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Molecular community analysis of arbuscular mycorrhizal fungi—Contributions of PCR primer and host plant selectivity to the detected community profiles

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ABSTRACT

Various primers targeting different regions of nuclear ribosomal DNA (rDNA) are commonly used in studies addressing diversity of soil- or root-associated fungi including the arbuscular mycorrhizal fungi (AMF) from the phylum Glomeromycota. Nevertheless, direct comparisons of the different primers remain rare. In this study, AMF community profiles were generated by 454 pyrosequencing of amplicons resulting from direct (i.e., single) PCR amplification with three commonly used primer pairs. Root DNA extracts from four different plant species growing in the same field soil were included into the study to address the following aspects: (1) specificity of the primers for Glomeromycota, (2) structure of the detected AMF communities, and (3) efficiency of the different primers for detecting specific AMF genera. The magnitude of the effect due to PCR selectivity was then compared with the well documented effect of host plant identity on the structure of AMF communities. The primers targeting the small ribosomal subunit (SSU) yielded almost exclusively glomeromycotan sequences. The primers targeting the large ribosomal subunit (LSU) were reasonably selective for Glomeromycota (75% of the operational taxonomic units [OTUs] detected), whereas only about 25% of the OTUs obtained by the primers targeting the internal transcribed region (ITS) of the rDNA belonged to the Glomeromycota. There was good agreement in detecting AMF community dominants between the different PCR primers. In comparison to the AMF selectivity due to host plant identity, the variation due to PCR primer choice was even larger. This was partly due to a large fraction of (mainly ITS) sequences that could not be assigned to validly described AMF taxa. The community profiles generated by the SSU and LSU primers also differed significantly from each other, mainly due to considerable SSU primer selectivity. The SSU primers in fact missed 4 rare AMF genera out of at least 11 genera present in the analyzed AMF community. Our study thus highlights the magnitude of possible bias in AMF community profiling that can occur using single-PCR amplification coupled with next-generation sequencing.

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1. Introduction

Arbuscular mycorrhizal fungi (AMF, phylum Glomeromycota) are obligate symbionts of 70–90% of land plant species (Smith and Read, 2008). These fungi play a crucial role in a number of ecosystem processes, such as movement of mineral nutrients, carbon, and water between soil and plants (van der Heijden et al., 2003); stabilization of soil aggregates (Leifheit et al., 2014), plant coexistence (van der Heijden et al., 1998), interactions of plants with pathogens (Newsham et al., 1995; Vigo et al., 2000), and plant tolerance to drought (Aroca et al., 2007). A number of AMF taxa coexist in virtually every soil and ecosystem, including agricultural monocultures, strongly suggesting that the various taxa play

Abbreviations: OTU, operational taxonomic unit; rDNA, nuclear ribosomal DNA; AMF, arbuscular mycorrhizal fungi; LSU, large ribosomal subunit; SSU, small ribosomal subunit; ITS, internal transcribed spacer; ANOVA, analysis of variance; PerMANOVA, permutational multivariate analysis of variance; PCR, polymerase chain reaction.

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different roles (Jansa et al., 2008; Koide, 2000) and the community thus cannot easily and without consequences be replaced by a single taxon (but see Mathimaran et al., 2005). Characterization of AMF communities thus appears to be of crucial importance not only for bioindication (Jansa et al., 2014) but also for understanding the importance of these fungi in ecosystem functioning.

Traditionally, description of AMF communities' composition was based solely on spore morphologies. This had its limitations due to insufficient numbers of morphological features in some AMF taxa, occurrence of non-sporulating species, or dependence of spore production on environmental conditions and physiological parameters of the various AMF taxa (Oehl et al., 2004, 2009). The morphology approach has more recently been supported by using biochemical features such as isozymes (Dodd et al., 1996; Hepper et al., 1986; Rosendahl and Sen, 1992; Rosendahl et al., 1989), which enable separation of taxa at different (even genotype) levels. Further, AMF taxonomy has been greatly stimulated by the development of molecular methods enabling phylogenetic reconstructions, very sensitive separation of closely related taxa, and AMF identification/quantification. These methods do not require spores and can easily be deployed to analyze environmental samples such as roots or soils (Dumbrell et al., 2010; Öpik et al., 2010). Moreover, our knowledge of the AMF distribution and diversity substantially increased with the implementation of high-throughput sequencing technologies, and particularly of 454 amplicon pyrosequencing (Margulies et al., 2005).

Various nuclear ribosomal DNA (rDNA) regions have commonly been used in AMF molecular ecology studies since the advent of molecular approaches (e.g., Clapp et al., 1995; Helgason et al., 1998). To describe the diversity of indigenous AMF communities in environmental samples, the small ribosomal subunit (SSU) rDNA region has now most frequently been targeted (Öpik et al., 2006; Öpik et al., 2010; Öpik et al., 2013). Thus, SSU provides us with the largest reference sequence data set compared with the other genomic regions (Öpik et al., 2010). Additionally, various parts of the large ribosomal subunit (LSU) rDNA region have been used in AMF community studies based on end-point PCR (Jansa et al., 2003; van Tuinen et al., 1998), pyrosequencing (e.g., Lekberg et al., 2012), or quantitative PCR (Jansa et al., 2014). Internal transcribed spacer (ITS) region is overly variable in Glomeromycota and has been claimed to provide only a limited power to resolve closely related species (Stockinger et al., 2010). Nevertheless, both the LSU rRNA genes and ITS have frequently been used in previous taxonomic studies of AMF, even as the focus on SSU rRNA clearly dominates in recent ecological studies (Öpik et al., 2014). On the other hand, recent research has identified the ITS region to be the superior universal barcoding region for fungi including the Glomeromycota (Schoch et al., 2012), and the first studies are now being published on the glomeromycotan sequences being pulled from much larger fungal ITS sequence data sets and analyzed separately (e.g., Maestre et al., 2015).

Different primers have been used in the past to amplify various parts of the fungal rDNA region for purposes of community ecology of fungi in general and the AMF in particular. Moreover, it is increasingly recognized that the choice of primers has a significant impact on the detected community profiles due to PCR and/or cloning bias (Bellemain et al., 2010; Morgan et al., 2010; Polz and Cavanaugh, 1998; Tedersoo et al., 2010). However, there is still only limited quantitative information as to how much the different primers distort the AMF community profiles – apart from the evidence that they do so. Moreover, direct comparisons evaluating the reproducibility/comparability of the different primer pairs in AMF community studies are sparse. Recently, Kohout et al. (2014) compared several commonly used primer combinations targeting different rDNA regions. Some of the amplifications included several rounds of PCR using a nested PCR approach, and the study was based on cloning and Sanger sequencing instead of on massive parallel sequencing technologies. Apart from significant shifts in AMF community profiles due to the primers employed (i.e., overdominance by Glomeraceae in the profiles generated by both SSU and LSU primers as compared to those targeting the ITS region), they recorded differential selectivity of the various primers for Glomeromycota. Van Geel et al. (2014) evaluated six primers targeting the SSU or LSU (though not the ITS) regions which have previously been used in AMF community analyses for their usefulness in 454 pyrosequencing of amplicons generated from apple tree roots. They found their LSU primers to have lower selectivity for Glomeromycota. On the other hand, these authors detected three of the four tested SSU primer pairs to be powerful for AMF community characterization but to vary in detecting particular AMF families. Stockinger et al. (2010) compared several rDNA regions to identify possible candidates for DNA barcoding of AMF. They identified the LSU-D2 and ITS2 regions of the rDNA to be most suitable for high-throughput 454 GS-FLX Titanium pyrosequencing.

The aim of the present study was to directly compare the distortion of AMF community profiles due to PCR primer choice with the well described selective effect of host plant species (Helgason et al., 2002; Jansa et al., 2002a). To this end, we included three primer pairs, each targeting one of the three major regions of interest (i.e., SSU, ITS, and LSU) and which have previously been used frequently in amplification of fungi/AMF from roots and/or soil samples. The primers were tested on roots of different plant species growing in the same (nonsterile) field soil. Unlike most previous studies, we aimed to reduce PCR bias by employing a single (direct) PCR amplification rather than a nested PCR approach, which a priori excluded some of those PCR protocols relying solely on nested PCR (e.g., Kohout et al., 2014). In particular, we aimed to (a) evaluate selectivity of the different primer pairs for the AMF, (b) determine their efficiency to detect specific AMF genera, (c) compare the contributions of the different primer pairs and plant selectivity to the detected variation in AMF community profiles, and (d) identify significant associations between certain plant and AMF taxa.

2. Material and methods

2.1. Experimental soil

The composition of AMF communities was analyzed in alluvial soil under permanent and natural (unfertilized, not mown) grassland in Litoměřice, Czech Republic (N 50°31′54.53″, E 14°06′37.10″). About 10 kg of topsoil (0–15 cm depth) was collected from four spots within a 20 × 20 m quadrant on 3 April 2013 and mixed to homogenize. The altitude of the collection site was 170 m above sea level. For physicochemical analyses, the soil was dried at room temperature for 14 days. Soil acidity (pH) as assessed in a water slurry (1:10, w:v) was 7.88. Total phosphorus (P) content as measured by a Malachite green method (Ohno and Zibilske, 1991) in concentrated HNO_3 extract of dry ashed soil (550 °C for 12 h) was 797 mg kg^{-1}. Water extractable P content (soil slurry 1:10 w:v, shaken end over end for 18 h and passed through a 0.2 μm filter) was 3.3 mg kg^{-1}, and the soil mineral fraction (after removal of organic matter with H_2O_2) contained 42% clay and 40% sand. Organic carbon content as assessed by CN analyzer (Flash EA 2000, ThermoFisher Scientific, Waltham MA, USA) was 2.26%, and the total nitrogen content was 0.13%.

2.2. AMF trap cultures

The nonsterile and naturally moist soil described above and with large roots removed (the soil having been shaken off) was

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