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Characterization and spatial distribution of ectomycorrhizas colonizing aspen clones released in an experimental field

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Abstract Ectomycorrhizas (EM) from aspen clones released on an experimental field were characterized by morphotyping, restriction analysis and internal transcribed spacer (ITS) sequencing. In addition, their community structure and spatial distribution was analyzed. Among the 23 observed morphotypes, six mycobionts dominated, forming roughly 90% of all ectomycorrhizas: *Cenococcum geophilum*, *Laccaria* sp., *Phialocephala fortinii*, two different Thelephoraceae, and one member of the Pezizales. The three most common morphotypes had an even spatial distribution, reflecting the high degree of homogeneity of the experimental field. The distribution of three other morphotypes was correlated with the distances to the spruce forest and deciduous trees bordering the experimental field. These two patterns allowed two invasion strategies of ectomycorrhizal fungi (EMF) to be recognized, the success of which depends on adaptation of the EMF to local ecological conditions.

Keywords Ectomycorrhiza · ITS-sequences · Morphotyping · Spatial patterns · Populus

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Introduction

For most trees in temperate and boreal forests, establishment, growth and survival are dependent on colonization by ectomycorrhizal fungi (EMF) (Smith and Read 1997). EMF belong to the Basidiomycota, Ascomycota and also Zygomycota, and their estimated diversity is above 5,000 species (Molina et al. 1992). Below ground, ectomycorrhiza (EM) communities are often species-rich, with anything from 20 to over 50 EM species colonizing single tree species at a given stand (Horton and Bruns 2001). In this context, the starting point of each investigation in the field is characterization of EM at the species level. This goal can be achieved by combining detailed light-microscope-based morphological and anatomical description—morphotyping (Agerer 1991)—with molecular approaches. Molecular characterization is based on PCR amplification of the internal transcribed spacer (ITS) region within the rDNA, which has been shown to be a suitable species marker for EMF (Buscot et al. 2000 and references therein). This method has, therefore, been used in many studies on EM community structure since the pioneering work of Gardes et al. (1991). To date, the majority of studies have focused on EM of coniferous trees, mainly of the genera *Picea* and *Pinus*. In the *Colour Atlas of Ectomycorrhizae* (Agerer 1987–1998), the largest available systematic collection of EM morphotype descriptions, the keys for *Picea* and *Pinus* EM contain 91 and 67 morphotypes, respectively, while for fast-growing deciduous trees like *Eucalyptus*, *Populus* or *Salix* only 3–4 morphotypes are included.

In the last decade, *Populus* has become one of the most interesting trees for biotechnology. Besides being of commercial importance, e.g., for the paper industry, poplars combine many biotechnological advantages, such as rapid growth, simple in vitro propagation and the existence of genetic transformation systems (Fladung and Ahuja 1996). In the near future, genetically modified (transgenic) poplars could be cultivated in large plantations. Therefore, it becomes necessary to characterize their mycobionts, especially with a view to assessing the

mycorrhization pattern of transgenic trees under field conditions in comparison to that of wild type trees. In a recent study, we initiated such a comparative study on *Populus* (Kaldorf et al. 2002) within the frame of the first release experiment involving transgenic trees in Germany (Fladung and Muhs 2000).

Several mechanisms, such as host plant specificity (Cullings et al. 2000), clonal versus sexual spread (Fiore-Donno and Martin 2001), competition (Wu et al. 1999) and ecophysiological preferences (Bruns 1995) of EMF, have been discussed to explain the formation of spatial patterns in EM communities. In the present work, given that in the experiment mentioned above defined aspen lines were released in a homogeneous field, we take the opportunity to complete the detailed description of aspen EM morphotypes by microscopic and molecular methods and to evaluate the impact of ecological factors not related to host plant effects on the formation of the local EM community.

Materials and methods

Description of the field site

The experimental field of approximately 1,500 m^2 was located at the Federal Research Center for Forestry and Forest Products (Großhansdorf, Germany; Fladung and Muhs 2000). To obtain homogeneous growth conditions, the loamy sand soil was ploughed and harrowed prior to planting. The experimental field was flat, thus excluding the influence of altitude or slope. However, the surrounding vegetation was heterogeneous, with a spruce forest at the south and west borders, deciduous trees at the north and grassland at the east border (Fig. 1). To minimize edge effects, four additional rows of aspen were planted on the southern side of the field, while one row of additional aspen was planted on each of the other three sides. These additional aspen were not included in this study.

In this context, the northern part of the field clearly received more sunlight than the southern one. This was confirmed by measurements of the light intensity with a LI-COR Quantum/Radiometer/Photometer, Model LI-185B (LI-COR Biosciences, Lincoln, Neb.), taken with five replicates 1 m above ground at three points (close to the spruce forest, central and close to the deciduous trees) within each sector of the experimental field on a sunny summer day (see Results). This may explain why, during the field work, we often observed a high soil moisture content in the shady southern part of the field.

Plant material

The plant material investigated consisted of seven lines of hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) planted out in 1996. One line was the parental wild type clone Esch5, from which six transgenic lines were derived and multiplied by micropropagation before planting out (Fladung et al. 1997). Four of these transgenic lines carried the *rolC* gene from *Agrobacterium rhizogenes* under the control of the 35S promoter of cauliflower mosaic virus on the plasmid pPCV002-35S-*rolC* (Spena et al. 1987) as a morphologically selectable marker to analyze transgene stability in the field. These 35S-*rolC* transgenic aspen with constitutive *rolC* expression were characterized by altered levels of several endogenous growth regulators, resulting in reduced plant height, shortened internodes and smaller leaves (Fladung et al. 1997). Two further lines were obtained by transformation with the plasmid pPCV002-rbcS-*rolC* (Schmülling et al. 1993), in which *rolC* is under the control of the light-inducible rbcS promoter from potato. Under field conditions, rbcS-*rolC* transgenic trees, expressing the *rolC* gene mainly in the leaves, were not different phenotypically from the parental aspen line Esch5 (Fladung and Muhs 2000).

Each aspen line was represented in the field by four randomized blocks with 8 plants in each (in total 32 plants per line). The experimental field was divided into four sectors (Fig. 1), each of them containing one block of each aspen line. By this planting design, an even spatial distribution of wild type, 35S-*rolC* transgenic and rbcS-*rolC* transgenic aspen was obtained.

Sampling strategy

Each EM sample consisted of one root fragment of approximately 5–8 mm diameter and 100–120 cm length taken together with its derived fine rootlets. Most samples contained between 250 and 400 root tips with fully developed EM, all of which were characterized by morphotyping as described below. The root fragments were dug out, starting from trunks, in order to unambiguously assign each of them to individual trees. Most trees were sampled only once during the field experiment in order to reduce disturbance of the root systems, especially of the small 35S-*rolC* transgenic plants. However, for a few trees double sampling was performed to check whether the distribution of different morphotypes observed within the single samples was representative for a given tree. The root samples were stored at 4°C in plastic bags filled with water for up

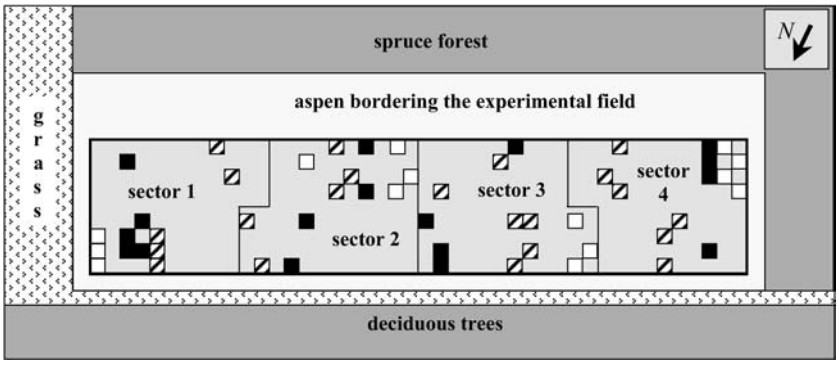

Fig. 1 Schematic map of the experimental field at Großhansdorf (Germany) on which different lines of wild type and transgenic hybrid aspen were released. The central part, from which all samples were taken, had a size of 44×11.5 m. Each square represents one single tree, from which a root sample was investigated. ❑ Wild type aspen line Esch5, ◪ rbcS-*rolC* transgenic trees, ■ 35S-*rolC* transgenic trees. The four sectors correspond to repeats in the planting design

to 4 weeks until analysis (Kaldorf et al. 2002). Samples were collected 12 times between September 1998 and October 2001 to include all morphotypes independent of seasonal or successional effects. At each sampling date, between 9 and 15 root samples were taken, covering at least three of the four sectors of the experimental field with samples from at least one wild type, one 35S-*rolC* transgenic and one rbcS-*rolC* transgenic tree per sector.

In 1998/1999, the work was focused on the morphotype characterization, while the data collected in 2000/2001 concerned the spatial distribution of the different morphotypes. Overlapping of spatial by temporal effects was reduced by excluding the data collected in 1998/1999 from spatial pattern analysis, as the EM community changed considerably between 1998/1999 and 2000/2001 (data not shown). Additionally, statistical analyses of spatial patterns were performed for the eight morphotypes that were found in at least five of the six samplings in 2000/2001. Linear and multiple regression analyses were performed to test correlations between spatial patterns and environmental factors for statistical significance.

Morphotyping of EM

Morphotypes were described on the basis of fresh EM root tips as recommended by Agerer (1991). The first step was to examine morphological characters such as color, shape, branching patterns, and surface structure under a dissecting microscope. In addition, the anatomy of the hyphal mantle and Hartig net was described based on tangential sections through the mantle observed with a Zeiss Axioplan light microscope at 400×–1,000× magnification. Photographs of the mycorrhizal habitus and of cross-sections were taken with an MC100 microscope camera (Zeiss, Oberkochen) using Kodak EPY64T film.

Characterization of morphotypes by PCR-RFLP

Extraction of genomic DNA for PCR was performed as described by Doyle and Doyle (1990) from single EM roots tips (fresh weight 0.1–1 mg) homogenized with micropestles in 100 µl CTAB DNA extraction buffer. Purified DNA was dissolved in 100 µl sterile water and stored at 4°C. The PCR assays contained 5 µl 10× *Taq* polymerase reaction buffer (Promega, Heidelberg, Germany), 4 µl 25 mM $MgCl_2$, 10 nmol of each deoxynucleotide, 50 pmol of each of the primers ITS1 and ITS4 (White et al. 1990) and 1 µl template DNA in a total volume of 50 µl. After 10 min of denaturation at 95°C, PCR was started by adding 2 U *Taq* DNA polymerase (Promega). The PCR program comprised 32 cycles (40 s at 92°C, 40 s at 52°C, 40 s at 72°C) using an OmniGene HB-TR3 thermocycler (MWG-Biotech, Ebersberg, Germany). PCR products were cleaved, in single enzyme digests, with *Alu*I, *Eco*RI, *Bsu*RI, *Hinf*I or *Msp*I (all from MBI Fermentas, St. Leon-Rot, Germany). The lengths of amplification products and restriction fragments were determined by electrophoresis on 2% agarose gels run at 10 V/cm.

Cloning and sequencing

PCR products were cloned into the pCR4-Topo Vector (Invitrogen, Karlsruhe, Germany) and transformed into TOP10 Chemically Competent *Escherichia coli* following the manufacturer's protocol provided with the TOPO TA Cloning Kit. Sequencing was performed on a LI-COR DNA Sequencer Long Reader 4200 using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia, Little Chalfont, UK). Sequence data were compared with GenBank entries using the BLASTN program (Altschul et al. 1997).

Results

A total of 38,219 EM from 102 aspen trees was investigated in four subsequent vegetation periods. Among the aspen EM, 23 morphotypes were differentiated and are described below. A common feature of all aspen EM was the formation of a Hartig net restricted to the anticlinal walls of the rhizodermis cells. The habitus of the 12 most common morphotypes is documented in Fig. 2, and computer-edited images of tangential sections through the hyphal mantles of mycorrhizal root tips are shown in Fig. 3. A ranked abundance curve for the morphotypes is given in Fig. 4. In addition to the 23 morphotypes that showed no dimorphism within their hyphal mantles or emanating hyphae and were apparently formed by only one fungal partner, four mixed types were found in which the same root tip was obviously colonized by two mycobionts.

Morphotypes with few or no emanating hyphae; cystidia and rhizomorphs missing

EM 6.2: dominating morphotype, abundance 31.4% of all EM. Branched EM; white to beige; mantle with 5–7 layers of hyphae, plectenchymatic (similar to mantle type B-H, according to Agerer 1991); emanating hyphae rare, with clamps. (*Tomentella* sp., see Figs. 2e, 3e).

EM 6.1: very common, abundance 10.5%. Unbranched EM; white to beige; mantle 5–7 layers, plectenchymatic (similar to type H), similar to EM 6.2; emanating hyphae rare, with clamps. (*Laccaria* sp., see Figs. 2h, 3h).

EM 2: common, abundance 4.7%. Branched EM; dark brown; mantle 1–3 layers, plectenchymatic (similar to type H); emanating hyphae missing. (Pezizales, see Figs. 2a, 3a).

EM 10: scattered, abundance 1.5%. Branched EM; reddish brown; mantle 1–3 layers, pseudoparenchymatic (similar to type O-P); emanating hyphae rare, without clamps. (Pezizales, see Figs. 2b, 3b).

EM 15: rare, abundance 0.6%. Branched EM; gray; mantle 5–7 layers, plectenchymatic (similar to type B); emanating hyphae rare, with clamps. (*Laccaria* sp.).

EM 1: very rare, abundance 0.1%. Unbranched EM; beige; mantle 3–5 layers; outer hyphal layers pseudoparenchymatic (similar to type L), inner layers plectenchymatic (type H-E); emanating hyphae missing.

EM 21: very rare, abundance 0.01%. Branched EM; dark brown; mantle 2–4 layers, plectenchymatic (similar to type C-E); emanating hyphae missing.

Morphotypes with well developed emanating hyphae; cystidia and rhizomorphs missing

EM 5: very common, abundance 14.0%. Unbranched EM; black; mantle 3–5 layers, plectenchymatic (similar to type B-E); emanating hyphae septate without clamps. (*Phialocephala fortinii*, see Figs. 2c, 3c).

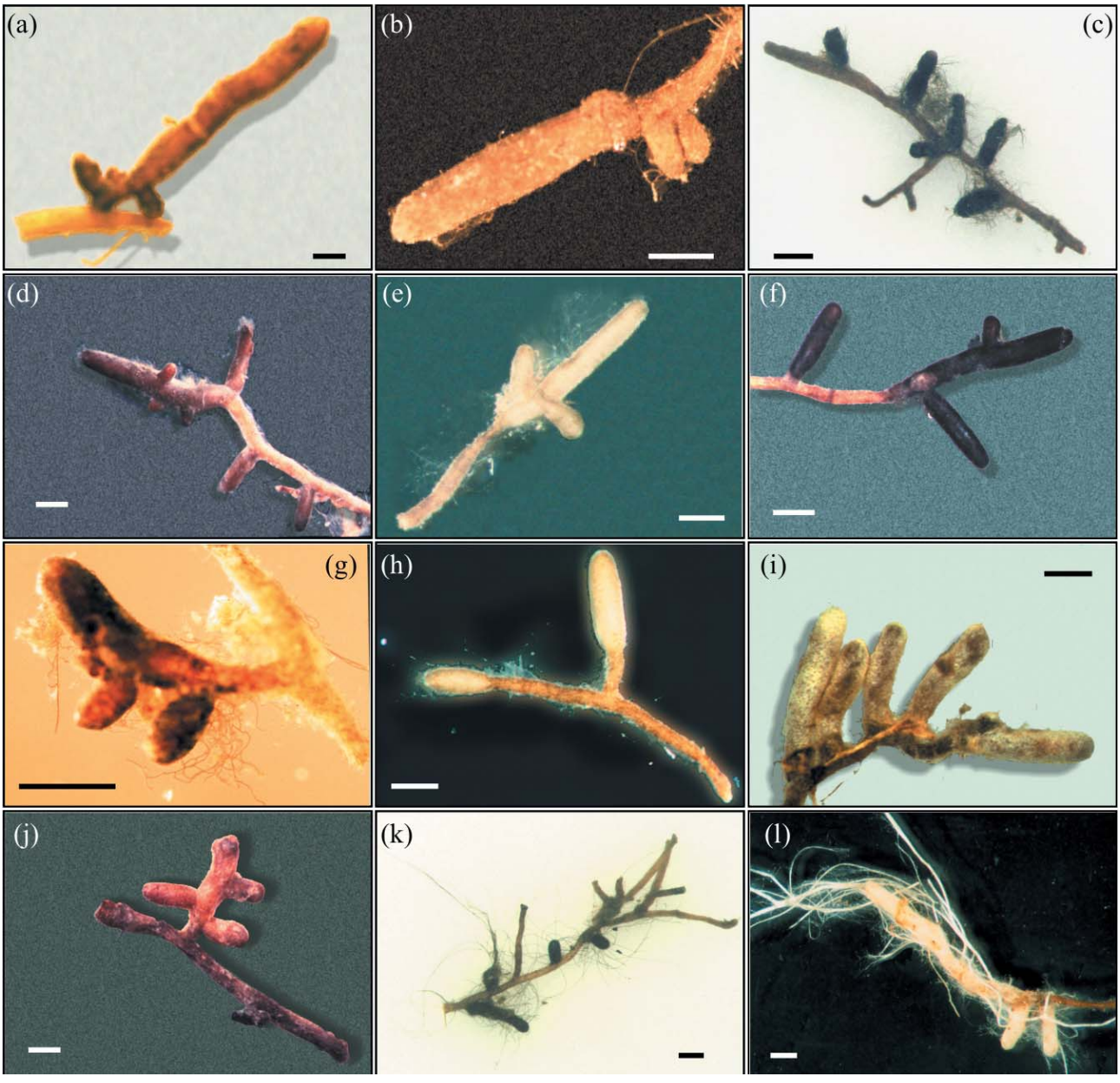

Fig. 2a–l Habitus of the 12 most common ectomycorrhiza (EM) morphotypes of hybrid aspen at the experimental field. **a** Pezizales EM 2, **b** Pezizales EM 10, **c** *Phialocephala fortinii* (EM 5), **d** Thelephoraceae EM 14, **e** *Tomentella* sp. (EM 6.2), **f** Thelephoraceae EM 20, **g** *Lactarius* sp. (EM 3), **h** *Laccaria* sp. (EM 6.1), **i** Boletaceae EM 16, **j** *Tuber* sp. (EM 17), **k** *Cenococcum geophilum* (EM 18), **l** Agaricales EM 22. Images were computer edited to optimize contrast and color reproduction. *Bars* 500 μm

EM 14: common, abundance 5.2%. Branched EM; brown; mantle 3–5 layers, plectenchymatic (similar to type E-H); emanating hyphae septate with clamps. (Thelephoraceae, see Figs. 2d, 3d).

EM 18: common, abundance 4.9%. Unbranched EM, often spherical; black, shining; mantle 2–3 layers, plectenchymatic (similar to type G); emanating hyphae much longer than mycorrhizal roots, septate without clamps. (*Cenococcum geophilum*, see Figs. 2k, 3k).

EM 3: scattered, abundance 1.3%. Branched EM with short lateral roots; brown; mantle 3–5 layers, pseudoparenchymatic (similar to type M); emanating hyphae septate with clamps. (*Lactarius* sp., see Figs. 2g, 3g. As *Lactarius* EM usually do not possess clamps, the "emanating hyphae" were possibly foreign hyphae growing on the mantle).

EM 20: rare, abundance 0.9%. Branched EM with long lateral roots; blackish brown; mantle 3–5 layers, pseudoparenchymatic (similar to type L-M); emanating hyphae septate with clamps. (Thelephoraceae, see Figs. 2f, 3f).

EM 7: rare, abundance 0.5%. Branched EM; light brown; mantle 2–4 layers, plectenchymatic (similar to

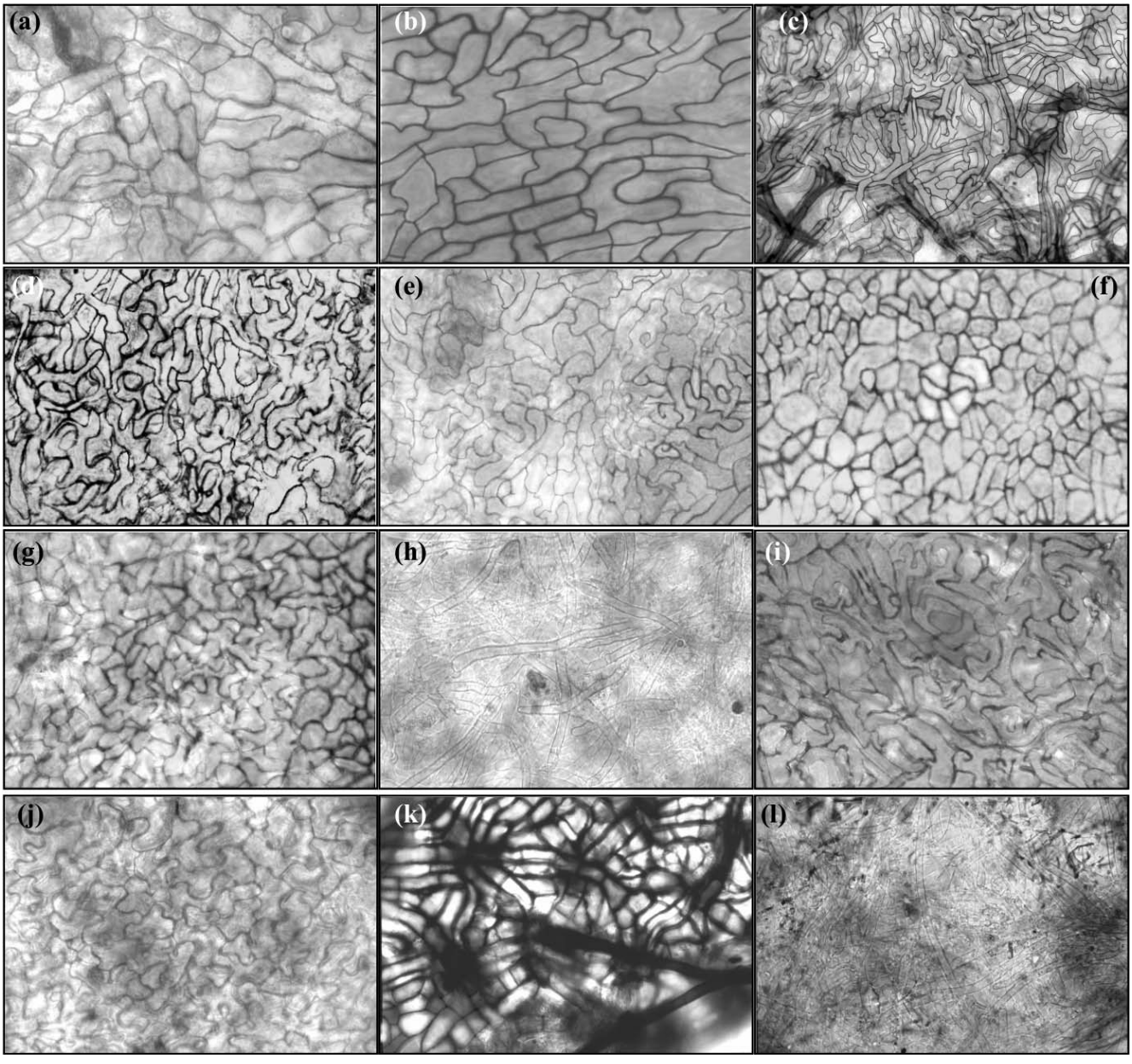

Fig. 3a–l Hyphal mantle structure of the most common EM morphotypes of hybrid aspen at the experimental field. Images of tangential sections of the hyphal mantles at 1,000× magnification were digitized and computer-edited to improve brightness, contrast and sharpness. **a** Pezizales EM 2, **b** Pezizales EM 10, **c** *P. fortinii* (EM 5), **d** Thelephoraceae EM 14, **e** *Tomentella* sp. (EM 6.2), **f** Thelephoraceae EM 20, **g** *Lactarius* sp. (EM 3), **h** *Laccaria* sp. (EM 6.1), **i** Boletaceae EM 16, **j** *Tuber* sp. (EM 17), **k** *C. geophilum* (EM 18), **l** Agaricales EM 22

type B-E); emanating hyphae woolly, septate with clamps.

EM 11: rare, abundance 0.4%. Unbranched EM; beige; mantle 2–3 layers, plectenchymatic (similar to type A); emanating hyphae not septate.

EM 12: very rare, abundance 0.1%. Unbranched EM; brown; mantle 4–6 layers, pseudoparenchymatic (similar to type Q); emanating hyphae septate without clamps.

EM 13: very rare, abundance 0.1%. Branched EM; brown; mantle 2–3 layers, plectenchymatic (similar to type B); emanating hyphae septate with clamps.

EM 9: very rare, abundance 0.1%. Unbranched EM; brown; mantle 1–2 layers, plectenchymatic (similar to type H-M); emanating hyphae septate without clamps.

EM 4: very rare, abundance 0.04%. Branched EM; brown; mantle 3–5 layers, pseudoparenchymatic (similar to type L); emanating hyphae septate without clamps.

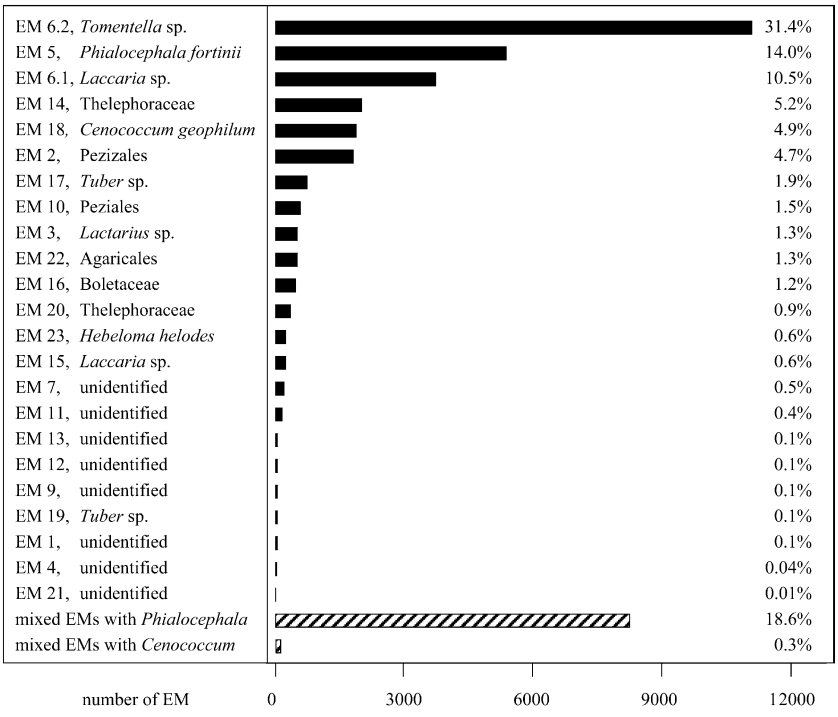

Fig. 4 Ranked abundance curve of the EM morphotypes colonizing different hybrid aspen lines. The absolute number of EM assigned to each EM morphotype between 1998 and 2001 and the corresponding percentage are given

Morphotypes with cystidia

EM 17: scattered, abundance 1.9%. Branched EM, often globular; orange-brown; mantle 4–6 layers, pseudoparenchymatic (similar to type L-M); few needle-shaped cystidia, other emanating hyphae absent. (*Tuber* sp., see Figs. 2j, 3j).

EM 19: very rare, abundance 0.1%. Unbranched EM; brown; mantle 2–4 layers, plectenchymatic (similar to type D); many needle-shaped cystidia, other emanating hyphae absent. (*Tuber* sp.).

Morphotypes with rhizomorphs

EM 22: scattered, abundance 1.3%. Branched EM; silver-white; mantle 5–6 layers, plectenchymatic (similar to type A-B); emanating hyphae septate with clamps; well developed rhizomorphs. (Agaricales, see Figs. 2l, 3l).

EM 16: scattered, abundance 1.2%. Branched EM; yellow; mantle 8–10 layers, plectenchymatic (similar to type A); emanating hyphae rare, septate without clamps; well developed, strong rhizomorphs. (Boletaceae, see Figs. 2i, 3i).

EM 23: Rare; abundance 0.6%. Branched EM; silvery; mantle 4–6 layers, outer layers plectenchymatic (similar to type H), inner layers nearly pseudoparenchymatic; emanating hyphae septate with clamps; fine rhizomorphs formed by only few hyphae. (*Hebeloma helodes*).

EM formed by two mycobionts

All mixed EM belonged to the white/beige morphotypes EM 6.1 (*Laccaria* sp.) or EM 6.2 (*Tomentella* sp.), secondarily colonized by the mycobiont of either EM 5 (*P. fortinii*) or EM 18 (*C. geophilum*). The mixed type EM 6.1/5 (*Laccaria*/*Phialocephala*) was very common (abundance 15.9%), with developmental levels of *Phialocephala* ranging from single hyphae colonizing the EM surface to nearly closed layers of hyphae. In contrast, the mixed type EM 6.2/5 (*Tomentella*/*Phialocephala*), characterized by a loose colonization with *Phialocephala* hyphae, occurred scattered (abundance 2.7%). In the case of the rare mixed types between EM 6.1/18 (*Laccaria*/*Cenococcum*, abundance 0.2%) and EM 6.2/18 (*Tomentella*/*Cenococcum*, abundance 0.1%), secondary colonization was mostly restricted to the tip of the mycorrhizas.

Assessment and identification of morphotypes by PCR-RFLP and ITS sequencing

RFLP patterns of the ITS regions of eight common morphotypes have been published previously (Kaldorf et al. 2002). Additional analyses were performed in the present study to obtain RFLP patterns for all of the 14 most frequent morphotypes (Table 1), which represented over 98% of all EM. Nine of these (EM 3, EM 5, EM 14, EM 16, EM 17, EM 18, EM 20, EM 22, and EM 23) gave reproducible RFLP patterns. PCR-RFLP analyses were carried out in duplicate for the unmistakable morphotypes EM 16, EM 18, EM 20, and EM 23 and at least in

Table 1 Restriction fragment length (in bp) of PCR-amplified internal transcribed spacer (ITS) regions of the 14 most common ectomycorrhiza (EM) morphotypes colonizing different hybrid aspen lines grown on an experimental field in Großhansdorf (Germany). Fragment length was determined from the ITS sequences in all cases except EM 3, EM 10 and EM 15, for which it was estimated after PCR-RFLP analysis (Kaldorf et al. 2002)

Morphotype	EM 2	EM 3	EM 5	EM 6.1	EM 6.2	EM 10	EM 14	EM 15	EM 16	EM 17	EM 18	EM 20	EM 22	EM 23
PCR product	632	ca. 650	510	692	706	ca. 630	705	ca. 690	1,006	575	585	704	1,001	732
*Alu*I	632	ca. 450 ca. 200	510	398 143 94 57	262 179 119 92 54	ca. 630	438 123 100 54	ca. 380 ca. 150 ca. 100 (ca. 50)	691 147 82 54 17 15	575	400 185	439 121 90 54	282 186 185 149 76 75 48	330 211 191
*Eco*RI	324 308	ca. 350 ca. 300	279 231	352 340	378 328	ca. 320 ca. 310	380 325	ca. 350 ca. 340	554 452	354 221	305 280	378 326	642 359	369 363
*Bsu*RI	472 160	ca. 420 ca. 230	287 160 57 6	692	386 233 79 8	ca. 630	462 243	ca. 690	511 137 133 82 80 63	383 192	477 108	463 241	744 257	469 186 77
*Hinf*I	334 290 8	ca. 330 ca. 180 ca. 140	263 239 8	203 187 157 118 8	337 217 144 8	ca. 330 ca. 290	334 219 144 8	ca. 350 ca. 200 ca. 140	281 258 175 122 83 79 8	230 217 120 8	197 156 132 92 8	335 217 144 8	388 338 267 8	372 352 8
*Msp*I	459 126 47	ca. 650	375 90 45	692	473 233	ca. 460 ca. 130 (ca. 40)	705	ca. 690	792 214	384 191	435 98 52	704	1,001	732

Table 2 Identification of the mycobionts of EM morphotypes colonizing different hybrid aspen lines

Morpho-type	Identified by	Accession No.	Best BLAST hit		Classified as
EM 2	ITS sequence	AJ510267	Pezizales sp. d334, AF266709	(82% identity)	Pezizales EM 2
EM 3	PCR-RFLP	–	–		*Lactarius* sp.
EM 5	ITS sequence	AJ510268	*Phialocephala fortinii*, AY078141	(99% identity)	*P. fortinii*
EM 6.1	ITS sequence	AJ510269	*Laccaria laccata*, AF204814	(96% identity)	*Laccaria* sp.
EM 6.2	ITS sequence	AJ510270	*Tomentella ellisii*, AF272913	(94% identity)	*Tomentella* sp.
EM 10	PCR-RFLP, anatomy	–	–		Pezizales EM 10
EM 14	ITS sequence	AJ510271	Thelephoraceae sp. C.t.-3, AF184742	(94% identity)	Thelephoraceae EM 14
EM 15	PCR-RFLP, anatomy	–	–		*Laccaria* sp.
EM 16	ITS sequence	AJ510272	*Xerocomus pruinatus*, AF402140	(82% identity)	Boletaceae EM 16
EM 17	ITS sequence, anatomy	AJ510273	*Tuber maculatum*, AF106889	(86% identity)	*Tuber* sp.
EM 18	ITS sequence, anatomy	AJ510274	*Cenococcum geophilum*, AY112935.1	(99% identity)	*C. geophilum*
EM 19	Anatomy	–	–		*Tuber* sp.
EM 20	ITS sequence	AJ510275	Thelephoraceae sp. C.t.-3, AF184742	(94% identity)	Thelephoraceae EM 20
EM 22	ITS sequence	AJ510276	*Entoloma nitidum*, AF335449	(85% identity)	AgaricalesEM 22
EM 23	ITS sequence	AJ510277	*Hebeloma helodes*, AF124710	(99% identity)	*H. helodes*

triplicate for EM 3, EM 5, EM 14, EM 17, and EM 22. Only one PCR product was obtained in the case of EM 15. The RFLP patterns of EM 2 and EM 10 differed only by the presence or absence of one *Bsu*RI restriction site (Table 1). The occurrence of this *Bsu*RI site did not always coincide with the morphological characters used to separate EM 2 and EM 10. The RFLP pattern originally obtained from EM 2 was found in three specimens of EM 2 and also in three from EM 10 (as identified by morphotyping), while six specimens of EM 10 and four of EM 2 gave the RFLP pattern originally obtained from EM 10. In the dominating group of smooth EM with white to beige color and plectenchymatic structure of the hyphal mantle, two morphotypes (EM 6.1 and EM 6.2) had been separated. In RFLP analysis of seven PCR products from EM 6.1 and nine from EM 6.2, a third RFLP pattern was found for two EM classified as EM 6.1 and EM 6.2. Thus, a third EMF may have been present forming similar EM, which could not be separated from EM 6.1 and EM 6.2 by morphotyping.

ITS sequences were obtained for 11 morphotypes, which allowed their rough identification based mainly on BLAST hits (see Table 2). Different taxa levels were reached, depending on the degree of identity between EM ITS sequences and the best BLAST hits. In the case of identities above 98%, the species name was assigned to the morphotype. Identifications at the genus level were based either on ITS sequence identities above 90% or on combined judgment of anatomical and molecular characters (e.g. EM 17). The three morphotypes not sequenced (EM 10, EM 15 and EM 19) were classified based on similarities in both morphological and RFLP characters, with Pezizales (EM 2), *Laccaria* sp. (EM 6.1) and *Tuber* sp. (EM 17), respectively. The comparison of EM RFLP patterns with those obtained from fruiting bodies collected in the experimental field allowed EM 3 to be identified as *Lactarius* sp. (Table 2).

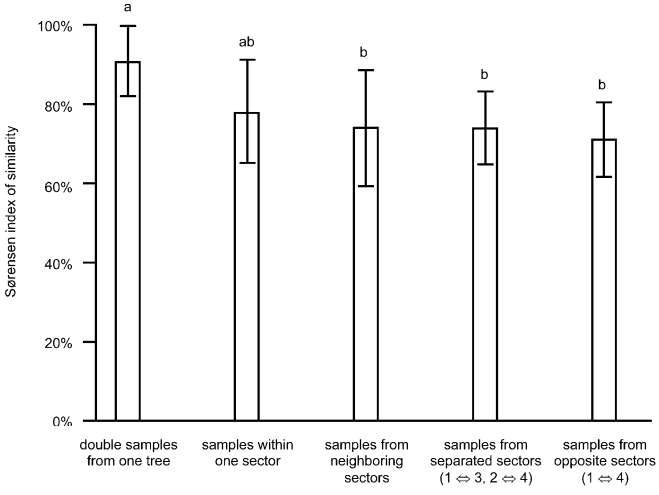

Fig. 5 Mean±SD of the Sørensen index values obtained by pairwise comparison of EM morphotype composition in single root samples from hybrid aspen, in relation to the spatial distance between the samples. Different letters indicate significant differences ($P<0.05$, student's *t*-test). For the field design and sector separation, see Fig. 1

Spatial patterns of EM distribution

The EM community composition of different root samples was compared by pairwise calculation of the Sørensen index of similarity for five different sampling situations (Fig. 5). When two samples from one tree were analyzed, high Sørensen index values of about 90% were obtained. This indicates that the single root samples analyzed in most cases were highly representative of the EM community of the corresponding individual tree. With increasing distance between trees, the similarity of the EM communities decreased significantly, indicating an uneven spatial distribution of at least some morphotypes over the experimental field. Nevertheless, the average Sørensen index value of 70.9% between samples from opposite sectors of the experimental field reflected a high degree of homogeneity in the EM community of the

Table 3 Average light exposure of 12 spots at the experimental field. Measurements of light intensity were taken over a sunny summer day with five replications between 9:00 a.m. and 3:00 p.m. 1 m above ground. For the organization of the field and sector delimitation see Fig. 1

Location	Light intensity (μE m^{-2} sec^{-1})
Sector 1, close to spruce forest	680s
Sector 2, close to spruce forest	520
Sector 3, close to spruce forest	540
Sector 4, close to spruce forest	330
Sector 1, central	1,300
Sector 2, central	1,240
Sector 3, central	1,220
Sector 4, central	900
Sector 1, close to deciduous trees	1,140
Sector 2, close to deciduous trees	1,100
Sector 3, close to deciduous trees	1,200
Sector 4, close to deciduous trees	890

whole field. As each of the four sectors of the experimental field (Fig. 1) had the same composition of wild type, rbcS-*rolC* transgenic and 35S-*rolC* transgenic aspen, the uneven spatial distribution demonstrated by the significantly reduced Sørensen index values was not related to the distribution of the different aspen lines.

Therefore, the influence of three other parameters—distance from the spruce forest, distance from the deciduous trees growing outside of the experimental field and light exposure (Table 3)—on the spatial distribution of morphotypes was checked by simple and multiple linear regression calculation. As expected from their apparently random patterns (Fig. 6a–e), the distributions of *P. fortinii* (EM 5), *Laccaria* sp. (EM 6.1), *Tomentella* sp. (EM 6.2), Pezizales EM 10 and *Tuber* sp. (EM 17) were not significantly correlated with any of the three factors tested, either alone or in combination. Thelephoraceae EM 14 (Fig. 6g) and *H. helodes* (EM 23, data not shown) were rare (EM 14) or missing (EM 23) in both the northern and the southern corner of the field, but even this distribution pattern could not be related significantly to the environmental factors tested.

In contrast, an uneven spatial distribution related to the factors mentioned above was observed for three morphotypes. The occurrence of *C. geophilum* (EM 18, Fig. 6f) showed a highly significant positive correlation with the distance from the deciduous trees (r^2=0.285, P<0.001) as well as a highly significant negative correlation with the light exposure (r^2=0.210, P<0.001) and the distance from the spruce forest (r^2=0.266, P<0.001). An opposite distribution pattern was confirmed for the only two morphotypes with differentiated rhizomorphs, namely Agaricales EM 22 (Fig. 6h) and Boletaceae EM 16 (Fig. 6i). Their occurrence was significantly positively correlated with the distance from the spruce forest (r^2=0.164, P=0.003 for EM 22 and r^2=0.132, P=0.007 for EM 16) and significantly negatively correlated with the distance from the deciduous trees (r^2=0.152, P=0.004 for EM 22 and r^2=0.098, P=0.021 for EM 16). The influence of light exposure on the distribution of Agaricales EM 22 and Boletaceae EM 16 was not significant.

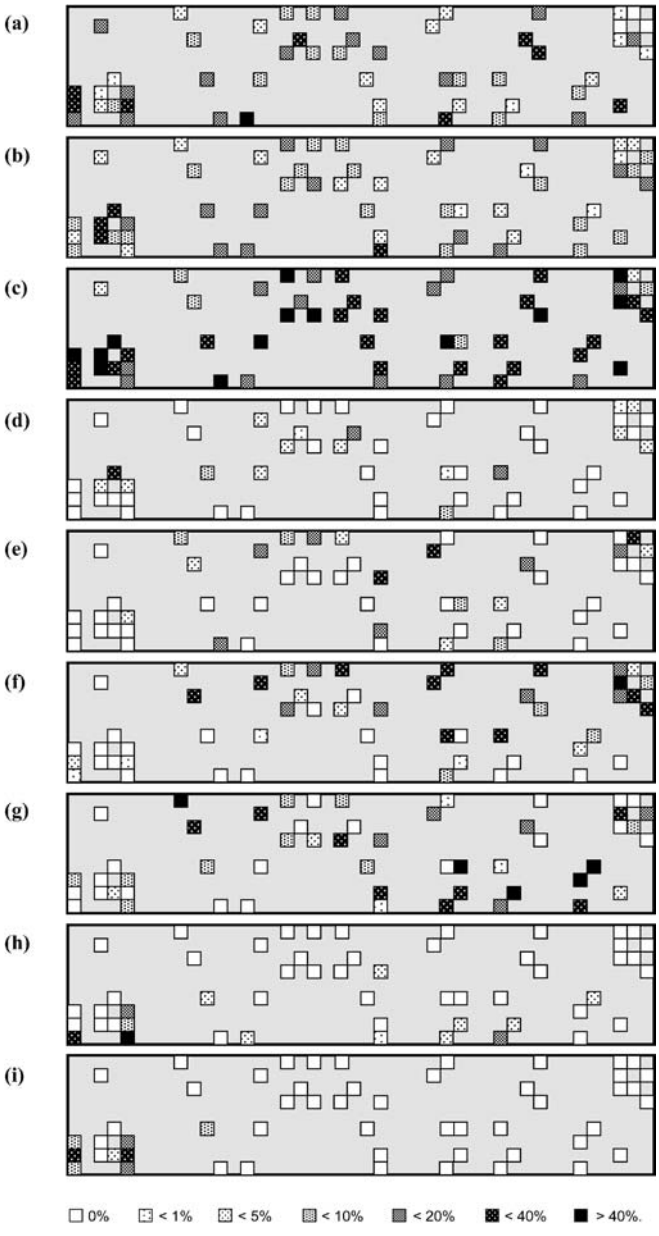

Fig. 6a–i Distribution maps of eight abundant EM morphotypes. **a** *Phialocephala fortinii* (EM 5), **b** *Laccaria* sp. (EM 6.1), **c** *Tomentella* sp. (EM 6.2), **d** Pezizales EM 10, **e** *Tuber* sp. (EM 17), **f** *Cenococcum geophilum* (EM 18), **g** Thelephoraceae EM 14, **h** Agaricales EM 22, **i** Boletaceae EM 16. Each small square represents one root sample taken from one individual tree. The abundance of the morphotypes in each sample is represented by the following scale under the figure. For field design, see Fig. 1

Among the rare morphotypes, EM 1, EM 9 and EM 21 occurred only once in a single root sample. Other rare types (e.g. EM 4, EM 12, EM 13, EM 19) were found in less than five samples (abundance per sample below 10%), located in two or three different sectors in all four cases. For these rare morphotypes, no spatial preferences within the field were apparent. Even *Lactarius* sp.

(EM 3), EM 11 and Thelephoraceae EM 20 were restricted to three or less samples, but in contrast to the other rare morphotypes mentioned above, these morphotypes were then dominant within samples (maximal abundance of 44.4% for EM 11, 65.2% for EM 20 and 75.1% for EM 3).

Discussion

Community structure of aspen EM

The combination of morphotyping and PCR-based analyses of parts of the rDNA is a well-accepted method to describe EM and analyze their community structure (Horton and Bruns 2001). The community observed at our experimental field seems to be typical for a young monoculture of trees. The number of EM morphotypes detected—23— is in accordance with the 16 to 24 morphotypes reported in young stands of alder, birch, pine and spruce (Hashimoto and Hyakumachi 2000; Jonsson et al. 1999; Kårén and Nylund 1997; Pritsch et al. 1997).

Species within the Thelephoraceae and Russulaceae are among the most frequent and abundant EMF in Europe and North America (Horton and Bruns 2001). Forming 38% of all EM, the Thelephoraceae (*Tomentella* sp. EM 6.2, Thelephoraceae EM 14 and Thelephoraceae EM 20) were dominant on our field, while the Russulaceae represented only 1.3% of the EM with one morphotype (*Lactarius* sp. EM 3). The two black morphotypes (EM 5 and EM 18) were formed by two widespread fungi, *P. fortinii* and *C. geophilum*, respectively. *P. fortinii* (EM 5) occurs in the entire temperate zone of the northern hemisphere without apparent host specificity (Addy et al. 2000, and references therein). It belongs to the artificial group of dark septate endophytes, for which interactions with plant roots ranging from parasitic to mycorrhizal have been described (Jumpponen et al. 1998; Wang and Wilcox 1985). *C. geophilum* (EM 18) is considered as a nearly ubiquitous EMF with worldwide distribution and low, or no, host specificity (Kovács et al. 2000; Wurzburger and Bledsoe 2001). These two fungi were involved in the formation of about 40% of all EM at our field site, either alone, or in mixed EM with *Tomentella* sp. (EM 6.2) or *Laccaria* sp. (EM 6.1). *Laccaria*, which formed 10.5% of all EM, has also been described as one of the genera colonizing deciduous trees like birch at early stages of EM succession (Mason et al. 1983). The last common morphotype found, Pezizales EM 2, was identified only at the order level. Consequently, its ecological abilities cannot be discussed. Together, these 6 EM fungi formed nearly 90% of all mycorrhizas, while none of the other 17 morphotypes had an abundance above 2%.

Spatial patterns

EM morphotypes often occur as clusters (Horton and Bruns 2001, and references therein), making it difficult to establish correlations between their distribution and local variations of factors such as pH, temperature, moisture or nutrient availability in the soil. Using in vitro cultures, Sanchez et al. (2001) demonstrated that changes in pH, water supply and temperature had different effects on the growth of eight EMF species. In a field study, increasing atmospheric nitrogen deposition was found to correlate with a drastic change in EM community structure (Lilleskov et al. 2002). Both observations demonstrate that EMF species with ecophysiological specificity exist; such specificity has been suggested to be an important factor for EMF biodiversity and community structure (Bruns 1995). In normal field situations however, attempts to analyze such relationships is traditionally hampered by overlapping heterogeneities in the soil and the host plants community with small-scale microclimatic variations.

A central goal of the field release experiment in Großhansdorf was the assessment of mycorrhizal colonization of transgenic aspen. This aspect was considered in a former article showing that in the first 2 years of the experiment (1998/1999), the expression of the *rolC* gene from *A. rhizogenes* in transgenic aspen had no influence on their mycorrhization rates. Additionally, other than a reduced compatibility between *P. fortinii* (EM 5) and one of the four 35S-*rolC* transgenic aspen lines, no differences in EM community composition were observed (Kaldorf et al. 2002). In this context, the release of genetically closely related aspen clones on a homogeneous field offered a unique opportunity to follow the establishment of an EM community and to analyze the effect of a few varying ecological factors such as the light and drought regime or the influence of neighboring forest stands.

Due to the context of the experiment, the sampling technique used had to allow for correct assignment of each root tip to an individual tree and should not endanger the survival of the saplings. Analyses of EM were therefore based on a 1-m-long sector of a single lateral root per tree. This procedure is uncommon, as randomly taken soil cores are used in most studies on EM community structure (Horton and Bruns 2001). When two samples from the same tree were compared, very similar EM compositions were found, as indicated by high Sørensen index values. This demonstrates that our single sample procedure correctly reflected the EM community of each tree analyzed. Therefore, analysis of the spatial EM distribution was possible, and two patterns in the establishment of EMF were recognized.

The first pattern results from an invasion starting from mycorrhizal trees adjacent to the experimental field and can be termed “vicinal invasion”. This concerns on the one hand the morphotypes Boletaceae EM 16 and Agaricales EM 22, whose occurrence, based on the r^2 values, appeared to be linked to the proximity of deciduous trees. On the other hand, it applies to the area

colonized by *C. geophilum* (EM 18), which correlated with the distance to the bordering spruce forest. *C. geophilum* has been described as one of the most drought-resistant EMF (Pigott 1982). Most likely, the presence of *C. geophilum* (EM 18) mainly on the wettest part of our experimental field was not related to this ecological trait. However, this does not exclude that the extent of a vicinal invasion can be influenced by local ecological factors. Even if not proven by the correlation analysis, the extension of Boletaceae EM 16 and Agaricales EM 22 at the more sunny and dry part of the field fits well with the fact that these morphotypes were the only ones with a hydrophobic hyphal mantle surface and well differentiated rhizomorphs, two characters typical of drought-resistant EMF with medium- to long-distance exploration strategies (Agerer 2001; Unestam and Sun 1995). As indicated by r^2 values below 0.3, additional environmental factors (e.g., temperature or soil moisture) as well as spread of EM fungi by chance may have additionally contributed to the formation of spatial patterns.

The second pattern of EM establishment observed in the field can be termed "random invasion". This pattern is well documented by the extension of the three most common morphotypes, EM 5 (*P. fortinii*), EM 6.1 (*Laccaria* sp.) and EM 6.2 (*Tomentella* sp.), which were found randomly distributed on over 90% of the root samples. The origin and the time of the invasion cannot be determined, but several sources can be hypothesized: (1) an invasion with the plants themselves if they were already mycorrhizal in the greenhouse prior to their outplanting; (2) a massive invasion via immediately germinating spores or via any propagules resting in the soil prior to planting. For the three morphotypes mentioned above, the invasion can be considered as massive and the mycobionts as well-adapted to the ecological conditions of a young plantation. A random invasion can also result in a more discrete extension of EMF. This was the case for rare morphotypes detected only a few times on different independent trees (EM 4, EM 12, EM 13, EM 19) or even only once on a single tree (EM 1, EM 9 and EM 21). These morphotypes did not spread after their punctual random invasions in 1998–2000 and therefore seemed not to be competitive compared to the dominating morphotypes. Scarce random invasion of more competitive EMF is illustrated by the distribution patterns of *Lactarius* sp. EM 3, EM 11 and Thelephoraceae EM 20, as these morphotypes were very abundant on the few trees on which they were detected.

The observation of two establishment strategies of EM on our field site (vicinal invasion and random invasion) is a first documentation that the filter theory proposed to explain assembly rules in the regeneration of plant communities (Fattorini and Halle, in press) could also be suitable for microorganisms such as EMF. According to this theory, the two factors determining the successful establishment of an organism would be its arrival at the plot and its ability to pass a "filter" constituted by the local ecological constraints. Verifying this kind of theory in the field is especially difficult in the case of EMF. However, our study indicates that it becomes possible when working under simplified conditions with clonal plants on roughly homogenized fields.

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