreus	1088	1088	100%	0.0	100.00%	589	OR497735.1
Aspergillus terreus strain OR28F17 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene an...	Aspergillus terreus	863	863	99%	0.0	93.87%	584	MT510016.1
Aspergillus terreus YLA12 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence	Aspergillus terreus	861	861	99%	0.0	93.73%	950	LC496504.1
Aspergillus terreus isolate AS1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and int...	Aspergillus terreus	857	857	96%	0.0	94.54%	640	MW594168.1
Aspergillus terreus isolate CUZF40HIK internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene...	Aspergillus terreus	857	857	98%	0.0	93.72%	593	OM980659.1
Aspergillus terreus isolate T46 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and int...	Aspergillus terreus	856	856	98%	0.0	93.69%	593	MN180819.1
Aspergillus terreus strain ZYL050009 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...	Aspergillus terreus	856	856	98%	0.0	93.69%	583	MF374803.1
Aspergillus sp. strain KG_20 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter...	Aspergillus sp.	856	856	98%	0.0	93.69%	576	MG647852.1
Fungal sp. isolate F32M4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal ...	fungal sp.	856	856	98%	0.0	93.97%	602	OQ835489.1
Aspergillus terreus isolate T50 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and int...	Aspergillus terreus	854	854	98%	0.0	93.68%	584	MN180820.1
Aspergillus terreus strain GKF27 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Aspergillus terreus	854	854	97%	0.0	93.96%	561	MK713430.1
Aspergillus terreus strain CA-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and int...	Aspergillus terreus	854	854	96%	0.0	94.38%	572	MH918670.1
Aspergillus terreus strain NPF3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and int...	Aspergillus terreus	854	854	98%	0.0	93.69%	569	MH421849.1
Aspergillus terreus isolate Asp-7861 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a...	Aspergillus terreus	854	854	97%	0.0	93.96%	649	MF152908.1
Aspergillus sp. PE-2014 strain GM3921 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gen...	Aspergillus citrin...	854	854	96%	0.0	94.38%	578	KP175278.1
Aspergillus sp. PE-2014 strain GM2137 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gen...	Aspergillus citrin...	854	854	96%	0.0	94.38%	581	KP175275.1
Aspergillus sp. PE-2014 strain GM2025 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gen...	Aspergillus citrin...	854	854	96%	0.0	94.38%	577	KP175274.1
Aspergillus sp. PE-2014 strain GM676 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Aspergillus citrin...	854	854	96%	0.0	94.38%	578	KP175264.1
Aspergillus sp. PE-2014 strain GM673 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Aspergillus citrin...	854	854	96%	0.0	94.38%	583	KP175263.1
Aspergillus sp. PE-2014 strain GM541 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Aspergillus citrin...	854	854	96%	0.0	94.38%	584	KP175262.1

Figure (1-3) shows the conformity of the Aspergillus terreus isolate with global isolates in GenBank.

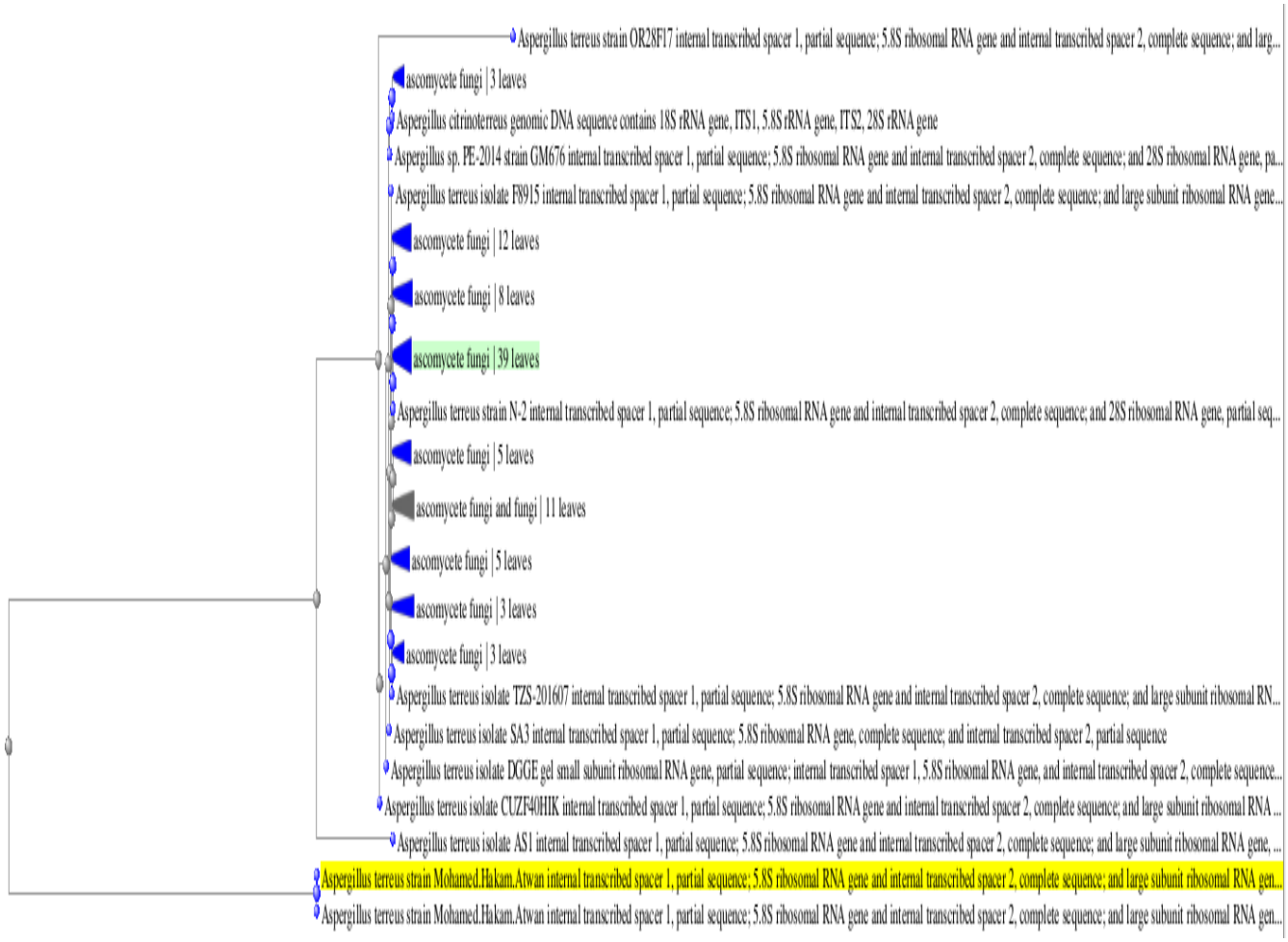

Figure (1-4): The genetic tree of the fungus Aspergillus terreus (marked in yellow), which was created based on the sequences of its nitrogenous bases for the ITS-rDNA region, in addition to the sequences of known global strains of the same fungus obtained from the GenBank data repository. Genetic distances were calculated using the neighbor-joining method.

Table (3). shows the percentage of genetic identity between the local isolates under study and the global isolates registered in the fungal gene bank.

Species **Local aerobic fungi**	**number** **Join the gene bank**	**Sequences of homologous species in NCB_BLAST**		
		Types of global aerobic fungi	GenBank accession number	Percentage match
Aspergllus quadrilieatus	OR492487	*Aspergllus quadrilieatus*	Mk108389	**%100**
Aspergllus terreus	OR497735	*Aspergllus terreus*	MT510016	**%100**

The results of the analysis showed a clear convergence between the original fungal species and the remaining cosmopolitan species, as demonstrated by the genetic tree analysis.

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