Research Article

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Occurrence and genetic diversity of *Cryptosporidium* spp. in wild foxes, wolves, jackals, and bears in central Europe

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Abstract: Parasites of the genus *Cryptosporidium* Tyzzer, 1910 are one of the most common protistan parasites of vertebrates. Faecal samples from 179 red foxes (*Vulpes vulpes* [Linnaeus]), 100 grey wolves (*Canis lupus* Linnaeus), 11 golden jackals (*Canis aureus* Linnaeus), and 63 brown bears (*Ursus arctos* Linnaeus) were collected in the Czech Republic, Poland and Slovakia. Samples were examined for the presence of *Cryptosporidium* spp. using microscopy and PCR/sequence analysis. Phylogenetic analysis based on the small subunit ribosomal RNA (SSU), actin and 60-kDa glycoprotein (gp60) genes using the maximum likelihood method revealed the presence of *Cryptosporidium tyzzeri* Ren, Zhao, Zhang, Ning, Jian et al., 2012 (n = 1) and *C. andersoni* Lindsay, Upton, Owens, Morgan, Mead et Blackburn, 2000 (n = 2) in red foxes, *C. canis* Fayer, Trout, Xiao, Morgan, Lai et Dubey, 2001 (n = 2) and *C. ubiquitum* Fayer, Santín et Macarisin, 2010 (n = 2) in grey wolves, and *C. galli* Pavlásek, 1999 in brown bears (n = 1) and red foxes (n = 1). Subtyping of isolates of *C. ubiquitum* and *C. tyzzeri* based on sequence analysis of gp60 showed that they belong to the XIId and IXa families, respectively. The presence of specific DNA of *C. tyzzeri*, *C. andersoni* and *C. galli*, which primarily infect the prey of carnivores, is probably the result of their passage through the gastrointestinal tract of the carnivores. Finding *C. ubiquitum* XIId in wolves may mean broadening the host spectrum of this subtype, but it remains possible this is the result of infected prey passing through the wolf – in this case deer, which is a common host of this parasite. The dog genotype of *C. canis* was reported for the first time in wolves.

Keywords: PCR, carnivores, genotyping, SSU, gp60, microscopy, Czech Republic, Poland, Slovakia

Cryptosporidium Tyzzer, 1910 is a genus of single-celled parasites that infect the gastrointestinal and respiratory tracts of a diverse range of vertebrate hosts (Fayer 2010, Ryan 2010, Kváč et al. 2014). Infections can result in the diarrhoeal disease, cryptosporidiosis, which can be chronic and even fatal in the absence of a competent immune response; however, no clinical signs are present in many wild animals (Kváč et al. 2014). Early efforts to detect infections with species of *Cryptosporidium* were based on the description of oocyst morphology, the detection of developing stages in epithelial scratches, or the identification of surface antigen or specific antibodies (Nichols et al. 1991, Nina et al. 1992, Ogunkolade et al. 1993). Nevertheless, these methods lacked the resolution necessary

to differentiate morphologically identical, closely related species (Ryan and Xiao 2014).

Due to recent progress in molecular diagnostic techniques, our knowledge of the diversity within *Cryptosporidium* has markedly increased, with a total of 45 species recognised as well as a similar number of genotypes having been documented to date (Ryan and Xiao 2014, Holubová et al. 2020). Whereas some groups of hosts – mainly humans, livestock and pets – are studied intensively, other host groups, including wild carnivores, have so far remained neglected and we know relatively little about the occurrence and diversity of *Cryptosporidium* spp. in these groups (Kváč et al. 2014, Robertson et al. 2014).

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Table 1. Identification of *Cryptosporidium* Tyzzer, 1910 in wild wolves, coyotes, foxes, and bears worldwide using microscopy⁽¹⁾, coproantigen test⁽²⁾, and PCR⁽³⁾.

Host (common name)	Country	<i>Cryptosporidium</i> taxa	No. of positive/ examined	Reference
<i>Canis lupus</i> Linnaeus (grey wolf)	Canada	<i>Cryptosporidium</i> sp. ⁽¹⁾	26/1558	Bryan et al. (2012)
	Canada	<i>Cryptosporidium</i> sp. ⁽¹⁾	7/601	Stronen et al. (2011)
	Poland	<i>Cryptosporidium</i> sp. ⁽¹⁾	28/51	Kloch et al. (2005)
	Poland	<i>C. parvum</i> Tyzzer, 1912 ⁽³⁾	5/14	Paziewska et al. (2007)
	Croatia	<i>Cryptosporidium</i> sp. ^(1,2)	8/400	Hermosilla et al. (2017)
<i>Canis latrans</i> Say (coyote)	USA	<i>C. canis</i> coyote genotype ⁽³⁾	5/22	Trout et al. (2006)
	USA	<i>C. muris</i> Tyzzer, 1907 ⁽³⁾	1/22	
	USA	<i>Cryptosporidium</i> sp. ^(1,3)	4/18	Oates et al. (2012)
<i>Vulpes vulpes</i> (Linnaeus) (red fox)	Slovakia	<i>Cryptosporidium</i> sp. ⁽²⁾	24/62	Ravaszová et al. (2012)
	Ireland	<i>C. parvum</i> ⁽¹⁾	2/464	Nagano et al. (2007)
	UK	<i>Cryptosporidium</i> sp. ⁽²⁾	22/184	Sturdee et al. (1999)
	Norway	<i>Cryptosporidium</i> sp. ⁽¹⁾	2/269	Hamnes et al. (2007)
	Iran	<i>Cryptosporidium</i> sp. ⁽¹⁾	2/62	Razmjoo et al. (2014)
	Spain	<i>C. hominis</i> Morgan-Ryan, Fall, Ward, Hijjawi, Sulaiman, Fayer, Thompson, Olson, Lal et Xiao, 2002 ⁽³⁾	4/197	Barrera et al. (2020)
		<i>C. canis</i> Fayer, Trought, Xiao, Morgan, Lal et Dubey, 2001 ⁽³⁾	3/197	
		<i>C. parvum</i> ⁽³⁾	2/197	
		<i>C. ubiquitum</i> Fayer, Santin et Macarasin, 2010 ⁽³⁾	1/197	
		<i>C. suis</i> Ryan, Monis, Enemar, Sulaiman, Read et al., 2004 ⁽³⁾	1/197	
<i>Vulpes</i> sp.	USA	<i>Cryptosporidium</i> sp. ⁽³⁾	1/197	Zhou et al. (2004)
		<i>C. canis</i> fox genotype ⁽³⁾	4/76	
		<i>C. canis</i> dog genotype ⁽³⁾	1/76	
<i>Vulpes lagopus</i> (Linnaeus) (arctic fox)	Canada	<i>Cryptosporidium</i> sp. ⁽¹⁾	1/76	Elmore et al. (2013)
<i>Urocyon cinereoargenteus</i> (Schreber) (grey fox)	USA	<i>Cryptosporidium</i> sp. ⁽¹⁾	9/95	Davidson et al. (1992)
<i>Ursus arctos</i> Linnaeus (brown bear)	Slovakia	<i>Cryptosporidium</i> sp. ⁽²⁾	3/157	Ravaszová et al. (2012)
<i>Ursus americanus</i> Pallas (American black bear)	USA	<i>C. canis</i> ⁽³⁾	35/63	Xiao et al. (2000)

Several studies reporting the presence of *Cryptosporidium* spp. in wild wolves, coyotes, foxes, or bears have been published, but most of these works lack genotyping data (for more detail see Table 1). Given the limited scope and number of studies published so far, the present account aimed to describe the occurrence and genetic diversity of *Cryptosporidium* spp. in wild foxes, wolves, jackals, and bears across the Czech Republic, Poland and Slovakia.

MATERIALS AND METHODS

In 2015–2018, faecal samples from wild grey wolves (*Canis lupus* Linnaeus), golden jackals (*Canis aureus* Linnaeus), red foxes (*Vulpes vulpes* [Linnaeus]), and brown bears (*Ursus arctos* Linnaeus) were collected in the Czech Republic, Poland and Slovakia. Faecal samples from foxes were collected from the rectum of animals shot during the hunting season. Wolves, jackals and bears were tracked and their faeces were collected from the ground on the trails or around the lair. The samples were collected in various territories to avoid repeated sampling of the same animals, but it cannot be ruled out that some wolf, jackal or bear was examined repeatedly. Each sample was placed into a separate airtight sterilised container labelled with the animal ID, kept at 4–8°C without fixative, and delivered to the laboratory for parasitological and molecular examination. Faecal consistency (loose if it took the form of the container and solid if it maintained its original shape) was noted at the time of sampling.

Presence of oocysts of *Cryptosporidium* spp. in faecal samples was screened using a light microscope (Olympus IX50, Olympus, Tokyo, Japan) following modified Sheather's sugar flotation method (Eckert et al. 1995) and aniline-carbol-methyl violet staining (Miláček and Vítovec 1985). Total DNA was extracted from 0.2 g of each faecal sample using the Exgene Stool SV Minikit (GeneAll, Seoul, Korea), which was preceded by homogenisation of the sample by glass beads as previously reported (Sak et al. 2008). Nested PCR protocols were used to amplify a partial region of the small subunit ribosomal RNA (SSU) (Jiang et al. 2005), actin (Sulaiman et al. 2002), and 60-kDa glycoprotein (gp60) (Alves et al. 2003, Li et al. 2014) genes.

Molecular grade water and the DNA of the *Cryptosporidium* chipmunk genotype were included in each PCR amplification as a negative and positive control, respectively. All samples were analysed in duplicate. Secondary PCR products were separated on an agarose gel, visualised under UV illumination using ethidium bromide staining, purified using the Gen Elute Gel Extraction Kit (Sigma, St. Louis, MO, USA), and directly sequenced by the commercial company SeqMe (Dobříš, Czech Republic).

The nucleotide sequences of each gene obtained in this study were edited using the ChromasPro 2.4.1. (Technelysium, Pty, Ltd., South Brisbane, Australia) and aligned with each other and with reference sequences from GenBank (<https://www.ncbi.nlm.nih.gov/pubmed>) using the MAFFT version 7 online server with the Q-INS-I algorithm (<http://mafft.cbrc.jp/alignment/software/>).

Table 2. Diversity of species of *Cryptosporidium* Tyzzer, 1910 in faecal samples of red foxes (*Vulpes vulpes* [Linnaeus]), grey wolves (*Canis lupus* Linnaeus), golden jackals (*Canis aureus* Linnaeus), and brown bears (*Ursus arctos* Linnaeus) detected by microscopy (MIC) and PCR analysis of the small subunit ribosomal RNA (SSU), actin, and 60 kDa glycoprotein (gp60) genes in the Czech Republic (CZE), Poland (POL), and Slovakia (SVK).

Host	Country	Number of screened/ MIC/PCR positive specimens	Animal ID	Molecular characterisation of <i>Cryptosporidium</i> spp.		
				SSU	actin	gp60
<i>Vulpes vulpes</i> (Linnaeus) (red fox)	CZE	58/0/1	13950	<i>C. tyzzeri</i>	<i>C. tyzzeri</i>	IXaA8
	POL	74/0/2	17238	<i>C. andersoni</i>	<i>C. andersoni</i>	-
	SVK	47/0/1	21802	<i>C. andersoni</i>	<i>C. andersoni</i>	-
	SVK	47/0/1	13517	<i>C. galli</i>	<i>C. galli</i>	-
	subtotal	179/0/4				
<i>Canis lupus</i> Linnaeus (grey wolf)	CZE	17/0/1	17601	<i>C. ubiquitum</i>	<i>C. ubiquitum</i>	XIIId
			29818	<i>C. canis</i> dog genotype	<i>C. canis</i> dog genotype	-
	SVK	83/0/3	29819	<i>C. canis</i> dog genotype	<i>C. canis</i> dog genotype	-
			31129	<i>C. ubiquitum</i>	<i>C. ubiquitum</i>	XIIId
	subtotal	100/0/4				
<i>Canis aureus</i> (Linnaeus) (golden jackal)	CZE	3/0/0	-	-	-	-
	SVK	8/0/0	-	-	-	-
	subtotal	11/0/0				
<i>Ursus arctos</i> Linnaeus (brown bear)	POL	15/0/0	-	-	-	-
	SVK	48/0/1	24444	<i>C. galli</i>	<i>C. galli</i>	-
	subtotal	63/0/1				

Phylogenetic trees were inferred by the maximum likelihood (ML) method, with the substitution model that best fit the alignment selected using Bayesian information criterion in the MEGA7 software. Bootstrap support for branching was based on 1,000 replications. Obtained phylograms were edited for style using CorelDrawX7. Sequences have been deposited in GenBank under the accession numbers MT810803–MT810811 and MT822822–MT822833.

RESULTS

A total of 353 faecal samples of red foxes (179), grey wolves (100), golden jackals (11), and brown bears (63) from the Czech Republic, Poland and Slovakia were examined by microscopy and molecular analysis for the presence of parasites of the genus *Cryptosporidium* (Table 2). Whereas microscopic examination did not reveal the presence of *Cryptosporidium* oocysts in any of the samples, *Cryptosporidium*-specific DNA was detected in five (2.6%) samples from red foxes, three (3.6%) samples from grey wolves and one (2.3%) sample from brown bear (Table 2).

All isolates were successfully sequenced at the SSU and actin genes. ML trees constructed from the alignments of SSU and actin sequences revealed the presence of *Cryptosporidium tyzzeri* Ren, Zhao, Zhang, Ning, Jian et al., 2012 and *C. andersoni* Lindsay, Upton, Owens, Morgan, Mead et Blackburn, 2000 in red foxes; *C. canis* Fayer, Trout, Xiao, Morgan, Lai et Dubey, 2001 and *C. ubiquitum* Fayer, Santín et Macarisin, 2010 in grey wolves; and *C. galli* Pavlásek, 1999 in a brown bear and a red fox (Table 2, Figs. 1, 2). At the SSU locus, *C. tyzzeri*, *C. andersoni*, *C. ubiquitum*, and *C. galli* isolates shared 100% sequence identity with *C. tyzzeri* (AF112571), *C. andersoni* (EU245042), *C. ubiquitum* (EU827424), and *C. galli* (HM116388), respectively. The isolates of *C. canis* identified in grey wolves were identical to the *C. canis* dog genotype (AB210854) and differed from *C. canis* fox, coyote and racoon genotypes (Fig. 1).

At the actin locus, the *C. tyzzeri* isolate was identical to *C. tyzzeri* variant A1 (JQ073406), differing from the variants A2 (JQ073388) and A3 (JQ073414; data not shown). The isolates of *C. canis* shared 100% identity with the *C. canis* dog genotype (EU754837) and differed from the *C. canis* fox and coyote genotypes (Fig. 2). Isolates of *C. andersoni*, *C. ubiquitum* and *C. galli* were identical to the previously reported sequences (Fig. 2).

The gp60 gene was successfully amplified and sequenced only from samples positive for *C. ubiquitum* and *C. tyzzeri*. Sequences of *C. ubiquitum* were identical to *C. ubiquitum* subtype family XIIId (JX412922) and *C. tyzzeri* sequence clustered together with the *C. tyzzeri* subtype family IXa (Fig. 3). On the basis of the nomenclature for gp60 subtypes (Sulaiman et al. 2005), we detected subtype IXaA8. No loose consistency of faeces was observed in the examined faecal samples.

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DISCUSSION

In the present study, the overall prevalence of *Cryptosporidium* spp. in wild foxes, wolves and bears was low (0.6–4.0%), which is similar to previous reports from Canada, Croatia, Iran, Ireland, Norway, Spain, UK, and USA, where the prevalence ranged from 0.4% to 16% in these hosts (Sturdee et al. 1999, Hamnes et al. 2007, Nagano et al. 2007, Stronen et al. 2011, Bryan et al. 2012, Razmjoo et al. 2014, Hermosilla et al. 2017, Barrera et al. 2020). In contrast, a few studies from Poland and Slovakia have reported the prevalence of *Cryptosporidium* spp. to be higher than 35% in these hosts (Kloch et al. 2005, Paziewska et al. 2007, Ravaszová et al. 2012).

Differences in prevalence among studies are often due to differences in infection rate in individual regions and also different methodological approaches. However, the highest prevalence was observed in studies where the oocysts

Fig. 1. Molecular phylogenetic tree of *Cryptosporidium* spp. detected in wild carnivores in this study (highlighted) and other *Cryptosporidium* spp. available in GenBank using a Maximum Likelihood analysis of partial sequences of the small subunit ribosomal RNA gene. The evolutionary history was inferred based on the Tamura 3-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree with the highest log likelihood (-3,000.39) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches (1,000 replicates). Bootstrap values for the nodes with more than 50% support are shown. The branch length scale bar indicates the number of substitutions per site. Sequences from this study are identified by an isolate number (e.g., 13517), host species and region (CZE for the Czech Republic, POL for Poland and SVK for Slovakia). The GenBank accession number for each sequence is mentioned in square brackets. The sequence of *Plasmodium falciparum* [JQ627151] was used as an outgroup.

were detected by microscopy or immunochromatographic methods, which are less sensitive than PCR. The high prevalence in studies from Poland and Slovakia may be a result of local focal infections in the studied populations.

In this study, we did not detect the presence of oocysts of species of *Cryptosporidium* in any of the examined faecal samples by microscopic methods, although subsequent molecular analyses did reveal their presence. These findings are consistent with results from experimental and field studies performed on a variety of wildlife (Ježková et al. 2016, Čondlová et al. 2018, Kváč et al. 2018). Although cryptosporidiosis is often associated with intestinal disease, similar to previous studies, we did not find any relationship between the occurrence of *Cryptosporidium* and

clinical cryptosporidiosis in wild carnivores (e.g., Sturdee et al. 1999, Zhou et al. 2004, Barrera et al. 2020).

Most of species and genotypes of *Cryptosporidium* are host-specific (Kváč et al. 2014, Ryan and Xiao 2014). To date, only two studies have genotyped *Cryptosporidia* in wild bears. Duncan et al. (1999) detected *Cryptosporidium parvum* Tyzzer, 1912 in tissue sections from the small intestine of a dead black bear cub and Xiao et al. (2000) described the *Cryptosporidium* bear genotype in a black bear. Canids are considered to be specific hosts of *Cryptosporidium canis* with distinguishable fox, dog and coyote genotypes (Morgan et al. 2000). Whereas fox and coyote genotypes of *C. canis* have been previously found exclusively in foxes and coyotes, respectively, the *C. canis* dog genotype has been previously described in dogs, foxes and

Fig. 2. Molecular phylogenetic tree of *Cryptosporidium* spp. detected in wild carnivores in this study (highlighted) and other *Cryptosporidium* available in GenBank using a Maximum Likelihood analysis of partial sequences of the actin gene. The evolutionary history was inferred based on the General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree with the highest log likelihood (-5,595.23) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches (1,000 replicates). Bootstrap values for the nodes with more than 50% support are shown. The branch length scale bar indicates the number of substitutions per site. Sequences from this study are identified by an isolate number (e.g., 13517), host species and region (CZE for the Czech Republic, POL for Poland, and SVK for Slovakia). The GenBank accession number for each sequence is mentioned in square brackets. The sequence of *Eimeria maxima* [XM013478337] was used as an outgroup.

minks (Morgan et al. 2000, Zhou et al. 2004, Trout et al. 2006). In the present study, it was found in wolves for the first time. The dog genotype has seemingly a broader host specificity than the other genotypes of *C. canis*, but this may not be true due to the small number of studies.

Most studies of carnivore hosts, including this one, have described the presence of *Cryptosporidium* spp. that are host-specific for animals other than carnivores. The presence of *Cryptosporidium tyzzeri* and *Cryptosporidium muris* Tyzzer, 1907 in the faeces of a red fox and coyotes in this study and that by Trout et al. (2006), respectively, is probably the result of the transfer of these parasites from prey species through the digestive tract. Likewise, the presence of the *Cryptosporidium muskrat* genotype I (a genotype specific for small rodents) in fox faeces in the study by Zhou et al. (2004) is probably the result of the mechanical passage of oocysts that originated from infected prey. Rodents, which are the typical hosts of these species of *Cryptosporidium* are common prey of foxes and coyotes (Stur-

ee et al. 1999). Similarly, the presence of specific DNA of *Cryptosporidium suis* Ryan, Monis, Enemar, Sulaiman, Read et al., 2004 in fox faeces in the study by Barrera et al. (2020) and *C. galli* in fox and bear faeces in the present study is most likely the result of consuming the carcasses of a wild boar and a bird, as these are the typical hosts of these parasites (*C. suis* and *C. galli*, respectively) (Němejc et al. 2012, Nakamura and Meireles 2015).

Cryptosporidium andersoni is widely considered a cattle-specific parasite (Lindsay et al. 2000), but has also been found in camels, sheep, goats, various rodents, and non-human primates (Kváč et al. 2016). The presence of *C. andersoni* in the faeces of two foxes shot on a farm with beef cattle can be considered a transfer of the parasite from a contaminated environment. Mechanical passage of non-host-specific species and genotypes of *Cryptosporidium* has been described in the past in a variety of mammals, birds and reptiles (Crawshaw and Mehren 1987, Graczyk et al. 1996, Xiao et al. 2004, Němejc et al. 2013).

Fig. 3. Molecular phylogenetic tree of *Cryptosporidium* spp. detected in wild carnivores in this study (highlighted) and other *Cryptosporidium* available in GenBank using a Maximum Likelihood analysis of partial sequences of the 60 kDa glycoprotein gene. The evolutionary history was inferred based on the General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree with the highest log likelihood (-6,836.73) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches (1,000 replicates). Bootstrap values for the nodes with more than 50% support are shown. The branch length scale bar indicates the number of substitutions per site. Sequences from this study are identified by an isolate number (e.g., 13517), host species and region (CZE for the Czech Republic, POL for Poland and SVK for Slovakia). The GenBank accession number for each sequence is mentioned in square brackets.

Unlike the above-mentioned *Cryptosporidium* spp., *C. ubiquitum* is characterised by broad host specificity. Li et al. (2014) originally suggested that *C. ubiquitum* gp60 subtype families have different host specificity: subtype XIIa is specific to ruminants and subtype families XIIb–XIIId to rodents. However, subtype XIIa has been detected in American minks (*Mustela vison* Schrebe) and long-tailed chinchillas (*Chinchilla lanigera* [Molina]) (Kellnerová et al. 2017). Subtype XIIId, which was detected in grey wolves in the present study, was identical to those previously found in red deer (*Cervus elaphus* Linnaeus), raccoon (*Procton lotor* [Linnaeus]), and crab-eating macaque (*Macaca fascicularis* [Raffles]) (Li et al. 2014, Kotková et al. 2016, Chen et al. 2019). This suggests that the *C. ubiquitum* subtype XIIId has a broader host range than previously reported; yet, the possibility cannot be entirely

ruled out that it was only passing through the wolf after it ate an infected deer.

In conclusion, the results of the present and previous studies show that the use of molecular techniques is very sensitive, enabling even a very small amount of specific DNA to be detected in faecal samples (Lindergard et al. 2003). As experimental studies have shown, the presence of specific DNA without detectable oocysts in the faeces can either indicate active but low intensity infection (Ježková et al. 2016, Kváč et al. 2018), or the passage of oocysts through the gastrointestinal tract (Xiao et al. 2004). However, distinguishing between active infection and the passage of cysts using PCR methods is impossible. Host specificity of the parasite, food preferences of the host and the environment in which the host lives should be used as helpful indicators in deciding between passage and active infection.

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