

ROOT-ENDOPHYTIC FUNGI DIVERSITY OF FUZI (*ACONITUM CARMICHAELII*) AND THEIR ANTI-FUNGAL ACTIVITY

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Abstract. Endophytic fungi (EFs) are one of the important and quantifiable components of fungal biodiversity. In the study, 171 endophytic fungi were isolated from the roots of *Aconitum carmichaelii*, a traditional Chinese medicine, collected from six production sites of Yunnan Province in southwestern China. These EFs were categorized to belong to 28 taxa by molecular identification, and *Chaetomium* and *Fusarium* were the dominant genera with a relative frequency of 16.96% and 16.37% respectively. *Cladosporium* and *Pochonia* were found to be unique dominant genera from the roots of *A. carmichaelii* in Huize and Yulong counties, respectively. The Shannon index ranged from 1.68 to 2.05 and the Simpson's diversity index ranged from 0.81 to 0.92 for the EFs of *A. carmichaelii*, indicating the rich diversity of EFs in *A. carmichaelii*. Preliminary screening of the growth inhibition of three severe fungal pathogens by the isolated EFs was conducted and 17 genera of EFs were found to inhibit the growth of the tested pathogens. For example, *Acrostalagmus luteoalbus*, *Phoma* sp., and *Thielavia* sp. each displayed good anti-fungal activity against *Alternaria alternate*, *Fusarium oxysporum* f. sp. *cucumerinum*, and *Phytophthora drechleri*. The investigation of the root-EFs diversity of *A. carmichaelii* provides additional fungal resources with the potential to biologically control pathogenic fungi.

Keywords: *traditional Chinese medicine, fungal biodiversity, community composition, ITS sequence, antagonism against fungal pathogens*

Introduction

Endophytic fungi (EFs), well-studied to be potential resources for producing bioactive natural products (Aly et al., 2008), asymptotically infect healthy plant tissues for all, or at least a significant part of their life cycles (Hyde and Soyong, 2008). The diversity of endophytes has been fully investigated in the case of many plant taxa (Sun et al., 2011; Silva et al., 2012; Kohout et al., 2013; Fernandes et al., 2015; Nascimento et al., 2015), and endophytes were shown to increase ecological adaptation by protecting plants against biotic and abiotic stresses (Liu et al., 2009; Morsy et al., 2010; Bittleston et al., 2011).

Nowadays, drug-resistance among bacteria and fungi is spreading significantly, and therefore further search is urgently needed for new and effective antimicrobial agents. Endophytes have the potential to produce bioactive compounds, such as alkaloids, resulting in the improvement of the evolutionary fitness of their host by providing resistance against micro-pathogens (Strobel et al., 2004; Kusari and Spiteller, 2012; Newman, 2015). EFs can produce the antimicrobial compounds with inhibitory activity toward pathogenic fungi (Li et al., 2005), and therefore can be helpful in fungal biocontrol in agricultural production (Mishra et al., 2012).

Aconitum L. is a large genus of more than 400 species, mainly distributed in the temperate regions of the northern hemisphere (Xiao et al., 2006). *Aconitum carmichaelii*, a perennial plant of the Ranunculaceae family, is one of the famous traditional Chinese medicines (TCM) fuzi (附子). As one of the most important TCMs, it has been used extensively by humans as a cardiogenic, to protect cardiomyocytes, as an analgesic and to treat arrhythmia (Zhou et al., 2015). However, there has been only one preliminary study about the EFs in *Aconitum* (Li et al., 2009). We aimed to isolate and identify naturally-occurring EFs in *A. carmichaelii* roots collected from the main production area of Yunnan Province, China, and explore their potential inhibitory activity against three primary fungal pathogens, *Alternaria alternate*, *Fusarium oxysporum* f. sp. *cucumerinum*, and *Phytophthora drechsleri*.

Materials and methods

Plant materials

The roots of *A. carmichaelii* were sampled in September 2014 across six selected counties including Heqing, Huize, Luquan, Malong, Panlong, and Yulong in Yunnan Province, southwestern China (Fig. 1), in order to maximize the endophytic fungal diversity. These *A. carmichaelii* were cultivated by local people. Five plants were randomly selected from each site, from which 3-5 lateral roots were collected. The roots and associated soil were packaged separately in sealable plastic bags, immediately transported to the laboratory, and then stored at 4 °C for isolating of endophytic fungi.

Endophyte isolation and primary classification based on morphological traits

The collected roots were washed under running tap water for 5 min, sterilized in 75% ethanol for 1 min, rinsed once in sterile water, 2% NaOCl for 5 min, and then rinsed three times in sterile water. After drying the roots on sterile paper, they were cut into segments, each about 1cm long. From each of the six collections, we randomly selected 45 segments, and divided into them into three groups with 15 segments each to comprise a replicate sample. The samples were placed in Petri dish containing potato

dextrose agar (PDA) medium amended with 0.5 g/L streptomycin sulfate, with one segment per plate. The plates were incubated at 28 °C and checked every other day for 3 weeks; and the fungi growing out of the roots were transferred to fresh PDA plates. Primary classification was carried out based on mycelia and colony morphological traits and secretion. After that, at least 50% isolates in each group were randomly selected for the molecular identification.

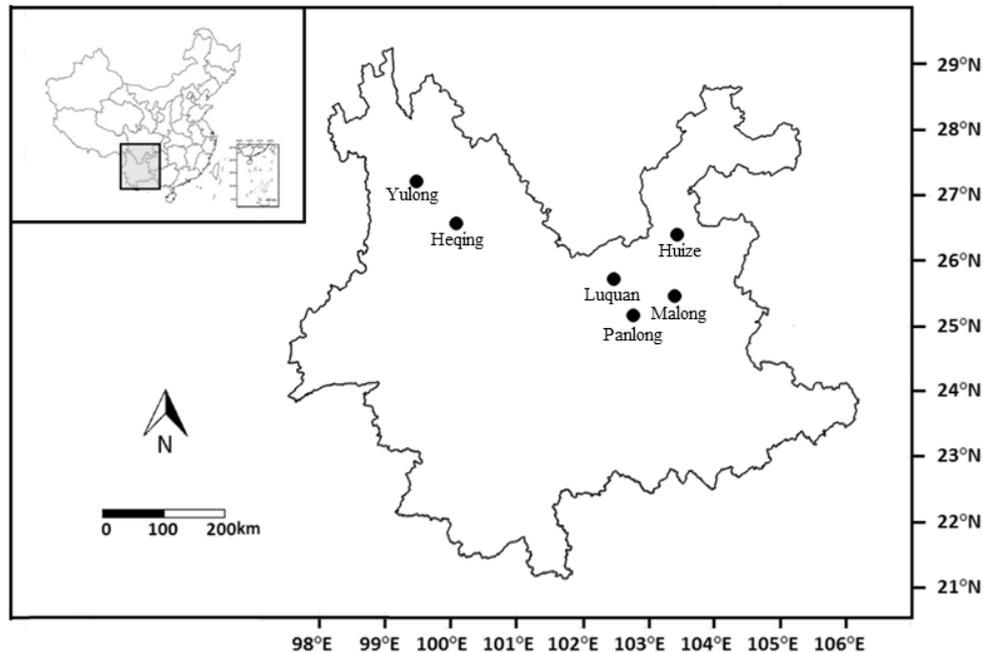


Figure 1. The geographical locations from left to right represent Yulong, Heqing, Luquan, Panlong, Malong, and Huize counties

Fungal identification by DNA analysis

Fungal DNA was extracted from pure culture mycelia using a 1 × cetyltrimethylammonium bromide (CTAB) method (Niu et al., 2010). The internal transcribed spacer (ITS) region of nuclear rDNA was amplified with the universal eukaryotic primer pair ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR amplifications were performed in a reaction mixture containing 2 × PCR EasyTaq PCR SuperMix, DNA extracts, and the primer pair. Thermocycler settings were 5 min initial denaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C, 50 s at 53 °C, 1 min at 72 °C, with a 10 min final extension at 72 °C. Sequences were manually inspected and edited using Chromas 2.4.1. Putative identification of sequences was carried out by comparing consensus sequences to the National Center for Biotechnology Information (NCBI) DNA database using the Basic Alignment Search Tool (BLAST). The following parameters were used to determine significance of BLAST results: alignment score > 500, $E < 0.0001$, and over 97 % sequence similarity were identified as the same species. The ITS sequences of those isolates were deposited in NCBI (MF962929-MF963015). Through sequences analysis in comparison with each other, phylogenetic tree figure of the isolated EFs was constructed using neighbor-joining (NJ) methods with 1000 boot-strap replications using MEGA 6.0.

Analyses of relative frequency and diversity index

The colonization rate was calculated as follows (Eq. 1) (Su et al., 2010), where N_i is the total number of segments colonized by endophytes, and N_t is the total number of segments.

$$\text{colonization rate (\%)} = \frac{N_i}{N_t} \times 100 \quad (\text{Eq.1})$$

The relative frequency (RF %) (Yuan et al., 2010) was calculated according to the following formula (Eq. 2), where n_i is the number of isolates of one species divided, and the N is the total number of isolates.

$$\text{RF(\%)} = \frac{n_i}{N} \times 100 \quad (\text{Eq.2})$$

The Shannon index (H') (Shannon and Weiner, 1963) was calculated according to the following formula (Eq. 3), where k is the total species number of one plot and P_i is the relative abundance of endophytic fungal species in the community.

$$H' = -\sum_{i=1}^k (P_i \times \ln P_i) \quad (\text{Eq.3})$$

The fungal dominance was determined by Camargo's index (Eq. 4) (Hunter and Gaston, 1988), where S was species richness = the number of species present in any given sample. A species was defined as dominant if $P_i > \text{Camargo's index}$.

$$\text{Camargo's index} = \frac{1}{S} \quad (\text{Eq.4})$$

Simpson's index (D) and Simpson's diversity index ($1-D$) (Camargo, 1993), where D was calculated as (Eq. 5). D could range between 0 (infinite diversity) and 1 (no diversity).

$$D = \sum_{i=1}^k \left(\frac{n_i \times (n_i - 1)}{N \times (N - 1)} \right) \quad (\text{Eq.5})$$

Anti-fungal activity assays

The anti-fungal activity of the EFs was evaluated against three fungal pathogens: *Alternaria alternate*, *Fusarium oxysporum* f. sp. *cucumerinum*, and *Phytophthora drechsleri* (all provided by Professor Zhiwei Zhao of the State Key Laboratory for Conservation and Utilization of Bio-Resources, Yunnan University). A dual-culture technique was modified to study antagonism (Gao et al., 2002). The pathogen was inoculated on PDA medium center and four different isolated fungi were inoculated around pathogen, incubated at 28 °C for a week, and the anti-fungal zone were measured by ruler (mm).

Results

Colonization rates and diversity of EFs

Colonization rate is an indication of the number of EFs in host plants and varies with the altitude, humidity, precipitation, temperature and plant community (Hashizume et al., 2010). In the present study, the colonization rate of the EFs from six sites ranged from 46.7% to 73.3%, and the highest colonization rate appeared in Malong, while the lowest was appeared in Panlong (Table 1). To further investigate the biodiversity of the EFs, we calculated the Shannon diversity index (H'), Simpson's index (D) and Simpson's diversity index (1-D). The Shannon diversity indexes (H') of the endophytic community in the six different sites were 1.98, 1.88, 1.82, 1.76, 2.05 and 1.68, respectively. The Simpson's diversity index is between 1 and 0, with 1 representing infinite variety of diversity, and 0 representing no diversity. The Simpson's diversity index showed a similar pattern for the six sites, which were 0.92, 0.85, 0.88, 0.89, 0.88 and 0.81, respectively.

Table 1. Number, colonization rate, Shannon-Weiner index (H') and Simpson's diversity index (1-D) of the root- endophytic fungi (EF) of *A. carmichaelii* from the six regions of Yunnan, China

Collection site	No. of EFs isolated	Colonization rate (%)	H'	1-D
Heqing	26	48.9	1.98	0.92
Huize	44	60.0	1.88	0.85
Luquan	38	64.4	1.82	0.88
Malong	38	73.3	1.76	0.89
Panlong	26	46.7	2.05	0.88
Yulong	39	53.3	1.68	0.81
Total	171	57.8		

Community composition of EFs

A total of 171 isolates were obtained from collected roots, and identified into 28 taxa by molecular identification based on rDNA ITS sequence analysis (Table 2). A total of 10 endophytic taxa from Heqing; 12 taxa were identified from Huize; 10 taxa from Luquan; 12 taxa from Malong; 11 taxa from Panlong; and 10 taxa from Yulong (Table 2). The dominant taxa determined by the Camargo's index and P_i were *Chaetomidium arxii*, *Chaetomium* sp., and *Phoma* sp. in Heqing; *Chaetomium* sp., *Cladosporium* sp., and *Fusarium* sp. in Huize; *Chaetomidium arxii*, *Plectosphaerella* sp., and *Pochonia* sp. in Luquan; *Chaetomium* sp. and *Plectosphaerella* sp. in Malong; *Chaetomidium arxii*, *Chaetomium globosum*, *Ilyonectria* sp., *Phoma* sp., and *Plectosphaerella* sp. in Panlong; and *Fusarium oxysporum* and *Ilyonectria* sp. in Yulong. Only *Chaetomidium arxii* and *Plectosphaerella* sp. were identified in all six sites (Table 2). The rates of colonization for *Chaetomium* sp. in Huize and *Fusarium oxysporum* in Yulong were higher than in other sites (Table 2). Endemic endogenous fungi were isolated from five habitats of *A. carmichaelii*, including: Huize (*Cladosporium* sp., *Cylindrocarpon* sp., *Mucor racemosus*, and *Verticillium tricorpus*); Luquan (*Bjerkandera* sp., *Pochonia* sp., and *Thielavia* sp.); Malong (*Acrostalagmus luteoalbus* and *Trametes versicolor*); Panlong (*Daldiniafissa* sp.); and Yulong (*Clonostachys rosea* and *Paraphaeosphaeria sporulosa*). The phylogenetic tree indicated that *Chaetomium* sp., *Fusarium* sp. and *Plectosphaerella* sp. were the

dominant EFs in this medicinal plant. In this study, phylogenetic analysis revealed that all sequences clustered in at least 18 discrete sequence groups (Fig. 2). The majority of clones (73.08%) fell into the G 1 (Group 1), G 2 (Group 2) and G 4 (Group 4), which were distributed across most root samples.

Anti-fungal activity assays

The diversity and antimicrobial potential of EFs from a number of medicinal plants have been studied (Qin et al., 2009; Egamberdieva et al., 2017; Xiang et al., 2017). Our screening of anti-fungal activity of the isolated EFs identified 17 genera which are resistant to the three fungal pathogens (*Alternaria alternata*, *Fusarium oxysporum* f. sp. *cucumerinum*, and *Phytophthora drechsleri*) in agricultural production (Table 3). *Acrostalagmus luteoalbus* (LD34), *Phoma* sp. (MC6), and *Thielavia* sp. (LQ14) displayed good anti-fungal effects, and the anti-fungal zone for *Alternaria alternata*, *Fusarium oxysporum* f. sp. *cucumerinum*, and *Phytophthora drechsleri* were 4, 3, and 2 mm; 5, 3, and 3 mm; and 4, 3, and 3 mm, respectively, although they accounted for the small proportion of the colonization (Table 3). In addition, *Chaetomium* sp. and *Fusarium* sp., two dominant strains obtained in this experiment, also showed some anti-fungal activity (Table 3).

Table 2. Number, taxa and relative frequency (RF) of the root-EFs of *A. carmichaelii* collected from six regions of Yunnan, China

Taxa	No. of strains isolated from each regions (RF %)						Total (RF %)
	Heqing	Huize	Luquan	Malong	Panlong	Yulong	
<i>Acrostalagmus luteoalbus</i>				1(2.63)			1(0.58)
<i>Alternaria</i> sp.			1(2.63)		1(3.85)		2(1.17)
<i>Bjerkandera</i> sp.			1(2.63)				1(0.58)
<i>Chaetomidium arxii</i>	3(11.54)	2(4.55)	6(15.79)	1(2.63)	3(11.54)	1(2.56)	16(9.36)
<i>Chaetomium globosum</i>		2(4.55)			3(11.54)		5(2.92)
<i>Chaetomium</i> sp.	3(11.54)	12(27.27)		6(15.79)	1(3.85)	2(5.13)	24(14.04)
<i>Cladosporium</i> sp.		6(13.64)					6(3.51)
<i>Clonostachys rosea</i>						2(5.13)	2(1.17)
<i>Cryptococcus</i> sp.	1(3.85)		1(2.63)				2(1.17)
<i>Cylindrocarpon</i> sp.		1(2.27)					1(0.58)
<i>Daldinia fissa</i>					1(3.85)		1(0.58)
<i>Fusarium oxysporum</i>	1(3.85)	2(4.55)		3(7.89)		11(28.21)	17(9.94)
<i>Fusarium</i> sp.	2(7.69)	4(9.09)		2(5.26)	1(3.85)	2(5.13)	11(6.43)
<i>Geomyces</i> sp.				1(2.63)	1(3.85)		2(1.17)
<i>Ilyonectria</i> sp.	4(15.38)		2(5.26)	1(2.63)	1(3.85)	9(23.08)	17(9.94)
<i>Irpex lacteus</i>				1(2.63)		1(2.56)	2(1.17)
<i>Mortierella</i> sp.		1(2.27)		1(2.63)			2(1.17)
<i>Mucor racemosus</i>		1(2.27)					1(0.58)
<i>Paraphaeosphaeria sporulosa</i>						1(2.56)	1(0.58)
<i>Penicillium</i> sp.	1(3.85)	1(2.27)	2(5.26)				4(2.34)
<i>Phoma</i> sp.	3(11.54)				6(23.08)	2(5.13)	11(6.43)
<i>Plectosphaerella</i> sp.	2(7.69)	3(6.81)	5(13.16)	6(15.79)	6(23.08)	1(2.56)	23(13.45)
<i>Pleosporales</i> sp.				1(2.63)	1(3.85)		2(1.17)
<i>Pochonia</i> sp.			7(18.42)				7(4.09)
<i>Thielavia</i> sp.			3(7.89)				3(1.75)
<i>Trametes versicolor</i>				2(5.26)			2(1.17)
<i>Trichocladium</i> sp.	2(7.69)		2(5.26)				4(2.34)
<i>Verticillium tricorpus</i>		1(2.27)					1(0.58)
Total taxa recovered	10	12	10	12	11	10	28



Figure 2. Neighbor-joining tree of ITS sequence of endophytic fungi associated with *Aconitum carmichaelii* constructed with a 1000 boot-strap replications. Bar: 0.5% sequence divergence. Numbers above the nodes indicate bootstrap support in neighbor-joining analysis. Representative sequences with Anti-black triangles (▼), black triangles (▲), white pentagram (☆), black pentagram (★), white square (□), black square (■) represent EFs originating from Heqing, Huize, Luquan, Malong, Panlong, Yulong. Arabic numbers in parentheses indicate the number of sequences (above slash) and the number of sequenced clones (below slash). §: represents the ratio of the numbers of sequences in a given clade to the total sequenced clones

Table 3. The anti-fungal activity of EF collected from *A. carmichaelii*

Taxa	No.	Pathogen fungi (mm)		
		<i>Alternaria alternate</i>	<i>Fusarium oxysporum</i> f. sp. <i>Cucumerinum</i>	<i>Phytophthora drechsleri</i>
<i>Acrostalagmus luteoalbus</i>	ML20	3	3	3
<i>Alternaria</i> sp.	PL16	1	1	2
<i>Chaetomidium arxii</i>	LQ30	1	1	2
<i>Chaetomidium arxii</i>	PL1	1	2	1
<i>Chaetomidium arxii</i>	PL18	1	1	1
<i>Chaetomidium arxii</i>	YL15	1		2
<i>Chaetomium</i> sp.	HZ34	1	1	1
<i>Chaetomium</i> sp.	HZ35	2		
<i>Chaetomium</i> sp.	HZ36	1	1	1
<i>Chaetomium</i> sp.	ML7	2		2
<i>Chaetomium</i> sp.	ML11	1	1	2
<i>Clonostachys rosea</i>	YL32	1		1
<i>Cylindrocarpon</i> sp.	HZ15	1		1
<i>Fusarium oxysporum</i>	YL17		2	1
<i>Fusarium</i> sp.	HQ1			2
<i>Fusarium</i> sp.	HZ11	1		1
<i>Ilyonectria</i> sp.	ML4	2	1	1
<i>Ilyonectria</i> sp.	PL13	2		2
<i>Ilyonectria</i> sp.	YL34	3	3	4
<i>Mortierella</i> sp.	HZ39	1	1	1
<i>Paraphaeosphaeria sporulosa</i>	YL30	2		1
<i>Phoma</i> sp.	HQ6	3	3	5
<i>Plectosphaerella</i> sp.	HZ43	1	1	1
<i>Plectosphaerella</i> sp.	PL17	1	2	2
<i>Pleosporales</i> sp.	PL20		2	1
<i>Thielavia</i> sp.	LQ14	2	3	4
<i>Trichocladium</i> sp.	HQ24	1	1	
<i>Trichocladium</i> sp.	LQ22	2	2	
<i>Verticillium tricorpus</i>	HZ41		1	2

The values of the anti-fungal zone in the table are the average of the three experiments. HQ, HZ, LQ, ML, PL, and YL are indicative of Heqing, Huize, Luquan, Malong, Panlong, and Yulong, respectively and the number after abbreviations showed the isolation order of the endophytic fungi

Discussion

Endophytic fungi were isolated from *Aconitum carmichaelii*, one of the most important TCMs, which laid the foundation for future biological control research. In this study, roots of *A. carmichaelii* were collected from six sites in Yunnan Province, the People's Republic of China for the isolation, identification and antibacterial analysis of EFs. Colonization rate and Shannon diversity index (H') of the EFs in different areas (Table 1) indicated that there were rich root-fungal endophytes existing in *A. carmichaelii* in Yunnan. In all isolates, only *Chaetomidium arxii* and *Plectosphaerella* sp. were identified in all six sites (Table 2), suggesting that these two endophytic fungi

are host-generalists. The rates of colonization for *Chaetomium* sp. in Huize and *Fusarium oxysporum* in Yulong were higher than in other sites (Table 2). This may be due to the fact that the roots are persistent, and can form unique endophytic fungal community. In addition, Huize has been documented heavy metal contamination (Lu et al., 2014), and *Chaetomium* sp. is known as a soil and contaminant fungi (Abdel-Lateff, 2008). Endemic EFs were isolated from five habitats, and the population structure of endophytic fungi normally represents a certain degree of regional specificity (Jia et al., 2016). In contrast to the endemic EFs, dominate endophytic fungi lived in a wide variety of plants such as *Phoma* sp. (Nisa et al., 2015), which was also isolated from *A. carmichaelii* with a RF as 6.43%.

Screening of anti-fungal activity of the isolated EFs identified 29 isolates, such as *Chaetomium* sp. (Table 3), with wide-spectrum antibacterial activity (Li et al., 2009). Previous research showed that *Chaetomium* sp. suppressed the growth of bacteria and fungi through direct competition, mycoparasitism, and antibiosis (Rodríguez et al., 2002). Two antibacterial furano-polyenes, (-) - musanahol and 3-epi-aureonitol, were isolated from cultures of *Chaetomium* sp. (Marwah et al., 2007). Dutch elm disease, caused by the fungal pathogen *Ceratocystis ulmi*, can be controlled by the endophytic fungus *Phomopsis oblonga* (Webber, 1981). Leutou et al. (2016) concluded that the microbial transformation of anthranilic acid by the marine-mudflat-derived fungus *Thielavia* sp. produced an antibacterial polycyclic quinazoline alkaloid, thielaviazoline. *Fusarium oxysporum* is well represented in the rhizosphere microflora (Lecomte et al., 2016). Some isolates can induce wilt or root rots while others are considered as nonpathogenic taxa. For example, nonpathogenic *F. oxysporum* in soil can suppress *Fusarium* wilts (Fravel et al., 2003), and the strain has also attracted interest for soil bioremediation and water purification due to its capability to detoxify and colonize polluted environments (Steinberg et al., 2016). Ratnaweera et al. (2015) found that equisetin, isolated from endophytic *Fusarium* sp., exhibited antibacterial activities against the Gram-positive bacteria.

Some recent studies have shown that *Lentzea* sp., *Nigrospora* sp. and *Streptomyces* sp. isolated from *A. carmichaelii* have antibiotic activity (Qiu et al., 2015; Zhang et al., 2016). EFs isolated from medicinal plants sometimes produce similar compounds as their hosts (Newman, 2015). Diterpene alkaloids are the dominant secondary metabolites of *Aconitum* species (Zhou et al., 2015), and other diterpene alkaloids have been reported to be produced by endophytic fungi of the genus *Epichloë* in perennial ryegrass (Philippe, 2016). Based on the anti-fungi activity of diterpene alkaloids (Kobayashi et al., 1998), we hypothesize that certain EFs from *A. carmichaelii* could produce aconitum alkaloids that inhibit these three fungal pathogens. We plan to examine the chemistry of these EFs in the future.

In this study, 171 strains of EFs were isolated and identified from six parts of *A. carmichaelii* roots, of which 29 isolates in 17 genera showed some level of anti-fungal activity. This study helps to lay a foundation for the potential association of EFs and disease resistance in *A. carmichaelii*.

Conclusion

Isolated from the roots of *A. carmichaelii* from six different production sites in Yunnan province, China, the root-endophytic fungi are assigned to 28 taxa. Among these identified taxa, *Chaetomium* and *Fusarium* were the dominant genera in all six

sites while *Cladosporium* and *Pochonia* were unique dominant genera in Huize and Yulong counties. *Acrostalagmus luteoalbus*, *Phoma* sp., and *Thielavia* sp. displayed good anti-fungal activity against *Alternaria alternate*, *Fusarium oxysporum* f. sp. *cucumerinum* and *Phytophthora drechsleri*, providing new biological control clues for these pathogenic fungi. In subsequent experiments, further investigation on whether the endophytic fungi have the same fungal pathogen resistance *in vivo* was necessary. In addition, exploring the chemical composition against these fungal pathogens of endophytic fungi would be helpful for identifying the active leading chemical compounds for fungal disease control.

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