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Endolichenic fungi in lichens of Champawat district, Uttarakhand, northern India

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Abstract Eleven lichen species belonging to five families (two fruticose and nine foliose) growing on the bark of *Quercus leucotrichophora* trees from four forests of Champawat district, Uttarakhand state, northern India were studied for their endolichenic fungal assemblage. We obtained 942 isolates of endolichenic fungi belonging to 33 morpho-species from these lichens. *Xylaria* spp. dominated the assemblage of 6 of the 11 lichens; it was the most common endolichenic fungus and constituted 31.4% of the total isolates. In co-culture experiments where 33 *Xylaria* isolates and select endolichenic fungi were cultured together, 39% of the combinations showed mutual stasis, while in 36% of the challenges, *Xylaria* overgrew the other endolichenic fungus co-cultured with it; *Xylaria* was inhibited only in 6% of the challenges. Of the 62 isolates tested by bioautogram, 45 and 37 isolates exhibited antialgal and antifungal activity, respectively, while 30 isolates showed both antialgal and antifungal activities. While production of such metabolites by the endolichenic fungi could aid them in overcoming competition within the lichen thallus, it is possible that they also alter the ecological fitness of lichens.

Keywords Endophytes · Lichen symbionts · Lichen · *Xylaria*

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Introduction

Lichens are generally thought to be a result of a symbiotic association of a filamentous fungus with a photobiont; however, a recent discovery shows that a yeast forms the third partner of a lichen thallus (Spribille et al. 2016). Thus, lichens constitute ‘miniature ecosystems,’ as they support diverse bacteria and fungi, both cultivable and non-cultivable, which are probably vested with some functional roles (Selbmann et al. 2013). Apart from the obligate fungal partners which go to make up a lichen thallus, a lichen supports three other ecological groups of fungi: (i) the lichenicolous fungi, which sporulate on the lichen thallus (Lawrey and Diederich 2003), (ii) incidental fungi on thallus surfaces and (iii) the endolichenic fungi, which also reside inside the lichen thallus in close association with the photobionts and are relatively rare in the mycobiont-dominated cortex and medulla (Miadlikowska et al. 2004; Arnold et al. 2009).

The endolichenic fungi are a group of endosymbionts and are known from every lichen species tested to date at sites ranging from the Arctic to the tropics (Suryanarayanan et al. 2005; Li et al. 2007; Arnold et al. 2009; Kannangara et al. 2009; Tripathi et al. 2014a, b, c; Tripathi and Joshi 2015). Like endophytic fungi of plants, their colonisation is asymptomatic, many are horizontally transmitted and are hyperdiverse (Arnold et al. 2009; Kannangara et al. 2009; U’Ren et al. 2012). Endolichenic fungi synthesise novel metabolites, including terpenoids, naphthalene derivatives, heptaketides, diphenyl ethers, polyketides, alkaloids and chromone derivatives, exhibiting interesting bioactivities (Paranagama et al. 2007; He et al. 2012; Yang et al. 2012; Zhang et al. 2012; Wang et al. 2012, 2013; Yuan et al. 2013, 2016; Li et al. 2015; Zhao et al. 2014).

Studies on endolichenic fungi in India were initiated by Suryanarayanan et al. (2005), who investigated some corticolous lichens and reported that there was little overlap

between their endolichenic fungal assemblages and the endophytes in the leaves of trees which supported these lichens. Recently, Tripathi et al. (2014a, b, c) and Tripathi and Joshi (2015) studied the diversity of endolichenic fungi of some Kumaun Himalayan macrolichens collected from Almora district of Uttarakhand. U'ren et al. (2010, 2012) showed that the incidence, diversity and composition of endolichenic fungal assemblages depend on the interaction of climatic patterns, geographic separation, lichen type and lineage. Considering the vast lichen diversity in the Himalayan biogeographic zone of India and the virtual lack of studies on endolichenic fungi from India, we decided to study some of these lichens for their endolichenic fungal assemblages. Furthermore, we hypothesised that the endolichenic fungi would necessarily elaborate some antialgal and antifungal metabolites to survive the competition within the holobiont lichen and, hence, screened them for such metabolites.

Materials and methods

Lichens

Eleven lichen species belonging to five families (two fruticose and nine foliose types) growing on the bark of *Quercus leucotrichophora*, trees were collected from Kranteshwar Temple forest, Chinnapani forest, Siddh Temple forest and Hingla Devi Temple forest of Champawat district, Uttarakhand and screened for endolichenic fungal diversity (Table 1).

Isolation and incubation procedures

Fresh and disease-free lichen thalli were collected and washed in running tap water. Each lichen species was then cut into 100 segments (approximately 0.5 cm²) and surface sterilised

following the protocol of Suryanarayanan et al. (2005). The segments were dipped in 70% ethanol for 5 s, followed by immersion in 4% NaOCl for 90 s and then washed in sterile water for 10 s. After surface sterilisation, the segments were plated on antibiotic-amended (chloramphenicol 150 mg L⁻¹) potato dextrose agar (PDA) medium in 9 cm diameter Petri dishes (10 segments/dish). The Petri dishes were sealed using Parafilm™ and incubated in a light chamber with a 12 h light:12 h dark cycle for 28 days at 26 ± 2 °C (Bills and Polishook 1992; Suryanarayanan 1992). The light chamber had a bank of three 4-foot Philips Daylight fluorescent lamps.

The efficacy of surface sterilisation was tested by the method of Schulz et al. (1998). The surface-sterilised segments were gently pressed onto an agar growth medium and removed, incubated and observed for the growth of fungi. An absence of fungal growth following this procedure indicated that the surface sterilisation was adequate and resulted in the isolation of endosymbionts only.

The tissue segments were observed periodically and the fungi growing out of them were scored, isolated and cultured on PDA slants. They were identified using standard taxonomic keys (Ellis 1971, 1976; Subramanian 1971; Barnett and Hunter 1972; von Arx 1974). A few sterile isolates which could not be assigned to any taxonomic group were given codes based on culture characteristics (Bills and Polishook 1994; Suryanarayanan et al. 1998). Sterile forms with different culture characteristics were assumed to represent different taxonomic species (Bills and Polishook 1994).

Interaction between endolichenic fungi

Since *Xylaria* spp. dominated the assemblage of fungal isolates from 6 of the 11 lichens and also occurred most commonly, we tested the interaction of one endolichenic

Table 1 List of lichen species sampled for endolichenic fungi

Lichens and their codes	Coordinates	Metres above sea level	Family
<i>Canoparmelia texana</i> (CT)	N 29°20.966' E079°59.305'	2071	Parmeliaceae
<i>Heterodermia diademata</i> (HD)	N 29°19.275' E080°04.025'	1870	Physciaceae
<i>Heterodermia podocarpa</i> (HP)	N 29°19.275' E080°04.025'	1870	Physciaceae
<i>Leptogium askotense</i> (LA)	N 29°19.275' E080°04.025'	1870	Collembataceae
<i>Lobaria kurokawae</i> (LK)	N 29°18.414' E080°07.844'	2193	Lobariaceae
<i>Parmotrema hababianum</i> (PH)	N 29°18.414' E080°07.844'	2194	Parmeliaceae
<i>Parmotrema tinctorum</i> (PT)	N 29°18.781' E080°07.132'	1816	Parmeliaceae
<i>Phaeophyscia hispidula</i> (PhH)	N 29°22.303' E080°04.020'	2018	Physciaceae
<i>Punctelia rudenta</i> (PR)	N 29°18.492' E080°07.303'	1859	Parmeliaceae
<i>Ramalina conduplicans</i> (RC)	N 29°18.781' E080°07.132'	1816	Ramalinaceae
<i>Usnea</i> sp. (U)	N 29°18.781' E080°07.132'	1816	Parmeliaceae

Samples were collected by M. Tripathi between 19 and 22 March, 2015 and have been deposited in the herbarium collection of Kumaun University

Table 2 Colonisation frequency (CF%), number of isolates (bold) and species, and diversity index of endolichenic fungi

Fungi	CT	HD	HP	LA	LK	PH	PhH	PR	PT	RC	U
<i>Acremonium</i> sp.					1			1	2		
<i>Alternaria</i> sp.						1					
<i>Aspergillus</i> sp.							1				
<i>Aureobasidium pullulans</i>	4	4	19	23	23	7	13	16	17	11	22
<i>Bispora</i> sp.	2										
<i>Botrytis</i> sp.									1	2	
<i>Chaetomium</i> sp. 1		13		3		1	8	6			
<i>Chaetomium</i> sp. 2	1		3		8				1		
<i>Chaetomium</i> sp. 3	8		2		14				2		
<i>Chaetomium</i> sp. 4	1				3						
<i>Cladosporium</i> sp.					1						
<i>Geotrichum</i> sp.	1	4			6				6		
<i>Humicola</i> sp.		5	2		1	1		1	2		3
Hyphomycetous form 1								1	1		
Hyphomycetous form 2	1										
Hyphomycetous form 3	1										
<i>Monilia</i> sp.		1			1						
<i>Nigrospora oryzae</i>				1							
<i>Penicillium</i> sp.				1	1					2	1
<i>Periconia</i> sp.			5	16	16	5	22	13	7		
<i>Sordaria</i> sp.		1	3	47	7		4	6	5		5
<i>Spegazzinia lobulata</i>								1			
<i>Sporormiella minima</i>	6	12	8		16	3	10	14	7	8	5
Sterile form 1	2	2	2	4	1	1	2	5	1	1	5
Sterile form 2	1	2	1		2	3		2	1		5
Sterile form 3	1	2	1		1	1					
<i>Talaromyces</i> sp.				2	1						
<i>Torulomyces</i> sp.	1		2	12	6	2	15	12			
<i>Trichoderma</i> sp.							1	2			
<i>Xylaria</i> sp. 1	2	8		13	28	18		15	20	10	34
<i>Xylaria</i> sp. 2			17				20	15			
<i>Xylaria</i> sp. 3			28				2	1			
<i>Xylaria</i> sp. 4		5	11	3	8	13	14	10	8	1	7
No. of isolates	32	59	104	125	145	56	112	105	81	36	87
No. of species	14	12	14	11	20	12	12	15	15	8	9
Fisher's α	9.49	4.55	4.36	2.91	6.29	4.69	3.41	4.79	5.42	3.19	2.52

Xylaria isolated from each lichen with three other endolichenic fungi isolated from the same thallus by co-culturing them on nutritive agar medium. A mycelial plug (5 mm diameter) cut from the growing margin of a colony of an endolichenic *Xylaria* sp. and a similar plug obtained from a non-xylariaceous endolichenic fungus isolated from the same lichen thallus were placed on opposite edges in the Petri dish containing PDA medium. The interaction between the endolichenic fungus and *Xylaria* after 5–7 days was assessed by the method of Schoeman et al. (1996), which grades the interaction as described in Table 3.

Bioautogram for the visualisation of antialgal and antifungal metabolites

An endolichenic fungus was grown in potato dextrose liquid medium supplemented with XAD™ Amberlite™ resin (Rohm and Haas, Philadelphia, PA, USA) (400 mg/flask) at 26 °C for 21 days. The XAD™ was collected and washed in methanol and acetone (1:1).

The concentrated secretome (50 μ L) was spotted on a silica gel coated aluminium thin-layer chromatography (TLC) sheet (layer thickness 0.1 mm, Merck, Germany) (Schulz et al. 1995). After air-drying, the TLC plate was sprayed with a suspension of *Chlorella fusca* cells (for antialgal activity) or conidia of *Cladosporium cucumerinum* (for antifungal activity). The chromatogram was incubated at 26 °C in light (for *C. fusca*) or in darkness (for *C. cucumerinum*) for 3–5 days. As a control, a methanol and acetone (1:1) extract of uninoculated medium was run and tested. The appearance of an inhibition zone on the chromatogram indicated the presence of bio-active secondary metabolite(s).

Statistical analysis

The colonisation frequency (CF%) of endolichenic fungi was calculated by the method of Hata and Futai (1995). The species diversity index (Fisher's α) was calculated using the method of Fisher et al. (1943).

Results

We obtained 942 isolates of endolichenic fungi belonging to 33 morphospecies from 11 lichen species (Table 2). The maximum number of isolates (145) and species (20) of these fungi were isolated from the thallus of *Lobaria kurokawae*; *Canoparmelia texana* yielded the lowest number of isolates (32) and *Ramalina conduplicans* supported the minimum number of endolichenic fungal species (8) (Table 2). *Aureobasidium pullulans*, *Xylaria* spp. and a sterile fungus SF1 were isolated from all the lichen species screened. *Aureobasidium pullulans* constituted 16.8% of the total number of endolichenic isolates and dominated the endolichenic fungal assemblage in *Punctelia rufecta*. *Xylaria* spp. dominated the assemblage of 6 of the 11 lichens (*Heterodermia podocarpa*, *L. kurokawae*, *Parmotrema hababianum*, *Parmotrema tinctorum*, *Phaeophyscia hispidula* and *Usnea* sp.) and the genus was the most common one, constituting 31.4% of the total isolates. *Chaetomium* sp. 1 and sp. 3 and *Sordaria* sp. had the highest CF% in *Heterodermia diademata*, *C. texana* and *Leptogium askotense*, respectively (Table 2). The species diversity index of endolichenic fungi was the highest for *C. texana* (9.49) and lowest for *Usnea* sp. (Table 2). A species accumulation curve did not reach an asymptote as the lichen species sampled increased (Fig. 1).

In co-culture experiments involving 33 *Xylaria*/endolichenic fungal challenges, 39% of the combinations showed mutual stasis, while in 36% of the challenges, *Xylaria* overgrew the other endolichenic fungus co-cultured along with it; *Xylaria* was inhibited only in 6% of the challenges (Table 3). The screening of the secretomes of the other endolichenic fungi for their antialgal and antifungal activities revealed that, of the 62 isolates tested by bioautogram, secretomes of 45 and 37 isolates exhibited antialgal and antifungal activity, respectively, while 30 isolates showed both

antialgal and antifungal activities (Table 4). Of the 12 *Xylaria* isolates screened, ten showed antialgal activity and eight were positive for antifungal activity.

Discussion

When compared to endophytic fungi, there are very few studies on endolichenic fungi, which reside inside the lichen thalli as endosymbionts and constitute an important ecological group distinct from the obligate mycobiont or the lichenicolous fungi associated with lichens. U'Ren et al. (2010) showed that endolichenic fungi are neither casual residents of lichen thalli nor merely saprotrophic fungi trapped by lichens. In this study, we isolated species of *Acremonium*, *Chaetomium* and *Xylaria* as endolichenic fungi, genera which are also common endophytes of plants. Although some endophytic species could also occur as endolichenic forms (Tripathi et al. 2014a, b, c), these two ecological groups of endosymbionts are quite distinct from each other (U'Ren et al. 2010). Earlier, Suryanarayanan et al. (2005) reported that there is little overlap between the fungal endophytic assemblages of the leaves of trees and the endolichenic fungal assemblages found in the lichens which grew on these trees, attesting the uniqueness of endolichenic fungi. In the present study, no distinct pattern of the occurrence of endolichenic fungi with regard to the taxonomy of the lichens or their location could be discerned, probably reflecting the generalist nature of the endolichenic fungi. This is similar to the results obtained by Chagnon et al. (2016), although our sample size is minimal when compared to this study. A species accumulation curve did not reach an asymptote, indicating that endolichenic fungi are hyperdiverse in these forests and increased sampling is needed for estimating their diversity.

Generally, the fungal endosymbiont assemblages of lichens are dominated by members of Pezizomycetes and those of vascular plants by the more recently evolved fungal classes,

Fig. 1 Species accumulation curve for endolichenic fungi isolated from 11 different lichens. The dotted lines represent 95% confidence interval limits for species observed. Data were randomised 100 times for plotting the graph

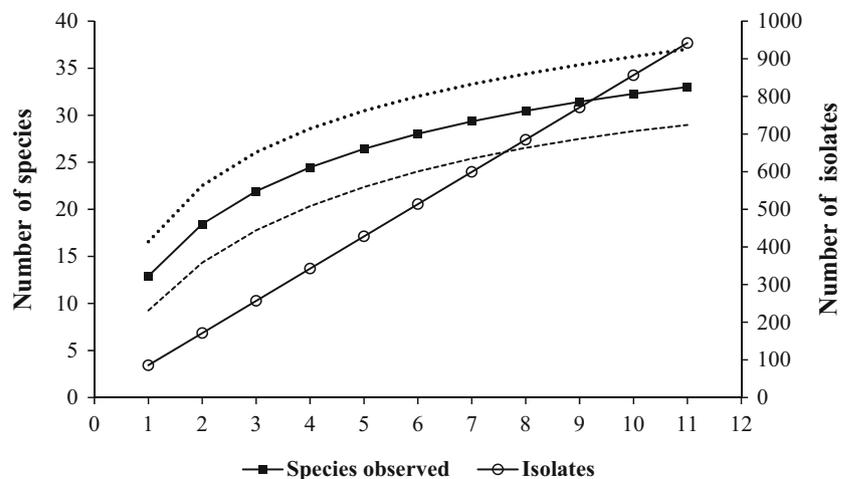


Table 3 Interaction between endolichenic *Xylaria* and endolichenic fungus isolated from the same lichen thallus

<i>Xylaria</i> sp. from	Fungus 1	Fungus 2	Fungus 3
CT	<i>Sporormiella minima</i>	a↑ <i>Chaetomium</i> sp. 4	a↑ <i>Humicola</i> sp. b
HD	<i>Chaetomium</i> sp. 1	b <i>S. minima</i>	c <i>Humicola</i> sp. b
HP	<i>Chaetomium</i> sp. 2	b <i>Humicola</i> sp.	a↑ <i>Chaetomium</i> sp. 3 b
LA	<i>Periconia</i> sp.	d Sterile form 2	b <i>Penicillium</i> sp. a↑
LK	<i>Talaromyces</i> sp.	c <i>Geotrichum</i> sp.	b <i>Chaetomium</i> sp. 3 a↓
PH	<i>Periconia</i> sp.	a↑ <i>S. minima</i>	b <i>Alternaria</i> sp. b
PhH	<i>Nigrospora oryzae</i>	a↑ <i>Chaetomium</i> sp. 2	a↑ <i>Trichoderma</i> sp. a↓
PR	<i>Spegazzinia lobulata</i>	b <i>Periconia</i> sp.	a↑ Sterile form 2 b
PT	<i>S. minima</i>	a↑ <i>Sordaria</i> sp.	a↑ <i>Chaetomium</i> sp. 2 a↑
RC	<i>S. minima</i>	a↑ Sterile form 2	N <i>Penicillium</i> sp. b
U	<i>Xylaria</i> sp. 4	c <i>Chaetomium</i> sp. 4	b <i>S. minima</i> c

a: Growth of one fungus over the colony of the other (↑ *Xylaria* growing over other endolichenic fungus, ↓ endolichenic fungus growing over *Xylaria*)

b: Mutual stasis of fungal growth

c: Appearance of a clear zone between colonies where growth was mutually inhibited

d: Coloured contact zone indicative of a reaction

N: No clear result observed

such as Sordariomycetes (U'Ren et al. 2010). However, our current results corroborate our earlier study (Suryanarayanan et al. 2005), where we observed that species of *Xylaria* (Sordariomycetes) were present in all the lichens and were dominant in many lichen species. Xylariaceous fungi are one of the generalist and dominant endophytic fungi of higher plants (Govinda Rajulu et al. 2013); however, we did not encounter other fungal genera, such as *Colletotrichum*, *Phyllosticta* and *Phomopsis*, which are more commonly isolated as endophytes from higher plants. Although *Xylaria* endophytes, due to host jumping, infect taxonomically unrelated plants (Davis et al. 2003; Tejesvi et al. 2009), molecular studies would confirm if the same species of *Xylaria* exist as endophytes and endolichenic fungi in plants and lichens, respectively.

Endophytes (Schulz et al. 2002; Suryanarayanan et al. 2010) and particularly xylariaceous endophytes (Liu et al. 2008; Oliveira et al. 2011; Stadler 2011; Schueffler and Anke 2011) are prolific producers of novel metabolites exhibiting different bioactivities, such as antifungal and antibacterial activities. It is envisaged that the production of such metabolites *in planta* by the endophytes could result in a

Table 4 Antialgal and antifungal activity of lichen fungi by bioautogram method

Lichen code	Fungi	Antialgal	Antifungal
PT	<i>Acremonium</i> sp.	–	–
HD	<i>Aureobasidium pullulans</i>	–	–
HP	<i>Aureobasidium pullulans</i>	+	–
LK	<i>Aureobasidium pullulans</i>	+	–
PH	<i>Aureobasidium pullulans</i>	+	–
PhH	<i>Aureobasidium pullulans</i>	–	–
CT	<i>Bispora</i> sp.	–	–
PT	<i>Botrytis</i> sp.	+	+
HD	<i>Chaetomium</i> sp. 1	+	+
PhH	<i>Chaetomium</i> sp. 1	+	+
HP	<i>Chaetomium</i> sp. 2	–	–
LK	<i>Chaetomium</i> sp. 2	–	+
LK	<i>Chaetomium</i> sp. 3	–	+
LK	<i>Chaetomium</i> sp. 4	–	+
LK	<i>Geotrichum</i> sp.	–	–
HP	<i>Humicola</i> sp.	+	+
PT	Hyphomycetous form 1	–	–
RC	Hyphomycetous form 1	+	+
HD	<i>Monilia</i> sp.	+	+
PhH	<i>Nigrospora oryzae</i>	+	+
LK	<i>Penicillium</i> sp.	+	–
LA	<i>Periconia</i> sp.	+	+
PH	<i>Periconia</i> sp.	+	+
PR	<i>Periconia</i> sp.	–	+
PT	<i>Periconia</i> sp.	+	+
HP	<i>Sordaria</i> sp.	+	–
LA	<i>Sordaria</i> sp.	+	–
LK	<i>Sordaria</i> sp.	+	+
PhH	<i>Sordaria</i> sp.	–	–
PR	<i>Sordaria</i> sp.	+	–
PT	<i>Sordaria</i> sp.	+	–
U	<i>Sordaria</i> sp.	+	+
PR	<i>Spegazzinia lobulata</i>	+	+
HD	<i>Sporormiella minima</i>	+	–
LK	<i>Sporormiella minima</i>	+	–
PT	<i>Sporormiella minima</i>	+	+
RC	<i>Sporormiella minima</i>	+	+
U	<i>Sporormiella minima</i>	–	+
HD	Sterile form 1	+	+
LK	Sterile form 1	+	+
PR	Sterile form 1	+	+
PT	Sterile form 1	+	–
U	Sterile form 1	+	+
LK	Sterile form 2	+	–
PR	Sterile form 2	+	+
PT	Sterile form 2	–	–
PH	Sterile form 3	+	+
LK	<i>Talaromyces</i> sp.	–	+
PH	<i>Trichoderma</i> sp.	+	+
PhH	<i>Trichoderma</i> sp.	+	+
HD	<i>Xylaria</i> sp. 1	+	–
LA	<i>Xylaria</i> sp. 1	+	+
LK	<i>Xylaria</i> sp. 1	+	+
PH	<i>Xylaria</i> sp. 1	+	+
PhH	<i>Xylaria</i> sp. 3	+	+
HP	<i>Xylaria</i> sp. 3	+	+
PR	<i>Xylaria</i> sp. 3	–	–
HP	<i>Xylaria</i> sp. 4	+	+
LA	<i>Xylaria</i> sp. 4	+	+
PH	<i>Xylaria</i> sp. 4	+	–
PhH	<i>Xylaria</i> sp. 4	+	–
PR	<i>Xylaria</i> sp. 4	–	+
	Positive strains	45	37

+: Inhibition zone observed

–: No activity

condition similar to soil antibiosis, culminating in the elimination of competitors and in the survival of select fungal species as endophytes in a given plant tissue (Suryanarayanan 2013). *In vitro* studies by Schulz et al. (2015) support this hypothesis; they opine that the inhibitory secondary metabolites produced by endophytes may play a role in maintaining the endophyte community in plant tissue through multiple balanced antagonisms.

We posit that a similar balanced antagonism mechanism is involved in maintaining an endolichenic fungal community, as we observed that many of the endolichenic fungi, including *Xylaria* spp., produce antifungal and antialgal metabolites. This is demonstrated by the competitive ability of endolichenic *Xylaria* spp. (Stadler 2011), which overgrew their co-endolichenic fungal species in the co-culture experiments in our study. Lichens produce antifungal metabolites active against a wide variety of fungi (Halama and Van Haluwin 2004). Some obligate fungal pathogens tolerate and even degrade such compounds, enabling them to infect lichen thalli (Lawrey et al. 1999). It is possible that the endolichenic fungi also have evolved mechanisms to tolerate/degrade such antifungal compounds of lichens to establish infection and to compete with co-occurring endolichenic organisms. It is also conceivable that the production of such metabolites alters the chemical profile of the host lichen, such that its susceptibility to fungal parasites and animal grazers is altered (Asplund et al. 2016).

Earlier studies, (Suryanarayanan et al. 2005; U'Ren et al. 2010; Tripathi et al. 2014a, b, c; Tripathi and Joshi 2015), along with this study show that the inner region of a lichen thallus serves as an ecological niche for some fungal species, and this niche needs to be explored for fungal species, metabolite diversity and functional role in the lichen symbiosis.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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