

*Full Length Research Paper*

# **Molecular phylogeny and genetic diversity studies of some potent endophytic fungi isolated from medicinal plants (*Calotropis procera* and *Catharanthus roseus*) using 18S rRNA and RAPD analysis**

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**Molecular identification of four endophytic fungi (*Penicillium singorense*, *Curvularia geniculata*, *Aspergillus neoflavipes* and *Alternaria alternata*) from two important medicinal plants, *Calotropis procera* (L.) R.Br. and *Catharanthus roseus* (L.) G.Don. was carried out by 18SrRNA sequencing. Genetic diversity using two RAPD primer viz. OPB-07 and OPC-06 showed good result with high polymorphism between the four strains. The sample DRC1 Accession No. MG322179 indicated strong homology with *Penicillium singorense*, strain DI16-118 (Accession No. LT558940), sample DRC2 Accession No. MG322180 with *Curvularia geniculata* strain F1 (Accession No. KX022497), sample DRC3 Accession No. MG322181 with *Aspergillus neoflavipes* strain AJR1 ribosomal gene (Accession No. KX218387), sample DRC4 Accession No. MH021686 with *A. alternata* strain AE1 18SrRNA gene, partial sequence, ITS1, 5.8S rRNA gene and ITS 2 complete sequence (Accession No. KY676196). Phylogenetic tree showing relatedness of sample DRC1 Accession No. MG322179 with 19 strains of *Penicillium* sp, DRC2 Accession No. MG322180 with 34 strains of *Curvularia* sp, DRC3 Accession No. MG322181 with 24 strains of *Aspergillus* sp and DRC4 Accession No. MH021686 with 16 strains of *Alternaria* sp of diverse origin. The present investigation thus gave an insight for the detection and genetic diversity study among the fungal endophytes isolated from two important Indian medicinal plants (*C. procera* and *C. roseus*) and it would probably the first report of its own kind from West Bengal, India.**

**Key words:** *Calotropis procera*, *Catharanthus roseus*, National Center for Biotechnology Information (NCBI), homology search, endophytic fungi, DNA marker, Random amplification of polymorphic DNA (RAPD) analysis, 18SrRNA sequencing.

## **INTRODUCTION**

Medicinal plants are the reservoir of biopharmaceutical compound traditionally used for the treatment of human ailment. Deforestation or constant use of medicinal plants makes them threatened or endangered (Chinedu, 2017). Conservation of medicinally important plant through

sustainable practices is one of the methods for their availability. Another alternative approach for the production of secondary metabolites or biopharmaceutical is the search for fungal endophytes having medicinal importance. The long association of fungi with plants in

mutuality's mode probably makes fungi rich sources for bioactive compounds (Pimentel, 2011).

The living organism is made up of DNA, RNA and protein, similar organism has the similar type of these molecules. Molecular phylogeny generally made with such data to infer a "relationship tree" that shows the probable evolution of various organisms. After the discovery of Sanger sequencing in 1977 it became possible to isolate and identify these molecular structures (Sanger and Coulson, 1975; Sanger et al., 1977). Studies on the diversity of fungal endophytes are expected to yield potential sources of natural products and bioactive compounds for medicinal, agricultural and industrial uses, such as new antibiotics as well as novel biocontrol agents (Molina et al., 2012). Molecular identification and phylogeny of an organism generally accomplished by the help of hereditary molecular differences, mainly in DNA sequences. DNA sequencing is therefore specific identification of an individual than conventional culture-based methods: identification is refined by including internal transcribed spacer (ITS) sequence analyses with phenotypic characterization. Nuclear-encoded ribosomal DNA (rDNA) gene has been the gene of interest to analyze phylogenetic relationships and resolve taxonomic problems in different taxonomic levels (Cai et al., 2005; Jeewon et al., 2004). The internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) have become a more popular marker for systematic and phylogenetic studies of closely related species of animals, plants and fungi (Von der Schulenburg et al., 2001), recently phylogenetic and taxonomic analysis in 100 endophytic fungi from *Chenopodium quinoa* has been carried out (Gonzalez-Teuber et al., 2017). Endophytic fungi often possess 'cryptic' morphological characters (Ganley et al., 2004). The cryptic morphological identity exhibited by fungal endophytes can be resolved with the help of molecular genetic marker-based identification (Bhagobaty and Joshi, 2011).

Two common medicinal plant, namely *Calotropis procera* and *Catharanthus roseus* commonly available in tropical area, mainly West Bengal has potential to provide various active ingredients of pharmaceutical importance. In the natural product research, secondary compounds leads to search an alternative way to obtain already existing active ingredients and new active phytochemical from the mutualistic symbionts mainly fungi, has gained momentum rapidly (Carvalho et al., 2016).

In the present investigation, an attempt has been made to identify endophytic fungi from *C. procera* (L.) R.Br. and *C. roseus* (L.) G.Don., the two important medicinal plant and phylogenetic classification with the collective patterns generated by partial 18S rDNA sequencing and random amplification of polymorphic DNA (RAPD).

## MATERIALS AND METHODS

### Genomic DNA isolation

Genomic DNA extraction from pure culture isolated from *C. procera* and *C. roseus* of *Alternaria* sp (Sample No.1), *Penicillium* sp (Sample No. 3), *Curvularia* sp ( Sample No. 4) and *Aspergillus* sp (Sample No. 5) was performed following the standard protocol (Gontia- Mishra et al., 2013) with little modification. In brief fungal mycelium (800 mg of each) were taken from pure and fresh fungal culture, crushed with (3.2 ml) extraction buffer (0.1 M Tris HCl pH 8, 10 mM EDTA pH 8, (2.5M) NaCl, 3.5% CTAB, 150 µl of 20 mg/ml of proteinase K) with sterilized fine sand in preautoclaved mortar pestle. The mixture was transferred to 10 ml centrifuge tube (Tarsons) vortexed vigorously, then incubated in a water bath at 65°C for 30 min. The samples were then centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was collected and equal volume of phenol-chloroform- isoamyl alcohol (25:24:1) was added mix thoroughly, followed by centrifugation at 10,000 rpm for 10 min at room temperature. Supernatant was collected and equal volume of chloroform-isoamyl alcohol (24:1) was added, mix thoroughly followed by centrifugation at previous condition. Supernatant was then collected, equal volumes of chilled isopropanol added, allowed to precipitate at - 20°C freezers for overnight. Samples were the again centrifuge at 13,000 rpm for 15 min to form the DNA pellet. Supernatant was discarded; DNA pellet washed thoroughly with 1 ml of absolute ethanol by centrifugation at 500 rpm for 5 min. DNA pellet was dried under Laminar Air Flow, dissolved in 200 µl of sterile high performance liquid chromatography (HPLC) grade water. The genomic DNA was treated with RNase A according to manufacturer's instruction to remove RNA and the purified DNA was quantified spectrophotometrically (NanoDrop 8000 spectrophotometer) to know their concentration and quality.

### Amplification of 18S rRNA gene with ITS 1 and ITS 4 primer

Polymerase chain reaction (PCR) amplification of the 18S rRNA gene of four fungal isolates was performed using the respective genomic DNA with the primers ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCGCTTATTGATATGC-3' (White et al., 1990). PCR was performed in 25 µl reaction volume containing 2.5µl of 10X Taq DNA polymerase, 2.0 µl of 2.0 mM dNTP mix, 15 pmol of each primer ITS1 and ITS4, 50 ng template DNA and 1.5U of Taq polymerase (Promega) using the following program: initial denaturation at 94°C for 1 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53.5°C for 45 s, extension at 72°C for 1 min and a final extension at 72°C for 5 min on a thermal cycler (ABI Veriti Thermal Cycler).

### 18 SrRNA detection and sequencing of amplicon

The amplicon generated were resolved in 1.5% agarose gel with ethidium bromide containing a (100 bp) DNA ladder. The amplicon was the enzymatically purified followed by EDTA-Ethanol purification method. The bi-directional DNA sequencing reaction of PCR amplicon was carried out with 18S sequencing primers (1F CTGGTGCCAGCAGCCCGGYAA, 4R CKRAGGCATYACWGACCTGTTAT) using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The PCR condition

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was 94°C 3 min, 94°C 30 s, 48°C 30 s, 72°C 1 min, 72°C 7 min, 4°C forever, the total cycles were 30.

#### Submission of 18S rRNA gene sequence in the NCBI database and construction of phylogenetic tree

The partial 18S rRNA gene sequence of selected fungal antagonists was submitted to NCBI GenBank for obtaining Accessions. Parsimony Methods were conducted in MEGA 5 software. The evolutionary history was inferred by using the Maximum Likelihood (Tamura et al., 2004). Evolutionary Genetics Analysis was performed using Maximum Likelihood, Evolutionary Distance (Tamura et al., 2011). The bootstrap value was calculated and shown in the dendrogram.

#### RAPD polymerase chain reaction

PCR was carried out in a final reaction volume of 25 µl in ABI Veriti Thermal Cycler with three RAPD primers: OPB-07 5'-GGTGACGCAG -3' (10 nts.) OPC-06 5'- GAACGGACTC-3' (10 nts.) and OPC-07 5'- GTCCCGACGA-3' (Gonita-Mishra et al., 2013). Composition of reaction mixture for PCR is : Nuclease free water to make-up volume for 25 µl, DNA 50 ng, Primer (10 pmole) 1.0 µl, 2X PCR Master Mix 10 µl, total Volume 25 µl. PCR tubes containing the mixture were tapped gently and spin briefly. The PCR tubes with all the components were transferred to thermal cycler. The PCR protocol designed for 40 cycles for the primers used is: initial denaturation 94°C, 4 min; denaturation 94°C, 1 min; annealing 37°C, 1 min; extension 72°C 2 min and final extension 72°C, 5 min.

#### Visualization of PCR product

To confirm the targeted PCR amplification, 10 µl of PCR product from each tube was mixed with 2 µl of 6X gel loading dye and electrophoreses on 2.0% agarose gel containing ethidium bromide and co run with 100 bp DNA ladders, at the constant 5 V/cm for 45 min in 1X TAE buffer. The amplified products were visualized under UV light and documented by a gel documentation system (BIORAD).

#### Data analysis and construction of dendrograms from RAPD profile

Clear and distinct bands amplified by RAPD primers were scored for the presence (1) and absence (0) of the corresponding band through OPC-06 and OPB-07 primers, in the gDNA of the sample. The data were entered into the binary matrix and subsequently analyzed. Using FreeTree Software, cluster analysis was performed by agglomerative technique using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean, replication no. 3) method. Relationships between the samples were graphically represented in the form of dendrograms.

## RESULTS

#### Qualitative and quantitative estimation of genomic DNA

Nanodrop reading of genomic DNA of four samples under

study viz: 1,3,4 and 5 showed high yield and good quality 847.4 ng/ µl (*Alternaria* sp), 469.8 ng/ µl (*Penicillium* sp), 811.3 ng/ µl *Curvularia* sp and 240.3 mg/µl (*Aspergillus* sp) respectively.

#### Amplification of 18S rRNA using ITS1 and ITS 4 primer

Amplification of the rDNA regions of four sample under study with ITS1 and ITS 4 oligonucleotide primers yielded amplicon of approximately 900 bp as estimated by 1.5% agarose gel electrophoresis using known standard DNA ladder.

#### Sequencing of 18SrRNA gene

The four 18SrRNA amplicon subjected to sequencing namely Sample No.1 (*Alternaria alternata* DRC 4), Sample No. 3 (*Penicillium* sp DRC1), Sample No.4 (*Curvularia* sp DRC2), Sample No. 5 (*Aspergillus* sp DRC 3) gave a good sequence reading of standard fragment size. The sequences used for the final homology search, phylogenetic analysis were done after manual counting trimming and corresponded to the ITS 1 and 2 complete regions, 5' portion of the 18S gene, 5.8 S complete sequences and the 3' end of the 28S gene.

#### GenBank accession followed by homology searching

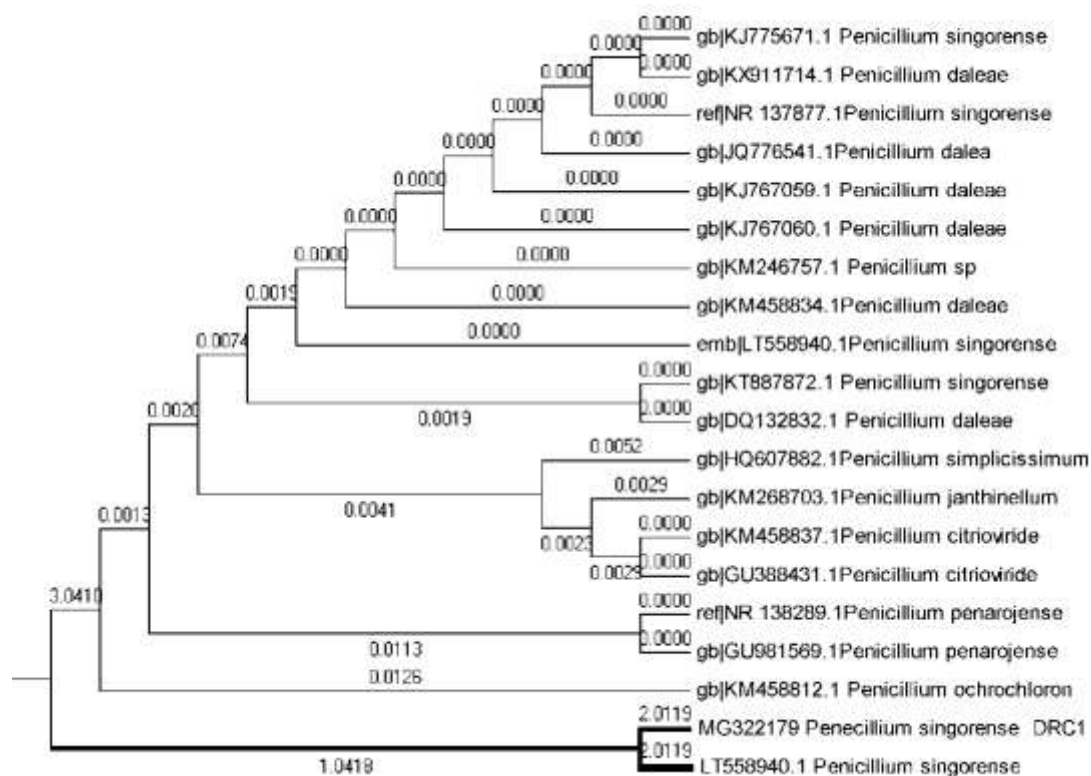
The FASTA format of four (sample DRC1, DRC2, DRC3, DRC4) 18 SrRNA sequence after submitting in NCBI database, the GenBank Accession were obtained (DRC1 MG322179, DRC2 MG322180, DRC3 KX218387, DRC4 MH021686) respectively. Sample 3 that is, DRC1 Accession No. MG322179 showed strong homology with *Penicillium singorense*, strain D116-118, sample 4 that is, DRC2 Accession No. MG322180 showed strong homology with *Curvularia geniculata* strain F1, sample 5 that is, DRC3 Accession No. MG322181 showed strong homology with *Aspergillus neoflavipes* strain AJR1 ribosomal gene, sample 1 that is, DRC4 Accession No. MH021686 showed strong homology with *Alternaria alternata* strain AE1 18SrRNA gene, partial sequence, ITS1, 5.8S rRNA gene and ITS 2 complete sequence Accession No. KY676196 (Table 1). The phylogenetic analysis of these four isolated and identified strain reveals their relatedness with respective species (Figures 1, 2, 3 and 4).

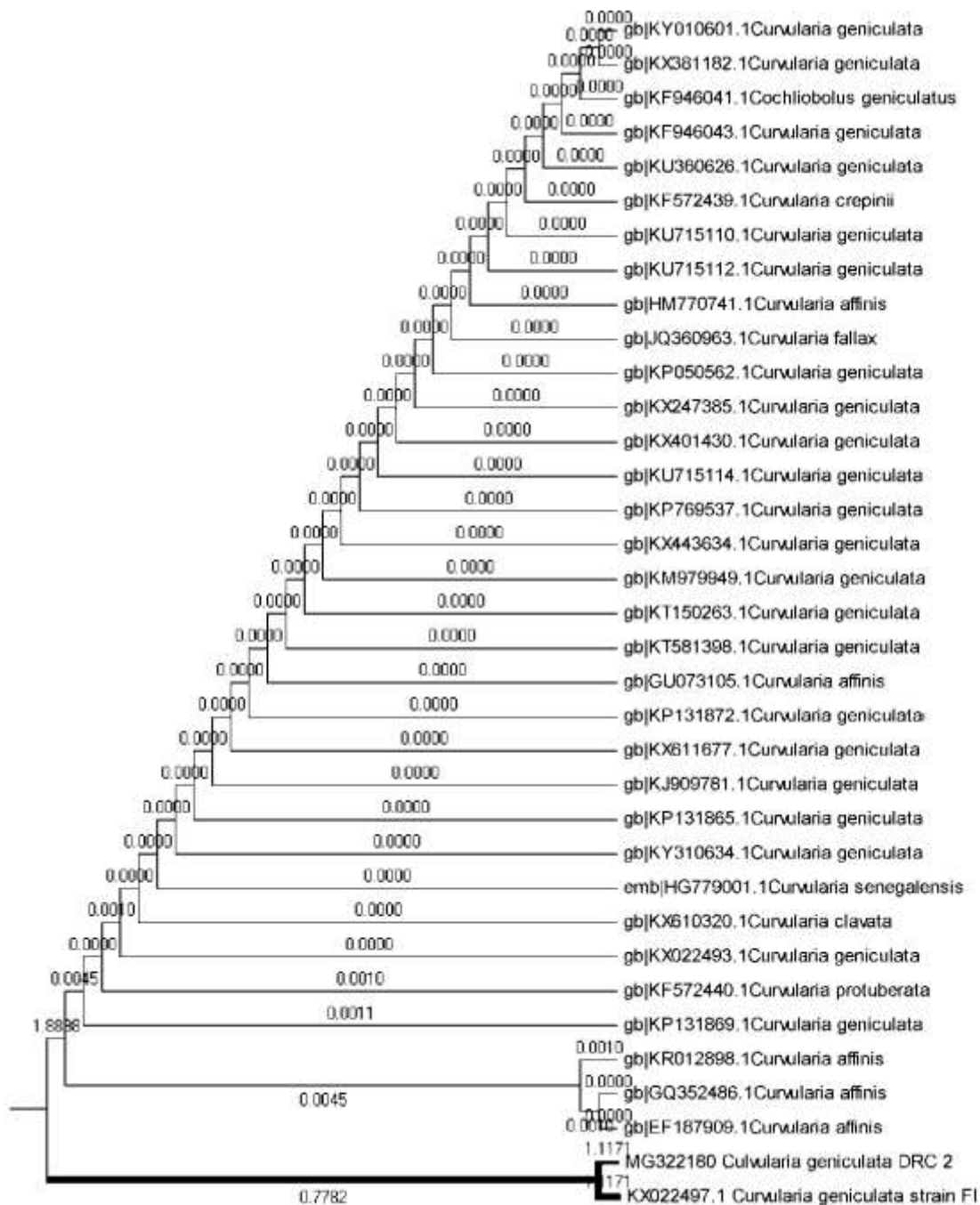
#### RAPD analysis

Among the three RAPD primer (OPB- 07, OPC-06 and OPC-07) tested to identify genetic diversity good

**Table 1.** Homology and annotation of 4 GenBank Accession of isolated endophytic fungi strains.

Strain code	NCBI No	Accession	Homology	Length (bp)	Annotation	Max score	Total score	E-value	Identical (%)
DRC1	MG322179	LT558940		599	<i>Penicillium singorensis</i> , strain DI 16-118 genomic DNA contains ITS1, 5.8SrRNA gene, ITS2, 28SrRNA gene	1033	1033	0.0	99
DRC2	MG322180	KX022497		597	<i>Curvularia geniculata</i> strain F1 18SrRNA gene, partial sequence, ITS1, 5.8S rRNA gene & ITS 2 complete sequence.	1055	1055	0.0	99
DRC3	MG322181	KX218387		896	<i>neoflavipes</i> strain AJR1 18S rRNA gene, partial sequence.	1594	1594	0.0	99
DRC 4	MH021686	KY676196			<i>Alternaria alternata</i> strain AE1 18SrRNA gene, partial sequence, ITS1, 5.8S rRNA gene & ITS 2 complete sequence.	1061	1061	0.0	100

**Figure 1.** Phylogenetic tree showing relatedness of DRC1 Accession No. MG322179 with 19 strains of *Penicillium* sp. of diverse origin showing highest similarity with *Penicillium singorensis*, strain DI16-118 (Accession No. LT558940).

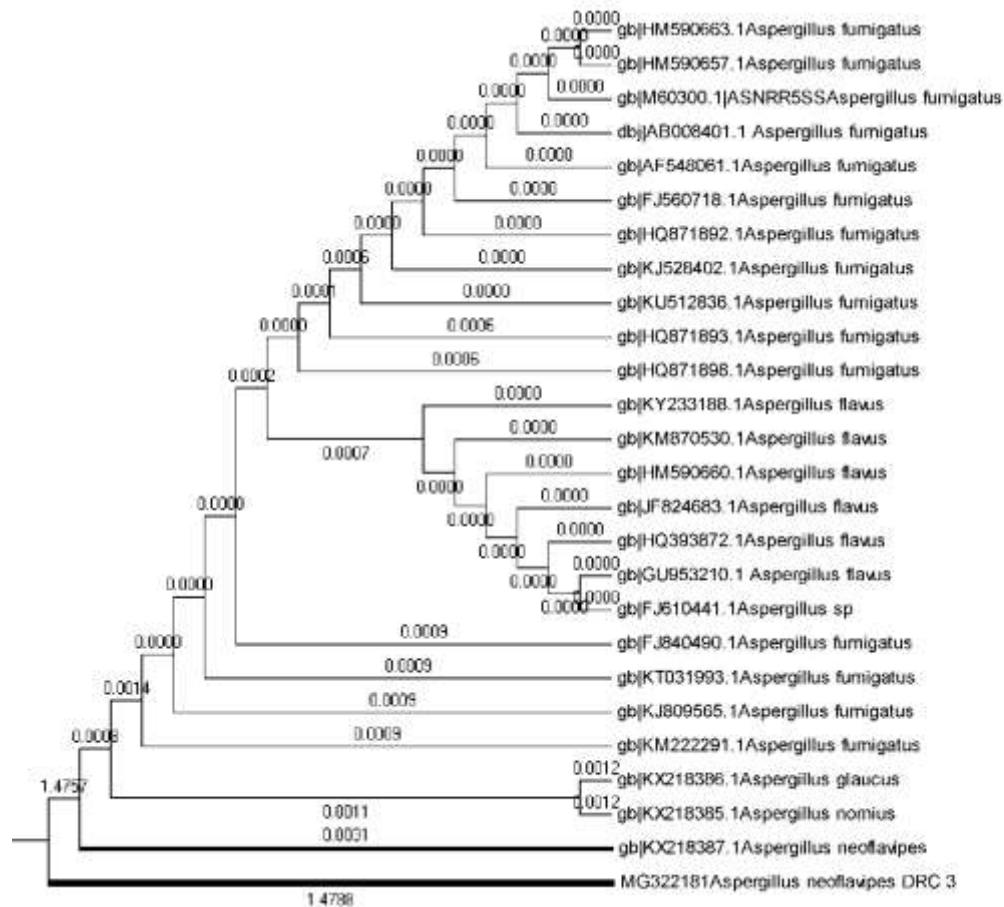


**Figure 2.** Phylogenetic tree showing relatedness of DRC2 Accession No. MG322180 with 34 strains of *Curvularia* sp of diverse origin showing highest similarity with *Curvularia geniculata* strain F1 (Accession No. KX022497).

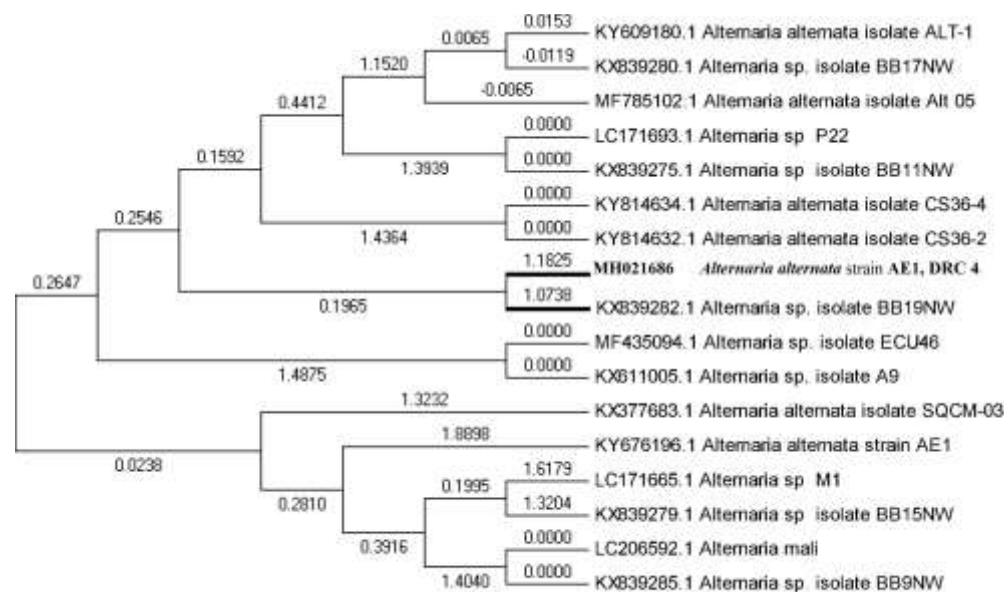
amplification was responded by two primers namely OPB-07 and OPC-06. The RAPD profile of four samples showed polymorphism and genetic diversity among them with fragment size of approximately 150 to 1.5 kb (Figure 5). The highest amplification was recorded with OPB-07 primer. RAPD marker has also been used (Gontia-Mishra et al., 2013) to amplify the genomic DNA of several species of *Aspergillus* and showed similar result.

### Dendrograms from RAPD profile

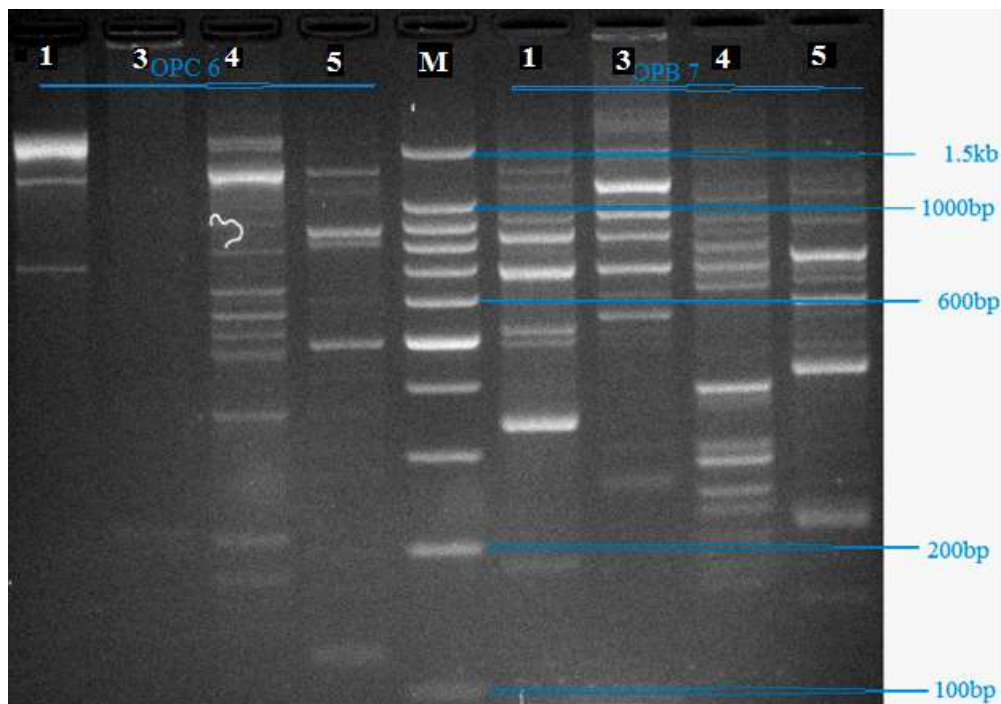
Dendrogram constructed from two RAPD profile depicted in Figures 6 and 7 reveals their relationship. In both the tree there are two groups showing the close relationship with sample number 1 (*Alternaria* sp.) and three (*Penicillium* sp.) and other with sample number 4 (*Curvularia* sp.) and 5 (*Aspergillus* sp.). Both the groups



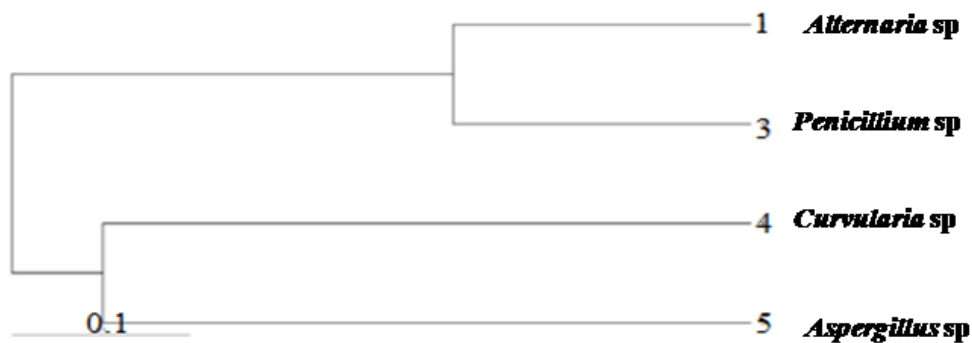
**Figure 3.** Phylogenetic tree showing relatedness of DRC3 Accession No. MG322181 with 24 strains of *Aspergillus* sp. of diverse origin showing highest similarity with *Aspergillus neoflavipes* strain AJR1 ribosomal gene (Accession No. KX218387).



**Figure 4.** Phylogenetic tree showing relatedness of DRC4 Accession No. MH021686 with 16 strains of *Alternaria* sp. of diverse origin showing highest similarity with *Alternaria alternata* strain AE1 18S rRNA gene (Accession No. KY676196).



**Figure 5.** 2% Agarose gel electrophoresis showing gel image of 1, 3, 4, 5 g DNA with primer OPC-06 and OPB-07. Lane M: 100bp DNA ladder.



**Figure 6.** Total homology search using RAPD Primer OPB-07 of the four DNA samples (Sample No. 1 *Alternaria* sp. sample No. 3 *Penicillium* sp. sample No. 4 *Curvularia* sp. and sample No. 5 *Aspergillus* sp.); cluster analysis was performed by agglomerative technique using the unweighted pair group method with arithmetic mean (UPGMA) method showing relationships between the samples.

in other hand distantly related in the case of both primers (Figures 6 and 7).

Total homology searching between the four fungal isolates namely *Penicillium* sp, *Curvularia* sp, *Aspergillus* sp. and *Alternaria* sp. revealed that *Alternaria* sp., *Penicillium* sp. and *Curvularia* sp., *Aspergillus* sp. are also closely related to each other. The molecular analyses proved that these four fungal isolates to the diverse genera.

## DISCUSSION

After thorough investigation for the presence of fungal endophytes from two important Indian medicinal plants (*C. procera* and *C. roseus*), four pure fungal isolates of *Penicillium* sp., *Curvularia* sp., *Aspergillus* sp. and *Alternaria* sp. has been identified by using molecular tools and techniques. From the perusal of literature it has been revealed that the four isolated fungi have great



**Figure 7.** Total homology search using RAPD Primer OPC-06 of the four DNA samples (sample No. 1 *Alternaria* sp., sample No. 3 *Penicillium* sp. sample No. 4 *Curvularia* sp. and sample No. 5 *Aspergillus* sp.); cluster analysis was performed by agglomerative technique using the unweighted pair group method with arithmetic mean (UPGMA) method showing relationships between the samples.

medicinal value, as they produce several bioactive compounds of pharmaceutical importance. *Penicillium singorense* is a species of the genus *Penicillium* which was isolated from house dust (Visagie et al., 2014). *Curvularia* is a hyphomycete fungus which is a facultative saprophyte of many plant species and common in soil. Most *Curvularia* are found in tropical to temperate regions. However literature reveals that *Curvularia affinis*, *Curvularia fallax*, and *Curvularia senegalensis* are synonymous to *C. geniculata*. The presence of the mycotoxins, fumifungin, fumiquinazoline A/B and D, fumitremorgin B, gliotoxin, sphingofungins, subrutines, and verruculogen in *Aspergillus fumigatus* a close relative of *A. neoflavipes* has been reported (Tamiya et al., 2015). The presence of isochromenone, from an endophytic fungus *Aspergillus fumigatus* isolated from *Bacopa monnieri* plant and proof its antioxidant and antitubercular activity has been investigated (Thakur et al., 2015). The methanol extract of *A. fumigatus* inhibits the growth of the virulent strain of *Mycobacterium tuberculosis* HRV with minimum inhibitory concentration 500 µg/ml.

Fungal endophytes are reported to secrete a diverse group of biomolecules extracellularly which are capable of reducing metal salts at a rapid scale under optimized conditions. One such endophyte is isolated from healthy leaves of *Andrographis paniculata* and employed for rapid synthesis of silver nanoparticles. The synthesized nanoparticles were evaluated for bactericidal activity against significant human pathogens (Azmath et al., 2016). The presence of paclitaxel from newly described endophytic fungus *Pestalotiopsis microspora*, isolated from the pine needles of *T. wallichiana* has been reported (Strobel et al., 1996). This study indicates that a large scale production of the natural product paclitaxel can be

made at a lower cost just by mass production of the fungus in culture. Paclitaxel can be obtained from Yew (*Taxus* sp.), but such obtaining requires destruction of trees. Chemical synthesis of paclitaxel by cell and tissue culture from the *Taxus* sp are cost effective and production is not up to the mark (Guenard et al., 1993). Recently, paclitaxel as well as other toxins have been detected in shells and leaves of *Corylus avellana* and also in leaves of *Ocimum basilicum* (Ottaggio et al., 2008; Gangadevi and Muthumary 2017). It is well known that the tissues of living plant residue the endophytic fungi which possess analogical ability to perform a biosynthesis of secondary metabolites as the host plant. The ability of the endophytic fungus of Hazel plant *Penicillium aurantiogriseum* NRRL 62431 to independently synthesize paclitaxel was detected by liquid chromatography-mass spectrometry and proton nuclear magnetic resonance. The presence of Taxol from *Phoma* sp isolated from *Calotropis gigantea* has been detected (Hemamalini et al., 2015). The genome of *Penicillium aurantiogriseum* NRRL 62431 was sequenced and gene candidates that may be involved in paclitaxel biosynthesis were identified by comparison with the 13 known paclitaxel biosynthetic genes in *Taxus* (Yang et al., 2014). Two new metabolites, from a soil fungus, *Curvularia affinis* strain HS-FG-196. pyrenocine J (1) and pyrenochaetic acid D (2), together with two known metabolites, pyrenocine A (3) and pyrenochaetic acid A (4) has reported (Zhang et al., 2012). Pyrenocine J showed cytotoxic activity against the human hepatic cancer cell line HepG2 with an IC (50) value of 28.5 µg/ml. The presence of different mycotoxins, gliotoxin, sphingofungins, subrutines, and verruculogen in *Aspergillus fumigatus* a close relative of *A. neoflavipes* has also been reported (Tamiya et al., 2015) which are of



great commercial value. The capacity of producing Vinblastin, an anticancerous drug from *Alternaria* sp isolated from *C. roseus* was reported (Guo et al., 1998). The paclitaxel a highly valued anticancerous compound was also produced by *A. alternata* TPF6 isolated from *Taxus chinensis* var. *mairei* (Tian et al., 2006).

The present study highlights the detection and genetic diversity among the fungal endophytes isolated from two important Indian medicinal plants. The endophytic fungi are expected to be a potential source of many natural bioactive products including paclitaxel and other taxanes. The search for a bioactive compound from fungal endophytes gaining momentum, thus the identification of fungal endophytes would be of great importance to the discovery of the newly active compound. The present study would probably the first report of its own kind from West Bengal, India.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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