

Pioneer dwarf willow may facilitate tree succession by providing late colonizers with compatible ectomycorrhizal fungi in a primary successional volcanic desert

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Summary

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- To advance understanding of the contribution of ectomycorrhizal (ECM) fungi to tree successional processes, natural establishment patterns of secondary colonizing hosts and their ECM fungal communities were investigated with special reference to pioneer hosts.
- In the volcanic desert on Mount Fuji, Japan, vegetation is sparsely distributed, resembling islands in a sea of scoria. Of 509 vegetation islands in the research area, 161 contained *Salix reinii* (*Salix*), the first colonizing ECM host species. The spatial coincidence between secondary colonizing timber species and *Salix* was analysed, and ECM fungal communities were studied using molecular identification methods.
- I found 39 and 26 individuals of *Betula ermanii* and *Larix kaempferi*, respectively. Without exception, these individuals were all accompanied by *Salix*. The ECM fungal communities of these timber species showed high similarity to that of *Salix* and were dominated by generalists that were compatible with two or more plant families.
- In this desert, available ECM propagules are limited. Pioneer *Salix* may contribute to tree succession by providing adjacent late colonizers with compatible ECM fungal symbionts.

Key words: common mycorrhizal networks, ectomycorrhizal (ECM) fungi, facilitation, generalists vs specialists, host ranges, host specificity, internal transcribed spacer (ITS) terminal RFLP, primary succession.

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Introduction

Ectomycorrhizal (ECM) fungi are symbiotic microorganisms that colonize the roots of many tree species. These fungi usually dominate forest soils and effectively scavenge soil nutrients that are subsequently transferred to the host plants. This nutrient supply from ECM fungi appears to be a requisite for normal growth in many host plants (Smith & Read, 1997). In single forest stands, tens to hundreds of ECM fungal species coexist. Many of these microorganisms are generalists that are compatible with many different tree species (Molina *et al.*, 1992). Because generalists usually dominate ECM communities, their spores and living mycelia are ubiquitous in forest soil. Hence regenerating seedlings of host species can be colonized rapidly by these ECM fungi (Jonsson *et al.*, 1999; Matsuda & Hijii, 2004).

Disturbance can reduce or change the availability of ECM sources and alter the ECM communities on regenerating hosts (Perry *et al.*, 1987 and references therein), but usually not to the extent that ECM association is impossible. For example, after a stand-replacing forest fire, *Rhizopogon* showed increased dominance on *Pinus muricata* seedlings relative to the prefire forest (Horton *et al.*, 1998; Baar *et al.*, 1999). Even in a gradually receding glacier area (Helm *et al.*, 1999), a sand dune (Ashkannejhad & Horton, 2006), and a mining district (Ingleby *et al.*, 1985), seedlings were readily associated with some ECM fungi. Thus host growth is not suppressed by the complete absence of ECM associations in most potential forest areas. It is therefore difficult to elucidate the contribution of ECM associations to ecological processes because of the difficulty in preparing non-mycorrhizal controls under natural conditions in most habitats.

Successful ECM colonization is problematic in some primary successional sites, especially in severely devastated volcanic deserts (Allen *et al.*, 1992). If nonmycorrhizal controls are available in natural settings, studies in these sites can provide important and fundamental knowledge concerning the role of ECM symbioses in ecological processes such as succession. In the volcanic desert on Mount Fuji, Japan, the first ECM plant to colonize is an alpine dwarf willow, *Salix reinii* (hereafter *Salix* unless otherwise specified; Nara *et al.*, 2003a). Although all established *Salix* shrubs are associated with ECM fungi (Nara *et al.*, 2003b), nonmycorrhizal seedlings that are transplanted into most parts of this area remain uncolonized by any ECM fungi until the end of the first growing season because of limited propagule availability (Nara & Hogetsu, 2004). Current-year *Salix* seedlings can easily develop ECM associations only beside established *Salix* shrubs via the spread of ECM mycelia from the established shrubs (Nara & Hogetsu, 2004; Nara, 2006). The growth of *Salix* seedlings is significantly improved once the seedlings are connected to the established ECM systems of most fungal species (Nara, 2006). In this way, early-established *Salix* shrubs facilitate the subsequent establishment of conspecific seedlings by providing ECM fungal symbionts.

ECM fungi on established *Salix* may also aid in the establishment of secondary colonizing tree species, thereby facilitating tree succession in the volcanic desert. A previous transplanting experiment showed improved ECM colonization on seedlings of late-colonizing tree species beside established *Salix*, but did not detect any positive effects of ECM associations on the growth of the secondary colonizers (Nara & Hogetsu, 2004), probably because of nutrient competition with the early-established *Salix*. However, this does not necessarily mean that ECM fungi do not contribute to primary tree succession. Because any manipulative approach in the field must be restricted in time and space, such an approach may not be suitable for evaluating primary tree succession, which requires at least several centuries to proceed. Because of the longevity of tree species, the rare establishment of single trees could have large long-term effects during primary succession. To understand the contribution of ECM fungi to such rare events, studies of the natural establishment patterns of late colonizers may overcome the shortcomings of manipulative approaches (Nara & Hogetsu, 2004) because rare events within the whole research area are temporally integrated.

Another important aspect of ECM symbioses in primary succession of trees is host–fungus compatibility. Because ECM propagules are limited in this volcanic desert, few ECM sources other than that of symbionts on early-established hosts can be used by late-colonizing plants (Nara & Hogetsu, 2004; Nara, 2006). If ECM fungi on early-established hosts are not compatible with late colonizers, the late colonizers must wait for the stochastic arrival of compatible ECM propagules. ECM communities on naturally established saplings of late colonizers, with reference to host ranges of individual fungal species, should

provide valuable information on the contribution of ECM fungi to primary succession of trees.

To advance our knowledge on the role of ECM fungi in tree successional processes, the spatial patterns were analysed of naturally established saplings of secondary colonizing timber species 300 yr after the last volcanic eruption, with special reference to pioneer ECM shrubs in the volcanic desert on Mount Fuji, Japan. The ECM communities of two timber species were compared with those of early-established shrubs. The host ranges of individual fungal species were also evaluated. Finally, the importance of ECM fungi in primary succession is discussed.

Materials and Methods

Research site

Mount Fuji, the highest and best known mountain in Japan, erupted in 1707, and its south-eastern area was completely covered with scoria (tephra, typically 2–30 mm in diameter), up to 10 m deep. The existing vegetation was completely destroyed, and is now recovering. Whereas the treeline is generally located at 2500 m asl on the other sides of the mountain, it is located at *c.* 1300 m on the south-eastern slope, and continues to increase in altitude after 300 yr of vegetation recovery. The study area was located above the treeline at 1450–1600 m asl.

At the study site vegetation is distributed patchily, forming isolated vegetation islands in a sea of volcanic desert. Each vegetation patch is normally initiated by the perennial herb *Polygonum cuspidatum*. This plant species is usually nonmycorrhizal, but on rare occasions it is colonized by arbuscular mycorrhizal fungi at low levels if accompanied by other arbuscular mycorrhizal hosts in the volcanic desert (Wu *et al.*, 2004). Individuals of *P. cuspidatum* spread vegetatively to 10 m in diameter, providing stable habitats for subsequent plant species on the unstable scoria desert (Adachi *et al.*, 1996). Many plant species are able to invade this stable habitat (see list of species and their frequencies in Nara *et al.*, 2003a). *Salix reinii* usually establishes beside a *P. cuspidatum* patch, where water and light conditions are favourable. This dwarf *Salix* is not significantly different from early-established *P. cuspidatum* in terms of plant height (usually <1 m) and leaf mass. *Salix* is the first pioneer ECM host species in the research site, and all established shrubs of this species, without exception, are intensively colonized by ECM fungi (Nara *et al.*, 2003a). In total, 31 ECM fungal species have been shown to be associated with *Salix* (Nara *et al.*, 2003a,b).

Because ECM tree species such as *Fagus*, *Quercus*, *Abies* and *Tsuga* dominate other areas at the same elevation on Mount Fuji (Ohsawa, 1984), it is likely that the research site will eventually become occupied by forests of these species in the distant future. Two timber species, *Betula ermanii* and *Larix kaempferi* (hereafter referred to as *Betula* and *Larix*, respectively), are the ECM host plants that follow *Salix* in the successional sequence. As in the case of *Salix* establishment,

these secondary colonizing plants always establish beside a vegetation patch, where the soil is poorly developed compared with inside a vegetation patch.

Although nitrogen-fixing plants play an important role in some areas of primary succession (Walker & del Moral, 2003), this is not the case on Mount Fuji. In this area, there were two N-fixing plant species. A relatively late-colonizing legume, *Hedysarum vicioides*, was observed as a minor constituent in c. 20% of vegetation patches. An alpine alder, *Alnus maximowiczii*, is also rare, and only two saplings were found in 21 ha of research area. The total area covered by these two N-fixing plants was <0.001% of the total vegetation. The absence of N-fixing plants is common at the initial stage of primary succession (Sprent & Sprent, 1990).

Spatial patterns of timber establishment

Because naturally established saplings of timber species were rare, I used a larger study area (c. 21 ha; Fig. 1) than the 5.5-ha quadrat used in previous studies (Nara *et al.*, 2003a, 2003b). The position of all vegetation patches >1 m in smallest diameter distributed in the study area was recorded using GPS. The area of each vegetation patch was estimated from the largest and smallest diameters.

All vegetation patches were surveyed in October 2004 for established *Betula* and *Larix* individuals. Because vegetation patches are physically separated by some distance by scoria desert, one patch is unlikely to affect another. This condition allowed examination of the natural establishment patterns of timber trees, and the conditions for this establishment, by determining the differences between patches that contained timber trees and those that did not. This study focused on the presence and absence of early-established ECM hosts (*Salix* shrubs) in each vegetation patch.

Sampling and treatment of ECM root tips

In early October 2004, 10 saplings of each of the two timber species were selected. The range of stem diameters of selected *Larix* saplings was 15–40 mm at ground level. For *Betula*, in most cases multiple stems had sprouted from each individual, and the largest stem diameters ranged from 14 to 46 mm. The age range of selected saplings was estimated at 7–20 yr for *Betula* and 7–22 yr for *Larix* by analysing the growth rings of four individuals of each species (data not shown).

A 20-cm section of main root (c. 3–5 mm diameter) that was traced to the stem was sampled in two different positions for each sapling. Each main root section and all attached root tips were placed in a plastic bag with a small amount of scoria, and kept at 4°C until use. If *Salix* roots were present in the same sampling positions, they were treated in the same manner. In total, I sampled 19, 20 and 14 root systems of *Betula* (no traceable root found at one position), *Larix* and *Salix*, respectively.

Each root system was washed carefully with tap water. I arbitrarily subsampled c. 100 root tips from a root system. If a root system contained <100 root tips, all root tips were used. The subsamples were observed under a dissecting microscope to determine the percentage of ECM root tips. ECM tips were then classified into morphotypes based on surface colour, texture, emanating hyphae and rhizomorphs, using the method from a previous study (Nara *et al.*, 2003b).

Molecular identification of ECM fungi

Two to five ECM root tips in a morphotype in each root system, depending on the number of root tips in the morphotype, were individually placed into 2.0-ml microtubes as replicates. I tried to use at least duplicate samples for molecular

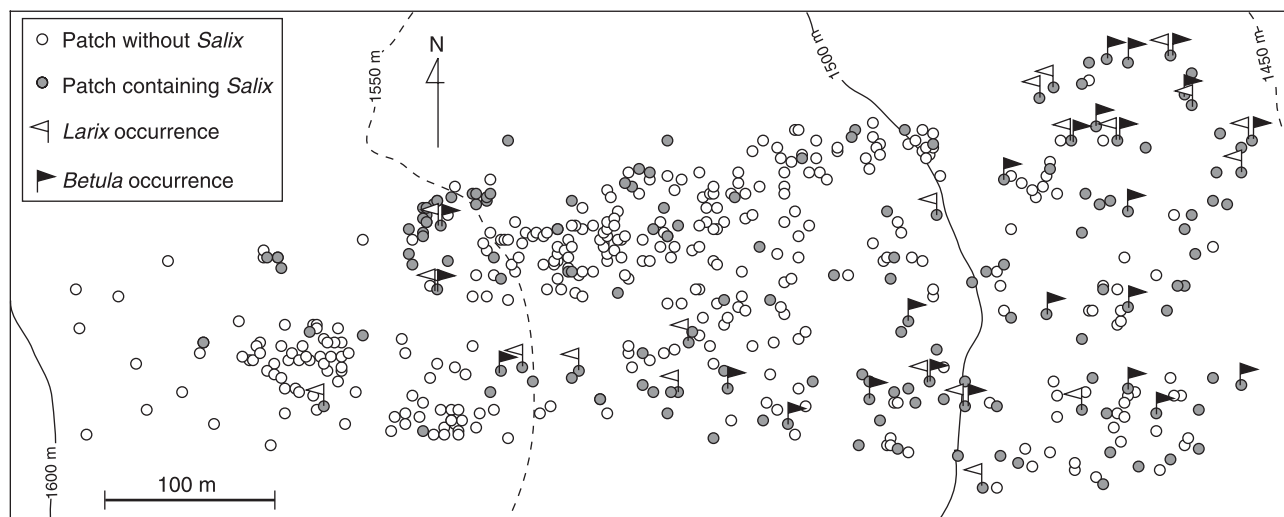


Fig. 1 Distribution pattern of vegetation patches and occurrence of timber species (*Betula ermanii* and *Larix kaempferi*), with special reference to early-established willow (*Salix reinii*) shrubs in the volcanic desert on Mount Fuji, Japan.

identification, but in some cases only one tip was available. These replicate ECM samples were dried and used for DNA extraction, as described by Nara *et al.* (2003b). Polymerase chain reaction (PCR) was conducted to amplify the internal transcribed spacer (ITS) region of rDNA of ECM fungi using a KOD plus kit (Toyobo, Osaka, Japan). The PCR primers were ITS1F and ITS4 (Gardes & Bruns, 1993), which were labelled with fluorescent Beckman dyes (Proligo Japan Co., Kyoto, Japan) D4 and D3, respectively. To adjust the fluorescence intensity between D4 and D3, the labelled ITS1F primer was diluted with the same amount of nonlabelled ITS1F primer. The PCR product (2 μ l) was digested in 10 μ l *Hinf*I solution (1061A, Takara Shuzo, Shiga, Japan) at 37°C for 8 h, and diluted twice with sterilized MQ water (Elix UV10, Millipore, Billerica, MA, USA). As another fragment, I used a PCR product that was amplified using the ITS3 and ITS4 primer set; ITS3 was labelled with D2 fluorescent dye (Proligo). The latter PCR product was diluted five times with sterilized MQ water. Then 1 μ l of both diluted solutions were mixed as a sample solution for terminal restriction fragment length polymorphism (T-RFLP) analysis (Dickie *et al.*, 2002b; Zhou & Hogetsu, 2002; Nara *et al.*, 2003b).

DNA fragments in sample solutions were purified and diluted in 25 μ l SLS solution (PN608082, Beckman Coulter, Fullerton, CA, USA) containing 0.25% of the 600-bp size standard mix (PN608095, Beckman Coulter). For T-RFLP, capillary gel electrophoresis was conducted in a sequencer (CEQ8800, Beckman Coulter) using the default setting for 600-bp fragment analysis. Three separate peaks, which represented D2-, D3-, and D4-binding fragments, were obtained from each sample in most cases. In some cases, a sample contained multiple peaks for a fluorescent dye because of contaminating soil fungi that appeared irrespective of morphotype. If the contaminating peaks were indistinguishable, all peaks of this dye were excluded from further analysis.

To identify the ECM fungal species, each T-RFLP pattern was compared with the patterns of sporocarps described in a previous study (Nara *et al.*, 2003b). If the T-RFLP patterns did not match any of the sporocarp species, they were grouped into T-RFLP types. T-RFLP patterns with all three fragments showing similar sizes within ± 2 bp were regarded as single T-RFLP types. Some samples showed no peaks in T-RFLP analysis because of unsuccessful PCR amplification. These samples were regarded as the same fungal species identified within the same morphotype of each root system or, rarely, within the same morphotype of different root systems.

Two different samples from each T-RFLP type were selected for sequencing. PCR products using nonlabelled ITS1F and ITS4 primers were cleaned and used for sequencing reaction with DTCS Quick Start Master Mix solution (PN608120, Beckman Coulter). Three sequencing primers (ITS1F, ITS3 and ITS4) were used individually for each sample. Sequence data obtained were combined for each sample using ATGC ver. 4.2 (Genetyx Co., Tokyo, Japan). The combined sequence data

were compared with the sequences of known species in the DDBJ/EMBL/GenBank database using BLAST.

In total, 7192 root tips were observed microscopically, 587 ECM tips were used for T-RFLP analyses, and 42 were sequenced.

Data analyses

The species richness of ECM fungi on each host species was estimated from the observed species counts using Chao2 and bootstrap estimators, using ESTIMATE S ver. 7.5 (Colwell, 2005; Mao & Colwell, 2005). To compare the number of observed species against species known to exist in this area, a species-accumulation curve was created using ESTIMATE S, where the mean of the expected number of species in pooled samples was plotted with 95% confidence ranges after 50 randomizations with replacement (Colwell *et al.*, 2004). Significant differences in richness among the three host species were tested using ANOVA. Simpson's and Shannon's indices were used to analyse the diversity of ECM fungi on each host species. Similarities in the ECM fungal communities on *Betula* and *Salix*, and on *Larix* and *Salix*, were evaluated using the Sørensen similarity index after abundance-based estimation for unobserved species using ESTIMATE S (Chao *et al.*, 2005).

Because the three host species examined in this study belong to three different families, ECM fungal species that appeared on two or three host species were regarded as generalists. ECM fungi shown to colonize *Salix* in previous studies were also included in determining host specificity (Nara *et al.*, 2003b). In contrast to generalists, ECM fungi that appeared on only one host species were separated into two groups, specialists and unknowns. The specialists comprised species the specific associations of which have been phylogenetically demonstrated in other studies (Molina *et al.*, 1992; Kretzer *et al.*, 1996; Bruns *et al.*, 2002; Grubisha *et al.*, 2002; den Bakker *et al.*, 2004). The remaining ECM fungi were included in the unknown category. Because the frequency of each ECM fungus in the unknown category was low, their occurrence on only one host species may be an effect of limitations in sample size (Horton & Bruns, 2001). To test for significant differences in ECM fungal composition (generalists vs specialists) among the three host species, the exact χ^2 test was performed using SPSS 11.5 (using the exact test option).

Results

Spatial pattern of timber species establishment

There were 509 vegetation patches >1 m in smallest diameter. The total area of vegetation patches (A_{total}) was 12 743 m², indicating that 6% of the ground was covered by vegetation. Of the 509 vegetation patches, 161 contained *Salix* (Fig. 1). The total area (A_{Salix}) covered by vegetation patches containing *Salix* was 8461 m².

In total, 39 individuals of *Betula* were found in 24 vegetation patches (Fig. 1). All these were accompanied by early-established *Salix* shrubs. To test for a significant effect of *Salix* presence on *Betula* establishment, I assumed the null hypothesis that *Betula* individuals had established evenly in all vegetation patches, but were detected only in *Salix*-containing patches by chance. The probability of this null hypothesis was as follows: $P_{Betula} = (A_{Salix}/A_{total})^N$, where N represents the number of *Betula* individuals. Note that A_{Salix} was used instead of the total area of *Salix* cover because *Salix* roots usually extend far from the above-ground cover, and sometimes spread through the whole area of a vegetation patch. Because $N = 39$ and $A_{Salix}/A_{total} = 0.664$, P_{Betula} was 0.00000012. Thus the null hypothesis was rejected ($P < 0.001$).

Twenty-six individuals of *Larix* were found in 20 vegetation patches that contained *Salix* (Fig. 1). The same probability analysis was conducted, and the null hypothesis for *Larix* was also rejected ($P_{Larix} = 0.000024$).

Another ECM timber species, *Pinus densiflora*, also appeared in the research area. Only two individuals were found, and both were accompanied by early-established *Salix*.

Salix that had recruited secondarily colonizing tree species ranged from 0.03 to 89 m² in coverage area. These *Salix* shrubs were substantially larger than the recruited secondary colonizer individuals in all cases, indicating the earlier establishment of *Salix*. *Salix* shrubs that were accompanied by secondary colonizers were significantly larger than those lacking secondary colonizers ($P = 0.006$, exact Mann–Whitney test). Vegetation patches that contained secondary colonizers were significantly larger than those containing no secondary colonizers (92.7 ± 11.5 vs 19.9 ± 1.5 m², respectively; $P < 0.001$, exact Mann–Whitney test).

ECM fungal communities

Molecular identification of ECM tips on 54 root systems found 36 fungal species from the three host species (Table 1; Appendix). The total species richness values estimated using Chao2 and bootstrap methods were 37.3 (36.2–44.9, 95% confidence range) and 39.9, respectively. These estimated values of species richness in the study area were close to from 36 (the actual

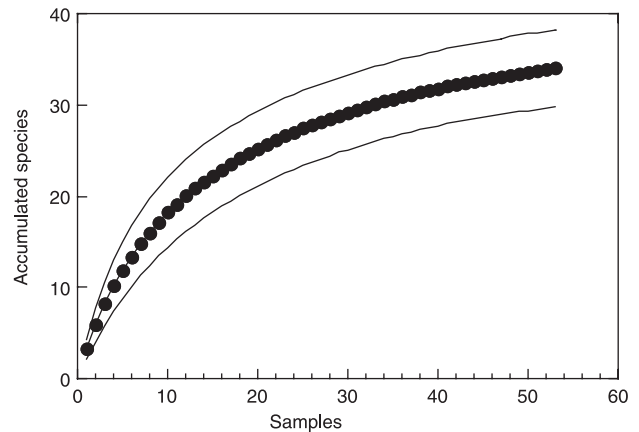


Fig. 2 Species-accumulation curve for ectomycorrhizal (ECM) fungi in the early successional volcanic desert on Mount Fuji, Japan. The mean of the accumulated number of expected species in pooled samples was plotted with 95% confidence ranges after 50 randomizations with replacement using ESTIMATE S ver. 7.5. Because most ECM fungi were shared among the three host species, all samples (root systems) of the three host species were combined.

number of species identified). The species-accumulation curve started to level off, indicating that most ECM fungal species were detected (Fig. 2). The species richness per root sample was not significantly different among host species ($P = 0.312$; Table 1).

Twenty-one ECM fungal species were found associated with *Salix* (Table 1). Most of these were also detected in a previous study (Nara *et al.*, 2003b), whereas four additional species were found in the present study (Table 2).

Betula was associated with 26 ECM fungal species (Table 1). Among these, *Laccaria amethystina* was the most abundant, occupying 29.4% of ECM tips, followed by *Tomentella* sp. 1 (20.2%) and *Laccaria murina* (11.1%; Table 2). *Laccaria amethystina* was also the most frequently observed species, appearing on nine of 19 root systems (Table 2). The ECM community of *Betula* was quite similar to that of early-established *Salix* (Sørensen similarity index = 0.798).

Twenty-three ECM fungal species were detected in *Larix* (Table 1). *Laccaria amethystina* was the most abundant (Table 2). Two *Larix*-specific fungi, *Suillus laricinus* and *Suillus grevillei*, followed *L. amethystina* in relative abundance

Table 1 Species richness (S) of ectomycorrhizal (ECM) fungi colonizing three host species during early primary succession on Mount Fuji, Japan

Hosts	ECM tips examined	Observed species	Species per sample*	Estimated S by Chao2†	Estimated S by bootstrap
<i>Betula ermanii</i>	2581	26	3.2 ± 0.3 ns	26.0	28.0
<i>Larix kaempferi</i>	2481	23	2.8 ± 0.3 ns	23.1	25.1
<i>Salix reinii</i>	1636	21	3.4 ± 0.2 ns	21.1	22.8
Total	6698	36	3.1 ± 0.2	37.3	39.9

*Number of ECM species detected in a root system shown as mean ± SE.

†Estimated values at the actual number of samples after 1000 randomization without replacement using ESTIMATE S ver. 7.5.

ns, Not significantly different among the three host species ($P = 0.312$, ANOVA).

Table 2 Relative abundance (RA) and frequency of ectomycorrhizal (ECM) fungi colonizing three host plants in the early successional volcanic desert on Mount Fuji, Japan

ECM fungal species*	<i>Betula ermanii</i>		<i>Larix kaempferi</i>		<i>Salix reinii</i>	
	RA (%)	Frequency (/19)	RA (%)	Frequency (/20)	RA (%)	Frequency (/16)
First-stage fungi						
<i>Inocybe lacera</i> (Fr.) Kumm.	0.0	0	3.5	1	2.7	2
<i>Laccaria amethystina</i> Cooke	29.4	9	27.2	9	22.8	9
<i>Laccaria laccata</i> (Scop. Fr.) Berk. & Br.	1.6	4	2.4	3	12.1	5
Second-stage fungi						
<i>Laccaria murina</i> Imai	11.1	4	5.0	3	9.1	5
<i>Scleroderma bovista</i> Fr.	0.4	1	0.0	0	0.4	1
Third-stage fungi						
<i>Boletus</i> cf. <i>rubellus</i> Krombh.	0.7	1	0.0	0	0.0	0
<i>Cenococcum geophilum</i>	0.0	0	0.6	2	0.0	0
<i>Cortinarius decipiens</i> (Pers. Fr.) Fr.	0.7	2	0.0	0	2.7	2
<i>Hebeloma leucosarx</i> Orton	0.0	0	4.5	3	1.7	2
<i>Hebeloma mesophaeum</i> (Pers.) Quél.	2.1	5	5.4	4	1.5	1
<i>Hebeloma pusillum</i> Lange	0.0	0	0.0	0	2.3	2
<i>Hebeloma</i> sp. 1	0.3	1	0.0	0	0.0	0
<i>Inocybe calospora</i> Quél.	2.5	2	1.0	1	2.3	1
<i>Inocybe dulcamara</i> (Pers.) Kumm.	1.4	2	0.0	0	4.1	1
<i>Inocybe</i> sp. 1	0.0	0	0.0	0	4.4	1
<i>Inocybe</i> sp. 2	1.0	2	2.4	4	2.0	2
<i>Laccaria</i> sp. 1	0.7	1	0.0	0	0.0	0
<i>Russula pectinatoides</i> Peck	0.2	1	2.0	2	0.0	0
<i>Russula sororia</i> (Fr.) Romell	2.3	2	4.2	3	0.0	0
<i>Sebacina</i> sp. 1	3.3	1	0.0	0	3.4	2
<i>Tomentella</i> sp. 1	20.2	6	1.6	3	2.4	3
<i>Tomentella</i> sp. 2	5.1	3	0.8	1	1.8	2
Unidentified D1	1.9	2	1.2	1	1.3	1
Later-stage fungi						
<i>Cortinarius</i> sp. 1	0.5	2	0.5	1	0.0	0
<i>Inocybe</i> sp. 3	0.0	0	2.0	1	0.0	0
<i>Leccinum</i> sp. 1	3.8	2	0.0	0	0.0	0
<i>Sebacina</i> sp. 2	4.6	1	0.0	0	0.0	0
<i>Suillus grevillei</i> (Klotzsch: Fr.) Sing.	0.0	0	8.7	2	0.0	0
<i>Suillus laricinus</i> (Berk.) O. Kuntze	1.1	1	16.1	5	0.0	0
Thelephoraceae sp. 1	0.0	0	3.3	2	0.0	0
Thelephoraceae sp. 2	1.2	1	5.1	1	6.1	1
<i>Tomentella</i> sp. 3	0.4	1	0.0	0	5.7	2
<i>Tomentella</i> sp. 4	2.5	2	1.8	1	0.0	0
Unidentified D2	0.0	0	0.0	0	8.1	2
Unidentified D3	0.0	0	0.6	1	3.3	1
Unidentified L1	1.0	1	0.2	1	0.0	0

The relative abundance of an ECM fungus at a sampling point was pooled and used to calculate RA at all sampling points for each host species to reduce the effect of differences in the number of ECM root tips between sampling points. Frequency, number of sampling points that contained each fungus in relation to the total number of sampling points (in parentheses) for each host species.

*ECM fungi colonizing the pioneer host plant species *Salix reinii* show a clear successional pattern with its growth. The sere starts with first-stage fungi, followed by second- and third-stage forms. Thus all fungi in these stages were confirmed to be compatible with *S. reinii*. See Nara *et al.* (2003a, 2003b) for details. ECM fungi that did not appear in the above stages were regarded as later-stage forms.

(Table 2). *Laccaria amethystina* was the most frequently observed species, occurring in nine of 20 root samples (Table 2). As in the case of *Betula*, the ECM community of *Larix* showed high similarity to that of *Salix* (Sørensen similarity index = 0.646).

The majority of ECM fungal species on each host were generalists that were detected in least two plant families: 24 of 26, 20 of 23 and 18 of 21 fungal species in *Betula*, *Larix* and

Salix, respectively (Table 2). The relative abundance of generalists in the total ECM tips reached 92, 70 and 85% for *Betula*, *Larix* and *Salix*, respectively (Fig. 3). In contrast, specialists were limited to one *Leccinum* and two *Suillus* species on *Betula* and *Larix*, respectively (Table 2). The relative abundance of specialists was significantly greater for *Larix* than for the other host species ($P < 0.001$; Fig. 3).

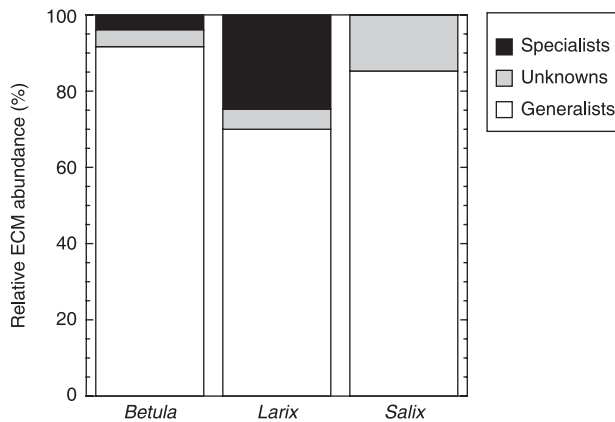


Fig. 3 The relative abundance of generalist and specialist ectomycorrhizal (ECM) fungi colonizing three host plant species during early primary succession on Mount Fuji, Japan. Generalists were ECM fungi that were compatible with two or more host plant species belonging to different families. ECM fungi observed on only one host plant species were classified as specialists or unknowns. See text for details.

Suillus laricinus was unexpectedly detected from a root sample of *Betula* that was accompanied by a *Larix* sapling. However, this fungus was regarded as *Larix*-specific (see Discussion). This is the first example in which a *Larix*-associated *Suillus* species was found to colonize a neighboring 'nonhost' species.

Shannon's diversity indices for ECM fungi on *Betula*, *Larix* and *Salix* were 2.41, 2.53 and 2.66, respectively. Simpson's diversity indices on *Betula*, *Larix* and *Salix* were 6.65, 8.05 and 10.11, respectively. Although the variation in estimates of diversity was not as great when using Shannon's index, both indices suggest that ECM fungal diversity was highest on early-established *Salix* (on which species richness was lowest) because of higher evenness in individual abundances of ECM fungi.

Discussion

ECM fungal community on secondarily colonizing timber species

In the sere of vegetation succession in the volcanic desert on Mount Fuji, the establishment of *Betula* and *Larix* is very important as an initial stage of forest formation. In a previous study, ECM fungi detected on transplanted current-year seedlings of both tree species were common to those on early-established *Salix* at this site (Nara & Hogetsu, 2004). Here, I found that ECM fungi on naturally established saplings of *Betula* and *Larix*, mostly >10 yr old, were still dominated by ECM fungi common to *Salix*. This indicates that other ECM fungal species that are associated preferentially with secondary colonizers are not dominant fungi, even a decade after their establishment.

ECM associations of current-year seedlings in this volcanic desert are mainly accomplished via extramatrical mycelia that radiate from established hosts (Nara & Hogetsu, 2004; Nara, 2006). The ECM fungal communities on naturally established saplings of the timber species were quite similar to that on *Salix*. This indicates that these timber species may have been connected to *Salix* shrubs by common mycelia of the same ECM fungus, called common mycorrhizal networks (CMNs: Newman, 1988; Simard & Durall, 2004). Because *L. amethystina* was the most abundant and frequent species on each plant species, CMNs of this fungus would have the highest possibility of occurrence. In a previous study, the effects of CMNs on experimentally connected *Salix* seedlings varied significantly among ECM fungal species (Nara, 2006). Interestingly, *L. amethystina* showed no positive effects on *Salix* seedlings via the CMN, whereas the other 10 ECM fungi improved the N absorption and growth of connected seedlings to varying extents (Nara, 2006). Whereas nutrients absorbed by ECM fungi are shared among different host species in a CMN, there is preferential transport to highly compatible hosts, rather than to less compatible hosts (Finlay, 1989). If *L. amethystina* is physiologically more compatible with *Betula* and *Larix* than *Salix*, the CMNs of this fungus would be favorable for *Betula* and *Larix*.

Host ranges of ECM fungal species during primary succession

Observations of sporocarp–host associations in the field have been used repeatedly to evaluate the ecological specificity and host ranges of a variety of ECM fungi (Molina *et al.*, 1992 and references therein). These studies have accumulated a large amount of information on sporocarp–host specificity; however, an association at one site does not necessarily indicate the same association at other sites because field conditions may differ (Molina *et al.*, 1992). To consider the practical effects of ECM fungi on ecological processes, ECM specificity and compatibility should be examined at each site (Harley & Smith, 1983; Molina *et al.*, 1992). A sporocarp approach is unsuitable in most sites for various reasons, including limited sporocarp production; co-occurrence of multiple host species in close vicinity; or great disparity between sporocarp and underground ECM communities. Instead of the sporocarp approach, a molecular approach is now available to confirm ECM colonization on individual hosts species under natural settings (Gardes & Bruns, 1993; Horton & Bruns, 2001). This enables studies of the specificity of ECM fungi between co-occurring tree species belonging to the same family (Horton & Bruns, 1998; Cullings *et al.*, 2000), and an evaluation of host ranges by comparing ECM fungi on taxonomically distant hosts (Horton *et al.*, 1999; Kennedy *et al.*, 2003; Richard *et al.*, 2005).

Generalists are usually defined as ECM fungi that are compatible with various plant families. I defined generalists as ECM fungi that were compatible with at least two of the three

plant families examined (Betulaceae, *Betula*; Pinaceae, *Larix*; Salicaceae, *Salix*). In contrast to this robust definition of generalists, the definition of specialists varies among studies, and the demonstration of specificity is usually difficult. This is partly because specific ECM associations include various levels of host specificity (species, genus or family; Molina *et al.*, 1992). Moreover, it is difficult to determine whether a specific occurrence is caused by real specificity or insufficient sample sizes, because ECM communities are usually composed of many rare species detected in only one or a few samples (Horton & Bruns, 2001; Richard *et al.*, 2005). Molecular phylogenetic studies of some fungal genera have demonstrated that specific ECM associations are common on related taxa within a genus that have evolved from a common ancestor (Kretzer *et al.*, 1996; Grubisha *et al.*, 2002; den Bakker *et al.*, 2004). These studies would be valuable for the definition of specialists.

Although the host ranges of dominant ECM fungi after severe disturbance have not been well studied, specialists (including *Rhizopogon* species) often dominate the postdisturbance ECM community (Horton & Bruns, 1998; Baar *et al.*, 1999; Bruns *et al.*, 2002). *Rhizopogon* and phylogenetically related genera such as *Suillus* show high specificity to the Pinaceae (Molina & Trappe, 1994; Kretzer *et al.*, 1996; Bruns *et al.*, 2002) and adapt well to disturbances by forming dormant spore banks (Baar *et al.*, 1999). The persistence of dormant spores of other ECM genera has not been demonstrated clearly. Thus the dominance of specialists may be restricted to areas where the Pinaceae can dominate following disturbance. Although the effect of specialist dominance on tree succession remains unknown, it should prevent the invasion of incompatible plant species. Therefore, *Pinus*–*Rhizopogon* relationships may allow both partners to continue to prosper in repeatedly disturbed sites.

In the volcanic desert on Mount Fuji, however, generalists clearly dominated the ECM community. This is completely different from specialist-dominated ECM communities after disturbance. Because there is no dormant spore bank in primary successional settings, the specialist–generalist patterns of ECM fungi may differ fundamentally between primary and secondary succession. In addition, the host-range patterns may vary with pioneer host species, where conifers are associated with specialists and broad-leaved species are associated with generalists. Although little is known about the effects of generalist dominance on succession, this work shows that the dominance of generalists contributes to ECM associations in secondary colonizing *Betula* and *Larix*, and possibly in late-successional tree species such as *Fagus*, *Quercus*, *Abies* and *Tsuga*.

The presence of a few specialists was also confirmed: *Leccinum* sp. 1 was found on *Betula*, and two *Suillus* spp. were found on *Larix*. *Suillus laricinus*, a notable *Larix*-specific fungus, was also detected from a root system of *Betula* that was associated with a *Larix* tree. Under suitable experimental conditions, especially if exogenous sugars are abundant, ECM fungi can colonize ecologically nonhost plants (Finlay, 1989; Molina

et al., 1992), although such ecologically incompatible associations are not fully functional (Finlay, 1989). Thus the mycelia of *S. laricinus* may infect *Betula* roots because of carbon support from the neighboring *Larix*. In accordance with other studies, I defined this fungus as a specialist because its occurrence was significantly biased to *Larix* ($P < 0.001$, exact χ^2 test where *S. laricinus* ECM root tips in *Betula* and *Salix* were pooled and compared with those in *Larix*), and phylogenetic studies of related taxa support its specificity (Kretzer *et al.*, 1996; Bruns *et al.*, 2002; Grubisha *et al.*, 2002). Consequently, specialists (two *Suillus* species) represented 25% of the relative abundance of ECM root tips in *Larix*. Because these specialists were not shared with early-established *Salix*, *Larix* could receive exclusive benefits from these fungi. Moreover, specialists may transfer more N to hosts than generalists (Hobbie *et al.*, 2005). Therefore, the relative contribution of specialists to timber establishment may not be proportional to their relative abundance in ECM communities.

Contribution of ECM fungi to tree succession

Under secondary successional settings, remaining host plants sometimes facilitate subsequent recolonization of ECM trees (Perry *et al.*, 1987). This facilitation can be derived from many biotic and abiotic factors (Callaway & Walker, 1997). ECM fungal symbionts may contribute partly to establishment because ECM fungal communities on seedlings near the remaining hosts are different from those on distant seedlings (Horton *et al.*, 1999; Kranabetter, 1999; Dickie *et al.*, 2002a; Ashkannejhad & Horton, 2006). Many remote seedlings, however, are usually colonized by some ECM fungi, irrespective of the early-colonizing hosts in these studies. Thus the facilitated seedling establishment cannot easily be attributed to the effect of ECM fungi unless different ECM communities are experimentally shown to have different effects at each site.

In contrast to these secondary successional sites, ECM colonization itself is nearly impossible for current-year seedlings of host species in the primary successional volcanic desert on Mount Fuji, unless seedlings are accompanied by early-established *Salix* (Nara & Hogetsu, 2004). Furthermore, facilitated seedling establishment of *Salix* was solely attributable to ECM colonization in a field inoculation experiment in which all abiotic and biotic conditions were uniform, except for ECM fungi (Nara, 2006). However, the ECM contribution to vegetation succession was uncertain because established *Salix* did not improve the performance (aside from ECM colonization) of *Betula* and *Larix* seedlings in a transplant experiment (Nara & Hogetsu, 2004). Here, I found only 39 and 26 established individuals of *Betula* and *Larix*, respectively, in the 21 ha of the study area approx. 300 yr after the last volcanic eruption. Although the establishment of *Betula* and *Larix* appears to have accelerated in recent years, the natural establishment of both timber species appears to be too episodic to be studied using experimental approaches.

The natural establishment of *Betula* and *Larix* coincided spatially with *Salix*, without exception. What is the most likely mechanism that explains the observed establishment pattern? On average, secondary colonizers occurred more often in association with large *Salix* shrubs and in large vegetation patches. Because soil nutrient availability and organic matter content are correlated with patch development in this volcanic desert (Hirose & Tateno, 1984), soil development may be attributable partly to the observed establishment patterns. However, a very small *Salix* patch (0.03 m²) within a small patch (8.4 m²) was confirmed to have recruited a *Larix* seedling (1 yr old) where the soil had developed poorly. Thus soil development alone may not be a requisite for the establishment of timber species. Because both *Betula* and *Larix* depend obligately on ECM fungi, these timber species must be colonized by some ectomycorrhizal fungi during early establishment. Therefore, compatible and accessible ECM fungi provided by early-established *Salix* would be an important mechanism that potentially could explain the observed pattern of timber establishment.

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Appendix

Table A1 Ectomycorrhizal fungi identified using ITS-T-RFLP and ITS sequence analyses

Fungal species	ITS terminal fragment length			Accession no.†	Closest match*		
	ITS1F– <i>Hinfl</i>	<i>Hinfl</i> –ITS4	ITS3–4		Accession no.	Species	e
<i>Boletus cf. rubellus</i>	272–276	157	682–683				
<i>Cenococcum geophilum</i>	–	–	334–335	<i>AB211277</i>			
<i>Cortinarius decipiens</i>	324.0	307–308	376				
<i>Cortinarius</i> sp. 1	324–325	314–315	382–384	AB244040	AJ534712	<i>Cortinarius</i> sp.	e-134
<i>Hebeloma leucosarx</i>	398	336–337	408–409	AB211268			
<i>Hebeloma mesophaeum</i>	401–402	334–338	404–409	AB211272			
<i>Hebeloma pusillum</i>	399–400	334–335	405–406	AB211274			
<i>Hebeloma</i> sp. 1	397–398	329–331	401–402				
<i>Inocybe calospora</i>	370–371	311–313	378–381				
<i>Inocybe dulcamara</i>	428–429	337	407–408				
<i>Inocybe lacera</i>	387–390	331–333	400–404	AB211269			
<i>Inocybe</i> sp. 1	160–161	304–305	371–372				
<i>Inocybe</i> sp. 2	402–403	318	382–383				
<i>Inocybe</i> sp. 3	382	175–176	376	AB244041	AY751558	<i>Inocybe</i> sp.	7e-95
<i>Laccaria amethystina</i>	387–388	335–339	406–412	AB211270			
<i>Laccaria laccata</i>	391–392	329	398	AB211273			
<i>Laccaria murina</i>	388–390	328–329	399–400	AB211271			
<i>Laccaria</i> sp. 1	352	313	385	AB244042	AF204814	<i>Laccaria laccata</i>	0.0
<i>Leccinum scabrum</i>	160	420	489–492	AB244043	AY538849	<i>Leccinum scabrum</i>	0.0
<i>Russula pectinatoides</i>	373	233–234	413–415	AB211276			
<i>Russula sororia</i>	374–375	347	419	AB211275			
<i>Scleroderma bovista</i>	283–285	247–248	428–433	AB211267			
<i>Sebacina</i> sp. 1	323–324	321	389–391	AB244044	AY296254	Sebacinaceae sp.	0.0
<i>Sebacina</i> sp. 2	162	327	396–398	AB244045	AY112923	Sebacinaceae sp.	4e-52
<i>Suillus grevillei</i>	320–321	217–218	423	AB244046	M91616	<i>Suillus grevillei</i>	0.0
<i>Suillus laricinus</i>	225	197	419–421	AB244047	L54102	<i>Suillus laricinus</i>	0.0
Thelephoraceae sp. 1	362–363	202–203	411–414	AB089959			e-150
Thelephoraceae sp. 2	360–361	337	402	AB244048	AF184744	Thelephoraceae sp.	
<i>Tomentella</i> sp. 1	358–361	198–200	407–413	AB089960	U83480	<i>Tomentella ramosissima</i>	e-162
<i>Tomentella</i> sp. 2	358–361	200	404	AB244049	AF272913	<i>Tomentella ellisi</i>	e-102
<i>Tomentella</i> sp. 3	360	335–336	403–405	AB244050	U83482	<i>Tomentella</i> sp.	e-179
<i>Tomentella</i> sp. 4	357–359	343–345	412–414	AB244051	U83482	<i>Tomentella</i> sp.	0.0
UD-1, Sordariaceae?	177	278–279	343	AB244052	–		
UD-2, Sordariaceae?	229	279	342–343	AB244053	–		
UD-3	391–392	172–173	328				
UL-1	328	258	398				

*If ITS-T-RFLP patterns did not match any sporocarp sample (Nara *et al.*, 2003a) or any species in my T-RFLP database (Nara *et al.*, 2003b), the sequence data were compared with sequence data of known fungal species in DDBJ/EMBL/GenBank. The accession number, species name and e value of the closest match in BLAST search are shown for each unknown T-RFLP type. Sequences were also studied to confirm the correctness of T-RFLP analyses.

†Accession numbers in italics are from our previous studies (Nara *et al.*, 2003b; Nara, 2006).



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