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ORIGINAL ARTICLE

FIRST REPORT OF GENUS *Cryptosporidium* IN CERVIDS SPECIES: *Mazama americana, Mazama nana* AND *Blastocerus dichotomus*

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Abstract

We analyzed *Cryptosporidium* spp. in fecal samples of wild cervids (*Ozotoceros bezoarticus, Blastocerus dichotomus, Mazama nana, Mazama americana*, and *Mazama bororo*) from many Brazilian regions, a fact unprecedented in the literature. Sniffer dogs were used to collect 936 fecal samples of cervids from 14 Brazilian localities. Cervids species were identified using polymerase chain reaction (PCR) performed from genomic DNA extracted from 563 fecal samples of *Ozotoceros bezoarticus, Blastocerus dichotomus, Mazama nana, Mazama americana,* and *Mazama bororo. Cryptosporidium* spp. oocyst screening was performed using malachite green negative staining. Nested PCR (nPCR) protocols targeting the 18S rRNA and GP60 genes followed by genetic sequencing were performed for *Cryptosporidium* spp. detection and *Cryptosporidium parvum* subtyping, respectively. Nested PCR targeting actin gene and genetic sequencing were performed in samples with non-identified *Cryptosporidium* species by 18S rRNA amplicon sequencing. The association between the occurrence of *Cryptosporidium* and the presence of bovines in the same locality was evaluated using Fisher's exact test. The positivity rates of diagnostic methods were compared by McNemar test and the Kappa correlation coefficient. The prevalence rates of *Cryptosporidium* spp. in cervids were 1.42% (8/563) and 0.36% (2/563) by nPCR and malachite green negative staining, respectively. *C. parvum* IIaA16G3R1 isolate was identified in three fecal samples from *M. americana,* two from *M. nana* and one from *B. dichotomus*. *Cryptosporidium ryanae* were found in one sample from *B. dichotomus*. We identified a new *Cryptosporidium* genotype, named *Cryptosporidium* deer genotype BR, from one *M. americana* fecal sample.

Keywords Coccid · *Cryptosporidium ryanae* · Deer · Species · Oocysts · Wildlife

Introduction

Parasites of *Cryptosporidium* genus are protozoans with a great capacity for dissemination through the environment. The transmission of these parasites occurs through the ingestion of water or food contaminated with sporulated oocysts, which are eliminated in the feces of infected hosts (Fayer et al. 2000). The genus *Cryptosporidium* is composed of approximately 42 species and more than 40 genotypes (Feng et al. 2018; Zahedi and Ryan 2020), identified in a variety of hosts, such as mammals, birds, reptiles, amphibians and fish (Rieux et al. 2013).

Currently, there are numerous reports of the occurrence of *Cryptosporidium* species and genotypes in deers from different regions of the world as following: *Cryptosporidium bovis* (García-Presedo et al. 2013), *Cryptosporidium ryanae*

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(García-Presedo et al. 2013; Koehler et al. 2016), *Cryptosporidium andersoni* (Huang et al. 2018), *Cryptosporidium ubiquitum* (Robinson et al. 2011; Feng et al. 2012; Koehler et al. 2016; Kotková et al. 2016; Huang et al. 2018,); *Cryptosporidium* deer genotype (Robinson et al. 2011; Santin and Fayer 2015; Wells et al. 2015; Kato et al. 2016; Kotková et al. 2016; Huang et al. 2018; Xie et al. 2019; Tao et al. 2020); *Cryptosporidium parvum* (Perz and Le Blancq 2001; Wells et al. 2015; Huang et al. 2018); *Cryptosporidium muris* (Kotková et al. 2016; Huang et al. 2018), *Cryptosporidium hominis* (Koehler et al. 2016), *Cryptosporidium xiaoi* (Zhao et al. 2020), *Cryptosporidium* Muskrat II genotype (Perz and Le Blancq 2001), *C. hominis*-like (Jellison et al. 2009) and *Cryptosporidium suis-like* (Koehler et al. 2016; Huang et al. 2018).

In Brazil, there are few studies related to the infection by *Cryptosporidium* spp. in cervids, mostly based on diagnosis using conventional microscopy techniques performed on feces of animals kept in captivity (Reginatto et al. 2010; Ludwig and Marques 2011). To date, only one study based on molecular diagnosis has been carried out on free-living cervids in Brazil. However, the aforementioned study evaluated the presence of oocysts in feces of only one species of deer (*Mazama gouazoubira*) (Teixeira et al. 2021).

Currently, there are eight species of native cervids (*Odocoileus virginianus, Ozotoceros bezoarticus, Blastocerus dichotomus, Mazama nemorivaga, M. gouazoubira, Mazama nana, Mazama americana* and *Mazama bororo*) distributed beyond the Brazilian territory (Duarte 1996; Duarte and Merino 1997; Duarte and Jorge 2003); most of them (with the exception to *M. gouazoubira*) are declining in number due to illegal hunting and infectious diseases (Machado et al. 2006; Szabó et al. 2009; Araújo et al. 2010; Piovezan et al. 2010). In this context, we investigated the prevalence of *Cryptosporidium* spp. in fecal samples of *Ozotoceros bezoarticus, Blastocerus dichotomus, Mazama nana, Mazama americana*, and *Mazama bororo,* a fact unprecedented in the literature.

Material and methods

Fecal samples collection

A total of 936 fecal samples from wild cervids were collected in 14 Brazilian locations (Table 1) with the help of sniffer dogs (Duarte 2005).During sample collections, the presence of cattle (one or more animals) in the physical environments where deer fecal samples were collected was evaluated.

Each fecal sample was divided into two aliquots. One aliquot was stored at -20°C for DNA extraction to perform *Cryptosporidium* research using nested PCR (nPCR), and another one was stored in a tube containing 100% ethanol to perform oocyst screening using centrifugal sedimentation

Table 1 Number of samples obtained from each deer species in different Brazilian locations

Region	*Mazama nana**	*Mazama bororo**	*Mazama americana**	*Ozotoceros bezoarticus**	*Blastocerus dichotomus**	Total
	Nº (%)	Nº (%)	Nº (%)	Nº (%)	Nº (%)	
Turvo State Park, RS	2 (22.2)	-	7 (77.8)	-	-	9
Serra do Tabuleiro State Park, SC	-	3 (100)	-	-	-	3
Serra do Itajaí National Park, SC	9 (81.8)	2 (18.2)	-	-	-	11
Araucárias National Park, SC	10 (90.9)	-	1 (9.1)	-	-	11
Mata Preta Ecological Station, SC	5 (3.4)	-	144 (96.6)	-	-	149
Iguaçu National Park, PR	54 (30.2)	-	125 (69.8)	-	-	179
São Camilo State Park, PR	10 (100)	-	-	-	-	10
Perobas Biological Reserve, PR	-	-	9 (100)	-	-	9
Vila Rica State Park, PR	24 (100)	-	-	-	-	24
Cajuru, SP	-	-	10 (100)	-	-	10
Jataí Ecological Station, SP	-	-	-	-	50 (100)	50
Alegria Farm, MS	-	-	-	44 (100)	-	44
Nhumirim Farm, MS	-	-	-	45 (100)	-	45
Emas National Park, GO	-	-	-	9 (100)	-	9
Total	**114 (20.2)**	**5 (0.9)**	**296 (52.6)**	**98 (17.4)**	**50 (8.9)**	**563**

Nº (%): Number of animals (percentage).

Brazilian States—RS: Rio Grande do Sul, SC: Santa Catarina, PR: Paraná, SP: São Paulo, MS: Mato Grosso do Sul, GO: Goiás.

* Cervid species were identified using PCR/RFLP (González et al. 2009; De Souza et al. 2013).

in water-ether followed by negative malachite green staining technique.

Genomic DNA extraction

Fecal samples stored at -20°C were submitted to genomic DNA extraction using the commercial kit QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's guidelines.

Cervids species identification

Identification of cervids species was carried out by PCR followed by hydrolysis with the restriction enzymes SspI, AflIII and BstN (González et al. 2009; De Souza et al. 2013), in all samples of genomic DNA. After these analyses, as they were not part of the main objective of this study, 373 fecal samples of *M. gouazoubira* were identified and excluded. We selected 563 samples belonging to the *Ozotoceros bezoarticus, Blastocerus dichotomus, Mazama nana, Mazama americana*, and *Mazama bororo* (Table 1).

Fecal samples purification and microscopic examination

All samples stored in ethanol and selected during cervids species identification were purified by centrifugal sedimentation in water-ether (Meloni and Thompson 1996) and the resulting sediment was used for *Cryptosporidium* spp. oocyst screening by microscopy using malachite green negative staining technique (Elliot et al. 1999).

PCR amplification and molecular characterization

DNA samples of the selected cervids species were subjected to amplification of a fragment of the 18S rRNA subunit gene of *Cryptosporidium* spp. by nested PCR (nPCR) using the primers 5´-TTCTAGAGCTAATACATGCG-3 ‘ and 5 ‘-CCC ATTTCCTTCGAAACAGGA-3 ‘ in the primary reaction, and the primers 5 ‘-GGAAGGGTTGTATTTATTAGATAA AG-3 ‘ and 5´-AAGGAGTAAGGAACAACCTCCA-3´ in the secondary reaction (Xiao et al. 2000). Both reactions were carried out under the following conditions: initial DNA denaturation at 94ºC for 3 min, followed by 34 cycles, each consisting of denaturation at 94ºC for 45 s, 45 s of annealing at 55ºC and 60 s of extension at 72º C, with a final extension at 72º C for 7 min.

The amplifications were confirmed by electrophoresis on a 2% agarose gel, followed by visualization of the amplified fragments (830 bp) in an ultraviolet light transilluminator. All samples positive for *C. parvum* were subjected to subtyping by a nested PCR targeting the GP60 gene using the primers 5′-ATAGTCTCC GCTGTATTC-3′ and 5′-GGA AGGAACGATGTATCT-3′ in the primary reaction and the primers 5′-TCCGCTGTATTCTCAGCC-3′ and 5′-GCAGAG GAACCAGCATC-3′ in the secondary reaction (Alves et al. 2003). The reactions consisted of an initial DNA denaturation at 95ºC for 3 min, followed by 40 cycles consisting of denaturation at 94ºC for 45 s, annealing at 50ºC for 45 s and extension at 72º C for 60 s, followed by a final extension at 72º C for 10 min.

When it was not possible to identify the species of *Cryptosporidium* by genetic sequencing of the 18S rRNA gene amplicon, a nested PCR protocol targeting the actin gene was performed using the primers 5’-ATGAGGATG AAGAAGATAAGCTATCAAGC-3’ and 5’- AGAAGACAC TTTTCTGTGTGACAAT-3’, in the primary reaction and the primers 5’-CAAGCATTTGAGTTGTTGATCAA-3’ and 5’-TTTCTGTGTGACAATATGCATTGG-3’ in the secondary reaction, under the following reaction conditions: in the primary reaction the samples were subjected to initial DNA denaturation at 94ºC for five minutes, followed by 35 cycles, each consisting of denaturation for 45 s at 94ºC, annealing at 50ºC for 45 s and extension to 72ºC for 60 s, with a final extension at 72ºC for 10 min. In the secondary reaction, the same reaction conditions as the primary reaction were used, except for the annealing step that was carried out at 45ºC for 45 s (Sulaiman et al. 2002).

All products amplified by nPCRs were quantified by spectrophotometry and purified using the QIAquick ™ Gel Extraction Kit (Qiagen). Afterward, the samples that presented a good quality and quantity of DNA (superior to 10 ng per uL) were sequenced using ABI Prism Dye terminator Cycling Sequence kit (Applied Biosystems) on an automatic sequencer ABI 3730XL (Applied Biosystems).

DNA sequences were assembled using the Codoncode Aligner version 7.1.1. software (CodonCode Corporation). The homology of products amplified by PCR to GenBank sequences was assessed using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Homologous sequences were aligned with the consensus sequences by Muscle (Edgar 2004) and BioEdit Sequence Alignment Editor (Hall 1999) software.

Phylogenetic analyses were conducted in MEGAX (Kumar et al. 2018) using maximum likelihood analysis based on the Tamura 3-parameter model (Tamura 1992) and the general time-reversible model (Nei and Kumar 2000) for 18S rRNA and actin genes, respectively. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. Substitution models and optional parameter sets were chosen using the model selection option in MEGAX. Trees were rooted with sequences from *Cryptosporidium molnari* HM243548.1 and

HM365219.1 for 18S rRNA and actin genes, respectively. The sequences that were chosen for 18S rRNA phylogenetic analysis were those that presented both higher genetic similarity to the sequences from this report and resulted in a phylogenetic tree with the best bootstrap support. This selection was performed by comparing the sequences obtained in this study with the sequences previously published in GenBank, using the BLAST tool of the NCBI. In this way, were selected to compose the phylogenetic trees sequences that showed similarity above 85%, in addition to those representing the main species of the genus *Cryptosporidium*, especially those that have been previously diagnosed in deer in the world.

Nucleotide sequences generated in this study were submitted to the GenBank database under the accession numbers MT327131.1, MT327132.1, MT327133.1, MT327134.1, MT327135.1, MT327136.1 and MT327137.1.

Statistical analysis

The data analysis was performed by McNemar test to verify the paired proportions and Kappa correlation coefficient (Landis and Koch 1977) to evaluate the agreement between nPCR (18S rRNA) and malachite green negative staining techniques. Fisher's exact test was used to analyze the association between bovines and *Cryptosporidium* prevalence in each locality. All statistical results were considered significant when $p < 0.05$.

Results

The prevalence of *Cryptosporidium* spp. in fecal samples of cervids by nPCR (18S rRNA) and malachite green negative staining were 1.42% (8/563) and 0.36% (2/563), respectively, with a significant statistical difference ($p = 0.0313$) between the results of these two diagnostic techniques by McNemar test and the Kappa correlation coefficient $p = 0.3966$ (Table 2). We detected *Cryptosporidium* spp. in three cervids species from 35.7% (5/14) of the localities by nPCR (18S rRNA), according to the following indexes positivity by species: 4% (2/50) in *B. dichotomus*, 1.7% (2/114) in *M. nana* and 1.3% (4/296) in *M. americana* (Table 2 and Fig. 1). The presence of bovines was observed in 28.6% (4/14) of the sampled locations (Fig. 1). There was no association between the presence of bovines and infection by *Cryptosporidium* spp. in cervids by Fisher's exact test ($p = 0.6426$).

Sequence analysis of the 18S rRNA gene amplicons allowed the identification of *Cryptosporidium* species in seven of eight positive samples by nPCR.

Table 2 Results obtained by the techniques of microscopy, nested PCR and sequencing (18S rRNA, Gp60 and Actin genes) performed on deer fecal samples of Brazilian cervids

Region	Deer species	Results						
		Microscopy*	Total positivity	nested PCR (18S rRNA)	Total positivity	Sequencing (18S rRNA)	Sequencing (Gp60)	Sequencing (Actin)
Mata Preta Ecological Station, SC	*M. americana*	-	2	+	8	*C. parvum*	IIa A16 G3 R1	b
Araucárias National Park, SC	*M. americana*	+		+		*C. parvum*	IIa A16 G3 R1	b
	M. nana	+		+		*C. parvum*	IIa A16 G3 R1	b
Jataí Ecological Station, SP	*B. dichotomus*	-		+		*C. ryanae*	b	b
	B. dichotomus	-		+		*C. parvum*	IIa A16 G3 R1	b
São Camilo State Park, PR	*M. nana*	-		+		*C. parvum*	IIa A16 G3 R1	b
Iguaçu National Park, PR	*M. americana*	-		+		*C. parvum*	b	b
	M. americana	-		+		c	b	deer genotype BR
	P value	0.0313[1]						

(1) Comparison between microscopy (malachite green negative staining) and nPCR (18S rRNA) techniques by McNemar test.

* malachite green negative staining.

-: negative result.

+: positive result.

b: analysis not performed.

c: species not identified by 18S rRNA gene sequencing.

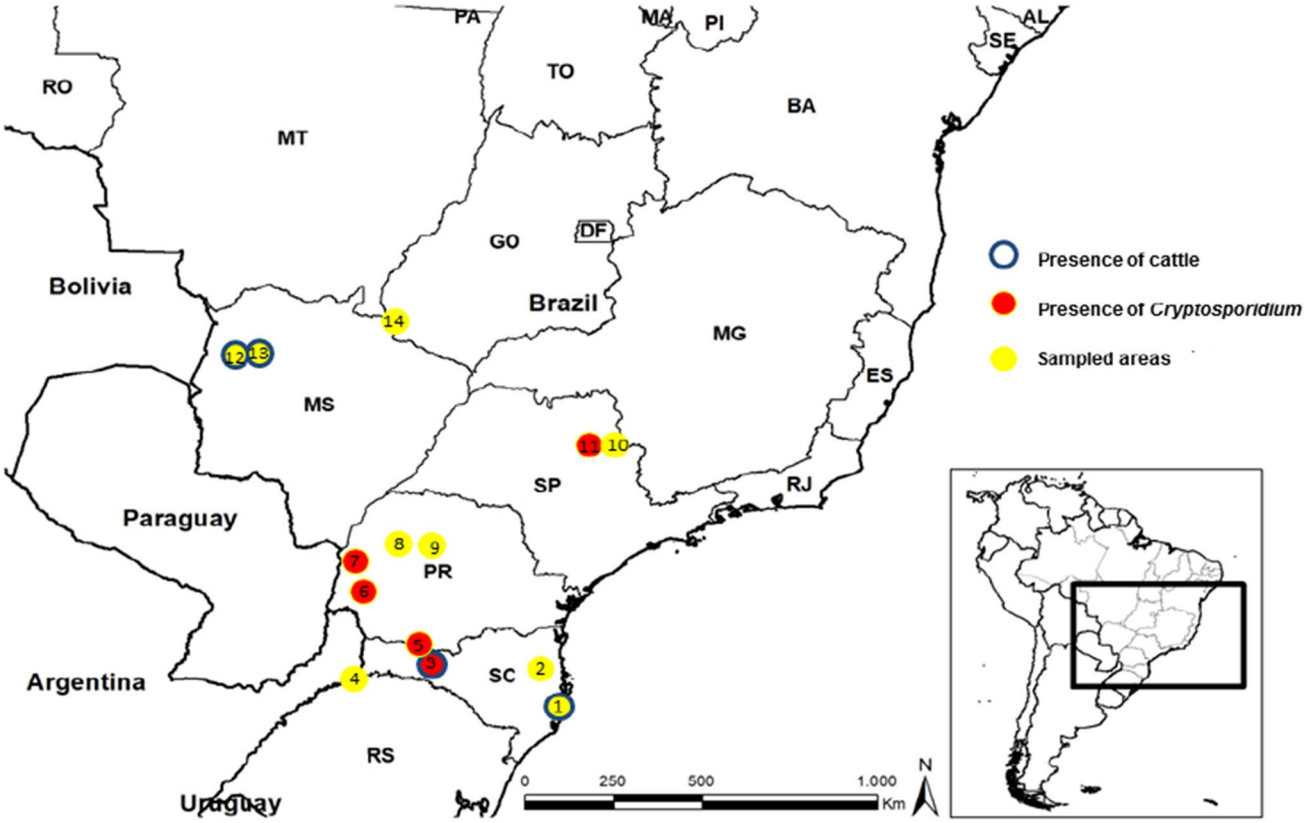

Fig. 1 Locations where faecal samples of deer were collected according to positivity for *Cryptosporidium* spp. and the presence of bovines. 1- Serra do Tabuleiro State Park, SC; 2- Serra do Itajaí National Park, SC; 3- Araucarias National Park, SC; 4- Turvo State Park, RS; 5- Mata Preta Ecological Station, SC; 6- Iguaçu National Park, PR; 7- São Camilo State Park, PR; 8- Perobas Biological Reserve, PR; 9- Vila Rica State Park, PR; 10- Cajuru, SP; 11- Jataí Ecological Station, SP; 12- Alegria Farm, MS; 13- Nhumirim Farm, MS; 14- Emas National Park, GO

Cryptosporidium ryanae (100% of genetic similarity to sequence EU410344.1) was detected in a fecal sample of *B. dichotomus* and *C. parvum* (100% of similarity to sequence AF093490.1) in three fecal samples of *M. americana,* two of *M. nana,* and one of *B. dichotomus*. All *C. parvum* positive samples (6/6) were identified by sequencing of GP60 gene as subtype IIaA16G3R1 isolate (100% of similarity to sequence MH511485.1) (Table 2).

In a fecal sample of *M. americana*, a new *Cryptosporidium* genotype named deer genotype BR was identified by sequencing the 18S rRNA gene amplicon, with 99.2% similarity to *C. ryanae* (EU410344), 98.8% to *Cryptosporidium bovis* (AY741305.1) and 98.6% to *Cryptosporidium xiaoi* (FJ896053). Sequencing of actin gene amplicon of deer genotype BR showed 91.1% similarity to *C. ryanae* (EU410345.1) and 88.2% to *Cryptosporidium* sp. deer genotype (LC18998.1), with no significant similarity to *C. bovis* (AY741307.1). The results regarding the phylogenetic analyzes of the 18S rRNA and actin genes are illustrated in Figs. 2 and 3, respectively.

Discussion

Among the results obtained in the present study, we highlight the unprecedented report of *Cryptosporidium* spp. in feces of *M. americana*, *M. nana,* and *B. dichotomus*. As well as the occurrence of *C. ryanae* in feces of *B. dichotomus*, in addition to the evidence of a new *Cryptosporidium* genotype. It is relevant to inform that a variety of species and genotypes of *Cryptosporidium* have already been identified in fecal samples of cervids in many world regions (Robinson et al. 2011; García-Presedo et al. 2013; Santin and Fayer 2015; Wells et al. 2015; Kato et al. 2016; Kotková et al. 2016; Tao et al. 2020) however, there are few reports in Brazil (Reginatto et al. 2010; Ludwig and Marques 2011; Teixeira et al. 2021).

C. parvum was the most prevalent species in fecal samples of *M. americana, M. nana* and *B. dichotomus*. The occurrence of *C. parvum* in wild cervids has already been reported in different cervids species worldwide (Deng and

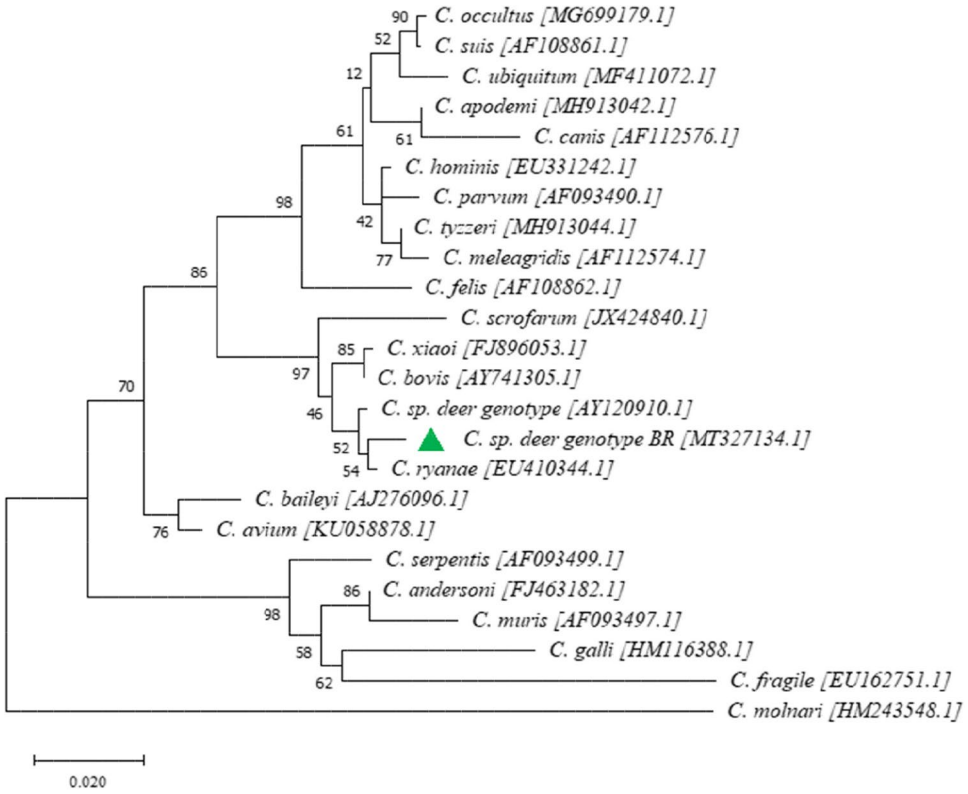

Fig. 2 Phylogenetic trees of the 18SrRNA gene sequences (736 base positions in the final dataset) from *Cryptosporidium* sp. deer genotype BR from this manuscript (green triangle) and selected *Cryptosporidium* species according to the maximum likelihood analysis based on the Tamura 3- parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1062)). Numbers on the left of the supported nodes indicate the bootstrap values (1000 replicates). The branch length scale bar, indicating the number of substitutions per site, is given in the tree. The tree was rooted with the sequence of *C. molnari*

Cliver 1999; Hajdušek et al. 2004; Wells et al. 2015), however, to date, it has not yet been reported in *M. americana, M. nana* and *B. dichotomus*. Prior to this study, there is only one report of subtype IIaA16G3R1 in deers (Teixeira et al. 2021). Subtype IIaA16G3R1 have been previously described in fecal samples from humans (Nazemalhosseini-Mojarad et al. 2011; Stensvold et al. 2015), bovines (Xiao et al. 2007; Brook et al. 2009; Nazemalhosseini-Mojarad et al. 2011; Rieux et al. 2013; Lee et al. 2016) sheeps, goats (Díaz et al. 2015), and yaks (Mi et al. 2013).

For the first time, *C. ryanae* was detected in *B. dichotomus*. Commonly diagnosed in cattle (Fayer et al. 2008), *C. ryanae* was described in deers (*Capreolus capreolus*) for the first time in Spain (García-Presedo et al. 2013). Previous reports of the occurrence of *C. ryanae* and *C. parvum* in deers, as well as the fact that cattle are the primary host of these parasites, motivated us to evaluate the correlation between the presence of cattle and the occurrence of *C. ryanae* in deers. It is important to note that the occurrence of *Cryptosporidium* in deers in the present study did not show a statistically significant relationship ($p < 0.05$) with the presence of cattle in the different locations where the samples were obtained. Such evidence was also observed at the Jataí Ecological Station, where the presence of fecal samples of *B. dichotomous* positive for *C. parvum* and *C. ryanae* was evidenced, although the presence of bovines was not observed in the locality.

Despite the negative relationship with the presence of cattle observed in the present study, Teixeira et al. (2021) observed a greater positivity for *Cryptosporidium* sp. in samples of *M. gouazoubira* obtained from localities where there was a large flow of cattle. Given such inferences, it is clear the need to assess the role of deer in the epidemiology of cryptosporidiosis in a given region, as well as the real influence that the concomitant presence of other hosts in the same habitat can influence *Cryptosporidium* infection in deers.

The sequencing, alignment, and phylogenetic analysis 18S rRNA and actin genes allowed the identification of a new *Cryptosporidium* genotype in a fecal sample of *M. americana*, named deer genotype BR. In phylogenetic tree, deer genotype BR grouped with intestinal *Cryptosporidium* species, with high bootstrap support, in the monophyletic group of *C. ryanae* (EU410344.1), *C. bovis* (AY741305.1), and *C. xiaoi* (FJ896053.1). In the actin gene phylogenetic tree, deer genotype BR grouped into the monophyletic group of *C. ryanae* (EU410345.1), with high bootstrap support. Phylogenetic analysis of *Cryptosporidium* spp. at the 18S rRNA and actin genes, showed that gastric and intestinal species of this genus tend to separate forming monophyletic groups among themselves (Xiao et al. 2004). This result may help future studies on the parasitic dynamics of the *Cryptosporidium* deer genotype BR.

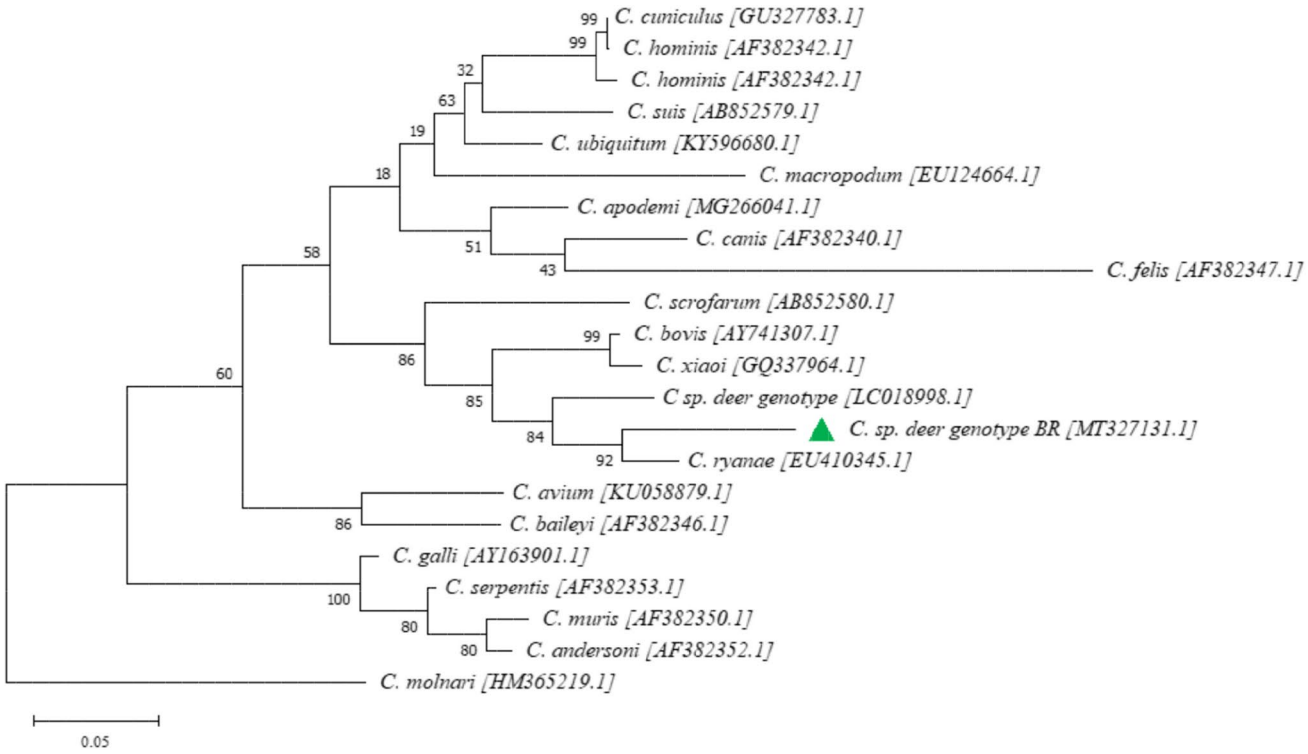

Fig. 3 Phylogenetic tree of the actin gene sequences (737 base positions in the final dataset) from *Cryptosporidium* sp. deer genotype BR from this manuscript (green triangle) and selected *Cryptosporidium* species according to the maximum likelihood analysis based on the general time-reversible model. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4195)). Numbers on the left of the supported nodes indicate the bootstrap values (1000 replicates). The branch length scale bar, indicating the number of substitutions per site, is given in the tree. The tree was rooted with the sequence of *C. molnari*

Cryptosporidium deer genotype BR presented 99.2% of genetic similarity (GS) with a sample identified in sika deer from China as *Cryptosporidium* deer genotype (Tao et al. 2020) at the 18S rRNA gene (MN056199). High genetic similarity (88%) was also observed at the actin gene (LC018998) between deer genotype BR and a *Cryptosporidium* deer genotype identified in *Cervus nippon yesoensis* from Japan (Kato et al. 2016). According to Kváč et al., (2016), in the actin locus, *Cryptosporidium proliferans* is 99.4% similar to *Cryptosporidium muris*. In comparison, *C. parvum* and *Cryptosporidium erinacei* share 99.5% similarity at this locus. Regarding the 18S RNA locus, *C. proliferans* is 99.4% and 98.3% similar *to C. muris* and *C. andersoni*, respectively; this difference is comparable to the similarities between *C. hominis* and *Cryptosporidium cuniculus* (98.9%), *C. parvum* and *C. erinacei* (99.5%), and *C. bovis* and *C. xiaoi* (99.5%), showing that different species of *Cryptosporidium* can present high rates of genetic similarity at the 18S rRNA gene.

Similarity indices obtained in the present study between deer genotype BR and *C. ryanae* (FJ463206, MT507487, and EU410345) did not exceed 91.1%, being even lower (88.2%) when compared to *Cryptosporidium* sp. deer genotype (LC018998). Such inferences, together with the genetic distances observed between the deer genotype BR and the different species and genotypes included in phylogenetic analyses of the actin and 18S RNA genes, allowed us to suggest that the *Cryptosporidium* isolate identified in this study in *B. dichotomus* is a new genotype.

Positivity for *Cryptosporidium* spp. in fecal samples of cervids by nPCR (1,42%; 8/563 samples) was statistically superior to that obtained using the microscopy technique (0,36%; 2/563 samples). Although microscopy techniques are more used in laboratory routines, generally they have lower levels of sensitivity and specificity than molecular techniques (Meireles et al. 2011; Homem et al. 2012). Low sensitivity rates of microscopy techniques in the diagnosis of cryptosporidiosis are widely reported (Elliot et al. 1999; Meireles et al. 2011; Homem et al. 2012), usually leading to false-negative results when compared to more sensitive techniques, as observed in this study. In addition to being more sensitive, the use of molecular techniques in the present study enabled the detection and differentiation of *Cryptosporidium* species.

Although the highest number of positive samples was diagnosed in *M. americana* (four cases), the analyzes of

the number of positive samples among the total number of samples collected from each deer species, showed a highest infection rate in *B. dichotomus* with 4% positivity in 50 samples analyzed, followed by *M. nana* with 1.7% in 114 samples, and *M. americana* with 1.3% in 296 samples. The highest occurrence in *B. dichotomus* is probably due to the feeding habits of these species. Unlike *M. nana* and *M. americana*, *B. dichotomus* have a "pasture-pruner" eating habit, ingesting tender and soft grasses together with shoots and legumes (Tomas and Salis 2000; Duarte 2001), which may have facilitated the ingestion of *Cryptosporidium* oocysts in the environment.

Epidemiological studies on the occurrence and distribution of *Cryptosporidum* spp. in fecal samples of different deer species from Brazil are especially important for programs for the preservation of cervids species in the country. Cryptosporidiosis can cause severe clinical signs, mainly in immunosuppressed animals infected with *C. parvum*, the most prevalent *Cryptosporidium* species in the present study. In addition, two deer species (*B. dichotomus* and *M. nana*), in which we have detected the presence of *Cryptosporidium*, are currently threatened with extinction (IUCN 2020). This fact is pointing to the need to evaluate the host-parasite relationship between *Cryptosporidium* and Brazilian wild cervids, which is still unknown in the worldwide literature.

Conclusion

In this study, we describe the first report of *Cryptosporidium* spp. in *M. americana, M. nana* and *B. dichotomus* species. In an unprecedented way, *C. ryanae* was isolated in a fecal sample of *B. dichotomus*. Sequences from actin and 18S rRNA genes from *Cryptosporidium* deer genotype BR identified in this study allowed us to infer that these sequences are related to a new *Cryptosporidium* genotype.

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Authors contribution Conceptualization: WFPT, JMBD; Methodology: WFPT, MLO, PHFP, BCMO; Formal analysis and investigation: WFPT, WBN, AMAJ; Writing—original draft preparation: WFPT, DSV; Writing—review and editing: WFPT, DSV, ELD, MVM, WDZL, KDSB; Funding acquisition: WFPT, JMBD, KDSB; Resources: JMBD, MVM, KDSB; Supervision: JMBD, MVM, KDSB.

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Data availability The datasets in this study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethical committee All the samples from animals used in this research followed ethical principles in animal research and welfare. This is study was performed and approved according to Ethical Principles of Animal Experimentation of the São Paulo State University, College of Agricultural and Veterinary Sciences, Jaboticabal (FCAV/UNESP), protocol number 15134/16.

Conflict of interest Authors declare that there is no conflict of interest.

Consent to participate All authors participated and helped voluntarily in the research.

Consent for publication All authors read and approved the final manuscript.

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