Annals of Microbiology, **58 (4)** 585-595 (2008)

Detection of soil fungal communities in an alpine primary successional habitat: Does pooling of DNA extracts affect investigations?

Irmgard OBERKOFLER*, Ursula PEINTNER

Institute of Microbiology, University of Innsbruck, Technikerstr. 25, 6020 Innsbruck, Austria

Received 21 August 2008 / Accepted 23 September 2008

Abstract - Our main aim was to understand whether the use of pooled DNA extracts bias the results of investigations in soil fungal communities in an alpine habitat. To avoid bias by DNA extraction methods, we used a commercial DNA extraction kit and consecutively pooled 1-4 DNA extracts from 0.25 g soil, each, before cloning. We expected to detect abundant species in all approaches, and the same diversity in clone libraries from pooled extracts. Soil fungal communities of primary successional sites mainly consist of rare species. Irrespective of pooling, no significant differences of species richness or diversity were detected between clone libraries.

Key words: fungi, dsDNA, glacier forefront, clone libraries.

INTRODUCTION

Ecological questions usually require a large, statistically relevant amount of representative samples to be analysed. This applies also and especially to the study of soil communities with molecular tools. One basic problem of the analysis of soil fungal communities is the generally high spatial heterogeneity of fungal mycelia in the soil (Taylor, 2002; Genney *et al*., 2006; Kang and Mills, 2006; Kjõller, 2006). This can be a critical issue especially in primary successional sites (Jummponen, 2003). It is therefore important to detect the sample size or size range that produces the most consistent results and the lowest variance (Kang and Mills, 2006). Soil sample size usually positively affects the yield of DNA, but strong variations were observed between replicates of smaller samples (< 1 g soil) (Ranjard *et al*., 2003). Therefore, Ranjard *et al*. (2003) suggested the use of sampling aliquots of ≥ 1 g soil for investigations of fungal communities. It is however, not always advising to use standard DNA extraction methods, especially in soils with generally low DNA concentration, due to the low or varying recovery rate (Martin-Laurent *et al*., 2001). Moreover, the method of DNA or RNA extraction can bias diversity studies (Kirk *et al*., 2004). Harsh extraction methods can shear the nucleic acids, leading to problems in subsequent PCR detection (von Wintzingerode *et al*., 1997). Even dispersion of soil by gentle shaking can result in strong shearing forces as particles grind against one another (Giller *et al*., 1997). Commercial DNA extraction kits are usually very efficient, but only small amounts (≤ 0.25 g) of soil can be used per extraction, leading to comparable small amounts of DNA extract. As for every new soil the best amount of DNA extract for a subsequent PCR has to be ascertained, often a higher amount of DNA extract is required. Therefore the main aim of our study was to understand whether in our comparatively young primary successional soils pooling of DNA extracts prior to PCR biases the investigations of fungal diversity and species richness. DNA pooling prior to PCR has also been recommended by other authors (Barrat *et al*., 2002; Sham *et al*., 2002). To study the effect of pooling on the analysis of fungal communities, we pooled up to four DNA extracts of soils with different plant cover and of a bare terrain sample. We expected to detect abundant and commonly occurring species in all approaches.

MATERIAL AND METHODS

Study site. The Rotmoosferner glacier forefront (46°50'N, 11°03'E) is located in the Ötz valley in the Stubaier Alpen (Austrian Central Alps) at an altitude ranging from 2280-2450 m a.s.l. The annual mean temperature is -1.3 °C (1997-1998) and the mean annual precipitation is 890 mm (1970-1986). Snow cover usually lasts from mid October to mid May. The parent material of the soil is mainly neoglacial moraine till and fluvioglacial sands with soils classified as Leptic and Eutric Regosols (Nicol *et al*., 2005). For our investigations we selected an area behind the moraine ridge of 1858 (with a soil development of 150 years). *Kobresia myosuroides*, *Salix herbacea*, and *Polygonum viviparum* occur together in this area in a patchy distribution.

* Corresponding author. Phone: (+43) 512 5076029;
Fax: (+43) 512 5072938; E-mail: Irmgard.Oberkofler@uibk.ac.at

The Rotmoosferner glacier forefront is one of the most intensively studied areas of its kind in the Austrian Central Alps. This site has been subject of several successional and ecological studies in recently deglaciated areas. Detailed descriptions of the geology (Hoinkes and Thöni, 1993), soil formation (Erschbamer *et al*., 1999), vegetation development (Raffl, 1999) and faunal succession (Kaufmann 2001, 2002; Kaufmann and Raffl, 2002; Kaufmann *et al*., 2002) are available. Below-ground microbial processes and enzyme activities have been investigated by Tscherko *et al*. (2003, 2005). The interactions of plants and rhizosphere microorganisms were studied by Hammesfahr (2002), ectomycorrhizal fungal communities by Mühlmann and Peintner (2008a, 2008b) and Mühlmann *et al*. (2008).

Soil sampling. Samples were taken on August 04th 2005. Mean soil temperatures were 11.3 °C (SD = 3.5, Min = 3.4 °C, Max = 19.8 °C) in 10 cm depth in July 2005.

One plot (1 x 1 m) was selected for each plant (*K. myosuroides, S. herbacea, and P. viviparum*) and from bare terrain as a control; from each plot five soil subsamples were taken and pooled. Soil samples were sieved using a 2 mm mesh and stored on ice until they were processed.

DNA extraction. DNA was extracted using the PowerSoil DNA Isolation Kit (MoBio, USA) according to the manufacturers instructions for 0.25 g amounts of soil. For each of the four plots, DNA extraction was carried out four times, and then pooled. Thereby equal volumes of two (II), three (III) or four (IV) randomly picked DNA extracts were mixed.

dsDNA concentrations. DNA concentrations were measured using the PicoGreen dsDNA Quantification Kit (Molecular Probes, Eugene, USA) and a Fluorescence Plate Reader f max (Molecular Devices). Measurements were conducted in triplicate.

PCR amplification and cloning. PCR amplification of fungal ITS-rDNA was carried out using the primer pair ITS1F (Gardes and Bruns, 1993) and ITS4 (White *et al*., 1990) using a Primus 96 thermal cycler (PeqLab) with 50 µl reaction volumes. The PCR mixtures contained between 5 µl and 10 µl of extracted template DNA, 20 pmol of each primer, 2.0 mM $MgCl_2$, 250 µM each dATP, dCTP, dGTP and dTTP, 10X buffer [1.5 mM $MgCl_2$, 10 mM TrisHCl, 50 mM KCl], 1 µl of a BSA solution (20 mg ml^{-1}) and 2.5 U of Taq DNA polymerase (PeqLab). Cycling parameters were 94 °C for 3 min followed by 10 cycles of 94 °C for 15 s, 53 °C for 30 s and 72 °C for 50 s. Twenty cycles of 94 °C for 15 s, 53 °C for 30 s and 72 °C for 50 s + 5 s followed the initial cycling, and a final extension at 72 °C for 7 min was used.

Negative controls (dd H_2O) were included in each PCR. Amplification products were electrophoresed in 0.8% (w/v) agarose gels (GenXpress) with ethidium bromide (10 µg $µl^{-1}$, SERVA 21238, Bioproducts, Boehringer-Ingelheim, Germany).

PCR amplification products were purified using ExoSAP-IT (USB Corporation, USA) before cloning with the TOPO TA Cloning Kit (Invitrogen, USA). Chemically competent *Escherichia coli* (TOP10 cells, Invitrogen) were used for the transformation. After 24 h of incubation, approximately 40 white colonies for each of the 15 clone libraries were picked out randomly and screened for the correctly sized inserts by carrying out PCR using the primer pair ITS1 (White *et al*., 1990) and ITS4. Colonies were picked with a toothpick and resuspended in 25 µl reaction volumes containing 20 pmol of each primer, 200 µM each dATP, dCTP, dGTP and dTTP, Enhancer [1.2 mM TrisHCl, 6.0 mM KCl, 12 mM EDTA, 50 % Glycerin], 10X buffer [1.5 mM $MgCl_2$, 10 mM TrisHCl, 50 mM KCl], 1.0 U of Taq DNA polymerase (PeqLab). Cycling parameters were 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 50 °C for 45 s and 72 °C for 2 min with a final extension of 72 °C for 5 min.

Twenty-seven colonies with positive inserts from each clone library were used for further investigations. Clone libraries were designated according to the plant cover (*Kobresia*, *Polygonum*, *Salix* and bare terrain) and number of pooled DNA extracts (I-IV).

RFLP patterns and sequencing of ITS PCR products. PCR products from all clones of each clone library were digested with the endonucleases *HinfI* and *EcoRI*, respectively. PCR products with similar RFLP patterns were digested additionally with the endonuclease *AluI* (all enzymes from Genecraft, Germany). The digestion was carried out with a modified protocol of Southworth (2000). The digested products were electrophoresed in 2.0% (w/v) agarose gels, stained with ethidium bromide (10 µg $µl^{-1}$) and visualised under UV light (Alpha DigiDocTM Imaging System, Biozym Scientific GmbH, Hess. Oldendorf, Germany). Patterns were distinguished using the GelCompar II V4.01 software (Applied Maths, St-Martens-Latem, Belgium) and controlled by the naked eye. For each distinct RFLP pattern, PCR products were purified using ExoSAP-IT (USB Corporation) and sequenced (MWG-Biotech, Germany) using the primer ITS1.

Sequence analysis. 218 clones from fifteen clone libraries were sequenced. DNA sequences were controlled and edited, and consensus sequences were obtained, using SEQUENCHER (version 4.6; Gene Codes Corporation). BLAST searches were performed using NCBI (National Center for Biotechnology Information) and UNITE (Kõljalg *et al*., 2005). At least three closely related sequences were downloaded as references.

Sequenced clones were regarded as identical at a minimum match of 97% and a minimum overlap of 90%. Clones with the same RFLP-patterns as sequenced clones were also considered as belonging to the respective operational taxonomic unit (OUT).

Analyses of soil parameters. The measurements of pH and the total amount of C and N were carried out using standard protocols as described by Schinner *et al*. (1996).

Statistics. Abundances of OTUs (species) in soils were investigated. Abundances are relative representations of species in different ecosystems. Usually they are specified as the percentage of a species (OTU) occurring in a sample (clone library).
To compare how well communities were sampled, rank-abundance curves for every soil sample were plotted. The OTUs were arranged based on their abundance in ascending order on the x axis, and the abundance of each OTU observed was plotted on the y axis (Hughes *et al*., 2002).

For a characterisation of the diversity and the equal distribution of species in the clone libraries the Shannon-Weiner diversity index (H) and equitability (E_H) (McCune and Grace, 2002) was used.

Principal Components Analysis (PCA) was carried out with PC-ORD Version 5.0 (McCune and Mefford, 1999) to explore / categorize clone libraries based on their species composition (abundances of OTUs).

A goodness-of-fit test for the normal distribution was carried out using the Kolmogorov-Smirnov test for each dataset. Significant differences ($p < 0.05$) between DNA yields of the un-pooled and pooled DNA extracts were determined using the

TABLE 1 - Number of clones and OTUs recovered from each clone library

Clone library[a]	Clones	OTUs	H[b]	E_H[c]	Total clones	Total OTUs	DNA[d] (µg g^{-1} dry soil)
Kobresia I	27	17	2.69	0.82	108	37	8.68 ± 0.37
Kobresia II	27	16	2.64	0.80			10.85 ± 0.50
Kobresia III	27	10	1.70	0.52			11.05 ± 0.39
Kobresia IV	27	16	2.59	0.78			11.42 ± 0.19
Polygonum I	27	14	2.31	0.70	108	32	4.87 ± 0.12
Polygonum II	27	16	2.64	0.80			4.62 ± 0.25
Polygonum III	27	11	1.80	0.55			4.20 ± 0.10
Polygonum IV	27	12	1.89	0.57			4.36 ± 0.05
Salix I	27	19	2.74	0.83	81	40	4.79 ± 0.29
Salix II	27	15	2.55	0.77			4.36 ± 0.14
Salix III	27	18	2.79	0.85			4.52 ± 0.41
bare terrain I	27	12	2.08	0.63	108	40	3.21 ± 0.25
bare terrain II	27	14	2.39	0.73			3.05 ± 0.07
bare terrain III	27	14	2.41	0.73			3.08 ± 0.09
bare terrain IV	27	18	2.73	0.83			3.02 ± 0.11

[a] I-IV = number of pooled DNA extracts.
[b] H = Shannon-Weiner diversity index calculated for each clone library using OTUs as a proxy for species.
[c] E_H = Shannon-Weiner equitability.
[d] Amounts of total double-stranded DNA (µg g^{-1} dry soil) in DNA extracts and pooled DNA extracts.
Results are mean values and standard deviations for three parallels.

Kruskal-Wallis and Mann-Whitney tests (both non-parametric). Statistical significances of H and E_H and significant differences between the number of OTUs in clone libraries generated from unpooled and pooled DNA extracts were calculated with Anova, Scheffé's procedure ($p < 0.05$). All statistical tests were carried out with SPSS (SPSS 14.0, © 2005, SPSS Inc., Chicago, USA).

Accession numbers. Sequences of all clones have been deposited in the GenBank database with accession numbers EF635637 to EF635844 and EF654512.

RESULTS

Soil parameters

In the four investigated plots, pH values ranged between 6.4 in *Kobresia* soils and 7.0 in bare terrain, while the C/N ratio ranged between 13.85 in *Polygonum* soils and 17.27 in bare terrain. The comparatively high alkalinity of these alpine soils is derived from the high carbonate content.

Concentration of double stranded (ds) DNA

DNA concentrations of extracts derived from the investigated alpine soils were comparatively low. The DNA concentrations ranged from 3.01 µg (in bare terrain IV) to 11.42 µg (in *Kobresia* IV) per gram dry soil (Table 1). DNA concentrations of all extracts from *Kobresia* soil and bare terrain plots clearly differed ($p < 0.001$) from the other soils. Standard deviations are very low between repeated measurements, causing these significant differences (Table 1). The differences between *Polygonum* and *Salix* soils were not statistically significant ($p = 0.643$) (Fig. 1). When comparing DNA concentrations of plots separately, concentrations did not differ significantly from each other in *Salix* and bare terrain plots (*Salix* $p = 0.186$, bare terrain $p = 0.715$), but in *Kobresia* and *Polygonum* plots, significant differences between pooled extracts were detected (*Kobresia* $p = 0.041$, *Polygonum* $p = 0.033$) (Fig. 1).

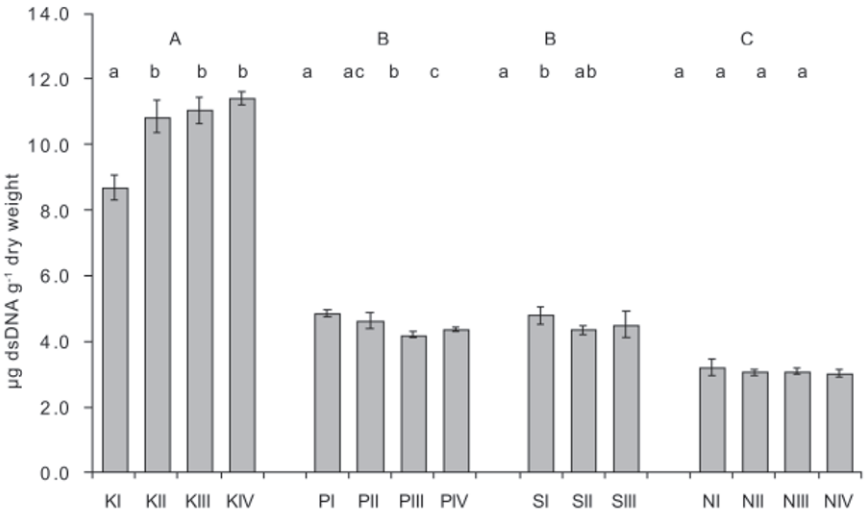

FIG. 1 - Amounts of double-stranded DNA (µg) per gram dry soil in DNA extracts (I) and pooled DNA extracts (II-IV) as found in four investigated alpine plots (K = *Kobresia,* S = *Salix,* P = *Polygonum* and N = bare terrain). Results are means and standard errors for three parallels. Bars with the same letter are not significantly different at the $p < 0.05$ level. Lower-case letters indicate differences within one plot, upper-case letters within all plots.

TABLE 2 - Fungal OTUs (rDNA ITS sequences) detected in the alpine soil samples with their respective GenBank Accession numbers and information on the closest Blast match (with Accession number), Blast-Score, and the number of clones detected in the four investigated plots with *Kobresia, Polygonum, Salix* covering and bare soil. OTUs are sorted based on their abundances (total number of clones detected) and then in alphabetical order based on their Blast match

OTU	GenBank Accession	Closest Blast match	Acc. no.	Blast-Score	No. of clones in different soils				Total no. of clones
					Kobresia	*Polygonum*	*Salix*	Bare terrain	
21	EF635637 EF635653 EF635661	unc. ECM *Thelephoraceae* 2	AJ893305	991	0	39	0	0	39
2	EF635640 EF635667 EF635669 EF635673 EF635675 EF635694 EF635702 EF635705 EF635779 EF635840	*Nectria mauritiicola* 1	AJ558115	1009	15	2	1	8	26
10	EF635644 EF635650 EF635810	*Mycocentrospora acerina*	AY266155	545[b]	0	0	0	23	23
9	EF635646 EF635690 EF635740 EF635765 EF635809	*Phoma* cf. *eupyrena*	AJ890436	948	6	1	2	11	20
25	EF635652 EF635663 EF635695	unc. Helotiales 1	AM181392	870	3	4	5	1	13
1	EF635654 EF635664	*Nectria* sp.	DQ779785	977	12	0	0	0	12
46	EF635676 EF635830	*Tetracladium maxilliforme*	DQ068996	977	9	0	0	1	10
23	EF635678 EF635682 EF635818 EF635822	*Tomentella* sp.	DQ974780	908	0	0	9	0	9
8	EF635645 EF635681 EF635688 EF635699 EF635734 EF635737 EF635766 EF635833	*Leptodontidium orchidicola* 1	AF486133	991	5	1	2	0	8
3	EF635685 EF635689 EF635693 EF635697 EF635701 EF635710 EF635728	*Verticillium* sp.	AY805596	1007	7	0	1	0	8
30	EF635706 EF635707	*Cortinarius rubricosus*	AY669673	997	0	0	7	0	7
14	EF635709 EF635714 EF635725	*Exophiala salmonis* 3	DQ344031	416[a,b]	2	0	4	1	7
19	EF635700 EF635721 EF635722	*Mortierella* sp. 2	DQ093725	1019	0	7	0	0	7
13	EF635733 EF635808	*Ramularia pratensis*	EU019284	833	0	0	0	7	7
35	EF635756 EF635795	*Thelephora caryophyllea*	UDB000119	1134	0	0	7	0	7
4	EF635749 EF635760 EF635787	*Truncatella angustata*	AF377300	1068	1	1	2	3	7
29	EF635763 EF635771	*Cenococcum geophilum*	EU427331	850	0	6	0	0	6
5	EF635764 EF635773	*Cladosporium cladosporioides*	EF405864	971	5	0	0	1	6
27	EF635767 EF635794	*Mortierella alpina* 1	AJ271629	1152	0	1	3	2	6
31	EF635835	*Mortierella* sp. 3	EF031108	1053	6	0	0	0	6
26	EF635778 EF635783 EF635784 EF635791 EF635793	*Sebacina incrustans*	EF655701	1100	0	0	0	6	6
40	EF635788 EF635796	unc. basidiomycete	AM902088	454[a,b]	0	0	0	6	6
34	EF635797 EF635800 EF635807	ascomycete sp. 1	AY590793	513[a,b]	1	0	0	4	5

OTU	GenBank Accession	Closest Blast match	Acc. no.	Blast-Score	No. of clones in different soils				Total no. of clones
					Kobresia	*Polygonum*	*Salix*	Bare terrain	
32	EF635801 EF635832	*Fusarium tricinctum*	AY188923	1007	1	3	0	1	5
37	EF635711 EF635829	*Inocybe rufofusca* 2	EU326156	396[a,b]	0	5	0	0	5
12	EF635727 EF635739	*Bovista cretacea*	DQ112610	1241	0	0	0	4	4
36	EF635743 EF635786 EF635827	*Inocybe rufofusca* 1	EF655704	1215	0	4	0	0	4
38	EF635755 EF635770 EF635790	*Sebacina* sp.	DQ974767	898	0	4	0	0	4
28	EF635772 EF635777 EF635780	unc. ECM *Inocybe* 2	AY634139	950	0	4	0	0	4
47	EF635816 EF635821 EF635842	*Coprinopsis echinospora*	AB071801	819[a]	3	0	0	0	3
55	EF635651 EF635662	*Cordyceps crassispora*	AB067714	985	3	0	0	0	3
61	EF635672 EF635687 EF635748 EF635785	*Cryptococcus victoriae*	AF444645	912	2	1	0	0	3
111	EF635746 EF635803	*Hebeloma ammophilum*	AY308585	1065	0	0	3	0	3
11	EF635683 EF635715 EF635720	*Phoma* sp.	EF589893	404[a,b]	0	0	0	3	3
16	EF635825 EF635826 EF635828	unc. fungus 2	AY970163	827	0	3	0	0	3
7	EF635750 EF635792	unc. Helotiales 3	EU195342	997	2	0	1	0	3
24	EF635752 EF635762 EF635774	unc. Helotiales 3	EU195342	553[b]	0	0	3	0	3
53	EF635754 EF635759	*Cryptococcus aerius*	AF444376	1168	2	0	0	0	2
41	EF635641 EF635648	*Cystodendron* sp.	DQ914672	908	1	1	0	0	2
92	EF635724 EF635732	*Dioszegia hungarica*	EU286796	864	0	2	0	0	2
103	EF635647 EF635781	*Leptosphaeria* sp.	DQ093682	912	0	0	2	0	2
18	EF635757 EF635776	*Myriosclerotinia caricis-ampullaceae*	Z99694	692	0	2	0	0	2
84	EF635726 EF635731	*Physconia muscigena*	DQ534478	565[a,b]	0	0	0	2	2
44	EF635708 EF635805	*Sarcosomataceae* sp.	AY465503	795	0	0	1	1	2
54	EF654512	*Sporobolomyces inositophilus*	AF444559	448[a,b]	2	0	0	0	2
20	EF635658 EF635704	*Tetracladium maxilliforme*	DQ068996	799	0	0	2	0	2
42	EF635638	*Tomentella fuscocinerea*	UDB000776	525[b]	0	2	0	0	2
39	EF635639	unc. ECM *Thelephoraceae* 1	AJ893296	1168	2	0	0	0	2
15	EF635642	unc. ECM *Thelephoraceae* 2	AJ893305	894	0	2	0	0	2
60	EF635643	unc. glomeromycete	EF619906	664	2	0	0	0	2
22	EF635649	unc. Helotiales 2	DQ182427	864	0	2	0	0	2
43	EF635655	unc. *Sebacinaceae*	DQ273405	468[a,b]	0	0	0	2	2
17	EF635656	unidentified fungus			0	2	0	0	2
33	EF635657	unidentified fungus			0	0	1	1	2
99	EF635659	unidentified fungus			0	0	2	0	2
102	EF635660	unidentified fungus			0	0	2	0	2
109	EF635665	unidentified fungus			0	0	2	0	2
6	EF635666	*Vermispora fusarina*	DQ494379	321[a,b]	2	0	0	0	2
64	EF635668	*Aleuria aurantia*	AF072090	466[a,b]	1	0	0	0	1
76	EF635670	*Amorphotheca resinae*	EU030275	432[a,b]	0	0	0	1	1
85	EF635671	ascomycete sp. 2	EF373584	303[a,b]	0	0	0	1	1
75	EF635674	basidiomycete sp. 1	DQ117964	682[a]	0	0	0	1	1
110	EF635677	basidiomycete sp. 2	DQ117964	785	0	0	1	0	1
97	EF635679	*Cadophora finlandica*	DQ485204	918	0	0	1	0	1
49	EF635680	*Cephalosporium gramineum*	AY428792	797	1	0	0	0	1
87	EF635684	*Cercophora caudata*	AY999135	589[b]	0	0	0	1	1
71	EF635686	*Cladophialophora* sp.	EU139132	751	0	0	0	1	1

OTU	GenBank Accession	Closest Blast match	Acc. no.	Blast-Score	No. of clones in different soils				Total no. of clones
					Kobresia	*Polygonum*	*Salix*	Bare terrain	
66	EF635691	*Cortinarius diasemospermus*	AJ889970	959	1	0	0	0	1
107	EF635692	*Cryptococcus terricola*	AF444377	1160	0	0	1	0	1
112	EF635696	*Diatractium cordianum*	EU541488	367[a,b]	0	0	1	0	1
72	EF635698	*Entrophospora* sp.	AY035666	807	0	0	0	1	1
48	EF635703	*Exophiala salmonis* 1	AM176667	888	1	0	0	0	1
119	EF635712	*Exophiala salmonis* 2	AY213652	886	0	0	1	0	1
56	EF635713	*Geomyces vinaceus*	AJ608972	1033	1	0	0	0	1
52	EF635716	*Helminthosporium solani*	DQ865090	759	1	0	0	0	1
93	EF635717	*Inocybe ochroalba*	EU326165	1003	0	1	0	0	1
116	EF635718	*Laetisaria fuciformis*	EU118639	870[a]	0	0	1	0	1
63	EF635719	*Lecythophora* sp.	AY219880	656	1	0	0	0	1
86	EF635723	*Leptodontidium orchidicola* 1	AF486133	892	0	0	0	1	1
83	EF635729	*Mortierella alpina* 2	AJ878532	954	0	0	0	1	1
117	EF635730	*Mortierella elongata*	AJ878504	900	0	0	1	0	1
65	EF635736	*Mortierella gamsii*	DQ093723	1094	1	0	0	0	1
67	EF635738	*Mortierella* sp. 1	AJ541799	375[a,b]	0	0	0	1	1
70	EF635741	*Mycosphaerella phacae-frigidae*	AY490758	607	0	0	0	1	1
59	EF635742	*Nectria mauritiicola* 1	AJ558115	670	1	0	0	0	1
57	EF635744	*Panaeolus sphinctrinus*	DQ182503	922	1	0	0	0	1
113	EF635745	Pezizales sp.	AF266709	339[a,b]	0	0	1	0	1
100	EF635747	*Phaeococcomyces chersonesos*	AJ507323	781	0	0	1	0	1
68	EF635751	*Phaeosphaeria padellana*	AF439496	886	0	0	0	1	1
79	EF635758	*Phaeosphaeriaceae* sp.	AY465459	597[b]	0	0	0	1	1
101	EF635761	*Phaeotellus griseopallidus*	U66436	999	0	0	1	0	1
90	EF635768	*Phialophora* sp.	EF160066	817	0	1	0	0	1
62	EF635769	*Resupinatus poriiformis*	AY571062	339[a,b]	1	0	0	0	1
58	EF635775	*Rhinocladiella* sp.	EU139137	418[a,b]	1	0	0	0	1
96	EF635782	*Seiridium unicorne*	AF377299	525[b]	0	1	0	0	1
78	EF635789	*Sporormiella isomera*	EU551184	385[b]	0	0	0	1	1
115	EF635798	*Stilbella byssiseda*	AF335453	632	0	0	1	0	1
118	EF635799	*Taphrina americana*	AF492078	807	0	0	1	0	1
89	EF635802	*Thelephoraceae* sp.	U83467	648	0	1	0	0	1
45	EF635804	*Tomentella atramentaria*	DQ974772	1156	0	1	0	0	1
106	EF635806	unc. ascomycete	AY970224	636	0	0	1	0	1
98	EF635811	unc. ECM *Inocybe* 1	AY634114	1197	0	0	1	0	1
105	EF635812	unc. fungus 1	AF504848	444[b]	0	0	1	0	1
114	EF635813	unc. Helotiales 1	AM181392	720	0	0	1	0	1
80	EF635814	unc. Pezizomycotina	DQ182459	398[a,b]	0	0	0	1	1
51	EF635815	unc. root-associated 1	EU144792	615[b]	1	0	0	0	1
77	EF635817	unc. root-associated 2	EU144795	886	0	0	0	1	1
73	EF635819	unc. soil fungus	DQ421304	1340	0	0	0	1	1
50	EF635820	unidentified fungus			1	0	0	0	1
69	EF635823	unidentified fungus			0	0	0	1	1
74	EF635824	unidentified fungus			0	0	0	1	1
81	EF635831	unidentified fungus			0	0	0	1	1
82	EF635834	unidentified fungus			0	0	0	1	1
88	EF635836	unidentified fungus			0	1	0	0	1
91	EF635837	unidentified fungus			0	1	0	0	1
94	EF635838	unidentified fungus			0	1	0	0	1
95	EF635839	unidentified fungus			0	1	0	0	1
104	EF635841	unidentified fungus			0	0	1	0	1
108	EF635843	unidentified fungus			0	0	1	0	1
120	EF635844	unidentified fungus			0	0	1	0	1

[a] BLAST matches were partial and did not span over the entire cloned sequence.

[b] E-value ≥ e^{-6}.

Number of OTUs in clone libraries.

A total of 405 colonies from 15 clone libraries were screened. Unique RFLP types were identified and sequenced, yielding a total of 120 OTUs (Table 2). Thirty-two OTUs were found for soils with *Polygonum* cover, 37 OTUs for soils with *Kobresia* cover, 40 OTUs for soils with *Salix* cover and 40 OTUs for bare terrain. Sample *Salix* IV was excluded from further analyses because it was impossible to obtain > 15 positive clones.

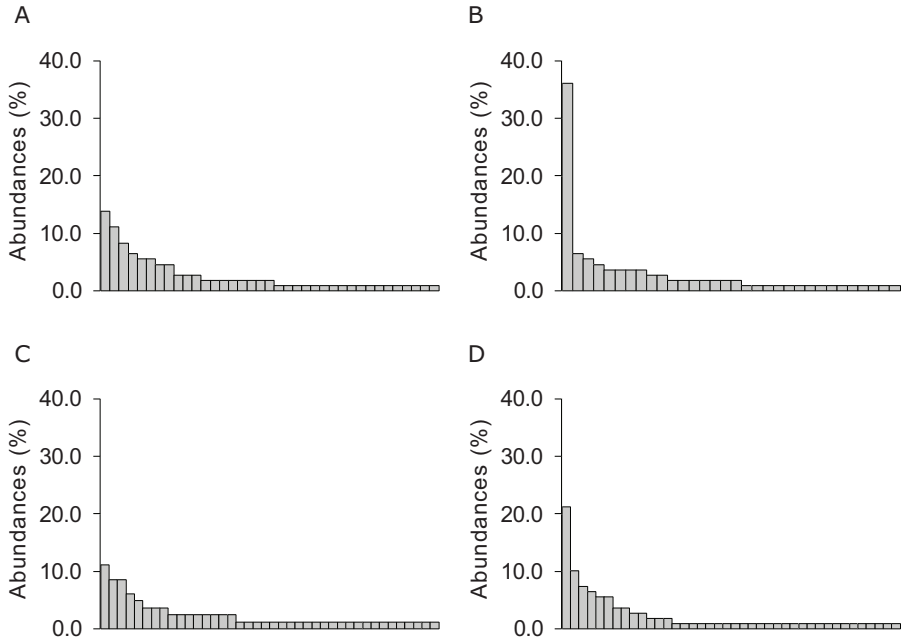

FIG. 2 - Rank-abundance plots. A: *Kobresia* soil, number of OTUs = 37, number of clones = 108; B: *Polygonum* soil, number of OTUs = 32, number of clones = 108; C: *Salix* soil, number of OTUs = 40, number of clones = 81; D: bare terrain, number of OTUs = 40, number of clones = 108.

Rank-abundance plots for the four soils (Fig. 2) confirmed the general trend that most of the observed OTUs occurred with low abundances. Therefore, we had to define OTUs as common when they either represented > 3% of the respective clone library or were detected as ≥ 4 clones per clone library.

Fungal communities of soils with different plant cover and of bare terrain

Soils with Kobresia *cover*

Out of the 37 detected OTUs in soils with *Kobresia* cover (Table 2), eight OTUs occurred with an abundance of more than 3.0%. These eight OTUs were: OTU 1 (*Nectria* sp.), OTU 2 (*Nectria mauritiicola* 1), OTU 3 (*Verticillium* sp.), OTU 5 (*Cladosporium cladosporioides*), OTU 8 (*Leptodontidium orchidicola* 1), OTU 9 (*Phoma* cf. *eupyrena)*, OTU 31 (*Mortierella* sp. 3) and OTU 46 (*Tetracladium maxilliforme*) (Fig. 3). All eight common OTUs were found in the clone library generated from the un-pooled DNA extract (*Kobresia* I), seven of the common OTUs were found in *Kobresia* IV, six OTUs in *Kobresia* II and four OTUs in *Kobresia* III. OTU 2 showed the highest abundances in soils with *Kobresia* cover. It yielded an abundance of 13.89% in all four *Kobresia*-clone libraries together, but it was detected only in two of the four

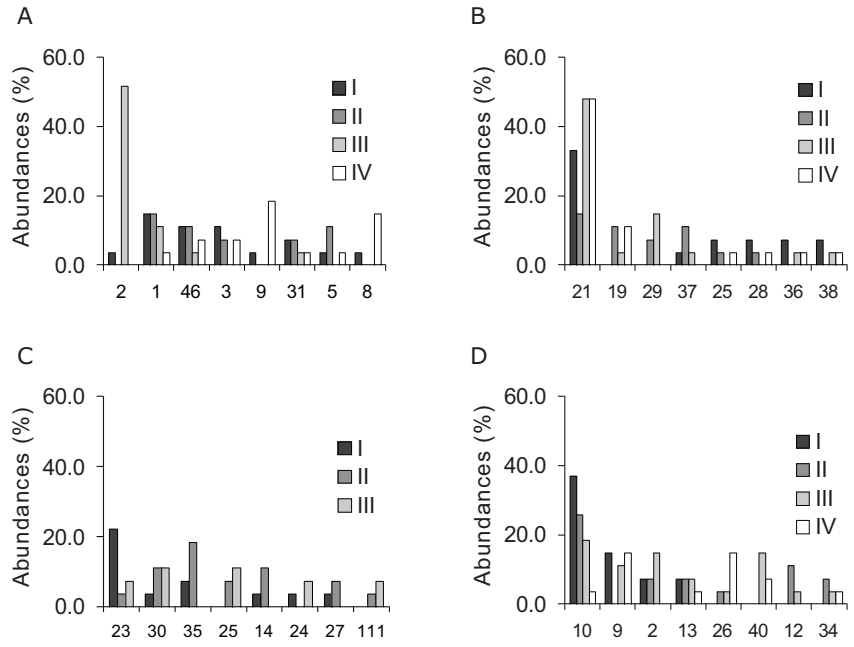

FIG. 3 - Abundances of the most common OTUs (≥ 4 clones) for the different clone libraries generated for *Kobresia* soil (A), *Polygonum* soil (B), *Salix* soil (C) and bare terrain (D). For identification of the respective OTU number please refer to Table 2.

clone libraries: in *Kobresia* I it was detected with an abundance of 3.70 %, in *Kobresia* IV with an abundance of 51.85%. OTUs 1, 31 and 46 were detected in all four *Kobresia*-clone libraries. OTUs 3, 5 and 55 (*Cordyceps crassispora*) were detected in three *Kobresia*-clone libraries, whereas OTU 55 was detected in three clone libraries with only one individual respectively (3.70% respectively). OTUs 2 and 9 were common also in bare terrain; all other OTUs were found frequently in soils with *Kobresia* cover only.

Soils with Polygonum *cover*

Out of the 32 detected OTUs in soils with *Polygonum* cover (Table 2), eight OTUs occurred with an abundance of more than 3.0%, namely OTU 19 (*Mortierella* sp. 2), OTU 21 [uncultured ectomycorrhiza (ECM) *Thelephoraceae* 2], OTU 25 (uncultured Helotiales 1), OTU 28 (uncultured ECM *Inocybe* 2), OTU 29 (*Cenococcum geophilum*), OTU 36 (*Inocybe rufofusca* 1), OTU 37 (*Inocybe rufofusca* 2) and OTU 38 (*Sebacina* sp.) (Fig. 3). Six of these eight common OTUs occurred in all four *Polygonum*-clone libraries respectively. OTU 21 showed the highest abundances in soils with *Polygonum* cover altogether (36.11% of all clones in soils with *Polygonum* cover) and also in every one of the four clone libraries respectively (48.15% of the clones in *Polygonum* III and *Polygonum* IV respectively, 33.33% of the clones in *Polygonum* I and 14.81% of the clones in *Polygonum* II). Besides OTU 21 no other OTU was detected in all four *Polygonum*-clone libraries. Seven OTUs were detected in three *Polygonum*-clone libraries: OTU 16 (uncultured fungus 2), OTU 19, OTU 25, OTU 28, OTU 36, OTU 37 and OTU 38, whereas OTU 16 was detected in three clone libraries with only one individual respectively. OTU 25 was common also in *Salix* soils, all other OTUs were found frequently in soils with *Polygonum* cover only.

Soils with Salix *cover*

Out of the 40 detected OTUs in soils with *Salix* cover (Table 2), eight OTUs occurred with an abundance of more than 3.0%, namely OTU 14 (*Exophiala salmonis* 3), OTU 23 (*Tomentella* sp.), OTU 24 (uncultured Helotiales 3), OTU 25 (uncultured Helotiales 1), OTU 27 (*Mortierella alpina* 1), OTU 30 (*Cortinarius rubricosus*), OTU 35 (*Thelephora caryophyllea*) and OTU 111 (*Hebeloma ammophilum)* (Fig. 3). All eight common OTUs occurred in *Salix* II, four of these five OTUs in *Salix* I and three OTUs in *Salix* III. OTU 23 showed the highest abundances in soils with *Salix* cover (11.11% of all clones in soils with *Salix* cover). OTUs 23 and 30 occurred in all three *Salix*-clone libraries.

Bare terrain

Out of the 40 detected OTUs in bare terrain (Table 2), eight OTUs occurred with an abundance of more than 3.0%, namely OTU 2 (*Nectria mauritiicola* 1), OTU 9 (*Phoma* cf. *eupyrena*), OTU 10 (*Mycocentrospora acerina*), OTU 12 (*Bovista cretacea*), OTU 13 (*Ramularia pratensis*), OTU 26 (*Sebacina incrustans*), OTU 34 (ascomycete sp. 1) and OTU 40 (uncultured basidiomycete) (Fig. 3). All eight common OTUs were found in bare terrain III. Six of these eight OTUs were found in bare terrain II and bare terrain IV respectively. Only four of the common OTUs were detected in bare terrain I. OTU 10 showed the highest abundances in bare terrain (21.30% of all clones in bare terrain). It occurred in all four bare terrain-clone libraries. In bare terrain I-III it was the most frequently detected OTU with abundances of 37.04, 25.93 and 18.52% respectively. In bare terrain IV it was detected with an abundance of 3.70%. Besides OTU 10 also OTU 13 was detected in all four bare terrain-clone libraries. OTUs 2, 9, 26 and 34 were detected in three out of four bare terrain-clone libraries.

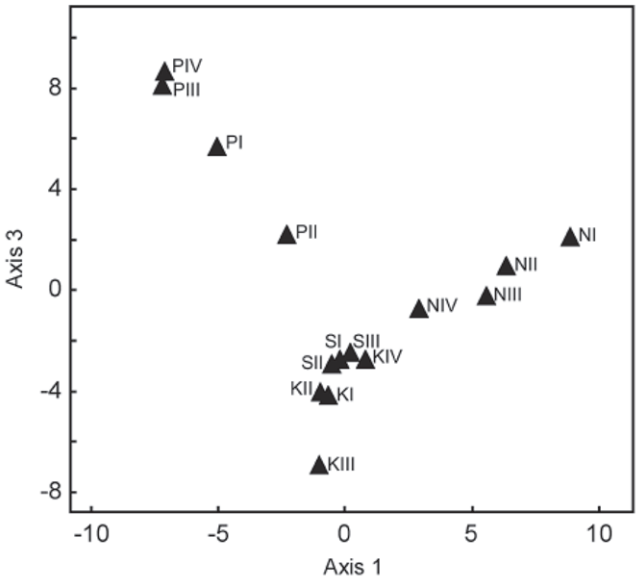

FIG. 4 - Principal Components Analysis (PCA) of DNA extracts (I) and pooled DNA extracts (II-IV) of four investigated soils (K = *Kobresia*, P = *Polygonum*, S = *Salix*, N = bare terrain). Axis 1 and axis 3 explain 34.926 and 14.788% of variance respectively.

Species richness and diversity of clone libraries

The respective clone libraries yielded a mean of 14.8 OTUs with values ranging between ten and 19 OTUs detected (Table 1). Species richness (as number of OTUs) was not affected by the number of pooled DNA extracts, nor by dsDNA concentration in this low concentration range: clone libraries from bare terrain with very low DNA concentrations yielded the same species richness (14.5 ± 2.5 OTUs) as clone libraries from *Kobresia* soil containing twice the amount of DNA (14.8 ± 3.2 OTUs).

Shannon diversity indices and equitability values (Table 1) did not differ significantly between clone libraries ($p = 0.748$ and $p = 0.748$). E_H was > 0.70 in most clone libraries, showing that OTUs were comparatively evenly distributed (exceptions: *Kobresia* III with OTU 2 dominating, *Polygonum* III and IV with OTU 21 dominating and bare terrain I with OTU 10 dominating). This pattern could be approved by using calculating a Principal Components Analysis (PCA; Fig. 4). The PCA showed that all clone libraries group together according to the plot. Clone libraries with one dominating OTU (as mentioned above: *Kobresia* III, *Polygonum* III and IV and bare terrain I) are more distinct from the other clone libraries of the same plot. Because of the even distribution of all OTUs, *Kobresia* I, II and IV and *Salix* I to III grouped together.

Comparing OTUs of clone libraries

Most OTUs detected in our clone libraries had low abundances, were rare and had a scattered distribution (Fig. 2) considering all clone libraries from this alpine site, 62 of the 120 OTUs (51.67%) were singletons (OTUs detected once). The percentage of singletons ranged from 46.88% in *Polygonum* soils to 67.50% in bare terrain, while the percentage of OTUs occurring in all four clone libraries from one plot ranged from 3.13% in *Polygonum* soils to 8.11% in *Kobresia* soils. Only eight OTUs were detected

frequently (> 3%) for each plot respectively (Fig. 3) and altogether only three OTUs were found frequently in two different soils (OTUs 2, 9 and 25). One to three OTUs were detected in each of the four clone libraries of a plot (OTUs 1, 31 and 46 for the *Kobresia* plot, OTU 21 in the *Polygonum* plot, and OTUs 10 and 13 for bare soil plots), two to four species were detected in three clone libraries from one plot (e.g. OTUs 23 and 30 for three *Salix* plots).

DISCUSSION

Primary successional sites have low concentrations of dsDNA

The concentrations of dsDNA in the different investigated alpine soil samples ranged from 3.02 µg to 11.42 µg. These DNA concentrations are low compared to forest soils (3.0 µg to 240.0 µg; O'Brien *et al*., 2005:), cultivated field soils (10.5 µg to 40.5 µg; Ranjard *et al*., 2003) and other soil types (2.0 µg to 20.1 µg in eight different soils; Zhou *et al*., 1996).

Kobresia soils showed significantly higher dsDNA concentrations than all other plots. Soil development is most advanced in *Kobresia* soils, and these dense plant cushions were characterised by the largest soil thickness (> 5 cm).

Spatial heterogeneity of fungal mycelia in the soil was indicated by different DNA concentrations of extracts from the same plot. Heterogeneity of DNA concentrations is generally most pronounced in soils with higher concentrations of fungal propagules/hyphae.

Does pooling of DNA extracts affect diversity of clone libraries?

To the best of our knowledge, this study is the first to investigate whether pooling of DNA extracts prior to cloning affects the analysis of fungal communities of an alpine habitat. Irrespective of pooling, no significant differences of species richness (number of OTUs) were detected between clone libraries: Species richness ranged between ten and 19 OTUs per clone library. Also Shannon diversity indices and equitability values were not affected by pooling of DNA extracts. Both showed about the same values in all clone libraries. In a PCA all clone libraries of the same plot grouped together, showing that soil fungal communities were similar.

We expected to find the same most abundant OTUs in all clone libraries of un-pooled and pooled DNA extracts of the investigated plots. However, due to the unexpected high diversity we found few OTUs occurring in all clone libraries from one plot (for *Kobresia* soils OTUs 1, 31 and 46, for *Polygonum* soils OTU 21, for *Salix* soils OTUs 23 and 30 and for bare soil OTUs 10 and 13). The majority of the 120 detected OTUs were only found in two or three subsamples. The most conspicuous was the case of OTU 2 in *Kobresia* soils. It was the most abundant OTU in clone libraries originating from this soil, but it was detected just in two subsamples: in *Kobresia* I, where it was found once, and in *Kobresia* III, where it represented approximately 50 % of the screened clones (Figure 3). This phenomenon can be explained by the generally low abundances of OTUs in these samples. Soil fungal communities of primary successional sites mainly consist of rare species with scattered distribution, thus, the number of clones analysed was probably not enough to completely categorize the community variability of each soil sample (Franklin and Mills, 2003). A higher number of clones could probably have lead to the detection of common species in all clone libraries.

Soil fungal communities of old soils with long established soil fungal communities, comprise few fungal species occurring with high abundances (e.g. Neubert *et al*., 2006; Peintner *et al*., 2007). The abundant, competitive fungal species dominate leading to comparatively low species richness and a low evenness. In soils with highly dominant species, rare species can often be masked or are difficult to detect with a reasonable amount of analysed clones. In contrast, in soils with mostly rare and no dominating species it is more likely to obtain a high number of species. The larger the soil sample size containing mostly rare species, the higher the number of OTUs detected with several cloning approaches (Ranjard *et al*., 2003).

Until better knowledge is available, we interpret our results as a characteristic of soils with very low DNA concentrations without dominating species: reproducibility is generally low in communities consisting predominantly of rare species. Summarising the above mentioned considerations, we did not see any effect on the detection of species richness and diversity by using un-pooled and pooled DNA extracts.

Rare species predominate in primary successional ecosystems

Receeding glaciers are valuable sites for studying primary succession and establishment of fungal communities. Cherrett (1989), who made a list of the 20 most important ecological concepts, set succession on position two of this list. He regarded only the ecosystem itself as more important. Low DNA concentrations and low fungal biomass, as detected in this study, appears to be one general characteristic of primary successional soil (Jumpponen, 2003). Moreover, fungal community assembly of glacier forefront ecosystems has a significant component of aerially deposited, dormant spore bank in the soil (Jumpponen, 2003). We speculate that this is probably the main reason for the predominance of "rare" OTUs (singletons) in our alpine soil. This also explains the high number of OTUs detected in bare terrain compared to soils with plant cover. In forest soil or other old stages of soil development with higher total DNA concentrations, such dormant spore banks are masked by more abundant, actively growing fungal mycelia.

Fungal lineages without reference sequences - are they new?

Detection of fungal lineages with no reference sequences in databases could be either unknown/new fungal taxa, or fungal taxa with no, inconspicuous or hypogeous fruit bodies. Moreover, many representatives of described fungal taxa have never been sequenced up to now, and/or are very difficult to collect and have therefore never been sequenced. One example is the Ascomycete *Moserella radicicola*, a hypogeous, apothecia-forming taxon without known affinities described from Austria (Pöder and Scheuer, 1994). Even after a concerted sequencing effort in fungi (Blackwell *et al*., 2006), and several barcoding initiatives (Dooh and Hebert, 2005), sequences from many fungal groups are still underrepresented in public databases relative to other fungal phyla (Vilgalys, 2003).

Conclusion and outlook

The main aim of the current investigation was to understand whether in these alpine soils with low DNA concentration, analysis of un-pooled and pooled DNA extracts would lead to different results in the investigation of soil fungal communities. We expected to detect the same diversity and abundant species in all approaches. Our experiment showed that the most abundant species were captured with both un-pooled and pooled DNA

extracts. We conclude that pooling of DNA extracts before cloning did not affect the diversity or the species richness of the resulting clone library.

Acknowledgments

We sincerely thank Ian Anderson and his team for assistance and guidance in the lab and for methodological training at the Macaulay Institute in Aberdeen. Regina Kuhnert-Finkernagel is acknowledged for her help with the analyses of soil parameters, and Margit Egg for her help with measuring the dsDNA concentrations. Thanks to Sebastian Waldhuber for determining the total amount of C and N in the different soils and to Rüdiger Kaufmann for providing soil temperature and moisture data of the Rotmoosferner glacier forefront.

The presented study was funded by the FWF Project P17910-B03 "Seasonal dynamics of underground fungal communities in an alpine environment".

REFERENCES

Barratt B.J., Payne F., Rance H.E., Nutland S., Todd J.A., Clayton D.G. (2002). Identification of the sources of error in allele frequency estimations from pooled DNA indicates an optimal experimental design. Ann. Hum. Genet., 66: 393-405.

Blackwell M., Hibbett D.S., Taylor J.W., Spatafora J.W. (2006). Research Coordination Networks: a phylogeny for kingdom Fungi (Deep Hypha). Mycologia, 98: 829-837.

Cherrett J.M. (1989). Key concepts: the results of a survey of our members' opinion. In: Cherrett J.M., Ed., Ecological Concepts, Blackwell, Oxford, pp. 1-161.

Dooh R., Hebert P.D.N. (2005). The Canadian Barcode of Life Network. URL http://www.bolnet.ca

Erschbamer B., Bitterlich W., Raffl C. (1999). Die Vegetation als Indikator für die Bodenbildung im Gletschervorfeld des Rotmoosferners (Obergurgl, Ötztal). Berichte der Naturwissenschaftlich-Medizinischen Vereins in Innsbruck, 86: 107-122.

Franklin R.B., Mills A.L. (2003). Multi-scale variation in spatial heterogeneity for microbial community structure in an eastern Virginia agricultural field. FEMS Microbiol. Ecol., 44: 335-346.

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

Genney D.R., Anderson I.C., Alexander I.J. (2006). Fine-scale distribution of pine ectomycorrhizas and their extramatrical mycelium. New Phytol., 170: 381-390.

Giller K.E., Beare M.H., Lavelle P., Izac A.M.N., Swift M.J. (1997). Agricultural intensification, soil biodiversity and agroecosystem function. Appl. Soil Ecol., 6: 3-16.

Hammesfahr U. (2002). Einfluss der Vegetation auf die mikrobielle Besiedlung des Gletschervorfeldes Rotmoosferner. In Institut für Landschafts- und Pflanzenökologie, University of Hohenheim, Germany.

Hoinkes G., Thöni M. (1993). Evolution of the Ötztal-Stubai, Scarl-Campo and Ulten basement units. In: Raumer J.F., Neubauer M., Eds., Pre-Mesozoic Geology in the Alps, Springer, Berlin.

Hughes J.B., Hellmann J.J., Ricketts T.H., Bohannan B.J.M. (2002). Counting the uncountable: Statistical approaches to estimating microbial diversity. Appl. Environ. Microbiol., 68: 448-448.

Jumpponen A. (2003). Soil fungal community assembly in a primary successional glacier forefront ecosystem as inferred from rDNA sequence analyses. New Phytol., 158: 569-578.

Kang S., Mills A.L. (2006). The effect of sample size in studies of soil microbial community structure. J. Microbiol. Meth., 66: 242-250.

Kaufmann R. (2001). Invertebrate succession on an Alpine glacier foreland. Ecology, 82: 2261-2278.

Kaufmann R. (2002) Glacier foreland colonisation: distinguishing between short-term and long-term effects of climate change. Oecologia, 130: 470-475.

Kaufmann R., Raffl C. (2002). Diversity in primary succession: The chronosequence of a glacier foreland. In: Körner C., Spehn E., Eds., Global Mountain Biodiversity: A Global Assessment, Parthenon, London, pp. 179-192.

Kaufmann R., Fuchs M., Gosterxeier N. (2002). The soil fauna of an Alpine glacier foreland: Colonization and succession. Arct. Antarct. Alp. Res., 34: 242-250.

Kirk J.L., Beaudette L.A., Hart M., Moutoglis P., Khironomos J.N., Lee H., Trevors J.T. (2004). Methods of studying soil microbial diversity. J. Microbiol. Meth., 58: 169-188.

Kjõller R. (2006) Disproportionate abundance between ectomycorrhizal root tips and their associated mycelia. FEMS Microbiol. Ecol., 58: 214-224.

Kõljalg U., Larsson K.H., Abarenkov K., Nilsson R.H., Alexander I.J., Eberhardt U., Erland S., Høiland K., Kjøller R., Larsson E., Pennanen T., Sen R., Taylor A.F., Tedersoo L., Vrålstad T., Ursing B.M. (2005). UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytol., 166: 1063-1068.

Martin-Laurent F., Philippot L., Hallet S., Chaussod R., Germon J.C., Soulas G., Catroux G. (2001) DNA extraction from soils: Old bias for new microbial diversity analysis methods. Appl. Environ. Microbiol., 67: 4397-4397.

McCune B., Mefford M.J. (1999). PC-Ord. Multivariate analysis of ecological data. Version 5.0. MjM Software, Gleneden Beach, Oregon, USA.

McCune B., Grace J.B. (2002). Analysis of ecological communities. MjM Software design, Gleneden Beach, Oregon, USA.

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

Mühlmann O., Peintner U. (2008a). Mycobionts of *Salix herbacea* on a glacier forefront in the Austrian Alps. Mycorrhiza, 18 (4): 171-180.

Mühlmann O, Peintner U. (2008b). Ectomycorrhiza of *Kobresia myosuroides* at a primary successional glacier forefront. Mycorrhiza, in press. doi:10.1007/s00572-008-0188-z.

Neubert K., Mendgen K., Brinkmann H., Wirsel S.G.R. (2006). Only a few fungal species dominate highly diverse mycofloras associated with the common reed. Appl. Environ. Microbiol., 72: 1118-1128.

Nicol G.W., Tscherko D., Embley T.M., Prosser J.I. (2005). Primary succession of soil Crenarchaeota across a receding glacier foreland. Environ. Microbiol., 7: 337-347.

O'Brien B.L., Parrent J.L., Jackson J.A., Moncalvo J.M., Vilgalys R. (2005). Fungal Community Analysis by Large-Scale

Sequencing of Enviromental Samples. Appl. Environ. Microbiol., 71: 5544-5550.

Peintner U., Iotti M., Klotz P., Bonuso E., Zambonelli A. (2007). Soil fungal communities in a *Castanea sativa* (chestnut) forest producing large quantities of *Boletus edulis* sensu lato (porcini): where is the mycelium of porcini? Environ. Microbiol., 9: 880-889.

Pöder R., Scheuer C. (1994). *Moserella radicicola* gen. et spec. nov., a new hypogeous species of Leotiales on ectomycorrhizae of *Picea abies*. Mycol. Res., 98: 1334-1338.

Raffl C. (1999). Vegetationsgradienten und Sukzessionsmuster in einem zentralalpinen Gletschervorfeld (Ötztaler Alpen, Tirol). In Department of Botany. Innsbruck, Austria: University of Innsbruck, Austria.

Ranjard L., Lejon D.P.H., Mougel C., Schehrer L., Merdinoglu D., Chaussod R. (2003). Sampling strategy in molecular microbial ecology: influence of soil sample size on DNA fingerprinting analysis of fungal and bacterial communities. Environ. Microbiol., 5: 1111-1120.

Schinner F., Öhlinger R., Kandeler E., Margesin R., Eds (1996). Methods in Soil Biology, Springer-Verlag, Berlin, Heidelberg.

Sham P., Bader J.S., Craig I., O'Donovan M., Owen M. (2002). DNA pooling: A tool for large-scale association studies. Nat. Rev. Genet., 3: 862-871.

Southworth D. (2000). RFLP Protocol Sheet. URL http://www.sou.edu/BIOLOGY/Faculty/Southworth/RFLP.htm

Taylor A.F.S. (2002). Fungal diversity in ectomycorrhizal communities: sampling effort and species detection. Plant Soil, 244: 19-28.

Tscherko D., Rustemeier J., Richter A., Wanek W., Kandeler E. (2003). Functional diversity of the soil microflora in primary succession across two glacier forelands in the Central Alps. Eur. J. Soil Sci., 54: 685-696.

Tscherko D., Hammesfahr U., Zeltner G., Kandeler E., Böcker R. (2005). Plant succession and rhizosphere microbial communities in a recently deglaciated alpine terrain. Basic Appl. Ecol., 6: 367-383.

Vilgalys R. (2003) Taxonomic misidentification in public DNA databases. New Phytol., 160: 4.

von Wintzingerode F., Gobel U.B., Stackebrandt E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev., 21: 213-229.

White T.J., Bruns T., Lee S., Taylor J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Michael A.I., Gelfand D.H., Sninsky J.J., White T.J., Eds., PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc., New York, pp. 315-322.

Zhou J.Z., Bruns M.A., Tiedje J.M. (1996) DNA recovery from soils of diverse composition. Appl. Environ. Microbiol., 62: 316-322.