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Sympatric western lowland gorillas, central chimpanzees and humans are infected with different trichomonads

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Abstract

We investigated intestinal trichomonads in western lowland gorillas, central chimpanzees and humans cohabiting the forest ecosystem of Dzanga-Sangha Protected Area in Central African Republic, using the internal transcribed spacer (ITS) region and SSU rRNA gene sequences. Trichomonads belonging to the genus *Tetratrichomonas* were detected in 23% of the faecal samples and in all host species. Different hosts were infected with different genotypes of *Tetratrichomonas*. In chimpanzees, we detected tetratrichomonads from 'novel lineage 2', which was previously reported mostly in captive and wild chimpanzees. In gorillas, we found two different genotypes of *Tetratrichomonas*. The ITS region sequences of the more frequent genotype were identical to the sequence found in a faecal sample of a wild western lowland gorilla from Cameroon. Sequences of the second genotype from gorillas were almost identical to sequences previously obtained from an anorexic French woman. We provide the first report of the presence of intestinal tetratrichomonads in asymptomatic, apparently healthy humans. Human tetratrichomonads belonged to the lineage 7, which was previously reported in domestic and wild pigs and a domestic horse. Our findings suggest that the ecology and spatial overlap among hominids in the tropical forest ecosystem has not resulted in exchange of intestinal trichomonads among these hosts.

Research Article

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Introduction

Trichomonads (Excavata: Parabasalia) are anaerobic, flagellated protists, which commonly inhabit the digestive, oral, or urogenital tract of various invertebrate and vertebrate hosts. Most of them are beneficial mutualists or commensals of termites, while only a minority has been described among vertebrates (Čepička *et al*., 2017). The trichomonads that inhabit the intestines of vertebrates are mostly considered commensals, however, notable exceptions exist including *Histomonas meleagridis*, an important pathogen of poultry, and *Dientamoeba fragilis*, whose pathogenic potential for humans is debated (Wong *et al*., 2018). In contrast, trichomonad species that have escaped the intestines into other internal organs (e.g. urogenital or respiratory tract) are often pathogenic for their hosts. The most important extraintestinal trichomonads causing diseases among humans and livestock are *Trichomonas vaginalis* (humans), *T. gallinae* (various birds) and *Tritrichomonas foetus* (cattle) (Honigberg, 1978, 1990). Historically, it was thought that most intestinal trichomonads had a rather limited host range, which is true for species infecting termites (e.g. Tai *et al*., 2015). In contrast, it has been shown that some trichomonad species from vertebrates can infect a broad range of hosts (for examples see Čepička *et al*., 2017). Interestingly, several bird-infecting trichomonads of the genus *Trichomonas* were recognized as close relatives of pathogenic trichomonads in humans including *T. vaginalis* and *T. tenax* (Maritz *et al*., 2014). These results call for further investigation of potentially zoonotic trichomonads in humans and their animal reservoirs.

Although trichomonad species can cause important diseases in humans and livestock, the diversity of trichomonads infecting vertebrates has been largely understudied. This is, surprisingly, true also for non-human primates (NHP), the closest relatives of humans. Particularly, the close phylogenetic relationship between great apes and humans results in high potential of

pathogen exchange (e.g. Calvignac-Spencer *et al.*, 2012). The historical records of trichomonads from NHP, summarized in Smejkalová *et al.* (2012), lack accurate morphological descriptions and had been published prior to the introduction of molecular methods, therefore the identity of these trichomonads remains doubtful. Smejkalová *et al.* (2012) recorded eight or nine trichomonad species in various captive NHP including chimpanzees based on the small subunit ribosomal RNA (SSU rRNA) gene sequences. Later studies confirmed the high prevalence of intestinal trichomonads in wild chimpanzees and other NHP (Rushmore *et al.*, 2015; dos Santos *et al.*, 2017). However, the information on the occurrence of intestinal trichomonads in NHP is still scarce.

In order to understand the diversity and zoonotic potential of primate intestinal trichomonads, we performed non-invasive monitoring of trichomonads in western lowland gorillas, central chimpanzees and humans co-inhabiting Dzanga Sangha Protected Areas (DSPA) in the south-western Central African Republic as part of long-term monitoring focused on pathogen transmission among gorillas and people in the natural tropical forest ecosystem (Sak *et al.*, 2013; Hasegawa *et al.*, 2014; Pafčo *at al.*, 2017, 2018).

Material and methods

Study area

The study was conducted in DSPA, Central African Republic. DSPA is comprised of the strictly protected Dzanga Ndoki National Park (1222 km^2) with restricted human access, and the Dzanga Sangha Dense Forest Special Reserve (3159 km^2), a multiple-use zone in which human activities are differentially controlled (Carroll, 1986; Blom *et al.*, 2001). In 1997, the DSPA launched the Primate Habituation Program (PHP), with the specific aim of habituating western lowland gorillas for tourism and research. There are no permanent inhabitants in the Park, but the Park and PHP employees from several local ethnicities (working as gorilla trackers, assistants and Park ecoguards), foreign researchers and volunteers stay temporarily in the PHP/ecoguard camps and move around the Park. Park and PHP staff live outside of the Park with their families, in the town of Bayanga, and villages of Yandoumbe and Mossapoula. For more information about the research site and sampled hosts see Hasegawa *et al.* (2014) and Sak *et al.* (2013).

Study subjects and sample collection

Ape sampling was carried out between 2007 and 2011 at the two PHP research camps in the Dzanga Sector of the National Park: (i) Bai Hokou, (ii) Mongambe and their surroundings. We collected faecal samples during follows of two habituated gorilla groups (Makumba, Mayele) and early in the morning (7–9 am) in the night nests of two gorilla groups under habituation, three unhabituated gorilla groups and two unhabituated chimpanzee groups. Stool samples from humans were obtained during regular health monitoring checks of Park and PHP employees. All sampled humans self-assessed themselves as healthy with no health problems or clinical signs of any disease. They regularly entered and stayed in the park. An additional sample of goat faeces was obtained from a household in Bayanga given the potential presence of trichomonads in livestock. Samples were either stored in 96% ethanol or (in 2010) inoculated into Dobell and Laidlaw's biphasic medium (Dobell and Laidlaw, 1926) and cultivated at 37 °C after transportation to the camp. Isolates were maintained in xenic cultures by serial transfer every 2nd or 3rd day for three or four times. Cultures were also continuously examined by using a light microscope and when trichomonads were observed, 500 μL of the culture was preserved in 96% ethanol. Faecal samples were fixed in 96% ethanol. Faecal samples and trichomonad cultures were transported to the Department of Zoology of Charles University, Prague, Czech Republic.

DNA isolation, amplification and sequencing

First, faecal samples and cultured isolates were washed three times in sterile phosphate-buffered saline buffer to remove ethanol from the sample. Then, total DNA was extracted from preserved cultured samples using the DNeasy Blood & Tissue Kit (Qiagen) and from faecal samples using the QIAamp DNA Stool Mini Kit (Qiagen). Primers 16Sl (TACTTGGTTGATCCTGCC; Tachezy *et al.*, 2002) and 16SRR (TCACCTACCGTTACCTTG; Cepicka *et al.*, 2005) were used to amplify the SSU rRNA gene of trichomonads. Primers ITSF (TTCAGTTCAGCGGGTCTT CC) and ITSR (GTAGGTGAACCTGCCGTTGG) (Cepicka *et al.*, 2005) were used to amplify the internal transcribed spacer (ITS) region (ITS1-5.8S rRNA gene-ITS2). Polymerase chain reaction (PCR) products were purified using the QIAquick PCR Purification Kit (Qiagen), and were directly sequenced on an ABI Prism 3130 Genetic Analyser (Applied Biosystems).

Phylogenetic analysis

We built two data sets comprising the ITS region (the first data set) and SSU rRNA gene (the second data set) sequences of trichomonads, including newly determined sequences. Sequences were aligned using MAFFT (Katoh *et al.*, 2002) on the MAFFT 7 server https://mafft.cbrc.jp/alignment/server/ with the G-INS-i algorithm at default settings. The alignments were manually edited using BioEdit 7.0.9.0 (Hall, 1999). Final data sets of unambiguously aligned characters consisted of 329 and 1522 positions, respectively, and are available upon request. Phylogenetic trees were constructed using both maximum likelihood (ML) and Bayesian methods. ML analysis was performed in RAxML 8.0.0 (Stamatakis, 2014) under the GTR + I + Γ model, which was for both data sets selected by the Akaike Information Criterion implemented in ModelGenerator v 85 (Keane *et al.*, 2004). Node support was assessed by ML analysis of 1000 bootstrap data sets. Bayesian analysis was performed in MrBayes 3.2.2 (Ronquist *et al.*, 2012) using the GTR + I + Γ + covarion model with four discrete categories. Four MCMCs were run for 3 000 000 and 2 000 000 generations, respectively, until the mean standard deviation of split frequencies based on last 75% of generations was lower than 0.01. The first 25% of trees were removed as burn-in.

Results

In total, 201 faecal samples (151 gorillas, 37 humans, 12 chimpanzees and a single goat) and 63 cultures established from fresh faeces (43 gorillas, 18 humans and two chimpanzees) were examined for the presence of trichomonads. Trichomonads were detected in 23% of the faecal samples using the ITS region amplification and 37% of the cultured samples were microscopically positive for actively swimming trophozoites. We determined sequences of the ITS region from ten chimpanzee samples, 45 gorilla samples, four human samples and one sequence from the goat faeces (Table S1).

All sequences of the ITS region obtained from chimpanzees were identical to the sequence of an isolate PAN6 from a captive chimpanzee (Smejkalová *et al.*, 2012) and clustered within the 'novel lineage 2' as defined by Smejkalová *et al.* (2012) (Fig. 1), which mostly comprises isolates from chimpanzees (Smejkalová

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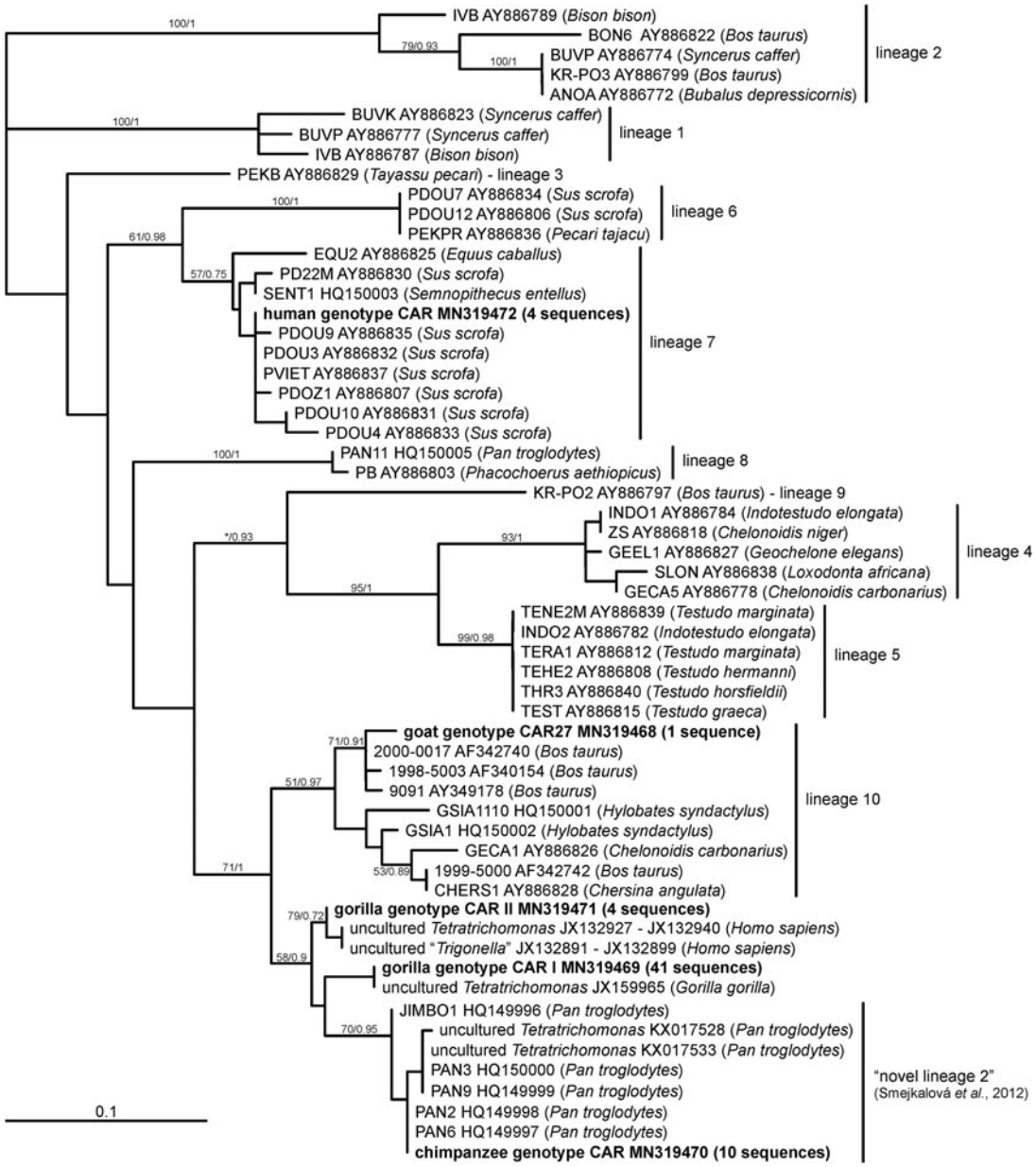

Fig. 1. Phylogenetic tree of the genus *Tetratrichomonas*, based on the ITS region sequences, constructed by the maximum likelihood in RAxML (GTR + I + Γ model). Values at branches represent statistical support in bootstrap values (RAxML)/posterior probabilities (MrBayes). Support values below 50/0.50 are not shown. Newly determined sequences are in bold.

et al., 2012; Rushmore *et al.*, 2014). Smejkalová *et al.* (2012) detected this lineage also in two samples of captive primates, which were kept in the same zoo pavilion as chimpanzees who harboured tetratrichomonads from 'novel lineage 2', namely siamang (*Symphalangus syndactylus*) and hanuman monkey (*Semnopithecus entellus*); but only the SSU rRNA gene sequence is available from them. Sequences of the ITS region of trichomonads from gorilla samples formed two genotypes. The most prevalent one, here referred to as the gorilla genotype CAR I, was represented by 41 sequences that were identical to the GenBank sequence JX159965 obtained from a faecal sample of a wild gorilla in Cameroon (Hamad *et al.*, 2014). The second less prevalent genotype, here referred to as the gorilla genotype CAR II, was represented by four sequences that shared a 99% similarity with the GenBank sequence JX132891 labelled as '*Trigonella* environmental sample', obtained from a French human patient suffering from anorexia nervosa (Gouba *et al.*, 2014). A mixed infection of both genotypes in a single sample from a habituated gorilla was revealed by the presence of double peaks in the chromatogram. The phylogenetic analysis (Fig. 1) showed that these two genotypes present in gorillas were not directly related to each other but formed an unsupported clan with the 'novel lineage 2' composed mostly of sequences from chimpanzees. The ITS sequence from the goat sample shared 99% similarity with the sequence of isolate *Tetratrichomonas* sp. '2000-0017', which was obtained from the prepuce of a domestic bull (Walker *et al.*, 2003) and branched within the *Tetratrichomonas* lineage 10 as defined by Cepicka *et al.* (2006), containing trichomonads from cattle, tortoises and captive siamangs. All four ITS region sequences obtained from human samples were identical to the sequence of isolate PDOU3 from a wild boar obtained in the Czech Republic (Cepicka *et al.*, 2006), and were placed to the *Tetratrichomonas* lineage 7, which contained also isolates from pigs, horses and captive langurs.

We determined the SSU rRNA gene sequences of trichomonads from three gorillas, two chimpanzees and one human (Fig. 2). The analysis confirmed the phylogenetic position of chimpanzee trichomonads (*Tetratrichomonas* 'novel lineage 2'),

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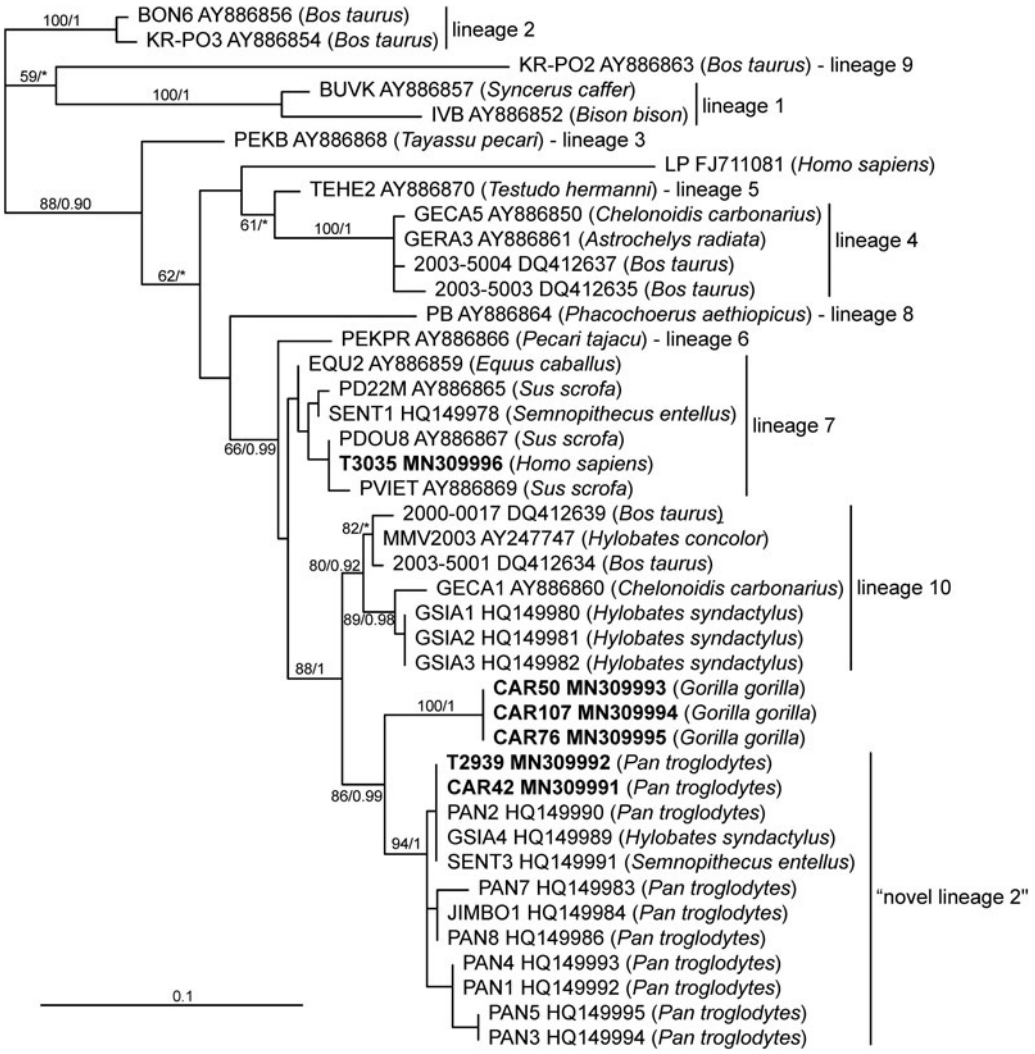

Fig. 2. Phylogenetic tree of the genus *Tetratrichomonas*, based on the SSU rRNA gene sequences, constructed by the maximum likelihood in RAxML (GTR + I + Γ model). Values at branches represent statistical support in bootstrap values (RAxML)/posterior probabilities (MrBayes). Support values below 50/0.50 are not shown or are represented by an asterisk (*). Newly determined sequences are in bold.

human trichomonads (*Tetratrichomonas* lineage 7) as well as the position of the more abundant trichomonad genotype from gorillas (i.e. the gorilla genotype CAR I). Gorilla isolates CAR76 and CAR107, from which ITS sequences were not available, had identical SSU rRNA gene sequences with the isolate CAR50. The newly determined sequences are deposited in GenBank under accession numbers MN309991–MN309996 and MN319468-MN319472.

Discussion

Intestinal trichomonads of NHP are poorly researched, limiting our understanding of their zoonotic and pathogenic potential. One reason for the limited knowledge to date may be because many trichomonad species do not produce cysts (see Čepička *et al*., 2017). Thus, standard coproscopic methods are not suitable for their detection. Trichomonads in general can be diagnosed based on the presence of motile trophozoites in fresh faeces, but this method does not allow accurate identification. Cultivation-dependent methods are also useful for the diagnosis of intestinal trichomonads (in NHP: Smejkalová *et al*., 2012). Particular species can be differentiated by a detailed morphological study or by an analysis of the SSU rRNA gene or ITS region sequences (Cepicka *et al*., 2006, 2010). Only a few studies on trichomonads in NHP have implemented such molecular approaches (Smejkalová *et al*., 2012; Rushmore *et al*., 2015; dos Santos *et al*., 2017).

Our results showed that intestinal trichomonads are a common part of the gastrointestinal microbiota of wild central chimpanzees and western lowland gorillas. For the first time, we also detected intestinal tetratrichomonads in apparently healthy humans (but see Gouba *et al*., 2014). All trichomonads detected in this study belonged to the genus *Tetratrichomonas* (specifically, group A *sensu* Cepicka *et al*., 2006); however, isolates from various hosts occupied different phylogenetic positions within this genus. *Tetratrichomonas* is one of the largest genera of intestinal trichomonads and its species have been found in a broad range of vertebrates and invertebrates (e.g. Cepicka *et al*., 2006; Smejkalová *et al*., 2012; Ibañez-Escribano *et al*., 2013). At least two *Tetratrichomonas* species have also been found in the respiratory tract of humans (Cepicka *et al*., 2005; Kutisova *et al*., 2005; Lopez-Escamilla *et al*., 2013). Although several studies reported transmission of trichomonads between different hosts (see Čepička *et al*., 2017), our results showed that apes and humans inhabiting the forest ecosystem of DSPA are infected with different trichomonad genotypes, with some likely being host-specific. We also obtained the first sequence data of tetratrichomonads isolated from a domestic goat; the phylogenetic analysis showed that the goat trichomonads were distinct from those obtained from humans and NHP. Our results indicate that the ecology and the

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level of spatial overlap among great apes and humans in the studied tropical ecosystem do not suggest exchange of intestinal non-cyst-forming trichomonads. Trichomonads are likely to require closer contact between hosts to allow cross-species transmission, even though two of studied groups of gorillas are habituated to human presence – meaning they are frequently exposed to close human observation. Smejkalová *et al.* (2012) indicated that the transmission of trichomonads in captive conditions is possible, particularly in the zoo environment, as animals can be in close proximity to other reservoir hosts.

All trichomonad-positive chimpanzees from DSPA were infected with tetratrichomonads from 'novel lineage 2' as defined by Smejkalová *et al.* (2012). Tetratrichomonads from the same lineage were recorded in faecal samples of wild chimpanzees from Kibale NP, Uganda (Rushmore *et al.*, 2015) and in all but one, isolates from captive chimpanzees from two Czech zoos by Smejkalová *et al.* (2012). Smejkalová *et al.* (2012) detected this lineage also in one sample of siamang (*Symphalangus syndactylus*) and one of hanuman monkey (*Semnopithecus entellus*). Those animals were kept in Ostrava Zoo in the same pavilion as the chimpanzees who harboured tetratrichomonads from 'novel lineage 2'. We conclude that chimpanzees are the main hosts and those trichomonads are able to persist in captive populations, but in zoo environment they can be transmitted from chimpanzees to other primate species.

Gorillas were infected by two different genotypes of *Tetratrichomonas*. Both genotypes are related to the chimpanzee 'novel lineage 2' as defined by Smejkalová *et al.* (2012). The ITS region sequence of the more frequent gorilla genotype is identical to the one identified by Hamad *et al.* (2014) in a faecal sample of a wild western lowland gorilla in south-central Cameroon; this tetratrichomonad thus may be specific for this host species. Hamad *et al.* (2014) identified this tetratrichomonad as *T. buttreyi*, however, according to Cepicka *et al.* (2006), *T. buttreyi* belongs to the tetratrichomonad lineage 7.

The second gorilla genotype was found only in four gorilla samples (two samples from the habituated groups and two from a group under habituation ranging nearby Bai Hokou). This genotype is almost identical to a sequence obtained from a French woman suffering from a severe form of anorexia nervosa with malnutrition (Gouba *et al.*, 2014). As there is no available information regarding how this woman became infected, it is difficult to interpret the occurrence of almost identical trichomonads in wild lowland gorillas.

With exception of the above described case (Gouba *et al.*, 2014), there have been no reports of intestinal tetratrichomonads in humans. We newly report the presence of the tetratrichomonads in stool samples of people of BaAka hunter-gatherer ethnic, employed as gorilla trackers. However, we did not detect tetratrichomonads in field assistants or ecoguards from various Bantu agricultural tribes living mostly in Bayanga. Humans were infected with tetratrichomonads from the lineage 7, which correspond with *T. buttreyi* that has been reported from domestic and wild pigs and a domestic horse in Europe (Cepicka *et al.*, 2006). Domestic pigs are kept only by Bantu inhabitants of Bayanga, while BaAka people do not keep any livestock. There are two species of wild suids occurring in DSPA, the red river hog (*Potamochoerus porcus*), and the giant forest hog (*Hylochoerus meinertzhageni*). Further studies in DSPA should focus on examination of more sympatric mammals including suids.

Interestingly, we did not detect *Pentatrichomonas hominis*, a well-known intestinal trichomonad of various mammals including captive NHP and humans (Wenrich, 1944; Flick, 1954; Reardon and Rininger, 1968; Myers and Kuntz, 1972; Smejkalová *et al.*, 2012; Li *et al.*, 2016), in any of examined hosts in DSPA. This raises a possibility that in the natural conditions intestinal ecosystems of hominids including humans keeping traditional hunter-gather lifestyle are inhabited by tetratrichomonads. Subsequently, the transition from traditional to modern, agricultural, or western-like lifestyles in humans and captive condition in NPH might have resulted in a gradual decline of tetratrichomonads and acquisition of *P. hominis* from reservoir hosts. Very old studies using traditional methods (for both parasitology and microbiology) found interesting associations among trichomonads, diet and bacteria (Ratcliffe, 1928), however those studies have been completely neglected by scientific community. A recent study on microbiome of coexisting BaAka and Bantu in DSPA has revealed that their distinct gut microbiomes reflect gradients of traditional subsistence strategies and comparison with US Americans suggests that agriculture and industrialization triggered the loss of traditional microbes (Gomez *et al.*, 2015). Clayton *et al.* (2016) showed that captivity has a parallel effect on the NHP gut microbiome to that of westernization in humans. Moreover, industrialization is also correlated with a nearly complete disappearance of intestinal parasites in humans and the lower bacterial diversity in industrialized countries could be partially an indirect consequence of the disappearance of gut eukaryotes (Chabé *et al.*, 2017; Rowan-Nash *et al.*, 2019). In the light of our findings and our knowledge on hominids' microbiomes, we suggest that further research on trichomonads in humans with different lifestyles and also wild and captive NHP should be conducted together with analyses of their microbiomes.

Our study has shown that hominids sharing the DSPA ecosystem harbour different lineages of tetratrichomonads. Based on our results and previous studies (Smejkalová *et al.*, 2012; Hamad *et al.*, 2014; Rushmore *et al.*, 2015), we suggest that tetratrichomonads detected in chimpanzees as well as the more prevalent genotype of gorilla tetratrichomonads are host-specific. However, the first detection of tetratrichomonads of the lineage 7 in humans, previously reported mostly from pigs, opens further questions about possible transmission of intestinal trichomonads among phylogenetically distant hosts inhabiting the DSPA. Screening of a wider host spectrum is needed to better understand the epidemiology of those neglected protists. Moreover, further studies examining trichomonads in wild (not only captive) NHP, other wildlife, livestock and humans with different lifestyles are warranted in order to increase our knowledge about their biology and diversity.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182019001343.

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Conflicts of interest. None.

Ethical standards. The research complied with the legal requirements of the Central African Republic and adhered to the research protocol of DSPA. All sample collection from humans was approved by the Anthropology Department Research Ethics and Data Protection Committee; University of Durham, U.K. Verbal non-recorded consent was obtained from all examined persons and the samples were anonymized. We also obtained verbal non-recorded permission from goat owners to collect animal faecal samples. The collection of faecal samples from gorillas, chimpanzees and livestock was non-invasive and did not cause any observable distress to the animals. Sample importation to the EU was approved by the State Veterinary Authority of the Czech Republic.

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