Endophytic fungi associated with lichens in Baihua mountain of Beijing, China

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Endophytic fungi of seven lichen species belonging to five families were investigated in Baihua mountain of Beijing. Various colonization rates (56.3-100%) of endophytic fungi were found among the lichens. A total of 32 taxa from 488 thallus segments were obtained, with 14 ascomycetes, 16 hyphomycetes, one ceolomycetes and one yeast. *Scopulariopsis* sp. was distributed in six out of seven lichens and was the dominant species in five lichens. *Geniculosporium serpens* was the dominant species in the lichen, *Dermatocarpon miniatum*. *Sporormiella* species only occurred in *Xanthoria mandschurica*. *Thielavia* fungi as endophytes were first reported. Different Shannon-Weiner diversity indices (0.48-1.85) for endophytic fungi were found in the seven lichens. Low endophyte composition similarities (*Cs*: 0-0.55) were obtained among the seven lichens. Results of this study suggest that abundant endophytic fungi are distributed within lichen thalli and endophyte composition is different among the lichens.

Key words: colonization rate, diversity, endophyte, lichen

Introduction

Lichens, as the pioneer organisms, are widely distributed and dominant in the early developmental stages of a variety of ecosystems undergoing primary or secondary succession (Burbanck and Platt, 1964; Foster, 1985; Sheridan, 1991). Lichens are also an important component of present-day ecosystems, particularly in Antarctic and Arctic regions, rock, arid and coastal regions (Awasthi, 2000) and are common in the tropics (Devarajan and Suryanarayanan, 2006). Some lichens are economically important as traditional medicine and food in some regions (Wei *et al*., 1982; Awasthi, 2000). They act as bio-indicators of air pollution (Vokou *et al*., 1999;

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McCune, 2000; Garty *et al*., 2002; Loppi and Frati, 2006; Loppi *et al*., 2006; Rossbach and Lambrecht, 2006).

Lichen thalli provide a mostly unexplored ecological niche for a wide variety of microorganisms including fungi (Gerson and Seaward, 1977). Fungi other than the obligate symbiotic mycobionts are frequently encountered during direct observation of lichen thalli, and their competitive presence often makes it difficult to isolate the mycobiont (Crittenden *et al*., 1995; Honegger, 1996), although successful culture of certain mycobionts has occasionally been reported in previous studies (Gams, 1971; Hawksworth and Jones, 1981; Crittenden *et al*., 1995). However, few studies have been conducted regarding culturable asymptomatic fungi existing within the lichen thalli (Petrini *et al*., 1990; Girlanda *et al*., 1997; Suryanarayanan *et al*., 2005).

In order to understand the status of lichen endophytes, a total of seven lichen species belonging to five families were collected from Baihua mountain of Beijing. The basic aim was to investigate whether there is high diversity of endophytic fungi within lichen thalli, and there is difference of endophyte composition between lichens in temperate region of Beijing, China.

Materials and methods

Sampling site and procedure

This study was conducted at Baihua mountain mixed woodland, 114 km west of Beijing, China (39º52'N, 115º37'E). The lichens were located at an altitude of *ca*. 1500 m above sea level. The mean annual temperature is 6-7ºC, and the mean annual precipitation is about 720 mm. Seven lichen species belonging to five families were collected in November 2005 (Table 1). These samples were immediately placed in plastic bags, labelled, and taken to the laboratory. Samples were stored at 4°C and processed within 7 days of collection.

Isolation and culture of endophytic fungi

The sampling regime was designed with the intention of isolating as many endophyte species as possible from lichens. Surface sterilization was performed using the modified method of Guo *et al*. (2003). The healthy-looking thalli of each lichen species were cleaned in tap water to remove excess earth and litter, and then thoroughly washed under running tap water. The lichen thalli were surface sterilized by consecutive immersion for 1 minute in 75% ethanol, 3 minutes in 2% sodium hypochlorite and 30 seconds in 75%

ethanol. The thalli were surface dried with sterile paper towels and were cut into segments of *ca*. 0.5 cm^2 (0.7 × 0.7 cm). Sets of four segments were then evenly placed in each 90 mm Petri dish containing malt extract agar (MEA, 2%) supplemented with Rose Bengal (30 mg L^{-1}) to slow down fungal growth, Streptomycin sulphate (50 mg L^{-1}) was added to suppress bacterial growth. Petri dishes were sealed, incubated for 2 months at 25°C, and examined periodically. When colonies developed, they were transferred to new Petri dishes with MEA. Subcultures were then incubated on potato dextrose agar (PDA, 2%). In addition, sterile branch segments of *Sophora japonica* L. were included to promote sporulation. Isolates were incubated at 25ºC, with cool white fluorescent light for a light regime of 12:12 hours light:dark to induce sporulation in culture.

Identification of endophytic fungi

Subcultures on PDA were examined periodically and identified based on morphological characteristics when isolates sporulated. Slides were mounted in lactophenol and sealed with nail varnish. These slides were labelled and colonies of sporulating species were dried and stored in the Herbarium Mycologicum Academiae Sinicae (HMAS) in Beijing. The remaining cultures that failed to sporulate after 2-months of incubation were named as mycelia sterilia. The living culture was deposited in the China General Microbiological Culture Collection Center (CGMCC) in Beijing, China.

Data analysis

Colonization rate (CR) was calculated as the total number of lichen segments infected by fungi divided by the total number segments incubated (Petrini *et al*., 1982). Colonization rate was expressed as percentages, as widely used in the past endophyte studies, therefore, colonization rates can be used for comparative purposes. Relative frequency (RF) was calculated as the total number of a taxon divided by the total number of taxa obtained from lichen thalli incubated. Relative frequency was expressed as percentages. The Shannon-Weiner biodiversity index (H') was calculated according to the formula $H' = -\sum_{i=1}^{k} pi \times \ln pi$, where k is the total number of fungal species, and pi is the proportion of individuals that species i contributes to the total (Pielou, 1975).

To evaluate the degree of community similarity of endophytic fungi between two lichen species, Sorenson's similarity coefficient (C_S) was

employed and calculated according to the formula: $C_S = 2j/(a+b)$, where j is the number of endophytic fungal species coexisted in both lichens, a is the total number of endophytic fungal species in one lichen, and b is the total number of endophytic fungal species in the other lichen.

Results

Colonization rate

High ranges of the colonization rates (56.3-100%) of endophytic fungi were obtained in the seven lichen species in the present study (Table 1). The highest colonization rate (100%) of endophytic fungi was found in *Cladonia coniocraea* and the lowest colonization rate (56.3%) of endophytic fungi was obtained in *Dermatocarpon miniatum*.

Endophytic fungus composition

A total of 310 isolates of endophytic fungi were recovered from 488 thallus segments of the seven lichens (Table 1). Of these, 190 isolates produced spores and were identified to 32 taxa, the other 120 isolates remained mycelia sterilia after sporulation under various conditions. Among the 32 taxa, eight were identified to species, and 20 were placed at genus level. Fourteen taxa were ascomycetes, 16 hyphomycetes, a ceolomycetes and a yeast (Table 2).

Various numbers of endophytic fungi were isolated from different lichens, i.e. three taxa from *Cladonia coniocraea*, ten from *Dermatocarpon miniatum*, 11 from *Melanelia sorediata*, five from *Parmelia* sp., 14 from *Punctelia borreri*, two from *Ramalina sinensis* and six from *Xanthoria mandschurica* (Table 2).

Of the 32 taxa, nine were recorded from more than one lichen, while the other 21 occurred in one lichen. *Scopulariopsis* sp. was distributed in six out of seven lichens, except for *Ramalina sinensis*, furthermore, it was the dominant species in five lichens, i.e. *Cladonia coniocraea*, *Melanelia sorediata*, *Parmelia* sp., *Punctelia borreri* and *Xanthoria mandschurica*. *Geniculosporium serpens*, as the dominant species, was only isolated from *Dermatocarpon miniatum*. *Sporormiella* species only occurred in *Xanthoria mandschurica* (Table 2).

The Shannon-Weiner diversity indices of endophytic fungi in seven lichens from high to low was *Melanelia sorediata* (1.85) > *Punctelia borreri* (1.76) > *Xanthoria mandschurica* (1.51) > *Dermatocarpon miniatum* (1.43) > *Parmelia* sp. (1.18) > *Cladonia coniocraea* (0.97) > *Ramalina sinensis* (0.48).

Table 1. Lichen materials and colonization rates (%) of endophytic fungi isolated from lichens in Baihua mountain of Beijing.

Lichen	No. of thalli	No. of tissue segments	No. of isolates	Colonization rate (%)	Habitat
Cladoniaceae					
Cladonia coniocraea (Flörke) Sprengel	2	16	15	100	Terricolous
Parmeliaceae					
Melanelia sorediata (Ach.) Goward & Ahti	6	48	53	85.4	Saxicolous, corticolous
Parmelia sp.	5	40	36	72.5	Saxicolous
Punctelia borreri (Smith) Krog	20	160	83	88.8	Saxicolous, corticolous
Ramalinaceae					
Ramalina sinensis Jatta	5	40	22	67.5	Saxicolous
Teloschistaceae					
Xanthoria mandschurica (Zahlbr.) Asahina	3	24	17	83.3	Saxicolous, corticolous
Verrucariaceae					
Dermatocarpon miniatum (L.) W. Mann	20	160	84	56.3	Saxicolous
Total	61	488	310		

Low similarities (*Cs*: 0-0.55) of endophytic fungus composition were found among the seven lichens (Table 3). The highest similarity (0.55) was between *Parmelia* sp. and *Xanthoria mandschurica*, and the lowest similarity (0) was between *Parmelia* sp. and *Ramalina sinensis* and between *Punctelia borreri* and *Ramalina sinensis*.

Discussion

Due to the spongy texture of lichen tissues, chemical surface sterilization was not applicable in the study of endophytic fungi (Petrini *et al*., 1990). Lichen thalli were only washed using sterile tap water, thus fungi isolated from lichens were not strict endophytes, but probably included some epiphytes (Petrini *et al*., 1990). Girlanda *et al*. (1997) investigated endophytic fungi associated with two lichens *Parmelia taractica* Krempelh. and *Peltigera praetextata* (Flk. ex Sommerf.) Zopf and treated lichen thalli using sterile water and H_2O_2, respectively. Suryanarayanan *et al*. (2005) compared the

Table 2. Relative frequency (%) of endophytic fungi isolated from lichens in Baihua mountain of Beijing.

Taxon	*Cladonia coniocraea*	*Dermatocarpon miniatum*	*Melanelia sorediata*	*Parmelia* sp.	*Punctelia borreri*	*Ramalina sinensis*	*Xanthoria mandschurica*
Acremonium sp.1		2.4					
Acremonium sp.2			1.9				
Chaetomium elatum Kunze			1.9				
C. globosum Kunze	13.3		9.4		3.6		
Chaetomium sp.					3.6		
Conidial stage of *Hypoxylon fuscum*					3.6		
Coniochaeta sp.		4.8		5.6			5.9
Geniculosporium serpens Chesters & Greenh.		9.5					
Nodulisporium hyalosporum S.C. Agarwal & J.K. Misra					1.2		
N. sylviforme Deighton			3.8				
Nodulisporium sp.		2.4	3.8		3.6		
*Phialophora bubaki*i (Laxa) Schol-Schwarz		6	3.8			4.5	
Phialophora sp.		1.2					
Phoma sp.					1.2		
Scopulariopsis sp.	53.3	4.8	37.7	50	36.1		52.9
Sporormiella minima (Auersw.) S.I. Ahmed & Cain							5.9
S. muskokensis (Cain) S.I. Ahmed & Cain							11.8
Sporormiella sp.1							5.9
Sporormiella sp.2							5.9
Sporothrix sp.		3.6		2.8		9.1	
Thielavia sp.1				2.8	1.2		
Thielavia sp.2					1.2		
Thielavia sp.3					1.2		
Thielavia sp.4					1.2		

Table 2 continued. Relative frequency (%) of endophytic fungi isolated from lichens in Baihua mountain of Beijing.

Taxon	*Cladonia coniocraea*	*Dermatocarpon miniatum*	*Melanelia sorediata*	*Parmelia* sp.	*Punctelia borreri*	*Ramalina sinensis*	*Xanthoria mandschurica*
Thielavia sp.5					1.2		
Thielavia sp.6			1.9				
Trichoderma sp.	33.3		1.9		2.4		
Trichobotrys sp.		1.2	1.9				
Yeast			26.4				
Hyphomycetes sp. 1		1.2					
Hyphomycetes sp. 2					2.4		
Hyphomycetes sp. 3				2.8			
Mycelia sterilia	0	63.1	5.7	36.1	36.1	86.4	11.8

Table 3. Sorenson's similarity coefficients of endophytic fungi isolated from seven lichens in Baihua mountain of Beijing.

	Cladonia coniocraea	*Dermatocarpon miniatum*	*Melanelia sorediata*	*Parmelia* sp.	*Punctelia borreri*	*Ramalina sinensis*	*Xanthoria mandschurica*
Cladonia coniocraea		0.15	0.43	0.25	0.35	0	0.22
Dermatocarpon miniatum			0.38	0.4	0.17	0.33	0.38
Melanelia sorediata				0.13	0.32	0.15	0.24
Parmelia sp.					0.21	0	0.55
Punctelia borreri						0	0.2
Ramalina sinensis							0.25
Xanthoria mandschurica							

effect of four methods and the results indicated that SSP4 (70% ethanol for 5 seconds, 4% NaOCl for 90 seconds and sterile water for 10 seconds) was the best in the isolation of lichen endophytes. In the present study, we also compared the effect of sterile water and chemical disinfectant in the removal of epiphytes, and similar result was obtained as Suryanarayanan *et al*. (2005) (data not shown). Thus the surface sterilization used in this study was effective in the isolation of lichen endophytes.

High colonization rates (56.3-100%) of endophytic fungi occurred in the seven lichens in this study. Girlanda *et al*. (1997) reported similar colonization

rates of endophytic fungi from lichens *Parmelia taractica* (90%) and *Peltigera praetextata* (70%). The colonization rate was comparable with other endophyte studies, e.g. 55-98% in leaves of *Rhizophora apiculata* Blume (Kumaresan and Suryanarayanan, 2002), 51-64% in leaves of *Cordemoya integrifolia* (Willd.) Pax in the Conservation Management Area plot at Maccabhé, Black River Gorges National Park, Mauritius (Toofanee and Dulymamode, 2002), 89% in leaves of *Tripterygium wilfordii* Hook. F. (Kumar and Hyde, 2004) and 20.9-45.8% of *Pinus tabulaeformis* Carr. in China (unpublished data).

A broad spectrum of endophytic fungi was isolated from the seven lichens in this study. Similar results were reported in previous endophyte studies. Petrini *et al*. (1990) investigated the fungi associated with 17 lichens in a forested area in the southern Black Forest and obtained 506 taxa including epiphytic and endophytic fungi. Fourteen and eight endophyte fungi were isolated from lichens *Parmelia taractica* and *Peltigera praetextata*, respectively (Girlanda *et al*., 1997). Suryanarayanan *et al*. (2005) isolated 24 endophytic fungi from five corticolous lichen species in Guindy National Park, Chennai, India.

Species of *Acremonium*, *Chaetomium*, *Fusarium*, *Nodulisporium*, *Phialophora*, *Phoma*, *Scopulariopsis Sporormiella* and *Trichoderma*, as endophytes, were isolated from other lichens (Petrini *et al*., 1990; Girlanda *et al*., 1997; Suryanarayanan *et al*., 2005) and also recovered from other hosts (Kumaresan and Suryanarayanan, 2001, 2002; Photita *et al*., 2001; Cannon and Simmons, 2002; Toofanee and Dulymamode, 2002; Santos *et al*., 2003; Kumar and Hyde, 2004). *Coniochaeta*, *Geniculosporium*, *Sporormiella* and *Sporothrix* species as endophytic fungi were reported also in woody plants (Petrini and Fisher, 1988; Sieber, 1989; Bills and Polishook, 1992; Sieber *et al*., 1999; Barengo *et al*., 2000; Cannon and Simmons, 2002; Wang and Guo, 2004; Sun *et al*., 2006). *Thielavia* species as endophytes were first reported in this study.

The Shannon-Weiner diversity index was employed to evaluate and compare the diversity of fungus community between different lichens, and various diversity indices (0.48-1.85) were obtained in the present study. Comparable Shannon-Weiner diversity indices of endophytic fungi were reported in lichens *Parmelia taractica* (1.6) and *Peltigera praetextata* (3.1) (Girlanda *et al*., 1997).

Sorenson's similarity coefficient was employed to test the similarity of endophytic fungus composition between lichens, and low Sorenson's similarity coefficients (0-0.55) were found between lichens in the present study. These data indicated that the distribution of the isolated fungal taxa on the seven lichens was different and some endophytes showed certain level of host

specificity or recurrence (Girlanda *et al*., 1997), while an opposing result was reported by Petrini *et al*. (1990).

The significance of the endophytic fungi within lichens remains unknown, however, abundant endophytes are harboured within lichen thalli, thus these fungi appear to play an important ecological role. These endophytes may assist lichen formation and growth and act antagonistically against insect herbivores, because some endophytes can produce bioactive substance as reported in the other studies (Webber, 1981; Carroll, 1986). These endophytes may exist in a living state and become pioneer decomposers after lichen senescence, as has been shown for other endophytes. Some endophytic fungi have also been shown to produce extracellular enzymes, which are indicative of their potential role in litter degradation (Kumaresan and Suryanarayanan, 2002).

Traditional techniques, e.g. culture-dependent methodologies, have usually been applied in the study of endophytes (Kumar and Hyde, 2004; Suryanarayanan and Thennarasan, 2004; Gonthier *et al*., 2006), however, molecular techniques have been recently employed in their detection and identification (Guo *et al*., 2000, 2001, 2003; Arnold *et al*., 2001; Photita *et al*., 2005; Promputtha *et al*., 2005; Wang *et al*., 2005). Sterile endophytes, which cannot be identified based on the morphological characteristics due to non-sporulation, were placed into different fungal groups using ITS sequence analyses (Guo *et al*., 2000, 2003; Lacap *et al*., 2003; Wang *et al*., 2005; Promputtha *et al*., 2005). Endophytic fungi have also been identified to different taxonomic levels by means of the sequence analyses of the ITS regions directly amplified from palm tissues (Guo *et al*., 2001). The endophyte diversity on leaves of *Magnolia liliifera* (L.) Baillon was assessed based on DGGE coupled with sequence analysis of 18S rRNA gene (Duong *et al*., 2006). These methods could also be applied in the study of lichen endophytes.

This primary study reports on the endophytic fungi associated with some lichen species in China. The life cycles and functioning of the lichen endophytes and the relationships between endophytic fungi and lichens is unknown. Further research is needed employing traditional and molecular techniques to address this relationships.

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