Plant and Fungal Diversity in Gut Microbiota as Revealed by Molecular and Culture Investigations

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Introduction

The human gut contains a wide variety of microorganisms known as the microbiota [1]. At birth, the human gut is sterile and is then colonized by bacteria originating from the mother, environment and diet [2,3]. Several studies have revealed the importance of gut microbiota in host health and the contribution of these microbes to diverse functions, including metabolism, immune function and gene expression [4]. Gut microbes produce a large arsenal of enzymes that are naturally absent from humans, which contribute to food digestion, energy harvesting and storage [5,6]. Two bacterial phyla, *Firmicutes* and *Bacteroidetes*, dominate in the gut microbiota. Some studies have shown a reduction in the relative proportion of *Bacteroidetes* in obese individuals compared to lean individuals [5,7]. Additionally, it has been observed that the microbiota of obese individuals extract more energy from the diet than the microbiota of lean individuals [1].

The gut microbiota is comprised of Viruses, Bacteria, Archaea and Eukaryotes [8]. Accordingly, there are much data available about the bacterial community. However, few studies have investigated eukaryotic communities in the human gut, resulting in a dearth of information about these communities. Previous studies that have used molecular methods to explore the eukaryotic community in the guts of healthy individuals detected only *Galactomyces* and *Candida* fungi and *Blastocystis hominis* as prevalent species [9,10]. Additional studies have reported increased fungal diversity in ill patients compared to healthy individuals [11–13].

Thus, our study aimed to examine the repertoire of plants and fungi in the gut of an obese human using both PCR-sequencing and culturing techniques.

Results

Molecular Detection

Mixing *Acanthamoeba castellanii* DNA and stool DNA yielded a positive amplification using specific primer pair for *Acanthamoeba* (JPD1/JDP2). Among the 25 primers pairs, 17 yielded an exact sequence with an appropriate positive control, whereas no positive control was available for 8 primer pairs (Table 1 & Table 2). Only 5 of these 25 eukaryotic PCRs yielded amplification product with the stool specimen, while the negative controls exhibited no amplification. The analysis of a total of 408 clones identified 7...
fungal species, 18 plant species and one Diatoms (Blastocystis sp.) species (Table 3). GenBank reference number of the best hit similarly to our sequences for each organism were: Galactomyces geotrichum (AY903644.1), Penicillium camemberti (GQ458039.1), Malassezia globosa (AY743604.1), Malassezia pachydermatis (AB18940.1), Malassezia restricta (AY743607.1), uncultured Chytridiomycota (GQ995333.1) Candida tropicalis (DQ513959.1).

Fungi Isolated Using Culture Media

In all experiments, the negative control plates remained sterile. A total 16 different fungal species were isolated (Table 4). Nine species of fungi (M. globosa, M. restricta, M. pachydermatis, Penicillium allii, Penicillium dipodomyicola, G. geotrichum, Cladosporidium sp., Climacocystis sp. and C. tropicalis) were cultured on Dixon agar medium. Three species of fungi (Penicillium sp./P. commune/P. camemberti, Aspergillus versicolor, Beauveria bassiana) were cultured on Potato Dextrose media. Two species of fungi (Aspergillus flavipes, Isaria farinosa) were cultured on CZAPEK medium. Two species of fungi (Hypocreales/Isaria chrysogenum, Penicillium brevicompactum) were cultured on both PDA and CZAPEK media, and C. tropicalis was cultured on both Dixon agar and PDA media. Five of the cultured species of fungi (G. geotrichum, C. tropicalis, M. pachydermatis, M. globosa, and M. restricta) were also identified by clone sequencing, while 11 fungi were detected only by culture (Figure 1). Penicillium, Aspergillus, Galactomyces, Beauveria, Candida, Cladosporidium, and Isaria are members of the Ascomycota phylum and Malassezia and Climacocystis are members of the Basidiomycota phylum.

Table 1. Eukaryotic and fungi primers selected in this study.

| Taxon          | Primer          | Target        | PCR product size (bp) | Annealing temperature and number of cycles | Reference |
|----------------|-----------------|---------------|-----------------------|------------------------------------------|-----------|
| Amoeba         | AmiF1/Ami9R     | 18S rRNA      | 670                   | 55˚C 30 s 40cycles                       | [47]      |
| Acanthamoeba   | JDP1/JDP2       | 18S rRNA      | 460–470               | 60˚C 40 s 40cycles                       | [48]      |
| Entamoeba      | JVF/DSPR2       | 18S rRNA      | 662–667               | 55˚C 60 s 40cycles                       | [49]      |
| Hartmanella    | HV1227F/HV1728R | 18S rRNA      | 502                   | 56˚C 30 s 40cycles                       | [50]      |
| Naegleria      | F/R             | ITS           | 376–388               | 55˚C 30 s 15 cycles                      | [51]      |
| Ciliophora     | 121F/1147R      | 18S           | 750–1000              | 55˚C 60 s 30 cycles                      | [52]      |
| Chlorophyta    | UCP1F/UCP1R     | Rps11-rpl2    | 384                   | 54˚C 60 s 30 cycles                      | [53]      |
| Didymos        | 18S/28R         | 18S rRNA      | 391                   | 56˚C 60 s 30 cycles                      | [54]      |
| Dinoflagellate | 18Scmoff1/Dino185R | 18S rRNA | 700–900               | 60˚C 30 s 30 cycles                      | [55]      |
| Diplomonads    | DimA/DimB       | 18S rRNA      | 650                   | 58˚C 60 s 40 cycles                      | [56]      |
| Euglenophyta   | EAF/EAF3        | 18S rRNA      | 1000                  | 62˚C 90 s 25cycles                       | [57]      |
| Kinetoplastidia| Kinokin1/knotokin2 | 18S rRNA | 600–650               | 56˚C 30 s 30 cycles                      | [58]      |
| Microsporidia  | V1/PMP2         | 18S rRNA      | 250–279               | 55˚C 30 s 30 cycles                      | [59]      |
| Rodophyta      | URP1_F/URP1_R   | rps10-dnA     | 464                   | 52˚C 60 s 30 cycles                      | [60]      |
| Trichomonads   | TF31/TFR2       | 5,8SrRNA, ITS | 338–391               | 60˚C 30 s 30 cycles                      | [61]      |
| Fungi          | MaF/MALR        | 26S           | 580                   | 55˚C 45 s 40cycles                       | [62]      |
| Fungi          | NS1/FR1         | 18S rRNA      | 1650                  | 48˚C 45 s 30 cycles                      | [63]      |
| Fungi          | ITS1F/ITS4R     | ITS           | Variable              | 50 45 s 40cycles                        | [64]      |
| Universal      | Euk1A/EUK516r   | 18S           | 500                   | 50˚C 30 s 30 cycles                      | [65]      |
| eukaryote      | EUK528/1391R    | 18S           | 1000–1300             | 55˚C 60 s 30 cycles                      | [66]      |
| Plant          | rbcLZ1/rbcL19b  | Chloroplast   | 157                   | 40˚C 30 s 40 cycles                      | [67]      |

Discussion

The PCR-based and culture-based results obtained here are validated by the fact that all the negative controls remained negative, precluding the possibility of cross contamination from the laboratory. Also, we ensured the absence of potential PCR inhibitors in the stool specimen. At last, the PCR systems yielded expected result with appropriate positive controls including Fungi which have been shown to be difficult to lyse [14]. Accordingly, we combined mechanical and enzymatic lysis to optimize recovery of DNA from Fungi as previously reported [9,14-15]. These data allowed to interpret negative results as true negatives. The 18S rRNA, ITS and chloroplast genes amplified in this study are molecular markers commonly used for eukaryotic screening [11,16-22]. These genes are conserved in all eukaryotes and contain variable regions suitable for primer design.

However, this is the first study to use a multiple set of primers for molecular approach to screen eukaryotic communities in a stool sample from an obese person. The combination of culture-dependent and culture-independent cloning and sequencing revealed a previously unsuspected diversity of eukaryotes among the human intestinal microbiota. Indeed, we detected a total of 37 eukaryotic species; only 16 of these species had been previously...
reported to be present in the gut microbiota. Interestingly, the culturing of the sample in using only three different culture media identified more than twice the fungal species than did the different PCR-based molecular methods (Table 5). Accordingly, culturing yielded A. flavipes, P. brevicompactum, B. bassiana, P. dipodomyicola, M. restricta, Climacocystis sp. and I. farisona, which have not been previously detected in human stool samples. This result differs from previous studies that cultured only one or two Candida spp. and Saccharomyces spp. from healthy individuals [9–12]. Our culture conditions were different from those used by Scanlan and Chen [9,12], as we incubated our cultures at 25°C for two weeks. We also did not use the same medium as Khait [23]. Our use of Dixon medium allowed us to isolate a wide variety of fungi (9 species). Our results can be explained by our subject’s obese status; it is possible that obese individuals harbor more fungi. Most of the fungi (11 species) identified in our study are known to be associated with cereal grains [29–31]. To the best of our knowledge, we are the first to report the presence of this species in a stool sample from an obese individual using a culture-dependent method. The A. versicolor species found in this stool sample is an environmental airborne fungal species [32]. A. versicolor and P. chrysogenum have also been previously isolated from dry cured meat products [33]. Accordingly, previous studies have detected these species in human stool samples [11,12]. The C. tropicalis sp. isolated from our subject’s stool sample is often found on fruit, such as grapes [34], and has been previously reported in stool samples [11].

The B. bassiana and I. farisona detected in this study are entomopathogenic fungi that are used as biocontrol agents in agriculture [35], which can explain their presence in the human gut. C. tropicalis, which was also isolated from our subject’s stool sample, has commonly been reported in human stool [23], in the intestine of normal individuals (up to 30%) and in the oral microbiome of healthy individuals [36]. The C. tropicalis sp. detected here is an edible fungus, which explains the detection of this fungus in this stool sample. This fungus was not found to be present in stool in previous studies.

The Malassezia species isolated from our subject’s stool sample are normal flora found on the skin of 77–80% of healthy adults [37]. These species were also found in scalp skin from healthy

### Table 2. Results of PCR testing with positive control. NA non available.

| Taxon                | Primers    | Positive control | PCR | Blast coverage% | Blast identity % | GenBank reference number |
|----------------------|------------|------------------|-----|-----------------|------------------|--------------------------|
| Amoeba               | AmF1/AmI9R | Acanthamoeba castellanii | Positive | 100 | 99 | A.castellanii (GU001160.1) |
| Hartmannella         | Hartmannella vermiciformis | Positive | 100 | 99 | H. vermiciformis (DQ123623.2) |
| Acanthamoeba         | JDP1/JDP2  | Acanthamoeba castellanii | Positive | 100 | 99 | A. castellanii (GU001160.1) |
| Entamoeba            | JVF/DSPR2  | NA               | NA  | NA              | NA               | NA                       |
| Hartmannella         | Hv1227F/Hv1728R | Hartmannella vermiciformis | Positive | 100 | 99 | H. vermiciformis (HM363627) |
| Naegleria            | F/R        | NA               | NA  | NA              | NA               | NA                       |
| Ciliophora           | 121 F/1147R | Colpoda steinii  | Positive | 100 | 99 | C. steinii (DQ388599.1) |
| Chlorophyta          | UCP1F/UCP1R | Chlorella vulgaris | Positive | 95 | 93 | C. vulgaris (AB001684.1) |
| Chlorophyta          | UCP2F/UCP2R | Chlorella vulgaris | Positive | 95 | 93 | C. vulgaris (AB001684.1) |
| Diatoms              | 185/28R    | NA               | NA  | NA              | NA               | NA                       |
| Dinoflagellates      | DinocomF1/Dino18SR1 | Poterioochromonas malhamensis | Positive | 100 | 98 | P. malhamensis (FN662745.1) |
| Diplomonads          | DimA/DimB  | NA               | NA  | NA              | NA               | NA                       |
| Euglenophyta         | EAF/EAF3   | NA               | NA  | NA              | NA               | NA                       |
| Kinetoplastidia      | Kinetokin1/kinetokin2 | Leshmania major  | Positive | 99 | 99 | L. major (FN677342.1) |
| Kinetoplastidia      | KinSSUF1/KinSSUR1 | Leshmania major  | Positive | 99 | 99 | L. major (FN677342.1) |
| Microsporidia        | V1/PMP2    | Encephalitozoon hellem | Positive | 100 | 99 | E. hellem (AF039229.1) |
| Rhodophyta           | URP1F/URP1R | NA               | NA  | NA              | NA               | NA                       |
| Rhodophyta           | URP2F/URP2R | NA               | NA  | NA              | NA               | NA                       |
| Trichomonads         | TRF1/TRF2  | NA               | NA  | NA              | NA               | NA                       |
| Fungi                | Malf/MalR  | Malassezia restricta | Positive | 100 | 98 | M. restricta (JN980105) |
| Fungi                | ITS1F/ITS4R | Candida albicans  | Positive | 100 | 99 | C. albicans (L28817.1) |
| Fungi                | NS1R/FR1   | Candida albicans  | Positive | 100 | 99 | C. albicans (JN940588.1) |
| Fungi                | FunF/FunR  | Candida albicans  | Positive | 100 | 99 | C. albicans (JN940588.1) |
| Universal Eukaryotes | euk528F/1391R | Acanthamoeba castellanii | Positive | 98 | 99 | A. castellanii (GU001160.1) |
| Chloroplast Plant    | rbcZ1/rbcL19b | Solanum sp.      | Positive | 98 | 94 | S. physalifolium (HQ23562) |

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volunteers [38]. However, *M. pachydermatis* and *M. globosa* were previously found in stool from healthy and ill subjects [12,13] by culture-independent methods. We report for the first time the detection of *M. restricta* in stool by molecular methods. The Malassezia species that were detected by culture-independent methods in this study were confirmed by culture. The presence of these fungi in our subject’s stool sample could be either a contaminant from the subject’s skin or a part of human gut flora, so more investigation is needed to confirm these results. The uncultured Chytridiomycota detected in this stool sample is a member of the Chytridiomycota family (Figure 2). Some Chytridiomycota species infect potatoes and tomatoes [39], which could explain the incidence of these fungi in the human gut. To the best of our knowledge, we are the first to report this species in a stool sample from an obese subject.

In addition to fungi, we detected 11 plant species, all of which are known to be associated with human food and traditional medicines. We identified the dietary plants *Solanum lycopersicum* (tomato), *Allium victorialis* (onion family), *Solanum tuberosum* (potato), *Citrus aurantium* (orange), *Cicer arietinum*, *Musa acuminata*/Ensete ventricosum* (banana), *Lactuca sativa*, *Humulus lupulus* (hops), *Pinus wallichiana*, *Helianthus annuus* (sunflowers) and *Brassica napus*. The sequences of *Nicotiana tabacum* and *C. arietinum* that we identified might be linked to the consumption of cigarettes by the patient. A previous study has also reported the presence of *N. tabacum* and *C. arietinum* in human stool [40].

The diversity of the plant species found in the stool sample can be explained by the patient’s diet. Because of her obesity, she may have a diet rich in plants. Some of the plant sequences found in this stool sample, such as *Atractylodes japonica*, *Fibraurea tinctoria*, *Angelica anomala* and *Mitella nuda*, are used as medicinal plants [41]. The genus *Atractylodes* has been found in the oral microbiome of healthy individuals [26]. The plants that we identified in this study are similar to those found in Nam’s study, which detected different plants from 10 Korean individuals [10]. We did not find the same plant species as those identified from Korean subjects because our obese subject did not have the same diet and lived in a different environment.

Finally, the *Blastocytis* sp. that we detected is commonly found in healthy microbiota [9,10] and is associated with irritable bowel syndrome.

| Table 3. Sequencing results on PCR products from clones. |

| Primers | clones | Sequences of Species | Blast Identity% and coverage% | Kingdom |
|---------|--------|----------------------|------------------------------|---------|
| ITS1F/ITS4R | 75 | 96% Galactomyces geotrichum | 99 and 99 | Fungi |
| MalFMalR | 57 | 28.07% Malassezia pachydermatis | 92 and 100 | Fungi |
| | | 17.54% Malassezia restricta | 100 and 99 | Fungi |
| | | 54.4% Malassezia globosa | 99 and 99 | Fungi |
| EUK1A/EUKS16r | 104 | 20.4% Blastocytis sp. | 99 and 99 | Protist |
| | | 0.96% Uncultured Chytridiomycota | 95 and 99 | Fungi |
| | | 0.96% Fibraurea tinctoria | 98 and 100 | Plant |
| | | 1.9% Allium victorialis | 98 and 100 | Plant |
| | | 3% Nicotiana tabacum | 99 and 99 | Plant |
| | | 0.96% Helianthus annuus | 96 and 100 | Plant |
| | | 0.96% Caprifoliaceae environmental | 98 and 99 | Plant |
| | | 0.96% Petrophile canescens | 98 and 99 | Plant |
| | | 60% Solanum lycopersicum | 99 and 99 | Plant |
| | | 5% Humulus lupulus | 98 and 100 | Plant |
| | | 3% Cicer arietinum | 99 and 98 | Plant |
| | | 0.96% Pinus wallichiana | 100 and 98 | Plant |
| | | 0.96% Mitella nuda | 100 and 98 | Plant |
| JV/DSPR2 | 141 | 94.32% Galactomyces geotrichum | 98 and 99 | Fungi |
| | | 0.71% Candida tropicalis | 98 and 99 | Fungi |
| | | 0.71% Citrus aurantium | 99 and 100 | Plant |
| | | 4.25% Atractylodes Japonica | 98 and 99 | Plant |
| | | 0.71% Pinus wallichiana | 99 and 100 | Plant |
| | | 78% Nicotiana undulate | 98 and 99 | Plant |
| rbcLZ1/rbcL19b | 31 | 3% Musa acuminata/Ensete ventricosum | 99 and 99 | Plant |
| | | 6.25% Lactuca sativa | 99 and 99 | Plant |
| | | 3% Solanum tuberosum | 100 and 99 | Plant |
| | | 3% Brassica napus/Arabidopsis lyrata | 100 and 99 | Plant |
| | | 6.25% Angelica anomala/Davidia involucrata/Aucuba japonica | 100 and 99 | Plant |

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Conclusions

Of 40 phyla of protists described in literature, eight phyla (Diatoms, Apicomplexa, Ciliate, Parabasalids, Fornicata, Amoeboza, Microsporidia, Fungi) have been previously detected in human gut [42]. However, most species including *Gardia intestinalis* (Parabasalids), *Blastocystis hominis* (Diatoms), *Cryptosporidium parvum* (Apicomplexa), *Balantidium coli* (Ciliates), *Dientamoeba fragilis* (Fornicata), *Entameba histolytica* (Archamoeba ), *Encephalitozoon intestinalis* (Microsporidia) and *Candida tropicalis* (Fungi) have been reported in patients with digestive tract disease [42–44]. Here, we showed that representatives of two of these eight phyla (Fungi and Blastocystis) can be also detected in one individual without digestive tract disease. Among 19 micro-eukaryotes found in this individual, five fungal species were detected using PCR-based and culture approaches, 16 fungal species were detected by culture and eight species including seven different fungi and one *Blastocystis* were detected by molecular methods. Accordingly, a total of 13 plants species and eight fungi including *Aspergillus flavipes*, *Beauveria bassiana*, *Laria farinosa*, *Penicillium brevicompactum*, *Penicillium dipodomyicola*, *Penicillium camemberti*, *Climacocystis sp.* and *Malassezia restricta* were detected for the first time in the human gut microbiota. These data illustrate that eukaryotes have to be searched in the digestive tract using a combined approach and that culture must be kept as a key approach. As a single stool sample was used herein, results here reported constitute a baseline for further studies to assess eukaryotic diversity in healthy and diseased individuals from various geographical origins.
Materials and Methods

Fecal Sample Collection

One stool specimen was collected in a sterile plastic container from a 27-year-old Caucasian woman, who weighed 120 kg with a body mass index (BMI) of 48.9 and lived in Marseille, France. After collecting the stool sample, 1 g aliquots were preserved in sterile microtubes stored at -80°C until use. The patient provided her written consent to participate in the study, and the agreement of the local ethics committee of the IFR48 was obtained (agreement number 09-022, Marseille, France). The subject did not take antibiotic or antifungal treatments in the month prior to the stool collection, but we were not given information about her diet.

DNA Extraction

DNA was extracted using the Qiamp® stool mini kit (Qiagen, Courtaboeuf, France) as has been previously described [9]. Briefly, 200 mg of stool was placed in a 2-mL tube containing a 200 mg mixture of 0.1–0.5 mm glass beads and 1.5-mL of lysis buffer.

Table 4. Fungi cultured using different culture media.

| PCR ITS from cultured fungi          | % Coverage and % Identity | Media for culture          |
|---------------------------------------|---------------------------|----------------------------|
| Penicillium sp./P. camemberti         | 99 and 100                | PDA                        |
| Hypocre Augusta/Paracutis camemberti  | 99 and 98                 | PDA/CZAPEK                 |
| Penicillium brevicompactum            | 95 and 97                 | PDA/CZAPEK                 |
| Penicillium allii                     | 99 and 99                 | Dixon agar                 |
| Penicillium dipodomyicola             | 99 and 100                | Dixon agar                 |
| Aspergillus flavipes                  | 100 and 99                | CZAPEK                     |
| Aspergillus versicolor                | 100 and 99                | PDA                        |
| Beauveria bassiana                   | 99 and 99                 | PDA                        |
| Isaria farinosa                       | 97 and 98                 | CZAPEK                     |
| Galactomyces geotrichum               | 100 and 100               | Dixon agar                 |
| Malassezia globosa                    | 100 and 99                | Dixon agar                 |
| Malassezia restricta                  | 100 and 99                | Dixon agar                 |
| Malassezia pachydermatis              | 100 and 93                | Dixon agar                 |
| Candida tropicalis                    | 99 and 100                | Dixon agar/PDA             |
| Cladosporium sp.                      | 100 and 99                | Dixon agar                 |
| Climacocystis sp.                     | 98 and 96                 | Dixon agar                 |

Table 5. Fungi cultured using different culture media.

| Cultured fungi          | PCR cloning sequencing-detected fungi |
|-------------------------|---------------------------------------|
| Galactomyces geotrichum | Galactomyces geotrichum                |
| Malassezia globosa      | Malassezia globosa                    |
| Malassezia restricta    | Malassezia restricta                  |
| Malassezia pachydermatis| Malassezia pachydermatis              |
| Candida tropicalis      | Candida tropicalis                    |
| Cladosporium sp.        |                                       |
| Climacocystis sp.       |                                       |
| Penicillium sp./P. camemberti | P. camemberti       |
| Hypocre Augusta/Paracutis camemberti |                   |
| Penicillium brevicompactum |                                 |
| Penicillium allii       |                                       |
| Penicillium dipodomyicola |                                   |
| Aspergillus flavipes    |                                       |
| Aspergillus versicolor  |                                       |
| Beauveria bassiana      |                                       |
| Isaria farinosa         |                                       |

Uncultured Chytridiomycota
PCR Amplification

A total of 25 eukaryotic primer pairs for PCR were selected from the literature and used to amplify the 18S rRNA gene, internal transcribed spacer (ITS) and a chloroplast gene (Table 1). Each set of primers was blasted against corresponding taxa of each phylum (Table 1). All PCRs were performed using the 2720 thermal cycler (Applied Biosystems, Saint Aubin, France). A reaction made as described above. Purified PCR products were sequenced in both directions using the Big Dye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems, Villebon-sur-Yvette, France) with the M13 forward and M13 reverse primers. These products were run on an ABI PRISM 3130 automated sequencer (Applied Biosystems). Eukaryotes were identified by comparing our obtained sequences with the sequences in the GenBank database using BLAST. The sequence alignments were performed using the clustalw algorithm for multiple sequence alignments (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html). Phylogenetic trees were constructed using the Mega version 5 bootstrap kimura2-parameter model [45].

Fungi Culture and Identification

One gram of stool was diluted in 9 mL of sterile phosphate-buffered saline (PBS), and a six-fold serial dilution from 10⁻¹ to 10⁻⁶ was prepared in PBS. Each dilution was spread in duplicate on potato dextrose agar (PDA) (Sigma-Aldrich, Saint-Quentin Fallavier, France), Czapeck dox agar (Sigma-Aldrich) supplemented with chloramphenicol (0.05 g/l) and gentamycin (0.1 g/l), and Dixon agar [46] supplemented with chloramphenicol (0.05 mg/mL) and cycloheximide (0.2 mg/mL). Dixon agar medium was prepared by adding 1 L of distilled water to a mixture of 36 g of malt extract, 6 g of peptone, 20 g of ox bile, 10 mL of Tween 40, 2 mL of glycerol, 2 mL of oleic acid and 12 g of agar (Sigma-Aldrich). The mixture was heated to boiling to dissolve all components, autoclaved (20 min at 121°C) and cooled to approximately 50°C. Agar plates made from this media were placed in plastic bags with humid gas to prevent desiccation and incubated aerobically at room temperature (~25°C) in the dark. The Dixon Agar medium plates were incubated aerobically at 30°C. Growth was observed for two weeks. The solution used for dilution of the sample was spread on the same media and

Cloning and Sequencing

PCR products were cloned separately using the pGEM®-T Easy Vector System Kit (Promega, Lyon, France) as described by the manufacturer. The presence of the insert was confirmed by PCR amplification using M13 forward (5'-GTAAACGACGCTATGAC-3') and M13 reverse (5'-AGGAAACGCTATGAC-3') primers (Eurogenetec) and an annealing temperature of 38°C. PCRs were performed as described above. Purified PCR products were sequenced using the Big Dye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems, Villebon-sur-Yvette, France) with the M13 forward and M13 reverse primers. These products were run on an ABI PRISM 3130 automated sequencer (Applied Biosystems). Eukaryotes were identified by comparing our obtained sequences with the sequences in the GenBank database using BLAST. The sequence alignments were performed using the clustalw algorithm for multiple sequence alignments (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html). Phylogenetic trees were constructed using the Mega version 5 bootstrap kimura2-parameter model [45].
incubated in the same conditions as a negative control. DNA extracted from colonies as described above was amplified with the fungal primers ITS 1F/ITS 4R and MalF/Mal R. The purified PCR products were submitted to direct sequencing using the ITS1R/ITS4 and MalF/Mal R primers with the Big Dye Terminus kit V1, Cycle Sequencing Kit (Applied Biosystems) as described above. When the peaks of the sequence overlapped, the amplicons were cloned as described above.

All sequences superior to 200 base pairs are available in GenBank with reference number KC143536–KC143757.

Author Contributions
Conceived and designed the experiments: MD DR. Performed the experiments: NG. Analyzed the data: MD. Contributed reagents/materials/analysis tools: DR. Wrote the paper: NG MD DR.

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24. Conined and designed the experiments: MD DR. Performed the experiments: NG. Analyzed the data: MD. Contributed reagents/materials/analysis tools: DR. Wrote the paper: NG MD DR.

All sequences superior to 200 base pairs are available in GenBank with reference number KC143536–KC143757.

Author Contributions
Conceived and designed the experiments: MD DR. Performed the experiments: NG. Analyzed the data: MD. Contributed reagents/materials/analysis tools: DR. Wrote the paper: NG MD DR.

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