Mycorrhiza (2008) 18:339–354
DOI 10.1007/s00572-008-0189-y

ORIGINAL PAPER

Diversity and structure of ectomycorrhizal and co-associated fungal communities in a serpentine soil

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Received: 12 February 2008 / Accepted: 9 July 2008 / Published online: 3 August 2008

Abstract The community of ectomycorrhizal (ECM) and co-associated fungi from a serpentine site forested with *Pinus sylvestris* and *Quercus petraea* was explored, to improve the understanding of ECM diversity in naturally metalliferous soils. ECM fungi were identified by a combination of morphotyping and direct sequencing of the nuclear ribosomal internal transcribed spacer region 2 and of a part of the large-subunit region. Co-associated fungi from selected ECM were identified by restriction fragment length polymorphism and sequencing of representative clones from libraries. Polymerase chain reaction with species-specific primers was applied to assess patterns of association of ECM and co-associated fungi. Twenty ECM species were differentiated. Aphyllophoralean fungi representing several basidiomycete orders and Russulaceae were dominant. *Phialocephala fortinii* was the most frequently encountered taxon from the diverse assemblage of ECM co-associated fungi. A ribotype representing a deeply branching ascomycete lineage known from ribosomal deoxyribonucleic acid sequences only was detected in some ECM samples. A broad taxonomic range of fungi have the potential to successfully colonise tree roots under the extreme edaphic conditions of serpentine soils. Distribution patterns of ECM-co-associated fungi hint at the importance of specific inter-fungal interactions, which are hypothesised to be a relevant factor for the maintenance of ECM diversity.

Keywords *Pinus sylvestris* · *Quercus petraea* · Ectomycorrhiza · ECM-co-associated fungi · *Phialocephala fortinii* · Serpentine · Rhizosphere · Diversity · Heavy metal toxicity

Electronic supplementary material The online version of this article (doi:10.1007/s00572-008-0189-y) contains supplementary material, which is available to authorized users.

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Introduction

Ectomycorrhizal (ECM) fungi (ECMF) are essential for host tree nutrition and growth, particularly in extreme environments. Among the benefits of mycorrhizal symbioses, the amelioration of toxicity in metalliferous soils has received particular interest. So far, most studies have focussed on anthropogenically polluted sites (Leyval et al. 1997; Markkola et al. 2002; Colpaert et al. 2004; Adriaensen et al. 2005, 2006), with biotechnological applications such as phytoremediation (Suresh and Ravishankar 2004; Krupa and Kozdrój 2007) in mind, while the question of ECM diversity in naturally metalliferous soils has been less studied, even though they are the primary candidate sites for the evolution of adaptations to heavy metal toxicity (Ernst 2000). Recent investigations of ECM communities

of serpentine (Panaccione et al. 2001; Moser et al. 2005) revealed specific assemblages of ECMF, apparently paralleling the patterns well known from vascular plants.

The serpentinite sites around Redlschlag and Bernstein (Austria) are part of a series of serpentine sites found along the Alps and the Balkans (Wenzel and Jockwer 1999). Serpentinite is a metamorphic rock, composed partly of the phyllosilicate serpentine ($(Mg,Fe)_3Si_2O_5(OH)_4$, magnesium iron silicate hydroxide). Due to its origin from earth mantle material, the chemistry of serpentine is unlike that of other minerals in the earth's crust. Serpentine is low in the plant nutrients K^+ and Ca^{2+} but contains high levels of potentially toxic elements such as Ni^{2+} and Cr^{3+}. Ni^{2+} is more bioavailable than Cr^{3+} (Barceloux 1999) and appears to be the most toxic element in the study site (Wenzel and Jockwer 1999) and in other ultramafic soils (Aggangan et al. 1998; Amir and Pineau 1998).

In plants, Ni^{2+} may competitively inhibit the uptake of divalent cations such as Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+} thereby inducing deficiencies that can result in characteristic plant chlorosis symptoms and reduced efficiency of photosynthesis (Marschner 1995). Ni^{2+} can impede root elongation and cell division in the root meristem and can disturb the plants control of the transpiration stream. In general, heavy metals can precipitate phosphate, which may decrease phosphorus availability (Gadd 1993).

Further stress factors characteristic of serpentine soils are an unfavourable Ca/Mg ratio (commonly about 0.1) and, particularly on slopes, phenomena associated with poor soil development, like high mobility, low water retention potential and low organic matter content (Brady et al. 2005).

Altogether, these factors severely restrict plant and microbial growth in serpentine sites and select for metal tolerance (Amir and Pineau 1998). As a result, plants that live on serpentinite are adapted to survive under these unusual chemical conditions, and many serpentinite sites host endemic plants (e.g. *Thlaspi goesingense*, *Alyssum murale*). The tree floras of many serpentine sites in the northern hemisphere are dominated by *Pinus* spp., which are rarely competitive on richer soils. Some evidence suggesting genetic adaptation to serpentine soils has been found for *P. ponderosa* (Wright 2007), *P. contorta* (Kruckeberg 1967), *P. balfouriana* (Oline et al. 2000) and *P. jeffreyi* (Furnier and Adams 1986), while the selection of serpentine ecotypes was not confirmed in *P. virginiana* (Miller and Cumming 2000).

The observation of specific ECM communities in serpentine soils (Panaccione et al. 2001; Moser et al. 2005) indicates that resistance to the adverse edaphic conditions of serpentine might also be acquired through the association with specialised mutualistic fungi. The high diversity and presumably shorter lifecycles of the fungal symbionts and their capability for long-distance spore transport are thought to increase their potential for genetic adaptation to heavy metal toxicity (Wilkinson and Dickinson 1995; Meharg and Cairney 2000; Markkola et al. 2002) compared to tree species.

In anthropogenically trace metal-polluted sites, several negative impacts on mycorrhizal communities are well documented. Extremely polluted sites are reported to have lower rates of mycorrhizal colonisation, fewer fungal propagules and lower fungus species diversity (Gadd 1993; Hartley et al. 1997; Leyval et al. 1997; Markkola et al. 2002). Heavy metal toxicity is a strong selection pressure leading to the evolution of specialised ECM genotypes (Hartley et al. 1997; Leyval et al. 1997; Markkola et al. 2002; Colpaert et al. 2004; Adriaensen et al. 2005), which can effectively alleviate the effects of heavy metal toxicity in their host trees (Jones and Hutchinson 1986; Dixon 1988; Dixon and Buschena 1988; Jones and Hutchinson 1988; Adriaensen et al. 2005, 2006) by providing a more balanced access to mineral elements, either by improving supply of essential elements or by reducing relative uptake of toxic elements (Marschner and Dell 1994). The 'toxic element filtering' hypothesis stating that at least some mycorrhizal fungi may protect host plants by limiting the transfer of toxic elements via the symbiotic exchange surfaces such as the Hartig net is well supported (Denny and Wilkins 1987; Turnau et al. 2001; Adriaensen et al. 2005).

The recent work of Kayama et al. (2006) demonstrates the complexity of the relationship of host resistance and ECM colonisation. Serpentine adapted *Picea glehnii* maintained a high level of ECM colonisation in serpentine soils, while ECM colonisation was found reduced in non-adapted *P. jezoensis* and *P. abies* in serpentine soils compared to control soils. This suggests that the adaptation to serpentine may depend upon the interaction of specialised genotypes of both host trees and ECMF.

Besides ECMF, ECM-co-associated fungi (i.e. fungi that can be detected in or attached to the ECM tissue) may also play a role in the host's performance in extreme environments, but the exact role of ECM-co-associated fungi is still barely known. The exploration of the ECM and co-associated fungal biodiversity of a naturally metalliferous soil may reveal organisms useful for bioremediation as well as potential model organisms for population studies and functional studies of adaptation to serpentine and Ni toxicity.

Materials and methods

Field site description and sampling of ectomycorrhizal roots

Samples were collected from a serpentine site in eastern Austria (16°18′52″ east, 47°26′21″ north), previously described as 'Redlschlag Ni/Cr site' by Wenzel and Jockwer (1999), who reported the soil characteristics: eutric leptosol,

pH_{CaCl2}6.55, $CaCO_3$ 19 g kg^{-1}, organic carbon 13 g kg^{-1}, C/N 16, cation exchange capacity 208 $mmol_{(+)}$ kg^{-1}, base saturation 100%, K_{ex} 4.1 $mmol_{(+)}$ kg^{-1}, Mg_{ex} 180 $mmol_{(+)}$ kg^{-1}, Mg_{ex}/Ca_{ex} 7.66, total (aqua regia extractable) Ni 2,580 mg kg^{-1}, total Cr 1,910 mg kg^{-1}, labile (1 M NH_4NO_3 extractable) Ni 5.81 mg kg^{-1}, labile Cr < 0.05 mg kg^{-1}.

Soil samples were taken along a transect of 150 × 20 m oriented in the slope line of a south–southwest-exposed versant where inclination (about 30–35%) impedes soil evolution and where high Ni values were recorded (Wenzel et al. 2003). Due to the shallow, eroded, sun-exposed soil poor in organic matter, drought may be an additional source of stress in this particular environment. The sampling site is sparsely wooded with old growth, autochthonous *Pinus sylvestris* and *Quercus petraea*; the scanty herb layer is composed of specialised serpentinophytes. For the identification of ECMF and cultivation-independent analysis of the ECM-co-associated fungal community, seven soil samples of 7 cm diameter and of about 8 cm depth were taken in June 2003. One thousand three hundred seventy seven ECM tips were analysed.

Identification of ECMF

ECM diversity was assessed both morphologically and molecularly. ECM tips were first sorted by morphotyping under a dissection microscope, assisted by inspection of representative mantle preparations with oil immersion microscopy (Agerer 1991). For each morphotype, samples of three to five fresh, thoroughly rinsed ECM tips were prepared for deoxyribonucleic acid (DNA) analysis and stored in DNA extraction buffer at −20°C, with several replicates for frequently occurring morphotypes, in order to control the reliability of the morphological classification. For isolation of DNA, ECM root tips were disrupted with Lysing Matrix A (Q BIOgene) in a FastPrep FP120 homogeniser (Q BIOgene). Further purification was done with the DNeasy Plant Min Kit (Qiagen). Recommended precautions were followed to prevent or detect potential cross-contaminations, i.e. frequent decontamination of lab surfaces, separation of pre- and post-polymerase chain reaction (PCR) workspace and reagents, use of filter tips, and inclusion of negative controls. Fungal-specific DNA was amplified with the primer pair internal transcribed spacer (ITS) 1F and TW13 (for primer features, see Table 1). In one case, where amplification with primer pair ITS1F/TW13 did not allow identification of the ECMF, primer pair ITS1F/ITS4B was applied. PCR products were separated on an agarose gel and major bands were excised from the gel, purified with the QIAEX II Gel Extraction Kit (Qiagen) and sequenced directly, using ITS3 and TW13 as sequencing primers. For details on primers for PCR amplification and sequencing, see Table 1. All amplifications were performed on a T3 Thermocycler (Biometra) with REDTaq ReadyMix PCR Reaction Mix (Sigma). The following thermocycling pattern was used: 95°C for 2 min and 30 s (one cycle); 94°C for 45 s—annealing *T* (adjusted according to melting temperatures as indicated in Table 1) for 45 s—72°C for 30 s to 1 min and 30 s (depending on the expected amplicon size, see Table 1; 35 cycles); and 72°C

Table 1 Properties of primers used for PCR and sequencing

Name	Sequence	Gene	Approx. amplicon size	T_m	Specificity	Reference
ITS1F	CTTGGTCATTTAGAGGAAGTAA	18S	ITS1F+TW13: ~1 kb	54	Fungi	(Gardes and Bruns, 1993a)
ITS3	GCATCGATGAAGAACGCAGC	5,8S			Eukaryota	(White et al., 1990)
ITS4	TCCTCCGCTTATTGATATGC	28S		58	Eukaryota	(White et al., 1990)
ITS4B	CAGGAGACTTGTACACGGTCCAG	28S	ITS1F+ITS4B: 0.8 kb	54	Basidiomycota	(Gardes and Bruns, 1993b)
TW13	GGTCCGTGTTTCAAGACG	28S		54	Eukaryota	(ODonnell, 1993)
nu-SSU-0817-5′	TTAGCATGGAATAATRRAATAGGA	18S	nu-SSU-0817-5′+Enig2: 1.6 kb	56	Fungi	(Borneman and Hartin, 2000)
Ph1	AGTGAGGCTACCGAACG	ITS1	Ph1+ITS4: 0.5 kb	58	*P. fortinii*, *A. applanata*	this study
Ph2	TGGAAACAGCGGTTAGGA	ITS1	Ph2+ITS4: 0.5 kb	58	*P. fortinii*, *A. applanata*	this study
Hym1	GGACGCTGGCCATCAACC	ITS1	Hym1+Hym2: 0.35 kb	65	*R. ericae*-aggr	this study
Hym2	CCGATGCTGGCCTGAACG	28S	Hym1+Hym2: 0.35 kb	65	*R. ericae*-aggr	this study
Cap1	CAATGACGGCGGCCTGTG	ITS2	Cap1+Cap2: 0.6 kb	65	*Capronia* sp.	this study
Cap2	ACCGATGTTGGCCTGGAC	28S	Cap1+Cap2: 0.6 kb	65	*Capronia* sp.	this study
Enig1	CGGACCGTTGGGTTGACC	ITS2	Enig1+Enig2: 0.3 kb	58	Enigmatic ascomyc.	this study
Enig2	ACCCGACTCTTCGAGGAC	28S	Enig1+Enig2: 0.3 kb	58	Enigmatic ascomyc.	this study

Gene Primer binding site in the ribosomal gene cluster

for 10 min (one cycle). Sequencing was done with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences) according to the manufacturer's instructions. Sequences were assembled and edited with VectorNTI sofware (Informax). Identification of sequenced fungi was based on the results of BLAST (Altschul et al. 1997) searches against the National Center for Biotechnology Information (NCBI) public database and subsequent phylogenetic placement. Species recognition was based on selected published DNA data regarded as authoritative and complemented with field observations on sporocarps. Identification at the species level was accepted if the query sequence and a reference sequence covering the most variable parts of the ITS2 region are identical (e.g. *Lactarius deliciosus*, Table 2) or nearly identical (typically 98% or 99% identity in the ITS2) and nested within a set of conspecific reference sequences (e.g. *Amanita citrina*, *Cenococcum geophilum*, Table 2). Thereby, species recognition was not based on an arbitrarily fixed threshold of sequence similarity, but information about the genetic variability of certain taxa (among them putative species complexes such as *C. geophilum*) was considered. Ribotype variants that could not be identified at the species level were regarded as representing different species, if they clustered with different lineages of a given kinship (Fig. 4). Vector NTI, ClustalX (Thompson et al. 1997), MAFFT (Katoh et al. 2005) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) were used to generate and edit alignments.

Phylogenetic analyses were performed with RAxML 7.0.0 (Stamatakis 2006) or with the Phylip package included in ARB (Ludwig et al. 2004).

Statistics

Species accumulation curves (Mao Tau) and estimators of species richness (Chao2, incidence-based coverage estimator, first-order and second-order Jackknife richness estimator, Bootstrap richness estimator and Michaelis–Menten richness estimators) were calculated with EstimateS (Version 8.0, R. K. Colwell, http://purl.oclc.org/estimates), either analytically or using 500 randomised runs without sample replacement. Patterns of association of ECM and co-associated fungi as revealed by the screening with specific primers were assessed using binomial statistics.

ECM ITS/LSU clone libraries

Fungal-specific PCR products from selected ECM samples were prepared as described for the identification of ECMF, then ligated individually into pCR4-TOPO (Invitrogen). *Escherichia coli* TOP10 (Invitrogen) was transformed with the ligation products according to the manufacturer's instructions. Depending on the total number of colonies obtained, 14 to 96 individual clones were picked. The inserts were amplified with the primers ITS1F and TW13 and cut with the restriction endonuclease *Bsu*RI (Fermentas), and the resulting fragments were separated on a high-resolution agarose gel. Representative clones of each restriction fragment pattern were sequenced with primers ITS3 and TW13. Downstream analysis of obtained sequences was done as described for the identification of ECMF. Sequence alignments were checked for the presence of chimeras using the Bellerophon server (Huber et al. 2004).

Detection of fungi with specific primers

Presence or absence of selected ribotypes of fungi co-associated with ECM samples was assessed by nested PCR amplifications with specific primers (see Table 1) following a first-round PCR with the primers ITS1F and TW13. PCR conditions were as described above. Specific primers were designed based on alignments including sequences from the clone libraries and BLAST search results, aided by the software FastPCR (Kalendar 2006). Primer specificity and efficiency were assessed in several ways: (1) BLASTn searches with search parameters adjusted for short input sequences, to test primer specificity against published sequence date, (2) comparison of direct and nested PCR to check the consistency of specific amplifications, (3) comparison of the amplification from ECM samples with different primer sets designed for the same taxon (*Phialocephala* primers only), (4) comparison of results from cloning and from PCR amplification from ECM samples and (5) restriction fragment length polymorphism (RFLP) digests and/or sequencing of specific PCR products, checking homogeneity and identity of amplicons.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the NCBI database under accession numbers EU046002–EU046087 and EU103612.

Results

Ectomycorrhizal fungi identified by DNA sequence analysis

From the seven soil samples, 29 morphologically preselected ECM samples were further processed for direct sequencing of the ECMF. The 29 ECM samples comprised 26 pine ECM and three oak ECM. This bias resulted most likely from the small sampling depth (8 cm), since the uppermost soil layer is predominantly occupied by fine roots of pine at that site. A total of 20 different ECMF could be identified by sequence analysis; 18 ECMF were

Table 2 Identification of ectomycorrhizal fungi by direct sequencing

RSEM no.	Tree	PCR	Identification	Acc.	BLAST ITS2+LSU % ID Acc.	BLAST ITS2 % ID Acc.	BLAST LSU % ID Acc.
27	Ps		*Cenococcum geophilum*	EU046029	98 AY394919	98 DQ474383	99 AY394919
01	Ps	a	*Sebacina* sp.	EU046002	99 AF465191	99 AF465191	99 AF465191
		b	Ascomycetes Group I sensu Schadt et al. (2003)	EU046003	93 AY394904	97 DQ068979	92 AY394904
06	Ps		Thelephoraceae 1 *(Thelephora* sp. ?)	EU046008	94 EF433965	94 AY969784	98 DQ835997
08	Ps		Thelephoraceae 1 *(Thelephora* sp. ?)	EU046009	94 EF433965	94 AY969784	98 DQ835997
10	Ps		Thelephoraceae 1 *(Thelephora* sp. ?)	EU046011	93 EF433965	94 AY969784	98 DQ835997
18	Ps		Thelephoraceae 1 *(Thelephora* sp. ?)	EU046021	93 EF433965	94 AY969784	96 EF433977
19	Ps		Thelephoraceae 1 *(Thelephora* sp. ?)	EU046022	93 EF433965	93 AY969784	96 EF433977
11	Ps		Thelephoraceae 2 *(Thelephora* sp. ?)	EU046012	93 EF433965	94 AY969784	98 DQ835997
13	Ps		*Tomentella* aff. *cinerascens*	EU046014	94 EF434149	99 AJ893302	97 AY586717
34	Qp		*Tomentella* aff. *lilacinogrisea*	EU046032	96 EF434149	100 DQ990853	98 AY586717
09	Ps		*Byssocorticium atrovirens*	EU046010		99 AJ889936	99 DQ273484
16	Ps	a	Atheliaceae	EU046017			97 AY751568
		b	*Phialocephala fortinii*	EU046018		98 EF433956	Missing
		c	Ascomycetes Group I sensu Schadt et al. (2003)	EU046019	91 AY394904	100 DQ068979	92 AY394904
15	Ps	a	*Cantharellus lutescens*	EU046015		missing	100 AF105304
		b	*Phialocephala fortinii*	EU046016	99 AY394921	100 AY394921	99 AY394921
26	Ps		*Cantharellus lutescens*	EU046028		99 AY082606	99 AF105304
30	Ps		cf. *Sistotrema alboluteum*	EU046030	97 AJ606042	96 AJ606043	98 AJ606042
02	Ps		*Russula* subg. *amoenula*	EU046004	89 DQ422018	91 AY061655	98 AB154742
22	Qp		*Russula* subg. *amoenula*	EU046025	91 DQ422018 89	AJ633583	97 AF287888
12	Ps		*Russula* subg. *ingratula* serie *pectinata* (*R. amoenolens* ?)	EU046013	92 DQ422026	99 AJ438037	99 AF325295
17	Ps		Related to *Russula* subsect. *laricinae*	EU046020	97 EF434048	96 AY061685	99 EF434048
20	Ps		Related to *Russula* subsect. *laricinae*	EU046023	97 EF434048	96 AY061685	98 EF434048
21	Ps		related to *Russula* subsect. *laricinae*	EU046024	97 EF434048	96 AY061685	98 EF434048
04	Ps		*Lactarius deliciosus*[a]	EU046006	96 DQ422002	100 AF249283	98 AF325280
05	Ps		*Lactarius deliciosus*[a]	EU046007	96 DQ422002	100 AF249283	98 AF325280
25	Qp		*Lactarius azonites*	see Table 3	95 AF335441	99 AY606954	98 AF325283
03	Ps		*Scleroderma* sp.	EU046005		100 DQ402508	97 DQ644138
23	Ps		*Xerocomus* aff. *subtomentosus*[a]	EU046026		Missing	98 AF514832
07	Ps	ITS1F+ITS4B	*Tricholoma albobrunneum*[a]	see Table 3	ITS1+ITS2	99 AB036894	
24	Ps		*Amanita citrina*[a]	EU046027		99 DQ990869	98 AF024446
33	Ps		*Entoloma nidorosum*[a]	EU046031		97 AJ938003	99 AF261296

Primer pair ITS1F+ITS4B was only used for amplification of the ECMF in sample RSEM07 (see text). In all cases, the highest scoring BLAST hit (Acc.) with the percentage identity is given. *RSEM no*. ECM sample number, *Tree*: *Ps Pinus sylvestris*, *Qp Quercus petraea*, *PCR* indication of individual bands on an agarose gel after PCR amplification with primer pair ITS1F/TW13 (a, b, c), *Identification* classification of the ECMF based on BLAST search results for ITS2 and LSU regions and phylogenetic placement. *Acc.* NCBI accession number, *BLAST ITS2+LSU* result of BLAST search with the complete sequence spanning the ITS2 region and the partial LSU region; when only partial matches either to the ITS2 region or the LSU region were found, no results are given, *BLAST ITS* result of BLAST search with the ITS2 sequence only, *BLAST LSU* result of BLAST search with the partial LSU sequence only

[a] Species recorded at the sampling site as sporophores

associated with *P. sylvestris*, and three species were identified in *Q. petraea* ECM (Table 2). *Russula* subg. *amoenula* (RSEM02 and RSEM22 in Table 2) and *C. geophilum* were found in both pine and oak samples (*C. geophilum* from oak was not sequenced).

The sample-based rarefaction curve (Fig. 1) does not reach saturation, suggesting that increased sampling effort would result in the detection of more species. Estimators of species richness give a rather wide range of values for species numbers: Chao2 estimates a total of 25 species, with the 95% confidence interval from 21 to 41. The means of other estimators also tend to converge in this value range, with the Bootstrap estimator giving the lowest value (24), the Jackknife estimators converging at around 30 and the Michaelis–Menten estimator (MMMeans) suggesting the presence of about 40 species.

The rank–abundance curve (Fig. 2) is indicative of a mature and rather diverse ECM community, with *Cantharellus lutescens*, the most abundant ECMF below ground, accounting for not more than 16.4% of ECM root tips. The ECM community was found to be composed of members from several fungal orders typically present in ECM associations, and non-agaricalean ECMF are particularly well represented (Fig. 3). Russulales are most diverse and abundant, (*Russula*, four species; *Lactarius*, two species). Furthermore, certain groups of non-gilled fungi accounted for much of the diversity and abundance of ECM root tips namely, Thelephoraceae (four species), Cantharellales (*C. lutescens*, the most abundant morphotype, and *Sistotrema* cf. *alboluteum*) and Atheliales. One species of *Sebacina* was detected, less than we would have expected, given the preference of many ECM Sebacinaceae for base rich soils (e.g. Urban et al. 2003; Murat et al. 2005; A. Urban, unpublished observations). Agaricales were represented by

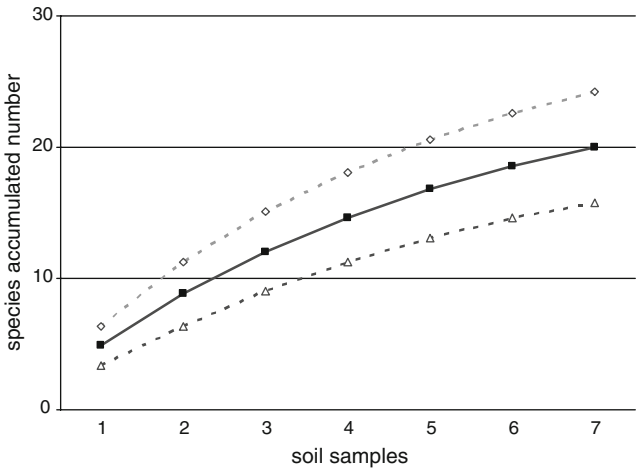

Fig. 1 Species accumulation and richness. Analytically computed species accumulation curve (Mao Tau, —■—) with upper (- -◇- -) and lower (- -△- -) 95% confidence levels. Values were calculated using EstimateS version 8.0

A. citrina, Entoloma rhodopolium and *Tricholoma albobrunneum* and accounted for only 10% of the ECM tips. *C. geophilum* was the most frequent and second most abundant ECM, while no other ECM ascomycetes could be detected. Two species of putative ECM-co-associated fungi were identified by purifying and sequencing multiple bands of the amplified DNA as revealed by gel electrophoresis: The dark septate endophyte (DSE) *Phialocephala fortinii* was found co-associated with *C. lutescens* (RSEM15) and with cf. *Amphinema* sp. (RSEM16), and an unidentified fungus from a new, deeply branching lineage of the ascomycota (Ascomycota Group I sensu Schadt et al. 2003, henceforth abbreviated as AG1, or Soil Clone Group I [SCGI] sensu Porter et al. 2008) was found co-associated with *Sebacina* sp. (RSEM01) and with cf. *Amphinema* sp. (RSEM16).

In two samples, RSEM07 and RSEM25, the PCR products could not be separated on an agarose gel; therefore, a cloning approach was undertaken to identify the ECMF and co-associated fungi (see below). Sequence analysis of an RFLP-based selection of 84 clones obtained from a PCR product from RSEM07 amplified with the primer pair ITS1F/TW13 (Table 3) yielded a high diversity of fungal clones, most of them with ascomycete affinities, but did not reveal a plausible candidate ECMF, since the morphotype was tricholomatoid. Therefore, fungal DNA from RSEM07 was amplified with primer pair ITS1F/ITS4B, the resulting products were cloned, and sequencing of selected clones allowed the identification of the ECMF from RSEM07 as *T. albobrunneum*. The high efficiency of PCR amplification with primer pair ITS1F/ITS4B as opposed to the low efficiency with primer pair ITS1F/TW13 (many bands with low intensities) suggests that in *T. albobrunneum*, the large-subunit (LSU) sequence deviates from the TW13-binding site consensus in a way that impedes PCR amplification.

In RSEM25, a sequence with 98% similarity to a reference sequence of *Lactarius ruginosus* was identified by molecular cloning.

Sequencing reactions of samples RSEM15 and RSEM26 (both *C. lutescens*) with primer ITS3 did not result in readable chromatograms. Comparison of the published *C. lutescens* ITS sequence (AY200806) with primer ITS3 revealed a mismatch at the 3′ position (C ≠ A) explaining the failure of cycle sequencing reactions with this primer.

Identification of ECM-co-associated fungi

A cloning approach was chosen to identify the fungi associated in multiple colonised ECM samples, as revealed by appearance of several individual bands on an agarose gel after PCR amplification of fungal-specific DNA with the

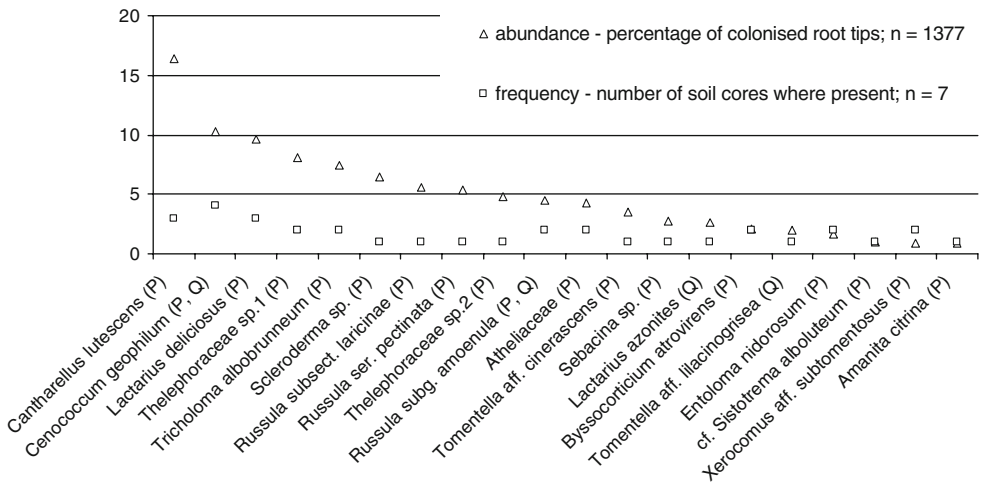

Fig. 2 Rank abundance and steadiness of ECMF. Rank abundance is given in percentage of investigated ectomycorrhizal root tips (n=1,377), and steadiness is given in number of soil samples (n=7) in which the indicated ECM type was found. The *letters in parentheses* behind the ECMF indicate the host tree species: *Pinus sylvestris* (*P*) or *Quercus petraea* (*Q*)

primer pair ITS1F and TW13. The following samples were selected for the identification of ECM-co-associated fungi:

- RSEM01, a sebacinoid ECM co-colonised by an unknown fungus from AG1 (see above)
- RSEM07, where no readable sequence could be obtained, apparently due to the superposition of different ribotypes (see above)
- RSEM15, from which two sequences were obtained from well separated bands by direct sequencing: one for the ECM basidiomycete *C. lutescens* and a second one for the endophytic ascomycete *P. fortinii*
- RSEM16, from which three sequences were obtained from three well separated bands, one related to the ECM basidiomycete *Amphinema byssoides* (Atheliaceae), a second one from the endophytic ascomycete *P. fortinii* and a third one from the 'enigmatic ascomycete'
- RSEM25, one of the three *Quercus* ECM among the samples, where direct sequencing failed, obviously due to the co-amplification of several fungi
- RSEM26, which is formed by *C. lutescens*, like RSEM15; *C. lutescens* was the most abundant ECMF found at the study site

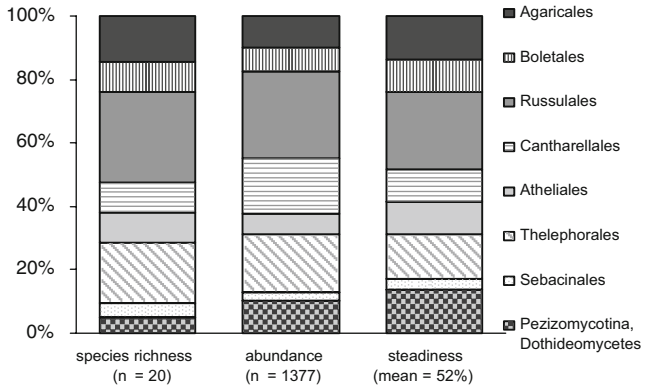

Fig. 3 Species richness, abundance and frequency at the ordinal level

Cloned PCR products contained ITS1, 5.8S ribosomal DNA (rDNA), ITS2 and partial 28S rDNA regions. ITS2 and partial 28S rDNA sequences were obtained allowing identification to the species level where reliable and conspecific ITS2 or LSU sequences are available in public databases. If nearly identical matches were missing, the assignment to higher taxonomic groups was based on phylogenetic analyses using 28S rDNA. Results from sequencing of selected clones are represented in Table 3.

Typically, about 90% of the analysed clones were from ECMF that had already been detected by direct sequencing of PCR products from ECM root tips except for sample RSEM16, where cf. *Amphinema* sp. (Atheliaceae) was represented by only 37% of the clones. In sample RSEM07, the ECMF was not among the clones from amplification with primer pair ITS1F/TW13. Besides the fungi already identified by direct sequencing of agarose gel purified bands, ascomycetes with helotialean, chaetothyrialean and hypocrealean affinities (*Rhizoscyphus ericae* agg. *Capronia* spp. and *Fusarium* spp., respectively) were most frequently found, along with dual colonisations of ECMF (see Table 3). Fungi with affinities to the Herpotrichiellaceae (Chaetothyriales) were most diverse in sample RSEM07. A phylogenetic analysis (Fig. 4) revealed that the detected ribotypes are affiliated to different lineages of the Chaetothyriales.

A fungus from AG1 (Schadt et al. 2003) could be detected in two samples by the cloning approach. In both samples, it had already been detected by the direct sequencing approach, confirming the specificity of the newly designed primers.

In sample RSEM07, where amplification of the ECMF with primer pair ITS1F/TW13 failed, 14 species of ECM-co-associated fungi could be detected by the analysis of 84 clones. In all other samples, the number of ECMF-co-associated fungi retrieved by cloning was small (one to

Table 3 Identification of ECM-associated fungi by cloning and sequencing

01	**Ps**	***Sebacina* sp.**	01_01	***Sebacina sp.***	EU046033	99 AF465191	100 AF465191	99 AF465191	92.9	14
			01_13	Ascomycetes Group I sensu Schadt et al. (2003)	EU046034	91 AY394904	99 DQ068979	93 AY394904	7.1	
07	**Ps**	***Tricholoma albobrunneum***	07_01	*Phialocephala fortinii*	EU046035	99 AY394921	99 EF446148	99 AY394921	33.3	84
			07_23	Herpotrichiellaceae ?*Capronia*	EU046044	99 DQ974822	99 DQ497937	100 DQ273471	17.9	
			07_02	Herpotrichiellaceae ?*Capronia*	EU046036	99 DQ974822	99 DQ497937	100 DQ273471		
			07_48	*Davidiella tassiana/Cladosporium cladosporioides*	EU046050		100 DQ458905	99 DQ008149	14.3	
			07_70	Pezizomycotina (Helotiales ?)	EU046054	99 AY394920	99 AY394920	99 AY394920	10.7	
			07_18	*Exophiala salmonis*	EU046043	99 AF050276	99 EF495231	99 AF050274	6.0	
			07_34	Herpotrichiellaceae	EU046049	96 AJ507323	98 DQ421063	97 AF346420	3.6	
			07_52	Helotiales 1a	EU046051	96 AF081443	98 DQ273333	99 EF434148	3.6	
			07_71	Helotiales 1a	EU046055	97 EF434148	100 DQ273333	99 EF434148		
			07_29	Hypocreales	EU046045	93 AF081480	100 AY833034	97 AY489720	2.4	
			07_11	*Tubeufia pezizula*	EU046041	97 EF434095	95 AY781217	99 AY856906	2.4	
			07_63	*Tubeufia pezizula*	EU046053	98 EF434095	97 AY781217	99 AY856906		
			07_03	*Capronia pulcherrima/Herpotrichiellaceae* ?	EU046037	94 AF050256	89 AF050255	97 AF050256	1.2	
			07_30	Ascomycete	EU046046	90 EF433960	99 DQ273328	99 DQ273460	1.2	
			07_31	*Cryptococcus terreus/elinovii/phenolicus*	EU046047	94 EF434116	100 AB032682	99 AF181523	1.2	
			07_33	*Cenococcum geophilum*	EU046048	99 AY394919	98 AY310839	99 AY394919	1.2	
			07_56	*Oidiodendron scytaloides/chlamydosporicum*	EU046052	94 EF434136	98 AF062804	96 EF434136	1.2	
		ITS1F+ITS4B	07_10B	***Tricholoma albobrunneum***	EU046039		99 AB036894		77.3	22
			07_10A	*Cryptococcus terreus/elinovii/phenolicus*	EU046038	ITS1 + ITS2	99 AF444367		13.6	
			07_10D	*Thelephoraceae 1 (Thelephora sp. ?)*	EU046040		90 EF433965		4.5	
			07_11E	*Cryptococcus podzolicus*	EU046042		98 AY254865		4.5	
15	**Ps**	***Cantharellus lutescens***	15_01	***Cantharellus lutescens***	EU046056		missing	99 AF105304	81.4	70
			15_02	*Phialocephala fortinii*	EU046057	99 AY394921	99 EF446148	99 AY394921	12.9	
			15_64	*Phialocephala fortinii*	EU046059	99 AY394921	99 EF446148	99 AY394921		
			15_04	*Gibberella fujikuroi* (= *Fusarium moniliforme*) complex	EU046058	99 AY188916	100 AY904065	99 AY628198	5.7	
16	**Ps**	**Atheliaceae**	16_10	Ascomycetes Group I sensu Schadt et al. (2003)	EU046062	91 AY394904	100 DQ068979	93 AY394904	44.4	27
			16_08	Ascomycetes Group I sensu Schadt et al. (2003)	EU046061	91 AY394904	98 DQ068979	93 AY394911		
			16_16	***Atheliaceae***	EU046063	93 EF434020	100 AB089818	97 AY586626	37.0	
			16_01	***Atheliaceae***	EU046060	93 EF434020	99 AB089818	96 EF434020		
			16_31	*Phialocephala fortinii*	EU046064	99 AY394921	99 AY606286	100 AY394921	11.1	
			16_35	*Cantharellus lutescens*	EU046065		99 AY082606	100 AF105304	7.4	
25	**Qp**	***Lactarius azonites***	25_01	***Lactarius azonites***	EU046066	95 DQ421988	100 EF560658	98 AF325283	88.5	96
			25_33	***Lactarius azonites***	EU046069	95 DQ421988	99 EF560658	98 AF325283		
			25_08	Helotiales 1b	EU046067	96 EF434148	97 DQ273333	99 EF434148	9.4	
			25_31	*Gibberella fujikuroi* (= *Fusarium moniliforme*) complex	EU046068	99 AY762364	99 EF556217	99 AY762371	2.1	
26	**Ps**	***Cantharellus lutescens***	26_17	***Cantharellus lutescens***	EU046070		99 AY082606	99 AF105304	91.3	46
			26_64	***Cantharellus lutescens***	EU046073		100 AY082606	99 AF105304		
			26_43	*Malassezia restricta*	EU046072		99 AJ437695	99 DQ365342	6.5	
			26_42	*Gibberella fujikuroi* (= *Fusarium moniliforme*) complex	EU046071	99 AY762364	99 EF556217	99 AY762366	2.2	

Two separate libraries were made for sample RSEM07: one library with primer pair ITS1F/TW13 and a second one with primer pair ITS1F/ITS4B.

RSEM no. Number of the ECM sample (same as in Table 2), *ECMF* identity of the ECMF based on sequencing (c.f. results from Table 2), *Clone no.* number of individual clones from the ECM libraries, ordered by abundance in the individual libraries (see "%" at the end of the table), *Identification*, *Acc.*, *BLAST ITS2+ LSU*, *BLAST ITS2*, *BLAST LSU* see Table 2, *Percent* percentage of clones with identical RFLP pattern; identical sequences were grouped together and the percentages combined to one number, *Number* number of clones in the individual libraries

three). Apparently, minor 'contaminant fungi' are preferentially detected by the PCR and cloning approach, if amplification of the quantitatively dominant ECMF fails. A second library of RSEM07-derived PCR products generated with primer pair ITS1F/ITS4B resulted in a distribution pattern comparable to the clone libraries from the other samples: The vast majority of the clones derived from the ECM forming fungus (i.e. *T. albobrunneum*), and only a minority of clones represented ECM-co-associated basidiomycetes (due to the use of the primer ITS4B).

Screening for selected ECM-co-associated fungi by direct PCR

To gain insight into distribution patterns of ECM-co-associated fungi, taxon-specific primers were developed to allow screening of all ECM samples for the presence of co-associated fungi. The following primer pairs were selected for this investigation:

1. Ph1/ITS4 and Ph2/ITS4. Both primer pairs are specific for *P. fortinii*, which seems to be a common ECM-co-associated fungi in the study site: it was detected in 2 out of 30 ECM samples by direct sequencing of agarose gel-separated bands and in three out of five ECM samples by cloning and sequencing; additionally, it was isolated by cultivation techniques from a *Quercus* ECM from the study site (Gorfer et al. 2007).

Both primer pairs yielded identical results for all samples. No conflict between the results from PCR amplification with ITS1F/TW13, cloning and amplification with *Phialocephala*-specific primers was detected. All amplicons

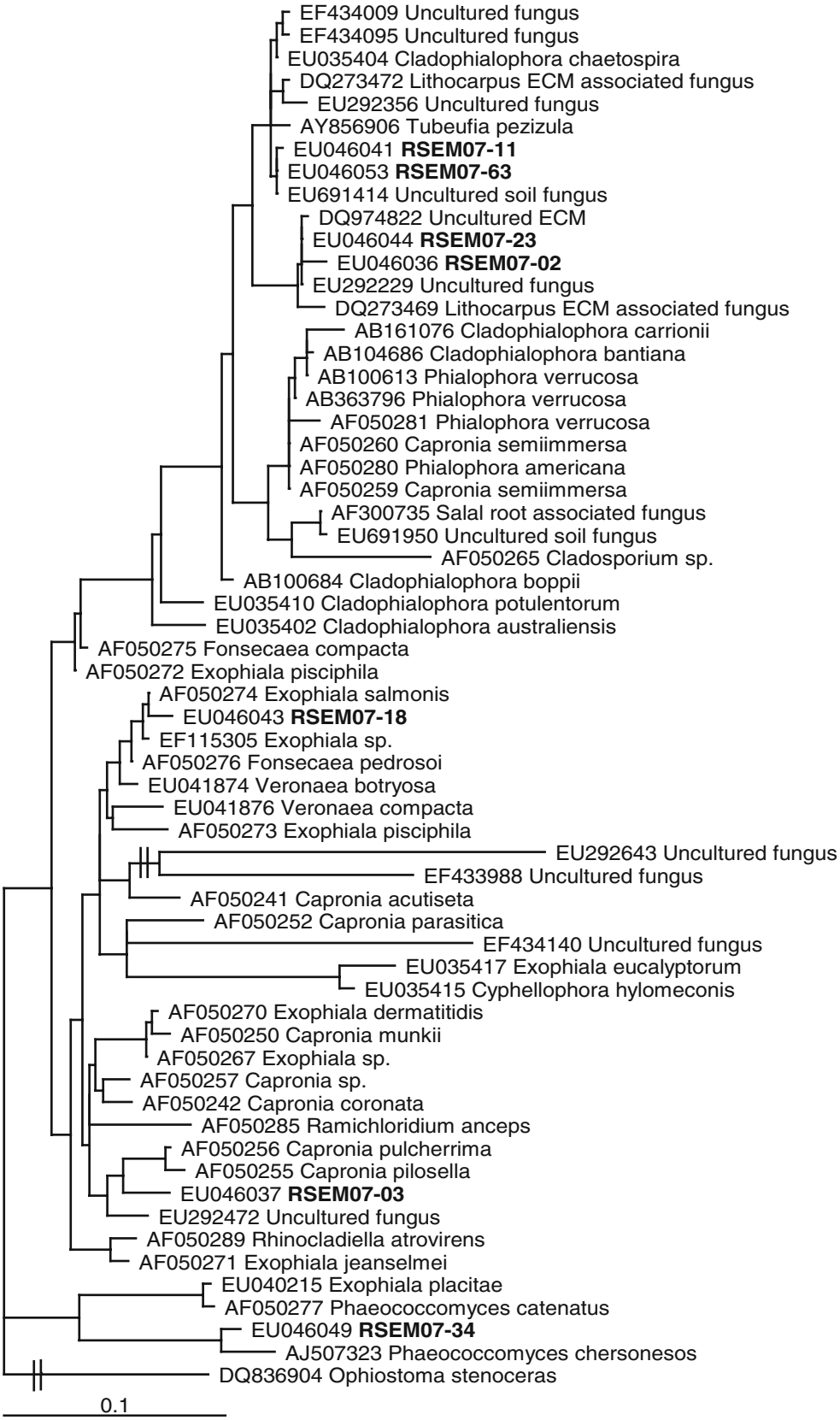

Fig. 4 Phylogenetic placement of ribotypes from ECM clone library RSEM07 belonging to the Chaetothyriales. Phylogeny calculated with RAxML 7.0.0 using the GTRMIX option. *Broken lines* are rescaled to one tenth of the original length

obtained with the specific primers were confirmed as *P. fortinii* by RFLP and sequence analysis.

The absence of *P. fortinii* in *Russula* ECM samples (represented by 6 of 29 analysed ECM samples; Table 4) is significantly different from the average frequency of *P. fortinii* in ECM samples (binomial statistics, $p<0.01$). It is unlikely that this result is due to a sampling bias, since the *Russula* ECM included in the screening are from four different soil samples (1, 2, 5, 6; Table 4), and in two of these samples (2, 3), *P. fortinii* was detected in ECM formed by other fungi.

2. Cap1/Cap2. These primers were designed to amplify DNA from a sub-group of *Capronia* (*Capronia semi-immersa* and related anamorphic *Capronia* spp., Herpotrichiellaceae), which is highly represented among rhizosphere environmental sequences according to GenBank search results (Fig. 4).

PCR amplification with Cap1/Cap2 was successful in RSEM07, where the target had been detected by the cloning approach, but also in three other samples (RSEM01, RSEM15, RSEM16), where the cloning approach had missed it.

In eight out of nine ECM samples, where *Capronia* was found, *P. fortinii* was detected as well (Table 4), suggesting a preferential co-occurrence.

3. Hym1/Hym2. This primer pair is specific for *R. ericae*-related fungi as detected in clone library samples.

Hym1/Hym2 detected *R. ericae*-related fungi in RSEM07 and RSEM25, where the targets (EU046051, EU046067) had been found by the cloning approach, as well as in RSEM01 and RSEM16, where the targets had been missed by cloning. PCR with Hym1/Hym2 was negative in the *C. lutescens* ECM (RSEM15, RSEM 26)

Table 4 Results of screening for ECM-associated fungi with taxon-specific primers

RSEM	Tree	ECMF	Order	Ph1/ITS4 Ph2/ITS4	Cap1/2	Hym1/2	Enig1/2	Soil sample
27	Ps	*Cenococcum geophilum*	Dothideomycetes	+	+	+	−	3
1	Ps	*Sebacina* sp.	Sebacinales	+	+/−	+	+	1
6	Ps	Thelephoraceae 1 (*Tomentella* sp. ?)	Thelephorales	+	−	−	−	2
8	Ps	Thelephoraceae 1 (*Tomentella* sp. ?)	Thelephorales	+	−	+	−	2
10	Ps	Thelephoraceae 1 (*Tomentella* sp. ?)	Thelephorales	+	+	+	−	2
18	Ps	Thelephoraceae 1 (*Tomentella* sp. ?)	Thelephorales	−	−	−	+	3
19	Ps	Thelephoraceae 1 (*Tomentella* sp. ?)	Thelephorales	+	−	+	−	3
11	Ps	Thelephoraceae 2 (*Tomentella* sp. ?)	Thelephorales	−	−	−	−	5
13	Ps	*Tomentella cinerascens*-related	Thelephorales	−	−	−	−	5
34	Qp	*Tomentella lilacinogrisea*-related (96% ITS2)	Thelephorales	−	−	+/−	−	7
9	Ps	*Byssocorticium atrovirens*	Atheliales	+	+	+	−	2
16	Ps	Atheliaceae	Atheliales	+	+	+	+	3
15	Ps	*Cantharellus lutescens*	Cantharellales	+	+	−	−	3
26	Ps	*Cantharellus lutescens*	Cantharellales	−	−	−	−	3
30	Ps	*Sistotrema alboluteum*-related (97%)	Cantharellales	−	−	−	−	3
2	Ps	*Russula* subg. *amoenula*	Russulales	−	−	−	−	2
22	Qp	*Russula* subg. *amoenula*	Russulales	−	−	−	−	6
12	Ps	*Russula* subg. *ingratula* serie *pectinata* (*R. amoenolens* ?)	Russulales	−	+	−	−	5
17	Ps	related to *Russula* subsect. *laricinae*	Russulales	−	−	+	−	3
20	Ps	related to *Russula* subsect. *laricinae*	Russulales	−	−	−	−	3
21	Ps	related to *Russula* subsect. *laricinae*	Russulales	−	−	+	−	3
4	Ps	*Lactarius deliciosus*	Russulales	−	−	−	−	2
5	Ps	*Lactarius deliciosus*	Russulales	+	−	−	−	2
25	Qp	*Lactarius azonites*	Russulales	−	−	+	−	3
3	Ps	*Scleroderma* sp.	Boletales	−	−	+	−	1
23	Ps	*Xerocomus subtomentosus*-related (98%)	Boletales	−	−	−	−	3
7	Ps	Tricholoma albobrunneum	Agaricales	+	+	+	−	2
24	Ps	*Amanita citrina*	Agaricales	+	−	+	−	3
33	Ps	Entoloma nidorosum	Agaricales	+	+	+	+/−	7

Cloning results and PCR results obtained with fungal-specific ITS1f/TW13 are included where available.
RSEM ECM sample number (same as in Table 2 and Table 3), *Tree*: *Ps Pinus sylvestris*, *Qp Quercus petraea*, *ECMF* identification of the ECMF based on sequencing (see Table 2), *Order* phylogenetic positioning of ECMF, for specificity of primer pairs see Table 1, + strong band with the expected size on agarose gel, +/− weak but clear band with the expected size on agarose gel, − no band with the expected size on agarose gel.

4. Enig1/Enig2. This primer pair is specific for a fungus from the enigmatic AG1. (RSEM01b, RSEM16c in Table 2 and RSEM01_13 in Table 3). The fungus was detected in four ECM samples. Results obtained by cloning and specific amplification with Enig1/Enig2 were perfectly congruent.

Primer pairs 1 to 2 amplify DNA from fungal taxa with the potential to interact with ericalean, grass and tree hosts to form mycorrhizal associations or colonise root systems as endophytes. Species of the *R. ericae* agg. (3) are known to be abundant and diverse in heavy metal-contaminated sites (Vralstad et al. 2002).

Optimal amplification conditions were established with the appropriate clones from cloning of ECM-co-associated fungi (see above) and, if available, with reference material from the in-house strain collection. Results from screening with specific primers are summarised in Table 4.

Amplification products with primer pairs Ph1/ITS4, Ph2/ITS4 (both specific for *P. fortinii*), Cap1/Cap2 (specific for *Capronia* sp.) and Hym1/Hym2 (specific for *R. ericae*) were cut with the restriction endonuclease *Bsu*RI (Fermentas) for further confirmation of the results. In all cases, the obtained restriction fragment pattern corresponded to in silico-generated patterns.

Representation of cryptic species of *P. fortinii*

P. fortinii was by far the most common fungus among the analysed samples. It was detected:

1. As a major ECM-co-associated fungus during direct sequencing of fungal DNA amplified from two ECM samples (RSEM15 and RSEM16)
2. In three samples out of six by sequence analysis of cloned fungal DNA (RSEM07, RSEM15 and RSEM16)
3. In 13 (out of 29) samples by screening with *P. fortinii*-specific primers (RSEM01, RSEM05, RSEM06, RSEM07, RSEM08, RSEM09, RSEM10, RSEM15, RSEM16, RSEM19, RSEM24, RSEM27, RSEM33)
4. By culturing from an oak ECM from the same site by standard isolation procedures (Gorfer et al. 2007)

The Redlschlag study site seems to be dominated by cryptic species (CSP) 3: 80% (16 out of 20) of the analysed sequences combined from direct sequencing of fungal DNA amplified from ECM samples, sequencing of clone libraries, sequencing of PCR products obtained with *P. fortinii* specific primers and the ITS sequence from one isolate belonged to CSP3; the remaining 20% (4 out of 20) belong to CSP2b; CSP1 was not detected. The recognition of CSP of *P. fortinii* follows Grünig et al. (2004). The alignment of *P. fortinii* ITS sequences is available as Electronic Supplementary Material (S1).

Phylogenetic placement of a representative of Ascomycota Group I

To explore the phylogenetic position of this fungus, the complete sequence of the insert ranging from the binding sites for ITS1F to TW13 was sequenced. Thereby, sequence information spanning ITS1/5.8S/ITS2 and ~0.6 kb from the 5′ end of the 28S rDNA was obtained. Additionally, a specific insert spanning ~0.8 kb of the 3′ end of the 18S rDNA together with the ITS1/5.8S/ITS2 region was amplified with the primer combination nu-SSU-0817-5′/Enig2 and completely sequenced. Separate NCBI database searches with all parts of the sequence (SSU/ITS/LSU) resulted in a range of good matching sequences (95–100%), exclusively from uncultured soil and mycorrhizosphere fungi. Phylogenetic analyses (Fig. S1) revealed that the enigmatic ribotype is part of a deeply branching lineage termed AG1 (Schadt et al. 2003) or SCGI (Porter et al. 2008).

Discussion

All major ECM basidiomycete orders except Gomphales contribute relatively evenly to the species assemblage detected in the serpentine study site, suggesting that there is a widespread ability among ECM basidiomycetes to cope with the extreme edaphic conditions caused by serpentine. The question if colonisation of serpentine sites by ECMF has resulted in speciation remains open. Species complexes such as the *Xerocomus subtomentosus* aggregate or *C. geophilum*, as well as systematically difficult groups such as *Russula* spp. and the aphyllophoralean ECMF detected in this study, are candidates to explore potential serpentine driven speciation. Genetic divergence in *C. geophilum* from serpentine and non-serpentine soils was detected with DNA markers (ITS-RFLP, amplified fragment length polymorphism [AFLP]; Panaccione et al. 2001). In contrast, Gonçalves et al. (2007) found significant variation in tolerance to and accumulation of Ni in isolates of *C. geophilum* but no correlation of AFLP patterns to the origin of strains from serpentine and non-serpentine soils in Portugal.

Is the ECMF diversity on serpentine outcrops reduced? Such a diagnosis might result from sporocarp inventories (Moser et al. 2005). At the study side, the production of sporophores could be observed in exceptionally wet seasons only, following relief of drought stress. The study of the belowground structure of the ECMF community appears to be less dependent upon rare events of good fruiting but is confronted with the difficulty to find an adequate control site for *P. sylvestris*-dominated forest on serpentine, since more mesic sites are typically dominated

by deciduous trees (unless transformed by forestry), which are reported to host more diverse ECM communities (e.g. Ishida et al. 2007). Fortunately, ECM communities associated with pine, particularly with Scots pine, are among the best studied worldwide. Comparing our results to other studies about pine ECM communities, it has to be considered that assessments of ECM communities are highly sampling and methods dependent (Koide et al. 2005a) and that ECM- and sporophore-based studies commonly supply different partial accounts of the true ECMF diversity.

Genney et al. (2006) recorded seven ECMF in four soil samples from a 125-year-old *P. sylvestris* stand on weakly podzolic sandy soil. Smit et al. (2003) detected a total of 13 ECMF associated with *P. sylvestris* in a drifting sand area in The Netherlands using a total soil DNA and cloning approach. Landeweert et al. (2005) found 14 basidiomycetes in the topsoil and 11 basidiomycetous ECMF on root tips in a *P. sylvestris* stand, again on drifting sand in The Netherlands. Gardes and Bruns (1996) found 21 ECMF in 16 soil cores sampled in a mature stand of *Pinus muricata* of about 40 years. Taylor and Bruns (1999) detected 20 different species of ECMF associated with a mature stand of *P. muricata*. Jonsson et al. (1999) found between 20 and 33 different ECM RFLP types in eight *P. sylvestris* stands affected or not by fire. They observed considerable site-specific and fire-dependent differences in species compositions, resulting in a total of 135 ECM RFLP types. Koide et al. (2005b) found 28 and 25 ECMF, as mycelia and ECM root tips, respectively, in a *Pinus resinosa* forest.

It can be concluded that despite the limitations of our sampling strategy, the recorded ECMF diversity (18 species sequenced from pine ECM) is within the admittedly wide range reported from other pine forests (7 to 33, most studies reporting about 20 ECMF).

A closer view on the properties of serpentine soils may reverse the expectation of a reduced ECM diversity associated with serpentine soils. While the unfavourable Ca/Mg ratio (Mg_{ex}/Ca_{ex}=7.66), high levels of bioavailable Ni (5.81 mg kg^{-1}) and the low water retention potential are certainly highly selective, other properties such as low N levels (C/N=16) and poorly developed organic layers are likely to enhance ECMF diversity (Smit et al. 2003) and to counterbalance the effects of trace metal toxicity (Moser et al. 2005). Significant within-species variation in Ni tolerance in ECMF (Blaudez et al. 2001; Gonçalves et al. 2007) and a variety of physiological mechanisms (Bellion et al. 2006) may be the basis for rapid adaptation to serpentine in a multitude of ECMF species.

A larger study including several pairs of serpentine sites and control sites would be required to assess the degree of specificity of serpentine ECM communities; here, we want to comment on a few observations, only. The absence of pezizalean ECM ascomycota, a group of fungi with a notorious preference for disturbed sites and base-rich soils (Tedersoo et al. 2006) and probably also for semi-arid environments (Danielson and Pruden 1989; Gehring et al. 1998), which is well represented belowground in various ECM communities (Urban, unpublished), may be due to the uncommon chemical composition of serpentine soils. The presence of species regarded as rare, such as *T. albobrunneum*, might be favoured by lower inter-specific competition in the absence of some commonly dominant species, analogous to vascular serpentinophytes. The frequency and abundance of the ECM generalist *C. geophilum* may be attributed to its drought resistance (di Pietro et al. 2007) and to the presence of Ni-tolerant ecotypes (Panaccione et al. 2001; Gonçalvez et al. 2007). *E. rhodopolium* (including var. *nidorosum*) is a common fungus with broad ecological amplitude and a somewhat ruderal tendency. Surprisingly, *Entoloma* spp. are relatively rarely reported in ECM inventories, and only a few species have been covered thus far (e.g. *E. nitidium*; Courty et al. 2005). According to the current infrageneric classification of the genus (Noordeloos, http://www.entoloma.nl/), *E. rhodopolium* belongs to the species-rich section *Rhodopolia* of the subgenus *Entoloma*. Supposing that this classification is evolutionarily meaningful, we would expect that many more of the forest-dwelling taxa of the sub-genus *Entoloma* are ECM.

ECM-co-associated fungi

P. fortinii was found to be the dominating co-associate of ECM root tips at the Redlschlag study site, a result consistent with the observation that *P. fortinii* is favoured in mineral-, humus-poor soils (Summerbell 2005). The *P. fortinii* CSP signature of the serpentine samples (prevalence of CSP3) is most likely due to the naturalness of the study site rather than linked to its particular edaphic conditions (Grünig et al. 2004, 2006).

Growth tests in the laboratory revealed the high Ni resistance of a *P. fortinii* isolate cultured from a *Q. petraea* ECM root tip (*P. fortinii* RSF-Q104). On malt extract agar, this strain is able to proliferate at a Ni concentration of 6 mM, whereas the growth of many other fungi (e.g. *Cadophora finlandia* PRF15 from a calamine site) is completely inhibited at 0.75 mM Ni (Gorfer, unpublished results). At present, the precise location of *P. fortinii* in the root tips is unknown. PCR-based results and the observation of clampless, more or less dematiaceous hyphae in basidiomycete ECM preparations, suggest that *P. fortinii* co-exists with certain ECMF. PCR signatures of DSE such as *P. fortinii and C. finlandia* have frequently been encountered in molecular ECM studies (e.g. Taylor and Bruns 1999; Korkama et al. 2006), and possibly, they were

mistaken for the ECMF in some cases. The availability of green fluorescence protein (GFP)-expressing fluorescent strains (Gorfer et al. 2007) of *P. fortinii* will allow to study its relationships with ECMF in more detail.

The ecological role of *P. fortinii* is still under intensive debate. Effects on host plants have been described as beneficial, neutral or adverse (Wilcox and Wang 1987; Jumpponen and Trappe 1998a,b). Kaldorf et al. (2004) found frequent *P. fortinii* ECM on aspen and, moreover, a high incidence of co-colonisation with typical ECMF. We found *P. fortinii* exclusively as an ECM-associated fungus. In repeated pure culture synthesis trials of *P. fortinii* isolate RSF-Q104 (CSP 3) with *P. sylvestris*, no diagnostic structures of ECM such as a mantle or Hartig net were observed. These observations were confirmed by pure culture syntheses with GFP-expressing transformants of *P. fortinii* RSF-Q104 (Gorfer et al. 2007). In some cases, intracellular infections of the host plant were observed, resulting in adverse effects on host vitality. With all the precautions necessary for negative results, it can be concluded that it is unlikely that *P. sylvestris* forms ECM with *P. fortinii* at the study site, while the co-colonisation of other ECM is very frequent. Possibly, the ability to form ECM is controlled by the host plant.

The co-occurrence of *P. fortinii* with ECMF and ECMF-associated fungi (Table 4) is not random and indicates a potential exclusion of *P. fortinii* by *Russula* species. Comparatively little is known about the nature and effects of fungal interactions in the mycorrhizosphere. However, it is reasonably safe to assume that there is competition among ECMF and that there is a continuum from commensalism to mutualism or parasitism between endophytic fungi and ECMF. Many of the ECM-associated fungi we detected are from fungal lineages which harbour a diversity of parasitic taxa (Fig. 4; Berbee 2001; Crous et al. 2007). The observed patterns suggest the existence of complex and specific biotic interactions among ECMF and co-associated fungi. We hypothesise that such interactions may drive the turnover of ECM root tips and play an essential role in the maintenance of ECM diversity, by establishing negative feedback loops that provide niches for the rarer fungi at the expense of the more dominant species, analogous to the effects of arbuscular mycorrhiza in grasslands on serpentine (Castelli and Casper 2003). Certainly, this might be a factor contributing to ECM diversity beyond the limits of serpentine sites. Localised diversity is thought to give important clues to niche and guild structure and thus about the functional significance of ECM diversity (Bruns 1995). The relations of ECMF and co-associated fungi may be another dimension of this complex issue. The importance of inter-fungal relationships has already been stressed by Bruns (1995) but without differentiating between ECMF and co-associated fungi. Sen (2001) reported on the complexity of multi-trophic interactions between host tree growth, ECMF and a parasitic *Rhizoctonia* sp. and described a potential feedback loop, albeit without referring to a diversity context. Summerbell (2005) observed a high degree of site specificity in ectomycorrhizosphere fungi and concluded that edaphic and overall microbial community conditions be much more significant than a potential symbiorhizosphere effect. Our results suggest that ECM tissues and their surfaces may be highly selective microhabitats, resulting in considerable specificity and diversity on a small scale.

Ascomycota Group 1—are these enigmatic fungi common co-associates of ECM?

AG1 is a monophyletic, genetically highly diverse group of fungi of cosmopolitan distribution detected in a broad variety of soils with PCR-based methods (Chen and Cairney 2002; Anderson et al. 2003; Schadt et al. 2003; Jumpponen and Johnson 2005; O'Brien et al. 2005; Porter et al. 2008), which could not be linked to any described fungal species. Hence, all available information about these fungi is inferred from environmental sequence data. Ribotypes belonging to AG1 have been repeatedly found in association with ECM (Rosling et al. 2003; Izzo et al. 2005; Menkis et al. 2005) and even detected from an *Acaulospora colossica* fungal spore (Pringle et al. 2000), suggesting a potentially biotrophic lifestyle. This hypothesis is compatible with the observation that SCGI has not been detected in clone libraries obtained from non-vegetated soil (Porter et al. 2008). Probably, these fungi play a significant role in the mycorrhizosphere but have gone undetected due to their inconspicuousness and due to the limitations of standard cultivation techniques.

Conclusions

This study demonstrates that the negative impact of high levels of toxic metals on ECM diversity may be limited to anthropogenically polluted sites, while naturally metalliferous serpentine soils host a rich and probably characteristic ECM community. This discrepancy underlines the need for protection of the primary sites of toxic metal resistance evolution. Most of the ECMF we detected belong to species complexes which require further study to make conclusions on the hypothesis of serpentine-driven speciation. Our observation of specificity in the association of ECMF and co-associated fungi needs to be tested in more sites, to assess if interactions between ECMF and mycorrhizosphere fungi are of more general significance in structuring ECM communities and maintaining ECM diversity.

Acknowledgements This work was supported by grant P15357 from the Austrian Science Foundation (FWF) and grant LS149 from the Vienna Science and Technology Fund (WWTF) to JS. The authors thank Anton Russell and two anonymous reviewers for critical reading of the manuscript and helpful suggestions.

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