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**Research article**

# Morphology, molecular identification and phylogenetic analysis based on internal transcribed spacer (ITS) of the ribosomal nuclear DNA (rDNA) sequence of a pathogenic fungal isolate *Aspergillus niger* LKO1

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**Abstract:** *Aspergillus niger* is the causal organism of "black mold" disease of fruits and vegetables. The objective of the present investigation was to characterize the pathogen morphologically *in vitro* and to confirm its molecular identity and phylogenetic position by the sequence of ITS region of rDNA. *A. niger* LKO1 was isolated from soil and cultured in PDB and PDA media. The characteristic of growth was monitored and the reproductive structures were analyzed. After 3 days of growth, the diameter of each colony was 1.5 cm, after which the colony was extended its diameter 1 cm per day and subsequently black conidiophores were formed with black pigmented spore heads carrying numerous conidia. Fungus was also characterized using molecular methods based on ITS-PCR (GenBank accession number MK696283). The amplified sequence was compared with the available sequences in the NCBI GenBank. The sequence showed 100% similarity with other isolates of *A. niger*. The isolation, morphological characterization and sequencing of ITS region of rDNA will help in further research on the management of this plant pathogen and its commercial utilization.

**Keywords:** *Aspergillus niger* - ITS sequence - Morphological characteristics - Plant pathogen.

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## INTRODUCTION

*Aspergillus* is the most abundant pathogenic fungus worldwide, which comprises of more than 837 species (Hawksworth 2011). They frequently cause the invasive allergic infections in humans such as bronchopulmonary diseases, mycotic keratitis, otomycosis, nasal sinusitis etc. Pitt & Hocking (1997) stated that *Aspergillus niger* van Tieghem is the most common species of *Aspergillus*. It was first described in 1867 in a manuscript entitled "Physiologie des mucédinées" by the French botanist Philippe (Dijksterhuis & Wösten 2013). He isolated this fungus from molded galls with the main aim to study the production of gallic acid by a process of fungal fermentation. It is a cosmopolitan fungus not very selective with respect to the environmental conditions. It thrives in the soil and on decaying plant material but is also abundant in man-made environments. It can be found on the floor, in carpet and mattress dust (Flannigan *et al.* 2011). It grows between temperature of 6 and 47ºC, pH 1.5 and 9.8 (Pitt & Hocking 2009).

Initially it has been reported as a pathogen of *Zingiber officinale* Roscoe plants (Pawar *et al.* 2008). Generally, it causes "black mold" disease in certain fruits, vegetables and food products such as onions, rice, coffee, nuts etc. It is responsible for post-harvest decay of guava, litchis, mangoes, papaya, pineapples, pomegranates, apples, pears, and grapes. During colonization, it may produce the mycotoxins ochratoxin A and fumonisins. Beside this *Aspergillus niger* has several beneficial applications. It is used for waste management and biotransformations. It is also used as a cell factory for the production of enzymes. These enzymes are used in a wide variety of applications ranging from clarification of fruit juices, lipid hydrolysis during cheese


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production, and degradation of phytate in animal feeds (Wösten *et al.* 2007). It is also widely used for the production of the food additives citric acid and gluconic acid. It produces large amounts of citric acid in a medium containing sugar (Currie 1917). It is the major source of citric acid; this organism accounts for over 99% of global citric acid production, or more than 1.4 million tonnes per year (Papagianni & Mattey 2006).

Proper identification of pathogenic fungus is very important to treat the diseased plants (Henry *et al.* 2000). Generally identification of the *Aspergillus* species is carried out by microscopic examination of the morphological characteristics of the colony. However, taxonomists faced the problems due to different hyphal thickness, similar sexual structures and sometimes lack of sexual structures (Henry *et al.* 2000). Although, molecular methods are more accurate quick and easy and thus became essential tools for identification of fungus (Henry *et al.* 2000, Shittu *et al.* 2016).

In the present study, we have isolated the fungal pathogen *Aspergillus niger*. We have identified the pathogen morphologically based on its vegetative and reproductive structures. We have also obtained the sequence of the internal transcribed spacer (ITS) of the ribosomal nuclear DNA (rDNA) with the PCR method using universal primers (Chen *et al.* 1992) for the molecular identification and phylogenetic analysis and sequence has been deposited in GenBank (NCBI).

## MATERIALS AND METHODS

*Collection and isolation of the fungus*

The sample was collected from soil samples in sterile capped bottles from Lucknow (26° 5′ N latitude, 80° 56′ E longitude), the capital of Uttar Pradesh. The collected sample was purified in sterile distilled water and maintained on PDB (potato dextrose broth) and solid media like PDA (potato dextrose agar) at 25ºC (Booth 1971). The isolate was identified with the help of keys provided by (Raper & Fennell 1965, Samson & Varga 2007, Bennett 2010) and also by sequence obtained from ITS-PCR.

*DNA isolation and PCR*

The microorganism was aseptically transferred from stock-culture into tubes containing inclined potato dextrose agar medium PDA obtained from Himedia Laboratories (India). The inoculated tubes were incubated at 30°C for 7 days and were conserved at 4°C. A 0.3% sterile solution of Tween 80 was added to a tube containing activation culture. Spores were manually removed by the platinum loop from the agar surface. A sample of 0.1 ml of the suspension was transferred to 90 mm Petri dishes containing PDA. Media were incubated in the dark at 30°C for 10 days.

The mycelium was then washed in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) and was kept at 320ºC for 24 hrs and DNA was extracted. Polymerase chain reactions were performed in 50 µl volumes containing 100 pmol of each of the universal primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC); 200 µM of each of the four dNTPs; 1.5 U of Taq polymerase (Invitrogen) and 300 ng DNA template in a PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl). The temperature cycling parameters were 95°C for 3 min for denaturation of the first cycle and 1 min for subsequent cycles, primer annealing for 1 min at 55°C and primer extension at 72°C for 1 min with a total of 35 cycles and a final extension at 72°C for 3 min. The sequence of the ITS region of the rDNA has been deposited at the GenBank (NCBI).

*Sequencing and phylogenetic analysis*

The PCR product was purified and sequenced (Chromous Biotech Pvt. Ltd., Bangalore). The sequence was compiled by ApE software (A plasmid Editor). The rDNA sequence was submitted to NCBI GeneBank. The sequence was also blasted (NCBI) to determine the percentage of similarity with related sequences. The BioEdit sequence alignment editor was used to obtain multiple alignments of nucleotides with related sequences for ITS. The blast output was processed for generating the phylogenetic tree in software MEGA7 by using 'Maximum Likelihood' method based on the Tamura-Nei model (Tamura & Nei 1993, Kumar *et al.* 2016).

## RESULTS AND DISCUSSION

*Growth, macroscopic features of colonies and microscopic characters for identification*

Dormant conidia (3.5 µm mean diameter) were increased in diameter of 4.0 µm in deionized water and in the PDB swelled to 6.5 to 7.0 µm before germ-tube outgrowth. The majority of conidia produced one or occasionally two germ tubes between 6 and 9 h after incubation at 30°C. The hyphae within the mycelium were divided by septa. After 3 days of growth, the diameter of each colony was 1.5 cm, after which the colonies were extended its diameter 1 cm per day. The diameter of each colony was 5 cm after 7 days of growth. Five

concentric zones were arbitrarily distinguished in the sandwiched colony. After vegetative growth it was produced conidiophores (the asexual reproductive structures). Previously, Levin *et al.* (2007) assessed the effect of substrate heterogeneity in colonies of *A. niger*. de Farias *et al.* (2010) compared the spore production in different media.

Macroscopically, white colonies were visible on agar surfaces and occasionally developed yellow tinges on which subsequently black conidiophores were formed. With a binocular, slender stalks bearing small white spherical vesicles that mature into black pigmented spore heads carrying numerous conidia on phialides and metulae have been observed. The round vesicles and the pronounced metulae can be regarded as a hallmark for *A. niger*. Recently, maturation of conidia on these asexual reproductive structures was studied by Teertstra *et al.* (2017). He found that pigmented conidia that had developed on conidiophores for 2, 5, and 8 days were resistant to heat.

Microscopic morphology of *A. niger* LKO1 showed large, globose, black conidial heads, which became radiate, tending to split into several loose columns with age. Conidiophores were smooth-walled, hyaline or turning dark towards the vesicle. Conidial heads were biseriate with the phialides born on brown, often septate metulae. Conidia were globose to subglobose, black and rough-walled (Fig. 1). The ability of conidia to produce conidiophores after germination depends on temperature and nutrients provided in the medium (Anderson & Smith 1971).

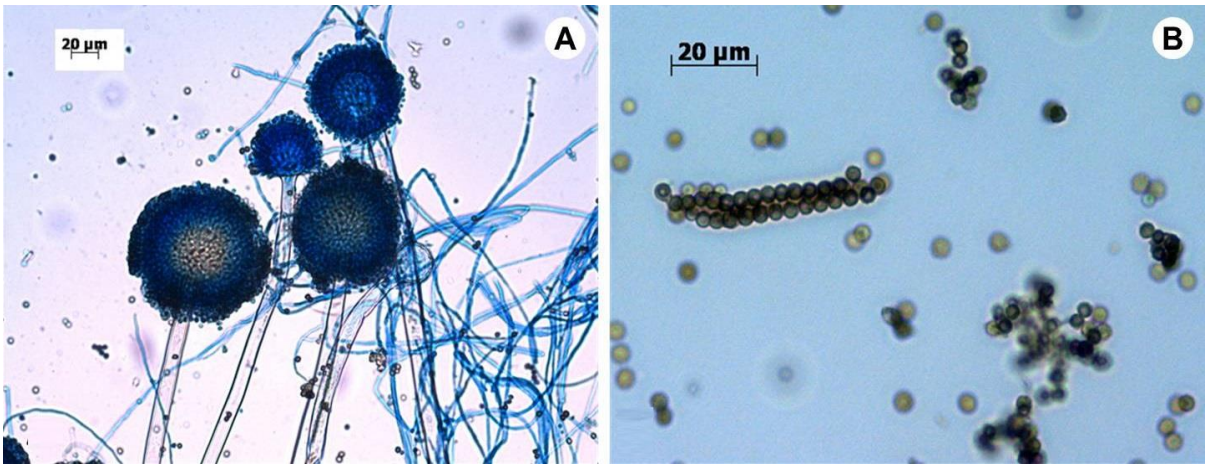


**Figure 1.** Vegetative and asexual reproductive structures of *Aspergillus niger*: **A**, Mycelia with conidiophores; **B**, Condia.

*Molecular identification based on the sequence of ITS region of rDNA*

In the present study, the partial ITS sequence of the flanking regions of the ribosomal nuclear DNA of *A. niger* LKO1 was PCR amplified using the specific forward and reverse primers with an amplicon size 541 base pairs. Sequence was submitted to NCBI GenBank (Accession number MK696283). Sequence of amplified ITS region was given below.

1 ccatccgtgt ctattatacc ctgttgcttc ggcgggcccg ccgcttgtcg gccgccgggg
61 gggcgccttt gccccccggg cccgtgcccg ccggagaccc caacacgaac actgtctgaa
121 agcgtgcagt ctgagttgat tgaatgcaat cagttaaaac tttcaacaat ggatctcttg
181 gttccggcat cgatgaagaa cgcagcgaaa tgcgataact aatgtgaatt gcagaattca
241 gtgaatcatc gagtctttga acgcacattg cgccccctgg tattccgggg ggcatgcctg
301 tccgagcgtc attgctgccc tcaagcccgg cttgtgtgtt gggtcgccgt ccccctctcc
361 gggggggacgg gcccgaaagg cagcggcggc accgcgtccg atcctcgagc gtatggggct
421 ttgtcacatg ctctgtagga ttggccggcg cctgccgacg ttttccaacc atttttttcca
481 ggttgacctc ggatcaggta gggatacccg ctgaacttaa gcatatcaat aaggcggggg

The amplified sequence was blasted in the NCBI. The sequence showed 100% similarity to partial sequence of internal transcribed spacer 1 (ITS 1) of 5.8S ribosomal RNA gene and internal transcribed spacer 2 (ITS 2) of large subunit ribosomal RNA gene with 4 sequences of *A. niger* (HM210842.1, EF134625.1, KT876702.1 and KP794197.1) and 99% similarity with other isolates of *A. niger* (MH055393.1, MH345876.1, MG759551.1 etc.) as well as with *A. tubingensis* (MH345877.1, MH055396.1, MG659603.1 etc.). Phylogenetic tree constructed from the blast result of NCBI also showed that the fungus is closer to both the fungus (Fig. 2A). However,

Taxonomic analysis based on NCBI GenBank database confirmed the fungus as a member of Ascomycetes (97 hits) and maximum similarity of sequence was matched with *A. niger* (53 hits) (Fig. 2B).

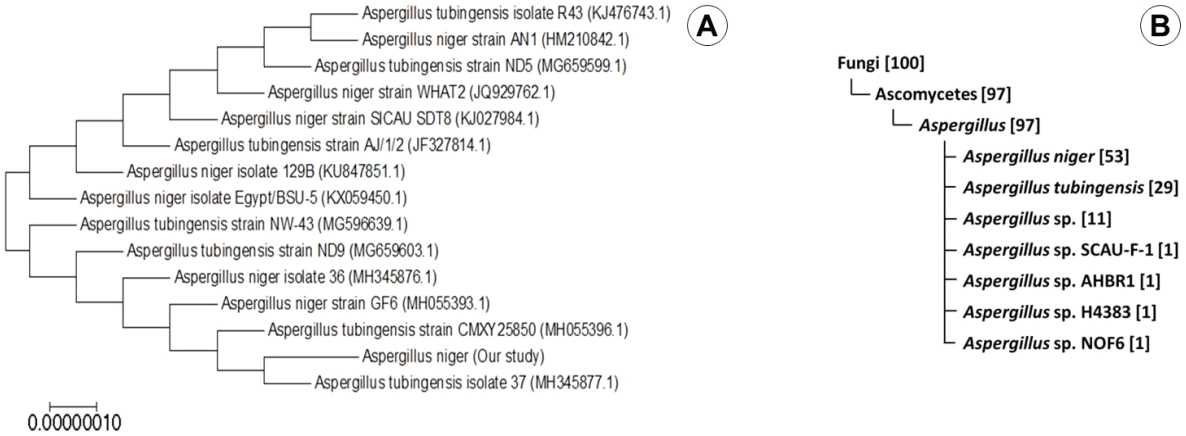


**Figure 2. A**, Molecular Phylogenetic analysis of *Aspergillus niger* based on the Tamura-Nei model by Maximum Likelihood method. The analysis is based on 14 nucleotide sequences from NCBI BLAST result; **B**, Taxonomic analysis based on NCBI GenBank database confirmed the fungus as a member of Ascomycetes (97 hits) and maximum similarity of sequence was matched with *A. niger* (53 hits).

In earlier studies, Henry *et al.* (2000) successfully identified 11 clinical isolates of *Aspergillus* from ITS amplicons ranged in size from 565 to 613 bp. Both ITS 1 and ITS 2 regions were needed for accurate identification of *Aspergillus* at the species level (Henry *et al.* 2000, Shittu *et al.* 2016).

## CONCLUSION

The causal organism of "black mold" disease of fruits and vegetables, *Aspergillus niger* LKO1 was isolated from Lucknow, India. The taxonomic identification was carried out by morphological diagnosis and molecular analysis. After the analysis of characteristics and dimensions of hyphae, conidiophores and conidia fungus was identified as *A. niger*. ITS sequence showed the 100% similarity (NCBI) with other isolates of *A. niger*. Phylogenetic analysis was revealed the Maximum Likelihood with two *Aspergillus* species *A. niger* and *A. tubingensis*. However, Taxonomic analysis based on NCBI GenBank database confirmed the fungus as *A. niger*, a member of Ascomycetes. The isolation and identification of this fungus will help in further research on accurate biological control for this plant pathogen and its commercial utilization.

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