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Factors shaping community structure of endophytic fungi–evidence from the *Pinus-Viscum*-system

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Abstract Endophytic fungal communities have been shown to be highly diverse in almost every host plant species analyzed so far. However, the factors shaping their compositions are largely unknown. To elucidate the impact of various factors, 10 independent replicates of DNA extracts from each of 17 surface-sterilized leaf and stem samples were analyzed by pyrosequencing of fungal ITS1 rRNA gene amplicons. Altogether, 154 fungal OTUs (operational taxonomic units), represented by 953,385 sequences, were found in at least 2 samples from *Viscum album* ssp. *austriacum* and/or its host *Pinus sylvestris*. Deviating from earlier, cultivation-based assessments, the communities were dominated by OTUs related to the genus *Mortierella* and OTUs not assignable to a certain fungal phylum. However, Ascomycota were still the most diverse group in terms of OTU richness and already hypothesized organ and host preferences of certain endophytic Xylariaceae isolated from the *Pinus-Viscum*-system could be confirmed. Host species and organ type were also the major factors shaping the detected fungal communities. The two plant species clearly differed according to the endophytic fungal communities, but only stems and needles of *Pinus* were inhabited by significantly different fungal assemblages. Interestingly, only the 1 and 3 year old stem sections differed according to the endophytic fungal community, while differently aged leaves of both plants were indistinguishable in this regard. Size of the organs had no impact on fungal communities in *Pinus*, but shorter internodes and smaller leaves showed at least a tendency to differ from the corresponding larger organs in *Viscum*. Fungal communities also differed slightly between the two sampling sites, lying 200 km apart, and between the three sampling campaigns. Because the samples were drawn within 15 days, this finding indicates that seasonal shifts clearly outweigh aging effects in host plant with perennial leaves. The results therefore provide strong evidence against a linear development of the endophytic fungal communities in *Pinus sylvestris* and *Viscum album* over the years. The communities seem to establish themselves already in the year the respective organs emerge. Further study is required to clarify whether they predominantly establish anew each year, or if the core community persists throughout subsequent years. The most abundant endophytic OTUs are known from soil and/or dead plant material and are supposed to represent latent decomposers. The study reveals for the first time that host and/or organ preferences of putatively saprotrophic fungi are predominantly responsible for compositional differences in the endophytic fungal communities between host plants and organs. While the analyses are shown to provide rather robust results, the significance of genetic abundance, as revealed by high-throughput sequencing analyses, remains an unsettled issue. This is discussed in detail, as well as the challenges in assigning taxonomic names to OTUs.

Keywords Endophytic fungi · Pyrosequencing · Host preference · Stems · Leaves · Organ age · Pinus sylvestris · Viscum album · Genetic abundance · Name assignment

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Introduction

The diversity of endophytic fungi in trees has been intensely studied throughout the last decades (for reviews see e.g.,

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Arnold 2007; Sieber 2007; Rodriguez et al. 2009; Porras-Alfaro and Bayman 2011; Suryanarayanan 2011; Unterseher 2011), revealing an enormous number of fungal taxa to be capable of growing inside living plant tissues. However, assessing the immense diversity of most endophytic communities in their entirety is a rather laborious task (e.g., Unterseher and Schnittler 2009), which becomes even more complex due to the diversity of factors shaping their compositions. Even within an individual tree, the communities may differ between and within organs and tissues (Sieber et al. 1991; Fisher et al. 1993; Bissegger and Sieber 1994; Hata et al. 2002; Ragazzi et al. 2003; Kumar and Hyde 2004), between the same but differently exposed organs (Unterseher et al. 2007), and in the course of the year (e.g. , Ragazzi et al. 2001; Osono 2008; Jumpponen and Jones 2010; Scholtysik et al. 2012; Wearn et al. 2012). Furthermore, individuals from different regions may host different fungal communities (Fisher et al. 1994; Collado et al. 1999; Santamaria and Diez 2005). Thereby, only few fungal species are restricted to one or few host tree species (Zhou and Hyde 2001; Weiß et al. 2011; Sun et al. 2012), which often results in a blurred distribution pattern with regard to host preference of the overall endophytic fungal community (Suryanarayanan 2011). With a few exceptions (Petrini and Fisher 1990; Unterseher et al. 2007; Hoffman and Arnold 2008; Wearn et al. 2012; Weig et al. 2013), clear patterns of host preference only emerged from meta-studies (Arnold 2007; Sieber 2007). These, again, could not account for the variability of the abovementioned factors among independent studies, which shape community structure within individual host plants. This is apparent for the intensely studied pine tree *Pinus sylvestris*: While Kowalski (1993) reported Leotiomycetes to dominate in its needles, Dothideomycetes (Zamora et al. 2008) and Sordariomycetes (Peršoh et al. 2010), respectively, were found to be most abundant at different sites. Accordingly, it remains unclear if *Pinus sylvestris* hosts a characteristic endophytic fungal community and if its composition differs between locations.

The system of *Pinus sylvestris* and its parasite *Viscum album* was re-analyzed (cf. Peršoh et al. 2010) in this study due to its benefits for elucidating the multiple factors shaping composition of the endophytic fungal community: 1) Both plants are phylogenetically only distantly related but spatially intimately connected. Being exposed to the same fungal inoculum, differences in the endophytic fungal communities may therefore be ascribed to host preferences of the fungi. 2) The age of leaves and stem sections is accurately determinable for both plants, which allows for considering organ age. Furthermore, the total age of mistletoes is determinable. 3) The existence of perennial leaves enables analyzing the factor 'organ age' independent of 'seasonality'. 4) An impact of mistletoe infection on the endophytic fungal community in the pine hosts, as previously reported (Giordano et al. 2009), may be ascertained. By applying high-throughput sequencing, potential biases of cultivation techniques were excluded as well as statistical uncertainties resulting from undersampling the fungal diversity (Amend et al. 2010; Unterseher et al. 2011). In return, the limitations of cultivation-independent biodiversity assessment are discussed.

Material and methods

Pinus sylvestris trees and *Viscum album* ssp. *austriacum* plants parasitizing the trees were sampled 4–6 m above ground in October 2011 at two distinct sites, >200 km apart. Thereby, uninfected pine trees were sampled in close proximity (distance ≤15 m) to infected trees at each of 5 plots per site:

Site 1: Germany, Bavaria, Landkreis Regensburg, Grain am Berg. 400 m alt. Mixed forest with *Fagus* (80 %), *Pinus* (10 %), *Picea* (5 %), and sporadic occurrences of *Betula*, *Hedera*, *Juniperus*, *Populus*, *Quercus*, *Rosa*, and *Salix*. Sampling dates: 2011/10/09 (A–C) and 2011/10/24 (D–E). Sampled pairs of tree individuals: A) 49° 7′ 39.317″ N, 11° 56′ 41.111″ E; B) 49° 7′ 40.357″ N, 11° 56′ 40.582″ E; C) 49° 7′ 41.185″ N, 11° 56′ 48.876″ E; D) 49° 7′ 39.176″ N, 11° 56′ 40.891″ E; E) 49° 7′ 38.456″ N, 11° 56′ 42.454″ E.

Site 2: Austria, Tirol, Bezirk Innsbruck-Land, Zirl. 720 m alt. Scots Pine wood (*Pinus* >90 %) with *Calluna vulgaris* as understory; sporadic *Amelanchier*, *Corylus*, *Crataegus*, *Fraxinus*, *Juniperus*, *Prunus*, and *Viburnum*. Sampling date: 2011/10/16. Sampled pairs of tree individuals: A) 47°16′46.369″ N, 11° 14′ 15.544″ E; B) 47° 16′ 45.912″ N, 11° 14′ 15.63″ E; C) 47° 16′ 44.674″ N, 11° 14′ 16.732″ E; D) 47° 16′ 45.584″ N, 11° 14′ 12.52″ E; E) 47° 16′ 42.179″ N, 11° 14′ 8.002″ E.

One individual of *Viscum*, *Viscum*-infected *Pinus sylvestris* trees and non-parasitized *Pinus* trees were sampled at each plot. Therefor, branches were cut off the plants at 5 year old stem sections. Thorough examination with binoculars (9× magnification) ensured the uninfected status of pine trees. The 30 samples were surface sterilized within 24 h following a previously approved protocol (Unterseher and Schnittler 2009; Peršoh et al. 2013): treatment with sterilized distilled water (dH_2O, 1 min) was followed by washing in 70 % EtOH (2 min), 1 % NaHClO (5 min), 70 % EtOH (1 min) and finally three times in sterilized dH_2O (1 min). The samples were enclosed in sterilized glass petri dishes (Ø=20 cm) for measuring length and diameter of each organ and age class (subsample) without risking contamination. Subsampling was also conducted under sterile conditions and the subsamples (organs of different age)

were shred to pieces of 1–2 mm^3. Needles from the current ('1 year old'), the previous ('2 years old') and the year before the last ('3 years old') were separated and compensating for their length (18–80 mm), 1.5–6 needles of each age and plant individual were pooled in a 2 ml reaction tube. Depending on their diameter (1–7 mm) fragments of 2–15 mm in length were subsampled from *Viscum* and *Pinus* stems of the 3 age categories. Of each *Viscum* leaf, a disc of 5.5 mm in diameter was punched from the center using a sterile cork borer and discs from 3 leaves were pooled per plant individual and age category (1 and 2 years). Altogether, 170 subsamples were obtained: 2 sampling sites × {[10 Pinus individuals × 2 organs × 3 ages] + [5 Viscum individuals × (3 stem ages + 2 leaf ages)]}.

Cells disruption was accomplished by adding a mix of ceramic (2.8 mm in diameter) and glass beads (1.4 and 0.5 mm in diameter) and the nucleic acid extraction buffer supplied with the DNA extraction kit to the subsample and 3 consecutive runs with the FastPrep®-24 Instrument (MP Biomedicals) at 5.5 ms^{-1} for 30 s. Total DNA was extracted using the Charge Switch® gDNA Plant Kit (Invitrogen) as recommended by the manufacturer. The fungal ITS1 region was amplified using the Primers ITS1F (5'-CTTGGT CATTTAGAGGAAGTAA, Gardes and Bruns 1993) and ITS1Univ-R (5'-GCTGCGTTCTTCATCGATGC, White et al. 1990). Primers of each pair were extended by one of 34 different sample-identifier-sequences (SIDs) of 10 bp in length at the 5'-ends. Each polymerase chain reaction (PCR) batch of 25 μl in total included 15.65 μl H_2O, 0.75 μl $MgCl_2$ (50 mM), 2.5 μl 10× PCR buffer (Invitrogen), 2.5 μl dNTPs (2 mM), 1.25 μl of each primer (10 μM), 0.1 μl Taq polymerase (5 U/μl, Invitrogen), and 1 μl DNA extract. Cycling reactions started with an initial denaturation of 2 min at 95 °C followed by 35 cycles of 20 s at 94 °C (denaturation), 1 min at 61 °C (annealing), 2 min at 72 °C (elongation) and finished with an extension step of 15 min at 72 °C. PCR products were purified using the ExoSAP-IT® kit (Affymetrix) and the relative DNA concentration was assed evaluating images of Agarose gels (0,8 %) after electrophoresis (2 μl PCR product and 1 μl of λ-Pst1 DNA ladder) using GeneTools (Syngene). PCR-products with different SIDs were equimolarly pooled to 5 supersamples. These were sent on dry ice to Beckman Coulter Genomics, who generated libraries with additional identifier sequences (LIDs) from each of these 5 supersamples prior to the 454-pyrosequencing run.

The 1,285,911 obtained sequences were delivered Tag-sorted (according to SIDs and LIDs) by Beckman Coulter Genomics. They were assembled to contigs using CLC Genomics Workbench (CLC bio), applying a bubble size of 50 and a minimum contig length of 110. Contigs of at least 85 % similarity were initially grouped by an hierarchical cluster analyses ('hclust') using the 'average linkage' method implemented in the R package 'RFLP Tools' R (v2.11.1, R Development Core Team 2008) and revised in ARB (www.arb-home.de) by creating consensus sequences of similar sequences (≥99 % similarity). Groups of which either the 5'-end of the 18S rRNA or the 3'-end of the 5.8S rRNA could not be located were analyzed in detail by downloading and aligning similar sequences. These ambiguous sequences were only retained if they definitely represented non-chimeric ITS1 sequences. The ITS1 regions were extracted and served as reference sequences, against which all 1,285,911 original sequences were mapped with thresholds for coverage and similarity set to 30 % and 95 %, respectively. Reference sequences were assigned to operational taxonomic units (OTUs) by grouping full length ITS1 sequences of at least 97 % similarity. Singletons (OTUs detected in only one of the 170 subsamples) were discarded as potential PCR-artifacts.

Names were assigned to OTUs by evaluating BLAST results as detailed previously (Peršoh et al. 2010). Briefly, the GenBank database (www.ncbi.nlm.nih.gov) was searched by MegaBLAST for sequences most similar to ITS1 sequences representative for each OTU. All hits which attained at least 90 % of the best alignment score were considered for name assignment. A taxonomic level unifying all names under which the related sequences were deposited was prepended to each OTU ID. Environmental sequences and clearly outlying names were disregarded, but documented as ambiguities and outliers, respectively (supporting information, Tab. S1). As discussed below, the name assignment merely served to associate taxon related information with OTU IDs and not to identify OTUs, i.e. taxonomic names were prepended to OTU IDs, but OTUs were not assigned to taxa.

A matrix coding the abundance of each OTU in each subsample (supporting information, Tab. S2) was imported in Primer6 (Plymouth Routines) and abundances within subsamples were standardized by total. Because preliminary Analyses of Similarity (ANOSIM) revealed one plot at each site to differ significantly (see "Results") subsequent analyses were conducted with two datasets: one including all subsamples and one without those from the deviating plots. Permutational multivariate analysis of variance (PERMANOVA), were used to elucidate which of the following fixed factors contribute to dissimilarities among the endophytic fungal communities: Host species, organ type, organ age, sampling site, and sampling date. The model applied regarded all factors as being crossed, except for 'sampling date' being nested within 'sampling site'. The impact of infection by *Viscum* on *Pinus* endophytes was added as sixth fixed factor, but the analyses were conducted separately, restricted to the pine samples. Impacts of the random factors '*Viscum* plant age', '*Viscum* gender', 'organ length' and 'stem diameter' were also calculated in separate analyses. To allow for a sufficient number of replicates, the factor 'organ age' was excluded

from the applied models, as it was revealed to have no impact by the former analyses. For analyses concerning not all samples, only the relevant samples (i.e. from *Viscum* and stems, respectively) were considered. ANOSIM may only account for two factors simultaneously. Therefore, each factor was analyzed in a crossed design against a combined factor including 'host species', 'organ type', and 'sampling date'. 'Organ age' was not included in the combined factor, as neither of numerous preliminary analyses revealed it to have an impact. Certain analyses were restricted to the corresponding subsets, as described above. In cases the factor of interest was part of the combined factor, the latter was reduced to the remaining two factors. Only samples from site 1 were considered for assessing the effect of 'sampling date' on fungal community composition.

Because abundances of ITS rRNA gene amplicons do usually not reflect biological abundances of the corresponding taxa (see "Discussion"), the absolute abundances were randomized among OTUs to test the robustness of the data. Therefore, the data were at first Log_{10}-transformed to reduce differences of abundances between OTUs. Subsequently, the abundance of each OTU was multiplied by a random factor (i.e. random decimal number between 0 and 1; 8 digits). This factor was fixed for each OTU, but random among OTUs. By replication this approach, 10 randomized abundance matrices were calculated for the full (10 plots) and the reduced (8 plots) datasets, each. These matrices were analyzed by PERMANOVA as described above.

SIMPER analyses (in Primer6) were used to determine the contribution of OTUs to the dissimilarity among categories. Correlations among distribution patterns of OTUs were assessed by calculating Spearman Rank Order Correlations in Statstica 7 (StatSoft Inc.). These calculations were exclusively based on the reduced dataset, from which samples of the two deviating plots had been excluded.

Results

Of the 1,285,911 obtained sequences 953,385 were assignable to 154 fungal OTUs detected in at least 2 samples ('non-singletons'). A negligible fraction (291 sequences) originated from host plant DNA and the remaining sequences represented either singletons or resulted from PCR artifacts. Half of the fungal OTUs were not further classifiable, even though highly similar sequences were found in the international sequence databases, as indicated by reasonable good alignment scores with the best matching sequence (Table 1). However, the majority (93 %) of their closest relatives, i.e. sequences obtaining at least 90 % of the alignment score of the best matching sequence, represent uncultured and undetermined fungi from environmental samples. A surprisingly small proportion of the sequences (14 %) was assignable to the Ascomycota, nevertheless representing 38 % of all OTUs. Basidiomycota were least abundant (10 % of the sequences) and less diverse (17 % of all OTUs) than the Ascomycota. The Zygomycetes were represented by only 5 OTUs (3 %), to which nevertheless as many sequences were assignable as to the other two fungal phyla together. All Zygomycota OTUs were assignable to the genus Mortierella (Mortierellaceae), which therefore constituted the most abundant taxon, at least in terms of ITS rRNA gene amplicon abundance (see "Discussion"). Helotiales and Ceratobasidiaceae were the most common taxa of the Ascomycota and Basidiomycota, respectively, each accounting for more than 2 % of the sequences (supporting information, Fig. S1). Only 6 of the further 37 families detected occurred at relative abundances >1 % (Russulaceae: 1.7 %, Herpotrichiellaceae: 1.6 %, Botryosphaeriaceae: 1.5 %, Xylariaceae: 1.4 %, Capnodiales inc. sed.: 1.3 %, Cantharellaceae: 1.3 %).

Two OTUs (Mycota-375, Mortierella-96) occurred in all samples, i.e. at a frequency of 100 %. Both occurred at similar abundances in the different organs, hosts, and age categories (cf. Fig. 1), as well as at the three sampling events. By contrast, the next frequent OTUs, Mortierella-434 and Mycota-1 (99 % and 83 %, respectively), differed notably in abundance between the sampling categories. The average abundance of Mortierella-434 ranged from 3 % (*Viscum* stems grown in current year) to 42 % (*Pinus* needles grown in current year). Mycota-1 was most abundant in 1 and 3 year old *Pinus* needles (13 % and 11 %, respectively) and least abundant 1-year old *Viscum* stems (<1 %). Noteworthy is also the fifth most frequent OTU, Mycota-160, because it occurred at moderate frequency (57 %), but consistently low abundance (<0.3 %).

Prior to comparative analyzes, the 5 plots of both sites, which are considered to represent independent replicates, were checked for homogeneity of fungal community composition. Thereby, Plot E of site 2 was revealed by ANOSIM to be exceptional ($p \leq 0.024$, $R = 0.155–0,272$) among the plots at this site, while the others did not differ with regard to fungal community composition ($p \geq 0.079$, $R \leq 0.135$). At site 1, plot C differed from D ($p = 0,028$, $R = 0,197$) and E ($p = 0,009$, $R = 0,240$), while the other plots were indistinguishable ($p \geq 0.144$, $R \leq 0.077$). Samples from the two plots (C from site 1, E from site 2) with deviating fungal communities were excluded from further analyses.

Only for testing data robustness, all 10 plots were analyzed with PERMANOVA in parallel to the 8 plots with the endophytic fungal communities considered to be representative for each site. The analyses of both datasets thereby yielded highly similar results (Table 2). Even the 20 matrices with randomized abundances among OTUs yielded results which were mostly accordance with those from the original dataset. In particular, the factor 'host plant' was consistently revealed to be a highly significant driver of

Table 1 Distribution of sequences and OTUs to fungal phyla

Taxon	Sequences	OTUs	Average alignment score	Best scoring sequences	Uncultured fungi among best scoring sequences [%]
Mycota (unclassifiable)	489,982	64	256	647	93
Ascomycota	131,986	59	282	3,488	34
Basidiomycota	97,171	27	286	1,569	74
Zygomycota	234,246	5	261	622	83

The average alignment score the ITS1 sequences obtained with the best matching sequence in GenBank is given as well as the total number of sequences obtaining at least 0.9 times the best alignment score. The percentage of sequences among the latter originating from uncultured organisms is noted in the last column

community composition, as it obtained *p*-values below 0.001 throughout all analyzes. By contrast, 'organ age' was never recognized as significant ($0.250<p<0.812$). Impacts of the factors 'organ type' and 'sampling site' again were significant according to the analyses of all abundance-randomized matrices, as indicated by *p*-values below 0.003 and 0.002, respectively. Only for 'sampling date', the results were inconsistent: while analyses of 16 matrices saw the factor as significant ($0.003<p<0.045$), it was not significant according to 4 analyses ($0.051\leq p<0.067$).

Analyses of the 136 samples from the 8 non-deviating plots revealed a strikingly similar number of 121 (site 'Grain') and 127 (site 'Zirl') non-singleton OTUs at the two **sampling sites**. Thereof, 105 OTUs occurred at both sites. By contrast, composition of the endophytic fungal communities differed significantly (Table 2), even though the OTUs restricted to a single site accounted together for only 4.2 % (site 'Grain') and 3,6 % (site 'Zirl') of all sequences detected at each site. Accordingly, the community composition mainly differed due to the abundance of the rather common OTUs *Mortierella*-434 and Mycota-153 (Table 3). However, also rarer OTUs, such as Helotiales-326 and Mycota-292 preferably occurred at one site. In addition to the geographical differences, the **sampling date** had a significant effect (Table 2). In particular, the endophytic communities at site 'Grain' differed in composition between the two sampling campaigns conducted 15 days apart (ANOSIM $p=0.007$, $R=0.111$). While the relative abundance of *Mortierella*-434 remained strikingly constant throughout this period of time, rarer fungi such as Mycota-1 and *Ceratobasidium*-394 increased in abundance (Table 3). In addition, 36 non-singleton fungal OTUs occurring at abundances <0.1 %, were only detected during one of the two sampling campaigns at site 'Grain'.

Taxonomic affiliation of the host plant had a major impact on fungal community composition, while neither **age** nor **gender of *Viscum*** individuals significantly separated the fungal communities and ***Pinus* plants infected** with mistletoes were not different from mistletoe-free plants (Table 2). The fungal communities differed only marginally between stems and leaves in general (Table 2), because the **organ type** had no effect in *Viscum* hosts (ANOSIM $p=0.308$, $R=0.020$). However, several OTUs preferred either stems (e.g., *Ceratobasidium*-394, *Phaeomoniella*-442, Mycota-282, Mycota-292) or needles (e.g., Mycota-1, Mycota-160) of *Pinus* (Fig. 1). This causes the organ type to have a significant impact on endophytic fungal community composition within *Pinus* ($p<0.001$, $R=0.257$). '**Host species**' separates the fungal communities best ($p<0.001$, $R=0.443$, Fig. 2). While endophytic communities in the leaves of the two hosts still differed ($p=0.004$), but largely overlapped ($R=0.217$), the stems hosted clearly separate communities ($p<0.001$, $R=0.661$). The latter was predominantly ascribable to host preferences of the common OTUs *Mortierella*-434 and Mycota-153 (Fig. 2), but also the abovementioned OTUs preferring either stems or needles of *Pinus*, were clearly less abundant or even absent in *Viscum* (Fig. 1). With regard to an earlier study (Peršoh et al. 2010), it is noteworthy that only 2 of the 7 OTUs assignable to the Xylariaceae were detected by cultivation earlier. Thereof, *Hypoxylon fuscum*-227 preferably colonized mistletoes and *Biscogniauxia nummularia*-301 was restricted to organs with photosynthetically active tissues, i.e. leaves of both plants and *Viscum* stems (Fig. 1).

Organ size, i.e. needle length or dimensions (length and diameter) of stem sections grown in 1 year, had no impact on fungal community composition in the pine hosts ($p\geq0.361$, $R<0.002$). While communities within *Viscum* stems did also not differ according to their diameter ($p=0.914$, $R<0.001$), those within *Viscum* internodes of less and more than 3.5 cm in length differed ($p=0.021$, $R=0.306$). However, the difference became insignificant when a single sample was excluded from the analyses, in which *Phaeobotryosphaeria visci*-356 was exceptionally more abundant (78 %) than in all other samples (≤14 %). *Viscum* leaves of intermediate size were not different from large or small leaves ($p\geq0.303$, $R\leq0.034$), while smaller leaves of *Viscum* (<3 cm in length) showed a tendency ($p=0.087$,

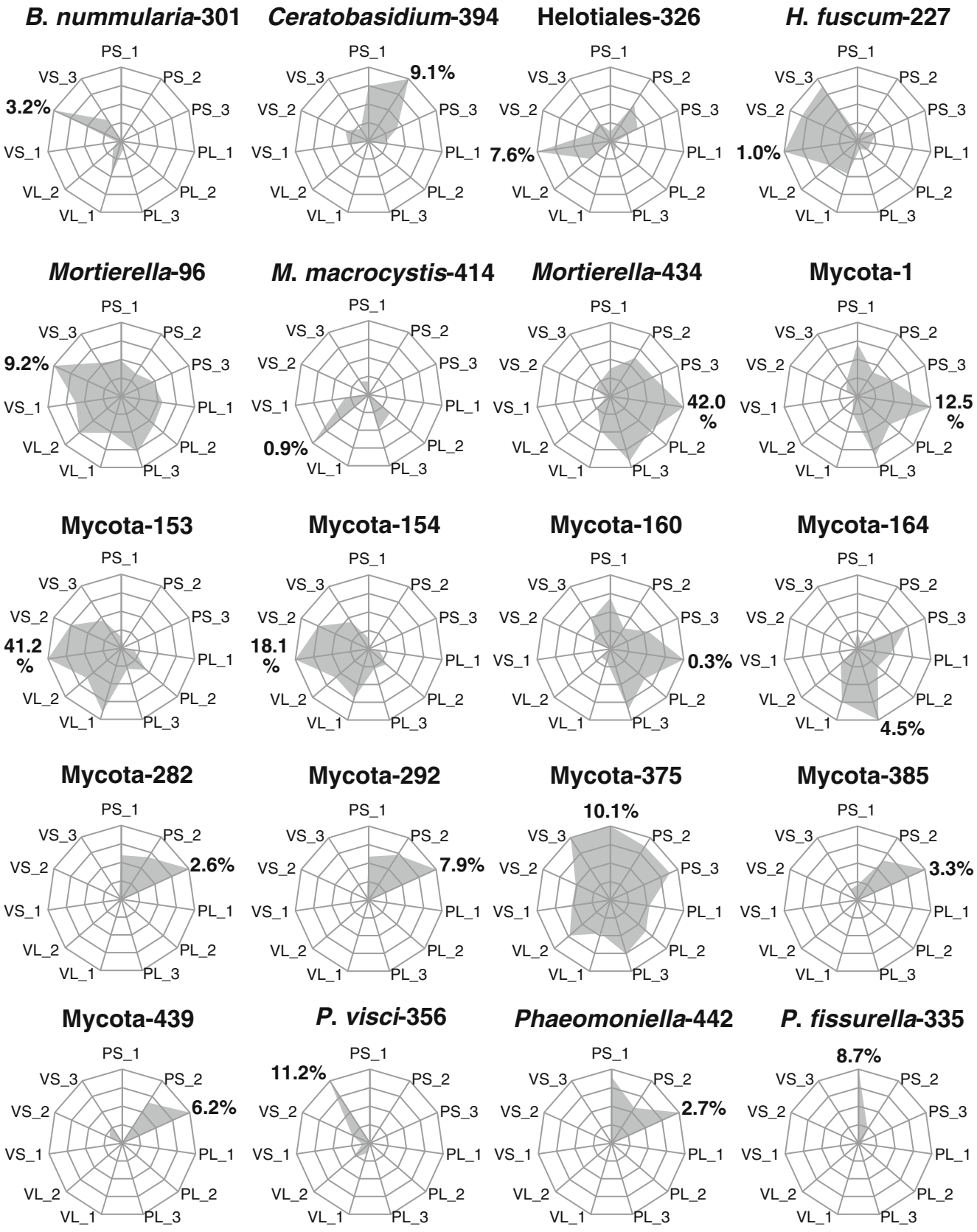

Fig. 1 Distribution patterns of selected OTUs. The relative abundance in 1, 2, and 3-years old stems (S) and leaves (L) of *Viscum album* (V) and *Pinus sylvestris* (P) is indicated in relation to the noted maximal relative abundance. Names of the following OTUs are abbreviated: *Biscogniauxia* nummularia-301, *Hypoxylon fuscum*-227, *Phaeobotryosphaeria visci*-356, *Phaeotheca fissurella*-335

Table 2 Differences between fungal communities according to the analyzed factors

Factor	$ANOSIM_8$		$PERMANOVA_8$		$PERMANOVA_{10}$	
	p	*R*	*p*	Pseudo-*F*	*p*	Pseudo-*F*
Sampling site	<0.001	0.138	<0.001	5.028	<0.001[5]	5.141
Sampling date	0.007	0.111	0.004[3]	3.204	0.001[4]	3.587
Host plant	<0.001	0.443	<0.001[1,3]	13.347	<0.001[2,4,5]	15.857
Organ type	<0.001	0.225	0.008[1]	2.742	0.008[2]	2.767
Organ age	0.410	0.004	0.248	1.180	0.570	0.896
Organ length	<0.001	0.274	<0.001	2.543	<0.001	2.920
Stem diameter	0.235	0.043	0.538	0.901	0.353	1.067
Mistletoe gender	0.632	<0.001	0.963	0.369	0.989	0.312
Mistletoe age	0.963	<0.001	0.233	1.255	0.508	0.927
Infection of pine by mistletoe	0.437	0.001	0.120	1.559	0.244	1.209

The *p*-values obtained from the analyses of the models detailed in the text are given as well as the corresponding R- and PSEUDO-*F* -values from ANOSIM and PERMANOVA analyses, respectively. In addition to the analyses of samples from the 8 non-deviating plots ($ANOSIM_8$, $PERMANOVA_8$), PERMANOVA analyses were conducted under consideration of all samples ($PERMANOVA_{10}$, final columns). Significant inceractions were detected between 'host plant' and 'organ type' ([1]: p=0.012, Pseudo-F=2.555; [2]: p=0.011, Pseudo-F=2.617), 'host plant' and 'sampling date' ([3]: p<0.001, Pseudo-F=6.488; [4]: p=<0.001, Pseudo-F=8.599), and 'host plant' and 'sampling site' ([5]: p=0.017, Pseudo-F= 2.406). Detailed results of the PERMANOVA analyses are provided as supplementary data

R=0.194) to differ from larger leaves (>4 cm in length). This was mostly ascribable to the higher abundance of Mycota-153 and Mycota-154 in smaller leaves (57 % and 19 % vs. 22 % and 9 %, respectively), while *Mortierella*-434 was more abundant the larger leaves (2 % vs. 21 %).

Organ age had no significant impact on fungal community composition in general (Table 2) and for leaves in particular ($p \geq 0.158$, $R \leq 0.031$). Stems from the previous year were indistinguishable from younger and older ones, but 1 and 3 year old stem sections differed in *Viscum* (p=0.016, R=0.180) and *Pinus* (p=0.023, R=0.103). While *Mortierella*-434 was more abundant in older stems of both host plants, the differences in mistletoes were predominantly caused by the higher abundances of Mycota-153 and Mycota-154 in young stems (Fig. 1). In pines, Mycota-1, *Ceratobasidium*-394, and *Phaeotheca fissurella*-335 were more abundant in young, and Mycota-292 in old stem sections. The richness of OTUs was thereby roughly similar in the differently aged organs of the three plant categories sampled at identical frequencies: *Viscum*, uninfected and mistletoe-infected *Pinus* plants (Fig. 3). However, two

Table 3 Average relative abundance of selected OTUs in samples from the different sampling campaigns

Sampling date and site / OTU	Oct. 9th Grain[A]	Oct. 24th Grain[B]	Oct.16th Zirl[C]
n	34	34	68
Ceratobasidium-394	0.54	3.17	4.29
Helotiales-326	3.55	3.18	1.05
Mortierella-434	29.56	29.78	18.75
Mycota-1	5.02	11.54	4.78
Mycota-153	8.55	12.62	18.09
Mycota-154	3.29	5.05	7.04
Mycota-292	1.18	0.30	3.89
Mycota-375	7.40	7.44	7.53

Different letters in superscript indicate significantly different groups. The number of samples per category (n) is given in addition

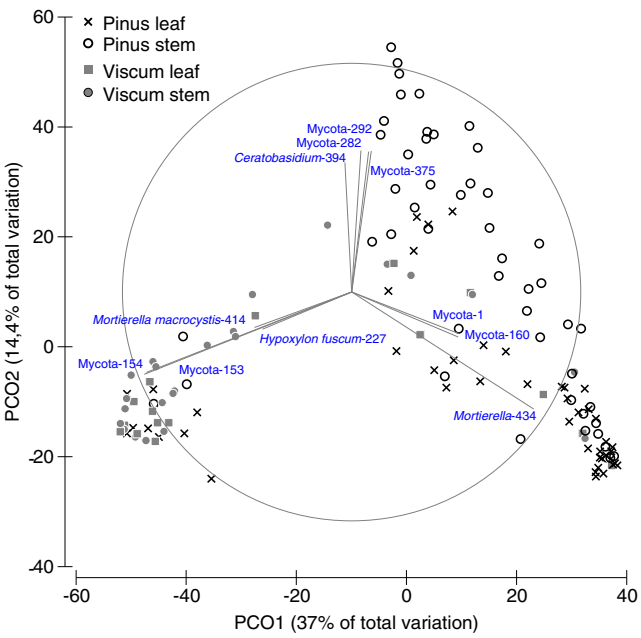

Fig. 2 OTUs correlating with sample similarity according to the overall fungal community composition. The similarity among the samples is visualized by a Principal Coordinate Analysis (PCO). The correlation of the best correlating OTUs (Pearson correlation >0.4) is superimposed on the PCO ordination

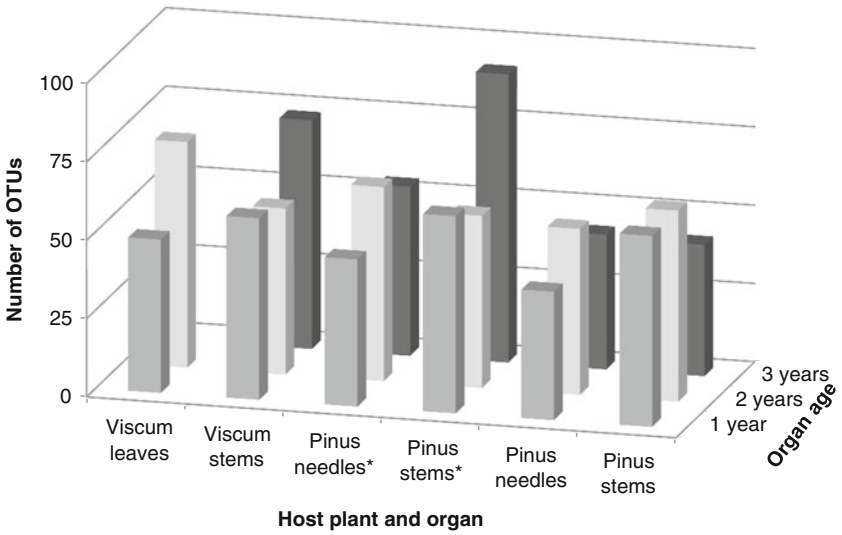

Fig. 3 OTU richness in stems and leaves of different age. The absolute number of OTUs detected in 1, 2, and 3 year old stems and leaves is plotted for the 10 analyzed individuals of *Viscum album* and *Viscum*-infected (*) and -uninfected *Pinus sylvestris* trees

trends were observed: First, leaves grown in the current year host fewer OTUs than older leaves. Second, in mistletoes and pine trees infected by them, more OTUs were found in the 3 year old stems as compared to the younger stems. However, the OTUs responsible for these differences occur at low frequencies (1.1–1.6 occurrences in 8 samples per category) and low abundances (0.1–0.7 %), on average. For comparison, OTUs found in all age classes of one organ occurred in 2.9–3.6 samples and accounted for 2.1–4.5 % of the sequences, on average.

Distribution patterns of the OTUs Mycota-153 and Mycota-154 ($p<0.001$, $r^2=0.88$), as well as those of Mycota-282 and Mycota-292 ($p<0.001$, $r^2=0.71$) were strongly correlated (Table 4). Each pair of the OTUs is also phylogenetically closely related and may therefore represent different genotypes of the same species. Abundances of

Table 4 Correlations among distribution patterns of selected OTUs

	Mortierella-96	Mortierella macrocystis-414	Mortierella-434	Ceratobasidium-394	Biscogniauxia nummularia-301	Hypoxylon fuscum-227	Helotiales-326	Mycota-153	Mycota-154	Mycota-160	Mycota-282	Mycota-292	Mycota-375	Gremmeniella abietina-257	Khuskia oryzae-255	Microcyclospora-175	Phaeobotryosphaeria visci-356	Phaeomoniella-442	Phaeosclera dematioides-258
Mor-96		58.07	95.10	*5.82*	9.71^{VS}	82.71	*57.20*	16.74	29.52	**1.43**	***1.60***	***2.49***	11.31	16.2^{PS}	37.2^{PL}	*66.2*^{VS}	70.6^{V}	89.8^{S}	*85.7*^{PS}
Mor-414	0.00		***0.00***	81.69	63.5^{VS}	**0.00**	**0.00**	**0.00**	**0.00**	***0.95***	*24.27*	*61.79*	*22.46*	*73.8*^{PS}	*59.6*^{PL}	7.83^{VS}	*15.3*^{V}	*12.6*^{S}	16.0^{PS}
Mor-434	0.00	***0.18***		***0.42***	38.5^{VS}	***0.00***	***0.00***	***0.00***	***0.00***	0.00	***3.30***	***1.65***	***2.90***	69.6^{PS}	*67.1*^{PL}	*18.1*^{VS}	40.6^{V}	*35.7*^{S}	*96.2*^{PS}
Cer-394	*0.03*	0.00	***0.06***		99.7^{VS}	36.36	*72.60*	*52.29*	*78.96*	*5.94*	**0.01**	**0.00**	**0.27**	24.7^{PS}	*59.6*^{PL}	*45.4*^{VS}	*5.51*^{V}	7.72^{S}	**4.14**^{PS}
Bis-301	0.13^{VS}	0.01^{VS}	0.04^{VS}	0.00^{VS}		59.0^{VS}	*44.9*^{VS}	*24.4*^{VS}	*12.7*^{VS}	**1.27**^{VS}	*42.1*^{VS}	*73.2*^{VS}	79.2^{VS}	n.a.	**0.85**^{VS}	*65.9*^{VS}	*72.9*^{VS}	*65.9*^{VS}	n.a.
Hyp-227	0.00	**0.27**	***0.13***	0.01	0.01^{VS}		**0.00**	0.00	0.00	***0.04***	*21.20*	*61.69*	*7.40*	*73.8*^{PS}	*67.2*^{PL}	10.5^{VS}	*20.9*^{V}	*35.2*^{S}	92.5^{PS}
Hel-326	*0.00*	**0.12**	***0.14***	*0.00*	*0.03*^{VS}	**0.15**		**0.00**	**0.00**	***0.07***	*29.21*	*61.10*	*23.85*	*59.5*^{PS}	**0.21**^{PL}	11.8^{VS}	*38.0*^{V}	*12.7*^{S}	*45.3*^{PS}
Myc-153	0.01	**0.27**	***0.27***	*0.00*	*0.06*^{VS}	0.30	**0.20**		0.00	***0.01***	***0.03***	***0.36***	***0.05***	*61.6*^{PS}	*91.5*^{PL}	30.2^{VS}	*57.4*^{V}	***4.06***^{S}	*56.0*^{PS}
Myc-154	0.01	**0.29**	***0.29***	*0.00*	*0.11*^{VS}	0.31	**0.20**	**0.88**		***0.00***	***0.59***	***1.73***	***0.18***	*61.6*^{PS}	*20.4*^{PL}	77.1^{VS}	*52.1*^{V}	*12.9*^{S}	83.4^{PS}
Myc-160	**0.04**	***0.05***	**0.17**	*0.03*	**0.26**^{VS}	***0.09***	***0.08***	***0.10***	***0.13***		*22.77*	***4.63***	19.22	*68.9*^{PS}	54.8^{PL}	*57.0*^{VS}	*61.3*^{V}	*73.1*^{S}	*91.8*^{PS}
Myc-282	***0.04***	*0.01*	***0.03***	**0.11**	*0.03*^{VS}	*0.01*	0.01	***0.10***	***0.06***	*0.01*		**0.00**	**0.00**	32.4^{PS}	*71.7*^{PL}	**0.66**^{VS}	51.9^{V}	**0.50**^{S}	11.5^{PS}
Myc-292	***0.04***	*0.00*	***0.04***	**0.13**	*0.01*^{VS}	*0.00*	0.00	***0.06***	***0.04***	***0.03***	**0.71**		**0.00**	32.6^{PS}	**0.01**^{PL}	**4.83**^{VS}	9.48^{V}	**0.24**^{S}	**4.38**^{PS}
Myc-375	0.02	*0.01*	***0.04***	**0.07**	0.00^{VS}	*0.02*	0.01	***0.09***	***0.07***	0.01	**0.14**	**0.14**		54.5^{PS}	21.1^{PL}	*66.2*^{VS}	*97.6*^{V}	6.65^{S}	57.3^{PS}
Gre-257	0.04^{PS}	*0.00*^{PS}	0.00^{PS}	0.03^{PS}	n.a.	*0.00*^{PS}	*0.01*^{PS}	*0.01*^{PS}	*0.01*^{PS}	*0.00*^{PS}	0.02^{PS}	0.02^{PS}	0.01^{PS}		n.a.	n.a.	n.a.	55.1^{PS}	**0.45**^{PS}
Khu-255	0.02^{PL}	*0.01*^{PL}	*0.00*^{PL}	*0.01*^{PL}	**0.29**^{VS}	*0.00*^{PL}	**0.19**^{PL}	*0.00*^{PL}	*0.03*^{PL}	0.01^{PL}	*0.00*^{PL}	**0.27**^{PL}	0.03^{PL}	n.a.		*83.7*^{VS}	*43.3*^{VL}	**2.21**^{PL}	n.a.
Mic-175	*0.01*^{VS}	0.14^{VS}	*0.08*^{VS}	*0.03*^{VS}	*0.01*^{VS}	0.12^{VS}	0.11^{VS}	0.05^{VS}	0.00^{VS}	*0.02*^{VS}	**0.30**^{VS}	**0.17**^{VS}	*0.01*^{VS}	n.a.	*0.00*^{VS}		*45.4*^{VS}	*83.7*^{VS}	n.a.
Pha-356	0.00^{V}	*0.05*^{V}	0.02^{V}	*0.10*^{V}	*0.01*^{VS}	*0.04*^{V}	*0.02*^{V}	*0.01*^{V}	*0.01*^{V}	*0.01*^{V}	0.01^{V}	0.07^{V}	0.00^{V}	n.a.	*0.03*^{VL}	*0.03*^{VS}		24.0^{VS}	n.a.
Pha-442	0.00^{S}	*0.03*^{S}	*0.01*^{S}	0.04^{S}	*0.01*^{VS}	*0.01*^{S}	*0.03*^{S}	***0.06***^{S}	*0.03*^{S}	*0.00*^{S}	**0.11**^{S}	**0.13**^{S}	0.05^{S}	0.01^{PS}	**0.11**^{PL}	*0.00*^{VS}	0.07^{VS}		77.0^{PS}
Pha-258	*0.00*^{PS}	0.04^{PS}	*0.00*^{PS}	**0.09**^{PS}	n.a.	0.00^{PS}	*0.01*^{PS}	*0.01*^{PS}	0.00^{PS}	*0.00*^{PS}	0.05^{PS}	**0.09**^{PS}	0.01^{PS}	**0.16**^{PS}	n.a.	n.a.	n.a.	0.00^{PS}	
Syd-276	0.00^{S}	0.02^{PS}	0.03^{S}	*0.00*^{S}	*0.02*^{VS}	*0.02*^{S}	***0.07***^{S}	*0.02*^{S}	*0.05*^{S}	0.03^{S}	0.00^{PS}	**0.14**^{PL}	0.04^{PS}	*0.01*^{PS}	*0.51*^{PL}	*0.00*^{VS}	*0.06*^{VL}	**0.24**^{PL}	*0.02*^{PS}

R^2-values according to Spearman Rank Order Correlations are given in the lower left half, the corresponding p-values [%] in the upper right. Significant correlations are highlighted in bold, those with r^2-values >0.2 are gradually shaded. Negative correlations are indicated as italics. Superscript letters indicate analyses restricted to Viscum (^{V}), Pinus (^{P}), leaves (^{L}) and/or stems (^{S}). Names of OTUs are abbreviated in the row headers, but written out in the Column headers, except for *Sydowia polyspora*-276 (Syd-276). Values below 0.005 are noted as "0.00"

Mortierella-434 were positively correlated with those of Mycota-160, but negatively correlated with those of most other OTUs. Thereby its distribution was opposed to Mycota-153 and Mycota-154, in particular. No significant correlations of the OTU were only found with *Mortierella*-96, *B. nummularia*-301 and the potential phytopathogenic OTUs. While the abundance of *B. nummularia*-301 only correlated positively with Mycota-160 and the potential pathogen *Khuskia oryzae*-255, distribution of a second Xylariaceae, *H. fuscum*-227, correlated negatively (*Mortierella*-434, Mycota-160) or positively (*Mortierella macrocystis*-414, Helotiales-326, Mycota-153, Mycota-154) with most of the abundant OTUs. Correlations between saprotrophic and potential phytopathogenic OTUs were rare in general, and negative correlations were even rarer. However, the distribution of Mycota-292 was positively correlated to that of five potential pathogens and *Khuskia oryzae*-255 was also positively correlated to 5 OTUs. It co-occurred with *Sydowia polyspora*-276 in particular ($p<0.001$, $r^2=0.51$).

The number of sequences obtaining at least 90 % of the alignment score of the best matching sequence ranged from 3 to 321 for the OTUs correlating best with the overall similarities among samples according to the fungal community composition (Fig. 2). Thereby, only the relatives of *Hypoxylon fuscum*-227 and Mycota-375 were predominantly detected in living aboveground plant parts so far (Table 5). Relatives of the other OTUs were mostly detected in samples from bulk soil and there, when further specified, predominantly in the organic layers.

Discussion

Assignment of names to OTUs

A reliable identification of fungal taxa by sequence data heavily depends on the availability and quality of reference data, i.e. sequence data of suitable taxonomic marker genes from reliably identified strains or specimens. In addition, coverage of the intraspecific variability of the marker gene by the reference data is desirable for species delimitation (Bergsten et al. 2012). Providing such highly valuable reference data is the goal of barcoding initiatives (e.g., ibol.org, www.fungalbarcoding.org), which are, however, still in their infancy (Chase and Fay 2009). Molecular ecological studies are therefore currently faced with only about 14 % of the known (Nilsson et al. 2009) and less than 1 % of the expected fungal diversity (Hawksworth 2012) being represented by reference sequences in public sequence databases so far. Due to a significant proportion of misidentifications among the published sequences (Bridge et al. 2003; Nilsson et al. 2006), morphological and molecular re-analyses of reference specimens and strains might be required to ascertain reliable sequence identification of even quite common species, such as *Xylaria hypoxylon* (Peršoh et al. 2009). Among the possible marker genes for fungal identification, the ITS region matches the requirements for high-throughput sequencing projects best, because it's the by far most common region among the available reference data (Begerow et al. 2010), amplification success is highest

Table 5 Sunstrates of origin and number of sequences related to selected OTUs

Substrate OTU	Bulk soil (organic/mineralic)	Plant root (mycorrhizal)	Plant leaf or stem (endophytic)	Wood	Fungus	Other substrates
Ceratobasidium-394	15 (4/0)	12 (9)	3 (0)	0	0	Air (1)
Hypoxylon fuscum-227	0	0	9 (7)	4	0	Unknown (1)
M. macrocystis-414	104 (1/1)	0	0	0	3	0
Mortierella-434	281 (15/2)	22 (16)	0	4	3	Insect gut (3), unknown (8)
Mycota-1	38 (3/0)	0	0	0	0	0
Mycota-153	28 (0/0)	5 (2)	0	0	0	0
Mycota-154	27 (0/0)	4 (2)	0	0	0	0
Mycota-160	37 (3/0)	0	0	0	0	0
Mycota-282	2 (1/0)	0	0	0	0	Lichen (1)
Mycota-292	2 (1/0)	0	3 (0)	0	0	0
Mycota-375	0	0	2 (0)	0	0	Air (1)

Sequences related to each OTU, i.e. those obtaining at least 90 % of the alignment score of the best matching sequence, are categorized according to the substrates they were obtained from. Sequences closest related to the OTUs corresponding best with sample similarities were selected (Fig. 2). The number of related sequences detected in bulk soil is given in column 2. Thereby, it is differentiated between sequences originating from covering organic layers and mineral soil horizons, when traceable. Column 3 notes the detections of related sequences in plant roots, including those in mycorrhizal roots. The number of detections in aboveground plant parts is listed in column 4, with those from surface-sterilized material noted in parentheses. The final three columns indicate the number of related sequences originating from dead wood, fungal stromata, and other substrates

across the fungal taxa, and it allows for species identification in most fungal groups (Schoch et al. 2012). However, a universal similarity threshold for species delimitation is not applicable, due to major differences in infra- and interspecific sequence variability across the fungal phylum, which would require phylogenetic rather than similarity-based analyses for species identification (Begerow et al. 2010). Furthermore, the ITS region is conserved among species of several large genera, which renders ITS-based identification of these species *a priori* impossible (Schoch et al. 2012). Against these limitations, the vast majority of species identifications based on sequence data alone have to be regarded as hypotheses, to be tested in monographic treatments.

To nevertheless allow for the association of taxon-linked information to the molecularly defined OTUs, the concept of Peršoh et al. (2010) was followed here. This approach does not seek to identify sequences, but simply prepends a taxonomic hypothesis (i.e. a taxon name) to the otherwise unique OTU ID. The process of 'name assignment' thereby accounts for the uncertainties caused by the current limitations discussed above. This is predominantly achieved by the selection of sequences considered for generating a consensus name prepended to each OTU ID. By taking all sequences into account which obtain an alignment score of at least 90 % of that the OTU obtained with the best matching sequence, the number of considered sequences depends from the genetic distance to the best matching published sequence. Accordingly, the more distant the queried sequence is from any published sequences, the higher is the genetic variability considered, which eventually results in a consensus name of higher taxonomic rank. In case of the presence of highly similar or identical sequences, again, the range of considered sequences is limited to close relatives. Unidentified sequences are ignored for generation of the consensus name, but documented as 'ambiguities' (cf. Tab. S1). They therefore do not interfere with the name assignment in the presence of identified reference sequences, but their consideration prevents false name assignments, by indicating the lack of identified reference sequences for OTUs clustering among exclusively unidentified sequences. In the presence of misidentified sequences, strict generation of a consensus name would often be accompanied by a significant loss of information. Therefore the applied concept allows for assigning obviously misidentified sequences as outliers (cf. Tab. S1). An example is OTU '*Cladosporium*-419': Among its best matching sequences, 44 were identified as *Cladosporium* spp. (Dothideomycetes) and one as *Trichoderma koningii* (Sordariomycetes). Accordingly, the OTU would be called 'Pezizomycotina-419', applying a strict consensus name. By classifying and documenting *'T. koningii'* as an outlier, the resulting name becomes more informative, as it indicates the possible association with a certain genus. With regard to misidentified sequences, the number of considered sequences matching the assigned name becomes a highly valuable quality criterion, because a name assignment based on many identified sequences (e.g., 91 for OTU '*Aureobasidium pullulans*-407') appears quite reliable, while other names rely on the correct identification of a single reference (e.g., '*Phaeomoniella zymoides*-440'). However, considering the corresponding quality criteria (i.e. alignment score, number of matching, outlying, and ambiguous names), the assignment of names to OTUs may substantially improve molecular environmental studies with regard to comprehensibility, interpretability, and comparability with non-molecular studies. The corresponding sequences should nevertheless be deposited as 'environmental samples' in public databases, and not as representatives of a certain taxon.

Genetic versus biological abundances

Pyrosequencing (i.e. 'Next Generation Sequencing') yields an immense number of sequence data, thereby also detecting the rare biosphere (Pedrós-Alió 2012). Restricting the interpretation of such data to the presence or absence of OTUs in samples (i.e. frequency) would overestimate the role of the rare and underestimate the role of the abundant data. For endophyte research, a frequency-based evaluation of pyrosequencing data seems particularly inappropriate, because preference rather than strict specificity for a certain host and/or tissue is the major driver of biodiversity (Rodriguez et al. 2009; Sun et al. 2012). Therefore, high-throughput sequencing approaches have to rely on abundance data for interpretation of the results. Cultivation-based approaches again usually plate multiple fragments of a sample and detect a single fungal strain in each fragment. Even though such approaches may be biased against biotrophic and towards fast growing taxa, they detect a more or less random subset from the actual diversity (Unterseher et al. 2013). Thereby, establishing the presence of a certain fungus in a sample becomes more likely with decreasing size and increasing number of plated fragments (Peršoh and Rambold 2012). This applies for endophytic fungi in particular, because they predominantly form highly localized infections (Lodge et al. 1996; Johnston et al. 2006). Reducing fragment size to a maximum by homogenization of the samples, as realized by the dilution-to-extinction technique (Collado et al. 2007), therefore appears to be most appropriate for cultivation-based biodiversity research. Even though the degree of fragmentation is limited to sizes below which viability of the fungi is compromised, the technique has the potential to also detect the rare biosphere (Unterseher and Schnittler 2009). Therefore it is faced with the same question as cultivation-independent approaches: If frequency is an inadequate measure, does abundance reflect natural conditions?

Biomass is the most commonly applied abundance measure in environmental studies. However, it is impossible to

deduce from an isolated culture the biomass a certain fungus possessed in the sample it was isolated from. Molecular studies again have to be aware of the relation between biomass and abundance of molecular marker genes being mostly unpredictable (Baldrian et al. 2013). However, if abundance of the same taxon is compared among similar samples, abundances of ITS rRNA gene amplicons were shown to be at least approximately quantitative (Amend et al. 2010; Davey et al. 2013). Sufficient similarity among the samples processed here is thereby indicated by the considerable overlap (i.e. low *R*-values) between fungal communities in different sampling categories (Table 2). However, abundance of ITS amplicons does not reflect natural abundances among different taxa (Amend et al. 2010; Baldrian et al. 2013). While sequence data allow for biomass inference in theory, establishing this ratio requires rather laborious studies for calibration and each strain has to be cultivated (Raidl et al. 2005; Tellenbach et al. 2010). Because such an approach is not feasible for the numerous uncultured OTUs usually detected by high-throughput molecular methods, one has to accept that some details of community structure, such as dominance, are currently not inferable from DNA sequence data alone. Nevertheless, the enormous number of sequences facilitates comparisons among samples due to the high dynamic range of detected amplicon quantities. Thereby, correlations among OTUs of significant different abundance may be detected, such as between *Hypoxylon fuscum*-227 and Mycota-153, accounting for a total of 2,977 and 132,615 sequences, respectively (cf. Table 4 and S2). When distribution patterns among samples are exhibited by multiple OTUs, their detection is less sensitive to the abundance of single OTUs. This is supported by the congruent results from PERMANOVA analyses of the original abundance matrix and artificial matrices calculated by randomization of abundances among OTUs. Accordingly, patterns among samples revealed by high-throughput sequencing appear rather robust, while extreme care should be taken when single OTUs are compared according to their abundance.

When searching for a suitable marker of OTU abundance within plant tissues, it has also to be considered that many endophytic fungi enter a quiescent state after invading the host plant, at least for a limited period of time (Sieber 2007). Biomass and genomic data, as well as colony forming units (CFUs), reflect the potential of these fungi often representing latent pathogens or decomposers, but cannot tell inactive from active fungi. Thereby, predominantly the latter are of interest for analyzing symbiotic plant-fungus interactions (e.g., Hamilton et al. 2012). Such metabolically active fungi may be selectively quantified by targeting the transcriptome instead of the genome. Thereby the ITS regions in the short-lived precursor rRNA constitute suitable phylogenetic markers reflecting a certain fungus' activity in general and protein coding mRNAs reflect specific metabolic processes (Peršoh et al. 2012).

Accordingly, taxon abundances assessed by different approaches are not necessarily comparable among each other and their respective biological and ecological significances remain to be clarified. For reasons of simplicity, the terms 'rare' and 'abundant' are nevertheless used in the following and applied in their common sense.

Endophytic fungi in the *Pinus*-*Viscum*-system

The detected fungal diversity in the *Pinus*-*Viscum*-system is in so far in accordance with the general pattern of endophytic diversity (Rodriguez et al. 2009), as the majority of OTUs were assignable to the Ascomycota, followed by Basidiomycota and Zygomycota (Table 1). However, the most abundant OTU represents a not further determinable species of Mortierella, many OTUs were unclassifiable and Basidiomycota were almost as abundant as Ascomycota. This is in contrast to all earlier studies on the diversity of endophytic fungi of *Pinus* (Kowalski 1993; Arnold 2007; Zamora et al. 2008; Peršoh et al. 2010). Because the results of this study were highly consistent throughout the 3 independent sampling campaigns and all reasonable measures had been undertaken to avoid contamination, the reason for the deviating spectra certainly lies in the different methodologies applied or in natural variations of the fungal communities. While the latter are certainly of importance, as discussed below, methodological issues may explain the most striking differences, as the earlier studies on endophytic fungi of Pinaceae used cultivation-based approaches. Since almost half of the detected sequences predominantly matched with sequences from other cultivation-independent studies (Table 1) it is likely that the corresponding OTUs only grow reluctantly in culture. The relatively low proportion of Basidiomycota obtained in cultivation-based studies may again be ascribed to the fact that the slower growing Basidiomycota are usually outcompeted on standard isolation media by Ascomycota and Zygomycota (Ainsworth 1995; Thorn et al. 1996; Lindahl and Boberg 2007). However, species of *Mortierella* are generally considered to be readily cultivatable (Domsch et al. 2007). Even though the some of the closest relatives to *Mortierella*-434 (i.e. "verticillata–humilis clade" sensu Petkovits et al. 2011) and *Mortierella macrocystis* were only efficiently isolatable from environmental samples at low temperatures (i.e. 0 °C, Carreiro and Koske 1992), these psychrophilic taxa also grow rapidly in culture at higher temperatures (Domsch et al. 2007). The data obtained here indicate in addition that endophytic occurrences of *Mortierella*-related OTUs are not restricted to local regions, but widely distributed. Their absence from earlier surveys (l.c.) seems even more puzzling because endophytic fungi were isolated from *Pinus sylvestris* by different working groups, certainly not all of which discarded *Mortierella* strains as possible contaminations. A contamination of the plant organ surfaces by adhering DNA again is

unlikely because the applied surface sterilization method was shown to efficiently remove DNA from epiphytic fungi (Peršoh et al. 2013) and the different *Mortierella*-related OTUs showed clear and deviating host and/or organ preferences (Fig. 2). As a working hypothesis for further study, I therefore suggest that endophytic *Mortierella* species assume a quiescent state in plant tissues (cf. Sieber 2007) and require a certain signal to become active again, i.e. to complete their life cycle. In the light of the study by Carreiro and Koske (1992), frost events may be such a trigger.

However, only a subset of the endophytic fungi may be isolated under standard conditions and this fraction is supposed to be biased towards the rare taxa in addition (Pedros-Alio 2006). These so-called satellite taxa are expected to be preferably isolated if the majority of the core community does not readily grow in culture, which seems to be the case in the *Pinus*-*Viscum*-system. Assuming that this applies for pines in general, it may explain the highly deviating endophytic spectra reported earlier (l.c.), because the satellite community is supposed to be predominantly subjected to temporal fluctuations (Magurran and Henderson 2003). This is in accordance with the rapid changes in the endophytic fungal community of the *Pinus*-*Viscum*-system being predominantly ascribable to rare OTUs. Nevertheless, preferences of such satellite taxa detected earlier by cultivation (Peršoh et al. 2010) could be confirmed. Strains of *Hypoxylon fuscum*, for example, were previously regularly isolated from *Viscum* stems and leaves, and the OTU *H. fuscum*-227 was 4 times more abundant in *Viscum* than in *Pinus* (Figs. 1 and 2). *Biscogniauxia nummularia*, again, was exclusively isolated from leaves of *Pinus* and *Viscum* earlier, and while *B. nummularia*-301 was detected now in addition in *Viscum* stems, it was still absent from stems of *Pinus*. This indicates that the fungus is either restricted to photosyntetically active tissues, or depends on the presence of stomata to invade the host species locally. Because the distribution patterns of certain readily cultivatable fungi also significantly correlate with those of abundant OTUs responding to the analyzed factors (Table 4), the cultivatable fraction alone also largely reflects the distribution patterns of the endophytic fungal community as a whole.

OTUs related to fungal taxa most frequently isolated by other authors (i.e. Kowalski 1993; Zamora et al. 2008) constituted only a minor fraction of the cultivation-independent fungal spectrum. In particular, OTUs related to *Alternaria*, *Cladosporium*, and *Lophodermium*, accounted for <0.1 %, 0.5 %, and <0.1 %, respectively. From the species *Anthostomella formosa*, *Cenangium ferruginosum*, and *Cyclaneusma minus*, which accounted together for >64 % of the strains obtained by Kowalski (1993), however, are no ITS sequences available. This illustrates again the above discussed challenges involved in conclusive comparison between biodiversity patterns assessed by cultivation-based and cultivation-independent methods, i.e. the need for reference data and basic research on the biological significance of method-dependent abundance data.

The OTUs predominantly responsible for differences in endophytic fungal community composition (cf. Fig. 2) are either related to known saprotrophic fungi or their closest unidentified relatives have previously been detected in soil and decaying plant material (Table 5). This further strengthens the hypothesis that a considerable part of the endophytic fungal community is involved in the decomposition of plant litter (e.g., Osono 2006; Promputtha et al. 2007; Purahong and Hyde 2011; Peršoh et al. 2013). Beyond that, the results reveal for the first time that the 'latent decomposers' are not randomly distributed among host plants. By selecting for certain decomposer fungi during their endophytic life stage, the host plant may therefore actively affect the litter decomposition process. However, neither of the common OTUs was restricted to a certain host species, indicating that no basic incompatibility exists between host plants and endophytic fungi. Instead, common correlations among the distribution patterns of the non-pathogenic fungi (Table 4) suggest variations in their competitiveness in dependence from the host plant species and/or tissue. By contrast, correlations between saprotrophic taxa and potential plant pathogens were rare and weakly pronounced when negative. A positive effect of endophytic fungi against plant pathogens therefore seems to be exceptional (Arnold et al. 2003). Moreover, the negligible correlations between potential saprotrophic and pathogenic taxa indicate the co-existence of two mostly independent fungal communities.

The clearly larger overlap of the leaf-inhabiting communities between the two hosts, as compared two those inhabiting the stem, renders an infection through the stomata likely. This is also supported by the fungal communities in the stomata bearing stems of the mistletoes being indistinguishable from those in the leaves, while the photosynthetically inactive stems without stomata of the pines host a community which is significantly different from that within the needles. Fungal infection through the stomata would also be in accordance with the *Viscum*-preferring OTUs Mycota-153 and -154 being more abundant in the smaller mistletoe leaves, while *Mortierella*-454, preferring *Pinus*, was more abundant in the larger mistletoe leaves. Due to the higher growth rates, larger organs are expected to bear a higher stomatal density (Masarovlčová 1991; Pääkkönen et al. 1998). Therefore, larger organs might provide more entry points for localized infections of ubiquitous fungi, and the relative abundance of fungi preferring the respective host decreases according to the higher infection rate of non-selective fungi. While this is just a preliminary working hypothesis, infection pathways and the impact of organ size on endophytic fungal community composition certainly deserves closer attention in future studies.

The similarity among the communities within leaves of different age indicates that the communities already

establish themselves within the first year. They may either remain unchanged in the following years or establish anew every year. The major fact opposed to the former option is, that composition of the endophytic communities is known to undergo pronounced seasonal changes (e.g., Osono 2008; Mishra et al. 2012), and it already changed within the *Pinus-Viscum*-system within 2 weeks (Tables 2 and 3). However, these changes were predominantly ascribable to rare OTUs, i.e. satellite taxa. It may therefore not be excluded that the core community remains stable for years. Nevertheless, a re-establishment of the endophytic fungi during each vegetation period is also conceivable. Both scenarios would be in accordance with the consistently lower OTU richness detected in leaves grown in the year of sampling. These leaves only start to grow at the beginning of the sampling season and are thus exposed to the fungal inoculum for a shorter period of time in the current year than the older leaves are. In the stems, the community already changes significantly but faintly from 1- to 3-years-old sections, indicating that the difference may increase with increasing age. However, the data do not allow for differentiating between interannual changes within an established community and possible alterations in penetrability of maturing stem tissues for invading fungi. Interannual shifts in stem-inhabiting communities should therefore be analyzed against the background of seasonal shifts in future studies.

Mistletoe infection was reported earlier to have an impact on the endophytic fungal community in sapwood of pines (Giordano et al. 2009) and three species were found to preferably inhabit infected individuals (*Thanatephorus cocumeris*, *Phoma herbarum* and *Mucor plumbeus*). A similar effect could not be observed in the present study (Table 2) and the responsible taxa were not detected. Even OTUs assignable to related taxa (Cantherellales and Pleosporales) showed no significant differences in frequency or abundance between infected and uninfected trees. It has also been hypothesized that certain endophytic fungi may invade pine trees via the tissues of the mistletoe parasite (Peršoh et al. 2010). This hypothesis may be rejected due to missing evidence for restriction of any endophytic fungi to mistletoe infected pine trees. Actually, the only difference detected was that the 3 year old stem sections of infected pine trees hosted a higher diversity of fungi than those of uninfected pine trees (Fig. 3). Even though the additional OTUs occurred at low abundances there, it may not be excluded that the difference increases with age. In particular the species detected by Giordano et al. (2009) near the stem base possibly only colonize rather old stem sections.

Conclusion

The study showed that interannual changes in endophytic community composition are less pronounced than short-term (i.e. seasonal) changes, at least within leaves of the *Pinus-Viscum*-system. At a certain time, the composition depends from host species and organ type, while locality has a minor impact. The compositional differences were predominantly ascribable to saprotrophic fungi, i.e. latent decomposer fungi. A full understanding of endophytic community structure and dynamics therefore requires knowledge of the full life cycles of the fungi involved. Thereby, simultaneous studies of phyllosphere and pedosphere seem inevitable to get deeper insights into the ecological significance of the endophytic life habit.

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