Characterization and Phylogeny of Fungi from Industrial Wastewater Using Highly Conserved Multiple Gene Loci

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Abstract

The aim of this study was isolation and molecular characterization of fungi from untreated industrial effluent by multigene phylogenetic analyses. The Fungi isolated were characterized based on PCR amplification and genomic sequencing of the internal transcribed spacer region (ITS), partial β-tubulin (Ben A), calmodulin (CaM), and DNA-directed RNA polymerase second large subunit (RPB2) genes, along with morphological characterization and species diversity. Fungal DNA extraction kits and primers sets for the selected genes were purchased and used following the manufacturer's instructions. The obtained sequences were subjected to BLAST analysis and the corresponding fungal isolates were assigned species names after comparison with representative sequences available in GenBank. All the sequences from this study were deposited in GenBank and the accession number assigned. Phylogenetic trees of the fungal isolates were drawn for each gene by the Maximum Likelihood method using MEGA 7.0 software. Fifteen (15) Fungi species belonging to four genera of Aspergillus, Penicillium, Fusarium and Trichoderma with Aspergillus as the predominant genus were identified.

1. Introduction

Fungi are diverse group of eukaryotic organisms characterized as heterotrophic, saprophytic, symbiotic and parasitic due to their achlorophyllous nature. Their cell walls are made up of β-glucans and chitin (Manoharachary et al., 2010). They are known as the second largest after kingdom Animalia with estimate of over 5 million species (Raja et al., 2017). The kingdom fungi is composed of nine phyla mamely; Opisthosporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Zoopagomycota, Mucoromycota, Glomeromycota, Basidiomycota and Ascomycota (Simeos et al., 2015; Yakop et al., 2019). The Ascomycota or sac fungi, are monophyletic and account for approximately 75% of all described fungi. Generally, fungi are significant both economically and medicinally as they are the major decomposers of dead organic matter and contribute significantly in recycling of nutrients in natural and modified ecosystems. They are also found in edible forms, producers of antibiotics, cheese formers and few of them are pathogenic.

Apart from known enormous importance of Fungi, the taxonomy of these organisms is still challenging due to a lack of reliable and advanced techniques for their identification and systematic studies. Morphological or conventional approaches to fungal systematics have some difficulties in determining species boundaries as these techniques of identification rely primarily on phenotypic analysis. For instance they describe colour, texture, spores, colony size and utilization of carbon and nitrogen compounds (Das et al., 2014). Thus other characteristics are needed for identification to species level. Gene sequence based identification of fungi has many advantages over the conventional methods as it does not require viable organisms or sporulation. In order to resolve the difficulties on identification at species level, many advanced techniques have been employed for the identification of fungi with emphasis on house-keeping genes and other target genes through the use of PCR and automated sequencing techniques.

Few of the several genetic markers for rapid classification of fungi having conserved sequences falling within definite microbial clades include internal transcribed spacer regions (ITS), Beta-tubulin genes (Ben A), Calmodulin (CaM) and RNA polymerase II gene (RPB2) (Das et al., 2014; Raja et al., 2017; Yin et al., 2017; Rajeshkumar et al., 2019).

The internal transcribed spacer regions (ITS) are used as official universal DNA barcode for fungi (Das et al., 2014; Yin et al., 2017). The ITS1, ITS2, and ITS4 have been proven to be useful for the identification of yeasts and some fungi such *Aspergillus, Penicillium, Talaromyces, Cryptococcus, candida* and *Trichosporon* among many others (Adeniyi et al., 2018; Rajeshkumar et al., 2019; Yin et al., 2017). However, ITS sequences cannot be used for phylogenetic analyses of unrelated taxa due to low variability and slow evolution. Also, ITS sequences do not always allow correct species differentiation especially among *Aspergillus* and *Penicillium* genera (Houbraken & Samson, 2011; Visagie et al., 2014). Hence, additional gene markers are essential for correct species delineation. Secondary molecular markers such as beta tubulin; calmodulin and RPB2 have been successfully used in fungal genomics (Kolanlarli et al., 2019; Raja et al., 2017; Rajeshkumar et al., 2019; Visagie et al., 2014). Asan et al., 2011 reported that these protein-encoding genes contained highly variable intron regions which contain highly variable introns that evolve at a faster rate compared to ITS.

Beta-tubulin genes are found in all eukaryotes encoding for polypeptide proteins. They have been used for phylogenetic analysis in fungi from the entire kingdom to the species level. Four Beta tubulin genes are found in all fungi; two α-tubulin (tub A) and two β-

tubulin (tub B) genes. Tub A is responsible for the production of two alpha tubulin polypeptides (alpha 1 and alpha 2) while Tub B produces one alpha polypeptide (alpha 2) (Das et al., 2014). Reports have it that beta-tubulin gene sequences contain 3.5-fold more phylogenetic information than the small sub-unit (SSU) rRNA gene, thus it has been reported that it is an ideal marker for analysis of deep-level phylogenies and for complex species groups (Raja et al., 2017; Visagie et al., 2014).

Calmodulin (CaM) is a small acidic protein present in all eukaryotic cells and shown to be highly conserved both functionally and structurally (De Carvalho et al., 2003; De Cássia et al., 2001). Its primary role is to serve as an intracellular Ca^{2+}receptor which signals proliferation, motility, and cell cyclic progression. Ca^{2+}-CaM complexes act by controlling the activity of numerous intracellular proteins such as phosphodiesterase, Ca^{2+}-ATPase, serine protein kinases, and protein phosphatases. It also acts on several metabolic pathways and gene expression regulation in many eukaryotic organisms including fungi (De Carvalho et al., 2003).

Transcription of the eukaryotic nuclear DNA into RNA is carried out by RNA polymerases using ribonucleoside triphosphates as substrates (Gerber et al., 2020; Malkus *et al.,* 2006). RNA polymerase II gene (RPB2) encodes for second largest protein subunit in eukaryotes which synthesizes mRNA precursors and functional non-coding RNAs (Malkus *et al.,* 2006). Vetrovsky *et al.* (2015) reported that RPB2 gene is a viable alternative molecular marker for the analysis of environmental fungal communities due its discriminative power, quantitative representation of community composition and suitability for phylogenetic analyses. Therefore this study was aimed at isolation and molecular identification of fungi from waste water using the selected molecular markers discussed.

2. Materials And Methods

2.1 Collection of untreated refinery effluents

Samples of untreated effluent were collected from waste water channel of Kaduna Refinery and Petrochemical Company (KRPC), Kaduna State, Nigeria. The samples were collected in ten-litre new plastic jerry cans and transported to the laboratory for physicochemical, heavy metal analysis and biosorption studies. The pH and temperature of the effluent were determined at the point of collection.

2.2 Isolation and molecular characterization of test fungi from untreated refinery effluent

2.2.1 Isolation of fungi from untreated waste water

The effluent samples collected were kept to stand at room temperature (30^{o}C) on a sterile laboratory work bench. 10ml of the samples in duplicates were aseptically dispensed in sterile centrifuge tubes and centrifuged at a speed of 250rpm for 10minutes to concentrate the samples. After decanting the supernatant, 0.1ml of the residue of each sample was spread-plated on sterile potato dextrose agar and Malt Extract Agar (MEA) plates in duplicate (containing 50µg/L of chloramphenicol) using sterile bent glass rod. The plates were incubated at room temperature for 7days.

2.2.2 Colony morphology and microscopic characterization of fungal Isolates

Colonies grown on each medium were distinguished on the basis of their surface characteristics such as texture, colour, zonation, sporulation and diameters. The distinguishable colonies were sub-cultured on PDA slant and incubated at room temperature (25^{o}C) for 7days to obtain pure isolates. The microscopic characteristics were carried out by mounting small portion of the growing region

of the fungi on a clean grease free slide with a drop of lacto phenol cotton blue, covered with a cover slip and examined under electron microscope using ×40 objective lens. The isolates were characterized on the basis of the sexual reproductive structures, presence or absence of septation, presence of foot cells and chlamydospores. The isolates were identified using taxonomic guide (Barnett and Hunter; 1999; Hakeem and Bhatnagar 2011; Asan et al., 2011). The pure cultures were maintained in PDA slants and stored in refrigerator for further identification.

2.2.3 Molecular identification of fungal isolates

2.2.3.1 Extraction of fungal genomic DNA

Each of the isolates was grown on potato dextrose agar at room temperature (25^{o}C) for 5 days. This was followed by sub-culturing each isolates into a 250 mL Erlenmeyer flask containing 100 mL potato dextrose broth and incubating for 5 days. The mycelial mass produced by each isolate were separated from the broth by filtration through sterile No. 5 Whatman filter paper. The mycelial mass were crushed using porcelain mortar and transferred to Eppendorf tubes for extraction.

The genomic DNA extraction was carried out using ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's manual instructions. (Viegas et al., 2015; Khandavilli et al., 2016; Yin et al., 2017; Adeniyi et al., 2018).

2.2.3.1 PCR amplification of the target genes

Primers specific for internal transcribed spacer region (ITS), beta-tubulin gene (benA), calmodulin gene (CaM) and RNA polymerase II second largest subunit (RPB2) loci are presented in Table 1.

PCR amplification of the extracted DNA was performed in a 20 µL reaction mixture as follow: 1 µL gDNA template, 0.2 µL DNA polymerase, 0.5 µL each forward and reverse primers, 1µL dNTPs and sterile double distilled water to a final volume of 20 µL.

The thermocycler was programed for the following PCR conditions: initial denaturation at 94 ^{o}C for 5 min, 35 cycles of denaturation at 94^{o}C for 45 s, annealing at 55 ^{o}C for 45s, and extension at 72 ^{o}C for 1 min, with a final extension at 72 ^{o}C for 10 min. For the amplification of RPB2 gene region, touch-up PCR conditions of 5 cycles with annealing temperature 48 °C followed by 5 cycles at 50 °C and final 25 cycles at 52 °C were used. After complete amplification, the PCR products were analyzed for gel electrophoresis by using 1% agarose gel (1g of agarose in 100ml of Tris buffer) with ethidium bromide as the staining agent (Houbraken & Samson, 2011).

2.3 Sequencing and phylogenetic analysis

The fungal isolates were identified by DNA sequencing according to standard protocols. Sequencing was carried out in a 28µl reaction mixture as follows: 4µl of each primer, 8µl of purified DNA and 16µl of PCR water and the samples was sequenced with the Di- Deoxy Terminator sequencer. The contigs (formed from forward and reverse sequences) obtained were analyzed using BioEdit 7.2.5 software and aligned using Clustal W of MEGA 7.0 software (Goujon et al., 2010; Kumar et al. 2015; Yin et al., 2017). The corresponding fungal isolates were assigned species names after comparison with representative sequences available in NCBI

(National Center for Biotechnology Information). The newly obtained sequences were deposited in GenBank and accession number assigned.

The evolutionary history of the fungi was analyzed using the Maximum Likelihood (ML) method based on the Tamura-Nei model of MEGA 7 (Kumar et al. 2015; Sidiq et al., 2016). The bootstrap tree formed from 1000 replicates represents the evolutionary history of the taxa analyzed The percentage associated taxa clustered together in the bootstrap test (1 000 replicates) is shown next to the branches (Houbraken et al., 2010)

3. Results And Discussions

3.1 Cultural and microscopic characteristics of fungal isolates

Fifteen (15) fungal isolates consisting of four genera; *Aspergillus, Penicillium, Fusarium* and *Trichoderma* were obtained in this study with *Aspergillus* being the predominant genera (Table 2, Figures 1a and 1b).The results revealed the cultural features of the isolates (F1 to F23) in terms of colour, surface characteristics, reverse, edge and diameter. The microscopic features of the isolates are presented in Figures 2(a) and 2(b) showing the conidia, spores and conidiophores. *Aspergillus* species had septate hyphae, hyaline conidiophores and radial conidial head bearing the spores (Figure 2a). *Penicillium* species appeared as septate hyphae with conidiophores and secondary branches (metulae). The metulae bear flasked shaped phialides with unbranched chains of round conidia (Figure 2b). *Fusarium* spp showed septate hyphae, multiseptate canoe shaped macroconidia attached to the conidiophores (Figure 2b). *Trichoderma* spp appeared as septate hyphae, short conidiophores which are flask shaped clustering together at the end of each phialides (Figure 2b).

The genus *Aspergillus* is one of the most well researched fungi genera with over 200 officially recognized species (Oyebanji et al., 2018). The ubiquitous nature of *Aspergillus* may be due to their saprophytic feeding habit as well as their ability to grow in a wide range of environment (Machido et al., 2014). This observation sturdily indicates that members of these fungal genera isolated, have the capacity to survive and withstand toxic effects of polycyclic aromatic hydrocarbons (Ezeonuegbu et al., 2014).

3.2 Polymerase Chain Reaction (PCR) of fungal isolates obtained

The fungal isolates from this study were further subjected to molecular identification, by amplifying the internal transcribed spacer (ITS), beta tubulin gene (Ben A), calmodulin gene (CMD) and RNA Polymerase II Second Largest Subunit (RPB2) genes of the fungal isolates (Figures 3-6).

3.2.1 Amplification of internal transcribed spacer

Thirteen out of the fifteen isolates were positive to PCR amplification of ITS regions with amplicon sizes of 600 base pairs (Figure 3). *Aspergillus niger* (F3) and *P. simplicissimum* (F23) represented by lanes 4 and 16 respectively were negative. Although, the ITS region is widely used as universal primers for fungi, it is not sufficient for identifying most fungi to specie level due to their low variability and slow evolution (Houbraken & Samson, 2011; Tiwari et al., 2011). Visagie et al. (2014) however suggested the use of other molecular markers for accurate identification of fungal species and phylogenetic relationships. Other secondary identification markers for *Aspergillus* and *Penicillium* species (and other ascomycetes) used in this study were beta tubulin; calmodulin and RPB2. These protein-encoding genes contained highly variable intron regions (Asan et al., 2017; Ozdil et al., 2017; Yin et al., 2017).

3.2.2 Amplification of beta-tubulin gene

Amplification of the beta tubulin gene revealed isolates represented by lanes 2, 4, 7, 10, 12 respectively were positive with amplicon sizes of 600 base pairs (Figure 4). Isolates represented by lanes 3, 5 and 15 respectively had amplicon sizes of approximately 550bp. Also, isolates represented by lanes 9, 11, 14 and 16 respectively had amplicon sizes of 500bp. Fungi represented by lanes 6, 8 and 13 respectively were negative (Figure 4).

Amplification of the partial beta-tubulin gene was successful in thirteen isolates (13) out of the fifteen (15) isolates amplified. The amplicon sizes obtained were in the range of 480-600bp. This results were similar to those obtained in previous studies. For instance, Ashtiani et al., (2017), Eulalia *et al.* (2018) and Kamarudin & Zakaria, (2018) who amplified *Aspergillus* fragments of beta tubulin genes with amplicon sizes in the range of 550 to 600bp. Samson et al. (2014) and Erika et al. (2012), obtained beta tubulin gene amplicon sizes of ranging from 432 to 550bp for *Aspergillus, Penicillium* and other fungal species. Beta-tubulin genes are found reliably in all eukaryotes and have been used for phylogenetic analysis in fungi from the entire kingdom to the species level. It is an ideal marker for analysis of deep-level phylogenies and for complex species groups. Reports have shown that beta tubulin genes have more resolution compared to the ITS region. This amount of variation is suitable for phylogenetic relationship among closely related species of *Penicillium* and *Aspergillus* species (Asan et al., 2019; Samson et al., 2014).

4.6.3 Amplification of RPB2 gene

The amplified partial RPB2 genes of the isolates revealed that only two isolates, *P. citrinum* (F19) and *P. citrinum* (F19D) represented by lanes 5 and 7 were positive with amplicon sizes of approximately 650 and 600 base pairs respectively (Figure 5). This result is in agreement with the studies of Houbraken & Samson, (2011) who identified *Penicillium citrinum* using RPB2 genes.

4.6.4 Amplification of Calmodulin gene

The result of the amplified calmodulin genes of the isolates showed that *A. niger* (F5), *A. niger* (F10) and *P. citrinum* (F19) represented by lanes 5, 9 and 15 had sizes of 500bp, 550 bp and 500bp respectively (Figure 6).

The calmodulin gene has been considered important for the identification of *Aspergillus* species, and some reports have even stated it should be used as the primary gene for identification of *Aspergillus* species (Ashtiani et al., 2017; Samson et al., 2014).

4.7 Gene Sequences of Fungal Isolates

The identifications based on cultural features were confirmed by sequence analysis of the isolates. Basic Logical Alignment Search Tool (BLAST) results of ITS region, Beta-tubulin, RPB2 gene and calmodulin gene sequences of this study in National Center for Biotechnology Information (NCBI) provided indications of possible relationships and similarities with reference sequences in GenBank. The amplified sequences of each gene were submitted to GenBank, and their accession numbers are shown in Table 3 which revealed that most isolates had above 95% similar identity to reference sequences of GenBank.

There has been little or no extensive research on identification of the Fungi using different molecular marker approach in Nigeria. Focus has been more on macroscopic and microscopic features.

4.8 Phylogenetic Tree

Phylogenetic trees of the fungal isolates revealed that the isolates were clustered in grouping pattern of close resemblance. The percentage of trees in which the associated taxa are clustered is shown next to the branches. Sequences from this study are shown in red colours while sequences from GenBank are shown in black. Test of phylogeny was bootstrap of 1000 replications. Phylogenetic tree based on ITS gene revealed that the alignment matrix contained 54 nucleotide sequences with 209 positions in the final dataset. All isolates of *Aspergillus* and *Penicillium* species were clustered with ex-type strains from Genbank with cluster identity of above 95%. The tree was out grouped by *T. erinaceum* (Figure 7).

Beta-tubulin gene alignment matrix contained 52 nucleotide sequences with with 19 positions in the final dataset. All the fungal species had above 85% cluster similarity with ex-type from GenBank while *P. simplicissimum* was placed in the out group (Figure 8).

Phylogenetic tree based on partial RPB2 gene revealed that the alignment matrix involved 19 nucleotide sequences with a total of 404 positions in the final dataset. The two positive isolates of *Penicillium citrinum* shared 90% cluster similarities with sequences from GenBank (Figure 9).

Phylogenetic tree based on calmodulin gene had an alignment matrix of 14 sequences. *A. niger* (F5D) shared 87% cluster similarity while the two isolates of *A. sydowii* (F7 and F7D) had equal (98%) cluster similarities with sequences from GenBank. *A. niger* (F5) falls in the outgroup (Figure 10). The phylogenetic trees revealed that related species are clustered together indicating a clear and well resolved classification and evolutionary history of the isolates (Asan et al., 2019; Kamarudin & Zakaria, 2018; Ozdil et a, 2017).

Conclusion

1. There has been little or no extensive research on identification of the Fungi using different molecular marker approach in Nigeria. Focus has been more on cultural and microscopic features. The fungal isolates from this study were further subjected to PCR amplification coupled with DNA sequencing of four molecular genes markers.
2. The fungal species isolated consist of the following genera; *Aspergillus, Penicillium, Fusarium* and *Trichoderma* with *Aspergillus* being the predominant genera. Sequence results obtained revealed above 95% similarities between the isolates in this study and those found in GenBank.
3. The identification and molecular characterization of the fungal isolates to specie level, gave a better results by PCR amplification and sequencing of ITS region, partial beta tubulin, calmodulin and RPB2 genes. Therefore should be used as molecular markers for species level identification of fungi (especially *Aspergillus* and *Penicillium* as proved by this study).

Declarations

Conflict of interest

The authors declare that they have no competing financial interests or personal relationships that could have influence the work reported in this paper.

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Tables

Table 1: Primers used for the amplification of specific genes in the fungal isolates

Locus	Primer	Direction	Oligonucleotide Sequence (5'–3')	Length (bp)	Reference
Internal Transcribed Spacer (ITS)	ITS1 ITS4	Forward Reverse	TCC GTA GGT GAA CCT GCG G TCC TCC GCT TAT TGA TAT GC	600	White *et al.* 1990 (Houbraken & Samson, 2011) (Yin et al., 2017)
β-tubulin (BenA)	Bt2a Bt2b	Forward Reverse	GGT AAC CAA ATC GGT GCT GCT TTC ACC CTC AGT GTA GTG ACC CTT GGC	550	(Houbraken et al., 2010) (Houbraken & Samson, 2011) (Yin et al., 2017)
Calmodulin (CaM)	CMD5 CMD6	Forward Reverse	CCG AGT ACA AGG ARG CCT TC CCG ATR GAG GTC ATR ACG TGG	580	(Houbraken & Samson, 2011) (Yin et al., 2017)
RNA polymerase II second largest subunit (RPB2-1)	5F 7CR	Forward Reverse	GAY GAY MGW GAT CAY TTY GG CCC ATR GCT TGY TTR CCC AT	700	(Houbraken et al., 2010) (Houbraken & Samson, 2011) (Yin et al., 2017)

Table 2: Cultural characteristics of fungal isolates from untreated refinery effluent

Isolate Code	Colour	Surface characteristics	Edge	Reverse colour	Colony diameter mm) (mean±SD)	Identity of isolates
F1	mint green	powdery	white, circular	cream	0.75±0.21	*Aspergillus flavus*
F2	Black	granular	white, irregular	cream	2.50±1.02	*Aspergillus japonicus*
F3	brownish-black	black	grey, irregular	black	2.70±0.14	*Aspergillus niger*
F5	Black	granular	black, irregular	cream	1.90±1.02	*Aspergillus niger*
F6	pale pink	granular	light pink, irregular	white	2.35±0.07	*Aspergillus melleus*
F7	dark-green	cottony	white, irregular	white	1.35±0.14	*Aspergillus sydowii*
F8	White	smooth	white, circular	cream	1.20±0.28	*Fusarium incarnatum*
F10	black	granular	white, irregular	cream	2.80±0.97	*Aspergillus niger*
F12	whitish gray	smooth	white, circular	cream	0.90±0.00	*Penicillium shearii*
F13	whitish-green	granular	circular	white	8.00±0.00	*Trichoderma erinaceum*
F14	white	smooth	white irregular	cream	1.80±0.00	*Aspergillus quadrilineatus*
F16	greenish blue	smooth	white, circular	white	1.15±0.07	*Aspergillus fumigatus*
F18	white	cottony	white, irregular	cream	1.85±0.07	*Aspergillus sydowii*
F19	bluish-green	cottony	white, irregular	white	1.30±0.00	*Penicillium citrinum*
F23	Dark green	cottony	white, irregular	white	1.25±0.07	*Penicillium simplicissimum*

Table 3. Accession numbers of amplified nucleotide sequences from fungal. Isolates

Fungi	Isolate	ITS		Beta-tubulin		Calmodulin		RPB2	
		Identity (%)	Accession No.	Identity (%)	Accession No.	Identity (%)	Accession No.	Identity (%)	Accession No.
Aspergillus flavus	F1	96.89	MK828704	100	MH180047	-	-	-	-
A. flavus	F1D	-	-	100	MG517775	-	-	-	-
A. japonicus	F2	99.11	MK840963	100	MH208743	-	-	-	-
A. japonicus	F2D	97.53	MK840964	-	-	-	-	-	-
A. niger	F3	100	MK828713	99.79	HQ632731	-	-	-	-
A. niger	F3D	-	-	100	MH781323	-	-	-	-
A. niger	F5	98.99	MK840965	100	MH781323	98.48	JX500080	-	-
A. niger	F5D	97.75	MK840966	99.59	LC389053	98.87	MG991517	-	-
A. melleus	F6	96.71	MK840967	-	-	-	-	-	-
A. sydowii	F7	98.95	MK828705	100	MH426599	96.86	LN898812	-	-
A. sydowii	F7D	99.45	MK828710	100	MH644075	96.63	LN898808	-	-
Fusarium incarnatum	F8	-	-	98.83	KT374271	-	-	-	-
F. incarnatum	F8D	-	-	98.90	KJ020856	-	-	-	-
A. niger	F10	99.41	MK828708	100	MH781319	-	-	-	-
A. niger	F10D	-	-	100	MH208814	-	-	-	-
A. fumigatus	F11	99.27	MK816855	-	-	-	-	-	-
Penicillium shearii	F12	98.96	MK840968	-	-	-	-	-	-
P. shearii	F12D	95.83	MK828709	-	-	-	-	-	-
T. erinaceum	F13	98.53	MK840969	-	-	-	-	-	-
A. quadrilineatus	F14	97.98	MK840970	-	-	-	-	-	-
A. fumigatus	F16	-	-	100	MH781343	-	-	-	-
A.fumigatus	F16D	-	-	100	MH781334	-	-	-	-
A. sydowii	F18	99.41	MK828707	100	LC367596	-	-	-	-
A. sydowii	F18D	97.34	MK828706	97.34	MK828706	-	-	-	-
P. citrinum	F19	99.27	MK828711	99.63	MG991339	-	-	99.27	MK828711
P. citrinum	F19D	99.54	MK840969	-	-	-	-	99.27	MK828711
P. simplicissimum	F23	98.93	MK840973	99.02	GU981631	-	-	-	-
P. simplicissimum	F23D	99.27	MK828712	99.32	GU981632	-	-	-	-

"-"denotes no clear PCR products were obtained using primers from Table 1

Figures

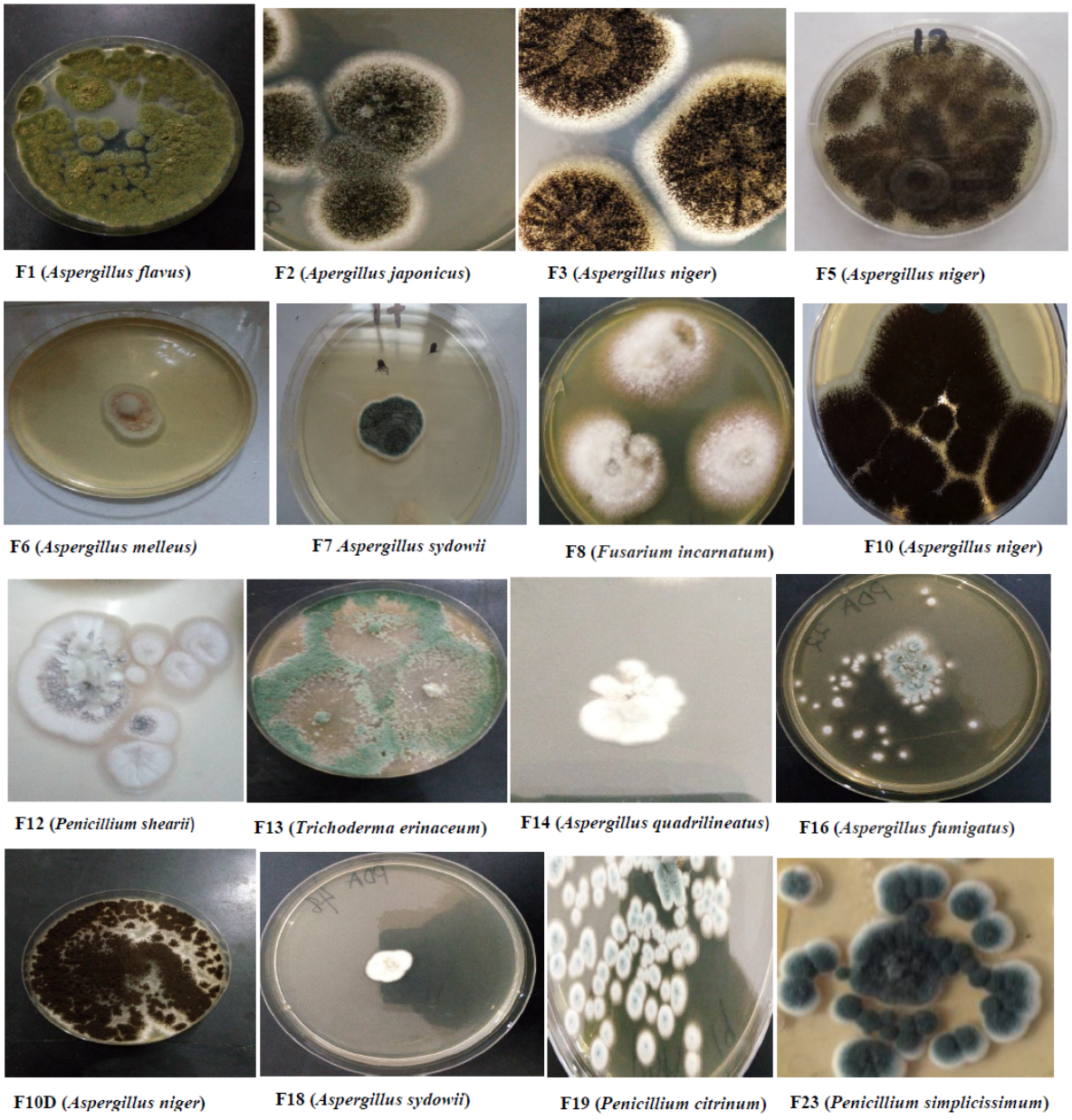

Figure 1

(a): Cultural pictures of isolated fungi (b): Cultural pictures of isolated fungi

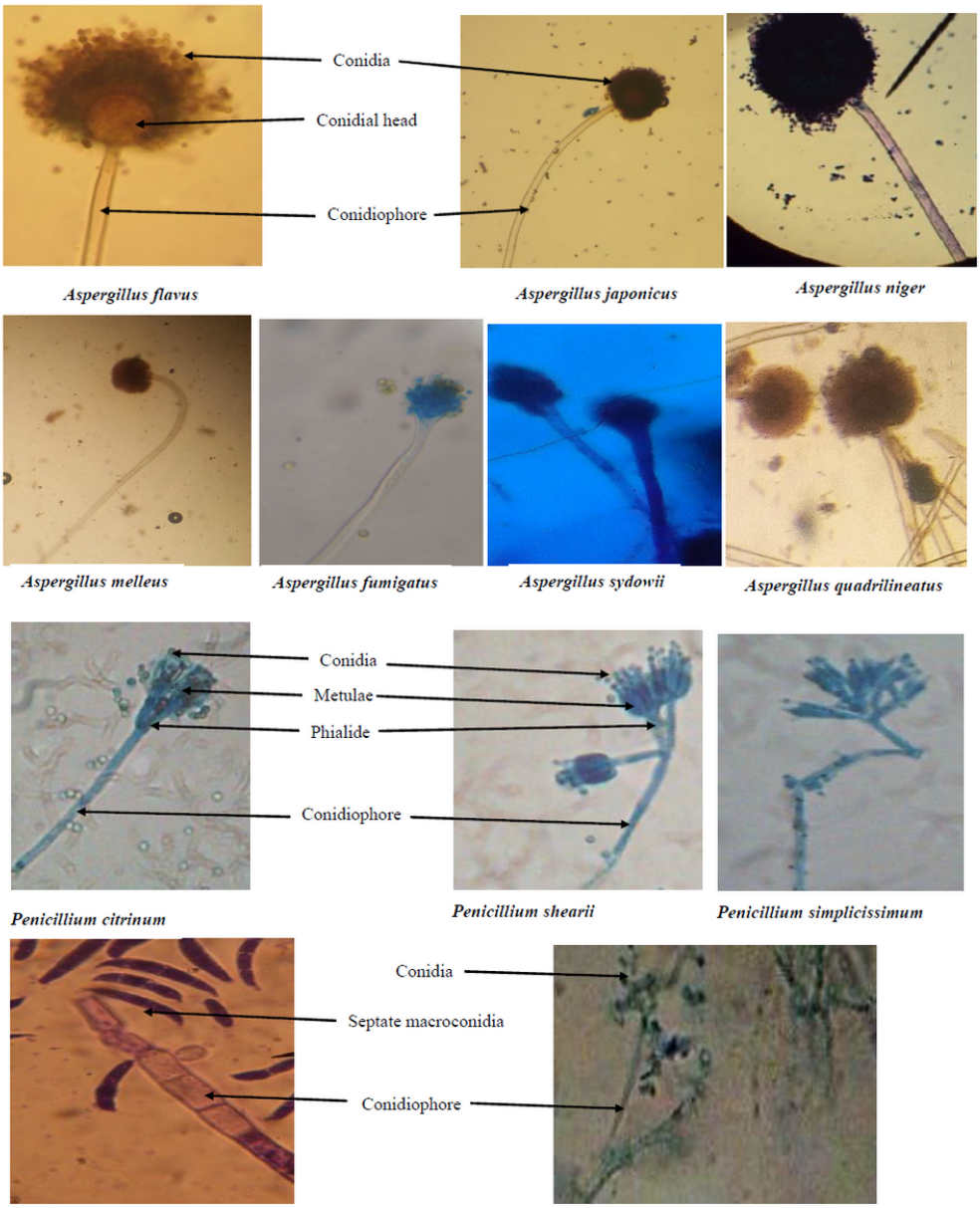

Figure 2

(a): Microscopic features of Aspergillus species (x40 magnification) (b): Microscopic features of Penicillium, Fusarium and Trichoderma species (x40 magnification)

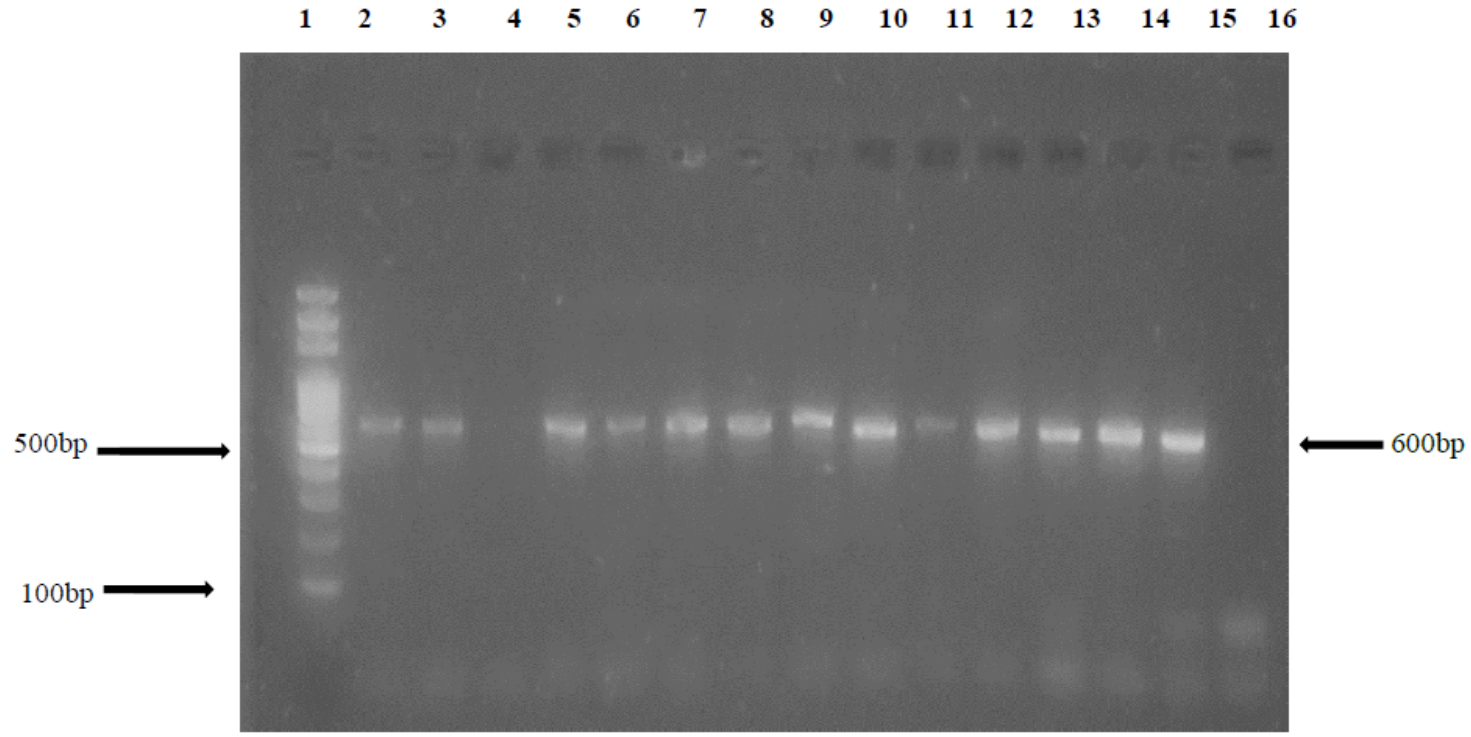

Figure 3

Agarose gel electrophoresis of PCR products amplified from genomic DNA of fungal isolates using ITS (ITS1-ITS4). Keys: Lane 1= 100bp solisbiodyne DNA Ladder; Lane 2 – Aspergillus flavus (F1); Lane 3=A. japonicus (F2D); Lane 4= A. niger (F3); Lane 5= A. niger (F5); Lane 6= A. melleus (F6); Lane 7= A. sydowii (F7); Lane 8=Fusarium incarnatum (F8); Lane 9= A. niger (F10); Lane 10= Penicillium shearii (F12); Lane 11=Trichoderma erinaceum (F13); Lane 12=A. quadrilineatus (F14); Lane 13= A. fumigatus (F16); Lane 14= A. sydowii (F18); Lane 15= Penicillium citrinum (F19); Lane 16= Penicillium simplicissimum (F23); bp= base pair; ITS=internal transcribed spacer region

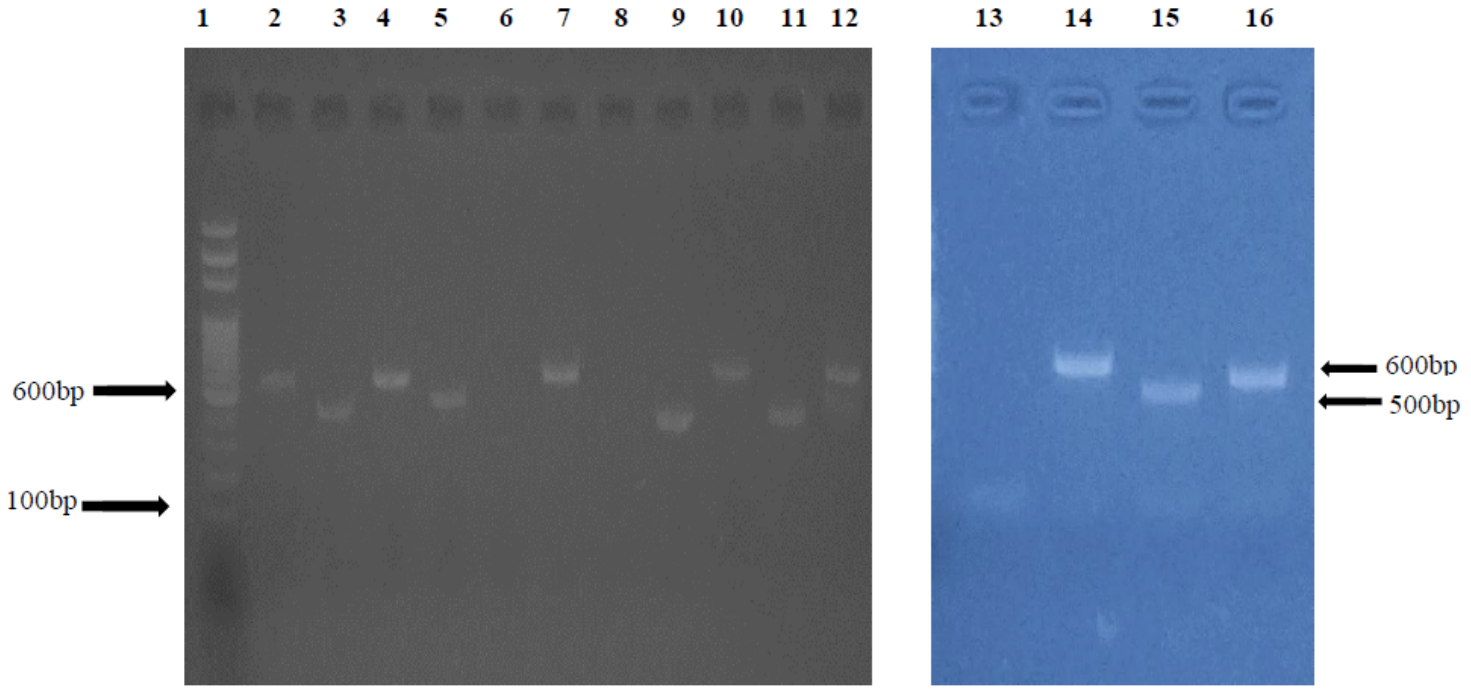

Figure 4

Agarose gel electrophoresis of PCR products amplified from genomic DNA of fungal isolates using β-tubulin genes (Bt2a-Bt2b). Keys: Lane 1= 100bp solisbiodyne DNA Ladder; Lane 2=Aspergillus. flavus (F1); Lane 3=A. japonicus (F2D); Lane 4= A. niger (F3); Lane 5= A. niger (F5); Lane 6= A. melleus (F6); Lane 7= A. sydowii (F7); Lane 8=Fusarium incarnatum (F8); Lane 9= A. niger (F10); Lane 10= Penicillium shearii (F12); Lane 11=Trichoderma erinaceum (F13); Lane 12=A. quadrilineatus (F14); 13= A. fumigatus (F16); Lane 14= A. sydowii (F18); Lane 15= Penicillium citrinum (F19); Lane 16= Penicillium simplicissimum (F23); bp= base pair

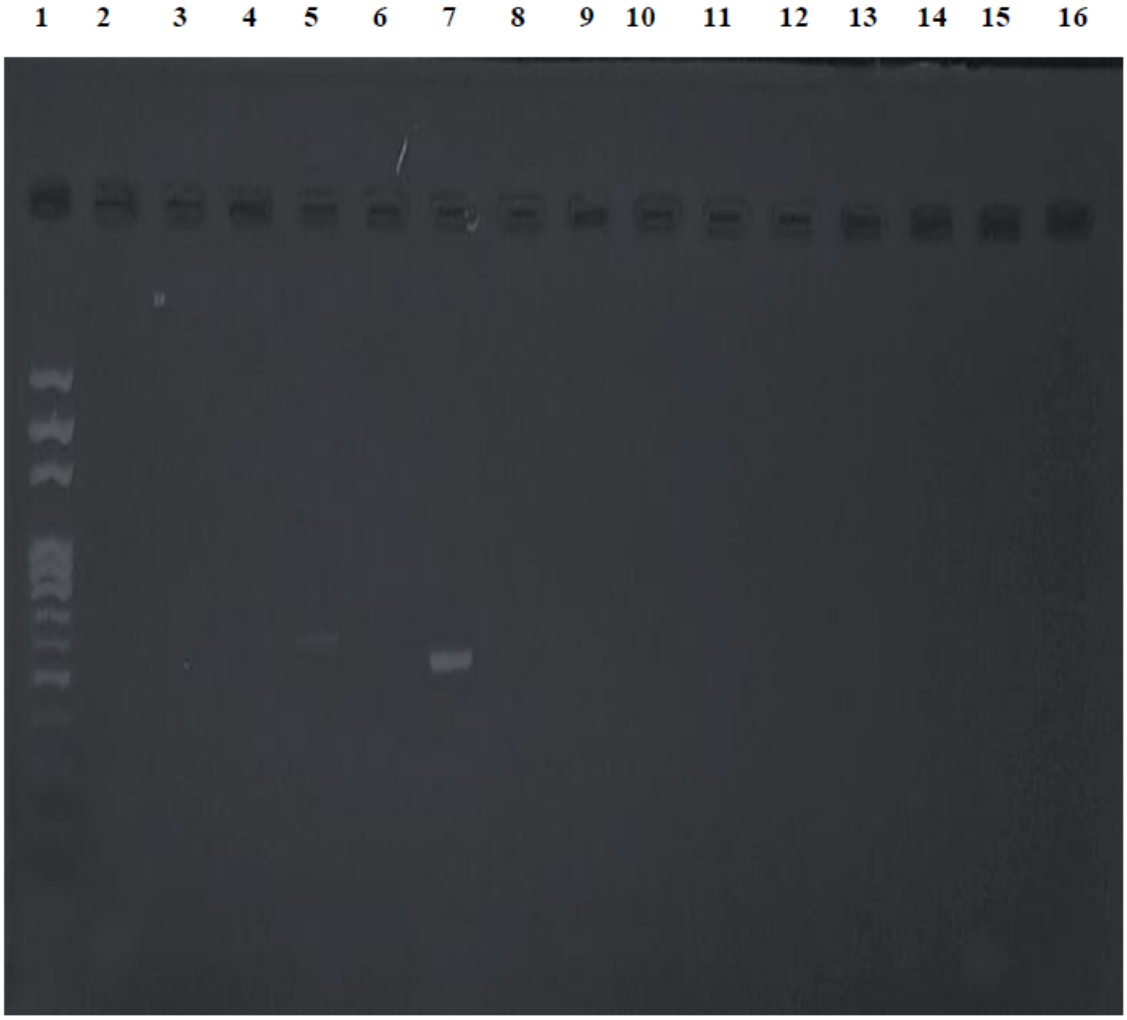

Figure 5

Agarose gel electrophoresis of PCR products amplified from genomic DNA of fungal isolates using RPB2 genes (5F-7CR). Keys: Lane 1= 100bp solisbiodyne DNA Ladder; Lane 2= Aspergillus flavus (F1); Lane 3=Aspergillus japonicus (F2D); Lane 4=Aspergillus niger (F5D); Lane 5= Penicillium citrinum (F19); Lane 6= Penicillium shearii (F12); Lane 7= Penicillium citrinum (F19D); Lane 8= Aspergillus melleus (F6); Lane 9= Fusarium incarnatum (F8); Lane 10= Aspergillus niger (F10); Lane 11=Trichoderma erinaceum (F13); Lane 12= Trichoderma erinaceum (F13); bp = base pair, RPB2= RNA Polymerase II Second Largest Subunit

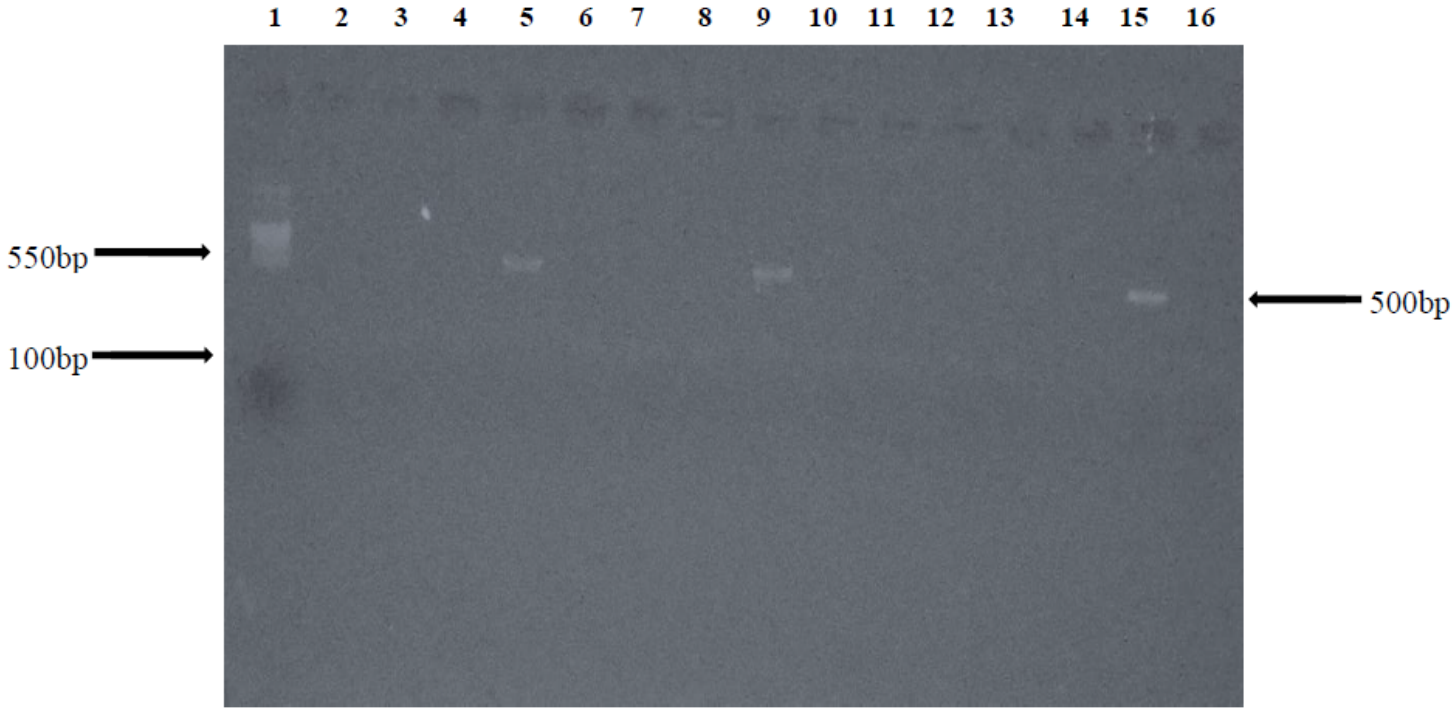

Figure 6

Agarose gel electrophoresis of PCR products amplified from genomic DNA of fungal isolates using calmodulin gene primers (CMD5-CMD6). Keys: Lane 1= 100bp solisbiodyne DNA Ladder; Lane 2 – Aspergillus flavus (F1); Lane 3=Aspergillus japonicus (F2D); Lane 4= Aspergillus niger (F3); Lane 5= Aspergillus niger (F5); Lane 6= Aspergillus melleus (F6); Lane 7= Aspergillus sydowii (F7); Lane 8=Fusarium incarnatum (F8); Lane 9= Aspergillus niger (F10); Lane 10= Penicillium shearii (F12); Lane 11=Trichoderma erinaceum (F13); Lane 12=Aspergillus quadrilineatus (F14); Lane 13= Aspergillus fumigatus (F16); Lane 14= Aspergillus sydowii (F18); Lane 15= Penicillium citrinum (F19); Lane 16= Penicillium simplicissimum (F23); bp= base pair

Figure 7

Phylogenetic tree of partial ITS gene sequences by maximum likelihood

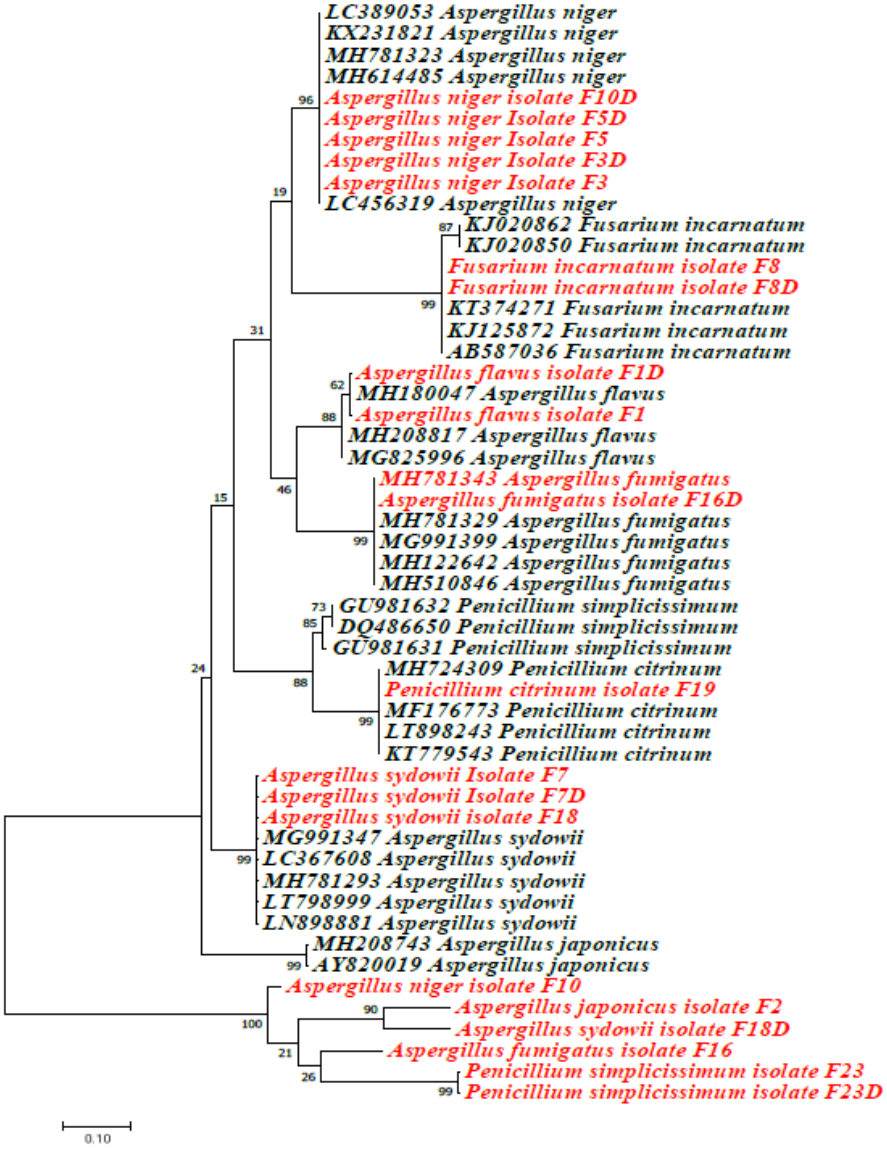

Figure 8

Phylogenetic tree of partial β-tubulin gene sequences by maximum likelihood

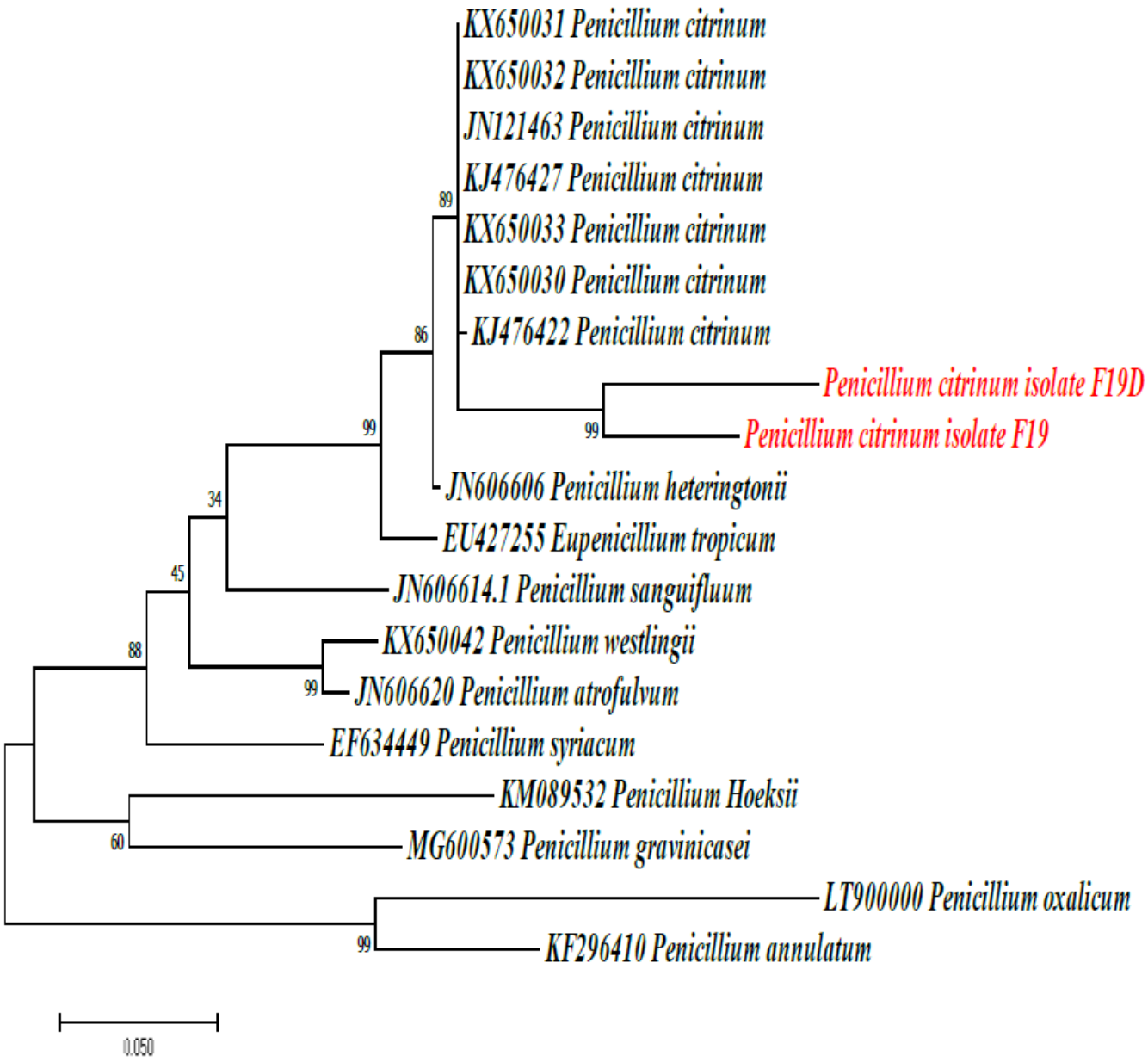

Figure 9

Phylogenetic tree of partial RPB2 gene sequences by maximum likelihood

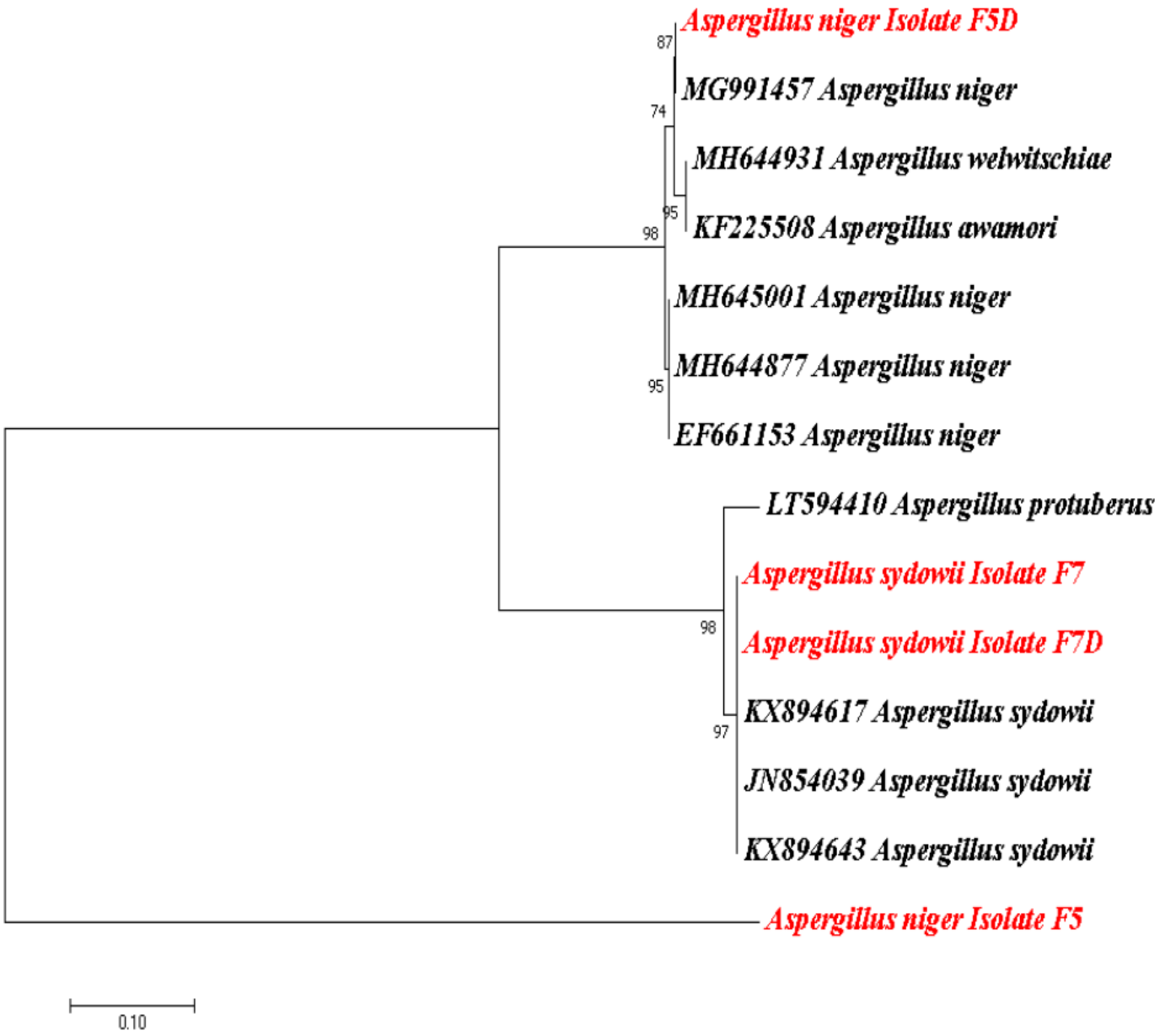

Figure 10

Phylogenetic tree of calmodulin gene sequences by maximum likelihood