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Molecular Identification of Ascomycota Fungi Using Its Region as DNA Barcodes

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Abstract

We studied the material collected from agricultural land. Four Ascomycota fungi were isolated from the soil sample. The preliminary identification based on morphological characters using taxonomic keys was done only up to the genus level, due to overlapping characters, making identification difficult. Therefore the Internal transcribed spacer (ITS) region also called as fungal DNA barcode was sequenced. The obtained results were compared with GenBank, the NCBI database, and identification was made up to species level based on the similarity search result. The phylogenetic relationship among the species and between species was studied using a neighbor-joining tree. The genetic distance between the study fungi was computed through the maximum composite likelihood model. These sequences were deposited in GenBank, NCBI, and accession numbers were allotted by GenBank to the submitted sequences. Based on the morphological characters and the DNA sequence analysis results, the isolated fungus was identified as *Aspergillus terrus and Aspergillus flavus*.

Keywords: Fungi, Ascomycota, Internal transcribed spacer (ITS), DNA barcode, Aspergillus *terrus, Aspergillus flavus*

Introduction

Soil is an important region in the earth's crust, which gives shelter to many organisms from microbes to plants and animals (Anisimova *et al*., 2019). Diseases that arise from soil-borne pathogens are a major problem in the reduction of cucumber yield (Wu *et al*., 2006; Shahreza *et al*., 2019; Aloufi *et al*., 2022). Fungi are an exceptionally flexible class of organisms constituted mainly of saprophytes, which flourish on the dead biological matter. The identification of fungi depends mainly on their external and biological characteristics (Mathew & Victório, 2020). Conversely, the distinctive characteristics of fungi make snags in identification and cataloging based on morphology. Accordingly, lone well-trained specialists can properly identify fungi species based on their morphology (Samson *et al.*, 2010).

Currently, numerous molecular methods have been developed over the years which include techniques of modern technologies like fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), terminal length restriction fragment polymorphism (T-RFLP), DNA array hybridization, and DNA sequencing, pulsed-field gel electrophoresis (PFGE), etc. The method based on DNA sequencing is the most widely used one (Ali *et al.*, 2016). Many species of animal have been barcoded using molecular markers (Shabeer Ahmed & Jaffar Ali, 2015). Many species of organisms found in shallow water to the deep sea (Shabeer Ahmed & Jaffar Ali, 2016) have been successfully barcoded. Insilico analyses of barcoded marine organisms (Shabeer Ahmed & Jaffar Ali, 2019) have been made in recent years. Identification based on DNA sequencing in the mitochondrial genome has some limitations, due to the presence of pseudogenes (Shabeer Ahmed & Jaffar Ali, 2016). Internal Transcribed Spacer (ITS) region of nuclear DNA (rDNA) is the most often sequenced region to identify fungal taxonomies at the species level and even within species (Watanbe *et al.*, 2011). This novel concept for the swift and exact documentation of an unknown fungal specimen using ITS as the molecular marker is referred to as DNA barcodes for the fungal kingdom (Geiser *et al.*, 2004). Hence in the present study, two fungi isolated from the soil were identified by sequencing the ITS region (Albureikan, 2020; Triantafyllopoulos *et al*., 2022).

Materials and Methods

Collection of Soil Sample

A pre-sterilized beaker was carried to the site of collection and with the help of a pre-sterilized spatula sample was collected. Serial dilutions of up to 10^{-10} dilutions were made in 10 ml of Ringer's solution.

Fungi Culture

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From the serially diluted sample, 0.1 ml was spread on potato dextrose agar (PDA) media and was allowed to grow at 28°C for 7 days in the dark.

Observation of Morphological Characters

7 days of grown fungus was observed under the microscope, Leica DM14000B (Germany) fitted with a camera.

DNA Extraction

7 days of grown fungus was subjected to DNA extraction using Hi PurA™ (Himedia) kit following the protocol given in the manual. The concentration and purity of the extracted DNA were checked using a Nanodrop Lite UV-Visible spectrophotometer.

DNA Amplification, Sequencing, and Phylogenetic Study

ITS region of the ribosomal RNA was amplified following the method of White *et al*, 1990. Sequencing of the amplicons was performed in a genetic analyzer at the sequencing center. Sequence alignment and the phylogenetic study were computed using MEGA 5 Software. All the sequences were deposited in the GenBank, NCBI, to which accession numbers were provided by GenBank.

Results and Discussion

Morphological Identification

Aspergillus terrus

The morphological characters expressed were distinct, with its exterior appearance shady to coffee-colored colonies, pear-shaped vesicles, and conidial heads in biserial arrangement, followed by columnar conidial arrangement. Good sporulation was observed on PDA. Following the Keys of Klich (2002) the microscopic and macroscopic characteristics were observed and studied which were in accordance with the descriptions of *A. terreus.*

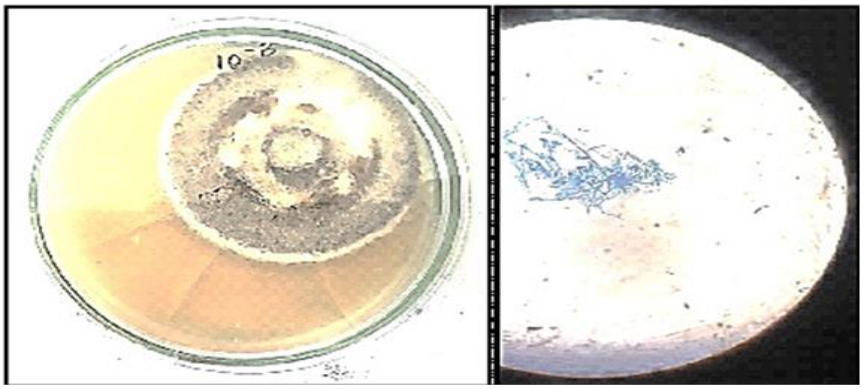


Figure 1. Micromorphological characters of *Aspergillus terrus*

Aspergillus flavus

It has the appearance of furry, mold exhibiting yellow to green or brown color mold with sandy fawn or colorless reverse. The matured colonies appeared thick green. The form had some radial wrinkles Conidiophores were dense-walled, toughened, colorless, generally less than 1 mm in length, located underneath the globose vesicles. Vesicles at a young are extended and later become globose, varying in length from 10 to 65 mm in diameter. The length of the primary branch is up to 10 mm, and the secondary up to 5 mm (Ruiqian *et al*., 2004; Hedayati *et al*., 2007).



Figure 2. Micromorphological characters of *Aspergillus flavus*

Molecular Identification

Table 1. Homology result

Study Sequence	Homologous Sequence	E- Value	Query Coverage	% identity	Accession Number
ICBTS1	*Aspergillus terrus*	0.0	100%	100%	MT558939
ICBTSS4	*Aspergillus terrus*	0.0	100%	100%	MT558939
ICBTSS3	*Aspergillus terrus*	0.0	100%	100%	MT558939
ICBTSS5	*Aspergillus flavus*	0.0	100%	100%	MT541875

Table 2. Genetic Distance result

Fungal sequences	Positions			
	1	**2**	**3**	**4**
Aspergillus terrus ICBTSS4				
Aspergillus terrus ICBTS1	0.007			
Aspergillus terrus ICBTSS3	0.007	0.00		
Aspergillus flavus ICBTSS5	0.118	0.128	0.128	

Mean Value = 0.065

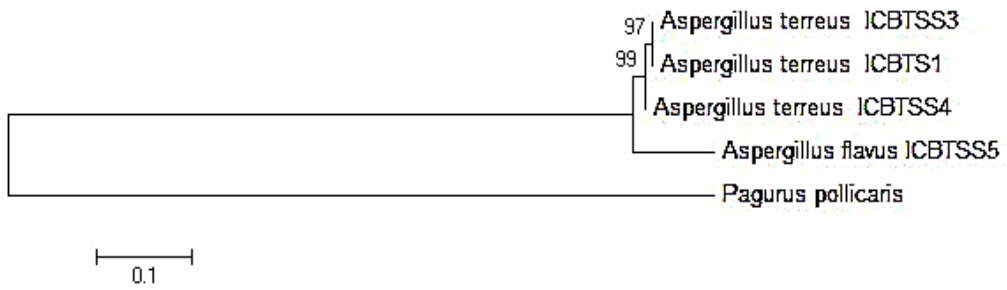


Figure 3. Phylogenetic relationship between study fungi

Four fungal strains were isolated from the soil sample collected from the agricultural land. The micromorphological characters were studied **(Figures 1 and 2)** and compared with the standard fungal taxonomical keys for identification. Morphological identification of *A. terreus* strains was based on colony appearance and color, like coffee with an arrangement of biseriate conidial heads in a columnar shape. *A. terreus* though was isolated from different substrates but its identification was based on its microscopic and macroscopic characteristics (Afzal *et al*., 2013) which produced alike morphological characteristics. Identification of *A. terreus* samples through molecular markers substantiated with identification of other *A. terreus* species using morphology thereby exhibiting 100% similarity with *A. terreus* **(Table 1)**. The genetic distance between the three *A. terreus* species was found to be 0.7% **(Table 2)**. The phylogenetic study revealed all the study fungi samples were clustered in one clade **(Figure 3)**. Furthermore, the efficiency of molecular markers can be proven with the phylogenetic studies of other researchers (Varga *et al.*, 2007; Samson *et al.*, 2011) where their findings presented *A. terreus* as a distinct clade, separated from other Aspergillus species.

The fungus labeled as ICBTS1 was identified to be *Aspergillus terrus*, after a combined analysis of morphological characters and ITS sequence similarity results. Sample ICBTSS3 and ICBTSS4 displayed differing characters in the external (phenotypical) appearance, but on morphological examination and ITS sequencing, it matched *Aspergillus terrus*, confirming its exact identity. All three sequences of *A. terreus* were deposited in GenBank, NCBI under the accession numbers, MW940968, MW945404, and MZ160915.

Descriptive taxonomic keys were used as the preliminary fungal identification criteria for the selection of presumptive *Aspergillus flavus*. Preliminary findings showed colony growth starting as a white mycelium that developed radially to cover the entire surface of the media. As sporulation ascended, a yellowish-green or dark green color of the conidia transacted the white colony color from the midpoint outwards, finally covering the entire surface. This observation was constant with other findings where a white color of the mycelia produced an olive or dark green conidia delimited with a white ring. Colonies had soft (downy) to crocheted (wool-like) textures regularly with a floccose center and cream color on

the reverse. Similar characteristics were described by scientists like Bastianelli and Le Bas and Odhiambo *et al*. Some species with wavering morphological characters were easily identified with the sequencing of the ITS region (Nariyampet *et al.*, 2022). The isolated fungus was confirmed based on the presence of conidiophores, a vital feature of the *Aspergillus* spp that belonged to the *Aspergillus* genera. On similarity searches it exhibited a 100% match with the *Aspergillus flavus* in Genbank **(Table 1**). The mean genetic distance value between *A. flavus* and the three species of *A. terreus* was found to be 12.4% **(Table 2)**, which is in accordance with the phylogenetic result, where *A. flavus* forms a separate branch **(Figure 3)**. *A. flavus* morphological identification was in concord with ITS sequence and phylogenetic analysis. This sequence was deposited in the GenBank, NCBI under the accession number MW940969.

Conclusion

Several molecular methods have been developed in recent times for the identification of microbes including fungi. The most effective method proven was DNA barcoding due to its accuracy and swiftness. In the case of fungi, the nuclear ribosomal Internal transcribed (ITS) region has been the most widely sequenced to identify fungi at the species level. Hence in the current study, the ITS region proved as an effective molecular marker in the identification of the soil – isolated fungi.

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