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RESEARCH ARTICLE

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Identification of potentially zoonotic parasites in captive orangutans and semi-captive mandrills: Phylogeny and morphological comparison

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Abstract

Cysts and trophozoites of vestibuliferid ciliates and larvae of *Strongyloides* were found in fecal samples from captive orangutans *Pongo pygmaeus* and *P. abelii* from Czech and Slovak zoological gardens. As comparative material, ciliates from semi-captive mandrills *Mandrillus sphinx* from Gabon were included in the study. Phylogenetic analysis of the detected vestibuliferid ciliates using ITS1-5.8s-rRNA-ITS2 and partial 18S ribosomal deoxyribonucleic acid (rDNA) revealed that the ciliates from orangutans are conspecific with *Balantioides coli* lineage A, while the ciliates from mandrills clustered with *Buxtonella*-like ciliates from other primates.

Abbreviations: BI, Bayesian inference; BLAST, Basic Local Alignment Search Tool; CIRMF, Centre Interdisciplinaire de Recherches Médicales de Franceville; GI, gastrointestinal; HVR, hypervariable regions; ITS, internal transcribed spacer; ML, maximum likelihood; PSD, pairwise sequence distance.

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Morphological examination of the cysts and trophozoites using light microscopy did not reveal differences robust enough to identify the genera of the ciliates. Phylogenetic analysis of detected L1 larvae of *Strongyloides* using partial *cox1* revealed *Strongyloides stercoralis* clustering within the *cox1* lineage A infecting dogs, humans, and other primates. The sequences of 18S rDNA support these results. As both *B. coli* and *S. stercoralis* are zoonotic parasites and the conditions in captive and semi-captive settings may facilitate transmission to humans, prophylactic measures should reflect the findings.

KEYWORDS

Balantioides coli, *Buxtonella*-like, *Mandrillus sphinx*, molecular phylogeny, *Pongo abelii*, *Pongo pygmaeus*, semi-captive animals, *Strongyloides stercoralis*, zoo animals

1 | INTRODUCTION

Significant numbers of the gastrointestinal (GI) parasites reported in primates in both captive and free-ranging populations are potentially zoonotic (Islam et al., 2022; Labes et al., 2011; Sak et al., 2013). The close phylogenetic relationship between humans (*Homo sapiens*) and other primates further escalates the risk of potential pathogen transmission (including GI parasites). Such transmission occurs easily in conditions of close human-other primates contact, especially in captivity (Keita et al., 2014). In the wild, the risk of primate infection by pathogens originating from humans increases with increasing human pressure (Dunay et al., 2018; Kooriyama et al., 2013). Proximity to other animal species in captive settings may facilitate transmission of parasites with low host specificity and these animals may serve as reservoirs of infection for primates (Modrý & Escalante, 2018). Additionally, limited space in captive settings leading to close contact with animals creates opportunities for parasite transmission (Sanchez & Pich, 2018).

Vestibuliferid ciliates, traditionally referred as *Balantidium coli*, are highly prevalent GI parasites in captive orangutans (*Pongo pygmaeus*, *P. abelii*) (Nurcahyo et al., 2017). Until recently, the vestibuliferid ciliates (Ciliophora: Litostomatea: Vestibuliferida) of primates were all treated as a single species, *B. coli* (Malmstein, 1857), recently reclassified to the genus *Balantioides* (Chistyakova et al., 2014). However, Pomajbíková et al. (2013) confirmed the occurrence of another *Buxtonella*-like vestibuliferid ciliate species in primates. Using molecular tools, *Balantioides coli* has been detected in captive hamadryas baboons (*Papio hamadryas*), captive macaques (*Macaca fascicularis*, *M. mulatta*), captive African great apes (*Gorilla gorilla*, *Pan troglodytes*, *P. paniscus*) and free-ranging mountain gorillas (*Gorilla beringei beringei*) (Barbosa et al., 2017; Hassell et al., 2013; Pomajbíková et al., 2013). Other studies, including reports on orangutans revealed the presence of *B. coli* based on microscopic examination only (Kuze et al., 2010; Mul et al., 2007; Setchell et al., 2007; Supriadi et al., 2012) and the exact taxonomic determination may be incorrect. In contrast, *Buxtonella*-like ciliates have never been reported in apes using molecular analyses, but only in primates of the family Cercopithecidae (Pomajbíková et al., 2013; Yan et al., 2018). Molecular analyses of vestibuliferid ciliates traditionally relied on amplification of 18S ribosomal deoxyribonucleic acid (rDNA) and internal transcribed spacer (ITS) (Barbosa et al., 2017; Norman Grim et al., 2015; Pomajbíková et al., 2013; Ponce-Gordo et al., 2011). While the 18S rDNA gene reveals basic classification and relatedness of taxa on a higher level (Wright et al., 1997), the ITS regions is a highly polymorphic marker suitable for determining the species and evaluating intraspecific diversity (Pomajbíková et al., 2013; Ponce-Gordo et al., 2011).

Balantioides coli is classified as an intestinal commensal (Schuster & Ramirez-Avila, 2008), but can be pathogenic under certain conditions and cause clinical symptoms in humans and captive primates (Schovancová et al., 2013; Strait et al., 2012). In primates, infection may be asymptomatic or accompanied by dehydration, bloody or mucous diarrhea, weight loss, anorexia, lethargy, tenesmus, and rectal prolapse (Cockburn, 1948; Strait et al., 2012; Teare & Loomis, 1982). Some cases of balantidiosis can be even fatal (Lankester et al., 2008; Strait et al., 2012). The intensity of infection can be affected by a diet rich in starch (Schovancová et al., 2013) and also by stress, which generally leads to immunosuppression (de Oliveira et al., 2022). *Balantioides coli* is transmitted directly between individuals via the fecal–oral route, but one of the main sources of the infection can be domestic pigs (*Sus scrofa domestica*) and wild boars (*Sus scrofa*) (Schuster & Ramirez-Avila, 2008). Despite some knowledge about *B. coli* in primates, no information is available about whether infections of *Buxtonella*-like ciliates can manifest clinically in primates.

The genus *Strongyloides* (Nematoda: Chromadorea, Rhabditida) includes at least 50 species of parasitic rhabditid nematodes (Speare, 1989). *Strongyloides stercoralis*, *S. fuelleborni*, *S. cebus*, and several unidentified *Strongyloides* spp. are known to infect primates; however, the species greatly differ in their host spectrum (Bradbury et al., 2021). While *S. stercoralis* has a broad host specificity and has been detected in a range of hosts including humans, carnivores, and (mainly captive) primates, *S. fuelleborni* is restricted to primates (mostly free-ranging) with occasional spillover to humans

(Bradbury et al., 2021). Studies using genotyping concluded that *S. stercoralis* likely originated in dogs (*Canis lupus familiaris*) and later adapted to human and other primate hosts (Barratt et al., 2019; Jaleta et al., 2017). The most commonly used markers for molecular analysis of *Strongyloides* include two hypervariable regions (HVR) of the 18S rDNA gene (HVR-I and HVR-IV) and a selected portion of the mitochondrial cytochrome c oxidase subunit 1 gene (*cox1*). These markers have been compared in different hosts and geographic regions, and haplotypes of the HVR-IV of 18S rDNA have been found to match *cox1* haplotypes (Bradbury et al., 2021). A few studies have used 28S rDNA and parts of ITS genes for *Strongyloides* detection (e.g., Labes et al., 2011; Solórzano-García & Pérez-Ponce de León, 2017), but the number of such sequences is rather small and therefore these markers are usually not used for further analyses.

Strongyloides infection can be asymptomatic, while uncomplicated disease is manifested by GI, pulmonary, and dermatological symptoms. In immunocompromised human patients, infection can result in a serious systemic disease with fatal consequences (Hasegawa & Pafčo, 2018; Nutman, 2017). There are no reports on the clinical outcomes of *Strongyloides* infections in free-ranging apes or other primate species, but reports describe fatal cases in captive animals (Hasegawa & Pafčo, 2018; Strait et al., 2012). Kleinschmidt et al. (2018) reported fatal strongyloidiasis in a 5-month-old Sumatran orangutan (*P. abelii*). A fatal course of infection has been also reported in young chimpanzees (*P. troglodytes*) and gorillas (*G. gorilla*) (Penner, 1981). Orangutans below the age of 5 years appear to be the most prone to clinical disease (Labes et al., 2011; McClure et al., 1973; Uemura et al., 1979).

Our study aimed to survey a spectrum of parasites found in captive Bornean orangutans (*P. pygmaeus*) and Sumatran orangutans (*P. abelii*) in Czech and Slovak zoological gardens. Special attention was paid to potentially zoonotic GI parasites, specifically *Strongyloides* spp. and vestibuliferid ciliates. We further determined if the orangutans were infected with zoonotic vestibuliferid ciliates *B. coli* or probably nonzoonotic *Buxtonella*-like ciliates and identified *Strongyloides* species/haplotypes. As comparative material for the vestibuliferid ciliates, we used fecal samples from semi-free ranging mandrills (*Mandrillus sphinx*) from Gabon. First, we predict that the dominant detected GI parasites in studied captive orangutans from Czech and Slovak zoological gardens are nematodes of the genus *Strongyloides* and vestibuliferid ciliates, since these parasites were most frequently found in captive orangutans (Nurcahyo et al., 2017). Second, we assumed that potentially zoonotic *B. coli* and *S. stercoralis* are present in captive orangutans studied, since these species have already been identified in other captive primates using molecular analyses (Nurcahyo et al., 2017; Pomajbíková et al., 2013). Last, if wild primates of the family Cercopithecidae harbor potentially nonzoonotic *Buxtonella*-like ciliates (Pomajbíková et al., 2013; Yan et al., 2018), we predict that these ciliates might be present in semi-free living mandrills. Detection and exact identification of parasites in captive settings is important, as unattended infections can cause serious health problems or even death in mostly young, old and weak individuals (Sanchez & Pich, 2018). Moreover, parasites with zoonotic potential may pose a risk to keepers and other staff working with captive animals. Detailed information about the parasites of captive animals is crucial to evaluate their impact on the health of free-ranging individuals, which is necessary for good conservation management.

2 | METHODS

2.1 | Animal ethics statement

Fecal samples from captive orangutans were obtained via a formal agreement with the zoo management, mandrill samples were obtained during routine health examinations. Noninvasive sample collection was accomplished following individual national regulations or guidelines for animal care. Each facility received the results of the parasitological examination. The research adhered to the American Society of Primatologists Principles for the Ethical Treatment of Nonhuman Primates.

2.2 | Sampling and coproscopic examination

Individual fresh fecal samples ($N = 14$) were collected from captive Bornean orangutans (*P. pygmaeus*) ($N = 7$), Sumatran orangutans (*P. abelii*) ($N = 6$), and one hybrid housed in Czech and Slovak zoological gardens by the zookeepers in March-July 2019. Animal health is monitored, and any clinical signs were recorded. Individual fecal samples ($N = 6$) from semi-free ranging mandrills (*M. sphinx*) housed at the Centre Interdisciplinaire de Recherches Médicales de Franceville (CIRMF) in Gabon were collected as a comparative material for analyses targeting the vestibuliferid ciliates in June–July 2017. A portion of each orangutan and mandrill fecal sample was divided and stored in two preservation media: 10% formalin (orangutan and mandrill samples), 96% ethanol (orangutan samples), and 90% ethanol (mandrill samples), while a third portion of the same orangutan sample was kept fresh for later analyses. All samples were then shipped to the laboratories of the Department of Pathology and Parasitology, University of Veterinary Sciences Brno, Czech Republic. Part of the fresh individual orangutan sample was examined by Baermann larvoscopy to collect *Strongyloides* larvae and another part of the same sample fixed in formalin was examined microscopically using modified Sheather's flotation and formalin-ether sedimentation method for detection and identification of GI parasites (Jirků-Pomajbíková & Hůzová, 2018; Pafčo, 2018). Cysts and trophozoites of vestibuliferid ciliates were measured under a light microscope (Olympus BX41) at 400 × magnification and photographed using Olympus AX70 with Nomarski differential contrast (Olympus, headquarters). Samples positive for vestibuliferid ciliates and *Strongyloides* sp. were selected for further DNA barcoding and phylogenetic analyses.

2.3 | DNA isolation, PCR, sequencing, and cloning

Fecal samples microscopically positive for vestibuliferid ciliates (orangutans $N = 4$, mandrills $N = 6$) preserved in ethanol were dried

overnight at 37°C before DNA isolation to evaporate the ethanol. The total DNA was extracted using DNeasy PowerSoil Kit (QIAGEN) following the manufacturer's protocol. The partial 18S rDNA gene (1270 bp) of the ciliates was amplified using EukA forward and SSUrBB reverse primers (Norman Grim et al., 2015) using Emerald AMP polymerase (Takara), while the ITS1-5.8s-rRNA-ITS2 region (ITS; 400 bp) was amplified using B5D forward and B5RC reverse primers (Ponce-Gordo et al., 2011) using Top Bio PPP polymerase (Top-Bio, s.r.o.). The total volume of 25 µL PCR mixture for 18S rDNA contained 12.5 µL polymerase, 1.25 µL of 10 µM each primer. The total volume of PCR mixture for ITS was the same and contained 12.5 µL polymerase and 1 µL of 10 µM each primer. Template DNA volume was 2 µL.

Individual *Strongyloides* larvae ($N = 15$) were collected from orangutan fecal samples ($N = 4$) after the Baermann larvoscopy method (Pafčo, 2018), placed in microcentrifuge tubes and frozen. Invisorb® Spin Forensic Kit (STRATEC) was used to isolate DNA from individual *Strongyloides* larvae following the manufacturer's protocol. Three genetic markers were selected for amplification: *cox1* and two HVR of 18S rDNA (HVR-I and HVR-IV) according to Barratt et al. (2019). Two primer pairs: TJ5207 forward and TJ5208 reverse (Jaleta et al., 2017) and SPPcox1F forward and SPPcox1R reverse (Barratt et al., 2019) were used for amplification of 650 and 217 bp of *cox1*, respectively. The total volume of 25 µL PCR mixture for *cox1* contained 12.5 µL Top Bio PPP polymerase (Top-Bio, s.r.o.), 0.5 µL of 10 µM each primer (TJ5207, TJ5208) or 12.5 µL PrimeStar polymerase (Takara) with 1.25 µL of 10 µM each primer (SPPcox1F, SPPcox1R). Template DNA volume was 2 µL. Two 18S rDNA HVR were amplified using primer pairs New HVRI F forward and New HVRI R reverse (HVR-I, 434 bp) and New HVR-IV F forward and New HVRIV R reverse (HVR- IV, 255 bp) (Barratt et al., 2019) using PrimeStar polymerase (Takara). The total volume of 25 µL PCR mixture for HVR-I contained 12.5 µL PrimeStar polymerase (Takara), 1.5 µL of 10 µM each primer. For HVR-IV, the total volume of PCR mixture was the same and contained 12.5 µL PrimeStar polymerase (Takara) and 0.5 µL of 10 µM each primer. Template DNA volume was 2 µL.

Amplification of all targeted *Strongyloides* and vestibuliferid ciliates markers was performed using Biometra T-personal thermocycler (Schoeller) under conditions specified in Table 1. The PCR products were separated by electrophoresis in a 1.5% agarose gel with Midori Green Advance (Nippon Genetics) and visualized on a UV transilluminator. The PCR products of the expected size were purified either directly or after cutting the band from the gel using Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech). All amplicons were Sanger sequenced in both directions commercially by Macrogen Europe (Amsterdam, Netherlands). If we detected mixed signals, the PCR product was cloned using pGEM® -T Easy Vector Systems (Promega Corporation) and GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). The resulting cloning products were also sent for Sanger sequencing. All obtained sequences were checked and manually trimmed in Geneious 9.1.5 (www.geneious.com) and the identity was checked using BLAST (Altschul et al., 1990).

2.4 | Phylogenetic analyses

All sequences obtained were aligned with sequences from GenBank using Muscle alignment in Geneious 9.1.5 (Kearse et al., 2012). For the 18S rDNA of vestibuliferid ciliates, the alignment (1629 bp) consisted of seven sequences obtained during the present project and

TABLE 1 PCR primers and reaction conditions.

Primers	Sequence 5′→ 3′	Reaction conditions	Region of gene	Parasite	References
EukA	AACCTGGTTGATCCTGCCAGT,	10 min at 94°C; 35 cycles of 1 min at 94°C, 90 s at 54.9°C, 90 s at 72°C; and 5 min at 72°C.	18S rDNA	Vestibuliferid ciliates	Norman Grim et al. (2015)
SSUrBB	AAATACATAGTCCCTCTAAGAAGTC				
B5D	GCTCCTACCGATACCGGGT,	10 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 64°C, 1 min at 72°C; and 5 min at 72°C.	ITS1-5.8s-rRNA-ITS2	Vestibuliferid ciliates	Ponce-Gordo et al. (2011)
B5RC	GCGGGTCATCTTACTTGATTTC				
TJ5207	TTTGATTGTTACCTGCTTCTATTTT	2 min at 94°C; 35 cycles of 20 s at 94°C, 15 s at 50°C, 90 s at 72°C; and 7 min at 72°C	*cox1*	*Strongyloides* sp.	Jaleta et al. (2017)
TJ5208	TTTTACACCAGTAGGAACAGCAA				
SPPcox1F	TTTGATCCTAGTTCTGGTGGTAATCC	2 min at 98°C; 45 cycles of 10 s at 98°C, 10 s at 63°C, 40 s at 72°C; and 4 min at 72°C	*cox1*	*Strongyloides* sp.	Barratt et al. (2019)
SPPcox1R	GTAGCAGCAGTAAAATAAGCACGAGA				
HVRI F	GCTCATTATAACAGCTATAGACTACACGGTA		18S rDNA (HVR-I)	*Strongyloides* sp.	Barratt et al. (2019)
HVRI R	CCACAACAATCATTTTATGCACTTGG				
HVRIV F	CGGGCCGGACACTATAAGG		18S rDNA (HVR-IV)	*Strongyloides* sp.	Barratt et al. (2019)
HVRIV R	ATCTCTAAACAGGAACATAATGATCACTAC				

Abbreviations: HVR, hypervariable region; ITS, internal transcribed spacer; PCR, polymerase chain reaction; 18S rDNA, ribosomal deoxyribonucleic acid.

82 sequences from Genbank. *Epispathidium papilliferum* (DQ411857) was used as an out-group. The final alignment of vestibuliferid ciliates ITS (534 bp) region sequences consisted of 11 newly obtained sequences and 94 sequences from the Genbank database representing genera *Balantioides*, *Buxtonella*, *Buxtonella*-like and *Balantidium* with *Balantidium ctenopharyngodoni* (KU170972) used as an out-group. Nodal support was assessed to 10^6 replicates. The phylogenetic relationships were reconstructed by Bayesian inference (BI) in MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001) with the substitution model GTR + G.

Four *Strongyloides cox1* sequences newly obtained from orangutan fecal samples were aligned with 56 homologue sequences representing the genus *Strongyloides* from GenBank with the length of the final alignment being 722 bp. *Necator americanus* (AJ417719) was used as an out-group. Nodal support was assessed to 10^6 replicates. Phylogenetic relationships were reconstructed by BI and Maximum likelihood (ML) approaches in MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001) and PhyML 3.3.2 (Guindon et al., 2010) programs. Substitution model GTR + G was used as it was selected by Modelgenerator (Keane et al., 2006).

3 | RESULTS

3.1 | Potentially zoonotic parasites detected in captive orangutans

In total, 14 fecal samples of individual orangutans from five Czech and Slovak zoos were microscopically examined. Four samples were negative for any parasites/commensals (Table 2). Cysts and trophozoites of vestibuliferid ciliates, tentatively identified as *B. coli* based on morphology, were found in four orangutan samples. Further, cysts of *Entamoeba coli*, *Chilomastix* sp., and *Giardia* sp. were detected (Table 2). *Entamoeba coli* was the most prevalent parasite, with cysts found in seven samples (50%), although in low quantities. Cysts of *Chilomastix* sp. and *Giardia* sp. were detected in a single individual each (Table 2). Baermann larvoscopy revealed *S. stercoralis* L1 larvae in four orangutan fecal samples. Both *B. coli* and *S. stercoralis* were detected in three samples, while two orangutans were each infected with *S. stercoralis* or *B. coli* only.

3.2 | No clinical signs related to parasitic infections were observed

None of the individuals positive for vestibuliferid ciliates and/or *Strongyloides* and no animals in the group showed any clinical signs at the time of sampling, or long term.

3.3 | Morphology of the vestibuliferid ciliates

Cysts and trophozoites corresponding morphologically to *B. coli* were found in four orangutan samples. Three samples contained only cysts, while both cysts and trophozoites were found in one sample, though trophozoites were in the minority (approx. 10%). The cysts were spherical or slightly subspherical with clearly visible vacuole

TABLE 2 Gastrointestinal parasites detected by microscopical observation of fecal samples of captive orangutans from Czech (CZ) and Slovak (SK) zoos.

Country/location	Sex	Host species/sample ID	*Strongyloides stercoralis*	*Balantioides coli*	*Entamoeba coli*	*Chilomastix* sp.	*Giardia* sp.
CZ/DKnL	F	*Pongo pygmaeus*/1	+	+	+	–	–
	F	*P. pygmaeus*/2	+	+	–	–	–
CZ/UnL	F	*P. pygmaeus*/3	–	–	+	–	–
	F	*P. pygmaeus*/4	–	–	–	–	–
	M	Hybrid/5	–	–	–	–	–
SK/Bojnice	M	*P. pygmaeus*/6	–	–	+	–	–
	F	*P. pygmaeus*/7	–	–	+	–	–
	M	*P. pygmaeus*/8	–	–	–	–	–
CZ/Praha	F	*P. abelii*/9	–	–	+	+	–
	F	*P. abelii*/10	+	–	+	–	+
	M	*P. abelii*/11	–	–	+	–	–
SK/Bratislava	M	*P. abelii*/12	+	+	–	–	–
	F	*P. abelii*/13	–	+	–	–	–
	M	*P. abelii*/14	–	–	–	–	–
Total prevalence			28.6% (4/14)	28.6% (4/14)	50% (7/14)	7.1% (1/14)	7.1% (1/14)

Abbreviations: DKnL, Dvůr Králové nad Labem; UnL, Ústí nad Labem; +, positive sample; –, negative sample.

containing ingested starch (Figure 1a). In total, 98 cysts were measured with a mean length of 50 µm (range 34.2–68.0) and width of 49 µm (range 35.1–70.9). The trophozoites were ovoid with visible somatic cilia and apparent cytostome (Figure 1b). Five intact trophozoites were measured with a mean length of 52 µm (range 45.0–57.5) and a mean width of 45 µm (range 36.8–63.7).

Vestibuliferid ciliates were found in all six mandrill samples examined. Either cysts or trophozoites, or mixture of both were observed. Examined cysts ($N = 98$) were spherical or slightly subspherical and measured a mean of 44 µm (range 26.3–69.4) × 42 µm (range 24.8–66.9). The cysts had a thick transparent wall tightly encompassing the visible trophozoite inside. The somatic ridges on the surface of the encysted trophozoites ran in parallel rows, resembling a fingerprint (Figure 1c). A vacuolar structure was also present centrally or sub-centrally in the majority of the observed cysts. Trophozoites were ovoid, covered by rows of somatic cilia with cytostome localized at the anterior end of the body and contained food vacuoles (Figure 1d). The trophozoites ($N = 33$) measured a mean of 68 µm (range 38.7–105.9) × 40 µm (range 15.6–71.5). When visible, their cytostome measured a mean of 17 µm (range 12.1–19.7) long.

3.4 | Phylogenetic analysis of vestibuliferid ciliates

Seven sequences (1145–1170 bp) of partial vestibuliferid ciliates 18S rDNA were obtained (two from Bornean orangutans, one from Sumatran orangutan, and four from mandrill samples). All sequences from orangutans were identical. In mandrills, one sequence differed by 2.8%. Sequences from orangutans differed from mandrill-derived sequences by 3.3–4.2%.

The resulting BI tree showed 19 separate clades corresponding to individual genera (Figure 2). All the isolates thought to be *B. coli* from orangutans clustered within a highly supported (1.00 BI) sub-clade within the genus *Balantioides* clade comprising mainly isolates from other captive primates: western lowland gorillas (*Gorilla gorilla gorilla*), chimpanzees (*Pan troglodytes*), a bonobo (*P. paniscus*), and a hamadryas baboon, a few sequences from pigs (*Sus scrofa domestica*), from an ostrich (*Struthio camelus*), a common rhea (*Rhea americana*), and a Guinea pig (*Cavia porcellus*) (Figure 2). The other *Balantioides* sub-clade comprised sequences mainly from pigs, an Asian elephant (*Elephas maximus indicus*), a giant anteater (*Myrmecophaga tridactyla*), and a chimpanzee. The pairwise sequence distance (PSD) within the *Balantioides* clade did not reach over 2.1%.

In contrast, the sequences originating from mandrills clustered within a highly supported (0.9 BI) clade comprising only sequences labeled as *Buxtonella*-like and originating from various cercopithecids. One sequence from a mandrill (OM423619) clustered separately from all other isolates in a highly supported (1.00 BI) sub-clade (Figure 2). The sequence differed from the sequences within the major sub-clade by 2.7–3.2%, while the PSD within the major sub-clade was below 0.3%.

Eleven sequences (420–514 bp) of ITS were obtained during the current study (two sequences from Bornean orangutans, five clones of an isolate from a Sumatran orangutan, and four sequences from mandrill samples). All sequences from Bornean orangutans were 100% identical. In the Sumatran orangutan, the cloned sequences differed by 0.2–1.6%. In mandrills, one sequence differed by 6%. Sequences from orangutans differed from mandrill-derived sequences by 8.4–11%. The ITS BI tree topology was similar to that of the 18S rDNA but showed significant intraspecific variability, mainly in the genera *Balantioides* and *Buxtonella*-like. The resulting tree showed four separated clades corresponding to the genera *Balantioides*, *Buxtonella*-like, *Buxtonella*, and *Balantidium* (Figure 2). All isolates from orangutans clustered within a highly supported sub-clade (1.00 BI) corresponding to the sub-clade A *sensu* Ponce-Gordo et al. (2011). This sub-clade A comprises sequences obtained from captive primates, one free-ranging mountain gorilla (HF545448), pigs,

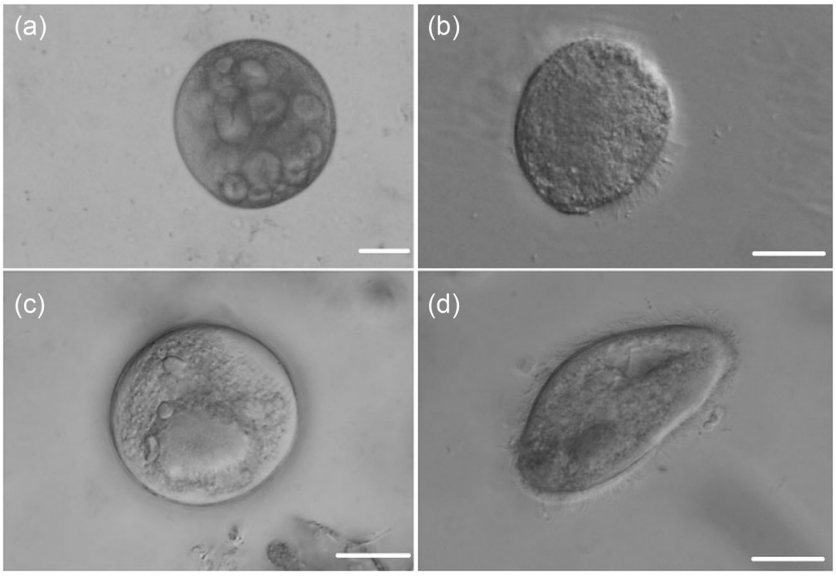

FIGURE 1 Life stages of vestibuliferid ciliates found in formalin-preserved fecal samples from captive orangutans from Czech and Slovak zoos and semi-captive mandrills from Gabon; scale bar = 20 µm. (a) *Balantioides coli* cyst from a Bornean orangutan with clearly visible granule of ingested starch; (b) *B. coli* trophozoite, Bornean orangutan; (c) *Buxtonella*-like cyst from a mandrill with a visible large "vacuole" inside; (d) Trophozoite of *Buxtonella*-like ciliate, mandrill.

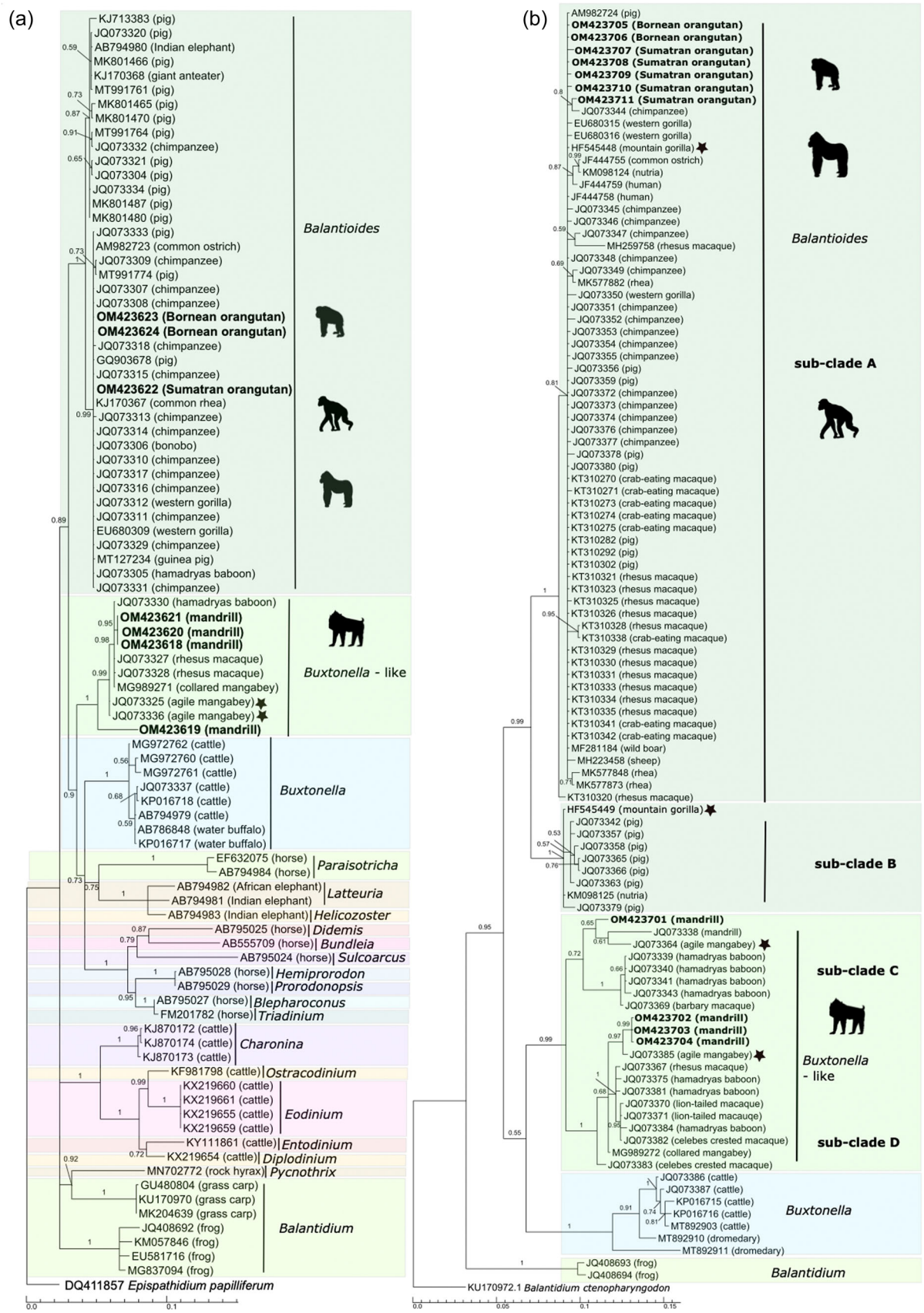

FIGURE 2 Bayesian inference phylogenetic tree inferred from (a) 18S rDNA (1629 bp) and (b) ITS region (534 bp). Both trees were calculated from a muscle-constructed alignment using GTR + G model for nucleotide substitutions. Numbers at the branches indicate Bayesian posterior probability based on 10^6 replicates. Sequences obtained during the current study are in bold. Branch lengths indicate expected numbers of substitutions per nucleotide site. The asterisk indicates free-ranging hosts.

wild boars (*Sus scrofa*), paleognath birds, humans, sheep (*Ovis aries*), and nutria (*Myocastor coypus*). Sub-clade B *sensu* Ponce-Gordo et al. (2011) was further divided by a polytomy and comprised mostly closely clustering sequences from domestic pigs and then rather distant sequences from a nutria, free-ranging mountain gorilla (HF545449) and one more pig sequence. The clades *Balantioides* and *Buxtonella*-like differed by 2.4–5.2%, while the PSD observed among other genera varied between 2.9% and 11.3%.

The four ciliate sequences originating from mandrills clustered with other ciliates from cercopithecids within a highly supported (0.99 BI) clade of *Buxtonella*-like ciliates, which was further divided into two further structured sub-clades which we labeled C and D (Figure 2). Sub-clade C included closely clustering sequences from captive hamadryas baboons and barbary macaque (*Macaca sylvanus*) and rather distant cluster of one of our mandrill-derived sequences (OM423701, originating from the same sample as the different 18S rDNA sequence—OM423619), another sequence from a captive mandrill and a free-ranging agile mangabey (*Cercocebus agilis*) from the Central African Republic. The sequences within sub-clade C differed by 0.4–6.3%. Sub-clade D comprised sequences from captive baboons, captive macaques, captive collared mangabey, and the remaining sequences from our mandrill samples which clustered closely with a sequence from wild agile mangabey (Figure 2). PSD in this sub-clade did not exceed 11.4%, while sub-clades C and D differed by 5–11.1%. All sequences of vestibuliferid ciliates obtained in this study were uploaded to GenBank under the accession numbers OM423618–OM423624 (18S rDNA); OM423701–OM423711 (ITS1-5.8s-rRNA-ITS2) (see Supporting Information: Table S1 for details).

3.5 | Haplotypes and phylogenetic analysis of *S. stercoralis*

Four sequences of partial *cox1* (263–627 bp) and four sequences of each 18S rDNA HVR-I (391–457 bp) and HVR-IV (235–281 bp) were obtained from individual *S. stercoralis* larvae extracted from orangutan fecal samples (two from *P. pygmaeus* and two from *P. abelii*). A BLAST search showed that all four sequences of HVR-IV corresponded to haplotype A in Barratt et al. (2019), while in HVR-I region, haplotype VI was detected in *P. pygmaeus* and haplotype II in *P. abelii*, following Barratt et al. (2019). The two *S. stercoralis cox1* sequences from *P. abelii* were identical, while the two sequences from *P. pygmaeus* differed at a single-nucleotide polymorphis site. The PSD between the *cox1* sequences from the two orangutan species was 2%. Both BI and ML *cox1* phylogenetic trees were well-resolved with highly supported nodes and yielded the same general topology (Figure 3). Clades formed corresponding to individual *Strongyloides* species. All *S. stercoralis* sequences obtained from orangutan fecal samples clustered in the highly supported (1.00 BI) clade corresponding to lineage A *sensu* Jaleta et al. (2017) (highlighted in darker blue in Figure 3) infecting dogs, humans, and other primates. The clade of lineage A was further divided into several sub-clades. The sequences from Bornean orangutans were placed in a different sub-clade than sequences from Sumatran orangutans and these two sub-clades differed by 1.7–3.6%. The PSD within the sub-clades comprising the Bornean orangutan sequences and Sumatran orangutan sequences was below 2.9% and 3.1%, respectively. All sequences of *Strongyloides* obtained in this study were uploaded to GenBank under the accession numbers OM423625–OM423632 (18S rDNA); OM392049–OM392052 (*cox1*) (see Supporting Information: Table S1 for details).

4 | DISCUSSION

Regular monitoring of parasites in captive primates including their molecular identification and genotyping should be an important part of health management, which can lead to reduction of the risk of parasite transmission between humans and other primates. However, genetic analyses are not routinely performed, and surveys focused on parasite infection in captive primates are rarely published and shared with a broad scientific audience. Here, we present a study focused on selected GI parasites with zoonotic potential in orangutans housed in Czech and Slovak zoos and provide important DNA analysis-based data for future research.

Parasites of orangutans are overall much less studied than those of African great apes (Modrý & Escalante, 2018). Captive, semi-captive, and wild orangutans are commonly diagnosed with protists and helminths with relatively low host specificity often in combination with orangutan-specific parasites (Modrý & Escalante, 2018). We detected *Giardia intestinalis*, which is a typical parasite with low host specificity. Infections in primates can occur asymptomatically or with clinical symptoms such as diarrhea or vomiting (Strait et al., 2012) that were not observed during our study. We also detected *E. coli* and *Chilomastix* sp., which are also protists commonly occurring in great apes (Jirků-Pomajbíková & Vlčková, 2018; Kváč & McEvoy, 2018). Both protists occur naturally in primates and are not pathogenic to the host (Jirků-Pomajbíková et al., 2016; Kváč & McEvoy, 2018). However, the most commonly detected GI parasites in captive and free-ranging orangutans are nematodes of the genus *Strongyloides* and vestibuliferid ciliates *B. coli* (Foitová et al., 2009; Nurcahyo et al., 2017), both of which were among the most common parasites in our study, consistent with our first hypothesis.

The common occurrence of ciliates identified as *B. coli* is reported in most of the microscopy-based studies focused on parasitofauna of orangutans (e.g., Kilbourn et al., 2003; Kuze et al., 2010; Labes et al., 2011; Mul et al., 2007). Studies of mandrills, the source of our comparative material, also mention detection of *B. coli* (Poirotte et al., 2016; Setchell et al., 2007). However, recent studies (Chistyakova et al., 2014; Jirků-Pomajbíková & Modrý, 2018; Pomajbíková et al., 2013; Yan et al., 2018) and our results show that at least two taxa of vestibuliferid ciliates, specifically *B. coli* and *Buxtonella*-like ciliates, occur in primates. Due to the lack of clear identification structures on the ciliate's cysts, the two taxa can hardly be distinguished by their morphology. Thus, some of the published

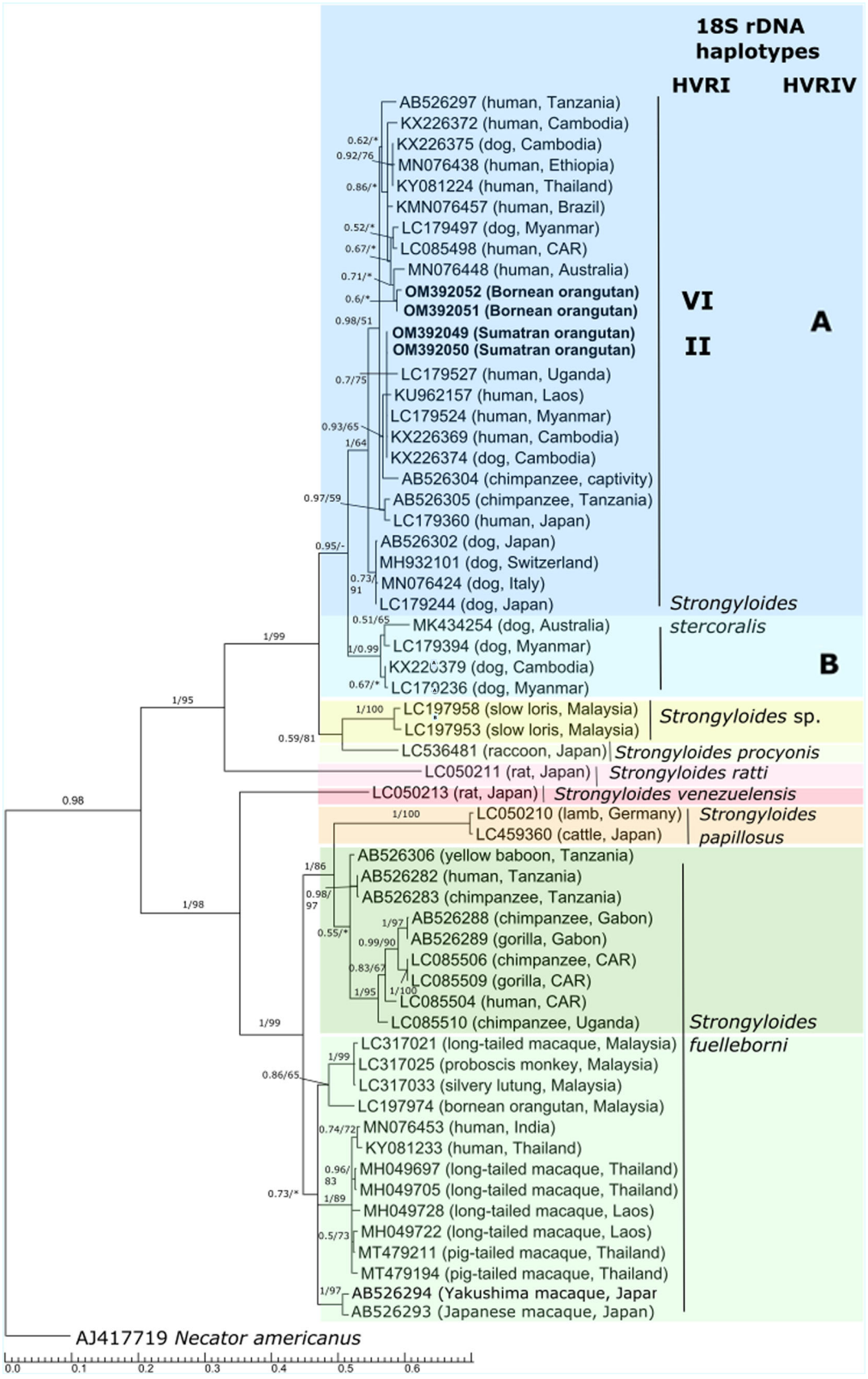

FIGURE 3 (See caption on next page)

records of *B. coli* in primates may represent the latter group of vestibuliferids (*Buxtonella*-like ciliates).

Our phylogenies of the 18S rDNA and ITS regions showed almost identical results and clearly assigned the vestibuliferid ciliates of orangutans in the sub-clade A of *B. coli*, consistent with our second hypothesis. This was previously detected in various primates, including captive African great apes, several captive macaque species (Barbosa et al., 2017; Pomajbíková et al., 2013), and one isolate from a free-ranging mountain gorilla (Hassell et al., 2013), extending the spectrum of this sub-clade hosts to captive Bornean and Sumatran orangutans. In contrast, the ciliates from our comparative samples from mandrills clustered with *Buxtonella*-like ciliates from other cercopithecids, consistent with our last hypotheses.

Although many studies report the occurrence of *B. coli* in captive/semi-captive and free-ranging orangutans (Nurcahyo et al., 2017), supporting molecular data is lacking. Here, we, for the first time, performed molecular-phylogenetic analyses and confirmed the occurrence of *B. coli* in captive Bornean and Sumatran orangutans. In the past, it was impossible to distinguish whether orangutans (including captive, semi-captive, and wild) are infected by *Buxtonella*-like or *B. coli*. *Balantoides coli* is often detected in captive primates, including great apes (Pomajbíková et al., 2010). In contrast, *Buxtonella*-like ciliates seem to be restricted to primates of the family Cercopithecidae both in captivity and in the wild (Pomajbíková et al., 2013; Yan et al., 2018), which is supported by our results for semi-captive mandrills. So far, it appears that *B. coli* is less host-specific and may occur in various primates as well as in humans, while *Buxtonella*-like infects mostly primates other than great apes. Implementation of molecular phylogeny is necessary to clarify the distribution and host specificity of the two vestibuliferid ciliates in wild, semi-captive and captive primate populations.

The occurrence of potentially pathogenic and zoonotic *B. coli* in captive primates in zoos and occasionally in sanctuaries is noteworthy and deserves further attention. The parasite appears to be rarely detected in free-ranging great apes (Hassell et al., 2013), whereas in captivity, there may be factors that increase the susceptibility of primates to infection. In case of *B. coli*, there is a positive correlation recorded between the number of ciliates in fecal samples of captive chimpanzees and starch content in the diet (Schovancová et al., 2013). Optimal nutrition of great apes in (semi)-captive settings is of utmost importance to prevent metabolic diseases, dental problems, or abnormal behavior patterns (Edwards & Ullrey, 1999; Kuhar et al., 2013; Plowman, 2013). Therefore, the role of diet in orangutans infected with *B. coli* should be investigated in future studies.

Sequencing confirmed the existence of the two types of ciliates in our sample set. However, the question arises as to the identification of the two taxa based on their morphological characteristics observed under the microscope. In general, the cysts formed by vestibuliferid ciliates vary in size and shape even within a species, which complicates their identification (Kuze et al., 2010; Mul et al., 2007; Pafčo et al., 2018; Yan et al., 2018). Although the cysts appeared slightly different in microscopy (see large vacuole in the cysts of *Buxtonella*-like vestibuliferids, Figure 1c), we did not find robust features that distinguish between *B. coli* and *Buxtonella*-like ciliates in primate fecal samples using microscopic analyses.

From the clinical perspective, strongyloidiasis is the most important parasitosis of captive and semi-captive orangutans (Kleinschmidt et al., 2018; Labes et al., 2011). It is assumed that wild primates are infected with *S. fuelleborni*, while *S. stercoralis* is typical for captive animals, and is probably transmitted to primates from other susceptible hosts (Bradbury et al., 2021). A study providing exact *Strongyloides* species determination in free-ranging, semi-captive, and captive Bornean orangutans using ITS and 18S rDNA analysis revealed that free-ranging animals were infected with *S. fuelleborni*, while *S. stercoralis* was identified only in captive individuals (Labes et al., 2011). Moreover, Frias et al. (2018) found only *S. fuelleborni* in free-ranging Bornean orangutans in the Lower Kinabatangan Wildlife Sanctuary, Sabah, Malaysia. In contrast, *S. fuelleborni* has never been found in orangutans kept in zoological gardens. However, the transmission of *S. fuelleborni* between primates and animal caretakers in captive conditions has been demonstrated between Southern pig-tailed macaques (*Macaca nemestrina*) and their owners in Southern Thailand (Janwan et al., 2020) and transmission is also very possible in case of *S. stercoralis* between captive primates and the staff and *vice versa* (Bradbury et al., 2021).

In all orangutans from both Slovak and Czech zoos, *S. stercoralis* was detected by coproscopic detection of L1 larvae and amplification of the partial sequence of *cox1*, HVR-I, and HVR-IV regions of 18S rDNA confirmed its identity, which supported our hypothesis. Based on *cox1*, two lineages A and B of *S. stercoralis* are distinguished, with lineage A occurring in dogs, humans, and other primates, while lineage B has been recorded in dogs only (Bradbury et al., 2021). All *cox1* sequences obtained in the present study clustered within the lineage A (highlighted in dark blue in Figure 3). Similarly, only haplotype A of HVR-IV, so far detected in humans, chimpanzees, and dogs (Bradbury et al., 2021) was detected in our samples. In contrast, we found haplotypes II and VI of the HVR-I. Although the significance of the HVR-I is not yet known, our results indicate possible interspecific variability. In general, our results support the hypothesis that *S. stercoralis* occurs not only in dogs and humans, but also in

FIGURE 3 Phylogenetic tree derived by Bayesian inference and maximum likelihood for *Strongyloides* spp. *cox1* (722 bp) sequences obtained in this study (in bold) and available in the GenBank database. The tree was constructed from a muscle alignment and calculated using the GTR + G model for nucleotide substitutions. Branch lengths indicate expected numbers of substitutions per nucleotide site. Numbers at the nodes show posterior probabilities under the Bayesian inference/bootstrap support values for maximum likelihood. Nodal supports <50 are marked with an asterisk. A total of 18S rDNA haplotypes are marked in the tree.

captive primates, whereas *S. fuelleborni* is restricted to free-ranging primates.

We detected zoonotic parasites with possible pathogenic potential, *S. stercoralis* and *B. coli*, in Bornean and Sumatran orangutans kept in several Czech and Slovak zoological gardens. Application of molecular, sequencing, and phylogenetic tools for exact identification of vestibuliferid ciliates is crucial as morphological distinction is difficult. We provided sequence data for *S. stercoralis* from captive orangutans and contributed to knowledge of the genetic diversity of the parasite as data from primates are very rare in public databases (Bradbury et al., 2021). Although none of the animals exhibited any clinical signs, these infections can lead, under some circumstances, to serious illness and even to death (Strait et al., 2012). Parasite monitoring is crucial for institutional health programs and reducing the risk of transmission to humans working with captive primates. Effective management, which includes monitoring of parasitic diseases, is a paramount component of the preventative medicine program of any facility that wants to reduce the risk of clinical illness in animals as well as the risk of zoonotic transmission (Sanchez & Pich, 2018). Regular parasite screening provides a base for targeted treatment, while regular and blind application of antiparasitic drugs can lead to resistance to the products and reduce their effect in the future, as known in livestock (e.g., Geurden et al., 2015).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data availability—sequences from *Strongyloides stercoralis*, *Balantioides coli*, and Buxtonella-like are available and uploaded to GenBank under the accession numbers OM392049–OM392052 (*cox1*); OM423701–OM423711 (ITS1-5.8s-rRNA-ITS2); OM423618–OM423632 (18S rDNA): see Supporting Information: Table S1.

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