Notes S1 - Comparison of ITS PCR approaches for plant root samples

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Contents

Summary

The characterization of plant root-associated fungi communities using universal internal transcribed spacer (ITS) PCR approaches typically causes two problems: the PCR primers co-amplify plant ITS sequences and/or recover only low levels of arbuscular mycorrhizal fungi (AMF). To select the best suited PCR approach, three setups were evaluated by profiling the same DNA extracts of Petunia roots (4 replicates):

1) ITS1F and ITS2 by McGuire et al. (2013) [1]
2) fITS7 and ITS4 by Ihrmark et al. (2012) [2]
3) ITS1F with the reverse complement of fITS7 (this study)

Separate community profiles were produced and inspected for the proportions of plant and AMF sequences as well as for fungal diversity. Although a PCR approach covering the ITS2 region would be preferred due to longer amplicon and enhanced taxonomic information, the approach with fITS7 and ITS4, whilst efficiently working on wheat roots [2,3], was insufficient to avoid Petunia plant sequences. Therefore, we opted for the ITS1F primer, which is also commonly employed for soil, rhizosphere and plant root studies [1,4,5] and is a fungal specific primer that was efficient in avoiding the amplification of plant sequences. While this primer is known to exclude basal lineages and Tulasnella fungi (https://unite.ut.ee/primers.php), which are wood- or litter-associated saprotrophs, important orchid mycobionts and some may form ectomycorrhizal associations, this constraint appears not too critical in our context, as we do not study forest ecosystems or ectomycorrhiza forming plant species.

Methods

PCR approaches

PCR approach ITS1F:ITS2:
The PCR approach with ITS1F and ITS2 was prepared according to cycling protocol from McGuire et al. using the polymerase of the 5Prime Hot Master mix [1]. This primer pair amplifies the ITS region 1.

PCR approach fITS7:ITS4:
The PCR primer fITS7 was developed to amplify the ITS region 2 in combination with primer ITS4 [2]. The PCR approach with fITS7 and ITS4 was prepared following cycling protocol from Ihrmark et al. and we also used their DreamTaq polymerase.

PCR approach ITS1F:fITS7rc:
In addition, we tested the idea of using the reverse complement of the primer fITS7 (known to exclude plant sequences; works well with wheat, see Ihrmark et al.) to amplify the ITS region 1 together with primer ITS1F. We utilized the cycling protocol and DreamTaq polymerase from Ihrmark et al. for this PCR approach.

Bioinformatics

PCR products were sequenced at the Functional Genomics Centre Zurich (MiSeq run04). The samples of the three PCR approaches ITS1F:ITS2, fITS7:ITS4 and ITS1F:fITS7rc were treated separately but processed with the same bioinformatic protocol as described in the main manuscript. Briefly, after quality filtering and demultiplexing, the sequences of each PCR approach were clustered into OTUs at 97% sequence identify using UPARSE [6] and their taxonomy was determined using the SYNTAX classifier with the UNITE database. Here in this script we then analyzed the separate community profiles (OTU and taxonomy tables).

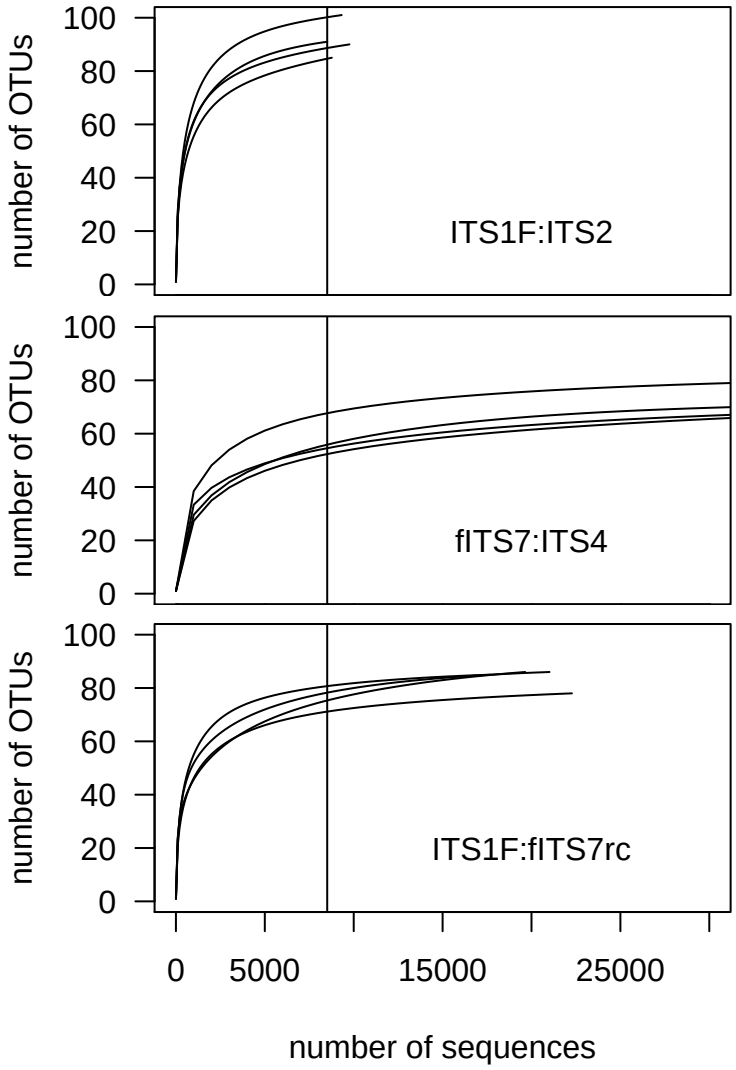
Results

Sampling depth

Number of sequences per sample and PCR setup:

	Rep1	Rep2	Rep3	Rep4	Total
ITS1F:ITS2	8768	9323	9759	8513	**36363**
fITS7:ITS4	31478	46347	47488	40535	**165848**
ITS1F:fITS7rc	22273	17715	21013	19643	**80644**

Overview sampling depths - rarefaction analysis for each PCR approach:

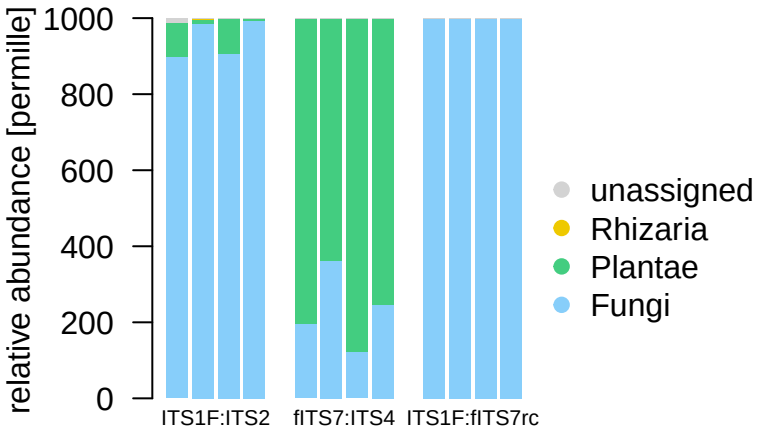


CONCLUSION: The sampling depths for the three PCR approaches were sufficient as a saturation is reached with a plateau at around 60 to 80 OTUs for all three setups.

Domain level analysis

We first tested for the proportion of co-amplified plant sequences in root samples.

The OTUs of the dataset were normalized by the sampling depth in each sample (TSS normalization), expressed as permilles and were summed up in each sample for a given Domain.



Summary (mean relative abundance of each Domain):

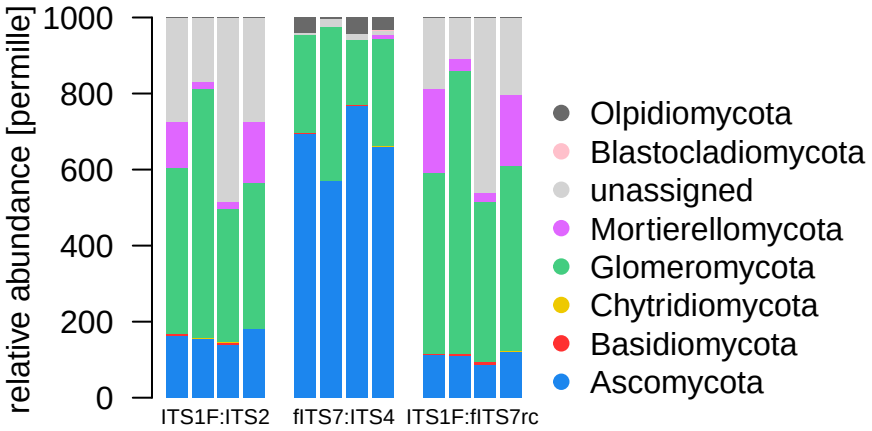
	ITS1F:ITS2	fITS7:ITS4	ITS1F:fITS7rc
Fungi	946.1	231.7	999.9
Plantae	**49.12**	**768.3**	**0.06103**
Rhizaria	1.564	0	0
unassigned	3.168	0	0

CONCLUSION: The PCR approach fITS7:ITS4 amplifies a large proportion (~75%!) of plant sequences, wherase the PCR setups ITS1F:ITS2 and ITS1F:fITS7rc primarily permit to amplify Fungi.

Phylum level

We then tested for the proportion of Glomaromycota sequences amplified by the different PCR approaches.

The dataset was subsetted to *Fungi* by removing all *Plant*, *Rhizaria* and *unassigned* OTUs. Fungal OTUs of a given Phylum were summed up in each sample, normalized by the sampling depth in each samples (TSS normalization) and expressed as permilles.



Summary (mean relative abundance of each Phylum):

	ITS1F:ITS2	fITS7:ITS4	ITS1F:fITS7rc
Ascomycota	159.4	673.5	107.7
Basidiomycota	3.171	1.463	4.772
Chytridiomycota	0.8584	0	0
Glomeromycota	**456.3**	**279.6**	**531.6**
Mortierellomycota	78.93	2.847	115.1
unassigned	300.9	12.64	240.8
Blastocladiomycota	0.4239	0	0
Olpidiomycota	0	29.97	0

CONCLUSION: The Glomeromycota are better amplified with PCR approaches ITS1F:ITS2 and ITS1F:fITS7rc compared to the setup fITS7:ITS4.

Richness analysis (Fungal OTUs only)

Finally, we inspected the fungal diversity amplified by the different PCR approaches.

For this analysis we utilized the dataset, which was subsetted to *Fungi*, and we subsampled it 100x (by rarefying) to the minimal sampling depth of all tested samples (5840) and each time the OTU richness was determined. The means of the OTU richness were calculated from the 100 iterations for each sample and employed for statistical comparison.

Table 4: Analysis of Variance Model

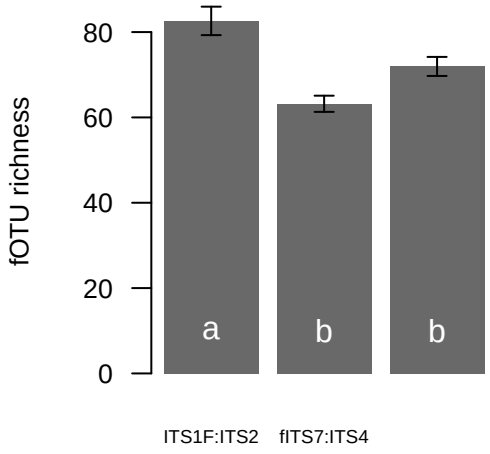
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
richness_stat_df$PCR	2	757.3	378.7	14.41	0.001563
Residuals	9	236.4	26.27	NA	NA

CONCLUSION: The fungal richness differs significantly between the community profiles amplified by the different PCR setups.

- **richness_stat_df$PCR**:

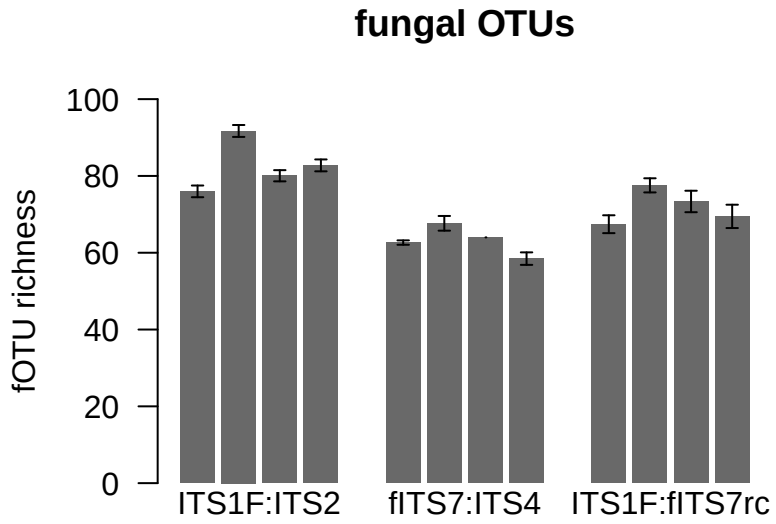
	diff	lwr	upr	p adj
fITS7:ITS4-ITS1F:ITS2	-19.43	-29.55	-9.308	0.001187
ITS1F:fITS7rc-ITS1F:ITS2	-10.67	-20.79	-0.5533	0.0394
ITS1F:fITS7rc-fITS7:ITS4	8.755	-1.364	18.87	0.08968

CONCLUSION: The fungal richness amplified with the approach ITS1F:ITS2 is significantly different from the two other PCR setups (fITS7:ITS4 and ITS1F:fITS7rc).



CONCLUSION: The fungal richness amplified with the approach ITS1F:ITS2 is significantly higher that with the setups fITS7:ITS4 or ITS1F:fITS7rc.

Plot with the individual mean richness values (±SD):



Conclusions

1) A sampling depth of 5840 sequences per sample is sufficient for all three PCR approaches to capture the fungal diversiy in Petunia roots.
2) The PCR approach with fITS7:ITS4 is not suitable to work with Petunia roots as it co-amplifies a large proportion of plant sequences.
3) The Glomeromycota are best amplified with the PCR approaches ITS1F:fITS7rc or ITS1F:ITS2 but not with the fITS7:ITS4 setup.
4) The PCR approach with ITS1F:ITS2 amplifies a significantly higher fungal richness than the setups with fITS7:ITS4 or ITS1F:fITS7rc.

In summary, we conclude that the PCR approach with *ITS1F:ITS2* is the best choice because it (i) captured low levels of plant sequences, with a (ii) good coverage of AMF and (iii) with highest levels of OTU richness. Moreover, this approach has been used in many studies.

References

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