Rapid and real-time identification of fungi up to the species level with long amplicon Nanopore sequencing from clinical samples.

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ABSTRACT

The availability of long-read technologies, like Oxford Nanopore Technologies, provides the opportunity to sequence longer fragments of the fungal ribosomal operon, up to 6 Kb (18S-ITS1-5.8S-ITS2-28S), and to improve the taxonomy assignment of the communities in real-time and up to the species level. We assess amplicons targeting a 3.5 Kb region (V3 18S-ITS1-5.8S-ITS2-28S D2) and a 6 Kb region (V1 18S-ITS1-5.8S-ITS2-28S D12) with the What's in my pot (WIMP) classifier. We used the ZymoBIOMICS™ mock community and different fungal cultures as positive controls. Long amplicon sequencing correctly identified *Saccharomyces cerevisiae* and *Cryptococcus neoformans* from the mock community, as well as *Malassezia pachydermatis, Microsporum canis, Aspergillus fumigatus* from the microbiological cultures, and identified *Rhodotorula graminis* as the species mislabelled as *Candida spp* in the previous culture.

We applied the same approach to communities, such as external otitis in dogs. *Malassezia* spp was found as the dominant fungi in the ear skin. We identified *M. pachydermatis* as the main species in the healthy sample, and a higher representation of *M. globosa* and *M. sympodialis* in otitis affected samples. We demonstrate the suitability of this approach to characterize the fungal community of complex samples, either healthy samples or samples with clinical signs of infection.

INTRODUCTION

Fungi is a vast kingdom of organisms with a range between 1.5 and 6 million species (Hawksworth and Lücking 2017) but only a modest part, around 140.000 species, is phenotypical and genetically described (Hibbett et al. 2016; Wurzbacher et al. 2018). Normally, fungi have been identified by morphology or pure cultures in agar medium. The main problem is that a lot of species are difficult to isolate, to culture, and even to classify (Arbefeville et al. 2017; Usyk et al. 2017).

Taxonomic classification is needed in clinical approaches and in research application to assign each taxa to its functional trait (Dayarathne et al. 2016; Raja et al. 2017); problems of unknown branches of fungal phylogenies still occur, due to considerable gaps in genetic knowledge and to old species description (Tedersoo et al. 2018).

Sequence-based methods allow to better classify the fungi kingdom but it is known that the choice of the methodology used to study the mycobiome, or even the intrinsic characteristics of a specific fungus, can have an impact on the data generated and the results reached (Usyk et al. 2017). Public databases for fungal identification are noteworthy but still limited due to new updates for a best nomenclature and identification of species (Prakash et al. 2017). Taxonomic revisions are still ongoing and the main databases for fungi classification are Species Fungorum (www.speciesfungorum.org), MycoBank (www.mycobank.org), UNITE (Abarenkov et al. 2010) and International Nucleotide Sequence Database consortium (https://www.ncbi.nlm.nih.gov/taxonomy).

One of the preferred markers for taxonomy assignment is the fungal ribosomal operon, that is almost 6,000 bp length. It contains three conserved units, the 18S rRNA gene (small subunit, SSU), 5.8S rRNA gene and 28S rRNA gene (large subunit, LSU), and two hypervariable units as internal transcribed spacers regions (ITS1 and ITS2). The ITS units flank the 5.8S RNA gene, and better represent the high variability among taxonomic levels of fungi; showing a superior species discrimination and PCR success rates (Kalan and Grice 2017). The variable domains located at the conserved 18S (V1-V9) and 28S rRNA genes (D1-D12) (Figure 1) are also worth to consider to refine the taxonomy assignment (Tedersoo et al. 2015).

Primers sets to amplify the fungal operon regions have been described in different manuscripts, starting from 1990 until 2018 (White et al. 1990; Vilgalys lab 1992; Lee et al. 2008; Ihrmark et al. 2012; Tedersoo and Lindahl 2016; Tedersoo et al. 2018). These sets of primers allow to target the appropriate operon fragments and proceed to sequencing short fragments, using the massive parallel sequencing (or 2nd generation sequencing) as Ion Torrent or Illumina, or longer fragments with single molecule sequencing (or 3rd generation sequencing) as PacBIO or Oxford Nanopore Technologies.

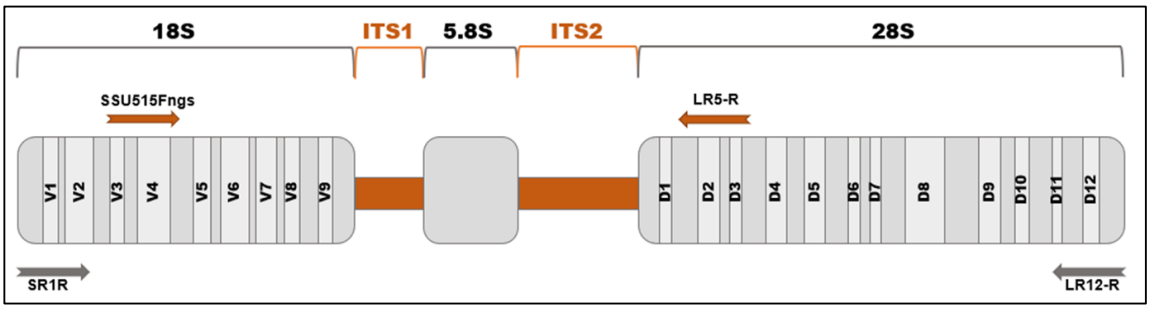

Figure 1. Fungal ribosomal operon: two hypervariable internal transcribed spacers regions (ITS1 and ITS2, marked in orange) and three conserved one (18S, 5.8S and 28S rRNA, marked in grey) that contain variables domains, nine for the 18S and twelve for the 28S rRNA genes. Primers set used for the amplification of the ITS region (3.5 Kb) are shown in orange in the upper part of the operon, and the ones for amplification of the full operon (6 Kb) are shown in grey in the lower part (Vilgalys lab 1992; Tedersoo et al. 2015).

Taxonomy with short reads is focused on ITS1 and ITS2 regions, considered as the official barcoding markers for species-level identification in fungi, due to their easy amplification, conserved primers sites, widespread use (Schoch et al. 2012) and available databases, such as UNITE or Mycobank. Normally, the ITS1 and ITS2 regions provide the taxonomy within-genus and within-species level, but debates on which one provides the best resolution are still discussed (Blaalid et al. 2013).

Alternative markers located in the small and large subunits of the rRNA genes are used to address the phylogenetic diversity, depending on the species (Blaalid et al. 2013; Tedersoo et al. 2015). The SSU and the LSU are used when taxonomy is investigated up to family level, while lower taxonomy level analysis requires the ITS regions. Also when primers sets include the D1-D2 regions of LSU subunit, fragments obtained from the amplification can be assigned up to the species level (Raja et al. 2017).

Here, we aim to investigate on the taxonomic resolution of long-amplicon PCR approaches to detect and identify at the species level the fungal microbiota present on complex microenvironments. We use microbiological fungal cultures phenotypically characterized as positive controls, and clinical samples of canine otitis.

MATERIALS and METHODS

Samples and DNA extraction.

LETI laboratories (LETI Animal Health) kindly provided a total of eight microbiological fungal cultures in petri dish; four cultures were classified up to genus level (*Alternaria spp*, *Aspergillus spp*, *Candida spp* and *Malassezia spp)* and four other ones classified up to species level: three of *Malassezia pachydermatis* and one of *Microsporum canis.* Also, fungal DNA of the ZymoBIOMICS™ mock community containing *Saccharomyces cerevisiae* and *Cryptococcus neoformans* was included in the study as positive control. The DNA from all fungal samples was extracted by ZymoBIOMICS™ Miniprep kit following the manufacturer's instructions.

As complex microbial communities, four canine otitis samples were analysed. Two of them were collected from a Petri dish, divided in two halves parts, to culture fungi from both ears of a dog, one of the ears was healthy and the other one shown clinical signs for external otitis (S02_healthy; S03_affected). The DNA samples were extracted by using ZymoBIOMICS™ Miniprep kit, as for the cultures. The other two complex samples (S01_affected; S04_affected) were clinical samples collected by swabbing the inner pinna of the ear of two dogs with clinical signs of external otitis, using Sterile Catch-All™ Sample Collection Swabs (Epicentre Biotechnologies). In those cases, DNA was extracted by QIAGEN-DNeasy PowerSoil Kit.

DNA quality control was checked by Nanodrop and Qubit™ Fluorometer (Life Technologies, Carlsbad, CA).

MinION sequencing.

Two set of primers were chosen (Table 1; Figure 1): the first set targeting the ribosomal operon from V3 region of 18S RNA gene to D3 region of 28S RNA gene (≈3,500 bp), and the second one targeting the complete ribosomal operon from V1 region of 18S RNA gene to D12 region of 28S RNA gene (≈6,000 bp). The primers, both forward and reverse, included the Nanopore Universal Tags.

Two PCR were performed: the first for the amplification of the fragment, and the second one to add the specific barcode to each sample. PCR final volume was 50 µl and contained 5 ng DNA, 10 µl of Phusion® High Fidelity Buffer (5x), 5 µl of dNTPs (2 mM), 0.5 µM of primer forward and reverse, and 0.02 U/µl of Phusion® Hot Start II Taq Polymerase (Thermo Scientific). PCR profile included an initial denaturation of 30 s at 98 °C, followed by 25 cycles of 10 s at 98 °C, 30 s at 62 °C, 80 s at 72 °C, and a final extension of 10 min at 72 °C. Amplicons obtained were purified with Agencourt AMPure XP beads, at 0.4X ratio for the fungal amplicon; then, they were quantified by Qubit™ fluorometer (Life Technologies, Carlsbad, CA).

Table 1. Primers targeting the full ITS region (3.5 Kb) and the full fungal operon (6 Kb). The Nanopore Universal Tag is shown in bold type.

NAME	SEQUENCE (5'-3')	TARGET	AMPLICON	REFERENCE
SSU515Fngs-F	**TTTCTGTTGGTGCTGATATTG** **C**GCCAGCAACCGCGGTAA	18S-V3	3.5 Kb	(Tedersoo et al. 2015)
LR5-R	**ACTTGCCTGTCGCTCTATCTT** **C**TCCTGAGGGAAACTTCG	28S-D3	3.5 Kb	(Tedersoo et al. 2015)
SR1R-Fw	**TTTCTGTTGGTGCTGATATTG** **C**TACCTGGTTGATQCTGCCAGT	18S-V1	6 Kb	(Vilgalys lab 1992)
LR12-R	**ACTTGCCTGTCGCTCTATCTT** **C**GACTTAGAGGCGTTCAG	28S-D12	6 Kb	(Vilgalys lab 1992)

Following the PCR Barcoding kit protocol (SQK-PBK004), 0.5 nM per each sample were required for the second PCR, in which barcodes of PCR barcoding kit (EXP-PBC001) will be added. The final volume of second PCR is 100 µl, containing 20 µl of DNA template from the previous PCR at 0.5 nM, 2 µl of specific barcode and 78 µl of mixture that include: 20 µl of 5× Phusion® High Fidelity Buffer, 10 µl of dNTPs (2 mM) and 2 U/µl of Phusion® Hot Start II Taq Polymerase. PCR profile included an initial denaturation of 30 s at 98 °C, 15 cycles of 10 s at 98 °C, 30 s at 62 °C, 80 at 72 °C and final step of 10 min at 72 °C. The amplicon obtained were purified again with Agencourt AMPure XP beads, at 0.4X ratio and quantified by Qubit™ fluorometer (Life Technologies, Carlsbad, CA).

We proceeded then to the Library preparation with the Ligation Sequencing kit (SQK-LSK109, Oxford Nanopore Technologies). Barcoded samples (1.5 µg) were pooled in 47 µl of nuclease-free water and the library was prepared following the manufacturer conditions.

With a final step of Agencourt AMPure XP beads 0.4X, the DNA library was cleaned and ready to be loaded into the flow cell. We used two SpotON Flow Cells (FLO-MIN106) for three MinION runs, primed with a mixture of sequencing buffer and Flush buffer according to the manufacturer's instructions. A quality control of sequencing pores was done before each run. Libraries were mixed with Sequencing Buffer and Loading Beads in a final volume of 75 µl. The final mix was added, by dropping, in the SpotON sample port.

Sequencing runs were between 16h and 19h, using the MinKNOWN 2.2 v18.07.2 and the MinKNOWN v18.12.9.

Data Analysis.

The sequencing of the 3.5 Kb amplicon generated fast5 files that were basecalled and demultiplexed by Albacore v2.3.3 for the 3.5 Kb amplicon or guppy 2.3.5 for the 6 Kb amplicon. Using Porechop (https://github.com/rrwick/Porechop) barcodes and adapters were removed. For the taxonomy assignment, we applied the cloud-based analysis *What's in my pot* (WIMP) application from the EPI2ME platform (Methricor), which is based in Centrifuge (https://ccb.jhu.edu/software/centrifuge/manual.shtml).

RESULTS

Our aim was to develop a long-amplicon PCR approach to detect and identify fungal microbiota present on complex microenvironments, and to apply it to clinical samples (canine otitis). As positive controls, we chose microbiological fungal cultures and fungal strains from a mock community. Some of the cultures were previously classified by classical microbiology up to genus level as *Alternaria spp*, *Aspergillus spp*, *Candida spp* and *Malassezia spp;* while others were classified up to species level as *Malassezia pachydermatis* and *Microsporum canis* at LETI laboratories (LETI Animal Health). The ZymoBIOMICS™ mock community fungal strains are *Saccharomyces cerevisiae* and *Cryptococcus neoformans.*

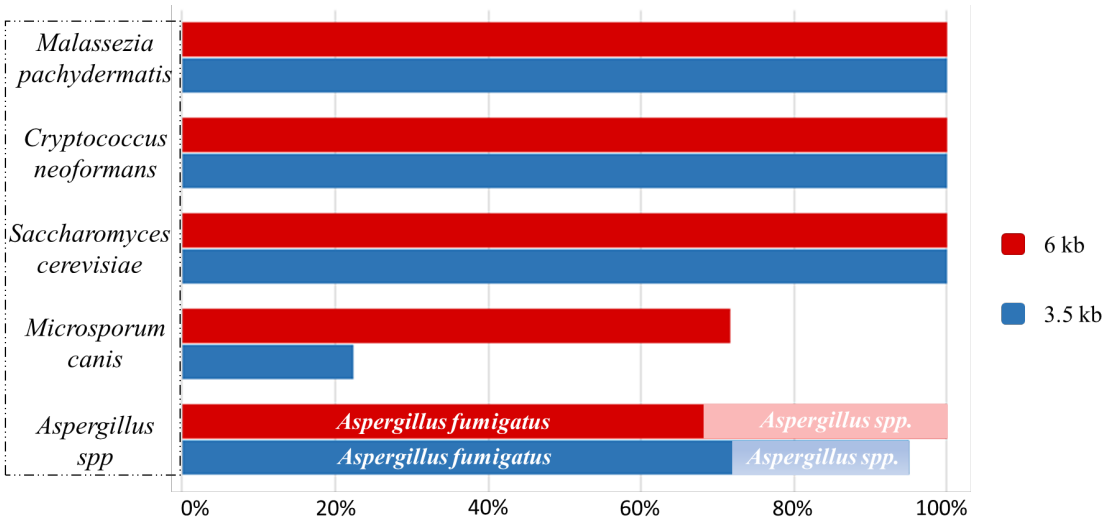

Figure 2: ZymoBIOMICS mock community (*S. cerevisiae* and *C. neoformans*) and microbiological cultures of fungi after taxonomical classification of the 3.5 Kb and 6 Kb ribosomal amplicons.

Identification of microbiological cultures and mock community with long amplicons.

All samples were amplified for both amplicon sizes, 3.5 Kb and 6 Kb. In the 3.5 Kb amplicon we included those domains that better help in taxonomic classification of fungi (as shown in Figure 2).

Both amplicons correctly detected and identified the ZymoBIOMICS™ mock community fungal strains *Saccharomyces cerevisiae* and *Cryptococcus neoformans,* and also *Malassezia pachydermatis* and *Microsporum canis* from the microbiological cultures. Looking in detail, up to 100% of *S. cerevisiae*, *C. neoformans* and *M. pachydermatis* 3.5 Kb and 6 Kb reads were correctly classified, while *M. canis* was better classified by the 6 Kb amplicon.

The reads from both amplicons were classified at the genus level for the *Aspergillus* microbiological culture, and determined *A. fumigatus* as the most abundant species found in the culture.

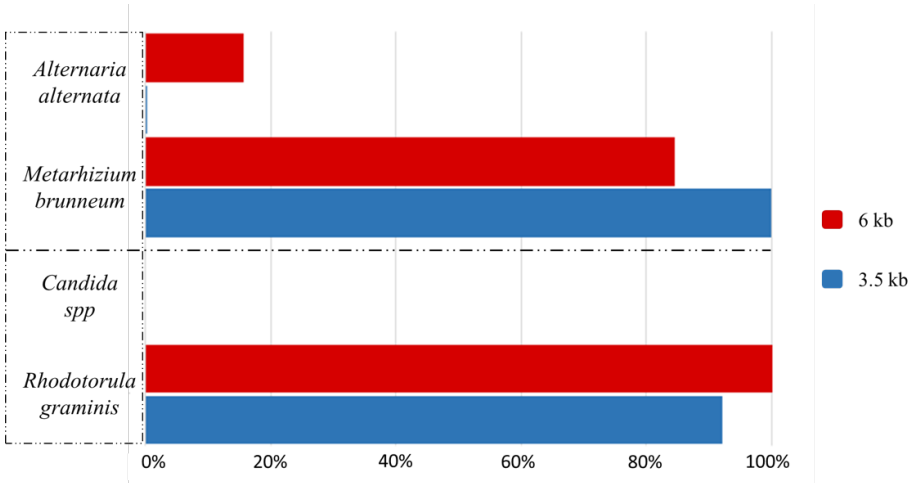

Figure 3. Fungal microbiological cultures showed unexpected results in the taxonomical classification after sequencing. Few reads from the *Alternaria* culture belonged to *Alternaria spp,* and it was classified at species level as *A. alternata*, but the most abundant fungus found was *Metarhizium brunneum*. No reads from the *Candida* culture were classified as *Candida spp* because of the presence of *Rhodotorula graminis*.

Alternaria spp and *Candida spp* showed different results of what we expected after the microbiological culture classification (Figure 3). For the *Alternaria* culture, most of the reads were classified as *Metarhizium brunneum,* while low relative abundance reads corresponded to *Alternaria alternata* (0.2% for 3.5 Kb and 15.6% for 6 Kb amplicons respectively). We found no similarity between these fungi: they belong to different order, Pleosporales and Hypocreales. Looking at the nomenclature of this fungus, *Metarhizium brunneum* belonged to *Metarhizium anisopliae* strain

(Tiago and Oliveira 2014; Yousef et al. 2018) but no correlation with *Alternaria alternata* were found. *Alternaria* is a ubiquitous filamentous fungi, which can be found in soil air and human/animal skin (Pastor and Guarro 2008); *Metarhizium* is commonly found as parasite of insects and symbiont of plant (Samish et al. 2014; Tiago and Oliveira 2014). It is noteworthy that the microbiological cultures were obtained by sampling dog skin, and therefore environmental species are often cultured and can be taxonomically misclassified, as it seems to be the case in our study.

For the *Candida* culture, the colonies in the petri dish were red/orange at the time of DNA extraction, and sequences revealed the presence of *Rhodotorula graminis* and only few reads were classified as *Candida spp. Rhodotorula* is a carotenoid biosynthetic yeast, part of the Basidiomycota phylum, easily identifiable by distinctive yellow, orange or red colonies (Yadav et al. 2014). This yeast is commonly associated with plants and it produces three major carotenoids: b-carotene, torulene and torularhodin (http://www.antimicrobe.org/f16.asp#t1). In this case, we could confirm the presence of two yeast species in the microbiological culture.

Identification of fungi in complex canine otitis samples.

We sequenced two other microbiological cultures of *M. pachydermatis* as positive controls and 4 clinical samples from canine external otitis targeting the 3.5 Kb amplicon. We run WIMP for fungal profiling: the positive controls were identified as *M. pachydermatis*, while the clinical otitis samples showed other *Malassezia* species (Table 2).

Table 2: Relative abundance of *Malassezia* species found in *M. pachydermatis* microbiological culture, and in four complex samples belonged to three different dogs affected by otitis. Samples S02 and S03 belong to the same dog, while S04 and S01 belong to two different dogs.

	M. pachydermatis	*M. globosa*	*M. sympodialis*	*Malassezia spp*	*Other*
M01	98.9%	0.2%	0.2%	0.2%	0.5%
M02	99.0%	0.1%	0.2%	0.2%	0.5%
M03	98.9%	0.2%	0.3%	0.2%	0.4%
S02_healthy	98.2%	0.1%	0.2%	0.2%	1.3%
S03_affected	95.1%	1.1%	1.7%	0.4%	1.7%
S04_affected	78.8%	4.0%	5.0%	3.9%	8.3%
S01_affected	73.7%	4.7%	5.1%	5.3%	11.2%

Two of the samples corresponded to the same dog, one from a healthy ear (S02) and the other one (S03) with clinical signs compatible with otitis externa, and *M. pachydermatis* is the main fungal species detected in both ears. The other two samples (S01 and S04) came from the ear with otitis externa of two other dogs. In that cases, other *Malassezia* species were detected together with *M. pachydermatis*, such as *M. globosa* and *M. sympodialis.*

DISCUSSION

Our first approach with Oxford Nanopore Technologies sequencing was aimed to understand if long amplicons are suitable markers to analyse the mycobiome in dog skin, and which size could be the best in the analysis of mycobiome. The microbiological cultures were essential for the study as positive controls, because their genome sequences were used to validate the correct detection of fungi in WIMP.

Primers used to amplify domains of the fungal ribosomal operon should be chosen depending on the fungus, but no standard markers are defined yet. Due to different sequence lengths of fungi, longest amplicons should be considered to describe the communities at lower taxonomy classification (Tedersoo et al. 2018; Wurzbacher et al. 2018). *Malassezia spp*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Microsporum canis* and *Aspergillus spp* were correctly detected and identified from the microbiological cultures. However, the microbiological cultures corresponding to *Alternaria spp* and *Candida spp* were misidentified as per classical microbiology, and other fungi were detected. It is noteworthy that the samples plated came from dog skin, which is prone to environmental contamination, as has been previously described in skin microbiome of healthy dogs (Cuscó et al. 2017; Cuscó et al. 2019).

Most of the reads from the *Alternaria* culture were classified as *Metarhizium brunneum.* This fungus belongs to the same phylum of Ascomycota, but differ at lower taxonomy levels. It was discovered in Spain and it is commonly used as herbicide against the fly *Bactrocera oleae* (Yousef et al. 2018). The *Candida* microbiological culture was misclassified, likely due to the presence of other fungi, *Rhodotorula graminis,* that was the causal agent of the colonies colour.

Clinical otitis in dogs are associated to *Malassezia* overgrowth, even when *M. pachydermatis* has been reported as the most abundant species in the ear canal of healthy dogs (Korbelik et al. 2018). We found *M. pachydermatis* as the dominant fungi in the ear skin of the dogs tested, whereas *M. globosa* and *M. sympodialis* were increasing their percentage in the samples with clear clinical signs

of otitis. Further analyses will assess which *Malassezia* species "compete" in the ear skin of dogs affected with otitis.

In conclusion, this is a first approach to assess nanopore sequencing and the long-read amplicon approach for the analysis of fungi in complex samples. We assess the taxonomical power of two different amplicons targeting 3.5 Kb and 6 Kb of the ribosomal operon respectively, with the longest one providing a better taxonomy assignation. We used positive controls from the ZymoBIOMICS mock community and from microbiological cultures, that not always can be considered as pure cultures. We demonstrate the suitability of this approach to characterize the fungal community of complex samples, either healthy samples or samples with clinical signs of infection, such as otitis. Next steps involve the analysis of complex samples from different origins to detect the causal agent of the disease in a clinical metagenomics approach.

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