

Ectomycorrhizal fungal community in alkaline-saline soil in northeastern China

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Abstract Alkaline-saline soil is widespread in arid and semiarid regions of the world and causes severe environmental and agricultural problems. To advance our understanding of the adaptation of ectomycorrhizal fungi (EMF) to alkaline-saline soil, we investigated EMF communities on Mongolian willow (*Salix linearistipularis*) growing in alkaline-saline soil (up to pH 9.2) in northeastern China. In total, 75 root samples were collected from 25 willow individuals over 4.7 ha. To identify fungal species in ectomycorrhizal root tips, we used terminal restriction fragment length polymorphism and sequencing analyses of the internal transcribed spacer region of ribosomal DNA. We detected 11 EMF species, including species of *Inocybe*, *Hebeloma*, and *Tomentella* of the Basidiomycota and three Ascomycota species. The EMF richness of the study site was estimated to be 15–17 using major estimators. The most abundant species was *Geopora* sp. 1, while no *Geopora*-dominated EMF communities have been reported so far. Phylogenetic analysis showed that the phylogroup including *Geopora* sp. 1 has been found mostly in alkaline soil habitats, indicating its adaptation to high soil pH.

Because EMF are indispensable for host plant growth, the EMF species detected in this study may be useful for restoration of alkaline-saline areas.

Keywords Alkaline-saline soil · Ectomycorrhizal fungal (EMF) community · *Geopora* · *Salix linearistipularis* (syn. *Salix mongolica*) · Terminal restriction fragment length polymorphism (T-RFLP)

Introduction

Associations with ectomycorrhizal fungi (EMF) are widely observed in many dominant tree species of cool-temperate to tropical forest ecosystems. These associations improve host performance by enhancing nutrient and water uptake from the soil and protecting host roots from pathogens and toxic compounds (Smith and Read 1997). Such beneficial effects would vary greatly under different environmental conditions, because each EMF has different physiological and ecological traits (Duñabeitia et al. 2004; Diédhiou et al. 2005; Nara 2006a). While EMF symbiosis is supposed to help host plants to grow under stressful conditions (van der Heijden and Kuyper 2001), the outcome would largely depend on the composition of EMF or EMF communities. Although we know little about EMF communities in devastated habitats with extremely stressful conditions, such information is potentially important for the reforestation of these habitats.

The extent of devastated lands with stressful environments has been rapidly increasing due to human impacts, causing severe local and global environmental problems. Saline soil is one of the most common types of devastated land caused by human activities, and 932 million hectares in arid and semiarid regions are estimated to be affected by

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severe salt accumulation (Summer et al. 1998). Because accumulated salts often include alkaline compounds such as Na_2CO_3 and CaCO_3 , soil salination is frequently accompanied by an increase in soil pH (Hillel 2004). Because both alkalinity and salinity in soil significantly reduce plant growth by inducing iron deficiencies and water and ion imbalances (Shannon 1997; López-Berenguer et al. 2006), vegetation development on alkaline-saline soil is usually poor. The growth of EMF is also reduced under alkaline conditions (Kernaghan et al. 2002) because most EMF inhabit moderately acidic to acidic soils in forest ecosystems. Although no detailed information is available about EMF in alkaline soils, such communities may be significantly different from those found in other habitats.

In northeastern China, the area of alkaline-saline soil on the Songnen plain is expanding. Large-scale clear-cuts and subsequent intensive grazing have induced soil erosion and increased evapotranspiration, resulting in the accumulation of salts from groundwater on the soil surface (Wang and Ripley 1997). In this area, a shrub willow (Mongolian willow), *Salix linearistipularis* K.S. Hao (syn. *Salix mongolica*) is the sole EMF host species and is intermixed with herbaceous halophytes. We investigated the EMF community on this dwarf willow using molecular identification techniques as the first study of EMF in alkaline-saline soil.

Materials and methods

Study site

The study was conducted at the Andah field station (46°27' N, 125°22' E) of the Northeast Forest University in Heilongjiang Province, northeastern China. This field station is located in the Songnen plain, which extends approximately 500 km × 300 km. Mean temperatures are 22.3°C in July and -19.6°C in January (in Harbin, the nearest large city, <http://www.harbin.gov.cn>). Annual precipitation is around 500 mm. The Songnen plain is dominated by *Aneurolepidium chinense*, a grass ideal for grazing and forage, with some *Calamagrostis epigeios*, *Puccinellia tenuiflora*, *Aeluropus litoralis*, and *Suaeda corniculata* (Gao et al. 1998). Degrees of salination and alkalization depend on the intensity of grazing pressure. Salt concentration, exchangeable sodium percentage, and pH vary between 0.01 and 0.51 g kg⁻¹, 6.3 and 53.5, and 7.6 and 10.1, respectively, on the surface 30-cm layer of soil (Shang et al. 2003). Mosaic distributions or patches of vegetation are observed at the study site, reflecting the vegetation gradient along the alkaline-saline soil gradient. Shrubs of Mongolian willow establish sporadically inside the vegetation patches (34.3 individuals ha⁻¹). Further descriptions of the Songnen plain can be found in Wang and Ripley (1997), Gao et al. (1996, 1998), and Shang et al. (2003).

Sampling, morphotyping, and molecular analysis

To determine the soil pH around ectomycorrhizal roots, soil samples were collected under 25 individuals of Mongolian willow in early September 2004. Two soil samples each were also collected from the inside and outside of vegetation patches lacking willows. Soil pH was measured within 48 h after sampling using a handheld pH meter (B-212, HORIBA, Ltd., Kyoto, Japan) by mixing 7.5 ml of soil with 25 ml of distilled water. Electrical conductivity (EC) was also measured for 16 soil samples collected under willows with a handheld conductivity meter (B-175, HORIBA, Ltd.).

In October 2006, we collected three soil cores (~500 cm³) from beneath each of 25 willows found over 4.7 ha. The sampled soil cores were placed separately in plastic bags and kept at 4°C. Root tips in the soil cores were observed under a dissecting microscope after careful washing in tap water. Each EMF root tip was assigned to a morphotype based on its surface color, texture, and emanating hyphae by examination under a dissecting microscope. A compound light microscope was also used when confirming EMF colonization and morphotypes. We used one or two representative EMF root tips from each morphotype from each root system for molecular identification. Root tips were placed individually in 2.0-ml tubes and dried for DNA extraction. A total of 3,291 root tips from 64 root systems were examined under a dissecting microscope, and 62 root tips were used in molecular identification. Eleven other root systems did not contain active root tips. We also collected seven sporocarps in early September 2004, which were also subjected to DNA analyses.

Molecular identification of fungi

The molecular identification methods used were described in Ishida et al. (2007). In brief, terminal restriction fragment length polymorphism (T-RFLP) analysis of the internal transcribed spacer (ITS) region in ribosomal DNA (rDNA) was conducted using a capillary sequencer (CEQ8800; Beckman Coulter, Fullerton, CA, USA) after digesting fluorescent-dye-labeled PCR products (ITS1F-ITS4; White et al. 1990; Gardes and Bruns 1993) with *Hinf*I (Takara Shuzo, Shiga, Japan, or Toyobo, Osaka, Japan). When samples had fragments within ±2 bp in all four fragments, they were considered to be the same T-RFLP type. When more than two fragments were observed for a fluorescent dye in one sample, the highest peak was used.

A PCR product amplified using ITS1F and ITS4 was purified using a PCR product pre-sequencing kit (USB Co., Cleveland, OH, USA). The obtained sequences were compared to those of known species in either the UNITE

database (Köljalg et al. 2005) or DDBJ/EMBL/GenBank using Blast. We normally trusted the results of UNITE, but the DDBJ/EMBL/GenBank results are given when UNITE did not show similar sequences.

Analyses

The numbers of root tips colonized by individual species were determined for each root system first. Then, the data of all root systems from a host plant were pooled, and the combined data for each host were used as an independent sampling unit. Thus, a total of 25 independent data units were used in further analyses. The relative abundance of each EMF species in the community was determined by averaging the relative abundance of colonized root tips among all the host plants. The frequency of each EMF species was the total number of host plants in which a given EMF was found.

Species richness estimators, including Chao2, Jackknife1, and Jackknife2, were calculated using ESTIMATES version 8.0 (Colwell 2006) based on the presence/absence of EMF species in each host. Simpson's diversity ($1/D$) and Shannon–Wiener information (H') indices were also calculated.

The most dominant EMF species found at the study site is uncommon in other EMF communities. To determine its relationship to other EMF species and the origin of its preference for alkaline-saline soil, we conducted a molecular phylogenetic analysis using accompanying environmental information. We derived all available ITS sequences of known species in the same genus and closely related sequences of environmental samples from GenBank using a Blast search. These sequences were aligned using the Clustal W algorithm and manually edited at misaligned sites. The aligned matrix contained 709 sites, among which 289

were variable. The aligned sequence matrix is available from the authors on request. Using MEGA4 (Tamura et al. 2007), we constructed a neighbor-joining phylogenetic tree using the maximum composite likelihood nucleotide substitution model with branching supports calculated from 1,000 bootstrap replicates. Gaps were eliminated in a pairwise fashion.

Results

The pH of soils in which Mongolian willow was growing ranged from 7.8 to 9.2 with a mean of 8.4 ± 0.3 (SD). The mean soil pH under willow shrubs was significantly lower than that under other herbaceous plants inside vegetation patches (9.2 ± 0.4 ; $P=0.002$ by ANOVA) and that outside vegetation patches (bare ground; 10.4 ± 0.1 , $P<0.0001$). The soil EC was 158 ± 51 mS cm⁻¹ under willow shrubs.

Ectomycorrhizal fungal colonization was generally poor in Mongolian willow at this site. No ectomycorrhizal colonization was observed in 17 of 64 root systems, resulting in three of 25 host plants having no ectomycorrhiza. These uncolonized plants were also included for further analyses because we treated the host plants as independent sampling units. On average, 57.7% of the root tips were not colonized by EMF.

A total of 11 T-RFLP types were found from 57 ectomycorrhizal root tips that were successfully analyzed (Table 1). Blast analyses enabled to identify eight of these T-RFLP types to genus level with high similarities with known fungal species in the database. Other two T-RFLP types were given conservative taxon names (Ascomycetes sp. 1 and Pezizales sp. 1) due to the short ITS sequences obtained and the resultant poor values in Blast results. Only

Table 1 Ectomycorrhizal fungi observed on *Salix linearistipularis* growing at an alkaline-saline soil in northeastern China

Tentative identification	Closest match in UNITE	E-value	Fr ^a	RA ^b	Accession number
<i>Geopora</i> sp. 1	<i>Geopora</i> cf. <i>cervina</i> ^c	0	14	23.6	AB369538
<i>Tomentella</i> sp. 1	<i>Tomentella fuscocinerea</i>	0	8	6.8	AB369539
<i>Tomentella</i> sp. 2	<i>Tomentella lateritia</i>	1E–139	4	2.2	AB369540
<i>Inocybe</i> sp. 1	<i>Inocybe fuscidula</i>	3E–91	4	0.8	AB369545
<i>Tomentella</i> sp. 3	<i>Tomentella fuscocinerea</i>	0	3	0.8	AB369541
<i>Hebeloma</i> sp. 1	<i>Hebeloma velutipes</i>	1E–164	2	2.8	AB369547
<i>Hebeloma</i> sp. 2	<i>Hebeloma populinum</i>	0	2	1.2	AB369543
Ascomycetes sp. 1	<i>Genea hispidula</i>	4E–24	1	2.2	Not deposited ^d
Pezizales sp. 1	<i>Geopora</i> cf. <i>cervina</i> ^c	1E–44	1	1.2	Not deposited ^d
<i>Inocybe</i> sp. 2	<i>Inocybe leptocystis</i>	1E–121	1	0.3	AB369542
Unknown sp. 1			1	0.4	

^a Frequency, the number of host individuals that the species was found among 25 host individuals

^b Relative abundance (%) of the root tips that the species was found

^c Closest match in DDBJ/EMBL/GenBank was shown since that in UNITE showed less homology

^d Sequences were not deposited in the Genbank since they were short (<250 bps) and included ambiguous bases. Accordingly, conservative taxa were given to these T-RFLP types

one T-RFLP type was left unknown because of the difficulty in sequencing (Table 1). Although different T-RFLP types showed the same best match in each of two T-RFLP pairs using a Blast search (Table 1), the sequence similarity in each pair was low (<96% in sequence homology). Thus, these T-RFLP types were regarded as different species and were assigned different names. Two T-RFLP types were obtained from the seven EMF sporocarps, corresponding to *Inocybe* sp. 1 and *Hebeloma* sp. 2. Representative sequences for most T-RFLP types were deposited in the DDBJ under accession numbers AB369538–AB369547 (Table 1).

Tomentella was the most species-rich genus, with three species detected, followed by *Inocybe* and *Hebeloma*, each

of which had two species. The remaining three EMF belonged to the Ascomycota. The EMF richness per host ranged from 0 to 4, with an average (± 1 SD) of 1.6 ± 1.2 . Low EMF species diversity was also indicated by the estimated species richnesses, 15.0, 14.8, and 16.8 using Chao2, and Jackknife 1 and 2, respectively. The Simpson's and Shannon–Wiener information indices were 2.72 and 1.45, respectively.

The most dominant species was *Geopora* sp. 1, which was detected in 14 of 25 willow individuals. Its relative abundance on all examined root tips was 23.6%, and it was found on 55.8% of all ectomycorrhizal root tips. The second most abundant species was *Tomentella* sp. 1, which was observed on eight host plants and occupied 6.8% of all

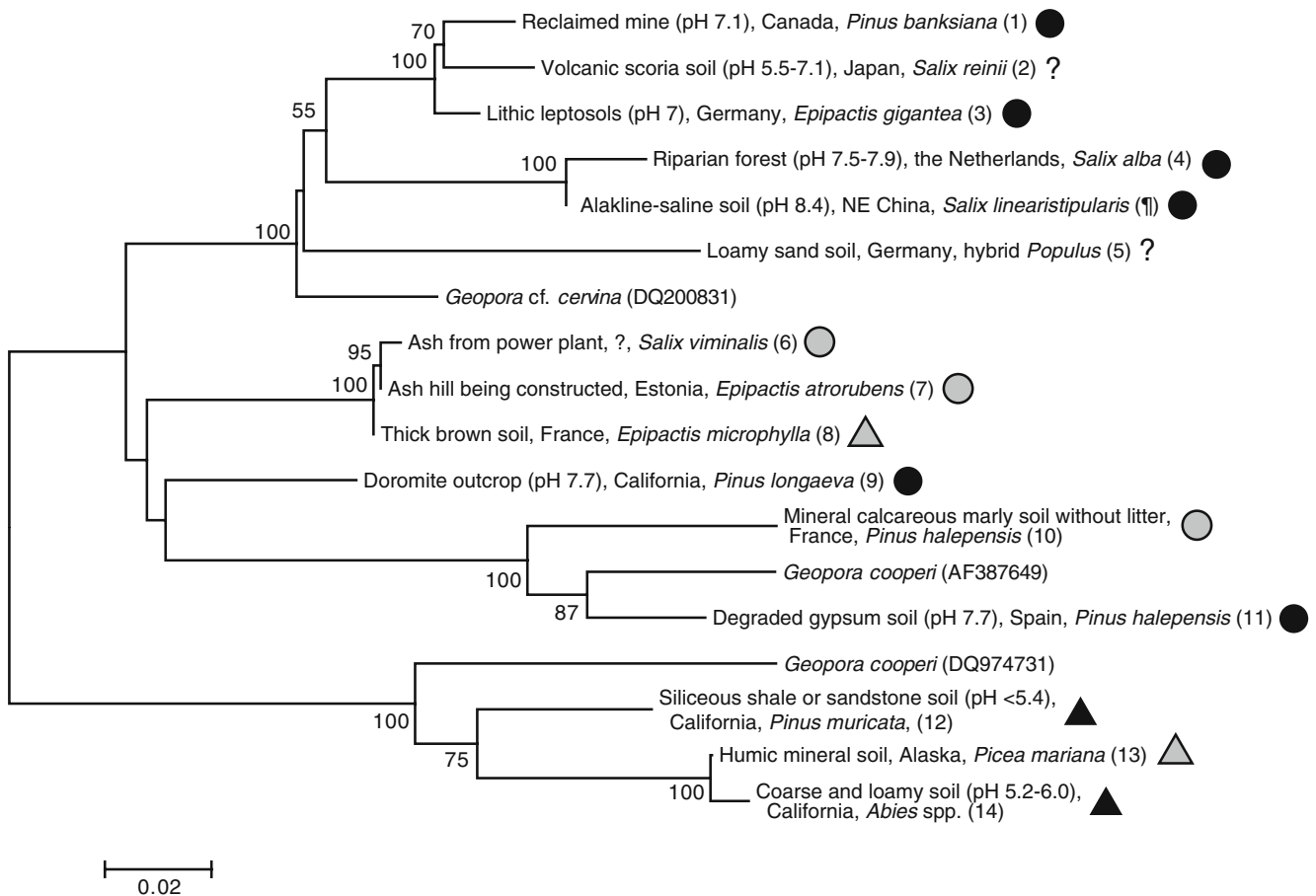


Fig. 1 The phylogenetic relationship between the dominant ectomycorrhizal fungus of alkaline-saline soil in northern China, *Geopora* sp. 1, and other known taxa using internal transcribed spacer (ITS) sequences of rDNA. Bootstrap values of 50% or higher are shown in the neighbor-joining tree. The numbered (1–14) sequences were obtained from ectomycorrhizal samples from various environments and relevant information such as soil or habitat type, soil pH, country or region, and host plant, is shown whenever available. Sequences of *Geopora* sporocarps were also included in the analysis. All sequences were derived from GenBank using a Blast search. Symbols indicate soil pH: alkaline (pH > 7, closed circle), presumably alkaline (gray circle), acidic (pH < 6, closed triangle), presumably acidic (gray triangle), and

unknown or variable (question mark). The references of the numbered operational taxonomic units are as follows: 1 AY192163 (Bois et al. 2005); 2 AB096869 (Nara et al. 2003; pH from Tateno and Hirose 1987); 3 AY634125 (Bidartondo et al. 2004); 4 AJ920026 (Parádi and Baar 2006); 5 AJ510267 (Kaldorf et al. 2004); 6 EU795365 (Hryniewicz, unpublished data); 7 EU195343 (Shefferson et al. 2008); 8 AY351625 (Selosse et al. 2004); 9 AF266709 (Bidartondo et al. 2001); 10: AJ410862 (El Karkouri et al. 2004); 11 EF484934 (Rincón et al. 2007); 12 DQ822805 (Peay et al. 2007; pH from Grogan et al. 2000); 13 EF434150 (Taylor et al. 2007); 14 AY702785 (Izzo et al. 2005); 15 EF195555 (Ducic et al. unpublished data); paragraph mark: AB369538 (this study)

root tips. The other EMF species were less abundant, colonizing less than 3% of all root tips (Table 1).

We conducted a phylogenetic analysis using the *Geopora* sp. 1 sequence, with related sequences from GenBank (14 sequences from ectomycorrhizal root tips and three from sporocarps). In this data set, soil pH was available for nine studies: six soils were alkaline (pH>7.0), two were acidic (pH<6.0), and one had a wide range of pH (5.5–7.1). For the soil types of unknown pH, we regarded three as presumably alkaline (ash soils and calcareous soil; Fisher and Binkley 2000) and the other two as acidic (humic soil and thick brown soil). The phylogroup that included *Geopora* sp. 1 was recorded mostly in alkaline soil and was widespread in the Northern Hemisphere and associated with various host plants (Fig. 1).

Discussion

Several studies have examined the culture of EMF under alkaline and/or saline conditions using EMF isolated from normal acidic forest soils (Chen et al. 2001; Kernaghan et al. 2002; Bois et al. 2006). Although these studies have successfully identified some EMF species that can grow in vitro under alkaline/saline conditions, the EMF species native to alkaline-saline soils remained unknown. The present study is the first to describe the EMF community of an alkaline-saline soil. The soil pH of our study site (7.8–9.2 around ectomycorrhizal roots) is the highest among existing studies of EMF communities.

In this extreme environment, an uncommon EMF, *Geopora*, was dominant. This result is in contrast to those of previous studies of *Salix* in severe habitats, which usually found the dominant EMF to be in the Basidiomycota (Nara et al. 2003; Staudenrausch et al. 2005; Obase et al. 2007; Hryniewicz et al. 2008). Our phylogenetic analysis included *Geopora* sp. 1 in a phylogroup composed of many taxa from high soil pH. Thus, *Geopora* sp. 1 and its close allies may have adapted to alkaline habitats. Since our data set based on ITS region with a small number of OTUs is not enough for the rigorous examination on the evolutionary history of this genus, further study is needed to infer the evolution of niche preference in this fungal group.

Another reason for the dominance of *Geopora* sp. 1 may be the general toxicity of alkaline and saline conditions to most EMF. Previous studies have demonstrated that EMF exhibit optimal growth at a pH of 5 or 6, and growth is depressed at higher pH (van der Heijden and Kuyper 2001; Kernaghan et al. 2002; Yamanaka 2003). High salinity is less toxic to most EMF, but critical to some EMF (Chen et al. 2001; Bois et al. 2006). Thus, the negative effects of alkaline-saline soil may render most EMF inactive or less

competitive. The low level of EMF colonization and low EMF diversity observed in the present study indicate severe effects associated with alkaline-saline soil. The resultant open niches (uncolonized fine roots) may enable colonization by less competitive but well adapted EMF such as *Geopora* sp. 1.

Although the EMF community in the alkaline-saline study soil was poor, the association with EMF may be necessary for the growth of host plants in this soil. Ectomycorrhizal *Pinus* seedlings grew better than non-mycorrhizal seedlings in an alkaline soil (pH 7.7; Rincón et al. 2005), even though EMF colonization was low. In a saline soil, EMF improved the uptake of potassium and phosphorus in *Coccoloba* seedlings, while sodium and chloride concentrations remained low (Bandou et al. 2006). In addition to such benefits for colonized hosts, EMF on established hosts may facilitate new seedling establishment in nearby soil by providing compatible EMF for seedlings, as demonstrated in primary succession (Nara 2006b). In addition to EMF associations, the lowered soil pH observed around *Salix* roots in this study may also contribute to vegetation development by alleviating the toxicity of alkaline soil.

Understanding which EMF are critical for the survival and growth of host plants under extremely stressful conditions is an important step toward the practical use of EMF in restoration activities. Although further research is necessary, the present study provides fundamental information about EMF species that have adapted to alkaline-saline soil.

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