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# Diversity of *Cryptococcus* and *Dioszegia* yeasts (Basidiomycota) inhabiting arbuscular mycorrhizal roots or spores

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**Abstract**

The genera *Cryptococcus* and *Dioszegia* contain basidiomycetous yeasts found in a wide range of habitats. Primers to amplify the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA) of arbuscular mycorrhizal fungi (AMF) also allow detecting members of this yeast group. Here we report the results of a sequence analysis using maximum parsimony on a set of 50 ITS sequences of yeasts associated with AMF structures (roots of 26 plant species, AM spores) from six field sites in Central Germany. Among 10 separated taxa, respectively five in the Tremellales and two in the Filobasidiales had unknown sequences. Therefore it was not possible to assign these sequences to any known species. The study indicates that exploring the diversity of *Cryptococcus* and *Dioszegia* in soil habitats with molecular methods might enlarge the actually estimated biodiversity of the group.


*Keywords:* Arbuscular mycorrhizal fungi; Biodiversity; *Cryptococcus*; *Dioszegia*; Filobasidiales; Rhizosphere; Soil fungi; Tremellales

## 1. Introduction

Basidiomycetous yeasts of the genus *Cryptococcus* have a world-wide distribution reaching from the tropics to the arctic and antarctic regions and are able to colonize phylloplane and stems of plants [1–3], different kinds of feces [4–6], or aquatic habitats [7,8]. Recent studies have also revealed *Cryptococcus* as a dominating fungal group in soils [9,10].

Classical microbiological methods and physiological profiles only separate a limited number of species, but molecular techniques have shown some of them (e.g. *Cryptococcus albidus* and *Cryptococcus laurentii*) to correspond to species groups [11,12], leading to the description of new taxa [13–15]. Recent studies have revealed the paraphyletic character of *Cryptococcus* that appeared to encompass species within the hymenomycetous Tremellales and Filobasidiales, but also some in the Trichosporonales and Cystofilobasidiales [16,17]. To solve some of the resulting taxonomic problems, the genus *Dioszegia* within the Tremellales was emended based on analysis of the internal transcribed spacer (ITS) of the nuclear ribosomal DNA (nrDNA), and three species, i.e. *D. hungarica*, *D. aurantiaca* and *D. crocea*, formerly belonging to *Bullera* or *Cryptococcus*, were designated to belong to *Dioszegia* [18].

In order to identify arbuscular mycorrhizal fungi (AMF) on roots by analysis of the ITS, we recently developed a nested PCR approach [19]. From its design, we knew that the primer pair for the first PCR has 100% specificity for AMF of the phylum Glomeromycota and also 100% compatibility for Basidiomycota within the Tremellales and Filobasidiales, to which *Cryptococcus* and *Dioszegia* belong. It was therefore to be expected

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that such yeasts could be detected if they colonize the root systems that we currently investigate for AMF. An alignment of the primers showing their compatibility with other fungi is given in Renker et al. [19]. In the present article we have gathered all sequences of those yeasts and submitted them to a sequence analysis. Based on the ITS region we detected sequences which represent unknown *Cryptococcus* and *Dioszegia* species in this so far unexplored habitat that root systems and AM structures represent.

## 2. Materials and methods

### 2.1. Investigated plant and fungal material and field sites

The examined materials consisted of putative arbuscular mycorrhizal (AM) roots (see plant list in Table 1) and spores of AM fungi, collected at sites situated in Thuringia (Germany). Field site No. 1 is a grassland surrounding a former fertilizer plant located in the Central Saale Valley, 13 km north of Jena (11°40′51″E/51°00′46″N, elevation 150–180 m above sea level). The phosphorus content of the soil is high (between 26 and 121 g $kg^{-1}$; detailed description can be found in [20]). An anthropogenic salt marsh near the stream Pöltschbach, east of Berga (12°12′14″E/50°45′31″N, elevation 260 m above sea level), represents site No. 2. Site No. 3 is a fresh meadow near a stream in Friedmannsdorf (12°13′33″E/50°45′25″N, elevation 300 m above sea level). Two mountain meadows in the Thüringer Schiefergebirge, one extensively and the other intensively farmed, represent field site No. 4 (extensively used meadow near Grumbach: 11°30′48″E/50°25′22″N, elevation 710 m above sea level) and 5 (intensively used meadow near Schlegel: 11°37′31″E/50°24′32″N, elevation 640 m above sea level), respectively. Field site No. 6 is a calcareous forest in the Central Saale Valley 10 km north of Jena (11°39′25″E/50°59′31″N, elevation 150 m above sea level). pH values ranged from 5.5 at site No. 4 to 7.7 at site No. 1, i.e. from acidic to alkaline conditions.

### 2.2. DNA extraction and nested PCR

Total plant root systems were removed from the field, and fine roots were chosen randomly. DNA was extracted from single 2-cm long root fragments which were chopped by a razor blade into little pieces and transferred into a 0.5-ml Eppendorf tube. After addition of NaOH (20 μl of 0.25 M) roots were incubated at 90 °C for 10 min. Afterwards Tris–HCl (10 μl 0.5 M) and HCl (20 μl 0.25 N) were added and the extraction mix was incubated for further 10 min. The supernatant was separated from the root fragments by centrifugation, diluted 1:100 in TE buffer and used as PCR template.

Spores of AMF were isolated from soil samples by wet sieving, crushed under a dissecting microscope and used directly in PCR.

Amplification of the ITS region by PCR was performed on a Hybaid Ltd. OmniGene TR3 CM220 Thermo Cycler (MWG-Biotech, Ebersberg, Germany) in a total volume of 50 μl containing 2 U *Taq* DNA polymerase (Promega, Heidelberg, Germany), 5 μl of 10× *Taq* polymerase reaction buffer (Promega), 4 μl of 25 mM $MgCl_2$, 10 nmol of each dNTP (MBI-Fermentas, St. Leon-Rot, Germany), 50 pmol of each of the two primers and 1 μl of the DNA extract. The reactions were performed as hot-start PCR with 10 min initial denaturation at 94 °C before adding the *Taq* polymerase at 80 °C. The PCR program comprised 32 cycles (40 s at 94 °C, 30 s at 54 °C, 40 s at 72 °C). A final elongation of 10 min at 72 °C followed the last cycle.

In the first reaction of nested PCR, the primer pair SSU-Glom1/LSU-Glom1 [19] was used, while the second step of the PCR was performed with the primers ITS5/ITS4 [21].

### 2.3. Cloning, sequencing and sequence analyses

PCR products were cloned into the pCR4-Topo Vector following the manufacturer's protocol of the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany) and transformed into TOP10 Chemically Competent *Escherichia coli*. Sequencing was done on an LI-COR DNA Sequencer Long Reader 4200 using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, UK). After sequencing data were compared with GenBank using the BLASTN program (http://www.ncbi.nlm.nih.gov/blast) to get a first rough estimation of the systematic position of the revealed yeasts.

DNA sequences of the full nrITS were submitted to the EMBL database under the accession numbers given in Figs. 1 and 2 as bold type. Additional sequences were taken from GenBank to clarify the systematic position of the sequences gathered from the roots. Thus, the ITS dataset we used consisted of sequences of 72 basidiomycetous yeasts (50 sequences obtained in this study and 22 sequences published previously by others). ITS1-5.8S-ITS2 sequences were manually aligned in BioEdit version 5.09 [22]. Ambiguously aligned positions were excluded from the phylogenetic analyses.

Maximum parsimony analyses were performed with PAUP* 4.0b10 [23] using the heuristic search mode with 10 random-addition sequence replicates, tree bisection-reconnection branch swapping, MULTrees option on and collapse zero-length branches off. All characters were treated as unordered and equally weighted. Strict consensus trees were calculated including all MP trees.

Table 1
Sequences of yeasts of the Tremellales and Filobasidiales from different field sites

| Best hit | Classification and Accession No. of our sequences | Site No. | Source | Date |
|---|---|---|---|---|
| *Cryptococcus aerius* | | | | |
| AB032666 | *Cryptococcus* sp. Cluster 7 AJ581032-33 | 5 | Roots of *Trifolium repens* | 02.09.02 |
| *Cryptococcus cellulolyticus*/*Bullera pseudoalba* | | | | |
| AF444399 | *Cryptococcus* sp. Cluster 1 AJ581031 | 1 | Roots of *Vicia hirsuta* | 17.06.02 |
| AF444442 | *Cryptococcus* sp. Cluster 1 AJ581034-35 | 4 | Roots of *Trifolium pratense* | 02.09.02 |
| *Cryptococcus podzolicus* | | | | |
| AF444321 | *Cryptococcus* sp. Cluster 1 AJ581037 | 1 | Spore of AMF | 17.06.02 |
| AF444321 | *C. podzolicus* AJ581036 | 4 | Roots of *Briza media* | 24.06.02 |
| *Cryptococcus saitoi* | | | | |
| AF444372 | *Cryptococcus* sp. Cluster 6 AJ581038 | 3 | Roots of *Lolium perenne* | 03.07.02 |
| *Cryptococcus statzelliae* | | | | |
| AY029343 | *Cryptococcus* sp. Cluster 4 AJ581039-42 | 3 | Roots of *Phalaris arundinacea* | 03.07.02 |
| AY029343 | *Cryptococcus* sp. Cluster 4 AJ581043-44 | 3 | Roots of *Plantago lanceolata* | 03.07.02 |
| *Cryptococcus victoriae* | | | | |
| AY188380 | *C. victoriae* AJ581045 | 1 | Roots of *Sonchus arvensis* | 13.08.01 |
| AF444645 | *C. victoriae* AJ581047-48 | 2 | Roots of *Puccinellia distans* | 28.05.02 |
| AF444645 | *C. victoriae* AJ581049 | 5 | Spore of AMF | 02.09.02 |
| AY040655 | *Cryptococcus* sp. Cluster 3 AJ581050 | 1 | Roots of *Atriplex sagittata* | 02.10.02 |
| *Dioszegia crocea* | | | | |
| AF444406 | *D. crocea* AJ581051-54 | 1 | Roots of *Euonymus europaeus* | 13.03.02 |
| AF444406 | *D. crocea* AJ581055 | 1 | Roots of *Galium aparine* | 13.03.02 |
| AF444406 | *D. crocea* AJ581056-59 | 6 | Roots of *Oxalis acetosella* | 09.04.02 |
| AF444406 | *D. crocea* AJ581060-62 and AJ581066-67 | 6 | Roots of *Pulmonaria obscura* | 09.04.02 |
| AF444406 | *D. crocea* AJ581063 | 6 | Roots of *Veronica hederifolia* | 09.04.02 |
| AF444406 | *D. crocea* AJ581064-65 | 6 | Roots of *Ranunculus auricomus* | 09.04.02 |
| AF444406 | *D. crocea* AJ581068 | 1 | Roots of *Myosotis ramosissima* | 02.05.02 |
| AF444406 | *D. crocea* AJ581069 | 1 | Roots of *Mentha arvensis* | 02.05.02 |
| AF444406 | *D. crocea* AJ581070 | 1 | Roots of *Inula conyza* | 02.05.02 |
| AF444406 | *D. crocea* AJ581071-72 | 1 | Roots of *Arrhenatherum elatius* | 21.05.02 |
| AF444406 | *D. crocea* AJ581073 | 1 | Roots of *Trifolium campestre* | 21.05.02 |
| AF444406 | *D. crocea* AJ581074-75 | 1 | Roots of *Poa pratensis* | 21.05.02 |
| AF444406 | *D. crocea* AJ581076 | 2 | Roots of *Angelica sylvestris* | 28.05.02 |
| AF444406 | *D. crocea* AJ581077 | 1 | Roots of *Galium album* | 04.06.02 |
| AF444406 | *D. crocea* AJ581078 | 3 | Roots of *Lolium perenne* | 03.07.02 |
| AF444406 | *D. crocea* AJ581079 | 1 | Roots of *Artemisia vulgaris* | 17.06.02 |
| AF444406 | *D. crocea* AJ581080 | 1 | Spore of AMF | 17.06.02 |
| *Dioszegia hungarica* | | | | |
| AB049614 | *Cryptococcus* sp. Cluster 5 AJ581081 | 5 | Roots of *Lamium album* | 22.07.02 |

Column 1 gives the accession number of the best hit with identified species based on comparisons with GenBank using the BLASTN program. Species identities of the best hits are given once in italics. Column 2 gives the classification and accession number of the species revealed in this study. Numbers in column 3 refer to the field sites from which root and soil samples were taken as described in Section 2. All sites are located within Thuringia (Germany). Column 4 gives the source from which yeasts were isolated (AMF: arbuscular mycorrhizal fungi). The sampling date is given in column 5.

The confidence of branching was assessed using 1000 bootstrap resamplings.

The data set used to reconstruct the tree of the Tremellales (Fig. 1) contained 507 characters of which 406 were constant, 32 parsimony uninformative and 69 parsimony informative. The data set used to reconstruct the tree of the Filobasidiales (Fig. 2) contained 396 characters of which 233 were constant,

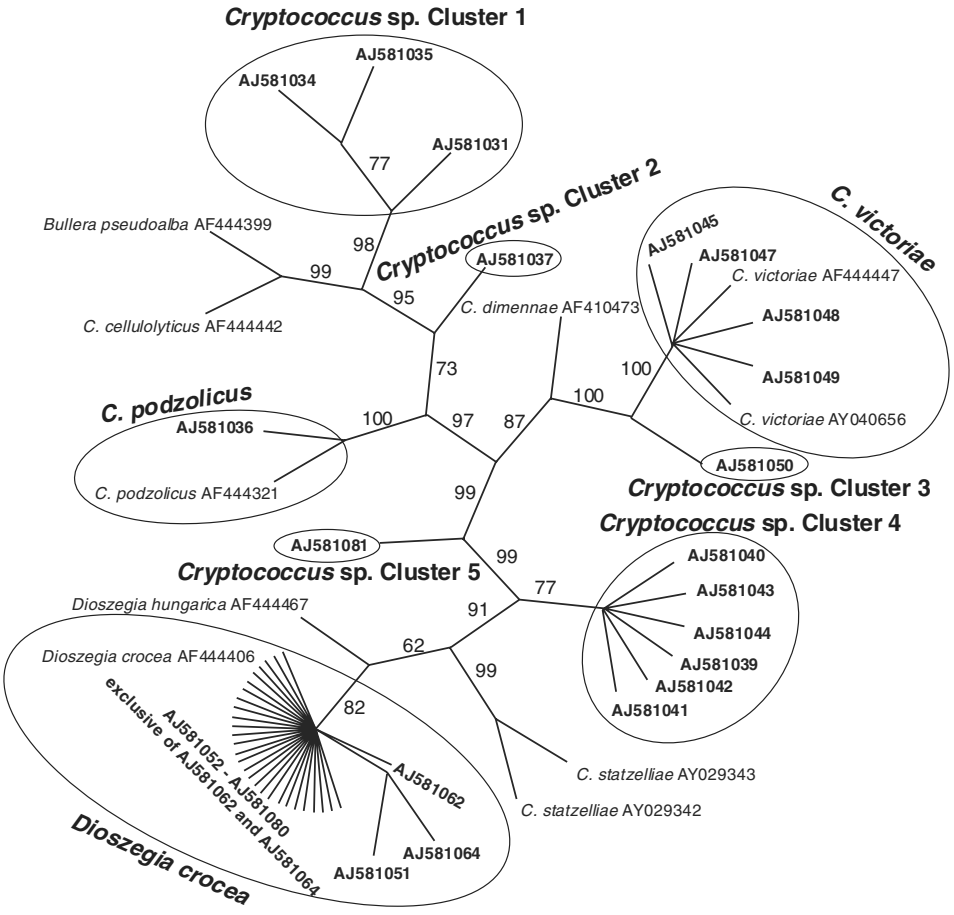


Fig. 1. Strict consensus tree of the Tremellales based on 47 ITS sequences obtained in this study (given in bold) and 10 previously published sequences obtained from GenBank. Sequence clusters related to our data and which can be regarded as one species are marked with circles. If attributable to known species their name is given.

59 parsimony uninformative and 104 parsimony informative.

## 3. Results

Yeasts were detected in AM roots and spores. Compared to the obtained AM sequences, the proportion of PCR products corresponding to yeasts varied between 0% and 20% according to the different field sites. Altogether 50 yeast sequences belonging to *Cryptococcus* and *Dioszegia* were gathered in this study (Table 1). As shown in the phylogenetic reconstructions, these sequences represented eight distinct clusters or lineages within the Tremellales (Fig. 1) and two additional within the Filobasidiales (Fig. 2). Noteworthy in the constructed trees is the fact that six sequence clusters found in the phylogenetic analysis are clearly distant from their best hits as revealed by BLAST search (Table 2). In the Tremellales, this is the case for sequence AJ581081 against *D. hungarica*; for sequences in cluster 1 against *C. cellulolyticus* or *Bullera pseudoalba*, for which conspecificity has been suggested [17]; for sequence AJ581037 against *C. podzolicus*; for sequences in cluster 4 against *C. statzelliae*; and for sequence AJ581050 against *C. victoriae* (Fig. 1). Within the Filobasidiales (Fig. 2) sequence AJ581038 is clearly separated from its best hit *C. saitoi*, as well as all sequences in cluster 7 from *C. aerius*. These cases of branching might indicate that the concerned clusters correspond to so far unknown species.

Yeasts were detected on root samples of 26 plant species belonging to thirteen families (Table 1). *Dioszegia crocea* was the most common species with 30 sequences from sixteen root systems belonging to sixteen different plant species, representing eleven families. *Dioszegia crocea* was detected at four of the six sites under study. *Cryptococcus victoriae* was found at three sites. All other species were detected at only one site and in most cases on a single root sample.

Besides sequences of *Cryptococcus* and *Dioszegia*, five sequences of yeasts belonging to other phylogenetic

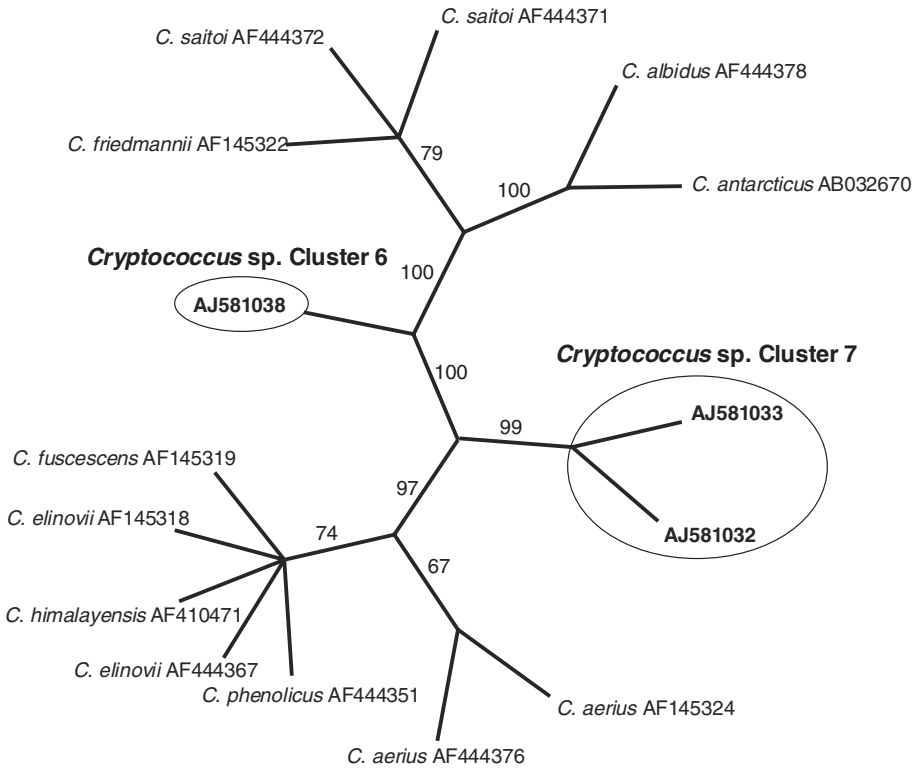


Fig. 2. Strict consensus tree of the Filobasidiales based on three ITS sequences obtained in this study (given in bold) and 12 previously published sequences obtained from GenBank. Sequence clusters related to our data and which can be regarded as one species are marked with circles.

Table 2
Identity of sequences gathered in this study in comparison with sequences available in GenBank

| Classification of own sequences | Best hit using BLASTN | Identity (%) | No. of aligned bp (including gaps) | No. of variable positions | No. of gaps |
|---|---|---|---|---|---|
| *Cryptococcus podzolicus* | *C. podzolicus* AF444321 | 99 | 518 | 1 | 5 |
| *Cryptococcus victoriae* | *C. victoriae* AY188380 or AF444645 | 99–100 | 495/520 | 2–7 | 0–2 |
| *Cryptococcus* sp. Cluster 1 | *C. cellulolyticus* AF444442 or *Bullera pseudoalba* AF444399 | 86–89 | 548–550 | 48–69 | 8–13 |
| *Cryptococcus* sp. Cluster 2 | *C. podzolicus* AF444321 | 77 | 528 | 101 | 18 |
| *Cryptococcus* sp. Cluster 3 | *C. victoriae* AY188380 | 87 | 497 | 61 | 4 |
| *Cryptococcus* sp. Cluster 4 | *C. statzelliae* AY029343 | 90–91 | 487 | 40–44 | 5 |
| *Cryptococcus* sp. Cluster 5 | *Dioszegia hungarica* AB049614 | 84 | 507 | 66 | 13 |
| *Cryptococcus* sp. Cluster 6 | *C. saitoi* AF444372 | 76 | 644 | 129 | 24 |
| *Cryptococcus* sp. Cluster 7 | *C. aerius* AB032666 | 94 | 630 | 34 | 6 |
| *Dioszegia crocea* | *D. crocea* AF444406 | 99–100 | 476 | 1–5 | 0–1 |

Column 1 gives the classification of our sequences revealed in this study. Column 2 gives the best hit of our sequences with available sequences in GenBank using the BLASTN program. Column 3 gives the identity of our sequences with the best hit (calculated as value in column 4 minus values in columns 5 and 6, divided by values in column 4). Column 4 gives the number of base pairs of the pairwise sequence alignments between our sequences and the corresponding sequence of the best hit. Column 5 gives the number of variable positions and column 6 gives the number of gaps in these alignments.

groups were detected. Two different sequences with 95% identity over 593 bp to each other from field site 5 had their best hit with a *Trichosporon* spec. (AF444446), to which they showed identities of 62 and 63% (over 566 and 563 bp, respectively). They had been collected from a mycorrhizal spore and roots of *Trifolium pratense*, respectively. On field site 4 we found a sequence from roots of *Trisetum flavescens* which has its best hit with *Fellomyces distylii* AF444475 (76% over 556 bp). Two different sequences with 78% identity over 661 bp (compared to each other) had there best hit with *Itersonilia perplexans* AB072233, and were detected on roots of *Poa trivialis* on field site 1 and roots of *Lolium multiflorum* on field site 5. While the first sequence had only

77% identity (over 627 bp), the latter showed 100% identity (only three variable nucleotides over 624 bp) with this sequence of *I. perplexans*. Because of their single character no further analysis was performed for these sequences.

## 4. Discussion

The molecular approach used in this work constitutes a step forward in the analysis of *Cryptococcus* yeasts, because it allows studying the diversity of these fungi directly on field material without time-consuming and hazardous cultivation steps.

Compared to former studies in soils which mainly used morphological and physiological features, the resolution of the taxonomic level was increased with our molecular approach. For example, Sláviková and Vadkertiová [9] have detected only four different *Cryptococcus* species in soils of three different forests and some of their species (i.e., *C. albidus* and *C. laurentii*, see above) might have represented species groups which could have been unravelled with molecular tools. Other difficulties using physiological and morphological tests concern the often considerable variability of these features within species [16].

Performing phylogenetic analysis based on ITS sequences of species being as variable as yeasts might seem awkward, but Fell et al. [16] and Scorzetti et al. [17] recommend the use of ITS sequences for species separation because this is one possible way to split up typologically clearly distinct species which sometimes would appear identical based on sequences of the D1/D2 domains of the coding large subunit (LSU) of the rDNA. Based on their observations, Fell et al. [16] have stated that the taxonomy of some *Cryptococcus* species might be clarified by ITS analysis.

As shown in Figs. 1 and 2 the obtained sequence data set indicates that in putative AM roots and in spores we detected eight different species belonging to the Tremellales and two species in the Filobasidiales, which are placed in clades with significant bootstrap support. Five species in the Tremellales and both within the Filobasidiales seem to represent species for which no sequences were available so far, although substantial sequencing programs have been performed for hymenomycetous yeasts [16,17]. However, without a cultivation step, which was not the focus of our study, it is not possible to definitively conclude that new yeast species were detected. Estimates indicate that the number of known yeast species may represent about 1% of the species existing in this group of fungi in nature [16]. Therefore, new species might be represented by the unknown sequences, thus reinforcing the presumption that the diversity of basidiomycetous yeasts is far from being analysed exhaustively and that exploring new habitats with molecular tools is a promising approach.

The low variability within the sequences of the *Cryptococcus* clusters 1–7 makes it likely that these clusters represent new species (Table 2), and also indicate a low intraspecific variability. Fell et al. [16] have stated that strains differing by more than one or two nucleotides in the D1/D2 region represent different taxa. Unfortunately, they do not give an estimation for differences in the ITS region necessary to separate strains. The observed identities of 77% to maximal 94% between different clusters containing our sequences and the corresponding best hits within GenBank on the one hand, and sequence identities of 99–100% within clusters (Table 2) on the other hand, indicate the distinct character of the clusters.

Besides these potentially new species, members of three already known yeasts were detected in the study. This concerns sequence AJ581036 which is close to that of *C. podzolicus* (AF444321), and all sequences of the *D. crocea* cluster. The third case of identification concerns *C. victoriae*. Here, it is noteworthy that up to 100% identity to a reference sequence (AF 444447) was found for four sequences (AJ581045-49) from three field sites. *C. victoriae* has originally been described from Antarctica [13]. Species closely related have recently been isolated from soil samples in Iceland [15]. *C. statzelliae*, another recently isolated yeast from Antarctica, was described as a yeast species with a close phylogenetic relationship to *D. crocea* [24]. Together with our sequence AJ581081 and the sequences of cluster 4 they form a monophyletic group of distinct taxa (see Fig. 1).

The antibacterial capacity of some yeasts, e.g. *C. laurentii*, is well established [2,25]. Some species, namely *C. neoformans* but also *C. albidus, C. curvatus* and *C. laurentii*, are identified as human pathogens, especially in AIDS patients [26–29]. The present work shows that species in *Cryptococcus* and *Dioszegia* are not uncommon on putative AM roots and AM spores in nature. Such relationships between human pathogenic fungi and soil organisms might be of epidemiological importance. In a recent paper we have detected some skin disease-causing fungi of the genus *Malassezia* in association with soil nematodes [30].

The examined materials were not sufficient to draw any conclusions on the specificity of *Cryptococcus* and *Dioszegia* for certain plant roots, AM species or even field sites. Similarly, the present investigation only allows to speculate on the nature of the interaction between the yeast and their host structure. Recent studies considering the interactions of soil microbiota have shown a growth-promoting effect of certain yeasts on AMF [31]. Whether the revealed yeasts are beneficial, neutral or antagonistic to AMF remains to be established.

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