Endophytic fungal community of *Dysphania ambrosioides* from two heavy metal-contaminated sites: evaluated by culture-dependent and culture-independent approaches

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Summary

Endophytic fungal communities of *Dysphania ambrosioides*, a hyperaccumulator growing at two Pb-Zn-contaminated sites, were investigated through culture-dependent and culture-independent approaches. A total of 237 culturable endophytic fungi (EF) were isolated from 368 tissue (shoot and roots) segments, and the colonization rate (CR) ranged from 9.64% to 65.98%. The isolates were identified to 43 taxa based on morphological characteristics and rDNA ITS sequence analysis. Among them, 13 taxa (30.23%) were common in plant tissues from both sites; however, dominant EF were dissimilar. In culture-dependent study, 1989 OTUs were obtained through Illumina Miseq sequencing, and dominant EF were almost same in plant tissues from both sites. However, some culturable EF were not observed in total endophytic communities. We suggest that combination of both culture-dependent and culture-independent methods will provide more chances for the precise estimation of endophytic fungal community than using either of them. The tissue had more influence on the culturable fungal community structure, whereas the location had more influence on the total fungal community structure (including culturable and unculturable). Both culture-dependent and culture-independent studies illustrated that endophytic fungal communities of *D. ambrosioides* varied across the sites, which suggested that HM concentration of the soil may have some influence on endophytic fungal diversity.

Introduction

The soil is important and essential for supporting life and planetary functions such as primary production, the regulation of biogenic gases and the earth’s climate, biogeochemical and water cycling, and the maintenance of biodiversity (Abhilash et al., 2012). Rapid and continuous worldwide industrialization, urbanization and modern agriculture practices have introduced excess of the heavy metals (HMs) into the soil. Due to their persistence in soil and their toxic nature, HMs adversely impact the ecosystem, agriculture, water quality, soil microbiota and human health (Rajkumar et al., 2010; Kidd et al., 2012; Wei et al., 2014). To mitigate the negative effects of HMs, the remediation of contaminated soils is gaining considerable momentum. Among all remediation methods, phytoremediation is considered as the most promising technology for its low tech-savvy technique, cost-effectiveness, sustainability and environment friendliness (Weyens et al., 2009a; Li et al., 2012a; Parmar and Singh, 2015). However, phytoremediation has some constraints, such as phytotoxicity, slowed plant growth, low biomass production, slow degradation of HMs, limited contaminant uptake and evapotranspiration of volatile contaminants; therefore, application of phytoremediation is limited in most circumstances (Gerhardt et al., 2009; Weyens et al., 2009b; Deng and Cao, 2017). Microbe-assisted phytoremediation can effectively reduce this problem. For some microorganisms, it can effectively improve the plant growth by transformation of nutrient elements, production of phytohormones, or provide iron to reduce the deleterious effects of metal contamination to plants (Rajkumar et al., 2010).

Endophytic fungi (EF) can be defined as fungi that reside asymptotically in the interior of host plant...
tissues (Hyde and Soytong, 2008). They are ubiquitous in nature and have been successfully isolated from wide range of hosts belonging to a wide range of environmental conditions (Redman et al., 2002; Bashyal et al., 2005; Rosa et al., 2009; Mishra et al., 2012). It was estimated that there are at least one million species of EF worldwide (Ganley et al., 2004). Interestingly, diverse EF are also found in highly HM contaminated environments (Xiao et al., 2010; Deng et al., 2011; Choo et al., 2015; Yamaji et al., 2016), and recent advances suggest that the EF can enhance HMs accumulation and tolerance capacity of host plants (Khan and Doty, 2011; Li et al., 2012c; Shen et al., 2013; Yamaji et al., 2016). The possible mechanism of increased tolerance to HM stress in the host plant by endophytes involves enhancements of antioxidative system, changing HM distribution in plant cells and detoxification of HM (Wang et al., 2016).

*Dysphania ambrosioides* (L.) Mosyakin & Clemants, previously known as *Chenopodium ambrosioides*, is an invasive plant in China. Previous studies have indicated that it is a dominant plant species in some Pb–Zn contaminated sites in Huize County, Yunnan Province, Southwest China (Li et al., 2012c, 2016), and it was reported as a Pb-hyperaccumulator (Wu et al., 2004). Our previous studies have revealed that *D. ambrosioides* growing in Pb–Zn contaminated locations have high diversity of EF, and some of them showed better Pb, Zn, Cd tolerance and could enhance host plant growth and affect its HMs accumulation (Li et al., 2016; Sun et al., 2018). To find out the role of endophytes in host plants’ HM adaptation and explore them in phytoremediation, the understanding of endophytic community is critical. Recent advances in the modern molecular phylogenetic and high-throughput DNA sequencing have provided an inclusive method to study culture-independent microbial community (Shakya et al., 2013; Senés-Guerrero and Schüßler, 2016; De Corte et al., 2018). Comparison of the abundance of culturable and unculturable endophytes by direct sequencing of *Deschampsia flexuosa* well established that unculturable endophytes are common and potentially more abundant than the culturables (Tejesvi et al., 2010). In this study, we aim to investigate both the culturable and total (including culturable and unculturable) endophytic fungal community of *D. ambrosioides* collected from two HM-contaminated through culture-dependent and culture-independent approaches.

**Results**

**Plant and soil properties**

The two investigated sites were situated in the area where Pb–Zn mining has been carrying out for more than 300 years. Consistent with this, soils and plants from both sampling sites were heavily polluted by Pb, Zn

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**Table 1.** Heavy metal content of plants and soils (mean ± SD).

| Sample site     | Plants (mg kg⁻¹, dry weight) | Soils (mg kg⁻¹, dry weight) |
|----------------|-----------------------------|-----------------------------|
|                | Total HM | Bio-available HM | Pb   | Zn     | Cd | Pb   | Zn | Cd |
| Slag heap      | 152.65 ± 24.65     | 1648.93 ± 27.12            | 1896.88 ± 24.65      | 61.27 ± 0.67 |
| Wasteland      | 264.13 ± 5.88      | 21436.25 ± 48.49           | 4262.37 ± 15.36      | 48.95 ± 0.15 |
| Standard       | 0.3 ± 0.05         | 200 ± 0.05                 | 500 ± 0.05           | 500 ± 0.05 |

Different letters in the same column indicate a significant difference at *P* < 0.05. Different symbols in the same column indicate a significant difference at *P* < 0.05.
and Cd (Table 1) (GB13106-1991, 1991; GB15618-1995, 1995; GB2762-2012, 2012). Overall, the level of HMs in the soils from the slag heap was comparatively lower than that from the wasteland. However, the bio-available (DTPA-TEA extractable fractions) concentrations of HMs in the soils from the slag heap were significantly lower (<0.05) than that from the wasteland. Consistent with this, the level of HMs in the plants from the slag heap was significantly lower (<0.05) than that from the wasteland. Except the available P, other soil physico-chemical characteristics, that is organic matter, total N, P, K, the available K and hydrolysable N, of the soils from the slag heap were dominantly lower than those of the soils from the wasteland (Table 2).

**Culturable endophytic fungal community**

A total of 237 EF were isolated from 368 tissue segments of *D. ambrosioides* growing naturally in the slag heap and wasteland (Table 3). The colonization rate (CR) was calculated as the total number of plant segments infected by one or more fungi divided by the total number of segments incubated (Sun et al., 2011). The colonization rate (CR) of different plant tissues from two sites ranged from 9.64% to 65.98% (Fig. 1). The total CR of plants from the slag heap (35.2%) was significantly (<0.05, chi-square test) lower than that of the plants from the wasteland (57.67%; Fig. 1). Similarly, the CR of the shoots from the slag heap (9.64%) was significantly lower (<0.05, chi-square test) than that of the shoots from the wasteland (65.98%). However, the CR of the roots from the slag heap (57.29%) was a little higher than that of roots from the wasteland (48.91%; <0.05, chi-square test) (Fig. 1).

The endophytic fungal isolates were identified to 43 taxa based on morphological characteristics and ITS sequence analysis (Table 3), and the rDNA ITS sequences of the fungi subjected to molecular identification in this study were deposited in GenBank (Accession numbers are KT291413, KT291414, KT291415, KT291416, KT291418, KT291419, KT291420, KT291422, KT291423, KT291428, KT291426 and KT291432). Among them, 13 taxa (30.23%) co-existed in plants from both sites, with a total of 27 and 29 taxa recorded in plants from the slag heap and wasteland respectively (Fig. 2a, Table 3). The relative frequency (RF) was calculated as the number of isolates of one species divided by the total number of isolates (Yuan et al., 2011a). The dominant EF of plants from the slag heap were *Plecosphaerella* sp., *Cladosporium* sp. 2 and *Verticillium* sp., showing RF of 18.07%, 10.84% and 8.43% respectively. The dominant EF of plants from the wasteland were *Phoma* sp., *Peyronelleta* sp., *Alternaria* sp. and *Cladosporium* sp. 1, showing RF of 20.13%, 12.34%, 11.69% and 7.14% respectively (Table 3).

The endophytic fungal community of shoots from two locations was clustered together, and similarly that of the roots together (Fig. 3). The endophytic fungal diversity was evaluated using the Shannon index (*H*), which has two main components, evenness and the number of species (Spellerberg and Fedor, 2003). The Simpson index (1−D) estimates the probability that two randomly selected individuals from a community belong to different species (Simpson, 1949). The *H* and Simpson (1−D) of EF from the slag heap and wasteland were 2.927 and 2.823, and 0.926 and 0.911 respectively (Table 3).

**Total endophytic fungal community**

A dataset was developed that consisted of 46 172 filtered high-quality and classified unique fungal ITS2 gene tags with a maximum length of 442 bp and minimum length of 238 bp (Table 4). All the sequences were clustered with the representative sequences, and >97% sequence identity cut-off was used; all tags of ITS2 region were classified at each level. The number of operational taxonomic units (OTUs) per sample ranged from 625 to 1168 (Table 4). In the plants from the slag heap, the OTUs of roots (625) were less abundant than that of shoots (679); however, in the plants from the wasteland, the OTUs of roots (1168) were more abundant than that of shoots (866) (Table 4).

The OTUs were analysed at different taxonomic level. Ascomycota was found to be the most abundant (62.5% to 79.2%) across all the samples analysed, followed by Basidiomycota (20.7% to 37.4%), while Chytridiomycota and Zygomycota were found to be rare and incidental (Fig. 4a). The relative abundance of *Cladosporium* sp.

**Table 2. Physico-chemical characteristics of soils.**

| Sample site  | pH  | Organic matter (g kg⁻¹, dry weight) | Total N | Total P | Total K | Hydrolysable N | Available P | Available K (mg kg⁻¹, dry weight) |
|-------------|-----|------------------------------------|---------|---------|---------|----------------|-------------|----------------------------------|
| Slag heap   | 7.79| 37.79 ± 0.35a                       | 0.77 ± 0.14a | 1.08 ± 0.03a | 3.1 ± 0.15a | 42.75 ± 9.32a | 21.01 ± 4.88a | 113.93 ± 9.36a                  |
| Wasteland   | 6.19| 109.29 ± 0.36b                      | 1.57 ± 0.03b | 2.03 ± 0.11b | 4.03 ± 0.15b | 88.63 ± 1.08b | 18.75 ± 1.56b | 181.43 ± 6.87b                  |

Mean ± standard deviation from three replicates. Different letters in the same column indicate a significant difference at *P* < 0.05.
was recorded to be highest across all the samples followed by Cryptococcus victoriae and Purpureocillium lilacinum (Fig. 4b). Other abundant species recorded were Aureobasidium pullulans, Aureobasidium sp., Alternaria alternate, Fusarium tricinctum, Filobasidium floriforme, Bullera coprosmae and Cryptococcus carnescens, while relatively large number (>12%) of species were remained unclassified (Fig. 4b). The relative abundance of the total EF of D. ambrosioides in different tissues and locations was shown in the heat maps (Fig. 5). It was observed that the location had more influence over tissue on the community structure of EF, as shoots and roots of plants from the slag heap clustered together, and in the same way shoots and roots of plants from the wasteland (Fig. 5).

The computational analysis of α-diversity estimated the richness and diversity of plant tissues from two sites at OTU cut-offs of 0.03 distance units (Table 4). Among them, Chao1 estimated minimum number of OTUs, and inverse Simpson diversity index indicated the richness of...
the communities (Akinsanya et al., 2015). Shannon index used in this study was as an expression or index of some relation between number of species and number of individuals (Spellerberg and Fedor, 2003). It was found that both the Chao1 and Simpson diversity of the shoots and roots of the plants from the slag heap were significantly lower ($P < 0.05$) than that of plants from the wasteland (Table 4). However, the Shannon indices ($H$) of the shoots and roots of plants from the slag heap were significantly higher than that of plants from the wasteland (Table 4). Beta diversity analysis indicated that the microbial structures of the shoots and roots of plants from slag heap clustered to one group, while the shoots and roots of plants from the wasteland clustered to another group, same as that observed in the heat maps (Fig. 5).

The raw sequencing data generated from this study have been deposited in NCBI SRA (http://www.ncbi.nlm.nih.gov/sra) under the accession number SRA510221.

Discussion

The colonization rate (CR) of culturable endophytic fungi (EF) of *D. ambrosioides* from two sites was 35.2% and 57.67% respectively. They were significantly lower than those reported in other environments without HM stress, which were usually ranged from 95% to 100% (Gambo and Bayma, 2001; Arnold et al., 2007; Rhoden et al., 2012). In addition, it was found that although the two sampling sites were close to each other (1.5 to 2 km), and the environmental conditions were similar except for soil physico-chemical characteristics (Tables 1 and 2); however, the CR of EF of plants from the wasteland was significantly higher than that of the slag heap (Fig. 1). Moreover, the dominant genera of culturable EF of *D. ambrosioides* from two sites were different, too. In the slag heap, the dominant EF were *Plectosphaerella* sp., *Cladosporium* sp. 2 and *Verticillium* sp., while, in the plants from the wasteland, the dominant EF were *Phoma* sp., *Peyronellaea* sp., *Alternaria* sp. and *Cladosporium* sp. 1 (Table 3). This difference may be due to the difference in the HM concentration of the soils at two sites (Table 1). The result was consistent with previous studies that the endophytic diversity can vary with the level of the pollution (Helander et al., 1993; Li et al., 2012c; Schmidt et al., 2018).

Four fungal phyla were recovered by culture-independent method, while only two fungal phyla (Ascomycota,
Zygomycota) were detected by culture-dependent method. Ascomycota was found to be the most common EF in plants by both culture-dependent and culture-independent methods. This is consistent with previous finding that Ascomycota was the dominant group in soils, marine environments, mangroves and endophytic community (Gazis and Chaverri, 2010; Persoh et al., 2010; Simões et al., 2015; Khan et al., 2017). Forty-three taxa

| Colour Key | Value      | 0 | 10 | 25 |
|------------|-----------|---|----|----|

| Relative frequency | 0% | 20% | 40% | 60% | 80% | 100% |
|--------------------|----|-----|-----|-----|-----|------|
| S1S | W2S | S1R | W2R |

Dendryphion_sp  
Ascomycota_sp  
Discosia_sp  
Pseudosphaeria_sp  
Fusarium_sp_4  
Fusarium_sp_5  
Fusarium_sp_6  
Moniliella_sp  
Hanesia_sp  
Fusarium_sp_2  
Fusarium_sp_3  
Pleurotus_sp  
Mucor_sp  
Cephalosporium_sp_1  
Unidentified_2  
Fusarium_sp_1  
Monilia_sp  
Microphoma_sp  
Colletotrichum_sp_2  
Chrysosporium_lobatum  
Geotrichum_sp  
Alternaria_fenuissima  
Unidentified_1  
Septoria_sp  
Rhynchosporum_sp  
Microspora_sp  
Verticillium_sp  
Epicoccum_nigrum  
Cladosporium_sp_2  
Nodulisporium_sp  
Girlandella_sp  
Plectosphaerella_sp  
Penicillium_sp  
Humicola_fusca  
Unidentified_3  
Phomopsis_columnaris  
Chaetomium_globosum  
Fusarium_sp_6  
Macrophoma_sp  
Verticillium_sp  
Phialophora_sp  
Cladosporium_sp_1  
Cladosporium_sp_1  
Peyronellecta_sp  
Altenaria_sp  
Phoma_sp  

Fig. 3. Heat maps of the relative abundance of culturable fungal endophytes of D. ambrosioides in different tissues and locations. Different colour means the different RF of the taxa in the all four samples (green means high RF) (S1 = Slag heap, W2 = Wasteland; S = Shoot, R = Root). The black and grey bars below show the phylum of the fungal endophytes isolated.

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were recovered of culturable endophytes, while 1989 OTUs were obtained in Illumina Miseq sequencing, which supported the fact that only a very small portion of endophytes can be cultured (Fig. 6) (Torsvik and Øvre, 2002; Alain and Querellou, 2009). In addition, culture-dependent study showed that the diversity of EF of roots was higher than that of shoots at both sites; however, the results of the culture-independent method were reverse (Fig. 6). Moreover, the diversity of total EF of both shoots and roots from the slag heap was higher than that of plants from the wasteland, which was also different from the results of culture-dependent method (Fig. 6). These results suggested that to understand the endophytic community comprehensively, combination of both culture-dependent and culture-independent methods are necessary.

The culturable EF of shoots from two locations clustered to one group, while the EF of roots from two locations clustered to another group, which suggested that the tissue has more influence on the endophytic fungal community than the tissue (Fig. 5). Beta diversity analysis and Bray–Curtis cluster analysis also supported this inference. As environmental parameters of two locations were almost similar except the soil physico-chemical characteristics (Tables 1 and 2), therefore, we suggest that the HM concentration of the soil may have some influence on the endophytic fungal diversity. This is consistent with previous findings that the metal contamination and other pollutants can affect the fungal diversity and community structure (Danti et al., 2002; Op De Beeck et al., 2015; Gly nou et al., 2016).

The dominant EF of different plant tissues from two locations found by culture-independent method were almost the same (Fig. 4b). However, the dominant culturable EF of D. ambrosioides differed with the locations (Table 3). Despite this, some genera were found to be dominant EF in both culture-dependent and culture-independent methods, such as Cladosporium sp. and Alternaria sp. (Table 3, Fig. 4b). Contrary to this, some culturable EF were not detected in culture-independent study, such as Chaetomium and Macrophoma. This may be repercussion of the low ratio of these fungi in the plants and thus were possibly below the detection level.

### Table 4. The unique tags and α diversity of endophytic fungi from *D. ambrosioides* (distance < 0.03)

| Sample site     | Sample ID | Unique tags | Number of OTU | Chao 1     | Shannon | Simpson |
|-----------------|-----------|-------------|--------------|------------|---------|---------|
| Slag heap       | S1S       | 3807        | 679          | 1457.010   | 4.462   | 0.038   |
|                 | S1R       | 4542        | 625          | 1287.625   | 4.066   | 0.062   |
| Wasteland       | W2S       | 12 380      | 866          | 2033.481   | 3.323   | 0.095   |
|                 | W2R       | 25 443      | 1168         | 2486.037   | 3.149   | 0.093   |

R = Root; S = Shoot; S1 = Slag heap, W2 = Wasteland.

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Fig. 5. Heat maps of the relative abundance of the total fungal endophytes of *D. ambrosioides* in different tissues and locations. Different colour indicates difference in relative abundance (Log$_{10}$) of the taxa in all four samples (S1 = Slag heap, W2 = Wasteland; S = Shoot, R = Root).
of Illumina Miseq sequencing. However, they were recovered profusely on artificial media for fast-growing characteristics (Mohamed et al., 2010). The same phenomenon was observed in other works (Premalatha and Kalra, 2013).

There were 12% to 17.98% OTUs that remained unclassified (Fig. 4). In a previous study, the endophytic fungal assemblage in stems of wild rice in China was characterized using a combination of morphology and molecular techniques and observed that 30% of the total taxa recovered remained sterile and unidentifiable (ITS sequence similarity 83%–94%) even to the genus level (Yuan et al., 2011b). There could be two reasons for this: first and foremost, the unidentified species may represent lineages new to the fungal biota, and second, a large number of species are undescribed and uncharacterized in the database or size of the OTUs was not enough for the 100% query coverage. These endophytes need more attention in the future study.

Plants in association with microbes can be applied to remove the labile/bio-available pool of inorganic contaminants from a site, remove or degrade organic contaminants, stabilize or immobilize contaminants (Megharaj and Naidu, 2017). The associated microbes can enhance host plant growth, increase solubility and bioavailability of contaminant and alter heavy metal accumulation through IAA, siderophores, organic acids and biosurfactant production (Li et al., 2012a; Ullah et al., 2015a,b; Tirry et al., 2018). Moreover, microbes can indirectly enhance phytoremediation by stimulating soil microbial communities (Burges et al., 2017). Endophytes have been demonstrated to play a key role in host plant adaptation to polluted environments and that they can enhance phytoremediation by mobilizing/degrading or immobilizing contaminants in the soil, promoting plant growth, decreasing phytotoxicity and improving plants’ metal tolerance, as well as in other ways (Li et al., 2012a). Therefore, endophytes of *D. ambrosioides* may also have enhanced host plant phytoremediation. Future experiments will be necessary to further evaluate the roles and the mechanisms of the endophytes.

### Experimental procedures

#### Description of site and sampling

The investigated sites were situated in Zhehai, Huize County, Yunnan Province, Southwest China (25°48′–27°04′N, 103°03′–103°55′E), where Pb–Zn mining has been carrying out for more than 300 years. The region belongs to temperate monsoon climate, with short, mild, dry winters and warm, rainy summers. The average elevation is 2099 m, and the annual mean temperature and annual rainfall are 12.6°C and 847.1 mm respectively. The frost-free period lasts for approximately 202 days. This region is full of slag heaps and wastelands, which are covered with sparse vegetation. *D. ambrosioides* was one of the dominant plant species at the sampling sites (Qin et al., 2013).

Healthy plants of *D. ambrosioides* were collected from both slag heap and wasteland sites that were about 1.5–2 km apart from each other. At each sampling location, randomly 15 healthy *D. ambrosioides* plants were collected with each plant at least 30 m apart from another, and the adjacent soils were also collected at a depth of 5–10 cm and mixed thoroughly. All the collected samples were immediately stored in a sterile polythene bags, labelled accordingly and brought to the laboratory under refrigerated conditions. The plant samples were processed within 24 h for the isolation of endophytic fungi and total DNA extraction.

#### Physico-chemical characteristics and heavy metal concentration analysis

Soil samples were air-dried (25°C) and then crushed and sieved with a 0.15-mm mesh to get fine powders. Then, soil organic matter, total nitrogen, total phosphorus, total potassium, alkaline hydrolysable nitrogen, available phosphorus, available potassium and pH (soil: H2O = 1:2.5) were measured according to previously described methods (Lin et al., 2017). For HM concentration analysis, plant samples were washed with distilled water to remove surface element trace and then were...
HClO₄ was added to continue digesting at 100°C for 30 min, followed by 120°C for 1 h. Finally, the digests were diluted to 50 ml with triple deionized water in a volumetric flask. The concentrations of bio-available Pb, Zn and Cd in soils were extracted by diethylenetriaminepentaaetic acid-triethanolamine (DTPA-TEA) (Huang et al., 2006). All the samples were prepared in triplicates. The concentrations of Pb, Zn and Cd in plant and soil digests were determined by flame atomic absorption spectrometry (Li et al., 2014).

The mean and standard deviation of the HM concentrations was calculated using three replicates of a mixed plant and soil samples from two sites respectively. A t-test was performed to determine the differences in mean HM concentration between samples from the two sites, and P was set at <0.05.

Endophytic fungal community of D. ambrosioides

The fungal endophytic communities in different tissues of D. ambrosioides from two sites were evaluated using both cultivation-dependent and cultivation-independent approaches.

Surface sterilization of plant tissues

The plants were washed with running tap water to remove the adhered soil particles and other contaminants. Thereafter, five leaves, five stems and five roots segments (about 6 cm long) were randomly selected from each plant, and thus, overall 75 leaves, 75 stems and 75 roots fragments were selected from the sampled plants from each site. The surface sterilization was carried out by sequentially dipping the fragments in 75% ethanol for 2 min, followed by 5% sodium hypochlorite for 2 min and finally 3–5 times rinsed with sterile distilled water (Li et al., 2012b). The surface sterilized fragments were dried on sterilized filter paper, and the efficacy of the surface sterilization process was confirmed by making imprints of disinfected plant fragments on Petri dish containing PDA (potato dextrose agar); the absence of any fungal growth was observed as an effective surface sterilization (Schulz et al., 1998).

Culturable endophytic fungal community

Fungal endophytes isolation and identification

The surface sterilized fragments were cut down to small segments of 0.5 × 0.5 mm using a sterile blade under aseptic conditions. Then, 100 root segments and 100 shoot segments (50 stems and 50 leaves) from each sampling site were placed on Petri dishes containing PDA supplemented with 0.5 g l⁻¹ streptomycin sulphate. The plates were incubated at 25 ± 1°C and checked every alternate day for 45 days; the emerging fungal mycelia from the plant tissues were transferred to fresh PDA plates. All the isolates were deposited in Medical School of Kunming University of Science and Technology.

Fungal morphological identification was based on the morphology of the colony as well as the mechanism of spore production and spore characteristics (Barnette and Hunter, 1987; Ellis, 1988). For frequently occurring morphotypes that were either sterile or sporulating structures that were difficult to identify to genus level, molecular identification was attempted using the internal transcribed spacer (ITS) region and a reference database. A total of 61 isolates from 12 morphotypes were selected to conduct molecular analysis. To produce fresh biomass of pure mycelium for DNA extraction, isolates were transferred to fresh PDA plates and incubated at 25°C for 1–2 weeks. Then, some mycelium were scraped off and DNA was extracted using PowerSoil® DNA Isolation Kit (Mobio: Carlsbad, California, USA) and amplified with the primers ITS1 and ITS4 (Khan and Lee, 2013). PCR products were purified using Cyclepure Kit (Biotek: Beijing, China) according to the manufacturer’s protocol and were sent to Sangon Biotech Co., Ltd. (Shanghai, China) for sequencing. Finally, the sequences obtained in this study were uploaded to GenBank database (http://www.ncbi.nlm.nih.gov/) and the similarities of them with the published sequences in GenBank database were determined by BLAST.

Data analysis

The colonization rate (CR) corresponds to the number of endophytic fungi colonized inside host plants and was calculated as the total number of plant segments infected by one or more fungi divided by the total number of segments incubated (Sun et al., 2011). The relative frequency (RF) was calculated as the number of isolates of one species divided by the total number of isolates (Yuan et al., 2011a). The Shannon index (H') was calculated according to the following formula: \[ H' = \sum P_i \times \ln P_i, \] where \( k \) is the total species number of one plot and \( P_i \) is the relative abundance of endophytic fungal species in one plot (Spellerberg and Fedor, 2003). Simpson index \( (1 - D) \) was calculated according to the following formula: \[ 1 - [D = \sum (n/n)^2] \] where
The number of distinct species \((n)\) and the abundance of each species in the community (Simpson, 1949).

SPSS software ver. 17.0 was used for statistical analysis. Chi-squared test was used to compare the differences in the CR of endophytes from two sites.

The total endophytic fungal community

**Total genomic DNA extraction and sequencing**

The surface sterilized root and shoot segments were transferred to sterilized mortars and homogenized in liquid nitrogen individually. The total genomic DNA was extracted from approximately 0.2 mg powdered sample using PowerSoil™ DNA Isolation Kit (Mobo, USA) and was verified by gel electrophoresis (1% agarose, 120 V, 30 min) (Khan and Lee, 2013). The fragment of the ITS2 region of about 360 bp length was targeted using a forward primer (5'-GATGGAAGAACGYAGYRAA-3') combined with a reverse primer (5'-TCCTCCGCTTATTGATATGC-3') for fungal community analysis. The PCR contained 2.5 µl Takara 10 × Ex Taq Buffer (Takara, China), 1.5 µl MgCl\(_2\) (25 mM MgCl\(_2\)), 2 µl dNTP Mixture (2.5 mM each), 0.25 µl Takara Ex Taq DNA Polymerase (2.5 units/µl), 1 µl Template DNA (20 ng), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM) and 16.75 µl Sterilized ddH\(_2\)O in a volume of 25 µl. PCR amplification was performed in a thermal cycler with the following cycling parameters: an initial denaturation at 94°C for 2 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 2 min, followed by 30 s, extension at 72°C for 30 s and a final extension at 72°C for 5 min. The amplified PCR products were verified by electrophoresis in a 1% agarose gel and visualized under a UV transilluminator. DNA band with the expected size was excised from the agarose gel with a clean and sharp scalpel. The PCR products were purified from agarose gels using a QIAGEN Plasmid Mega Kit 25 (Qiagen: Hilden, Germany) according to the manufacturer's protocol. The DNA concentration was measured in a micro-spectrophotometer ND-1000 (Nanodrop Technologies: Wilmington, USA), after which equimolar concentrations of the barcoded amplicons were collected per library and diluted to 100 ml using TE buffer. The library was bi-directionally sequenced using an Illumina MiSeq Desktop at Guangzhou Gene Denovo Biological Technology Company (Guangzhou, China). The sequence analysis was carried out using the BLAST algorithm in GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/blast/).

**Sequencing data analysis**

The raw Illumina Miseq sequencing data were obtained in FASTA files along with sequencing quality files. The files were accessed using MOthur v.1.34.0 bioinformatics software (Schloss et al., 2009) for further processing and analyses (Schloss et al., 2011). All sequences were denoised before barcodes, and primers were removed. The cleaned-up sequences were aligned and classified along known sequences in the SILVA rRNA database (Pruesse et al., 2007). Chimeric sequences together with known mitochondria and chloroplast sequences were filtered, and the remaining sequences were assigned to operational taxonomic units (OTUs) based on a 97% similarity criterion. Rarefaction curve of the OTUs were performed to check the sample adequacy using a 50 sequence increment. The representative sequence for each OTU was provided taxonomical annotation by Naive Bayesian based classifier, the RDP classifier [http://rdp.cme.msu.edu/classifier/classifier.jsp] at 0.5 confidence threshold (Wang et al., 2007).

To indicate the microbial diversity in plant tissues, the \(\alpha\)-diversity indices (including Chao1, Simpson and Shannon indices) were quantified in terms of OTU richness. OTU expression spectrum image was performed using R software. Subsequently beta diversity analysis was carried out calculating Whittaker's using formula \(\beta = (S \div \alpha) - 1\), where \(S\) represents the total number of OTU in two samples and \(\alpha\) represents the total of OTU of the two samples (Whittaker, 1960). The hierarchical clustering of Bray-Curtis was carried out with R software.

**Conclusion**

High-throughput sequencing study demonstrated that D. ambrosioides harbour more EF than culture-dependent method can estimate, and the location has more impact on the endophytic fungal community than the tissue type. The dominant EF of plants from two locations were almost similar: Cladosporium sp. was the most dominant EF followed by Cryptococcus victoriae and Purpureocillium lilacinum. However, the dominant culturable EF of D. ambrosioides differed with the locations. Although Ascomycota was observed to be the most dominant phylum followed by Basidiomycota in both culture-dependent and culture-independent studies, however, Chytridiomycota and Zygomycota were only observed in culture-independent studies. On the contrary, some culturable EF were not detected by culture-independent method. We suggest that the combination of culture-dependent and culture-independent methods more precisely reveals the structure of the endophytic fungal community than does either method alone. Both culture-dependent and culture-independent studies illustrated that endophytic fungal communities of D. ambrosioides varied across the slag heap and wasteland sites, which indicated that the HM concentration of the soil may have some influence on the endophytic fungal.
diversity. Having better insight of total endophytic fungal community structure of the *D. ambrosioides* from HM contaminated sites could be useful in future studies to elucidate functional role of EF in the HM tolerance of the host plant.

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**Conflict of interests**

The authors declare no conflict of interests.

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