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Comparison of commonly used primer sets for evaluating arbuscular mycorrhizal fungal communities: Is there a universal solution?

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ABSTRACT

Different primer systems have been developed to characterize arbuscular mycorrhizal fungal (AMF) communities; however, a direct comparison of their specificity, potential to describe diversity and representation of different phylogenetic lineages is lacking. Using seven root samples, we compared four routinely used AMF-specific primer systems for nuclear ribosomal DNA covering i) the partial small subunit (SSU), ii) the partial large subunit (LSU), iii) the partial SSU and internal transcribed spacer (ITS; "Redecker") and iv) the partial SSU–ITS–partial LSU region ("Krüger"). In addition, a new primer combination v) covering the ITS2 region (ITS2) was included in the comparison. The "Krüger" primers tended to yield the highest AMF diversity and showed a significantly higher Shannon diversity index than the SSU primers. We found a strong bias towards the Glomeraceae in the LSU and SSU primer systems and differences in the composition of AMF communities based on the "Redecker" primer system. Our results confirm the crucial role of the choice of target rRNA marker region for analysing AMF communities. We also provide evidence that nested-PCR based data can be interpreted semi-quantitatively and that the extent of observed AMF community overdominance largely depends on the choice of primer.

1. Introduction

Fungi from the phylum Glomeromycota (Schüssler et al., 2001) are an important component of ecosystems because they form arbuscular mycorrhiza, the most widespread type of symbiosis in the plant kingdom. In natural conditions, plants are colonized by communities of arbuscular mycorrhizal fungi (AMF), whose diversity and identity influence the structure and functioning of plant communities (van der Heijden et al., 1998).

The development of molecular methods for identifying AMF directly within plant roots has boosted the research of AMF communities (Simon et al., 1992; Helgason et al., 1998; Öpik et al., 2009). In contrast to diversity studies based on morphological determinations of soil-born spores (Johnson et al., 1991), DNA-based approaches enable the identification of AMF taxa, which directly interact with plants (Clapp et al., 1995; Hempel et al., 2007).

Different molecular markers have been described to identify AMF species or to investigate the AMF phylogeny. Most studies routinely use nuclear ribosomal RNA (rRNA) gene sequence markers. Three rRNA regions, individually or in combination, are used as molecular markers: the partial small subunit (SSU) rRNA gene, the internal transcribed spacers (ITS1, 5.8S and ITS2) and the partial large subunit (LSU) rRNA gene. The choice of rRNA region is crucial because rRNA regions differ in their ability to distinguish closely related AMF species (species resolution power) and in the extent to which well-determined sequences are represented in public sequence databases (Stockinger et al., 2010; Schoch et al., 2012). Moreover, the primer systems used for their amplification often discriminate certain AMF lineages or co-amplify DNA from non-target organisms (Stockinger et al., 2010), reflecting the difficulty to develop primers exclusively for AMF.

The SSU rRNA gene is the most frequently used molecular marker (e.g. Helgason et al., 1999; Lee et al., 2008; Öpik et al., 2008).

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The relatively low variability of AMF SSU sequences, compared to the ITS region, makes it possible to align the whole Glomeromycota into a single dataset, which facilitates phylogenetic analyses. Compared to the other primer systems in use, this region also provides semi-quantitative information about AMF communities because it can be amplified in a single-step PCR. However, previously designed primer systems for the SSU rRNA gene may exclude basal lineages of the Glomeromycota (Lumini et al., 2010), or co-amplify plant DNA (Alguacil et al., 2011) or non-target fungal groups (Liu et al., 2011).

The ITS rRNA region offers large sequence variability within the Glomeromycota and consequently a high discriminative power down to the species level (Stockinger et al., 2010). Currently, most Glomeromycota diversity studies which use the ITS marker employ a system of family-specific primers developed by Redecker (2000) and Redecker et al. (2003). Although it is more labour intensive than systems using a single AMF-universal primer pair, its higher sensitivity for less abundant AMF lineages, which may remain undiscovered by a single primer system on sites dominated by *Glomus* species (e.g. Hijri et al., 2006), presents a clear advantage.

The LSU resolution power at the species level is based on the D2 variable region and is comparable to that of ITS (Stockinger et al., 2010). However, results obtained by the most commonly used FLR4/FLR3 primers (Gollotte et al., 2004) might suffer from a considerable bias towards the Glomeraceae (Gamper et al., 2009). Despite this disadvantage, they are frequently used for analyses of AMF communities (e.g. Bainard et al., 2011; Meadow and Zabinski, 2012), especially by terminal restriction fragment length polymorphism (tRFLP) according to the system proposed by Mummey and Rillig (2007).

To overcome the above-mentioned problems and improve molecular species characterization of the Glomeromycota, Krüger et al. (2009) designed a mixed primer set, which amplifies an AMF rRNA fragment of approximately 1500 bp covering the partial SSU, the whole ITS and the partial LSU including the variable D1 and D2 regions. This primer combination, however, has so far been tested only in a very limited number of field studies (Wang et al., 2011; Fahey et al., 2012). Because of the length of the amplified DNA fragment, it is not suitable for next generation sequencing. For 454 sequencing-based studies, Stockinger et al. (2010) therefore recommended a combination of their primer system with nested-PCR amplification of a short variable fragment such as ITS2-partial LSU regions. Still, no suitable AMF-specific primer combination exists for its amplification.

Whereas most AMF diversity data are based on cloning and Sanger sequencing of clones, several recent studies have adopted the new 454-sequencing approach (e.g. Öpik et al., 2009; Lumini et al., 2010). This high-throughput technology overcomes the cloning step and provides orders of magnitude more data while saving time, labour and financial costs. The choice of primers and PCR conditions, however, remain crucial for obtaining an unbiased picture of the fungal community (Lumini et al., 2010; Tedersoo et al., 2010).

Though previous studies point at the limitations of certain specific primer systems in terms of exclusion or discrimination of certain lineages within the Glomeromycota (e.g. Daniell et al., 2001; Gamper et al., 2009), a direct comparison, which would systematically assess their relevance in diversity studies is missing. The aim of our study was therefore to compare AMF communities described by the most commonly used AMF primer systems in a set of field-collected root samples of arbuscular mycorrhizal plants. The specific goals of our comparison were the following: i) to evaluate specificity of the selected primer systems to AMF, ii) to determine differences in the detected spectra of AMF taxa, iii) to compare semi-quantitative information about the relative abundance of AMF molecular operational taxonomic units (MOTUs) and iv) to critically assess the suitability of different primer systems for AMF diversity studies.

Our comparison was mainly based on the primer system developed by Krüger et al. (2009) because the length of the amplified fragment enables a direct comparison with previously used SSU-ITS-based and LSU-based primer systems (Redecker, 2000; Gollotte et al., 2004). Additionally, we amplified the ITS2 region using a newly proposed primer combination. Unfortunately, the fragment amplified by the primers of Krüger et al. (2009) does not cover the SSU region used in most diversity studies (Helgason et al., 1998, 1999; Lee et al., 2008), thus precluding a direct comparison of the detected taxa between these two systems. We nevertheless included the SSU region in our study to compare the obtained diversity with results attained for other markers, focussing on the representation of the main AMF lineages and relative abundance of AMF taxa.

2. Materials and methods

2.1. Sampling and study sites

Six plant species from different locations and biotopes were chosen for this comparative study based on our preliminary results, which indicated that these plants differ in AMF taxon richness and community composition (see Table 1). A single adult plant per each species/biotope combination was sampled and transported to the laboratory, where the root system was washed, cut into pieces, frozen in aliquots of 50–100 mg in Eppendorf tubes and stored at −80 °C until use.

2.2. DNA extraction and polymerase chain reactions (PCR)

Root samples were ground in liquid nitrogen using a mortar and pestle. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen,

Table 1
List of samples included into the study.

Sample code	Host plant	Locality	Biotope	Coordinates	Altitude (a.s.l.)	Sampling time
LIT	*Littorella uniflora*	Mjåvatn, Rogaland S, Norway	Freshwater oligotrophic lake	58°22′53″N, 6°06′46″E	50 m	August 2010
LOB	*Lobelia dortmanna*	Mjåvatn, Rogaland S, Norway	Freshwater oligotrophic lake	58°22′53″N, 6°06′46″E	50 m	August 2010
TAN	*Tanacetum vulgare*	Hradec Králové, E Bohemia, Czech Republic	Species-poor field abandoned for ≥10 years	50°13′18″N, 15°52′49″E	237 m	November 2010
LEU	*Leucanthemum ircutianum*	Haratice, N Bohemia, Czech Republic	Species-rich mountain meadow	50°41′18″N, 15°19′59″E	490 m	November 2010
BMG	*Briza media*	Malešov, N Bohemia, Czech Republic	Seminatural dry grassland	50°30′02″N, 14°18′55″E	242 m	July 2010
BPG	*Brachypodium pinnatum*	Malešov, N Bohemia, Czech Republic	Seminatural dry grassland	50°30′02″N, 14°18′55″E	242 m	July 2010
BPF	*Brachypodium pinnatum*	Malešov, N Bohemia, Czech Republic	Adjacent field abandoned for about 20 years	50°30′05″N, 14°18′56″E	242 m	July 2010