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Autochthonous microbial community associated with pine needle forest litterfall influences its degradation under natural environmental conditions

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Abstract The slow natural degradation of chir pine (*Pinus roxburghii*) needle litterfall and its accumulation on forest floors have been attributed to its lignocellulosic complexities of the biomass. The present study offers a microbiological insight into the role of autochthonous microflora associated with pine needle litterfall in its natural degradation. The denaturing gradient gel electrophoresis (DGGE) fingerprinting indicated actinomycetes (*Saccharomonospora* sp., *Glycomyces* sp., *Agrococcus* sp., *Leifsonia* sp., *Blastocatella* sp., and *Microbacterium* sp.) as a dominant microbial community associated with pine needle litterfall with the absence of fungal decomposers. On exclusion of associated autochthonous microflora from pine litterfall resulted in colonization by decomposer fungi identified as *Penicillium chrysogenum* and *Aspergillus* sp., which otherwise failed to colonize the litterfall under natural conditions. The results, therefore, indicated that the autochthonous microbial community of pine needle litterfall (dominated by actinomycetes) obstructs the colonization of litter-degrading fungi and subsequently hinders the overall process of natural degradation of litterfall.

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Introduction

Within the Indo-Pacific region, Himalayan subtropical pine forests are the largest and are widely spread in Himachal Pradesh, a region of India with chir pine (*Pinus roxburghii*), as the dominant species (Singh and Kushwaha 2011; Bisht et al. 2014). The pine stands nearly account for about 2–3 ha^{-1} $year^{-1}$ needle litterfall on forest floors (Rawat et al. 2001; Tiwari et al. 2016). In natural environmental conditions, the natural decomposition of needle litterfall is a very slow process because of lignocellulosic complexities (Klotzbucher et al. 2011; Soong et al. 2015). Documented literature reveals that pine litter has less decomposition rates in comparison to litter from mixed forest systems (Cornwell et al. 2008; Weedon et al. 2009; Sheffer et al. 2015) and litter from oaks (Arslan et al. 2010). The time-consuming decomposition of forest litter results in accumulation of large volumes of pine needles as forest litter, a phenomenon occurring throughout the world (Safi et al. 2004; Vestgarden et al. 2004) which has serious implications on our ecosystem bio-geochemical cycling and energy flow (Zhou et al. 2015). On the forest floor, the thick layer of needles is a major source of ground fires, recurring annually in India (Sharma 2009). The thermal decomposition of pine needles results in the generation of volatiles which are hazardous to the environment (Safi et al. 2004). Their release into the atmosphere

could result in changing of the oxidative capacity and disturbance in the balance of greenhouse gases (Isidorov et al. 2016). In addition to the above-cited problems, pine needles are shown to possess allelochemicals which show phytotoxicity towards other species, which poses hindrance in the establishment of secondary successional species in the forest ecosystem (Navarro-Cano et al. 2010; Kimura et al. 2015).

All of the above-cited problems associated with the accumulation and degradation of pine needle litterfall on forest floors warrant better understanding of mechanisms that determine its decomposition under natural environment conditions. Documented literature highlights the significance of bacteria and fungi, in litter decomposition, especially in forest ecosystems (Persson et al. 1980; Van der Heijden et al. 2008). A major factor governing the litter decomposition rates on forest floors, is the interaction of microbes (fungal: bacterial associations) with the forest litter, which inturn depends on the nature and lignocellulosic quality of the litter. (Bray et al. 2012; Newman et al. 2015; Isidorov et al. 2016) However, an important aspect, relating to litter decomposition, which has not been investigated in the past, is the need to understand the role of forest litterfall-associated autochthonous microbial community in litter breakdown, which forms the basis of the present investigation. For this, in the present study, we employ the use of culture-independent molecular technique using denaturing gradient gel electrophoresis (DGGE) to evaluate the hypothesis that whether or not autochthonous microbial community of pine needle litter together with the lignocellulosic complexities, hinders its degradation under natural environmental conditions. The application of DGGE to study bacterial and fungal diversity has been widely reported by several researchers (Lyautey et al. 2005; Das et al. 2007; Mahajan et al. 2016). In the present investigation, a two-step validation experiment for testing the hypothesis for the role of autochthonous microflora was planned. In the first step, the autochthonous pine needle litter-associated microbial community was dislodged; it was followed by microbiological observation of pine needles for microbial succession, especially by decomposer fungi, under natural environmental conditions. Findings in the present study employing culture-independent analysis of microbiota and its phylogenetic analysis contribute to our knowledge of autochthonous microbiome of pine needle litter and its role in natural degradation of pine needle litter on forest floors.

Materials and methods

Study site

The pine needles were collected from the subtropical Kandaghat pine forest range (31° 00′ 57″ N; 77° 04′ 17″ E). The study site is one of the five forest ranges of Solan Forest Division, having strands of chir pine which mostly confirm to Champion and Seth's Forest type 9 C1a-Lower or Shiwalik chir pines and known for frequent forest fires (Sharma and Singh 2010). The dried pine needles were collected in the months of March to June, 2014 and 2015, and stored in airtight cartons; transported to laboratory; and processed immediately.

Holocellulose and lignin estimations

Determination of holocellulose (TNI-A-9 test method; Laboratory manual of CPPRI; Laboratory method manual 2001) and lignin content (T222 test method; Technical Association of the Pulp and Paper Industry (TAPPI), Atlanta, GA, USA) was done as per the standard methods (Ferris 1963).

DNA extraction and PCR amplification

In an attempt, to gain an insight into the role of autochthonous microbial community in pine needle litterfall breakdown, metagenomic DNA was extracted using standardized protocol of Dempster et al. (1999) with slight modification in methodology (Mahajan et al. 2016). The extracted metagenomic DNA was used as a template to amplify *Eucarya* (fungi) 18S–28S ITS genes and bacterial 16S ribosomal RNA (rRNA) genes using, PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 0.2 mM dNTP (dATP, dCTP, dGTP, and dTTP), 1.5 mM $MgCl_2$, 1.0 μM of each primer, and Pfu DNA polymerase at a concentration of 1.0 U (Thermo Fisher Scientific). DNA template (20–100 ng) was used for amplifications. The details on the use of primers and PCR reaction conditions are presented in Table 1.

DGGE analysis

The DGGE analysis of test DNA samples, resulted in separation of the DGGE-PCR-amplified products in polyacrylamide gels with denaturing gradients ranging from 20 to 45 % (7 M urea and 40 % (*v*/*v*) deionized formamide resulted in 100 % denaturing conditions)

Table 1 A description of the primers used in the study

Domain	Primer sequence (5′–3′)	Primer name	PCR conditions	Reference
Bacteria	**GC**-ACTCCTACGGGAGGCAGCAG[a] ATTACCGCGGCTGCTGG	Eub338 Eub518	• (Initial denaturation at 94 °C for 300 s) × 1 cycle • (Denaturation at 95 °C for 60 s; annealing at 53 °C for 60 s; extension at 72 °C for 120 s) × 30 cycles • (Final extension at 72 °C for 600 s) × 1 cycle • Hold at 4 °C	(Lane 1991; Muyzer et al. 1993)
Fungi	TCCGTAGGTGAACCTGCGG (3′ end of the 18S rDNA adjacent to the ITS1, F primer) 5′- TCCTCCGCTTATTGATATGC-3′ (5′ end of the 28S rDNA adjacent to the ITS2, R primer)	ITS1 ITS4	• (Initial denaturation at 94 °C for 300 s) × 1 cycle • (Denaturation at 95 °C for 45 s; annealing at 53 °C for 60 s; extension at 72 °C for 150 s) 30 cycles • (Final Extension at 72 °C for 600 s) × 1 cycle • Hold at 4 °C	(White et al. 1990)

rDNA ribosomal DNA

[a] GC-clamp: CGCCCGGGGCGCGCCCCGGGCGGGCGGCACGGGGGG

(Mahajan et al. 2016). Electrophoresis was carried out in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.0) at constant voltage of 65 V for 12 h using a DCode system (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were stained for 10 min. in TAE buffer containing ethidium bromide (0.5 mg/l), followed by 5 min of washing in distilled water, and then photographed (Alpha Imager System, Alpha Innotech, San Leandro, CA, USA) at a wavelength of 302 nm, under UV light. The prominent DGGE bands were excised and macerated in 20 μl of nuclease-free water and stored at 4 °C for 24 h. The samples were then centrifuged at 10,000*g* for 3 min and the supernatant (3 μl) was used as template, and PCR amplification was carried out under the conditions as described above. The amplified DNA was observed on agarose gel and purified using Real Genomics DNA purification kit and then sequenced. The use of culture-independent molecular techniques provides an opportunity to characterize microbial community associated with forest litterfall and therefore provide a better resolution of the microbial taxa.

Phylogenetic analysis and sequence data accession number

In an attempt to identify the microbial species, associated with autochthonous microbial community in pine needle litterfall, the *16S rRNA* and 18S–28S ITS gene sequences were analyzed using BLAST search (Altschul et al. 1990), to identify the closest matching sequences from NCBI, GenBank database (http://www.ncbi.nlm.nih.gov). The representing sequences and the test samples were further analyzed using Clustal Omega, a multiple alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic analysis was carried out on multiple sequence alignments, in order to construct phylogenetic trees using neighbor-joining method (CLC Main Workbench 8.0; http://www.clcbio.com). Bootstrap confidence (100 permutations) values were employed to statistically validate the phylogenetic tree. The nucleotide gene sequences, sequences in the present study have been deposited in the GenBank database under accession numbers KR258726–KR258736.

Hypothesis validation

We hypothesized that the autochthonous microbial community of pine needle (as revealed by DGGE analysis) together with the lignocellulosic complexities, hinders its natural degradation. A two-step validation experiment for testing the hypothesis was therefore planned. Firstly, the autochthonous pine

needle litter-associated microbial community was dislodged. Two treatment sets of pine needles were maintained, the first set (S_0) designated as control, where natural pine microbial community was not dislodged and the second set (S_1) of pine needles was autoclaved at 121 °C for 15 min for three consecutive days to remove autochthonous microbial community. This was followed by microbiological observation of pine needles for microbial succession, especially by decomposer fungi, under natural environmental conditions. The isolated decomposer fungi were molecularly identified by phylogenetic analysis of 18S–28S ITS gene sequences.

Results and discussion

Lignocellulosic composition

The major and most significant components of plant litter that are known to influence its natural decomposition, include organic compounds, hemicellulose, cellulose, and lignin; however, significant differences have been observed in their relative contents, which largely depend on the plant tissue being sampled (leaves, stem, etc.) and also on the plant species that actually contributes to the forest litter (Ding et al. 2012). In the present study, dried pine needle litter, collected from forest floors accounted for the presence of 18.05 % holocellulose content, 23.36 mg/g of acid-soluble lignin and 475 mg/g of acid-insoluble residue. In pine needles, a major portion of the cellulose is associated with the complex crystalline structures, which make it difficult for its enzymatic degradation. Similarly, because of the structurally complex (cross-linked polymers of phenolic monomers) nature of lignin, its natural degradation is difficult. In the present study, the presence of high lignin content in pine needle litter is in concomitance with previously well-established findings, that lignocellulosic complexities pose hindrance in natural degradation of pine needle litter. Purahong et al. (2015) reported that the major hindrance in the process of litter decomposition is the inability of lignin to decompose naturally under environmental conditions. Extensive review, in this regard as documented by Berg et al. 2015 highlights the fact that degradation of lignin is the major factor that determines the fate of pine needle litter decomposition.

Autochthonous pine needle litterfall-associated microbial community

The culture-independent studies of total bacteria, based on PCR-DGGE profiling of 16S rDNA fragments, resulted in identification of nine prominent bands. The phylum *Actinobacteria* was the dominant phylum followed by *Firmicutes* (Fig. 1a). Representatives from phylum *Actinobacteria* and *Firmicutes* belonged to *Saccharomonospora* sp., *Glycomyces* sp., *Psychrobacillus* sp., *Agrococcus* sp., *Leifsonia* sp., *Blastocatella* sp., *Microbacterium* sp., and *Paenibacillus* sp. The phylogenetic relatedness of the fragment post sequencing is presented in Fig. 1b. The results presented in Table 2 highlight the nearest homologues to the autochthonous pine microbial community. The results in the present study are in corroboration with previous reports, where researchers have reported dominance of actinomycete communities in forest soils using culture-dependent methods (Zvyagintsev et al. 1996). Newman et al. (2015) using culture-independent techniques also observed actinomycete assemblages on litter along with bacteria and fungi. The major degraders of fully lignified plant tissues are filamentous fungi, which are known for their abilities of cellulose and lignin degradation via their efficient hydrolytic enzymatic systems (Martinez et al. 2008). However, in the present study, fungal-specific primers (ITS1 and ITS4; Table 1) for internal-transcribed spacer (ITS) region did not result in any amplification. The dominance of actinomycetes associated with pine needles suggests the possible antagonistic impact of actinomycetes on decomposer fungal species. A few reports are available on the influence of actinomycetes on lignocellulosic degrading fungi and their involvement in inhibition of southern pine sapwood decomposition (*Lenzites sepiaria*, *Polyporus versicolor*, and *Lentinus lepideus*) by *Streptomyces* sp. (De Groot 1971). In vitro and microcosm studies carried out by Jayasinghe and Parkinson (2008) have also highlighted the antagonistic potential of actinomycetes against fungi, with a known ability of lignocellulosic decomposition. Dângelo et al. (2016) reported inhibition of filamentous fungal species as a result of secondary metabolites produced by actinomycetes. A molecular, culture-independent microbiological insight into pine litter and the structural complexity of pine needles, as studied in here, therefore, signify the

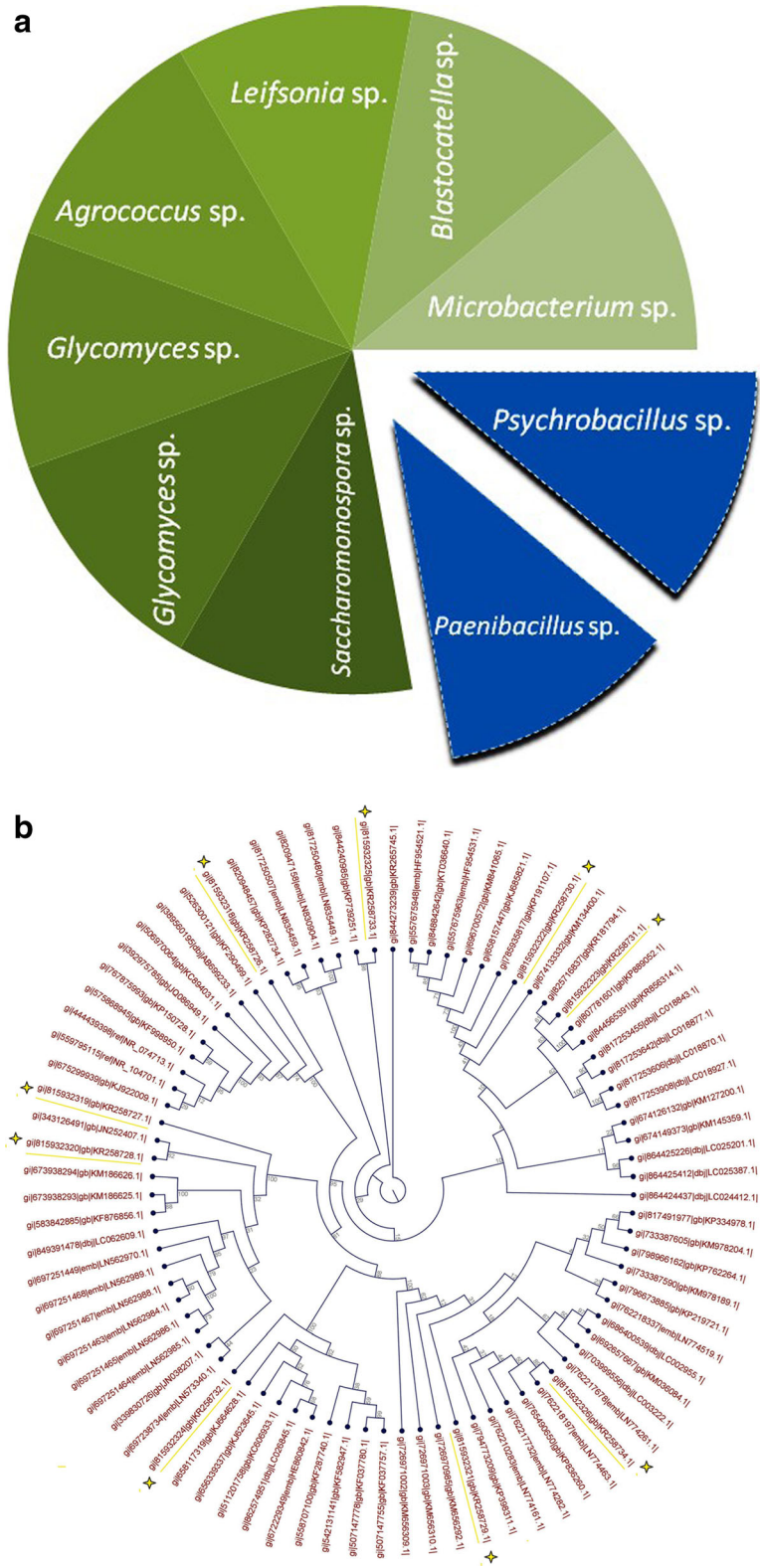

Fig. 1 Autochthonous microbial community associated with pine needles. **a** Actinobacteria was the dominant phylum followed by Firmicutes. **b** Phylogenetic relatedness of 16S rDNA gene sequences of DGGE gel bands associated with natural microbiota of pine needles (*underlined* and *marked*). Bootstrap confidence values were generated using 100 permutations of the data set to derive the nucleotide sequence similarities

Table 2 Phylogenetic relatedness of autochthonous pine microbial community

Microflora associated with pine needles (accession number)	Closest homologue				
	Taxonomic name (accession number)	Percent identity	Alignment score	Isolation source	Country
Uncultured *Saccharomonospora* sp. (KR258726)	Uncultured *Saccharomonospora* sp. clone 3_4_B8_b (JQ086949)	98.00	99.06	Push core sediment sample from the vadose zone of a hydrocarbon-contaminated aquifer	Germany: Leuna
Uncultured *Glycomyces* sp. (KR258727)	*Glycomyces* sp. AB82-M (JN252407)	98.00	98.25	Salt soil	Korea
Uncultured *Glycomyces* sp. (KR258728)	*Glycomyces* sp. AB82-M (JN252407)	100.00	100.00	Salt soil	Korea
Uncultured *Psychrobacillus* sp. (KR258729)	*Psychrobacillus psychrodurans* isolate 0312TES32F1 (LN774519)	98.00	98.31	Air sample	Spain: Malaga, Cueva del Tesoro
Uncultured *Agrococcus* sp. (KR258730)	*Agrococcus* sp. 9_99 (HF954531)	100.00	100.00	Soil sample	Spain
Uncultured *Leifsonia* sp. (KR258731)	*Plantibacter* sp. L395 (KR181794)	100.00	100.00	Forest litter	Czech Republic
Uncultured *Blastocatella* sp. (KR258732)	Uncultured Acidobacteria bacterium clone FWB3C1-74 (KF582947)	100.00	100.00	Freshwater ballast collected from a transoceanic-general cargo vessel	USA: Duluth-Superior Harbor, Duluth, MN
Uncultured *Microbacterium* sp. (KR258733)	*Microbacterium radiodurans* strain 9-G (KP739251)	100.00	100.00	Coffee fermentation	Brazil
Uncultured *Paenibacillus* sp. (KR258734)	*Psychrobacillus psychrodurans* isolate 0312TES14N1 (LN774463)	100.00	100.00	Air sample	Spain: Malaga, Cueva del Tesoro

Table 3 Phylogenetic relatedness of *Penicillium chrysogenum* DST-RFBR1 (KR258735) and *Aspergillus* sp. DST-RFBR2 (KR258736)

Phylogenetically related isolates/strains	Percent identity	Alignment score	Isolation source	Country	Accession number
Closest homologues to *Penicillium chrysogenum* DST-RFBR1 (KR258735)					
P. chrysogenum isolate 0311MAR40N4	100.00	100.00	Paper scraps	Spain: Huelva, Gruta de las Maravillas	LN809047
P. chrysogenum isolate *0311MAR33G7*	100.00	100.00	Plant debris	Spain: Huelva, Gruta de las Maravillas	LN809043
P. chrysogenum isolate 0312TES21U1	100.00	100.00	Air sample	Spain: Malaga, Cueva del Tesoro	LN809008
P. chrysogenum isolate 05D	100.00	100.00	Documentary material and indoor airborne of archive buildings	Bogota (Colombia)	KP067252
P. chrysogenum isolate 39D	100.00	100.00	Documentary material and indoor airborne of archive buildings	Bogota (Colombia)	KP067251
P. chrysogenum isolate 28A	100.00	100.00	Documentary material and indoor airborne of archive buildings	Bogota (Colombia)	KP067250
P. chrysogenum isolate DC09	100.00	100.00	Documentary material and indoor airborne of archive buildings	Bogota (Colombia)	KP067248
P. chrysogenum isolate DC05	100.00	100.00	Documentary material and indoor airborne of archive buildings	Bogota (Colombia)	KP067247
P. chrysogenum isolate DC01	100.00	100.00	Documentary material and indoor airborne of archive buildings	Bogota (Colombia)	KP067246
Closest homologues to *Aspergillus* sp. DST-RFBR2 (KR258736)					
Aspergillus sp. MBL1612	100.00	100.00	–	Pakistan	KM924435
Aspergillus fumigatus strain 3T-EGY	99.00	96.43	–	Egypt	KP140961
A. fumigatus isolate BP1	99.00	96.97	–	China	JF957862
A. fumigatus strain KCCM60331	99.00	96.26	Traditional starter cultures (Nuruks). Used for rice wine	South Korea	HQ285617
A. fumigatus isolate 13-F1	99.00	96.26	–	China	GU266273
A. fumigatus isolate Z 2	99.00	98.36	Flavi and Fumigati sections	Switzerland	KJ175459
A. fumigatus Zag1	99.00	97.86	–	Egypt	AB976023
A. fumigatus strain Afu0214M	100.00	94.65	Cave rock and water	Poland: Lower Silesia	KP670425
A. fumigatus strain HA1	100.00	96.26	Cereal	Egypt	KP081777

En dashes indicate information not available in the NCBI database

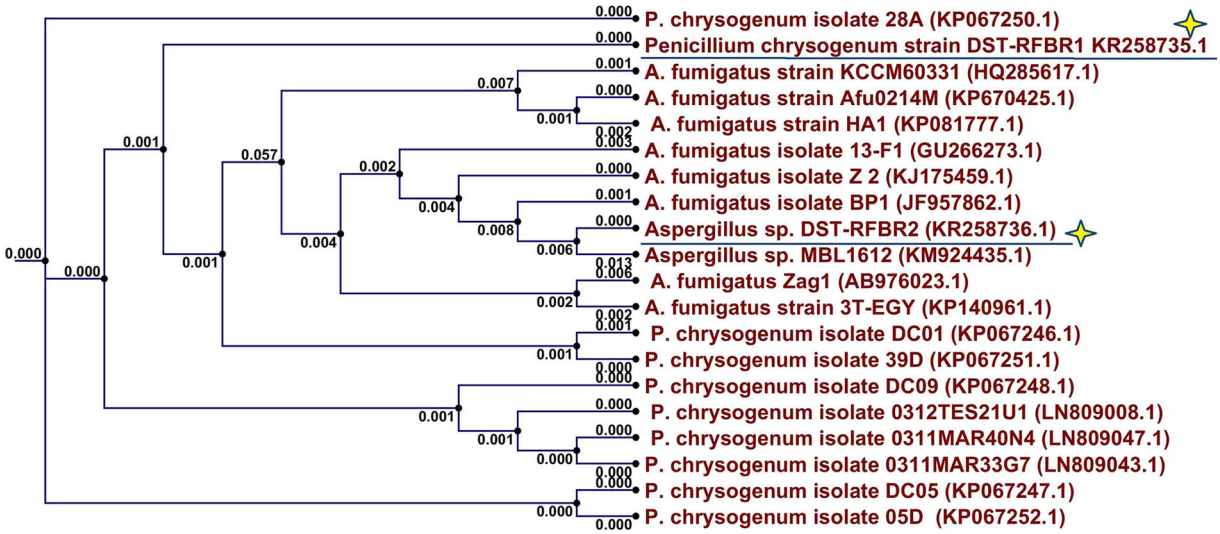

Fig. 2 Phylogenetic relatedness of fungal isolates (*underlined* and *marked*) observed as a result of microbial succession once the pine-associated autochthonous microbial community was dislodged. Bootstrap confidence values were generated using 100 permutations of the data set to derive the nucleotide sequence similarities

combined role of lignocellulosic content and dominant role of actinomycetes impeding the natural degradation of pine needle litter.

Validation of the test hypothesis

We hypothesized that the autochthonous microbial community of pine needle litterfall (dominated by actinomycete) obstructs colonization of litter-degrading fungi and subsequently hinders the overall process of natural degradation of litterfall. The results based on the experimentation for the validation of the test hypothesis, revealed extensive mycelial colonization in sample S_1 (treatment set of pine needles where autochthonous microbial community was dislodged) after 60 days of incubation, under natural environmental conditions. However, no mycelial growth was observed in sample S_0 where the autochthonous microbial community was not dislodged. The microbiological examination of sample S_1 resulted in isolation of two predominant fungal isolates, molecularly identified as *Penicillium chrysogenum* strain DST-RFBR1 and *Aspergillus* sp. DST-RFBR2 (Table 3, Fig. 2). Waing et al. (2015) reported the role of *Penicillium* and *Aspergillus* sp. along with other fungi in degradation of leaf litter. Fungi are considered to be the most influential of all the microorganisms in the process of litter degradation, due to the release of enzymes with known abilities of lignin degradation (Purahong et al. 2014). The *Penicillium* sp. and *Aspergillus* sp. are fast-growing fungal species; however, we did not observe their growth in the natural environment of pine needles and in sample S_0 where the autochthonous microbial community was not dislodged. The findings, therefore, support the overall hypothesis that autochthonous microbial community of pine needle litterfall determines the ability of fungal degraders to colonize the litter and thereby influence the pine leaf litter degradation. The plausible and logical rationale behind the findings can be attributed to (a) the inability of decomposer fungi to dwell in organic material, where significantly higher actinomycetes population is already present. (b) The dominance of actinomycete (present study) could plausibly result in high concentration of antifungal compounds and hyperparasitism that eventually results in inability of fungal species to colonize pine needles. Our findings are in corroboration with findings previously reported by Jayasinghe and Parkinson (2008) who reported that because of the known abilities of actinomycetes to synthesize antifungal metabolites and cause hyperparasitism, the population of decomposer fungi can be significantly lower in litter. In vitro studies have shown that when fungi and actinomycetes are co-cultured, there is a significant effect on the morphological characteristics of fungi such as fungal hyphae lysis, germinating spores with distorted hyphae, and hyphal stunting (Getha and Vikineswary 2002; Jayasinghe and Parkinson 2008; Loqman et al. 2009). In the present

study, after exclusion of autochthonous microbial community, microbiological observations revealed that the fungal species colonized pine needle litter due to their ability to secrete lignocellulolytic enzymes (Sharma et al. 2015).

Conclusions

The management of pine needle litter on forest floors is a major challenge considering its impact on socio-economic dynamics of forest management. The study emphasizes that in addition to lignocellulosic complexities, the dominance of actinomycetes as an autochthonous microbial population in pine litter restricts the colonization of decomposer fungi. The culture-based approaches with subsequent functional tests are needed, in order to develop a microbiological-based system for pine needle litter management on forest floors. The strategy of managing pine needle-associated microbiome is of much promise as it offers a futuristic yet biologically sustainable strategy for managing pine forest litter.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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