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Pathogenic Eukaryotes in Gut Microbiota of Western Lowland Gorillas as Revealed by Molecular Survey

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Although gorillas regarded as the largest extant species of primates and have a close phylogenetic relationship with humans, eukaryotic communities have not been previously studied in these populations. Herein, 35 eukaryotic primer sets targeting the 18S rRNA gene, internal transcribed spacer gene and other specific genes were used firstly to explore the eukaryotes in a fecal sample from a wild western lowland gorilla (*Gorilla gorilla gorilla*). Then specific real-time PCRs were achieved in additional 48 fecal samples from 21 individual gorillas to investigate the presence of human eukaryotic pathogens. In total, 1,572 clones were obtained and sequenced from the 15 cloning libraries, resulting in the retrieval of 87 eukaryotic species, including 52 fungi, 10 protozoa, 4 nematodes and 21 plant species, of which 52, 5, 2 and 21 species, respectively, have never before been described in gorillas. We also reported the occurrence of pathogenic fungi and parasites (i.e. *Oesophagostomum bifurcum* (86%), *Necator americanus* (43%), *Candida tropicalis* (81%) and other pathogenic fungi were identified). In conclusion, molecular techniques using multiple primer sets may offer an effective tool to study complex eukaryotic communities and to identify potential pathogens in the gastrointestinal tracts of primates.

The microbial communities residing in the gastrointestinal tracts of primates are complex and may play important roles in health and disease. The interactions between microbial cells and primate host cells could be either commensal or parasitic, and it is known that these interactions have an effect on the metabolic, developmental, and immunological status of the host[1,2]. The compositions and constituents of these communities are influenced by several factors, such as the host diet, geography, physiology, and disease state[3,4].

Gorillas share a close phylogenetic relationship with humans, resulting in a high potential for pathogen exchange involving bacteria, viruses and gastrointestinal parasites[5–7]. Moreover, the presence of these pathogens in wild primates may also have negative consequences for public health and wildlife conservation management[8]. To date, there have been no studies examining the entire eukaryotic community residing in the intestinal tract of the gorilla; rather, most studies focus on the parasitological aspects of these eukaryotic communities using coprological studies to survey the presence of intestinal parasites in wild gorilla populations. Several such studies have been conducted in both mountain gorillas (*Gorilla gorilla beringei*) and western lowland gorillas (*Gorilla gorilla gorilla*) in different geographical locations[9–13]. The intestinal microbiota appears to be the same among social groups and individual gorillas living in the Bwindi Impenetrable Forest in Uganda and that the flora is largely dominated by entodiniomorph ciliates and helminths, while amoebae and flagellates appear to be absent[9,10]. The coprological studies involving western gorillas also reported many species of parasites, particularly entodiniomorph ciliates and strongylates[11]. Two studies examined fecal samples from western lowland gorillas living in the Dzanga-Ndoki National Park and at Bai Hokou, Central African Republic, and found that most of individuals were infected with strongylates, whereas ascaroids and threadworms were only moderately present[12,13]. Low prevalence of *Entamoeba coli*, *Balantidium coli*, and *Iodamoeba butschlii* have also been recorded in western lowland gorillas along with trichomonads, which were the only protozoans that were present in all gorilla age-sex classes[12,13].

Despite these studies, the diversity of eukaryotic communities in primates, and particularly in gorillas, remains to be elucidated, particularly with regard to intestinal fungal diversity. In addition, the morphological descriptions of these eukaryotes are typically insufficient and thus cannot be considered in taxonomical studies. In this study, we present firstly, an extensive molecular data set of the occurrence of gastrointestinal eukaryotic microbiota including some human eukaryotic pathogens in a single fecal sample from a wild western lowland gorilla from

Cameroon, and then followed by molecular detection of potential human eukaryotic pathogen in gastrointestinal tracts of wild population of gorillas.

Results

Eukaryotes retrieved from gut microbiota of gorilla. In total, 35 existing primer sets targeting the 18S rRNA and internal transcribed spacer (ITS) genes and other specific eukaryotic genes were used to explore the diversity of the eukaryotes that were found in a fecal sample that was obtained from a wild gorilla in Cameroon (Supplementary Tables 1). Seventeen positive PCR products were obtained. Two of them (TFR1/TFR2 and 18ScomF1/Dino18SR1) were sequenced directly as obtained sequences from these two primers yield no trouble sequences, but the remaining were problematic and were thus cloned. Overall, 1,572 clones were obtained from all of the cloning libraries that were constructed in this study (Supplementary Tables 2), resulting in the detection of 87 eukaryotic species in the fecal sample (Fig. 1). Most of the species that were present were fungi (52 species, Table 1), while 10 were identified as protozoa (Table 2), 4 as nematodes (Table 3), and 21 as plants (Table 4).

Fungal diversity. Fifty-two different species of fungi were retrieved from the gorilla fecal sample following the analysis of 428 fungal clones (accounting for 59.8% of the total species detected and 27.2% of the total clones retrieved in this study). These species were detected from different cloning libraries that were generated using various primers (Table 1, Fig. 1). Most of the detected taxa belonged to the phylum Ascomycota (36 species, 69.2% of the identified taxa), followed by taxa from the Basidiomycota (15 species, 28.8% of the detected taxa). The remaining 2% of the taxa were affiliated with the phylum Zygomycota (Table 1). Only 5 fungal species were isolated through culture-dependent methods, including *Alternaria alternata*, *Cladosporium* sp., *Malassezia restricta*, *Malassezia globosa,* and *Malassezia pachydermatis*.

All of the 171 sequenced clones from the fungal ITS clone library that were generated using the ITS1-F/ITS-4R primer set were related to fungal sequences that were found in the GenBank databases with the exception of one clone belonging to the Viridiplantae (green plants) (Tables 1 and 4, Fig. 1). This amplification alone allowed for the identification of 25 different fungal species (roughly half of the fungal species detected and 28.7% of all eukaryotic species recovered in this study) (Fig. 1). The majority of the fungal sequences in this clone library were of ascomycete origin (91.2% of the total number of clones and 80.8% of the species detected); the species were assigned to different taxonomic groups, including Saccharomycetales, Pleosporales, Capnodiales, Eurotiales, Hypocreales, and Xylariales (Table 1). Only 6.4% of the sequences that were retrieved from this ITS library were related to the basidiomycetes, belonging to both Tremellales and Corticiales (Table 1). Our ITS results also showed the presence of a few zygomycetes-related sequences that were represented by *Mortierella* sp. (Table 1).

The primer set NSI/FRI, targeting the fungal 18S rRNA gene, was also used in this study, resulting in a total of 17 fungal species (16 plus one species that was retrieved from fungal ITS amplification) (Table 1, Fig. 1). In contrast to the ITS clone library, the BLAST results for the 96 clones that were obtained from this library revealed that 58 sequences (60.4%) were most closely related to the basidiomycetes, whereas 38 sequences (39.6%) were ascomycetes (Table 1).

In addition to the 41 fungal species that were recovered by the aforementioned fungal-specific primer sets, 11 were identified in other clone libraries when universal eukaryotic primers targeting 18S rDNA were used (Table 1, Fig. 1). Among these 11 species, 8 were detected using the universal eukaryotic primer set Euk1A/Euk516r.

The use of different specific and universal 18S clone libraries revealed a total of 30 fungal species in the stool sample (Fig. 1), while PCR methods using fungal ITS genes enabled the detection of 25 species (Fig. 1). Only 3 species, *A. alternata*, *Candida rugosa* and *Hanseniaspora occidentalis*, were detected in both the ITS and 18S amplifications, thus suggesting the complementarity of these approaches for the assessment of fungal communities in the gorilla intestinal tract.

Protozoal diversity. Ten different species of protozoans were detected in the gorilla fecal sample following both the sequencing of 246 protozoal-related clones (15.6% of all clones in this study) that were obtained from 8 different cloning libraries and the direct sequencing of the positive PCR product that were obtained with the TFR1/TFR2 primer set (Table 2, Fig. 1). Six species belonging to Ciliophora were detected from two of the cloning libraries, including *Blepharocorys curvigula*, *Cycloposthium bipalmatum*, *Cycloposthium ishikawai*, *Parentodinium* sp., *Triplumaria selenica* and *Troglodytella abrassarti* (Table 2, Fig. 1). Ninety-six sequences amplified by *Leishmania* specific primers were assigned to Trypanosomatidae family (Fig. 1, Table 2) as the best hit of BLAST results of these sequences was *Leishmania* sp. (with sequence coverage ranged between 69–79%). The remaining protozoans were identified as *Blastocystis* sp., *Iodamoeba* sp., and *Tetratrichomonas buttreyi*, belonging to the three taxa Stramenopiles, Amoebozoa and Parabasalia, respectively (Table 2, Fig. 1).

Helminthic diversity. Despite the use of several primers to detect the occurrences of trematodes, cestodes and nematodes in the gorilla fecal sample (Supplementary Tables 1), only the PCR amplifications using the latter primer pairs (NC1/NC2) yielded positive results; these PCR products were used to construct the nematode clone libraries (Table 3). Four nematode species, *Necator americanus*, *Libyostrongylus douglassi*, *Oesophagostomum* sp., and *Oesophagostomum stephanostomum,* were obtained from sequencing 192 positive clones in this library (Table 3, Fig. 1). The human parasitic nematode, *N. americanus*, accounted for 55.7% of this clone library (Table 3).

Plant diversity. Twenty-one plant species (Table 4) were retrieved in this study from 13 different cloning libraries that were generated using primer pairs targeting the 18S rRNA, ITS and chloroplast genes (Supplementary Tables 2). The plant-related clones constituted roughly 44.9% of all clones that were sequenced in this study. The majority of plant sequences that were detected in most of the cloning libraries belonged to *Manilkara zapota* and *Musa basjoo*, which comprised 28.6% and 16.7% of the total plant-related sequences, respectively (Table 4, Fig. 1). Only two plant species, *Schima superba* and *Davidia involucrata*, were detected from the chloroplast clone libraries that were constructed using the primer set rbcLZ1/rbcL19b; the 19 remaining plant species were retrieved from the 18S rRNA cloning libraries that were constructed from various 18S rRNA primer pairs (Table 4, Fig. 1).

Eukaryotic human pathogens in fecal samples of gorillas. Real time PCR examination (Supplementary Table 3) of 48 fecal samples from 21 individual of wild gorillas, sampled from Minton and Messok location in Cameroon, showed that 41 (18 gorillas, 86%) and 11 (9 gorillas, 43%) of gorillas' fecal samples harbor *Oesophagostomum bifurcum* and *N. americanus* respectively (Table 5). The results also showed that human pathogenic fungi such as *Candida tropicalis, Candida parapsilosis, C. rugosa, M. restricta, M. globosa*, *Trichosporon* spp. and *Trichosporon asahii* were also detected in feces of these wild animals as shown in Table 5. However, *Blastocystis sp.*, *Candida albicans*, *M. pachydermatis* and *Rhodotorula mucilaginosa* were not detected in any gorillas' feces.

Figure 1 | The eukaryotic species that were retrieved from the gut of *Gorilla gorilla gorilla* according to the different primers used. A box indicates that the species was positive with the primer set used. Blue color = fungi; red color = protozoa; orange color = helminths; and green color = plants. The trees were constructed using the free software MEGA 5 and sequences that were retrieved from GenBank (ITS sequences for fungi and helminths and 18S rDNA for protozoa and plants).

Discussion

Previous studies focusing on the detection and identification of eukaryotic communities residing in the non-human primate gastrointestinal tract analyzed the gut microbiota using the morphological features of the flora[9–13], limiting the acquisition of knowledge regarding the real eukaryotic intestinal contents of primates. Regardless of some limitations that are associated with culture-independent methods[14], these methods have recently been used to explore the eukaryotic diversity of the human gut[15–20], and these techniques have played a crucial role in providing novel insight into the true diversity and composition of the gut microbiota. Because very little is known about both the diversity of eukaryotic organism occurring in the digestive tract of non-human primates and the presence of potential pathogens in their guts, two general approaches were used in our investigation; firstly, culture dependent and independent methods were carried out in a single stool sample from gorilla then additional

Table 1 | Fungal species obtained from different cloning libraries in this study

Closest relative in NCBI	Similarity%	Taxa	Taxonomic group	Targeted gene	Primer (No. of clones/total)
Alternaria alternata	99	a	Pleosporales	18s rRNA, ITS	NSI/FRI (1/96); Euk1A/Euk 516r (20/126); ITS F/ ITS-4R (15/171)
Alternaria raphani	99	a	Pleosporales	18s rRNA	NSI/FRI (3/96)
Aspergillus sp.	99	a	Eurotiales	ITS	ITS F/ITS-4R (2/171)
Aspergillus versicolor	99	a	Eurotiales	ITS	ITS F/ITS-4R (4/171)
Bionectria setosa	98	a	Hypocreales	ITS	ITS F/ITS-4R (2/171)
Boeremia exigua	99	a	Pleosporales	18s rRNA	NSI/FRI (2/96)
Candida entomophila	99	a	Saccharomycetales	18s rRNA	Euk1A/Euk 516r (1/126)
Candida orthopsilosis	99	a	Saccharomycetales	ITS	ITS F/ITS-4R (94/171)
Candida quercitrusa	99	a	Saccharomycetales	ITS	ITS F/ITS-4R (4/171)
Candida rugosa	99	a	Saccharomycetales	18s rRNA, ITS	NSI/FRI (7/96); Euk1A/Euk 516r (28/126); E528F/ Univ1492RE (17/96); ITS F/ITS-4R (13/171)
Candida tropicalis	99	a	Saccharomycetales	ITS	ITS F/ITS-4R (6/171)
Ceriporiopsis pannocincta	99	b	Polyporales	18s rRNA	Euk1A/Euk 516r (1/126)
Cladosporium sp.	99	a	Capnodiales	18s rRNA	Euk1A/Euk 516r (1/126)
Climacodon septentrionalis	99	b	Polyporales	18s rRNA	NSI/FRI (2/96)
Cochliobolus kusanoi	99	a	Pleosporales	18s rRNA	E528F/Univ1492RE (2/96)
Cryptococcus terricola	99	b	Tremellales	18s rRNA	NSI/FRI (7/96)
Cylindrocladium sp.	99	a	Hypocreales	ITS	ITS F/ITS-4R (4/171)
Davidiella tassiana	99	a	Capnodiales	ITS	ITS F/ITS-4R (6/171)
Devriesia strelitziicola	99	a	Capnodiales	ITS	ITS F/ITS-4R (3/171)
Eupenicillium crustaceum	99	a	Eurotiales	18s rRNA	121F/1147R (2/96)
Filobasidium elegans	98	b	Filobasidiales	18s rRNA	NSI/FRI (4/96)
Filobasidium globisporum	99	b	Filobasidiales	18s rRNA	NSI/FRI (5/96)
Galactomyces geotrichum	99	a	Saccharomycetales	18s rRNA	NSI/FRI (5/96); Euk1A/Euk 516r (3/126); 121F/ 1147R (1/96); E528F/Univ1492RE (27/96)
Hanseniaspora occidentalis	98	a	Saccharomycetales	18s rRNA, ITS	FunF/FunR (4/123); ITS F/ITS-4R (48/171)
Hanseniaspora thailandica	99	a	Saccharomycetales	ITS	ITS F/ITS-4R (5/171)
Issatchenkia hanoiensis	98	a	Saccharomycetales	ITS	ITS F/ITS-4R (7/171)
Issatchenkia orientalis	98	a	Saccharomycetales	ITS	ITS F/ITS-4R (2/171)
Leptosphaeria maculans	99	a	Pleosporales	18s rRNA	ITS F/ITS-4R (2/126)
Limonomyces culmigenus	99	b	Corticiales	ITS	ITS F/ITS-4R (5/171)
Malassezia globosa	99	b	Malasseziales	18s rRNA	NSI/FRI (13/96)
Malassezia restricta	99	b	Malasseziales	18s rRNA	NSI/FRI (8/96)
Mortierella sp.	99	z	Mortierellales	ITS	ITS F/ITS-4R (3/171)
Penicillium allii	99	a	Eurotiales	ITS	ITS F/ITS-4R (3/171)
Phanerochaete sp.	99	b	Polyporales	18s rRNA	NSI/FRI (2/96)
Phoma exigua	99	a	Pleosporales	18s rRNA	E528F/Univ1492RE (3/96)
Phoma sp.	98	a	Pleosporales	18s rRNA	Euk1A/Euk 516r (2/126)
Pichia sorbitophila	98	a	Saccharomycetales	18s rRNA	Euk1A/Euk 516r (20/126)
Pleospora papaveracea	99	a	Pleosporales	ITS	ITS F/ITS-4R (14/171)
Pleosporales sp.	98	a	Pleosporales	18s rRNA	Euk1A/Euk 516r (1/126)
Saccharomycopsis crataegensis	99	a	Saccharomycetales	ITS	ITS F/ITS-4R (7/171)
Serpula lacrymans	99	b	Boletales	18s rRNA	NSI/FRI (6/96)
Setosphaeria monoceras	99	a	Pleosporales	18s rRNA	NSI/FRI (12/96)
Sordaria fimicola	99	a	Sordariales	18s rRNA	NSI/FRI (7/96)
Sympodiomycopsis paphiopedili	99	b	Microstromatales	18s rRNA	NSI/FRI (5/96)
Teratosphaeria miniata	99	a	Capnodiales	ITS	ITS F/ITS-4R (4/171)
Termitomyces albuminosus	99	b	Agaricales	18s rRNA	Euk1A/Euk 516r (23/126)
Trichosporon asahii	98	b	Tremellales	ITS	ITS F/ITS-4R (4/171)
Trichosporon fecale	99	b	Tremellales	ITS	ITS F/ITS-4R (2/171)
Tyromyces fumidiceps	99	b	Polyporales	18s rRNA	Euk1A/Euk 516r (2/126); NSI/FRI (6/96); E528F/ Univ1492RE (1/96)
Williopsis saturnus	99	a	Saccharomycetales	18s rRNA	NSI/FRI (2/96)
Xylaria adscendens	99	a	Xylariales	ITS	ITS F/ITS-4R (2/171)
Xylaria laevis	99	a	Xylariales	ITS	ITS F/ITS-4R (1/171)

a, Ascomycetes; b, basidiomycetes; z, zygomycetes; ITS, internal transcribed spacer.

stool samples of wild gorillas were screened for pathogens by specific PCRs.

Fungal diversity and detection of human fungal pathogens. Culture-dependent approaches limited the number of fungi that were isolated, revealing less fungal diversity because of difficulties that were encountered in their growth using routine laboratory culture[21]. In contrast, the culture-independent methods were more effective and revealed a larger diversity of fungi[16].

Although the molecular-based methods potentially detected a wide range of fungi in this study, but it is still difficult to predict whether they are the real symbiotic fungal components of gut in these animal or potential environmental fungal contaminants. The immediate collection of feces from animal in forest is a complicate process especially with these endangered wild animals and therefore the difficulties in preventing direct contact of feces bulk with its surrounding environment during and after defecation time are a major drawback in such studies. Moreover, the nature of some ascomyce-

Table 2 | Protozoal species detected by various clone libraries in this study

Closest relative in NCBI	Similarity%	Taxa	Targeted gene	Primer (No. of clones/total)
Blastocystis sp.	98	Stramenopiles	18s rRNA	Ami6F1/Ami9R (4/96); BlastF/BlastR (39/48); DimA/DimB (9/96); E528F/Univ1492RE (8/96)
Blepharocorys curvigula	99	Ciliophora	18s rRNA	121F/1147R (7/96); FunF/FunR (5/123)
Cycloposthium bipalmatum	98	Ciliophora	18s rRNA	121F/1147R (8/96); FunF/FunR (6/123)
Cycloposthium ishikawai	98	Ciliophora	18s rRNA	121F/1147R (1/96)
Iodamoeba sp.	98	Amoebozoa	18s rRNA	JVF/DSPR (44/96)
Trypanosomatidae	98	Kinetoplastida	ITS1	LeF/LeR (96/96)
Parentodinium sp.	99	Ciliophora	18s rRNA	121F/1147R (2/96); FunF/FunR (2/123)
Tetratrichomonas buttreyi	99	Parabasalia	18s rRNA	TFR1/TFR2 (Direct sequencing)
Triplumaria selenica	98	Ciliophora	18s rRNA	121F/1147R (1/96)
Troglodytella abrassarti	99	Ciliophora	18s rRNA	121F/1147R (14/96)

ITS, internal transcribed spacer.

tous and basidiomycetous species that were detected in this study are considered to be saprobes and thus they possibly representing environmental fungal contaminants as they are often found in association with plants, animals and their interfaces[22]. For example several members of Saccharomycetales such as *Hanseniaspora* spp., *Saccharomycopsis crataegensis*, *Pichia* spp., *Issatchenkia* spp. and *Candida quercitrusa* regard as plant associated fungi and many of them described as common fermentative spoilage yeast[23–27]. *Candida entomophila* characterized by its ability to ferment glucose and D-xylose therefore it usually has been isolated from wood-inhabiting insects and decaying wood[28]. Furthermore, some members of orders Eurotiales, Hypocreales, Xylariales and Pleosporales that have been identified in this study are regarded as the most common environmental fungi and they abundantly occur in forest soils or on fading leaves of herbaceous and woody plants such as cosmopolitan genus of *Penicillium* (Eurotiales) which has been found to play important role as decomposers of organic materials in soil[29], several species of *Bionectria* (Hypocreales) which can be found as common saprophytes on dead broad-leaf trees in forest[30], along with members of the genus *Xylaria* (Xylariales) that usually occur as saprobes or as parasite on flowering plants in lowland forests[31] and finally some species of Pleosporales that occur as saprobic fungi on decaying leaf or animal dung[32].

Another explanation for occurrence of these fungi in feces of gorillas is the fact that these apes are herbivore in their behavior and they feed on different parts of plants in which different fungi coexist. Therefore these fungi could also represent transient contaminants in the gut of gorillas. Moreover, several of the ascomycetous yeast that were detected in this study, such as *Candida orthopsilosis*, *C. rugosa*, *C. tropicalis* and *Galactomyces geotrichum*, have been previously described in the human gut[15,19,33]. Some of the basidiomycetous yeasts that were detected in our sample, such as the *Malassezia* and *Trichosporon* species, are regarded as human pathogens[34,35]. The remaining basidiomycetous fungi that were identified in our study including the saprotrophics, wood decomposers and symbiotic fungi, such as *Termitomyces*, which is generally regarded as a nutritional source for termites[36] that are in turn regarded as a source of food, and particularly of protein and vitamins, for wild gorillas[37]. Finally, using real-time PCR, many human fungal pathogens were detected in gorilla feces including species in the genera *Candida, Malassezia* and *Trichosporon* with high prevalence of *C. tropicalis* (81%), *M. globosa* (43.5%) and *C. parapsilosis* (38%) through our survey (Table 5).

Protozoal Diversity. Numerous studies have been performed on the intestinal parasites of wild non-human primate species, especially on gorillas[9–13]; however, this study, to the best of our knowledge, is the first molecular study attempting to detect both parasitic and commensal protozoans in the gastrointestinal tract of the wild western lowland gorilla. In the present study, the majority of the intestinal protozoa that were detected in the fecal sample belonged to four different phyla: the Ciliophora, Amoebozoa, Parabasalia and Stramenopiles. The most prevalent protozoal species that were found in this study were ciliates; approximately 6 species were detected in the fecal sample, which is in agreement with results from previous studies that identified entodiniomorph ciliates in the majority of fecal samples that had been collected from wild western lowland gorillas at Bai Hokou, Dzanga-Ndoki National Park (Central African Republic) and the Lopé Reserve (Gabon)[11–13]. However, our results conflict with those from the study by Modry *et al.*[38], in which *T. abrassarti* was the sole entodiniomorph ciliate that was detected in captive western lowland gorillas at the Prague Zoo in the Czech Republic. *T. abrassarti* has also been morphologically observed in fecal specimens from wild lowland gorillas[39], where it appears to play an important role in digestion because of its ability to ferment polysaccharides in the hindguts of primates[40].

Our study described the first report of *B. curvigula*, *Parentodinium* sp. and Cycloposthiidae species, including *C. bipalmatum*, *C. ishikawa* and *T. selenica* in the gastrointestinal tract of the wild gorilla.

Members of the Amoebozoa were detected at low frequencies despite the use of many primer sets that target the 18S rRNA genes of the major groups in this phylum (Supplementary Tables 1). Only *Iodamoeba* spp. were found in the present study. The low abundance of amoebae reported here agrees with studies by Freeman *et al.*[13] and Lilly *et al.*[12], both of whom reported the low prevalence of amoebae in fecal samples of wild lowland gorillas. However, our results disagree with the study by Sleeman *et al.*[10], who reported the high prevalence of amoebae in mountain gorillas. *Blastocystis* sp. was retrieved from the intestinal tract of one gorilla in this study. Screening the 48 samples from 21 individual of gorillas has demonstrated that this protozoan is not frequent within western lowland gorillas in

Table 3 | Nematode species detected by the NC1/NC2 clone library in this study

Closest relative in NCBI	Similarity%	Taxa	Targeted gene	Primer (No. of clones/total)
Libyostrongylus douglassi	99	Nematoda	ITS	NC1/NC2 (40/192)
Necator americanus	99	Nematoda	ITS	NC1/NC2 (107/192)
Oesophagostomum sp.	99	Nematoda	ITS	NC1/NC2 (39/192)
Oesophagostomum stephanostomum	99	Nematoda	ITS	NC1/NC2 (6/192)

Table 4 | Plant species retrieved from the fecal sample of a wild gorilla

Closest relative in NCBI	Similarity%	Targeted gene	Primer (No. of clones/total)
Barclaya longifolia	98	18s rRNA	Ami6F1/Ami9R (2/96)
Canna indica	99	18s rRNA	Ami6F1/Ami9R(1/96); JVF/DSPR (3/96)
Cicer arietinum	99	18s rRNA	JVF/DSPR (2/96)
Clematis armandii	99	18s rRNA	BlastF/BlastR (4/48); DimA/DimB (13/96); Euk1A/Euk 516r (5/126); JVF/DSPR (41/96)
Cratylia argentea	99	18s rRNA	FunF/FunR (2/123); 121F/1147R (2/96); E528F/Univ1492RE (1/96)
Fibraurea tinctoria	99	18s rRNA	DimA/DimB (54/96)
Grevillea robusta	98	18s rRNA	E528F/Univ1492RE (1/96)
Guadua angustifolia	99	ITS	ITS1-F/ITS-4R (1/171)
Hemiphylacus alatostylus	99	18s rRNA	DimA/DimB (2/96)
Manilkara zapota	98	18s rRNA	FunF/FunR (79/123); 121F/1147R (36/96); Euk1A/Euk 516r (17/126); E528F/Univ1492RE (23/96); E528F/Univ1391RE (24/72); EUKA/EUKB(23/72); 18ScomF/Dino18SR1 (direct sequencing)
Marantochloa atropurpurea	99	18s rRNA	Ami6F1/Ami9R (74/96); FunF/FunR (15/123); 121F/1147R(11/96); E528F/Univ1391RE (3/72)
Musa basjoo	99	18s rRNA	BlastF/BlastR (5/48); DimA/DimB (18/96); E528F/Univ1492RE (12/96); E528F/Univ1391RE (33/72); EUKA/EUKB (49/72)
Panax japonicus	98	18s rRNA	JVF/DSPR (6/96)
Pongamia pinnata	98	18s rRNA	FunF/FunR (1/123)
Pouteria macropoda	99	18s rRNA	FunF/FunR (1/123)
Trifolium repens	99	18s rRNA	FunF/FunR (1/123)
Trigonopleura malayana	99	18s rRNA	Ami6F1/Ami9R (3/96)
Xantolis siamensis	99	18s rRNA	Ami6F1/Ami9R (12/96); FunF/FunR (7/123); 121F/1147R (10/96); E528F/Univ1391RE (11/72)
Schima superba	99	rbcLgene*	rbcLZ1/rbcL19b (72/96)
Davidia involucrata	98	rbcLgene*	rbcLZ1/rbcL19b (24/96)
Delarbrea michieana	98	18s rRNA	E528F/Univ1391RE (1/72)

*chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit.

Cameroon (Table 5). *Blastocystis* can be found in both humans and nonhuman primates[41,42]. In humans, it appears to be a causative agent of irritable bowel syndrome (IBS) in certain circumstances[41], while in non-human primates, its role remains unclear.

Either none or very few enteric flagellate protozoa have been observed in the fecal samples of wild gorillas[9–13]; additionally, only some members of the trichomonads and *Giardia* sp. have been recorded in both captive and wild gorillas, respectively[10,43]. These previous studies are in partial agreement with our study, in which only *T. buttreyi*, a trichomonad, was detected. *T. buttreyi* has also been detected in ruminant feces, and it appears to be harmless to its host[44].

Helminthic diversity and detection of human nematode pathogens. We attempted to determine the presence of trematodes, cestodes and nematodes in the fecal samples of the gorillas. Only parasites belonged to the phylum Nematoda were identified; we did not report the presence of any other groups of helminths. Previous studies that were conducted in wild lowland gorilla populations in Gabon and the Central African Republic showed also an absence of cestodes and a scarcity of trematodes in fecal samples[11–13].

The presence of nematode species, such as *O. stephanostomum,* in the intestinal tract of gorillas has been morphologically confirmed by Sleeman *et al.*[10]. *Oesophagostomum* spp. can also infect ruminants, pigs and monkeys[45]. Some reports have described human infections with *Oesophagostomum* spp., particularly in northern Togo and Ghana where they have been known to cause serious health problems[45,46]. Another nematode that was recorded in our study was *N. americanus*, which is an obligate hookworm parasite that is responsible for most common chronic infections in humans, particularly in areas of rural poverty in the tropics and subtropics[47]. This hookworm is generally transmitted through contact with contaminated soil and resides in the intestinal tract of its host[47]. The hookworm's infection with *N. americanus* has been described previously in intestinal tract of both mountain gorillas (*G. g. beringei*) inhabiting at Bwindi Impenetrable National Park, South West Uganda[48] and western lowland gorillas (*G. g. gorilla*) residing at Dzanga-Sangha Protected Areas, Southwest of Central African Republic[49]. Finally, an ostrich-specific nematode (*L. douglassi*) was also detected in our sample. As this parasite commonly infects the ostrich proventriculus and can cause libyostrongylosis, which has a high mortality rate among juvenile birds[50], the detection of this nematode in gorilla's

Table 5 | Eukaryotic Human Pathogens detected by Real Time PCR in 48 fecal samples from wild gorillas (21 individuals)

Real time PCR	Target	No. of positive fecal samples	No. of positive gorilla	Frequency
Necator americanus	ITS	11	9	43%
Oesophagostomum bifurcum	18s	41	18	86%
Candida parapsilosis	ITS	15	8	38%
Candida rugosa	ITS	4	2	9.5%
Candida tropicalis	ITS	25	17	81%
Malassezia globosa	26s	13	9	43%
Malassezia restricta	26s	14	8	31%
Trichosporon asahii	ITS	2	2	9.5%
Trichosporon spp.	ITS	13	7	33%

feces in this study could be resulted either from environmental contamination or consumption of contaminated food items. Analyzing of more fecal samples is needed to further explaining the presence of this parasite in feces of this animal. In our survey, high percentage of both human parasitic worms; *O. bifurcum* and *N. americanus* (86% and 43% respectively) was discovered in the stool samples of western lowland gorillas from Cameroon (Table 5).

Residual plants in gut of gorilla. Gorillas are largely herbivorous and consume a wide variety of plant species (between 50 and 300)[51,52]. Studies of western gorillas have shown that fruit is an essential part of their diet[53,54], but they also eat leaves, shoots, flowers, and the woody parts of plants[51,52]. In this study, we detected 21 different plant species in the fecal sample of a wild gorilla collected around the village of Minton. Primer sets targeting both 18S rRNA and the chloroplast *rbcL* genes were used to identify the residual plant species in the gorilla feces. Unexpectedly, only two phylotypes of plants were detected when the primer targeting the chloroplast *rbcL* gene was used. These results are in agreement with those of Bradle *et al.*[55], who also detected few plants in western gorilla feces using the same primer set, which preferentially amplifies DNA from chloroplast-rich tissues, such as leaves or stems, rather than fruits, flowers and seeds[55]. Not surprisingly, roughly 19 plant species were co-amplified along with other eukaryotes in this study when universal eukaryotic primers targeting 18S rRNA were used. These plant species belong to different families that may be consumed by wild western lowland gorillas.

In conclusions, this is the first study to characterize fecal eukaryotic diversity, including fungi, in non-human primates using a comprehensive extended molecular analysis. The multiple primer set approach used herein enabled us to recover a high diversity of eukaryotes from the intestinal tract of the wild lowland gorilla, which may include human pathogens as revealed by our real-time PCR assessments in gorillas' feces. Although the detection of fungi species should be interpreted cautiously because the possibility of environmental contamination, the presence of human parasites in gorillas should be viewed as an important public health concern, particularly for surrounding rural villages where habitat overlap is frequent. Additional studies from other geographic locations and using the methodological strategy presented here are required for detailed descriptions of the occurrences and abundances of eukaryotes, including pathogens, in the guts of non-human primates, which have until now been poorly described.

Methods

Source of fecal samples. A total of 48 fecal samples were collected from 21 individual western lowland gorillas (*G. g. gorilla*) in this study (Supplementary Tables 4). One fecal sample was collected in a site near Minton village which located in south-central Cameroon and was used in this study for exploring the occurrence of gut eukaryotes in gorilla intestinal tract through using PCR-based amplification using various primers, followed by cloning and sequencing, while the rest of 47 fecal samples were collected from different sites around Messok village which located in the south-east Cameroon and were used in this study for investigating the presence of human eukaryotic pathogens in gut of gorillas. The sample collection protocol was described previously[56]. The GPS position, time and date were recorded for all samples. The fecal samples were preserved in RNA*later* (Ambion, Austin, TX) and kept at room temperature at base camps for less than 3 weeks then transported to a central laboratory and kept at −80°C.The collection of the fecal samples was approved by the Ministry of Scientific Research and Innovation of Cameroon. No other permit was required for the described field as this research was non-invasive work and the collection of the samples from soil did not disrupt the wild fauna.

DNA extraction. Total DNA was extracted from the frozen fecal samples using a modification of the Qiagen stool procedure and the Qiamp® DNA Stool Mini Kit (Qiagen, Courtaboeuf, France)[16]. The inner part of the fecal bulk was used for extraction to avoid as much as possible an eventual contamination with soil organisms and/or environmental species during collection as previously described by[14]. Aliquots of 200 mg of this part were added into tubes containing a 200 mg mixture of 0.1, 0.5, and 2 mm zirconium beads and 1.5 ml of ASL buffer (Qiagen). The samples were mixed vigorously by agitation in a FastPrep BIO 101 agitator (Qbiogene, Strasbourg, France) at 3,200 rpm for 90 seconds. Agitation was followed by heating at 95°C for 10 min to increase both the yield of DNA and proteinase K digestion before the DNA was bound to a column, washed, and eluted in TE buffer.

Genomic amplification. All universal and specific eukaryotic primers targeting both the ITS and 18S rRNA genes that were used in this study were adopted from previously published studies (Supplementary Tables 2). The 50 μL PCR reaction mixture contained 5 μL of dNTPs (2 mM of each nucleotide), 5 μL of DNA polymerase buffer (Qiagen, Courtaboeuf, France), 2 μl of $MgCl_2$ (25 mM), 0.25 μL of HotStarTaq DNA polymerase (1.25 U) (Qiagen, Courtaboeuf, France), 1 μL of each primer, and 5 μL of DNA. The PCR cycling conditions for all amplifications were as follows: 1 cycle at 95°C for 15 min, 40 cycles at 95°C for 0.5 min, 48–60°C for 0.5–2 min (Supplementary Tables 2), and 72°C for 1–2 min, followed by a final cycle at 72°C for 5 min. All amplifications were performed in a PCR system 2720 thermal cycler (Applied Biosystems, Courtaboeuf, France). Amplification products were visualized on a 1.5% agarose gel that was stained with ethidium bromide and viewed under a UV light source. The PCR products were purified using the Nucleo-Fast® 96 PCR Kit (Marcherey-Nagel, Hoerdt, France) according to the manufacturer's instructions.

Cloning, Sequencing and phylogenetic analyses. The cloning and sequencing reactions were performed as previously described[16]. The PCR products were cloned separately using the pGEM® -T Easy Vector System Kit (Promega, Madison, USA). Aliquots (150 μl) of cell suspensions were plated onto LB (Luria-Bertani Broth) agar plates that were supplemented with ampicillin (100 mg/mL), X-GAL (80 mg/mL) and IPTG (120 mg/mL), and the plates were incubated overnight at 37°C. Positive clones were suspended in 25 μL of distilled water and stored at −20°C. The presence of the insert was confirmed by PCR amplification using the M13 forward (5′-GTAAAACGACGGCCAG-3′) and M13 reverse (5′-AGGAAACAGCTATGAC-3′) primers (Eurogentec, Seraing, Belgium). The purified PCR products were sequenced in both directions using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing products were run on an ABI PRISM 3130 automated sequencer (Applied Biosystems). Finally, intestinal eukaryotes were identified by comparing the resulting sequences with those that were deposited in GenBank using the basic local alignment search tool (BLAST), which is available at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/). Phylogenetic analyses were performed using MEGA5.04 and a distance matrix neighbor-joining (NJ) approach[57].

Real-Time PCR Assay for Detection of human pathogen. Primers and probes specific to some human eukaryotic pathogens were used as described previously (Supplementary Table 3). For the primers and probes used for first time in this study, sequences corresponding to each species were collected in GenBank and aligned using multiple sequence alignment ClustalW2, and the PRIMER 3 software[58] was used to design primer sets in the conserved regions of aligned sequences. The specificity of each primer was tested using the basic local alignment search tool (BLAST), which is available at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/). The real-time PCR reactions were conducted using 25 μL total volumes and analyzed for 44 cycles using a CFX96™ Real-Time PCR Detection System (BIO-RAD, Life Science, Marnes-la-Coquette, France) following methods recommended by the manufacturer. Amplification reactions were done as follows: 95°C for 15 min, 60°C for 0.5 min, and 72°C for 1 min.

Species confirmation and Microsatellite analyses. The DNA was extracted from gorilla fecal samples in order to determine the number of individuals that carrying human eukaryotic pathogens. Total of 48 samples were genotyped at 7 polymorphic loci (D18s536, D4s243, D10s676, D9s922, D2S1326, D2S1333 and D4S1627) as described previously[56]. The gender of gorillas was determined by amplification of a region of the amelogenin gene that contains a deletion in the X, but not the Y chromosome[56]. To exclude the allelic dropout, all loci were amplified four times. Aliquot 1 μL of PCR products was mixed with 10 μL of formamide and 0.25 μL of the ladder marker (ROX GeneScan 400HD, Applied Biosystem), The resulting amplifications were analyzed by 3130xl Genetic Anlayser sequencer (Applied Biosystem, Foster City,CA). Amplification products were visualized and sized using Genemapper 3.7 software (Applied Biosystems).

Culturing and identification of fungal species. The fecal samples were serially diluted, and six-fold dilutions were spread onto potato dextrose agar (Fluka® Analytical, France), Czapek dox agar (Fluka® Analytical, France) and Dixon agar. The plates were incubated aerobically at room temperature. The colonies exhibiting different morphologies were restreaked to obtain pure cultures. The fungi were amplified using fungal primers (ITS1-F/ITS-4R) and identified as previously described[16].

Nucleotide sequence accession numbers. All sequences obtained in this work have been deposited in GenBank database with the accession numbers JX158488 to JX159965.

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Author contributions

D.R. and F.B. designed the experiments; I.H., M.K. conducted the experiments; I.H., M.K., M.P., E.D., D.R. and F.B. analyzed the results; I.H. and F.B. prepared the figure; I.H. and F.B. wrote the manuscript. All authors reviewed the manuscript.

Additional information

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