



## *Blastocystis* occurrence and subtype diversity in wild European terrestrial mammals – The case of Białowieża Primeval Forest (NE Poland)

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### ABSTRACT

*Blastocystis* cf. *Blastocystis hominis* is the most common unicellular parasite found in human and animal intestines. Little is known about the life cycle, transmission and mechanisms of pathogenesis for this parasite. The aim of this study was to obtain new data on the genetic diversity of *Blastocystis* in selected species of mammals found in the Białowieża Primeval Forest (BPF), Poland, the best preserved lowland forest in temperate Europe. 113 faecal samples were collected in the period 2018–2020 from seven species of wild mammals occurring within the Polish part of BPF and its surroundings. *Blastocystis* was detected by molecular amplification and sequencing the small subunit rRNA gene. The overall prevalence of *Blastocystis* in animals was 8.9%. A larger number of stool samples (90) were collected from European bison and only for this species was it possible to calculate the prevalence of infection (5.6%). The isolates obtained from European bison were classified as ST1, ST3, ST5, ST7. *Blastocystis* was also detected in the grey wolf (*Canis lupus*) (ST5), wild boar (*Sus scrofa*) (ST5) and red deer (*Cervus elaphus*) (ST1). In conclusion, for the first time we have demonstrated the presence of *Blastocystis* in wild European bison. Preliminary studies have shown that *Blastocystis* is present in at least 4 species of wild mammals in the BPF area and that it exhibits great genetic diversity.

### 1. Introduction

*Blastocystis* cf. *Blastocystis hominis* is a common unicellular protozoan inhabiting the intestinal tracts of humans and many animals, both domestic and wild (Stensvold et al., 2009; Stenzel and Boreham, 1996). The human *Blastocystis* infection rates range from 10% in developed countries to 100% in developing countries (Cian et al., 2017; El Safadi et al., 2014; Ramírez et al., 2014).

High genetic diversity has been found among isolates from humans and animals. Based on nucleotide differences in small subunit ribosomal RNA genes (SSU rDNA) of *Blastocystis* sp., 31 subtypes (STs) have been found so far, however, the separateness of some of them (STs 18–20 and ST22) raises doubts (Maloney et al., 2021; Stensvold and Clark, 2020). Ten subtypes (STs 1–9 and ST12) have been identified in humans to date (Maloney et al., 2019). Since *Blastocystis* subtypes from animals include

the same subtypes as are found in humans (with the exception of ST9), this indicates a zoonotic potential (Stensvold et al., 2009). There are some reports of the possibility of transmission of this parasite between humans and animals (Salim et al., 1999). Infection with the same subtypes of *Blastocystis* has been demonstrated in both humans and animals in zoo owners, butchers, and humans using the same water sources as livestock (Lee et al., 2012; Parkar et al., 2010). Transmission of *Blastocystis* from animals to humans has been described in various ways: directly or through contaminated water or food (Lee et al., 2012). There are reports of the occurrence of *Blastocystis* in companion and farm animals, but little data on the occurrence of this protist in wild animals.

So far, there have been few studies on the prevalence and genetic diversity of *Blastocystis* in animals in Poland. The first reports of animal blastocystosis were described in chickens in 2014 (Bobusia and Gawel, 2014) and two years later by (Lewicki et al., 2016). In 2018, Kaczmarek

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**Table 1**  
Animal samples collected from study hosts and subtype results from sequencing positive samples.

Host	Latin name	Sample number	Infected	ST1	ST3	ST5	ST7
<b>Artiodactyla</b>							
Wild boar	<i>Sus scrofa</i>	2	1	–	–	1	–
Moose	<i>Alces alces</i>	2	0	–	–	–	–
Red deer	<i>Cervus elaphus elaphus</i>	8	2	2	–	–	–
European bison	<i>Bison bonasus</i>	90	5	1	1	2	1
<b>Carnivora</b>							
Grey wolf	<i>Canis lupus</i>	7	1	0	0	1	–
Red fox	<i>Vulpes vulpes</i>	1	0	–	–	–	–
Martens	<i>Martes martes</i> and/or <i>M. foina</i>	3	0	–	–	–	–
<b>Total</b>		<b>113</b>	<b>9</b>	<b>3</b>	<b>1</b>	<b>4</b>	<b>1</b>

fresh stool specimens, swabs of samples were collected with a disposable sterile polystyrene-viscose swab with carbon-containing transport medium (Equimed, cat. No. 7.012.211.110). The carbon-containing transport medium protected the material against air access, drying out and other unfavourable conditions that could affect the survival of *Blastocystis* until the samples were delivered to the laboratory.

## 2.2. Molecular identification and sequencing

DNA isolation was performed using Genomic Mini kits (A&A Biotechnology, Gdynia, Poland). Fragments of the small subunit rRNA gene were amplified by PCR using the primers B118SPPF1 and B118SR2PP (Poirier et al., 2011).

The obtained products were visualized by electrophoresis on a 2% agarose gel, the excised bands were purified using the Gel-Out kit (A&A Biotechnology, Gdańsk). Sequencing was performed using the Sanger method at the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences.

Chromatograms were analysed using CLC Main Workbench v 20.0.1 software (QIAGEN Aarhus A/S). The obtained sequences without the primer sequences were compared with the sequences deposited in the GenBank database using the BLAST tool (Sayers et al., 2019).

Phylogenetic analysis using Bayesian inference was performed (Huelsenbeck and Ronquist, 2005; Miller et al., 2010) taking into account 41 reference sequences representing the *Blastocystis* ST1–ST17 subtypes (Kaczmarek et al., 2017; Mattiucci et al., 2016; Rivera, 2008). *Proteromonas lacertae* (GenBank: U37108) was used as an outgroup. The sequences have been deposited with GenBank (accession numbers: MW173219–MW173229). *Blastocystis* subtype nomenclature according to Stensvold et al. (2007).

## 3. Results

Molecular diagnosis of 113 stool samples revealed *Blastocystis* infection in 9 samples. Total prevalence of *Blastocystis* infection was 8.9%. The protozoan was detected in faecal samples from: grey wolf (*Canis lupus*) (1 positive), wild boar (*Sus scrofa*) (1 positive), red deer (*Cervus elaphus*) (2 positive) and European bison (*Bison bonasus*) (5 positive) (see Table 1). A larger number of faecal samples (90) were collected from European bison and only for this species was it possible to calculate the prevalence of infection, which was 5.6%. Based on the phylogenetic analysis, the obtained isolates were identified as the subtypes ST1, ST3, ST5 and ST7. The ST5 subtype was the most common (Table 1, Fig. 2).

## 4. Discussion

The results obtained in this study are the first world data on the occurrence of *Blastocystis* in wild wolves and European bison and the first data on wild boar and red deer in Poland. Moreover, it should be emphasized that these data come from a forest complex, unique in Europe, which is the Białowieża Primeval Forest, inhabited by a diverse community of wild mammals. In our study *Blastocystis* ST5 was the most commonly identified subtype. In the literature *Blastocystis* ST5 subtype has been reported worldwide in both humans and animals, especially in farm animals, such as cattle, goats and pigs. In Poland, *Blastocystis* ST5 subtype was confirmed in zoo animals (no species listed) (Wesołowska et al., 2019), in hens (Kaczmarek et al., 2019), and in pigs (Rudzińska et al., 2020). Pig studies have shown a high *Blastocystis* infection prevalence of 46.97% (Rudzińska et al., 2020).

The ST1 and ST3 subtypes are the most commonly reported subtypes of *Blastocystis* in humans. The ST3 is considered typically anthroponotic, although in the literature it was reported from many non-humans host such as rodents and cattle (Alfellani et al., 2013; Ramírez et al., 2014). In Poland, so far ST3 subtype has only been detected in humans. A common feature of the examined wild mammals is their use of space. They mainly live in the forest, but often forage in forest meadows and field crops, which are subject to anthropogenic changes. (Kuijper et al., 2014). It is likely that the infection of wild animals with *Blastocystis* occurred while foraging in cultivated fields in contact with soil fertilized with manure. Organic fertilizer, such as manure, is a mixture of solid and liquid animal excrements and litter, for improving the chemical and biological properties of the soil. However, if it is poorly composted, it can be a potential source of infection with microorganisms, including parasites (Bintsis, 2018). Wild animals can be reservoirs of pathogens and transport them over long distances. Comprehensive understanding of the transmission of *Blastocystis* is complicated, given the wide distribution of this protozoan in the environment. The lack or a very small number of studies on the occurrence of *Blastocystis* in wild living animals should be emphasized, which makes it difficult to assess how this parasite is transmitted.

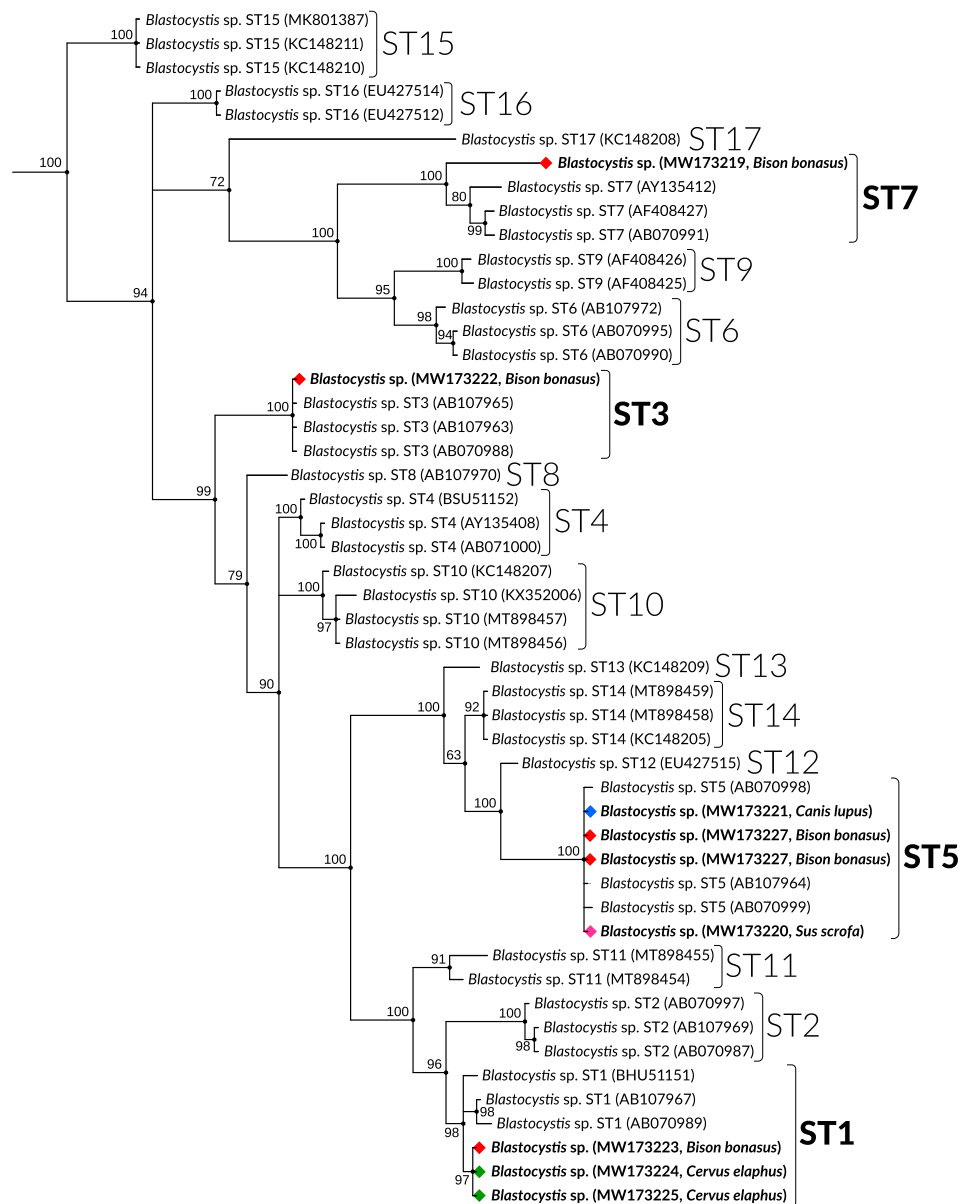
### 4.1. Mammalian orders and families infected with *Blastocystis*

#### 4.1.1. Arctiodactyla

4.1.1.1. *Bovidae – European bison (Bison bonasus)*. European bison is a poorly studied group of mammals in terms of *Blastocystis* infection. So far, the ST10 subtype has been detected only in captive European bison from a UK zoo (Betts et al., 2018, 2020). Previously, Alfellani et al. (2013) tested only one sample from European bison from Poland, but they obtained a negative result. Our research showed that the prevalence of *Blastocystis* infection in the wild European bison population in the BPF is 5.6%.

The *Blastocystis* sequences obtained as a result of analysis of the European bison faecal samples from BPF belong to four different subtypes – ST1, ST3, ST5 and ST7. The ST1 and ST3 detected in European bison individuals are among the most common of *Blastocystis* in humans, while ST5 and ST7 are also found in humans, but more often in animals (Kaczmarek et al., 2017). So far, the ST3 subtype has not been detected in animals in Poland. Subtypes ST10 and ST14, other than those found in our population of European bison, were found in the species related to European bison – the American bison from the French zoo in La Palmyre (Cian et al., 2017). It is known that the resettlement of European bison from BPF, resulting from the history of the species and the current management of its populations, may cause transmission of diseases to various parts of Europe (Vadlejch et al., 2017). Every new pathogen found in this vulnerable species may pose a real threat and should be continuously monitored.

4.1.1.2. *Suidae – wild boar (Sus scrofa)*. The ST5 subtype found in our studies in wild boar populations is characteristic for Suidae; previously



**Fig. 2.** Bayesian inference tree based on fragment of sequences obtained from the small subunit rRNA gene (SSU rDNA) of *Blastocystis* isolates of the present study, performed using MrBayes 3.2.7a. The Bayesian posterior probabilities are shown adjacent to branch nodes.

ST5 was also detected in wild boar in Spain (Rivero-Juarez et al., 2020), in the same study ST5 subtype (as well as ST1 and ST3) was detected in Black Iberian pig. *Blastocystis* ST5 is also found in other animals, but it can infect humans as well (Rudzińska et al., 2020; Stensvold et al., 2009).

The study conducted by Ithoi et al. (2011) indicated that the faeces of wild boar and other wild animals were probably the source of *Blastocystis* contamination in the Malaysia River. We can expect a similar situation in Poland; in the only PCR study of river water conducted so far in Poland, the presence of *Blastocystis* ST1 and ST3 in north-west Poland was detected (Adamska, 2020). Under the conditions of central and eastern Europe, wild boar is a species living in relatively high densities, which may affect the rapid transmission of diseases and pathogens within groups (Podgórski et al., 2018). However, the transmission of parasites, including *Blastocystis*, by wild boars can also be favoured by low densities of this species. Such a situation is currently observed in eastern Poland as a result of the high mortality rate caused by African swine fever (ASF) (Morelle et al., 2020). It is fostered by the method of

fighting ASF, relying on carcass disposal from the forest (wild boars also feed on carrion). The scarcity of food may increase the mobility of live individuals, which contributes to the spread of diseases and pathogens (Pepin et al., 2020).

**4.1.1.3. Cervidae – red deer (*Cervus elaphus elaphus*).** *Blastocystis* has already been detected in red deer - the ST4 and ST10 subtype in Great Britain (Betts et al., 2018), the ST4 subtype in Australia (Roberts et al., 2013), and the ST10 subtype in China and Malaysia (Li et al., 2020; Rauff-Adedotun et al., 2020; Zhao et al., 2017, 2018). The present study found infection with the ST1 *Blastocystis* subtype in BPF red deer. In our opinion, among all the mammals in which we detected *Blastocystis* in the Białowieża Primeval Forest, the red deer is the species with the lowest possibility of transmitting diseases and pathogens over long distances. The unpublished results of the hunting inventory indicate that its density has not changed significantly in recent times. However, some authors show that red deer contribute to the transmission of certain diseases (Vikøren et al., 2019).

#### 4.1.2. Carnivora

**4.1.2.1. Canidae – Grey wolf (*Canis lupus*).** So far, the only subtype of *Blastocystis* detected in wolves is ST3 in an individual from a French zoo (Cian et al., 2017). Until now, no one has described *Blastocystis* in wolves in stool samples derived from wild animals. Thus, this study is the first in the world to confirm the *Blastocystis* infection in this group of Canidae (ST5). Wolves can migrate long distances, being an important reservoir of this pathogen in the environment. In recent years, wolves have been colonizing new areas in Europe (Szewczyk et al., 2019), which may contribute to the spread of *Blastocystis* across Europe. In the literature, parasitological studies of wolves have been reported (Great Britain, China, Basque Country), but no *Blastocystis* infection was found (Betts et al., 2020; Zhao et al., 2017, 2018). In the case of samples from zoos it is important to keep in mind the ability to easily transfer various pathogens between different animals. But in nature it cannot be excluded that wolves become infected with *Blastocystis* from other animals, for example with ST5 after eating bison or wild boar.

Several studies have shown the presence of *Blastocystis* in Canidae. One case of *Blastocystis* infection with ST7 has been reported in dog in Poland (Kaczmarek et al., 2020). However Cian et al., based on their research on *Blastocystis* infection in various groups of animals, suggest that dogs are not natural hosts for this unicellular protozoan (Cian et al., 2017), as do Wang et al. (2013).

#### 5. Conclusion

Białowieża Primeval Forest is the best-preserved forest complex in the European lowlands, characterized by a high diversity of wild mammals and numerous moist habitats. The area of the BPF is also inhabited by people and their pets, therefore intra-species and inter-specific transmission of *Blastocystis* in this area is highly probable. Our preliminary studies have shown that *Blastocystis* is present in at least 4 species of wild mammals in the area and that it exhibits great genetic diversity. The limited number of tested mammalian species and collected stool samples did not allow for obtaining comprehensive data on the presence of *Blastocystis* in wildlife of BPF.

Therefore, we believe that research in this field should be continued, covering a wider range of hosts, as well as other regions of Poland in the future.

#### Declaration of competing interest

None.

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