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# ISOLATION OF AN ASCOMYCOTA FUNGUS FROM SOIL AND ITS IDENTIFICATION USING DNA BARCODE

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## ABSTRACT

In this study Ascomycota fungi was isolated from the soil collected from cucumber field. The micromorphological characters of the isolated fungus were muddling with overlapping characters and identification was unwhole. Hence, nuclear ribosomal internal transcribed spacer (ITS) region, the ideal molecular marker, accepted as DNA barcode for fungal identification was sequenced and compared with the existing GenBank, NCBI, database to identify the species. This sequence was deposited in GenBank, NCBI under the accession number MW945403. Based on the homology and phylogenetic analysis, the isolated fungus was identified as *Acrothecium nigrum* which is the first report of its presence in the cucumber field.

**Keywords:** Internal transcribed spacer (ITS) region, DNA barcode, Ascomycota fungi, *Acrothecium nigrum*.

## 1. INTRODUCTION

Fungi are one of the most important eukaryotic genera, with species numbers comparable to animals but exceeding those of plants [1] and ranging from 1.5 to 5.1 million species [1-3]. Fungi are highly successful soil dwellers because of their high plasticity and their ability to assume different forms in response to adverse or unfavorable conditions [4]. Due to their ability to produce a variety of extracellular enzymes, they are able to degrade all types of organic matters, decompose soil components, and thereby regulate the balance of carbon and nutrients [5]. The diversity and activity of fungi is controlled by various biotic (plants and other organisms) and abiotic (pH, moisture, salinity, structure, and temperature of the soil) factors [6, 7]. Fungi can be found in almost any environment and can live in a wide range of pH and temperature [8]. Fungal populations are strongly influenced by the diversity and composition of the plant community and, in turn, influence plant growth through mutualism, pathogenicity, and their effects on nutrient availability and cycling [9-11]. Phytopathogenic species can infect wild grasses and staple crops such as rice, maize, wheat and sorghum, leading to significant losses in agricultural production [12-14].

Identification of fungi is primarily based on their phenotypic and morphological characteristics. However, the unique characteristics of fungi lead to difficulties in morphology-based identification and classification. Therefore, only well-trained experts are able to correctly identify fungal species based on fungal morphology alone [15]. In addition, a well-trained technician may also be able to identify specimens using step-by-step instructions from the morphological "key" book. However, in many cases, experienced and professional taxonomists are needed. Therefore, accurate and rapid identification of fungi (especially marine fungi) is critical. Nowadays, many molecular methods have been developed to identify fungal species, including DNA barcoding, a short, highly variable and standardized DNA region about 700 nucleotides long that is used as a unique pattern to identify living organisms [16]. The concept of DNA barcoding was first proposed by Hebert, Ball, and Jeremy [17]. In 2003, Hebert et al. proposed a 648 base pair (bp) mitochondrial gene cytochrome c oxidase I (COI) as a standard barcode for animals. Since then, the use of COI for animal species identification has proven to be very effective. Until recently, only the Internal Transcribed Spacer (ITS) region of nuclear DNA (rDNA) was the

most frequently sequenced region for identification of fungal taxonomies at the species level and even within species [18]. The ITS region exhibits a higher degree of variation than the other regions of rDNA (SSU and LSU). Therefore, the ITS region has recently been referred to as the DNA barcode for the fungal kingdom [19]. Although in some cases, such as soil-dwelling Fusarium, sequencing of additional genes, such as the b-tubulin gene (b-Tub) and the aminoadipate reductase gene (LYS2), has been proposed to obtain correct taxonomic identification at the species level [20], while Translation Elongation Factor is also suggested [21]. Therefore, in the present study, the ITS region was sequenced to identify the fungus isolated from agricultural soils.

## 2. MATERIAL AND METHODS

### 2.1. Soil Collection and serial dilution

Soil samples were collected from the agricultural field of Tirupatthur district in Tamil Nadu. Soil was collected in a pre-sterilized beaker using a pre sterilized spatula and transferred to laboratory. 1 gm of the soil sample was taken in 10 ml of Ringer's solution and was serially diluted to $10^{-10}$ dilutions.

### 2.2. Fungal Culture

A 0.1 ml of the serially diluted sample was spread on petridishes containing potato dextrose agar (PDA) under aseptic condition at 28°C and allowed to grow for 7 days at 28°C in dark.

### 2.3. Morphological Examinations

Colony morphology of the 7 days grown fungus on PDA was examined with a Leica DM14000B (Germany) microscope equipped with a camera.

### 2.4. DNA Extraction

Fungal strain grown for 7 days in potato dextrose agar (PDA) at 28°C in dark was used for DNA extraction. DNA extraction was performed using Hi PurA™ Fungal DNA Mini Kit (Himedia) according to the manufacturer's protocol. The concentration and purity of extracted DNA was determined using Nanodrop Lite UV-Vis Spectrophotometer.

### 2.5. Amplification, Sequencing and phylogenetic Analysis

Amplification of ITS region of the ribosomal RNA gene cluster was performed using the method of White et al, 1990 [22]. Sequencing of the amplicons was performed in a 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, USA) in the sequencing Centre. The obtained sequence was aligned using Bioedit tool [23] and phylogentic analysis was performed using MEGA 5 Software [24]. The good quality sequence was deposited in the GenBank, NCBI under the accession number given in the result section.

## 3. RESULTS AND DISCUSSION

The fungi isolated from the agricultural soil exhibited different micromorphological characters, when examined under microscope. The colony appeared similar to Curvularia Americana sp, greyish green in colour [25]. The micromorphological characters were difficult to examine due to overlapping characters.

For molecular identification a 531 bps of the ITS region was sequenced and deposited in the GenBank, NCBI under the accession number MW945403. On performing BLAST, it exhibited 99-100% similarity with different fungal strains (Table 1). All these similar sequences were amplified in the ITS region. On computing genetic distance, the overall mean value between these sequences was 0.003 (Table 2). For the phylogenetic tree analysis of the ITS region K2+G+I model in MEGA5 was followed. The study sequence formed a cluster with *Athroceium nigrum, Curvularia verruculosa, C. lunata* and C. *Americana* species (Fig. 1). Both the analyses proved the BLAST result. This ambiguity in identification through ITS as molecular marker may be due to factors such as PCR bias [26], less accuracy of identification in case of invasive fungal infections [27]. According to Bellemian et al, 2010 [27], primers ITS1-F, ITS1 and ITS5 are biased towards amplification of Basidomycetes, while primers ITS2, ITS3 and ITS4 are biased towards Ascomycetes. Here in the present study, the primers used were even- handed, since both ITS1 and ITS4 were used for amplification. Further, this bias can be ruled out due to the homology results obtained, where all the most similar matches were with only Dikarya: Ascomycota. However, according Mayer et al 2010, the ITS region allowed only 75% of all fungi to be correctly identified.

In such scenario, where the identification of the study species totally relies on molecular markers in the absence of morphological identifications, it is suggested to combine the primary barcode (ITS) with the secondary barcodes such as Translational Elongation Factor 1 α (TEF1α), Glyceraldehyde-3-phosphate dehydrogenase (gdp) gene, fragments of ribosomal large subunit RNA (LSU) and the RNA polymerase II subunit rpb2, to ensure accurate identification of all taxa analyzed.

**Table 1: Homology searches result**

| Most similar Sequence | % identity | Accession Number |
|---|---|---|
| *Acrothecium nigrum* strain CBS 105.28small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1,5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene,partial sequence | 100.00% | MH854940.1 |
| *Curvularia verruculosa* isolate RH0019small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer2, partial sequence | 100.00% | MN068858.1 |
| *Curvularia lunata* isolate CC08-45-1_ModV8 18S ribosomal RNA gene,partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 100.00% | JX960590.1 |
| *Curvularia lunata* strain 25C internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 100.00% | KU715134.1 |
| *Curvularia americana* genomic DNA containing 18S rRNA gene, ITS1, 5.8SrRNA gene, ITS2 and 28S rRNA gene, strain UTHSC 10-1276 | 100.00% | HG779020.1 |

**Table 2: Genetic Distance**

| Fungal sequences | Positions | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| MH854940.1 Acrothecium nigrum strain CBS | | | | | | |
| MN068858.1 Curvularia verruculosa isolate RH0019 | 0.00 | | | | | |
| JX960590.1 Curvularia lunata isolate CC08-45-1_ModV8 | 0.00 | 0.00 | | | | |
| MH037565.1 Curvularia americana strain S5S263 | 0.00 | 0.00 | 0.00 | | | |
| MW081342.1 Curvularia lunata strain 4-F22 | 0.008 | 0.008 | 0.008 | 0.008 | | |
| Study Sequence ICBTS2 | 0.00 | 0.00 | 0.00 | 0.00 | 0.008 | |

*Overall Mean distance 0.003*

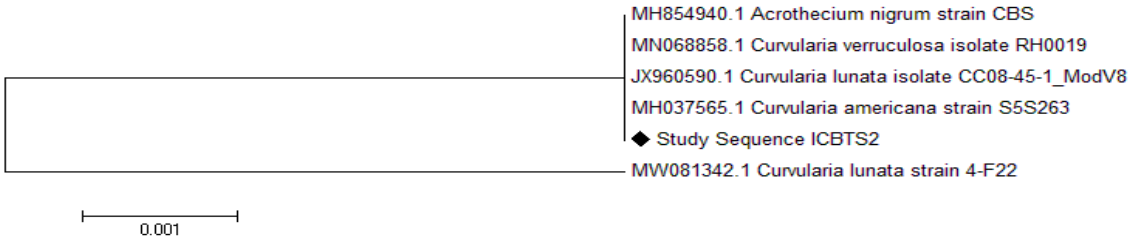


**Fig. 1: Maximum likelihood phylogeny of Ascomycota fungi, *Acrothecium nigrum***

## 4. CONCLUSION

It is concluded that in the present study, identification is done tentatively as *Acrothecium nigrum* based on homology results highest match. This species can be further confirmed by amplifying it with secondary barcodes discussed above to prove the species identity. As of present, this is the first report of *Acrothecium nigrum* from agricultural soil identified through ITS, a universal DNA barcode marker for fungi.

### *Conflict of interest*

None declared

### *Source of funding*

None declared

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