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Evaluation of six primer pairs targeting the nuclear rRNA operon for characterization of arbuscular mycorrhizal fungal (AMF) communities using 454 pyrosequencing

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ABSTRACT

In the last few years, 454 pyrosequencing-based analysis of arbuscular mycorrhizal fungal (AMF; Glomeromycota) communities has tremendously increased our knowledge of the distribution and diversity of AMF. Nonetheless, comparing results between different studies is difficult, as different target genes (or regions thereof) and primer combinations, with potentially dissimilar specificities and efficacies, are being utilized. In this study we evaluated six primer pairs that have previously been used in AMF studies (NS31–AM1, AMV4.5NF–AMDGR, AML1–AML2, NS31–AML2, FLR3–LSUmBr and Glo454–NDL22) for their use in 454 pyrosequencing based on both an in silico approach and 454 pyrosequencing of AMF communities from apple tree roots. Primers were evaluated in terms of (i) in silico coverage of Glomeromycota fungi, (ii) the number of high-quality sequences obtained, (iii) selectivity for AMF species, (iv) reproducibility and (v) ability to accurately describe AMF communities. We show that primer pairs AMV4.5NF–AMDGR, AML1–AML2 and NS31–AML2 outperformed the other tested primer pairs in terms of number of Glomeromycota reads (AMF specificity and coverage). Additionally, these primer pairs were found to have no or only few mismatches to AMF sequences and were able to consistently describe AMF communities from apple roots. However, whereas most high-quality AMF sequences were obtained for AMV4.5NF–AMDGR, our results also suggest that this primer pair favored amplification of Glomeraceae sequences at the expense of Ambisporaceae, Claroideoglomeraceae and Paraglomeraceae sequences. Furthermore, we demonstrate the complementary specificity of AMV4.5NF–AMDGR with AML1–AML2, and of AMV4.5NF–AMDGR with NS31–AML2, making these primer combinations highly suitable for tandem use in covering the diversity of AMF communities.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) form a root symbiosis with approximately 80% of the terrestrial plant species and improve nutrient and water uptake as well as pathogen resistance in their hosts in exchange for plant assimilated carbon (Smith and Read, 2008). It is therefore increasingly acknowledged that AMF play a key role in ecosystem functioning, and therefore, quantifying and understanding their distribution and diversity is of major importance (Rillig and Mummey, 2006; van der Heijden et al., 2008). All AMF belong to the phylum Glomeromycota (Schüβler et al., 2001), which is divided in four orders (Diversisporales, Glomerales, Archaeosporales and Paraglomerales) and ten families. Most AMF species belong to the families of Diversisporaceae, Acaulosporaceae, Gigasporaceae, Claroideoglomeraceae and Archaeosporaceae (Redecker et al., 2013).

Molecular methods have become the standard for studying AMF communities (Gorzelak et al., 2012). Especially high throughput sequencing technologies such as 454 amplicon pyrosequencing (Margulies et al., 2005), enabling highly efficient characterization of microbial communities by sequencing medium-sized (200–600 bp) amplicons are currently often used (e.g. Öpik et al., 2009, 2013; Lekberg et al., 2012; De Beenhouwer et al., 2014). For molecular identification of AMF, the nuclear ribosomal RNA (rRNA) operon is commonly used, mainly due to its high resolving power and alignment capability across broad taxonomic groups (Stockinger et al., 2010; Schoch et al., 2012). Whereas the internal transcribed spacer (ITS) region has been suggested as the standard fungal barcode (Schoch et al., 2012), this region is exceptionally variable for AMF and does not resolve closely related species (Stockinger et al., 2010). Therefore, Stockinger et al. (2010) recommended a 1500 bp region, including a segment of the small subunit (SSU) rRNA gene, the entire ITS region, and a portion of

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the large subunit (LSU) rRNA gene for AMF DNA barcoding. However, so far the SSU region has been most commonly used for studying AMF communities (Öpik et al., 2013).

Different primer pairs have been used to amplify different parts of the SSU region to investigate AMF communities. Öpik et al. (2009) were the first studying AMF communities using 454 pyrosequencing and used the NS31–AM1 primer pair, one of the first primer pairs designed for the detection and identification of AMF (Simon et al., 1992; Helgason et al., 1998). Whereas this primer pair has been frequently used in AMF studies, it was also shown to pick up non-AMF species and to not detect all species from the basal AMF families Ambisporaceae, Archaeosporaceae and Paraglomeraceae (Daniell et al., 2001). Lumini et al. (2010) used AMV4.5NF and AMDGR (Sato et al., 2005) for 454 pyrosequencing-based AMF investigation, and showed that this primer pair resulted in a higher proportion of AMF sequences and detection of a broader spectrum of Glomeromycota when compared to NS31–AM1. In order to overcome the disadvantages of the NS31–AM1 primer pair, Lee et al. (2008) designed primer pair AML1–AML2 showing a better in silico specificity and coverage across the Glomeromycota. For 454 pyrosequencing-based AMF studies, the reverse primer AML2 has been combined with NS31 (Davison et al., 2012).

In addition to the SSU region, segments of the LSU region have also been used for studying AMF communities using pyrosequencing. More specifically, Stockinger et al. (2010) recommended the LSU-D2 region, marked by the primers FLR3 and LSUmBr. Lekberg et al. (2012) exploited this region to study shifts in AMF communities using a novel forward primer, Glo454, in combination with the reverse primer NDL22 designed by van Tuinen et al. (1998).

AMF community analysis using next-generation sequencing has tremendously increased our knowledge of the diversity and the spatial and temporal variation of AMF communities. However, comparing results between studies is often difficult, as there has been little consistency in target genes and primer sets utilized. Moreover, each combination of target region and primer pair, with potentially different specificities and amplification kinetics (Kohout et al., 2014), may bias the description of the fungal community sampled. Therefore, the objective of this study was to critically evaluate the performance of six different AMF primer pairs, previously used in AMF studies, for use in high-throughput sequencing-based AMF community analysis. Different regions of the rRNA gene were evaluated in silico with respect to their effectiveness to resolve AMF species; the in silico coverage of Glomeromycota was assessed; and the primer pairs were compared by evaluating their ability to characterize AMF communities in field samples using 454 pyrosequencing. Primers were evaluated with respect to (i) the number of high-quality sequences obtained, (ii) selectivity for AMF species, (iii) reproducibility and (iv) ability to accurately describe AMF communities. In the framework of an ongoing study on the distribution of AMF across apple orchards of different management types, we focused on the AMF communities of cultivated and wild apple trees in the central and eastern part of Belgium. Previous work has shown that cultivated apple trees harbor a high diversity of AMF species and genera (Purin et al., 2006).

2. Materials and methods

2.1. Primer selection

Six SSU- or LSU-targeting primer pairs, commonly used in AMF studies, were selected for this study (Table 1; Fig. 1). Primer combinations included four primer pairs previously used in 454 pyrosequencing-based AMF community analyses: NS31–AM1, AMV4.5NF–AMDGR, NS31–AML2 and Glo454–NDL22. Additionally, we included FLR3–LSUmBr and AML1–AML2 in our comparison.

2.2. In silico target region evaluation

In order to assess the capacity of the different rRNA gene regions targeted by the selected primers to distinguish different AMF species, the nucleotide diversity (Pi) was calculated using 458 (SSU) and 614 (LSU) aligned AMF reference sequences belonging to almost 100 AMF species (retrieved from Krüger et al., 2012). Pi is the average number of nucleotide differences in a 10 base window between two sequences (Nei, 1987).

2.3. In silico primer evaluation

In order to assess the ability of the selected primers to efficiently and specifically amplify AMF sequences, all primer sequences were individually subjected to an in silico analysis using the PrimerProspector software (Walters et al., 2011). Each primer sequence was screened against all sequences from the Krüger et al. (2012) database, representing the ten major families in the Glomeromycota, including the Acaulosporaceae, Ambisporaceae, Archaeosporaceae, Claroideoglomeraceae, Diversisporaceae, Glomeraceae, Geosiphonoceae, Gigasporaceae, Pacisporaceae and Paraglomeraceae. PrimerProspector scores were calculated as [non-3′ mismatches * 0.40] + [3′ mismatches * 1.00] + [3′ terminus mismatch = True, + 3.00] + [non 3′ gaps * 1.00] + [3′ gaps * 3.00], giving larger penalties to gaps and mismatches in the 3′ end (last 5 bp) of the primer, which represents the most crucial region for primer extension (Lefever et al., 2013). For

Table 1
Primer pairs used in this study.

	Primer pair[a]	Sequence (5′ → 3′)	Fragment size	Target region[b]	Reference
1.	NS31 (F)	TTGGAGGGCAAGTCTGGTGCC	550 bp	SSU	Simon et al. (1992)
	AM1 (R)	GTTTCCCGTAAGGCGCCGAA			Helgason et al. (1998)
2.	AMV4.5NF (F)	AAGCTCGTAGTTGAATTTCG	300 bp	SSU	Sato et al. (2005)
	AMDGR (R)	CCCAACTATCCCTATTAATCAT			Sato et al. (2005)
3.	AML1 (F)	ATCAACTTTCGATGGTAGGATAGA	800 bp	SSU	Lee et al. (2008)
	AML2 (R)	GAACCCAAACACTTTGGTTTCC			Lee et al. (2008)
4.	NS31 (F)	TTGGAGGGCAAGTCTGGTGCC	550 bp	SSU	Simon et al. (1992)
	AML2 (R)	GAACCCAAACACTTTGGTTTCC			Lee et al. (2008)
5.	FLR3 (F)	TTGAAAGGGAAACGATTGAAGT	370–436 bp	LSU	Krüger et al. (2009)
	LSUmBr1 (R)	DAACACTCGCATATATGTTAGA			Krüger et al. (2009)
	LSUmBr2 (R)	AACACTCGCACACATGTTAGA			Krüger et al. (2009)
	LSUmBr3 (R)	AACACTCGCATACATGTTAGA			Krüger et al. (2009)
	LSUmBr4 (R)	AAACACTCGCACATATGTTAGA			Krüger et al. (2009)
	LSUmBr5 (R)	AACACTCGCATATATGCTAGA			Krüger et al. (2009)
6.	Glo454 (F)	TGAAAGGGAAACGATTGAAGT	350 bp	LSU	Lekberg et al. (2012)
	NDL22 (R)	TGGTCCGTGTTTCAAGACG			van Tuinen et al. (1998)

[a] F, Forward primer; R, Reverse primer.
[b] SSU, small subunit rRNA gene; LSU, large subunit rRNA gene.