

Original Research Article

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## Association and Variation of Endophytic Fungi among Some Macrolichens in Central Western Ghats, Southern India

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### ABSTRACT

#### Keywords

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Association of endolichenic fungi with eleven lichen species belongs to five families distributed in Bhadra Wildlife Sanctuary, South India were investigated during May, 2013 to January, 2014. A total of 30 endolichenic taxa from 486 segments were isolated. The colonization rate ranged from 61.5% to 90.6 % among lichen host species. *Chaetomium globosum* was occurred in eight, among 11 species of lichen, it was the dominant species of endolichenic fungi in thalli of lichens screened. The *Ramalina pacifica* and *Parmotrema tinctorum* showed maximum colonization by the endolichenic fungi. Out of 30 species of fungi 24 were sporulated and they were identified up to genus/species level. *Chaetomium globosum*, *Fusarium* spp., *Xylaria* sp. were isolated from thalli of 8, 5, 6 lichen species respectively. The Shannon index was 0.18-0.26 and Simpson species abundance was 0.004-0.015. Results of this study revealed that fungi were associated within lichen thalli other than symbiotic fungi and their compositions were varied with lichen species.

### Introduction

Microbial endophytes have been found in the leaves, bark and xylem of higher plants (Schulz *et al.*, 1993; Arnold and Lutzoni, 2007; Murali *et al.*, 2007; Krishnamurthy *et al.*, 2008) including grasses (White and Cole, 1985; Krishnamurthy and Hemalatha, 2003) also in lower forms like algae

(Hawksworth, 1988) and mosses (Dreyfuss, 1986). Endolichenic fungi also reported as parasymbionts in lichens (Petrini *et al.*, 1990). A number of fungi grow and produce fruiting bodies on the surface of lichen thalli with identical fructifications. The hyphae of these fungi penetrate the lichen thalli and

form intimate union with cells of the algal symbiont (Ahmadjian, 1967). There are about 300 genera and 1000 species of obligatory lichenicolous fungi known which form parasitic, saprophytic, parasymbiotic and obligatory symbiotic mode of biological relationships on lichen species. Endolichenic fungi are fungi that live within their host plants causing any noticeable symptoms of disease (Hawksworth and Hill, 1984). These are distinct from lichen mycobionts or any other previously recognized fungal associates of lichens. They represent the same major lineages of Ascomycota as do fungal endophytes, largely parallel the high diversity of endophytes from the arctic to the tropics (Arnold *et al.*, 2009). For about 32 taxa of endophytic fungi with 14 ascomycetes, 16 hypomycetes, each of coelomycete and yeast were reported (Wen-Chao *et al.*, 2007). *Scopyleriopsis* was distributed in all 6 species of macrolichens. About 70 cultures of black fungi belong to Chaetothriomycetidae and Dothideomycetidae from 14 different species of lichen thalli, they showed that some lineages of black fungi can opportunistically grow on lichens (Harutyunyan *et al.*, 2008). A recovery of endolichenic fungi from tissue segments of lichen thalli showed that these fungi not found on surface sterilized lichen thalli (Arnold *et al.*, 2009). The colonization of fungi was considered environmental contaminants (Frankland, 1974). Fungi belong to soil and litter symbiosis or pathogenic also isolated from fruticose lichens which were symbionts or pathogens of higher plants and lichens inhabiting fungi (Petrini *et al.*, 1990). Although there are several studies on endophytic microorganisms of higher plants, very few studies were carried out on the isolation of endolichenic fungi from the lichen thallus in India (Suryanarayanan *et al.*, 2005). Therefore, we have examined some lichen species for isolation of endolichenic fungi

from the internal tissues of macrolichens which commonly occur in forests of Bhadra Wildlife Sanctuary, Southern India.

## **Materials and Methods**

### **Sampling of Lichens**

A total of eleven lichen specimens were collected from different habitats of Bhadra wildlife sanctuary, (75°15' and 75°50' E to 13°25' and 13°50' N) Karnataka, Southern India (Table 1). Apparently healthy looking lichen thalli were brought to the laboratory, in paper bags stored in refrigerator and processed within 24 hours. The lichen specimens were identified by using the manual (Awasthi, 1988). These lichen samples are stored in the herbarium of Department of Applied Botany, Kuvempu University, Karnataka, India.

### **Isolation of Endolichenic Fungi from Lichen Thalli**

Surface sterilization of lichen thalli was performed using the modified method (Guo *et al.*, 2003). Healthy looking thalli of each lichen specimens were cleaned in tap water to remove dirt and then thoroughly washed under running tap water. These lichen thalli were surface sterilized by consecutive immersion for one minute in 75% ethanol, 3 minute in 2% Sodium hypochlorite and 3 seconds in 75 % ethanol and washed several times with sterile distilled water. The thalli were surface dried with sterile paper and cut into 1cm sized lichen segments and then plated on nutrient medium consisted of 2% malt extract, 0.4 yeast extract and 2% agar to which 50mg/l chlortetracycline used as antibacterial antibiotic (Petrini *et al.*, 1990). The plates were incubated at 25°C± 2 for 10-15 days under Philips fluorescent tube light for 12/12 h day and night alternate light conditions. They were examined regularly

and colonies developed were transferred immediately to new petri dishes with PDA medium for identification. Sterilized tissue segments were pressed down on to the surface of PDA medium to check back the efficacy of surface sterilization procedure and to confirm endolichenic isolations only from internal tissues of lichen segments. Absence of growth of any fungi on the medium confirmed that surface sterilization procedure was effective in removing the surface fungi as reported in a previous study (Schulz *et al.*, 1993). Morphology of subcultures on PDA were examined periodically and identified when isolates were sporulated (Domsch and Gams, 1972; Nagamani *et al.*, 2006). The fungal cultures that failed sporulation even after two months of incubation were named as mycelia sterilia (Schulz *et al.*, 1993). Pattern of fungal association with lichens on nutrient rich and poor media was also done in order to understand influence of different medium on fungal growth. The surface sterilized lichen segments were incubated on 3 such media *viz.*, PDA (Dextrose-20g, Agar- 15g, potato 200g/ 1000 ml pH-5.6±0.2), MYA (Malt extract-10g, Yeast extract- 4g, Glucose-4g and Agar 5g pH-7.5±0.2) and CZA (Sucrose-30g, NaNO<sub>3</sub>-3g, K<sub>2</sub>HPO<sub>4</sub>-1g, KCl-0.5g, MgSO<sub>4</sub>.7H<sub>2</sub>O-0.01g, Agar-15g, distilled water 1000ml, pH 6.2±0.3). Incubation conditions maintained were as mentioned earlier.

### Data Analysis

The Relative Frequency (RF %) of occurrence of endolichenic fungi was calculated as the total number of taxon divided by the total number of taxa obtained from lichen thalli incubated (Arnold *et al.*, 2009). The Shannon-Weiner diversity index (H') was calculated according to the formula  $H' = -\sum p_i \times \ln p_i$  to assess species richness and Simpson diversity index (D') was calculated

according to the formula  $D' = 1/\sum (p_i)^2$ , where  $k$  is the total number of fungal species and  $p_i$  is the proportion of individual that species  $i$  contributes to the total (Pielou, 1975). Simpson index was calculated to understand species diversity of endolichenic fungi occurred in lichen thalli.

### Results and Discussion

A total of 30 different endolichenic fungal species were recovered from 11 species of foliose and fruticose lichens (Table 2). Twenty four species of fungi have produced fructifications in culture and have been identified to genus or species level and rest were categorized as sterile forms (Table 3). The high colonization rate (61.5-90.6%) of endolichenic fungi was observed among eleven lichen species in this study. Maximum colonization rate was observed in *Parmotrema cristiferum* (90.6%) and *P. reticulatum* (90.3%) and moderate in *Cladonia fruticulosa* (61.5%). Among the 30 isolates, 24 species were identified in that most of them were belonging to ascomycota and all other isolates categorized as *Mycelia sterilia* (6). A moderate to high per cent of endolichenic fungi occurred in this study from eleven species of macrolichens. After a thorough surface sterilization protocol, and incubation, all the fungi expressed from the cut ends of the thalli. This is confirmed as sterilized thalli were pressed on to the medium to check back the possibility of contaminants on the surface of thallus to know effectiveness of surface sterilization.

The fungal species associated with thalli of lichen species such as *Chaetomium globosum* (8 species of lichens), *Cladosporium cladosporioides* (5), *Colletotrichum dematium* (5), *Fusarium heterosporum* (4), *Phoma* sp. (3) and *Xylaria* (6). *C. globosum* species showed high rate of relative frequency in the lichen

host where as *Parmotrema* sp. as evidenced by maximum number of isolates of endolichenic fungi (12.82 %). Endolichenic fungi grew on different nutrient rich and poor media showed that there is no much variation in the appearance of colonies as well as density of sporulation or occurrence of some different strains. However MYA influenced expression of more number of colonies (77) than PDA (69) and CZA (65). Per cent occurrence varied from 0.5 to 50 on all three media (Table 4). Shannon diversity index showed higher value in *Parmotrema reticulatum* (0.26) and lower in *Parmotrema tinctorum* (0.18). Simpson's index also showed species abundance higher in *Parmotrema reticulatum* (Figure1). It is a fact that lichen species such as *P. reticulatum* and *P. tinctorum* harbored about 90% of endolichenic fungi. This data was based on number of segments encountered with these fungi against total number of segments incubated.

The terms lichenicolous or endolichenic fungi are different because of their occurrence or association on surface or within tissues of internal parts. Some researchers claim the term endolichenic because they isolated from internal tissues (Petrini *et al.*, 1990; Suryanarayanan *et al.*, 2005) and others isolated endolichenic fungal strains that lived along in the lichen thallus *Pseudocephalaria* sp. *Usnea* sp. and *Parmotrema* sp. (Kannangara *et al.*, 2009). The associations of fungi other than their obligate mycobiont also existed. Experimental evidence demonstrates that these endolichenic fungi distinctly different from lichenicolous fungi (Suryanarayanan *et al.*, 2005; Girlanda *et al.*, 1997; U'Ren *et al.*, 2009). Lichenicolous fungi are lichen forming fungi which are one of the important components of symbiosis (Hawksworth and Hill, 1984). Some of the fungi isolated in this study from lichen host may also represent lichenicolous fungi. The

Shannon index is employed to evaluate and compare the diversity of fungal community between different lichen species. One of the researcher, he reported that the Shannon-Weiner diversity index was 0.48-1.85 (Wen-Chao *et al.*, 2007). In the present study, the results had showed the higher diversity between the lichen species.

In a study, isolation of about 506 species of endolichenic fungal species from 17 lichen species was carried out and it was illustrated that some fungi were surface colonizers and rests were true endophytes (Petrini *et al.*, 1990). The colonization rate of endolichenic fungi from lichens such as *Parmelia taractica* (90%) and *Peltigera praetextata* (70%) was reported (Girlanda *et al.*, 1997). Several authors have suggested that lichenicolous or parasymbiotic fungi are especially diverse in tropical forests (Frohlich and Hyde, 1999; Arnold *et al.*, 2000). One of the researcher, he recovered 24 endolichenic fungi from five corticolous lichens such as *Dirinaria picta*, *Heterodermia diademata*, *Physcia aipolia*, *Pyxine cocoes* and *Rocella montagnei* and he concluded that these fungi are not contaminants (Suryanarayanan *et al.*, 2005). Endolichenic fungi have an intimate association and live in the thalli of lichens that are analogous to plant endophytic fungi (Arnold and Lutzoni, 2007). In a recent study on lichen thalli through surface sterilization, the endolichenic fungi occurred within tissues rather than upon lichen thalli. Scanning electron microscopy results of six lichen thalli made no evidence of extra spores within the lichen thalli. The anatomical studies confirmed that endolichenic fungi associated with green algal photobiont layer (Arnold *et al.*, 2009).

The investigation of this study revealed that association of *C. globosum*, *C. cladosporioides*, *C. dematium*, *Fusarium* spp., *Phoma* sp. and *Xylaria* sp., are other

than lichenicolous fungi with lichen thalli collected from forests. These endolichenic fungi may assist in lichen thallus formation and growth also act as antagonistically against insect herbivores. Some endophytic microorganisms were characterized known to produce secondary metabolites are of

valuable pharmaceutical substance which are having biotechnological and industrial interest (Culberson, 1969; Strobel and Long, 1996). Results of several studies demonstrated that lichen associated fungi are a good resource for new bioactive natural products.

**Table.1** Lichens studied for isolation of endolichenic fungi

Lichens	Family	Form	Habitat
<i>Cladonia fruticulosa</i> Kremp.	Cladoniaceae	Fruticose	Terricolous
<i>Heterodermia dendritica</i> (Pers.) Poelt.	Physciaceae	Foliose	Corticolous
<i>H. incana</i> (Stirton) D.D.Awasthi	Physciaceae	Foliose	Corticolous
<i>Parmotrema reticulatum</i> (Taylor) Choisy	Parmeliaceae	Foliose	Saxicolous
<i>P. tinctorum</i> (Despr.ex Nyl.) Hale	Parmeliaceae	Foliose	Corticolous
<i>P. cristiferum</i> (Taylor) Hale	Parmeliaceae	Foliose	Corticolous
<i>Ramalina arabum</i> (Ach.) Meyen & Flot.	Ramalinaceae	Fruticose	Corticolous
<i>R. pacifica</i> Asahina	Ramalinaceae	Fruticose	Corticolous
<i>Teloschistes flavicans</i> (Sw.) Norm.	Teloschistaceae	Fruticose	Corticolous
<i>Usnea stigmatoides</i> G. Awasthi	Parmeliaceae	Fruticose	Corticolous
<i>U. galbinifera</i> Asahina	Parmeliaceae	Fruticose	Corticolous

**Table.2** Colonization of endolichenic fungi in eleven species of lichens

Lichen species	No. of tissue segments	No. of isolates	Colonization rate (%)
<i>Cladonia fruticulosa</i>	13	8	61.5
<i>Heterodermia dendritica</i>	20	16	80.0
<i>H. incana</i>	26	21	80.8
<i>Parmotrema reticulatum</i>	31	28	90.3
<i>P. tinctorum</i>	120	90	75.0
<i>P. cristiferum</i>	64	58	90.6
<i>Ramalina arabum</i>	46	41	89.1
<i>R. pacifica</i>	98	83	84.7
<i>Teloschistes flavicans</i>	11	8	72.7
<i>Usnea stigmatoides</i>	29	20	68.9
<i>U. galbinifera</i>	36	28	77.7
Total	486	409	

**Table.3** RF (%) of endolichenic fungi isolated from lichen species

Sl. No.	Fungal species	Cl	Hd	Hi	Pr	Pt	Pc	Ra	Rp	Tf	Us	Ug
1	<i>Alternaria alternata</i> (Fr.) Keissler	2.6			12	10.6						
2	<i>Aspergillus niger</i> Van. Tieghem						9.3	7.6		3.6		14.5
3	<i>Chaetomium globosum</i> Kunze & Schm	4.0	1.8		8	6.4	3.2		12.2	2.2	6	
4	<i>C. elatum</i> Kuze				10			7.6				5.8
5	<i>Chaetomium</i> sp.			5	30	16	14	11.5	9.8			
6	<i>Colletotrichum dematium</i> (Fr.) Grove		4.3			3.5		4.6	8.1		6	
7	<i>Cladosporium cladosporioides</i> (Fres.) de vries.			6.6				11.5		11	18	9.6
8	<i>Chalara</i> sp.				9.2			7.6			18	
9	<i>Curvularia lunata</i> (Wakker) Boedijn		5.2						12.2		12	
10	<i>Fusarium heterosporum</i> Nees ex Fr.	2.6					5.6		7.5			5.8
11	<i>F. lateritium</i> Nees ex Fries		13	10				11.5		11	12	
12	<i>F. solani</i> (Mart.) Sacc.				20		14		4.4		9	
13	<i>Hypoxylon</i> sp.	8			10							
14	<i>Phoma</i> sp.			10		9.1				2.2		
15	<i>P. leveillei</i> Boerema & Bollen	8	6.5		20			9.2				
16	<i>Phialophora verrucosa</i> Medlar.			10					6.1			2.4
17	<i>Penicillium chrysogenum</i> Thom.		8.6		15						9	
18	<i>Pestalotiopsis hypodermia</i> (Niessl) Steyaert				5.2	3.2				1.2		
19	<i>Rhizoctonia solani</i> Kuhn.			2.8			9.3				7.2	
20	<i>Trichoderma viride</i> Per. ex Fries	1.6				7.1		15.3			7.2	
21	<i>Thielavia</i> sp.		6.5	6.6								
22	<i>T.basicola</i> (Berk. & Br.) Zopf.				15			6.5				5.8
23	<i>Xylaria</i> sp.	2.0			12		4.6	7.6		5.5		2.0
24	<i>X. vaporaria</i> Berk.				17.1		7	15.3	8.1			
25	Mycelium Sterile- 1		8.6	4		6.4		15.3				3.6
26	Mycelium Sterile -2	2.6			17.1				7			
27	Mycelium Sterile -3		8.6							3.6	5.1	
28	Mycelium Sterile -4		5.2	5						1.8	2.7	
29	Mycelium Sterile -5	2.6		6.6					6.5			
30	Mycelium Sterile -6		13		24		7		4.4	2.2		7.2

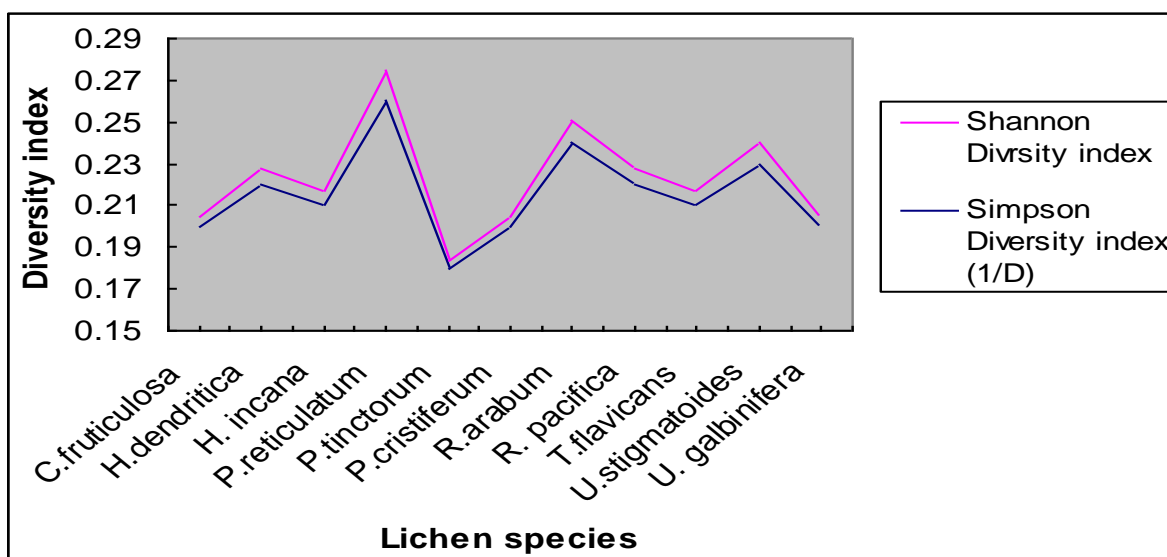
(Cl- *C.fruticulosa*, Hd- *H.dendritica*, Hi- *H.incana*, Pr- *P.reticulatum*, Pt- *P.tinctorum*, Pc- *P.cristiferum*, Ra- *R.arabum*, Rp- *R.pacifica*, Tf- *T.flavicans*, Us- *U.stigmatoides*, Ug- *U.galbinifera*)

**Table.4** Influence of different nutrient media on pattern of endolichenic fungal isolation

a) Fungal species	MYA (%)	PDA (%)	CZA (%)
<i>Alternaria alternata</i>	2.0	1.0	1.0
<i>Aspergillus niger</i>	2.5	2.5	1.0
<i>Chaetomium globosum</i>	0.5	1.0	-
<i>Colletotrichum dematium</i>	-	1.0	-
<i>Cladosporium cladosporioides</i>	12.5	1.0	1.0
<i>Curvularia lunata</i>	-	1.0	-
<i>Fusarium heterosporum</i>	0.5	-	0.5
<i>Hypoxylon</i> sp.	-	-	0.25
<i>Penicillium chrysogenum</i>	50.0	50.0	37.5
<i>Penicillium</i> sp.	-	0.5	0.75
<i>Pestalotiopsis hypodermia</i>	30.0	30.0	25.0
<i>Trichoderma viride</i>	42.5	35.0	32.5
<i>Xylaria</i> sp.	-	2.0	-
Mycelium Sterile- 1	0.50	-	-
Mycelium Sterile -2	0.75	0.25	1.0
Mycelium Sterile -3	1.0	-	-
Mycelium Sterile -4	0.75	0.5	2.0
Mycelium Sterile -5	-	1.0	2.0
Mycelium Sterile -6	-	2.0	-
<b>b) Total isolates</b>	<b>77</b>	<b>69</b>	<b>65</b>

MYA- Malt Yeast extract Agar, PDA- Potato Dextrose Agar, CZA- Czapek Dox Agar

**Fig.1** Shannon and Simpson diversity index studied in eleven species of lichens



The endolichenic fungi like *Pestalotiopsis* sp. known to produce antifungal metabolites

such as ambuic acid and torreyanic acid from lichen *Clavarioides* sp. (Ding *et al.*,

2009). Endolichenic fungal strains isolated from lichen thalli *Pseudocephalaria* sp., *Parmotrema* sp. and *Usnea* sp. showed antifungal activities against common plant pathogenic fungi. They were also capable of utilizing the substrates by producing specific enzymes (Pielou, 1975). An unidentified lichenicolous strain LL-RB 0668 yielded lichenicolous A and B which are Bisnaphthopyrones (He *et al.*, 2005). An endolichenic fungus *Neurospora terricola* produce allenyl phenyl ethers and alkynyl phenyl ethers (Zhang *et al.*, 2009). Antifungal activity of a lichenicolous fungus against *Collectrichum acutatum* of hot pepper also established (Wei *et al.*, 2008). One of the researcher, he isolated obligate lichenicolous fungi associated with *Parmelia taractica* and *Peltigera praetextata* in Italy and he found that, the clear host specificity of some mycobionts with lichens (Girlanda *et al.*, 1997).

In conclusion, the association of endolichenic fungi was reported in many parts of the world from wide variety of host plants from cryptogams to higher plants. Association of endolichenic fungi is of quite interesting type of host-fungal interactions. As the lichens composed of mutual relationship, these non-obligatory endolichenic fungal presence in lichen thalli attracts further study regarding lichen-fungi inter-relationship and their significance in symbiosis, parasitism or they use lichen thalli as refugia.

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