Fungal Diversity (2011) 47:85–95
DOI 10.1007/s13225-010-0086-5

Community composition of endophytic fungi in *Acer truncatum* and their role in decomposition

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Received: 1 December 2010 / Accepted: 13 December 2010 / Published online: 1 January 2011

Abstract The mycota and decomposing potential of endophytic fungi associated with *Acer truncatum*, a common tree in northern China, were investigated. The colonization rate of endophytic fungi was significantly higher in twigs (77%) than in leaves (11%). However, there was no significant difference in the colonization rates of endophytic fungi between lamina (9%) and midrib (14%) tissues. A total of 58 endophytic taxa were recovered using two isolation methods and these were identified based on morphology and ITS sequence data. High numbers of leaf endophytes were obtained in the method to determine decomposition of leaves by the natural endophyte community (35 taxa) as compared to disk fragment methodology (9 taxa). The weight loss in *A. truncatum* leaves decomposed by endophyte communities increased with incubation time; the weight loss was significantly higher at 20 weeks than at 3 and 8 weeks. Both common and rare endophytic taxa produced extracellular enzymes in vitro and showed different leaf decay abilities. Our results indicated that the composition and diversity of endophytic fungi obtained differed using two isolation methods. This study suggests that endophytic fungi play an important role in recycling of nutrients in natural ecosystems.

Keywords Endophyte · Isolation method · Leaf decomposition · Extracellular enzymes

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Introduction

Endophytic fungi have been associated with plants for over 400 million years (Krings et al. 2007) and have been isolated from many different plants such as mosses (Jakucs et al. 2003; Davey and Currah 2006), ferns (Swatzell et al. 1996), grasses (Muller and Krauss 2005; Su et al. 2010), shrub plants (Barrow et al. 2004; Olsrud et al. 2007), deciduous and coniferous trees (Guo et al. 2008; Albrectsen et al. 2010; Mohamed et al. 2010), and lichens (Suryanarayanan et al. 2005; Li et al. 2007). Because of the high diversity of endophytic fungi (Arnold and Lutzoni 2007; Hyde and Soytong 2007, 2008), their ability to produce various bioactive chemicals (Aly et al. 2010; Xu et al. 2010) and promotion of host growth and resistance (Cheplick et al. 1989; Ting et al. 2008; Saikkonen et al. 2010), the study of endophyte has become one of the hottest research focuses in mycology.

Endophytic fungi are thought to play important roles in recycling of nutrients in natural ecosystems (Whitoon and Hyde, unpublished). Endophytic fungi were shown to be capable of decomposing Norway spruce needles in vitro and in situ by Müller et al. (2001) and Korkama-Rajala et al. (2008). Promputtha et al. (2010) found that some endophytic species were morphologically and phylogenetically similar to saprobes and that strains isolated from both lifestyles produced the same leaf degrading enzymes. Molecular phylogeny studies also suggest that some endophytic species change their ecological strategies and adopt a saprobic lifestyle following senescence (Sokolski et al. 2006; Promputtha et al. 2007; Korkama-Rajala et al. 2008). However, further studies are needed to understand the role of endophytes in litter decomposition in natural ecosystems (Promputtha et al. 2010; Whitoon and Hyde, unpublished).

Acer truncatum (Aceraceae) is broadly distributed and is the main woody tree species of northern Chinese forests. They contribute significantly to the economy and ecology of forests in China. However, we know little concerning their endophytic fungi and the role of these fungi in litter decomposition. The aims of the present study were to (1) investigate the composition and diversity of fungal endophytes associated with *A. truncatum* in Dongling mountain mixed woodland using two isolation methods, (2) identify the endophytes using morphology and ITS sequence data, (3) investigate the production of extracellular degrading enzymes, and (4) show that endophytes are involved in the decomposition of *A. truncatum* leaf litter.

Materials and methods

Sampling site

The study was carried out in the Dongling mountain mixed woodland of the Forest Ecosystem Research Station of the Chinese Academy of Sciences, located 117 km west of Beijing, China (39º58′N, 115º26′E). The warm temperate sampling site is located at an altitude of 1211 m a.s.l. The mean annual temperature is 4.8 C, and the mean annual precipitation is 611.9 mm.

Isolation of endophytes using disk fragment methodology

In late August 2005, 20 mature individuals of *A. truncatum* were randomly selected and one branch with leaves was collected from each individual. Samples were immediately placed in plastic bags, labeled, and returned to the laboratory. Samples were stored at 10°C and processed within 2 days of collection.

Each selected branch was divided into three age-classes (i.e. 1-, 2-, and 3-yr-old branches) and cut into 5 mm long fragments. Leaves were removed from branches and 5 mm diam discs were cut from lamina and midrib tissues. Eight fragments from each branch age class and eight leaf discs from both tissue types were randomly selected for isolation. In total, 800 fragments (8 fragments×5 tissues×1 branch×20 trees) were used in this study.

Endophytic fungi were isolated using traditional disk methodology used in most previous endophyte studies (Guo et al. 2000). The plant fragments were surface sterilized by consecutive immersion for 1 min in 75% ethanol, 3 min in 3.25% sodium hypochlorite and 30 s in 75% ethanol. The fragments were surface dried with sterile paper towels. Sets of four fragments were then evenly placed in a 90 mm Petri dish containing malt extract agar (MEA, 2%). Benzylpenicillin sodium (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 PDA slants for morphological observation and preservation when colonies developed. Sterilised branch fragments of host plants were also included to promote sporulation as outlined by Guo et al. (1998). Subcultures on PDA were examined periodically and sporulating isolates identified based on their morphological characteristics. The nonsporulating cultures were designated as *mycelia sterilia*, which were divided into different "morphotypes" according to cultural characteristics such as colony colour, texture, and growth rate on MEA (Guo et al. 2000). All morphological observations and measurements were made in sterile water, with a mean of 30 measurements for each character under a compound microscope (Nikon E600, Japan). The living cultures are deposited in China General Microbiological Culture Collection Center (CGMCC) in Beijing, China.

Decomposition of leaves by single endophyte strains

The capability of single endophyte strains to decompose leaves was determined using a modified method of Osono and Takeda (2006) (Fig. 1a). Fresh leaves of *A. truncatum* were oven-dried at 40 C for 1 week and then sterilized by exposure to formaldehyde gas at room temperature for 1 week, followed by oven heating for 2 days at 40 C to dispel residual formaldehyde gas. Sterilized leaves were placed individually on the surface of Petri dishes (9 cm diam) containing 20 ml agar (2%). Twenty-one selected endophyte strains comprising 12 rare and 9 common taxa were investigated. Inocula for each assessment were cut from the margin of colonies on PDA plates, and placed on the leaf. The plates were sealed and incubated at 25 C. Twelve plates were tested for each strain and three replicate leaves were retrieved to determine the mass loss at 4, 8, 12 and 16 weeks of incubation. The retrieved leaves were oven-dried at 40 C for 1 week and weighed. Twelve surface sterilized fresh leaves were also oven-dried at 40 C for 1 week and weighed to determine original mass. Weight loss was determined as a percentage for each replicate. Plates inoculated with blank agar squares were used as control for comparison.

Decomposition of surface sterilized leaves by the natural endophyte community

The flask incubation method (Fig. 1b) was used to test the decay abilities of naturally occurring endophyte communities within leaves. Leaf decomposition by endophyte communities was tested using a modified method of Müller et al. (2001). The microcosms were prepared by filling a 250 ml Erlenmeyer flask with 150 mL vermiculite–humus

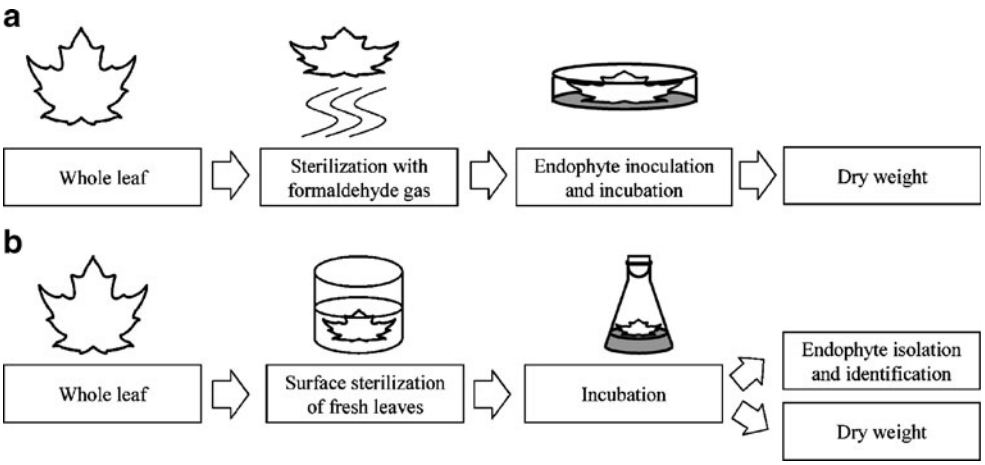

Fig. 1 Schematic outline of the procedure used for isolation of endophytic taxa from *Acer truncatum* and their leaf decomposition ability. **a** Procedure used to determine leaf decomposition by single endophytic strains. **b** Procedure used to isolate endophyte community from the whole surface sterilized leaves and determine leaf decomposition abilities

layer mixture (1:1) moistened to full capacity. The flasks were closed with cotton plugs and foil and autoclaved four times at 121 C for 30 min. Fresh healthy leaves of *A. truncatum* were collected from the sampling site in 2005 and surface sterilized as described above. One surface sterilized leaf was placed into each flask and incubated at 25 C. A total of 72 flasks were used in this study. Twenty-four flasks were sampled at 3, 8 and 20 weeks of incubation. Of these, 12 flasks were used to measure mass loss and the other 12 flasks were used to isolate the endophytic fungi present. Weight loss of leaves was determined as for the Petri dish methods.

Detection of extracellular enzyme production

The methods to detect extracellular enzyme production by endophytes are similar to that used by Kumaresan and Suryanarayanan (2002). The same 21 endophytic strains, including 9 common fungi and 12 rare fungi, were used as in the method to determine decomposition of leaves by single endophyte strains. Production of amylase, cellulase, laccase, lipase, pectate transeliminase, pectinase, protease and tyrosinase were determined (Table 1).

DNA extraction and amplification

Genomic DNA was extracted from fresh cultures of representative isolates of each morphotype following the method of Guo et al. (2000). The ITS (ITS1, 5.8 S, ITS2) region was amplified using primer pairs ITS4 and ITS5 (White et al. 1990). Amplification was performed in a 50 μl reaction volume which contained PCR buffer (20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 20 mM Tris–HCl, pH 8.4), 200 μM of each deoxyribonucleotide triphosphate, 15 *p*mols of each primer, *c*. 100 ng template DNA, and 2.5 units of *Taq* DNA polymerase (Biocolor BioScience & Technology Company, Shanghai, China). The thermal cycling program was as follows: 3 min initial denaturation at 95 C, followed by 35 cycles of 40 s denaturation at 94 C, 50 s primer annealing at 52 C, 1 min extension at 72 C, and a final 10 min extension at 72 C. A negative control using water instead of template DNA was included in the amplification process. Four microliters of PCR products from each PCR reaction were examined by electrophoresis at 75 V for 2 h in a 0.8% (W/V) agarose gel in 1×TAE buffer (0.4 M Tris, 50 mM NaOAc, 10 mM EDTA, pH 7.8) and visualized under UV light after staining with ethidium bromide (0.5 μg ml^{-1}).

Sequencing and analyses

PCR products were purified using PCR Cleanup Filter Plates (MultiScreen® $PCR_{\mu 96}$, Millipore, USA) according to the manufacturer's protocol. Purified PCR products were directly sequenced with primer pairs as mentioned above in the ABI 3730-XL DNA sequencer (Applied Biosystems, Inc., USA). Contigs were assembled using Sequencher 4.0 (GeneCodes Corp., Ann Arbor, MI, USA). A value of 97% ITS region identity (excluding flanking 18 S and 28 S rDNA sequences) was used as a DNA barcoding criterion (O'Brien et al. 2005). The ITS sequences were submitted to NCBI. BlastN searches were performed in the GenBank public sequence databases to provide at least tentative identification for non-sporulating fungi.

Data analysis

Colonization rates (CR) were calculated as the total number of plant tissue fragments infected by one or more fungi divided by the total number fragments incubated (Kumar and Hyde 2004). Relative frequency (RF) was calculated as the number of isolates of certain species divided by the total number of isolates. Endophytes were categorized as common taxa when RF≥1% and as rare taxa when RF< 1%, as in previous endophyte studies (Guo et al. 2008; Sun et al. 2008).

Table 1 Bioassays for extracellular enzyme activities of endophytic taxa

Enzyme	Medium	Detection of extracellur emzyme
Amylase	0.2% soluble starch in GYP (1 g glucose+0.1 g yeast extract+0.5 g peptone, 16 g agar per liter distilled water) medium at PH6	Flood the plate with iodine solution after 3–5 days of colony growth. Appearance of yellow areas around the fungal colony in an otherwise purple medium indicated amylase activity
Cellulase	0.5% Na-carboxy-methylcellulose in YP medium (0.1 g yeast extract+0.5 g peptone, 16 g agar per 1 l distilled water)	Flood the plate with 0.2% aqueous congo red solution and destained with 1 M NaCl (15 min each) after 3–5 days of colony growth. Appearance of yellow areas around the fungal colony in an otherwise red medium indicated cellulase activity
Laccase	0.05 g/L 1-naphthol in GYP medium at PH6	Colourless medium turns blue as the fungus grow indicated laccase oxidation of 1-naphthol
Lipase	1 ml autoclaved Tween 20 was added to 100 ml of sterile, cooled agar medium (Peptone 10 g, NaCl 5 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, Agar 20 g per liter distilled water at PH6)	Clear or precipitation around the fungal colony indicated lipase activity
Pectate Transeliminase	1 g yeast extract, 5 g pectin, 15 g agar per liter distilled water in PH7	Flood the plate with 1% aqueous solution of hexadecyltrimethylammonium bromide after 3–5 days of colony growth. A clear zone formed around the fungal colony indicated pectate transeliminase activity
Pectinase	1 g yeast extract, 5 g pectin, 15 g agar per liter distilled water in PH5	Flood the plate with 1% aqueous solution of hexadecyltrimethylammonium bromide after 3–5 days of colony growth. A clear zone formed around the fungal colony indicated pectinase activity
Protease	0.4% gelatin in GYP medium at PH6	Flood the plate with saturated aqueous solution of ammonium sulphate. Appearance of clear zone around the fungal colony in an otherwise opaque medium indicated protease activity
Tyrosinase	GYP medium	Formation of red brown colour around the fungal colony after addition of 1.08 g/L *p*-cresol solution with 0.05% glycine to the surface of fungal colony indicated tyrosinase activity

ANOVA was performed using the SPSS for windows version 11.5 (SPSS Inc.). The Kruskal-Wallis test was utilized to analyze the difference in the colonization rates of the endophytes between leaves and twigs and between five different (three age-class) tissues (Sokal and Rohlf 1981). When results of Kruskal-Wallis test suggested a significant difference, LSD was used to analyze potentially significant differences among all possible pairs of compared variables.

Results

Colonization rates of endophytic fungi

A total of 465 endophyte strains were isolated from 800 plant tissue fragments of *Acer truncatum* using disk fragment methodology. Of these strains, 418 were recovered from branches and 47 from leaves (Table 2). The overall colonization rate of endophytic fungi in samples was 50%. The colonization rate of endophytic fungi was significantly higher in twigs (77%) than in leaves (11%). However, there was no significant difference of the colonization rates of endophytic fungi between lamina (9%) and midrib (14%) tissues. The colonization rates of endophytic fungi were significantly lower in 1 year old twigs than in 2 and 3 year old twigs.

Community composition of endophytic fungi

Sporulating endophytic strains (374) were identified according to their morphological characteristics, while 91 non-sporulating strains were grouped into 20 morphotypes in the isolation of endophytes using disk fragment methodology. Endophyte strains (130) were recovered from 36 surface sterilized leaves in the method to determine decomposition of leaves by the natural endophyte community. Of these, 31 sporulating strains were identified based on their morphological characteristics and the other 99 non-sporulating strains were grouped into 36 morphotypes. A total of 56 morphotypes isolated from the two methods were then identified based on the ITS sequence data (Table 3).

Table 2 Colonization rates of endophytic fungi in leaves and twigs of *Acer truncatum*

	Leaf			Twig				Overall
	Lamina	Midrib	Total	1-yr	2-yr	3-yr	Total	
No. of samples	160	160	320	160	160	160	480	800
No. of isolates recovered	21	26	47	96	169	153	418	465
Colonization rate (%)	9	14	11	75	99	88	77	50

A total of 38 endophytic taxa were identified from *A. truncatum* using disk fragment methodology; nine taxa were isolated from leaves and 35 from twigs (Table 2). *Alternaria alternata* and *Phomopsis archeri* were the dominant endophyte species in *Acer truncatum*. Of the 12 most common endophytic taxa (RF≥1%), *Ascochytopsis vignae*, *Coniothyrium olivaceum*, *Coniothyrium* sp., *Paraconiothyrium brasiliense*, *Phoma glomerata*, *Phomopsis archeri*, and *Phomopsis* sp. 3 were obtained only from twigs. The other five taxa occurred in both leaves and twigs. Of these taxa, *Septoria* sp. mainly occurred in leaves, but *Alternaria alternata*, Coelomycete sp. 6, *Microsphaeropsis arundinis* and *Phomopsis* sp. 1 predominated in twigs (Table 2).

In addition, 35 endophytic taxa were obtained from leaves of *Acer truncatum* using the method to determine the decomposition of leaves by the natural endophyte community (Table 4). *Alternaria alternata* predominated in this community. A total of 58 taxa were isolated by disk fragment methodology and the method to determine decomposition of leaves by the natural endophyte community. Of the 58 taxa, 15 were recovered using both methods; five overlapping species were recovered from leaves using both methods (Table 4).

Leaf decomposition by endophytic taxa

Acer truncatum leaves were decomposed when inoculated with single endophyte strains, including 12 rare and 9 common taxa (Fig. 2). The weight loss of leaves inoculated with the twelve rare species ranged from 5 to 30% ($\bar{x}$ = 16%) following 4 weeks of incubation, from 6 to 38% ($\bar{x}$ = 16%) following 8 weeks of incubation, from 11 to 41% ($\bar{x}$ = 23%) following 12 weeks of incubation, and from 9 to 44% ($\bar{x}$ = 24%) following 16 weeks of incubation. The weight loss of leaves inoculated with the nine common species ranged from 6 to 18% ($\bar{x}$ = 13%) following 4 weeks of incubation, from 7 to 37% ($\bar{x}$ = 22%) following 8 weeks of incubation, from 11 to 51% ($\bar{x}$ = 31%) following 12 weeks of incubation, and from 28 to 47% ($\bar{x}$ = 37%) following 16 weeks of incubation.

Of the twelve rare species inoculated, three rare species caused more than 25% leaf weight loss between 4 and 12 weeks of incubation, while four of the rare species caused more than 25% weight loss of leaves after 16 weeks of incubation. However, none of the common species caused more than 25% leaf weight loss after 4 weeks of incubation, but more common fungi (4–9 taxa) caused more than 25% weight loss of leaves after 8 to 16 weeks of incubation.

The weight losses in *Acer truncatum* leaves colonized by natural endophyte communities increased with incubation time (Fig. 3). The mean weight loss was 22% after 3 weeks, 23% after 8 weeks, and 37% after 20 weeks of incubation, respectively. The weight loss was significantly higher at 20 weeks than at 3 and 8 weeks, but there was no significant difference between 3 and 8 weeks. The average of weight losses caused by endophyte community was higher than that by single endophyte inoculations at the end incubation, but the difference was not significant.

Extracellular enzymes produced by endophytic fungi

Amylase, cellulase, laccase, lipase, pectate transeliminase, pectinase, protease and tyrosinase activities were assayed for 21 selected endophyte taxa (Table 4). Seventeen species demonstrated amylase activity, 16 cellulase activity, 17 laccase activity, 19 lipase activity, 15 pectate transeliminase activity, 15 pectinase activity, 19 protease activity, and 10 tyrosinase activity.

Discussion

Endophyte colonization rates

The colonization rates of endophytic fungi in twigs were higher than in leaves in the present study. Similar results have been reported in numerous other endophyte studies (Fisher et al. 1994; Wang and Guo 2007; Gong and Guo 2009). For example, Collado et al. (2000) reported that the colonization rates of endophytic fungi were 25% and 36% in leaves and 76% and 82% in twigs of *Quercus ilex* and *Q. faginea*, respectively. Gond et al. (2007) found that endophyte colonization rates of *Aegle marmelos* in India were higher in bark than in leaves. Sun et al. (2008) indicated that the colonization rates of endophytic fungi associated with six medical plant species in China were

Table 3 Molecular identification of sterile endophytic morphotypes from *Acer truncatum* based on blastN queries in NCBI

Taxon	GenBank accession No.	Closest blast match (GenBank accession No.)	Query/reference ITS length (Similarity%)
Agaricales sp.	FJ025303	*Marasmius* sp. (AY916733)	583/663 (87)
Alternaria alternata	FJ025362	*Alternaria alternata* (FJ618522)	481/481 (100)
Bipolaris heveae	FJ025295	*Bipolaris heveae* (EF432245)	498/501 (99)
Bipolaris zeae	FJ025283	*Bipolaris zeae* (AF081452)	499/500 (99)
Coniothyrium sp.	FJ025351	*Coniothyrium* sp. (AM901685)	495/506 (97)
Cryptodiaporthe salicella	FJ025361	*Cryptodiaporthe salicella* (EU199183)	500/512 (97)
Cryptodiaporthe sp.	FJ025280	*Cryptodiaporthe aesculi* (EU199179)	478/507 (94)
Cyclothyrium sp.	FJ025297	*Cyclothyrium* sp. (FJ025227)	479/484 (98)
Diaporthe phaseolorum	FJ025336	*Diaporthe phaseolorum* (AF001018)	490/493 (99)
Diaporthe phaseolorum var. *meridionalis*	FJ025345	*Diaporthe phaseolorum* var. *meridionalis* (FJ357155)	479/493 (97)
Dothiorella gregaria	FJ025311	*Dothiorella gregaria* (EU520233)	454/454 (100)
Epicoccum nigrum	FJ025271	*Epicoccum nigrum* (FM991735)	456/457 (99)
Gibberella sp.	FJ025342	*Gibberella avenacea* (EU255801)	475/475 (100)
Glomerella miyabeana	FJ025324	*Glomerella miyabeana* (EF452727)	494/495 (99)
Guignardia vaccinii	FJ025308	*Guignardia vaccinii* (FJ538353)	551/564 (97)
Helminthosporium velutinum	FJ025338	*Helminthosporium velutinum* (AF145704)	479/483 (99)
Leptosphaeria sp.	FJ025302	*Leptosphaeria* sp. (AJ608969)	494/499 (98)
Leptosphaeriaceae sp.	FJ025340	*Leptosphaeria biglobosa* subsp. *thlaspii* (AJ550891)	460/504 (91)
Melanconis carthusiana	FJ025354	*Melanconis carthusiana* (EU199196)	516/516 (100)
Microdiplodia hawaiiensis	FJ025341	*Microdiplodia hawaiiensis* (DQ885897)	498/513 (97)
Nigrospora oryzae	FJ025287	*Nigrospora oryzae* (EU436680)	466/468 (99)
Paraconiothyrium brasiliense	FJ025359	*Paraconiothyrium brasiliense* (EU295634)	516/517 (99)
Pezizomycetes sp.	FJ025339	Uncultured *Geopora* isolate (EU668289)	540/575 (80)
Phoma sp. 5	FJ025306	*Phoma* sp. (AB369501)	488/489 (99)
Phomopsis longicolla	FJ025298	*Phomopsis longicolla* (FJ462759)	487/491 (99)
Phomopsis sp. 1	FJ025327	*Phomopsis longicolla* (FJ462759)	462/495 (93)
Phomopsis sp. 3	FJ025346	*Phomopsis* sp. (AB302244)	489/492 (99)
Podosordaria tulasnei	FJ025274	*Podosordaria tulasnei* (AY572970)	452/486 (93)
Preussia sp.	FJ025301	*Preussia* sp. (FJ430778)	451/456 (98)
Pseudocercosporella sp.	FJ025335	*Pseudocercosporella* sp. (FJ425195)	456/459 (99)
Sclerostagonospora sp.	FJ025330	*Sclerostagonospora* sp. (FJ372393)	500/504 (99)
Septoria canadensis	FJ025343	*Septoria canadensis* (DQ019383)	423/424 (99)
Sirococcus clavigignenti-juglandacearum	FJ025355	*Sirococcus clavigignenti-juglandacearum* (EU199200)	501/512 (97)
Sordariomycetes sp. 1	FJ025267	*Mycoleptodiscus* sp. (FJ478407)	418/500 (84)
Sordariomycetes sp. 2	FJ025337	*Periconia macrospinosa* (FJ536208)	435/484 (90)
Xylaria sp. 1	FJ025292	*Xylaria* sp. (FN812861)	551/565; (98)
Xylaria sp. 2	FJ025307	*Xylaria* sp. (DQ780441)	492/492 (100)

higher in twigs than in leaves. Guo et al. (2008) also revealed higher colonization rates of endophytic fungi in twigs than in needles of *Pinus tabulaeformis*. Gong and Guo (2009) investigated endophytic fungi of the medical plants *Dracaena cambodiana* and *Aquilaria sinensis*, and higher colonization rates were observed in stems than in leaves of both plants. The differences might be caused by the fact that the structure and substrates are different between twig and leaf tissues, which influence the colonization of endophytic fungi (Carroll and Petrini 1983; Rodrigues 1994), or that the twigs are more permanent than leaves, so that the twigs accumulate more fungal propagules (Fisher et al. 1986; Wang and Guo 2007; Guo et al. 2008).

Table 4 Relative frequency (RF%) of endophytic taxa in different tissues of *Acer truncatum*

Taxon	DFM[a]			DEC
	Leaf	Branch	Overall	Leaf
Agaricales sp.				1
Alternaria alternata	0.9	28.6	29.6	28
Alternaria arborescens		0.5	0.5	7
Ascochytopsis vignae		1.4	1.4	
Bipolaris heveae				1
Bipolaris zeae				7
Cladosporium herbarum	0.2	0.2	0.5	1
Clypeopycnis aeruginascens		0.7	0.7	
Coelomycete sp. 21		0.2	0.2	
Coelomycete sp. 6	0.2	8.2	8.4	
Colletotrichum sp.				1
Coniothyrium olivaceum		7.3	7.3	4
Coniothyrium sp.		2.1	2.1	
Coprinellus radians		0.2	0.2	
Cryptodiaporthe salicella		0.2	0.2	
Cryptodiaporthe sp.		0.2	0.2	3
Cyclothyrium sp.				2
Diaporthe phaseolorum		0.7	0.7	2
Diaporthe phaseolorum var. *meridionalis*		0.2	0.2	
Discula sp.				1
Drechslera biseptata	0.2		0.2	4
Epicoccum nigrum				1
Fusarium sp. 1		0.2	0.2	3
Fusarium sp. 2				1
Geniculosporium serpens	0.2		0.2	1
Gibberella acuminata		0.9	0.9	
Glomerella miyabeana		0.2	0.2	
Guignardia vaccinii				1
Helminthosporium velutinum		0.5	0.5	
Leptosphaeria sp.				3
Leptosphaeriaceae sp.		0.7	0.7	
Melanconis sp. 1		0.9	0.9	
Melanconis sp. 2		0.9	0.9	
Microdiplodia hawaiiensis		0.2	0.2	
Microsphaeropsis arundinis	0.2	6.4	6.6	
Nigrospora oryzae				1
Paraconiothyrium brasiliense		3.0	3.0	10
Pezizomycetes sp.				1
Phoma sp. 3	0.5	0.5	0.9	
Phoma sp. 4		0.5	0.5	
Phoma sp. 5				1
Phoma glomerata		1.6	1.6	9
Phomopsis archeri		19.1	19.1	2
Phomopsis longicolla				1
Phomopsis sp. 1	0.2	3.0	3.2	
Phomopsis sp. 2				2
Phomopsis sp. 3		2.5	2.5	3
Phomopsis sp. 4				1
Podosordaria tulasnei				1
Preussia sp.				1
Pseudocercosporella sp.		0.9	0.9	
Sclerostagonospora sp.		0.5	0.5	
Septoria sp.	3.6	0.2	3.9	2
Sirococcus clavigignenti-juglandacearum		0.2	0.2	
Sordariomycetes sp. 1		0.2	0.2	
Sordariomycetes sp. 2		0.5	0.5	1
Xylaria sp. 1				1
Xylaria sp. 2				2

[a] *DFM* Disk fragment methodology; *DEC* Decomposition of leaves by the natural endophyte community

Effect of isolation and identification methods on endophytic diversity

It is unlikely that the entire endophyte diversity can be revealed using single isolation techniques (Hyde and Soytong 2007, 2008). In the present study, nine endophytic taxa were isolated using disk fragment methodology, but 35 taxa were isolated using the method to determine decomposition of leaves by the natural endophyte community. This confirms that the entire endophyte community cannot be revealed by a single technique. The endophytic taxa isolated are affected by the size of incubated plant fragments when traditional methodology is applied. Gamboa et al. (2003) reported that the number of endophytes isolated was strongly correlated linearly with the size of fragments. Unterseher and Schnittler (2009) acquired complementary endophyte compositions when applying fragment plating and extinction culturing, in which about two-thirds of the 35 fungal taxa were isolated using one cultivation technique. Isolation of endophytes would be also affected by the fitness of media and the growth rate of strains (Fisher and Petrini 1987; Bills 1996). Thus, it is unlikely that the investigator would acquire the entire endophyte community using a single isolation technique (Gamboa et al. 2003; Schulz and Boyle 2005).

The isolation of *mycelia sterilia* complicates endophyte identification, as these isolates are difficult to identify using morphological characters (Lacap et al. 2003; Hyde and Soytong 2008). A combination of inducing sporulation (Guo et al. 1998) and molecular identification has been used to solve the problem of

Table 5 Extracellular enzyme activities of endophytic fungi from *Acer truncatum*

Taxon	Amylase	Cellulase	Laccase	Lipase	Pectinase	Pectate transeliminase	Protease	Tyrosinase
Alternaria alternata	2[a]	0	+[b]	6	2	0	2	+
Alternaria arborescens	2	2	+	9	2	2	2	+
Ascochytopsis vignae	1	2	+	6	2	3	2	−
Coniothyrium olivaceum	2	1	+	4	0	2	9	+
Coniothyrium sp.	3	0	−	12	2	2	2	+
Diaporthe sp. 2	1	3.5	+	12	2	2	2	−
Drechslera biseptata	2	1	+	8	0	0	2	+
Glomerella miyabeana	2	2	+	10	2	2	2	−
Gnomoniella sp. 1	2	2	+	10	2	2	5	−
Helminthosporium velutinum	10	2	−	5	0	0	5	+
Leptosphaeria sp. 1	5	0	+	6	2	2	13	−
Melanconis sp. 1	0	0	+	20	5	10	0	−
Melanconis sp. 2	0	13	+	7	4	4	0	−
Microsphaeropsis arundinis	2	5	+	0	0	0	5	−
Paraconiothyrium brasiliense	2	3	+	0	0	0	6	+
Phoma sp. 4	2	1	+	5	0	2	5	+
Phoma glomerata	1	1	+	2	0	0	2	+
Pseudocercosporella sp.	0	0	−	5	6	6	2	−
Septoria sp.	2	3	−	10	2	2	6	+
Sirococcus clavigignenti-juglandacearum	1	5.5	+	7	8	3	2	−
Coelomycetes sp. 15	0	0	+	4	2	2	2	−

[a] Width of reaction rings (mm)

[b] + for positive,−for negative

species mycelia sterilia recognition (Arnold and Lutzoni 2007; Unterseher et al. 2007; Huang et al. 2009; Pinruan et al. 2010; Sakayaroj et al. 2010. Guo et al. (2000) recovered 778 endophytic taxa from *Livistona chinensis*, of which 650 strains were induced to sporulate on nutrient agar with added palm tissues, and 128 non-sporulating strains were grouped into 19 morphotypes identified at different taxonomic levels using ITS sequence data. In the present study, 405 sporulating strains were identified using morphological characteristics. The other 190 mycelia sterilia strains were grouped into 56 morphotypes and further identified based on the analysis of ITS sequence data. Therefore, high endophyte species diversity was revealed using a combination of morphology and molecular techniques.

Decomposition of endophytes

The roles of endophytes in ecosystems has been well speculated upon and well studied, but their specific role is yet to be established (Elmi and West 1995; Malinowski et al. 1999; Pinto et al. 2000; Mucciarelli et al. 2003; Ting et al. 2008). The role of endophytic fungi in decomposition in ecosystems has been demonstrated in previous studies (Müller et al. 2001; Kumaresan and Suryanarayanan 2002; Whitoon and Hyde, unpublished). Sokolski et al. (2006) showed that based on the analysis of morphology and molecular data, that an endophytic species of *Dwayaangam* associated with *Picea mariana* was also a freshwater hyphomycete saprobic fungus. Molecular data have also provided evidence to suggest that some endophytic fungi transform to a saprotrophic lifestyle after leaf fall (Promputtha et al. 2007). Increasing research is blurring the boundary between what is an endophyte and saprobe (Selosse et al. 2008).

The present study provides evidence for the decomposer role of endophytic fungi in ecosystems. Endophytes produced various extracellular enzymes in this study, and these enzymes have been shown to be important in litter degradation (Kumaresan and Suryanarayanan 2002; Promputtha et al. 2010). Endophytes generally decompose dead host leaves, not only as single species, but also as communities. This is evident from studies of saprobes on fallen leaves, where invariably several species can be found on a single decaying leaf. Some endophytes (e.g. *Colletotrichum* [anamophic *Glomerella*] and *Phomopsis* [anamophic *Diaporthe*]) were isolated from leaves of *Magnolia liliifera* in Thailand (Promputtha et al. 2005), and these fungi were also recovered

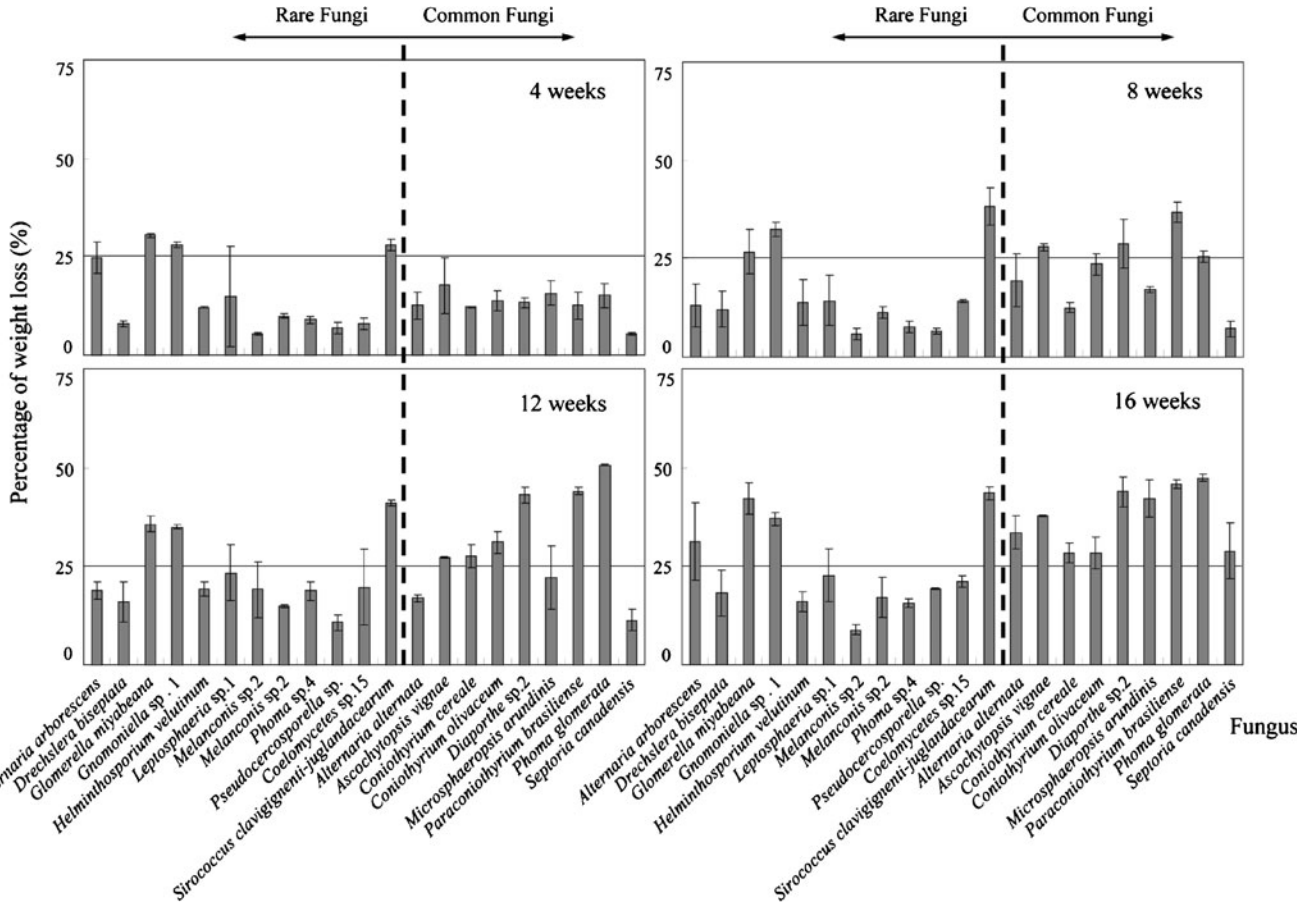

Fig. 2 Percentage weight loss of *Acer truncatum* leaves inoculated individually with endophytic strains. *Bars* indicate means and±SD

as saprobes from fallen leaves of *M. liliifera* (Promputtha et al. 2002). The endophytic and saprobic strains isolated from leaves of *M. liliifera* were similar in morphology and phylogeny based on the analyses of morphological characteristics and molecular data (Promputtha et al. 2005; Duong et al. 2006), and they produced the same degrading enzymes (i.e., cellulase, laccase, β-mannanase, polygalacturonase) and xylanase activity (Promputtha et al. 2010). Osono and Hirose (2009) reported that *Coccomyces* sp., an endophytic fungus of *Camellia japonica*, was first to decompose the litter and stimulated the subsequent decomposition by other fungi.

In general, the common species had a higher decomposing ability than the rare species, based on the results obtained in the present study. All nine common species caused more than 25% weight loss after 16 weeks of incubation, whereas only four of the twelve rare species did so. We hypothesize that a saprobic lifestyle may be characteristic of the common species, while the rare species may be less efficient decomposers producing less propagules and therefore becoming less common endophytes. It would be worthwhile to study the ecological roles and interaction between host of common and rare species in ecosystems, and to establish the evolutionay relationships of common and rare species in future work.

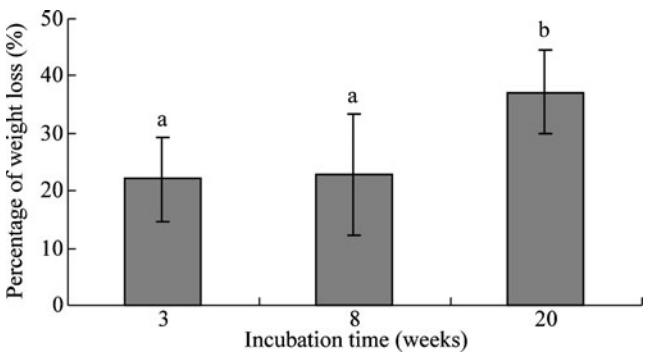

Fig. 3 Percentage weight loss of *Acer truncatum* leaves decomposed by endophyte communities. The error lines represent standard deviation. A different letter above the columns indicate a significant difference at the $p<0.05$ level

Acknowledgements This project is supported by the National Natural Science Foundation of China Grants (No. 30930005 and 30870087) and the Chinese Academy of Sciences Grant (No. KSCX2-YW-Z-0935).

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