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MOLECULAR CHARACTERIZATION AND PATHOGENICITY TEST ON CITRUS FRUITS BY GREEN MOLD AND BLUE MOLD

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ABSTRACT

Citrus fruits are essential for preventing various health conditions, including diabetes, neurological diseases, and cancer. Among the postharvest diseases affecting citrus fruits, *Penicillium digitatum* and *Penicillium italicum* are particularly significant. This study aimed to *characterize Penicillium spp.* isolated from three citrus varieties: orange (*Citrus sinensis*), small orange, and Malta. Pathogenicity tests confirmed that these *Penicillium* isolates were capable of infecting the tested citrus fruits. For molecular characterization, PCR amplification of the D1/D2 domain of 26S rDNA was performed using universal primers, which target the conserved regions of the nucleotide sequence. The PCR products were inserted into the pGEM-T Easy vector and transformed into *E. coli* Dh5α. The presence of the D1/D2 domain was verified by endonuclease digestion with EcoR1. Sequencing was conducted using the T7 promoter primer, and the resulting DNA sequences were analyzed with the DNAMAN analysis system. Sequence analysis revealed that the D1/D2 region of 26S rDNA from the orange isolate showed 99% similarity with *Penicillium sp*., while the D1/D2 region from the small orange isolate had 99.84% similarity with *P. digitatum* strain CBS 112082. The D1/D2 region from the Malta isolate showed 100% similarity with *P. digitatum*. Multiple sequence alignments among the three *Penicillium* isolates revealed a 98.81% identity. This study highlights the use of molecular techniques for understanding the pathogenicity of *Penicillium spp*. in citrus fruits.

Keywords: Pathogenicity, Sequencing, Ligation, *Penicillium* sp., Citrus Fruits

INTRODUCTION

Citrus fruits are among the most significant fruit crops globally, cultivated in over 135 countries under diverse climatic conditions **(Tayel *et al.*, 2016).** With an annual production of approximately 80 million tons, citrus fruits hold substantial economic value worldwide. In Bangladesh, the Department of Agriculture Extension (DAE) reported in 2021 that around 164,008 metric tons of citrus fruits were produced from 6,615 hectares of land. Citrus cultivation is widespread across the country, and the demand for these fruits continues to rise, making them readily available in local fruit markets. Postharvest diseases significantly impact citrus production, with green mold caused by *Penicillium digitatum* and blue mold caused by *Penicillium italicum* being particularly destructive. According to **Liu *et al.*, (2023),** *P. digitatum* and *P. italicum* are major pathogens affecting various citrus fruits, including orange, mandarin, grapefruit, and lemon. Globally, over 25% of citrus fruits are affected by postharvest losses, primarily due to fungal infections **(Tayel *et al.*, 2016).** Among these pathogens, green mold and blue mold are the most severe contributors to citrus decay (**Kai Chen *et al.*, 2019).**
Different races of *Penicillium* spp. exhibit varied pathogenic interactions with citrus varieties such as orange, Malta, and lemon. Traditional methods for identifying *Penicillium* species rely on morphological characteristics, which can be inadequate for precise identification. Conventional techniques often fail to provide comprehensive microbial taxonomy as they mainly describe shape, color, size, staining properties, motility, host range, pathogenicity, and carbon source utilization **(Prakash *et al.*, 2007)**. Additionally, fungi's slow growth and the complexity of conventional identification methods necessitate high expertise and can lead to ambiguities **(Siqueira and Rocas, 2005).**
The D1-D2 large subunit region of the 26S rDNA is recognized as a reliable marker for DNA-based species identification and is recommended for use alongside mitochondrial markers in broad-scale studies **(Sonnenberg *et al.*, 2007). Sugita and Nishikawa (2003)** demonstrated that the D1/D2 domain of 26S rDNA is more effective than the internal transcribed spacer (ITS) region for fungal identification, noting that more sequence data for D1/D2 26S rDNA are available in DNA databases compared to ITS data. This study focuses on identifying postharvest diseases in orange, small orange, and Malta citrus varieties caused by *Penicillium digitatum* and *Penicillium italicum*. To the best of our knowledge, this is the first research addressing postharvest rot in these fruits within the context of Bangladesh. This study aims to molecularly characterize various *Penicillium spp*. isolated from different citrus varieties. Additionally, it seeks to develop a phylogenetic tree to elucidate the relationships among these isolates and evaluate their pathogenicity through cross-inoculation experiments with different citrus varieties from postharvest decaying conditions. To the best of my knowledge, no such research on the postharvest rotting state of the mentioned fruits is available in the context of Bangladesh.
Therefore, in the proposed study, molecular characterization was carried out to identify the different *Penicillium* spp. from different varieties of citrus fruits. Moreover, a phylogenetic tree was developed to observe the relationship among the identified *Penicillium* spp., and their pathogenicity were evaluated by cross-inoculation with different varieties of citrus fruits.

MATERIALS AND METHODS

This chapter deals with the experimental aspect of the work. The materials used and methods followed in this experiment have been presented in this chapter.

Experimental Site

The experiment was conducted at Plant Disease Clinic (PDC) and Plant Pathology Laboratory of the Department of Plant Pathology, Patuakhali Science and Technology University (PSTU), Dumki, Patuakhali.

Plate 1 Blue mold infected orange, small orange and malta

Sample Collection and Preservation

Three types of green mold-infected citrus fruits—orange, small orange, and banana (Plate 1)—were collected from Pirtala Bazar, near Patuakhali Science and Technology University, Dumki, Patuakhali. The samples were stored in clean polythene bags at 4°C in the refrigerator until further use.

Isolation of Pathogenic Fungi

Penicillium spp. was isolated from the green mold-infected citrus fruits using the tissue planting method. The working area was sanitized with 70% ethanol, and the fruits were thoroughly washed to remove dust. The infected surfaces were cut into 5 mm pieces from the lesions' advancing edges and treated with a 10% Clorox solution for 1 minute. After surface sterilization, the pieces were rinsed with sterile distilled water three times.

The sterilized tissues were placed on Petri dishes containing sterile PDA medium (15–20 ml) and positioned 1 cm from the edge using flame-sterilized forceps. Five tissue pieces were placed on each PDA plate, labeled, wrapped with brown paper, and incubated at 25°C. After 3 days, fungal growth was observed, continuing for up to 10 days. Mycelial growth and sporulation around the tissue pieces were examined under a stereo binocular microscope. Pure fungal colonies were identified by their mycelia and spores. Marked mycelial tips were transferred with a cork borer into PDA plates or PDA slants and incubated at 25°C. Once pure fungal colonies grew, the PDA plates and slants were stored at 4°C for preservation and further use.

Extraction of Fungal Genomic DNA

DNA was extracted using DNAzol reagent (Molecular Research Center Inc., Cincinnati, OH) and the Cenis method. *Penicillium* isolates were initially cultured in liquid medium (potato dextrose broth) (Plate 2). Fifty milligrams of fresh mycelium from liquid culture were placed into a 2 ml Eppendorf tube, suspended, and lysed in 1 ml of DNAzol reagent by inverting 5–6 times. The mixture was incubated for 10 minutes at room temperature, with shaking every 3 minutes.

To efficiently remove tissue debris, phenol, proteins, and lipids, an additional elution step with 500 µL of chloroform was performed. After centrifugation at 10,000 rpm and 4°C for 10 minutes, the supernatant was transferred to a new 1.5 ml Eppendorf tube. Then, 0.5 ml of 100% ethanol per milliliter of lysate was added, mixed by inverting 5–6 times and incubated for 3 minutes at room temperature. To increase DNA yield, the mixture was centrifuged at 10,000 rpm for 5 minutes. The pellet was washed first with 500 µL of 70% DNAzol reagent and 30% ethanol, then with 500 µL of 70% ethanol. After discarding the supernatant, the pellet was exposed to air for 10 minutes and dissolved in 100 µL of sterile distilled water.

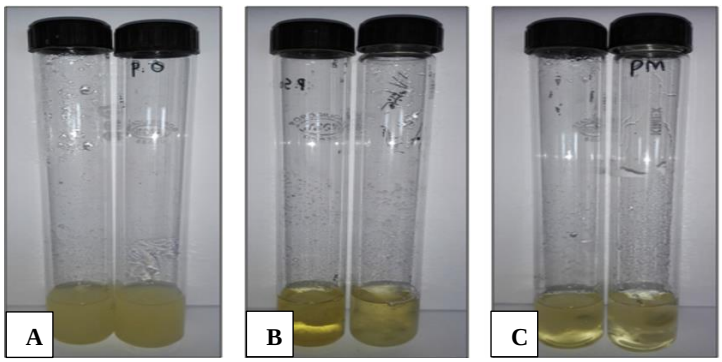

Plate 2 Liquid culture of *Penicillium* isolates from (A) orange, (B) small orange, and (C) malta

PCR for amplification of D1/D2 domains of fungal 26S rDNA

The D1/D2 domains of fungal 26S ribosomal DNA (rDNA), illustrated in Figure 1, were amplified using polymerase chain reaction (PCR). For this amplification, we employed the following universal primers:

Forward primer # 103 F 5'-ACCCGCTGAAYTTAAGCATAT-3′

Reverse primer # 103 R 5'-CTCCTTGGTCCGTGTTTCAAGAC-3′

Following chemical were used in the PCR mixture of 50 µL:

Table 1 Name of the chemicals and used doges

Sl. No.	Name of the chemicals	Amount
1	Fungal DNA (150 ng/ml)	3 µL
2	Forward primer #103F (10 pmol)	5 µL
3	Reverse primer #103R (10 pmol)	5 µL
4	dNTPs (mM)	5 µL
5	DNA polymerase	1 µL
6	10X buffer	5 µL
7	$MgCl_2$	5 µL
8	ddH_2O	21µL
	Total volume	50 µL

Table 1.1 PCR condition was as follows

Step		Time	Temperature	Comments
First		5 Minutes	95°C	
	Second	30 Seconds	95°C	Denaturation
		30 Seconds	51°C	Anneling
		45 Second	72°C	Anneling
Third		10 Minutes	72°C	
Fourth		∞	4°C	

Agarose Gel Electrophoresis and DNA Purification

The PCR products were separated by agarose gel electrophoresis. The gel was prepared with 1.5 grams of agarose per 100 milliliters of distilled water, and 6 microliters of ethidium bromide (EtBr) were added. The gel was run at 100 volts for 40 minutes, as illustrated in Plate 3. After electrophoresis, the gel was examined using a gel documentation system, where DNA bands were visualized under ultraviolet light. Bands corresponding to the desired DNA fragment, approximately 640 base pairs (bp) in size, were excised from the gel using a sterile blade and transferred to an Eppendorf tube. DNA purification was then performed using a DNA purification kit. The target product, estimated to be around 650 bp, was extracted from the gel with the Gel Purification Kit (FavorPrep GEL/PCR Purification Mini Kit, Favorgen Biotech Corp.).

Figure 1 Schematic representation of the fungal ribosomal RNA genes, highlighting the D1/D2 domains of 26S rDNA, the Internal Transcribed Spacer (ITS), and the Intergenic Spacer (IGS).

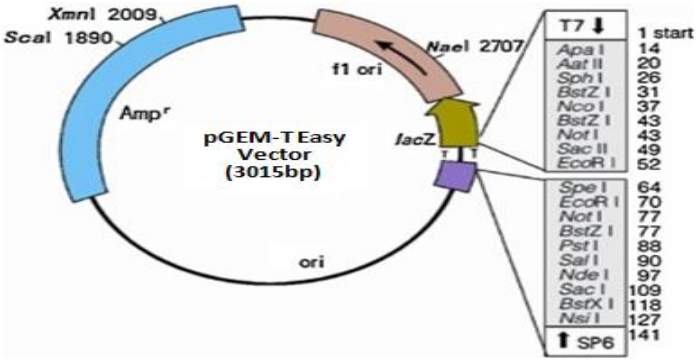

Figure 2 pGEM-T Easy vector having multiple cloning site (MCS) and marker gene and origin of replication (ori).

Cloning of D1/D2 domain of fungal 26S rDNA for phylogenetic reconstruction

D1/D2 domains of fungal 26S rDNA were cloned into the pGEM-T Easy vector (Promega, WI, USA) according to **Islam *et al*., 2010**. Initially the D1/D2 domains of 26S rDNA were cloned into pGEM-T Easy vector (Fig. 2) with the following ligation mixture.

Table 2 Name of the chemicals and used doges

Sl. No.	Name of the chemicals	Amount
1	pGEM-T Easy vector	1 µL
2	Amplified DNA	8 µL
3	T4 Ligase enzyme	1 µL
4	Ligase buffer (2X)	10 µL
	Total volume	50 µL

The above-mentioned mixture was kept at 4°C for ligation.

Transformation

The ligated vector containing the insert was transformed into *E. coli* DH5α cells following the protocol described by **Islam *et al.* (2010).** Competent *E. coli* DH5α cells were initially prepared and stored at -80°C. For the transformation procedure, 10 minutes before transformation, the competent cells were kept on ice. The ligation mixture was then added to the ice-cold competent cells and incubated on ice for 30 minutes. Following this, the mixture was subjected to a heat shock by placing it in a 42°C water bath for 1.5 minutes to facilitate DNA uptake. The cells were then rapidly cooled on ice for 10 minutes. Subsequently, 1 mL of LB broth was added to the Eppendorf tube containing the cells, and the mixture was incubated with shaking at 37°C for 1.5 hours. The transformed *E. coli* DH5α cells were plated on LAXI agar plates containing Luria-Bertani (LB) broth, ampicillin, X-gal (bromo-chloro-indolyl-galactoside), dimethylformamide (DMSO), and IPTG (isopropylthio-β-galactoside). The plates were incubated at 37°C overnight. Colonies that appeared blue and white the next day were analyzed. White colonies were indicative of successful insertion of the pGEM-T Easy vector with 26S rDNA, whereas blue colonies contained self-ligated pGEM-T Easy vectors. White colonies, along with a few blue ones, were selected from the LAXI plate. All cloning procedures followed the methods outlined by **Sambrook and Russell (2001).**

Plasmid Isolation and Confirmation of Recombination

Plasmid DNA from recombinant colonies was extracted using the FavorPrep Plasmid Extraction Mini Kit (Favorgen Biotech Corp.). The white colonies, which contained the recombinant plasmid, were cultured in LB broth supplemented with ampicillin and IPTG.

Sequencing and Analysis

Nucleotide sequencing of the D1/D2 domains of the 26S rDNA was performed using the dideoxy chain-termination method at the National Institute of Biotechnology, Savar, Dhaka. The obtained DNA sequences were analyzed with the DNAMAN analysis system. Sequence similarity searches for 26S rDNA were conducted using BLASTn on the NCBI website **(https://www.ncbi.nlm.nih.gov).**

Deposition of Nucleotide Sequences in Gene Bank

The DNA sequences of the D1/D2 domains of 26S rDNA from *Penicillium* sp. isolates were submitted to the National Center for Biotechnology Information (NCBI) for deposition in Gene Bank, and accession numbers were assigned.

Development of Phylogenetic Tree

Phylogenetic analysis was carried out using the neighbor-joining method. Bootstrap analysis was performed with data resampled 1,000 times using the DNAMAN analysis system. Reference sequences were sourced from the National Center for Biotechnology Information (NCBI) database.

RESULTS AND DISCUSSION

Citrus fruits are commonly affected by postharvest pathogens, particularly *Penicillium digitatum* and *Penicillium italicum*, throughout the fruit storage cycle **(Louw *et al.*, 2015).** Among these, *Penicillium* species are the primary culprits in citrus fruit spoilage **(Nishat *et al.*, 2017).** This study focused on the impact of *Penicillium* species on different varieties of oranges, including Citrus sinensis varieties such as orange, small orange, and malta. The study involved isolating *Penicillium* species from these fruits, evaluating their pathogenicity, and performing molecular characterization. Additionally, a phylogenetic tree was constructed to examine the relationship between the isolates and related *Penicillium* species.

Isolation of *Penicillium* Species

Penicillium species were isolated from three types of citrus fruits—orange, small orange, and malta—collected from local markets in Pirtala Bazar, Dumki, and Patuakhali (Plate 4). The isolates from oranges displayed irregular, rough surfaces with wavy colonies on PDA medium. In contrast, *Penicillium* isolates from malta formed round-shaped colonies with smooth surfaces on PDA medium. All isolates exhibited white mycelial margins, with notable sporulation. The fruit was extensively covered by white mycelium, followed by the appearance of green spores from *P. digitatum* and bluish spores from *P. italicum* **(Saleh *et al.*, 2020).**

Pathogenicity Test of *Penicillium* Isolates

The pathogenicity of all *Penicillium* isolates was assessed by inoculating them onto orange, small orange, and malta fruits (Plate 5). Results indicated that all isolates were capable of infecting the citrus fruits within 6 days of inoculation. Both *P. digitatum* and *P. italicum* were confirmed as significant pathogens affecting citrus fruits such as oranges, mandarins, grapefruits, and lemons. Research has shown that these pathogens can infect fruits during various stages including in the grove, packinghouse, and throughout distribution and marketing **(Louw *et al.*, 2015).**

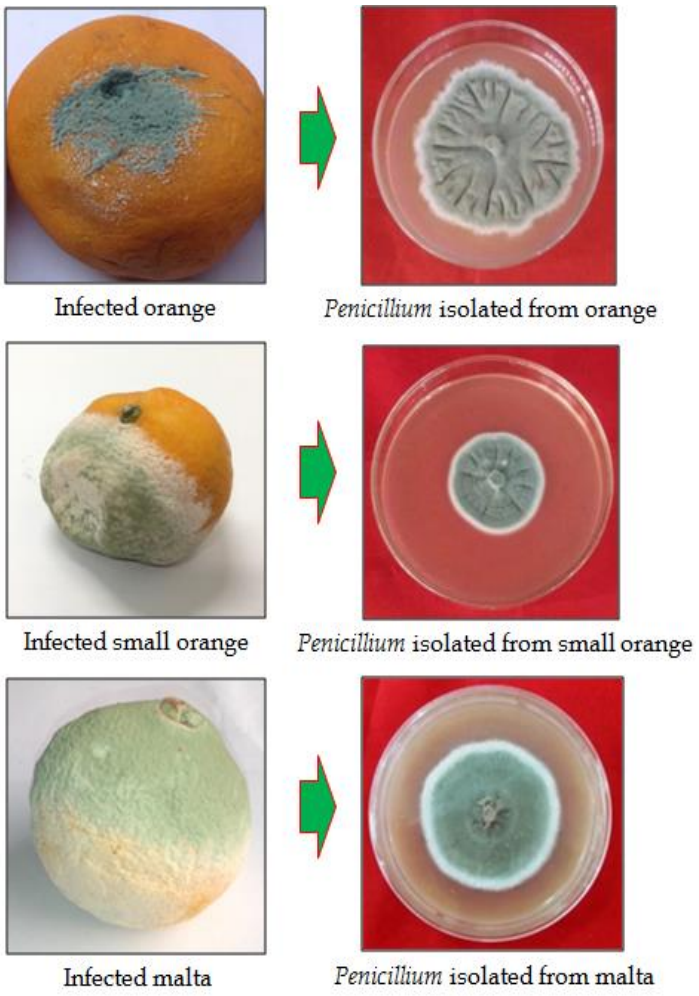

Plate 4 Isolates of *Penicillium* spp. from orange, small orange and malta

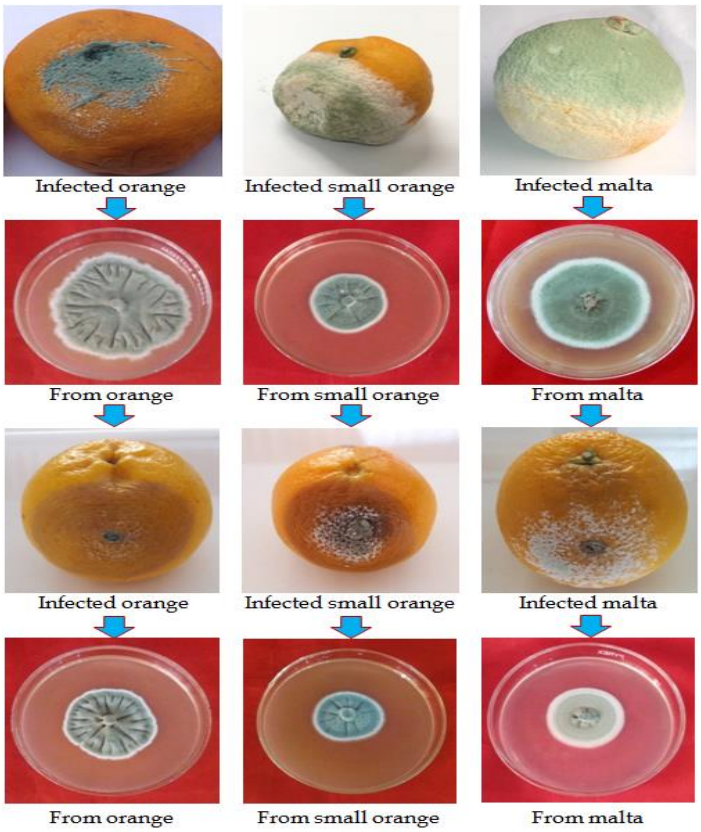

Plate 5 Pathogenecity test of the isolates of *Penicillium* spp. from orange, small orange and malta.

PCR amplification of the D1/D2 domain of 26S rDNA

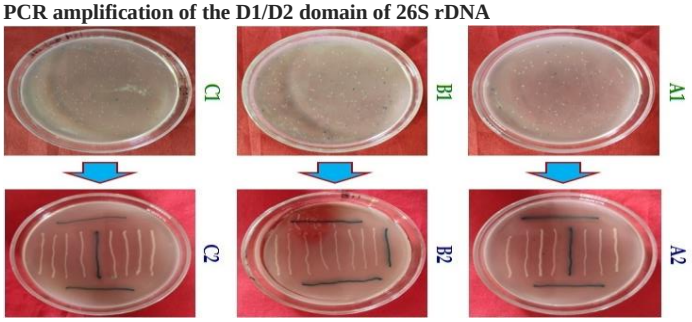

Plate 6 Recombinant *E. coli* Dh5α having only pGEM-T Easy vector (blue colonies) and pGEM-T Easy + D1/D2 of 26S rDNA (white colonies). White colonies having D1/D2 of 26S rDNA of *Penicillium* spp. from (A1) orange, (B1) small orange, and (C1) malta, and A2, B2, and C2 are the picked colonies, respectively.

PCR amplification of the D1/D2 domain of 26S rDNA was performed using the universal primers: forward primer #103F (5'-ACC CGC TGA AYT TAA GCA TAT-3') and reverse primer #103R (5'-CTC CTT GGT CCG TGT TTC AAG AC-3'). The amplification products were analyzed by agarose gel electrophoresis and visualized under ultraviolet light. DNA bands of approximately 640 base pairs (bp) were observed, as shown in Plate 6. This result is consistent with similar studies, such as Nwaiwu (2016), which identified *Saccharomyces cerevisiae* using a 600 bp PCR amplicon of the D1/D2 domain region of 26S rRNA.

Cloning of the D1/D2 domain of the fungal 26S rDNA and transformation into *E. coli* Dh5α

The PCR products, approximately 640 bp in size, were purified and subsequently cloned into the pGEM-T Easy vector using T4 DNA ligase. The resulting construct, pGEM-T Easy with the D1/D2 domain insert, is illustrated in Figure 3. This construct was then transformed into *E. coli* DH5α cells. The transformed cells were plated on LAXI agar plates containing LB broth, ampicillin, X-gal, and IPTG, and incubated overnight at 37°C to allow for colony growth.
Following incubation, blue and white colonies were observed on the LAXI plates. White colonies indicated the successful insertion of the D1/D2 domain into the pGEM-T Easy vector, while blue colonies contained only the self-ligated vector without the insert. Several white colonies, along with a few blue colonies, were selected from the LAXI plates for further analysis.

Plasmid isolation and conformation for recombination

The plasmids were isolated from the white colonies having pGEM-T Easy + D1/D2 of 26S rDNA. The pGEM-T Easy + D1/D2 of 26S rDNA were confirmed by the endonuclease digestion with *Eco*R1 (Plate 8). Two DNA bands were observed after digestion with *Eco*R1, one band was approximately 3 kb, which was most probably for pGEM-T Easy another was approximately 0.64 kb, which was most probably for D1/D2 of 26S rDNA. The plasmid having these two DNA bands were initially conformed that the plasmid having D1/D2 of 26S rDNA.

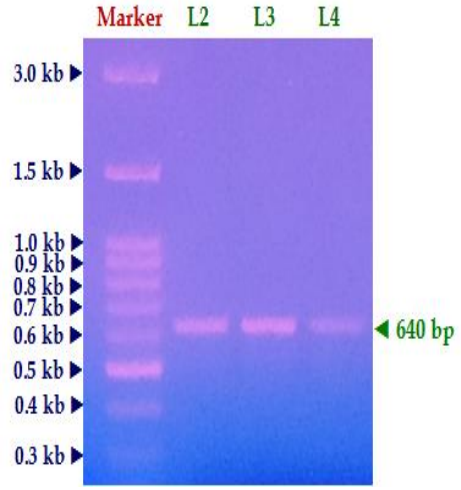

Plate 7 Agarose gel electrophoresis image showing PCR amplification of the D1/D2 domain of 26S rDNA from *Penicillium* spp. The gel lanes are as follows:

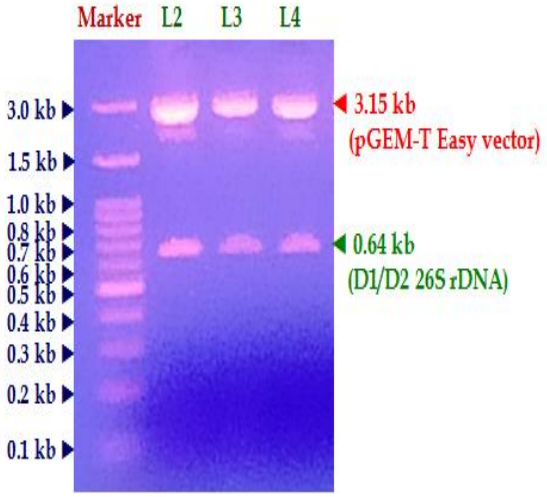

Plate 8 Agarose gel electrophoresis picture of endonuclease enzyme digested of pGEM-T Easy + D1/D2 of 26S rDNA with *Eco*R1 enzyme, where D1/D2 of 26S rDNA of *Penicillium* spp. from (L1) orange, (L2) small orange and (L3) malta.
Legend: L1: Molecular marker (100 bp ladder), **L2**: Amplified D1/D2 domain of 26S rDNA from orange, **L3**: Amplified D1/D2 domain of 26S rDNA from small orange, **L4**: Amplified D1/D2 domain of 26S rDNA from malta

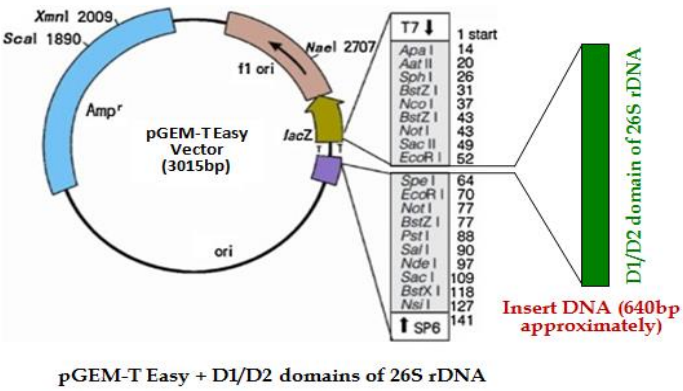

Figure 3 Schematic representation for the vector construction of the pGEM-T Easy with D1/D2 domains of 26Sr DNA.

Sequencing and Analysis

The confirmed pGEM-T Easy constructs containing the D1/D2 domain of 26S rDNA were sent to the National Institute of Biotechnology, Savar, Dhaka, for sequencing. Sequencing was performed using the T7 promoter primer (5'-TAA TAC GAC TCA CTA TAG GGG-3'). The sequences for the three *Penicillium* isolates are illustrated in Figure 4.

The DNA sequences were analyzed using the DNAMAN analysis system. Details for the D1/D2 domain of 26S rDNA from different isolates are as follows: From Orange (PLP-Org.1): The sequence was 642 bp long, with nucleotide composition of 26% A, 23% C, 32% G, and 20% T. The molecular weights were 199.52 kDa for ssDNA and 395.8 kDa for dsDNA. From Small Orange (PLP-Sor.1): The sequence was 639 bp long, with nucleotide composition of 26% A, 24% C, 32% G, and 19% T. The molecular weights were 198.49 kDa for ssDNA and 394.00 kDa for dsDNA. From Malta (PLP-Malt.1): The sequence was 637 bp long, with nucleotide composition of 25% A, 24% C, 32% G, and 19% T. The molecular weights were 197.91 kDa for ssDNA and 392.70 kDa for dsDNA.

Similarity Search for Matching

Similarity searches for the 26S rDNA sequences were conducted using BLASTn on the NCBI website (https://www.ncbi.nlm.nih.gov) to compare with existing DNA sequences in the NCBI Gene Bank and identify the fungal strains. The results were as follows: Orange (PLP-Org.1): The sequence showed the highest similarity of 98.75% with *Penicillium* sp. MG-2017a (accession number: LT898171), as depicted in Figure 5. Small Orange (PLP-Sor.1): The sequence exhibited 99.84% similarity with *Penicillium digitatum* strain CBS 112082 (accession number: MH874465), as shown in Figure 6. Malta (PLP-Malt.1): The sequence displayed 100% similarity with *Penicillium digitatum* strain CBS 112082 (accession number: MH874465), as illustrated in Figure 7. These results align with findings from other researchers who have used the D1/D2 domain of 26S rDNA for fungal strain identification **(Kurtzman and Robnett, 1998; Sonnenberg *et al.*, 2007; Dagar *et al.*, 2011).**

PLP-Org.1

Sequence Length: 642 base pairs (bp)
Composition: Adenine (A): 164 (26%), Cytosine (C): 148 (23%), Guanine (G): 204 (32%), Thymine (T): 126 (20%), Other: 0
Molecular Weight: Single-Stranded DNA (ssDNA): 199.52 kDa, and Double-Stranded DNA (dsDNA): 395.8 kDa
ORIGIN
1 ACCCCGCTGA AATTTAAGCA TATCAATAAA GCGGAGGGAA AAGAACCCAA CAGGGATTGC
61 CCCAGTAACG GCGAGTGAAG CGGCAAGAGC TCAAATTTGA AAGCTGGCTC CTTCGGGGTC
121 CGCATTGTAA TTTGCAGAGG ATGCTTCGGG AGCGGTCCCC ATCTAAGTGC CCTGGAACGG
181 GACGTCATAG AGGGTGAGAA TCCCGTATGG GATGGGGTGT CCGCGCCCGT GTGAAGCTCC
241 TTCGACGAGT CGAGTTGTTT GGGAATGCAG CTCTAAATGG GTGGTAAATT TCATCTAAAG
301 CTAAATATTG GCCGGAGACC GATAGCGCAC AAGTAGAGTG ATCGAAAGAT GAAAAGCACT
361 TTGAAAAGAG AGTTAAAAAG CACGTGAAAT TGTTGAAAGG GAGGCGCTTG CGACCAGACT
421 CGCTCGCGGG GTTCAGCCGG CATTCGTGCC GGTGTATTTC CCCGCGGGCG GGCCAGCGTC
481 GGTTTGGGCG GTCGGTCAAA GGCCCTCGGA AGGTAACGCC CCTAGGGGCG TCTTATAGCC
541 GAGGGTGCAA TGCGACCTGC CTAGACCGAG GAACGCGCTT CGGCTCGGAC GCTGGCATAA
601 TGGTCGTAAG CGACCCGTCT TGAAACACGG AACCAAAGGA GA

PLP-Sor.1

Sequence Length: 639 base pairs (bp)
Composition: Adenine (A): 163 (26%), Cytosine (C): 152 (24%), Guanine (G): 202 (32%), Thymine (T): 122 (19%), Other: 0
Molecular Weight: Single-Stranded DNA (ssDNA): 198.49 kDa and Double-Stranded DNA (dsDNA): 394.0 kDa
ORIGIN
1 ACCCCGCTGA AACTTAAGCA TATCAATAAG CGGAGGAAAA GAAACCAACA GGGATTGCCC
61 CAGTAACGGC GAGTGAAGCG GCAAGAGCTC AAATTTGAAA GCTGGCTCCT TCGGGGTCCG
121 CATTGTAATT TGCAGAGGAT GCTTCGGGAG CGGTCCCCAT CTAAGTGCCC TGGAACGGGA
181 CGTCATAGAG GGTGAGAATC CCGTATGGGA TGGGGTGTCC GCGCCCGTGT GAAGCTCCTT
241 CGACGAGTCG AGTTGTTTGG GAATGCAGCT CTAAATGGGT GGTAAATTTC ATCTAAAGCT
301 AAATATTGGC CGGAGACCGA TAGCGCACAA GTAGAGTGAT CGAAAGATGA AAAGCACTTT
361 GAAAAGAGAG TTAAAAAGCA CGTGAAATTG TTGAAAGGGA AGCGCTTGCG ACCAGACTCG
421 CTCGCGGGGT TCAGCCGGCA CTCGTGCCGG TGTACTTCCC CGCGGGCGGG CCAGCGTCGG
481 TTTGGGCGGT CGGTCAAAGG CCCTCGGAAG GTAACGCCCC TCGGGGCGTC TTATAGCCGA
541 GGGTGCAATG CGACCTGCCC AGACCGAGGA ACGCGCTTCG GCTCGGACGC TGGCATAATG
601 GTCGTAAGCG ACCCGTCTTG AAACACGGAA CCAAGGAGA

PLP-Malt.1

Sequence Length: 637 base pairs (bp)
Composition: Adenine (A): 162 (25%), Cytosine (C): 150 (24%), Guanine (G): 202 (32%), Thymine (T): 123 (19%), Other: 0
Molecular Weight: Single-Stranded DNA (ssDNA): 197.91 kDa, and Double-Stranded DNA (dsDNA): 392.7 kDa
ORIGIN
1 ACCCGCTGAA ATTTAAGCAT ATCAATAAGC GGAGGAAAAG AAACCAACAG GGATTGCCCC
61 AGTAACGGCG AGTGAAGCGG CAAGAGCTCA AATTTGAAAG CTGGCTCCTT CGGGGTCCGC
121 ATTGTAATTT GCAGAGGATG CTTCGGGAGC GGTCCCCATC TAAGTGCCCT GGAACGGGAC
181 GTCATAGAGG GTGAGAATCC CGTATGGGAT GGGGTGTCCG CGCCCGTGTG AAGCTCCTTC
241 GACGAGTCGA GTTGTTTGGG AATGCAGCTC TAAATGGGTG GTAAATTTCA TCTAAAGCTA
301 AATATTGGCC GGAGACCGAT AGCGCACAAG TAGAGTGATC GAAAGATGAA AAGCACTTTG
361 AAAAGAGAGT TAAAAAGCAC GTGAAATTGT TGAAAGGGAA GCGCTTGCGA CCAGACTCGC
421 TCGCGGGGTT CAGCCGGCAC TCGTGCCGGT GTACTTCCCC GCGGGCGGGC CAGCGTCGGT
481 TTGGGCGGTC GGTCAAAGGC CCTCGGAAGG TAACGCCCCT CGGGGCGTCT TATAGCCGAG
541 GGTGCAATGC GACCTGCCCA GACCGAGGAA CGCGCTTCGG CTCGGACGCT GGCATAATG
601 TCGTAAGCGA CCCGTCTTGA AACACGGACC AAGGAGA

Figure 4 Nucleotide sequence of D1/D2 domain of 26S rDNA from different strains of *Penicillium* spp. from orange, small orange and malta, respectively**.**

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Manage Columns Show 100

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 16491	1131	1131	99%	0.0	98.75%	LT898171.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 16481	1131	1131	99%	0.0	98.75%	LT898170.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15211	1131	1131	99%	0.0	98.75%	LT898169.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15210	1131	1131	99%	0.0	98.75%	LT898168.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15192	1131	1131	99%	0.0	98.75%	LT898167.1
Penicillium solitum strain 20-01 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer	1131	1131	99%	0.0	98.75%	JN642222.1
Penicillium sp. 5H1-P0-P5-3 strain 5H1_P0_P5_3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal R	1127	1127	99%	0.0	98.59%	KM232463.1
Penicillium aurantiocandidum strain CBS 162.81 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribos	1125	1125	99%	0.0	98.59%	MH861314.1
Penicillium aurantiocandidum strain CBS 294.48 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribos	1125	1125	99%	0.0	98.59%	MH856349.1
Penicillium aurantiocandidum strain CBS 292.48 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribos	1125	1125	99%	0.0	98.59%	MH856348.1
Penicillium cellarum isolate F727 internal transcribed spacer 2 and 28S ribosomal RNA gene, partial sequence	1125	1125	99%	0.0	98.59%	MG714818.1
Penicillium sp. 6 MGS-2017 genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15032	1125	1125	99%	0.0	98.59%	LT899786.1
Penicillium sp. 6 MGS-2017 genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15031	1125	1125	99%	0.0	98.59%	LT899785.1
Penicillium sp. 6 MGS-2017 genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15188	1125	1125	99%	0.0	98.59%	LT899784.1
Penicillium polonicum isolate 25R-4-F04 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed sp	1125	1125	99%	0.0	98.59%	KX958079.1

A

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Score            Expect   Identities       Gaps          Strand
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Sbjct  488   CAGTAACGGCGAGTGAAGCGGCAAGAGCTCAAATTTGAAAGCTGGCTCCTTCGGGGTCCG  547
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Sbjct  548   CATTGTAATTTGCAGAGGATGCTTCGGGAGCGGTCCCCATCTAAGTGCCCTGGAACGGGA  607
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Sbjct  1028  GTCGTAAGCGACCCGTCTTGAAACACGGA-CCAA-GGAG  1064
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B

Figure 5 Nucleotide BLAST search result for D1/D2 of 26S rDNA of *Penicillium* sp. isolated from small orange (denoted by PLP-Sor.1), where (A) matching with different strains, (B) sequence alignment with highest matching strain.

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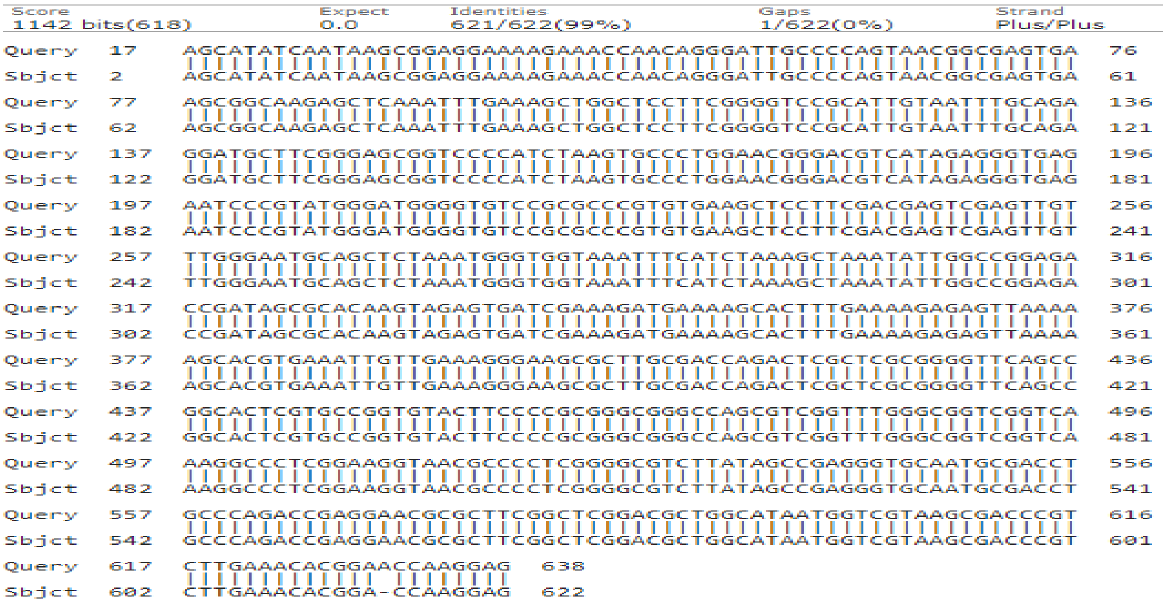
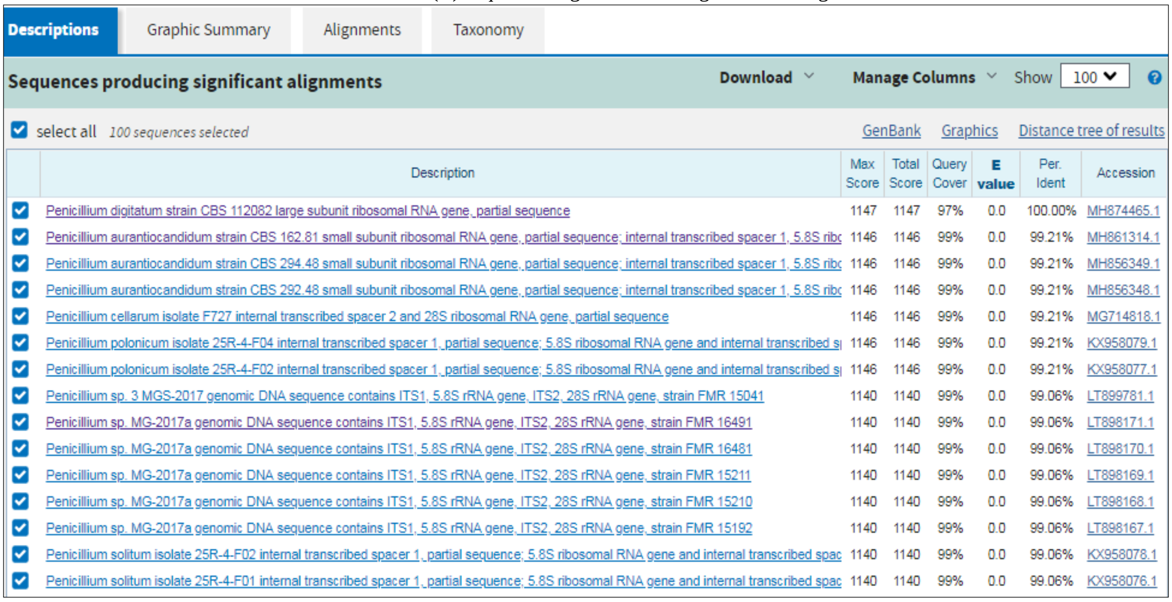
Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Penicillium aurantiocandidum strain CBS 162.81 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribc	1146	1146	99%	0.0	99.21%	MH861314.1
Penicillium aurantiocandidum strain CBS 294.48 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribc	1146	1146	99%	0.0	99.21%	MH856349.1
Penicillium aurantiocandidum strain CBS 292.48 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribc	1146	1146	99%	0.0	99.21%	MH856348.1
Penicillium cellarum isolate F727 internal transcribed spacer 2 and 28S ribosomal RNA gene, partial sequence	1146	1146	99%	0.0	99.21%	MG714818.1
Penicillium polonicum isolate 25R-4-F04 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed s	1146	1146	99%	0.0	99.21%	KX958079.1
Penicillium polonicum isolate 25R-4-F02 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed s	1146	1146	99%	0.0	99.21%	KX958077.1
Penicillium digitatum strain CBS 112082 large subunit ribosomal RNA gene, partial sequence	1142	1142	97%	0.0	99.84%	MH874465.1
Penicillium sp. 3 MGS-2017 genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15041	1140	1140	99%	0.0	99.06%	LT899781.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 16491	1140	1140	99%	0.0	99.06%	LT898171.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 16481	1140	1140	99%	0.0	99.06%	LT898170.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15211	1140	1140	99%	0.0	99.06%	LT898169.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15210	1140	1140	99%	0.0	99.06%	LT898168.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15192	1140	1140	99%	0.0	99.06%	LT898167.1
Penicillium solitum isolate 25R-4-F02 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spac	1140	1140	99%	0.0	99.06%	KX958078.1
Penicillium solitum isolate 25R-4-F01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spac	1140	1140	99%	0.0	99.06%	KX958076.1

A



B

Figure 6 Nucleotide BLAST search result for D1/D2 of 26S rDNA of *Penicillium* sp. isolated from small orange (denoted by PLP-Sor.1), where (A) matching with different strains, (B) sequence alignment with highest matching strain.



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Manage Columns — Show 100

select all 100 sequences selected — GenBank — Graphics — Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Penicillium digitatum strain CBS 112082 large subunit ribosomal RNA gene, partial sequence	1147	1147	97%	0.0	100.00%	MH874465.1
Penicillium aurantiocandidum strain CBS 162.81 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribo	1146	1146	99%	0.0	99.21%	MH861314.1
Penicillium aurantiocandidum strain CBS 294.48 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribo	1146	1146	99%	0.0	99.21%	MH856349.1
Penicillium aurantiocandidum strain CBS 292.48 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribo	1146	1146	99%	0.0	99.21%	MH856348.1
Penicillium cellarum isolate F727 internal transcribed spacer 2 and 28S ribosomal RNA gene, partial sequence	1146	1146	99%	0.0	99.21%	MG714818.1
Penicillium polonicum isolate 25R-4-F04 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed s	1146	1146	99%	0.0	99.21%	KX958079.1
Penicillium polonicum isolate 25R-4-F02 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed s	1146	1146	99%	0.0	99.21%	KX958077.1
Penicillium sp. 3 MGS-2017 genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15041	1140	1140	99%	0.0	99.06%	LT899781.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 16491	1140	1140	99%	0.0	99.06%	LT898171.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 16481	1140	1140	99%	0.0	99.06%	LT898170.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15211	1140	1140	99%	0.0	99.06%	LT898169.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15210	1140	1140	99%	0.0	99.06%	LT898168.1
Penicillium sp. MG-2017a genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain FMR 15192	1140	1140	99%	0.0	99.06%	LT898167.1
Penicillium solitum isolate 25R-4-F02 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spac	1140	1140	99%	0.0	99.06%	KX958078.1
Penicillium solitum isolate 25R-4-F01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spac	1140	1140	99%	0.0	99.06%	KX958076.1

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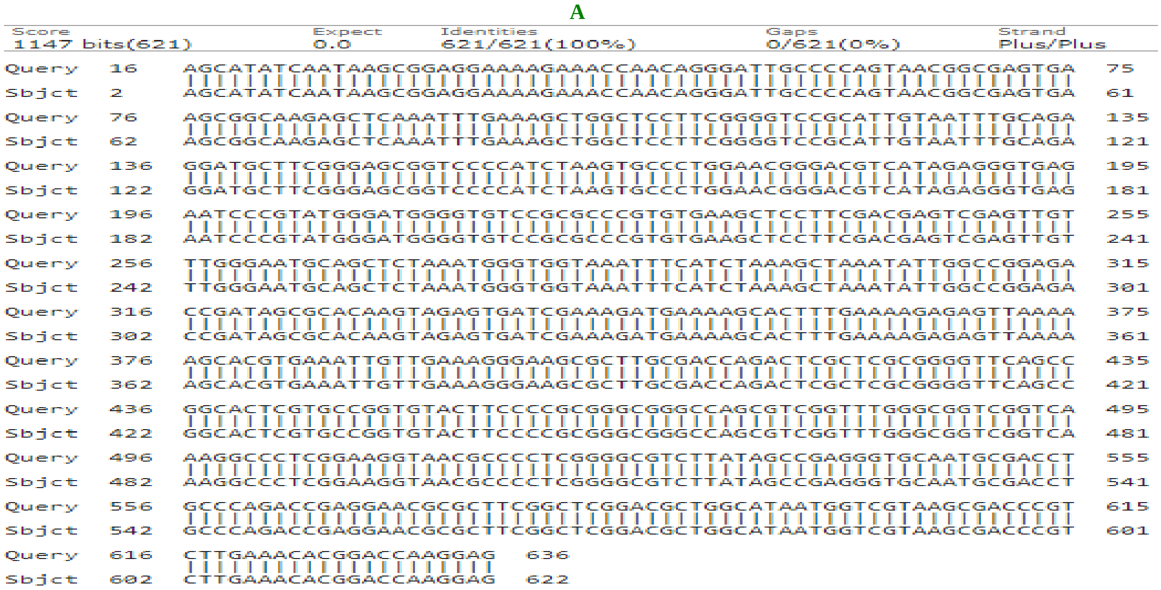

B

Figure 7 Nucleotide BLAST search result for D1/D2 of 26S rDNA of *Penicillium* sp. isolated from malta (denoted by PLP-Malt.1), where (A) matching with different strains, (B) sequence alignment with highest matching strain.

Phylogenetic Tree Development and Multiple Sequence Alignment

Phylogenetic analysis was conducted using neighbor-joining methods. Bootstrap analysis was performed with data resampled 1,000 times using the DNAMAN software (see Fig. 8). The resulting phylogenetic tree indicated that strains PLP-Malt.1 and PLP-Sor.1 are closely related to *Penicillium digitatum* strain CBS 112082 (Accession Number: MH874465). In contrast, strain PLP-Org.1 was found to be closely related to *Penicillium* commune strain 4.1 (Accession Number: KF880926), *Penicillium italicum* strain CBS 278.58 (Accession Number: MH869316), *Penicillium* crustosum strain CBS 133085 (Accession Number: MH877525), and *Penicillium sp.* MG-2017a (Accession Number: LT898171).Multiple sequence alignment of the three *Penicillium* isolates—PLP-Org.1, PLP-Sor.1, and PLP-Malt.1—showed a 98.81% sequence identity (see Fig. 9).

Deposition of Nucleotide Sequences in NCBI Gene Bank

The D1/D2 regions of 26S rDNA sequences for Penicillium sp. isolates were submitted to the National Center for Biotechnology Information (NCBI) Gene Bank. The accession numbers are as follows: **PLP-Org.1**: MN393491, **PLP-Sor.1**: MN393492 and **PLP-Malt.1**: MN393493.

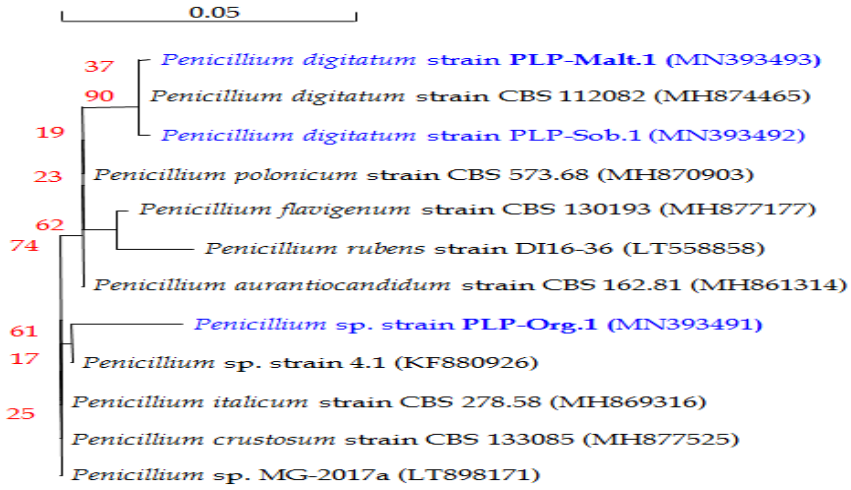

Figure 8 Phylogenetic tree of *Penicillium* spp. isolated from various citrus fruits based on the D1/D2 domain of 26S rDNA sequences. The numbers above each node represent the confidence levels (%) derived from 1,000 bootstrap replications. The scale bar indicates the number of nucleotide substitutions per sequence position.

Multiple_Sequence_Alignment
MAXLENGTH: 642
NAMES: PLP-Malt.1.txt PLP-Org.1.txt PLP-Sor.1.txt
MAXNAMELEN: 14
Identity: 98.81%
ORIGIN

```
PLP-Malt.1.txt .ACCCGCTGAAATTTAAGCATATCAAT.AAGCGGA.GGAAAAGAAACCAA     47
PLP-Org.1.txt  acCCCGCTGAAATTTAAGCATATCAATaAAGCGGAgGGAAAAGAAcCCAA     50
PLP-Sor.1.txt  acCCCGCTGAAAcTTAAGCATATCAAT.AAGCGGA.GGAAAAGAAACCAA     48
Consensus      cccgctgaaa ttaagcatatcaat aagcgga ggaaaagaa ccaa

PLP-Malt.1.txt CAGGGATTGCCCCAGTAACGGCGAGTGAAGCGGCAAGAGCTCAAATTTGA     97
PLP-Org.1.txt  CAGGGATTGCCCCAGTAACGGCGAGTGAAGCGGCAAGAGCTCAAATTTGA    100
PLP-Sor.1.txt  CAGGGATTGCCCCAGTAACGGCGAGTGAAGCGGCAAGAGCTCAAATTTGA     98
Consensus      cagggattgccccagtaacggcgagtgaagcggcaagagctcaaatttga

PLP-Malt.1.txt AAGCTGGCTCCTTCGGGGTCCGCATTGTAATTTGCAGAGGATGCTTCGGG    147
PLP-Org.1.txt  AAGCTGGCTCCTTCGGGGTCCGCATTGTAATTTGCAGAGGATGCTTCGGG    150
PLP-Sor.1.txt  AAGCTGGCTCCTTCGGGGTCCGCATTGTAATTTGCAGAGGATGCTTCGGG    148
Consensus      aagctggctccttcggggtccgcattgtaatttgcagaggatgcttcggg

PLP-Malt.1.txt AGCGGTCCCCATCTAAGTGCCCTGGAACGGGACGTCATAGAGGGTGAGAA    197
PLP-Org.1.txt  AGCGGTCCCCATCTAAGTGCCCTGGAACGGGACGTCATAGAGGGTGAGAA    200
PLP-Sor.1.txt  AGCGGTCCCCATCTAAGTGCCCTGGAACGGGACGTCATAGAGGGTGAGAA    198
Consensus      agcggtccccatctaagtgccctggaacgggacgtcatagagggtgagaa

PLP-Malt.1.txt TCCCGTATGGGATGGGGTGTCCGCGCCCGTGTGAAGCTCCTTCGACGAGT    247
PLP-Org.1.txt  TCCCGTATGGGATGGGGTGTCCGCGCCCGTGTGAAGCTCCTTCGACGAGT    250
PLP-Sor.1.txt  TCCCGTATGGGATGGGGTGTCCGCGCCCGTGTGAAGCTCCTTCGACGAGT    248
Consensus      tcccgtatgggatggggtgtccgcgcccgtgtgaagctccttcgacgagt

PLP-Malt.1.txt CGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAAATTTCATCTAAAG    297
PLP-Org.1.txt  CGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAAATTTCATCTAAAG    300
PLP-Sor.1.txt  CGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAAATTTCATCTAAAG    298
Consensus      cgagttgtttgggaatgcagctctaaatgggtggtaaatttcatctaaag

PLP-Malt.1.txt CTAAATATTGGCCGGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGAT    347
PLP-Org.1.txt  CTAAATATTGGCCGGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGAT    350
PLP-Sor.1.txt  CTAAATATTGGCCGGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGAT    348
Consensus      ctaaatattggccggagaccgatagcgcacaagtagagtgatcgaaagat

PLP-Malt.1.txt GAAAAGCACTTTGAAAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAGG    397
PLP-Org.1.txt  GAAAAGCACTTTGAAAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAGG    400
PLP-Sor.1.txt  GAAAAGCACTTTGAAAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAGG    398
Consensus      gaaaagcactttgaaaagagagttaaaaagcacgtgaaattgttgaaagg
```

```
PLP-Malt.1.txt GAAGCGCTTGCGACCAGACTCGCTCGCGGGGTTCAGCCGGCACTCGTGCC  447
PLP-Org.1.txt  GAgGCGCTTGCGACCAGACTCGCTCGCGGGGTTCAGCCGGCAtTCGTGCC  450
PLP-Sor.1.txt  GAAGCGCTTGCGACCAGACTCGCTCGCGGGGTTCAGCCGGCACTCGTGCC  448
Consensus      ga gcgcttgcgaccagactcgctcgcggggttcagccggca tcgtgcc

PLP-Malt.1.txt GGTGTACTTCCCCGCGGGCGGGCCAGCGTCGGTTTGGGCGGTCGGTCAAA  497
PLP-Org.1.txt  GGTGTAtTTCCCCGCGGGCGGGCCAGCGTCGGTTTGGGCGGTCGGTCAAA  500
PLP-Sor.1.txt  GGTGTACTTCCCCGCGGGCGGGCCAGCGTCGGTTTGGGCGGTCGGTCAAA  498
Consensus      ggtgta ttccccgcgggcgggccagcgtcggtttgggcggtcggtcaaa

PLP-Malt.1.txt GGCCCTCGGAAGGTAACGCCCCTCGGGGCGTCTTATAGCCGAGGGTGCAA  547
PLP-Org.1.txt  GGCCCTCGGAAGGTAACGCCCCTaGGGGCGTCTTATAGCCGAGGGTGCAA  550
PLP-Sor.1.txt  GGCCCTCGGAAGGTAACGCCCCTCGGGGCGTCTTATAGCCGAGGGTGCAA  548
Consensus      ggccctcggaaggtaacgcccct ggggcgtcttatagccgagggtgcaa
Continued-

PLP-Malt.1.txt TGCGACCTGCCCAGACCGAGGAACGCGCTTCGGCTCGGACGCTGGCATAA  597
PLP-Org.1.txt  TGCGACCTGCCtAGACCGAGGAACGCGCTTCGGCTCGGACGCTGGCATAA  600
PLP-Sor.1.txt  TGCGACCTGCCCAGACCGAGGAACGCGCTTCGGCTCGGACGCTGGCATAA  598
Consensus      tgcgacctgcc agaccgaggaacgcgcttcggctcggacgctggcataa

PLP-Malt.1.txt TGGTCGTAAGCGACCCGTCTTGAAACACGG.ACCAAGGAGA.       636
PLP-Org.1.txt  TGGTCGTAAGCGACCCGTCTTGAAACACGGaACCAAaGgaga      643
PLP-Sor.1.txt  TGGTCGTAAGCGACCCGTCTTGAAACACGGaACCAAGGAGA.      638
Consensus      tggtcgtaagcgacccgtcttgaaacacgg accaa g
```

Figure 9 Multiple sequence alignment of D1/D2 domain of 26S rDNA from different strains of *Penicillium* spp. from orange, small orange, and malta, respectively.

CONCLUSION

Laboratory experiments were performed to investigate the molecular characteristics of isolated *Penicillium* spp. from various citrus fruits, specifically orange, small orange, and Malta. The *Penicillium* isolates from orange and small, orange-produced colonies with irregular shapes, rough surfaces, and wavy margins on PDA medium. In contrast, the isolate from Malta formed round colonies with smooth surfaces. All isolates exhibited white, mycelial margins.

Pathogenicity tests demonstrated that all *Penicillium* isolates were capable of infecting the tested citrus fruits within six days of inoculation.

PCR amplification of the D1/D2 domain of 26S rDNA was conducted using the universal forward primer #103F (5'-ACC CGC TGA AYT TAA GCA TAT-3') and reverse primer #103R (5'-CTC CTT GGT CCG TGT TTC AAG AC-3'). The resulting DNA bands, approximately 640 bp in length, were purified and ligated into the pGEM-T Easy vector. The recombinant vector was then transformed into *E. coli* Dh5α. Plasmid isolation from white colonies containing the pGEM-T Easy vector with the D1/D2 domain of 26S rDNA was performed, and the presence of the insert was confirmed by endonuclease digestion with EcoRI, which revealed two distinct DNA bands: one at approximately 3 kb (representing the vector) and another at approximately 0.64 kb (corresponding to the D1/D2 domain).

Sequencing of the D1/D2 domain of 26S rDNA was carried out using the T7 promoter primer (5'-TAA TAC GAC TCA CTA TAG GGG-3'). Analysis of the sequences using the DNAMAN analysis system showed that the D1/D2 region of 26S rDNA from the orange isolate was 642 bp long, with a composition of 26% A, 23% C, 32% G, and 20% T. The D1/D2 region from the small orange isolate was 639 bp, with 26% A, 24% C, 32% G, and 19% T. The Malta isolate's D1/D2 region was 637 bp, with 25% A, 24% C, 32% G, and 19% T.

BLASTn searches on the NCBI website revealed that the D1/D2 sequence of the orange isolate (PLP-Org.1) showed the highest similarity (98.75%) to *Penicillium* sp. MG-2017a (accession number: LT898171). The D1/D2 sequence of the small orange isolate (PLP-Sor.1) showed 99.84% similarity to *Penicillium digitatum* strain CBS 112082 (MH874465). The Malta isolate (PLP-Malt.1) showed 100% similarity to *P. digitatum* strain CBS 112082.

Phylogenetic analysis indicated that isolates PLP-Malt.1 and PLP-Sor.1 are closely related to *P. digitatum* strain CBS 112082 (accession number: MH874465). In contrast, PLP-Org.1 is closely related to *Penicillium* sp. strain 4.1 (KF880926), *P. italicum* strain CBS 278.58 (MH869316), *P. crustosum* strain CBS 133085 (MH877525), and *Penicillium* sp. strain MG-2017a (LT898171). Multiple sequence alignments revealed a 98.81% identity among the three isolates (PLP-Org.1, PLP-Sor.1, and PLP-Malt.1).

The DNA sequences of the D1/D2 domain of 26S rDNA from these isolates have been deposited in the NCBI Gene Bank with the following accession numbers: MN393491 for *Penicillium* sp. strain PLP-Org.1, MN393492 for *Penicillium digitatum* strain PLP-Sor.1, and MN393493 for *Penicillium digitatum* strain PLP-Malt.1. Further research is needed to explore the diversity of citrus green molds and their potential control strategies for postharvest citrus fruit rot.

Acknowledgements: A special thanks to Dr. Shah Md Ashraful Islam for his invaluable guidance and supervision throughout these experiments. His expertise and support were instrumental in the successful completion of this research.

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