Bangladesh J. Plant Taxon. **29**(2): 297-312, 2022 (December) DOI: https://doi.org/10.3329/bjpt.v29i2.63531

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF MICROMYCETES ASSOCIATED WITH SEEDS OF SELECTED COTTON (*GOSSYPIUM HIRSUTUM* L.) VARIETIES

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Keywords: Mycoflora; Cotton varieties; ITS; PCR amplification; Sequence analysis.

Abstract

A total of 14 varieties (CB1-CB14) of cotton (*Gossypium hirsutum* L.) seeds were collected from Cotton Research, Training and Seed multiplication Farm, Sreepur, Gazipur to detect and identify the seed borne fungi by morphological and molecular techniques. The sequence results obtained using the ITS1 and ITS4 primers were compared with NCBI GenBank and BOL database using BLAST analysis. In the present investigation, a total of 29 fungal isolates were morphologically identified from different varieties of cotton seeds, of which 19 fungal isolates were identified by molecular techniques. Among the isolated fungi, *Aspergillus subramanianii, A. toxicarius, A. wentii, Penicillium aculeatum, P. citrinum, Rhizomucor* sp. and *Meyerozyma guilliermondii* have been reported as new records for Bangladesh.

Introduction

Cotton, unique among agricultural crops, provides food and fibre. Cotton is major natural fibre crop and also provides us edible oil and seeds by-products for livestock food. Cotton is cultivated in tropical and subtropical regions of more than seventy countries of the world, which represents 2.5% of the all cultivated land. Cotton is the second important cash crop in Bangladesh and it is also called white Gold.

Cotton is generally propagated by seeds and these are potential harbour of numerous micro-fungi which may impair seed germination resulting in the production of abnormal seedlings (Bateman and Kwasna, 1999; Khanzada *et al*., 2002). Most cotton diseases are transmitted through seeds which in most cases affect the quality of the fibre and seeds. Seed diseases may cause seed rot and damping-off of the seedlings reducing subsequently the number of stands. Various fungal seed borne pathogens have been reported in the world which reduce germination percentage and seedling vigour of cotton seeds (Jeyalakshmi *et al*., 1999; Eisa *et al*., 2007; Tomar *et al*., 2012).

In Bangladesh, so far, a total of 14 diseases of cotton were recorded of which 12 diseases are caused by fungal pathogens (BARI, 1990). Majority of cotton diseases are seed-borne *viz*., *Alternaria* blight, bacterial blight, *Fusarium* wilt, *Myrothecium* blight, *Cercospora* blight, *Exserohilum* blight etc. (BARI, 1990). In Bangladesh, *Alternaria tenuis, Aspergillus flavus, A. niger, A. fumigatus, Fusarium moniliforme* and *Rhizopus nigricans* were reported to be predominant in cotton seeds (Lutfunnessa and Shamsi, 2011). *Aspergillus flavus, A. niger, Curvularia lunata, Fusarium moniliforme* var. *subglutinans, F. sporotrichioides* and *Rhizoctonia solani* were found to be pathogenic for 3 varieties of Hill cotton (*Gossypium arboreum*) in Bangladesh (Naznin and Shamsi, 2018).

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The correct species name of a plant pathogenic fungi is most important for the development of effective disease control management, quarantine purposes and as a basis for making decisions to protect agricultural crops as well as other natural resources from fungal pathogens (Rossman and Palm-Hernandez, 2008). So far, no molecular identification report is available regarding fungi associated with cotton seeds in Bangladesh. Therefore, the present research work was undertaken to find out the pathogenic fungi associated with different cotton varieties following morphological as well as molecular identification.

Materials and Methods

Seed samples of CB1-14 were collected from Cotton Research, Training and Seed multiplication Farm, Sreepur, Gazipur after harvesting and kept in clean glass jars, labeled properly and preserved at room temperature for subsequent use.

Fungi associated with selected rice samples were isolated with following "Tissue planting method" on PDA medium (CAB, 1968), "Blotter method" and "Paper Towel method" (ISTA, 1996). Morphological identification of the isolates was determined following the standard literatures (Thom and Rapper, 1945; Rapper and Thom, 1949; Subramanian, 1971; Barnett and Hunter, 1972; Benoit and Mathur, 1970; Booth, 1971; Ellis, 1971, 1976; Sutton, 1980). Molecular identification was done following Amer *et al*. (2011) with some modification.

DNA extraction

For DNA extraction, the fungi were grown on PDA medium at 25± 2ºC for 15 days. With a sterile spatula one gm fungal mycelia were taken in 1.5 ml Eppendorf tubes from the petri plates. The mycelia were immediately grinded with a homogenizer machine in each Eppendorf with 400 µl sterile extraction buffers (200mM Tris- HCL, 250mM NaCl, 25mM EDTA, 0.5% SDS). Then 6 µl of 20 mg/ml RNase was added in each Eppendorf. The tubes were placed in 65ºC preheated water bath for 10 minutes. The samples were taken from the water bath and cooled down to room temperature. In each sample, 130 µl of 3M sodium acetate, pH 5.2 was added. Samples were vortexed for 30s at maximum speed to mix well and incubated at -20º C for 10 minutes. The samples were centrifuged at 13,000 rpm, 4º C for 15 minutes. The supernatants were transferred to fresh tubes and an equal volume of isopropanol was added to each sample, mixed well and were incubated at 4°C for one night. Samples were then centrifuged at 6000 rpm, 4ºC for 20 minutes. White coloured pellet was formed. The supernatant was discarded and the pellet was washed with 700 µl of 70% ethanol in two times. The DNA pellets were then air dried in an oven at 40°C for at least 10 min. The resultant DNA pellet was then resuspended in 100 µl of 1 x TE (10 mM Tris-HCl, 1 mM EDTA) buffer (pH 8.0). The DNA was dissolved overnight at 4 ºC in the refrigerator.

PCR amplification

Molecular identification of the isolates was completed using the internal transcribed spacer (ITS) region. PCR amplification was conducted using the ITS1 (5'-TCCGTAGGTGAACCTG CGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers for the ITS gene. The PCR was performed in 0.2 ml PCR tube with 25 reaction volume containing 2.00µl Template DNA, 12.5µl Master mix, 1.0µl Forward Primer, 1.0µl Reverse Primer and 8.5µl MilliQ H_2O. Reaction mixture was vortexed and centrifuged in a micro centrifuge. The PCR was introduced by an initial denaturation step at 94ºC for 5 minutes following 35 cycles of 94, 54 and 72ºC each for 30 sec, with a final extension step of 5 min at 72º C and ended with 4º C. PCR amplified products were stored in – 20º C freezer for analysis by resolving in 1% agarose gel. The gel was prepared using 1.0g agarose powder containing 8µl ethidium bromide. Agarose gel electrophoresis was conducted in 1× TAE buffer at 90 Volts and 300 mA for 60 minutes. Alongside the ITS reactions, one

molecular weight marker 1kb DNA ladder was electrophoresed. DNA bands were then photographed by a Gel Documentation system (model: DI-HD, UK).

Sequence analysis

The PCR amplified products were purified by alcohol precipitation and sequenced through automated sequencer in Centre for Advanced Research in Sciences (CARS), University of Dhaka. The obtained sequences were compared with already available sequences in the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) using BLAST program (http://blast.ncbi.nlm.nih.gov) to identify the genus and species of the isolates.

Results and Discussion

Morphological identification

Twenty-nine fungal species, representing 14 genera were found to be associated with 14 varieties of cotton seeds. The isolated fungi were *Aspergillus aculeatus* Lizuka, *A. flavus* Link, *A. fumigatus* Fresenius, *A. niger* Van Tiegh, *A. nidulans* Eidam, *A. subramanianii* Visagie, Frisvad & Samson, *A. tamarii* Kita G., *A. toxicarius* Murak, *A. wentii* Wehmer, *Curvularia lunata* (Wakker) Boedijn, *Colletotrichum gloeosporioides* Penz & Sacc, *C. gossypii* Southw., *Chaetomium globosum* Kunze., *Fusarium moniliforme* J. Shelden, *F. nivale* (Fr.) Sorauer, *F. oxysporum* Schlechtendal, *F. fujikuroi* Nirenberg, *F. solani* (Mart.) Sacc., *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Meyerozyma guilliermondii* (Wick.) Kurtzman & M. Suzuki., *Mucor* sp. P. Micheli ex L., *Penicillium aculeatum* Raper & Fennell, *Penicillium citrinum* Thom, *Rhizoctonia solani* Khun., *Rhizopus stolonifer* (Ehrenb.) Vuill., *Rhizopus oryzae* Went & Prins. Geerl., *Rhizomucor* sp. Lucet & Costantin, *Syncephalastrum racemosum* Cohn and *Trichoderma viride* Pers.

Based on morphological characteristics, these 29 fungal isolates were identified provisionally. In the present investigation, some fungal species were unable to identify up to species level based on the morphological features only. Hence, molecular characterization of the fungal species was performed for proper identification. For further confirmation of these 29 fungi, ITS sequence based molecular analysis was performed and 19 were confirmed up to species level.

Key morphological features of the nineteen fungi identified by molecular analysis:

***Aspergillus aculeatus* Lizuka**, Annls sci. nat. (Bot.), Ser. 5, **8**: 240 (1867) **(Fig. 1A)**

Aspergillus aculeatus is a ubiquitous species that usually isolated from rotting fruits and soil. Colonies effuse, brownish black. Mycelium well developed, septate, profusely branched and hyaline. Cells are multinucleate. Conidiophores are very long, often with a foot cell, straight or flexuous, swollen at the apex in to a spherical vesicle. Surface of vesicle is covered by closely packed, more or less clavate shaped branches. Conidia catenulate, dry, usually globose, echinulate, dark brown in colour.

Specimen examined: Six varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 04, 07 May 2018.

Aspergillus flavus Link. **(Fig. 1B)**

Colony colour on PDA medium is grayish powdery and fast growing. Conidial heads are yellow to green became brownish in edge. Conidiophores are less than 1.0 mm length and 10- 20 µm diameter, vesicle was glubose to subglubose. Conidia are glubose minutely accumulate and measured 2.5-3.5 µm. Mycelia well developed, septate, hyaline and profusely branched.

Conidiophores 300-600 µm long. Cells are multinucleate Vesicles 10 - 35 µm in diameter. Sterigmata 8 - 14 × 3 - 5 µm.

Specimen examined: Fourteen varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun, 09, 11 September, 2017.

Aspergillus fumigatus Fresenius. Beitragezur Mykologie **3**:81 (1863) **(Fig. 1C)**

Colonies flat, olivaceous green, mycelia well developed, septate. Cells are multinucleate. Conidiophores are long, often with a foot cell, straight or flexuous, swollen at the apex into a spherical vesicle. Surface of vesicle are covered by closely packed more or less clavate branches. Conidia catenulate, dry, usually globose, echinulate and smooth. Colonies of the fungus produced thousands of minute pale green conidia 2-3 µm.

Specimen examined: Isolated from fourteen varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun, 07 March 2017.

Aspergillus subramanianii Visagie, Frisvad & Samson: 66 (1877) **(Fig. 1D)**

Colonies yellow to yellow-orange, ochraceus or buff, powdery to granular. Conidial heads radiate, later splitting into several columns. Conidiophores brownish, 1-1.9 µm long, rough walled. Vesicles globose and phialides biseriate covering almost the entire surface of the vesicle. Conidia spherical to sub spherical, 2.5-3.5 µm in diameter, smooth walled to finely roughen. Sclerotia are pink to vinaceous-purple coloured, irregular shaped and up to 1 mm diam. It is a species with rough walled stipes, biseriate conidial heads, yellow to ochre conidia and sclerotia that do not turn black.

Specimen examined: Eight varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 07, 12 December 2017.

Aspergillus tamarii Kita G, in Centralb. F. Bakt., 37, No. 17/21, pp. 433-452. (1913) **(Fig. 1E)**

Aspergillus tamarii belongs in *Aspergillus* Section *Flavi*, and resembles *A. flavus* and *A. parasiticus*, but conidia colour is olive to brown and are larger, with thick, conspicuously roughened walls. Colonies on Czapek's solution agar spreading broadly at room temperature with vegetative hyphae mostly submerged, fruiting areas at first colourless, then passing through orange yellow shades to brown in old colonies. Not showing true green but often presenting a suggestion of green that is transient and limited to areas of young heads; reverse uncoloured or occasionally pinkish. On AFPA, it produces a deep brown reverse coloration, in contrast to the orange yellow of *A. flavus* and *A. parasiticus*. Conidial heads varying greatly in size in the same fruiting area, from more or less columnar but not completely globose and upto 30µ in diameter, with radiating chains and columns of conidia. Conidiophores arising from submerged hyphae upto 1or 2mm. in length, colorless with walls becoming abruptly thinner at the base of the vesicle. Vesicles globose to subglobose, 25 to 30µ in diameter. Sterigmata, in large heads. Conidia ranging from more or less pyriform, through subglobose to globose, commonly ranging from 1.5-3µ in diameter,

Specimen examined: Twelve varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 04, 05 March 2018.

Aspergillus wentii Wehmer **(Fig. 1F)**

Aspergillus wentii is an asexual, filamentous, endosymbiotic fungus. It produces single-celled, globose, conidia in unbranched, filamentous chains. Spores are smooth, colourless, and ellipsoidal,

approximately 1–2 µm in diameter. Conidia are darker yellow to brown in colour when mature and have a single wall. The elongating chains of conidia are dispersed through slightly pigmented, vase-shaped structures known as phialides that are around 6-8 µm in diameter. The conidial head or vesicle is yellow to darker coffee-coloured and 6.0–8.0 µm in diameter. The conidiophore can grow anywhere, 3-5 millimeters in length, has a glassy or hyaline appearance and although granular conidiophores have been found. It produces aerial hyphae, white or sometimes yellow in colour that can grow to a few millimeters in length. Foot cells have dense walls and are branched.

Specimen examined: Twelve varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 02, 07 July 2017.

Aspergillus toxicarius Murakami, (1971) **(Fig. 2A)**

Aspergillus toxicarius belongs in *Aspergillus* Section *Flavi*, and resembles *A. flavus*, but conidia of *A. toxicarius* are coloured olive to yellow, and are larger, with thick, conspicuously roughened walls. Conidiophores are less than 1.2 mm length and 10- 20 µm in diameter, vesicle was glubose to subglubose and 15-30 µm in diameter. Colonies effuse yellowish green. Mycelia well developed, septate, hyaline and profusely branched. Conidiophores 10-18 µm long. Cells are multinucleate. Vesicles 15 - 30 µm in diameter. Sterigmata 10-14 × 4-5 µm. Conidia greenish, catenulate, globose or pyriform, smooth, 4 - 5 µm in diameter. Colonies spreading broadly, dark cress green.

Specimen examined: Eight varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 14, 16 October 2017.

Curvularia lunata (Wakker) Boedijn. [Cochliobolus linatus Nelson & Haasis]. Ellis MB, Mycol. Pap. **106**: 2-43, 1966**.** **(Fig. 2B)**

Colonies are effuse, brown, grey or black, hairy, cottony or velvety. Stromata rarely formed in culture, colonies on PDA markedly zonate. Conidiophores are solitary, mostly unbranched, straight or slightly undulating, mostly flexuous geniculate, mid brown, septate up to 250 µm. Conidia are mostly 3-septate, dark brown, mostly curved, third cell from the base is broader and darker than others, broader cells are mid brown and other cells paler, smooth, 20.5 - 31.78 × 8.5 – 13.5 µm.

Specimen examined: Seven varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun, 06, 07 October 2017.

Fusarium oxysporum Schlecht, Flora berol. **2**: 139, (1824) **(Fig. 2C)**

Mycelium are delicate, white in color in the culture plate. Microconidia borne on simple phialides arising laterally on the hyphae. Microconidia generally abundant, variable, oval, ellipsoid, cylindrical, straight, 5-12 × 2.2-3.5 µm in size and macroconidia are thin walled, generally 3-5 septate, fusoid-subulate and pointed at both ends; 3 septate 7-14 × 3-5 µm, 5 septate 35-60 × 3-5 µm. The most commonly found spores are 3 septate.

Specimen examined: One variety of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 17 September 2017.

Fusarium moniliforme Sheldon 1904. Rep. Neb. Agric. Exp. Stn **17**:23-32 **(Fig. 2D)**

They are extensive and cottony, white, often with some tinge of pink mycelium. Reverse pinkish yellow. Mycelia are hyaline, profusely branched, septate. Conidiophores are hyaline, 0-2 septate. Phialides hyaline, 16 - 20 × 3 – 4 µm in diameter and conidia are hyaline, variable,

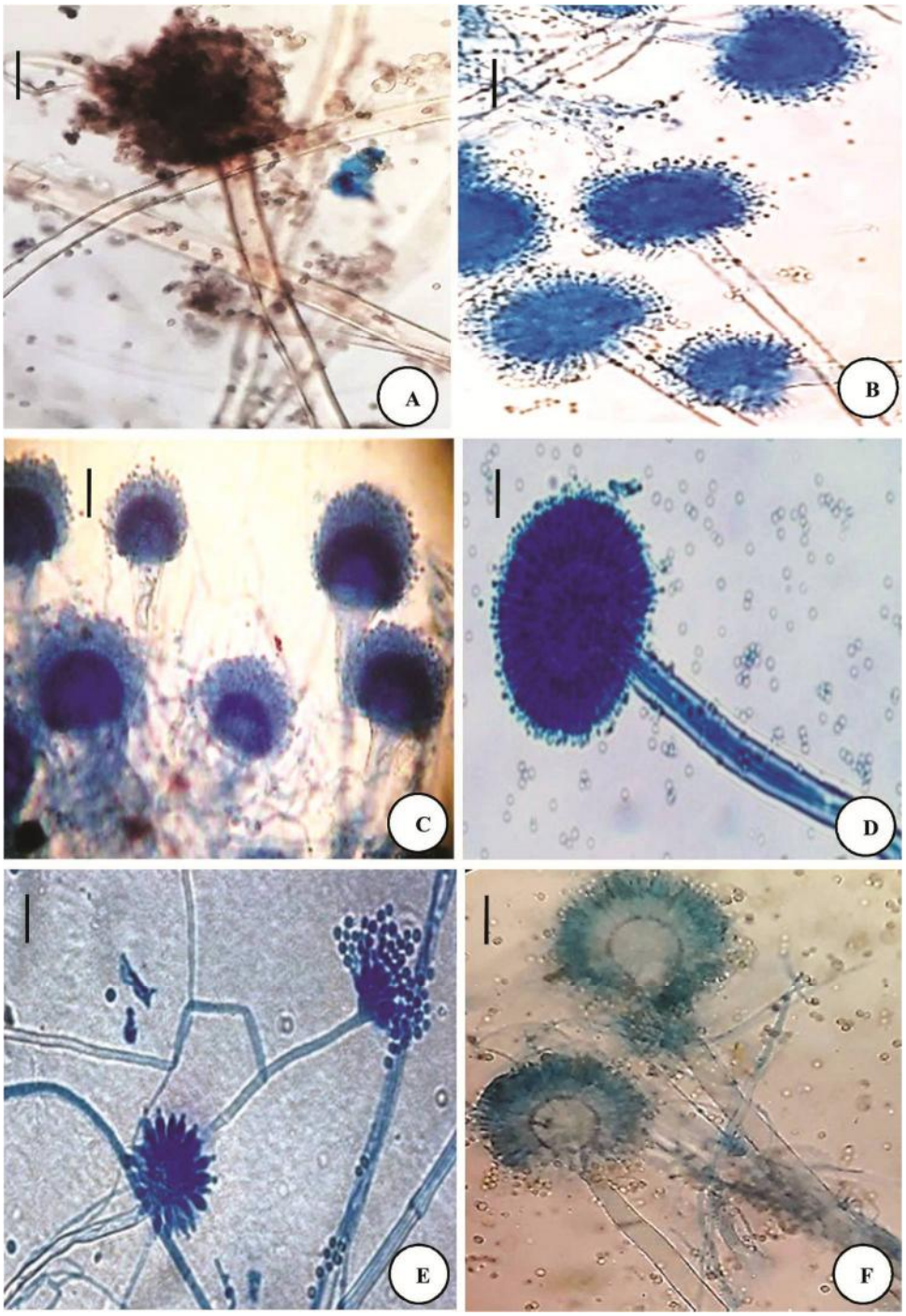

Fig. 1. Conidiophore and conidia of different fungi associated with cotton seeds. A. *Aspergillus aculeatus*, B. *A. flavus*, C. *A. fumigatus*, D. *A. subramanianii*, E. *A. tamarii* and F. *A. wentii* (Bar = 50 µm).

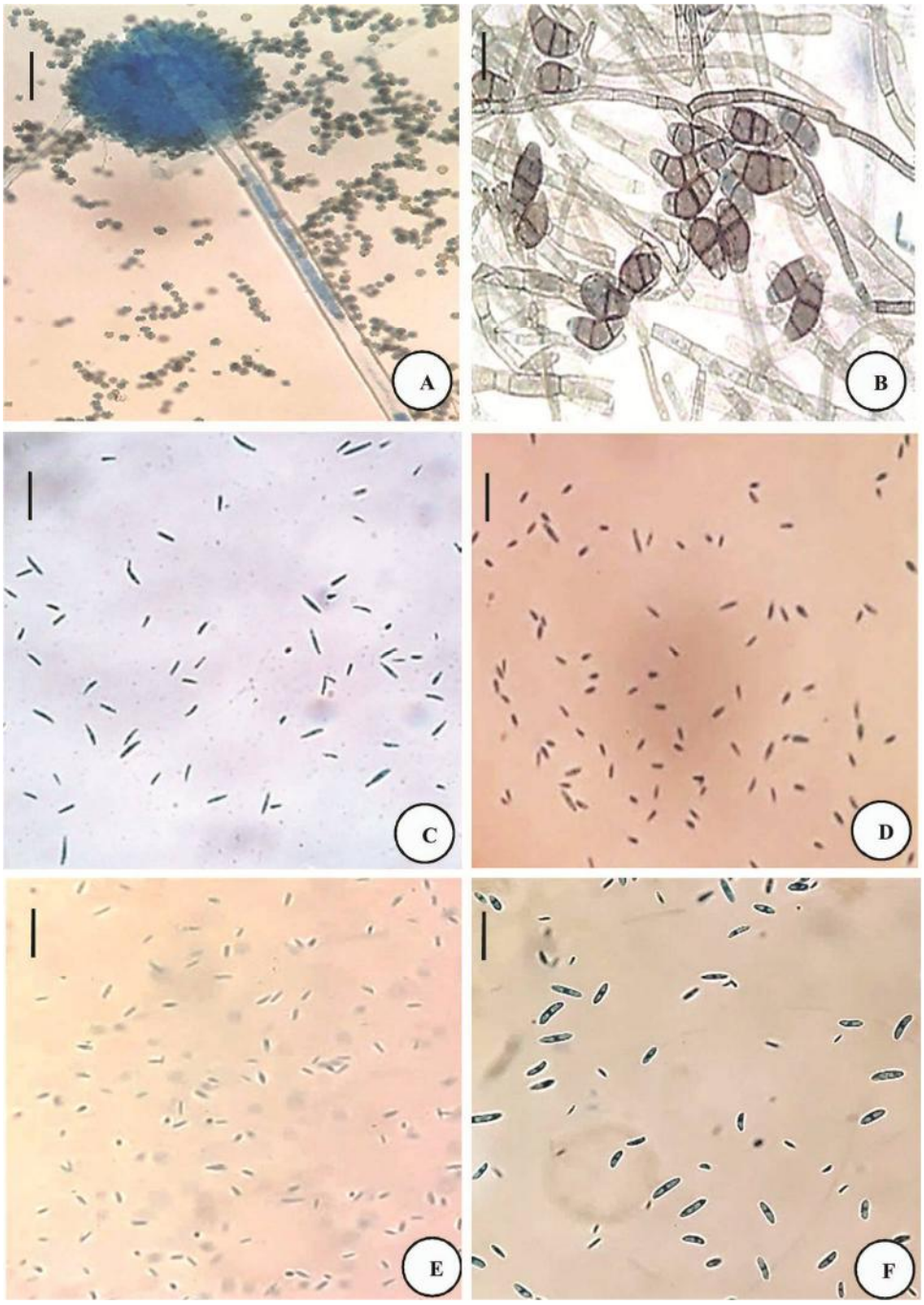

Fig. 2. Conidiophore and conidia of different fungi associated with cotton seeds. A. *Aspergillus toxicarius*, B. *Curvularia lunata*, C. *Fusarium oxysporum*, D. *F. moniliforme*, E. *F. fujikuroi*, and F. *F. solani* (Bar = 50 µm).

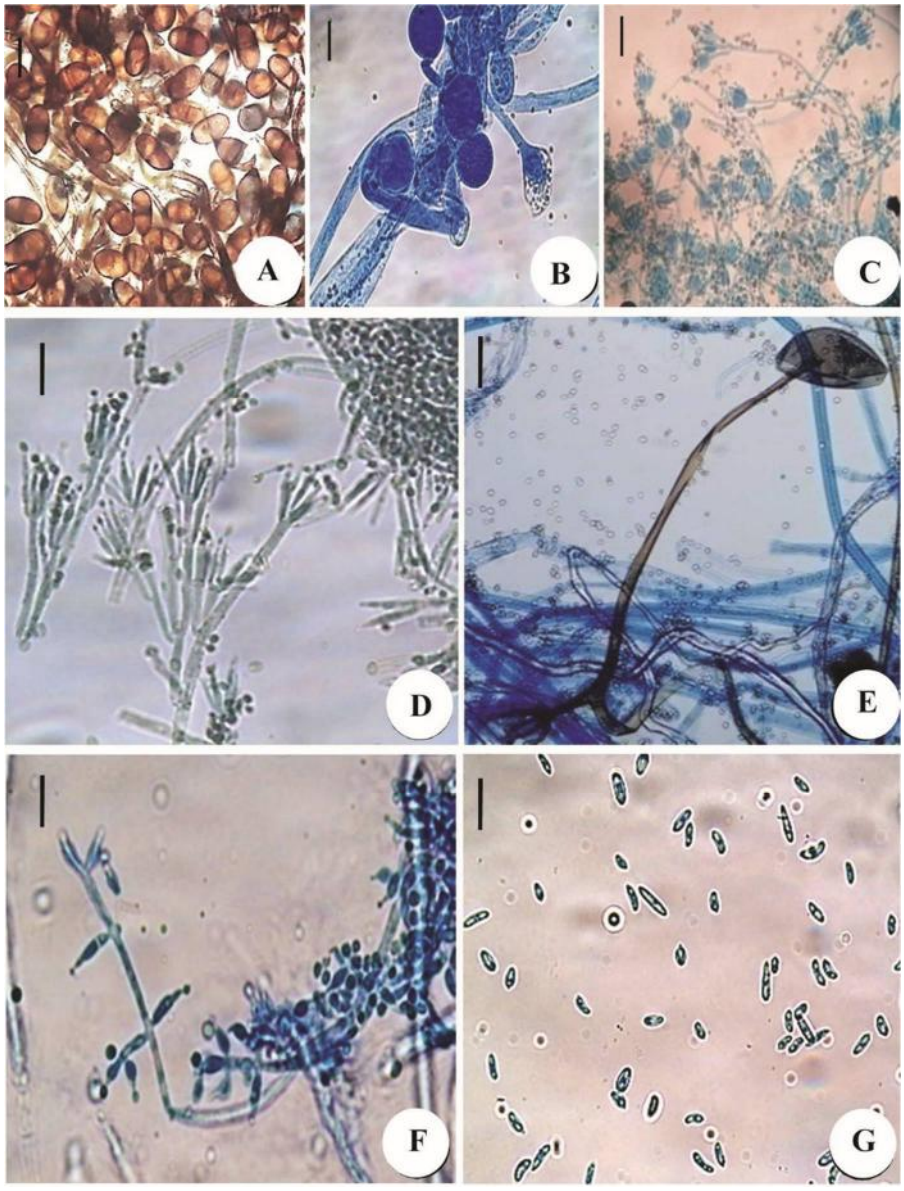

Fig. 3. Conidiophore and conidia of different fungi associated with cotton seeds. A. *Lasiodiplodia theobromae*, B. *Mucor* sp., C. *Penicillium citrinum*, D. *P. aculeatum*, E. *Rhizopus oryzae*, F. *Trichoderma viride* and G. *Meyerozyma guilliermondii* (Bar = 50 µm).

principally of two kinds. Microconidia and macroconidia. Microconidia hyaline, 1-celled, ovoid or oblong, borne singly or in chains, 5 - 15 × 2 - 3 µm. Macroconidia hyaline, several-celled, slightly curved or bent at the pointed ends, 3 - 5 septate, 3 septate conidia 25 - 35 × 3 - 4 µm, 5 septate conidia 30 - 50 × 3 - 5 µm.

Specimen examined: Three varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 25, 29 July 2017.

Fusarium fujikuroi *Gibberella fujikuroi* (Sawada) Wollenw., (1931) **(Fig. 2E)**

Colonies are white, floccus to slightly felt. Conidia are hyaline, fusiform, ovate or clavate; one or two celled, measured 26.7-73.6 × 8.1-17.1 µm. Mycelium sparse to densely floccose or felted. Conidiophores hyaline, usually 0-2 septate.

Specimen examined: Three varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 03, 05 September, 2017.

Fusarium solani (Mart.) Sacc., Michelia **2**: 296, 1881, emend. Snyder & Hansen pro. parte, Am. J. Bot. **26**:740, 41. **(Fig. 2F)**

Cottony whitish mycelium was found at the coller parts of the plant and the surrounding soil was infected wilt the pathogen. Growth rate is 3.2 cm; colony greyish-white and aerial mycelium striate, sparse to dense and floccose. Microconidia develops abundantly in the fresh isolates after 2-3 days. They are formed from lateral conidiphores. Microconidia of *F. solani* are also broader and more oval in shape with somewhat thicker walls; they are 8-16×2-4 µm. Macroconidia develop after four to seven days from initially simple but later from short multibranched conidiophores which soon merge to form effuse sporodochia. They are inequilaterally fusoid with many of the spores having the widest diameter in the penultimate cell.

Specimen examined: Two varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 19, 21 August 2018.

Lasiodiplodia theobromae (Pat.) Griff. & Maubl, Bull. Trimest. Soc. Mycol. Fr. **8**:136 (1892) **(Fig. 3A)**

Colonies are greyish brown, cottony, reverse brownish black. Hyphae septate, branched, dark chocolate brown. Pycnidia globose, dark brown, ostiolate. Conidiophore short, hyaline. Conidia dark brown, two-celled, ellipsoidal, 16−22 × 8−12 µm. Pycnidia formed with septate paraphyses between the conidiogenous cells. The conidia measured 20-21.8 × 9.1-10.9 µm. They are initially hyaline, thin-walled and aseptate, cylindrical to sub ovoid in shape.

Specimen examined: Three varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 07, 11 August 2018.

Mucor Fresen **(Fig. 3B)**

Colonies are typically white to beige or grey and are fast-growing. Older colonies become grey to brown in color due to the development of spores. *Mucor* spores or sporangiospores are simple or branched and form apical, globular sporangia that are supported and elevated by a column-shaped columella. *Mucor* can be differentiated from moulds of the genera *Absidia*, *Rhizomucor* and *Rhizopus* by the shape and insertion of the columella, and the lack of rhizoids. Some *Mucor* species produce chlamydospores. They produce mold with irregular, non-septate hyphae branching at wide angles. The tip of the sporangiophore swells to form a globose sporangium that contains uninucleate, haploid sporangiospores. An extension of the sporangiophore called the columella which protrudes into the sporangium. The sporangium walls are easily ruptured to release the spores, which germinate readily to form a new mycelium on appropriate substrates. They may germinate to form hyphae or a sporangium.

Specimen examined: Seven varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 07, 11 May 2018.

Penicillium citrinum Thom in US Dept. Agr. Bur. Amin, Ind Bul. 118, pp. 61-63. Fig. 22.1910. **(Fig. 3C)**

Penicillium citrinum produces septate, hyaline hyphae. Colonies are usually fast growing, in shades of green, sometimes white, mostly consisting of a dense felt of conidiophores. Microscopically, chains of single-celled conidia are produced in basipetal succession from a specialised conidiogenous cell called a phialide. They are 6.5-12.0 µm in diameter and conidia are 1.5-3.15 µm in diameter.

Specimen examined: Fourteen varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 09, 10, 17 August 2017.

Penicillium aculeatum Raper & Fennell (1948). **(Fig. 3D)**

It is characterized by very restricted and comparatively deep colonies on Czapek agar, variously buckeled and wrinkled, irregular in outline, medium sporing, often with a limited overgrowth of red- pigmented hyphae, growing margins 2-3 mm wide, white to slightly pink, odor almost lacking, reverse in vinaceous or purplish red in older areas. Conidiophores short, commonly about 50 µ, rarely upto 100 µ, with walls appearing somewhat granular. Penicilli are relatively shorter and broader than in the preceding species, usually appearing definitely inflated, sterigmata 9-15 µm by 1.5-3.0 µm and conidia are strictly globose to subglobose, 2-3.5 µm in diameter with walls comparatively heavy and strongly echinulate.

Specimen examined: Four varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 14 May 2018.

Rhizopus oryzae Went & Prins. Geerl., (1895) **(Fig. 3E)**

Rhizopus oryzae is a filamentous heterothallic microfungus that occurs as a saprotroph in soil, dung, and rotting vegetation. It differs from *R. oligosporus* and *R. microsporus* by its larger columellae and sporangiospores. It has variable sporangiosphoressuch as straight or curved, swollen or branched, and the walls can be smooth or slightly rough. sporangiosphores are pale brown to brown in colour. Sporangiosphores grow between 210-250 µm in length and 5-18 µm in diameter. The sporangia in *R. oryzae* are globose or subglobose, wall spinous and black when mature, 60-180 µm in diameter. The columellae are globose, subglobose or oval in shape. The wall is generally smooth and pale brown in colour. The average diameter growth ranges from 30-110 µm. It has abundant, root-shaped rhizoids. The stolons are smooth or slightly rough, almost colorless or pale brown, 5-18 µm in diameter. The chlamydospores are abundant, globose ranging from 10-24 µm in diameter, elliptical and cylindrical. Initially colonies are white becoming brownish with age and can grow to about 1 cm thick.

Specimen examined: Three varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 16, 21 May 2018.

Trichoderma viride Pers. (1794) **(Fig. 3F)**

Colony effuses, light green in colour. Conidiophores hyaline, much branched that cluster into fascicles, bearing phialides single or in groups. Broad and straight/flexuous branches. They may have conidial pigments that are either white or bright green in colour. Conidia are usually hyaline,

powdery mass, 1-celled, ovoid shaped and borne in small terminal clusters. It is used in the commercial production of enzyme cellulase.

Specimen examined: Ten varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 12, 14, 17 September 2017.

Meyerozyma guilliermondii (Wick.) Kurtzman & M. Suzuki **(Fig. 3G)**

Meyerozyma guilliermondii (formerly known as *Pichia guilliermondii*) is a species of yeast of the genus *Meyerozyma* whose asexual or anamorphic form is known as *Candida guilliermondii.* Colonies are flat, moist, smooth and cream to yellow in colour on Sabouraud dextrose agar. It does not grow on the surface when inoculated into Sabouraud broth. Pseudohyphae are short and few in number. Cell reproduces by budding, ellipsoidal, ovoidal and clavate, occur singly and in pairs, or short chains, pseudohyphae is formed. Colony flat, moist, smooth, cream to yellow in colour.

Specimen examined: Two varieties of cotton seeds (*Gossypium hirsutum* L.) A. Khatun 16 August 2018.

Molecular identification

Molecular characterization of the fungal species was performed according to Amer *et al.* (2011) with some modifications. For further confirmation of these 29 fungi, ITS sequence based molecular analysis was performed and 19 were confirmed up to species level (Fig. 5).

Genomic DNA was successfully isolated from the nineteen isolates. PCR was conducted using ITS1 (Forward) and ITS4 (Reverse) primers and ~600 bp DNA band was amplified (Fig. 4). Sequence analysis of the amplified DNA through BLAST search in GenBank was conducted and found 92.60-99.81% similarity with partial sequence of 18S ribosomal RNA gene, complete sequence of internal transcribed spacer 1, internal transcribed spacer 2, 5.8S ribosomal RNA gene and partial sequence 28S ribosomal RNA gene of different isolates (Table 1).

Analysis of the nucleotide sequences of the amplified fragments allowed the identification of the isolates at the species level (Table 1 and Fig. 4). ITS1 and ITS4 primers depicted isolate species identities more than 90% sequence similarity. All fungal isolates were identified using the sequences obtained through ITS1 and ITS4 primers. To confirm at the genomic sequence level, PCR amplified bands (**~** 600 bp) from nineteen samples were subjected to automated sequencing followed by BLAST analysis (Fig. 4).

ITS sequences of nineteen samples were analyzed through NCBI-BLAST program database search system. Results obtained from the BLAST database showed that 99.09% nucleotide identities with *Aspergillus aculeatus* isolate KUASN10; 98.93% nucleotide identities with *Aspergillus fumigatus* isolate HF11 and *Aspergillus wentii* strain CBS 131.49; 97.74% nucleotide identities with *Aspergillus flavus* isolate En14; 96.70% nucleotide identities with *Aspergillus tamarii* isolate MH3; 96.51% nucleotide identities with *Aspergillus toxicarius* strain CBS 129270; 99.11% nucleotide identities with *Aspergillus subramanianii* 18S rRNA gene (partial); 99.38% nucleotide identities with *Curvularia lunata* strain AME-83; 98.47% nucleotide identities with *Lasiodiplodia theobromae* strain E42F; 98.31% nucleotide identities with *Rhizopus oryzae* isolate EV62; 97.24% nucleotide identities with *Penicillium aculeatum* strain LP65; 94.97% nucleotide identities with *Penicillium citrinum* isolate 14R-2-F05; 99.74% nucleotide identities with *Fusarium moniliforme* isolate CJBB12-18; 98.15% nucleotide identities with *Fusarium solani* strain GuangX9 and *Fusarium oxysporum* isolate FLS 4; 92.60% nucleotide identities with *Fusarium fujikuroi* isolate EFS3; 92.86% nucleotide identities with *Mucor* sp. isolate 580816; 99.81% nucleotide identities with *Trichoderma viride* strain TVJ-S-1 and 98.25% nucleotide identities with *Meyerozyma guilliermondii* strain Q2 (Table 1).

Table 1. Identification of fungal isolates using ITS sequence comparison with data from GenBank through BLAST search.

Isolates No.	Length (Base pair)	Acc. No.	Description	Max score	Total score	Query coverage (%)	E-value	Identity (%)
1.	573	MN186997	*Aspergillus aculeatus* isolate KUASN10 small 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.	985	985	98%	0.0	99.09
2.	595	MN180857	*Aspergillus flavus* isolate En14 small 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	981	981	97%	0.0	97.74
3.	583	GU183175	*Aspergillus fumigatus* isolate HF11 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	1002	1002	98%	0.0	98.93
4.	608	FR733823	*Aspergillus subramanianii* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), culture collection CCF<CZE>:4008	1013	1013	95%	0.0	99.11
5.	603	MH562046	*Aspergillus tamarii* isolate MH3 small 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	952	952	97%	0.0	96.70
6.	611	MH865314	*Aspergillus toxicarius* strain CBS 129270 small 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.	942	942	98%	0.0	96.51
7.	589	MH856464	*Aspergillus wentii* strain CBS 131.49 small 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	1002	1002	98%	0.0	98.93
8.	620	MG571435	*Curvularia lunata* strain AME-83 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	867	867	79%	0.0	99.38
9.	510	KY425745	*Lasiodiplodia theobromae* strain E42F small 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.	917	917	99%	0.0	98.47
10.	596	MG601177	*Meyerozyma guilliermondii* strain Q2 small 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	996	996	96%	0.0	98.25

Table 1 (Contd.)

Isolates No.	Length (Base pair)	Acc. No.	Description	Max score	Total score	Query coverage (%)	E-value	Identity (%)
11.	545	KX958025	*Penicillium citrinum* isolate 14R-2-F05 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.	873	873	98%	0.0	94.97
12.	590	HQ392496	*Penicillium aculeatum* strain LP65 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, partial sequence	454	454	86%	0.0	97.24
13.	568	KY785016	*Fusarium solani* strain GuangX9 small 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	942	942	98%	0.0	98.15
14.	585	MK371768	*Mucor* sp. isolate 580816 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	845	845	100%	0.0	92.86
15.	560	KF439055	*Trichoderma viride* strain TVJ-S-1 28S ribosomal RNA gene, partial sequence.	942	942	100%	0.0	99.81
16.	598	KU671029	*Fusarium oxysporum* isolate FLS 4 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.	942	942	98%	0.0	98.15
17.	586	MH084746	*Fusarium fujikuroi* isolate EFS3 small 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	911	911	96%	0.0	92.60
18.	576	KC895528	*Gibberella moniliforme* isolate CJBB12-18 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.	993	993	96%	0.0	99.74
19.	621	MK108436	*Rhizopus oryzae* isolate EV62 small 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.	1033	1033	95%	0.0	98.31

From the comparison between morphological and molecular identification, it was clear that out of 19 fungal isolates morphological identification of one fungal isolate did not match with molecular identification. It was *Aspergillus ochraceous* which was identified as *Aspergillus subramanianii* by molecular identification (Table 2). Besides, there were four species of *Aspergillus*, two species of *Fusarium* and two species of *Penicillium* which were difficult to identify up to species label by morphological identifications. The species name of these fungi were easily identified by this molecular technique. Furthermore, one unidentified fungus was detected up to species level employing nucleotide sequences (Table 2).

Table 2. Comparison between morphological and molecular identification of 19 fungal isolates.

lsolates No.	Morphological identification	Molecular identification
1.	*A. flavus*	*A. flavus* isolate En14
2.	*A. fumigatus*	*A. fumigatus* isolate HF11
3.	*A. ochraceous*	*Aspergillus subramanianii* 18S rRNA gene (partial)
4.	*Aspergillus* sp. 1	*Aspergillus aculeatus* isolate KUASN10
5.	*Aspergillus* sp. 2	*Aspergillus tamarii* isolate MH3
6.	*Aspergillus* sp. 3	*Aspergillus wentii* strain CBS 131.49
7.	*Aspergillus* sp. 4	*Aspergillus toxicarius* strain CBS 129270
8.	*Curvularia lunata*	*Curvularia lunata* strain AME-83
9.	*Fusarium fujikuroi*	*Fusarium fujikuroi* isolate EFS3
10.	*Fusarium oxysporum*	*Fusarium oxysporum* isolate FLS 4
11.	*Fusarium* sp. 1	*Fusarium solani* strain GuangX9
12.	*Fusarium* sp. 2	*Fusarium moniliforme i*solate CJBB12-18
13.	*Lasiodiplodia theobromae*	*Lasiodiplodia theobromae* strain E42F
14.	*Mucor* sp.	*Mucor* sp. isolate 580816
15.	*Penicillium* sp. 1	*Penicillium citrinum* isolate 14R-2-F05
16.	*Penicillium* sp. 2	*Penicillium a*culeatum strain LP65
17.	*Rhizopus oryzae*	*Rhizopus oryzae* isolate EV62
18.	*Trichoderma viride*	*Trichoderma viride* strain TVJ-S-1
19.	Unidentified fungus	*Meyerozyma guilliermondii* strain Q2

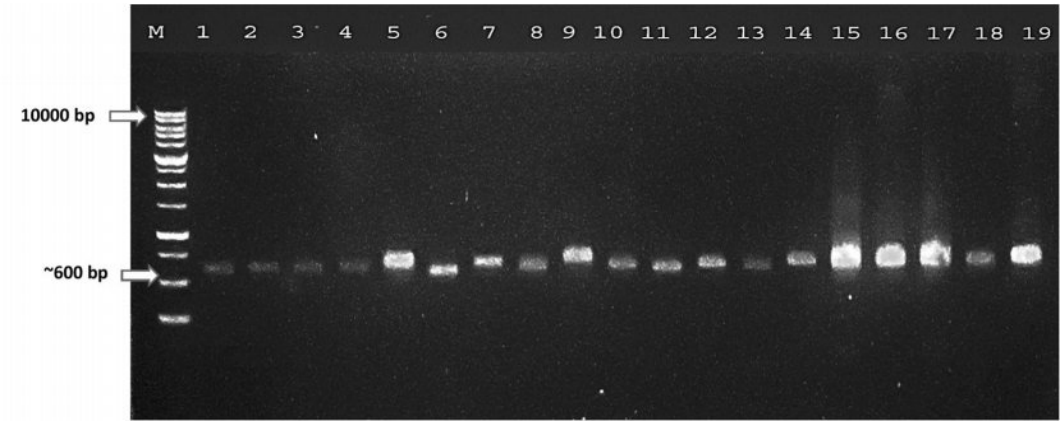

Fig. 4. Gel electrophoresis of the PCR products of 19 fungal isolates performed by ITS1 (F) and ITS4 (R) primers and showing ~600 bp amplification.

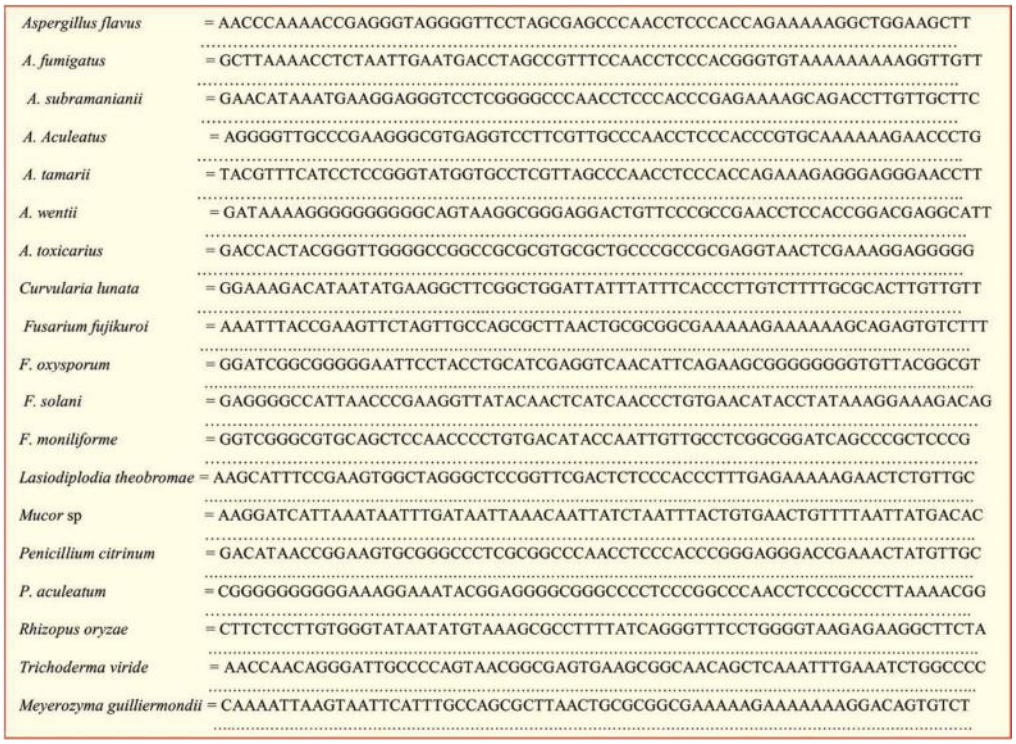

Fig. 5. DNA sequences of the PCR products of isolated fungi.

Among the isolated fungi, *Aspergillus subramanianii, A. toxicarius, A. wentii, Penicillium aculeatum, P. citrinum, Rhizomucor* sp. and *Meyerozyma guilliermondii* have been reported as new records for Bangladesh as these were not documented in relevant literature (Siddiqui *et al*., 2007; Shamsi S, 2017; Nahar *et al.*, 2019).

The present investigation suggests that molecular technique is more accurate and rapid means of fungal identification. ITS-based molecular identification methods might be an important complement to conventional mycological detection by culture.

Acknowledgements

The first author gratefully acknowledges to the "Ministry of Science and Technology", People's Republic of Bangladesh for providing financial support in her research through NST fellowship.

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(Manuscript received on 01 January, 2022; revised on 14 October, 2022)