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Diversity of endolichenic fungi within lichen genus *Parmotrema* from India

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Abstract: The lichens serve as an ecological niche for a group of unexplored fungal species residing inside their thallus without causing any noticeable symptoms and such fungi are termed as endolichenic fungi. The objective of the current study is to check the endolichenic fungal diversity within lichen genus *Parmotrema*. The study resulted in a total of 450 endolichenic fungi (ELF) isolates from 15 species of *Parmotrema*. Out of these, 73 sporulating isolates were identified using ITS sequences which resulted in 47 species under 23 genera. The phylogenetic assemblage of the fungi comprised Sordariomycetes (84.50%), Dothideomycetes (5.63%), Eurotiomycetes (7.04%), Pezizomycetes (1.40%), and Agaricomycetes (1.40%). *Daldinia eschscholtzii*, *Xylaria feejeensis*, *Nemania diffusa*, *Annulohypoxylon truncatum*, and *Nigrospora sphaerica* were frequently occurring isolates inhabiting at least five different species of *Parmotrema*. Furthermore, *Daldinia eschscholtzii* and *Nemania diffusa* were found to be with maximum colonization rate of 1.55% and relative frequency of 0.148. Sorenson's similarity coefficient was found to be highest between *P. hababianum* and *P. nilgherrense* with 0.36. The study encountered fungal taxa such as *Annulohypoxylon truncatum*, *Coprinellus radians*, *Cladorrhinum* sp., *Plectania rhytidia*, *Fimetariella rabenhorstii*, and *Liangia sinensis* earlier not reported as endolichenic fungi.

Key words: Colonization rate, endosymbionts, relative frequency, biodiversity index

1. Introduction

The lichens are a highly integrated system of mycobiont and photobiont (algae and/or cyanobacteria) communities. The lichen fungus requires specific photobiont in order to develop the symbiotic phenotypes. The lichen thallus, which resembles plant tissue, provides a fascinating biological environment for a variety of microorganisms (Zhang et al., 2016). In addition to their primary symbionts, lichens also have lichenicolous fungi, endolichenic fungi, and culturable and nonculturable nonphotosynthetic bacteria (Biosca et al., 2016; Muggia et al., 2014). Since lichens date back to over 600 million years (Yuan et al., 2005) and today they dominate approximately 10% of the planet's terrestrial ecosystems, lichens and their partners represent a successful style of symbiosis (Papazi et al., 2015). However, lichens are underexplored habitats for microbial diversity. It is profitable to investigate less-studied environmental conditions and habitats for microorganisms like fungi, in order to better understand microorganisms' biology and use their distinctive genes for technology (Suryanarayanan et al., 2017).

Sometimes the fungi live asymptomatically within the tissue of other organisms with no evident signs of infection (Kellogg and Raja, 2016). One such group that resides within the plant tissues is endophytic fungi, which is primarily comprised of the phylum Ascomycota (Arnold et al., 2009). After the discovery of paclitaxel (taxol), an important source of anticancer drug produced by *Taxomyces andreanae*, which resides inside *Taxus brevifolia* (Stierle et al., 1993), the interest in these endophytic fungi has intensified. Numerous articles have emphasised the diversity and potential of endophytic fungi as sources of natural pharmaceutically important compounds (Kaul et al., 2012; Nisa et al., 2015; Proksch et al., 2010; Strobel et al., 2004; Tan and Zou, 2001). These endolichenic fungi (ELF) are comparable to endophytic fungi, which also produce new compounds with intriguing bioactivities, including alkaloids, terpenoids, naphthalene derivatives, polyketides, diphenyl ethers, heptaketides, and chromenone derivatives (Paranagama et al., 2007; He et al., 2012; Zhang et al., 2012; Wang et al., 2013; Yuan 2013; 2016; Li et al., 2015; Zhao et al., 2014). About 500 ELF have been isolated of so far, but only 135 have been identified up to species level while a large number have either been partially identified up to genus level or treated

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as unidentified. Most of the studies focused on identifying bioactive molecules (Chakarwarti et al., 2020).

Every lichen species that has been researched so far, from the Tropics to the Arctics, contains the ELF (Suryanarayanan et al., 2005; Li et al., 2007; Arnold et al., 2009; Kannangara et al., 2008; Tripathi et al., 2014, Tripathi and Joshi, 2019). U'Ren et al. (2012) confirmed through a molecular investigation that ELF represent a unique ecological group and are not lichen colonizer by chance. The species diversity of ELF is still unknown, although a pyrosequencing investigation revealed the occurrence of various fungal groups within the lichen thalli (Bates et al., 2011). A fluorescence *in situ* hybridization and gene sequencing investigation found that host–parasite interactions change the microbiota (Wedin et al., 2016). In order to understand the pattern of diversity, distribution, host specificity and influence of biotic and abiotic factors, more research on ELF occurring within lichens growing in varied habitats is needed. Petrini (1990) was the first person who isolated endolichenic fungi from two fruticose lichens. Thereafter, about 50 articles are published globally, mostly focusing on bioprospecting aspects of ELF as indicated by 'Recent Literature on Lichens database'. In India, studies on ELF were first started by Suryanarayanan et al. (2005), who looked into several corticolous lichens. Their findings demonstrated that the endophytes found in the leaves of the trees that supported these lichens and the ELF assemblages had little in common. Tripathi et al. (2014; 2019) investigated the variety of ELF in macrolichens selected for the study from Almora district of Uttarakhand. From 12 macrolichens, they identified a total of 24 isolates of endolichenic fungi, claiming *Aspergillus flavus*, *Fusarium solani*, and *Alternaria alternata* to be the most frequently isolated fungi in some forests of Kumaun Himalaya. As the studies on ELF are scarce in India, the aim of the present study is to better understand the diversity and assemblage pattern of different ELF residing in different species of *Parmotrema* collected from various parts of the country.

2. Materials and methods

2.1. Collection of lichens

The collection of lichen samples was done from different parts of the country located mostly in Western Himalaya, Western Ghats, and North-East India (Figure S1). The samples were identified following standard procedures and keys (Divakar and Upreti, 2005). A total of 15 species of the genus *Parmotrema* were utilized in the present study, namely, *P. austrosinense* (Zahlbr.) Hale, *P. crinitoides* J.C. Wei, *P. crinitum* (Ach.) M. Choisy, *P. direagens* (Hale) Hale, *P. hababianum* (Gyeln.) Hale, *P. melanothrix* (Mont.) Hale, *P. nilgherrense* (Nyl.) Hale, *P. praesorediosum* (Nyl.) Hale, *P. pseudonilgherrense* (Asahina) Hale, *P. pseudotinctorum* (Abbayes) Hale, *P. reticulatum* (Taylor) M. Choisy, *P. saccatilobum* (Taylor) Hale, *P. stuppeum* (Taylor) Hale, *P. tinctorum* (Despr. ex Nyl.), and *P. thomsonii* (Stirt.) A. Crespo, Divakar & Elix. Most of these lichen thalli were found growing on the bark of the trees. The lichen samples were then transferred to polythene packets, labelled and tightly sealed to avoid moisture. A set of identified samples were accessioned and preserved in herbarium LWG of CSIR-NBRI, Lucknow (Table S1).

2.2. Isolation of endolichenic fungi

The lichen samples which were fresh and free from any sign of disease were chosen and cleaned under tap water. Each lichen sample was fragmented into 30 segments of size approximately 0.5 cm^2 and then their surface sterilisation was performed by immersing them sequentially in 30% hydrogen peroxide (H_2O_2) for 90 s, 70% ethanol (C_2H_5OH) for 5 s, 4% sodium hypochlorite (NaOCl) for 90 s and finally rinsing in distilled water for 10 s (Suryanarayanan et al., 2005). The segments were then put in potato dextrose agar (PDA) petri dishes and sealed using Parafilm™. The plates were then incubated in a light chamber with a 12:12 h light-dark cycle for 28 days at 26 ± 2 °C (Bills and Polishook, 1992; Suryanarayanan, 1992). The fungi that emerged from the tissue segments were separated, scrutinised, and individually cultivated on PDA slants. For the isolation, culture-dependent techniques were undertaken and nonsporulating fungal species (mycelia sterilia) were excluded.

2.3. Identification of isolates

Each lichen sample's fungal isolates were first segregated into morphospecies based on their physical features (e.g., texture and colour). The isolates were then identified through slide preparation by staining hyphae in cotton blue and using taxonomic keys (Ellis, 1971; Subramanian, 1971; Barnett and Hunter, 1972; Von Arx, 1974). The unique, representative colonies of morphospecies were selected for DNA-based identification. The DNA extraction from the fungi, amplification of genes, and sequencing was done following Sharma et al. (2016). The genomic DNA of ELF was extracted from actively growing hyphae in Czepak Dox medium using DNA extraction kit (G Sure fungal DNA extraction kit). The isolated DNA was amplified by polymerase chain reaction (PCR) using primers ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 (5'TCCTCCGCTTATTGATATGC3') sequences. The sequencing was performed via Sanger sequencing method. The sequences were matched with available sequences in NCBI GenBank through BLAST® analysis.

2.4. Phylogenetic analysis

Three phylogenetic trees were constructed separately for total isolates, abundant genera, and unique genera. The obtained sequences were edited manually using

GENIOUS PRIME v2022.2.2. All sequences, including those downloaded from NCBI GenBank were aligned using MUSCLE (Edgar, 2004) in MEGA X (Kumar et al., 2018). Phylogenetic tree was constructed using maximum likelihood (ML) analysis performed with RAxML v8.2.12 (Stamatakis, 2014). A rapid bootstrap with 1000 bootstrap replications and GTR GAMMA substitution matrix was used. *Mortierella elongata* (AB542112_1) was used as the outgroup in all the three phylogenetic trees. The resulting trees were visualized using FigTree v1.4.4 (Rambaut, 2012).

2.5. Data analysis

The colonisation rate (CR%) was determined by dividing the total amount of fungi-infected tissue segments by the total amount of tissue segments incubated, then multiplying that by 100. Relative frequency (RF) was determined by dividing the total amount of a particular taxon by the total taxa collected from all incubated thalli of lichens. Shannon–Weiner Biodiversity index (H') was determined by applying the formula (Tripathi et al., 2014):

H' = log Ni/N x 3.322 x log Ni/N,

where *Ni* is the amount of individual fungal species and *N* is the entire number of different fungi species.

Simpson Index of Diversity was used to determine the species abundance, evenness, and richness for each lichen (Hunter and Gaston, 1988). For calculating Simpson index, the following formula was used:

Simpson index (D) = 1- Σn(n-1)/N(N-1),

where *n* is the number of individual fungal species and *N* is the total number of fungal species.

Using the formula Cs = 2A/(B+C), where A is the number of fungal taxa coexisting in two lichen species, B is the total number of fungal taxa in one species, and C is the total number of fungal taxa in other species, the Sorenson's similarity coefficient was calculated to assess the similarity of endolichenic fungi among various lichen species of the genus *Parmotrema*. Besides this, using the PRISM-GraphPad (http://www.graphpad.com/features) software, a species accumulation curve and a unique species curve for endolichenic fungus were plotted following 100 randomizations (Rajulu et al., 2019).

3. Results

3.1. Identification of isolates

A total of 450 ELF were isolated from 15 species of *Parmotrema* from the present study. Out of these, 73 phenotypically distinct isolates were selected for identification using ITS marker which resulted in 47 species of ELF under 23 genera (Table 1). Furthermore, the ELF represented classes Sordariomycetes (84.50%), Dothideomycetes (5.63%), Eurotiomycetes (7.04%), Pezizomycetes (1.40%), and Agaricomycetes (1.40%) (Figure 1). Out of these 23 genera, the majority of isolates belonged to *Xylaria* (11), followed by *Daldinia* (9), *Nemania* (8), *Nigrospora* (7), *Aspergillus* and *Hypoxylon* (5), *Annulohypoxylon* (4), *Biscogniauxia* (3), *Alternaria* (2), *Chaetomium* (2), *Nodulisporium* (2), *Periconia* (2), *Trichoderma* (2), *Cladorrhinum* (1), *Cladosporium* (1), *Coniochaeta* (1), *Coprinellus* (1), *Diaporthe* (1), *Fimetariella* (1), *Fusarium* (1), *Liangia* (1), *Plectania* (1), *Preussia* (1).

The majority of ELF are from Ascomycota lineages, which are different from lichen mycobionts, lichenicolous fungi, or accidental fungi on the thallus surface. The diversity analysis performed for all isolates showed that *Daldinia eschscholtzii* and *Nemania diffusa* were frequently occurring fungi inhabiting at least five different species of *Parmotrema*, with highest colonization rate (CR) of 1.55%, relative frequency (RF) of 0.148, followed by *Nigrospora sphaerica* and *Xylaria feejeensis* inhabiting four different species of *Parmotrema* with CR of 0.88% and RF of 0.085 (Table 2). *Annulohypoxylon truncatum* and *Xylaria arbuscula* inhabited three different hosts with CR 0.66% and RF of 0.063. Four ELF (*Alternaria alternata*, *Biscogniauxia mediterranea*, *Nodulisporium* sp., *Periconia macrospinosa*) were found to occur in two different species hosts. A large number of ELF were found to occur in single lichen host viz. *Annulohypoxylon* sp., *Aspergillus aculeatus*, *A. chevalieri*, *A. flavus*, *A. ruber*, *Biscogniauxia petrensis*, *Chaetomium* sp., *C. globosum*, *Cladorrhinum* sp., *Cladosporium xanthochromaticum*, *Coniochaeta velutina*, *Coprinellus radians*, *Daldinia* sp., *Daldinia vernicosa*, *Diaporthe tulliensis*, *Fimetariella rabenhorstii*, *Fusarium* sp., *Hypoxylon* sp., *H. fendleri*, *H. lignicola*, *H. lividipigmentum*, *H. perforatum*, *Liangia sinensis*, *Nemania bipapillata*, *Nigrospora* sp., *N. chinensis*, *N. oryzae*, *Plectania rhytidia*, *Preussia* sp., *Trichoderma* sp., *Trichoderma viridescens*, *Xylaria* sp., *X. badia*, *X. grammica* and *X. longipes*,

The diversity analysis was performed for lichen hosts too. The colonization frequency, Shannon–Weiner biodiversity index (H') and Simpson index (D) was calculated for each fifteen lichens. *Parmotrema thomsonii* was found to be colonizing maximum number of isolates with a total no. of 10 isolates, H' with 0.418 and D with 0.145. *P. thomsonii* was followed by *P. hababianum*, *P. pseudotinctorum*, and *P. tinctorum* with a total number of 7 isolates, H' with 0.349 and D with 0.101. The values of Sorenson's similarity coefficients ranged from 0 to 0.36 between two species of *Parmotrema* (Table 3). The similarity was highest between *P. hababianum* (PH) and *P. nilgherrense* (PN) which was followed by similarity coefficient of 0.35 between *P. tinctorum* (PT) and *P. thomsonii* (PTH).

In phylogenetic analysis, the isolates were grouped according to their classes (Figure 2). Out of 73 isolates, 61 isolates were grouped under Sordariomycetes, 6 under Dothideomycetes, 4 under Eurotiomycetes, and

Table 1. Identified ELF and their GenBank accession number for ITS 1 and 4 regions.

Sr. No.	Lichen host	Isolate code	Endolichenic fungi identified in NCBI search BLAST	Percentage identity	GenBank accession No. for ITS 1 and 4 regions
1	*P. crinitoides*	PC 2	*Aspergillus flavus* Link	98%	ON945608
		PC 4	*Diaporthe tulliensis* R.G. Shivas, Vawdrey & Y.P. Tan	99%	ON945609
2	*P. melanothrix*	PM 7	*Daldinia vernicosa* Ces. & De Not.	100%	ON936071
		PM 8	*Aspergillus chevalieri* (L. Mangin) Thom & Church	100%	ON927186
3	*P. nilgherrense*	PN 1	*Ascomycota* sp.	99%	ON945601
		PN 3	*Xylariaceae* sp.	99%	ON945602
		PN4	*Nemania diffusa* (Sowerby) Gray	100%	ON927187
		PN 5	*Biscogniauxia mediterranea* (De Not.) Kuntze	100%	ON927188
4	*P. praesorediosum*	PP-1	*Nodulisporium* sp.	99%	ON862737
		PP-13	*Nodulisporium* sp.	99%	ON863894
		PP-16	*Daldinia eschscholtzii* (Ehrenb.) Rehm	100%	ON863895
		PP-72	*Daldinia eschscholtzii*	100%	ON863896
		PP-76	*Daldinia eschscholtzii*	99%	ON863897
		PP-87	*Xylaria badia* Pat.	98%	ON863898
5	*P. stuppeum*	J-1	*Xylaria feejeensis* (Berk.) Fr.	99%	ON797629
		J-2	*Xylaria feejeensis*	100%	ON863891
		J-3	*Daldinia eschscholtzii*	99%	ON863892
		J-4	*Daldinia eschscholtzii*	100%	OM501132
		J-5	*Xylaria feejeensis*	100%	ON863893
6	*P. reticulatum*	PR-1	*Nemania diffusa*	99%	ON945604
		PR-4	*Fimetariella rabenhorstii* (Niessl) N. Lundq.	99%	ON927189
		PR-5	*Nemania diffusa*	99%	ON945605
		PR-8	*Annulohypoxylon truncatum* (Starbäck) Y.M. Ju, J.D. Rogers & H.M. Hsieh	100%	ON945606
7	*P. saccatilobum*	PS-1	*Daldinia eschscholtzii*	99%	ON927190
		PS-2	*Xylaria feejeensis*	99%	ON945607
		PS-3	*Hypoxylon lividipigmentum* F. San Martín, Y.M. Ju & J.D. Rogers	99%	ON927191
8	*P. tinctorum*	PT-3	*Aspergillus aculeatus* Iizuka 1953	100%	ON926871
		PT-10	*Coprinellus radians* (Desm.) Vilgalys, Hopple & Jacq. Johnson	99%	ON982544
		PT-27	*Xylaria arbuscula* Sacc.	99%	ON927181
		PT-34	*Alternaria alternata* (Fr.) Keissl.	100%	ON927182
		PT-76	*Periconia macrospinosa* Lefebvre & Aar.G. Johnson	100%	ON927183
		PT-82	*Cladorrhinum* sp.	99%	ON927184
		PT-91	*Coniochaeta velutina* (Fuckel) Cooke	100%	ON927185
9	*P. pseudotinctorum*	PST-1	*Chaetomium globosum* Kunze	100%	ON876169
		PST-2	*Annulohypoxylon* sp.	99%	ON876170
		PST-4	*Chaetomium* sp.	99%	ON876171
		PST-5	*Xylaria grammica* (Mont.) Mont.	98%	ON907649
		PST-6	*Nemania diffusa*	100%	ON907650
		PST-7	*Biscogniauxia petrensis* Z.F. Zhang, F. Liu & L. Cai	99%	ON907651
		PST-8	*Nemania diffusa*	100%	ON907652
		PST-10	*Aspergillus ruber* (Jos. König, Spieck. & W. Bremer) Thom & Church	99%	ON907653

Table 1. (Continued)

10	*P. hababianum*	PH-1	*Nemania diffusa*	99%	ON936064
		PH-2	*Preussia* sp.	100%	ON936065
		PH-3	*Nigrospora sphaerica* (Sacc.) E.W. Mason	100%	ON936066
		PH-4	*Nigrospora* sp.	100%	ON936067
		PH-5	*Biscogniauxia mediterranea*	100%	ON936068
		PH-6	*Nigrospora chinensis* Mei Wang & L. Cai	100%	ON936069
		PH-7	*Hypoxylon fendleri* Berk. ex Cooke	99%	ON936070
11	*P. austrosinense*	PA-1	*Plectania rhytidia* (Berk.) Nannf. & Korf	99%	ON876164
		PA-3	*Xylaria arbuscula*	99%	ON876165
12	*P. crinitum*	CR-1	*Nigrospora sphaerica*	100%	ON935739
		CR-2	*Nigrospora oryzae* (Berk. & Broome) Petch	100%	ON935740
		CR-4	*Annulohypoxylon truncatum*	99%	ON935741
		CR-7	*Daldinia* sp.	100%	ON935742
		CR-8	*Nigrospora sphaerica*	98%	ON876162
		CR-71	*Nemania bipapillata* (Berk. & M.A. Curtis) Pouzar	100%	ON935743
		CR-12	*Liangia sinensis* H. Yu, Y.B. Wang, Y. Wang, Z.H. Chen & Zhu L. Yang	99%	ON876163
13.	*P. pseudonilgherrense*	PSN-1	*Trichoderma* sp.	100%	ON935736
		PSN-2	*Trichoderma viridescens* (A.S. Horne & H.S. Will.) Jaklitsch & Samuels	100%	ON935737
		PSN-4	*Nemania diffusa*	99%	ON935738
14.	*P. thomsonii*	PTH-1	*Hypoxylon* sp.	100%	ON907654
		PTH-2	*Alternaria alternata*	99%	ON907655
		PTH-3	*Daldinia eschscholtzii*	99%	ON907656
		PTH-4	*Annulohypoxylon truncatum*	99%	ON907657
		PTH-5	*Fusarium* sp.	99%	ON907658
		PTH-7	*Hypoxylon lignicola* Z.L. Luo, K.D. Hyde & H.Y. Su	100%	ON907659
		PTH-8	*Xylaria arbuscula*	99%	ON907660
		PTH-9	*Cladosporium xanthochromaticum* Sand.-Den., Gené & Cano	99%	ON907661
		PTH-11	*Periconia macrospinosa*	99%	ON907662
		PTH-12	*Nigrospora sphaerica*	100%	ON907663
15.	*P. direagens*	PD-1	*Xylaria* sp.	99%	ON876166
		PD-2	*Xylaria longipes* Nitschke	98%	ON876167
		PD-3	*Hypoxylon perforatum* (Schwein.) Fr.	99%	ON876168

Agaricomycetes and Pezizomycetes were represented by a single isolate. The phylogenetic analysis of unique genera exhibited distinct positions of isolates in the phylogenetic tree (Figure 3). The phylogenetic analysis of abundant genera, i.e. of *Daldinia* and *Nemania* showed that both of them were closely related (Figure S2). The evolutionary study also revealed that, despite having different lichen hosts, the majority of ELF species do not choose a specific *Parmotrema* species for their colonisation and survival. The species accumulation curve, which plots the total number of isolated species against sample size, did not flatten when more samples were screened (Figure 4). Similarly, unique species curve also did not flatten and it can be seen that the ELF taxa that have not been reported previously as endolichenic occurred in our study, sometimes in more than one lichen host (Figure 5).

4. Discussion

Most of the isolates recovered in the current study belonged to Ascomycota, but one of the isolates (*Coprinellus radians*)

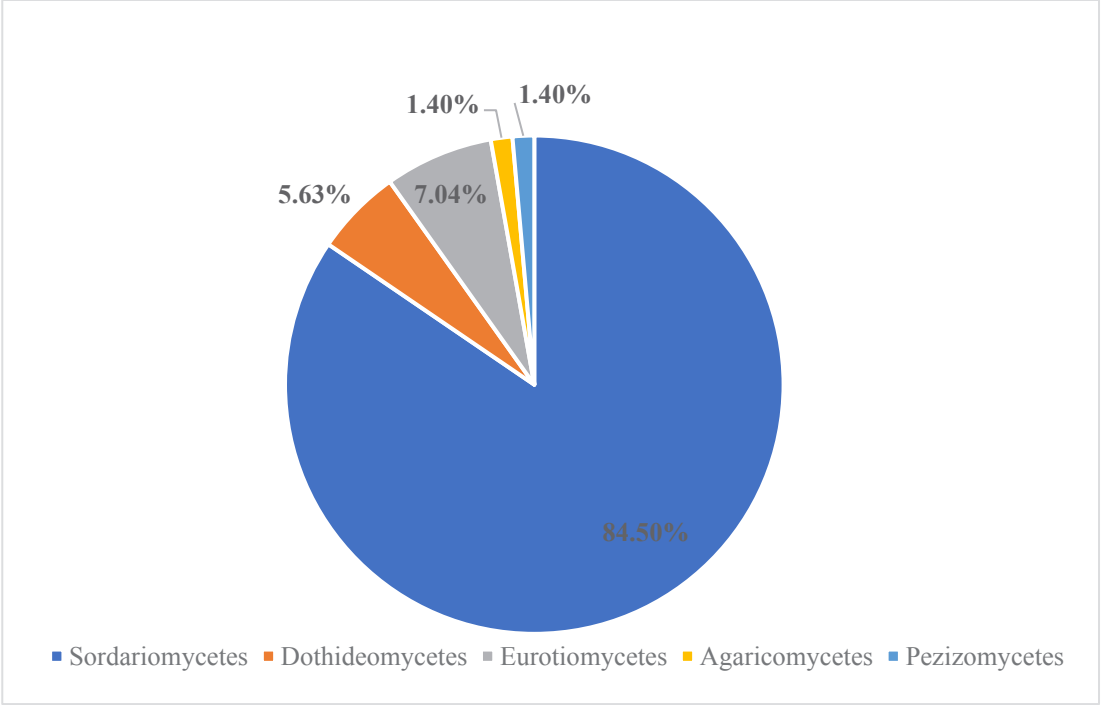

Figure 1. Percentage representation of ELF in *Parmotrema* species under different classes.

belonged to Basidiomycota. The studies on diversity of ELF isolated from different species of lichens have been performed since 1990 after Petrini pioneered this work. The majority of ELF previously isolated from different lichen species also belonged to Ascomycota followed by Basidiomycota, and very few belong to Mucoromycota (Chakarwarti et al., 2020). In a study by Zhang et al. (2016), lichen samples taken from Arctic habitats contained ELF species belonging to phyla Ascomycota, Basidiomycota, and Zygomycota. However, none of the ELF in the current study belonged to the phylum Zygomycota. The difference can be due to application of different cultural practices and sequencing methods as to recover both culturable and nonculturable organisms, they used 454-Next Generation sequencing platform, whereas only culturable isolates were studied using Sanger sequencing in the current investigation.

Among Ascomycota, Sordariomycetes has the highest number of ELF species (84.50%) which was followed by Eurotiomycetes (7.04%) and Dothideomycetes (5.63%). Pezizomycetes and Agaricomycetes were equal in number (1.40%). Some earlier investigations also demonstrated the dominance of Sordariomycetes in ELF species (Arnold et al., 2009; Zhang et al., 2016; Lagarde et al., 2018; Li 2007). However, other research revealed the predominance of Dothideomycetes, Leotiomycetes, or Pezizomycetes (Petrini, 1990; U'Ren et al., 2010, Girlanda, 1997).

Out of the four frequently occurring fungi, three ELF (*Daldinia eschscholtzii*, *Nemania diffusa*, *Annulohypoxylon truncatum*) belonged to order Xylariales, while *Nigrospora sphaerica* belonged to order Trichosphaeriales. The study corroborates with that of Rajulu et al. (2019), as wide host range and high colonisation frequency of Xylariales were observed by them in their findings. However, they explored different species of lichens other than *Parmotrema.* The *Xylaria* also infect plants as endophytes, but they are taxonomically different species due to host hopping (Davis et al., 2003).

The occurrence of different ELF from different hosts indicates that the ELF are not host-specific as many of these isolates were previously reported from different lichen genera (Tripathi et al., 2014; Maduranga et al., 2018; Rajulu et al., 2019; Santiago et al., 2021). Some of the ELF isolated in the present study were also reported as endophytes, e.g., *Alternaria alternata*, *Aspergillus flavus*, *Chaetomium globosum*, *Daldinia eschscholtzii*, *Fusarium* sp., *Hypoxylon* sp., *Diaporthe* sp., *Nigrospora sphaerica*, *Trichoderma* sp., *Xylaria* sp. (Wen et al., 2022).

There can be various reasons governing the occurrence and diversity of the ELF in lichens. The elevation from where the lichen host was collected can be one such reason. Lichen is an organism that depends on the atmosphere for its sustenance; therefore, the elevation affects the amount of dew and fog present in the air, which encourages lichen growth (Nash, 2008). At higher elevations, more fog and dew are present providing conducive conditions to the lichen and consequently to ELF. However, more research is

Table 2. Diversity analysis: colonization rate (CR%), relative frequency (RF) of endolichenic fungi isolated from lichen *Parmotrema* species and Shannon–Weiner index (H') and Simpson index (D) of lichen hosts.

ELF	PA	PC	CR	PD	PH	PM	PN	PP	PSN	PST	PR	PS	J	PTH	PT	TOTAL	Colonization rate (CR%)	Relative frequency (RF)
Alternaria alternata														1	1	2	0.44	0.042
Annulohypoxylon sp.										1						1	0.22	0.021
Annulohypoxylon truncatum			1								1			1		3	0.66	0.063
Ascomycota sp.							1									1	0.22	0.021
Aspergillus aculeatus															1	1	0.22	0.021
Aspergillus chevalieri						1										1	0.22	0.021
Aspergillus flavus		1														1	0.22	0.021
Aspergillus ruber										1						1	0.22	0.021
Biscogniauxia mediterranea					1		1									2	0.44	0.042
Biscogniauxia petrensis										1						1	0.22	0.021
Chaetomium globosum										1						1	0.22	0.021
Chaetomium sp.										1						1	0.22	0.021
Cladorrhinum sp.															1	1	0.22	0.021
Cladosporium xanthochromaticum														1		1	0.22	0.021
Coniochaeta velutina															1	1	0.22	0.021
Coprinellus radians															1	1	0.22	0.021
Daldinia eschscholtzii								3				1	2	1		7	1.55	0.148
Daldinia sp.			1													1	0.22	0.021
Daldinia vernicosa						1										1	0.22	0.021
Diaporthe tulliensis		1														1	0.22	0.021
Fimetariella rabenhorstii											1					1	0.22	0.021
Fusarium sp.														1		1	0.22	0.021
Hypoxylon fendleri					1											1	0.22	0.021
Hypoxylon lignicola														1		1	0.22	0.021
Hypoxylon lividipigmentum												1				1	0.22	0.021
Hypoxylon perforatum				1												1	0.22	0.021

Table 2. (Continued)

Hypoxylon sp.														1		1	0.22	0.021
Liangia sinensis			1													1	0.22	0.021
Nemania bipapillata			1													1	0.22	0.021
Nemania diffusa					1		1		1	2	2					7	1.55	0.148
Nigrospora chinensis					1											1	0.22	0.021
Nigrospora oryzae			1													1	0.22	0.021
Nigrospora sp.					1											1	0.22	0.021
Nigrospora sphaerica			2		1									1		4	0.88	0.085
Nodulisporium sp.								2								2	0.44	0.042
Periconia macrospinosa														1	1	2	0.44	0.042
Plectania rhytidia	1															1	0.22	0.021
Preussia sp.					1											1	0.22	0.021
Trichoderma sp.									1							1	0.22	0.021
Trichoderma viridescens									1							1	0.22	0.021
Xylaria arbuscula	1													1	1	3	0.66	0.063
Xylaria badia									1							1	0.22	0.021
Xylaria feejeensis												1	3			4	0.88	0.085
Xylaria grammica										1						1	0.22	0.021
Xylaria longipes				1												1	0.22	0.021
Xylaria sp.				1												1	0.22	0.021
Xylariaceae sp.							1									1	0.22	0.021
Total no. of isolates	2	2	7	3	7	2	4	5	4	8	4	3	5	10	7	**73**		
Total no. of species	2	2	6	3	7	2	4	2	4	7	3	3	2	10	7	64		
Shannon-Weiner index (H')	0.156	0.156	0.32	0.207	0.349	0.156	0.25	0.156	0.25	0.349	0.207	0.207	0.156	0.418	0.349			
Simpson index (D)	0.029	0.029	0.087	0.043	0.101	0.029	0.058	0.029	0.058	0.101	0.043	0.043	0.029	0.145	0.101			

Table 3. Sorenson's similarity coefficients of ELF isolated from 15 lichen species from genus *Parmotrema*.

Lichen hosts	*PA*	*PC*	*CR*	*PD*	*PH*	*PM*	*PN*	*PP*	*PSN*	*PST*	*PR*	*PS*	*J*	*PTH*
PC	0													
CR	0	0												
PD	0	0	0											
PH	0	0	0.14	0.2										
PM	0	0	0	0	0									
PN	0	0	0	0	0.36	0								
PP	0	0	0	0	0	0	0							
PSN	0	0	0	0	0.18	0	0.25	0						
PST	0	0	0	0	0.13	0	0.16	0	0.16					
PR	0	0	0.18	0	0.18	0	0.25	0	0.25	0.16				
PS	0	0	0	0	0	0	0	0.25	0	0	0			
J	0	0	0	0	0	0	0	0	0	0	0	0.2		
PTH	0.16	0	0.11	0	0.11	0	0	0.13	0	0	0.14	0.15	0.13	
PT	0.22	0	0	0	0	0	0	0	0	0	0	0	0	0.35

needed to be carried out for verifying these hypotheses (Santiago et al., 2021). The second reason can be techniques utilized for isolation of ELF (Hyde et al., 2008). Too harsh surface sterilization techniques can kill ELF. In contrast, ineffective sterilisation will promote the growth and contamination of ELF, which will make it harder to recover viable isolates on culture media (Ownley et al., 2008). Third, different types of media used for recovery of isolates can also have a significant impact on how well each fungus class is represented; for example, using MEA (Malt Extract Agar) as the isolation medium results in a low population of Eurotiomycetes (Arnold et al., 2009; U'Ren et al., 2010; 2012).

The lichens used in this study did not exhibit any signs of decay or disease; from this, it can be concluded within the lichen thallus, a large number of ELF species have a latent saprotrophic lifestyle. This phenomenon has also been seen with endophytic fungus in plants, when leaves fall to the ground, many endophytic fungi in the leaves change into saprotrophic fungi (Guerreiro et al., 2018). In the current investigation, *Daldinia* and *Nigrospora* species were among the most frequently found ELF species, which in previous investigations were also frequently isolated from the lichens (Vinayaka et al., 2016; Masumoto et al., 2019). Chan et al. (2015) mentioned that *Daldinia eschscholtzii*, within the core gene families, has an ATP-dependent molecular chaperone that aids in heat stress response and also aids in carbon assimilation in nutrient-limited situations (acid trehalases).

The study also resulted few species of fungi (*Aspergillus chevalieri* and *A. ruber*) which were reported earlier as opportunistic pathogens causing skin infections (Naidu et al., 1994). Furthermore, *A. ruber* has been isolated from coffee beans, tea, and soil (Chen et al., 2017). *Daldinia vernicosa* is regarded as pyroxylophilous fungus and known to occur on burnt wood (Rhoads, 2018). Among other ELF, *Hypoxylon lignicola* is a fresh water Sordariomycetes (Zong et al., 2019) while *H. fendleri* is a wood fungus (Intaraudom et al., 2019). *Hypoxylon perforatum* is of cosmopolitan distribution occurring on dead wood and is one of the few hypoxylaceae species (Ju and Rogers, 1996; Stadler et al., 2008; Sir et al., 2019). *Cladosporium xanthochromaticum* (Xu et al., 2021), *Coniochaeta velutina* (Xie et al., 2015), *Diaporthe tulliensis* (Wu et al., 2021), *Hypoxylon lividipigmentum* (Sanchez M et al., 2020), *Nemania diffusa* (Liu et al., 2016), *Periconia macrospinosa* (Azhari et al., 2021), *Trichoderma viridescens* (Khan et al., 2020), *Xylaria badia* (Deepthi et al., 2019), and *Xylaria longipes* (Büttner et al., 2019) are plant endophytic fungi and are being reported as ELF in the present study.

Besides these, the study also finds some of the fungi not reported previously as ELF. *Annulohypoxylon truncatum* has been reported previously as oak tree canker pathogen (Cha et al., 2018). *Coprinellus radians*, the only reported ELF under Basidiomycota in the current study, has been reported as a species of saprobic mushroom (Lu et al., 2020). *Cladorrhinum* sp. and *Plectania rhytidia* were reported to be used as biocontrol agent (Martin et al., 2019, Costa et al., 2020), *Fimetariella rabenhorstii*, an

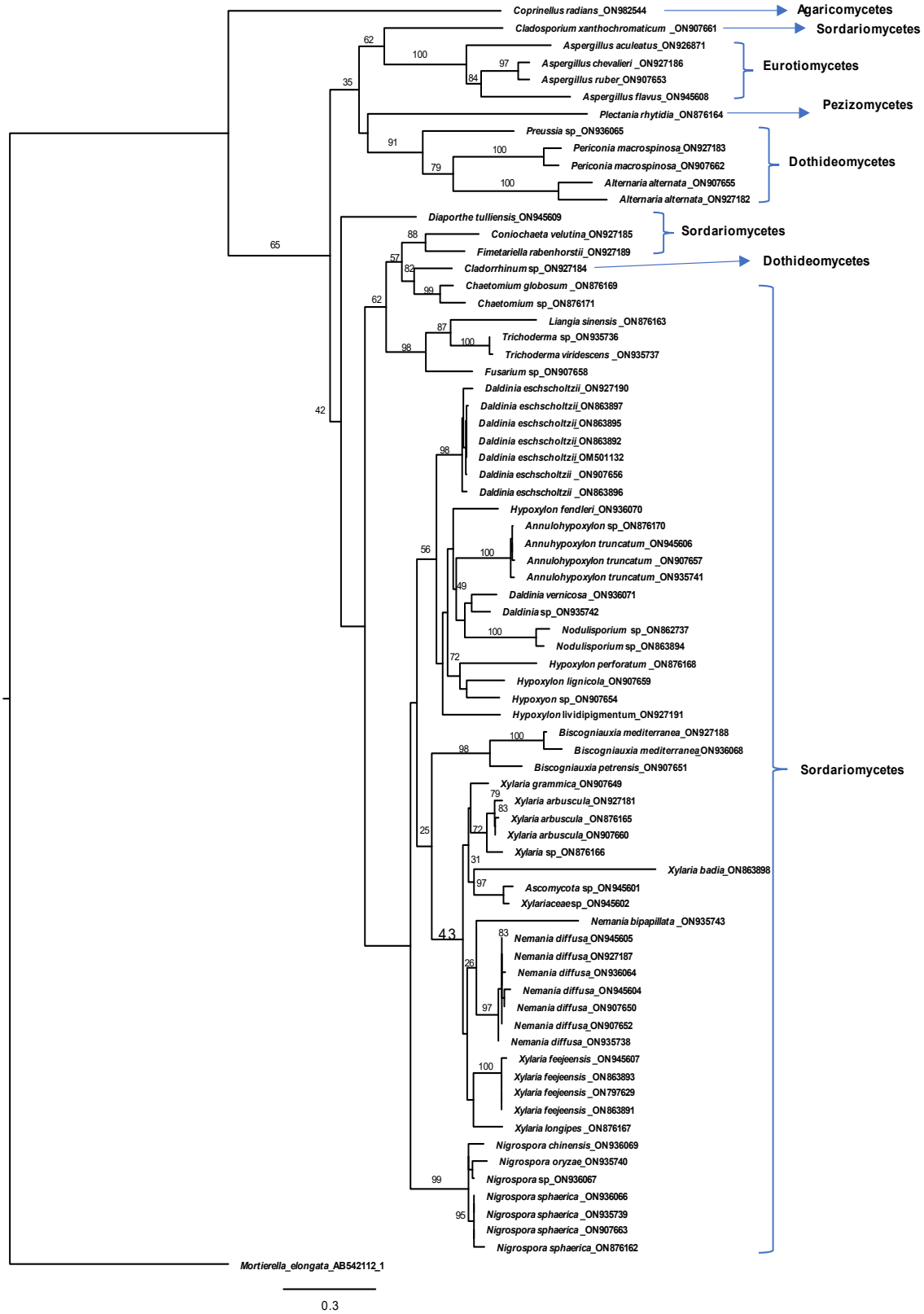

Figure 2. Phylogenetic tree based on maximum likelihood (ML) analysis of 73 endolichenic fungi isolated from fifteen species of lichen genus Parmotrema. Bootstrap percentages from ML analysis indicated on the branches.

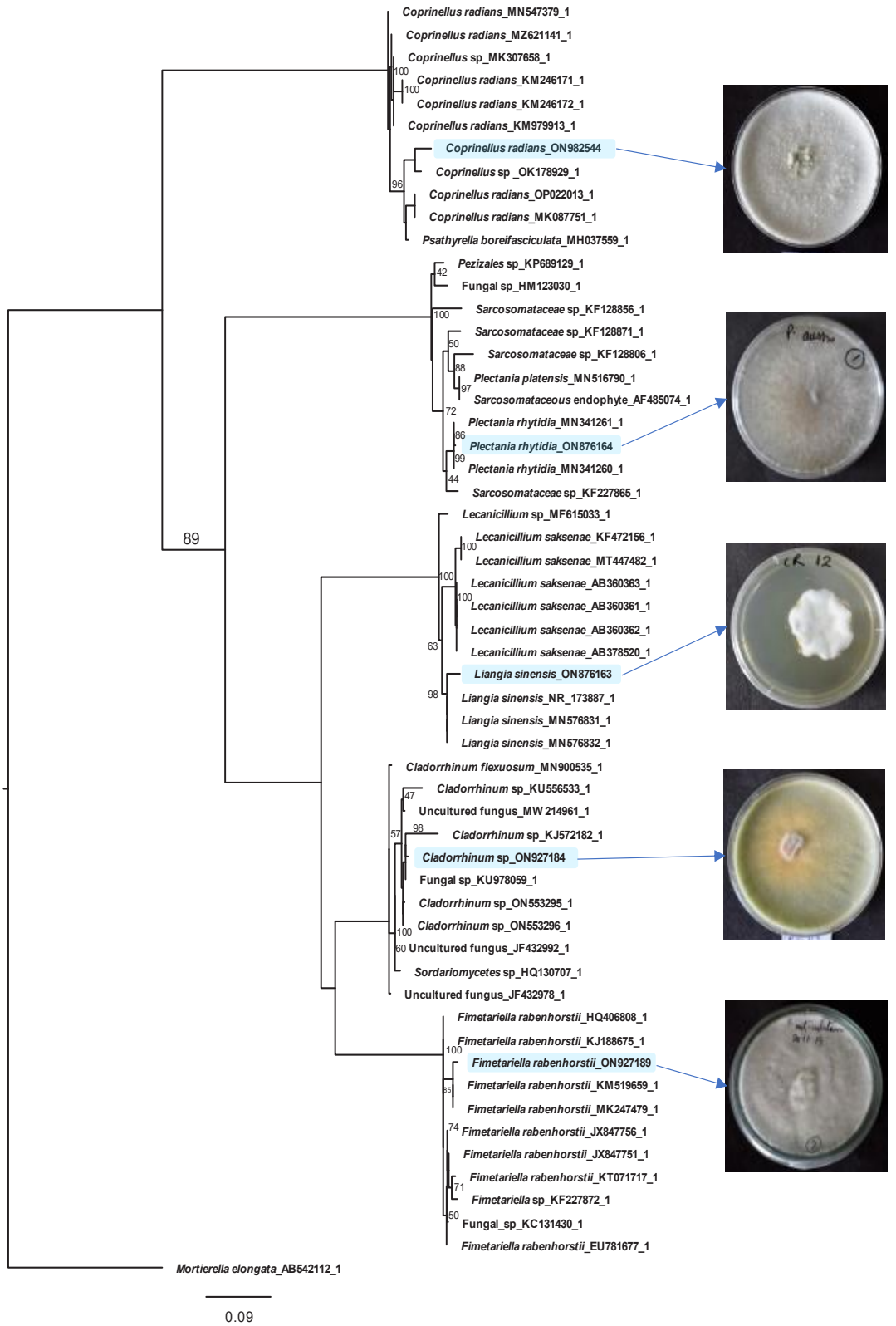

Figure 3. Phylogenetic tree showing distinct phylogenetic positions of unique genera isolated from genus *Parmotrema*.

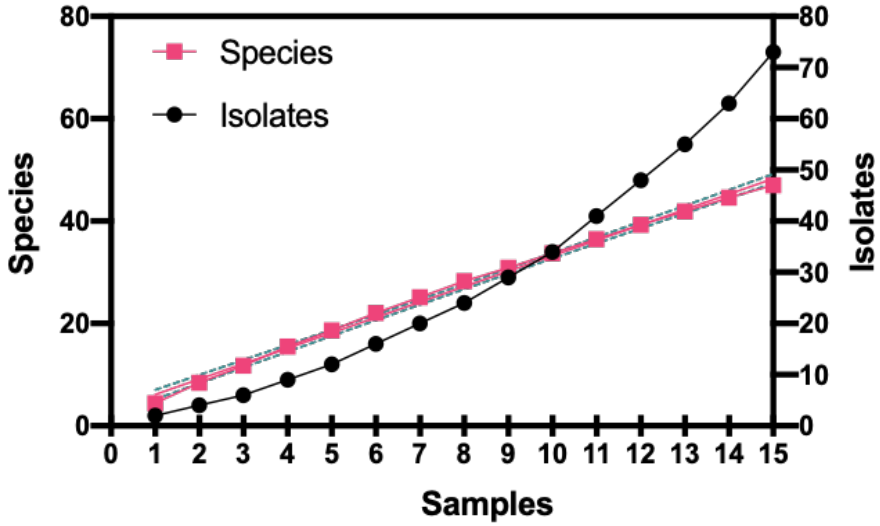

Figure 4. The graph representing the species accumulation of endolichenic fungi, which were isolated from fifteen different species of the genus *Parmotrema*. For the graph, data were randomly chosen 100 times. The 95% confidence interval is shown as a dotted line.

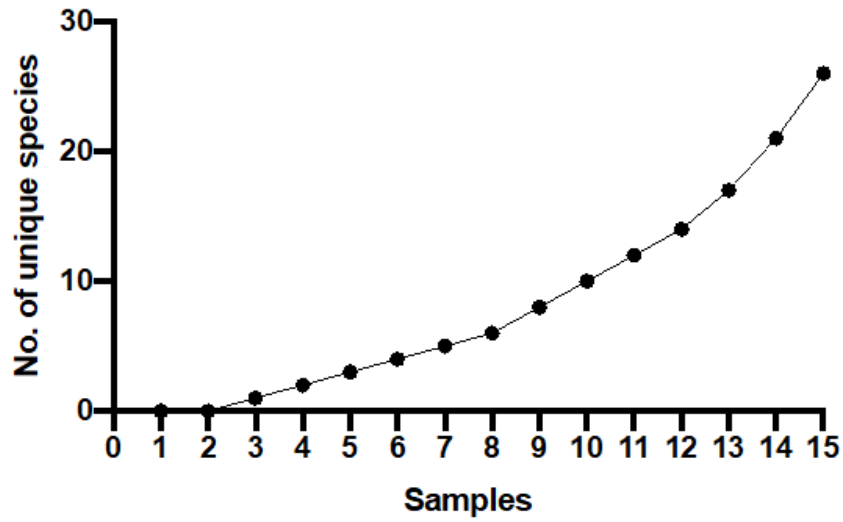

Figure 5. Fifteen distinct lichen thalli were used to isolate a large number of distinct species of endolichenic fungi. For the graph, data were 100 times randomised. A polynomial trend line is shown by the curve.

endophytic fungus (Tao et al., 2011) while *Liangia sinensis*, is a cordicipitoid fungus (Wang et al., 2020).

The ELF richness could be compared among the lichen genus *Parmotrema*. *P. thomsonii* was found to be colonizing maximum number of isolates (10), which was followed by *P. tinctorum*, *P. pseudotinctorum*, and *P. hababianum* (7 isolates), *P. crinitum* (6 isolates), *P. nilgherrense* (4 isolates), *P. direagens*, *P. praesorediosum*, *P. pseudonilgherrense*, *P. reticulatum*, *P. saccatilobum* (3 isolates) and the lowest number of isolates were found in *P. austrosinense*, *P. crinitoides*, *P. melanothrix*, and *P. stuppeum* (2 isolates). Different ecoregions and variable climatic conditions can be the factors influencing ELF richness.

In the phylogenetic tree, regardless of their lichen hosts, fungal species were grouped together, showing that lichen hosts play a little or no role in selecting their endolichenic species. According to Sorenson's similarity coefficients in the present study, most of the species of *Parmotrema*

do not share common isolates between them. While very few species of *Parmotrema* shared some common taxa of ELF, none are 100% similar, suggesting that host-related factor may have an influence on the endolichenic fungal communities. However, our sample size was insufficient to completely represent species specificity as indicated by raising species accumulation and unique species curve.

Although we isolated a variety of ELF species, there are certain fundamental limitations in our study that need to be kept in mind while interpreting the findings of the study. We used single type of media, i.e. PDA, which limited the number of isolated ELF species. The ELF diversity can be increased by using different media that include various nutritional contents and different sterilising techniques (Muggia et al., 2017). Moreover, this study solely focused on cultivable ELF. Several studies which included culture-independent techniques have shown a greater fungal diversity (Tedersoo et al., 2014; Buée et al., 2009). Our study clearly indicates that the culture-specific approach can also reveal the striking diversity of ELF.

5. Conclusion

In India, research on endolichenic fungi is still in its early stages, and more work is needed to be carried out. Studying the diversity of endolichenic fungi raises the possibility of finding new taxa of fungi, filling the gap between known and unknown species. The lichen thallus represents a microecosystem since it harbours a variety of microbes making it a suitable subject for studying microbial diversity and their interaction. The ELF are found in almost all of the lichen species that have been studied so far; however, they represent an important yet understudied area of lichenology. In the present study, few species and genera of ELF are found to be new and have not been reported previously. To comprehend the relationship between lichens and ELF, the ecological role these organisms play, and the metabolites produced in the symbiosis, a detailed research is needed at the molecular level.

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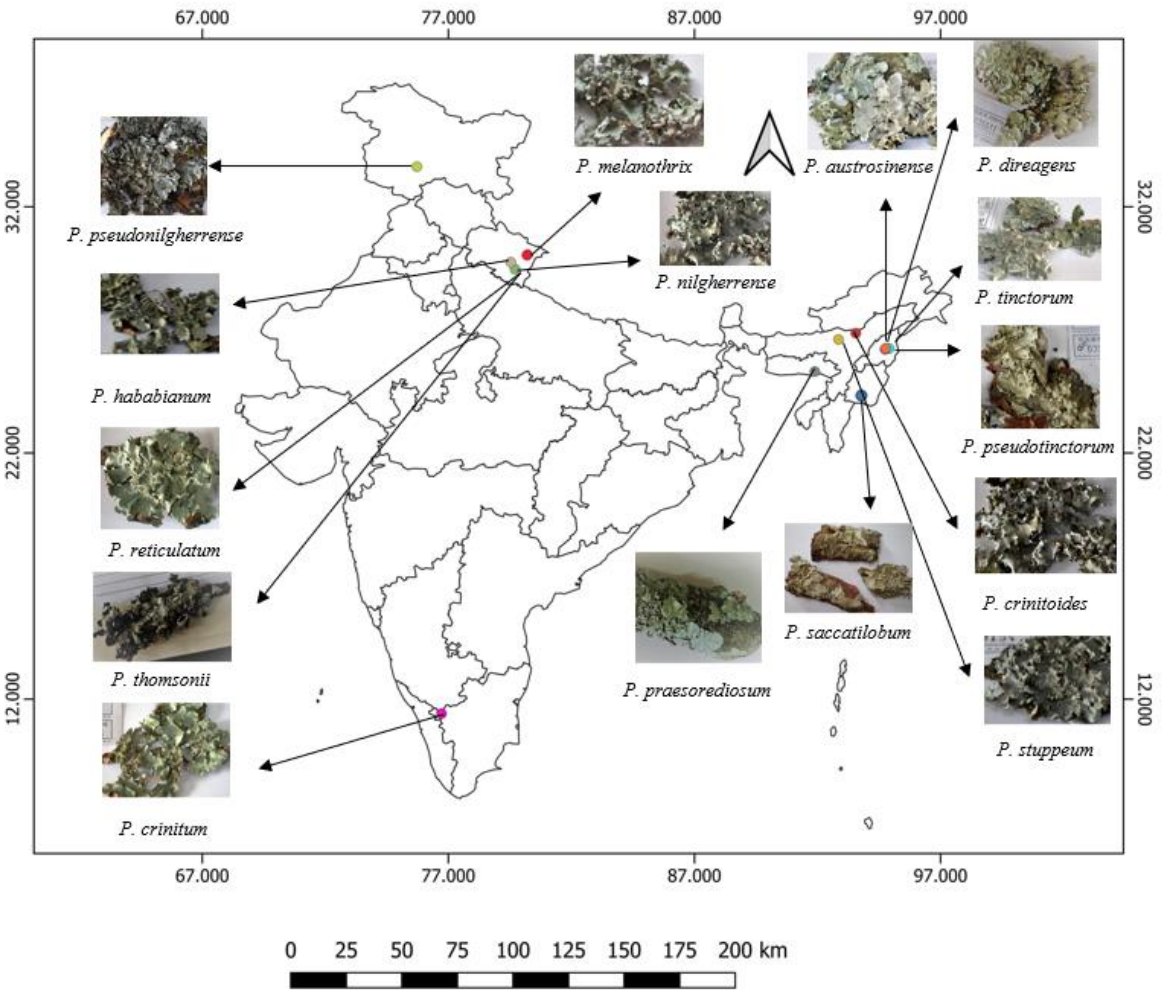

Figure S1. The collection sites of lichen *Parmotrema* species in India.

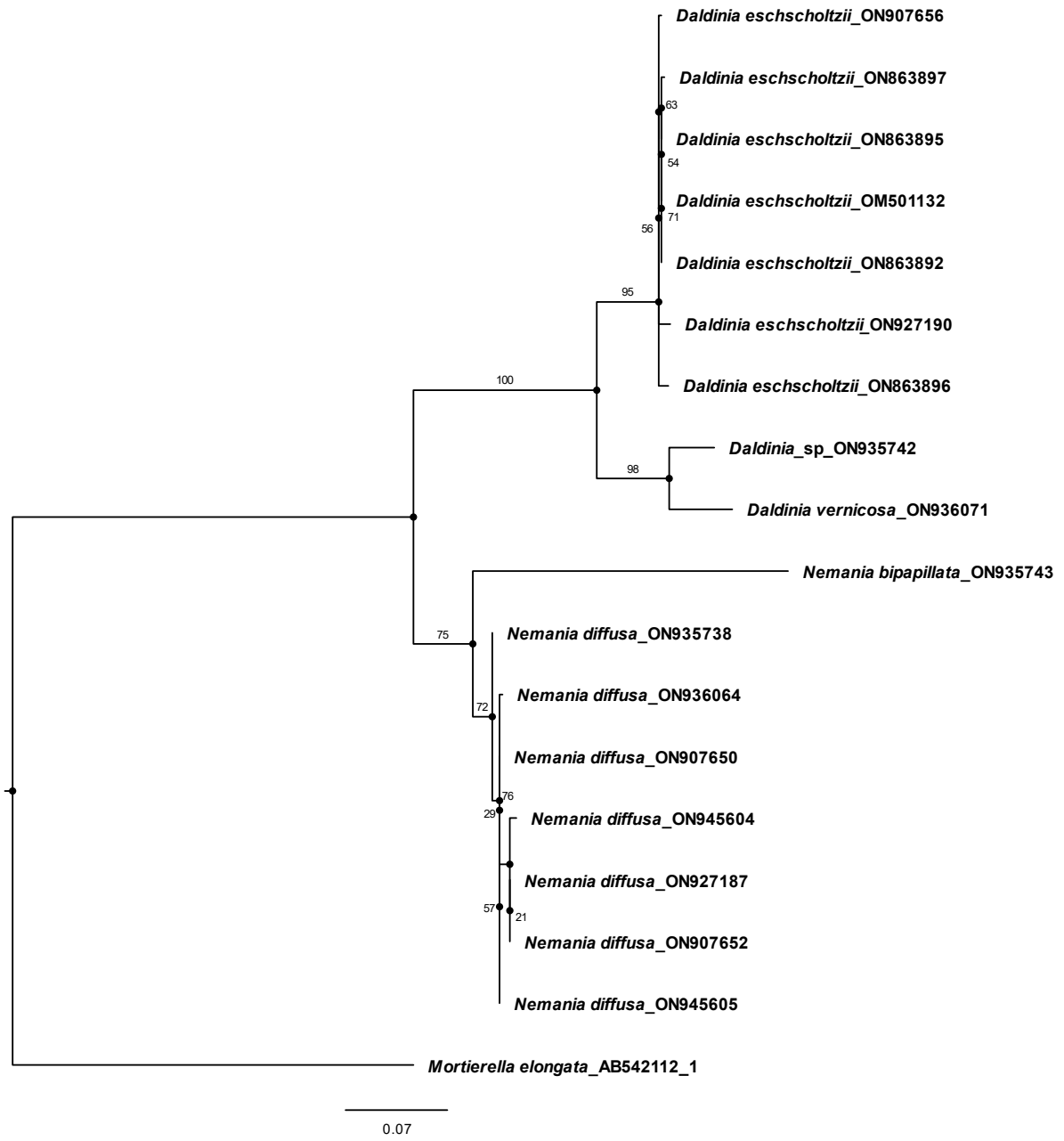

Figure S2. Phylogenetic tree of two frequently occurring isolates, i.e. *Daldinia eschscholtzii* and *Nemania diffusa* isolated from genus *Parmotrema*.

Table S1.

Sr. No.	Lichen	Collection site	Substratum	Vegetation/ climate	Accession no.
1	*Parmotrema crinitoides* J.C. Wei	**Assam**, Tezpur Distt., Siali Tapo, 82 m, N26°36′50.4″, E92°51′14.04″	On bark of the tree	Tropical, Monsoon Rainforest	LWG-36702
2	*Parmotrema melanothrix* (Mont.) Hale	**Uttarakhand**, Pithoragarh Distt., Munsiyari, 2774 m, N30°02′22.8″, E80°11′53.2″	On trunk and twigs of the tree	Temperate	LWG-36703
3	*Parmotrema nilgherrense* (Nyl.) Hale	**Uttarakhand**, Pithoragarh Distt., Munsiyari, near Kalamui Temple, 2774 m, N30°02′21.6″, E80°11′55.3″	On tree trunk	Temperate	LWG-36710
4	*Parmotrema praesorediosum* (Nyl.) Hale	**Meghalaya**, Pynursula, near Catholic Church, 1560 m, N25°18′36″, E91°53′25″	On rock surface	Tropical, warm and humid	LWG-36717
5	*Parmotrema stuppeum* (Taylor) Hale	**Manipur**, Churachandpur Distt., Khongjanglok, 857 m, N24°20′7.60″, E93°47′2.50″	On rock and bark of trees	Tropical, humid	LWG-36704
6	*Parmotrema reticulatum* Taylor M. Choisy	**Uttarakhand**, Gulati, Sattal, 2149 m, N29°24′53.56″, E79°32′18.75″	On tree trunk	Temperate	LWG-36708
7	*Parmotrema saccatilobum* (Taylor) Hale	**Assam**, Golaghat Distt., Bagori Range, 103 m, N26°51′65″, E93°32′90″	On trees and twigs	Tropical, Monsoon Rainforest climate	LWG-61397
8	*Parmotrema tinctorum* (Despr. ex Nyl.)	**Nagaland**, Tuensang Distt., Khudii village, near Panchayat court, N26°15′9.80″, E94°45′47.60″	On tree trunk, rocks and boulder	Tropical, Monsoon Rainforest climate	LWG-36714
9	*Parmotrema pseudotinctorum (Abbayes) Hale*	**Nagaland**, Tuensang Distt., Chingmei, way to Konya, N26°14′41.41″, E94°54′17.10″, 2079 m	On bark of trees	Tropical, Monsoon Rainforest climate	LWG-36713
10	*Parmotrema hababianum* (Gyeln.) Hale	**Uttarakhand**, Almora District, Mori village, 1918 m, N29° 27' 57.53" E79°42' 34.48"	On bark and twigs of trees	Temperate	LWG-59426
11	*Parmotrema austrosinense* (Zahlbr.) Hale	**Nagaland**, Tuensang Distt. Way to Konya, N26°14′41.41″, E94°54′17.10″, 2079m	On tree trunk	Tropical, Monsoon Rainforest	LWG-36709
12	*Parmotrema crinitum* (Ach.) M. Choisy	**Tamil Nadu**, Nilgiri Hills, Ootacamund- Kotagiri road, near Dodabetta tea estate, half mile down, 7000 ft	Over mosses on ground	Tropical	LWG-45660
13	*Parmotrema pseudonilgherrense* (Asahina) Hale	**Jammu & Kasmir**, Kishtwar Distt., Kishtwar, High altitude National Park, Qaderna, 2450 m, N33°37' 51.39", E75° 43'22.58"	On trees and boulders	Temperate	LWG-62149
14	*Parmotrema direagens* (Hale) Hale	**Nagaland**, Tuensang Distt. Way to Mokokechung, 2084 m, N26°12′31.70″, E94°44′40.00″	On tree bark	Tropical, Monsoon Rainforest	LWG-36705
15	*Parmotrema thomsonii* (Stirt.) A. Crespo	**Uttarakhand**, Pithoragarh Distt., Munsiyari, 2650 m, N36°03′33.3″, E80°13′13.4″	On tree trunk	Temperate	LWG- 36706