Ectomycorrhizal fungal communities on the endangered Chinese Douglas-fir (*Pseudotsuga sinensis*) indicating regional fungal sharing overrides host conservatism across geographical regions

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Abstract

Background and aims Ectomycorrhizal (ECM) fungal communities are affected by many abiotic and biotic factors, among which host identity is often regarded as the most significant. Although little about ECM fungal communities on endangered tree species is known, knowledge of their ECM associations could be a key to conservation given the functional importance of the symbiosis.

Methods We collected 73 soil samples from three relict forests dominated by endangered Chinese Douglas-fir (*Pseudotsuga sinensis*). Identity of fungal and host species in individual ECM tips was determined by morphotyping and DNA analyses.

Results Of the 86 ECM fungal species identified, 66 were observed on Chinese Douglas-firs. While the fungal composition did not significantly differ between coexisting trees ($p=0.843$), the composition was significantly separated based on location ($p=0.021$).

Moreover, the observed ECM fungal communities were dissimilar to those on the Japanese Douglas-fir ($p=0.001$), which is monophyletic to Chinese Douglas-fir.

Conclusions Our results indicate that ECM fungal communities are determined more by geographical location than host monophyly on this spatial (c.770–1,600 km) and geological time scale (c. 20–25 My). For conservation of endangered trees, it may be important to preserve local ECM fungal pools, irrespective of host species.

Keywords Ectomycorrhizal fungi · *Pseudotsuga sinensis* · Endangered species · Biogeography · Host effect

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Introduction

Ectomycorrhizal (ECM) associations are a prerequisite for many tree species to grow and survive in nature (Smith and Read 2008). This is mainly because host trees depend largely on colonizing ECM fungi for soil nutrients, despite their large expenditures of photosynthates to the fungi, as much as 22 % of net primary production (Hobbie 2006). ECM fungi are diverse not only in terms of species and lineage richness (Rinaldi et al. 2008; Tedersoo et al. 2010) but also in their physiological (Erland and Finlay 1992; Rineau and Courty 2011; Koide et al. 2014) and ecological traits (Nara et al. 2003; Bruns et al. 2009). In fact, host performance in the field is critically determined by colonizing ECM fungi (Nara 2006).

ECM trees dominate most boreal, temperate, and sub-tropical forests throughout the world, as well as some tropical forests (Smith and Read 2008; Tedersoo et al. 2012). In each of these forests, tens to hundreds of ECM fungal species per hectare are estimated to exist (Horton and Bruns 2001; Ishida et al. 2007; Miyamoto et al. 2014), forming diverse ECM fungal communities. Given that these ECM fungi function as a community and sustain host trees, the diversity and composition of ECM fungal communities is quite relevant to forest ecosystems. While many studies have described ECM fungal communities in this context (e.g. Tedersoo et al. 2012 and references therein), those of endangered tree species have rarely been documented (Tedersoo et al. 2007; Murata et al. 2013). To conserve an endangered tree species, it may be critically important to understand the ECM fungal communities in its remaining precious forests, which are on the verge of extinction.

While many potential determinants of ECM fungal communities have been proposed, such as climate (Bahram et al. 2012), soil (Cox et al. 2010; Jarvis et al. 2013; Roy et al. 2013), geographical position (Miyamoto et al. 2014), precipitation (Jarvis et al. 2013), nitrogen deposition (Lilleskov et al. 2002), heavy metals (Huang et al. 2014), and vegetation succession (Nara et al. 2003; Twieg et al. 2007), host effect may be the most significant factor, as has been demonstrated in many studies (Ishida et al. 2007; Tedersoo et al. 2008, 2012; Smith et al. 2009; Lang et al. 2011; Velmala et al. 2013; Põlme et al. 2013; Murata et al. 2013). If this is applicable to endangered hosts, they may be associated with unique ECM fungal communities that are different from those of coexisting and surrounding hosts.

The ECM host genus *Pseudotsuga* is a monophyletic conifer lineage composed of four extant species (some taxonomists accept more species; Wu and Raven 1999). It has a unique distribution pattern, that is, the intercontinental disjunction between Asia and North America. In North America, the Douglas-fir (*Pseudotsuga menziesii*) dominates a wide range of forests from the Pacific coast to the Rocky Mountains, representing typical forest ecosystems in these regions (Farjon 1990). This species produces good timber and may be the most economically important single tree species in the world (Eckenwalder 2009). Another North American species, *Pseudotsuga macrocarpa* is confined to small areas in California (Farjon 1990). In Asia, two *Pseudotsuga* species are accepted widely (Farjon 1990; Strauss et al. 1990): the Chinese Douglas-fir (*Pseudotsuga*

sinensis) in mainland China and Taiwan (*P. sinensis* var. *wilsoniana*), and the Japanese Douglas-fir (*Pseudotsuga japonica*) in Japan. Both Asian species are designated as endangered in the International Union for Conservation of Nature (IUCN) Red List (IUCN 2014), and remaining populations are small and fragmented (Kasai and Saito 2009; Fu 1992; Fu et al. 1999). According to fossil records and molecular phylogeny, Wei et al. (2010) estimated that *Pseudotsuga* originated in North America in the Eocene and migrated to Asia through the Bering land bridge approximately 32 million years ago (Mya), and diversified into Chinese and Japanese lineages c. 25–20 Mya.

Murata et al. (2013) studied ECM fungal communities in all of the major populations of Japanese Douglas-fir and found a significant difference in ECM fungal communities between co-existing hosts. However, no coevolved ECM fungal lineage has been confirmed to be associated with the Japanese Douglas-fir, except for one *Rhizopogon* species that was found only in soil spore banks. This is in sharp contrast to Douglas-fir ECM fungal communities in North America, where *Pseudotsuga*-specific lineages, such as *Rhizopogon* and *Suillus* (Molina et al. 1992; Kretzer et al. 1996; Horton and Bruns 1998; Horton et al. 2005; Twieg et al. 2007), account for a substantial portion of ECM fungal communities, as much as 25 % in relative abundance of ECM tips. Furthermore, there is little overlap of ECM fungal species between North American and Japanese Douglas-firs, indicating limited migration between the continents, as seen in *Pseudotsuga* (Wei et al. 2010) and many other plant lineages (Xiang et al. 1999). Considering the scarcity of coevolved fungal symbionts, host changes within the local ECM fungal pool could be important in structuring ECM fungal communities on the endangered tree species; however, further evidence is needed to confirm this. Given that the Chinese Douglas-fir is monophyletic with the Japanese Douglas-fir, and that both have been isolated for a long period on a geologic time scale, this subject is worthy of attention. Focused research will improve our understanding of how ECM fungal communities are structured with respect to local host changes, as well as host phylogeny, host biogeography, and ECM fungal biogeography.

In this study, ECM fungal communities associated with the Chinese Douglas-fir were characterized for the first time, a rare example of a study focusing on ECM

fungi on an endangered tree species. We also compared the communities with those of the Japanese Douglas-fir to address the similarity of the ECM communities between the two species, which are the closest extant relatives, although they have been separated for a long geological period, possibly c. 25–20 My. Based on our results, we discuss the importance of local host sharing in structuring ECM fungal communities in endangered, small, and fragmented host populations. We also discuss the biogeography of ECM fungi, which is largely host-independent on this geologic time scale.

Materials and methods

Sampling

We collected samples at three sites, all of which are protected by the Chinese government for conservation of the Chinese Douglas-fir. The Sanqing Mountain site (SQM) was located in Jiangxi province, China. The other two sites were located in Sangzhi, Zhangjiajie (ZJJ1 and ZJJ2), in Hunan Province, China. Geological and climate conditions are summarized in Table 1. Generally, the climate at these three sites (annual mean temperature: 16.3–18.2 °C) is warmer than that of Japanese Douglas-fir forests (annual mean temperature: 10.6–12.5 °C), and is characterized as subtropical. The Chinese Douglas-fir does not form mono-specific forests and is usually accompanied by other Pinaceae trees, such as *Tsuga*, *Pinus*, and *Abies*. Other tree families at the three sites included Ericaceae, Fagaceae, and Lauraceae. Aquifoliaceae, Theaceae, and Rosaceae were present at the SQM site (Guo et al. 2007), while Hamamelidaceae and Pteridiaceae were frequent at the ZJJ sites. At all three sites, most Chinese Douglas-fir individuals were in mature stages (Table 1), and they were the predominant components of the forests.

At each site, 23–25 Chinese Douglas-fir trees were selected, while maintaining a distance of >10 m between the selected trees for sample independence as in previous studies (Murata et al. 2013). A soil sample (5-×5-×10-cm, length×width×depth) was collected within about 2 m from each selected tree. In total, 73 soil samples were collected (23, 25, and 25 from SQM, ZJJ1, and ZJJ2, respectively) and stored at 4 °C until use. All roots in each soil sample were hand-picked, washed carefully under tap water on a 1-mm sieve, and examined under a dissecting microscope to collect all roots containing ECM tips. The ECM root tips were then classified into morphotypes based on their surface color, texture, and emanating hyphae, as described in Murata et al. (2013). Three to five replicate ECM root tips (or all, if fewer than three tips were available) were selected from each morphotype in each soil sample for molecular identification. In total, 1,005 root tips (305 cumulative morphotypes) were separately placed into 2-mL test tubes and used for DNA extraction.

Molecular analysis

Internal transcribed spacer (ITS) regions of fungal rDNA were amplified by polymerase chain reaction (PCR) using the ITS 1 F (Gardes and Bruns 1993) and LR 21 or LR 22 primer pairs (http://biology.duke.edu/fungi/mycolab/primers.htm), depending on the success of amplification. For host identification, the plastid trnL intron was amplified with the primers trnE (5′—GGTT CAAGTCCCTCTATCCC—3′) and trnF (5′—ATTT GAACTGGTGACACGAG—3′). We used AmpliTaq Gold 360 Master Mix (Applied Biosystems, CA, USA) for PCRs, however, when PCR products were faint or absent, the Multiplex PCR kit (Qiagen, GmbH, Hilden, Germany) was also used. Amplified PCR products were verified on 1.2 % agarose gels with Gel Red (Biotium, CA, USA) under UV light (Benchtop 2UV Transilluminator, UVP, UK) and then purified using a PCR product pre-sequencing kit (Exonuclease I, Shrimp Alkaline Phospatase, GE Healthcare, UK). Sequencing reactions were carried out using the BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), and sequencing was performed with a 3,130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All sequences were manually cleaned by checking the original chromatograms and eliminating ambiguous base-calls. The cleaned sequences were then grouped into operational taxonomic units (OTUs) based on a 97 % identity threshold in ITS regions (including the ITS1, 5.8S, and ITS2 regions) using ATGC (version 7, GENETYX, JAPAN). Representative sequences from each OTU were compared with known sequences in the international nucleotide sequence database (INSD: DDBJ/EMBL/NCBI) using the Megablast algorithm on the NCBI website. We only retained those OTUs that belong to ECM phylogroups (Tedersoo et al. 2010) by confirming phylogenetic relationships with available

Table 1 Geological and climate information of research sites

Site	MT[a] (°C)	MP[b] (mm)	Altitude (m)	Studied area (ha)	Center of sampling site	Mean DBH[c] (cm)
SQM	18.2	1857.7	1,530–1,560	–	N 28° 54′ 48″ E 118° 03′ 25″	–
ZJJ-1	16.3	1447.0	627–680	3.2	N 29° 39′ 30.03″ E 110° 11′ 33.69″	31.8 (n=28)
ZJJ-2	16.3	1447.0	658–692	1.5	N 29° 40′ 44.01″ E 110° 14′ 0.62″	39.8 (n=30)

[a] Mean annual temperature; b: Mean annual precipitation; c: Mean Diameter at Breast Height of *P. sinensis*; −: no data available

sequences in INSD, especially for genera containing both ECM and other trophic types such as *Entoloma* (Kinoshita et al. 2012) and *Sebacina* (Selosse et al. 2007). Theoretically, each OTU represents a different ECM fungal species; hereafter we use the term "species" unless otherwise specified (Murata et al. 2013). ECM fungal species names were assigned as the genus name followed by sequential numbers (e.g., *Amanita* sp.1) when ITS similarities were >95 % to known sequences in the Blast results. Family or order level classification was applied to those species showing the highest similarity of 90–95 % or <90 %, respectively, to known species in the INSD database. The identified sequences were deposited to DDBJ under the accession numbers AB981985- AB982069.

Analyses

The frequency of a fungus was defined as the number of soil samples in which that species occurred. The relative abundance of an ECM fungus was the percentage of ECM tips colonized by that fungus out of the total number of ECM tips observed. Soil samples containing no ECM fungi were excluded from the following analyses.

To estimate ECM fungal richness for Chinese Douglas-firs, for all hosts, and for each sampling site, we calculated Chao2 and Jackknife2 (Jack2) estimators using EstimateS version 9.0 (http://viceroy.eeb.uconn.edu/EstimateS), with 1,000 randomizations without sample replacement. The Sørensen similarity index value was used to calculate the similarity of the ECM fungal communities between Chinese Douglas-fir trees and the other hosts, based on frequency data.

To unravel the effects of host identity on ECM fungal community structure in our data set, we employed non-metric multidimensional scaling (NMS) implemented in PC-ORD ver. 6 (McCune and Mefford 2011). Each host

genus within a sampling site was regarded as a sampling unit in the ordination analyses, in which frequency data were used. Hosts represented by less than 10 % of the total soil samples were excluded from the analysis. We used the relative Sørensen distance measure for NMS analysis. To test the effect of hosts and geographical positions (i.e., sampling sites) on fungal community composition, we used the Adonis function in the Vegan package of R (Oksanen et al. 2011) with the following options: Hellinger-transformed species frequency, Bray–Curtis distance measure, and 999 permutations.

We also combined the present results with Japanese Douglas-fir data published previously (Murata et al. 2013), where the sampling and identification methods were basically the same as in the present study, to examine the effects of host monophyly and long-time host isolation on ECM fungal communities. NMS and Adonis analyses for the combined data set were performed as described above.

Results

Of the 73 soil samples, 65 contained ECM roots and 54 included ECM roots from Chinese Douglas-firs. The other dominant host genera were *Pinus* (*Pinus densata* and *Pinus massoniana*) and *Quercus* (*Quercus multinervis* and *Quercus phillyraeoides*), which were observed in 15 and 9 soil samples, respectively. *Tsuga diversifolia* was confirmed in 5 soil samples, while *Abies farges* and *Fraxinus americana* were each detected only once (Table 2). Eight soil samples did not contain ECM root-tips. In terms of ECM root abundance, the Chinese Douglas-fir was dominant, accounting for 61 % of the identified ECM root tips.

In total, we identified 86 ECM fungal species, of which 66 species were found on Chinese Douglas-fir roots. *Pinus* spp. and *Quercus* spp. were associated with

Table 2 Ectomycorrhizal fungi identified in Chinese Douglas-fir forests

Species	R.A[a]	Sites	F[b]	Host[c]	Accession No.	Best blast match	
						Acc. No.	% identity
Amanita sp.1	2.0 %	SQM	4	Pi, Ps	AB981985	KC581322.1	97 %
Amanitaceae sp.1	0.4 %	ZJJ-1	1	Ps	AB981986	HQ604824.1	93 %
Amanitaceae sp.2	0.8 %	ZJJ-1	2	Ps, −	AB981987	AB096059.1	92 %
Amphinema sp.1	1.2 %	ZJJ-2, SQM	2	−	AB981988	AB873186.1	99 %
Amphinema sp.2	2.3 %	ZJJ-1, ZJJ-2	4	Ps, −	AB981989	JN943925.1	99 %
Atheliaceae sp.1	0.4 %	SQM	1	−	AB981990	EU649087.1	95 %
Cantharellales sp.1	0.4 %	SQM	1	Ps	AB981998	KC876295.1	88 %
Cantharellales sp.2	0.4 %	SQM	1	Ps	AB981999	EU862224.1	82 %
Cenococcum geophilum	3.5 %	ZJJ-2, ZJJ-2, SQM	8	Pi, Ps, Qu, −	AB981991	DQ474376.1	98 %
Ceratobasidium sp.1	0.8 %	SQM	2	Pi	AB981992	AB831845.1	99 %
Clavulina sp.1	1.6 %	ZJJ-2	3	Pi, Ps, Qu, −	AB981993	KC679837.1	99 %
Clavulina sp.2	0.8 %	SQM	1	Ps	AB981994	KF476771.1	97 %
Clavulina sp.3	0.8 %	SQM	1	Ps	AB981996	FR852042.1	98 %
Clavulinaceae sp.1	0.8 %	ZJJ-2, SQM	2	Ps, −	AB981995	AB831847.1	92 %
Clavulinaceae sp.2	0.4 %	SQM	1	Ps	AB981997	AY534200.1	95 %
Cortinarius sp.1	0.4 %	ZJJ-2	1	−	AB982000	EU821665.1	97 %
Entoloma sp.1	0.4 %	ZJJ-1	1	Pi	AB982002	KC710063.1	96 %
Entoloma sp.2	0.8 %	ZJJ-2, SQM	1	Ps, Qu	AB982003	AB692005.1	100 %
Entolomataceae sp.1	0.8 %	ZJJ-1, ZJJ-2	2	Pi, Ps	AB982001	FM995558.1	92 %
Helotiaceae sp.1	0.4 %	SQM	1	Ps	AB982011	JQ711893.1	92 %
Hygrophorus sp.1	0.4 %	SQM	1	Ps	AB982004	FJ845410.1	96 %
Inocybaceae sp.1	2.7 %	SQM	3	Pi, Ps, −	AB982005	HQ604555.1	90 %
Inocybaceae sp.2	0.4 %	SQM	1	Qu	AB982006	FN550881.1	92 %
Lactarius sp.1	0.4 %	SQM	1	Ps	AB982008	KF432995.1	96 %
Lactarius sp.2	0.8 %	ZJJ-1, ZJJ-2	2	Ps	AB982009	KF432994.1	99 %
Piloderma sp.1	0.4 %	ZJJ-2	1	Ps, −	AB982010	JQ711935.1	96 %
Rhizopogon sp.1	0.4 %	ZJJ-1	1	Ps	−[d]	KC542888.1	94 %
Russula sp.1	5.5 %	ZJJ-2, SQM	5	Ps, Qu, −	AB982012	DQ778002.1	96 %
Russula sp.2	2.7 %	ZJJ-2	4	Ps, −	AB982013	HQ021944.1	96 %
Russula sp.3	1.6 %	ZJJ-1	3	Ps	AB982014	KF245524.1	97 %
Russula sp.4	1.2 %	ZJJ-2	1	Qu	AB982016	DQ422001.1	99 %
Russula sp.5	2.0 %	ZJJ-2	2	Pi, Ps, −	AB982017	AB507011.1	97 %
Russula sp.6	0.4 %	SQM	1	Ps	AB982020	AB636109.1	98 %
Russula sp.7	1.6 %	ZJJ-2, SQM	4	Ps, Qu, −	AB982022	JX425403.1	99 %
Russula sp.8	2.7 %	ZJJ-2, SQM	6	Pi, Ps, Qu, −	AB982023	JF908664.1	96 %
Russula sp.9	0.4 %	ZJJ-1	1	Ps	AB982025	AB629019.1	99 %
Russula sp.10	0.8 %	ZJJ-1	2	Ps, Ts	AB982026	JQ991824.1	96 %
Russula sp.11	0.4 %	ZJJ-2	1	Pi	AB982038	KF361762.1	96 %
Russula sp.12	0.4 %	ZJJ-2	1	Pi	AB982040	JF273535.1	99 %
Russula sp.13	0.4 %	ZJJ-1	1	Ps	AB982043	KC505578.1	99 %
Russulaceae sp.1	1.2 %	ZJJ-1	3	Ps	AB982007	KF432993.1	93 %
Russulaceae sp.2	2.0 %	ZJJ-2	2	Ps, −	AB982015	GU998132.1	91 %
Russulaceae sp.3	0.8 %	ZJJ-2	1	Ps	AB982018	DQ422006.1	91 %
Russulaceae sp.4	0.4 %	ZJJ-1	1	Ps	AB982019	EU303008.1	95 %
Russulaceae sp.5	0.4 %	ZJJ-1	1	Ps	AB982021	DQ273398.1	95 %

Table 2 (continued)

Species	R.A[a]	Sites	F[b]	Host[c]	Accession No.	Best blast match	
						Acc. No.	% identity
Russulaceae sp.6	0.4 %	SQM	1	Ps	AB982024	JX625306.1	91 %
Russulaceae sp.7	0.8 %	SQM	2	Ps	AB982027	KF002775.1	93 %
Russulaceae sp.8	0.4 %	SQM	1	Ps	AB982028	JF908664.1	94 %
Russulaceae sp.9	0.4 %	SQM	1	Pi	AB982029	HF674599.1	94 %
Russulaceae sp.10	0.4 %	ZJJ-1	1	–	AB982030	DQ384581.1	93 %
Russulaceae sp.11	0.4 %	SQM	1	Ps	AB982031	AF418641.1	94 %
Russulaceae sp.12	0.8 %	SQM	2	Ps	AB982032	KF002780.1	92 %
Russulaceae sp.13	0.4 %	ZJJ-1	1	–	AB982033	KF245505.1	95 %
Russulaceae sp.14	0.4 %	SQM	1	Qu	AB982034	FJ454969.1	94 %
Russulaceae sp.15	0.8 %	ZJJ-1	2	Ps	AB982035	JQ991786.1	90 %
Russulaceae sp.16	0.4 %	ZJJ-1	1	–	AB982036	EU598164.1	95 %
Russulaceae sp.17	0.4 %	ZJJ-2	1	Ps	AB982037	DQ422013.1	94 %
Russulaceae sp.18	0.4 %	ZJJ-1	1	Ps	AB982039	JQ991805.1	93 %
Russulaceae sp.19	0.4 %	ZJJ-2	1	Qu	AB982041	JQ991822.1	93 %
Russulaceae sp.20	0.4 %	ZJJ-2	1	Ps	AB982042	KF002780.1	93 %
Sebacina sp.1	10.5 %	ZJJ-1, ZJJ-2, SQM	16	Fr, Pi, Ps, Qu, Ts, –	AB982044	HQ154316.1	97 %
Sebacina sp.2	5.9 %	ZJJ-2, SQM	7	Pi, Ps, Qu, –	AB982045	HQ154299.1	96 %
Sebacina sp.3	1.6 %	ZJJ-1	3	Ps, –	AB982047	AB873204.1	99 %
Sebacina sp.4	1.2 %	ZJJ-2, SQM	3	Ps, Qu, –	AB982049	FR852338.1	98 %
Sebacina sp.5	0.4 %	ZJJ-2	1	Ts	AB982052	JQ665485.1	98 %
Sebacina sp.6	1.2 %	ZJJ-2	1	Ps, –	AB982054	KC702633.1	97 %
Sebacinaceae sp.1	4.7 %	ZJJ-1, SQM	6	Ps, –	AB982046	AF465191.1	93 %
Sebacinaceae sp.2	1.6 %	ZJJ-1	2	Ps, –	AB982048	AB506994.1	95 %
Sebacinaceae sp.3	3.9 %	ZJJ-1, ZJJ-2, SQM	9	Ab, Ps, –	AB982050	AB506923.1	93 %
Sebacinaceae sp.4	0.8 %	ZJJ-2	2	Ps	AB982051	HE814102.1	92 %
Sebacinaceae sp.5	0.4 %	ZJJ-2	1	Pi	AB982053	JQ420941.1	90 %
Sebacinaceae sp.6	0.4 %	ZJJ-2	1	Ts	AB982055	FR852369.1	94 %
Thelephoraceae sp.1	1.2 %	ZJJ-1	3	Ps, –	AB982056	JF273546.1	99 %
Thelephoraceae sp.2	1.2 %	SQM	2	Pi, Ps	AB982057	FJ196975.1	94 %
Thelephoraceae sp.3	0.8 %	ZJJ-1	2	Ps	AB982058	JQ991862.1	99 %
Thelephoraceae sp.4	0.4 %	SQM	1	Ps	AB982059	AB839400.1	99 %
Thelephoraceae sp.5	0.4 %	SQM	1	Ps	AB982060	HE687150.1	96 %
Thelephoraceae sp.6	0.4 %	SQM	1	Ps	AB982061	FR852209.1	93 %
Thelephoraceae sp.7	0.4 %	ZJJ-1	1	–	AB982062	AB259143.1	98 %
Thelephoraceae sp.8	0.4 %	ZJJ-1	1	Ps	AB982063	AB634265.1	95 %
Thelephoraceae sp.9	0.4 %	ZJJ-2	1	Ps	AB982064	EF411076.1	96 %
Thelephoraceae sp.10	0.4 %	ZJJ-2	1	–	AB982065	AB769926.1	95 %
Thelephoraceae sp.11	0.4 %	ZJJ-2	1	Ps	AB982066	JQ991887.1	96 %
Thelephoraceae sp.12	0.4 %	ZJJ-1	1	Ps	AB982067	FJ454933.1	97 %
Tremellodendron sp.1	3.5 %	ZJJ-2, SQM	5	Pi, Ps, Qu, –	AB982068	KC876350.1	96 %
Tuber sp.1	1.2 %	ZJJ-2, SQM	3	Ps	AB982069	JQ991906.1	99 %

[a] Relative abundance of ECM tips, b: Frequency (the number of soil samples), c: Ab: *Abies fargesi*, Fr: *Fraxinus americana*, Pi: *Pinus* spp., Ps: *Pseudotsuga sinensis*, Qu: *Quercus* spp., Ts: *Tsuga diversifolia*, d: available in a coming paper

17 and 14 ECM fungi, respectively. Of the 66 species identified on Chinese Douglas-firs, 39 species appeared only on Chinese Douglas-firs, while 11 and 9 species were shared with *Pinus* and *Quercus*, respectively. These shared species were relatively frequent in the forests (Table 2). An average of 2.8 ECM fungal species were detected per soil sample, with a maximum of 7 species.

At the family level, the relative abundances of Russulaceae and Sebacinaceae were 33 and 32 %, respectively. Russulaceae (35 spp.) was the most species-rich ECM fungal lineage, followed by Sebacinaceae (12 spp.), Thelephoraceae (12 spp.), and Clavulinaceae (5 spp.) (Table 2). At the species level, *Sebacina* sp.1, Sebacinaceae sp.3, and *C. geophilum* were dominant on Chinese Douglas-firs, and were found in 16, 9, and 8 soil samples, respectively. Only eight ECM fungal species were found in more than five soil samples. In contrast, 49 species were singletons, represented by a single soil sample (Table 2, Supplemental Fig. S1).

The richness estimator chao2 (±SD) indicated that at least 164±35 ECM fungal species are expected to inhabit these forests, while the estimator for Chinese Douglas-fir alone was 154±40. The observed ECM fungal richnesses (and Chao2 estimates) at the ZJJ1, ZJJ2, and SQM sites were 30 (67±43), 38 (111±46), and 39 (83±40), respectively. Species accumulation curves of ECM fungi for all hosts and for Chinese Douglas-fir alone did not approach asymptote at our maximum sampling effort (Fig. 1), indicating that additional species would be found with greater sampling effort. The curves for Chao2 became asymptotic at around 15 samples for all hosts and for Chinese

Douglas-fir alone, but then increased linearly because of the species-area relationship, that is, with greater sampling area, more species are found, as is the general ecological principle (MacArthur and Wilson 1967).

The Sørensen similarity index value indicated that the ECM fungal community of Chinese Douglas-fir was most similar to that of *Pinus* (0.27), followed by *Quercus* (0.21). The similarity value was 0.30 for the *Quercus–Pinus* pair. NMS ordination did not clearly separate ECM communities between host genera (Supplemental Fig. S2). The Adonis test also revealed that host identity had no significant effect on ECM fungal community composition ($F_{2,5}=0.838$, $p=0.843$). In contrast, the communities were significantly different between sampling sites ($F_{2,5}=1.417$, $p=0.021$).

Only eight ECM fungal species (*Amanita* sp.1, *Ceratobasidium* sp.1, *Entoloma* sp.2, *Russula* sp.2, *Russula* sp.9, Russulaceae sp.5, Thelephoraceae sp.4, Thelephoraceae sp.5) were shared between Chinese and Japanese Douglas-fir forests, after applying the 97 % ITS identity threshold to the combined data set. Of the eight species, *Amanita* sp.1, *Ceratobasidium* sp.1, and *Entoloma* sp.2 were not *Pseudotsuga* specific, and were found also in association with other coexisting hosts (Table 2). Although the other five species were found only on Chinese Douglas-fir in our study sites, their frequency was not sufficient to examine the biased occurrence on Chinese Douglas-firs. In addition, we found no *Pseudotsuga*-specific fungal lineages in our phylogenetic analyses using sequences found only on Chinese Douglas-fir and their related sequences in the

Fig. 1 Species accumulation curves for ectomycorrhizal (ECM) fungi found in Chinese Douglas-fir forests. Filled and open triangles represent observed species richness of ECM fungi from all host species, and Chinese Douglas-fir alone, respectively, with 95 % confidence intervals. Jackknife2 (*squares*) and Chao2 (*circles*) minimum species richness estimates of ECM fungi are shown for all host species and Chinese Douglas-fir and indicated by filled and open symbols, respectively
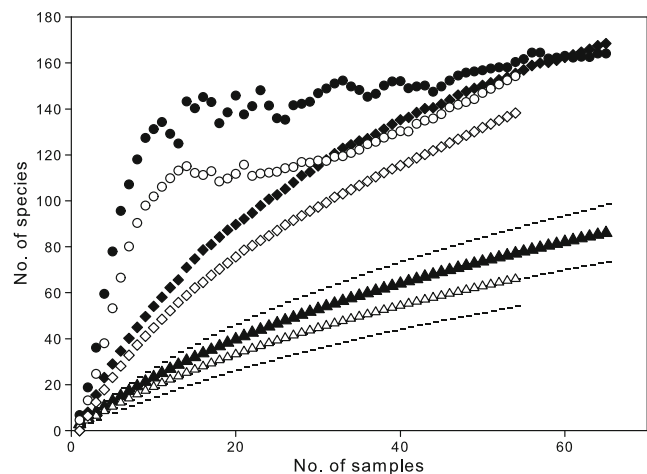
INSD, with the exception of one *Rhizopogon* sp.1 (Supplemental Figs. S3, S4). The NMS plot for the combined data set clearly demonstrates that ECM fungal communities found in Chinese Douglas-fir forests are dissimilar to those of Japanese Douglas-fir forests (Fig. 2). A significant regional effect (China vs. Japan) was also confirmed by the Adonis test ($F_{1,18}=3.851$, $p=0.001$), while the effect of host was not significant in the combined data set ($F_{2,17}=1.027$, $p=0.359$).

Discussion

In total, we identified 66 ECM fungal species associated with the Chinese Douglas-fir, while at least 154 ± 40 species were estimated to exist. The observed and estimated species richness values were similar to those obtained for other abundant host species using the same sampling method and analyses (Ishida et al. 2007; Miyamoto et al. 2014). Although ECM fungal richness in endangered tree species has rarely been reported, Tedersoo et al. (2007) found only 15 ECM fungal species in 16 samples collected from a small remaining population of *Vateriopsis seychellarum*

(Dipterocarpaceae) in Seychelles, and estimated a fungal richness of 29.5 species. This remaining *V. seychellarum* population may be too small (constituting ~15 trees) and too distant from other ECM forests to sustain rich ECM communities. Although individual Chinese Douglas-fir populations were comparably small, they were mixed with other common ECM trees that have wider distributions. Therefore, the Chinese Douglas-fir would be able to maintain rich ECM fungal communities by utilizing a larger regional ECM fungal pool.

Accordingly, Chinese Douglas-fir shared many ECM fungi with coexisting trees (e.g., *Pinus* and *Quercus*), and ECM fungal communities did not differ significantly between coexisting hosts. A similar pattern was also demonstrated in a North American mixed Douglas-fir forest (Horton and Bruns 1998), where 12 of 16 ECM fungal species were shared between *P. menziesii* and *Pinus muricata*. Molina et al. (1992) estimated that Douglas-fir and pines have a 72 % (or 1,800 species) overlap of compatible fungi. In addition, most of the ECM fungi found only in Chinese Douglas-fir were not significantly biased toward

Fig. 2 Nonmetric multidimensional scaling (NMS) plot for ECM fungal communities in Chinese Douglas-fir and Japanese Douglas-fir forests. Frequency data for ECM fungi on individual host genera at each research site were used for NMS ordination with the relative Sørensen distance measure. *Open symbols and filled symbols* represent ECM fungal communities in China and Japan, respectively. *Circles, triangles, inverted triangles, diamonds and squares* represent ECM fungal communities on *Pseudotsuga, Pinus, Abies, Tsuga* and *Quercus*, respectively
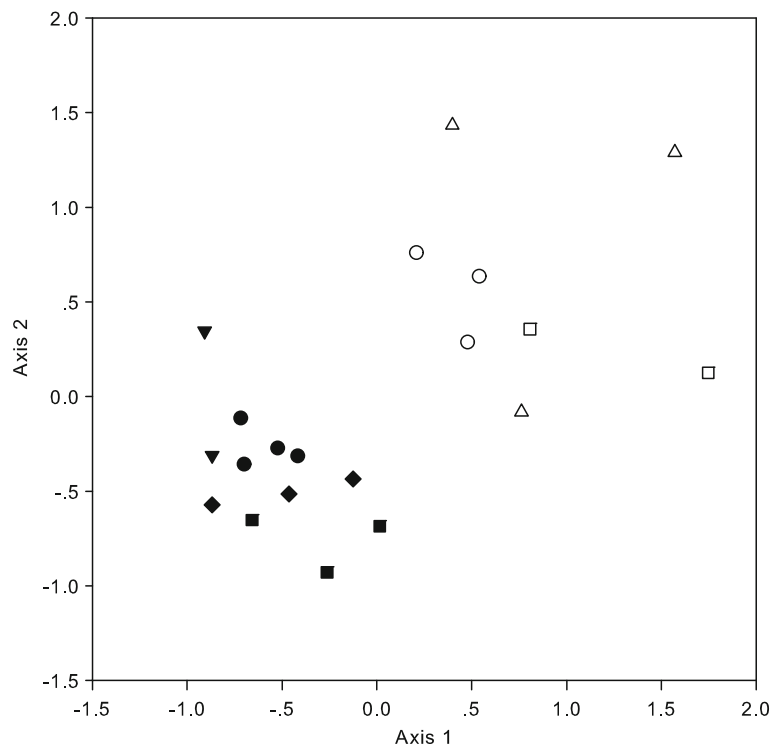
this host (i.e., exclusive observations occurred by chance) and did not belong to *Pseudotsuga*-specific lineages (i.e., no host conservatism during long evolutionary periods). Because of the existence of multi-host fungi and the prevailing host changes, an endangered tree species could acquire compatible ECM fungal symbionts if surrounded by other ECM host trees that have evolved in the same region.

There is sharp contrast between ECM fungal compositions in North American and Asian Douglas-fir forests. In North American Douglas-fir forests, co-evolved and specific ECM fungi, such as some *Rhizopogon* and *Suillus* species, often appear as major components in the communities (Horton et al. 2005; Luoma et al. 2006; Twieg et al. 2007). In Chinese Douglas-fir forests, no ECM fungi were confirmed to be phylogenetically specific to *Pseudotsuga*, except for one rare *Rhizopogon* sp.1 (Table 2, Supplemental Figs. S4). This is in agreement with ECM fungal communities in Japanese Douglas-fir forests, where no co-evolved ECM fungal lineages were found on mature trees (Murata et al. 2013). Although the contrast in ECM fungal communities between North American and Asian Douglas-fir forests may be related to differences in climate conditions, we think the small and isolated populations of Asian Douglas-firs, as indicated by their endangered status, could also account for the rarity of host specialists. As in all plant and animal species, each ECM fungal species exists as assemblages of populations. Small populations are vulnerable to extinction because of demographic fluctuation, limited genetic diversity and inbreeding depression, and random catastrophes (Lande 1993). In association with small and isolated host populations, host-specific ECM fungi would have difficulty in maintaining effective population sizes over a long geological time period so as to avoid extinction. Therefore, the richness and relative abundance of host-specific ECM fungi may be determined by the effective population sizes of hosts.

Co-evolved specialists are less represented in mature forests than in soil spore banks, as seen in Suilloid fungi in pine forests (Taylor and Bruns 1999). The spores of these specialists can survive disturbances and play important roles in seedling establishment after disturbances for pines (Baar et al. 1999; Izzo et al. 2006; Peay et al. 2009), and likely also for Douglas-fir (Cline et al. 2005). Indeed, while *Rhizopogon* species were a minor population on mature tree roots in Chinese and Japanese Douglas-fir forests, they were relatively frequent in spore banks in both forests (unpublished data). Thus, we should not underestimate the function of minor specialists in forest dynamics or regeneration. For conservation of endangered Asian *Pseudotsuga*, further research is necessary to clarify ECM fungal symbionts at all host life stages, especially in the seedling stage.

ECM fungal communities in Chinese Douglas-fir forests differed significantly among three sites, which were separated by a maximum of 770 km. The effect of geographical location on ECM fungal communities was more pronounced between the Chinese and Japanese Douglas-fir forests, which were >1,610 km apart, with a sea barrier. These results indicate that ECM fungal migration is infrequent on these spatial scales, even over long geological periods. In fact, Southern China and the main island of Japan have been separated for over 1.7 My by Watase's Line (Tokara Strait, maximum depth >1,000 m), which constrains the distribution of many plant and animal species (Hotta 1974; Ono 1989; Hiramatsu et al. 2001). Indeed, Chinese and Japanese Douglas-fir lineages have been separated without genetic exchange for c. 20–25 My (Wei et al. 2010). Similarly, many ECM fungal components in Chinese and Japanese Douglas-fir forests may have been separated for a comparable geological period; this was confirmed by the sequence divergence between the Chinese and Japanese *Rhizopogon* species specific to *Pseudotsuga* (Supplement Fig. S4).

Taken together, our results suggest that, except for rare specialists, most ECM fungi may have been able to migrate independently of the biogeography of endangered hosts, as far and as long as contiguous ECM forests of other species existed. In fact, most ECM fungal genera or families have global distributions while the distribution of hosts is not global (Tedersoo and Nara 2010). Thus, one might conclude that the lack of compatible ECM fungi is not problematic to endangered host trees. On the other hand, while artificially introduced trees suffer from a lack of compatible ECM fungi (Mikola 1973 and references therein, Pringle et al. 2009), some specificity likely exists after long periods of allopatric evolution in different regions. Therefore, with the aim of conserving endangered trees, we should

take into consideration regional ECM fungal pools that share long histories of evolutionary interactions.

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