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Journal of Plant Pathology, 2019, **101**: 743–748
Doi: 10.1007/s42161-019-00276-9
hal-02618173

Online version of this article (https://doi.org/10.1007/s42161-019-00276-9) contains supplementary material, which is available to authorized users.

Comparison and validation of Oomycetes metabarcoding primers for *Phytophthora* high throughput sequencing

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Abstract

Oomycetes are eukaryotic plant pathogens that require health monitoring. High-throughput sequencing (HTS) methods replace progressively cultivation-based approaches in soil surveys of Oomycetes, but very little control has been done from synthetic communities. Indeed, several potential biases do exist and need to be assessed for Oomycetes communities. We created a mock community by mixing DNA from 24 Phytophthora species. We amplified two barcode regions with Oomycete-specific primers before HTS. With this aim, we used three primer sets in nested PCR amplification, targeting the ITS-1 region or the RAS gene region. The three nested PCR strategies proved to be a reliable qualitative approach, identifying approximately 95% of the species after Illumina Miseq sequencing and bioinformatic analysis. However, quantitative proportions of each species showed distortions compared to the original mixture of the mock. In addition, we compared the two ITS primer sets on soil environmental DNA sampled from temperate forests. The ' oom18S-ITS7/18ph2f-5.8S-1R ' primer set, more specific to Phytophthora, was able to detect seven Phytophthora species, confirming what was expected for temperate forests. Using the ' DC6-ITS7/oom18S-ITS7 ' primer set that covers the broader Peronosporaceans, we detected only one Phytophthora species among the dominance of Pythium and Phytopythium species. We concluded that ' oom18S-ITS7/18ph2f-5.8S-1R ' primer set is a reliable tool for the qualitative description of environmental Phytophthora communities.

Keywords: Phytophthora. Metabarcoding. ITS region. RAS gene. Mock community

Oomycetes are fungus-like eukaryotic organisms, including widespread plant pathogens such as *Phytophthora infestans*, P. ramorum or P. cinnamomi (Hansen 2008; Kamoun et al. 2015). Many species of Phytophthora have co-evolved with their hosts in their native ecosystems and do not cause severe diseases in those environmental conditions. However, when introduced in a new environment, they may become invasive and represent important threats to the managed or natural ecosystems (Hansen 2008). Examples include *P. ramorum*, *P. uniformis* or *P. lateralis* which are important invasive pathogens in Europe and/or North America but do not appear to be significant pathogens in their native environments (Brasier et al. 2010; Aguayo et al. 2013; Turner et al. 2017). It is thus important to increase our knowledge of *Phytophthora* communities, in particular of species that do not cause conspicuous damages to their host and metabarcoding of environmental DNA represents a new valuable tool for this purpose.

Indeed, DNA metabarcoding and high throughput sequencing (HTS) have been used for addressing significant questions in ecology and biodiversity assessment (Taberlet et al. 2012). Recently, these culture-independent approaches have been applied to Oomycete communities, skipping the traditional steps of baiting on trap leaves, and isolation on Petri dish, and increasing the rate of data acquisition. Sequencing of Internal Transcribed Spacer (ITS) regions is the universal barcode for fungal diversity analysis (Schoch et al. 2012). This molecular marker has been used for Oomycetes also (Coince et al. 2013, Vannini et al. 2013; Sapkota and Nicolaisen 2015; Riit et al. 2016). Similar to the diversity of fungal primer pairs (Bellemain et al. 2010; Tedersoo et al. 2015), oomycetes communities have been investigated thanks different primer pairs without any validation from artificial mock. Interestingly, few studies focused on *Phytophthora* communities using the 18Ph2F- 5.8S-1R primer pair (Scibetta et al. 2012; Català et al. 2015). Nevertheless, metabarcoding revealed multiple biases, including sequence errors during PCR, library preparation, primer – template mismatches bias or primer specificity (Tedersoo et al. 2018). In recent studies focusing on the Phytophthora community, very little control has been done, needing a benchmarking effort for this ecological group. Mock communities have proved to be efficient for revealing potential biases involved along the methodological steps of metabarcoding studies targeting fun gal or protists communities (Bokulich et al. 2013; Egge et al. 2013; Taylor et al. 2016). Artificial communities are a particularly efficient tool to reveal mismatches between the read abundance and the initial proportion of species. To our knowledge, no study has used it yet for Phytophthora community studies. The aim of this study was to create mock communities of Phytophthora species, covering the nine most important clades, then to compare our three sets of primers targeting Oomycete sub-groups and validate some of them as tools for studying Phytophthora communities by HTS.

Mock communities were created from genomic DNAs of 24 Phytophthora species (Table 1) with or without back ground metagenomic DNAs from other microorganisms (Table S1). This additional microbial DNA (fungal or bacterial) aimed

to control the potential mismatch errors between *Phytophthora* DNA and other microbial DNA. The different mock communities were amplified using three sets of primers, targeting the *Phytophthora* genus at the ITS or RAS-Ypt loci, or the broader *Peronosporaceae* group at the ITS locus (Table 2). The two ITS sets of primers were also tested on environmental samples collected from forests of NE France.

Table 1. Phytophthora isolates used in the mock community. ITS sequences were deposited in GenBank under accession Nos. MH178327-MH178350. The sequences of RAS-Ypt regions have been deposited in the DRYAD database (http://datadryad.org/). DRYAD entry https://doi.org/10.5061/dryad.68tk49

Name	Species	Clade
PAU60	*Phytophthora uniformis*	7
PAM54	*Phytophthora xmultiformis*	7
Pc1A21	*Phytophthora xcambivora*	7
87_neb	*Phytophthora cinnamomi*	6
CIN4	*Phytophthora cinnamomi*	6
BBA65909	*Phytophthora cryptogea*	8
2KE4	*Phytophthora europaea*	7
PFF CSL	*Phytophthora fragariae*	7
gon1	*Phytophthora gonapodyides*	6
lat1	*Phytophthora lateralis*	8
PP2	*Phytophthora parasitica*	1
cit2	*Phytophthora pini*	2
resi1	*Phytophthora plurivora*	2
resi32	*Phytophthora pseudosyringae*	3
2KP7	*Phytophthora quercina*	NA
ram1	*Phytophthora ramorum*	8
soj1	*Phytophthora sojae*	7
resi73	*Phytophthora syringae*	8
Plac3	*Phytophthora lacustris*	6
27_neb	*Phytophthora heveae*	5
1288_neb	*Phytophthora castaneae*	5
1543_neb	*Phytophthora sp. neb_1543*	9
resi89	*Phytophthora sp. hungarica*	6
resi51	*Phytophthora obrutafolium*	6
NII72 – 5	*Phytophthora infestans*	1

Genomic DNA was extracted from 25 *Phytophthora* sp. isolates (INRA Nancy collection) corresponding to 24 species, then quantified with a Qubit® 2.0 Fluorometer (Life technologies) at 30 ng in 2 µl. Bacterial and eukaryotic genomic DNA used as background, was extracted from 306 isolates (Table S1) from INRA collections. Additionally, eight environmental samples were obtained from soils collected in April 2016 at the base of four trees each (four soil samples per tree from 5 to 10 cm depth) in two forest plots of north-eastern France. The sampled trees belonged to *Alnus glutinosa*, *Quercus* sp., *Fraxinus excelsior* and *Carpinus betulus*. Environmental DNA was extracted with the Fast DNA SPIN Kit for soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer ' s instructions.

Three amplicon libraries (hereafter referred to as PERO_ITS, PHYTO_ITS and PHYTO_RAS) were produced using different combinations of primers. The PERO_ITS library (targeting *Peronosporaceae*) that has resulted from a nested PCR amplification of the ITS1 and 18S region fragment, used first the DC6 – ITS7 primers (Cooke et al. 2000) and then the newly designed primer pair Oom18s – ITS7 (Fig. 1; Table 2). For the PHYTO_ITS library (targeting *Phytophthora*), the ITS1 was amplified by another nested PCR approach, using first the Oom18s – ITS7 primers pair and then the *Phytophthora*-specific primer pair 18Ph2F-5.8S-1R (Scibetta et al. 2012). For both ITS libraries, 2 µ l of the first-round amplicons diluted 10 times was used as DNA sample for the second round following the same PCR conditions. The second PCR round was conducted in triplicate, the amplicons being pooled and purified afterward. Finally, the PHYTO_RAS library (targeting *Phytophthora*, on the RAS-Ypt gene) was produced as described by Weir et al. (2015). For each PCR assay, 2 µ l of DNA material were added to 18 µ l of PCR reactive solution. Detailed temperature and duration for each PCR cycle are provided in Table S2. Multiplexing and Illumina MiSeq sequencing (Reagent Kit v3 chemistry, 2x250pb) were done by the GeT-PlaGe platform of Toulouse (http://get.genotoul.fr/).

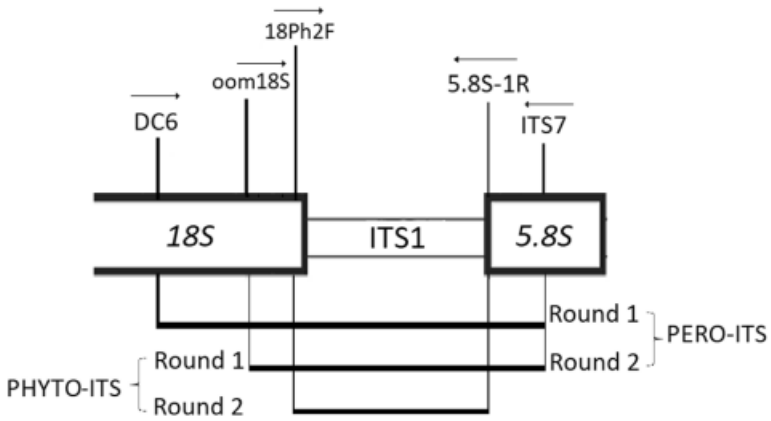

Fig. 1. Map of the 18S, ITS1, and 5.8S regions showing positions of different primer pairs

Sequences were sorted and trimmed according to their quality using Mothur (Schloss et al. 2009) and Usearch (Edgar 2010). Only reverse reads (R2) were retained to analyze both ITS sequencing data sets. The forward reads (R1) covered the 18S ribosomal DNA region, which is highly conserved and therefore lacks nucleotide polymorphism between *Phytophthora* species. Furthermore, the paired reads could be used with V3 chemistry (2x300pb) in future studies, because the average length of the amplified fragment is 480 bp. The R2 reads were dereplicated, sorted by decreasing abundance and the singletons were discarded using Usearch "sortbysize" command. Sequences were then clustered with a 99% similarity threshold in order to discriminate efficiently between species, using Usearch

cluster_otus command. Molecular Operational Taxonomic Units (OTUs) were taxonomically assigned using BLAST (Altschul at al. 1990) against a local database (all NCBI sequences assigned to Oomycetes with the “ITS” keyword in their GenBank definition), or a homemade database containing Sanger RAS-Ypt sequences from the mock isolates (Table 1). OTUs assigned to the same species were merged according to their taxonomic identity at the species level. Among the 27 samples, ten failed during the Miseq sequencing, because of very low yield or high proportions of contaminants from samples of other studies incorporated into this common Miseq run. The 17 remaining samples were submitted to a random subsampling at 471 reads by sample (minimum depth of the sample set). Because of putative PCR contaminants from other environmental samples in the same HTS run, only OTUs supported by >10 sequences were retained in the environmental samples (Miller et al. 2016).

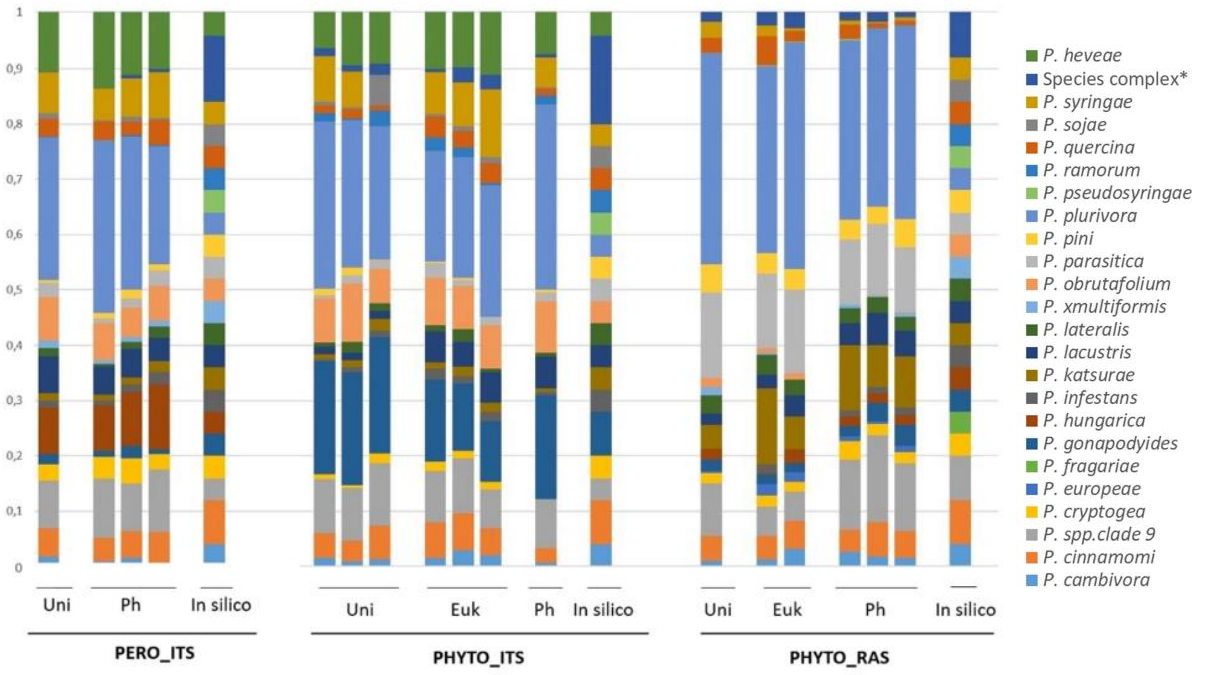

Fig. 2. Proportions of reads per Phytophthora species for each technical replicate, depending on the mock community composition and the PCR amplification strategy. Euk= Eukaryotic mock community; Ph= *Phytophthora*-only mock community; Uni = Eukaryotic and prokaryotic mock community. PERO_ITS is the library amplified by Peronosporales-specific primer set oom18S/ITS7; PHYTO_ITS is the library amplified by *Phytophthora*-specific primer set 18Ph2F/5.8 – 1.5R; RAS_ITS is the library amplified by *Phytophthora*-specific primer set Ypr1F/Ypr1R. The number of reads by sample was subsampled to 471 reads. *Species complex: represent the fusion of *P. uniformis*, *P. europeae* and *P. fragariae* for PERO_ITS and *P. uniformis*, *P. europeae*, *P. fragariae* and *P. ×multiformis* for PHYTO_ITS. « In silico » corresponds to expected OTUs composition based on the clustering at 99% similarity of 25 Sanger sequences from the RAS or ITS regions. Missing repetitions are due to low quality MiSeq sequencing. Differences in species proportions between samples were tested using the function ADONIS of the R vegan package (with 99,999 permutations)

Table 2. Primers pairs used in this study and previous ones. The PHYTO_ITS and PERO_ITS libraries were created using nested PCR (two consecutive amplifications): first using DC6/ITS7 then oom18S/ITS7 for the PERO_ITS library; first using oom18S/ITS7 then 18Ph2F/5.8S-1R for the PHYTO_ITS library. The PHYTO_RAS library was made using simple PCR

Primer pair	Region	Specificity	Forward sequence	Reverse sequence	Annealing temperature (°c)	Reference	Used in this study
DC6-ITS7	ITS	Oomycetes	GAGGGACTTTTGGGTAATCA	AGCGTTCTTCACTCGATGTGC	60	Cooke et al. (2000)	Yes
oom18S-ITS7	ITS	Peronosporal-es	GCGCATCGTGCTAGGGATAG	AGCGTTCTTCACTCGATGTGC	60	Cordier (designed for this study)	Yes
18Ph2f-5.8S1R	ITS	*Phytophthora*	GGATAGACTGTTGCATTTTCAGT	GCARRGACTTTCGTCCCYRC	53	Scibetta et al. (2012)	Yes
Yph1F-Yph2R	YPT1	*Phytophthora*	CGACCATKGGTGTGGACTTT	ACGTTCTCMCAGGCGTATCT	58	Weir et al. (2015)	Yes
ITS1oo-ITS3oo	ITS	Oomycetes	GGAAGGATCATTACCACACGGAAGGATCATTACCAC	AGTATGYYGTATCAGTG	NA	Riit et al. (2016)	No
ITS6-ITS7	ITS	Oomycetes	GAAGGTGAAGTCGTAACAAGG	AGCGTTCTTCACTCGATGTGC	NA	Cooke et al. (2000)	No

Some species with over 99% similarity in the studied amplicons clustered together. Thus, no more than 20, 22 and 23 species could be retrieved in the PHYTO_ITS, PERO_ITS, and PHYTO_RAS libraries, respectively (Fig. 2). The clustering process generated 75 and 222 *Phytophthora* OTUs for the PHYTO_ITS and PERO_ITS libraries respectively corresponding to 19 and 21 species, as one species from the original mix. Indeed, as confirmed with in silico analysis, *P. uniformis*, *P. europeae* and *P. fragariae* clustered together at 99% of similarity for PERO_ITS PCR strategy; and *P. uniformis*, *P. europeae*, *P. fragariae* and *P.* × *multiformis* were identified as one single group at 99% of similarity also for PHYTO_ITS PCR strategy (Fig. 2). *P. pseudosyringae* was never identified in both ITS libraries. The RAS amplification gave out 60 different OTUs belonging to 22 species, with *P. ramorum* missing. Proportions of reads from each species were significantly different from the proportions of the original mix regardless of the library ($p < 0.001$). However, proportions were conserved between technical replicates, and did not depend on the added presence of other Dikarya / Procarya DNA in the mock communities ($p = 0.99$). Species distribution patterns were significantly different between each library ($p < 0.001$). Seven different *Phytophthora* species were detected in environmental samples with the PHYTO_ITS library and just one species (*P. syringae*) with the PERO_ITS library (Table 3). Non-*Phytophthora* Oomycetes reads represented 5% and 98% of the total reads in the PHYTO_ITS and the PERO_ITS libraries respectively. Phytophthora represent- ed only 2% of the total reads of PERO_ITS; while 95% of the reads were assigned to Pythium and *Phytopythium* and sorted in 117 OTUs pertaining to 29 species. This result constituted 97% of *Peronosporomycete* reads with the PERO_ITS amplification. The two nested PCR approaches, DC6/ITS7 x Oom18S / ITS7 (PERO_ITS) and Oom18S/ITS7 × 5.8S1R/18ph2F (PHYTO_ITS), proved reliable for qualitative characterisation of mock Phytophthora communities without other Oomycete species. However, the second PCR strategy was much more effective for environmental samples, usually containing a much higher abundance of other *Peronosporaceans* such as *Pythium* (Cerri et al. 2017). Moreover, the number of amplified *Phytophthora* species corresponded to the order of magnitude of *Phytophthora* diversity in temperate forests, which is of about 2 to 8 species (Jung et al. 2010; Hansen and Delatour 1999). Thus, our study suggests that the Oom18S/ITS7 x 5.8- S1R/18ph2F primer set is an effective tool for qualitative description of environmental *Phytophthora* species. Nevertheless, quantitative distortions in the species proportions have been observed in HTS data obtained with the three primer sets, as described with other primers targeting fungal community (Nguyen et al. 2015; Buée et al. 2016). Possible causes include PCR steps and primer affinities for specific species, difference in ITS copy numbers in the genome (Bakker 2018), or errors in initial genomic DNA quantifications. This needs to be taken into account for quantitative studies. On the other hand, no mismatches bias was observed in the *Phytophthora* mocks supplemented with microbial metagenome background; and

finally, qualitative detection of *Phytophthora* species, in order to detect potential pathogenic agents, would be very effective.

Table 3. Number of environmental samples where *Phytophthora* species were detected, depending on the PCR amplification strategy. A total of 8 soil samples (corresponding to 2 sites × 4 trees) were analyzed. The PHYTO_ITS library was made using the 18Ph2F/5.8S-1R primer pair and the PERO_ITS library using the oom18S/ITS7 primer pair. Retrieved *Phytophthora* species belonged to 4 clades out of the 10 *Phytophthora* clades

Species	Number of samples / library		Clade
	PERO_ITS	PHYTO_ITS	
P. cactorum	0	5	1
P. europeae	0	3	7
P. pini	0	3	2
P. plurivora	0	2	2
P. pseudosyringae	0	4	3
P. sp. neb_1543	0	1	7
P. syringae	2	4	8

Although unreliable for the *Phytophthora* genus, the *Peronosporacean*-specific primer set enables the coverage of a wider Oomycete community, in particular *Pythium* and *Phytopythium*. The amplification of RAS-Ypt region showed good results on mock communities and could represent an alternative marker for barcoding as the ITS region presents low polymorphism in *Phytophthora*. Furthermore, it has been shown that single-copy markers can provide better relative abundance estimates of fungal taxa than the multi-copy ITS regions (Větrovský et al. 2016; Pérez-Izquierdo et al. 2017). Targeting single-copy marker could therefore improve relative abundance estimates for *Phytophthora* communities as well, but potentially at a cost of a lower detection threshold. The efficiency of the RAS gene remains to be validated on environmental samples. In conclusion, our study revealed a high concordance between the expected and recovered community composition of *Phytophthora* within mock communities. Our results show quantitative differences that can be caused by the selection of barcode and associated primer sets. Taken together, these results reveal that “Oom18S/ITS7 x 5.8-S1R/18ph2F” primer set is a robust tool for future qualitative studies of *Phytophthora* community, richness study and diversity monitoring using the third-generation sequencing generations.

Acknowledgements. We gratefully acknowledge E. Morin for her assistance in bioinformatics advices, and A. Gillet and L. Fauchery for their technical support. We also thank the members of the MetaBAR project (“Meta-omics and microbial ecosystems“ INRA Metaprogramme), who provided the non-*Phytophthora* microbial genomics DNA from INRA microbe collections. We thank also one anonymous

reviewer for providing helpful comments to improve our manuscript. JL holds PhD fellow- ships awarded by the “Agence Nationale de la Recherche” as part of the ANR Blanc program NEBEDIV (ANR-13-BSV7-0009) and by the INRA (Metaprogramme MEM). The UMR IAM is supported by a grant overseen by the French National Research Agency (ANR) as part of the “Investissements d’Avenir” program (ANR-11-LABX-0002-01, Laboratory of Excellence ARBRE).

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