

Occurrence and diversity of anaerobic gut fungi in wild forest elephants and buffaloes inhabiting two separated forest ecosystems in Central West Africa

Authors: Schulz, Doreen, Pšenková-Profousová, Ilona, Červená, Barbora, Procter, Miranda, Neba, Terence Fuh, et al.

Source: Journal of Vertebrate Biology, 71(21033)

Published By: Institute of Vertebrate Biology, Czech Academy of Sciences

URL: <https://doi.org/10.25225/jvb.21033>

BioOne Complete (complete.BioOne.org) is a full-text database of 200 subscribed and open-access titles in the biological, ecological, and environmental sciences published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Complete website, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Complete content is strictly limited to personal, educational, and non - commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

Occurrence and diversity of anaerobic gut fungi in wild forest elephants and buffaloes inhabiting two separated forest ecosystems in Central West Africa

Doreen SCHULZ^{1,2,3*}, Ilona PŠENKOVÁ-PROFOUSOVÁ^{1,4}, Barbora ČERVENÁ^{1,5}, Miranda PROCTER⁶, Terence FUH NEBA⁷, David MODRÝ^{2,8}, Klára J. PETRŽELKOVÁ^{5,8,9} and Moneeb A. QABLAN⁶

¹ Department of Pathology and Parasitology, University of Veterinary Sciences Brno, Brno, Czech Republic

² Department of Botany and Zoology, Faculty of Science, Masaryk University, Brno, Czech Republic; e-mail: doreenschulz@gmail.com, modryd@vfu.cz

³ Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Praha, Czech Republic

⁴ Ústí Zoo, Ústí nad Labem, Czech Republic; e-mail: ilona.psenkova@gmail.com

⁵ Institute of Vertebrate Biology, Czech Academy of Sciences, Brno, Czech Republic; e-mail: bara.cervena@gmail.com, petrzelkova@ivb.cz

⁶ Department of Veterinary Medicine, United Arab Emirates University, College of Agriculture and Veterinary Medicine, Al Ain, Abu Dhabi, United Arab Emirates; e-mail: m.procter@uaeu.ac.ae, m.qablan@uaeu.ac.ae

⁷ World Wildlife Fund, Primate Habituation Project, Dzanga-Sangha Protected Areas, Bangui, Central African Republic; e-mail: TNebe@wwfcar.org

⁸ Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice, Czech Republic

⁹ Liberec Zoo, Liberec, Czech Republic

Received 6 May 2021; Accepted 30 August 2021; Published online 9 December 2021

Abstract. Anaerobic gut fungi of the class Neocallimastigomycetes are of great importance for herbivorous animals. Their immediate colonization and mechanical breakdown of plant particles pave the way for highly efficient enzymatic fermentation of complex plant polysaccharides. Neocallimastigomycetes are found in a variety of herbivores, yet so far studies almost exclusively investigated domestic or captive animals. Here, the occurrence and diversity of Neocallimastigomycetes in two different populations of sympatric, wild African forest elephants and forest buffaloes were determined. In both hosts together, a total of 16 species-equivalent Operational Taxonomic Units (OTUs) (0.05 cut-off level) were generated. Buffaloes harboured four and elephants five anaerobic fungi genera or genus-equivalent taxa, respectively, with four genera occurring in both hosts. In elephants the majority of gut fungi group within a cluster of yet unknown Neocallimastigomycetes. Similarly, some anaerobic fungi found in buffaloes form a genus-equivalent cluster with likewise undescribed gut fungi. Sequences grouping in these two clusters could potentially qualify as representatives of new anaerobic fungi genera. Further, three sequences have not yet been encountered in any study and cannot be assigned to any genus or genus-equivalent Neocallimastigomycetes taxon. Whether these sequences also represent putative new lineages needs further investigation.

Key words: gut microbiome, mycobiome, wild herbivores, Neocallimastigomycetes

* Corresponding Author

Introduction

Microbes residing in the gastrointestinal tract, referred to as the gut microbiota, are of significant importance for their respective host. They influence the host's immune system development and function (Round & Mazmanian 2009, Chung et al. 2012), neural development and behaviour (Diaz Heijtz et al. 2011, Luna & Foster 2015), and even affect mating success (Brucker & Bordenstein 2013). Particularly for herbivores, the gut microbiome provides crucial functions in the digestion process. Herbivorous mammals lack cellulolytic and hemicellulolytic enzymes (Gruninger et al. 2014) and thus rely on microbial fermentation end products (such as short-chain fatty acids) for an adequate energy gain (e.g. Sekirov et al. 2010). Amongst microbes found in the gastrointestinal tract, anaerobic gut fungi (AGF) of the class Neocallimastigomycetes play a pivotal role. They are among the first to colonize plant particles (Edwards et al. 2008) and, immediately after colonization, their growing rhizoids penetrate plant cell walls mechanically, increasing the surface area for enzymatic breakdown (Ho et al. 1988, Grenet et al. 1989). Some of the highly efficient hydrolases of AGF are aggregated in extracellular complexes called cellulosomes (Joblin et al. 2010) which enable Neocallimastigales to degrade plant cell walls, weaken plant tissue, and reduce the size of plant particles (Doi & Kosugi 2004, Fontes & Gilbert 2010), consequently leading to an overall increase in microbial fermentation (Lee et al. 2000). Moreover, the degradation of plant cell walls results in the release of proteins and other nutrients bound within cell wall fibres, which later are accessible to the host's glandular digestion (Bauchop 1981). How crucial AGF are for herbivores is highlighted by findings that the occurrence of the most recent common ancestor of these fungi coincides with the transition of mammals from insectivore to herbivore (about 66 MYA; Wang et al. 2019).

Originally, AGF were discovered in the rumen of sheep and recognized as fungi in the 1970s (Orpin 1975), yet their classification as a phylum distinct from *Chitridiomyxota* was completed much later based on molecular, morphological, and ultrastructural data (James et al. 2006, Hibbet et al. 2007). The class Neocallimastigomycetes comprises only one order (Neocallimastigales). Initially, classification of AGF was based on challenging culture-dependent methods and

for a long time only six genera were recognized: *Neocallimastix*, *Caecomycetes*, *Orpinomyces*, *Piromyces*, *Anaeromyces* and *Cyllamyces*. Advances in culture-independent molecular methods and phylogenetic reconstruction based on amplification of the ITS (internal transcribed spacer) regions of the rRNA genes created a new frontier in AGF taxonomy and led to the discovery of several putative new lineages (Tuckwell et al. 2005, Liggenstoffer et al. 2010, Nicholson et al. 2010, Herrera et al. 2011, Kittelmann et al. 2012, Paul et al. 2018). In 2015 two new genera were classified: *Buwchfawromyces* and *Oontomyces* (Callaghan et al. 2015, Dagar et al. 2015). Followed by an additional three genera in the following years, namely *Pecoramyces*, *Feramyces*, and *Liebetanzomyces* (Hanafy et al. 2017, 2018, Joshi et al. 2018). Lately, Hanafy et al. (2020a), in their multi-year long intensive study on AGF in domestic, captive and wild hosts described further seven genera (*Agriosomyces*, *Aklioshbomyces*, *Capellomyces*, *Ghazallomyces*, *Joblinomyces*, *Khoyollomyces* and *Tahromyces*).

Molecular based studies also resulted in a better understanding about host variety, geographical distribution, and factors influencing the AGF community structure in different hosts. AGF are part of the gut microbiota of herbivores of all types of digestive systems, i.e. ruminant, pseudo-ruminant foregut, and non-ruminant hindgut fermenters (Liggenstoffer et al. 2010, Hanafy et al. 2020b). So far, it seems that there is no geographical limitation in AGF occurrence. For example, the genus *Cyllamyces* is widely distributed across continents (Sridhar et al. 2007, Liggenstoffer et al. 2010, Nicholson et al. 2010, Sirohi et al. 2013), and a recent phylogenetic census of Neocallimastigales revealed that *Orpinomyces*, *Caecomycetes*, *Piromyces* and *Neocallimastix* occur in herbivores from various geographic regions (Paul et al. 2018). Indeed, factors influencing the AGF community structure appear to be of the intrinsic host-related kind, such as gastrointestinal physiology and host phylogeny (Liggenstoffer et al. 2010).

The accumulated knowledge regarding AGF in domestic animals is expanding. However, studies targeting wild animals are scarce. Furthermore, comparative analyses of AGF in co-habiting herbivores with overlapping diet but with a different digestive physiology are missing. This study aims to analyse AGF in sympatric forest elephants (*Loxodonta africana cyclotis*) and forest buffaloes (*Syncerus caffer nanus*) from two discrete

forest ecosystems in West Central Africa to i) gather initial information on the diversity of AGF in the two different wild hosts and ii) address the influence of the host and the environment on AGF communities. Forest elephants are non-ruminant hindgut fermenters, feeding on a wide range of fruits, but with leaves and browse constituting the dominant food items (Short 1983, Blake 2002, Blake et al. 2009). Forest buffaloes are ruminant foregut fermenters, which rely mainly on grasses, but also frequently ingest browse (Kingdon 1997, Bekhuis et al. 2008). We predict: i) a higher diversity in AGF in ruminant buffaloes compared to elephants; and ii) variance in the occurrence of different anaerobic fungal genera in relation to the different type of fermentation (foregut *vs.* hindgut) in the two species rather than geographical differences between populations.

Material and Methods

Study site, subjects and sample collection

Faecal samples from sympatric forest elephants and forest buffaloes were collected at two field sites in Central West Africa: i) in a private ecotourism concession under development in Loango National Park (LNP; 2°10' S 9°34' E), Gabon (May to July 2014); and ii) in the surroundings of the research station in Bai Hokou (2°50' N, 16°28' E) located in the Sangha sector of the Dzanga-Ndoki National Park, Dzanga-Sangha Protected Areas (DSPA), Central African Republic (September 2014 to January 2015). As neither elephants nor buffaloes can be tracked in the forest, samples were collected opportunistically along elephant trails and in forest clearings. At both field sites, samples older than one day were not collected while freshness of faeces was confirmed by experienced trackers. Faeces were stored in 8 ml tubes containing 96% ethanol with an approximate ratio of 2/3 ethanol to 1/3 faeces to ensure proper fixation. All samples were collected non-invasively, adhering to site regulations and other health and safety protocols. All material was shipped to the Department of Pathology and Parasitology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic and stored at -20 °C.

Ethical note

At both field sites all samples were collected non-invasively with no contact or harm to the animals. Research in the Dzanga-Sangha Protected Areas, Central African Republic, was permitted by the Ministère de L'enseignement Supérieur et

de la Recherche Scientifique and the Ministère des Eaux, Forêts, Chasses, Pêches, chargé de l'Environnement. Permission to conduct research in Loango National Park, Gabon, was granted by the Centre National de la Recherche Scientifique et Technologique and the Agence National des Parcs Nationaux.

Sample processing

DNA isolation

For this study, 20 elephant and 20 buffalo samples from DSPA, as well as eight elephant and five buffalo samples from LNP were processed. Ethanol was evaporated from the sample tube overnight at 40 °C on a heating block. Subsequently, genomic DNA was isolated with the Fast DNA Spin Kit for Soil (MP Biomedicals, USA) following the manufacturer's protocol with the following alteration: homogenization by bead-beating was carried out twice for 30 sec each with samples placed on ice for 30 sec after each beating session (Cheng et al. 2009). DNA was eluted with 70 µl DES and isolates were stored at -20 °C until further use.

Amplification of fungal barcodes

The primer pair MN100 (TCCTACCCTTTGTG AATTG) and MNGM2 (CTGCGTTCCTTCATCG TTGCG) specific for anaerobic gut fungi (Nicholson et al. 2010) were used to amplify the ITS1 sequence as fungal barcodes for subsequent analysis. PCR reaction mixtures contained 12.5 µl Master Mix (PCRBIO Taq Mix Red, PCR Biosystems, UK), 9.5 µl H₂O, 0.01 µM of each primer, and 1 µl isolated DNA per sample. Fungal ITS1 fragments were amplified under the following touchdown cycling conditions: 95 °C for 5 min; 20 cycles with 95 °C for 30 sec, 65 °C for 30 sec with -0.5 °C per cycle, 72 °C for 30 sec; followed by another 20 cycles with 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec, and a final extension of 5 min at 72 °C. Amplified products were visualized on 1% agarose gels and fragments of expected length were purified with ExoSap (Affymetrix Inc., USA) and subjected to cloning.

Clone library construction

PCR products were further processed with the TOPO TA Cloning Kit for Sequencing (Life Technologies, USA) according to the manufacturer protocol. From each S.O.C. cell culture 70 µl were transferred on LB agar plates containing 50 µg/ml ampicillin and plates were incubated overnight. Liquid LB culture medium for subsequent incubation of selected clone colonies contained

70 µg/ml ampicillin. Isolation of plasmid DNA was performed using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, Germany) following the manufacturer's recommendations. Lastly, plasmid isolates were subjected to Sanger sequencing (SEQme s.r.o.; Czech Republic).

Sequence and phylogenetic analyses

Obtained sequences were edited with BioEdit software (version 7.2.3). Subsequently, Nucleotide BLAST search was applied to identify sequences which relate to deposited identified AGF or uncultured Neocallimastigales clone sequences. All potential AGF clone sequences were clustered at 95% similarity (MOTHUR; Schloss et al. 2009) based on a MAFFT alignment (online version 7, <http://mafft.cbrc.jp/alignment/server/>; Katoh & Standley 2013) generated with iterative E-INS-i setting. The Phylip formatted distance matrix was built with the calc = onegap setting. The 95% similarity level was chosen to generate species-equivalent Operational Taxonomic Units (OTUs) (Liggenstoffer et al. 2010).

Sequences representing generated OTUs were subjected to BLAST searches to find similar sequences. The top BLAST hits were included in the final dataset, along with sequences of described

genera within the Neocallimastigomycota (based on the dataset of Joshi et al. 2018) and representatives of the genus equivalent clades (as listed by Paul et al. 2018), with *Monoblepharella mexicana* as outgroup. The dataset was subsequently aligned using the online version of MAFFT using the E-INS-I iterative refinement method, as before. The best nucleotide substitution model for phylogenetic analysis was inferred using jModelTest (Darriba et al. 2012). MEGA 7 (Tamura 1992, Kumar et al. 2016) was used to perform maximum likelihood analysis for the aligned dataset by using the GTR + I + G substitution model and 500 bootstrap replicates. The tree generated was also viewed and edited in MEGA7. Bootstrap values below 50 were removed from the final phylogenetic tree. Bayesian Inference was conducted on the same dataset using MrBayes v. 3.2.6 (Ronquist et al. 2012). The analysis was run until the standard deviation frequencies reached a value below 0.01 (10 million generations), sampling every 1000th generation and burn-in value of 25%.

Results

Occurrence and distribution of AGF in the two host species

In total, 180 sequences were incorporated in the analysis, with 177 clone sequences identified as

Table 1. Occurrence of species-equivalent OTUs in the two investigated populations of forest elephants and forest buffaloes. Titles of the representative cloned sequence indicate origin and species, respectively: BH: origin Bai Hokou (DSPA); Lo: Loango National Park; _B: source buffalo faeces; _E: source elephant faeces.

OTU	representative clone	Occurrence				No. of sequences
		BH Buff	Lo Buff	BH Eleph	Lo Eleph	
1	BH_B7_2	•	•	•		71
2	BH_E1_3			•	•	71
3	Lo_B57_1	•	•		•	12
4	Lo_E53_5	•			•	7
5	BH_B21_4	•				3
6	BH_E5_1			•		2
7	Lo_E30_5	•			•	2
8	Lo_B29_4		•			2
9	BH_B20_5	•				2
10	BH_E12_4			•		2
11	BH_E22_2			•		1
12	Lo_B29_1		•			1
13	BH_E24_2			•		1
14	BH_B1_3	•				1
15	BH_B1_4	•				1
16	BH_B10_1	•				1

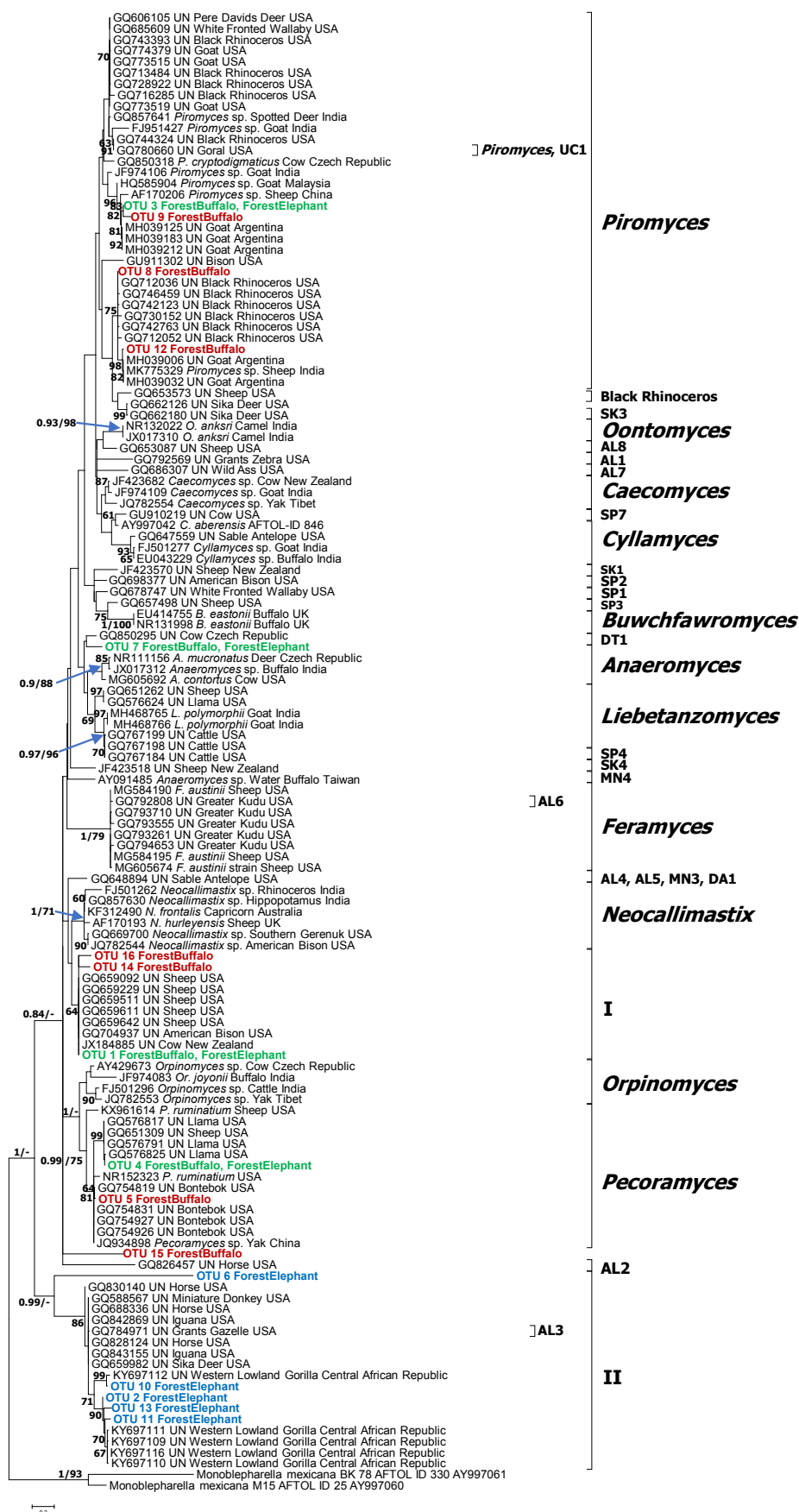


Fig. 1. Maximum likelihood (ML) phylogenetic tree constructed from ITS data. The tree was constructed using MEGA 7, with the GTR + I + G substitution model. Values on branches indicate Bayesian posterior probability values (≥ 0.9)/ML bootstrap values (≥ 50) from 500 replicates. Where only one value is present, it represents the ML bootstrap value. Posterior probability below 0.9 has been added to the backbone of the tree only, and has not been indicated below the nodes of genera/-equivalent lineages and lineages I and II. Taxon labels include host species and country of origin. *UN = Uncultured fungal clones.



potential AGF ITS1 sequence fragments and three sequences not matching significantly with any sequence in GenBank (Table S1). Of those 180 sequences, 68 sequences were obtained from 14 elephant samples from Central African Republic, 18 sequences from six elephant samples from Gabon, 79 sequences from 15 buffalo samples from Central African Republic and 15 sequences from four Gabonese buffalo samples. Cluster analysis revealed 16 distinct species-equivalent OTUs (Table 1), amongst which, 11 and nine OTUs were found in buffalo and elephant samples, respectively. Seven OTUs were unique for buffaloes and five for elephants. OTUs 1 and 2 were the most prevalent with each represented by 71 sequences. OTUs 1, 3, 4, and 7 occurred in both hosts. The two distant populations of buffaloes share just two OTUs (OTUs 1 and 3), while the two elephant populations shared only OTU2 (Table 1, Table S2).

Taxonomic assignment of Neocallimastigales clones from elephants and buffaloes

Overall, all included OTU-representative sequences from our study cluster with Neocallimastigales reference sequences in a monophyletic, and significantly supported clade (Fig. 1). Four of our fungal OTUs were assigned to multiple lineages within the polyphyletic *Piromyces*. Among them, OTU3 and OTU9 group with reference sequences generated from goat and sheep (Brookman et al. 2000, Fliegerova et al. 2021; HQ585904 F.O. Kok, unpublished data; JF974106 S.S. Dagar, unpublished data). OTU8 groups with references from black rhinoceros (Liggenstoffer et al. 2010) and OTU12 with another lineage of sequences being generated from goat (Fliegerova et al. 2021) and sheep (MK775329 A. Joshi, unpublished data). Distinct clades within the polyphyletic *Piromyces* that include sequences of the present study are sufficiently supported. The representative of DT1 (as per Paul et al. 2018) originating from cow, groups with weak support with OTU7, peripheral to the lineage consisting of *Anaeromyces* and *Liebetanzomyces*. Two OTUs, namely OTU4 and OTU5 were assigned to the genus *Pecoramyces*, with sequences isolated from bontebok, llama, and sheep (Liggenstoffer et al. 2010). The three OTUs 1, 14, and 16 form a distinct, though moderately supported clade with sequences originating from sheep, cow and American bison (clade I in Fig. 1). This clade includes no known genus or genus-equivalent representatives of the Neocallimastigales order. OTUs 2, 10, 11, and 13 cluster within AGF clade II (this study). They form

a nested clade and group with sequences obtained from Western lowland gorillas (Schulz et al. 2018), living in the same area as one of this study's sample collection site around Bai Hokou. The strongly supported clade II contains the AL3 representative (as per Paul et al. 2018), and sequences generated from horse, iguana, Sika deer, and miniature donkey (Liggenstoffer et al. 2010). OTU6 lies outside the larger cluster within clade II that contains representatives of the AL3 lineage and sequences generated from elephants. Last, OTU15 groups in a separate lineage, outside of any genus or genus-equivalent clade. Representative cloned AGF sequences are accessible in GenBank under the accession numbers MN565920-MN565935.

Discussion

Diversity and community composition of AGF in African forest elephants and forest buffaloes

The current study represents an initial attempt to investigate the diversity of AGF in wild populations of forest elephants and buffaloes using the highly variable, widely accepted rRNA ITS1 as a phylogenetic marker (Paul et al. 2018). Overall, 16 distinct species-equivalent OTUs were inferred with variable representation in both species. The results indicate that a slightly higher degree of diversity of AGF do circulate among buffaloes ($n = 11$) compared to elephants ($n = 9$). However, phylogenetic analysis suggests that OTUs occurring in buffaloes belong to only three AGF genera or genus-equivalent taxon, namely *Piromyces*, *Pecoramyces* and AGF clade I (this study). On the other hand, four AGF genera or genus-equivalent taxa were identified in elephants: *Piromyces* and *Pecoramyces* as well as clade I and clade II. This would suggest a slightly higher AGF diversity at the genus-level in elephants compared to buffaloes. *Piromyces* are widely distributed in herbivores across different continents (Paul et al. 2018), including species like black rhinoceros, white-fronted wallaby, and goat (Brookman et al. 2000, Liggenstoffer et al. 2010). Our results indicate that *Piromyces* are also part of the AGF community in wild ruminant and non-ruminant African animals. The taxon *Pecoramyces*, so far, has only been detected in various foregut fermenters (Hanafy et al. 2017). The assignment of OTU4 which is present in both investigated species to *Pecoramyces* is robustly supported and thus gives a strong indication that *Pecoramyces* might also occur in non-ruminant herbivores.



Three OTUs found in buffaloes and five OTUs found in elephants, group within clades consisting of reference AGF sequences not yet classified (Fig. 1: clade I and II). Among those are the most prevalent OTUs of both hosts (Table 1). A further two OTUs (OUT 7 and 15) could not be assigned to a known AGF taxon or genus-equivalent taxa. While AGF taxa that are known to be abundant in domestic and captive herbivores, such as *Neocallimastix* or *Caecomyces* (Liggenstoffer et al. 2010, Paul et al. 2018), were not detected in this study. This outcome contradicts previous findings where *Piromyces* appears to be the most abundant taxon, followed by *Neocallimastix* and *Caecomyces*, in various herbivores (Liggenstoffer et al. 2010, Paul et al. 2018). These findings indicate that AGF communities of wild herbivores might differ significantly from those of domestic animals. Similar differences have been observed in gut bacteria communities between captive and wild conspecifics (Schwab et al. 2009, Nakamura et al. 2011, Nelson et al. 2012).

Only one previous study investigated the occurrence of Neocallimastigales in wild African elephants and buffaloes, namely in the Savannah elephant (*Loxodonta africana*) and the Cape buffalo (*Syncerus caffer caffer*) in Zimbabwe (Nicholson et al. 2010). Based on a DGGE (denaturing gradient gel electrophoresis) approach this study described the occurrence of *Anaeromyces* in elephants and buffaloes, while *Orpinomyces* and AGF that were defined as novel group 2 were present in buffaloes only. A consecutive study (Kittelmann et al. 2012) demonstrated that this novel group 2 corresponds to the likewise novel group 8 of Liggenstoffer et al. (2010) which later was named AL8. Unfortunately, none of the more recent attempts to resolve AGF taxonomy included the only available sequences from Savannah elephants or Cape buffaloes. Sequences from forest elephants and forest buffaloes obtained in the present study did not group with *Anaeromyces*, *Orpinomyces* nor AL8. However, the possibility that these three AGF taxa occur in forest elephants and forest buffaloes cannot be excluded. In general, the applied cloning methodology has inherent limitations in obtaining a full picture of AGF diversity in a particular host. Thus, the AGF diversity described in this study most likely does not reflect the whole AGF community of either one of the host species. Nonetheless, choosing the Sanger approach has the advantage of specifically targeting microbes that occur in very low quantities

such as AGF which constitute only 8% of the gut microbiota (Theodorou et al. 1996). Recent next generation sequencing (NGS) -based mycobiome studies that included wild animals like primates with a high plant fibre intake, or ruminant roe deer, have not found AGF, although they likely comprise part of their intestinal fungal community (Sun et al. 2018, Barelli et al. 2020, Harrison et al. 2021). Another disadvantage of applying NGS in such studies is the lack of analytical methods especially designed for mycobiome studies (Halwachs et al. 2017).

Differences in AGF communities in the two sympatric host species

Remarkably, the overlap in AGF genera present in both species is much higher than would be expected by their different digestive physiology and diet (Liggenstoffer et al. 2010, Kittelmann et al. 2012, Kumar et al. 2013). Specifically, four described AGF or genus-equivalent taxa are present in both host species. This finding contradicts our prediction of marked differences in AGF between the two species. However, caution is warranted in the case of OTU1. Our cluster analyses assigned 71 sequences to this OTU with only one of them originating from an elephant. Yet, despite this possible restriction, forest elephants and forest buffaloes appear to have most AGF genera in common. One possible explanation is that the anaerobic fungal microbiome of the two species is functionally conserved (Muegge et al. 2011, Sharpton 2018). Tropical plants typically are of lower nutritional quality, with higher fibre content and a wide range of secondary compounds compared to temperate plants (Coley & Aide 1991, Coley & Barone 1996, Dal Pizzol et al. 2017). Given that diet is a major driver in evolution, these plant traits might play a pivotal role in conserving AGF community function in distantly related tropical herbivores. Also in line with this argumentation are findings of superior fibrolytic activities in wild and captive, non-domestic compared to domestic animals (Nagpal et al. 2009, Paul et al. 2010). Thus, the AGF harboured by both investigated species might provide crucial functions for digesting tropical plants. To investigate whether other herbivores living in the west of the Congo Basin have similar AGF communities certainly would help to address this question.

It is noteworthy that OTUs uniquely found in elephants and indexed sequences from western lowland gorillas cohabiting with elephants in the



Bai Hokou area (Schulz et al. 2018) form a nested clade within clade II. This result gives strong support to previous findings on the influence of digestive physiology and diet on AGF community structure (Liggenstoffer et al. 2010, Kittelmann et al. 2013, Kumar et al. 2013) as both species are hindgut fermenters and share a great deal of food sources (Blake 2002, Masi 2008).

Differences between the two host species become apparent considering the most prevalent OTU. In buffaloes OTU1 constituted 73% of the number of sequences obtained from the species. For elephants OTU2 made up 83% of the sequences. This result might reflect differences in diet (Bekhuis et al. 2008, Blake et al. 2009, Kittelmann et al. 2013), gastrointestinal physiology or host phylogeny (Liggenstoffer et al. 2010) or a combination of all of the above.

Between host population comparison of AGF communities

Comparison of AGF diversity between the geographically distant populations of the two study species is limited by low numbers of sequences generated from samples from Loango NP. The investigated buffalo populations share only two out of 11 OTUs. Forest elephants of the two populations have just one out of eight OTUs in common. These findings contradict the prediction that distant populations of the same species harbour similar AGF communities. Data generated in this study suggest that geographic distance and differences in habitats could result in differences in AGF community on the species level. This conclusion is in line with predictions of the metacommunity dynamic theory (Leibold et al. 2004) applied to a host microbial communities. Within the framework of this ecological theory hosts and their habitat constitute patches between which microbes are transferred (Hubbell 2001, Costello et al. 2012). With increasing distance between populations of the same host, species migration of microbial taxa via the host becomes less frequent. Taking genetic drift into account these processes lead to distinct microbial pools in distant habitats which ultimately lead to differences in microbial communities between isolated populations of the same host species (Fallani et al. 2010, Lankau et al. 2012). However, more sophisticated investigations are needed to confirm whether differences between populations found in the present study actually reflect reality.

Conclusion

The present study provides the first data on AGF diversity in two sympatric wild African herbivores. Most of the generated OTUs group within undescribed genus-equivalent AGF clades or cannot be associated with known AGF genera. These OTUs might be representatives of putatively new AGF taxa. Two of which might be characteristic for herbivores in the central West African region while others have a wide distribution in domestic and wild animals. Such an assumption is valid taking into consideration that finding new AGF taxa in wild herbivores is not unlikely (Nicholson et al. 2010, Hanafy et al. 2020a). This conclusion highlights the need for more studies on AGF diversity in wild animals to increase our knowledge of global AGF diversity and distribution.

Further, environmental factors such as diet and geographic distribution might play a greater role in influencing the AGF community structure than previously thought (Liggenstoffer et al. 2010). Our data suggest that AGF community composition in wild tropical animals might be functionally conserved rather than being determined by host phylogeny and gut morphology, as elephants and buffaloes share many AGF taxa which might be crucial for digesting tropical plants. The surprising result that the two distant populations of the hosts have very few OTUs in common raises questions about the importance of geographical distribution of populations. This finding again underlines the importance of studying wild animals in order to address questions about host-related and environmental factors that shape the AGF community.

Finally, this study shows that preservation of faecal samples in ethanol at ambient temperatures is a suitable method to obtain material for AGF surveys in wild animals. This result paves the way for future studies that include recently developed and more reliable DNA markers, namely the D1/D2 region (Hanafy et al. 2020b) combined with modern methods such as NGS, as well as -omics based approaches. Such data can bring crucial insights on host-gut microbiota co-evolution. Further, this information might even help in understanding the resilience of endangered animals to human disturbances (Amato et al. 2013, Barelli et al. 2015) and thus provide crucial information for the application of conservation measurements.



Acknowledgements

We thank the government of the Central African Republic and the *Ministre de l'Education Nationale, de l'Alphabetisation, de l'Enseignement Supérieur, et de la Recherche* for granting permission to conduct our research within the Dzanga-Sangha Protected Areas, Central African Republic. We are very grateful to the World Wildlife Fund and the associated Primate Habituation Project for their administrative and logistical support. We likewise thank the *Centre National de la Recherche Scientifique et Technologique* (permit number N°AR0010/14/MESRS/CENAREST/CG/CST/CSAR) and the *Agence National des Parcs Nationaux* (permit number N°AE140009/PR/ANPN/SE/CS/AEPN) for research authorization in the Loango National Park, Gabon. We thank SFM Safari Gabon for hosting this research and Matthew H. Shirley and Emilie Fairet for their kind assistance with logistics. We also express our deepest gratitude for the invaluable work of the tracker teams and field assistants at each field site

without whom the project would not have been possible. The project was financially supported by the Leakey Foundation (D. Schulz, K.J. Petrželková), by the project CEITEC (Central European Institute of Technology, CZ.1-05/1-1-00/02-0068) from the European Regional Development Fund (D. Modrý), by institutional support of Institute of Vertebrate Biology, Czech Academy of Sciences (RVO: 68081766) (K.J. Petrželková, D. Schulz, B. Červená) and cofinanced from the European Social Fund and the state budget of the Czech Republic (CZ.1-07/2-3-00/20-0300) (D. Schulz, I. Profousová-Pšenková, M.A. Qablan, D. Modrý, K.J. Petrželková). Author contributions: Sample collection of elephant and buffalo faeces in Bai Hokou has been carried out by D. Schulz, T.F. Neba and I. Profousová-Pšenková; sample collection in Loango NP by B. Červená. All laboratory work was done by D. Schulz. Data have been analysed by D. Schulz, M.A. Qablan and M. Procter. Writing the manuscript was done by D. Schulz, M.A. Qablan and K.J. Petrželková with contributions from B. Červená, D. Modrý and M. Procter.

Literature

- Amato K.R., Yeoman C.J., Kent A. et al. 2013: Habitat degradation impacts black howler monkey (*Alouatta pigra*) gastrointestinal microbiomes. *ISME J.* 7: 1344–1353.
- Barelli C., Albanese D., Donati C. et al. 2015: Habitat fragmentation is associated to gut microbiota diversity of an endangered primate: implications for conservation. *Sci. Rep.* 5: 14862.
- Barelli C., Albanese D., Stump R.M. et al. 2020: The gut microbiota communities of wild arboreal and ground-feeding tropical primates are affected differently by habitat disturbance. *mSystems* 5: e00061–20.
- Bauchop T. 1981: The anaerobic fungi in rumen fibre digestion. *Agric. Environ.* 6: 339–348.
- Bekhuis P.D.B.M., De Jong C.B. & Prins H.H.T. 2008: Diet selection and density estimates of forest buffalo in Campo-Ma'an National Park, Cameroon. *Afr. J. Ecol.* 46: 668–675.
- Blake S. 2002: The ecology of forest elephant distribution and its implications for conservation. *PhD thesis, University of Edinburgh, Edinburgh, UK.*
- Blake S., Deem L.S., Mossimbo E. et al. 2009: Forest elephants: tree planters of the Congo. *Biotropica* 41: 459–468.
- Brookman J.L., Mennim G., Trinci A.P.J. et al. 2000: Identification and characterization of anaerobic gut fungi using molecular methodologies based on ribosomal ITS1 and 18S rRNA. *Microbiology* 146: 2.
- Brucker R.M. & Bordenstein S.R. 2013: The hologenomic basis of speciation: gut bacteria cause hybrid lethality in the genus *Nasonia*. *Science* 341: 667–669.
- Callaghan T.M., Podmirseg S.M., Hohlweck D. et al. 2015: *Burwchfawromyces eastonii* gen. nov., sp. nov.: a new anaerobic fungus (Neocallimastigomycota) isolated from buffalo faeces. *MycoKeys* 9: 11–28.
- Cheng Y.F., Edwards J.E., Allison G.G. et al. 2009: Diversity and activity of enriched ruminal cultures of anaerobic fungi and methanogens grown together on lignocellulose in consecutive batch culture. *Bioresour. Technol.* 100: 4821–4828.
- Chung H., Pamp S.J., Hill J.A. et al. 2012: Gut immune maturation depends on colonization with a host-specific microbiota. *Cell* 149: 1578–1593.
- Coley P.D. & Aide T.M. 1991: A comparison of herbivory and plant defences in temperate and tropical broad-leaved forests. In: Price P.W., Lewinsohn T.M., Wilson Fernandes G.W. & Benson W.W. (eds.), *Plant-animal interactions: evolution ecology in tropical and temperate regions. John Wiley & Sons, New York, USA*: 25–49.
- Coley P.D. & Barone J.A. 1996: Herbivory and plant defenses in tropical forests. *Annu. Rev. Ecol. Syst.* 27: 305–335.
- Costello E.K., Stagaman K., Dethlefsen L. et al. 2012: An application of ecological theory toward an understanding of the human microbiome. *Science* 336: 1255–1262.
- Dagar S.S., Kumar S., Griffith G.W. et al. 2015: A new anaerobic fungus (*Oontomyces anksri* gen. nov., sp. nov.) from the digestive tract of the Indian camel (*Camelus dromedarius*). *Fungal Biol.* 119: 731–737.
- Dal Pizzol J.G., Ribeiro-Filho H.M.N., Quereuil A. et al. 2017: Complementarities between grasses and forage legumes from temperate and subtropical areas on *in vitro* rumen fermentation characteristics. *Anim. Feed Sci. Technol.* 228: 178–185.
- Darriba D., Taboada G.L., Doallo R. & Posada D. 2012: jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods* 9: 772.
- Diaz Heijtz R., Wang S., Anuar F. et al. 2011: Normal gut microbiota modulates brain development and behaviour. *Proc. Natl. Acad. Sci. U. S. A.* 108: 3047–3052.
- Doi R.H. & Kosugi A. 2004: Cellulosomes: plant-cell-wall-degrading enzyme complexes. *Nat. Rev. Microbiol.* 2: 541–551.
- Edwards J.E., Kingston-Smith A.H., Jimenez H.R. et al. 2008: Dynamics of initial colonization of nonconserved perennial ryegrass by anaerobic fungi in the bovine rumen: initial colonization of forage by ruminal anaerobic fungi. *FEMS Microbiol. Ecol.* 66: 537–545.
- Fallani M., Young D., Scott J. et al. 2010: Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breastfeeding and antibiotics. *J. Pediatr. Gastroenterol. Nutr.* 51: 77–84.
- Fliegerová K.O., Podmirseg S.M., Vinzelj J. et al. 2021: The effect of a high-grain diet on the rumen microbiome of goats with a special focus on anaerobic fungi. *Microorganisms* 9: 157.
- Fontes C.M. & Gilbert H.J. 2010: Cellulosomes: highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates. *Annu. Rev. Biochem.* 79: 655–681.



- Grenet E., Breton A., Barry P. & Fonty G. 1989: Rumen anaerobic fungi and plant substrate colonization as affected by diet composition. *Anim. Feed Sci. Technol.* 26: 55–70.
- Gruninger R.J., Puniya K., Callaghan T.M. et al. 2014: Anaerobic fungi (phylum Neocallimastigomycota): advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential. *FEMS Microbiol. Ecol.* 90: 1–17.
- Halwachs B., Madhusudhan N., Krause R. et al. 2017: Critical issues in Mycobiota analysis. *Front. Microbiol.* 8: 180.
- Hanafy R.A., Elshahed M.S., Liggenstoffer A.S. et al. 2017: *Pecoramyces ruminantium*, gen. nov., sp. nov., an anaerobic gut fungus from the feces of cattle and sheep. *Mycologia* 109: 231–243.
- Hanafy R.A., Elshahed M.S. & Youssef N.H. 2018: *Feramyces austinii*, gen. nov., sp. nov., an anaerobic gut fungus from rumen and fecal samples of wild Barbary sheep and fallow deer. *Mycologia* 110: 513–525.
- Hanafy R.A., Johnson B., Youssef N.H. & Elshahed M.S. 2020b: Assessing anaerobic gut fungal (Neocallimastigomycota) diversity using PacBio D1/D2 LSU rRNA amplicon sequencing and multi-year isolation. *Environ. Microbiol.* 22: 3883–3908.
- Hanafy R.A., Lanjekar V.B., Dhakephalkar P.K. et al. 2020a: Seven new Neocallimastigomycota genera from wild, zoo housed, and domesticated herbivores greatly expand the taxonomic diversity of the phylum. *Mycologica* 112: 1212–1239.
- Harrison X.A., McDevitt A.D., Dunn J.C. et al. 2021: Host-associated fungal communities are determined by host 1 phylogeny and exhibit widespread associations with the bacterial microbiome. *Proc. R. Soc. Lond. B* 288: 1957.
- Herrera J., Poudel R. & Khidir H.H. 2011: Molecular characterization of coprophilous fungal communities reveals sequences related to root-associated fungal endophytes. *Microb. Ecol.* 61: 239–244.
- Hibbett D.S., Binder M., Bischoff J.F. et al. 2007: A higher-level phylogenetic classification of the Fungi. *Mycol. Res.* 111: 509–547.
- Ho Y.W., Abdullah N. & Jalaludin S. 1988: Penetrating structures of anaerobic rumen fungi in cattle and swamp buffalo. *J. Gen. Microbiol.* 134: 177–181.
- Hubbell S.P. 2001: The unified neutral theory of biodiversity and biogeography. *Princeton University Press, Princeton, USA*.
- James T.Y., Kauff F., Schoch C.L. et al. 2006: Reconstructing the early evolution of fungi using a six-gene phylogeny. *Nature* 443: 818–822.
- Joblin K., Naylor G., Odongo N.E. et al. 2010: Ruminant fungi for increasing forage intake and animal productivity. In: Odongo N.E., Garcia M. & Viljoen G.J. (eds.), Sustainable improvement of animal production and health. *FOA, Rome, Italy*: 129–136.
- Joshi A., Lanjekar V.B., Dhakephalkar P.K. et al. 2018: *Liebetanzomyces polymorphus* gen. et sp. nov., a new anaerobic fungus (Neocallimastigomycota) isolated from the rumen of a goat. *MycKeys* 40: 89–110.
- Katoh K. & Standley D.M. 2013: MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30: 772–780.
- Kingdon J. 1997: African buffalo *Syncerus caffer*. In: Kingdon J. (ed.), The kingdom field guide to African mammals. *Academic Press, London, UK*: 503–504.
- Kittelman S., Naylor G.E., Koolaard J.P. & Janssen P.H. 2012: A proposed taxonomy of anaerobic fungi (class Neocallimastigomycetes) suitable for large-scale sequence-based community structure analysis. *PLOS ONE* 7: 5.
- Kittelman S., Seedorf H. & Walters W.A. 2013: Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLOS ONE* 8: 2.
- Kumar S., Dagar S.S., Sirohi S.K. et al. 2013: Microbial profiles, in vitro gas production and dry matter digestibility based on various ratios of roughage to concentrate. *Ann. Microbiol.* 63: 541–545.
- Kumar S., Stecher G. & Tamura K. 2016: MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33: 1870–1874.
- Lankau E.W., Hong P.Y. & Mackie R.I. 2012: Ecological drift and local exposures drive enteric bacterial community differences within species of Galapagos iguanas. *Mol. Ecol.* 21: 1779–1788.
- Lee S.S., Ha J.K. & Cheng K.-J. 2000: Relative contributions of bacteria, protozoa, and fungi to *in vitro* degradation of orchard grass cell walls and their interactions. *Appl. Environ. Microbiol.* 66: 3807–3813.
- Leibold M.A., Holyoak M., Mouquet N. et al. 2004: The metacommunity concept: a framework



- for multi-scale community ecology. *Ecol. Lett.* 7: 601–613.
- Liggenstoffer A.S., Youssef N.H., Couger M.B. & Elshahed M.S. 2010: Phylogenetic diversity and community structure of anaerobic gut fungi (phylum Neocallimastigomycota) in ruminant and non-ruminant herbivores. *ISME J.* 4: 1225–1235.
- Luna R.A. & Foster J.A. 2015: Gut brain axis: diet microbiota interactions and implications for modulation of anxiety and depression. *Curr. Opin. Biotechnol.* 32: 35–41.
- Masi S. 2008: Seasonal influence on foraging strategies, activity and energy budgets of western lowland gorillas (*Gorilla gorilla gorilla*) in Bai Hokou, Central African Republic. *PhD thesis, Università di Roma, Italy.*
- Muegge B.D., Kuczynski J., Knights D. et al. 2011: Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science* 332: 970–974.
- Nagpal R., Puniya A.K., Griffith G.W. et al. 2009: Anaerobic rumen fungi: potential and applications. In: Khachatourians G.G., Arora D.K., Rajendra T.P. & Srivastava A.K. (eds.), *Agriculturally important microorganisms. World Academic Press, Singapore*: 375–393.
- Nakamura N., Amato K.R., Garber P.A. et al. 2011: Analysis of the hydrogenotrophic microbiota of wild and captive black howler monkeys (*Alouatta pigra*) in Palenque National Park, Mexico. *Am. J. Primatol.* 73: 909–919.
- Nelson T.M., Rogers T.L., Carlini A.R. & Brown M.V. 2012: Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. *Environ. Microbiol.* 15: 1132–1145.
- Nicholson M.J., McSweeney C.S., Mackie R.I. et al. 2010: Diversity of anaerobic gut fungal populations analysed using ribosomal ITS1 sequences in faeces of wild and domesticated herbivores. *Anaerobe* 16: 66–73.
- Orpin C.G. 1975: Studies on the rumen flagellate *Neocallimastix frontalis*. *J. Gen. Microbiol.* 91: 249–262.
- Paul S.S., Bu D., Xu J. et al. 2018: A phylogenetic census of global diversity of gut anaerobic fungi and a new taxonomic framework. *Fungal Divers.* 89: 253–266.
- Paul S.S., Kamra D.N. & Sastry V.R.B. 2010: Fermentative characteristics and fibrolytic activities of anaerobic gut fungi isolated from wild and domestic ruminants. *Arch. Anim. Nutr.* 64: 279–292.
- Ronquist F., Teslenko M., Mark P.V.D. et al. 2012: MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61: 539–542.
- Round J.L. & Mazmanian S.K. 2009: The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9: 313–323.
- Schloss P.D., Westcott S.L., Ryabin T. et al. 2009: Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75: 7537–7541.
- Schulz D., Qablan M.A., Pšenková-Profousová I. et al. 2018: Anaerobic fungi in gorilla (*Gorilla gorilla gorilla*) feces: an adaptation to a high-fiber diet? *Int. J. Primatol.* 39: 567–580.
- Schwab C., Cristescu B., Boyce M.S. et al. 2009: Bacterial populations and metabolites in the feces of free roaming and captive grizzly bears. *Can. J. Microbiol.* 55: 1335–1346.
- Sekirov I., Russell S.L., Antunes L.C.M. & Finlay B.B. 2010: Gut microbiota in health and disease. *Physiol. Rev.* 90: 859–904.
- Sharpton T.J. 2018: Role of the gut microbiome in vertebrate evolution. *mSystems* 3: e00174–17.
- Short J.C. 1983: Density and seasonal movements of the forest elephant (*Loxodonta Africana cyclois Matschie*) in Bia National Park, Ghana. *Afr. J. Ecol.* 21: 175–184.
- Sirohi S.K., Choudhury P.K., Puniya A.K. et al. 2013: Ribosomal ITS1 sequence-based diversity analysis of anaerobic rumen fungi in cattle fed on high fiber diet. *Ann. Microbiol.* 63: 1571–1577.
- Sridhar M., Kumar M., Anandan S. et al. 2007: Occurrence and prevalence of *Cyllumyces* genus – a putative anaerobic gut fungus in Indian cattle and buffaloes. *Curr. Sci.* 92: 1356–1358.
- Sun B.H., Gu Z.Y., Wang X. et al. 2018: Season, age, and sex affect the fecal mycobiota of free-ranging Tibetan macaques (*Macaca thibetana*). *Am. J. Primatol.* 80: e22880.
- Tamura K. 1992: Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Mol. Biol. Evol.* 9: 678–687.
- Theodorou M.K., Mennim G., Davie D.R. et al. 1996: Anaerobic fungi in the digestive tract of mammalian herbivores and their potential for exploitation. *Proc. Nutr. Soc.* 55: 913–926.
- Tuckwell D.S., Nicholson M.J., McSweeney C.S. et al. 2005: The rapid assignment of ruminal

fungi to presumptive genera using ITS1 and ITS2 RNA secondary structures to produce group-specific fingerprints. *Microbiology* 151: 1557–1567.

Wang Y., Youssef N.H., Couger M.B. et al. 2019: Molecular dating of the emergence of anaerobic rumen fungi and the impact of laterally acquired genes. *mSystems* 4: e00247-19.

Supplementary online material

Table S1. Nearest BLAST Hits to uncultured Neocallimastigales clones generated in the study. Species and region of collected samples are indicated by the letters: BH – Bai Hokou, Lo – Lonago NP, _B – buffalo, and _E – elephant.

Table S2. Overview of the distribution of uncultured Neocallimastigales clones among host species across the two different populations.

(<https://www.ivb.cz/wp-content/uploads/JVB-vol.-71-2022-Schulz-et-al.-Table-S1-S2.xlsx>)