



Association of ectomycorrhizal fungi with *Picea crassifolia* (Pinaceae, Piceoidae) from high-altitude stands in Mount Helan Nature Reserve, China

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ABSTRACT. We investigated the diversity of ectomycorrhiza associated with the endemic *Picea crassifolia* in Mount Helan National Nature Reserve in Inner Mongolia, China. Toward this objective, we conducted morphological and molecular identification of ectomycorrhizae in soil cubes taken from pure *P. crassifolia* stands. Eleven types of ectomycorrhizal (ECM) organisms were separated, briefly described, and identified. Nine morphotypes belonged to the

phylum Basidiomycotina [*Amphinema byssoides*, *Cortinarius* sp (cf. *limonius*), *Cortinarius vernus*, *Inocybe* cf. *nitidiscula*, *Inocybe* sp 1, *Sebacina incrustans*, *Sebacina* sp, *Suillus luteus*, and *Piceirhiza tuberculata* x *Picea crassifolia* (comb. Nov.)], and two morphotypes to the phylum Ascomycotina (*Cenococcum geophilum* and *Helvella* sp). The diversity of ECM organisms in *P. crassifolia* was lower than that reported by other studies on spruce or pine forests, or on sporocarp diversity in the high-mountain forests of China. Most of the fungi in the rhizosphere did not correspond to species previously recorded as sporocarps above ground. Here, several new ectomycorrhiza morphotypes are proposed and described. We also confirmed the ectomycorrhizal status of the genus *Sebacina* (order Sebaciales).

Key words: Mount Helan (China); Diversity; Ectomycorrhizal; Morphological and molecular identification; ITS nrDNA sequencing; *Picea crassifolia* Kom. (Qinghai spruce) stand

INTRODUCTION

Picea crassifolia Kom. (Qinghai spruce) is an endemic species that is distributed in northwest Qinghai Province, Gansu Province, Ningxia Province, and the Helan Mountain Range in Inner Mongolia, China. Its distribution is limited to the arid areas of south-central Asia and the northern hilly margin of the Tibetan Plateau (Farjon, 1990). *P. crassifolia* is an important forest floristic element of central Asia. In the Mount Helan National Nature Reserve, it forms dominant conifer forests, mainly covering shaded and semi-shaded slopes in the boreal belt, at altitudes of 2100-3100 meters above sea level (MASL). *P. crassifolia* forests account for about 1% of the total forested area in Inner Mongolia and 90% of the total forest cover of the Helan Mountain Range. *P. crassifolia* has been under the protection of the International Union for Conservation of Nature (IUCN) since 1998, and is in the “low-risk” category (Conifer Specialist Group, 1998).

P. crassifolia, like many other spruce species and most boreal and temperate forest trees, is ectomycorrhizal (ECM) (Agerer, 1991; Lian et al., 2007). Many ECM fungi are associated with spruce (*Picea* spp), but the diversity of ECM fungi on endemic spruce in Inner Mongolia has received only limited attention. Previous attempts to document the ECM fungal diversity associated with *P. crassifolia* in China, specifically in the Jiangsu Province, Daqing, the Manhan Mountains, and the Helan Mountains, have been conducted solely as isolated sporocarp surveys (Bai et al., 2001; Lian et al., 2007). In addition, Song and Wang (1999) have published comprehensive lists of fungi recorded in spruce forests in China. All the lists are based on aboveground macroscopic sporocarp investigations and are likely to be biased against species that are undetected, small, hypogeous, or resupinate, and against all ECM fungi that were not producing sexual structures at the time of survey. No ECM status of listed species was available to the authors, except for a putative rhizomorph connection tracing (Bai et al., 2001). Such lists can indicate ECM community diversity in the area and confirm species presence, but they underestimate total ECM diversity. Several authors have pointed out that the above- and below-ground ECM communities do not necessarily correlate well (Gardes and Bruns, 1993). In China, particularly in nature reserves and national parks, research on below-

and above-ground ECM communities is limited; in particular, there have been no systematic and comprehensive studies on ECM fungi associated with *P. crassifolia*.

The research site in the Helan Mountain Range is a special geographic location that connects the flora and climate of the Mongolian Plateau in Northern China. Accordingly, it is significant for studying the ECM fungi communities in the Helan Mountains as a biodiversity hotspot linking two ecologically distinct areas. With this in mind, we attempted to provide the first insight into belowground *P. crassifolia* ECM diversity in this nutrient- and water-limited boreal area. We applied a morphological approach and a molecular method to identify ECM organisms associated with *P. crassifolia*, and aimed to compare the diversity of ECM organisms on *P. crassifolia* and on other *Picea* species from natural sites, and to discover potential species or site-specific genotypes in this area.

MATERIAL AND METHODS

Study site

For more than 200 km, the Helan Mountain Range runs between the eastern Yinchuan Plain and the western Alashan Plateau, and is bordered by Ningxia and Inner Mongolia (38°21'-39°22'N, 105°44'-106°42'E) (Figure 1). The Helan Mountain Range has an average altitude of 2000 m and “Obogda” is the highest peak (3556 MASL) (Liu et al., 2005). The Helan Mountains comprises a fringe of vegetation in northwest China, with various climates. The eastern side has climate and vegetation similar to that of the Steppe, whereas the western side has a desert climate and vegetation, with an alpine forest ecosystem. At higher altitudes, both have sandy, arid soils that are poor in nutrients. The average annual temperature at the foot of the mountains is 8.5°C. The annual rainfall is 202.8 mm in the south and 183.3 mm in the north.

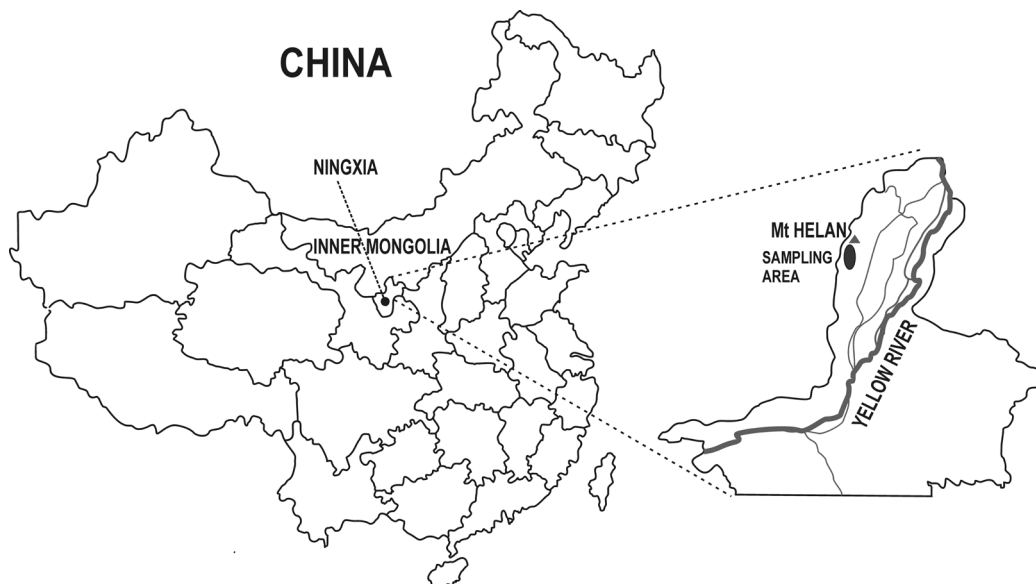


Figure 1. Location of the Helan Mountains in China with a more detailed ECM sampling location.

P. crassifolia grows in cool, dry climates with a mean annual air temperature of -0.9°C . In this alpine zone, the average annual rainfall is 420 mm, most of which occurs in summer. In winter, the area is covered with shallow but persistent snow (Liu et al., 2005).

Sampling was carried out in August a few days after rainfall. Pure *P. crassifolia* stands were selected for soil sampling, with other woody species at least 50 m away from the sampled spruce trees. Samples were taken from approximate altitudes of 2250, 2400, and 2600 MASL (± 50 m) to determine the altitudinal span of the species.

The soil samples were generally taken from gray forest soils to gray-brown desert soils (soil types in People's Republic of China; www.ocs.oregonstate.edu/prism), and were 20-30 cm deep on average. The organic content of the samples ranged from 5-10% and they had a C:N ratio of 14-20:1. The pH was measured by the authors and was close to neutral (6.5-7.0).

Soil and ECM sampling

Three trees were sampled at each altitude, with three soil samples taken at each sampling site as a repetition. Soil cores were gathered from the upper 20 cm along the trunk radial at distances of half the canopy (midpoint between the tree bole and the drip line of the canopy), at full canopy (drip line of canopy), and at 10 m from the drip line. The sampled trees were at least 5 m apart. Soil samples were taken in three directions from each trunk at the same distance. Each soil cube of 20 x 20 cm was cut with a sharp knife from the upper soil layer with minimum disturbance to the sample (Agerer, 1991). Altogether, 81 soil samples were obtained between 2007 and 2009.

The soil cubes were preserved at 4°C for no more than 1 week. Subsequently, the fine roots of the woody plants were gently washed in tap water to remove most of the soil and organic debris, minimizing any damage to ECM roots. Tightly adhering material was removed with forceps. The clean roots were cut into 2.5-cm long sections and placed on a Petri dish filled with tap water. Sections were randomly selected from the Petri dish for counting vital ECM root tips and identification of each morphotype. To standardize sampling, successive root sections were selected and analyzed until 300 fine root tips had been counted in each soil sample. A total of 24,300 vital or old and non-mycorrhizal ECM root tips were analyzed. Vital ECM root tips from all samples were separated according to morphological characteristics.

Morphotyping of ECM

Morphotypes were distinguished by their stereomicroscopic and microscopic characteristics. Each vital ECM root tip was examined under a stereomicroscope (6-90X magnification) to assess ECM color, shape, size, texture, branching, emanating elements, and other taxonomically relevant morphological features (Agerer, 1991). The key anatomical characteristics of the ECM mantle were assessed under a microscope (magnification up to 1000X). The morphotype was identified if its characteristics matched the ECM descriptions published by Agerer (1987-2008), Agerer and Rambold (2004-2010), Danielson and Visser (1989), or Shishido et al. (1996). Each morphotype was briefly described, photographed, and divided into subsamples for molecular analysis.

A representative amount of each morphotype was preserved in formaldehyde/alcohol/acetic acid (FAA) according to the method described by Agerer (1991), and stored in the reference collection at the Biological Science and Technology Institute (Baotou City, China) for further studies.

Molecular analysis

DNA extraction

DNA was extracted from 5-10 ECM root tips collected from the same ramifying systems as had been previously characterized at the morphological level. Fresh ECM root tips were placed in 2% CTAB buffer for short-term storage (up to 1 day) and subsequently treated following the DNA extraction protocol or using a Biospin fungal genomic DNA extraction kit (TIANGEN Bio-Chem Technology Group Company Ltd., China) according to the manufacturer instructions. Three parallel samples were extracted for each morphotype.

Polymerase chain reaction (PCR) and sequencing

The entire internal transcribed spacer (ITS) region was amplified with the fungal-specific primers ITS1F and ITS4 (Gardes and Bruns, 1993; Chang et al., 2013; Pei et al., 2014). The PCR mixture contained 2 μ L undiluted DNA template, 2 μ L each primer (10 μ M concentrations) and 25 μ L Master Mixture (containing Mg and 1U Expand™ High Fidelity Polymerase from Sangon Biotech, China). The reaction mixture was topped-up with sterile distilled H₂O to a total volume of 50 μ L. The PCR was run using a DNA-Engine thermocycler (MJ Research, USA) and the regimen was as follows: a pre-denaturation step at 94°C for 2 min; 35 cycles of denaturation for 40 s at 94°C, annealing for 40 s at 56°C, and extension for 45 s at 72°C; and a final extension at 72°C for 10 min.

Successfully amplified products were purified with a PCR purification kit (TIANGEN, Bio-Chem Technology Group Company Ltd.) and re-amplified following the PCR protocol described above. The direct cycle sequencing was carried out with an ABI PRISM 3.1 BigDye terminator kit (Applied Biosystems, Foster City, CA, USA), using the same primers as in the initial PCR. Electrophoresis was carried out on an ABI PRISM 3100 genetic analyzer. The obtained sequences were arranged using Sequencher® version 5.0 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI, USA) and deposited in the GenBank database.

Molecular identification

All obtained sequences were BLASTed against GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and UNITE databases (<https://unite.ut.ee/index.php>) to identify the ECM fungus genus, and to avoid the presence of contaminating (soil) fungi sequences (BLAST is an abbreviation for the Basic Local Alignment Search Tool, and UNITE is an abbreviation for the User-friendly Nordic ITS Ectomycorrhiza Database) (Abarenkov et

al., 2010). With the phylogenetic approach, only sequences covering the complete ITS region without ambiguous bases and with clear and reliable identification of the fungus were used. Sequences were aligned in MAFFT v. 6.903 (Katoh et al., 2009). jModelTest 0.1.1 was used to assess the nucleotide substitution model giving the lowest likelihood (loglk) value for each of the analyzed datasets (Posada, 2008). Maximum likelihood consensus phylogenetic trees for each genus were calculated in MEGA version 5. MEGA version 5 was also used to visualize and annotate the phylogenetic trees, collapse species or group samples, and add appropriate comments and corrections to samples and clade names.

RESULTS

The 81 samples yielded 4832 vital ECM organisms with morphological characteristics for identification/separation. The mean number of ECM types per tree analyzed was 3.85, with minimum one and maximum nine distinct types per tree. In total, 11 distinct ECM morphotypes were distinguished by morphological and anatomical identification approaches after nuclear ribosomal DNA (nrDNA) ITS sequencing and a brief phylogenetic approach (Table 1). The identified morphotypes belonged to the phylum Basidiomycetes (Sebacinaceae, Cortinariaceae, Boletaceae, Atheliaceae), and the phylum Ascomycetes [Helvellaceae, *Cenococcum* (*incertae sedis*), Gloniaceae cf.].

Amphinema byssoides (Pers.: Fr.) J., *Cenococcum geophylum* Fr., *Sebacina incrustans* (Pers.) Tul. and *Suillus luteus* (L.: Fr.) Gray ECM organisms on *P. crassifolia* corresponded to previously published ECM organisms on *Picea* spp or *Pinus* spp, and were confirmed by the molecular data.

A phylogenetic tree for the genus *Inocybe* revealed the close proximity of unidentified morphotype T1 to *Inocybe nitidiuscula* (Britz.) Sacc. (97% sequence identity with *I. nitidiuscula* sequences AM882911 and AM882913), and unidentified morphotype T5 to an *Inocybe* sp in section *Tardae* (94% sequence identity with *Inocybe tarda* FN550920) (Figure 2A). Three distinct ECM types were formed by fungi from the Sebacinaceae family, namely, unidentified morphotypes T7, T8, and T10 (Table 1). The unidentified morphotype T7 showed 95% sequence similarity with that of, and close phylogenetic proximity to, *Sebacina cystidata*. The sequence similarity was sufficient to conclude the genus of the unidentified morphotype T7 (*Sebacina* sp x *P. crassifolia*). The unidentified ECM organism on *P. crassifolia* T8 was grouped in the terminal clade with several *S. incrustans* sequences with 100% sequence similarity. The third Sebacinaceae ECM organism (unidentified morphotype T10) formed an isolated terminal clade close to *Sebacina* cf. *epigaea* (Figure 2B), but with low sequence similarity (91%), so it remained unidentified with the proposed name *Piceirhiza tuberculata* x *P. crassifolia* (Fan). Using the sequences obtained by BLAST in international databases, most additional types of ECM organisms from *P. crassifolia* were identified at the species level, namely *I. nitidiuscula*, *Cortinarius vernus* H. Lindstr. & Melotand *Cortinarius* cf. *limonius* (Fr.: Fr.) Fr. (Figure 2C), *S. luteus* (L.: Fries) Gray (Figure 2D), *C. geophilum* Fr. (Figure 2E), and *A. byssoides* (Pers.) J. Erikss (Figure 2F). *Helvella* sp x *P. crassifolia* (Figure 2G) remained at the genus level.

Table 1. Cumulative table for all identified types of ectomycorrhizae from *Picea crassifolia* from Mount Helian National Nature Reserve in Inner Mongolia, China, with codes corresponding to herbarium vouchers. Final identification or proposed names are given in full in addition to a brief morphological description and molecular identification based on the comparison with two public databases.

Herbarium voucher	Proposed name	Morphology and anatomy of ECM (brief description of key characteristics)					Molecular comparison with public databases					
		Color	Shape of mycorrhiza	Surface of mantle	Cystidia	Hypphae	Rhizomorphs	Structure of mantle	Reference comment	GenBank accession No.	Closest (identified) match - GenBank (% sequence similarity)	Closest (identified) match - UNITE (match in bits)
T1	<i>Inocybe cf. nitidiscalis</i> (Bretzelm.) Lapl.	White-gray, browning with age	Monopodial-pyramidal; Unramified ends straight to bent.	Cottony	Not observed	Frequent, septa without clamps	Lacking	OM ⁺ /plectenenchymatous, hyphae arranged net-like, septa simple anastomoses H-shape; IM densely plectenenchymatous, hyphae mostly arranged in parallel; Harig net palmetti-like, mantle type N.		FJ80392 7	Uncultured ectomycorrhiza (<i>Inocybe nitidiscalis</i>) AM882913 (97%)	<i>Inocybe nitidiscalis</i> UD8011885 (371 bits; 92%)
T2	<i>Cortinarius versus</i> H. Lindstr. & Melot	Silvery white, old parts becoming ochre to browning	Irregularly pinnate, dichotomous-like. Unramified ends bent to sinuous; not inflated and cylindrical	Rough	Not observed	Branched, septate with clamps	Frequent	OM ⁺ /IM ⁺ plectenenchymatous, hyphae irregularly arranged, or at some places forming ring-like strands, clamp in outer mantle layer lacking but present in emanating hyphae emerging from the mantle, mantle type AB		FJ80392 8	<i>Cortinarius arvenscolanus</i> JQ724024 (99%)	<i>Cortinarius arvenscolanus</i> UD8001011 (1049 bits; 98%)
T3	<i>Hevelia</i> sp.	Yellowish brown to brown, dark brown to black with age.	Monopodial-pyramidal, with up to 11 side-branches per lobe, tubercles present and almost roundish	Hairy	Not observed	Branched, no septae and clamps observed	Lacking	OM ⁺ /IM ⁺ pseudoparenchymatous, hyphae mesh-like arranged and tightly glued together, surface view liking epidermal cells, mantle type L/M		FJ80392 9	<i>Hevelia</i> cf. <i>accabulum</i> FJ25151 (92%)	<i>Hevelia dovreensis</i> UDB000177 (301 bits)
T4	<i>Amphinema byssoides</i> (Pers.) J. Erikss.	Gold-brown	Monopodial-pinnate, unramified ends straight to bent, inflated and cylindrical	Hairy	Not observed	Branched, septate with clamps	Infrrequent	OM ⁺ /IM ⁺ plectenenchymatous, infrequently forming irregular ring-like arrangements of single hyphae or hyphae bundles, mantle type P/Q	Weiss and Agerer 1988	FJ80393 1	<i>Amphinema byssoides</i> JQ711816 (97%)	<i>Amphinema byssoides</i> UDB0008315 (1009 bits)
T5	<i>Inocybe</i> sp. 1 (section <i>Tardae</i> M. Bon)	Creamy whitish to yellowish	Monopodial-pinnate or monopodial-pyramidal; unramified ends straight or bent, not inflated and the tip sharp gradually	Loosely cottony	Not observed	Infrrequent septae with clamps	Lacking	OM ⁺ /IM ⁺ plectenenchymatous, hyphae rather irregularly arranged and no special pattern discernible, or hyphae arranged net-like, repeatedly and squarrosely branched, mantle type B/E		FJ80393 5	Uncultured ectomycorrhiza (<i>Inocybe tarda</i>) FN550920 (94%)	<i>Inocybe sindonia</i> UDB002392 (315 bits)
T6	<i>Cenococcum geophilum</i> Fr.	Black	Unramified	Densely woolly	Not observed	Abundant	Lacking	OM ⁺ /IM ⁺ plectenenchymatous, hyphae star-like arranged and tightly glued together, mantle type G		FJ80393 2	<i>Cenococcum geophilum</i> EL498730 (99%)	<i>Abrothallus succicus</i> UDB003359 (268 bits)

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Table 1. Continued.

Herbarium voucher	Morphology and anatomy of ECM (brief description of key characteristics)										Molecular comparison with public databases		
	Proposed name	Color	Shape of mycorrhiza	Surface of mantle	Cystidia	Hyphae	Rhizomorphs	Structure of mantle	Reference /comment	GenBank accession No.	Closest (identified) match - GenBank (% sequence similarity)	Closest (identified) match - UNITE	Closest (identified) match - UNITE (match in bits)
T7	<i>Schactia</i> sp. x <i>Picea crassifolia</i> (Sebacinae)	Reddish-brown	Monopodial-pinnate, unramified ends straight	Cottony with soil particles, enveloping the whole mycorrhizal system	Coniform	Abundant	Lacking	OM/IMF plectenychymatous, hyphae rather irregularly arranged and no special pattern discernible, single hyphae dichotomous		FR80393 0	Uncultured ectomycorrhiza (<i>Schactia</i>) AY534907 (98%)	<i>Schactia arizana</i> UDB000975 (585 bits)	
T8	<i>Schactia incrustans</i> (Pers.) Tul. & C. Tul.	Brown, old parts dark brown	Monopodial-pinnate, inflated, cylindrical	Almost smooth with few emanating hyphae	Not observed	Few emanating hyphae, branched, septated	Lacking	OM/IMF densely plectenychymatous, hyphae typically bunched, but ring-like structures lacking, hyphae colorless, forming nodes at branching point, with backwards orientated ramifications		FR80393 4	<i>Schactia incrustans</i> EF644113 (100%)	<i>Schactia incrustans</i> UDB000118 (1183 bits/100%)	
T9	<i>Suillus luteus</i> (L.) Roussel	Lead or white	Monopodial-pinnate or monopodial-pyramidal, unramified ends straight or bent, not inflated and the tip sharp gradually	Distinct smooth, with silvery appearance, or with white dust	Not observed	None or few emanating hyphae	Lacking	OM/IMF plectenychymatous, hyphae mostly arranged in parallel, node net-like, squarrosely branched, gelatinous matrix lacking, mantle type B-E		FR80393 3	<i>Suillus luteus</i> AY 898620 (99%)	<i>Suillus luteus</i> UDB011435(187 bits/99%)	
T10	<i>Picea</i> x <i>tuberculata</i> x <i>Picea crassifolia</i> (Sebacinae)	Reddish-brown	Monopodial-pinnate, unramified ends bent or simous and tip sharp gradually	Hairy, with soil particles	Not observed	Branched, no septae and clamps	Lacking	OM/IMF plectenychymatous, hyphae star-like arranged and tightly glued together, radial, hyphae branched, no septae and clamps, cell wall thin, mantle type G		FR80393 6	Uncultured ectomycorrhiza (Sebacinae) AY 825519 (95%)	<i>Schactia</i> sp. UDB00073 (737 bits/90%)	
T11	<i>Cortinarius</i> sp (cf. <i>limonius</i>)	White, yellow to brown when old or damaged	Monopodial-pinnate, unramified ends bent or sharp gradually	Loosely cottony, white film-like on the surface	Tapered	Abundant	Infrequent	OM/IMF plectenychymatous, hyphae arranged irregularly		FR80393 7	<i>Cortinarius limonius</i> GQ159869 (97%)	<i>Cortinarius neofurvaesii</i> UDB001268 (311 bits/96%)	

^aOM/IM: outer mantle layers in ectomycorrhiza (ECM)/inner mantle layers in ECM.

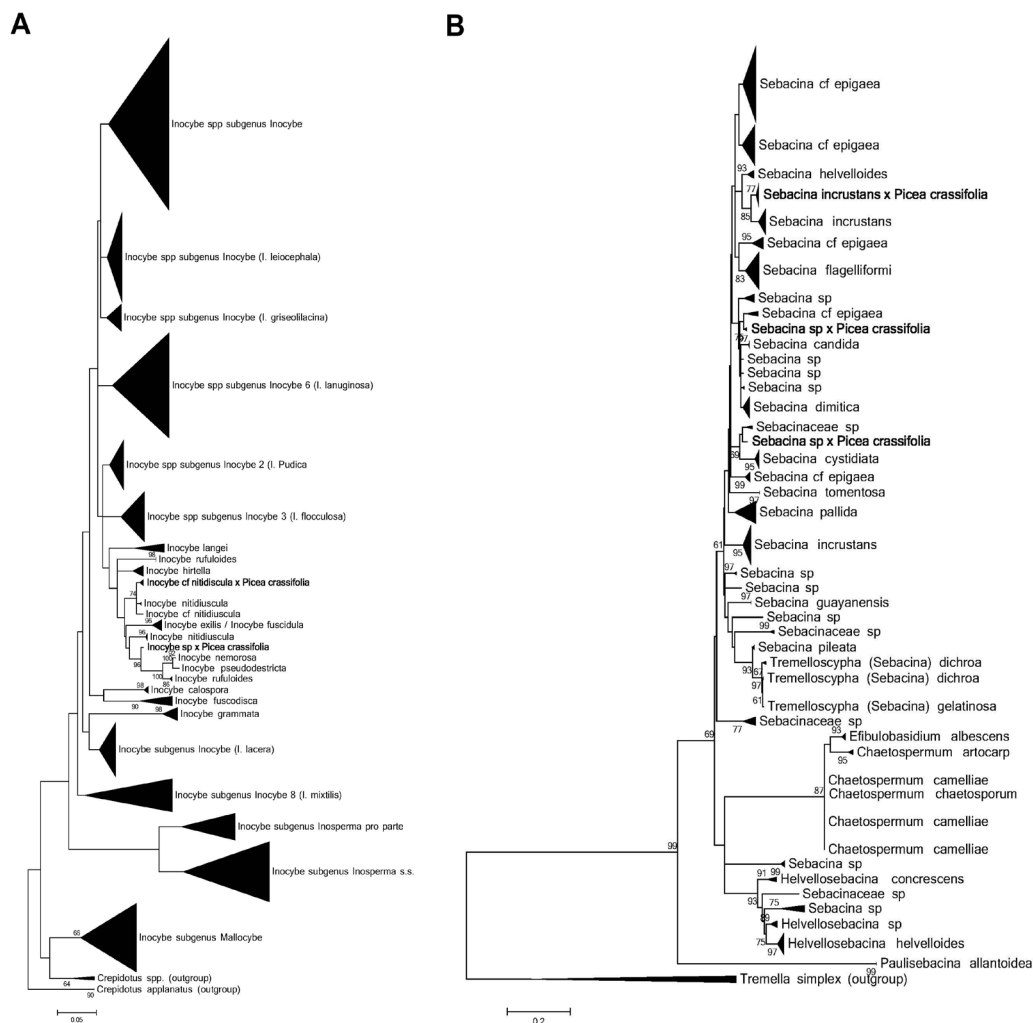
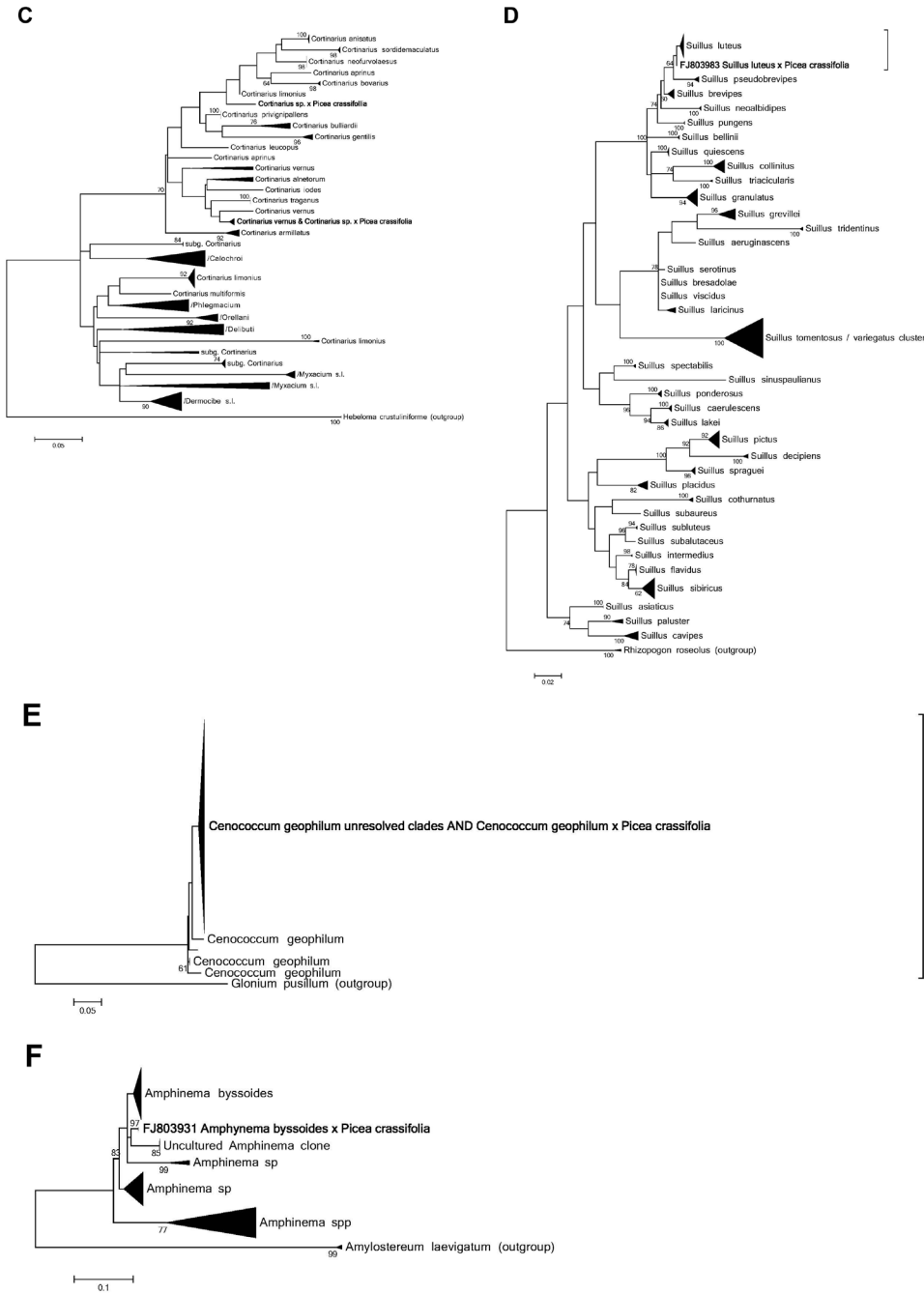


Figure 2. Phylogenetic trees for genera of the identified ectomycorrhizal root tips on *Picea crassifolia*, as identified after BLAST analysis. Bootstrap values over 60% for maximum likelihood trees are given and are based on 1000 bootstrap repetitions. Species or higher taxonomic groups within genus were collapsed for easier visualisation of clades with *Picea crassifolia* ectomycorrhiza sequences: **A.** Phylogenetic tree for the genus *Inocybe* with phylogenetic position of *Inocybe* cf. *nitidiuscula* (Britzelm.) Lapl. (T1) and *Inocybe* sp 1 (section Tardae M. Bon) (T5). *Crepidotus* spp was used as outgroup. **B.** Phylogenetic tree for the genus *Sebacina* with phylogenetic position of *Sebacina* sp x *Picea crassifolia* (Sebacinaceae), (T7), *Sebacina incrustans* (Pers.) Tul. & C. Tul. (T8), and *Piceirhiza tuberculata* x *Picea crassifolia* (T10). *Tremella simplex* was used as outgroup. **C.** Phylogenetic tree for the genus *Cortinari* with phylogenetic position of *Cortinari* *vernus* H. Lindstr. & Melot (T2) and *Cortinari* sp (cf. *limonius*) (T11). *Hebeloma crustuliniforme* was used as outgroup. **D.** Phylogenetic tree for the genus *Suillus* with phylogenetic position of *Suillus luteus* (L.) Roussel (T9). *Rhizopogon roseolus* was used as outgroup. **E.** Phylogenetic tree for the genus *Cenococcum* with phylogenetic position of *Cenococcum geophilum* Fr. (T6). *Glonium pusillum* was used as outgroup. **F.** Phylogenetic tree for the genus *Amphinema* with phylogenetic position of *Amphinema byssoides* (Pers.) J. Erikss. (T4). *Amylostereum laevigatum* was used as outgroup. **G.** Phylogenetic tree for the genus *Helvella* with phylogenetic position of *Helvella* sp (T3). *Helvella rivularis* was used as outgroup.

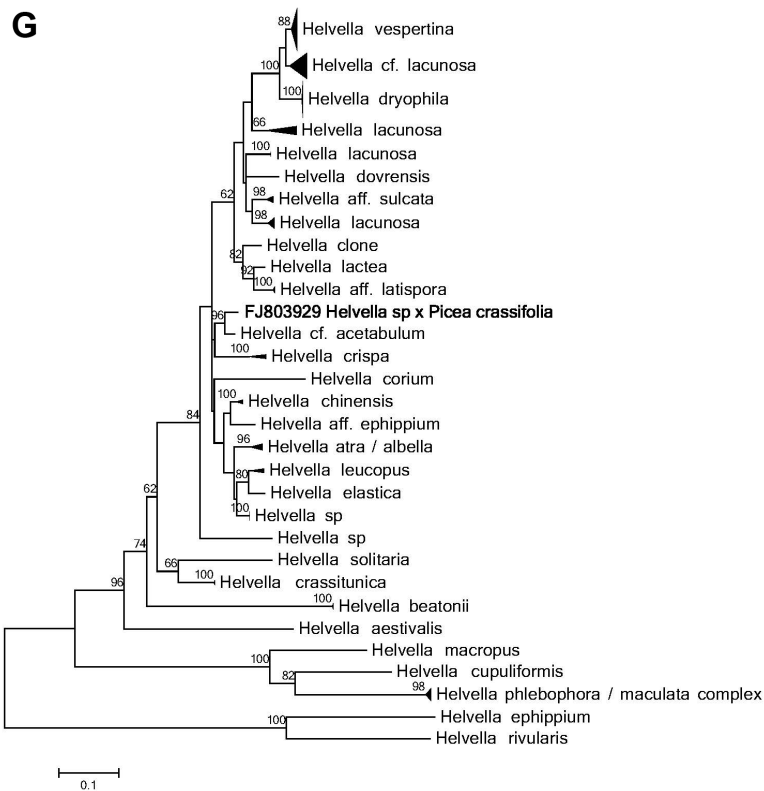
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DISCUSSION

Past studies of ECM organisms in China have been limited to above-ground sporocarp diversity and have only revealed the presence of a few ECM genera, namely *Cortinarius*, *Inocybe*, *Helvella*, and *Suillus*. No ECM species in our study matched the species identified on the basis of sporocarp production in pine and larch, previously recorded in the Helan Mountains and the Jiangsu Province (Bai et al., 2001; Lian et al., 2007). The same authors also listed a number of ECM organisms with various plant partners, including those in *P. crassifolia* forests in Daqing and the Manhan Mountains. However, the species differed considerably in comparison with those below ground in the Helan Mountains. The discrepancy at the species level was expected because above- and below-ground ECM fungus communities assessed by sporocarp mapping and molecular identification of ECM organisms from soil samples are often significantly different (Peter et al., 2001). However, ECM organism diversity at three pure *P. crassifolia* sampling stands was relative low, despite a comparable percentage of vital ECM organisms recorded on boreal stands of *Picea abies* (Trošt et al., 1999).

The diversity of ECM organisms on *P. crassifolia* is low in comparison with the cumulative number of morphotypes detected, and there is an increasing number of samples in pine forests (Taylor, 2002), compared with ECM diversity in other studies on ECM organisms

associated with plants in the high mountains of China (Gao and Yang, 2010). The lower number can be explained by the strict selection of pure *P. crassifolia* stands and the extreme conditions (sandy soil, low precipitation, high yearly temperature differences) of the sites that favored only a few morphotypes. The dominance of one or a few ECM organism types is a common pattern observed in extreme environments. A few types of ECM organisms, in comparison with some hyper-diverse ECM organism systems such as oak seedlings have also been observed on several other spruce species (Walker et al., 2005), for example, on partially cut *Picea glauca* trees from natural stands (Lazaruk et al., 2005) and on mature *P. abies* trees growing in forests with dolomite lime soils in otherwise optimal spruce habitats (Jonsson et al., 1999).

Species-by-species analysis, including a molecular identification approach to ECM fungi on *P. crassifolia*, revealed the expected presence of several generalist and stress-resistant species on spruce (*C. geophilum* and *A. byssoides*) (Rineau and Garbaye, 2009). *C. geophilum* is a cosmopolitan ECM organism, which is present in many ecosystems and on various plant hosts. It was described for the first time on *P. abies* (Agerer and Gronbach, 1988), so its presence in *P. crassifolia* was not surprising. In addition to the plant partners, sandy soils that are poor in nutrients, and forests with very low annual precipitation (below 500 mm of rainfall) (Liu et al., 2005) and high annual temperature differences indicate that the site can be considered to be under constant stress. Stress conditions can explain the abundance of *C. geophilum*, which is a stress-tolerant species, in the analyzed plots (Lobuglio, 1999). The limestone-rich soils of the analyzed sites developed on permo-carbon deposits account for the presence of ECM *A. byssoides*, which is known to increase in abundance after liming of otherwise acidic sites in pine forests (Veerkamp et al., 1997). However, *Russula* and *Lactarius* spp, cosmopolitan and widespread in the northern hemisphere (Dickie and Moyersoen, 2008), particularly at more acidic sites (Rineau et al., 2010), were not present in ECM *P. crassifolia*. A general lack of ECM *Russula* and *Lactarius* spp at the sites suggests that a combination of environmental conditions, such as the distinct soil conditions and low precipitation in the Helan Mountains, influences and reduces the presence of ECM organisms. In addition, the ECM *S. luteus*, collected and identified on *P. crassifolia*, indicated different conditions from other *Suillus* stands, where species from this genus form ectomycorrhiza on several species of pine and larch (Agerer and Rambold, 2004-2010). We suggest that under unfavorable conditions, *S. luteus* has a broader potential plant-partner selection from natural stands, as previously shown by *in vitro* inoculations of *Picea glauca* (Dixon and Buschena, 1988).

Members of the family Sebacinaceae are prominent in the ECM community. The family appeared common on *P. crassifolia* with ECM *S. incrustans* growing under the dry and cold conditions of the Helan Mountains. In addition, the identification of an ECM *Sebacina* sp in *P. crassifolia* (T7; Table 1), which was closely related to *S. cystidiata* (previously named *Tremellodendron*), confirmed the previous results by Rinaldi et al. (2008). This indicated that the ECM status of *Tremellodendron* with species of *Quercus*, *Pinus*, and *Tilia cordata*, is broader; in our case, a well established ECM was identified on *Picea*. *Tremellodendron* was recently synonymized with *Sebacina* (Oberwinkler et al., 2014). Other ECM fungi on *P. crassifolia* did not match available ECM descriptions. The *Sebacina* sp x *P. crassifolia* showed morphological characteristics similar to *Piceirhiza bicolorata* on *P. abies*, but there was a clear difference in the color of the ectomycorrhiza. The third ECM organism belonging to Sebacinaceae (*P. tuberculata* x *P. crassifolia*) could not be related to any available hit in the nucleotide databases, and showed only distant morphological similarity to several unidentified

types of ECM organisms on oak (Azul et al., 2006).

An ECM *Inocybe* sp from *P. crassifolia* belonged to section *Tardae*, but could not be identified at the species level. This indicates the putative existence of an endemic species with ecological demands similar to those of *I. tarda*, which requires dry conditions and poor calcareous soils (Ryberg et al., 2010). The ECM *I. cf. nitidiscula* (T1) requires calcareous soils but, in contrast to basidiocarp-based literature data for the northern temperate European species (*ibid.*), it does not occur in rich and wet soils in symbiosis with *P. crassifolia*. We can additionally conclude that the unidentified morphotype *Inocybe* sp (T5) represents a species of which the nrDNA ITS region has not been sequenced until date, and is likely to be a new species that is specific to Asia (China) or to the particular stressed environment.

The first insight into the diversity of ECM organisms on *P. crassifolia* is far from complete because the total sampled ECM roots and the sampling strategy limited our ability to accurately assess species richness, in particular, in view of the inherent structure of most ECM communities, with a few common species and a large number of rare species (Taylor, 2002). The latter remained underestimated but, with these encouraging preliminary results, we hope to facilitate future basic ECM studies in the region and on the particular host.

Conflicts of interest

The authors declare no conflict of interest.

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