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RESEARCH ARTICLE

Diversity of fungi associated with plants growing in geothermal ecosystems and evaluation of their capacities to enhance thermotolerance of host plants

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Diversity and heat-adaptation of endophytic fungi (EF) and rhizospheric fungi associated with plants growing in geothermal ecosystems, Southwest China, as well as their benefit in improving host plant thermotolerances were investigated. A total of 1589 culturable fungi belonging to 38 taxa were isolated, in which *Curvularia*, *Acrophialophora*, *Penicillium*, and *Aspergillus* were the dominant genera. The Shannon indices of EF and rhizospheric fungi ranged from 1.80 to 2.56 and 0.73 to 2.11, respectively. Phylogenetic analysis indicated that the EF have a close relationship with rhizospheric fungi. However, some fungi exhibited apparently species-specific habitat distribution patterns. Growth temperature tests indicated that 60.22% of the tested isolates were thermotolerant fungi and only 39.78% were mesophiles, and the number of heat-adapted fungi increased with increasing environmental temperatures. The strain G1-29, which was isolated from the roots of *Hedyotis diffusa* and identified as *Curvularia crepinii*, significantly improved host plant thermotolerance under laboratory conditions: the death rate of endophyte-infected plants was significantly lower than that of endophyte-free plants (*t*-test, $p = .0158$, $df = 4$). Our results suggested that the EF and rhizospheric fungi associated with plants growing in geothermal ecosystems are diverse, and many of them have adapted to the high environmental temperatures. Some fungi have come to be the dominant endemic inhabitants of specific niches, and some played an important role in improving host plant thermotolerances.

Keywords: geothermal ecosystem; endophyte; diversity; heat-adaptation; stress-tolerance

Introduction

The geothermal ecosystem is one kind of extreme environment, usually with high temperature, elevated concentrations of a range of minerals and extreme pH. Plant survival is difficult under these conditions. However, it has been demonstrated that many kinds of organisms, especially microorganisms, have adapted to this specific environment (Kvist et al. 2007; Lau et al. 2009; Pan et al. 2010). Moreover, extensive research has shown that microbial communities under these circumstances were significantly different than the microbial communities in other environments (Inskeep et al. 2013). Many new species have been isolated and some have been found to be able to produce unique high-temperature active metabolites (Asoodeh et al. 2010; Valsange et al. 2012; Inskeep et al. 2013; Giaveno et al. 2014). Therefore, the geothermal ecosystem can be considered to be an ideal environment to study the diversity of extremophiles and their special metabolites, as well as mechanisms of their adaptation (Hreggvidsson et al. 2012; Inskeep et al. 2013; Jay et al. 2014).

The majority of previous studies have mainly focused on bacteria and archaea in hot springs (Pan et al. 2010; Sahm et al. 2013; Hamamura et al. 2013). In contrast, the study of fungi has been insufficiently examined. This may be because the number of species of bacteria and archaea are much larger than the number of fungal species in hot springs (Tansey & Brock 1972). Nevertheless, recently it has been found that terrestrial environments in geothermal ecosystems are rich in fungi, and some of them play a very important role in improving host plant heat-adaptation (Redman et al. 2002; Bunn et al. 2009; Lau & Lennon 2012).

Endophytic fungi (EF), fungi causing asymptomatic infections in living plant tissues, have been found to be ubiquitous in the entire plant kingdom, and they are rich in species diversity (Hyde & Soytong 2008; Li et al. 2012a; Ghimire et al. 2011; Rivera-Orduña et al. 2011; Zhang et al. 2013). It has been demonstrated that EF can enhance their host plants' habitat-specific stress-tolerance, such as tolerance to heat, salt, disease, and drought (Márquez et al. 2007; Rodriguez et al. 2008; Li et al. 2012b; Hamilton et al. 2012). However, there has been comparatively little research on EF of the plants growing in geothermal ecosystems. One study which has considered the role of EF in geothermal ecosystems is the work of Redman et al. (2002) who reported that the EF of the grass *Dichanthelium lanuginosum*, collected from geothermal soils, could improve host plant adaptation to high-temperature soils. Because beneficial endophytic symbiosis may be important components of all stressful ecosystems, the EF in such ecosystems should be studied systematically, including factors such as the community structure, heat-adaptation mechanisms, and other ecological functions.

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Eryuan County, Yunnan Province, China (26°15′N, 99°59′E) is located in the Mediterranean-south Asia geothermal abnormal belt and is rich of geothermal ecosystems. There is sparse vegetation in geothermal soils which have annual temperature fluctuations ranging from about 20°C to 60°C. To examine the diversity, heat-adaptation, and possible ecological functions of endophytic and rhizospheric fungi associated with plants growing in this area, six dominant plant species were collected, and their EF as well as rhizospheric fungi were isolated. Simultaneously, heat-adaptation, phylogenetic relationships of fungi, as well as their benefits in improving host plant thermotolerances were studied.

Materials and methods

Field sampling

Six dominant plant species *Hedyotis diffusa*, *Trifolium repens*, *Digitaria ischaemum*, *Silene tenuis*, *Cynodon dactylon*, and *Alternanthera philoxeroides* were collected from geothermal ecosystems in Eryuan County, Yunnan Province, Southwest China (26°15′N, 99°59′E) in October 2011. The soil temperatures at sampling points were different and ranged from 40°C to 50°C, which were recorded at a depth of 5–8 cm from the surface.

For each plant species, 15 healthy dominant plants (plants without any apparent disease symptoms were recognized as healthy plants) were chosen at random and the plants were sampled for endophytes. Roots were sampled by taking soil cores (10 cm diameter and 20 cm deep) adjacent to the plants. Each sample was placed in a plastic bag and transported to the lab and processed within 24 h.

Fungal isolation

The rhizospheric soils from 15 individuals of each plant species were mixed thoroughly. Then, 10 g of soil was suspended in 80 ml of sterile water by mixing for 10 min with a magnetic stirrer. Finally, the soil suspensions were serially diluted, and 1 ml aliquots of 10^{-1}, 10^{-2}, and 10^{-3} dilutions were transferred to a Petri dish, after which 15 ml of melted potato dextrose agar (PDA, temperature 45°C) was added and mixed thoroughly; triplicate replicates for each dilution were prepared. The plates were incubated at 25°C for 45 d. Afterwards, colonies were counted and transferred to new PDA plates.

To isolate EF, one stem segment and one root segment (about 8 cm long) were selected at random from each plant; total of 15 stem segments and 15 root segments of each plant species were chosen. Stem and root pieces were washed in running tap water and processed as follows: the samples were cut into segments (about 5 × 5 mm) and surface-sterilized by sequentially dipping into 0.5% sodium hypochlorite (2 min) and 70% ethanol (2 min) (Li et al. 2012a). Then, 80 root segments and 80 stem segments from each plant species were placed in a Petri dish containing PDA supplemented with 0.5 g/l streptomycin sulfate, then incubated at 25°C and checked every other day for 45 d. The fungi growing out of the plant tissues were transferred to fresh PDA plates. The effectiveness of surface disinfection was controlled by making imprints of disinfected segments onto PDA plates (Schulz et al. 1998). All of the isolates were stored in freezers at the College of Life Sciences and Technology in Kunming University of Science and Technology, China.

Morphological and molecular identification of fungi

Fungal morphological identification was based on the morphology of the colony as well as the mechanism of spore production and spore characteristics (Sutton 1980; Barnett & Hunter 1987; Ellis 1988). For frequently occurring morphotypes that were sterile or were sporulating but difficult to identify to genus, molecular identification was attempted using the ITS region and a reference database. To produce biomass of pure mycelium for DNA extraction, isolates were cut to small pieces and seeded to a 250 ml flask with potato dextrose broth (PDB), then put on a shaker for 1−2 weeks. Some mycelium was removed, squeezed dry on a sterile paper towel, and DNA was extracted and amplified with the primers ITS1 and ITS4 (Yu et al. 2013). Polymerase chain reaction product was purified using Cycle-pure Kit (Bioteke) according to the manufacturer's protocol, and was sent to Sangon Biotech Company for Sanger DNA sequencing. Finally, the similarities of the sequences obtained in the present study with the published sequences in Genbank database (http://www.ncbi.nlm.nih.gov/) were determined by Basic local alignment search tool (Altschul et al. 1990).

Phylogenetic relationship analyses

The most appropriate relative sequences selected from Genbank and the sequences obtained in the present study were imported into MEGA 5.1, and a phylogenetic tree was constructed using the neighbor-joining method combined with bootstrap analysis setting with 1000 replications. Only bootstrap values above 50% were shown, and only those above 70% were considered significant (Liu et al. 2010). The phylogenetic tree was rooted using *Chytriomyces hyalinus* as an outgroup (GenBank accession number DQ536499.1).

Growth temperature test

Approximately one-fourth of the isolates of each dominant species (total 269 isolates belonging to 16 species) were selected at random for growth temperature tests (130 from rhizospheric soils, 91 from roots, and 48 from stems). They were inoculated in the center of PDA plates (diameter 90 mm) and incubated at 18°C, 28°C, 40°C, and 50°C in the dark. The colony diameter was measured every 2 d in two directions at right angles for one month, and the mean diameter was obtained from three replicates. The colony diameter growth rate was calculated as the mean diameter of the isolate divided by the days of its incubation (Osono et al. 2011).

Table 1. Numbers, taxa, and RFs of EF and rhizospheric fungi associated with six dominant plant species.

Taxa	From	No. of EF and rhizospheric fungi isolated (RF%)						Total (RF%)
		H. diffusa	*T. repens*	*D. ischaemum*	*S. tenuis*	*C. dactylon*	*A. philoxeroides*	
Acrophialophora fusispora[a]	Plant	3 (3.45)	13 (16.88)	28 (28.87)	8 (6.96)	35 (28.46)	1 (0.78)	88 (14.04)
	Soil	–	–	–	–	–	–	–
Acrophialophora nainiana[a]	Plant	–	–	–	–	–	–	–
	Soil	1 (0.59)	3 (1.59)	136 (60.18)	–	–	–	140 (14.55)
Alternaria sp.	Plant	–	–	–	1 (0.87)	–	–	1 (0.16)
	Soil	–	–	–	–	–	–	–
Apiocarpella sp.[b]	Plant	2 (2.30)	–	–	1 (0.87)	–	–	3 (0.48)
	Soil	–	–	–	–	–	–	–
Apodus oryzae[a]	Plant	2 (2.30)	–	–	–	–	–	2 (0.32)
	Soil	–	–	–	–	–	–	–
Aposphaeria sp.[b]	Plant	–	–	–	1 (0.87)	1 (0.81)	–	2 (0.32)
	Soil	–	–	–	–	–	–	–
Aspergillus fumigatus[a]	Plant	–	–	–	–	–	–	–
	Soil	21 (12.43)	5 (2.65)	17 (7.52)	–	10 (6.67)	3 (1.83)	56 (5.82)
Aspergillus niger[a]	Plant	–	10 (12.99)	18 (18.56)	–	–	1 (0.78)	29 (4.63)
	Soil	9 (5.33)	1 (0.53)	–	–	–	–	10 (1.04)
Aspergillus terreus[a]	Plant	–	3 (3.90)	–	1 (0.87)	–	–	4 (0.64)
	Soil	31 (18.34)	12 (6.35)	2 (0.88)	–	–	–	45 (4.68)
Aspergillus versicolor[a]	Plant	2 (2.30)	–	1 (1.03)	2 (1.74)	5 (4.07)	8 (6.25)	18 (2.87)
	Soil	–	–	–	–	–	–	–
Cochliobolus cynodontis[a]	Plant	–	–	–	3 (2.61)	–	–	3 (0.48)
	Soil	–	–	–	–	–	–	–
Colletotrichum trifolii[a]	Plant	2 (2.30)	–	–	3 (2.61)	–	–	5 (0.80)
	Soil	–	–	–	–	–	–	–
Cryptosporiopsis sp.[b]	Plant	–	–	–	4 (3.48)	1 (0.81)	–	5 (0.80)
	Soil	–	–	–	–	–	–	–
Curvularia crepinii[a]	Plant	1 (1.15)	–	–	1 (0.87)	7 (5.69)	6 (4.69)	14 (2.23)
	Soil	19 (11.24)	50 (26.46)	17 (7.52)	24 (37.50)	82 (54.67)	18 (10.98)	210 (21.83)
Curvularia protuberate[a]	Plant	4 (3.45)	5 (6.50)	11 (11.34)	10 (8.70)	23 (18.70)	23 (17.97)	76 (12.12)
	Soil	–	–	–	–	–	–	–
Dothiorella sp.[b]	Plant	34 (39.08)	6 (7.79)	1 (1.03)	–	–	7 (5.47)	48 (7.66)
	Soil	–	–	–	–	–	–	–
Ephelis sp.[a]	Plant	9 (10.34)	7 (9.09)	–	–	–	14 (10.94)	30 (4.78)
	Soil	1 (0.59)	30 (15.87)	2 (0.88)	–	–	–	33 (3.43)
Fusarium solani[a]	Plant	6 (6.90)	4 (5.19)	3 (3.09)	6 (5.22)	7 (5.69)	12 (9.38)	38 (6.06)
	Soil	10 (5.92)	21 (11.11)	10 (4.42)	–	–	–	41 (4.26)
Fusarium sp1.[a]	Plant	2 (2.30)	–	–	–	–	–	2 (0.32)
	Soil	–	–	–	–	–	–	–
Fusarium sp2.[a]	Plant	–	–	–	1 (0.87)	1 (0.81)	10 (7.81)	12 (1.91)
	Soil	–	–	–	–	–	–	–

(*Continued*)

Table 1. Continued.

Taxa	From	No. of EF and rhizospheric fungi isolated (RF%)						Total (RF%)
		H. diffusa	*T. repens*	*D. ischaemum*	*S. tenuis*	*C. dactylon*	*A. philoxeroides*	
Gibberella fujikuroi[a]	Plant	1 (1.15)	–	–	5 (4.35)	9 (7.32)	14 (10.94)	29 (4.63)
	Soil	–	–	–	–	17 (11.33)	98 (59.76)	115 (11.95)
Gloeosporium sp.[b]	Plant	3 (3.45)	1 (1.30)	5 (5.15)	18 (15.65)	3 (2.44)	11 (8.59)	41 (6.54)
	Soil	25 (14.53)	41 (21.69)	37 (16.37)	39 (60.94)	–	–	142 (14.72)
Glomerella magna[a]	Plant	–	–	–	2 (1.74)	2 (1.63)	2 (1.56)	6 (0.96)
	Soil	–	–	–	–	–	–	–
Melanconium sp.[a]	Plant	1 (1.15)	1 (1.30)	–	19 (16.52)	–	1 (0.78)	22 (3.51)
	Soil	–	–	–	–	18 (12.00)	–	18 (1.87)
Monilia sp1.[a]	Plant	–	–	–	–	7 (5.69)	–	7 (1.12)
	Soil	–	–	–	–	–	–	–
Monilia sp2.[a]	Plant	–	–	–	–	10 (8.13)	–	10 (1.59)
	Soil	–	–	–	–	–	–	–
Mucor sp.	Plant	–	–	–	–	–	–	–
	Soil	–	1 (0.53)	–	–	–	–	1 (0.10)
Penicillium chrysogenum[a]	Plant	6 (6.70)	24 (31.17)	26 (26.80)	15 (13.04)	1 (0.81)	2 (1.56)	74 (11.80)
	Soil	–	–	–	–	–	–	–
Penicillium griseofulvum[a]	Plant	–	–	–	–	–	–	–
	Soil	14 (8.28)	4 (2.12)	1 (0.44)	1 (1.56)	–	45 (27.44)	65 (6.76)
Penicillium pinophilum[a]	Plant	–	–	–	–	–	–	–
	Soil	34 (20.11)	11 (5.82)	–	–	23 (15.33)	–	68 (7.07)
Phialophora sp.[b]	Plant	3 (3.45)	1 (1.30)	5 (5.15)	18 (15.65)	3 (2.44)	11 (8.59)	41 (6.54)
	Soil	–	–	–	–	–	–	–
Phoma sp.[a]	Plant	–	–	–	1 (0.87)	1 (0.81)	1 (0.78)	3 (0.48)
	Soil	–	–	–	–	–	–	–
Pleurophragmium sp.[b]	Plant	–	1 (1.30)	1 (1.03)	–	1 (0.81)	–	3 (0.48)
	Soil	–	–	–	–	–	–	–
Rhizopycnis vagum[a]	Plant	7 (8.05)	–	1 (1.03)	–	–	–	8 (1.28)
	Soil	–	–	–	–	–	–	–
Sphaceloma sp.[b]	Plant	1 (1.15)	–	–	1 (0.87)	1 (0.81)	–	3 (0.48)
	Soil	–	–	–	–	–	–	–
Stilbum sp.[a]	Plant	–	–	–	–	–	–	–
	Soil	3 (1.78)	10 (5.29)	4 (1.77)	–	–	–	17 (1.77)
Trichoderma sp.	Plant	–	–	–	–	–	–	–
	Soil	1 (0.59)	–	–	–	–	–	1 (0.10)
Total	Plant	87 (100)	77 (100)	97 (100)	115 (100)	123 (100)	128 (100)	627 (100)
	Soil	169 (100)	189 (100)	226 (100)	64 (100)	150 (100)	164 (100)	962 (100)
Total taxa isolated	Plant	17	12	11	20	18	16	30
	Soil	12	12	9	3	5	4	15

Notes: All of the taxa listed above were morphological identified.

–, The taxa were not found in plants/soil.

[a,b]The taxa were further identified by molecular method and 'a' means that the isolates showed 97–99% similarity with their closest relatives, whereas 'b' means that the isolates showed <95% similarity with their closest relatives.

Table 2. Numbers, CFUs, and Shannon index (H') of fungi from the rhizospheric soils (RS) of six plant species.

Plant species	Temperature of RS (°C)	Moisture content of RS (%)	No. of fungi isolated	CFUs/g	H'
H. diffusa	40.0	72.71	169	29.19	2.11
T. repens	40.0	71.82	189	32.47	2.01
D. ischaemum	45.0	73.31	226	39.17	1.31
C. dactylon	45.5	73.61	150	26.04	1.30
A. philoxeroides	46.0	71.82	164	28.18	0.98
S. tenuis	50.0	76.06	64	11.27	0.73
Total		73.21	962	27.72	

Effects of endophytes on host plant thermotolerance

Thirteen isolates of *Curvularia* spp. and five of *Acrophialophora* spp., which exhibited apparently species-specific habitat distribution patterns, were selected at random to assess their effects on host plant thermotolerance as follows: the sterile seedlings of indica rice (*Oryza sativa* L. ssp. *indica* Var. 9311) were transplanted into culturing flasks (diameter 6.5 cm, two seedlings/flask) containing a mixture of Canadian sphagnum peat with perlite (7:1 volume, Fafard, Canada). Fungal endophytes were seeded to 250 ml flasks with PDB, then put on a shaker for 4–7 d. Thirty grams (wet wt) of mycelium were triturated and re-suspended in 90 ml sterile water and divided into two parts (part A and part B, and part B was sterilized). Then, part A was sprayed onto surfaces of indica rice seedlings until they were wet (endophyte-infected, E+), and mock-inoculated seedlings (sprayed with sterilized part B) were used as the control (endophyte-free, E−). To confirm fungal colonization inside inoculated plants, seedlings were collected at random after 3 d inoculation, and were washed under running tap water and surface-disinfected as above (fungal endophyte isolation). Finally, the seedlings were homogenized by grinding, and diluted suspensions were grown in PDA medium for detection of the strain inoculated. Colonization was confirmed by re-isolation of the strain from inoculated plants, but failure to isolate from water-inoculated control plants.

Five days after inoculation, the seedlings were transferred to an electric-heated thermostatic water bath (water temperature was 51°C, and the water depth was a little higher than the soil depth to keep the soil temperature at 50°C). Plants were watered twice everyday with 30 ml sterile water each time and fertilized weekly with Peter's General Purpose 20-20-20 fertilizer (Grace Sierra Horticultural Products, Milpitas, CA), and the number of seedlings that died was recorded.

Data analysis

The colonization rate (CR) was calculated as the total number of plant tissue fragments infected by one or more fungi divided by the total number of fragments incubated (Kumar & Hyde 2004). The relative frequency (RF) was calculated as the number of isolates of one species divided by the total number of isolates (Yuan et al. 2011). Endophytic fungal diversity was evaluated using the Shannon index (H'), which has two main components, evenness and the number of species. The Shannon index was calculated according to the following formula: $H' = -\sum_{i=1}^{k} P_i \times \ln P_i$, where k is the total species number in one plot and P_i is the relative abundance of endophytic fungal species in one plot (Spellerberg & Fedor 2003).

The soil moisture content (θ_m) was calculated using the following formula: $\theta_m = (W_1 - W_2/W_2) \times 100$, where W_1 is the weight of wet soil and W_2 is the weight of air-dried soil. The numbers of fungal colony forming units (CFUs) were calculated as the number of strains isolated from the sample divided by the weight of the sample and are expressed as CFUs/g. The death rate of plants was calculated as the total number of plants that died divided by the total number of plants cultured. SPSS software ver. 11.5 was used for statistical analysis. Student's t-test was used to compare the differences in death rates of plants in thermotolerance tests between endophyte-infected (E+) and uninfected plants (E−).

Results

Composition of fungi in rhizospheric soils

A total of 962 fungi were isolated from the rhizospheric soils associated with the six plant species (Table 1). The isolates were identified to 15 taxa, of which *Curvularia*, *Gloeosporium*, *Acrophialophora*, *Penicillium*, *Gibberella*, and *Aspergillus* were the dominant genera, and their RFs were

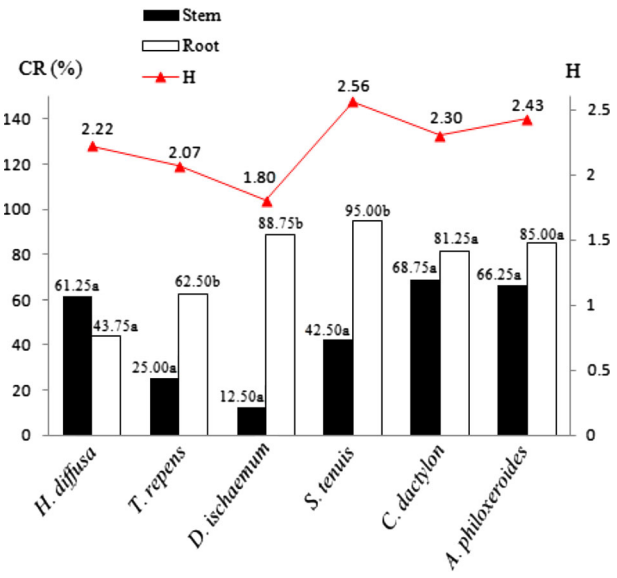

Figure 1. Number, CR, and Shannon index (H') of EF from six plant species. Values with different letters in the same row indicate a significant difference at the $p < .05$ level.

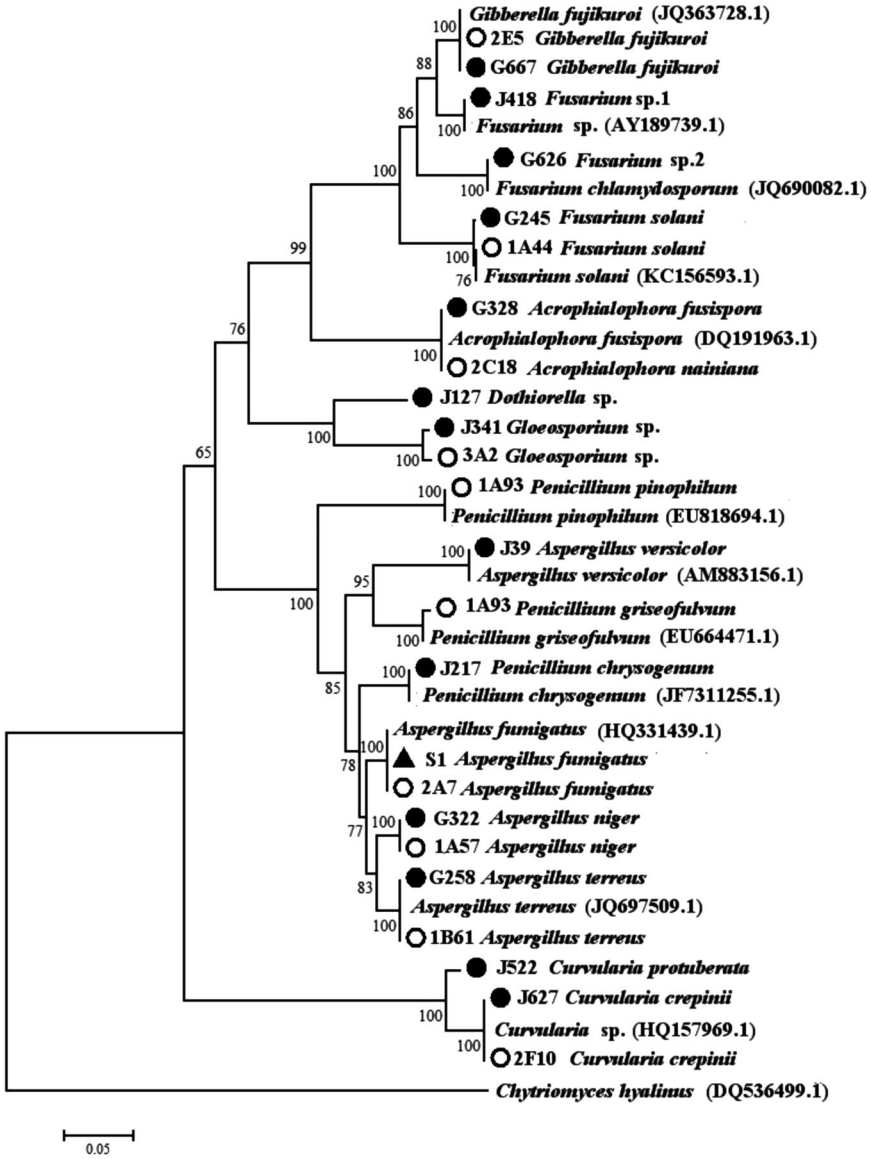

Figure 2. Neighbor-joining phylogenetic tree constructed using rDNA-ITS sequences (>550 bp) of dominant fungi from Eryuan geothermal ecosystems. Number of branch indicates bootstrap value (>50%) of neighbor-joining analysis from 1000 replicates. The scale bar represents 0.05 nucleotide substitutions per site. The black cycle, white cycle, and black triangle represent nucleotide sequences of fungi from plants, soil, and hot spring water, respectively.

21.83%, 14.76%, 14.55%, 13.83%, 11.95%, and 11.54%, respectively. The numbers of fungal CFUs obtained from rhizospheric soils of the six plant species differed greatly and the maximum number of CFUs could be found in *Digitaria ischaemum*, whereas significantly less were found in *Silene tenuis* (Table 2). The Shannon indices of fungi from rhizospheric soils of the six plant species ranged from 0.73 to 2.11, which showed a decreasing trend with the increasing soil temperature (Table 2).

Composition of fungal endophytes

A total of 627 EF were isolated from 960 tissue segments from the six plant species (232 from stems and 395 from roots). The CRs of the EF from the six plant species ranged from 43.75% to 75.63%. The highest CR appeared on *Alternanthera philoxeroides*, whereas the lowest was found for *Trifolium repens* (Figure 1).

The EF from the six plant species were identified to 30 taxa, among which *Curvularia*, *Acrophialophora*, *Penicillium*, *Fusarium*, *Aspergillus*, *Dothiorella*, *Gloeosporium*, and *Phialophora* were the dominant genera. These fungi were widely distributed in plants and their RFs were 14.35%, 14.04%, 11.80%, 8.29%, 8.13%, 7.66%, 6.54%, and 6.54% respectively (Table 1). In contrast, some endophytic fungal genera were rare, and only one isolate was found from them, such as *Alternaria*. The Shannon indices of EF from the six plant species ranged from 1.80 to 2.56 (Figure 1).

Phylogenetic relationships between fungi from plants and rhizospheric soils

A total of 43 strains from 33 morphological taxa (29 from plants, 13 from rhizospheric soils, and 1 from hot spring water) were further subjected to phylogenetic analysis.

Table 3. Growth temperatures of fungi.

Taxa	Origin of isolates	No. of strains tested	Growth temperature (°C)				Maximum growth rate of some isolates (mm/d)				
			18	28	40	50	Strains	18°C	28°C	40°C	50°C
Acrophialophora fusispora	Root	20	+	+	+	–	G537	0.31 ± 0.03	6.05 ± 0.59	9.73 ± 0.42	–
Acrophialophora nainiana	RS	20	+	+	+	–	2E1	0.42 ± 0.16	5.44 ± 0.35	11.00 ± 0.34	–
Aspergillus fumigatus	RS	10	+	+	+	–	2A7	2.29 ± 0.61	5.72 ± 0.38	3.85 ± 0.38	–
Aspergillus niger	Root	7	+	+	–	–	G322	3.00 ± 0.35	7.17 ± 0.76	–	–
	Stem	3	+	+	–	–	J212	4.33 ± 0.50	7.08 ± 0.38	–	–
Aspergillus terreus	RS	10	+	+	+	–	1B61	3.19 ± 0.61	6.78 ± 1.61	4.19 ± 0.72	–
Aspergillus versicolor	Root	5	+	+	–	–	G131	3.78 ± 0.41	6.89 ± 0.63	–	–
	Stem	4	+	+	–	–	J656	3.86 ± 0.74	7.01 ± 0.47	–	–
Curvularia crepinii	RS	20	+	+	+	–	1B31	5.00 ± 0.43	13.75 ± 0.25	8.73 ± 0.50	–
	Root	3	+	+	+	–	G518	7.33 ± 0.29	15.25 ± 0.25	1.00 ± 0.29	–
	Stem	5	+	+	+	–	J419	5.33 ± 0.52	17.08 ± 1.28	1.19 ± 0.26	–
Curvularia protuberata	Root	10	+	+	–	–	G649	7.08 ± 0.29	15.17 ± 0.29	–	–
	Stem	10	+	+	–	–	J35	5.58 ± 0.14	14.58 ± 0.14	–	–
Dothiorella sp.	Root	8	+	+	–	–	G116	5.67 ± 0.19	8.05 ± 0.22	–	–
	Stem	5	+	+	–	–	J130	4.79 ± 0.19	8.00 ± 0.25	–	–
Gibberella fujikuroi	RS	20	+	+	+	–	2E8	5.33 ± 0.46	8.73 ± 1.33	4.40 ± 0.20	–
	Root	10	+	+	+	–	G667	4.94 ± 0.25	11.73 ± 0.61	1.52 ± 0.13	–
	Stem	5	+	+	–	–	J651	6.61 ± 0.19	10.80 ± 0.40	–	–
Gloeosporium sp.	RS	20	+	+	–	–	1D55	1.58 ± 0.19	4.74 ± 0.23	–	–
	Root	6	+	+	–	–	G366	1.39 ± 0.10	5.19 ± 0.13	–	–
	Stem	6	+	+	–	–	J149	1.42 ± 0.14	5.07 ± 0.45	–	–
Penicillium chrysogenum	Root	14	+	+	+	–	G335	6.00 ± 0.62	10.83 ± 0.71	6.67 ± 0.88	–
	Stem	4	+	+	–	–	J437	5.52 ± 0.54	6.12 ± 0.37	–	–
Penicillium griseofulvum	RS	15	+	+	+	–	1A93	5.32 ± 0.51	5.87 ± 0.43	10.33 ± 0.73	–
Penicillium pinophilum	RS	15	+	+	+	–	1B44	5.52 ± 0.63	6.12 ± 0.78	4.94 ± 0.63	–
Phialophora sp.	Root	8	+	+	–	–	G115	4.63 ± 0.65	9.56 ± 0.35	–	–
	Stem	6	+	+	–	–	J633	4.30 ± 0.13	5.83 ± 0.73	–	–

Notes: '+' indicates that the strain could grow and '–' indicates that the strain could not grow.
'RS' means rhizospheric soil.

The dendrogram based on ITS sequence analysis showed that the 43 strains selected were distributed among most clades on the tree (Figure 2). Each species in our study clustered together with the same or a similar species that had been reported previously, and thus showed close genetic relatedness between them. Furthermore, it was found that the majority of the morphological taxa (e.g. *Gibberella fujikuroi*, *Curvularia crepinii*, *Fusarium solani*, *Acrophialophora fusispora*, and *Acrophialophora nainiana*, etc.) from different origins clustered together (Figure 2). The rDNA-ITS sequences of the fungi in this study were deposited in GenBank with the accession numbers KF572436–KF572478.

Growth temperature test

According to Cooney's definition (Cooney & Emerson 1964), among all 269 isolates tested, 60.22% of the isolates were thermotolerant fungi (total 162, 110 of which from soils, 47 from roots, and 5 from stems) (Table 3). They could grow at 18°C, 28°C, and 40°C but could not grow at 50°C; their optimum growth temperature was 28°C, except for *Acrophialophora fusispora*, *Acrophialophora nainiana*, and *Penicillium griseofulvum*, whose optimum growth temperature was 40°C (Table 3). In contrast, 39.78% of the isolates were mesophilic fungi (total 107, 20 of which from soils, 44 from roots, and 43 from stems). They could only grow at 18°C and 28°C and could not grow at 40°C and 50°C, and their optimum growth temperature was 28°C. In addition, it was found that the number of heat-adapted fungi increased with increasing environmental temperatures: 84.62% of the isolates from rhizospheric soils were thermotolerant fungi, whereas 51.65% of the isolates from plant roots and 10.42% from plant stems were thermotolerant fungi. Some isolates of the same genera/species from different niches showed different levels of thermotolerance; for example, all isolates of *Curvularia crepinii* could grow at 40°C, while none of the *Curvularia protuberata* isolates could grow at this temperature (Table 3).

Effects of endophytes on host plant thermotolerance

At primary screening tests, it was found that five isolates of *Curvularia* spp. showed activity in improving host plant thermotolerance, while, none of the *Acrophialophora* spp. isolates exhibited activity (Table 4). In further study, the strain G1-29, which was isolated from the root of *H. diffusa* and identified as *Curvularia crepinii*, showed better effects in improving host plant

Table 4. Effects of endophytes on host plant (indica rice) thermotolerance at primary screening tests.

Taxa	Isolates	Effects of endophytes on host plant thermotolerance		
		2 d[a]	4 d	6 d
Curvularia spp.	1F55	1[b]	0	0
	1B31	1	1	0
	2F10	2	1	1
	G518	2	1	1
	J627	2	1	1
	G649	2	0	0
	J522	1	0	0
	J113	2	2	1
	J627	1	1	0
	G63	2	1	0
	G129	3	3	2
	J532	2	0	0
	J64	0	0	0
Acrophialophora spp.	G63	1	0	0
	2C18	0	0	0
	2C35	1	1	0
	G228	0	0	0
	G346	0	0	0
	G536	0	0	0
	G517	1	0	0
	G124	2	0	0
	G226	1	0	0
Control	Sterile water	2	1	0

[a]The culturing time of seedlings with rhizosphere temperatures of 50°C.
[b]The number of seedlings alive. Four seedlings were cultured for each treatment.

thermotolerance: the death rates of endophytic-infected plants were significantly lower than that of endophytic-free plants (*t*-test, $p = .0158$, $df = 4$) with rhizosphere temperatures of 50°C for 4 d under laboratory conditions (Figure 3).

Discussion

The CR and Shannon index are indications of the number and diversity of EF in host plants and are known to vary with altitude, humidity, precipitation, temperature, host plant species, and communities (Spellerberg & Fedor 2003; Kumar & Hyde 2004; Yuan et al. 2011). In the present study, the CR and Shannon index of EF from six plant species ranged from 43.75% to 75.63% and 1.80 to 2.56, respectively, which were a little lower than those reported in non-extreme environments in Yunnan Province; CR and Shannon index of 96.88–100% and 1.80–3.22 for four bryophyte species from a sub-tropical rain forest (Zhou et al. 2015). Similarly, the number and diversity of fungi in rhizospheric soils in the present study were significantly lower than those reported in non-geothermal soils (Cabello & Arambarri 2002; Nesci et al. 2006). The reason for this may be due to high temperatures, high heavy metal content, sparse vegetation, and limited organic carbon, all of which are disadvantageous for fungal survival and selects against non-tolerant species (Redman et al. 1999; Cullings & Makhija 2001).

From Table 1 we see that almost all of the taxa found in soils also occur as endophytes of plants, and the dominant

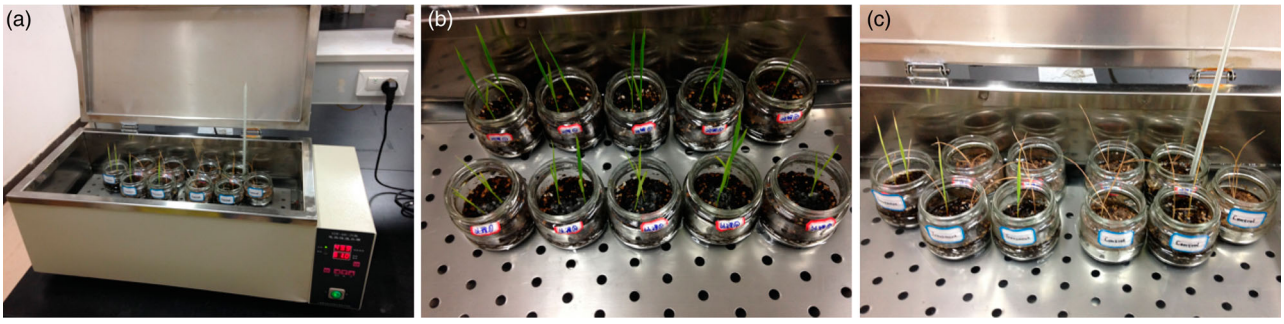

Figure 3. Representative symbiotic (with G1-29) and nonsymbiotic indica rice seedlings with rhizosphere temperatures of 50°C for 4 d under laboratory conditions. (a) Culture equipment; (b) seedlings at the beginning of culture with rhizosphere temperatures of 50°C; and (c) seedlings after 4 d culture with rhizosphere temperatures of 50°C (the five flasks on the left were treatments and the other five were controls).

genera in the rhizospheric soils were also the dominant genera in the plants, except for *Gibberella*. The dendrogram supports this finding; the isolates of the same species isolated from different origins were clustered together (Figure 2). However, we also noted that some species exhibited apparently species-specific habitat distribution patterns. For example, *Acrophialophora fusispora* was the most dominant fungus followed by *Curvularia protuberate* in plants, but soils were devoid of them. On the other hand, *Curvularia crepinii* was the most dominant species while *Acrophialophora nainiana* was the third most dominant fungus in soils. However, plants were almost devoid of them (Table 1). The same phenomenon was observed by Redman et al. (2002). They found that *Curvularia* sp. was the most common endophyte of plants growing in Yellowstone National Park, however, geothermal soils from the base of the plants were devoid of it. These results suggested that many endophytes in geothermal ecosystems have a close relationship with soil fungi, but some of them have evolved to adapt to that particular environment, and become dominant and endemic inhabitants of geothermal ecosystems.

Growth temperature tests indicated that 60.22% of the tested isolates were thermotolerant fungi, and the number of thermotolerant isolates has a positive correlation with environmental temperature (Table 3), which suggested that temperature may be one of the most important environmental factors shaping fungal heat-adaptation in the geothermal ecosystem.

Curvularia spp. and *Acrophialophora* spp. were the dominant fungi in the present study, and *Acrophialophora fusispora* has been reported as a thermotolerant soil fungus that could grow well at 45°C or higher temperatures (Al-Mohsen et al. 2000). Similarly, we found that all isolates of *Acrophialophora* spp. could grow well at 40°C (Table 3). However, different species of *Curvularia* showed different levels of thermotolerance: all isolates of *Curvularia crepinii* could grow at 40°C, while none of the *Curvularia protuberata* isolates could grow at this temperature. The reason for this requires further study. *Curvularia protuberata* has been demonstrated to enhance plant thermotolerance in geothermal ecosystems (Redman et al. 2002; Márquez et al. 2007). In our study, we also demonstrated that the isolate G1-29 (*Curvularia crepinii*) could significantly improve host plant thermotolerance. All these results suggested that species of *Curvularia* and *Acrophialophora* may be important fungi in geothermal ecosystems, and perhaps species or strains have evolved to adapt plants to different temperatures and perhaps may play an important role in improving host plant thermotolerance in the geothermal ecosystem.

In conclusion, our findings have provided evidence that endophytic and rhizospheric fungal communities associated with plants growing in geothermal ecosystems are diverse, and bear a close relationship to one another. Our evidence suggests that most of the fungi in geothermal ecosystems have adapted themselves to high environmental temperatures. *Curvularia* spp. and *Acrophialophora* spp. were the most dominant fungi and often exhibited species-specific habitat distribution patterns. Our data suggest that these species play an important role in improving host plant thermotolerances in geothermal ecosystems.

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