Multi-loci Molecular Characterisation of Endophytic Fungi Isolated from Five Medicinal Plants of Meghalaya, India

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The phylogenetic relationships of the most dominant and morphologically cryptic endophytic fungal isolates from each of five selected medicinal plants, namely *Potentilla fulgens*, *Osbeckia stellata*, *Osbeckia chinensis*, *Camellia caduca*, and *Schima khasiana* of the biodiversity rich state of Meghalaya, were assessed with random amplification of polymorphic DNA and PCR-restriction fragment length polymorphism profiles. Sequencing of the internal transcribed spacer 1, small subunit rRNA and partial β-tubulin gene fragments was also conducted to determine the phylogenetic relationships of these isolates with fungal sequences available in Genbank, NCBI. The identity of the fungal isolates is suggested based on the molecular phylogenetic data.

KEYWORDS : Cryptic morphology, Endophytic fungi, Identification, Medicinal plants, Molecular phylogeny

The hill state of Meghalaya in the north-eastern region of India (20.1° N~26.5° N latitude, 85.49° E~92.52° E longitude) is part of the Indo-Burma mega-biodiversity hotspot. Local communities in this region have traditionally used and relied on herbs and plants for treating various ailments [1]. These medicinal plants are mostly found in the traditionally preserved 'sacred forests,' which have remained untouched for centuries due to the religious beliefs of the indigenous tribal inhabitants. These private and community-managed sacred forests also represent the climax vegetation of the area and are home to many medicinal plant species [2]. Plants growing in unique habitats and that are used as ethno-medicines to treat human diseases among the local ethnic population are often likely to harbour novel endophytic fungi [3]. Based upon this rationale, five medicinal plants, i.e., *Potentilla fulgens*, *Osbeckia stellata*, *Osbeckia chinensis*, *Camellia caduca* and *Schima khasiana* were selected for a study of their associated endophytic mycoflora. Isolation followed by biological activity studies of some of the endophytic isolates from these plants indicates their potential for novel biotechnological applications [4]. Endophytic fungi often exhibit 'cryptic' morphological characters [5]. Our previous experience with identifying some of these isolates, particularly the first of the RS07PF series originally isolated from the tap roots of *P. fulgens*, indicates that the cryptic morphological identity exhibited by some of these fungal endophytes can be resolved with the help of molecular genetic marker-based identification [6]. The dominant and most frequently isolated endophytic fungal isolates from each of the five selected medicinal plants exhibited cryptic morphological characters, making it necessary to identify them using molecular tools and techniques. In the present investigation, we developed the most likely phylogenetic classification of the five endophytic fungi under study with the collective patterns generated by random amplification of polymorphic DNA (RAPD), PCR-restriction fragment length polymorphism (PCR-RFLP), internal transcribed spacer (ITS), partial 18S rDNA and partial β-tubulin gene sequencing.

Materials and Methods

Isolation of endophytic fungi from medicinal plants. Five selected ethno-medicinal plants i.e., *P. fulgens*, *O. stellata*, *O. chinensis*, *C. caduca*, and *S. khasiana* were collected from Nongkrem, Cherrapunji, Mawphlang and Umsaw sacred forests spread over the state of Meghalaya, India (Table 1). About 100 surface sterilised root and stem pieces of each of the five plants were air dried and flamed before removing the outer layers [7]. Two centimetre long pieces of these roots and stems were placed on

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Table 1. Details of the medicinal plants and their ethno-medicinal uses

Medicinal plants of Meghalaya, India	Sacred forest	Habitat	Ethno-medicinal usage	Plant parts from which isolation was undertaken
Potentilla fulgens	Nongkrem	Open grassland	Treatment of high blood sugar levels	Roots and stem
Osbeckia stellata	Cherrapunji	*Pinus kesiya* forest undergrowth	Treatment of cuts and wounds and as a remedy for toothaches	Roots and stem
Osbeckia chinensis	Cherrapunji	*Pinus kesiya* forest undergrowth	Treatment of watery diarrhoea, dysentery and excessive sputum production in coughing. Also used in the control of high blood sugar	Roots and stem
Camellia caduca	Mawphlang	Mixed forest floor	Used as an astringent, digestive, carminative and diuretic	Roots and stem
Schima khasiana	Umsaw	Mixed forest floor	Used in the treatment of stomach ache and allergies	Roots and stem

Table 2. Endophytic fungi isolated from the selected medicinal plants

Medicinal plant	Fungal endophytes[a] (No. of isolates)
Potentilla fulgens	*Talaromyces flavus* (167)
(total endophytic	*Penicillium verruculosum* (25)
isolates: 195)	Sterile mycelia (3)
Osbeckia stellata	*Mortierella hyalina* (85)
(total endophytic	*Syncephalastrum racemosum* (37)
isolates: 128)	Sterile mycelia (6)
Osbeckia chinensis	*Paecilomyces variabilis* (63)
(total endophytic	*Aspergillus* sp. (37)
isolates: 108)	*Penicillium pinophilum* (8)
Camellia caduca	*Penicillium* sp. (69)
(total endophytic	*Syncephalastrum racemosum* (19)
isolates: 126)	*Acremonium* sp. (17)
	Paecilomyces sp. (16)
	Trichoderma harzianum (3)
	Sterile mycelia (2)
Schima khasiana	*Penicillium* sp. (127)
(total endophytic	Sterile mycelia (10)
isolates: 145)	*Aspergillus chrysogenum* (7)
	Chaetomidium sp. (2)

Unique taxonomic groups are in italics. The fungal isolates that were identified to the genus level were different from similar isolates in different plants and, as such, are considered a separate taxonomic group with the exception of sterile mycelia (total taxonomic groups characterised: 16).
[a]Morphological identity.

Petri dishes containing water agar, as described by Strobel *et al.* [8] and incubated at 24°C for 7 days. After the incubation, hyphal tips of developing fungi were aseptically removed and placed on potato dextrose agar, according to Strobel *et al.* [9]. The most frequently isolated endophytic fungal isolates (RS07PF, RS07OS, RS07OC, RS07CC, and RS07SK) from each of the medicinal plants were selected for molecular characterisation (Table 2).

Fungal DNA extraction. DNA was extracted from the five endophytic fungi using the HiPer™ Fungal DNA mini kit (MBT032) (HiMedia Laboratories Private Ltd., Mumbai, India), according to the manufacturer's instructions.

RAPD analysis. RAPD analysis of the five endophytic fungi was conducted with GeNei™ RFu-C Fungal RAPD primers (Bangalore Genei, Karnataka, India). Ten primers i.e., primer 1~10 were screened with each of the endophytic fungal isolates. Of the 10 primers screened, primer Nos. 4 and 7 were selected for RAPD profile generation, based on the presence of scorable bands with these primers. Each sample was amplified at least three times to verify reproducibility. The reaction consisted of the following in a 25 μL volume: 1 μL (15 ng) template DNA, 1 μL primer DNA (5 μM stock), 0.375 μL dNTPs (10 mM stock), 1.5 μL Mg^{2+} (25 mM stock), 2.5 μL 10× Taq DNA polymerase reaction buffer (500 mM KCl, 100 mM Tris-HCl, 1.0% Triton X-100) and 0.36 μL Taq DNA polymerase (5 U/μL; Promega, Madison, WI, USA). The DNA samples were amplified in a GeneAmp® 9700 thermal cycler (Applied Biosystems Inc., Foster City, CA, USA), using the following thermal profile: 5 sec at 95°C; 1 min 55 sec at 92°C; followed by 45 cycles of 5 sec at 95°C, 55 sec at 92°C, 1 min at 35°C, and 2 min at 72°C; followed by 7 min at 72°C [10]. Amplification products were separated by electrophoresis on 1.8% agarose gels with 1× Tris-acetate-EDTA (TAE) buffer at 70 volts. The RAPD profiles generated were photographed using Kodak Gel logic 100 gel documentation system (Kodak, Rochester, NY, USA). The data analysis and dendrogram generation using the RAPD banding patterns was performed using the Numerical Taxonomy System NTSYSpc, ver. 2.2 for Windows XP (Exeter Software, Setauket, NY, USA).

PCR-RFLP. RFLP patterns of the PCR amplified ITS, small subunit (SSU) rDNA and β-tubulin gene products were generated with the *Msp*I, *Alu*I, *Nde*II, *Hae*III and *Hin*fI restriction enzymes (Bangalore Genei). Digestion was performed by incubating a 20 μL aliquot of the PCR products with 10 units of the respective restriction enzyme in a final reaction volume of 25 μL at 37°C for 2 hr [11]. Restriction fragments were separated by 1.8% agarose gel

electrophoresis in Tris-Borate-EDTA (TBE) buffer for approximately 45 min at 100 volts and visualised by staining with ethidium bromide in a Kodak Gel logic 100 gel documentation system. Dendrograms based on the restriction patterns were generated with the Numerical Taxonomy System NTSYSpc.

PCR amplification and sequencing of the ITS region. Amplification of the endophytic fungi ITS region was performed using fungal domain specific ITS1f and 5.8S primers [12]. The PCR reaction mixture was comprised of 10 μL fungal DNA, 5 μL 10× PCR buffer, 1.5 μL of 50 mM $MgCl_2$, 1 μL of 10 mM dNTP, 0.25 μL Taq polymerase, 40 pM each of the forward and the reverse primers in a total reaction volume of 50 μL [13]. Amplification was performed in a GeneAmp® 9700 Thermal Cycler (Applied Biosystems), and PCR conditions were 15 min at 95°C, followed by 40 cycles of 95°C for 1 min, 30 sec at the annealing temperature, and 72°C for 1 min [12]. Aliquots (10 μL) of each amplified product were electrophoretically separated on a 2% agarose gel in 1× TAE buffer and visualised using ethidium bromide under UV illumination in an Kodak gel logic 100 gel documentation system. A 100-bp DNA molecular weight ladder (Bangalore Genei) was included in each run. PCR amplicons were excised and purified using the QIA Quick Gel Extraction Kit (Qiagen, Hilden, Germany). The amplicons were then sequenced in an Applied Biosystems 3700 Genetic Analyser with BigDye Terminator ver. 3.1. Sequences (GenBank accession Nos.: HM569608, HM569609, HM569610, HM569611, and HM569612) were then searched for similarity with other deposited sequences in GenBank. Alignments and phylogenetic analyses were performed using MEGA4 software [14].

PCR amplification and sequencing of the SSU rDNA region. Amplification of the SSU rDNA region of the endophytic fungal genomes was conducted using the fungi specific primers nu-SSU-0817-5 and nu-SSU-1536-3 [15]. The PCR reaction mixture was comprised of 10 μL fungal DNA, 5 μL 10× PCR buffer, 1.5 μL of 50 mM $MgCl_2$, 1 μL of 10 mM dNTP, 0.25 μL Taq polymerase, 40 pM each of the forward and the reverse primers in a total reaction volume of 50 μL [13]. All reagents were combined and heated at 940°C for 2 min. Thirty-five cycles of PCR were then performed at 94°C for 0 sec, 56°C for 10 sec, and 72°C for 30 sec, followed by 72°C for 2 min [15]. PCR was performed in a GeneAmp® 9700 Thermal Cycler (Applied Biosystems). Aliquots (10 μL) of each amplified product were electrophoretically separated on a 2% agarose gel in 1× TAE buffer and visualised using ethidium bromide under UV illumination in an Kodak gel logic 100 gel documentation system. A 100-bp DNA molecular weight ladder (Bangalore Genei) was included

in each run. The PCR amplicons were excised and purified using a QIA Quick Gel Extraction Kit (Qiagen). The amplicons were then sequenced in Applied Biosystems 3700 Genetic Analyser with BigDye Terminator ver. 3.1. Sequences obtained (GenBank accession Nos.: HM581667, HM581668, HM581669, HM581670, and HM581671) were then searched for similarity with other deposited sequences in GenBank. Alignments and phylogenetic analysis were performed using MEGA4 software [14].

PCR amplification and sequencing of the partial β-tubulin gene. Amplification of the partial β-tubulin fragment of the endophytic fungal genomes was conducted using the universal primers btub3 and btub4r [16]. The PCR reaction mixture was comprised of 10 μL fungal DNA, 5 μL 10× PCR buffer, 1.5 μL of 50 mM $MgCl_2$, 1 μL of 10 mM dNTP, 0.25 μL Taq polymerase, 40 pM of each forward and the reverse primers in a total reaction volume of 50 μL [13]. The PCR amplification was performed in a GeneAmp® 9700 Thermal Cycler with the following thermal protocol: initial strand denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 1.5 min and a final extension step at 72°C for 7 min [17]. The PCR products were analysed by 2% agarose gel electrophoresis and visualised using ethidium bromide under UV illumination in a Kodak gel logic 100 gel documentation system. A 100-bp DNA molecular weight ladder was included in each run. The amplicons were then sequenced in an Applied Biosystems 3700 Genetic Analyser with BigDye Terminator ver. 3.1. Sequences obtained (GenBank accession Nos.: HM596779, HM596780, HM596781, HM596782, and HM596783) were searched for similarity with other deposited sequences in GenBank. Alignments and phylogenetic analyses were performed using MEGA4 software [14].

Results and Discussion

The genetic relatedness dendrogram generated by scoring of the presence or absence of reproducible bands of fungal specific RAPD primers Nos. 4 and 7 clearly showed the genetic variability between the five endophytic fungal species isolated from five different ethno-medicinally important plants in the sacred forests of Meghalaya (India) (Fig. 1). The endophytic fungus isolated from *O. chinensis* and *C. caduca* formed a cluster, which was closely related to the endophytic fungus isolated from *P. fulgens*. The endophytic fungus isolated from *S. khasiana* was distinct from the others but was related to the isolate obtained from *P. fulgens* (Fig. 1). The endophytic fungal isolate obtained from *O. stellata* was the most divergent in terms of the RAPD distance in relation to the other four endophytic fungal isolates investigated.

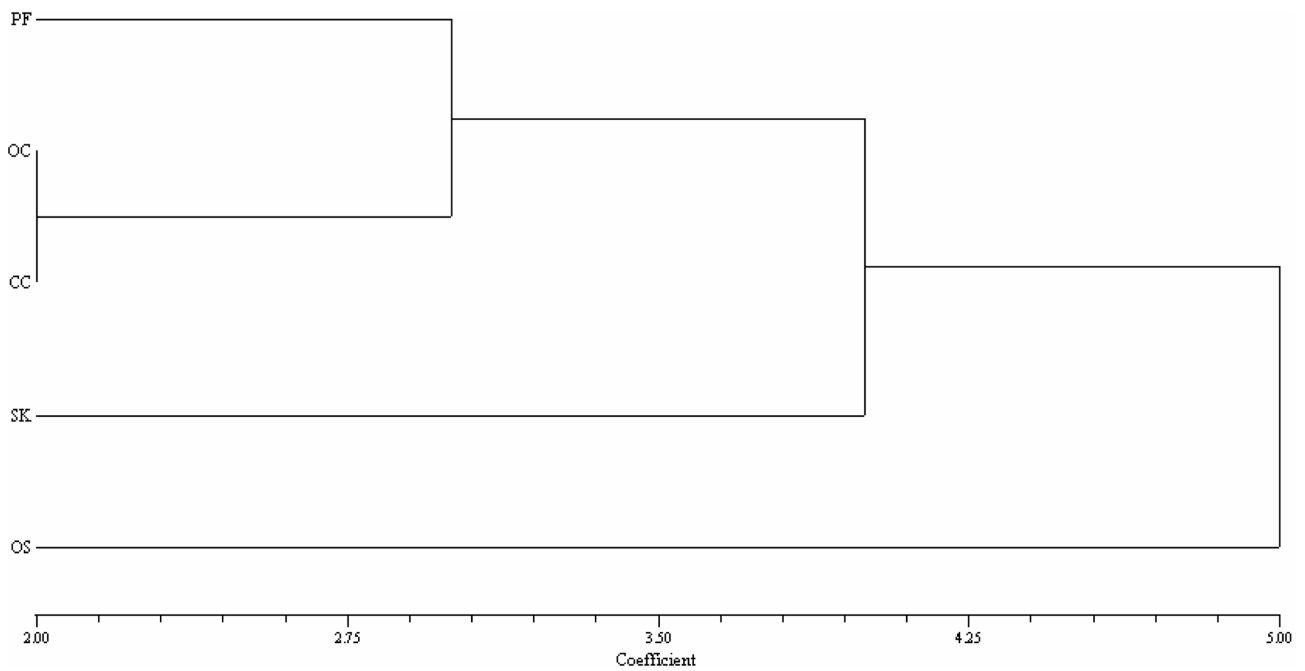RFLP profiles were generated for each of the ampli-

Fig. 1. Dendrogram of genetic relatedness generated by NTSYSpc ver. 2.0 from the random amplification of polymorphic DNA banding pattern analysis of the endophytic fungal isolates upon amplification by primers nos. 4 and 7. The neighbour-joining algorithm and Jaccard's similarity coefficient were used to generate the trees.
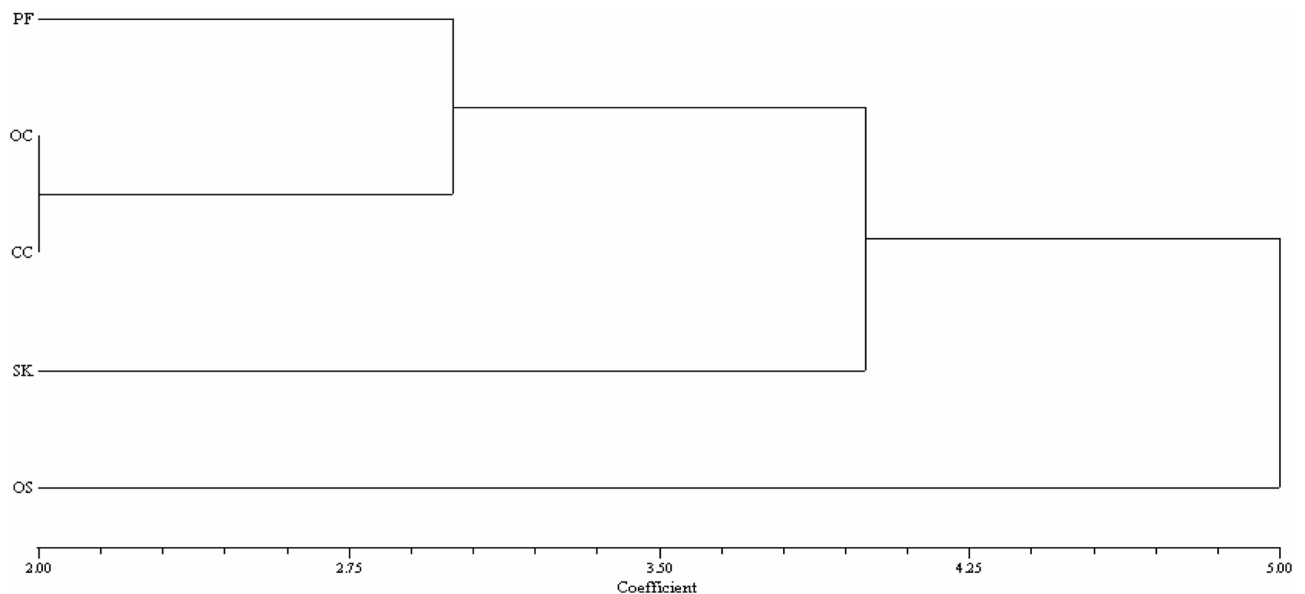

Fig. 2. Combined dendrogram of genetic relatedness generated by NTSYSpc ver. 2.0 from the PCR-restriction fragment length polymorphism banding pattern analysis of the PCR amplified internal transcribed spacer, small subunit rDNA and β-tubulin gene products with the *Msp*I, *Alu*I, *Nde*II, *Hae*III, and *Hin*fI restriction enzymes. The neighbour-joining algorithm and Jaccard's similarity coefficient were used to generate the trees.

cons using *Msp*I, *Alu*I, *Nde*II, *Hae*III, and *Hin*fI restriction enzymes. The overall genetic relatedness of the five endophytic fungal isolates based upon the combined dendrogram generated by the PCR-RFLP fingerprints is presented in Fig. 2.

Amplification of the target loci, i.e., ITS1, SSU rDNA and partial β-tubulin gene sequences of the five endophytes, led to the generation of amplicons that were near the expected size of the amplicons with the specific primers used. The genetic relatedness pattern for the five endophytic fungal isolates among themselves, based on the phylogenetic analysis of the ITS1, SSU rDNA and partial
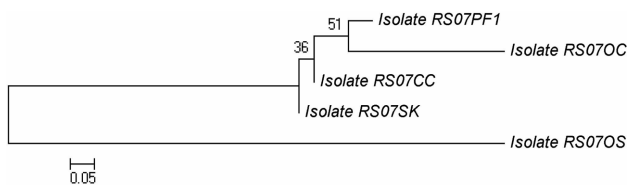
Fig. 3. Evolutionary relationships of the five endophytic fungal isolates based on internal transcribed spacer 1 sequence similarity.
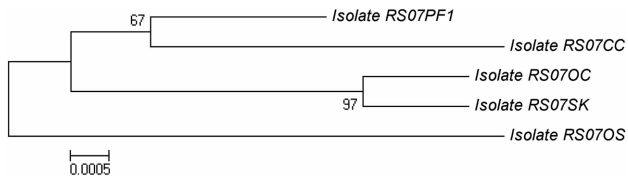

Fig. 4. Evolutionary relationships of the five endophytic fungal isolates based on small subunit sequence similarity of the 18S rRNA.

Fig. 5. Evolutionary relationships of the five endophytic fungal isolates based on β-tubulin gene sequence similarity.

β-tubulin gene sequences of the five endophytes using MEGA4 software is shown in Figs. 3~5. The intra-group sequence divergence in the ITS1 region among the five endophytic fungal isolates showed that the endophytic fungal isolate from *P. fulgens* was closely related to that of *O. chinensis* (Fig. 3).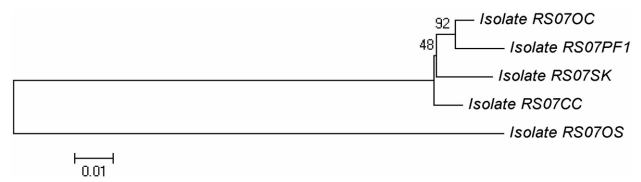 In contrast, the evolutionary simi-larity of the SSU of the 18S rRNA among the five iso-lates showed that the endophytic fungal isolate from *O. chinensis* was closely related to the endophyte isolated from *S. khasiana* with 97% bootstrap support for the grouping. The endophytic fungal isolate from *P. fulgens* was grouped with the isolate from *C. caduca* (67% boot-strap support) (Fig. 4). Analysis of the intra-group rela-tionships among the five isolates of endophytic fungi with evolutionary sequence similarity of the partial β-tubulin gene sequence, showed that the endophytic fungal isolate obtained from *P. fulgens* was closely related to the endo-phytic fungal isolate obtained from *O. chinensis*, with a bootstrap support of 92% (Fig. 5). In all three phyloge-netic trees, i.e., Figs. 3~5, the endophytic fungal isolate from *O. stellata* was the most distantly related to the other four endophytes. Although the overall picture generated by the analysis of specific DNA portions of the endo-
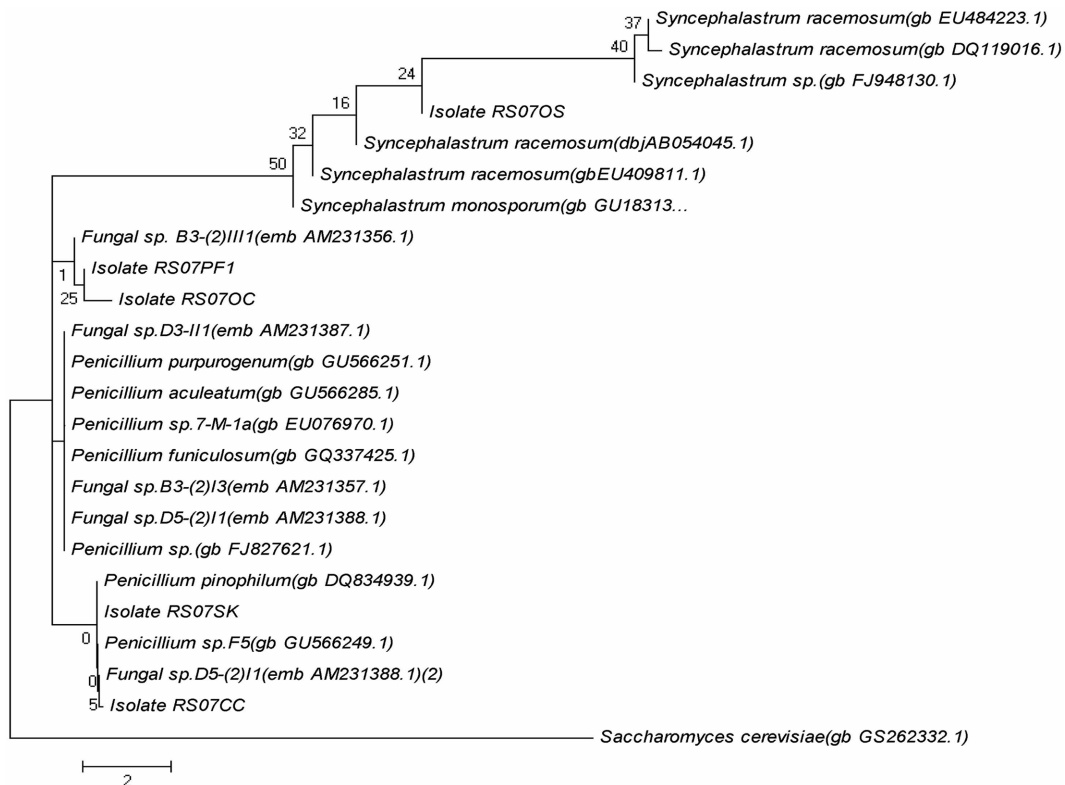

Fig. 6. Evolutionary positions of the five endophytic fungal isolates with other related fungal species based on internal transcribed spacer 1 sequence similarity.

phytic fungal isolates was quite similar to that generated by the RAPD and PCR-RFLP banding patterns, determining the sequences of particular gene fragments may provide a deeper insight into the genetic relatedness of these fungal isolates. Notably, the ITS1 and β-tubulin phylogenies represented a very similar picture of the genetic relationship of the five endophytic fungal isolates. We then tested the phylogenetic relationships of the five endophytic fungal isolates based on the ITS1, SSU of 18S rRNA and β-tubulin gene sequences to those fungal species having sequences deposited in public repositories

such as the NCBI nucleotide database. Major taxa showing the closest homology to our sequences were selected and aligned using the ClustalW program in the MEGA4 software. Phylogenetic trees were constructed using the neighbour-joining algorithm with 1,000 bootstrap replications. The phylogenetic trees generated from the ITS1, SSU of 18S rRNA and β-tubulin gene sequences are presented in Figs. 6~8, respectively.

The phylogenetic tree generated by the ITS1 sequences did not clarify the species identity of the endophytic fungal species, as the bootstrap support for these groupings
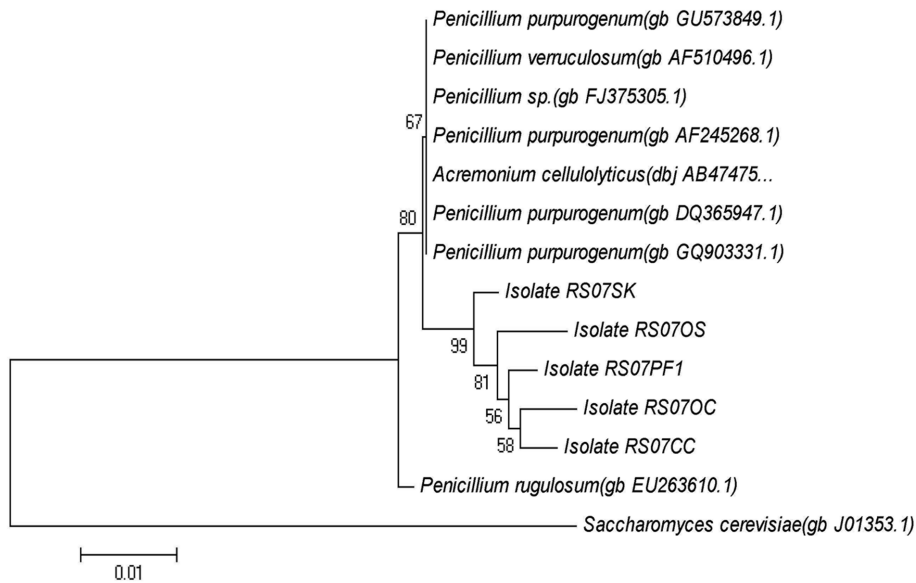

Fig. 7. Evolutionary positions of the five endophytic fungal isolates with other related fungal species based on small subunit sequence similarity of the 18S rRNA.
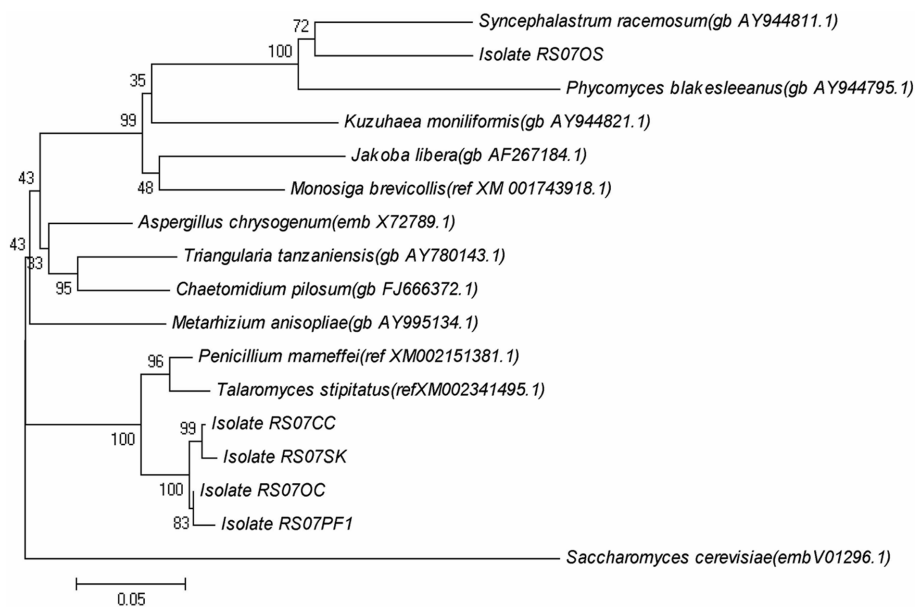

Fig. 8. Evolutionary positions of the five endophytic fungal isolates with other related fungal species based on β-tubulin gene sequence similarity.

was generally low (Fig. 6). Isolate RS07OS was placed in the fungal genus *Syncephalestrum*, whereas isolates RS07PF and PS07OC showed close homology with an unidentified fungal species B3-2(III) 1 (accession Nos.: emb AM231356.1). Isolate RS07SK was a species of *Penicillium* with close homology to *Penicillium pinophilum* (accession Nos.: gb DQ834939.1). Endophytic fungal isolate RS07CC was also unidentifiable, as it was closely related with unidentified fungal species D5-(2)/1 (accession Nos.: emb AM 231388.1) (Fig. 6). The phylogenetic tree generated by the analysis of the sequence information of the SSU region of the fungal 18S rRNA (Fig. 7) placed all endophytic isolates into *Penicillium*, and isolate RS07SK was the closest neighbour of *P. purpurogenum* (accession Nos.: gb AF245268.1). The four other endophytic isolates, namely RS07PF, RS07OC, RS07OS and RS07CC branched out from isolate RS07SK but were flanked by *Penicillium rugulosum* (accession Nos.: gb EU263610.1) (Fig. 7). Better bootstrap support values were obtained for the phylogenetic tree generated by the β-tubulin gene sequences of the five endophytic fungal isolates (Fig. 8). The endophytic fungal isolate obtained from *O. stellata*, i.e., RS07OS, was closely related to *Syncephalestrum racemosum* (accession Nos.: gb AY944811.1), whereas the β-tubulin gene sequences of the other endophytic isolates such as RS07PF, RS07CC, RS07OC and RS07SK were related to *Penicillium marneffei* and *Talaromyces stipitatus* (Fig. 8). Ribosomal loci with high and heterogeneous rates of change, such as ITS, IGS and mitochondrial SSU rDNA, can be used to distinguish taxa at the genus and species level [18]. The noncoding regions of these loci are prone to significant length variation, making alignment of distantly related taxa problematic. Therefore, fast evolving ribosomal genes are less useful in large-scale concatenated analyses involving higher-level phylogenetic relationships [18]. β-tubulin is abundant in eukaryotic cells and is the primary constituent of microtubules [16]. The β-tubulin gene sequence contains 3.5-fold more phylogenetic information than that of the SSU rRNA gene. Thus, the β-tubulin gene is an ideal marker to analyse deep-level phylogenies and complex species groups [19]. However, the precise homology defin-ing a species using phylogenetic evidence is speculative [20]. Endophytic fungi often exhibit 'cryptic' morphological characters [5]. Thus, to arrive at the most probable identity for our isolates, we correlated our previously observed morphological data with the molecular data generated by the ITS1, SSU rRNA and β-tubulin gene sequence analyses (Table 3). The isolates that showed close homology to *P. purpurogenum* based on SSU rDNA sequences may belong to a species complex (group) within *P. purpurogenum*. Therefore, the possibility that some of these isolates are novel fungal species cannot be completely ruled out. We believe that multi-loci molecular characterisation of endophytic fungi coupled with morphological validation will help fungal taxonomists unravel the yet un-explored world of cryptic fungal endophytes. Efforts are currently underway to deposit the isolates in microbial culture collections and to further elucidate their biotechnologically important genes and metabolites.

Acknowledgements

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Table 3. Identity of the five endophytic fungal isolates based on internal transcribed spacer 1 (ITS1), small subunit (SSU) rRNA and β-tubulin gene sequences

Endophytic fungal isolate	Morphological identity	Cryptic morphological characteristics	Identity based on ITS1, SSU rRNA and β-tubulin gene sequences
RS07PF	*Talaromyces flavus*	Shows resemblance to *Talaromyces* and *Mortierella*	*Penicillium purpurogenum*
RS07OS	*Mortierella hyalina*	None observed	*Syncephalestrum racemosum*
RS07OC	*Paecilomyces variabilis*	Colony appearance and colour similar to *Aspergillus* and *Paecilomyces*	*Talaromyces* sp.
RS07CC	*Penicillium* sp.	Colony morphology shows similarity with isolate RS07OS	*Penicillium* sp.
RS07SK	*Penicillium* sp.	None observed	*Penicillium purpurogenum*

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