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ARBUSCULAR MYCORRHIZAL SPORES DISTRIBUTION ACROSS DIFFERENT ECOSYSTEMS OF THE QINGHAI TIBETAN PLATEAU

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Abstract

Although arbuscular mycorrhizal fungal (AMF) spores diversity is well studied in several ecosystems, very little is known about AMF spore diversity in cold-dominated ecosystems of Qinghai-Tibetan Plateau. Overall 60 soil samples were collected from 15 sites, with 5 replicates of each including agriculture, forest, meadow, and shrub ecosystems. Both morphological and molecular methods were adapted for spore identification. At the generic level, differences among the four ecosystems were distinct. The agriculture ecosystem was particularly unique due to its greater uneven distribution of AMF genera. Four of the 11 genera, *Diversispora*, *Funneliformis*, *Claroideoglomus*, and *Acaulospora*, accounted for more than 90% of the total relative abundance. *Acaulospora koskei*, *Funneliformis sp.* and *Dentiscutata erythropa* were the key indicator species in agriculture, shrub and meadow ecosystems, respectively. Spore densities in the agriculture ecosystem were higher than in the other ecosystems while species richness were lower. Compared to other ecosystems Shannon index of diversity indicated the highest diversity of AMF in forest. Morphological and single spore identification provides the better differentiation in community distributions and occurrence as well as the diversity of AMF spores in various ecosystems.

Key words: Arbuscular mycorrhiza spores, Distribution, High altitude, Ecosystems, 18s rDNA, Specific primer.

Introduction

In the multifaceted world of symbiosis, arbuscular mycorrhizal fungi (AMF) signify a unique interaction between fungus and its host plant, leading to an overall enhancement of the fitness of interaction patterns (Karandashov & Bucher, 2005; Verbruggen *et al*., 2012), and contribution to plant resistance against environmental stresses (Sikes *et al*., 2009; Balliu *et al*., 2015; Xu *et al*., 2018). AMF maintain ecosystem stability and enhance ecosystem development (Fuhrman, 2009; Rosindell *et al*., 2011; Verbruggen *et al*., 2012). More specifically, AMF diversity and plants diversity are linked with each other (Lovelock & Ewel, 2005), and consequently there is an increasing interest in the assessment of biodiversity and function of AMF communities (Landis *et al*., 2004; Hashem *et al*., 2018). Although AMF diversity has been a major research topic in various terrestrial ecosystems,because very little is documented about the distribution of AMF in high altitude regions.

Previous studies reported diverse AMF taxa in different natural ecosystems (Husband *et al*., 2002). The investigation of agriculture or natural habitats revealed a high diversity of AMF (Öpik *et al*., 2008; Bonfim *et al*., 2016), where plant root-associated AMF communities had inclined to a lower diversity in agriculture ecosystems (Schnoor *et al*., 2011; Bainard *et al*., 2015). AMF traits evolution might be very much dependent on specific abiotic factors (e.g. hypoxia and soil temperature). Certain AMF are confined to severe habitats like as gypsum soils and geothermal (Alguacil *et al*., 2009; Helgason & Fitter, 2009; Dumbrell *et al*., 2010). Diversity of AMF species reported in forest, grasslands, and other ecosystems, mostly belong to the zone with temperate climatic conditions while little is stated in cold regions and low Arctic meadow (Pietikainen *et al*., 2007; Gai *et al*., 2009; Opik *et al*., 2010; Kotilinek *et al*., 2017). While most of the studies are focused about the colonization of mycorrhiza (Rasmussen *et al*., 2016; Bernaola *et al*., 2018), very few are addressed about the diversity of AMF (Manoharan *et al*., 2017), although AMF has been affecting multiple ecosystem functions and processes (Scheublin *et al*., 2007; Lee *et al*., 2013). Thus, the importance of AMF species occurrence and distribution of spores need to be addressed.

AMF species have been described based on the simple morphology and ontogeny of their asexual spores. Schüssler's Glomeromycota phylogeny (http://www.amf-phylogeny.com/) for current approximation about AMF species and number of Glomeromycota genera (most up to date resource; May 2018) are being used to assess AMF diversity. Genomic DNA sequencing of the AMF isolates in spore collections has been used to increase the understanding its phylogeny, which has assisted the construction of a more robust molecular-based taxonomy (Redecker *et al*., 2000; Öpik *et al*., 2016). Three main techniques has been used for identification of AMF taxa present in terrestrial ecosystems: 1) morphological and molecular identification of spores isolated from soils, 2) molecular identification of the hyphae and other structures (e.g. arbuscules) colonizing plant roots, and 3) molecular identification of the hyphae and spores isolated from the whole soil samples. AMF identification is usually based on observations of spores' samples, collected from soil, by microscopic approach (Schenck & Perez, 1990). Molecular techniques are used to examine the genetic variations of AMF and also their phylogenetic relationships. Numerous efforts have been done to use internal transcribed spacer (ITS) region/area of the fungal rDNA as a tool to identify AMF, and DNA extractions

and sequencing based phylogenetic studies on spores of AMF (Redecker *et al*., 1997; Ohsowski *et al*., 2014). This group of AMF has lately raised up as an innovative monophyletic phylum Glomeromycota, which is based on small subunit rDNA sequencing analysis.

Hence, we expected that AMF species and their traits in habitats with temperate regions might be different from those in ecosystems with high altitude. To better understand the AMF occurrence and distribution and its determinants in habitats with high altitude, AMF spore across the different ecosystem of East-Qinghai-Tibet Plateau, China was selected in this study. Based on morphological analyses, about 104 species of AMF have been identified from China, so far (Gai *et al*., 2009). Moreover, little is known about AMF spores on the basis of nucleotide sequences, in the study region. Thus, we used both single spores morphological and partial 18S rDNA gene amplification analyses to describe the AMF spore diversity present across different ecosystems. The specific questions addressed were: (1). What is the AMF spore abundance, composition and richness across four ecosystems in the Qinghai-Tibet Plateau? (2). Which genera are most frequent and abundant across four ecosystems? (3). To which extent are the AMF taxa present in this area is different from those in other ecosystems? (4) Do the molecular analyses of AMF spore consistent with morphological identification which allow us to compare and evaluate both these methods?

Materials and Methods

Soil collection/sampling: Soil samples were collected in September 2015 from the area surrounded by 4 different ecosystems divided into 12 sites, on East-Qinghai-Tibetan Plateau, China. The climate of this region includes it in the cold-dominated ecosystem. The sample plots were divided into 4 types of ecosystems viz. forest (F), shrub (S), meadow (M), and agriculture (A). Collection of soil samples was done randomly from shrub, forest and meadow ecosystems, while soil sampling of crop rhizosphere was done from agriculture ecosystem. Most common plant species in the forest ecosystem were *Rosa xanthina* Lindl. f. *normalis* Rehd. et Wils., *Berberis diaphana* Maxim., *Lonicera rupicola* J. D. Hooker & Thomson, *Paeonia veitchii* Lynch, *Fragaria orientalis* Losinsk., *Thalictrum aquilegifolium* L. var. sibiricum Regel et Tilling and *Bupleurum smithii* Wolff var. *parvifolium* Shan et Y. Li. The shrub ecosystem mainly dominated by *Potentilla fruticosa* (L.) Rydb., *Hippophae rhamnoides* L., *Saussurea hieracioides* Hook. f., *Swertia tetraptera* Maxim., *Potentilla ancistrifolia* Bunge var. dickinsii (Franch. Et Savat.) Koidz and *Potentilla anserine* (L.) Rydb. Meadow ecosystem was dominanted *Heteropappus hispidus* (Thunb.) Less., *Persicaria vivipara* (L.) Ronse Decr., *Thalictrum alpinum* L., *Swertia tetraptera* Pall., *Leontopodium leontopodioides* (Willd.) Nakai and *Astragalus polycladus* Bureau & Franchet. However, in agriculture ecosystem the only cultivated crop species was *Triticum aestivum* L.. A total of 60 soil samples were collected, sealed in zip-lock bags, then categorized and kept for one week for air-drying, and stored at 10°C for spore isolation.

Morphological identification of spores: AMF spores were extracted from 100 g air-dried soils per sample by wet sieving (a pair of sieves: 750 and 38-µm mesh) and sucrose centrifugation (Brundrett *et al*., 1994) and the densities of the spore cells were counted. The extracted spores initially clustered and then grouped according to spore morphology and color using a dissecting microscope. For morphological identification; color of spore, surface ornamentation, shape, wall structures and spore contents were observed while the latest taxonomic criteria was executed in accordance with the Schüßler & Walker, (2010) and Redecker *et al*., (2013), and from the web page (http://invam.caf. wvu.edu). Afterward, we further sub-divided the initial cluster of spores, removed the dead spores and recounted. This recorded data was used for AMF spore community analysis. For species identification, Melzer's reagent, and trypan blue were utilized and then permanent slides were fixed with polyvinyl-lacto-glycerol, subsequently airtight with nail varnish, and then kept for storage in the laboratory (Professor Huyuan Feng, Lab. N0. 324), at School of Life Sciences, Lanzhou University, China.

Molecular identification of spores: To study the spore morphology of INVAM spores by single spore method is quite difficult and error-prone. Therefore, single spores after morphological identification of spores, were separated and used to identify at a molecular basis, across all ecosystems.

Spore separation clustering: AMF spores were separated from 25 g soil, per soil sample by wet-decanter-sucrose gradient centrifugation and then morphological clustering was performed by using a stereo-microscope and photographed. Afterward, spores were transferred into a 2 mL centrifuge tube comprising 1.5 mL dd-H_2O and kept for 48 hours at 4°C and then moved to a petri dish. Freshly sprouted spores were re-filled into centrifuge tubes along with fresh distilled water and placed for 24 ~ 48 h at 4°C. The atypical spores were removed, washed with distilled water and again shifted to the centrifuge tube for short-term preservation and then for DNA extraction and single spore propagation.

Single spore DNA extraction: Spores were washed in the tube containing 1.5 mL dd H_2O in Ultrasonic Cleaners set 10 KHz for 5 minutes, then each spore moved into a 0.5 ml PCR tube having 10 µl sterilized water. A sterilized pipette was used to press spores in the PCR tube under the endoscopic microscope and then spores were crushed for extracting DNA. Subsequently, PCR tubes were moved into a water bath and kept for 7 minutes at 80°C and then stored at 4°C until ready for use.

Nested amplification to obtain the characteristic sequence in 18s-rDNA: Nested PCR was used to amplify the partial 18S rDNA fragments of AMF. First PCR was executed by using primers GeoA2 and Geo11 (Schwarzott

& Schußler, 2001), Template single spore DNA, 25 µl PCR system included: 2 µl template, 2 µl dNTP, primer 1 µl, buffer 2.5 µl, Taq DNA polymerase 0.3 µl, and ddH_2O 17.2 µl. PCR cycles were set at 94°C for 2 min, 35 X (at 94°C for 30 s, at 59°C for 1 min, at 72°C for 2.5 min), at 72°C for 10 min, and at 16°C for forever. DNA amplified by PCR was detected on 1.5 % agarose gel with ethidium bromide (EtBr) and tested under UV transilluminator.

First PCR products were utilized as a pattern to perform the second amplification by using universal eukaryotic NS31 (Simon *et al*., 1992) and AMF-specific primers AML2 (Lee *et al*., 2008) for 30 cycles (25 µl PCR system: 2 µl template, 2 µl dNTP, primer 2 µl, buffer 1.5 µl, Taq 0.3 µl, and ddH_2O 17.2 µl). PCR cycles were set at 94°C for 2 min, 35 X (at 94°C for 30 s, at 58°C for 1 min, at 72°C for 80 s), at 72°C for 10 min, and at 16°C for forever. DNA (about 560 bp) obtained after PCR amplification was detected on 1.5% agarose gel with EtBr and tested under UV transilluminator.

Clone library construction: PCR products-derived all spore were used to construct the clone libraries. PCR products with expected length (c. 560 bp) purified with Gel as well as PCR clear up system was named as Promega. Afterward a retailing technique, DNA product was ligated into the pGEM-T vector, Promega, and then cloned in *Escherichia coli*, and DH5α, Tiangen Biotech, following the instructions of manufacturer. Per clone library, 3 positive clones were selected using ampicillin, based on white screening, dipped into 30 mL ddH_2O and then exposed to three freezing and thawing cycles to prepare the plasmid template. Reamplification of inserts was done with NS31/AML2 primers following similar PCR settings as mentioned above. Later PCR products were further examined by 1.5% (w/v) agarose gel electrophoresis. The bands having a strong signal and, right position were selected and corresponding clones were sent for sequencing (Majorbio, Shanghai, China).

Processing and analysis of data: Composition of AMF spores across different ecosystems was estimated depending upon the density, species richness, relative abundance, frequency of occurrence, and importance value. Density of spore (spores in each 25 g air-dried soil) calculated by directly counting the spores. Total number of AMF species of spores in each 100 g air-dried soil was termed as species richness. Frequency of occurrence (FO) was evaluated as the samples percentage where spores were isolated on the basis of a specific genus and/or species.

$$FO = \frac{number\ of\ soil\ samples\ those\ AMF\ families, genera\ or\ species\ occurred}{total\ number\ of\ soil\ samples} \times 100\%$$

Relative abundance (RA) was described as the total number of spores belonging to a family, genus and/or species, divided by the whole number of spores.

$$RA = \frac{total\ number\ of\ spores\ of\ a\ family, genus\ or\ species}{number\ of\ total\ spores} \times 100$$

Important value (IV) was described, the total sum of frequency of occurrence and relative abundance. The dominant genus and species were determined according to the important values.

$$IV = (frequency + relative\ abundance)/\ 2$$

The Shannon-Weiner index was calculated as following the formula:

$$H = -\sum [P_i\ log_2(P_i)]\ P_i = n_i/N$$

where H is Shannon-Weiner index, P_i is the relative abundance of AMF spores of each site/area. The indicator species analyses (species with Indvalvalues > 0.3 and $P < 0.05$, are strong indicators) were performed to test whether there were specific AMF spores associated with the ecosystems, and the indicator value index was used to measure the associations. One-way ANOVA was used to analyze the data by SPSS 22.0 version and figures draw in Origin 9.0 (Microcal Software Inc.). Statistically significant differences were considered when P was ≤0.05. A Venn diagram based on unique and shared AMF spores were produced using R language (R version 3.3.2) to characterize the differences and similarities among spores. A single spore sequencing was aligned with MEGA 7.0 to construct a phylogenetic tree.

Results

AMF spore community composition: Spores were screened from 60 collected soil samples of 4 different ecosystems. A total of 31 species were identified with 13 genera and 6 families. There were 8 taxa identified belonging the genus *Funneliformis*; 3 to *Septoglomus*, *Acaulospora*, and *Diversispora*; 2 to *Claroideoglomus*, *Rhizophagus*, *Racocetra*, *Gigaspora*, and *Dentiscutata*; and 1 of *Glomus*, *Scutellospora*, and *Ambispora*, respectively (Table 1, Fig. S1). Among 29 taxa, 5.9% belonged to the Glomeraceae family although they are not yet identified at species level.

AMF spore density, richness, and diversity: Spore density diverged across 4 ecosystems, having the highest and lowest spore density in agriculture and forest

ecosystem, compared to meadow and shrub ecosystem, respectively (Fig. 1A). In the agriculture ecosystem the richness of the AMF spore population was significantly lower (Fig. 1B; $p<0.05$), while other ecosystems showed relatively higher richness but had no significant difference, indicating that spore population decreased with extensive land use practices than natural ecosystems (Fig. 1B). We further calculated the Shannon index as a measure of AMF spore diversity. In a forest ecosystem, the spore diversity was higher than agricultural whereas, in shrub and meadow, the spore diversity was similar (Fig. 2).

To evaluate and indicate the distribution and number of shared and specific AMF spores among the different ecosystems, Venn diagram was constructed. The presence of AMF spores at 31 is illustrated by Venn diagram. The number of species shared by the 4 ecosystems was 18. The number of AMF spores of meadow and forest samples shared largest 24, while meadow and shrub, shrub and forest, agriculture and meadow showed the same trends (22 species) followed by agriculture and shrub, and agriculture and forest (21 spores) (Fig. 3).

AMF spore frequency of occurrence (FO), relative abundance (RA) and important value (IV): Glomeraceae family occurred most frequently, at the generic level, where agriculture ecosystem was unique compared to the other three ecosystems, due to its more uneven distribution, (The total relative abundance of *Diversispora*, *Funneliformis*, *Claroideoglomus*, and *Acaulospora* was more than 90% of the total relative abundance, while remaining had less than 10%). Dispersion and spore number were considered simultaneously to determine the dominance of species in AMF community, so defined the dominance of AMF spore-based on, FO>50% and RA>5% (Table S1). It is evident that *Scutellospora* was dominant in agriculture while *Ambispora* was absent in agriculture and forest both, however, other AMF genera were found in all ecosystems (Table 1).

Table 1. Relative abundance (RA) and frequency of occurrence (FO) of the identified AMF species in the four ecosystems.

No.	Family	AMF species/Genera	RA (%)				FO (%)			
			A	F	M	S	A	F	M	S
	Glomeraceae	*Funneliformis*	21.6	14.5	31.9	18.4	95.8	85.7	100	88.0
1.		*Funneliformis mosseae*	14.3	3.6	3.7	6.9	66.7	16.7	38.1	64.0
2.		*Funneliformis coronatum*	2.6	14.4	-	3.4	29.2	58.3	-	20.0
3.		*Funneliformis caledonvus*	4.2	4.8	2.1	2.0	54.2	50.0	33.3	28.0
4.		*Funneliformis coronatus*	0.5	5.9	5.0	3.9	12.5	70.8	47.6	64.0
		Septoglomus	0.3	10.0	13.8	16.3	16.7	76.2	50.0	76.0
5.		*Septoglomus constrictum*	0.1	13.8	5.0	13.5	8.3	50.0	47.6	72.0
		Glomus	1.4	7.0	0.9	2.5	33.3	76.2	19.2	64.0
6.		*Glomus hoi*	1.4	0.9	7.0	2.5	33.3	50.0	47.6	64.0
		Rhizophagus	4.3	4.0	1.2	2.5	50.0	47.6	12.5	36.0
7.		*Rhizophagus aggregatus*	4.0	1.2	3.3	2.2	50.0	12.5	42.9	32.0
		Unknown genus	1.5	12.4	8.2	6.4	50.0	66.7	83.3	60.0
8.		Unknown sp.	1.4	8.2	12.4	6.4	50.0	83.3	66.7	64.0
	Gigasporaceae	*Scutellospora*	-	7.8	10.6	17.0	-	47.6	62.5	52
9.		*Scutellospora calospora*	-	10.6	7.8	17.0	-	62.5	47.6	56.0
		Racocetra	1.0	2.8	5.0	1.2	29.7	33.3	37.5	24.0
10.		*Racocetra coralloidea*	1.0	3.8	2.0	1.2	25.0	29.1	23.8	28.0
		Gigaspora	0.3	3.2	2.2	0.7	8.33	19.1	41.7	16.0
		Dentiscutata	0.3	1.2	3.8	0.6	8.33	28.6	54.2	21.0
	Claroideoglomeraceae	*Claroideoglomus*	14.4	13.2	4.3	5.5	62.5	81.0	58.3	40.0
11.		*Claroideoglomus claroideum*	13.5	0.2	7.5	5.5	41.7	8.3	47.6	44.0
12.		*Claroideoglomus* sp.	0.8	4.1	5.7	-	25.0	54.1	66.7	4.0
	Acaulosporaceae	*Acaulospora*	17.0	16.2	11.7	19.1	75.0	61.9	70.8	76.0
13.		*Acaulospora koskei*	16.8	1.9	4.2	5.1	75.0	20.8	33.3	56.0
14.		*Acaulospora kentinensis*	0.1	9.8	2.6	13.7	4.2	58.3	28.5	60.0
	Diversisporaceae	*Diversispora*	38.1	7.9	6.4	9.7	83.3	90.5	50.0	80.0
15.		*Diversispora aurantia*	28.5	0.1	2.4	-	33.3	4.2	42.9	-
16.		*Diversispora eburnea*	7.1	5.9	10.9	9.7	75.0	37.5	81.0	81.0
	Ambisporaceae	*Ambispora*	-	-	0.06	0.18	-	-	4.2	4.0

A, Agriculture ecosystem; F, Forest ecosystem; M, Meadow ecosystem; S, Shrub ecosystem

Table S1. Phylogenetic characteristic of the AM fungal species.

Families	Genera	AMF species	RA (%)	FO (%)	IV (%)	ID
Glomeraceae (41.4%)	*Funneliformis* (22.7%)	*Funneliformis mosseae*	8.18	46.81	27.49	1
		Funneliformis coronatum	5.54	27.66	16.60	2
		Funneliformis caledonvus	3.49	41.49	22.49	3
		Funneliformis coronatus	3.38	48.94	26.16	4
		Funneliformis geosporus	1.09	21.28	11.18	5
		Funneliformis geosporum	0.56	9.57	5.07	6
		Funneliformis sp.	0.43	9.57	5.00	7
		Funneliformis verruculosum	0.05	1.06	0.56	8
	Septoglomus (9.4%)	*Septoglomus constrictum*	7.88	43.62	25.75	9
		Septoglomus viscosum	0.86	15.96	8.41	10
		Septoglomus deserticola	0.63	8.51	4.57	11
	Glomus (2.3%)	*Glomus hoi*	2.25	50.00	26.13	12
	Rhizophagus (2.92%)	*Rhizophagus aggregatus*	2.68	32.98	17.83	13
		Rhizophagus intraradices	0.25	6.38	3.32	14
	Unknown genus (5.9%)	Unknown sp.	5.91	64.89	35.40	15
	Scutellospora (8.3%)	*Scutellospora calospora*	8.33	41.49	24.91	16
	Racocetra (2.3%)	*Racocetra coralloidea*	1.87	25.53	13.70	17
		Racocetra sp.	0.43	7.45	3.94	18
Gigasporaceae (13.3%)	*Gigaspora* (1.3%)	*Gigaspora* sp.	1.03	18.09	9.56	19
		Gigaspora margarita	0.22	6.38	3.30	20
	Dentiscutata (1.4%)	*Dentiscutata erythropa*	0.96	12.77	6.86	21
		Dentiscutata reticulata	0.43	15.96	8.19	22
Claroideoglomeraceae (9.2%)	*Claroideoglomus* (9.2%)	*Claroideoglomus claroideum*	7.12	34.04	20.58	23
		Claroideoglomus sp.	2.07	35.11	18.59	24
Acaulosporaceae (15.0%)	*Acaulospora* (15.0%)	*Acaulospora koskei*	8.19	46.81	27.50	25
		Acaulospora kentinensis	6.57	38.30	22.43	26
		Acaulospora morrowiae	0.23	7.45	3.84	27
Diversisporaceae (19.3%)	*Diversispora* (19.3%)	*Diversispora aurantia*	10.2	19.15	14.65	28
		Diversispora eburnea	7.96	68.09	38.02	29
		Diversispora tortuosa	1.21	11.70	6.46	30
Ambisporaceae (0.07%)	*Ambispora* (0.1%)	*Ambispora gerdemannii*	0.07	2.13	1.10	31

Note: the percentages6 in the brakets under families or genera present the relative abundance. RA, relative abundance; FO, frequency of occurrence; IV, important value. The species with grey background was set as the most important part to be analyzed

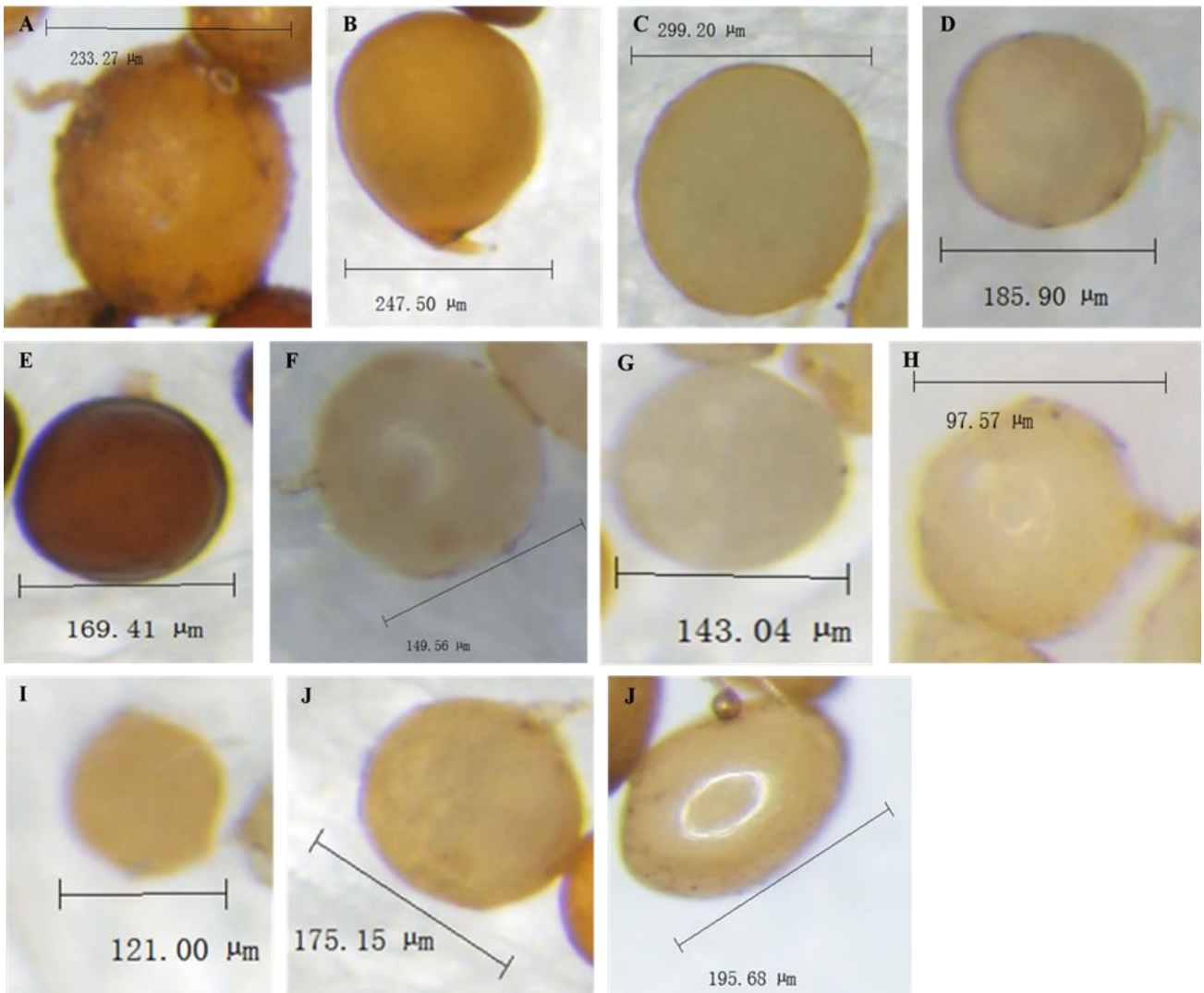

Fig. S1. Spore of arbuscular mycorrhizal fungi used in this study. (Accession number; family; sources) **A**. *Glomus coronatum* (AJ276086; Glomeraceae; M4), **B**. *Glomus caledonium* (Y17635; Glomeraceae; A2), **C**. *Glomus caledonium* (Glomeraceae; A4), **D**. *Funneliformis mosseae* (Glomeraceae; A2), **E**. *Funneliformis constrictum* (FR750212; Glomeraceae; F1), **F**. *Claroideoglomus lamellosum* (AJ276087; Charoideoglomeraceae; M4), **G**. *Diversispora sp. W3033* (FR686934; Diversisporaceae; F1), **H**. *Diversispora aurantia* (AM713432; Diversisporaceae; A4), **I**. *Diversispora sp*. (KY416573; Diversisporaceae; F1), **J**. *Scutellospora crenulata* (HQ202290; Gigasporaceae; A4), and **K**. *Scutellospora dipurpurescens* (KY416581; Gigasporaceae; F1).

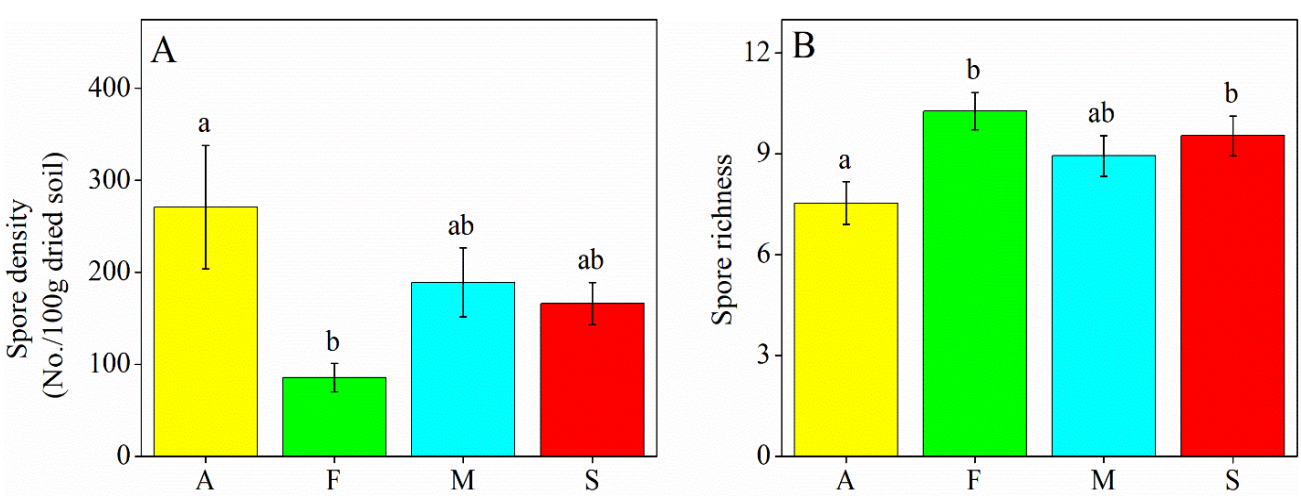

Fig. 1. (A) Average number of spore density (per 100 g soil) and (B) spore richness, under different ecosystem. The lowercase letters in each column represent significant difference (p<0.05) among different ecosystems. Error bars denote ± SE. A, agriculture; F, forest; M, meadow; S, shrub.

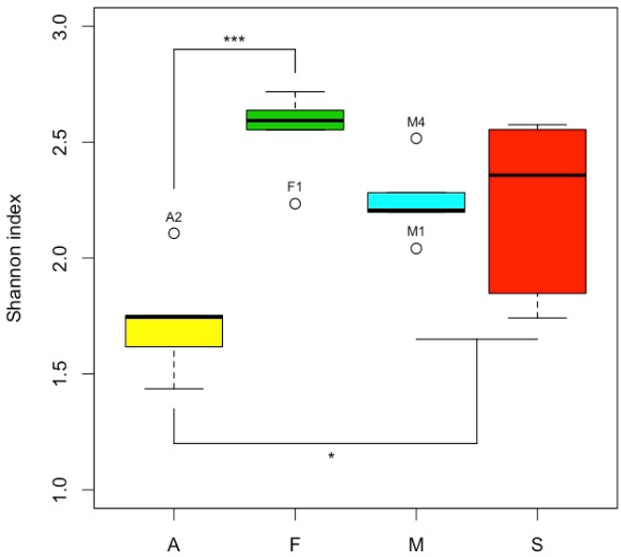

Fig. 2. Compositions of spore communities of arbuscular mycorrhizal fungi (AMF) across the ecosystems. Shannon index of spore communities in soil samples at different ecosystem. Asterisks denote significance differences among ecosystem for a given soil according to *Tukey, spost-hoctest* (*, p<0.05; **, p<0.01; ***, p<0.0).Error bars denote ± SE of the Shannon index observations. A, agriculture; F, forest; M, meadow; S, shrub.

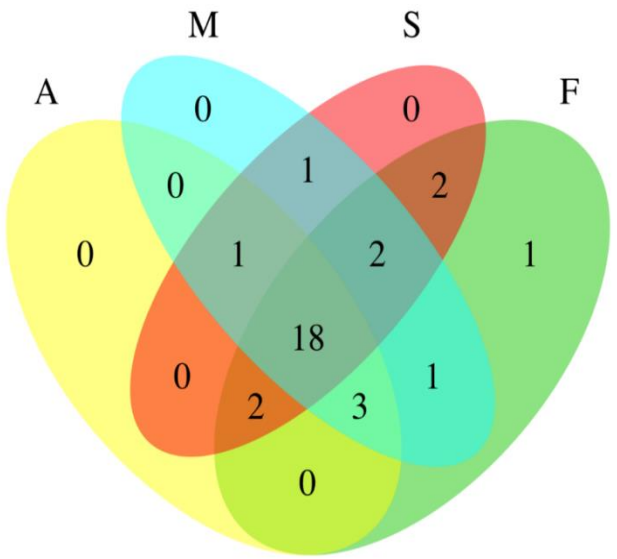

Fig. 3. Venn diagram illustrating partitioning of the variation in arbuscular mycorrhizal fungal (AMF) spore community profiles among the four groups of ecosystems. The size of circles is proportional to the variability in AMF communities as explained by different ecosystems; the overlap of the circles represents the variation shared across the ecosystems. Different colors represent different ecosystems. A, agriculture; F, forest; M, meadow; S, shrub.

The FO and RA of AMF spore species varied greatly across ecosystems. In agriculture ecosystem, the spores of *Funneliformis* were the most frequent (95%), followed by *Diversispora* (83 %), *Acaulospora* (75%), *Claroideoglomus* (63%), *Rhizophagus* and unidentified genus (50%), and *Glomus* (33%), and RA accounted for *Diversispora* was 38% of the total number of spores followed by *Funneliformis* (22%). The dominant species were *D. eburnean*, *A. koskei*, and *F. mosseae*. Among them, *A. koskei*, *F. mosseae*, and *D. eburnean* accounted for 17, 14, and 7% of the total number of spores, respectively. However, the important value (IV) of *A. koskei* was 46% followed by *D. eburnea* (41%), and *F. mosseae* (41%). In addition, *Scutellospora calospora* was absent in agricultural ecosystem (Tables 1 and 2). In a forest ecosystem, the FO of spores that belonged to *Glomus*, *Claroideoglomus*, *Diversispora*, and unidentified genus were higher, and those of *Funneliformis*, *Rhizophagus*, and *Acaulospora* decreased to 86%, 48%, and 62%, respectively. *Funneliformis coronatus*, *Septoglomus constrictum*, *Glomus*, unidentified sp., *Scutellospora calospora*, and *Acaulospora kentinensis* were the dominant species. Although the important value of *R. aggregatus*, *Claroideoglomus claroideum*, and *Diversispora aurantia* was less than 10% of the total soil samples, and they accounted for 1.15, 0.19 and 0.06% of the total of the spores (Tables 1 and 2). In meadow ecosystem, the spores of the *Funneliformis* were most frequent and were accounted for about 31% of the total number of spores, followed by *Septoglomus* (14%). The dominant species were *D. eburnea*, *G. hoi*, Unknown sp., and *Claroideoglomus sp*. among them, and Unknown sp., *D. eburnea* and *G. hoi* accounted for 12, 11, and 7% of the total number of spores, respectively. In addition, *F. mosseae*, *R. aggregatus*, *A. koskei* and *D. aurantia* were frequently detected, although their RA was less than 5% (Tables 1 and 2). In shrub ecosystem, the spores of *Funneliformis* were up to 18%, while those of *Diversispora*, *Scutellospora*, and *Septoglomus* were much higher compared to agriculture, forest and meadow ecosystem. *S. calospora*, which accounted for 17% of spores, was the most common species, followed by *S. constrictum*, *A. kentinensis*, and *D. eburnea*. Additionally, *D. eburnea*, *S. constrictum*, *F. mosseae*, *Funneliformis coronatus*, *S. calospora*, *A. koskei*, *A. kentinensis* and *G. hoi* were frequently isolated from this ecosystem. Remarkably, *D. eburnea* was the only species which had the important value such as 41.0%, 21.7%, 45.9% and 44.8% for agriculture, forest, meadow and shrub ecosystem respectively, and it held a high percentage of the total spores (Tables 1 and 2). Indicator species analysis detected that there were 4 indicator species (Indvalvalue ≤ 0.6, p≤0.05) of AMF, namely *A. koskei*, *Funneliformis* sp., *Dentiscutata erythropa* and *F. coronatum*. *A. koskei* is an indicator species in agriculture, *Funneliformis* sp., is an indicator species of shrub and *D. erythropa* and *F. coronatum* both are indicative species of meadow (Table 3).

Molecular identification of AMF spore: AMF spores grouped on the basis of morphological characteristics, and demonstrative spores from individual groups were designated. Specified spores based on single spore identification produced and were analyzed with NS31/AML2 primer pair. In total, 48 sequences screened from spores, where 44 sequences, contained a band of expected size (about 560 bp), deposited to GenBank database for molecular identification. Quite similar sequences obtained by sequence database from National Center for Biotechnology Information (NCBI) using BLAST search, and 31 sequences were confirmed, which belonged to AMF (Table S2). In this grouping numerous sequences, on the basis of morphological identification, were not consistent, primarily because of the restriction in the identification based on spores' morphological characteristics as well as inadequate AMF sequencing data in the database.

Table 2. Important value (IV) of the identified AMF species in the four ecosystems.

No.	Genera	AMF species	IV (%)			
			A	F	M	S
1.	*Funneliformis*	*Funneliformis mosseae*	40.5	10.1	20.9	35.5
2.		*Funneliformis coronatum*	15.9	36.3	-	11.7
3.		*Funneliformis caledonvus*	29.2	27.3	17.7	15.0
4.		*Funneliformis coronatus*	6.5	38.3	26.3	33.9
5.	*Septoglomus*	*Septoglomus constrictum*	4.2	31.9	26.3	42.7
6.	*Glomus*	*Glomus hoi*	17.3	15.0	41.6	33.3
7.	*Rhizophagus*	*Rhizophagus aggregatus*	27.0	6.82	23.1	17.0
8.	*Unknown genus*	Unknown sp.	25.7	45.8	39.5	35.2
9.	*Scutellospora*	*Scutellospora calospora*	-	36.5	27.7	36.5
10.	*Racocetra*	*Racocetra coralloidea*	13.0	16.5	12.9	14.6
11.	*Claroideoglomus*	*Claroideoglomus claroideum*	27.6	4.3	27.6	24.8
12.		*Claroideoglomus* sp.	12.9	29.2	26.2	2.0
13.	*Acaulospora*	*Acaulospora koskei*	45.9	11.4	18.8	30.5
14.		*Acaulospora kentinensis*	2.1	34.0	15.6	36.8
15.	*Diversispora*	*Diversispora aurantia*	30.9	2.1	22.6	-
16.		*Diversispora eburnea*	41.0	21.7	45.9	44.8

A, Agriculture ecosystem; F, Forest ecosystem; M, Meadow ecosystem; S, Shrub ecosystem

Table 3. Indicator species that were found to differ significantly across agricultural (A), shrub (S) and meadow (M) ecosystems.

No.	Indicator speceis of AMF spore	Ecosystem	Indicator value	*p*-value
1.	*Acaulospora koskei*	A	0.6927	0.009
2.	*Funneliformis* sp.	S	0.6400	0.012
3.	*Dentiscutata erythropa*	M	0.8627	0.002
4.	*Funneliformis coronatum*	M	0.7072	0.021

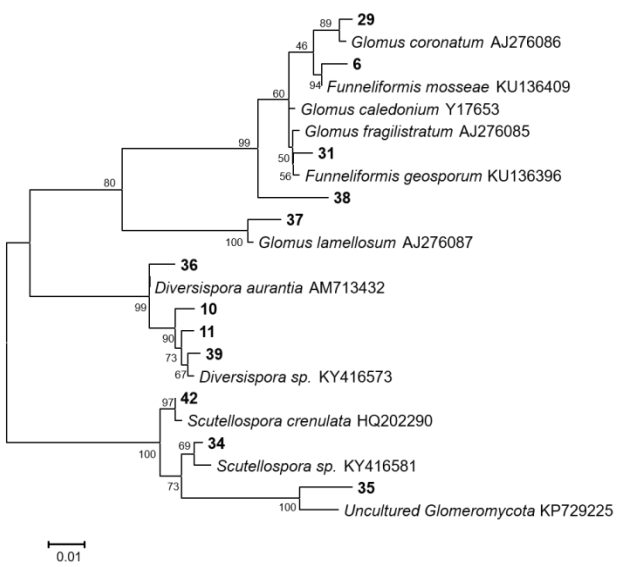

Fig. 4. The sequences of the single spores obtained in this study (corresponding to table3-5) and referenced sequences from Gen-Bank in the phylogenetic tree.

By constructing a phylogenetic tree based on known sequences, 4 families (Glomeraceae, Claroideoglomeraceae, Diversisporaceae, and Gigasporacee) were detected from 31 single spore analysis. *Glomus* consisted of *Glomus coronatum* Giovann., *Glomus caledonium* (T.H. Nicolson & Gerd.) Trappe & Gerd *Funneliformis mosseae*, and *Septoglomus constrictum*; *Claroideoglomus* had only one species *Claroideoglomus lamellosum*; *Diversispora* contained *Diversispora* sp. *W3033*, *D. aurantia*, *Diversispora* sp. and *Scutellospora crenulata* and *Scutellospora dipurpurescens*, (Fig. 4). Across all species, two sequences belonged to *G. coronatum*, twelve belonged to *G. caledonium* and 7 belonged to *F. mosseae*, respectively, whereas only one remaining sequence was detected in each of the remaining 7 species. Single spore sequencing results were inconsistent, such as No. 10 and 11 sequencing were similar to 10 (Table S2), while the results obtained by sequence alignment were not exactly same. Meanwhile, the sequencing of No. 34 and 35 was similar to the No. 34 spore as shown (Fig. 4; Table S2) though in blast result same genera belonged to different species.

Discussion

Current study was performed to evaluate how AMF spore communities were assembled in the soil with high altitude, particularly in Qinghai-Tibet Plateau, China. To better understand the distribution and diversity of AMF, samples were collected from 4 different ecosystems including agriculture, forest, meadow, and shrub. The morphological and molecular approach were used for spore identification. Since little evidence was provided about AMF spores on the basis of nucleotide sequences as most studies are based on the morphological analysis.

As far as we know, this is the major study to report AMF spore community affected by different ecosystems (agriculture, forest, shrub, and meadow) in Qinghai-Tibetan Plateau. Findings from our study are similar to those of other studies (Xiang *et al*., 2014), showing higher average AMF spores diversity in forest than in grasslands or shrubs. This might be linked with the relatively high plant diversity in forest. In the present study, spore richness in agriculture

ecosystem was relatively lower, compared to other ecosystems. It is well known that agriculture practices influence the interactions of AMF spores and plants (Douds & Millner, 1999; Carrillo-Saucedo *et al*., 2018). In general, AMF spore richness is often reduced by soil disturbance due to agricultural activities (Oehl *et al*., 2003; Chaudhry *et al*., 2015). As expected, the large number of AMF spore was shared by meadow and forest ecosystem soils, and the considerable number of spores were shared across 4 ecosystems. The current study thus contradicts with previous observations (Gai *et al*., 2006, 2009) that reported lesser abundance and diversity of AMF spores in unnatural ecosystem as compared to natural ecosystems (Boddington & Dodd, 2000).

Comparatively high diversity of AMF spores was found at high altitude across 4 ecosystems, in Qinghai Tibet Plateau. At whole 13 AMF genera were detected in four ecosystems, in current study, which were close to the number of southern and central Tibet Plateau detected by Gai *et al*., (2009) and Liu *et al*., (2011) though the climate was lesser extreme in our study areas. Present investigation proposes, a disparity in AMF spore community across sites of different ecosystem in the Qinghai Tibet Plateau as well as across sampling locations. Numerous AMF spore phylotypes were detected in this area that could be compared with those identified among temperate natural ecosystems, investigated in different studies (Öpik *et al*., 2006, 2010). Our study predicted, Glomeraceae as the "global species" and showed similar distribution discovered in another location of Tibetan Plateau. However, the Gigasporaceae and Acaulosporaceae had spatial specificity. A great ratio of novel AMF taxa identified in previous work which approves the interpretation that there are numerous taxa still needs to be explored (Helgason *et al*., 2002; Fitter, 2005; Opik *et al*., 2010), particularly from less-investigated geographical biomes and regions (Lumini *et al*., 2010).

Most of the detected AMF spores belongs to Glomeraceae that is the quite commonly wide spread family in managed and natural ecosystem (Zhao *et al*., 2017; Soka & Ritchie, 2018). Unpredictably, Diversisporaceae, a family poorly represented especially in other tropical grasslands (Kotilinek *et al*., 2017; Xu *et al*., 2017), was the most abundant in meadow ecosystem. Evidence has been found for ecosystem preference of various genera, *Funneliformis*, and *Diversispora* with higher frequency and abundance in meadow and forest ecosystem. Whereas *Glomus* was markedly increased in forest and shrub ecosystem and there were several lines of evidence supporting this view. For instance, in all ecosystems, *Glomus* spores were found, suggesting the generalized occurrence of *Glomus* to a wide range of ecosystem (Helgason *et al*., 1998; Lee *et al*., 2006; Dobo, 2016). At the genus level, differences among 4 ecosystem were clear, where agriculture was the most special, compared to the other three types, due to its more uneven distribution (The total relative abundance of *Diversispora*, *Funneliformis*, *Claroideoglomus*, and *Acaulospora* were more than 90%, while remaining 7 genera were less than 10%). Although other studies (Moora *et al*., 2014) displayed profound differences in the distribution of AMF genera across ecosystems (Kivlin *et al*., 2011). However, as per our understanding, it is the first time to document the variances across managed and natural ecosystems at morphological and single spore level of identification, emphasizing the presence of ecological specification for AMF spores.

The molecular analyses of AMF spore using phylogenetic and blast tree displayed inconsistent results with morphological identification. In molecular techniques, identification and segregation of AMF spore at low abundance, appear general, due to problems in PCR-amplification and/or DNA extraction. A poor illustration of taxa by low abundance in PCR-based methods is a common problem produced by experimental bias during sampling time and DNA extraction or during PCR and consequent experimental approaches (Shi *et al*., 2012; Guo & Zhang, 2013). Although Courtney *et al*., (2012) acknowledged an upright sensitivity of the PCR-approach applied for AMF taxa with low abundance, their approaches could be used for DNA extraction from pure spore samples and not from soil or roots.

In swift, both approaches (morphological and molecular) showed relative abundance where AMF spore dominated by *Funneliformi*, *Diversispora*, and *Glomus*. An increase in AMF spore diversity, within the ecosystem confirmed by morphological or single spore molecular identification, was linked to each ecosystem. The morphological approach was able to further distinguish this picture, which suggested the occurrence of fungal species of low abundance belonging to *Diversispora* and *Glomus*, but also to other AMF genera. An investigation by morphological spore documentation exposed clear difference of AMF spores while comparing across ecosystems. But on the other hand, practices of next-generation sequencing are speedily advanced at present, and primer sets are being progressively upgraded, so that some of the primers also comprise diverse species, or differ more openly from related species (Lee *et al*., 2006; Kruger *et al*., 2009; Kruger *et al*., 2012; Moora *et al*., 2014; Crossay *et al*., 2017).

Conclusion

To date, this is the most comprehensive study of AMF spore distribution within a natural and disturbed ecosystem in the cold-dominated region. For AMF spore morphological study, we determined relative abundance, frequency of occurrence, importance value, species richness, and Shannon index. We firmly state that species richness and Shannon index of AMF spores are strongly influenced by natural ecosystem but not by disturb ecosystem. However, in addition to the overarching picture of AMF spore communities with the use of molecular identification tools, the morphological methodology seems more consistent. Thus, identification of AMF at morphological level is quite, practicable approach to evaluate the AMF communities from the soil samples of natural or disturbed ecosystem.

Table S2. Sequence information about the single spore from NCBI.

ID	Accession number	Genbank definition	Length (μm)	Source
38	FR750212	*Funneliformis constrictum*	170.3	F1
6	KU136409	*Funneliformis mosseae*	176.3	A2
7		*Funneliformis mosseae*		
15	KU136409	*Funneliformis mosseae*	166.7	A2
16	KU136409	*Funneliformis mosseae*	177.5	A2
17	KU136409	*Funneliformis mosseae*		
18	KU136409	*Funneliformis mosseae*	150.7	A1
19	KU136409	*Funneliformis mosseae*	260.0	A1
9	KU136409	*Funneliformis mosseae*	228.0	A1
20	Y17653	*Glomus caledonium*	319.8	F3
21	Y17653	*Glomus caledonium*		
24	Y17635	*Glomus caledonium*	107.3	S1
25	Y17635	*Glomus caledonium*		
48	Y17635	*Glomus caledonium*	226.0	A2
41	Y17635	*Glomus caledonium*		
26	Y17635	*Glomus caledonium*	168.5	S2
27	Y17635	*Glomus caledonium*		
30	Y17635	*Glomus caledonium*	154.0	M5
31	Y17635	*Glomus caledonium*	268.0	A4
28	Y17635	*Glomus caledonium*	276.4	A4
40	Y17635	*Glomus caledonium*	193.6	A4
29	AJ276086	*Glomus coronatum*	220.1	M4
33	AJ276086	*Glomus coronatum*	228.3	M4
37	AJ276087	*Claroideoglomus lamellosum*	142.0	M4
10	FR686934	*Diversispora* sp. W3033	142.1	F1
11	JF340049	*Uncultured Diversispora*		
36	AM713432	*Diversispora aurantia*	96.7	A4
39	KY416573	*Diversispora* sp.	104.2	F1
34	KY416581	*Scutellospora* sp.	188.0	F1
35	KP729225	*Scutellospora dipurpurescens*		
42	HQ202290	*Scutellospora crenulata*	103.5	A4
8	KC236251	*Orchesellides sinensis*	158.8	A1
12	AY596362	*Hypogastrura* sp.	138.0	S2
13	AY596363	*Hypogastrura* sp.		
14	KM355995	*Agaricales* sp.	190.4	A2
22	AF426949	*Lachnella villosa* isolate AFTOL-ID 525	122.4	M2
23	AF426949			
32	KY382771	*Protaphorura fimata*	183.5	A1
5	DQ646535	*Marcelleina tuberculispora*	108.6	A4
43	JQ237143	*Basidiomycota sp.* S-MA-4	162.0	A1
44	AJ515166	Uncultured soil ascomycete	132.2	A1
45	EU368304	Uncultured ascomycete clone 76	102.2	A4
46	DQ646534	*Marcelleina persoonii*	180.3	A4
47	KM096371	*Geomyces* sp. MF584	150.3	A4

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