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ORIGINAL ARTICLE

Study on endolichenic fungal assemblage in *Parmotrema* and *Heterodermia* lichens of Shivamoga, Karnataka

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Abstract

Background Lichen is a symbiotic association of algae and fungi, recognized as a self-sustaining ecosystem that constitutes an indeterminant number of bacteria, actinomycetes, fungi, and protozoa. We evaluated the endolichenic fungal assemblage given the dearth of knowledge on endolichenic fungi (ELFs), particularly from part of the Central Western Ghats, Karnataka, and conducted a phylogenetic analysis of xylariaceous fungi, the most diversified group of fungi using ITS and ITS+Tub2 gene set.

Results Out of 17 lichen thalli collected from 5 ecoregions, 42 morphospecies recovered, belong to the class Sordariomycetes, Eurotiomycetes, Dothideomycetes, Leotiomycetes, Saccharomycetes. About 19 and 13 ELF genera have been reported from *Parmotrema* and *Heterodermia* thallus. Among the ecoregions EC2 showing highest species diversity (*Parmotrema* (1-D) = 0.9382, (H) = 2.865, Fisher-α = 8.429, *Heterodermia* (1-D) = 0.8038, H = 1.894, F-α = 4.57) followed the EC3 and EC1. Xylariales are the predominant colonizer reported from at least one thallus from four ecoregions. The morphotypes ELFX04, ELFX05, ELFX08 and ELFX13 show the highest BLAST similarity (> 99%) with *Xylaria psidii*, *X. feejeensis*, *X. berteri* and *Hypoxylon fragiforme* respectively. Species delimitation and phylogenetic position reveal the closest relation of Xylariaceous ELFs with plant endophytes.

Conclusions The observation highlights that the deciduous forest harness a high number of endolichenic fungi, a dominant portion of these fungi are non-sporulating and still exist as cryptic. Overall, 8 ELF species recognized based on phylogenetic analysis, including the two newly reported fungi ELFX03 and ELFX06 which are suspected to be new species based on the present evidence. The study proved, that the lichen being rich source to establish fungal diversity and finding new species. Successful amplification of most phylogenetic markers like RPB2, building of comprehensive taxonomic databases and application of multi-omics data are further needed to understand the complex nature of lichen-fungal symbiosis.

Keywords Diversity · *Xylariaceous endolichenic fungi* · Phylogeny · Species delimitation · ITS · Beta-tubulin

Abbreviations

ELF	Endolichenic fungi
ITS	Internal transcribed spacer
Tub2	Beta tubulin subunit 2
Tef1	α Translation elongation factor 1 alfa
PDA	Potato dextrose agar
MYA	Malt yeast extract agar
SDA	Sabouraud dextrose agar
CR	Colonization rate
EC	Ecoregion
OUT	Operational taxonomic unit
NMDS	Non metric multidimensional statistical analysis

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Introduction

Lichen is a symbiotic association of algae and fungi. After reporting the presence of multiple organisms, this assemblage was reconceptualized as a self-sustaining ecosystem [1]. A detailed view of the lichen-associated microbial community evidenced the presence of bacteria, actinomycetes, fungi, and protozoa [2, 3]. The mycobiont determines the thallus organization, which itself develops the structural modifications needed for the assemblage of secondary

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microbial partners [4]. Relationships of various partners with lichens may be obligatory or facultative. The secondary fungal component resides in the lichens categorized into lichenicolous and endolichenic fungi [5]. By definition, lichenicolous fungi are pathogens of lichens and cause various levels of virulence in the host. Whereas endolichenic fungi (ELF) are defined as mutualistic, asymptomatic fungi. Several studies suggest that ELFs produce chemicals to invade lichen parasites and establish mechanisms to coexist with dominant number of other fungi in the similar way that plant endophytes do [6]. The existence of ELFs reported from the lower Devonian period is evidenced by fossil studies [7]. ELFs are dominated within the non-lichenized lineage of Ascomycota; Fungi belong to Basidiomycota [8, 9], and Mucoromycota are also known to assemble with lichen. Phylogenetically they are distinct from the mycobiont, lichenicolous fungi and resemble endophytic fungi in many ways [10].

From the Arctic to the Tropics, ELFs are distributed and reside in lichens from all major environments of the world [5, 11–14]. Lichen From different continents evaluated for endolichenic fungal composition, Tan et al. [15] isolated nine ELFs in *Parmotrema rampoddense* collected from the Philippines. Yu et al. [16] isolated 61 endolichenic fungal species from 45 Antarctic lichen samples. Li et al. [17] isolated 32 endolichenic fungal taxa in 488 lichen segments collected from Baihua Mountain in Beijing, China. It has been reported that tropical lichens have a rich endolichenic fungal assemblage; Suryanarayanan et al. [18] isolated 942 isolates belonging to 33 species from Northern India. Kannangara et al. [19] Isolated 23 species of ELFs belonging to the genera *Acremonium*, *Broomella*, *Chysosporium*, *Cladosporium*, *Curvularia*, *Fusarium*, *Nigrospora*, *Penicillium*, *Periconia*, and *Phoma* in lichen species *Pseudocyphellaria*, *Usnea*, and *Parmotrema* collected from Sri Lanka. Tripathi et al. [20] isolated *Alternaria*, *Aspergillus*, and *Fusarium* species predominantly from the Himalaya. The central Western Ghat one of the biodiversity hotspots in India, is home to a wide variety of lichen species [21]. Concerning the diversity and distribution of ELFs, these lichens were unexplored. Lichen thallus belongs to the species *Parmotrema tinctorum* (PT), *P. grayanum* (PG), *P. praesorediosum* (PP), *P. cristiferum* (PC), *Heterodermia obscurata* (HA), *H. speciosa* (HS), *H. incana* (HI), *H. flabellate* (HF), *H. diodemata* (HD), and *H. hypocaesia* (HH) collected from Shivamoga district and subjected to isolation and enumeration ELFs.

To date, nearly 500 ELFs have been isolated around the world; however, only 135 isolates were identified at the species level [22, 23], indicating a dearth of taxonomic knowledge. The dominant portion of ELF is still tentatively identified, possibly because ELFs frequently lack spore-producing structures and the inaccuracy of molecular data. Xylariales, one of the diversified order of fungi in tropical regions, ecologically transit from saprophytic to endophytic lifestyle [24], also known to colonize inside the lichen thallus. The order Xylariales constitute of 22 family 110 genera [25]. The largest family Xylariaceae (32) and allied family Hypoxylaceae associated with taxonomically unrelated plants and comprise the most common endophyte. The order was the best example of unified nomenclature, One fungus One name [26]. They are identified using the multigene or polyphasic approach, which utilizes morphological, chemical and molecular data together for species delimitation [27–29]. Unfortunately, endosymbiotic Xylariaceous fungi rarely produce ascospores on culture media and the anamorph states of Xylariales are not suitable at all to assess the genus because they are rather characteristic of species on the one hand or families on the other hand [25]. Xylariaceous fungi originated from various plant hosts have been frequently subjected to phylogenetic analysis using one or more genes [30–31], but there are no previous attempts were made to utilize a multigene approach to recognize species identity and phylogenetic position the Xylariaceous endolichenic fungi. Members of Xylariaceae and Hypoxylaceae isolated from *Heterodermia* and *Parmotrema* thallus were subjected here to the delimitation of the species boundary and evaluation of the phylogenetic position. The lichen and the associated fungi are the greatest source to establish and study the fungal diversity, so it is important to understand the fungal diversity and compositional variation with respect to the mycobiont type, lichen habitat and nutrient media. Discussion and possible utilization of molecular information provide insights into the evolutionary relationships between lichen-associated fungi and their saprophytic counterparts. Which is the most needed study to understand the ecological transition, identity, ecosystem dynamics, and collective role of fungi in lichen.

Methods

Study region and lichen sampling

A total of 17 lichen samples belonging to the genus *Parmotrema* (10) and *Heterodermia* (7) are selected for the isolation and analysis of ELFs. The samples have been collected around Shivamoga district, which lies on the central Western Ghats of India. The sampling sites from which these thalli are collected represent a dry deciduous, moist deciduous, semievergreen, and evergreen forest (Supplementary Table 1A). The lichens are identified based on morphology and microchemical methods [33, 34] and a set of voucher specimens was deposited in the National

Herbarium CSIR-NBRI Lucknow (LWG) (Supplementary Table 1B).

Isolation and identification of endolichenic fungi

Collected samples have processed within 24 h, each thallus was washed in running tap water before surface sterilization with 75% alcohol for 30 s, followed by 4% NaOCl for 2 min, and 90% alcohol for 30 s [18], along with subsequent rinsing in sterile water. 10 surface-disinfected segments (size 5 mm X 5 mm) plated on ciprofloxacin (100 mg/L) suspended Potato Dextrose Agar (PDA), Malt Yeast Extract Agar (MYA), and Sabouraud Dextrose Agar (SDA) plates (HiMedia Laboratories, Mumbai) aseptically, and incubated for 45 days at 25 °C. Each segment was observed under a Carl Zeiss Stemi 2000C stereomicroscope, recorded the number of emergent isolates from each segment, and immediately carried pure culture on Malt Extract Agar (MEA) plates. Identification of fungi was done based on morphological characters [35], ELF isolates which failed to produce sporulating structures were grouped into morphotypes (Fig. 1) and subjected to molecular species delimitation.

DNA isolation and gene amplification

The total genomic DNA content was isolated from selected morphotypes by the 2X CTAB method with slight modifications [36]. Actively growing mycelium on MEA medium was scraped and grinded with a sterile plastic pestle in 500 µL CTAB extraction buffer (1.4 M NaCl, 100 mM Tris Cl [pH 8.0], 20 mM EDTA, 2% CTAB, 1.0% PVP, 0.2% 2-Mercaptoethanol) and incubated for 45 min at 60 °C. Centrifuged at 13,000 rpm for 30 min, the supernatant was then treated with RNase A and incubated for 10 min at 37 °C. Precipitated protein by phenol: chloroform: isoamyl alcohol (25:24:1) mixed briefly and centrifuged (10,000 rpm for 10 min). To the supernatant 600 µL of ice-cold isopropanol was added and incubated overnight, pelleted by centrifugation (5 min at 10,000 rpm), washed in 70% ice-cold ethanol, dried and eluted in 50 µL of sterile water. Three targeted loci namely, ITS, Tub2, and Tef1-α, are amplified using AMPLICON's ready-to-use Taq DNA polymerase 2 × Master Mix RED in Eppendorf Master cycler, suitable primers and amplification conditions are provided in supplementary Table 2. The resulting PCR amplicons are sequenced in Applied Biosystems 3730xL analyzer (Barcode Bioscience Private Limited, Bengaluru, India). Assured the sequence quality by inspecting the chromatograms, subjected to end trimming and created contigs of forward and reverse sequence with Sequencher V5.1 software. The consensus was subjected to BLAST against NCBI depositories for primary identification of the isolates. The sequences generated was deposited in GenBank (Supplementary Table 3).

Phylogenetic analysis

The sequences generated in this study are added to NCBI retrieved sequences (Supplementary Table 4) to perform MUSCLE sequence alignment in MEGA-X V.32. Model selection for each gene alignment was performed by running JModeltest. The best fit evolutionary model with the Akaike Information Criterion (AIC) is implemented in PAUP v.4. The phylogenetic relationship was inferred by reconstructions of individual and concatenated gene trees. The parsimony criterion analysis and maximum parsimony trees were constructed using PAUP v.4. with 1000 bootstrap replications. Whereas, the Bayesian inference trees are constructed in MrBayes-3.2.7-WIN. with 1,000,000 generation MCMC runs [37]. The FigTree V.1.4 application is used to accomplish the rooting and topological depiction of all the resulting trees.

Statistical analysis

All the statistical analyses are performed in PAST V. 3.4 software. The colonization frequency (CF) of ELFs was calculated using the formula, the total number of lichen segments infected by fungi divided by the total number of segments incubated. Further, alfa diversity was assessed in terms of the Shannon, Simpson, and Fisher-α indices [38]. Beta diversity among ecoregions, thallus types, and agar media was measured by non-metric multidimensional scaling (NMDS). Species dominance is represented by relative abundance and Important Value Index (IVI). The relative abundance (RA) was calculated by dividing the number of individuals representing species by the total number of individuals. IVI is the sum of relative frequency and relative density. Species richness in each thallus is represented by the species accumulation curve [39].

Results

From 1530 lichen segments, 980 ELF individuals of 42 morphospecies of ELFs are isolated. Belonging to 25 genera, 20 families, 10 orders, and 5 classes of Ascomycota. Out of which 19 genera successfully emerged from *Parmotrema* thallus, while 13 genera reported in *Heterodermia*. Since no discernible statistical significance was observed in individual sites, data from three trials and seven study sites are combined and tabulated into a single. The diversity of the ELFs was presented in terms of Shannon (1-D), Simson (H), and Fischer alfa (F-α) diversity indices. Among the three ecoregions (EC) from

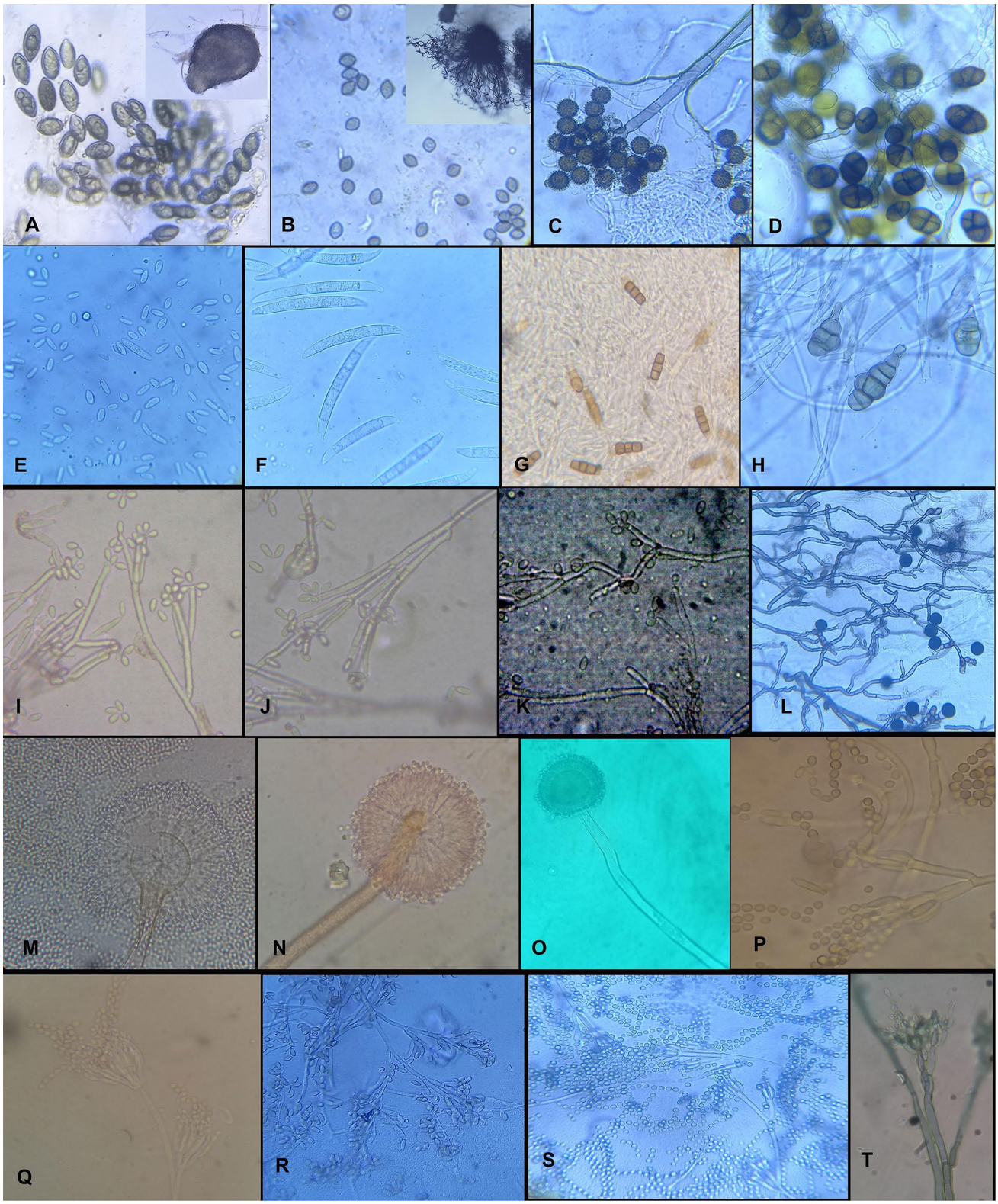

Fig. 1 Morphology of Endolichenic fungi isolated from *Parmotrema* and *Heterodermia* thallus; Spore producing structure,- **A** *Sordaria fimicosla*, **B** *Chaetomium globosum*, **C** *Periconia epigraphicola*, **D** *Ulocladium* sp., **E** *Fusarium sp2*, **F** *Fusarium oxysporum*, **G** *Pestalotiopsis* sp. **H** *Alternaria alternata*, **I–L.** Anamorphic spores of Xylariaceous fungi- **I** *Daldinia eschscholtzii*, **J** Nodulosporium like spore **K** Periconiella-like spores. **L** *Apiospora sp*., **M** *Aspergillus ochraceous*, **N** *A. subramanianii*, **O** *A. niger*, **P** *P. steckii*, **Q** *Purpureocillium lilacinum,* **L** *Clonostachys rosea,* **M** *Talaromyces* sp., **N** *Cladosporium cladosporioides*

which *Parmotrema* samples were collected, EC2 showed the highest species diversity [(1-D) = 0.9382, (H) = 2.865, Fisher-α = 8.429], followed by EC3 and EC1. In the *Heterodermia* thallus sampled from five ecoregions, the highest species diversity was observed in EC2 (1-D = 0.8038, H = 1.894, F-α = 4.57), while the least species diversity was observed in the thallus collected from EC4 and EC5 (Table 1).

Ecoregion and distribution of ELFs

The coordinates representing ecoregions fit distantly in the NMDS plot. (Fig. 2A). In *Parmotrema* thallus, 6, 18, and 8 species reported from EC1, EC2, and EC3, respectively. Xylariaceous fungi are the dominant emergent reported from all these sites; among them, *Xylaria sp. ELFX12* in the EC2, *Xylaria* sp. *ELFX03*, and *Xylaria berteri* from the EC3 emerge predominantly. Considering the *Heterodermia* thallus, the composition of ELFs in each ecoregion is as follows: In EC1, *Talaromyces sp*. presents dominantly, whereas *Sordaria fimicola* has a low relative frequency. In EC2, anamorphic Xylariales are the dominant emergent, while *Penicillium chrysogenum*, *Chaetomium globosum*, and *Sordaria fimicola* are reported to be the least abundant. In EC3, *Xylaria* and the anamorphic Xylariales (*ELFX20*) found dominantly, and *E. endophytica* reported with a low frequency. In EC4, only *Chaetomium* and *Fusarium* species emerged frequently. Whereas *Purpureocillium lilacinum* and anamorphic Xylariales are reported frequently from the *Heterodermia* thalli sampled from EC5.

Mycobiont and composition of endolichenic fungi

About 12 species of ELFs are exclusively present in *Parmotrema* whereas, 10 are exclusively reported only in *Heterodermia* thallus. About 9 species including *Xylaria feejeensis*, *X. berteri*, *Xylaria sp. ELFX03*, *Xylaria sp. ELFX06*, *Torula*, *Fusarium sp2*, *E. endophytica*, *Chalar sp*. *A. subramanianii* isolated from both *Parmotrema* and *Heterodermia* thallus, result in two thalli overlaps in NMDS ordination with good stress value (Fig. 2B). Indicating that, these species may be significant contributors to the overall ecological dynamics. Among the 19 species isolated from *Heterodermia* thallus, the important value index was high for *Fusarium sp*.,

Table 1 Shannon, Simpson and Fisher-α diversity indices values of endolichenic fungi

	Ecoregions	Simpson (1-D)	Shannon (H)	Fisher-α	CF%
Parmotrema	1	0.7513	1.562	2.338	46.11
	2	0.9382	2.865	8.429	78.14
	3	0.8727	2.159	3.692	54.8
Heterodermia	1	0.7604	1.448	1.528	83.3
	2	0.8038	1.894	4.57	80
	3	0.8307	1.87	2.322	23.7
	4	0.5128	0.68	0.4462	65.5
	5	0.8204	1.686	1.528	84.4

EC1-threes plots from moist deciduous forest; EC2- two plots from dry deciduous forest; EC3- two plots from semievergreen forest; EC4 and EC5 represent evergreen forest

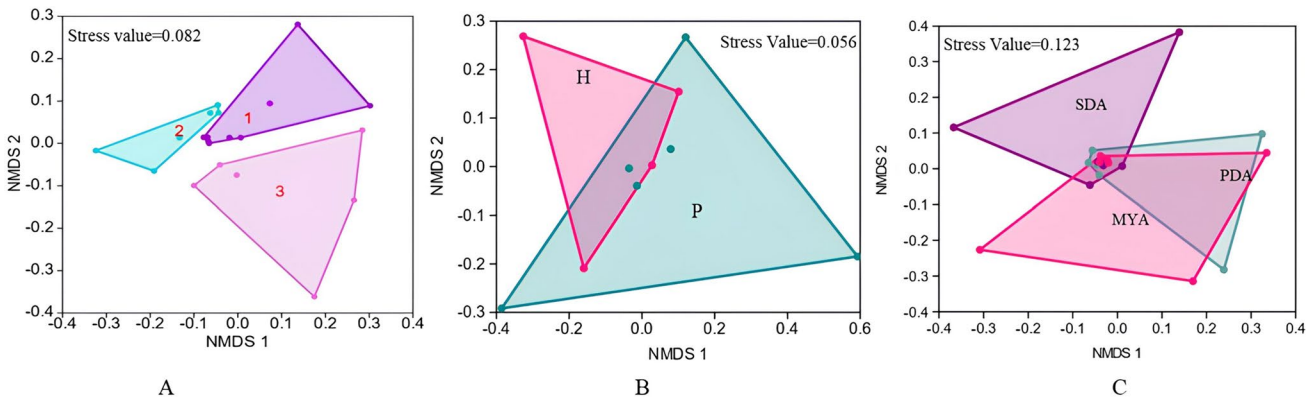

Fig. 2 Non metric multidimensional plot representing Beta-diversity of endolichenic fungi, **A** Ecoregions EC1, EC2 and EC3, **B** Between thallus *Heterodermia* and *Parmotrema*, **C** Media PDA, MYA and SDA

Xylaria feejeensis, *X. berteri*, and *Talaromyces sp*. (Table 2). Similarly, *Endomelanconiopsis endophytica*, *X. berteri*, and *Nodulosporium sp.* displayed high IVI among the 25 ELF species found in *Parmotrema* (Table 3). The average colonization frequency of ELF in *Parmotrema* thallus is 58.33%, *E. endophytica*, and *Nodulosporium sp*. are the predominant ELFs emerged from *Parmotrema* species. Whereas, *Heterodermia* has 72.2% average colonization frequency. *Fusarium sp2*. *Xylaria berteri* and *X. feejeensis* are predominant emergent in the *Heterodermia* thallus.

Nutrient media and emergence of endolichenic fungi

Among the three media used for the isolation of ELFs, PDA supports the growth of a diverse group of fungi with comparably high species richness. The NMDS ordination of PDA overlaps with SDA and MYA (Fig. 2C). About 6, 3, and 2 ELF species exclusively emerged on PDA, SDA and MYA apart. About 5 ELFs are common to the media PDA-SDA, 3 to SDA-MYA and 4 to the PDA-MYA. About 13 species commonly emerge from all media. Among the five classes reported, Dothideomycetes, Eurotiomycetes, Saccharomycetes and Sordariomycetes emerged from all the media, whereas Leotiomycetes emerged predominantly in the MYA medium (Fig. 3).

Phylogenetic tree construction and interpretation

ITS, beta-tubulin and Tef1 alfa sequences amplified from the endolichenic isolates. No appropriate homologous sequence of tef1 alfa is available for sequence alignment, therefore results of only ITS and Tub2 data are presented. The primary taxonomic delimitation of this sequence was based on the ITS sequence similarity searches in the BLAST program, which inferred that seven isolates belong to the Xylariaceae and one Hypoxylaceae. The aligned data matrix was compiled with the endolichenic sequences, their closely related

Table 2 Composition and relative abundance of endolichenic fungi associated with *Heterodermia*

Endolichenic fungi/study site	1	2	3	4	5	IVI/
Anamorphic						
Aspergillus subramanianii	0	2.63	0	0	0	3.21
Candida sp.	0	6.58	0	0	0	3.98
Chalara sp.	0	0	3.53	0	0	3.73
Endomelanconiopsis endophytica	0	2.631	1.76	0	0	6.17
Fusarium sp.*2*	0	0	0	51.28	0	12.9
Apiospora sp.	0	3.95	0	0	0	3.47
Penicillium chrysogenum	0	1.31	0	0	0	2.95
Purpureocillium lilacinum	0	0	0	0	25.37	7.07
Talaromyces sp.	30.67	0	0	0	0	8.61
Torula sp.	0	0	2.35	0	0	2.95
ELFX20[1]	24	36.84	12.94	0	28.36	29.3
ELFX20[2]	0	19.74	0	0	0	6.55
Teleomorph						
Chaetomium globosum	0	1.31	0	48.76	0	7.58
Sordaria fimicola	2.67	1.32	0	0	0	6.17
Xylariaceous morphotypes						
Xylaria psidii	0	2.63	0	0	0	3.21
X. feezeensis	0	7.89	0	0	0	9.26
X. berteri	0	0	25.29	0	0	9.12
Xylaria sp.[3]	29.3	10.52	36.47	0	10.46	12.34
Xylaria sp. *isolate ELFX03*	0	0	0	0	11.94	4.75
Xylaria sp. *isolate ELFX06*	0	0	1.76	0	0	2.95
Morphotypes	13.3	0	15.88	0	23.88	20.96

Relative abundance of endolichenic fungi based on 630 segments of lichen from 5 ecoregions; Number of segments used from EC1- 90, EC2- 90, EC3- 270, EC4- 90, EC5- 90. Thallus of *Heterodermia* used to isolate endolichenic fungi belongs to the genus *H. incana, H. speciosa, H. flabellate, H. diodemata*, *H. hypocaesia, H. obscurata*

ELFX20[1]**-** Nodulosporium like spores

ELFX20[2]: Periconiella-like spores

Xylaria[3]- ungrouped Xylaria morphotypes (Greenhalgh and Chesters 1968 [56])

Table 3 Composition and relative abundance of endolichenic fungi associated with *Parmotrema*

Study site/ELF composition	1	2	3	IVI
Anamorphic				
Alternaria alternata	0	2.37	0	3.13
Acremonium sp.	0	0.47	0	2.36
Aspergillus subramanianii	0	2.37	0	3.13
Aspergillus niger	0	0.95	0	2.55
Chalara sp.	0	0	0.67	2.36
Cladosporium cladosporioides	0	1.42	0	2.75
Daldinia eschscholtzii	0	9.48	0	5.98
Endomelanconiopsis endophytica	4.82	10.90	12.84	16.05
Fusarium oxysporum	2.41	0	0	2.94
Fusarium sp.	0	3.317	0	3.51
Clonostachys rosea	0	0	7.43	4.27
Myrothecium sp.	0	0	0.67	2.36
Nodulosporium sp.	3.61	1.42	7.43	10.3
Penicillium sp.	1.81	0.95	0	5.30
Pestalotiopsis sp.	0	0.47	0	2.36
Torula sp.	0	6.16	1.35	7.21
Trichoderma sp.	0	7.58	2.027	7.97
ELFX20[1]	31.32	7.11	1.35	19.67
Ulocladium sp.	0	3.317	0	3.51
Periconia epigraphicola	0	0.947	0	2.55
Xylariaceous morphotypes				
Xylaria berteri	0	0	22.29	8.46
X. feezeensis	0	6.63	0	4.84
X. psidii	0.60	3.317	0	5.88
Xylaria isolate ELFX03	0	0	14.19	6.17
Xylaria isolate ELX06	1.205	0	0	2.55
Xylaria isolate ELX21	0	11.37	0	6.75
Hypoxylon fragiforme	0	1.422	0	2.75
Xylaria sp.[3]	22.89	15.64	13.51	23.8
Morphotypes	31.32	2.369	16.21	20.44

Relative abundance of endolichenic fungi based on 900 segments of lichen from 3 ecoregions; Number of segments used from EC1- 360, EC2- 270, EC3- 270. Thallus of the *Parmotrema* utilized to isolate endolichenic fungi are *P. tinctorum, P. praesorediosum, P. grayanum, P. cristiferum*. *Xylaria*[3]-ungrouped morphotypes

sequences, and type sequences from the NCBI database to provide a phylogenetic context (Supplementary Table 4). The ITS dataset included 45 sequences from fungal specimens representing 22 species and had an alignment length of 661 characters, of which 314 characters were constant, 106 were variable and parsimony uninformative, and 241 were parsimony informative. The parsimony analysis yielded 100 equally parsimonious trees (TL = 1184, CI = 0.492, RI = 0.663, and RC = 0.327, HI = 0.508). GTR + I + G4 was determined to be the optimal model for the ITS dataset. Following 1,000,000 generations of MCMC run, the Bayesian analysis yields 5055 trees with split frequencies of 0.0479 and an effective sample size (ESS) of 1670.5 for the average ESS (avg ESS). The tree inferred from the ITS gene (Fig. 4) shows that two query sequences, *ELFX04* and *ELFX23* form well-supported clad with *Xylaria psidii*. *ELFX05* is inferred as *X. feejeensis*. Isolate *ELFX13* close to that of *Hypoxylon fragiforme*. The sequence *ELFX06* is close to the clade *X. psidii*. Sequences *ELFX08* and *ELFX21* are located in the clade corresponding to *X. berteri*.

The ITS + Tub2 combined alignment included sequences from 46 taxa representing 45 species. The dataset had an aligned length of 1088 characters, of which 489 characters were constant, 157 were variable and parsimony uninformative, and 442 were parsimony informative. The maximum parsimony analysis yielded fifteen equally parsimonious trees (TL = 2622, CI = 0.405, HI = 0.595, RI = 0.539, and RC = 0.219). The best model for the ITS + Tub2 dataset estimated and applied in the Bayesian analysis was GTR + I + G. The Bayesian analysis reached stationarity after 1,000,000 generations, resulting in a total of 5600 trees and an effective sample size average ESS (avg ESS) of 418.5. The Bayesian tree from the above analysis shows the nesting of related taxa with best root support (Fig. 5). Sequences of *ELFX06* nested into separate clade with 100% Posterior probability value, *ELFX13* fitted with Hypoxylon fragiforme have 100% root support, whereas sequence *ELFX08* and *ELFX21* split out from *X. berteri* clade.

Discussion

ELFs belong to 25 genera of Ascomycota recovered and identified from lichen thallus collected around the study area. We observed high variability in lichen samples and their fungal composition in the region. lichen thallus collected from the deciduous forest has the highest species richness and diversity, also abundant lichen thallus collected in this area. On the other hand, fewer lichens were obtained and the lowest species diversity was found in the evergreen environment, suggesting a correlation between the lichen habitat and ELF colonization [40]. Sordariomycetes are the most prevalent ELFs isolated from every site and the thallus. These observations corroborated previous studies on the tropical zone. In a recent study Chakarwati et al. [41] isolated 73 ELF isolates from the *Parmotrema* thallus collected across India, 84.50% of these ELF was belonging to the class Sordariomycetes. Maduranga et al. [42] isolated 171 endolichenic fungal strains from 32 lichen species ~ 56% of which are Sordariomycetes. The composition of endolichenic fungal community differed significantly among five ecoregions, U'Ren et al. [43] opined that the composition of ELFs changes dramatically over geographical distance, and the local climate in which lichen grows has a positive correlation with community similarity. It also suggested

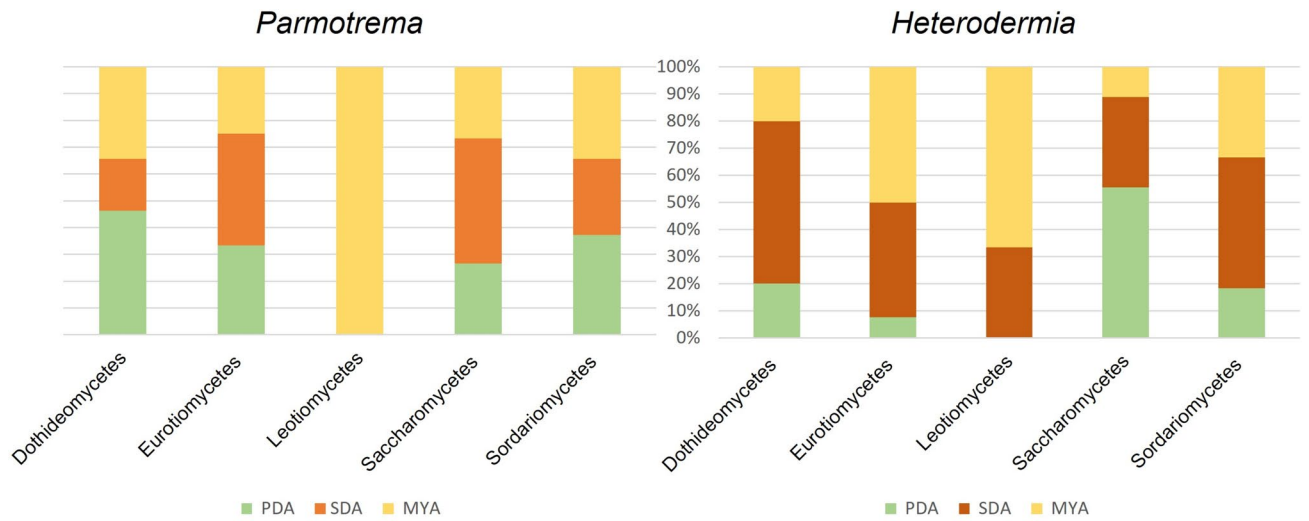

Fig. 3 Graph representing taxonomic composition of endolichenic fungi in different nutrient media- PDA, SDA and MYA: Data is generated based on endolichenic fungal emergence in 990 *Parmotrema* segments and 530 segments of *Heterodermia*

that the mycobiont taxonomy plays a significant role on the composition of ELF [44, 45]. ELF richness was comparable between *Heterodermia* and *Parmotrema*, among the two thallus low species accumulation was observed in *Heterodermia* thallus (95% confidence value: > 4) (Fig. 6). Despite being distinct, the NMDS coordinates of the two communities do overlap to some extent, most likely due to the fact ELF community is structured by the interaction of multiple factors, and distinguishing the relative influence of each factor is difficult [46]. Among the recovered taxa, the highest number of ELFs emerged from the PDA medium. Unlike the culture-independent techniques, which massively detect species, the culture-based method has limitations [47]. About 99% of the ELFs reported in culture media belong to the Ascomycota. The research findings unambiguously show a correlation between the makeup of growth media and the emergence of distinct fungal groups. Comparing the exact nutritional needs of the various fungal groups is still challenging, though, as several media elements are only available as extracts [48]. ELFs distributed throughout the lichen thallus unevenly and receive different effects upon surface sterilization [49]. Many studies have pointed out that the combination of alcohol and sodium hypochlorite gives effective surface sterilization. We observe a colonization rate of 23–84%, with the same surface sterilization protocol, Li et al. [17] reported a colonization rate of 53–100% or even a low colonization rate observed by other authors. Santiago et al. [50] found a colonization rate of 30–23% predominant number of these isolates belonged to the genera *Nemania* and *Xylaria*. A detailed literature search on the composition of ELFs around tropical zones shows Xylariales are the predominant emergent species in culture. It is noteworthy that among the 16 thalli subjected to ELF isolation, Xylariaceous fungi emerged from 15 thalli. Govindarajulu et al. [51] isolated *Xylaria apiculata*, *Xylaria primorskensis*, *Nodulisporium sp*. *Nemania bipapillata*, *Hypoxylon investiens*, *Daldinia eschscholtzii* from lichen collected around Southern Western Ghats. Xylariales belongs to the genera *Annulohypoxylon*, *Biscogniauxia, Daldinia, Hypoxylon, Nodulisporium* and *Xylaria* frequently reported from the Northern India [50, 52]. Indeed, Xylariales have high host acquisition capacity and can colonize in the taxonomically unrelated lichen regarded as the most diversified ELFs in the tropical region [53].

The most common observation in all the above research is that xylariaceous fungi in culture conditions lack appropriate taxonomic character. One way to identify such isolates is through the molecular method. All the xylariaceous sequences subjected to phylogenetic analysis in this study will not necessarily form monophyletic clades since the group has variable genetic distance. Although phylogenetic analysis based on ITS proved to be very practical concerning taxonomic identification at the genus and species level, a concatenated tree generated from ITS + beta-tubulin, on the other hand, gives good resolution. The low base support for some clade in phylogenetic trees may affected by sequence data availability. Adding more taxa will not increase the resolution of the tree instead creates a large topological difference. Hence, far-related clades were removed and only clades fitted with the query presented here. All the Xylariaceous morphotypes subjected to phylogenetic analysis belong to the genera *Xylaria* and *Hypoxylon*. It was expected that the two genera would nest since the families Xylariaceae and Hypoxylaceae are the most closely related groups within the order Xylariales [54, 55]. The query sequence *ELFX13* shows 99.6% similarity to *H. fragiforme*

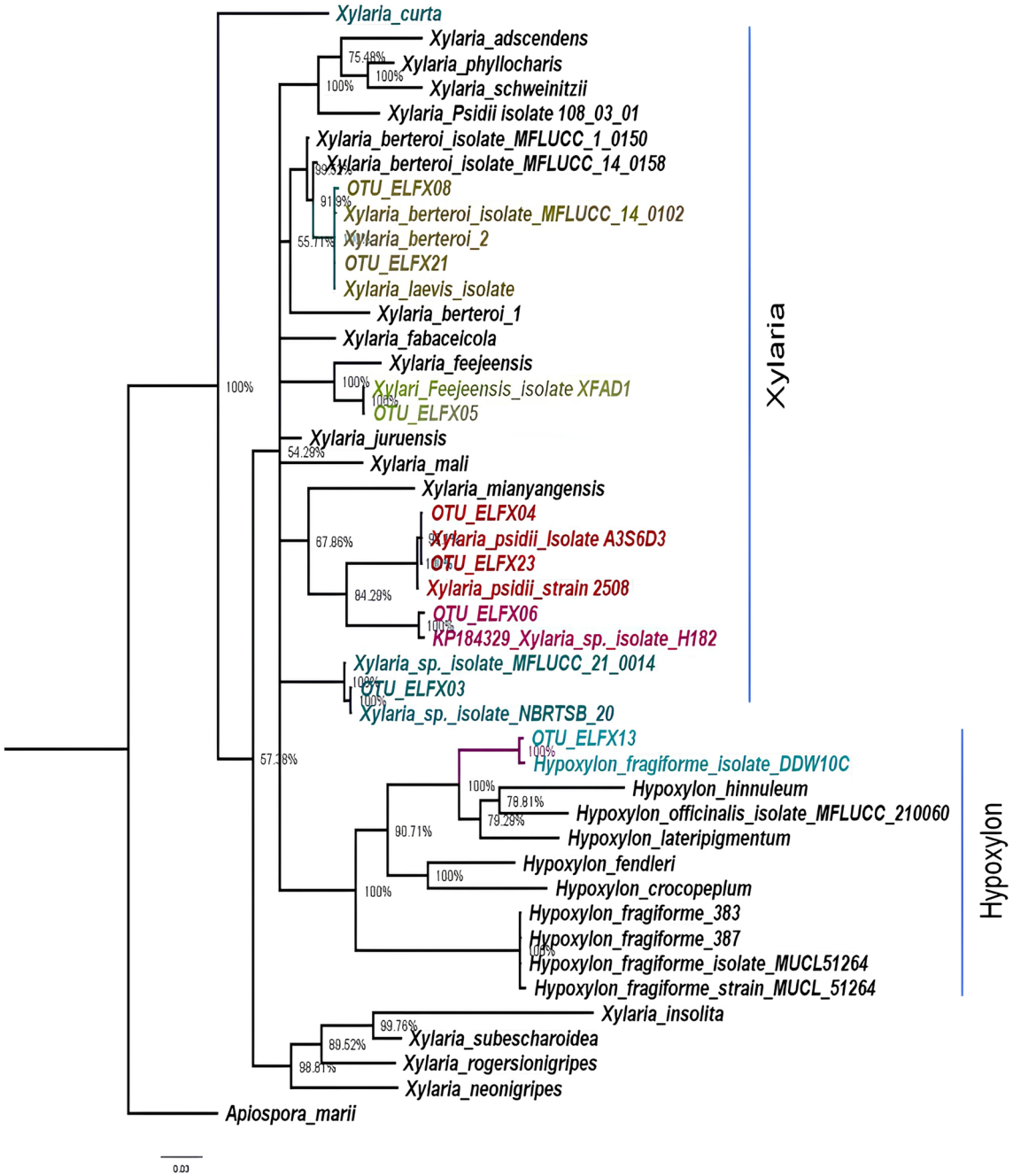

Fig. 4 ITS-Bayesian phylogenetic tree; obtained from 5 runs of 1,000,000 generations MCMC run. The query sequences and their closest homologous were highlighted with colors. *Apiospora marii* is selected as outgroup

and the clade representing the genus is paraphyletic nested with other species such as *H. hinnuleum, H. officinalis*, and *H. lateripigmentum*, with a high posterior probability value (> 75%) [57]. In this study genus *Xylaria* shows a polyphyletic origin. The clade representing *Xylaria psidii* included the sequences *ELFX04* and *ELF23* with 100% BLAST similarity. While the query *ELFX05* fitted with the clade *Xylaria feejeensis* received 100% posterior probability support in the ITS tree. However, the observation deviated in the combined tree, where the clade was nested with *X. neonigripes*, *X. rogersionigripes*, and *H. subescharidea*. Similar condition was observed for the query *ELFX08* and *ELF21* however, *ELFX08* has a 99% similarity with *X. berteri*. Beside the phylogenetic analysis, two endolichenic isolates, namely *ELFX03* and *ELFX06* (Fig. 7) will not form clades with any of the known species. The sequences of

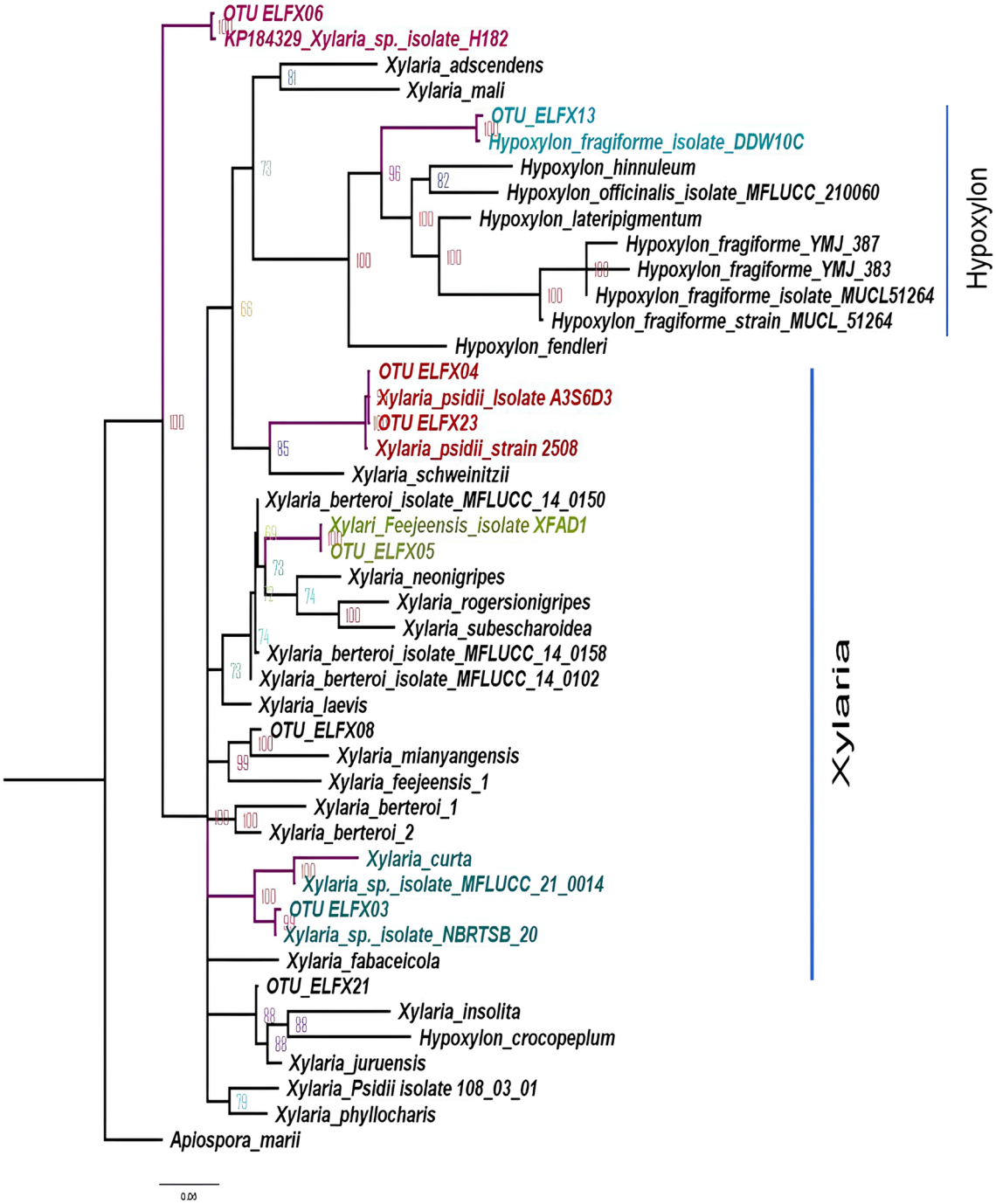

Fig. 5 A Concatenated tree of ITS+Tub2 based on Bayesian phylogenetic analysis; obtained from 5 cycles of 1,000,000 generations MCMC run. The query sequences and their closest homologous were highlighted with colors. *Apiospora marii* is selected as outgroup

ELFX03 share > 99% sequence identity with *Xylaria sp.* isolate MFLUCC 21–0014 (gap-0%, identity: 511/512 bp for ITS, 326/329 bp for Tub2). The two top BLAST hits are *X. cubensis* and *X. laevis*. *Xylaria sp.* isolate 21–0014 was introduced as new species by Ma et al. [30] based on phylogenetic analysis. The sequences of *ELFX06* on the other hand, phylogenetically close to only sequence *Xylaria sp.* H182, an endophyte of *Hevea brasiliensis* originated from Thailand [58]. Based on the present information we suspect that the isolate can be new species of *Xylaria*, However, the available morpho-molecular evidence was insufficient to confine the speciation event. Which highlights the importance of the comprehensive approach to accurately defining species boundaries and taxonomic studies. Generating

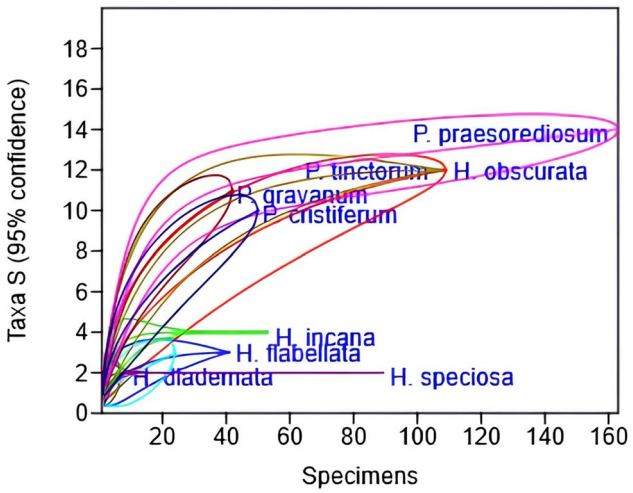

Fig. 6 Rarefaction curve, representing species richness in each thallus. H-*Heterodermia*, P-*Parmotrema*

morpho molecular data of cryptic fungal inhabitant from various habitat will be valuable asset to explore the hidden fungal diversity.

Conclusion

The study certainly proved that the lichen thallus can be great source to study the fungal diversity, large number of cryptic fungal assemblages reported from the lichen of Shivamoga district, Karnataka. Comparison among ecologically distinct sites show deciduous lichens exhibit more ELF species richness. All 42 morpho species isolated here belong to the phylum Ascomycota, which are explosively reported from the endosymbiotic life cycle and rarely reported as saprophytes. Sequence comparison and the nesting pattern of related queries in the phylogenetic tree render us to ask a question; Are endolichenic and endophytic lifestyles were shared? rather, being resembled each other. It was observed that mycobiont and lichen habitat are crucial factors that structure the ELF. The phylogenetic analysis resolves the identity of eight non-sporulating Xylariaceous endolichenic fungi, two of which were speculated to be new species. (Further research is needed to obtain more sequence data and morphological analysis to gain better understanding on identity of new species). Successful amplification of

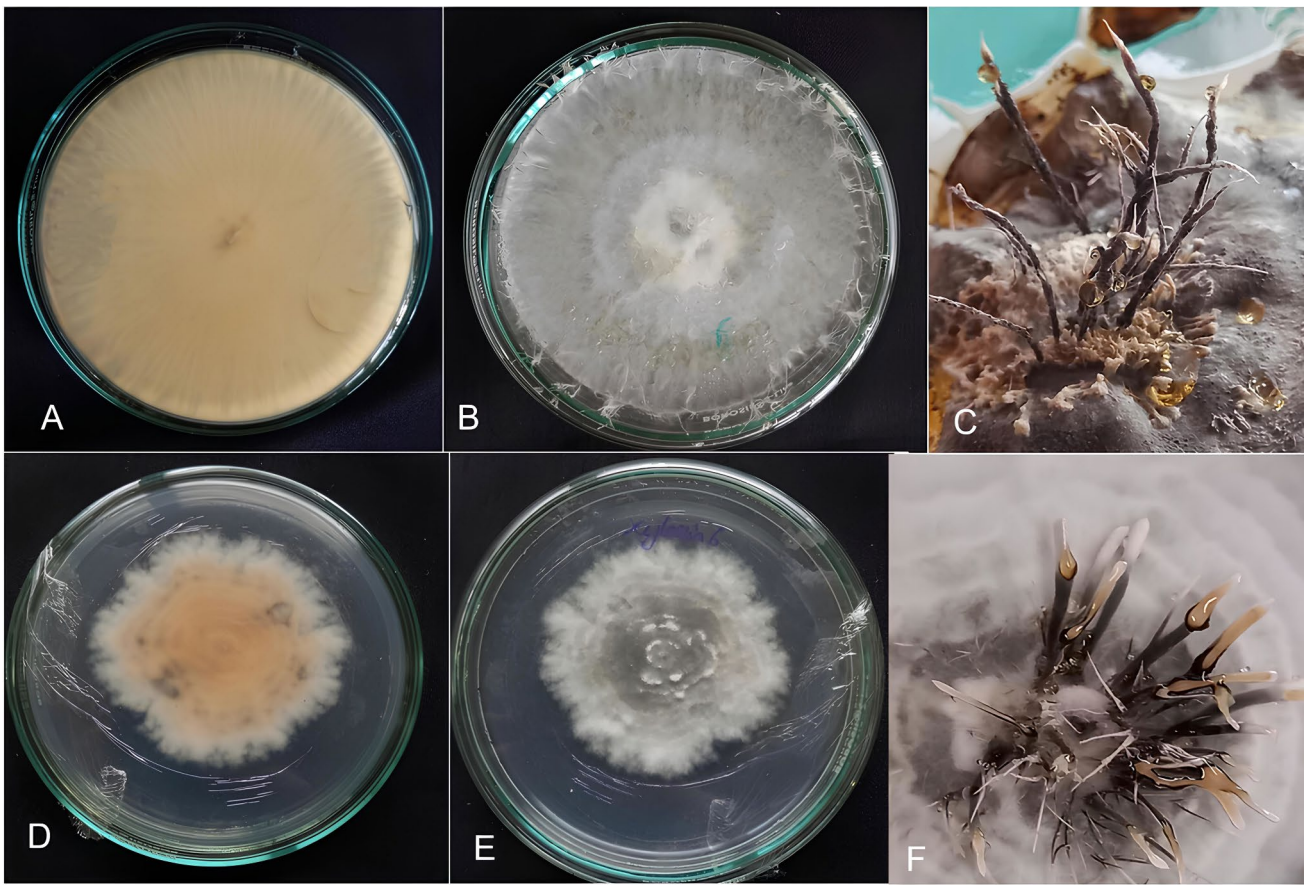

Fig. 7 Morphology of the *Xylaria sp. ELFX03*; on PDA medium, **A** reverse View, **B** front view, **C** Stromata like structures produced on OA medium. *Xylaria sp. ELFX06*; on PDA, **D** reverse View, **E** front View, **F** Stromata like structures produced on OA medium

most phylogenetically informative markers and building comprehensive taxonomic databases are further needed to bring light to more such a cryptic species.

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Author contributions DP generated, analyses the data and prepare draft, YLK prepare the outline of the study, analyze the data, and finalized the manuscript**.**

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Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval Not applicable.

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