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An improved high throughput sequencing method for studying oomycete communities

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ABSTRACT

Culture-independent studies using next generation sequencing have revolutionized microbial ecology, however, oomycete ecology in soils is severely lagging behind. The aim of this study was to improve and validate standard techniques for using high throughput sequencing as a tool for studying oomycete communities. The well-known primer sets ITS4, ITS6 and ITS7 were used in the study in a semi-nested PCR approach to target the internal transcribed spacer (ITS) 1 of ribosomal DNA in a next generation sequencing protocol. These primers have been used in similar studies before, but with limited success. We were able to increase the proportion of retrieved oomycete sequences dramatically mainly by increasing the annealing temperature during PCR. The optimized protocol was validated using three mock communities and the method was further evaluated using total DNA from 26 soil samples collected from different agricultural fields in Denmark, and 11 samples from carrot tissue with symptoms of *Pythium* infection. Sequence data from the *Pythium* and *Phytophthora* mock communities showed that our strategy successfully detected all included species. Taxonomic assignments of OTUs from 26 soil sample showed that 95% of the sequences could be assigned to oomycetes including *Pythium*, *Aphanomyces*, *Peronospora*, *Saprolegnia* and *Phytophthora*. A high proportion of oomycete reads was consistently present in all 26 soil samples showing the versatility of the strategy. A large diversity of *Pythium* species including pathogenic and saprophytic species were dominating in cultivated soil. Finally, we analyzed amplicons from carrots with symptoms of cavity spot. This resulted in 94% of the reads belonging to oomycetes with a dominance of species of *Pythium* that are known to be involved in causing cavity spot, thus demonstrating the usefulness of the method not only in soil DNA but also in a plant DNA background. In conclusion, we demonstrate a successful approach for pyrosequencing of oomycete communities using ITS1 as the barcode sequence with well-known primers for oomycete DNA amplification.

1. Introduction

The oomycota are fungal-like microorganisms that are currently classified under Stramenopiles. Although oomycetes are similar to fungi in relation to their mode of nutrition and growth, they are different in terms of cytology as well as biochemical pathways (Beakes et al., 2012). Phylogenetic studies have revealed that oomycetes are closely related to diatoms and seaweeds, and probably linked to the marine environment during evolution (Thines, 2014). Oomycetes are found in a wide range of ecosystems and can be found in terrestrial and aquatic environments. Important oomycetes include *Saprolegnia*, *Achlya* and *Lagenidium* which are mainly infecting animals, and *Phytophthora*, *Pythium*, *Aphanomyces* and *Peronospora* that are important plant pathogens (Thines, 2014). Many of these are soil-borne and infect through roots or seedlings (Kamoun, 2003). In the absence of suitable host plants or other substrates for saprophytic growth they may survive in the soil for long periods of time as thick-walled sexual oospores. Sporangia are asexual structures that may germinate to produce a germ tube or that may produce motile zoospores that are able to infect plants through roots or seedlings. Despite the economic importance of oomycetes, relatively few ecological studies have been conducted on this group of organisms. To date, most studies have focused on individual or a few pathogenic species, and there are only few studies that aim to understand interactions and dynamics in oomycete communities in soil (Cooke, 2007; Coince et al., 2013). Many of the plant diseases that are caused by oomycetes involve a complex of several species emphasizing the need to not only study single pathogens but also use a community approach in studies of disease. For instance, cavity spot in carrot is caused by a number of species within *Pythium* such as *Pythium violae*, *Pythium intermedium*, *P. sulcatum*, *Pythium sylvaticum* and others (Hermansen et al., 2007).

Molecular ecological studies of microbial communities in terrestrial habitats such as soils have revealed a huge diversity of previously unknown and non-culturable organisms (van Elsas and Boersma, 2011). Very few of these studies have included oomycetes, and efficient strategies for studying the diversity of oomycetes in the environment are non-existing. A study by Arcate et al. (2006) using cloning and

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sequencing of environmental DNA from the rhizosphere of several plant species revealed a huge diversity of oomycetes. Similarly, successful selective amplification of *Phytophthora* from environmental samples followed by cloning and sequencing has been reported recently (Scibetta et al., 2012). The ability to extract total DNA directly from soil and the advent of high throughput sequencing has revolutionized molecular ecology studies by allowing high resolution examination of community structures for a range of soil organisms such as fungi, bacteria and oomycetes. However, oomycete studies using high throughput sequencing are limited in comparison to fungal and bacterial studies, partly due to insufficient methods.

Currently one of the most widely accepted genome regions for species identification in oomycetes is the rDNA internal transcribed spacer (ITS) due to its high sequence variation and easy amplification using universal primers. ITS based sequence databases are rapidly increasing, and there are already dedicated reference databases for *Phythophthora* (Park et al., 2013), *Pythium* (Levesque and de Cock, 2004), *Peronospora* (Voglmayr, 2003) and several others. Several sets of primers had been developed and tested to target the ITS region of rDNA in oomycetes such as ITS6 and ITS7 (Cooke et al., 2000), ITS4 and ITS5 (White et al., 1990), and UN-UP18S42 and UN-Lo28S22 (Levesque and De Cock, 2004). ITS6 and ITS7 have been used to amplify the ITS1 region from pure oomycete cultures as well as from communities (Coince et al., 2013; Vannini et al., 2013). However, in a recent study using these primers, Coince et al. (2013) only obtained a limited proportion of oomycete related sequences whereas most of the sequences were assigned to basidiomycetes and plants, probably caused by a too low annealing temperature during PCR.

High throughput sequencing studies to explore oomycete community ecology in soils have just begun, and tools still have not been standardized for environmental samples. The objective of this study was to identify standard PCR procedures to optimize yields of oomycete derived sequences from a background of soil DNA and to ensure taxonomic coverage within oomycetes. For this, we used ITS6 and ITS7, and increased the annealing temperature during PCR amplification to increase the specificity towards oomycete DNA. We tested our optimized strategy on three mock communities composed of *Pythium* and *Phytophthora* species. We also tested the strategy using DNA from soils collected from 26 agricultural fields from different locations in Denmark and DNA from 11 carrots with symptoms of *Pythium* infection.

2. Materials and methods

2.1. Mock communities

Phytophthora and *Pythium* mycelium from plate cultures was homogenized in liquid nitrogen and DNA was extracted using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Mock communities were assembled by pooling DNA extracts from 9 strains belonging to two genera and seven species: *P. violae*, *P. sylvaticum*, *P. intermedium*, *P. sulcatum* and *Phytophthora cactorum* (2 strains), *P. fragariae* (2 strains) and *P. infestans* (Table 1). DNA extracts from cultures of *Pythium* and *Phytophthora* were mixed in different proportions as seen in Table 1 to make two replicate communities (samples 1 and 2), and one community where the concentration of *Phytophthora* species was reduced to 1/10th (sample 3). These three DNA mock communities were used as templates during amplification and pyrosequencing.

2.2. Soil sampling

In total, soils from 26 agricultural fields widely distributed in Denmark were selected in the autumn: from the fields, 20 subsamples (app. 2 kg) were randomly taken from the upper 15 cm soil layer, and were then pooled and mixed thoroughly. To prevent further microbial growth, soil samples were stored at −20 °C within 24 h of collection. Subsamples of approximately 100 g were taken and freeze dried for 48 h, and then larger particles were removed manually before samples were ground for five minutes in a mixer mill (Retsch MM301, Haan, Germany). A sample of 250 mg of this material was used for DNA extraction.

2.3. Sampling of carrot tissue with symptoms of cavity spot

Carrots with symptoms of *Pythium* infection were taken from 11 commercial fields in Denmark in 2013 and washed to remove soil and other material. Lesions of cavity spot were incised in conical shape and immediately frozen. Samples were then subjected to freeze drying for 48 h followed by grinding using a Geno/Grinder 2000 (SPEX CertiPrep, Metuchen, NJ, USA) at 1500 rpm for 3 × 30 s. The ground powder was subjected to DNA extraction using DNeasy (Qiagen, Hilden, Germany) following the manufacturer's instructions.

2.4. Soil DNA extraction and PCR conditions

Total soil DNA was extracted using the PowerLyzer™ PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions except that samples were further homogenized in a Geno/Grinder 2000 at 1500 rpm for 3 × 30 s, instead of the homogenizer recommended in the kit. DNA amplification from total DNA was done in a semi-nested approach to target the ITS1. The ITS region was first amplified using ITS6 and ITS4 primers for 15 cycles. The PCR product obtained was diluted to 1:10 before being used as a template for a second PCR using ITS6 and ITS7 for 25 cycles (Cooke et al., 2000). The PCR reaction mixture consisted of 1× PCR reaction buffer, 1.5 mM of MgCl2, 0.2 mM of dNTPs, 1 μM of each primer, and 1 U of GoTaq Flexi polymerase (Promega Corporation, Madison, USA) in a total volume of 25 μl containing 24 μl of reaction mixture and 1 μl of template. The thermal cycle for the first PCR was an initial denaturation at 94 °C for 5 min followed by 15 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final elongation at 72 °C for 10 min. The second PCR was identical to the first one except that an annealing

Table 1
List of strains and CBS (Centraalbureau voor Schimmelcultures) number used in making three mock communities of oomycetes.

Taxon name	CBS number	DNA (ng/μl)		Reads distribution % (number)		
		Samples 1 & 2	Sample 3	Sample 1	Sample 2	Sample 3
Pythium violae	–	0.222	0.222	0.19 (4)	0.14 (3)	0.88 (7)
Pythium sylvaticum	633.67	0.222	0.222	3.84 (79)	3.49 (75)	10.93 (87)
Pythium intermedium	102,607	0.222	0.222	17.53 (361)	14.35 (308)	49.37 (393)
Pythium sulcatum	604.073	0.144	0.144	0.10 (2)	0.09 (2)	0.25 (2)
Phytophthora cactorum	279.37	0.161	0.016	39.39 (811)	45.39 (974)	22.24 (177)
Phytophthora cactorum	435.34	0.222	0.022			
Phytophthora fragariae	209.46	0.156	0.016	23.26 (479)	23.35 (501)	8.42 (67)
Phytophthora rubi	109892	0.222	0.022			
Phytophthora infestans	13.3.50	0.222	0.022	15.69 (323)	13.19 (283)	7.91 (63)

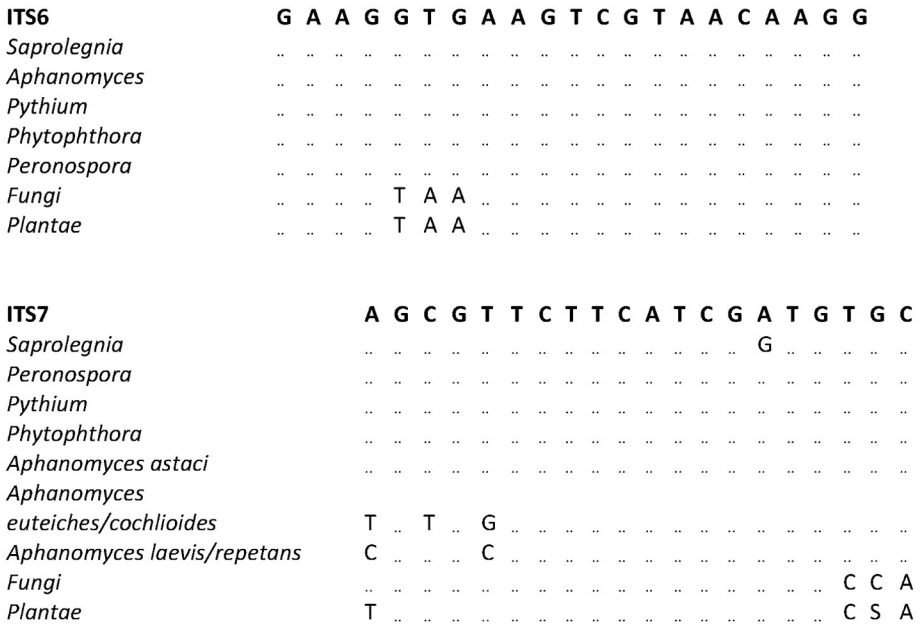

Fig. 1. ITS6 and ITS7 primers and consensus sequences with five genera of oomycetes, plantae and fungi. Dots indicate identical nucleotides to primers.

temperature of 59 °C was used and that 25 cycles were conducted. In order to evaluate the effect of the annealing temperature in the second PCR cycle, reactions were carried out with annealing temperatures of 55 °C, 57 °C, and 59 °C respectively, using one soil sample. Amplicons were most abundant using 59 °C during this step, as visualized by gel electrophoresis, and this temperature was chosen for all further experiments (data not shown).

2.5. Pyrosequencing

Sequencing was carried out in a total of 40 samples consisting of 26 soil samples, 11 carrot lesion samples, and 3 mock communities. An adaptor and a 10 base pair barcode sequence were added to the ITS6 primer for sequencing and for sample identification in downstream sequence analyses after pooling of samples. After the second PCR reaction, the concentration and amplicon size was estimated by resolving 5 μl of sample in a 1.5% agarose gel. Samples were then pooled, and concentrated by ethanol precipitation. The DNA was dissolved in 50 μl of TE buffer, electrophoresed in 1.5% agarose gels and visualized by ethidium bromide staining. The expected DNA smear of 300–450 base pairs was cut from the gel and extracted using the QIAquick Gel Extraction Kit (QIAGEN). The DNA concentration was estimated using a spectrophotometer (Nanodrop, ND-100, Thermo Fisher Scientific, Wilmington, DE, USA) and stored at −20 °C until shipped to Eurofins MWG for sequencing on a GS Junior 454 Sequencer (Roche Diagnostics).

2.6. Processing of pyrosequencing data

Data analysis was performed in QIIME v. 1.8 (Caporaso et al., 2010). Quality filtering was performed to remove reads mismatching to primer and MID sequences, and to remove homopolymer errors. Reads shorter than 150 bp were removed and sequences with homopolymers longer than 10 bp were filtered using a sliding window filter value of 50. To denoise reads, denoise_wrapper.py from QIIME was used (Reeder and Knight, 2010). The ITS1 region was extracted by ITSx extractor version 1.0.6 to remove 18S and 5.8S regions as removal of these conserved regions increases the sensitivity of clustering and taxonomy assignment (Bengtsson-Palme et al., 2013). This step also removed reads that are not derived from the ITS1. The Uclust algorithm was used for clustering of ITS sequences using 'pick OTUs' at 97% similarity level (Edgar, 2010). After clustering, operational taxonomic units (OTUs) were assigned by blasting our own built oomycete ITS reference database that is compatible with QIIME (see below), as well as GenBank (NCBI).

An ITS sequence library that is compatible with the QIIME pipeline was built for oomycetes for taxonomy assignments. This database includes widely studied species with multiple entries in the NCBI database. *Pythiales*, *Albuginales*, *Saprolegniales* and *Peronosporales* ITS sequences were downloaded from NCBI and ITS sequences were extracted using ITSx software in order to retain only the ITS sequence. The *Phythophthor*a database (PD) was downloaded from http://www.phytophthoradb.org/ (Park et al., 2013) and merged with the NCBI derived sequences. The taxon ID for each sequence consists of the accession number for all sequences except the *Phytophthora* entries which were as listed in PD.

3. Results

3.1. Amplification strategy

ITS6 and ITS7 have been evaluated previously for oomycete amplification (Cooke et al., 2000). An *in silico* analysis of primers showed that both primers perfectly align to sequences from genera of oomycetes such as *Pythium*, *Phytophtora*, *Peronospora* and *Saprolegnia*, supporting that these primers will amplify most oomycetes efficiently (Fig. 1). We only found a few *Aphanomyces* sequences that showed a few mismatches in the 3′ end of the priming region. When plant and fungal sequences were compared to ITS6 and ITS7, we found several mismatches (3 nucleotides in the ITS6 region and 3–4 nucleotides in the ITS7 region), supporting the assumption that these primers are oomycete specific. However, Coince et al. (2013) only obtained a low proportion of oomycete derived sequences with these primers using an annealing temperature of 53 °C. As the theoretical annealing temperature of the two primers is approximately 59–64 °C, the annealing temperature was raised to 59 °C in our study in order to increase the proportion of oomycete derived amplicons in our sequencing effort.

3.2. Mock communities

In order to assess any potential biases associated with our amplification and sequencing strategy, three mock communities including species of *Pythium* and *Phytophthora* were constructed. These mock communities were then PCR amplified and sequenced according to

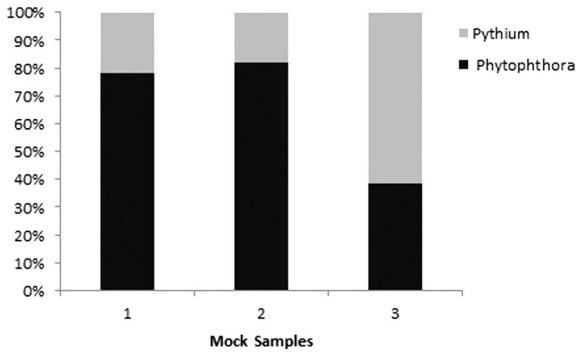

Fig. 2. Distribution of *Pythium* and *Phytophthora* sequences in mock communities.

our strategy. All OTUs representing singletons were removed resulting in 2061, 2150 and 796 ITS reads in samples 1, 2 and 3, respectively. The number of sequences obtained from each species in the mock communities can be seen in Table 1. All species from the mock communities were found in the retrieved sequences, however, in varying proportions. Although the genera *Phytophthora* and *Pythium* were mixed in 55% (5 species) and 45% (4 species) in mock communities 1 and 2, *Phytophthora* reads were found to be dominating in sample 1 (78.3%) and 2 (81.9%). Similarly, when the concentration of DNA from *Phytopthora* species was reduced to 1/10th in sample 3, the read distribution was found to be 61.4% for *Pythium* and 33.6% of *Phytophthora* (Fig. 2). Distribution of reads in the two replicate samples 1 and 2 were remarkably similar (Fig. 2, Table 1).

3.3. Oomycetes diversity in soil samples

We recovered a total of 73,740 quality filtered reads from the 26 soil samples, out of which 73,609 reads were identified as ITS sequences by the ITSx extractor. Read numbers in individual samples ranged from 82 to 10,645 with an average of 2829 reads per sample. The reads could be clustered into 112 OTUs at 97% similarity level excluding singletons

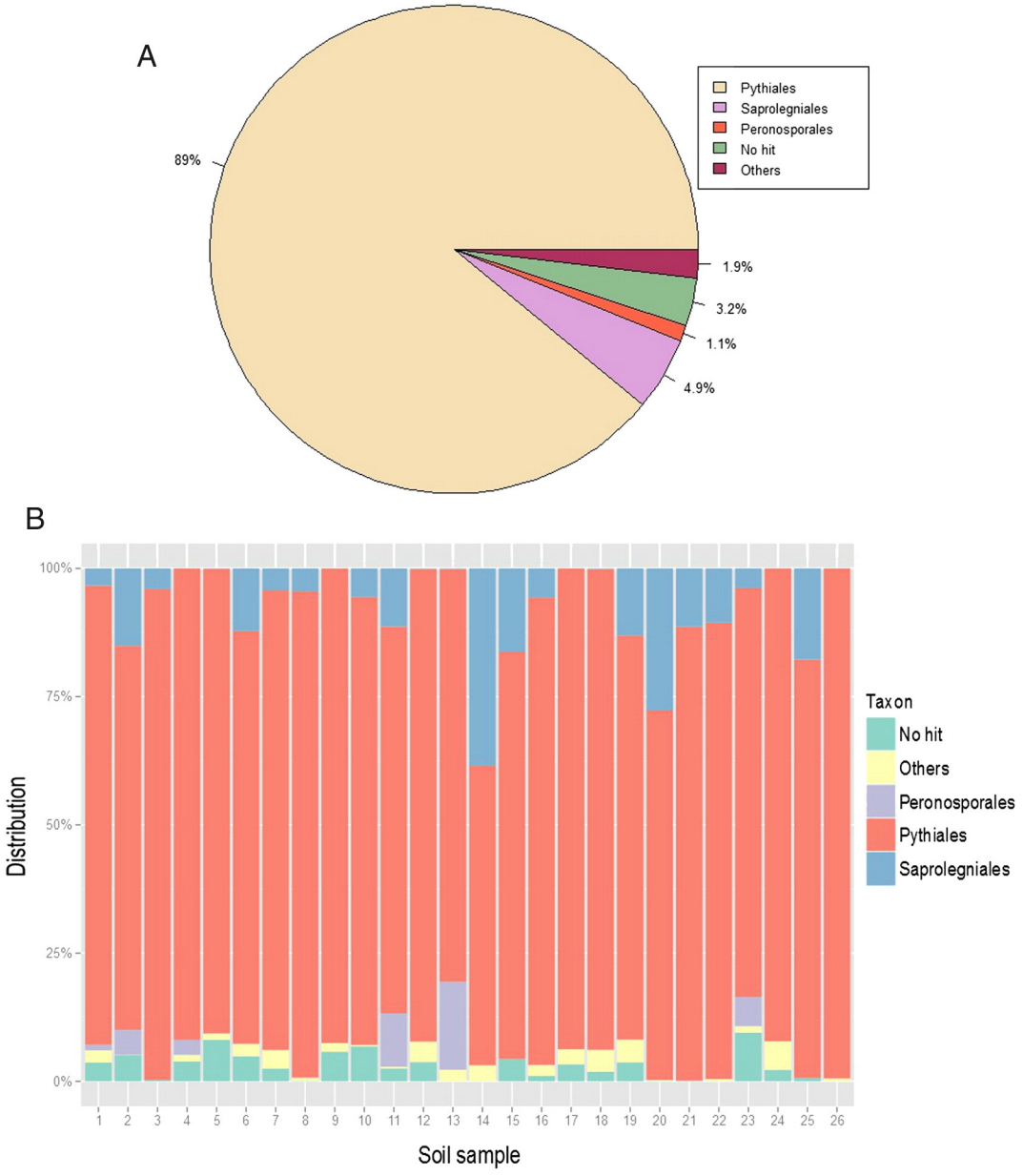

Fig. 3. Distribution of oomycetes in cultivated soil at order level. A. Distribution of sequences in 26 soil samples in total and B. Distribution of sequences assigned to oomycetes in each sample.

Table 2
Most dominant 20 OTUs, total number of reads and the assigned taxonomy in 26 soil samples.

OTU ID	Total reads	Taxonomy (coverage%/identity%)
denovo60	14,028	*Pythium attrantheridium*
denovo5	8388	*Pythium sylvaticum*
denovo143	5912	*Pythium heterothallicum*
denovo67	4399	*Pythium heterothallicum*
denovo136	4041	*Pythium apiculatum*
denovo111	3580	*Pythium monospermum*
denovo23	3222	*Pythium amasculinum* (96/77)[a]
denovo13	3146	*Pythium volutum*
denovo36	2734	*Pythium ultimum*
denovo125	2309	*Pythium rostratum* (100/92)[a]
denovo59	2231	*Pythium monospermum*
denovo142	1610	*Saprolegnia megasperma*
denovo52	1402	*No blast hit*
denovo61	1363	*Pythium intermedium*
denovo20	1359	*Pythium jasmonium*
denovo90	1200	*Pythium amasculinum*
denovo110	1084	*Pythium* sp.
denovo39	757	*Chlamydomonas gyrus* (100/82)[a]
denovo43	731	*Pythiogeton* sp. (40/90)[a]
denovo78	693	*Aphanomyces astaci* (88/76)[a]

[a] Coverage and identity percentage values were listed only if they were less than 97.

(Table S1). Taxonomy assignments were done using a custom database as well as the NCBI database. Hit results for OTUs with higher than 97% similarity or coverage level to best hit were assigned at species level, whereas below 97% hits were assumed to be members of a higher order depending on the best hit in NCBI database. Based on these taxonomic assignments, a total of 95% of the reads were assigned to oomycetes, whereas 5% of the reads belonged to others categories such as fungi, plantae and unclassified. A dominance of oomycete reads were consistently found in all 26 soil samples (Fig. 3). Within the oomycete reads, *Pythiales* were dominating in all soil samples (89% of total), followed by *Saprolegniales* (5%) and *Peronosporales* (1%) (Fig. 3). In total, 52 OTUs were assigned to the order *Pythiales*, 11 OTUS to *Saprolegniales* and 4 to *Peronosporales* (Table S1). Among the identified species, *Pythium attrantheridium*, *P. sylvaticum*, *P. heterothallicum*, *P. apiculatum* and *P. monosperum* were dominant. The 45 OTUs (5% of reads) which were not assigned to oomycetes mostly belonged to fungi, plantae including green algae (25 OTUs) and the remaining 20 OTUs failed to hit any sequence in NCBI database (Table S1).

3.4. Cavity spot community

In order to test our strategy of sequencing in a less complex habitat than soil and to test the strategy in a high plant DNA background, we analyzed samples from carrots showing symptoms of *Pythium* infection. Carrots from 11 different fields were collected and DNA was extracted from cavity spot symptoms. After quality control and ITS extraction, we retrieved 31,191 reads that clustered into 25 OTUs excluding singletons. After assigning taxonomy to the reads, we found that 94% of the reads were assigned to oomycetes, whereas 6% of reads belonged to plant and fungi with carrot sequences as the main contributor (5.76%). Out of 25 OTUS, 17 belonged to oomycetes and 8 OTUs belonged to others including fungi and carrot (Table S2). Of the 17 oomycete OTUs, 9 OTUs were identified as different species of *Pythium*, two as *Phytophthora* and five OTUs were not assigned to species level but were considered as member of oomycetes (Table 3). *P. intermedium*, *P. violae* and *P. sulcatum* were dominant, however distribution between single lesions of cavity spot was highly variable (Fig. 4).

4. Discussion

The aim of this study was to identify and validate a standard procedure for studying oomycete community diversity using next generation sequencing. We did this by exploring primers that have already proven to amplify a broad diversity of oomycetes to optimize yields of oomycete-derived sequences from a background of soil or plant DNA. The optimized procedure was validated in a soil matrix as well as in symptomatic plant tissue.

High-throughput sequencing of amplicons has enabled studies of microbial community ecology with a very high resolution, and has revolutionized fungal and bacterial ecology studies. However, studies in oomycete ecology studies are still limited despite the economic importance of *Pythium* and *Phytophthora* as pathogens in agriculture and forestry, and only a few studies have been published, mainly due to insufficient methods. One of the cornerstones of such sequencing-based ecological studies are the primers and the amplification protocol. ITS6 and ITS7 targeting the ITS1 region have successfully been applied to amplify many species of *Pythium* and *Phytophthora* in earlier studies (Cooke et al., 2000; Vannini et al., 2013; Vettraino et al., 2012) suggesting their usefulness to study oomycete ecology using next generation sequencing. However, a recent study of oomycete diversity from forest soils using ITS6 and ITS7 in combination with ITS4 resulted in less than 15% of sequences to be of oomycete origin (Coince et al., 2013). In silico assessment of ITS6 and ITS7 primers confirmed the specificity of primers (Fig. 1), however, this also indicated that the primers could amplify fungi and plant DNA at lower annealing temperatures which could be the reason for the low proportion of oomycete sequences retrived in the study by Coince et al. (2013) who used an annealing temperature of 53 °C. Another study of *Phytophthora* diversity in a chestnut forest using ITS6 and ITS7 and an annealing temperature of 55 °C successfully captured 15 *Phytophthora* species (and 18 *Pythium* OTUs) using a pyrosequencing approach, but the number of reads from ten soil samples were reported to be only 11,637 (Vannini et al., 2013). Based on these studies and the fact that the theoretical annealing temperature of the primer pair is 59–64 °C we hypothesized that by increasing the annealing temperature during amplification, the proportion of oomycete sequences could be significantly increased. This has been shown in other studies on the effects of primer annealing temperatures on primer mismatches (Sipos et al., 2007),

Our preliminary experiments using ITS6 and ITS4 as primers in a PCR followed by ITS6 and ITS7 in a semi-nested approach showed that an efficient amplification could be obtained from DNA from soils at 59 °C during primer annealing. We evaluated an amplification protocol at this annealing temperature using three mock communities that were assembled using *Phytophthora* and *Pythium* DNA. This showed that the method was successful in amplifying *Phytophthora* along with *Pythium* as all species from the mock community were retrieved, although in varying amounts. When the amount of *Phytopthora* DNA was reduced 10×, the number of *Phytophthora* reads were reduced, however not in

Table 3
Identified OTUs and total number of sequences in dataset from cavity spot.

#OTU ID	Total reads	Blast results
denovo9	21,754	*Pythium intermedium*
denovo12	6526	*Pythium violae*
denovo32	719	*Pythium sulcatum*
denovo11	125	*Phytophthora megasperma*
denovo14	106	*Pythium polymastum* (28/91)
denovo20	57	*Pythium irregulare*
denovo24	26	*Pythium macrosporum*
denovo15	12	*Phytophthora cactorum*
denovo29	9	*Pythiogeton* sp. (40/90)
denovo27	8	*Pythium* spp. (99/78)
denovo28	6	*Pythium attrantheridium*
denovo2	4	*Pythium heterothallicum*
denovo26	4	*Pythium intermedium* (96/97)
denovo37	4	*Pythium grandisporangium* (28/92)
denovo40	3	*Pythium amasculinum* (96/77)
denovo31	3	*Pythium volutum*
denovo42	2	*Pythium monospermum*

*Coverage and identity percentage values were listed only if they were less than 97.

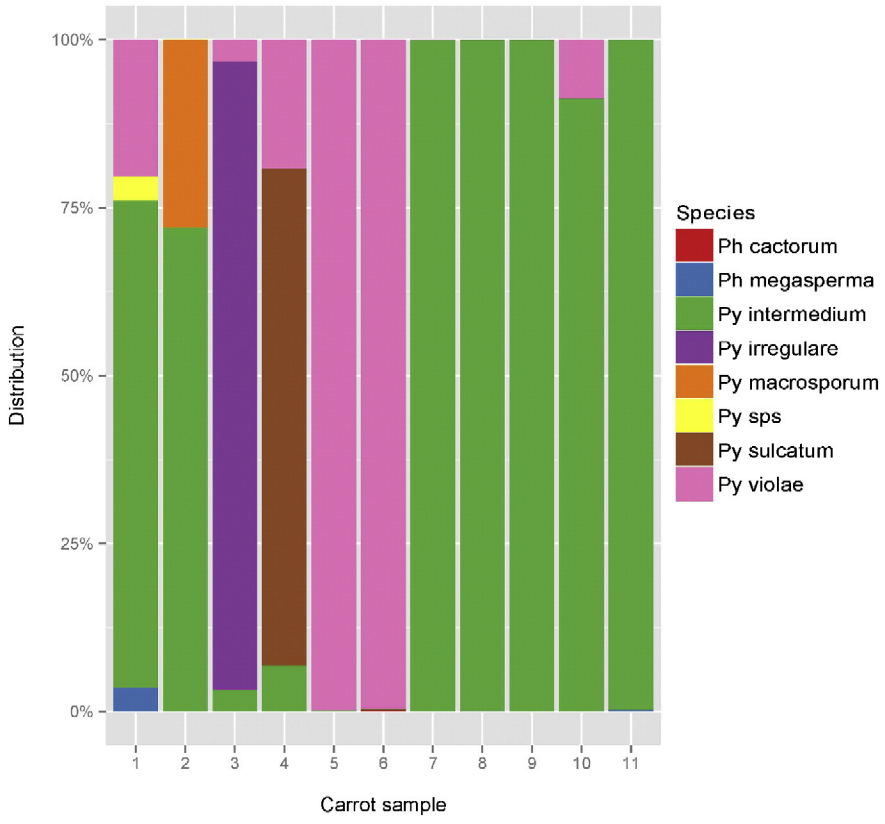

Fig. 4. Distribution of sequences of *Pythium* (Py) and *Phytopthora* (Ph) species in 11 cavity spot samples.

equivalent proportions (Table 1), showing that within-sample comparisons may be problematic and that quantitative data should be interpreted with caution. Similar conclusions have been made in related studies for other groups of organisms such as nematodes (Porazinska et al., 2010) and fungi (Amend et al., 2010). The two replicate communities were found to be very similar in read composition indicating the consistency of the procedure.

Using our amplification protocol on DNA from a set of 26 agricultural soil samples that were collected from various locations in Denmark, we were able to retrieve up to 95% oomycete sequences. A comparison to previously reported studies showed that our optimized strategy dramatically improved oomycete sequence recovery (Table 4). Most of the species detected in our soil samples belonged to the genus *Pythium* indicating its abundance in cultivated soils. Some OTUs with lower percentage of identity and coverage were assigned to *Pythium* but only at the genus level because of a relatively limited database. Although *Phytophthora* species were successfully detected in the mock communities, their presence was very low in the soil samples used in this study. Whether this low abundance of *Phytophthora* in the soils is caused by technical reasons such as low recovery of *Phytophthora* DNA from soil or by biological reasons is not known. However, the mock community studies demonstrated that our strategy is indeed able to retrieve *Phytophthora* sequences indicating that biological reasons may be responsible. Another study of oomycetes reported a dominance of *Pythium* along with several *Pythium* like clusters in the rhizosphere of several plant species (Arcate et al., 2006). Other studies using the same primer set (but at other annealing temperatures) showed different proportions of *Phythophthora* in different environments. Nelson and Karp (2013) found approximately 1% *Phytophthora* derived sequences among oomycetes in wetland soils whereas Vannini et al. (2013) found *Phytophthora* to dominate in forest soils with a known history of *Phytophthora* related diseases. In conclusion, data from the soil samples showed that our strategy covered a significant part of the diversity of oomycetes, as Pythiales, Saprolegniales and other Peronosporales were detected, taxa that are normally found to be dominant in the soil ecosystem, and Saprolegniales are usually found in fresh water (Beakes et al., 2012). Even higher diversity could probably have been found if samples from a wider range of habitats such as forests, grasslands and water had been included. The usefulness of the technique was also confirmed in a high plant DNA background from carrot tissue with symptoms of *Pythium* infection. These samples were dominated by *P. intermedium* and *P. violae*, although other species such as *Pythium irregulare* and *P. sulcatum* were found in to be dominating in a few single lesions. Communities varied between lesions and usually one species was found to be dominating in a single lesion. Other studies using classical approaches have been carried out in Canada (Boule et al., 2003), Norway (Hermansen et al., 2007) and Australia (Davison et al., 2003) to identify *Pythium* species involved in cavity spot, and *P. sulcatum*, *P. violae*, *P. intermedium* and several other species were suggested to

Table 4
Comparison of results from the current study with recent studies on oomycete diversity in soil.

Description	Coince et al. (2013)	Vannini et al. (2013)	This study
Country	France	Italy	Denmark
Environmental samples	Beech Forest soil	Chestnut Forest soil	Cultivated soil
Number of samples	20	10	26
DNA extraction	Fast DNA spin kit	Nucleospin Plant II kit	PowerLyzer PowerSoil Kit
PCR primer sets 1st cycle	ITS6/ITS4	ITS6/ITS7	ITS6/ITS4
PCR primer sets 2nd cycle	ITS6/ITS7	–	ITS6/ITS7
Annealing temperature (PCR)	53 °C	55 °C	59 °C
Sequencing	454 GS FLX	454 GS FLX	454 Junior
Number of reads	100,889	11,637	73,740
Oomycete reads (%)	14.65	78.77	95
Oomycete OTUs (excluding singletons)	10	23	67

be involved in cavity spot, supporting our results. *P. intermedium* has further been reported to cause ring rot in carrot in combination with *Phytopthora megasperma* (Hermansen et al., 2007), a species that was also found in this study, although in low abundance. Our assay thus successfully captured the diversity of *Pythium* species in the symptomatic lesions and further demonstrated that each lesion has its own profile of *Pythium* species.

We used the highly variable ITS1 which has been increasingly used as a barcode for species identification, also for oomycetes (Robideau et al., 2011). Moreover, extraction of the ITS1 region by removing conserved regions in both ends of reads was carried out in order to increase the sensitivity of clustering and successful identification of species (Bengtsson-Palme et al., 2013). In recent years, a large number of ITS sequences have been continuously deposited in reference databases resulting in a higher accuracy of taxonomic assignments. To overcome the risk of low quality sequences or taxonomic error associated with public DNA repositories, expert annotated and tested databases such as UNITE for fungal ITS, SILVA for small subunit (16S/18S, SSU) and large subunit (23S/28S, LSU) ribosomal DNA sequences are preferable. For oomycetes, a curated database for *Phytophthora* has already been developed (Park et al., 2013) which was used for taxonomy assignment in this study. The development of a curated ITS and perhaps also a cytochrome oxidase I (COI) database that includes also oomycetes will be useful for reliable taxonomy assignments in the future (Robideau et al., 2011).

In this study, we have improved and optimized a strategy for studying oomycete community ecology in soil using 454 pyrosequencing. The procedure developed in this study is simple, fast and reliable and gives useful insights into oomycete ecology, and it could be valuable as a tool to understand many soil-borne oomycete-related plant diseases, for example cavity spot in carrot as demonstrated here.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2015.01.013.

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