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Endolichenic fungal diversity associated with some lichens of the Western Ghats

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ABSTRACT

A total of 389 strains of fungi belonging to 38 species were isolated from 10 lichen species of the Western Ghats, southern India. All the lichens screened, irrespective of their growth forms or location, harboured endolichenic fungi. Most of the fungi belonged to the Ascomycotina. *Chaetomium* sp. 1, *Hypoxylon investiens, Nemania bipapillata, Nodulisporium* sp., *Paecilomyces* sp., and *Sporormiella intermedia* were the most common ones and were present in 5 or more lichen species. Of the 28 sporulating fungi, 7 belonged to Xylariales, and their total colonization frequency in all the lichens was also high. This observation further substantiates the high ecological amplitude of Xylariales, possibly due to overlapping ecological niches and their ability to inhibit co-occurring endolichenic fungi.

Introduction

By existing for more than 600 million y, lichens with their fungal partner and unicellular or filamentous algal or cyanobacterial partner, represent one of the most successful symbiotic systems [1]. A lichen represents a mini-ecosystem since apart from the obligate symbiont, the symbiome of lichen supports a basidiomycete yeast [2], nonculturable nonphotosynthetic bacteria [3], lichenicolous fungi [4], and endolichenic fungi [5]. The endolichenic fungi are nonobligate fungal component of lichen symbiome; they are similar to the endophytes of higher plants as they occur inside the lichen thallus, are nonpathogenic, and are transmitted horizontally [5–7]. Very few lichens across the globe have been screened for their endophytic fungal assemblages, and all of them invariably harbour these fungi [8]. These endolichenic fungi appear to produce many novel metabolites with technologically exploitable bioactivities [9–12]. Although India supports 2511 species of lichens [13], very few species have been screened for their endolichenic fungal assemblage, and that too mostly from temperate regions of Central Himalaya (Uttarakhand) [14]. Considering the paucity of information on endolichenic fungi [8] especially from India [15], we studied the endolichenic fungal assemblage in 10 lichen species (2 crustose, 4 each foliose and fruticose forms) occurring in the Western Ghats, southern India.

Results

Three hundred and eighty-nine strains of endolichenic fungi belonging to 38 species (including 10 sterile fungi) were isolated from 10 different lichen species irrespective of their growth forms or location (▶ **Tables 1**, **2**). The species accumulation curve, which represents the cumulative number of species isolated against sample size, did not flatten with more samples screened (▶ **Fig. 1**). Similarly, the unique species curve, which is a measure

▶ **Table 1** Lichen species screened for endolichenic fungi.

Lichen species	Code	Family	Growth form	Location	Co-ordinates
Arthonia sp.	AR	Arthoniaceae	Crustose	Mudumalai	11.63° N, 76.52° E
Bacidina sp.	BA	Ramalinaceae	Crustose	Mudumalai	11.63° N, 76.52° E
Dirinaria consimilis (Stirt.) D. D. Awasthi	DI	Physciaceae	Foliose	Masinagudi	11.57° N, 76.64° E
Evernia prunastri (L.) Ach.	EV	Parmeliaceae	Fruticose	Masinagudi	11.57° N, 76.64° E
Hypotrachyna crenata (Kurok.) Hale	HY	Parmeliaceae	Foliose	Mudumalai	11.63° N, 76.52° E
Parmotrema sp.	PA	Parmeliaceae	Foliose	Kargudi	11.56° N, 76.54° E
Parmotrema tinctorum (Despr. ex Nyl.) Hale	PT	Parmeliaceae	Foliose	Mudumalai	11.63° N, 76.52° E
Ramalina pacifica Asahina	RA	Ramalinaceae	Fruticose	Masinagudi	11.57° N, 76.64° E
Teloschistes flavicans (Sw.) Norman	TF	Teloschistaceae	Fruticose	Masinagudi	11.57° N, 76.64° E
Usnea sp.	US	Parmeliaceae	Fruticose	Kargudi	11.56° N, 76.54° E

of those species that occur in only 1 lichen sample species irrespective of their colonization frequency, did not flatten with sampling effort (▶ **Fig. 2**). The present study revealed that majority of the fungi belonged to the Ascomycotina. The colonization frequency (CF%) was lowest for the lichen *Evernia* sp. and maximum for *Bacidina* sp. Among the lichens studied, *Teloschistes flavicans* supported the highest species diversity of endolichenic fungi (▶ **Table 2**). Some fungi including *Chaetomium* sp. 1, *Hypoxylon investiens, Nemania bipapillata, Nodulisporium* sp., *Paecilomyces* sp., and *Sporormiella intermedia* were present in 5 or more lichen species (▶ **Table 2**). Of the 28 sporulating fungi, 7 belonged to Xylariales whose total CF% in all the lichens was 191. Of these, *N. bipapillata* and *H. investiens* occurred in 7 and 6 lichen species with a total CF% of 83 and 20, respectively; *Nodulisporium* sp. was present in 6 lichen species with a total CF% of 29.

Xylariaceous fungi isolated from lichens were identified using molecular techniques. Amplification of ITS1–5.8S-ITS2 region of rRNA using universal primer pair ITS-1 and ITS-4 resulted in differently-sized PCR products ranging from approximately 531–659 bp in size. Out of 6 fungal isolates identified, LX1was approximately 581 bp in size; LX2 ~ 658 bp, LX3 ~ 565 bp, LX4 ~ 531 bp, LX5 ~ 659 bp and LX7 ~ 626 bp in size. Analysis of different ITS sequences corresponding to different fungal isolates using the BLASTn program revealed that the LX1 sequence was closely similar to *Xylaria primorskensis* (97–99%). Similarly, LX2 and LX5 showed close similarity to *Hypoxylon investiens* (97–99%), LX3 to *Daldinia eschscholtzii* (99–100%), LX4 to *Nemania bipapillata* (99–100%) and LX7 to *Xylaria apiculata* (99%). Phylogenetic analysis separated all the identified xylariaceous fungi into 5 different groups based on bootstrap values. These fungi were deposited in GenBank (MN310382–MN310387) (▶ **Fig. 3**, **Table 3**).

Discussion

The majority of the studies on endolichenic fungi relate to their secondary metabolite spectrum because of the desirable properties exhibited by these metabolites [9, 11, 12]. Although similar to the endophytic fungi of higher plants, the function of endolichenic fungi in maintaining the complex lichen association is not known.

As observed in the few earlier studies, including in Antarctica [16, 17], the Arctic [18], and China [19], the endolichenic fungi isolated in the present study belonged to the Ascomycota [5, 7, 19–21]. We did not observe any host specificity among the endolichenic fungi. This was in conformity with the study of Chagnon et al. [22] who conclude that endolichenic fungi are generalists and are not strictly host-specific. Despite this commonality, endolichenic fungi are not random associates of lichens as revealed by the molecular studies of U'Ren et al. [21]. It appears that the habitats that the lichens occupy and the chemical milieu of their thalli determine their endolichenic fungal composition. In temperate forests, Ascomycota were dominant among the lichen-associated fungi, while Basidiomycota dominated the fungal assemblages of plant roots suggesting the operation of a selection mechanism [17, 23, 24]. Furthermore, altitude and geographic location also influence the composition of lichen-associated fungi as revealed by 18S rDNA sequencing study [18].

We observed that both the species accumulation curve (▶ **Fig. 1**) and the unique species curve (▶ **Fig. 2**) did not reach an asymptote with increasing sample size, indicating that the endolichenic diversity was high and our sample size was not adequate to capture it fully. Additionally, we add the caveat that the ITS sequencing method, though a well-established molecular method for fungal identification, may not be absolutely reliable since some of the sequence data available in databases are from wrongly-named isolates; furthermore, it is recommended to compare the sequence data with those of the type species to confirm the identity. Since we did not attempt the latter, it is likely that some of the species reported have been tentatively identified [25].

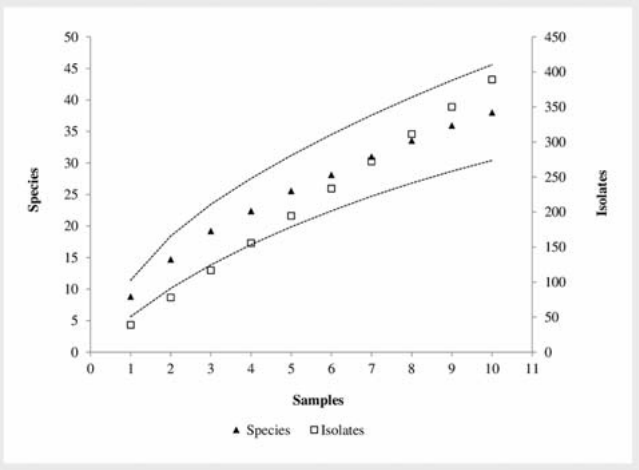
The broad host range as well as the high colonization frequency of Xylariales observed in the present study is noteworthy and corroborated with Yu et al. [16]. It has also been proven several times that members of Xylariales establish an endophytic association with taxonomically unrelated plants, which are geographically isolated [26–30]. Indeed the host acquisition ability of *Xylaria* as an endophyte is high, and it has been isolated from plants belonging to 3 divisions (including liverworts and higher plants) and occurring on 2 continents [31]. In our investigations in the forests near the locations from which the lichens were col-

▶ **Table 2** Species composition, diversity, and Colonization Frequency (CF %) of endolichenic fungi isolated from lichen thalli (Refer to ▶ **Table 1** for host code).

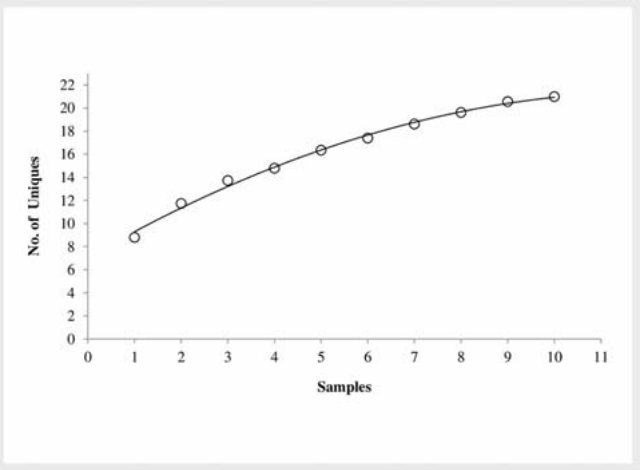
Endolichenic Fungi	AR	BA	DI	EV	HY	PA	PT	RA	TF	US	Total
Aureobasidium pullulans		4							1		5
Botrytis sp.			1								1
Chaetomium sp. 1		2	2	1		3				14	22
Chaetomium sp. 2			5								5
Cladosporium sp.	3				8		8				19
*Daldinia eschscholtzii**			24								24
Fusarium sp. 1		2									2
Fusarium sp. 2	1										1
*Hypoxylon investiens**			3	2	2	3	7	3			20
Lasiodiplodia theobromae		4			2						6
*Nemania bipapillata**		33	3		15	2	13		1	16	83
Nigrospora oryzae							1				1
Nodulisporium sp.	4	6		1	8		4		6		29
Paecilomyces sp.			3	2	1	1	6	1	1		15
Penicillium sp. 1							1				1
Penicillium sp. 2						1			1		2
Pestalotiopsis sp.		9									9
Phialophora sp.							3		1		4
Phoma sp.	1										1
Sporormiella intermedia	1		4		4	5	4	7	3		28
Sterile fungus 1	2										2
Sterile fungus 2	1										1
Sterile fungus 3	6										6
Sterile fungus 4	1										1
Sterile fungus 5	11				6						17
Sterile fungus 6		8									8
Sterile fungus 7					13						13
Sterile fungus 8	1				1						2
Sterile fungus 9							1				1
Sterile fungus 10						1					1
Taeniolella sp.									1		1
Talaromyces sp.							10	1			11
Torulomyces sp.	3	4			2						9
*Xylaria apiculata**	13										13
*Xylaria primorskensis**					1	2		7		6	16
Xylariaceous form							4			2	6
Yeast form					1						1
Zygosporium sp.							2				2
Total CF%	48	72	45	6	64	18	64	19	15	38	389
Total No. of Species	13	9	8	4	13	8	13	5	8	4	38
Fisher's alpha	5.9	2.7	2.8	5.2	4.9	5.5	4.9	2.2	7.0	1.1	

Note = * molecular identification by ITS sequencing

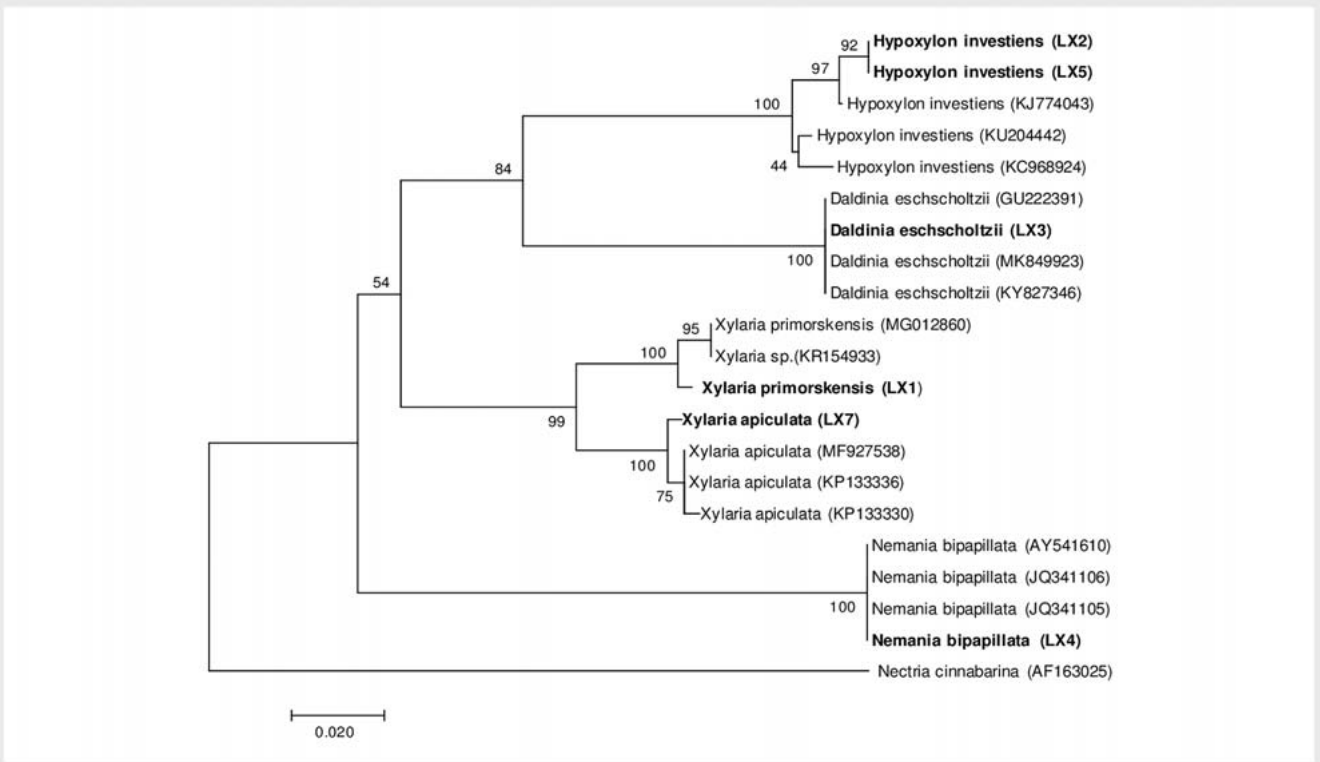
lected in the present study, we isolated species of *Xylaria* as foliar endophytes [30]. *Xylaria apiculata*, occurring as endolichenic fungus in *Arthonia* in the present study, exhibits both decomposer and endophytic life strategies in tropical forests [28]. Yu et al. [16] speculate that in Antarctica, fungal endophytes of bryophytes exist as endolichenic fungi to survive extreme environmen-



▶ **Fig. 1** Species accumulation curve for endolichenic fungi isolated from ten different lichen species. Data were randomized 100 times for plotting the graph. Dotted line represents 95% confidence interval.



▶ **Fig. 2** Number of unique species of endolichenic fungi isolated from ten different lichen thalli. Data were randomized 100 times for plotting the graph. The curve represents a polynomial trend line.



▶ **Fig. 3** Neighbour-joining tree from ITS sequences showing the relationship between of the present study Xylaria isolates and other closely related Xylaria species retrieved from the GenBank. Bootstrap values (1000 replicates) are shown on the branches. Bar = 2 nucleotide substitutions per 100 nucleotides.

tal conditions. These observations of host jumps and shifts in life styles, as well as the current finding that Xylariales are common endolichenic fungi underscore the high ecological amplitude of this group of fungi, possibly determined by overlapping ecological niches [32] as well as the ability of endolichenic Xylariales to inhibit co-occurring endolichenic fungi [10]. It is established that host jumping among plant pathogens is an evolutionary strategy to diversify and to ensure survival [33]; it is conceivable that the symp-

▶ **Table 3** Identity of different *Xylaria* species and their GenBank accession numbers (Refer to ▶ **Table 1** for host code).

Xylaria Code	Fungus	Host Code	VINSTROM Culture Collection	GenBank Accession Numbers
LX1	*Xylaria primorskensis*	RA	2010	MN310382
LX2	*Hypoxylon investiens*	RA	2011	MN310383
LX3	*Daldinia eschscholtzii*	DI	2012	MN310384
LX4	*Nemania bipapillata*	BA	2013	MN310385
LX5	*Hypoxylon investiens*	HY	2014	MN310386
LX7	*Xylaria apiculata*	AR	2015	MN310387

tomless endophytic fungi also adopt this strategy for diversification.

Endolichenic fungi as an ecological group possibly determined the evolution of endophytic life style of fungi colonizing higher plants [34]; although similar to endophytic fungi in being non-pathogenic associates and in their mode of dispersal, it is not known if endolichenic fungi contribute to stress tolerance (abiotic and biotic) of their lichen hosts like the endophytes. Since very few of the 18 500 lichen species [35] have been investigated for their endolichenic fungi, basic investigations focusing on their species diversity and interaction with their lichen hosts and with other partners of the lichen microbiome would help realize better technological potential of endolichenic fungi. Furthermore, the use of different isolation media and sterilization procedures along with molecular methods to account for nonculturable and slow-growing fungi would capture the real species diversity of endolichenic fungi in the study area [36].

Materials and Methods

Study area

The Western Ghats mountain range is a mega-biodiversity hotspot [37] and runs along the western coast of India for over a distance of 1600 km and is over 100 km wide. Ten different species of lichens growing on tree barks were collected from 3 locations in southern Western Ghats (▶ **Table 1**) and screened for their endolichenic assemblage.

Isolation of endolichenic fungi and incubation procedures

Fresh and disease-free lichen thalli were collected and washed in water for removing soil particles and debris. Each lichen species was cut into 100 segments (approximately 0.5 cm^2) and surface sterilized as follows [5]. The segments were dipped in 70 % ethanol for 5 s and then in 4 % NaOCl for 90 s and finally washed in sterile water for 10 s. The segments were plated on chloramphenicol (150 $mg \cdot L^{-1}$) containing potato dextrose agar (PDA) medium (20 mL) in 9 cm diameter Petri dishes (10 segments/dish). The Petri dishes were incubated in a light chamber and exposed to 12 h light:12 h dark cycle for 28 days at 26 ± 2 °C to induce sporulation. The imprinting method of Schulz et al. [38] was used to confirm the efficacy of surface sterilization in killing the epithalline microbes. The fungi growing out of the tissue segments then were isolated, purified, and identified. Those fungi that did not sporulate in culture and that were distinct from one another were treated as sterile fungi; they were given numbers and considered to represent different species.

Genomic DNA isolation and PCR

The fungal isolates obtained were grown on PDA layered with sterile cellophane sheets and incubated at 28 °C under dark conditions for 7–14 days. Each fungal mycelium thus obtained was scraped from cellophane sheets and crushed using pestle and mortar in liquid nitrogen. Genomic DNA was isolated as described by Allen et al. [39]. The DNA isolated was used as a template for amplification of ITS region of rRNA gene spanning ITS1–5.8S-ITS2 using universal fungal primers, ITS-1 (5′-TCCGTAGGT-GAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) [40]. The PCR mixture consisted of 50 ng DNA template, 1 × PCR buffer, 0.2 mM dNTPs mix, 0.5 µm each primer, 1 mM $MgCl_2$, and 2.5 units of Taq DNA polymerase (Invitrogen). PCR amplification was performed in Veriti 96-well Thermal Cycler (Applied Biosystems, USA) and the PCR program consisted of an initial denaturation at 98 °C for 5 min followed by 35 cycles of denaturation at 98 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min followed by final extension at 72 °C for 7 min.

Sequencing and sequence analysis

The PCR products obtained corresponding to different fungal strains were run on agarose gel electrophoresis to determine their size. The PCR products were then purified using GeneJET PCR purification kit (Thermo Fisher Scientific) according to manufacturer's instructions. Purified PCR products were then sequenced using Sanger's sequencing (1st BASE DNA sequencing division, Apical Scientific Sequencing, Malaysia). Nearest possible sequences similar to the ITS sequences of endolichenic fungal strains obtained using Sanger's sequencing were searched in GenBank of NCBI database using BLASTn program to find the closely related taxa. Sequences thus obtained were aligned using software MAFFT v 6.240 program, and alignments were manually corrected. Phylogenetic tree was constructed using MEGA5 software [41]. Estimation of evolutionary distance was performed using Kimura 2 parameter model [42]. The phylogenetic reconstruction was done using the neighbor-joining algorithm using 1000 bootstrap replicates.

Statistical analysis

The CF% of each endolichenic fungal species was calculated as the number of segments colonized for every 100 bits sampled. The species diversity (Fisher's α) of the endolichenic fungi was calculated by the method of Fisher et al. [43]. A species accumulation curve and unique species curve for the fungi were plotted using the software EstimateS after 100 randomizations [44].

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Conflict of Interest

The authors declare that they have no conflict of interest.

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