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Mycobionts of *Salix herbacea* on a glacier forefront in the Austrian Alps

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Abstract Dwarf willows (e.g. *Salix herbacea*) are among the earliest ectomycorrhizal (EM) plants colonising primary successional sites such as glacier forefronts in the Tyrolean Alps. EM of *S. herbacea* were sampled at the Rotmoos glacier forefront (Ötz Valley, Austria) three times a year during the growing season and once a year during winter when plants were covered with snow in 2005 and 2006. EM were investigated using morphological methods and by sequencing the rDNA ITS region. The degree of EM mycorrhization was high throughout both years (93%). We distinguished 21 EM morphotypes and identified 19 fungal species. *Cenococcum geophilum*, *Sebacina* spp., *Tomentella* spp. and *Cortinarius* spp. dominated the mycobiont community of *S. herbacea*. The observed species richness in this about 150-year-old soil was at least 59% of the estimated species richness. Fungal communities differed significantly between consecutive years, and spatial heterogeneity was high. These differences made it difficult to detect seasonal impacts. Abundances of *C. geophilum* EM increased throughout the 2-year sampling period. *Sebacina incrustans* EM were very abundant in 2005, but nearly disappeared in 2006, whilst its fruitbodies were still frequent in the sampling area. This suggests that the mycorrhizae were displaced from the roots by an outcompeting species, whereas the mycelium was still present in the soil.

Keywords *Salix herbacea* · Ectomycorrhizae · Seasonal aspects · Spatial variation · Glacier forefront · Primary succession

Introduction

Fundamental knowledge about the diversity of soil fungal communities and their functional impact, especially for ectomycorrhizal (EM) fungi in alpine habitats, is still scarce. However, this belowground fungal diversity is essential for the establishment and maintenance of EM plant communities.

Glacial forefronts are not only fascinating and interesting habitats, but can also be regarded as model systems for primary succession (Jumpponen et al. 2002; Cázares et al. 2005). Primary successional ecosystems are young, and settling of plants and fungi was enabled within comparatively short, usually well-known time spans. Diversity of plant and fungal communities is low, and the supply of fungal EM inoculum is limited in e.g. recently deglaciated alpine areas (Jumpponen et al. 2002; Cázares et al. 2005) allowing more thorough and exhaustive investigations.

Dwarf willow species, such as *Salix herbacea* L. (snowbed willow), are important EM host plants in Northern hemisphere, circumpolar, arctic and alpine habitats. In the Alps, this mat-forming shrub can be found at altitudes of up to 3,300 m, whilst it grows at sea level in the Arctic. The EM fungal communities of dwarf willows (e.g. *S. herbacea*, *S. retusa*, *S. arctica*) occurring in arctic or alpine environments have been studied using various methods. Fruitbody sampling and identification have been often used for the assessment of fungal diversity of various plant species: Senn-Irlet (1993) reported 30 ectomycorrhizal species occurring in alpine mire communities rich in *Salix*, and Graf

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(1994) reported 55 species suspected to form EM associations with *S. herbacea* growing in snowbeds in the Swiss Alps; Cripps (2002) detected 60 species of EM fungi occurring with *Salix* spp. on an alpine vegetation zone in the Rocky Mountains (USA); Jumpponen et al. (2002) found 13 fungal EM species occurring with shrub willows on the Lyman glacier forefront in the northern Cascade mountains of Washington (USA).

Data concerning the mycorrhizal status of dwarf willows have been reported in several studies (e.g. Haselwandter and Read 1980; Read and Haselwandter 1981; Gardes et al. 2000; Graf and Brunner 1996; Treu et al. 1996; Cázares et al. 2005) but none of them has focussed on the ectomycorrhizal fungal community. To our knowledge, this is the first study to examine the EM fungal community of *S. herbacea* in a glacier forefront. We selected an approximately 150-year-old moraine at the Rotmoosferner (Ötz Valley, Tyrol, Austria) as sampling area. We investigated the EM status of *S. herbacea* using both morphological and molecular methods that are recommended generally for EM community analyses (Sakakibara et al. 2002; Harrington and Mitchell 2005a, b; Clemmensen and Michelsen 2006; Tedersoo et al. 2006; Obase et al. 2007), and especially important for EM host plants growing in arctic/alpine habitats (Trowbridge and Jumpponen 2004; Mühlmann et al. 2008). Our specific objectives were to (1) quantify the degree of EM colonisation of *S. herbacea*, (2) estimate EM fungal diversity of *S. herbacea* growing at this 150-year-old alpine primary successional site based on DNA molecular analysis of morphotypes and (3) identify dominant fungi colonising dwarf willows at this site.

Because the seasonal dynamics of EM fungi are poorly understood, we also examined the seasonal and developmental dynamics of EM fungi on the roots of *S. herbacea* over four seasons in 2005 and 2006, including sampling dates when plants were under snow cover.

Materials and methods

Study site

Sampling was performed on the Rotmoos glacier forefront, a primary successional site in the Tyrolean Alps (Ötz Valley, Austria). This glacier forefront in the Rotmoos valley lies 2,280–2,450 masl. The sampling area of about 100×200 m was chosen at the moraine of 1858 (46°50′ N, 11°01′ E), thus approximately 150 years without ice cover. Plant cover varied between 50% and 90%, and the vegetation at this site was described in detail by Erschbamer et al. (1999). Briefly, the plant community is considered a grassland (Raffl and Erschbamer 2004; Raffl et al. 2006) with the following ectomycorrhizal plants: *S. herbacea*, *S. retusa*, *Kobresia myosuroides* and *Polygonum viviparum*. Soil composition is very heterogeneous due to dependence on microhabitats; the content of organic matter is about 7% (Erschbamer et al. 1999).

Sampling

Each sampling year, five sampling plots (S1 to S5, 1×1 m each) were selected at the study site where *S. herbacea* occurred as one of the dominant plants. *S. retusa* had a rare, scattered distribution at the study site, therefore, we cannot exclude that roots of this dwarf willow were occasionally sampled together with *S. herbacea* roots. From each sampling plot, five samples under and at the margin of the *Salix* plant cover were excavated with a small scoop including their roots and surrounding soil. The resulting holes measured approximately 5×5×5 cm. Samples were stored in the original soil at 4°C until further treatment for no longer than 2 weeks. Sampling was conducted four times a year in 2005 and 2006: spring samples were taken 2 weeks after snow melt (15 June 2005 and 27 June 2006), summer samples were taken in August (4 August 2005 and 22 August 2006), fall samples were taken on 15 September 2005 and 3 October 2006 and winter samples were taken under an approximately 40 cm high snow cover (14 December 2005 and 12 December 2006).

To obtain reference sequences for species-level identification of mycorrhizal fungi, we harvested all occurring fruitbodies in the study site during 2005 and 2006. Voucher material was deposited in the Herbarium IB (University of Innsbruck). Furthermore, earlier IB voucher material from this study area was investigated.

Environmental data

Soil temperature and relative values of moisture data were kindly provided by Dr. Rüdiger Kaufmann (University of Innsbruck, Institute of Ecology). Data loggers were buried at a soil depth of 10 cm at the study site. The mean values 2 weeks before each sampling date were calculated (Table 1).

Table 1 Mean temperature (T; °C) and moisture (relative values) 2 weeks before the samplings spring, summer, fall and winter in 2005 and 2006

	T (°C)		Moisture	
	2005	2006	2005	2006
Spring	9.2*	13.2*	0.538	0.525
Summer	12.4*	8.7*	0.537*	0.476*
Fall	10.3	8.3	0.555*	0.447*
Winter	−0.6	−0.3	0.315	0.388

Values differing significantly between the years are marked with an asterisk.

Sample processing

After careful washing, fine roots were separated from *Salix* main roots. For each of the five sampling plots, 100 mycorrhizal root tips were randomly selected yielding a total of 500 root tips from each sampling date. Root tips were examined at 10- to 100-fold magnification and sorted into morphotypes (MTs) based on colour, emanating elements, mantle layer, and hyphal anatomy (Agerer 1991; Supplementary Table 1 of the Electronic Supplementary Material).

At least three representatives of each MT were transferred into CTAB buffer and stored at −20°C until later molecular investigations. At least three samples of each MT were analysed. If only one sequence pattern (operational taxonomical unit [OTU]) was obtained, the MT was considered as this OTU. Otherwise, additional root tips of this MT were sequenced. The most abundant OTU was then regarded as the mycorrhizal partner.

Molecular methods

DNA was extracted from single root tips according to Southworth (2000). PCR was performed as described by Mühlmann et al. (2008) using the primer combinations ITS1F x LR15 or ITS1F x NL4 (O'Donnell 1993; Gardes and Bruns 1993; Vilgalys 2005). DNA isolation and sequencing protocols for fruitbody processing followed those of a previous study (Peintner et al. 2001). Primers used for PCR amplification and sequencing were ITS1 combined with LR15 or NL4 (White et al. 1990; O'Donnell 1993; Vilgalys 2005). Purified PCR products were sequenced by MWG AG Biotech (Germany) or Genecust (Custom Services for Research, France) with the primer ITS1. Sequences were analysed using the Sequencher software (version 4.6; Gene Codes, Ann Arbor, MI, USA).

Sequence analyses

BLAST searches were carried out against the public sequence databases National Center for Biotechnology Information (NCBI) and UNITE (Kõljalg et al. 2005). OTUs were defined as sequences with at least 97% similarity and regarded as belonging to one species.

Furthermore, sequences of the EM were compared to those generated from reference fruitbodies found in the study site.

Statistical analyses

Statistical analyses were carried out with relative abundances of MTs. We calculated the abundance of MTs (including senescent and non-mycorrhizal root tips) as the percentage of investigated root tips per plot. Frequency of MTs was calculated as the percentage of plots where the individual MT was detected. We divided the MTs into main MTs and rare MTs: main MTs were detected in ≥10% of all samples and colonised ≥5% of all investigated root tips, whereas rare MTs occurred in <10% of all samples and showed abundances of <5% of all root tips.

To analyse seasonal and spatial variation of the EM fungal communities associated with *Salix* spp., we calculated richness (*S*) for total number of species, evenness (*E*) for their distribution and Simpson and Shannon's diversity indices according to McCune and Grace (2002) with PC-ORD Version 5.0 (McCune and Mefford 1999).

Analysis of variance (ANOVA) was performed with the SigmaStat Software (SigmaStat for Windows 3.5, 2006, Systat Software) using several datasets: the whole dataset including data of both sampling years and reduced datasets using data for each year, separately: As the dependant variable, we used either Shannon and Simpson's diversity indices or relative abundances of the most frequent MTs (MT 14, MT 11, MT 3, MT 6, MT 8 and senescent root tips) and the pooled abundances of the remaining MTs. After passing the normality test, one-way ANOVA was calculated with either "season" or "plot" as independent variable. If normality test failed, Kruskal–Wallis one-way ANOVA on ranks was used for the detection of significant differences between the groups. The pairwise multiple comparison procedures (Tukey test) were applied to detect groups differing significantly from the others ($p>0.050$).

To minimise underestimates of species richness (because of the sampling methodology itself), the true richness was estimated with four methods for the data from both years and all seasons combined: (1) abundance-based coverage estimator of species richness (ACE), which relies on the abundances of rare species for estimation of the true species richness; (2) incidence-based coverage estimator of species richness (ICE), which is based on the presence or absence of data; (3) Chao estimators (Chao1 and Chao2), which use common species and singletons and doubletons to estimate the number of missing species and (4) the second-order jackknife richness estimator (Jack2), which is very sensitive to the number of rare species and can perform poorly with a small sample size. Samples were randomised without replacement. All estimations were calculated in EstimateS 8.0 (Colwell 2006).

Results

Of the 4,000 investigated *S. herbacea* root tips, 93% were colonised by EM fungi. Altogether, 21 EM MTs were detected and characterised by well-developed mantle layers (Supplementary Table S1 of the Electronic Supplementary Material).

MT 14 was identified as *Cenococcum geophilum* based on morphological characters. Ten other MTs could be assigned to fungal species or genera based on rDNA ITS sequence homology of at least 97% to reference sequences from public databases (Table 2) or from reference material (fungal fruitbodies collected at the study site; Table 3). Four of these MTs were formed by several species of one genus each: five species of *Cortinarius* formed MT 8, four species of *Tomentella* formed MT 3 and two species of *Inocybe* formed MT 12 and MT 16.

The remaining ten MTs could not be unambiguously identified: based on BLAST searches, three MTs were related to Xylariaceae, Helotiales and *Tetracladium*, but it is doubtful that they are mycobionts of *Salix* sp. Six other vital MTs remained unidentified because amplification or sequencing of these comparatively rare MTs failed. Molecular analyses of MT 2 resulted in controversial or often heterogeneous sequences. This MT was "unhealthy looking", characterised by a gaunt surface. Therefore, we defined MT 2 as degenerating or senescent EM root tips.

Thus, 19 fungal species belonging to the genera *Cadophora*, *Cortinarius*, *Inocybe*, *Sebacina*, *Tarzetta*, *Thelephora* and *Tomentella* were unambiguously identified as ectomycorrhizal partners of dwarf willows (Table 2). Basidiomycetes dominated the species composition of *S. herbacea* mycobionts. Species richness was most pronounced in the genera *Cortinarius*, *Inocybe* and *Tomentella* (including *Thelephora*) with five species each. *Sebacina* was represented by two species. Ascomycetes were represented by three taxa, one species each of the genera *Cenococcum*, *Cadophora* and *Tarzetta*, but the latter was identified based on a sequence homology of 92% only.

Estimates of actual species richness (based on MTs) ranged between the observed 22 MTs (including senescent root rips) and a maximum of 38 MTs as estimated by Jack2 (ACE=22, ICE=33.43, Chao1=22, Chao2=30.78, Jack2=37.55) (compare Fig. 1).

Frequencies and abundances of MTs

Five main MTs and 16 rare MTs were detected in all samples (Supplementary Table S2 of the Electronic Supplementary Material). The five main MTs (MT 14 *C. geophilum*, MT 11 *Sebacina* sp. 1, MT 3 *Tomentella* spp., MT 6 *Sebacina incrustans* and MT 8 *Cortinarius* spp.) dominated root tip colonisation (Table 4), representing between 72.7% of the mycorrhizal root tips in 2005 and 91.2% in 2006. *C. geophilum* occurred in 45% (2005) and 85% (2006) of the samples and colonised 5.1% (2005) and 39.3% (2006) of all root tips. *Sebacina* sp. 1 was present in

Table 2 Ectomycorrhizal mycobionts of *S. herbacea* detected at a primary successional site

Root tip ID	GenBank Accession No.	Length	Reference	Score	Identities (%)	Name
981	EU326169	982	DQ233900	1,507	98	*Cadophora* sp.
1049	EU326171	679	AY669664	1,324	99	*Cortinarius* sp. 1
1050	EU326172	697	DQ102673	1,368	99	*Cortinarius* sp. 2
1045	EU326170	687	AY669673	1,255	98	*Cortinarius* sp. 3
1124	EU326176	340	UDB002176	664	99	*Cortinarius* sp. 4
113	EU326159	431	EF434114	841	99	*Cortinarius* sp. 5
1061	EU326174	638	AM181392	888	99	Helotiales sp.[b]
1051	EU326173	679	EU326177		100[a]	*Inocybe johannae*
254	EU326165	280	EU326178		100[a]	*Inocybe ochroalba*
82	EU326156	598	EF655704		100[a]	*Inocybe rufofusca*
97	EU326157	717	EF655703		100[a]	*Inocybe substraminipes*
140	EU326161	683	AY310819	426	89	*Inocybe* sp. 1
78	EU326155	594	EF655701		99[a]	*Sebacina incrustans*
947	EU326168	689	DQ974767	1,130	96	*Sebacina* sp. 1
715	EU326167	488	DQ974820	383	92	*Tarzetta* sp.
295	EU326166	634	EF434086	1,051	96	*Tetracladium* sp.[b]
105	EU326158	642	EF655705		100[a]	*Thelephora caryophyllea*
135	EU326160	207	EF218826	370	97	*Tomentella* sp. 1
154	EU326162	259	EF655702		100[a]	*Tomentella* sp. 2
241	EU326163	756	AJ893339	1,411	99	*Tomentella* sp. 3
1064	EU326175	753	AF430289	1,065	93	*Tomentella* sp. 4
243	EU326164	646	AF284130	858	98	Xylariaceae sp.[b]

Root tip ID, GenBank accession number of rDNA ITS sequences, sequence length, best BLAST match (reference) and score, identities (%) and identification on genus, family or order level are shown.
[a] Samples identified based on reference fruitbody material.
[b] Doubtful results.

Table 3 Species list of fruitbodies collected at the Rotmoos glacier forefront used as reference material for the identification of *S. herbacea* mycorrhizae, including species names, voucher numbers, GenBank accession numbers and sequence length

Species	Voucher No.	GenBank Accession No.	Sequence length
Inocybe johannae	IB20050451	EU326177	565
Inocybe ochroalba	IB20050452	EU326178	651
Inocybe substraminipes	IB20050457	EF655703	675
Inocybe rufofusca	IB20040114	EF655703	629
Sebacina incrustans	IB20060213	EF655701	942
Tomentella sp. 2	IB20060231	EF655702	910
Thelephora caryophyllea	IB20060087	EF655705	508

45% (2005) and 70% (2006) of the samples and was found on 15.1% (2005) and 19.0% (2006) of all root tips. *Tomentella* spp. were found in 80% (2005) and 85% (2006) of all samples and accounted for 12.1% (2005) and 21.5% (2006) of the root tips. *S. incrustans* was found in 95% (2005) and 25% (2006) of the samples and accounted for 27.5% (2005) and 3.2% (2006) of the root tips. *Cortinarius* spp. was found in 65% (2005) and 55% (2006) of the samples and showed abundances of 13.1% (2005) and 8.4% (2006).

The 16 rare MTs occurred irregularly at most in 15% of the samples a year with abundances ≤2.0% in a year (compare Supplementary Table S2 of the Electronic Supplementary Material). In addition to these 21 MTs, senescent root tips were detected with a frequency of 80% (2005) and 10% (2006) and accounted for 8.4% (2005) and 1.9% (2006) of the analysed root tips (Table 4). Non-mycorrhizal root rips were observed with frequencies of 80% (2005) and 45% (2006) and with abundances of 9.6% (2005) and 4.7% (2006).

Differences of species diversity and abundances between sampling years

Species richness varied in seasons between 8 and 12 species (2005) and 4 and 9 (2006) species, and in plots between 6 and 15 species (2005) and 4 and 7 (2006) species.

Richness and diversity indices (richness, evenness, Shannon diversity index and Simpson's diversity index) of both sampling years for *S. herbacea* mycorrhizal morphotypes in each season (plot pooled) and each plot (seasons pooled) are given in Table 5. Simpson's diversity index and evenness were highest in spring and lowest in winter (both years) when rare MTs were comparatively infrequent. Shannon and Simpson's diversity indices were significantly different between the two sampling years (Shannon: p=0.005; Simpson: p=0.017). For each year separately, statistically significant differences between seasons could be found only in 2006: between spring and winter for both indices ($p \leq 0.015$) and between summer and winter for Shannon (p=0.024). Differences between plots were found

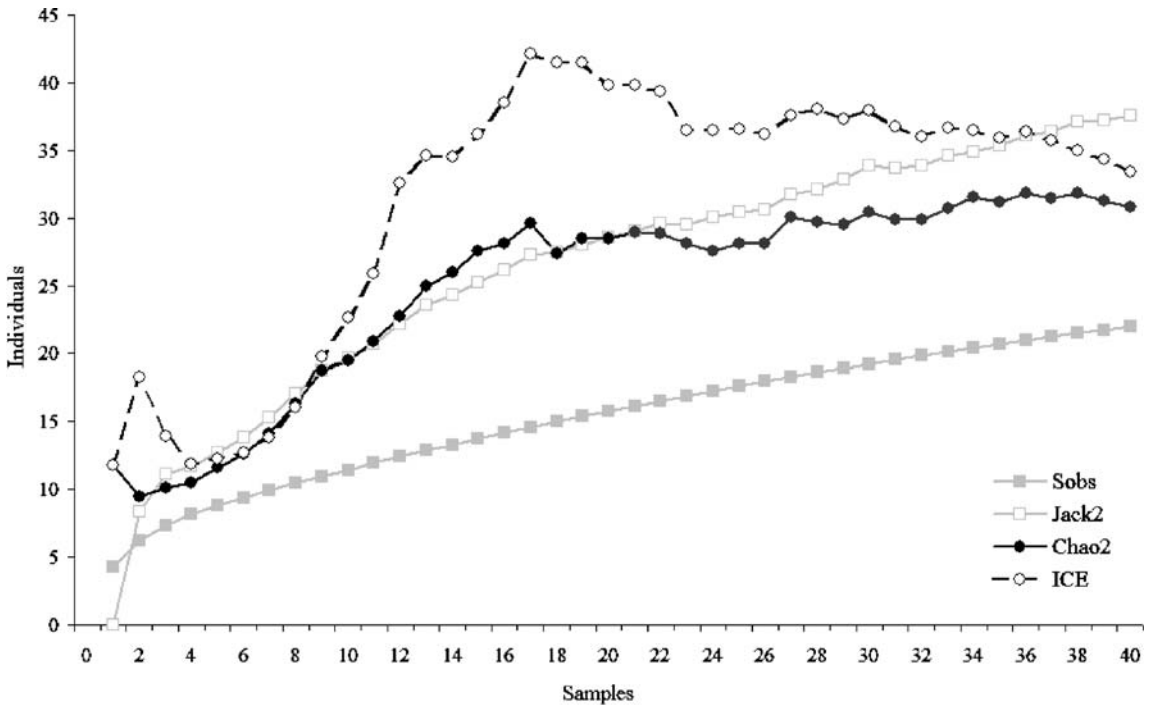

Fig. 1 Species richness estimation curves of *S. herbacea* ectomycorrhizae in all samples of the years 2005 and 2006. *Sobs* species observed, *ICE* incidence-based coverage estimator of species richness, *Chao2* Chao estimator 2, *Jack2* second-order Jackknife richness estimator

Table 4 Relative abundances and frequencies (both in percent) of the main *S. herbacea* ectomycorrhizal MTs including taxon names, senescent root tips and non-mycorrhizal root tips are presented for each season (spring, summer, fall, winter), for each plot (S1 to S5) and in total for both sampling years (2005 and 2006) separately

	MT 14		MT 11		MT 3		MT 6		MT 8		Senescent root tips		Non-mycorrhizal root tips	
	Cenococcum geophilum		*Sebacina* sp.1		*Tomentella* spp.		*Sebacina incrustans*		*Cortinarius* spp.					
	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006
Abundances														
Spring (5)	2.6	21.6	6.6	30.8	16.8	14.0	20.0	8.6	12.4	8.8	12.0	7.4	12.6	8.8
Summer (5)	5.6	35.4	41.2	13.0	9.4	24.8	17.2	2.8	7.0	9.4	4.4	0.0	5.2	5.4
Fall (5)	5.8	34.0	5.4	20.8	10.4	30.0	33.2	1.2	21.0	9.6	8.6	0.0	8.0	4.4
Winter (5)	6.2	66.0	7.2	11.4	11.6	17.0	39.4	0.0	11.8	5.6	8.4	0.0	12.4	0.0
S1 (4)	1.3	43.0	6.0	2.5	16.3	41.3	53.3	6.5	0.0	0.0	6.8	3.5	14.3	0.8
S2 (4)	3.5	13.5	15.5	41.3	11.0	19.5	11.8	5.8	16.8	6.3	12.0	5.8	8.0	4.8
S3 (4)	0.0	62.8	36.8	21.3	4.8	6.3	31.0	1.5	6.8	0.0	5.0	0.0	6.0	8.3
S4 (4)	0.5	33.8	10.8	24.0	3.0	15.5	21.3	0.0	30.0	16.5	12.3	0.0	18.3	4.5
S5 (4)	20.0	43.3	6.5	6.0	25.3	24.8	20.0	2.0	11.8	19.0	5.8	0.0	1.3	5.0
Total (20)	5.1	39.3	15.1	19.0	12.1	21.5	27.5	3.2	13.1	8.4	8.4	1.9	9.6	4.7
Frequencies														
Spring (5)	20	80	20	80	100	80	100	60	80	60	80	20	80	100
Summer (5)	40	80	100	60	60	100	80	20	40	60	60	0	100	40
Fall (5)	40	80	20	80	60	100	100	20	80	60	60	0	60	40
Winter (5)	60	100	40	60	100	60	100	0	60	40	100	0	80	0
S1 (4)	25	100	25	25	100	100	100	25	0	0	25	25	100	25
S2 (4)	50	25	50	75	100	50	75	50	75	75	75	25	75	50
S3 (4)	0	100	100	100	50	100	100	25	50	0	75	0	75	50
S4 (4)	25	100	25	75	50	75	100	0	100	100	75	0	100	50
S5 (4)	75	100	25	50	100	100	100	25	75	100	75	0	50	50
Total (20)	45	85	45	70	80	85	95	25	65	55	80	10	80	45

Number of samples is given in parentheses. MTs are sorted based on their abundances in all samples.

in 2005: both indices for the plots S1 and S5 ($p \leq 0.014$); in 2006, Simpson's values differed in plots S3 and S5 ($p=0.037$).

Abundances of main MTs and senescent root tips also differed significantly between the two sampling years: MT 14 ($p=0.001$), MT 3 ($p=0.043$), MT 6 ($p<0.001$), MT 8 ($p=0.037$) and senescent root tips ($p=0.039$).

Seasonal and spatial variations of MT abundances within each sampling year

In the first year, abundance of *Sebacina* sp. (MT 11) in summer differed significantly ($p<0.001$) from its abundances in the other seasons, and *S. incrustans* (MT 6) abundance in winter differed significantly from those in spring ($p=0.021$) and summer ($p=0.009$). In 2006, abundance of *C. geophilum* (MT 14) in spring was significantly different from its abundance in winter ($p=0.015$).

The abundances of MTs in plots showed the following significant differences: *C. geophilum* (MT 14) in 2005 in plot S5 vs. the other four plots ($p<0.001$); in 2006, in plot S2 vs. S3 ($p=0.024$). *Sebacina* sp.1 (MT 11) in 2005 in plot S3 vs. the other four plots ($p \leq 0.003$); in 2006, in plot S2 vs. S1 and S5 ($p \leq 0.010$). *Tomentella* spp. (MT 3) in 2005 in plot S5 vs. S2, S3 and S4 ($p<0.030$) and in plot S1 vs. plot S4 ($p=0.045$). *S. incrustans* in 2005 in plot S1 vs. all other plots ($p \leq 0.026$). *Cortinarius* spp. (MT 8) in 2005 in plot S4 vs. plots S1, S3 and S5 ($p \leq 0.038$); in 2006, plots S4 and S5 vs. S1, S2 and S3 ($p \leq 0.020$).

Seasonal changes in species composition over the sampling period

All main MTs were found in every season (Table 4). *Tomentella* spp. were abundant in all seasons (between 9.4% and 30.0%). *Cortinarius* spp. did not show any seasonal preference and varied in relative abundances between 5.6% and 21.0%. Rare MTs were observed during all seasons and 14 MTs occurred in only one season.

The following changes were observed: *C. geophilum* (MT 14) increased throughout the 2 years (relative abundances from 2.6% in spring 2005 to 66.0% in winter

Table 5 Diversity indices for *S. herbacea* mycorrhizal morphotypes for all samples in each year separately (2005, 2006), for all samples in both years together and for seasons or plots (S1 to S5) and in each year separately

		Richness	Evenness	Shannon	Simpson
2005	All samples	5.0	0.841	1.323	0.6756
2006		3.5	0.821	1.007	0.5635
Both years		4.2	0.831	1.165	0.6204
2005	Spring	5.2	0.894	1.470	0.7333
	Summer	5.2	0.807	1.270	0.6478
	Fall	4.2	0.899	1.273	0.6825
	Winter	5.4	0.765	1.279	0.6389
	S1	3.3	0.790	0.931	0.5316
	S2	6.8	0.865	1.628	0.7532
	S3	5.0	0.765	1.231	0.6420
	S4	4.5	0.850	1.231	0.6687
	S5	5.5	0.937	1.594	0.7826
2006	Spring	5.0	0.870	1.396	0.7136
	Summer	4.4	0.850	1.266	0.6569
	Fall	3.8	0.841	1.084	0.6052
	Winter	2.6	0.731	0.645	0.3926
	S1	3.5	0.856	1.011	0.5913
	S2	4.0	0.887	1.137	0.6215
	S3	3.8	0.652	0.869	0.4566
	S4	4.3	0.823	1.203	0.6147
	S5	4.3	0.898	1.269	0.6765

2006 and frequency from 20% to 100%). *Sebacina* sp. 1 (MT 11) was usually abundant and frequent, but frequencies were low in spring and autumn 2005 (20% each). *S. incrustans* (MT 6) was very abundant and frequent in 2005, but rare in 2006.

Senescent root tips were most abundant in spring 2005 (12.0%) and lacking in summer, fall and winter 2006. Non-mycorrhizal root tips were most abundant in spring and winter 2005 (12.6% and 12.4%) with lower abundances during the other seasons (between 0.0% and 8.8%)(Table 4).

Discussion

This was the first intensive study of the ectomycorrhizal community of dwarf willow in an alpine primary successional site. Dwarf willows are especially important in these habitats because they are one of the first perennial plants and, therefore, play an important role in soil development and stability.

A high degree of EM colonisation (93%) was observed over the 2 years of investigation. EM fungal diversity was comparatively high at this alpine primary successional site. We distinguished 22 EM fungal taxa on *Salix* root tips based on morphotyping 4,000 root tips and sequencing 213 root tips. A similar investigation on the mycorrhizal status of *Polygonum viviparum* at the same site (Mühlmann et al. 2008) also showed high EM colonisation (100%) and similar EM diversity (18 species). Based on various species richness estimators, our sampling effort was intensive enough to detect at least 59% of the estimated species richness based on Jack2 and more when based on other estimators (71% based on Chao2 and 66% based on ICE; Fig. 1). These results agree with the multi-study analysis of Dickie (2007); he calculated similar values (observed richness reaches 64% of Jack2 values) for fungal communities on single host species. Our data differ, however, from other studies conducted at various primary successional sites. Obase et al. (2007) reported EM root colonisation of 17% to 42% and observed four EM MTs on *S. hultenii* and *S. sachhalinensis* growing on a volcanic successional site of Mt. Usu in Japan 4 years after eruption. Likewise, Trowbridge and Jumpponen (2004) reported an average of 25% EM colonisation and differentiated 12 EM fungal species on shrub willows (*S. commutata*, *S. phylicifolia*) at the forefront of Lyman Galcier (USA), which was deglaciated for 90 years at most. Successional age of soil is an important factor influencing EM fungal species diversity; percentage of root tip colonisation and species diversity of EM host plants generally increase with successional age (Trowbridge and Jumpponen 2004; Cázares et al. 2005). Similar patterns have been reported for *Dryas octopetala* (Harrington and Mitchell 2002, 2005b) and *Arctostaphylos uva-ursi* (Krpata et al. 2007) in artic/alpine ecosystem types.

Seasonal changes

A recent study with *Kobresia myosuroides* on alpine tundra soils in Colorado (USA) showed seasonal variation in soil fungal biomass and that biomass reaches maximum annual levels during late winter under the snow pack (Schadt et al. 2003). Our study is the first to investigate seasonal changes of the *S. herbacea* mycobiont composition in situ. Although we included root samples from frozen winter soil, we observed no significant seasonal changes in the mycobiont community. Non-mycorrhizal root tips occurred throughout the year with a trend to higher abundance in spring. This reflects the surge in plant root growth in spring. The most apparent seasonal changes were exhibited by *C. geophilum* and *Sebacina* mycorrhizae. The observed trend of *C. geophilum* abundances to the increase in winter samples could be explained by its high stress tolerance (e.g. Corbery and LeTacon 1997; Jany et al. 2003; Rincón et al. 2005). Species of Sebacinaceae are fairly common mycobionts in various ectomycorrhizal plant communities (Urban et al. 2003), but the physiological features of these fungi as mycorrhizal partners need further investigations.

Species composition

Gardes and Dahlberg (1996) reviewed important arctic and alpine ectomycorrhizal fungal genera mostly based on fruitbody occurrence. The authors described fungal species belonging to *Amanita*, Boletales, *Cenococcum*, *Cortinarius*, *Hebeloma*, *Inocybe*, *Laccaria*, *Lactarius*, *Russula* and hypogeous fungi as important arctic/alpine mycobionts based on their occurrence in these habitats. Meanwhile, several studies (Massicotte et al. 1998; Harrington and Mitchell 2005a, b; Krpata et al. 2007; Mühlmann et al. 2008) described and identified EM morphotypes and mycobionts of plants in arctic and alpine environments. These studies provide a new understanding of arctic and alpine mycobiont species diversity. *Inocybe* and *Cortinarius* mycobiont species diversity is generally high in arctic/alpine habitats, as estimated by fruitbody data and EM species diversity. *Cortinarius* spp. were abundant and evenly distributed on *S. herbacea* roots. But based on fruitbody abundance data, the importance of *Hebeloma*, *Laccaria*, *Lactarius* and *Russula* spp. as mycobionts was highly overestimated (Graf 1994), whilst inconspicuous species in *Tomentella*, *Sebacina* and other corticoid genera were not considered. Fungi belonging to these genera were dominant mycobionts of dwarf willows at our sampling site: *Sebacina* (two species), *Tomentella* (four species) and *C. geophilum* had the highest EM abundances throughout the year. The same three fungal genera also dominated on the roots of *Polygonum viviparum* growing at this site (Mühlmann et al. 2008). Moreover, Trowbridge and Jumpponen (2004) found two *Tomentella* species on the roots of shrub willows on the Lyman glacier forefront (USA), and Obase et al. (2007) described four *Tomentella* species on the roots of *Salix* spp. 4 years after volcanic eruption.

Spatial heterogeneity of the sampling plots

Our study site was characterised by a patchy structure of soil and plant cover (Erschbamer et al. 1999). The observed plot level differences are likely to result from this spatial heterogeneity. The impact of spatial heterogeneity on the EM community has been reported for alpine or similar sites. Göbl (1995) showed clear differences in dry weight of mycorrhizal root tips and the variability of mycorrhizal morphotypes depending on various microhabitats (e.g. mounts or troughs) of a subalpine spruce forest pasture. These microclimatic and microstructural conditions highly affect the spatial distribution of mycorrhizal fungi. Clumped distributions of EM fungi are considered common (Taylor 2002). The mycobiont community of *S. herbacea* showed higher spatial than seasonal variation (abundances of MTs). Furthermore, our 2-year investigation clearly demonstrated EM dynamics and unpredictability: Fungal communities differed significantly between subsequent years. Species dominating in the first year nearly disappeared the next year (e.g. *S. incrustans*) or vice versa (e.g. *C. geophilum*). Moreover, spatial heterogeneity was high (differences between plots), e.g. *Cortinarius* spp. were never detected in plot S1, but always in plot S4. These differences made it very difficult to clearly detect seasonal impacts on the ectomycorrhizal diversity. However, we observed a trend of species diversity and evenness to decrease in winter when abundance of *C. geophilum* increased.

Several reasons could account for the observed differences between years and spatial heterogeneity: (1) Fluctuations in climatic conditions: we observed significant differences between the 2 years for soil temperatures in spring and summer and for soil moisture in summer and fall (compare Table 1). (2) Heterogeneous microhabitats and scattered distribution of fungal mycelia in the soil. (3) Different persistence of fungal species on the roots depending on the competitive ability and/or stress tolerance of fungal species.

Fungal propagules deposited on the site, mycelial development and interactions between fungal species increase with successional age. We observed abundances and frequencies of *C. geophilum* to increase throughout the 2-year sampling period. This increase could be either a general increase due to the establishment of this competitive species in the sampling site or a temporal increase due to its stress tolerance to the comparatively dry weather conditions in 2006.

A rapid turnover of EM fungal species on the root systems is assumed in the absence of any obvious major disturbances of the ecosystem (Guidot et al. 2001). Ectomycorrhizal fungi can be eliminated as mycorrhizal symbionts and as mycelia in the soil within 1 year, as Guidot et al. (2003) demonstrated for *Hebeloma cylindrosporum* mycobionts in a *Pinus pinaster* stand. Our data show a similar situation: *S. incrustans* EM were very abundant in the first year and nearly disappeared in the second year. But in the second sampling year, we found many fruitbodies of this species in the sampling area (23 collections). One possible explanation besides the influence of abiotic factors is that the mycorrhizae were displaced from the roots by a competitive species (very likely by *C. geophilum*), whereas the mycelium was still present in the soil.

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References

Agerer R (1991) Characterization of ectomycorrhiza. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology. Academic, London, UK, pp 27–73

Cázares E, Trappe JM, Jumpponen A (2005) Mycorrhiza–plant colonization patterns on a subalpine glacier forefront as a model system of primary succession. Mycorrhiza 15(6):405–416

Clemmensen KE, Michelsen A (2006) Integrated long-term responses of an arctic-alpine willow and associated ectomycorrhizal fungi to an altered environment. Can J Bot 84:831–843

Colwell RK (2006) Estimates, version 8.0: Statistical estimation of species richness and shared species from samples (software and user's guide)

Corbery Y, LeTacon F (1997) Storage of ectomycorrhizal fungi by freezing. Ann Sci For 54:211–217

Cripps C (2002) Alpine macrofungi of North America (Rocky Mountains). International Mycological Congress, 11–17 August, Oslo, Norway

Dickie I (2007) Host preference, niches and fungal diversity. New Phytol 174(2):230–233

Erschbamer B, Bitterlich W, Raffl C (1999) Die Vegetation als Indikator für die Bodenbildung im Gletschervorfeld des Rotmoosferners (Obergurgl, Ötztal). Ber Naturwiss Med Ver Innsbr 86:107–122

Gardes M, Bruns T (1993) ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Gardes M, Dahlberg A (1996) Mycorrhizal diversity in arctic and alpine tundra: An open question. New Phytol 133:147–157

Gardes M, Charcosset J, Dahlberg A (2000) High species richness and dominance of *Cortinarius* taxa in belowground communities of ectomycorrhizal fungi from the Canadian Arctic. MSA 2000 Abstract. Inoculum 51:30

Göbl F (1995) Investigations on mycorrhizae and fine roots in an area used as forest pasture. Ber Forst Bundesversuchsanstalt Wien 87:201–213

Graf F (1994) Ecology and sociology of macromycetes in snowbeds with *Salix herbacea* L. in the alpine valley of Radönt (Grisons, Switzerland). Diss Bot 235:1–242

Graf F, Brunner I (1996) Natural and synthesized ectomycorrhizas of the alpine dwarf willow *Salix herbacea*. Mycorrhiza 6:227–235

Guidot A, Debaud JC, Marmeisse R (2001) Correspondence between genet diversity and spatial distribution of above- and below-ground populations of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Mol Ecol 10:1121–1131

Guidot A, Debaud JC, Effosse A, Marmeisse R (2003) Below-ground distribution and persistence of an ectomycorrhizal fungus. New Phytol 161:539–547

Harrington TJ, Mitchell DT (2002) Characterization of *Dryas octopetala* ectomycorrhizas from limestone karst vegetation, western Ireland. Can J Bot 80:970–982

Harrington TJ, Mitchell DT (2005a) Ectomycorrhizas associated with a relict population of *Dryas octopetala* in the Burren, western Ireland. I. Distribution of ectomycorrhizas in relation to vegetation and soil characteristics. Mycorrhiza 15:425–433

Harrington TJ, Mitchell DT (2005b) Ectomycorrhizas associated with a relict population of *Dryas octopetala* in the Burren, western Ireland II. Composition, structure and temporal variation in the ectomycorrhizal community. Mycorrhiza 15:435–445

Haselwandter K, Read DJ (1980) Fungal associations of roots of dominant and sub-dominant plants in high-alpine vegetation systems with special reference to mycorrhiza. Oecologia 45 (1):57–62

Jany JL, Martin F, Garbaye J (2003) Respiration activity of ectomycorrhizas from *Cenococcum geophilum* and *Lactarius* sp. in relation to soil water potential in five beech forests. Plant Soil 255:487–494

Jumpponen A, Trappe JM, Cázares E (2002) Occurrence of ectomycorrhizal fungi on the forefront of retreating Lyman glacier (Washington, USA) in relation to time since deglaciation. Mycorrhiza 12(1):43–49

Kõljalg U, Larsson KH, Abarenkov K, Nilsson RH, Alexander IJ, Eberhardt U, Erland S, Høiland K, Kjøller R, Larsson E, Pennanen T, Sen R, Taylor AFS, Tedersoo L, Vrålstad T, Ursing BM (2005) Unite: A database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytol 166(3):1063–1068

Krpata D, Mühlmann O, Kuhnert R, Ladurner H, Göbl F, Peintner U (2007) High diversity of ectomycorrhizal fungi associated with *Arctostaphylos uva-ursi* in subalpine and alpine zones: Potential inoculum for afforestation. For Ecol Manag 250(3):167–175

Massicotte HB, Melville LH, Peterson RL, Luoma DL (1998) Anatomical aspects of field ectomycorrhizas on *Polygonum viviparum* (Polygonaceae) and *Kobresia bellardii* (Cyperaceae). Mycorrhiza 7:287–292

McCune B, Grace JB (2002) Analysis of ecological communities. MjM Software, Gleneden Beach, OR, USA

McCune B, Mefford MJ (1999) Pc-ord. Multivariate analysis of ecological data. Version 5.0. MjM Software, Gleneden Beach, OR, USA

Mühlmann O, Bacher M, Peintner U (2008) *Polygonum viviparum* mycobionts on an alpine primary successional glacier forefront. Mycorrhiza 18(2):87–95

O'Donnell K (1993) *Fusarium* in near relatives. In: Reynolds D, Taylor J (eds) The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. CBA International, Washington, DC, USA, pp 225–233

Obase K, Tamai Y, Yajima T, Miyamoto T (2007) Mycorrhizal associations in woody plant species at the Mt. Usu volcano, Japan. Mycorrhiza 17:209–215

Peintner U, Bougher NL, Castellano MA, Moncalvo JM, Moser MM, Trappe JM, Vilgalys R (2001) Multiple origins of sequestrate fungi related to *Cortinarius* (Cortinariaceae). Am J Bot 88 (12):2168–2179

Raffl C, Erschbamer B (2004) Comparative vegetation analyses of two transects crossing a characteristic glacier valley in the Central Alps. Phytocoenologia 34(2):225–240

Raffl C, Mallaun M, Mayer R, Erschbamer B (2006) Vegetation succession pattern and diversity changes in a glacier valley, Central Alps, Austria. Arct Antarct Alp Res 38(3):421–428

Read DJ, Haselwandter K (1981) Observations on the mycorrhizal status of some alpine plant communities. New Phytol 88:341–352

Rincón A, Priha O, Lelu-Walter MA, Bonnet M, Sotta B, LeTacon F (2005) Shoot water status and ABA responses of transgenic hybrid larch *Larix kaempferi* x *L decidua* to ectomycorrhizal fungi and osmotic stress. Tree Physiol 25:1101–1108

Sakakibara SM, Jones MD, Gillespie M, Hagerman SM, Forrest ME, Simard SW, Durall DM (2002) A comparison of ectomycorrhiza identification based on morphotyping and PCR-RFLP analysis. Mycol Res 106:868–878

Schadt CW, Martin AP, Lipson DA, Schmidt SK (2003) Seasonal dynamics of previously unknown fungal lineages in tundra soils. Science 301:1359–1361

Senn-Irlet B (1993) The mycoflora of Alpine mire communities rich in *Salix*. Bibl Mycol 150:235–249

Southworth D (2000) CTAB DNA extraction protocol. Available at http://www.sou.edu/BIOLOGY/Faculty/Southworth/CTAB.htm

Taylor AFS (2002) Fungal diversity in ectomycorrhizal communities: sampling effort and species detection. Plant Soil 244: 19–28

Tedersoo L, Suvi T, Larsson E, Kõljalg U (2006) Diversity and community structure of ectomycorrhizal fungi in a wooded meadow. Mycol Res 110:734–748

Treu R, Laursen GA, Stephenson SL, Landolt JC, Densmore R (1996) Mycorrhizae from Denali national park and preserve, Alaska. Mycorrhiza 6:21–29

Trowbridge J, Jumpponen A (2004) Fungal colonization of shrub willow roots at the forefront of a receding glacier. Mycorrhiza 14:283–293

Urban A, Weiss M, Bauer R (2003) Ectomycorrhizas involving sebacinoid mycobionts. Mycol Res 107:3–14

Vilgalys R (2005) Conserved primer sequences for PCR amplification and sequencing from nuclear ribosomal RNA. Available at http://www.biology.duke.edu/fungi/mycolab/primers.htm#Internal%20transcribed%20spacer%20(ITS)%20region%20primers

White T, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics.. In: Innis MA, Gelfand D, Sninsky J, White T (eds) PCR Protocols: a guide to methods and applications. Academic, New York, pp 315–322