Research Article

Taxonomic Divergence of Medically Important and Toxigenic *Aspergillus minisclerotigenes* from *Aspergillus flavus*

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Abstract | Molds produce noxious mycotoxins and cause more than 30% yield losses. The aflatoxins producer *Aspergillus minisclerotigenes* and *Aspergillus flavus* are morphologically similar species that belong to the *Aspergillus* section *Flavi*. *A. minisclerotigenes* and *A. flavus* were isolated from soybean and okra seeds, respectively. The isolated species were first identified morphologically. ITS1–5.8S–ITS4 primers sequence and amplification of ISSR nucleotide sequences using three primers [P01 $(AGAG)_4$ G, P02 $(GTG)_5$, and P03 $(GACA)_4$] confirmed that *A. minisclerotigenes* and *A. flavus* are two genetically distinct strains. Furthermore, both strains were qualitatively analyzed for aflatoxins (AFB1 and AFB2) production by thin-layer chromatography (TLC). A polyphasic strategy as adopted for the current study is a reliable and reproducible means to differentiate *A. minisclerotigenes* from *A. flavus*, indeed essential in interpretations of taxonomic and nomenclature of *A. flavus* group that may allow prior diagnosis and selection of effectual antifungal agents.

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1. Introduction

Various toxigenic strains of *Aspergillus* section *Flavi* produce lethal aflatoxins (G1, G2, B1 and B2) in agricultural commodities (Ismaiel and Papenbrock, 2015) and are a frequent cause of infections in humans and animals (Elad and Segal, 2018). The section *Flavi* included 33 species, and the species relationship within the section is still unclear. The classical means for the identification of these species still primarily depend on cultural and morphological traits. However, it is often tricky to differentiate these species because the phenotypic differences are not divergent and are easily ostentatious by the surroundings and are also mystified by the high degree of intra- and interspecies variations (Lee *et al.*, 2004). Among different species within section *Flavi*, *A. minisclerotigenes* exhibited a close phylogenetic relationship with *A. flavus*.

A. flavus is an extremely competitive cosmopolitan, notorious plant pathogen with wide host range, which has been initially described two centuries ago (Link, 1809). *A. flvaus* produces only produce B type, but there are also reports indicated the production of G type aflatoxins toxin as well (Frisvad *et al.*, 2019). *A. minisclerotigenes* has been described 10 years back (Pildain *et al.*, 2008), and is present in Central, East and Southern Africa and Australia (Probst *et al.*, 2014). It can grow on many substrates like maize, almond, groundnut and spices and produce both B and G aflatoxins (Makhlouf *et al.*, 2019).

For food safety purposes, correct species identification is of high importance and by using a polyphasic

strategy based on the combination of phenotypic and genotypic characteristics may contribute to the differentiation of toxigenic *Aspergillus* species within Flavus group. The current study was aimed to employ a polyphasic strategy that included phenotypic as well as genomic criteria (based on ITS and ISSR analysis) to discriminate the *A. minisclerotigenes* from *A. flavus*.

2. Materials and Methods

2.1 Isolation and identification

Soybean (*Glycine* max) and okra (*Abelmoschus esculentus*) seeds from storage house, Lahore Pakistan during 2014, were found contaminated by morphologically similar molds. These seeds after surface sterilization with Clorox for one minute thoroughly washed with distilled water and incubated on moist blotter paper for 5 days at 27 °C. The grown spores were transferred to Malt Extract Agar (MEA) and Czapek Dox Agar (CZA) media and incubated for 3-4 days at 30 °C. The pure cultures were used for pathogen identification using macroscopic and microscopic features (Pildain *et al.*, 2008).

2.2 Extrolite analysis

Isolated pathogens were preliminary characterized for their aflatoxigenicity based on emission of blue or green fluorescence after UV light excitation at 365 nm after growth on coconut cream agar (CCA) medium (Lin and Dianese, 1976).

A portion of CCA medium (6-7 cm) without fungal mycelium was cut and put into the 250 mL of Erlenmeyer flask filled with 50 mL of chloroform, incubated at 27 °C in shaking incubator at 200 rpm for 3 hours. Chloroform contents were filtered (Whatman No. 1) and separated into separate bottles. Extracts were allowed to dry at 35 °C for 5 days and dissolved into 2 mL of commercial methanol and aflatoxins of different isolates were saved at 4°C for qualitative analysis of aflatoxins by thin–layer chromatography (Guezlane-Tebibel *et al.*, 2013).

Both strains were analyzed by spotting crude extract (55 µL) of aflatoxins along with the standard of AFBs (AFB1 and AFB2). The TLC plates used were coated with silica gel 60 F254 on aluminum sheet, 20 x 20 cm. TLC plates were developed in chloroform and acetone (90:10, v/v) solvent system (Reddy *et al.*, 2004). The mobile phase was allowed to run 3/4 of the TLC plate. The plates were dried in the dark and then observed under UV light at 365 nm and samples spots were compared with standard aflatoxins spotted on the same plate.

2.3 Genetic analysis

Method of Weigand *et al.* (1993) was used for the isolation of genomic DNA from fungal species. Using genomic DNA as a template, ITS1/ITS4 [ITS1 forward (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 reverse primer (3′-TCC TCC GCT TAT TGA TAT GC-5′)] regions of the genome were amplified (White *et al.*, 1990). The amplified fragments were separated in 1% agarose gel by electrophoresis. PCR products were purified by using a PCR purification kit (Enzynomics) and the fragments were sequenced in both orientations from Macrogen, Korea by using ITS forward and reverse primers. Three primers P01, P02 and P03 were used for ISSR amplification (Table 1) and the amplified PCR products were separated by gel electrophoresis and analyzed.

Table 1: ISSR primers to amplify fungal DNA.

Primer name	Primer sequence
P1	5′- AGA GAG AGA GAG AGA GG -3′
P2	5′- GAG AGA GAG AGA GAG AT -3′
P3	5′- GAG AGA GAG AGA GAG AC -3′

3. Results and Discussion

Two post-harvest fungal strains of *A. flavus* group named *A. minisclerotigenes* and *A. flavus* were subjected to a polyphasic approach for authentic identification.

3.1 Morphological characterization

The colonies of *A. miniseclerotegenious* were dull green to greyish green in color and yellow at reverse on MEA (Figure 1a and c Am), 50–65 mm in diameter without zonation and displayed sclerotia production, while colonies on CZA attained a diameter of 30-40 mm and sclerotia were present (Figure 1b and d Am). Uni and biseriate conidial heads bearing long conidiophores (0.9-1.2 mm) and globose vesicles (25-40 µm). The size of metulae and phialides were 5-8 µm with 8-12 µm, respectively, while globose conidia (3.5-5 µm diameter) were pale green or olive green and smooth-walled to echinulate (Figure 1e-f).

A. flavus colonies were 50-60 mm in diameter (without zonation) and exhibited sclerotia production on MEA (Figure 1a and c Af). On CZA medium, fungal colonies were slow-growing, attained diameter

of a 30-40 mm (without zonation), having sclerotia, that were heavily produced in the center of each colony (Figure 1b and d Af). Conidial heads were typically radiate, splitting into several poorly defined columns. Subglobose to globose (25-45 µm) vesicles were hyaline, while both metulae and phialides were present. Metulae with 6.5-10 × 3-4.5 µm dimensions completely covered vesicle surface, however, phialides were 8-12 × 3-5 µm in size. Subglobose to globose (3.5-4.5 µm) Conidia were pale green and conspicuously echinulate (Figure 1h-j).

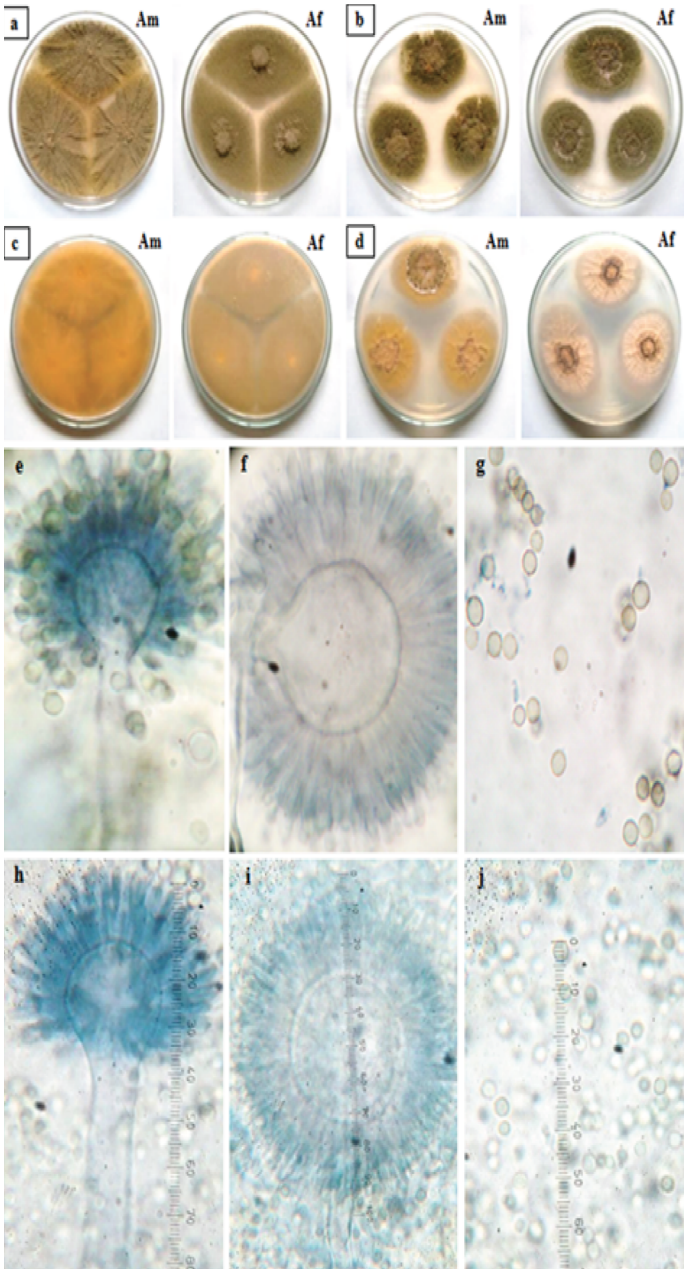

Figure 1: Comparison of colonies grown on MEA front and reverse (a and c) and on CZ (b and d). Microscopic study of *A. minisclerotigenes* (e-g) and *A. flavus* (h-j) showing seriation (uniseriate and biseriate) and conidial attachment. Am: *A. minisclerotigenes*; Af: *A. flavus*.

A vial of a pure culture of *A. miniseclerotegenious* (FCBP-1353) and *A. flavus* (FCBP-0529) were deposited in the First Fungal Culture Bank of Pakistan.

3.2 Aflatoxins production

The culturing of both strains on CCA medium revealed that both *Aspergillus* species were capable of producing aflatoxins AFBs (Figure 2). Aflatoxins analysis on TLC also confirmed that *A. minisclerotigenes* (FCBP-1353) and *A. flavus* (FCBP-0529) were toxinogenic with consistent mycotoxigenic profile. Both were produced AFBs (AFB1 and AFB2) and showed clear bands on the TLC plate under UV light (Sultan and Magan, 2010) (Figure 3).

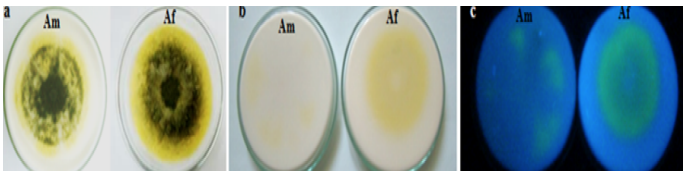

Figure 2: Comparative screening of aflatoxin production by *A. minisclerotigenes* and *A. flavus* grown on CCA. a: colony from front side; b: reverse colony; c: reverse colony under UV light. Am: *A. minisclerotigenes*; Af: *A. flavus*.

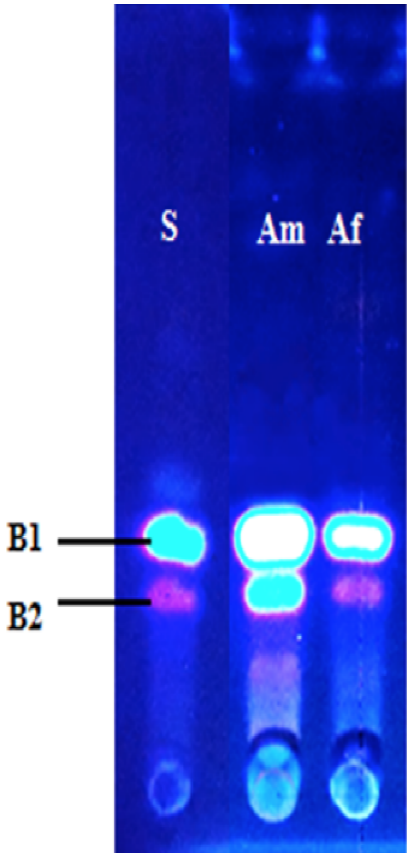

Figure 3: Aflatoxins production on TLC. S: AFBs Standard, Am: *A. minisclerotigenes* and Af: *A. flavus*.

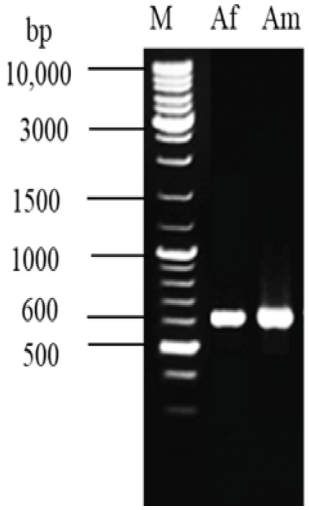

Figure 4: Amplified ITS region of strains, M=1kb DNA marker; Af: *A. flavusus* and Am: *A. minisclerotigenes.*

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FCBP1365    1   TCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGG  60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. mini.  100   TCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGG  159

FCBP1353   61   AGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTA  120
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. mini.  160   AGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTA  219

FCBP1353  121   AAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT  180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. mini.  220   AAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT  279

FCBP1353  181   AACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCC  240
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. mini.  280   AACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCC  339

FCBP1353  241   CTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGT  300
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. mini.  340   CTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGT  399

FCBP1353 301    GTGTTGGGTCGTCGTCCCCTCTCCggggggACGGGCCCCAAAGGCAGCGGCGGCACCGC  360
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. mini   400   GTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGC  459

FCBP1353  361   GTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGC   414
                ||||||||||||||||||||||||||||||||||||||||||||||||||||
A. mini.  460   GTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGC   513
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Figure 5: ITS sequence alignment of *A. minisclerotigenes.*

The BLAST results revealed 100% identity of *A. minisclerotigenes* FCBP1353 to the 8 strains including G5 (KF841549.1), E76 (JX456215.1), E74 (JX456193.1), E44 (JX292091.1), E21 (JX292090.1), CS5 (JF412778.1), NRRL 29002 (JF412775.1), CS2 (JF412776.1) and some other *A. minisclerotigenes* strains.

3.3 Genetic analysis

The obtained nucleotide sequence of PCR product of both species were sent for DNA sequencing and identified as 551 bp of ITS region of *A. minisclerotigenes* and 536 bp of *A. flavus* (Figure 4). The ITS sequence of *A. minisclerotigenes* and blast results in Figure 5 also showed 100% identity to the 8 strains of *A. minisclerotigenes* available in GenBank including G5 (KF841549.1), E76 (JX456215.1), E74 (JX456193.1), E44 (JX292091.1), E21 (JX292090.1), CS5 (JF412778.1), NRRL 29002 (JF412775.1), CS2 (JF412776.1) and some other *A. minisclerotigenes* strains. Likewise, *A. flavus* (FCBP–0529) blast analysis showed 100% identity with more than 25 strains including KJ473711.1, KJ013417.1, KF753952.1,KF656712.1,KF723010.1,KJ123911.1, GU172440.1, GU076485.1, KF031021.1 and some other *A. flavus* in GenBank (Figure 6). The nucleotide sequence of *A. minisclerotigenes* (FCBP-1353) *A. flavus* (FCBP-0529) were deposited to GenBank under the accession no. KJ564033 and KJ999747, respectively. The uniformity of ITS fragment size in several fungal groups builds nucleotide sequencing of ITS fragments obligatory to expose interspecific, and in some cases, also intraspecific variation (Hinrikson *et al.*, 2005; Inglis and Tigano, 2006). The ITS region was very functional in resolving taxonomic difficulties in many fungal genera as verified by Driver *et al.* (2000) and Inglis and Tigano (2006). Hinrikson *et al.* (2005), revealed that the small variation in band size probably made ITS an unreliable parameter for separating *Aspergillus* species. Unlike ITS, ISSR profile has significant importance as an assisting tool for identification, genetic diversity analysis and differentiation among strains (Batista *et al.*, 2008; Zhang *et al.*, 2013). ISSR analysis has also been shown usefulness in population genetics, epidemiological surveys and ecological studies of *A. flavus* (Batista *et al.*, 2008). Amplification of ISSR with three primers confirmed (Figure 4) genetic differences between *A. minisclerotigenes* and *A. flavus* (Hatti *et al.*, 2010).

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FCBP0529    1   ACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGC  60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. flavus[  59   ACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGC  118

FCBP0529   61   CGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTA  120
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. flavus  119   CGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTA  178

FCBP0529  121   GTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGT  180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. flavus  179   GTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGT  238

FCBP0529  181   TCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGT  240
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. flavus  239   TCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGT  298

FCBP0529  241   GAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTC  300
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. flavus  299   GAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTC  358

FCBP0529  301   CGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCgg  360
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. flavus  359   CGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGG  418

FCBP0529  361   ggggggACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTT  420
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. flavus  419   GGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTT  478

FCBP0529  421   TGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGG  480
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
A. flavus  479   TGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGG  538

FCBP0529  481   TTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATAT   524
                ||||||||||||||||||||||||||||||||||||||||||||
A. flavus  539   TTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATAT   582
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Figure 6: ITS sequence alignment of *Aspergillus flavus.*

The BLAST results revealed 100% identity of *A. flavus* (FCBP0529) to the more than 25 strains including S19 (KJ473711.1),BC-212(KJ013417.1),LPSC1183(KF753952.1), PTN13 (KF656712.1), KVCET2 (KF723010.1), G49 (KJ123911.1), UPM A8 (GU172440.1), A2 (GU076485.1), KAR-8 (KF433946.1) and J8M-40 (JN226905.1), PW2961 (KF562204.1), PW2953 (KF562196.1), MDU-5 (KC914096.1), JP44MY8 (KF031021.1) and some other *A. flavus* strains.

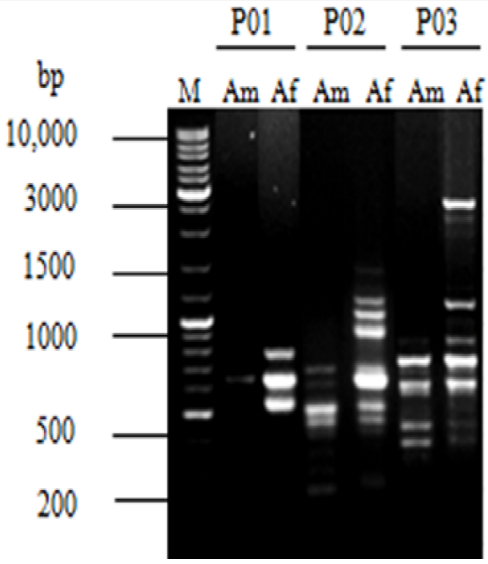

Figure 7: DNA banding profile of PCR-ISSR amplification product. M: DNA marker; Am: *A. minisclerotigenes* and Af: *A. flavus*.

4. Conclusions

In the current study, high relatedness between two medically important strains of *A. flavus* group concluded that the process of differentiating them needs an under-species classification accomplished by a number of different tactics including morphological basis, amplified ITS fragment, ISSR molecular markers, which is actually a supplementary tool for genetic characterization and could be useful in distinguishing between strongly correlated species or strains.

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Author's Contribution

Amna Shoaib: Supervised research and wrote the manuscript.
Zoia Arshad Awan: Performed experiments and collect the data.
Naureen Akhtar: Supervised research and wrote the manuscript

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