

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

Nina Gouba, Didier Raoult, Michel Drancourt*

Aix Marseille Université, URMITE, UMR63, CNRS 7278, IRD 198, Inserm 1095, Marseille, France

Abstract

Background: Few studies describing eukaryotic communities in the human gut microbiota have been published. The objective of this study was to investigate comprehensively the repertoire of plant and fungal species in the gut microbiota of an obese patient.

Methodology/Principal Findings: A stool specimen was collected from a 27-year-old Caucasian woman with a body mass index of 48.9 who was living in Marseille, France. Plant and fungal species were identified using a PCR-based method incorporating 25 primer pairs specific for each eukaryotic phylum and universal eukaryotic primers targeting 18S rRNA, internal transcribed spacer (ITS) and a chloroplast gene. The PCR products amplified using these primers were cloned and sequenced. Three different culture media were used to isolate fungi, and these cultured fungi were further identified by ITS sequencing. A total of 37 eukaryotic species were identified, including a Diatoms (*Blastocystis* sp.) species, 18 plant species from the Streptophyta phylum and 18 fungal species from the Ascomycota, Basidiomycota and Chytridiomycota phyla. Cultures yielded 16 fungal species, while PCR-sequencing identified 7 fungal species. Of these 7 species of fungi, 5 were also identified by culture. Twenty-one eukaryotic species were discovered for the first time in human gut microbiota, including 8 fungi (*Aspergillus flavipes*, *Beauveria bassiana*, *Isaria farinosa*, *Penicillium brevicompactum*, *Penicillium dipodomyicola*, *Penicillium camemberti*, *Climacocystis* sp. and *Malassezia restricta*). Many fungal species apparently originated from food, as did 11 plant species. However, four plant species (*Atractylodes japonica*, *Fibraurea tinctoria*, *Angelica anomala*, *Mitella nuda*) are used as medicinal plants.

Conclusions/Significance: Investigating the eukaryotic components of gut microbiota may help us to understand their role in human health.

Citation: Gouba N, Raoult D, Drancourt M (2013) Plant and Fungal Diversity in Gut Microbiota as Revealed by Molecular and Culture Investigations. PLoS ONE 8(3): e59474. doi:10.1371/journal.pone.0059474

Editor: Yolanda Sanz, Institute of Agrochemistry and Food Technology, Spain

Received: July 12, 2012; **Accepted:** February 18, 2013; **Published:** March 15, 2013

Copyright: © 2013 Gouba et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: michel.drancourt@univmed.fr

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

fungus 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* (JN903644.1); *Penicillium camemberti* (GQ458039.1), *Malassezia globosa* (AY743604.1), *Malassezia pachydermatis* (AB118940.1), *Malassezia restricta* (AY743607.1), uncultured *Chytridiomycota* (GQ995333.1) *Candida tropicalis* (DQ515959.1).

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 dipodomycicola*, *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 (*Hypocrea lixii*/*Penicillium 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.

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

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

Taxon	Primer	Target	PCR product size (bp)	Annealing temperature and number of cycles	Reference
<i>Amoeba</i>	AmiF1/AmiR9	18S rRNA	670	55°C 30 s 40cycles	[47]
<i>Acanthamoeba</i>	JDP1/JDP2	18S rRNA	460–470	60°C 60 s 40cycles	[48]
<i>Entamoeba</i>	JVF/DSRP2	18S rRNA	662–667	55°C 60 s 40cycles	[49]
<i>Hartmannella</i>	HV1227F/HV1728R	18S rRNA	502	56°C 30 s 40cycles	[50]
<i>Naegleria</i>	F/R	ITS	376–388	55°C 30 s 35cycles	[51]
<i>Ciliophora</i>	121F/1147R	18S	750–1000	55°C 60 s 30cycles	[52]
<i>Chlorophyta</i>	UCP1F/UCP1R	Rsp11-rpl2	384	54°C 60 s 35cycles	[53]
	UCP2F/UCP2R	Rsp11-rpl2	391	56°C 60 s 35cycles	
<i>Diatoms</i>	18S/28R	18S-28S rRNA	700–900	60°C 30 s 35cycles	[54]
<i>Dinoflagellate</i>	18ScomF1/Dino18SR1	18S rRNA	650	58°C 60 s 40cycles	[55]
<i>Diplomonads</i>	DimA/DimB	18S rRNA			[56]
<i>Euglenophyta</i>	EAF/EAF3	18S rRNA	1000	62°C 90 s 25cycles	[57]
<i>Kinetoplastidia</i>	Kinetokin1/kinetokin2	18S rRNA	600–650	56°C 60 s 35cycles	[58]
	KinSSUF1/KinSSUR1	18S rRNA	427–600	60°C 60 s 35cycles	[59]
<i>Microsporidia</i>	V1/PMP2	18S rRNA	250–279	55°C 30 s 35cycles	[60]
<i>Rhodophyta</i>	URP1_F/URP1_R	rps10-dnaK	464	52°C 60 s 35cycles	[53]
	URP2_F/URP2_R	rps10-dnaK	1772	52°C 60 s 35cycles	
<i>Trichomonads</i>	TFR1/TFR2	5.8S rRNA, ITS	338–391	60°C 30 s 35cycles	[61]
<i>Fungi</i>	MalF/MALR	26S	580	55°C 45 s 40cycles	[62]
<i>Fungi</i>	NS1/FR1	18S rRNA	1650	48°C 45 s 35cycles	[63]
	ITS1F/ITS4R	ITS	Variable	50°C 45 s 40cycles	[9]
<i>Fungi</i>	FunF/funR	18S	1000	52°C 30 s 40cycles	[12]
<i>Universal</i>	Euk1A/EUK516r	18S	560	50°C 30 s 35cycles	[9]
<i>eukaryote</i>	EUK528/1391R	18S	1000–1300	55°C 60 s 30cycles	[64]
<i>Plant</i>	rbCLZ1/rbCL19b	Chloroplast	157	40°C 30 s 40 cycles	[16]

doi:10.1371/journal.pone.0059474.t001

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	AmiF1/Ami9R	<i>Acanthamoeba castellanii</i>	Positive	100	99	<i>A.castellanii</i> (GU001160.1)
		<i>Hartmannella vermiformis</i>	Positive	100	99	<i>H. vermiformis</i> (DQ123623.2)
<i>Acanthamoeba</i>	JDP1/JDP2	<i>Acanthamoeba castellanii</i>	Positive	100	99	<i>A. castellanii</i> (GU001160.1)
<i>Entamoeba</i>	JVF/DSR2	NA				
<i>Hartmannella</i>	Hv1227F/Hv1728R	<i>Hartmannella vermiformis</i>	Positive	100	99	<i>H. vermiformis</i> (HM363627)
<i>Naegleria</i>	F/R	NA				
<i>Ciliophora</i>	121 F/1147R	<i>Colpoda steinii</i>	Positive	100	99	<i>C. steinii</i> (DQ388599.1)
<i>Chlorophyta</i>	UCP1F/UCP1R	<i>Chlorella vulgaris</i>	Positive	95	93	<i>C. vulgaris</i> (AB001684.1)
<i>Chlorophyta</i>	UCP2F/UCP2R	<i>Chlorella vulgaris</i>	Positive	95	93	<i>C. vulgaris</i> (AB001684.1)
<i>Diatoms</i>	18S/28R	NA				
<i>Dinoflagellates</i>	DinocomF1/Dino18SR1	<i>Poterioochromonas malhamensis</i>	Positive	100	98	<i>P. malhamensis</i> (FN662745.1)
<i>Diplomonads</i>	DimA/DimB	NA				
<i>Euglenophyta</i>	EAF/EAF3	NA				
<i>Kinetoplastidia</i>	Kinetokin1/kinetokin2	<i>Leshmania major</i>	Positive	99	99	<i>L. major</i> (FN677342.1)
<i>Kinetoplastidia</i>	KinSSUF1/KinSSUR1	<i>Leshmania major</i>	Positive	99	99	<i>L. major</i> (FN677342.1)
<i>Microsporidia</i>	V1/PMP2	<i>Encephalitozoon hellem</i>	Positive	100	99	<i>E. hellem</i> (AF039229.1)
<i>Rhodophyta</i>	URP1F/URP1R	NA				
<i>Rhodophyta</i>	URP2F/URP2R	NA				
<i>Trichomonads</i>	TFR1/TFR2	NA				
<i>Fungi</i>	MalF/MalR	<i>Malassezia restricta</i>	Positive	100	98	<i>M. restricta</i> (JN980105)
<i>Fungi</i>	ITS1F/ITS4R	<i>Candida albicans</i>	Positive	100	99	<i>C. albicans</i> (L28817.1)
<i>Fungi</i>	NSR1/FR1	<i>Candida albicans</i>	Positive	100	99	<i>C. albicans</i> (JN940588.1)
<i>Fungi</i>	FunF/FunR	<i>Candida albicans</i>	Positive	100	99	<i>C. albicans</i> (JN940588.1)
Universal Eukaryotes	euk528F/1391R	<i>Acanthamoeba castellanii</i>	Positive	98	99	<i>A. castellanii</i> (GU001160.1)
	Euk1A/Euk516r	<i>Acanthamoeba castellanii</i>	Positive	100	99	<i>A. castellanii</i> (GU001160.1)
Chloroplast Plant	rbcLZ1/rbcL19b	<i>Solanum</i> sp.	Positive	98	94	<i>S. physalifolium</i> (HQ23562)

doi:10.1371/journal.pone.0059474.t002

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. dipodomycicola*, *M. restricta*, *Climacocystis* sp. and *I. farisana*, 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 medium as Khatib [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 dietary sources. In particular, *G. geotrichum* and *P. canemberti* are used as starters for the production of many cheeses [24–25]. Accordingly, *G. geotrichum* has been commonly reported in human stool samples [9–12]. *P. brevicompactum*, which was also identified in our study, has been previously reported to be part of the oral microbiome in healthy individuals, but it has not been identified among the gut microbiota [26]. *P. brevicompactum* is frequently isolated from smoked dry-cured hams [27]. The *P. dipodomycicola* species that was identified in this study has also been reported in

food [28]. The *A. flavipes* and *P. allii* species are usually found 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 *Cladosporidium* 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. farisana* 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 *Climacocystis* 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 3. Sequencing results on PCR products from clones.

Primers	clones	Sequences of Species	Blast Identity% and coverage%	Kingdom
ITS1F/ITS4R	75	96% <i>Galactomyces geotrichum</i>	99 and 99	Fungi
		4% <i>Penicillium camemberti</i>	99 and 99	Fungi
MalF/MalR	57	28.07% <i>Malassezia pachydermatis</i>	92 and 100	Fungi
		17.54% <i>Malassezia restricta</i>	100 and 99	Fungi
		54.4% <i>Malassezia globosa</i>	99 and 99	Fungi
EUK1A/EUK516r	104	20.4% <i>Blastocystis</i> sp.	99 and 99	Protist
		0.96% <i>Uncultured Chytridiomycota</i>	95 and 99	Fungi
		0.96% <i>Fibraurea tinctoria</i>	98 and 100	Plant
		1.9% <i>Allium victorialis</i>	98 and 100	Plant
		3% <i>Nicotiana tabacum</i>	99 and 99	Plant
		0.96% <i>Helianthus annuus</i>	96 and 100	Plant
		0.96% <i>Caprifoliaceae environmental</i>	98 and 99	Plant
		0.96% <i>Petrophile canescens</i>	98 and 99	Plant
		60% <i>Solanum lycopersicum</i>	99 and 99	Plant
		5% <i>Humulus lupulus</i>	98 and 100	Plant
		3% <i>Cicer arietinum</i>	99 and 98	Plant
		0.96% <i>Pinus wallichiana</i>	100 and 98	Plant
		0.96% <i>Mitella nuda</i>	100 and 98	Plant
JVF/DSPR2	141	94.32% <i>Galactomyces geotrichum</i>	98 and 99	Fungi
		0.71% <i>Candida tropicalis</i>	98 and 99	Fungi
		0.71% <i>Citrus aurantium</i>	99 and 100	plant
		4.25% <i>Atractylodes Japonica</i>	98 and 99	Plant
		0.71% <i>Pinus wallichiana</i>	99 and 100	Plant
		78% <i>Nicotiana undulate</i>	98 and 99	Plant
rbcLZ1/rbcL19b	31	3% <i>Musa acuminata/Ensete ventricosum</i>	99 and 99	Plant
		6.25% <i>Lactuca sativa</i>	99 and 99	Plant
		3% <i>Solanum tuberosum</i>	100 and 99	Plant
		3% <i>Brassica napus/Arabidopsis lyrata</i>	100 and 99	Plant
		6.25% <i>Angelica anomala/Davidia involucrata/Aucuba japonica</i>	100 and 99	Plant

doi:10.1371/journal.pone.0059474.t003

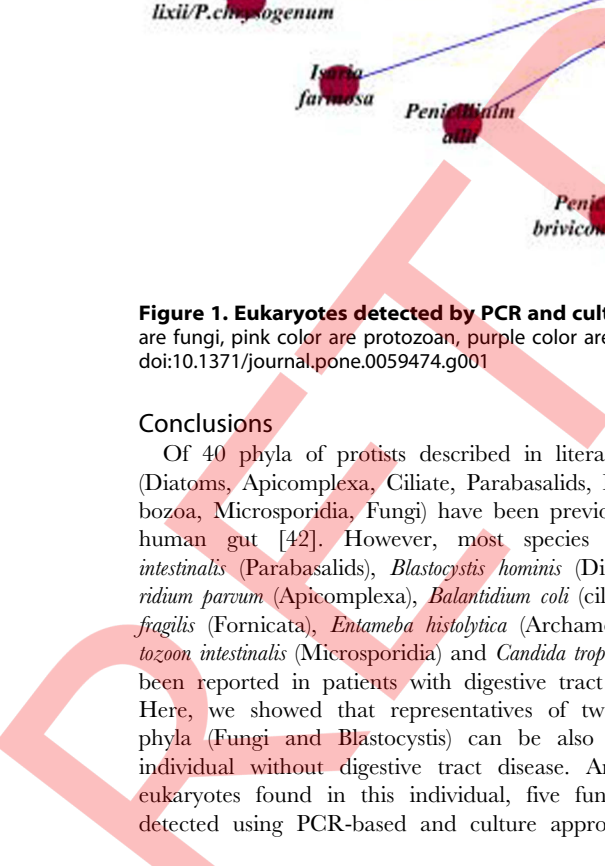
volunteers [38]. However, *M. pachydermatis* and *M. globosa* were previously found in stool from healthy and ill subjects [12,13] by culture-independent methods. We report for the first time the detection of *M. restricta* in stool by molecular methods. The *Malassezia* species that were detected by 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 *Nicotiana undulate* 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 *Blastocystis* sp. that we detected is commonly found in healthy microbiota [9,10] and is associated with irritable bowel syndrome.



Conclusions

PLOS ONE | www.plosone.org

5

Table 4. Fungi cultured using different culture media.

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

doi:10.1371/journal.pone.0059474.t004

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 Qiaamp® 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 5. Cultured fungi compared to fungi detected by PCR and sequencing.

Cultured fungi	PCR cloning sequencing-detected fungi
<i>Galactomyces geotrichum</i>	<i>Galactomyces geotrichum</i>
<i>Malassezia globosa</i>	<i>Malassezia globosa</i>
<i>Malassezia restricta</i>	<i>Malassezia restricta</i>
<i>Malassezia pachydermatis</i>	<i>Malassezia pachydermatis</i>
<i>Candida tropicalis</i>	<i>Candida tropicalis</i>
<i>Cladosporium</i> sp.	
<i>Climacocystis</i> sp.	
<i>Penicillium</i> sp./ <i>P. camemberti</i>	<i>P. camemberti</i>
<i>Hypocrea lixii</i> / <i>Penicillium chrysogenum</i>	
<i>Penicillium brevicompactum</i>	
<i>Penicillium allii</i>	
<i>Penicillium dipodomyicola</i>	
<i>Aspergillus flavipes</i>	
<i>Aspergillus versicolor</i>	
<i>Beauveria bassiana</i>	
<i>Isaria farinosa</i>	
	Uncultured Chytridiomycota

doi:10.1371/journal.pone.0059474.t005

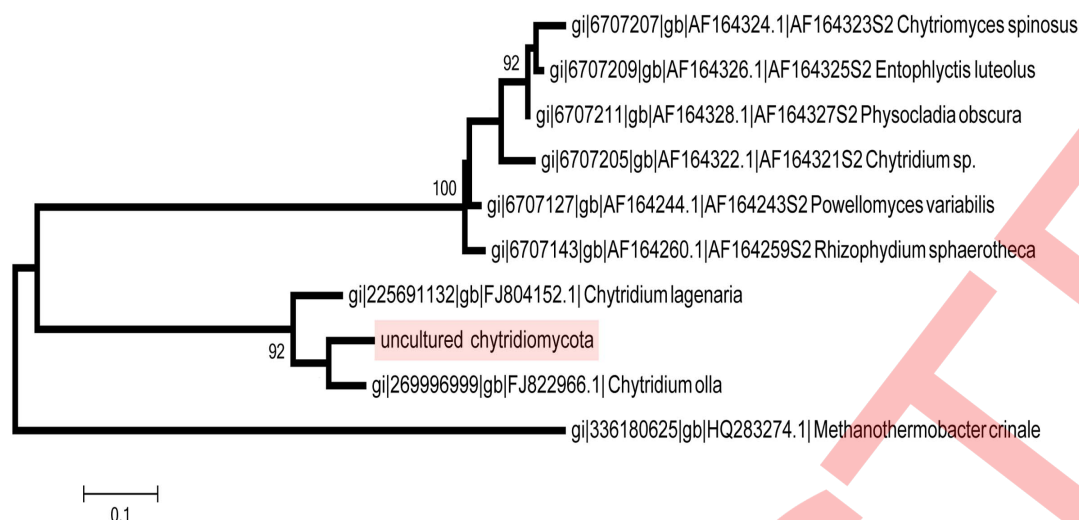


Figure 2. Phylogenetic tree of 18S rRNA gene sequences of uncultured Chytricomycota.
doi:10.1371/journal.pone.0059474.g002

(ASL) (Qiagen). Mechanical lysis was performed by bead-beating the mixture using a FastPrep BIO 101 apparatus (Qiagen, Strasbourg, France) at level 4.5 (full speed) for 90 s. A minor modification was made to the manufacturer's procedure by increasing the proteinase K incubation time to 2 h at 70°C. For all DNA extractions, 200 µL of distilled water was used as a negative control. The extracted DNA was stored at -20°C until use.

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 in nucleotide BLAST program from the National Center for Biotechnology Information (NCBI) to test its ability to amplify the corresponding phylum. The sets of primers were selected on the basis of a 100% coverage and a 100% identity shown by at least one of the primer from a set. Primers which yielded negative PCR were tested using positive controls specific for each phylum (Table 2). For each eukaryotic primer pair, the 50 µL PCR reaction mixture contained 5 µL of dNTPs (2 mM of each nucleotide), 5 µL of DNA polymerase buffer (Qiagen) 2 µL of MgCl₂ (25 mM), 0.25 µL HotStarTaq DNA polymerase (1.25 U) (Qiagen), 1 µL of each primer (Eurogentec, Liège, Belgium) and 5 µL of DNA. PCR was performed with a 15 min initial denaturation at 95°C followed by cycles of 95°C for 30 sec. The initial extension was performed at 72°C for 1 min, and the 5 min final extension was performed at 72°C. The annealing temperature and the number of cycles used for each primer are presented in Table 1. All PCRs were performed using the 2720 thermal cycler (Applied Biosystems, Saint Aubin, France). A reaction made up of buffer without DNA was used as a negative control for each PCR run. Amplified products were visualized with ethidium bromide staining after electrophoresis using a 1.5% agarose gel. The PCR products were purified using the Nucleo-Fast® 96 PCR Kit (Marcherey-Nagel, Hoerd, France) according to the manufacturer's instructions. To test for potential PCR inhibitors, 1 µL of *A. castellanii* was added to 4 µL of stool DNA prior to PCR amplification.

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'-GTAAACGACGGC-CAG-3') and M13 reverse (5'-AGGAAACAGCTATGAC-3') primers (Eurogentec) and an annealing temperature of 58°C. PCRs were performed 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

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® Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems) as described above. When the peaks of the sequence overlapped, the amplicons were cloned as described above.

References

- Tilg H, Moschen AR, Kaser A (2009) Obesity and the microbiota. *Gastroenterology* 136: 1476–1483.
- Backhed F (2011) Programming of host metabolism by the gut microbiota. *Ann Nutr Metab* 58 Suppl 2: 44–52.
- Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI (2005) Host-bacterial mutualism in the human intestine. *Science* 307: 1915–1920.
- Tsai F, Coyle WJ (2009) The microbiome and obesity: is obesity linked to our gut flora? *Curr Gastroenterol Rep* 11: 307–313.
- Ley RE (2010) Obesity and the human microbiome. *Curr Opin Gastroenterol* 26: 5–11.
- DiBaise JK, Zhang H, Crowell MD, Krajmalnik-Brown R, Decker GA, et al. (2008) Gut microbiota and its possible relationship with obesity. *Mayo Clin Proc* 83: 460–469.
- Million M, Maraninchi M, Henry M, Armougom F, Richet H, et al. (2011) Obesity-associated gut microbiota is enriched in *Lactobacillus reuteri* and depleted in *Bifidobacterium animalis* and *Methanobrevibacter smithii*. *Int J Obes Lond* 36: 817–825.
- Rajilic-Stojanovic M, Smidt H, de Vos WM (2007) Diversity of the human gastrointestinal tract microbiota revisited. *Environ Microbiol* 9: 2125–2136.
- Scanlan PD, Marchesi JR (2008) Micro-eukaryotic diversity of the human distal gut microbiota: qualitative assessment using culture-dependent and -independent analysis of faeces. *ISME J* 2: 1183–1193.
- Nam YD, Chang HW, Kim KH, Roh SW, Kim MS, et al. (2008) Bacterial, archaeal, and eukaryal diversity in the intestines of Korean people. *J Microbiol* 46: 491–501.
- Ott SJ, Kuhbacher T, Musfeldt M, Rosenstiel P, Hellmig S, et al. (2008) Fungi and inflammatory bowel diseases: Alterations of composition and diversity. *Scand J Gastroenterol* 43: 831–841.
- Chen Y, Chen Z, Guo R, Chen N, Lu H, et al. (2011) Correlation between gastrointestinal fungi and varying degrees of chronic hepatitis B virus infection. *Diagn Microbiol Infect Dis* 70: 492–498.
- Li Q, Wang C, Zhang Q, Tang C, Li N, et al. (2012) Use of 18S ribosomal DNA polymerase chain reaction-denaturing gradient gel electrophoresis to study composition of fungal community in 2 patients with intestinal transplants. *Hum Pathol* 43: 1273–1281.
- Fredricks DN, Smith C, Meier A (2005) Comparison of Six DNA Extraction Methods for Recovery of Fungal DNA as Assessed by Quantitative PCR. *J Clin Microbiol* 43(10): 5122–5128.
- Griffiths LJ, Anyim M, Doffman SR, Wilks M, Millar MR, et al. (2006) Comparison of DNA extraction methods for *Aspergillus fumigatus* using real-time PCR. *J Med Microbiol* 55 (9): 1187–1191.
- Poinar HN, Kuch M, Sobolik KD, Barnes I, Stankiewicz AB, et al. (2001) A molecular analysis of dietary diversity for three archaic Native Americans. *Proc Natl Acad Sci U S A* 98: 4317–4322.
- Stensvold CR, Lebbad M, Verweij JJ (2011) The impact of genetic diversity in protozoa on molecular diagnostics. *Trends Parasitol* 27: 53–58.
- Slapeta J, Moreira D, Lopez-Garcia P (2005) The extent of protist diversity: insights from molecular ecology of freshwater eukaryotes. *Proc Biol Sci* 272: 2073–2081.
- Pounder JL, Simmon KE, Barton CA, Hohmann SL, Brandt ME, et al. (2007) Discovering potential pathogens among fungi identified as nonsporulating molds. *J Clin Microbiol* 45: 568–571.
- Atkins SD, Clark IM (2004) Fungal molecular diagnostics: a mini review. *J Appl Genet* 45: 3–15. 178.
- Dong W, Liu J, Yu J, Wang L, Zhou S (2012) Highly variable chloroplast markers for evaluating plant phylogeny at low taxonomic levels and for DNA barcoding. *PLoS One* 7(4): e35071.
- Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol Biol* 17: 1105–1109.
- Khatib R, Riederer KM, Ramanathan J, Baran J Jr. (2001) Faecal fungal flora in healthy volunteers and inpatients. *Mycoses* 44: 151–156.
- Georges S, Mounier J, Rea MC, Gelsomino R, Heise V, et al. (2008) Commercial ripening starter microorganisms inoculated into cheese milk do not successfully establish themselves in the resident microbial ripening consortia of a South German red smear cheese. *Appl Environ Microbiol* 74: 2210–2217.
- Giraud F, Giraud T, Aguilera G, Fournier E, Samson R, et al. (2010) Microsatellite loci to recognize species for the cheese starter and contaminating strains associated with cheese manufacturing. *Int J Food Microbiol* 137: 204–213.
- Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, et al. (2010) Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathog* 6: e1000713.
- Asafa DT, Gjerde RO, Sidhu MS, Langsrud S, Kure CF, et al. (2009) Moulds contaminants on Norwegian dry-cured meat products. *Int J Food Microbiol* 128: 435–439.
- Rodriguez A, Rodriguez M, Andrade MJ, Cordoba JJ (2012) Development of a multiplex real-time PCR to quantify aflatoxin, ochratoxin A and patulin producing molds in foods. *Int J Food Microbiol* 155: 10–18.
- Klich MA (2009) Health effects of *Aspergillus* in food and air. *Toxicol Ind Health* 25: 657–667.
- Saleh A Kabli (2007) Studies on Fungal Communities Associated with Litter of Plant Cover at Al-Taif Province, Saudi Arabia. *Met env and Arid Land Agric, Sci* 18: 87–98.
- Valdez JG, Makuch MA, Ordovini AF, Frisvad JC, Overy DP, et al. (2009) Identification, pathogenicity and distribution of *Penicillium* spp. isolated from garlic in two regions in Argentina. *Plant pathology* 58: 352–361.
- Inoue Y, Matsuaki Y, Shin SH, Ponikau JU, Kita H (2005) Nonpathogenic, environmental fungi induce activation and degranulation of human eosinophils. *J Immunol* 175: 5439–5447.
- Sonjak S, Lilen M, Frisvad JC, Gunde-Cimerman N (2011) The mycobiota of three dry-cured meat products from Slovenia. *Food Microbiol* 28: 373–376.
- Bragulat MR, Abarca ML, Cabanes FJ (2008) Low occurrence of patulin- and citrinin-producing species isolated from grapes. *Letters in Applied Microbiology* 47: 286–289.
- Westwood GS, Huang SW, Keyhani NO (2005) Allergens of the entomopathogenic fungus *Beauveria bassiana*. *Clin Mol Allergy* 3: 1.
- Ann Chai LY, Denning DW, Warn P (2010) *Candida tropicalis* in human disease. *Crit Rev Microbiol* 36: 282–298.
- Yim SM, Kim JY, Ko JH, Lee YW, Choe YB, et al. (2010) Molecular analysis of *Malassezia* microflora on the skin of the patients with atopic dermatitis. *Ann Dermatol* 22: 41–47.
- Lee YW, Byun HJ, Kim BJ, Kim DH, Lim YY, et al. (2011) Distribution of *Malassezia* species on the scalp in Korean seborrheic dermatitis patients. *Ann Dermatol* 23: 156–161.
- Abdullahi I, Koerber M, Stachewicz H, Winter S (2005) The 18S rDNA sequence of *Synchytrium endobioticum* and its utility in microarrays for the simultaneous detection of fungal and viral pathogens of potato. *Appl Microbiol Biotechnol* 68: 368–375.
- Pandey PK, Siddharth J, Verma P, Bavdekar A, Patole MS, et al. (2012). Molecular typing of fecal eukaryotic microbiota of human infants and their respective mothers. *J Biosci* 37: 221–226.
- Hong MH, Kim JH, Bae H, Lee NY, Shin YC, et al. (2010) *Atractylodes japonica* inhibits the production of proinflammatory cytokines through inhibition of the NF-kappaB/IkappaB signal pathway in HMC-1 human mast cells. *Arch Pharm Res* 33: 843–851.
- Parfrey LW, Walters WA, Knight R (2011) Microbial eukaryotes in the human microbiome: ecology, evolution, and future directions. *Front Microbiol* 2: 153.
- Wright SG (2012) Protozoan infections of the gastrointestinal tract. *Infect Dis Clin North Am* 26 (2): 2323–2339.
- Kullberg BJ, Oude Lashof AM (2002) Epidemiology of opportunistic invasive mycoses. *Eur J Med Res* 7(5): 183–191.
- Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16: 111–120.
- Leeming JP, Notman FH (1987) Improved methods for isolation and enumeration of *Malassezia furfur* from human skin. *J Clin Microbiol* 25: 2017–2019.
- Thomas V, Herrera-Rimann K, Blanc DS, Greub G (2006) Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. *Appl Environ Microbiol* 72: 2428–2438.
- Schroeder JM, Booton GC, Hay J, Niszl IA, Seal DV, et al. (2001) Use of subgenomic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* from humans with keratitis and from sewage sludge. *J Clin Microbiol* 39: 1903–1911.
- Santos HL, Bandea R, Martins LA, de Macedo HW, Peralta RH, et al. (2010) Differential identification of *Entamoeba* spp. based on the analysis of 18S rRNA. *Parasitol Res* 106: 883–888.

50. Kuiper MW, Valster RM, Wullings BA, Boonstra H, Smidt H, et al. (2006) Quantitative detection of the free-living amoeba *Hartmannella vermiformis* in surface water by using real-time PCR. *Appl Environ Microbiol* 72: 5750–5756.
51. Pelandakis M, Pernin P (2002) Use of multiplex PCR and PCR restriction enzyme analysis for detection and exploration of the variability in the free-living *Amoeba*, *Naegleria* in the environment. *Appl Environ Microbiol* 68: 2061–2065.
52. Dopheide A, Lear G, Stott R, Lewis G (2008) Molecular characterization of ciliate diversity in stream biofilms. *Appl Environ Microbiol* 74: 1740–1747.
53. Provan J, Murphy S, Maggs CA (2004) Universal plastid primers for *Chlorophyta* and *Rhodophyta*. *Eur J Phycol* 39: 43–50.
54. Senapin S, Phiwsaiya K, Kiatmetha P, Withyachumnarnkul B (2010) Development of primers and a procedure for specific identification of the diatom *Thalassiosira weissflogii*. *Aquacult Int* 19: 693–704.
55. Lin S, Zhang H, Hou Y, Miranda L, Bhattacharya D (2006) Development of a dinoflagellate-oriented PCR primer set leads to detection of picoplanktonic dinoflagellates from Long Island Sound. *Appl Environ Microbiol* 72: 5626–5630.
56. Kolisko M, Silberman JD, Cepicka I, Yubuki N, Takishita K, et al. (2010) A wide diversity of previously undetected free-living relatives of diplomonads isolated from marine/saline habitats. *Environ Microbiol* 12: 2700–2710.
57. Mullner AN, Angeler DG, Samuel R, Linton EW, Triemer RE (2001) Phylogenetic analysis of phagotrophic, photomorph and osmotrophic euglenoids by using the nuclear 18S rDNA sequence. *Int J Syst Evol Microbiol* 51: 783–791.
58. Desquesnes M, McLaughlin G, Zougrana A, Davila AM (2001) Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. *Int J Parasitol* 31: 610–614.
59. Callahan HA, Litaker RW, Noga EJ (2002) Molecular taxonomy of the suborder *Bodonina* (Order *Kinetoplastida*), including the important fish parasite, *Ichthyobodo necator*. *J Eukaryot Microbiol* 49: 119–128.
60. Chabchoub N, Abdelmalek R, Mellouli F, Kanoun F, Thellier M, et al. (2009) Genetic identification of intestinal microsporidia species in immunocompromised patients in Tunisia. *Am J Trop Med Hyg* 80: 24–27.
61. Hayes DC, Anderson RR, Walker RL (2003) Identification of *Trichomonadid protozoa* from the bovine preputial cavity by polymerase chain reaction and restriction fragment length polymorphism typing. *J Vet Diagn Invest* 15: 390–394.
62. Mirhendi H, Makimura K, Zomorodian K, Yamada T, Sugita T, et al. (2005) A simple PCR-RFLP method for identification and differentiation of 11 *Malassezia* species. *J Microbiol Methods* 61: 281–284.
63. White T, Burns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide and applications*. New York: Academic Press, Harcourt Brace Jovanovich. 315–322.
64. Stoeck T, Hayward B, Taylor GT, Varela R, Epstein SS (2006) A multiple PCR-primer approach to access the microeukaryotic diversity in environmental samples. *Protist* 157: 31–43.