

# Diversity and dynamics of fungal endophytes in leaves, stems and roots of *Stellera chamaejasme* L. in northwestern China

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**Abstract** This study was conducted to explore fungal endophyte communities inhabiting a toxic weed (*Stellera chamaejasme* L.) from meadows of northwestern China. The effects of plant tissue and growth stage on endophyte assemblages were characterized. Endophytes were recovered from 50 % of the samples, with a total of 714 isolates. 41 operational taxonomical units (OTUs) were identified, consisting of 40 OTUs belonging primarily to Ascomycota and 1 OTU belonging to Basidiomycota. Pleosporales and Hypocreales were the orders contributing the most species to the endophytic assemblages. The total colonization frequency and species richness of endophytic fungi were higher in roots than in leaves and stems. In addition, for the plant tissues, the structure of fungal communities differed significantly by growth stages of leaf emergence and dormancy; for the plant growth stages, the structure of fungal communities

differed significantly by plant tissues. This study demonstrates that *S. chamaejasme* serves as a reservoir for a wide variety of fungal endophytes that can be isolated from various plant tissues.

**Keywords** *Stellera chamaejasme* L. · Ascomycota · Ecology · Fungal diversity · Plant growth stage · ITS

## Introduction

Fungal endophytes commonly infect host plants asymptotically and reside within internal tissues of living plants (Hyde and Soyong 2008). Recent studies of a broad range of plant species have demonstrated that endophytic fungi were ubiquitous in various geographic and climatic zones and have been found within all examined plants (Arnold 2007; Ghimire et al. 2011; Rivera-Orduña et al. 2011). It has been shown that some endophytic fungi may protect their host plants from fungal pathogens, insect pests and nematodes (Martin et al. 2012; Menjivar et al. 2012). Several studies have demonstrated that some endophytic fungi have the ability to increase the fitness of plant hosts by enhancing their resistance to abiotic (Sieber 2007; Hyde and Soyong 2008) and biotic stress (Arnold et al. 2003). Although interest in the ecological roles of endophytic fungi has increased in recent years, it has been hampered by little sampling

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and lack of characterization of fungal diversity (Hyde et al. 2007).

Much attention is now being paid to endophytic biodiversity, chemistry and bioactivity of endophytic metabolites, as facets of the relationship between endophytes and their host plants (Schulz et al. 2002; Tao et al. 2008). While, there is little information on whether culturable endophyte communities change seasonally, the limited evidence suggests that this might occur in both roots and leaves of plant (Quilliam and Jones 2010). Furthermore, the effect of seasonal changes on endophytic fungi remains unclear. Seasonal patterns have been detected among taxa that were not host specific, but other studies could not confirm any seasonality among endophytes (Widler and Müller 1984; Sieber and Hugentobler 1987; Joshee et al. 2009). Moreover, endophytic fungi colonize all plant parts such as roots, stems, leaves, bark and floral organs. The plant-associated habitat is a dynamic environment in which many factors affect the structure and composition of species that colonize different tissues (Rivera-Orduña et al. 2011). Surveys of endophytic communities in both above and below ground plant organs simultaneously are extremely limited so far (Saunders and Kohn 2009; Wearn et al. 2012).

Fungal endophytes have been recognized as a potential repository of novel natural products for pharmaceutical, agricultural and industrial uses (Hyde and Soyong 2008). Secondary metabolites extracted from plants are usually considered to have been produced by the plant. However, some of these metabolites may in fact have been produced by endophytic fungal colonizers in the plant (Stierle et al. 1993; Mishra et al. 2012). So, endophytic fungi are good sources of novel secondary metabolites that possess antibacterial, antifungal and anticancer properties (Ding et al. 2008; Kharwar et al. 2009; Kumara et al. 2012). In recent years, there has been particular emphasis on studying endophytes of medicinal plants in order to discover novel compounds (Zhou et al. 2007; Huang et al. 2009).

*Stellera chamaejasme* L., a toxic perennial weed, is a multi-stemmed herbaceous plant belonging to the family Thymelaeaceae. It has a wide geographical range from southern Russia to northern China and Mongolia, southwards as far as to the dry regions of the western Himalayas, the Tibetan Plateau and southwestern China (Zhang et al. 2011). *S. chamaejasme* has spread in recent decades and is now abundant

in northern and western China (Sun et al. 2009; Zhang et al. 2011), and has become one of the most serious weeds damaging ecological balance of grasslands (Sun et al. 2009). It has increased the grassland degradation and the desertification process, and caused food poisoning of cattle due to eating stems and leaves. *S. chamaejasme* is listed as one of the most serious weeds in China (Sun et al. 2009; Zhang et al. 2011). Previous studies on this toxic weed in our lab indicated that root extracts of *S. chamaejasme* showed significant nematicidal activity (Guo et al. 2011), and several purified metabolites that were extracted from roots of *S. chamaejasme* exhibited phytotoxic activities (Yang et al. 2011). Thus, to learn if any endophytic fungi may be producing one or more of a plethora of bioactive substances known from *S. chamaejasme*, it would first be crucial to do a systematic study on the endophytic fungi of this plant and to know their composition and diversity in both above (leaves and stems) and below ground (roots) plant organs in the whole growing seasons. In addition, it is important to determine the species that can be cultured because this allows for the subsequent manipulation of endophytic fungi activity and natural products extraction (Wearn et al. 2012).

The aim of the present study was to characterize endophytic fungi in leaves, stems and roots of *S. chamaejasme* over the course of an entire growing season. There were two specific objectives: one was to isolate and taxonomically identify leaf, stem and root-associated fungal endophytes of *S. chamaejasme*; the other is to evaluate these endophyte communities over the course of a growing season to provide insight into symbiont community dynamics. We used a traditional technique to isolate the endophytes, and a combination of morphological and molecular methods to identify these fungal isolates (Wang et al. 2005). The study will create a reservoir of strains that may be assayed for potential applications. To the best of our knowledge, this is the first report of the endophyte community associated with *S. chamaejasme*.

## Materials and methods

### Sampling site and procedure

The plant samples were collected at the Cuiying mountain area (35°56'N, 104°08'E) in the Yuzhong

campus of Lanzhou University, province of Gansu (Northwest China). It covers 201 ha with loessial soil, and the current vegetation is dominated mainly by *Melica przewalskyi*, *Agropyron cristatum*, *Artemisia frigida* and *S. chamaejasme*. The climatic characteristics of the study area can be classified as temperate, semi-arid, continental climate with a mean annual temperature of 6.7 °C and mean annual precipitation of 381.8 mm. The average elevation of various sampling sites is 1,965.8 m above sea level (Yang et al. 2010).

The developmental stages of *S. chamaejasme* at Cuiying mountaintop were leafing (leaf emergence) in early May (LE), flowering (full bloom) in early June (FB), fruiting (seed maturation) in mid-July (SM) and dormancy in early November until the following March (D). To study the fluctuation of endophyte communities with different developmental stages of *S. chamaejasme*, plant samples were collected in the middle of May, June and July of 2011, and mid-January of 2012 according to the distribution patterns of *S. chamaejasme*.

Healthy, symptomless leaves (L), stems (St) and roots (R) were collected from six individual plants of *S. chamaejasme* at each development stage, the distance between single plants varied from 30 to 50 m depending on the distribution within the vegetation cover. One stem (length, 10–15 cm; diameter, 1–2 mm) with leaves and two root segments (length, 8–12 cm; diameter, 2–4 mm) were randomly collected from each plant in each of the four growth stages, respectively. All root samples were taken at a depth of at least 15–20 cm below ground level. These plant samples were immediately brought to the laboratory in ice boxes, stored at 4 °C and processed within 48 h.

#### Isolation and identification of endophytic fungi

The sampling regime was designed with the intention of isolating as many endophyte species as possible from the different tissues samples. Samples were washed thoroughly in running tap water, rinsed with distilled water, and surface-sterilized according to Zamora et al. (2008). Samples were dipped in 70 % ethanol for 1 min, immersed in aqueous solution of NaOCl (2 % available chlorine) for 5 min (leaves) and 8 min (stems and roots). The samples were then rinsed in double-distilled sterile water three times and dried under aseptic condition. Each leaf sample (leaves were

removed from stem), stem and root was cut into 20 segments, measuring approximately 5 × 5 mm for leaves or 5 mm in length for stems and roots. In total, 1,440 segments (20 segments × 3 tissues × 6 individual plants × 4 growth stages) were used in this study. Sets of 20 segments were then evenly placed in a 90 mm Petri dish containing potato dextrose agar (PDA) supplemented with streptomycin (200 mg l<sup>-1</sup>) to suppress bacterial growth. The effectiveness of the surface sterilization was controlled by making imprints of disinfected sample fragments on PDA plates (Schulz et al. 1998). Petri dishes were sealed, incubated for 45–60 days at 25 ± 1 °C, and examined periodically.

When colonies developed, actively growing fungal mycelia were transferred to new PDA plates for purification and identification. Fungal isolates were selected and grouped together according to morphological characters such as spore production, spore length and morphology, aerial mycelium color, texture and form, exudates and the growth rate (Sun et al. 2011), but in addition, specimens of most isolates were also identified with internal transcribed spacer (ITS) region sequences. All isolates are maintained in Key Laboratory of Chemistry of Northwestern Plant Resources, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences.

#### DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from fresh cultures following the protocol of Sim et al. (2010). The ITS (ITS1, 5.8S, ITS2) region was amplified using primer pairs ITS4 and ITS5 (White et al. 1990). PCR reactions were set-up using the following ingredients for each 50 µl reaction: PCR buffer (20 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 20 mM Tris-HCl, pH8.4), 200 µM of each deoxyribonucleotide triphosphate, 15 pmols of each primer, a maximum of 25 ng µl<sup>-1</sup> of template DNA, and 2.5 units of *Taq* DNA polymerase (Biocolor BioScience & Technology Company, Shanghai, China). PCR amplifications were performed using the following conditions: 4 min initial denaturation at 94 °C, then 1 cycle consisting of denaturation (1 min at 94 °C), annealing (20 s at 55 °C), extension (50 s at 72 °C), and followed by 37 cycles of 20 s at 94 °C, 20 s at 55 °C, 50 s at 72 °C, and a final 4 min at 72 °C. A negative control using deionized water instead of template DNA was included in each batch

of the PCRs. PCR products were checked electrophoretically in a 0.8 % (W V<sup>-1</sup>) agarose gel. The band of size approximately 550 bp in the electrophoresis pattern was excised and purified with the DNA Fragment Quick Purification/Recover Kit (Dingguo, Beijing, China) following the protocol. Purified PCR products were directly sequenced with primer pairs as mentioned above in the ABI-Prism 377 automated sequencer (Applied Biosystems, Inc. USA).

#### Taxonomic assignments and phylogenetic analysis

The ITS sequences were checked for chimeras using the CHECK\_CHIMERA PROGRAM (Cole et al. 2007) and Pintail (Ashelford et al. 2005) programs. Assembly and editing of good sequences were performed using BioEdit Software (Hall 1999). Operational taxonomic units (OTUs) were defined with the MOTHUR program ([http://www.mothur.org/wiki/Silva\\_reference\\_alignment](http://www.mothur.org/wiki/Silva_reference_alignment)). The uncorrected pairwise distances between aligned sequences were calculated and the OTUs were defined using the furthest neighbour-clustering algorithm at evolutionary distances of 3 % (that is on the basis of 97 % similarity between sequences), and a representative sequence was chosen from each OTU by a majority decision (Schloss et al. 2009). A representative sequence for each OTU was used as query sequence to search for similar sequences in GenBank with BLASTN program to provide at least tentative identification for the fungus. A value of 97 % ITS region identity was used as a DNA barcoding threshold (O'Brien et al. 2005). The ITS sequence of a representative in each OTU was aligned in ClustalW for phylogeny analysis. The alignments were further used for phylogenetic trees construction using MEGA software version 5.0 (Tamura et al. 2011). The neighbor-joining (NJ) method was used to infer the evolutionary history of the fungal isolates and the bootstrapping was carried out using 1,000 replications. All of the sequences have been submitted to the GenBank. The accession numbers are the following; sequences from leaf samples: KC292821–KC292843, stem samples: KC292886–KC292914, root samples: KC292844–KC292885.

#### Data analysis

The colonization frequency (CF) was calculated as the total number of segments colonized by endophytic fungi

divided by the total number of incubated segments. The relative species frequency (RF) was calculated as the number of isolates of one species divided by the total number of isolates (Yuan et al. 2010).

The Shannon–Weaver diversity index ( $H'$ ) (Shannon and Weaver 1963) was used to show diversity of the endophytic fungal species and was calculated as:  $H' = -\sum p_i \ln p_i$ . Shannon evenness ( $E$ ) was calculated as  $H'/H_{\max}$ , where  $H_{\max} = \ln(S)$ ,  $S$  is the total number of OTUs in the subsample. Simpson's diversity ( $D = 1 - \sum p_i^2$ , where  $p_i$  is the proportion of isolates assigned to the  $i$ th OTU, that is  $P_i = n_i/N$ ) (Simpson 1964) was calculated to compare the species richness. A Simpson diversity index is close to 1 means that the sample is highly diverse.

Difference in Shannon diversity indices of any two fungal communities were compared using a Student's  $t$  test at a 95 % confidence level. Differences were considered statistically significant at  $p < 0.05$  (Kutorga et al. 2012). Principal component analysis (PCA) was conducted to explore the compositions of endophytic fungal communities at different growth stages. Principal component models were built with full leverage correction and based on percentage abundance of endophytic fungal isolates at each growth stage. The statistical significance for PCA was assessed by analysis of variance of PCA scores among different growth stages (Beraldi-Campesi et al. 2012). The statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

## Results

### Isolation and identification of endophytic fungi

A total of 714 endophyte strains were isolated from 1,440 plant tissue segments of *S. chamaejasme* during LE, FB, SM and D growth stages. Of these strains, 224 were recovered from leaves, 236 from stems and 254 from roots. These fungal strains were sorted into 91 OTUs (22 OTUs for leaves, 29 OTUs for stems, and 40 OTUs for roots). The colonization rate of endophytic fungi was higher in roots (53 %) than in leaves (47 %) and stems (49 %). The colonization rate of endophytic fungi was significantly lower at LE stage (26 %) than at FB (56 %), SM (57 %) and D (60 %) stages.

The fungal isolates were mainly composed of Ascomycota (711, 99.6 % of the isolates), including

**Table 1** Colonization frequency (%) of fungal classes, with Shannon, Evenness and Simpson diversity indices for different growth stages of *S. chamaejasme*

Plant organ and growth stage	Fungal classes (%)							Colonization frequency (%)	Shannon ( <i>H'</i> )	Evenness ( <i>E</i> )	Simpson ( <i>D</i> )
	Aga-	Asc-	Dot-	Eur-	Leo-	Muc-	Sor-				
<b>Leaves</b>											
LE	–	–	37.5	–	–	–	3.3	40.8	0.95	0.24	0.51
FB	–	–	28.3	–	–	–	–	28.3	1.29	0.37	0.71
SM	–	8.3	2.5	–	–	–	45.0	55.8	1.69	0.40	0.78
D	–	5.8	18.3	–	–	–	37.5	61.7	1.73	0.40	0.79
Total	–	3.5	21.7	–	–	–	21.5	46.7	1.67	0.27	0.98
<b>Stems</b>											
LE	–	1.7	4.2	–	9.2	–	–	15.0	1.09	0.38	0.57
FB	2.5	–	53.3	–	–	–	–	55.8	0.66	0.16	0.31
SM	–	11.7	12.5	5.0	–	–	28.3	57.5	2.62	0.62	0.91
D	–	16.7	15.0	–	1.7	–	35.0	68.3	1.11	0.25	0.63
Total	0.6	7.5	21.3	1.3	2.7	–	15.8	49.2	1.68	0.27	0.97
<b>Roots</b>											
LE	–	–	3.3	5.8	0.8	–	10.8	20.8	2.29	0.71	0.88
FB	–	–	20.0	1.7	14.2	–	49.2	85.0	2.13	0.46	0.85
SM	–	–	–	19.2	5.8	–	31.7	56.7	2.47	0.59	0.90
D	–	–	–	5.8	–	21.7	21.7	49.2	1.47	0.36	0.74
Total	–	–	5.8	8.1	5.2	5.4	28.3	52.9	2.12	0.34	0.99

LE leaf emergence; FB full bloom; SM seed maturation; D dormancy; Aga- Agaricomycetes; Asc- unclassified Ascoymcota; Dot- Dothideomycetes; Eur- Eurotiomycetes; Leo- Leotiomyces; Muc- Mucoromycotina; Sor- Sordariomycetes

a high relative abundance of Sordariomycetes (303, 42.6 %, 38 OTUs) and Dothideomycetes (240, 33.8 %, 30 OTUs). The unclassified Ascoymcota (53 isolates, 5 OTUs), Eurotiomycetes (45 isolates, 10 OTUs), Leotiomyces (36 isolates, 4 OTUs) and Mucoromycotina (34 isolates, 3 OTUs) constituted only 7.5, 6.3, 5.1 and 4.8 %, respectively, of the isolated ascomycetes (Table 1). A single basidiomycete (Agaricomycetes, 3 isolates, 1 OTUs) was identified and the OTU was detected in 0.4 % of the total samples (Table 1). In the entire dataset, *Alternaria* (dominated in leaves and stems) and *Fusarium* (dominated in roots) were the most frequently isolated genera. *Fusarium acuminatum* was the most abundant taxon, followed by *Alternaria* sp., *Ascomycota* sp., *Dothideomycetes* sp. and *Alternaria tenuissima*.

Endophyte community in leaf, stem and root tissues

The leaf endophyte community was composed of 224 fungal isolates that belong to 22 OTUs (Table 2;

Fig. 1a). The representation of individual species in the community was variable, as some species were recovered frequently in two or three collections (e.g. *Alternaria tenuissima*, *Alternaria alternata*, *Ascomycota* sp., *Dothideomycetes* sp., *Fusarium acuminatum*) and others were recovered infrequently and/or from a single collection (e.g. *Alternaria brassicae*, *Aporospora terricola*, *Fusarium sporotrichioides*, *Sordaria lappae*). The number of isolates recovered from different growth stages varied, with 49, 34, 67 and 74 isolates obtained at LE, FB, SM and D stage, respectively. At LE stage, the fungal isolates were grouped into 4 OTUs, the majority of the isolates were *Alternaria tenuissima* (65.3 %), followed by *Alternaria alternata* (22.4 %). At FB stage, the fungal isolates were also grouped into 4 OTUs, the majority of the isolates were *Dothideomycetes* sp. (35.3 %) and *Sporormiella* sp. (35.3 %), followed by *Alternaria solani* (17.6 %) and *A. tenuissima* (11.8 %). At SM stage, the fungal isolates were grouped into 7 OTUs, the majority of the isolates were *Fusarium acuminatum* (35.8 %), followed by *Acremonium exuviarum*

**Table 2** Comparisons of species diversity of fungal endophyte communities recovered from leaves (L), stems (St) and roots (R) of *Stellera chamaejasme* at four growth stages

Parameter	Comparisons	Number of		Shannon index ( $H'$ )	$t$ value	df	$P$ value
		Isolate	OTUs				
Leaves	LE vs. FB	49 vs. 34	4 vs. 4	0.59 vs. 1.29	5.78	81	<0.001
	LE vs. SM	49 vs. 67	4 vs. 7	0.59 vs. 1.69	6.71	114	<0.001
	LE vs. D	49 vs. 74	4 vs. 7	0.59 vs. 1.73	6.45	121	<0.001
	FB vs. SM	34 vs. 67	4 vs. 7	1.29 vs. 1.69	4.12	99	<0.001
	FB vs. D	34 vs. 74	4 vs. 7	1.29 vs. 1.73	4.34	106	<0.001
	SM vs. D	67 vs. 74	7 vs. 7	1.69 vs. 1.73	0.63	139	>0.500
Stems	LE vs. FB	18 vs. 67	4 vs. 4	1.09 vs. 0.66	4.19	83	<0.001
	LE vs. SM	18 vs. 69	4 vs. 17	1.09 vs. 2.62	7.40	85	<0.001
	LE vs. D	18 vs. 82	4 vs. 4	1.09 vs. 1.11	0.50	98	>0.500
	FB vs. SM	67 vs. 69	4 vs. 17	0.66 vs. 2.62	8.36	134	<0.001
	FB vs. D	67 vs. 82	4 vs. 4	0.66 vs. 1.11	4.07	147	<0.001
	SM vs. D	69 vs. 82	17 vs. 4	2.62 vs. 1.11	6.22	149	<0.001
Roots	LE vs. FB	25 vs. 102	11 vs. 10	2.29 vs. 2.13	0.89	125	>0.100
	LE vs. SM	25 vs. 68	11 vs. 14	2.29 vs. 2.47	0.92	91	>0.100
	LE vs. D	25 vs. 59	11 vs. 5	2.29 vs. 1.47	5.97	82	<0.001
	FB vs. SM	102 vs. 68	10 vs. 14	2.13 vs. 2.47	3.55	168	<0.001
	FB vs. D	102 vs. 59	10 vs. 5	2.13 vs. 1.47	4.94	159	<0.001
	SM vs. D	68 vs. 59	14 vs. 5	2.47 vs. 1.47	6.76	125	<0.001
LE	L vs. St	49 vs. 18	4 vs. 4	0.59 vs. 1.09	4.91	65	<0.001
	L vs. R	49 vs. 25	4 vs. 11	0.59 vs. 2.29	8.70	72	<0.001
	St vs. R	18 vs. 25	4 vs. 11	1.09 vs. 2.29	4.96	41	<0.001
FB	L vs. St	34 vs. 67	4 vs. 4	1.29 vs. 0.66	5.11	99	<0.001
	L vs. R	34 vs. 102	4 vs. 10	1.29 vs. 2.13	3.03	134	<0.001
	St vs. R	67 vs. 102	4 vs. 10	0.66 vs. 2.13	7.52	167	<0.001
SM	L vs. St	67 vs. 69	7 vs. 17	1.69 vs. 2.62	5.08	134	<0.001
	L vs. R	67 vs. 68	7 vs. 14	1.69 vs. 2.47	4.43	133	<0.001
	St vs. R	69 vs. 68	17 vs. 14	2.62 vs. 2.47	0.81	135	>0.100
D	L vs. St	74 vs. 82	7 vs. 4	1.73 vs. 1.11	4.62	154	<0.001
	L vs. R	74 vs. 59	7 vs. 5	1.73 vs. 1.47	2.16	131	<0.001
	St vs. R	82 vs. 59	4 vs. 5	1.11 vs. 1.47	4.30	139	<0.001
Total	L vs. St	224 vs. 236	22 vs. 29	1.67 vs. 1.68	0.26	458	>0.500
	L vs. R	224 vs. 254	22 vs. 40	1.67 vs. 2.12	4.49	476	<0.001
	St vs. R	236 vs. 254	29 vs. 40	1.68 vs. 2.12	4.25	488	<0.001

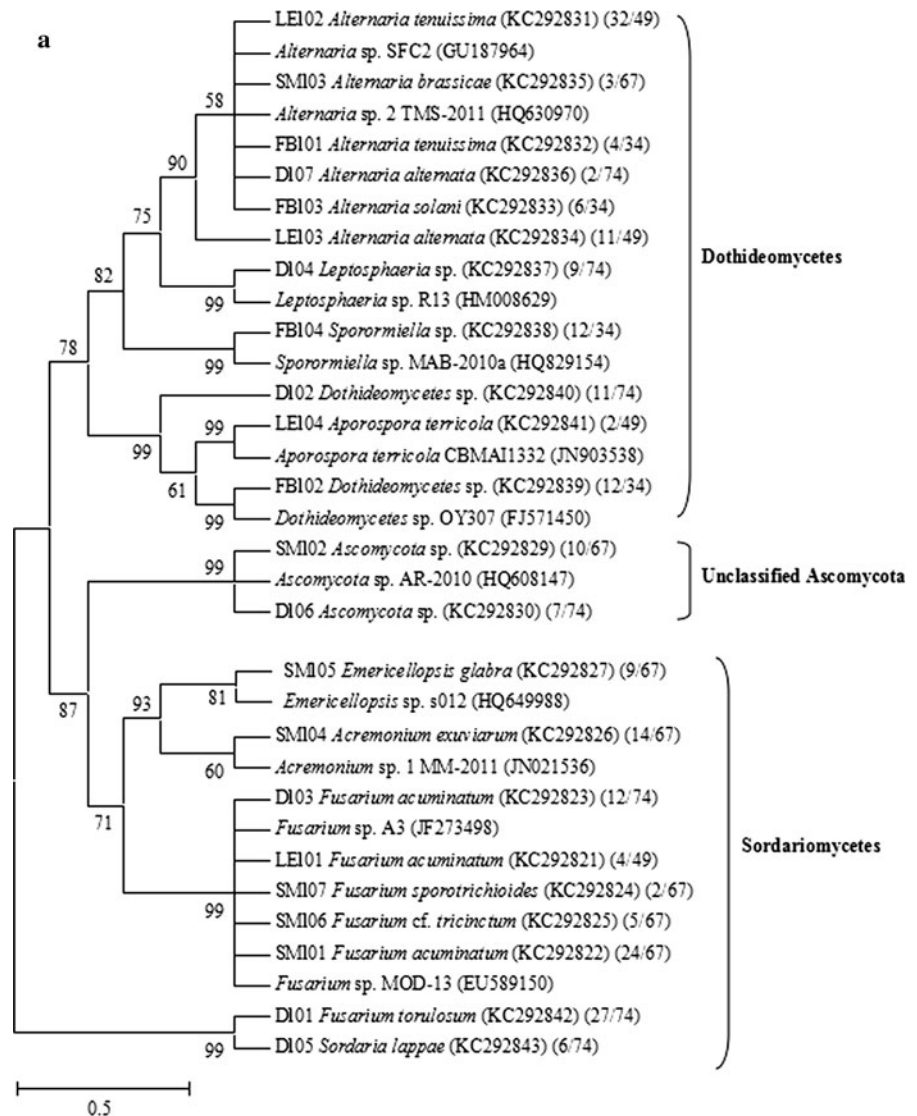
LE leaf emergence; FB full bloom; SM seed maturation; D dormancy; L leaf; St stem; R root;  $t$  value, Student's  $t$ -test value,  $t$  test is about the Shannon diversity indices;  $df$  degrees of freedom;  $P$  Probability

(20.9 %) and *Ascomycota* sp. (14.9 %). The genus of *Fusarium* was predominant in the leaf tissues at SM stage. At D stage, the fungal isolates were grouped into 7 OTUs, the majority of the isolates were *Fusarium torulosum* (36.5 %), followed by *Fusarium acuminatum* (16.2 %) and *Dothideomycetes* sp. (14.9 %). As

for SM stage, the genus of *Fusarium* was predominant in the leaf tissues.

The stem endophyte community was composed of 236 fungal isolates that belong to 29 OTUs (Table 2; Fig. 1b). As for the leaf tissues, a number of fungal isolates obtained from stem tissues varied among

**Fig. 1** Phylogenetic tree constructed using the neighbor-joining method based on sequences of the ITS region showing closest relatives of endophytic filamentous fungi isolated from the leaves (a), stems (b) and roots (c) of *S. chamaejasme* at different growth stages. Bootstrap values ( $\geq 50\%$ ) from 1,000 replicates are shown. The culture collection accession numbers of all reference strains are shown. The number in parentheses of each isolate indicates accession number of the gene sequences deposited in the GenBank sequence library, and the relative proportions of the fungal isolates are also presented (LE leaf emergence; FB full bloom; SM seed maturation; D dormancy; l leaves; st stems; r roots.)



growth stages. The highest number of isolates were obtained from D stage ( $n = 82$ ) and the least from LE stage ( $n = 18$ ). A total of 67 and 69 isolates were obtained from FB and SM stages, respectively. As observed for leaf, the representation of individual species in the stem endophyte community was variable. At LE stage, the fungal isolates were grouped into 4 OTUs, the majority of the isolates were *Botryotinia fuckeliana* (61.1 %), followed by *Aporospora terricola* (16.7 %). The genus of *Botryotinia* was predominant in the stem tissues at LE stage. At FB stage, the fungal isolates were also grouped into 4 OTUs, the majority of the isolates were *Alternaria* sp. (82.1 %), followed by *Dothideomycetes* sp. (9.0 %). The genus

of *Alternaria* was predominant in the stem tissues at FB stage. At SM stage, the fungal isolates were grouped into 17 OTUs, the majority of the isolates were *Ascomycota* sp. (20.3 %), followed by *Fusarium* cf. *acuminatum* (14.5 %) and *Fusarium avenaceum* (7.2 %). The genus of *Fusarium* was predominant in the leaf tissues at SM stage. At D stage, the fungal isolates were grouped into 4 OTUs, the majority of the isolates were *Fusarium acuminatum* (51.2 %), followed by *Ascomycota* sp. (24.4 %) and *Dothideomycetes* sp. (22.0 %). As for SM stage, the genus of *Fusarium* was predominant in the stem tissues.

The root endophyte community was composed of 254 fungal isolates that belong to 40 OTUs (Table 2;

Fig. 1 continued

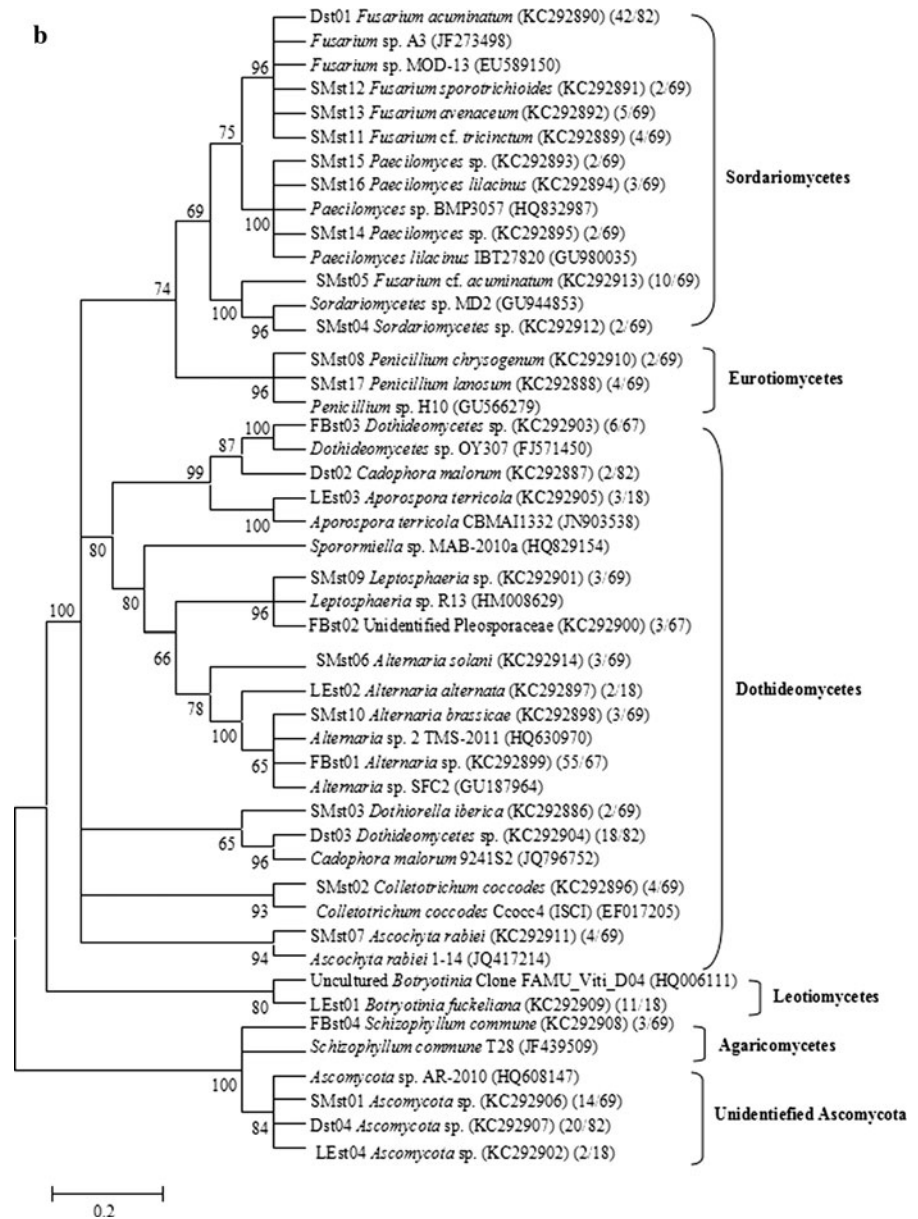
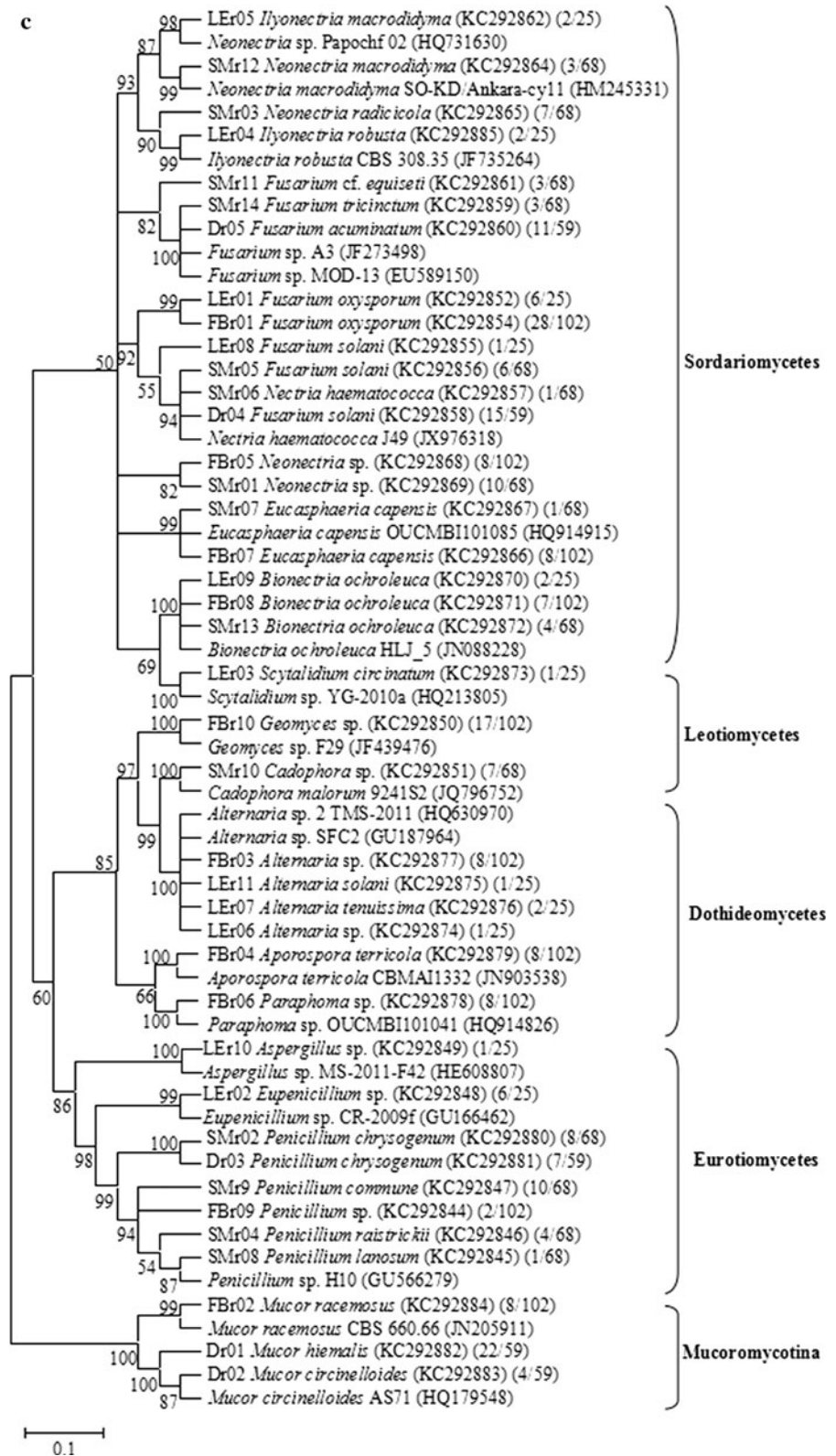


Fig. 1c). As for the leaf and stem tissues, a number of fungal isolates were obtained varied among growth stages. The highest number of isolates were obtained from FB stage ( $n = 102$ ) and the least from LE stage ( $n = 25$ ). A total of 68 and 59 isolates were obtained from SM and D stages, respectively. As observed for leaf and stem, the representation of individual species in the root endophyte community was variable. *Bionectria ochroleuca*, *Fusarium solani*, *Penicillium chrysogenum*, etc. were recovered frequently in two or four collections, and *Eucasphaeria capensis*,

*Penicillium lanosum*, *Scytalidium circinatum*, etc. were recovered infrequently and/or from a single collection. At LE stage, the fungal isolates were grouped into 11 OTUs, the majority of the isolates were *Eupenicillium* sp. (24.0 %) and *Fusarium oxysporum* (24.0 %). The genus of *Fusarium* was predominant in the root tissues at LE stage. At FB stage, the fungal isolates were also grouped into 10 OTUs, the majority of the isolates were *Fusarium oxysporum* (27.5 %), followed by *Geomyces* sp. (16.7 %). As for LE stage, the genus of *Fusarium* was predominant at



Fig. 1 continued



FB stage. At SM stage, the fungal isolates were grouped into 14 OTUs, the majority of the isolates were *Neonectria* sp. (14.7 %) and *Penicillium commune* (14.7 %), followed by *Penicillium chrysogenum* (11.8 %) *Cadophora* sp. (10.3 %). The genus of *Penicillium* was predominant in the root tissues at SM stage. At D stage, the fungal isolates were grouped into 5 OTUs, the majority of the isolates were *Mucor hiemalis* (37.3 %), followed by *Fusarium solani* (25.4 %) and *Fusarium acuminatum* (18.6 %). The genera of *Mucor* and *Fusarium* were predominant in the root tissues at D stage.

#### Diversity of endophytic fungi

The species diversity indices for different fungal communities were presented in Table 1. There was a higher species richness of endophytic fungi in roots than in leaves and stems. The Shannon diversity indices and Shannon evenness indices were the highest in roots and lowest in leaves. The Simpson's diversity index was the highest in roots and lowest in stems.

The leaf fungal community from D stage had the highest species diversity whereas the least diversity was found in LE stage community. Species diversity of leaf-associated fungi was rather similar between SM and D stages ( $P > 0.500$ ), but all the other communities from leaf differed in species diversity ( $P < 0.001$ ) (Table 2). The stem fungal community from SM stage had the highest species diversity whereas the least diversity was found in FB stage community. All pairwise comparisons from stem fungal community differed in species diversity except between LE and D stage communities (Table 2). As for root fungal community, SM stage had the highest species diversity and D stage had the least. Species diversity were similar between LE and FB stages, and between LE and SM stages ( $P > 0.100$ ), the remaining pairwise comparisons from root fungal community differed in species diversity (Table 2).

All fungal communities from leaf, stem and root tissues at different growth stages differed in species diversity except between stem and root communities at SM stage ( $P > 0.100$ ) (Table 2). Species diversity was rather similar between leaf-associated fungi and stem-associated fungal community ( $P > 0.500$ ), but both differed from that found in root-associated fungal community (Table 2).

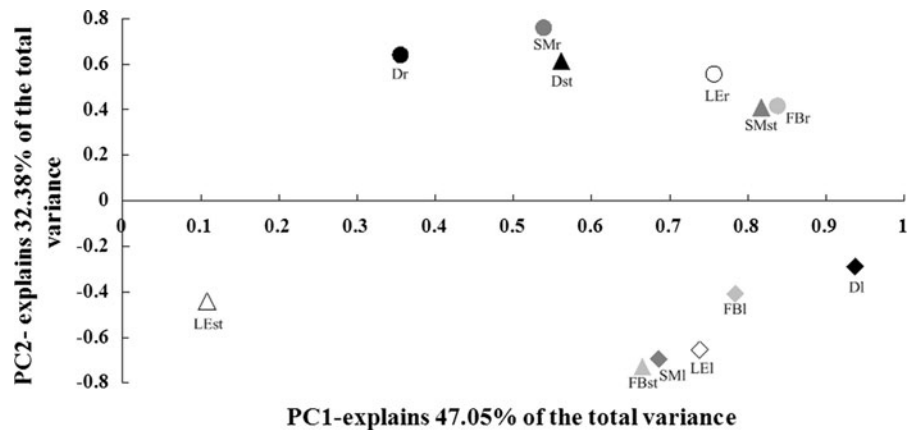
#### PCA analysis

Results from PCA of different growth stages based on fungal community composition of leaves, stems and roots of *S. chamaejasme* were presented in Fig. 2. The first two principal components (PCs) represent 79.43 % of the variance. A two-dimensional scores plot was performed, from the X axis defined by PC1 (47.05 %), the scores for each of the three tissue samples from the same growth stage were different. The score for stem sample at LE stage was lower (0.11) than the similar scores for leaf sample (0.74) and root sample (0.76), while at D stage, the score for leaf sample was higher (0.94) than the similar scores for stem sample (0.56) and root sample (0.36). The scores for leaf, stem and root samples from FB and SM stages were similar. Leaf samples and root samples were easily distinguished from the Y axis, defined by PC2 (32.38 %), since the scores of the root samples at four different growth stages were all more than zero, whereas those of leaf samples were all less than zero. The score for stem samples were divided into two groups, one group included LE and FB stages, the scores were less than zero; the other group included SM and D stages, the scores were more than zero (Fig. 2). PCA showed that, for the plant organs, the structure of endophytic fungal communities differed significantly by growth stages of LE and D; for the plant growth stages, the structure of endophytic fungal communities differed significantly by plant organs (Fig. 2).

#### Discussion

This study constitutes the first research on the endophytic fungal community of *S. chamaejasme*. The study's analysis of endophytic diversity was performed at four growth stages characterized by different developmental stages and seasonal variation. The fungal communities were studied with a cultivation-based approach on samples from three different plant tissues, the leaves, stems and roots. The isolated fungal species were identified using molecular methods. The culture method was chosen in order to be in a position to conduct experiments on the ecological role of these fungi (especially for their ability to produce natural secondary metabolites which can be applied as biocontrol agents) (Wearn et al. 2012).

**Fig. 2** Principal component analysis (PCA) of all tissue samples of *S. chamaejasme* at different growth stages. X axis was defined by PC1 which represented 47.05 % of the total variance and Y axis was defined by PC2 which represented 32.38 % of the total variance (*LE* leaf emergence; *FB* full bloom; *SM* seed maturation; *D* dormancy; *l* leaves; *st* stems; *r* roots.)



The total colonization frequency and species richness of endophytic fungi were higher in the below ground part (roots) than in the aboveground parts (leaves and stems) in our study. One likely reason is soil-borne fungi are typically much more prevalent and diversified than those that infect aerial plant tissues (Ghimire et al. 2011). The other reason is the main sources of easily accessible substrate are roots, and roots might be considered as a relatively stable environment adequate for many fungal species (Garbeva et al. 2004). It may be that the roots of *S. chamaejasme* release a wide variety of compounds/exudates into the surrounding soil and create a nutrient rich environment favoring fungal survival and coexistence in roots organs. Similar results have been reported in previous endophytes studies (Ghimire et al. 2011; Angelini et al. 2012). However, this point has disagreement with some other studies. For example, Mishra et al. (2012) reported that frequency of colonization and species richness of fungal communities associated with *Tinospora cordifolia* were highest in the leaves and lowest in the roots. In *Holcus* leaves and roots, the number of species identified and the Shannon index were very similar for both organs (Sánchez Márquez et al. 2010). In our study, the total colonization frequency and species richness of endophytic fungi were slightly higher in the stems than that in the leaves. This is in agreement with other previous studies (Guo et al. 2008; Sun et al. 2012). This may be due to the structure and substrates are different between stem and leaf tissues, which influence the infection of endophytic fungi (Rodrigues 1994), or that the stems are more permanent than leaves, so that the stems accumulate more fungal species (Guo et al. 2008; Sun et al. 2012). In addition, the relative abundance of each endophytic

species identified in *S. chamaejasme* reflects a very unequal distribution of isolate richness among species. Such unequal distributions of endophytic fungal species have been reported in other plants (Sánchez Márquez et al. 2010; Ghimire et al. 2011).

The isolation-based study and molecular identification showed that the total fungal diversity was relatively high, consisting of 40 OTUs belonging primarily to the Ascomycota (99.6 % of the total number of isolates). Only a few basidiomycota isolates were obtained, and represented by a single OTU. This finding is consistent with the results of previous studies in which a predominance of ascomycetes were reported as a characteristic of endophytic mycobiota (Rakotoniriana et al. 2008; Angelini et al. 2012). Basidiomycetes are generally recorded only rarely as stem endophytes. The single basidiomycete species (*Schizophyllum commune*, 0.4 % of the total number of isolates) in our study was found only associated with the stem tissues at D stage.

Pleosporales (25.6 % of the total number of isolates) and Hypocreales (42.4 % of the total number of isolates) were the orders contributing the most species to the endophytic assemblages of *S. chamaejasme*. This result was consistent with the previous studies in many semiarid plants (Porrás-Alfaro et al. 2007; Herrera et al. 2010). It seems that these fungi form important, intimate and long lasting relationships with *S. chamaejasme*.

In the present study, *Alternaria* was a dominant genus associated the leaf and stem tissues of *S. chamaejasme*. Although species of *Alternaria* are mainly saprophytes that are commonly found in soil or on decaying plant tissues, many recent studies have

demonstrated that species belonging to the *Alternaria* genus are true endophytes associated with temperate and tropical plants (Morakotkarn et al. 2006) and an Antarctic grass (*Deschampsia antarctica*) (Rosa et al. 2009). The genus *Fusarium* represented the most abundant endophytic fungi recovered from the roots of *S. chamaejasme* at almost all growth stages, but was also present in leaf and stem tissues at FB and SM stages. The species of *Fusarium* often are found among the most frequently isolated endophytes in tropical plants (Vega et al. 2010). The plant tissue becomes highly resistant to subsequent invasions by other fungi when occupied by species of *Fusarium*. This was because it is a protected niche in which the primary colonizer has an increased capability to resist replacement by other endophytic fungi (Lockwood 1981).

The aboveground parts (leaf and stem) and belowground part (root) are the interfaces which interact most dynamically with the environment due to the very different ecological niches (different water content, organic matter, light and UV radiation levels) they represent. It is reasonable to expect that different plant organs are inhabited by distinctive endophytic communities (Arnold 2007; Angelini et al. 2012). As expected, tissue specificity of endophytes was demonstrated in this present study. For example, *Sporormiella* sp. and *Sordaria lappae* were only isolated from leaves; *Botryotinia fuckeliana*, *Schizophyllum commune*, *Dothiorella iberica* and *Cadophora malorum* were only isolated from stems; and *Bionectria ochroleuca*, *Ilyonectria* sp., and *Mucor* sp. were only isolated from roots. Some previous studies in other plants have also indicated that endophytes exhibit tissue specificity (Rodrigues 1994; Kumar and Hyde 2004; Sun et al. 2012). Particular endophytes found only in one tissue suggest that certain species survive within a specific chemistry or texture of different tissues (Rodrigues 1994).

Endophyte assemblages were also influenced by growth stages of *S. chamaejasme* in our study. There were not only differences in colonization frequency and species richness (Table 1), but also differences in fungi in certain orders (Table 2) throughout the growing season. The variations observed indicate that for most endophytes, individual infections are not persistent. Ongoing turnover of endophyte populations within a single plant organ may occur (Joshee et al. 2009). One possibility for this variation may be that

there are very few endophytes in newly developed leaves and stems of *S. chamaejasme*, but as the leaves and stems mature, the density and diversity of fungi increase. Over a period of several months, fungal diversity declines, but density continues to increase. In addition, the root tissues of *S. chamaejasme* remain more persistent throughout the growing season, while leaf and stem tissues of senescing or pre-senescent have lower nutrient content, which could be less suitable for endophytic growth. The other reason for this variation may be environmental factors, such as temperature, humidity, and ecological niches. These factors may determine spread and germination success of endophytic fungal spores (Schulthess and Faeth 1998).

Endophytic fungi are considered potential sources of novel antimicrobials metabolites after the discovery of taxol from *Tinospora andreanae*, an endophyte of *Taxus brevifolia* (Stierle et al. 1993; Mishra et al. 2012). Species of the genera *Chaetomium*, *Penicillium* and *Streptomyces* which were isolated from *Oryza sativa* exhibited antifungal activity against various phytopathogens (Shankar et al. 2007). *Alternaria* sp., *Nigrospora oryzae* and *Papulospora* sp. which were isolated from medicinal plants showed inhibitory activity against both Gram positive and Gram negative bacteria and also against *Candida albicans* (Raviraja et al. 2006). Endophytic *Penicillium* spp. are well known to produce a plethora of secondary metabolites with antibiotic, anticancer and/or cytotoxic activity (Wang et al. 2010; Mishra et al. 2012). Some endophytic *Penicillium* species also produce plant growth hormones such as gibberellins (Hamayun et al. 2010). At present, more than 150 bioactive compounds were isolated during a screening of 6,500 endophytic fungi, and more than 50 % of these compounds were significantly active against various pathogenic microbes (Hyde and Soyong 2008). The endophytes isolated from *S. chamaejasme* in this study offer an excellent platform for the discovery of novel bioactive (antibacterial, antifungal, anticancer and cytotoxic) compounds.

Although the ITS region is typically variable and rich in informative sites and has the highest probability of successful identification for the broadest range of fungi, it is unlikely that a single-marker barcode system will be capable of identifying every specimen or culture to species level. Furthermore, the limitations of ITS sequences for identifying species in some

groups and the failure of the universal ITS primers to work in a minority of other groups will have to be carefully documented (Oechsler et al. 2009; Schoch et al. 2012). Taking these factors into account, the species level identification of *Fusarium* isolates from ITS sequences were tentative, and phylogenetic species were recognized if they received  $\geq 50$  % bootstrap values from 1,000 replicates in this study.

This study shows that *S. chamaejasme* harbours a wide diversity of fungal endophytes, and provides a reservoir of strains for the search of bioactive secondary metabolites which can be screened against different drugable targets. Based on this purpose, the fungal communities were determined using a culture-based technique. However, this approach may have overlooked the presence of uncluturable or slow-growing species (Hyde and Soyong 2008). Culture-independent (direct molecular analysis) approaches can be used to more comprehensively and systematically explore the fungal endophytes associated with *S. chamaejasme*.

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