

Potential zoonotic transmission and environmental factors of *Blastocystis* sp. infection in wild roe deer (*Capreolus capreolus*) from Central Poland

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Abstract

Blastocystis sp. is one of the most common protists colonizing the gastrointestinal tract of humans and animals worldwide.

The aim of this study was to analyze the occurrence of *Blastocystis* sp. in wild roe deer (*Capreolus capreolus*) from Central Poland, as well as to determine its zoonotic potential and the influence of selected environmental factors on the infection level.

A total of 151 fecal samples were collected and analyzed using PCR amplification followed by DNA sequencing. To identify predictors of *Blastocystis* sp. occurrence, data on animal characteristics, season, and landscape structure were compiled. Variables were analyzed using a binary generalized linear model with AIC selection. *Blastocystis* sp. was detected in 49 samples (32.5%). Sequence analysis revealed three subtypes: ST14 (53.1%), ST13 (32.7%), and ST10 (10.2%), while two samples (4.0%) remained classified at the genus level.

Roe deer present on wind farms and in areas with a higher percentage of settlements had a significantly lower probability of infection. Conversely, the probability of *Blastocystis* sp. presence increased with a higher proportion of arable land and surface water.

Zoonotic threat exists, its potential in the studied population was low. The transmission risk may be influenced by factors related to landscape structure and anthropogenic pressure.

INTRODUCTION

Blastocystis sp. (*Blastocystis*) is one of the most common protists colonizing the gastrointestinal tract of humans and animals worldwide [1, 2]. The presence of *Blastocystis* in humans has been associated mainly with gastrointestinal symptoms, as well as being commonly found in asymptomatic individuals. Therefore, its pathogenicity is controversial [2, 3]. It has also been suggested that *Blastocystis* colonization could be, in some individuals, associated with a healthy gut microbiota [2, 4].

Blastocystis isolates are indistinguishable morphologically; however, they show high genetic diversity. Based on polymorphism of the small subunit ribosomal RNA (18S rRNA) gene, 44 *Blastocystis* subtypes (STs) have been reported in the literature [5–7]. Of these, 28 subtypes (ST1–ST17, ST21, ST23–ST32) have been acknowledged as legitimate subtypes according to the criteria of having SSU rDNA sequences that differ by 4% or more [3, 8]. The vast majority of human colonization reported worldwide are caused by ST1–ST4 [9]; nonetheless, the low host specificity demonstrated by some subtypes shared between humans and animals implies potential bidirectional transmission [3, 10, 11].

Blastocystis transmission is via the fecal-oral route, through contact with infected humans/domestic and wild animals or through ingestion of contaminated water and food [12, 13]. Colonization with the same subtypes of *Blastocystis* has been demonstrated in both zoo animals and their animal keepers, as well as in humans using the same water sources as livestock [12, 14]. The same colonization was also found

in both humans and in recreational waters contaminated with feces (from both human and/or non-human hosts) [15]. In Poland, most published studies have focused on animals under human care - in zoos, farms and veterinary practices [16, 17]. However, wild ungulate communities, including deer populations, can live close to domestic animals and near human settlements in a given area.

Blastocystis has been identified from a wide range of wild cervidae worldwide [1], but there are no studies on the *Blastocystis* subtype occurrence in deer from Poland, and very limited data in general for wildlife in this country are available [18]. Better knowledge of *Blastocystis* subtype distribution in wildlife, especially wild ungulate populations, is necessary to understand the potential role of these animals as the source for human colonization in the protist transmission dynamics.

The roe deer species (*Capreolus capreolus*) is the most abundant wild cervid taxon in Europe [19]. It is a small to moderate-sized deer, with a body weight of 15–35 kg, a length of 95–138 cm, and a shoulder height of 60–70 cm [20]. Roe deer are a mobile species that can move from their original area wherever suitable habitat is available, although their spread range may be limited by various natural barriers [20]. Over the course of the last few decades, roe deer have evolved from occupying a typical forest edge and have successfully adapted to a variety of habitats (i.e. forests, fields, urban areas) [21–23]. Furthermore, as a typical synanthropic species, it is well adapted to modern agricultural landscapes where the energy supply is sometimes even higher than in pure forest areas [24–28].

The current population of roe deer in Poland, according to the Statistical Yearbook of Forestry, is estimated to have exceeded 900,000 individuals [29]. Its population is increasing on average by 15% every 5 years, which results in their excessive density, through limiting the areas of its natural occurrence by human interference, mostly due to the conversion of forested and agricultural lands into suburban areas that improve habitat conditions for those wild animals [29, 30]. The species is expanding their habitat into human populations, often appearing looking for food and living near or in suburban areas, where they frequently cross paths with humans, as well as encounter domestic pets and livestock [31]. These circumstances have resulted in cross-species transmission of various pathogens from the roe deer to humans or domestic animals. Therefore, it has become significant to determine the infectious diseases carried by roe deer to prevent pathogen transmission from deer to humans and livestock. The focus on the most common intestinal protist, *Blastocystis*, increases worldwide as novel data appear on the number of subtypes, host specificity, and impact on host gut microbiota. This consequently has an impact on host health and disease [32].

It has been reported that roe deer can carry zoonotic intestinal protozoa, such as *Giardia* and *Cryptosporidium*, that spread from animals to humans [33]. However, studies focusing on the occurrence of *Blastocystis* in forest roe deer remain limited, and the exact role of this species as a reservoir for other animals and humans requires further clarification.

The aim of the present work is to provide the first comprehensive study on the occurrence of *Blastocystis* in wild roe deer (*Capreolus capreolus*) in Poland, as well as to determine the zoonotic potential and the influence of selected environmental factors on the infection level.

RESULTS

Subtype distributions of Blastocystis in roe deer

In this study, 151 stool samples were examined, of which 49 (32.5%) tested positive for *Blastocystis*. A total of three subtypes were identified, with subtype ST14 being the most prevalent (n = 26), followed by ST13 (n = 16) and ST10 (n = 5). In two cases, the subtype could not be determined. The presence of *Blastocystis* was confirmed in all three study locations. In Location 1 (Węgrów), the dominant subtype was ST14 (n = 12), followed by ST13 (n = 7); no cases of ST10 were detected. In Location 2 (Rawa Mazowiecka), ST14 was also the most frequent (n = 9), while ST10 and ST13 were detected at the same frequency (n = 2 each). In Location 3 (Iłża), ST13 was the most common subtype (n = 7), followed by ST14 (n = 5) and ST10 (n = 3) (Table 1).

Table 1
Distribution of *Blastocystis* subtypes in wild roe deer (*Capreolus capreolus*) in Poland.

Factor	Category	No. (%) Examined	No. (%) Positive	ST10	ST13	ST14	ST?
Region	Locality 1 Węgrów	43 (28.5)	19 (44.2)	-	7	12	-
	Locality 2 Rawa Mazowiecka	45 (29.8)	14 (31.1)	2	2	9	1
	Locality 3 Iłża	63 (41.7)	16 (25.4)	3	7	5	1
Gender	Female	84 (55.6)	26 (31.0)	5	8	12	1
	Male	67 (44.4)	23 (34.3)	-	8	14	1
Season	Autumn	25 (16.6)	8 (32.0)	2	2	3	1
	Winter	64 (42.4)	20 (31.3)	3	6	11	-
	Summer	47 (31.1)	19 (40.4)	-	8	11	-
	Spring	15 (9.9)	2 (13.3)	-	-	1	1
Age group	Yearling	17 (11.3)	3 (17.6)	-	1	2	-
	Adult	134 (88.7)	46 (34.3)	5	15	24	2
Total		151 (100)	49 (32.5)	5	16	26	2

Genetic diversity of Blastocystis subtypes

Phylogenetic analysis (Fig. 1), based on partial small subunit ribosomal RNA (SSU rRNA) sequences of *Blastocystis*, demonstrated that the sequences obtained in this study clustered with reference sequences within their respective subtype clades.

Two isolates WE003, WE006 (GenBank: PV982223, PV982225), showed 100% similarity to subtype 14 from China (MW850533, host: sheep; ON796564, host: goat) and Bangladesh (PP581326, host: cattle). Isolate IL025 (GenBank: PV982221) showed nearly 100% similarity to subtype from China (PP626101, host: black goat; ON796564, host: goat; MW850533, host: sheep). Moreover, isolate RA018 (GenBank: PV982222) showed 100% similarity to subtype 14 from United Kingdom (MN526815, host: elk) and nearly 100% similarity to subtype from Senegal (MT621804, host: homo sapiens).

According to subtype 13, three isolates: IL023, WE020 and RA014 (GenBank: PV982220, PV982228, PV982244), showed 99,14–100% similarity to the subtype from the United Kingdom (MF186700, host: muntjac) and China (MT764943, host: goral). In relation to subtype 10, two isolates: IL022 and IL013 (Gen Bank: PV982219, PV982234) showed 100% similarity to the subtype from China (MK930353, host: sika deer; MT672787, host: sheep; MK930353, host: sika deer). Furthermore, isolate RA019 (Gen Bank: PV982236) showed 100% similarity to subtype 10 from China (MF541104, host: goat), Malaysia (MK240480, host: cattle) and nearly 100% similarity to the subtype from the United Kingdom (MN526919, host: bongo).

In turn, the phylogenetic analysis including only various cervid species revealed that the representative sequences obtained in this study showed high similarity to reference sequences of *Blastocystis* and were assigned to ST10, ST13, and ST14 (Table 2). Subtype 10 clustered with sequences derived from elk, red deer, sika deer, and white-lipped deer. Subtype 13 clustered with sequences from muntjac, reindeer, and forest musk deer, whereas subtype 14 grouped with sequences isolated from sika deer and elk (Fig. 2). The subtypes (ST10, ST13, and ST14) reported in our study have low zoonotic potential. However, studies by other authors have confirmed zoonotic potential in roe deer, including subtypes ST1, ST2, and ST5 (Table 3).

Table 2
Comparison of the obtained sequences with GenBank sequences including cervid species.

Representative sequence ID/Isolate	Closest match ID	Host	Origin	Percentage match	Query cover
ST10	MK930356	Sika deer	China	100%	100%
PV982219/IL022	MK930352	White-lipped deer	China	100%	100%
					100%
ST10	MK930356	Sika deer	China	100%	100%
PV982234/IL013	MK930352	White-lipped deer	China	100%	100%
ST10	MK930356	Sika deer	China	99,81%	100%
PV982241/RA019	MK930357	Sika deer	China	99,81%	100%
ST13	MF186700	Muntjac	United Kingdom	100%	100%
PV982220/IL023	MH325366	Reindeer	China	99,14%	100%
ST13	MF186700	Muntjac	United Kingdom	100%	100%
PV982228/WE020	MH325366	Reindeer	China	99,14%	100%
ST13	MF186700	Muntjac	United Kingdom	100%	100%
PV982244/RA014	MH325366	Reindeer	China	99,07%	100%
ST14	MK357783	Sika deer	China	98,95%	100%
PV982221/IL025	MF186664	Elk	United Kingdom	98,95%	100%
ST14	MK357783	Sika deer	China	99.63%	100%
PV982222/RA018	MF186664	Elk	China	100%	100%
ST14	MK357783	Sika deer	China	98,78%	100%
PV982223/WE003	MF186664	Elk	China	98,78%	100%
ST14	MK357783	Sika deer	China	98,78%	100%
PV982225/WE006	MF186664	Elk	China	98,78%	100%

Table 3
Occurrence and zoonotic potential of *Blastocystis* subtypes in *Cervidae* in Europe.

Host latin name	Host common name	Prevalence % (no. positive /total)	<i>Blastocystis</i> subtypes	Country	References
<i>Capreolus capreolus</i>	Roe deer	32.5 (49/151)	ST10' , ST13, ST14'	Poland	This study
		100 (1/1)	ST10'	Denmark	Stensvold et al., 2009
		32.5 (13/40)	ST5 ♦, ST10' , ST13, ST44, ST49	Portugal	Figueiredo et al., 2025b
		100 (1/1)	ST10'	Portugal	Santin et al., 2023
		12.9 (12/93)	ST2* , ST10' , ST13, ST24, ST49	Spain	Figueiredo et al., 2025b
		50 (1/2)	ST5 ♦	United Kingdom	Alfellani et al., 2013b
<i>Alces alces</i>	Elk	40 (2/5)	ST4* , ST10' , ST14'	United Kingdom	Betts et al., 2018; Betts et al., 2020
<i>Rangifer tarandus</i>	Reindeer	100 (1/1)	ST10'	United Kingdom	Betts et al., 2020
<i>Muntiacus reevesi</i>	Reeves's muntjac	100 (2/2)	ST13, ST14'	United Kingdom	Betts et al., 2018; Betts et al., 2020
<i>Cervus elaphus</i>	Red deer	50 (2/4)	ST4* , ST10'	United Kingdom	Betts et al., 2018; Betts et al., 2020
		40.4 (38/94)	ST2* , ST5 ♦, ST10' , ST21, ST24, ST25	Portugal	Figueiredo et al., 2025b
		11.9 (39/329)	ST5 ♦, ST10' , ST13, ST14' , ST21, ST23' , ST24, ST43, ST44	Spain	Figueiredo et al., 2025b
		100 (1/1)	ST25, ST42	Spain	Santin et al., 2023
		25 (2/8)	ST1*	Poland	Kaczmarek et al., 2021
<i>Dama dama</i>	Fallow deer	100 (1/1)	ST5 ♦	Italy	Gabrielli et al., 2021
		5.2 (4/96)	ST5 ♦, ST10' , ST14' , ST21, ST24, ST25,	Spain	Figueiredo et al., 2025b

Host latin name	Host common name	Prevalence % (no. positive /total)	<i>Blastocystis</i> subtypes	Country	References
			ST26, ST30, ST42, ST43, ST44		

Potentially zoonotic subtypes are in bold. *Frequent subtype shared between humans and animals; ♦Moderate common in human; †Rare common in human

Contributing factors

Statistical analysis showed that the best-fitting model included five variables: presence on a wind farm, percentage of arable land, percentage of settlements, percentage of water, and the study area (Table 4). Roe deer on wind farms had a significantly lower probability of *Blastocystis* occurrence than those outside the area (Wald Chi² = 5.759, p = 0.016). The probability of *Blastocystis* occurrence in roe deer decreased with increasing percentage of settlements (Wald Chi² = 4.460, p = 0.035) but increased with increasing percentage of arable land and water in the area occupied by them (Wald Chi² = 6.295, p = 0.012 and Wald Chi² = 5.080, p = 0.024 respectively). Other variables, sex and age of animals, and season did not significantly impact the *Blastocystis* occurrence in roe deer and were excluded during model selection.

Table 4
Effects of wind farm (WF), arable land cover (ARABLE), settlement cover (SETTLEMENTS), surface water cover (WATER), and study area (AREA) on the probability of *Blastocystis* presence in roe deer in a generalized linear binary model; other variables (sex, age of animals, and season) were excluded during model selection.

Parameter	Estimate	St. error	Lower CI	Upper CI	Wald Chi ²	P-value
Intercept	-15.247	5.937	-26.883	-3.610	6.595	0.010
WF (yes)	-1.300	0.542	-2.362	-0.238	5.759	0.016
WF (no)	0*					
ARABLE	0.305	0.122	0.067	0.543	6.295	0.012
SETTLEMENTS	-1.100	0.521	-2.122	-0.079	4.460	0.035
WATER	1.970	0.874	0.257	3.683	5.080	0.024
AREA (Iłża)	3.374	1.861	-0.273	7.020	3.288	0.070
AREA (Rawa M.)	-1.001	0.541	-2.069	0.051	3.479	0.062
AREA (Węgrów)	0*					

DISCUSSION

Blastocystis is a common protozoa of humans, which is also frequently observed in wild and domestic animals [1]. However, the role of wildlife in *Blastocystis* transmission is not well explored, especially in Poland. Roe deer are a common wildlife species with habitats that may overlap with humans and other domestic and wild animals, which creates the potential for roe deer to act as reservoirs for pathogens among these populations. Although the role of *Blastocystis* in causing intestinal disease remains debated, since many infected humans and animals show no symptoms [34, 4], research on this organism has intensified because of its zoonotic potential and considerable genetic diversity [35]. Evidence indicates bidirectional transfer between animals and humans, particularly in settings with close contact, such as among zoo workers, laboratory staff, and individuals exposed to livestock or shared water sources [10, 35, 36]. Identical or highly similar subtypes have been detected in both humans and animals, supporting this link [12, 14]. Experimental studies have further confirmed human-to-animal transmission [37]. The protist's low host specificity and wide subtype diversity highlight the significance of cross-species transmission among livestock, wildlife, domestic animals, and humans sharing the same environment [1].

Our study represents the largest analysis of free-ranging roe deer (*Capreolus capreolus*) for *Blastocystis* in Europe to date (n = 151). The prevalence of 32.5% is comparable to the infection rate previously documented in wild roe deer populations in Portugal (32.5%) while being noticeably higher than that observed in Spain (12.9%) [38]. The remaining reports from Europe (Denmark, Portugal, United Kingdom) involved only a few individuals [35, 39, 40].

Furthermore, the presence of *Blastocystis* in Europe has already been confirmed in several other cervid species, including elk (*Alces alces*), reindeer (*Rangifer tarandus*), Reeves' muntjac (*Muntiacus reevesi*), red deer (*Cervus elaphus*), and fallow deer (*Dama dama*) [18, 34, 38, 39, 41, 42]. The isolates obtained in our study align with this trend, indicating close phylogenetic connections with various cervid species. Within the identified ST13, ST10, and ST14 clades, the sequences from Polish roe deer clustered with subtypes isolated from wild ruminants in other parts of the world, including British muntjac, red deer, and elk; as well as Chinese reindeer and sika deer. This relatively high infection rate underscores the need for ongoing surveillance of *Blastocystis* transmission at the wildlife-livestock-human interface. Their adaptable feeding behavior and habitat use can increase infection burden and facilitate cross-species transmission via direct contact or environmental contamination [27, 28, 43, 44].

Forty-four *Blastocystis* subtypes have been reported in the literature [5–7, 38]. Of those, sixteen subtypes (ST1-ST10, ST12, ST14, ST16, ST23, ST35, and ST41) have been described in humans [6, 45, 46]. To date, about seventeen STs, including ST1, ST2, ST4, ST5, ST10, ST13, ST14, ST21, ST23, ST24, ST25, ST26, ST30, ST42, ST43, ST44, and ST49, have been identified in European *Cervidae*. Eight *Blastocystis* STs, including ST2, ST5, ST10, ST13, ST14, ST24, ST44, and ST49, were identified in *Blastocystis*-positive samples from European wild roe deer (Table 2.).

In the present study, three subtypes in wild roe deer, ST10, ST13 and ST14 were identified, and in two samples the subtype was not identified. The most prevalent subtype was ST14 (26/49; 53,1%), followed

by ST13 (16/49; 32.7%) and ST10 (5/49; 10.2%). In comparison to the study by Figueiredo et al. (2025) [38], ST13 was identified as the predominant subtype among roe deer in Portugal (58.8%) and Spain (43.8%), while the frequency of ST10 was 5.9% and 18.8%, respectively, and subtype ST14 was not detected.

Phylogenetic analysis confirmed that the obtained isolates clustered within the clades of their respective subtypes (ST10, ST13, and ST14). The isolates belonging to subtype ST14 showed nearly 100% similarity to isolates obtained from domestic ruminants (goats, sheep, and cattle) from Asia, while one of the isolates clustered with a human sequence from Senegal. Similarly, the ST10 isolates showed similarity to both human strains from Senegal and wild cervids from the United Kingdom. In contrast, the ST13 isolates formed a highly stable, monophyletic clade closely related (99.14–100% similarity) to wild ungulates, including Tibetan antelope, goral, reindeer, and muntjac. Contrary to earlier beliefs, *Blastocystis* subtypes do not appear to be host-specific. In humans, ST1–ST9 and ST12 are commonly reported, with occasional cases of ST10, ST14, and ST16; whereas ST13 has not yet been detected in humans [46–48]. Various transmission routes, human-to-human, zoonotic, and waterborne, have been investigated [12, 40, 49, 50], and the presence of certain subtypes in both humans and animals suggests their zoonotic potential [11, 35].

According to the subtypes detected in this study, subtype 10 has been previously found in human volunteers in Thailand [45] and both subtype 10 and 14 were found in Senegalese children [48]. Both are typical cattle subtypes [51–53]. The study by Jinatham et al. (2021) suggests possible zoonotic transmission of some subtypes, including ST10 [45], but the lack of sharing between animals and their owners indicates that environmental sources such as water or soil are the more likely origin of *Blastocystis* in the examined community [45]. The authors underline that many *Blastocystis* subtypes were detected in untreated, unboiled rainwater used for drinking and food washing, likely contaminated by wildlife and inadequate filtration or chlorination, facilitating transmission of various subtypes [50, 54–57]. The mode of transmission of ST10 to humans remains unclear and may be through either direct contact with livestock or indirect contact with wild animals, such as waterborne transmission, as ST10 has already been detected in river water samples [58].

Analysis by Jiménez and colleagues (2022) [59] showed several associations between the same subtype with different hosts, and it was also demonstrated that the same host could have significant associations with multiple subtypes; for example, cattle were found to be associated with four subtypes, including ST14, which supports the hypothesis that interactions involving direct contact among animals of the same or different species, humans, and the surrounding environment may constitute an important pathway for *Blastocystis* transmission [59]. The hypothesis of multiple transmission routes, including anthroponotic links, applies to human-associated subtypes, alongside human-to-human, animal-to-human, and likely environmental transmission. Defining subtype-specific pathways requires further study, including environmental sampling to better understand the *Blastocystis* life cycle. Other numerous reports show ST10 and ST14 as predominant generally in wild and domesticated ruminants [9, 16, 60–

62]. There appears to be some degree of host adaptation amongst ungulates, with frequent identification of ST10 followed by ST14 in bovids, cervids, camelids, ovids, and equids [1].

Additionally, the researchers underline that ST13, although rarely reported in animals, might exhibit a wider host range, having been identified in primates, marsupials, and artiodactyls. Originally ST13 was recognized in Australia in the quokka, a marsupial (*Setonix brachyurus*) [14], followed by the western grey kangaroo [63], the Java mouse-deer [51], as well as in reindeer in China [64]. Furthermore, in the Polish Gdańsk Zoo, this subtype was detected in all tested lutungs and one guereza [16].

Furthermore, this study investigated the potential effects of anthropogenic pressure (distance to the nearest wind turbine, built-up areas) and landscape structure (arable land, surface water cover) on *Blastocystis* infection levels in roe deer. Positive correlation between the percentage of arable land and the probability of *Blastocystis* occurrence in roe deer is probably associated with exposure of wildlife to fecal-borne pathogens associated with agricultural management. Roe deer frequently forage in cultivated fields, utilizing agroecosystems as a rich food resource. However, this synanthropization of behavior increases the risk of contact with soil amended with manure or slurry. As indicated by Akdur-Öztürk et al. (2025) and Kuijper et al. (2014) [65, 66], agricultural areas are characterized by a higher level of environmental contamination with infective stages of parasites. Insufficiently composted natural fertilizers, consisting of livestock excreta and litter, serve as key reservoirs and sources of microbial contamination [67]. Furthermore, the application of liquid slurry promotes the long-term persistence of *Blastocystis* cysts in the topsoil, from where they can be easily ingested by foraging roe deer. Moreover, cultivated fields promote roe deer aggregation during foraging, leading to greater fecal contamination of the environment and consequently increasing the risk of protist transmission [67]. Sharing habitats, such as pastures and cropland, with domestic ruminants (cattle, sheep) can lead to the transmission of pathogens from livestock to wildlife [18]. An emerging body of scientific evidence also confirms that the soil itself in intensively managed agricultural areas becomes an independent and significant transmission route [45].

Blastocystis can occur in aquatic environments, suggesting a role for surface waters as a reservoir and source of infection. A high proportion of water favors environmental transmission, especially in the coexistence of wild and farm animals. Waterborne transmission of *Blastocystis* has long been recognized [12, 50] as it was identified in the water supply of a rural community in central Thailand as well as in schoolchildren that consumed it [55] and in untreated drinking water in Peninsular Malaysia [50]. *Blastocystis* has been identified in rivers that supply water to village communities [12, 58]. In Malaysia, *Blastocystis* was identified in river water as well as in other water sources in the village, such as tap, stored, or well water [58]. In Nepal, these same subtypes were identified from a river and in humans and animals in the same village, providing further evidence of the occurrence of waterborne transmission [12, 68].

Roe deer on wind farms had a significantly lower probability of *Blastocystis* occurrence than those outside the area. No direct studies link *Blastocystis* in wildlife to wind farms, though habitat changes and

fragmentation may indirectly affect transmission by altering animal behavior (avoidance of the area and selection of alternative migration routes), access to water, and contact patterns [18, 65, 69]. In one of the studied areas, a lower density of roe deer was found at the wind farm in summer compared to the adjacent area [70]. Similarly to the wind farms, the probability of *Blastocystis* occurrence in roe deer decreased with the increasing percentage of settlements. The increasing percentage of settlements correlates with higher habitat fragmentation and a significant reduction in the availability of continuous, natural habitats suitable for roe deer. In highly fragmented or suburban landscapes, roe deer populations often exhibit lower local densities or altered spatial distribution to avoid human disturbance and infrastructure [22, 71].

In conclusion, this study for the first time determined the occurrence and subtype distribution of *Blastocystis* in wild roe deer in Poland. The data showed that three *Blastocystis* subtypes occur in wild roe deer. Although two of these subtypes ST10 and ST14 can be shared with humans, their zoonotic potential remains low. Nevertheless, these findings provide baseline data for monitoring and exploring the transmission dynamics of this protist at the animal-human interface. The increasing interaction between humans and animals, promoted by factors such as agriculture, urbanization, industrialization, and globalization, can influence cross-species transmission and the spread of zoonotic diseases such as blastocystosis.

Moreover, our results demonstrate that *Blastocystis* infection in wild roe deer within the study area is influenced by landscape structure and anthropogenic factors.

Thus, it is crucial to carry out more studies evaluating the presence of *Blastocystis* in more animal species, both domestic and wild, from different geographic locations to improve our knowledge of its epidemiology and host specificity of subtypes in order to understand potential zoonotic transmission patterns.

MATERIALS AND METHODS

Source and sample collection

During the project a total of 151 fecal samples from hunter-killed roe deer were collected. The study was conducted in accordance with national guidelines. According to the Animal Protection Law in Poland (Act of the Polish Parliament dated 21 August 1997), the use of samples of animal origin from game animals for scientific research is not a procedure, and the consent of the ethics committee is not required.

The material was obtained in 2023/2024 from three locations: location 1 (WE-Węgrów, n = 43), location 2 (RA-Rawa, Skierniewice, and Brzesko, n = 45), and location 3 (IL-Radom and Lipsko, n = 63) (Fig. 3). Approximately 10 g of fecal samples were collected from a section of the large intestine during autopsy and placed in a sterile tube with 70% alcohol. The prepared material was stored at -80°C until processing.

Molecular analysis

Prior to molecular analysis, 200 mg of feces from each sample was washed three times with sterile phosphate-buffered saline (PBS) using 2.0 mL microcentrifuge tubes (centrifugation at 2800×g for 5 min). The commercial QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used for genomic DNA isolation. The extracted DNA was dissolved in 40 µL of TE buffer and stored at -20°C for further studies.

Molecular identification of *Blastocystis* was performed by PCR targeting a 607-base pair fragment of the small subunit ribosomal RNA (SSU rRNA) gene, amplified with specific primers RD5 (5'-ATCTGGTTGATCCTGCCAGT-3') and BhRDr (3'-GAGCTTTTAACTGCAACAACG-5'). PCR amplification was conducted on a Mastercycler® nexus thermal cycler (Eppendorf, Hamburg, Germany) in standard 0.2 mL microtubes according to the following composition of a 25 µL reaction mixture: 12.5 µL of 2 × PCR Master Mix Plus (A&A Biotechnology, Gdynia, Poland), 1 µL of each 10 µM primer, 2 µL of genomic DNA and a nuclease-free water. The PCR reaction conditions were as follows: initial denaturation at 94°C for 1 min, and then 30 cycles of denaturation, annealing and extension for 1 min at 94°C, 59°C and 72°C, respectively, and a final extension step of 2 min at 72°C. The PCR products were electrophoresed on 1.5% agarose gels stained with Midori Green Advance dye (Nippon Genetics, Japan) and then visualized using a Molecular Imager® Gel DocTM XR System (Bio-Rad, Hercules, California, USA).

The positive PCR products were purified with Clean Up (A&A Biotechnology, Gdynia, Poland) and then subjected to bidirectional sequencing by the Macrogen Humanizing Genomics Europe (Amsterdam, The Netherlands). The obtained nucleotide sequences were assembled using BioEdit Sequence Alignment Editor v. 7.2 and subsequently analyzed by the BLAST-NCBI program (<https://blast.ncbi.nlm.nih.gov/Blast>). The nucleotide sequences obtained in this study are available in the GenBank database under accession numbers PV982219–PV982267.

Phylogenetic analyses

The phylogenetic analyses for *Blastocystis* were based on the SSU rRNA (small subunit ribosomal RNA). The trees were constructed using 10 representative sequences. Three sequences belong to subtypes 10 and 13, and 4 sequences belong to subtype 14. A *Proteromonas lacertae* sequence was used as an outgroup to root the trees. Sequences were aligned using the ClustalW algorithm in Mega X and trimmed at the ends using BioEdit v. 7.2 [72]. The trees were constructed using Mega X software [72]. The maximum likelihood (ML) method was applied using the Tamura 3-parameter (T92 + G). The analysis involved 37 and 29 nucleotide sequences, respectively. Accordingly, there were 190 and 460 positions in the final dataset. In both analyses, the internal node support was assessed by 1,000 bootstrap sampling. The scale bar indicates the equivalence of distance between sequences.

Data elaboration and statistics

Based on the hunters' reports, basic information was determined for each sample: age of roe deer, sex of roe deer, season of sample collection, and study area (Iłża, Węgrów, Rawa Mazowiecka). Based on the roe deer location, their presence in the wind farm area was determined. The wind farm area was defined as the interior of the area surrounded by a compact group of wind turbines and within a buffer of 700 m from the outermost wind turbine, in accordance with previously assumed distances indicating the impact of this infrastructure on roe deer [73–75]. Next, for each hunting district where roe deer were hunted, the percentage of selected land cover types was calculated. Land cover structure was calculated based on the publicly available Corine Land Cover (CLC) database. For the study, four main land cover types were selected: 1) arable land, 2) forests, 3) water, and 4) settlements. The percentage of each cover type was calculated in QGIS 3.22 by overlaying the hunting district boundaries and the acquired CLC layers. The correlation of the percentage of calculated cover types was then assessed to eliminate collinearity between explanatory variables in the model. A standard threshold correlation coefficient of $|r| = 0.7$ was adopted. Forests showed a high correlation with arable land; therefore, forests were excluded from further analysis.

A statistical analysis of the *Blastocystis* occurrence in roe deer was performed using a generalized linear binary model in SPSS software. The dependent variable in the model was the presence or absence of *Blastocystis* in roe deer, expressed as a binary variable. The probability of the *Blastocystis* occurrence in roe deer was assessed based on eight variables including animal characteristics, season, sampling location, and environmental conditions: a) roe deer sex, b) roe deer age, c) season, d) study area, e) presence of roe deer in the wind farm area, f) percentage of arable land, g) percentage of water, h) percentage of settlements. We performed a full model selection procedure for all model variants (with various variable numbers and compositions), including the null, intercept-only model, to determine the model that best fit the data. We assessed the quality of the models using the Akaike Information Criterion (AIC), where the lower its value, the better the model fit. Ultimately, we presented the best-fitting model, i.e., with the set of variables that resulted from the analysis of the AIC values.

Declarations

Competing interests

The authors declare no competing interests.

Author Contribution

JK and ML: wrote the main manuscript text; JK: prepared figures 1-3 and tables 1,3, molecular analysis; ML: prepared table 2;DK: prepared table 4, statistical analysis; MK and MS: collecting the samples. All authors reviewed the manuscript.

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Data Availability

The nucleotide sequences generated in the present study have been deposited in GenBank (<https://www.ncbi.nlm.nih.gov/>) under accession numbers PV982219–PV982267. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

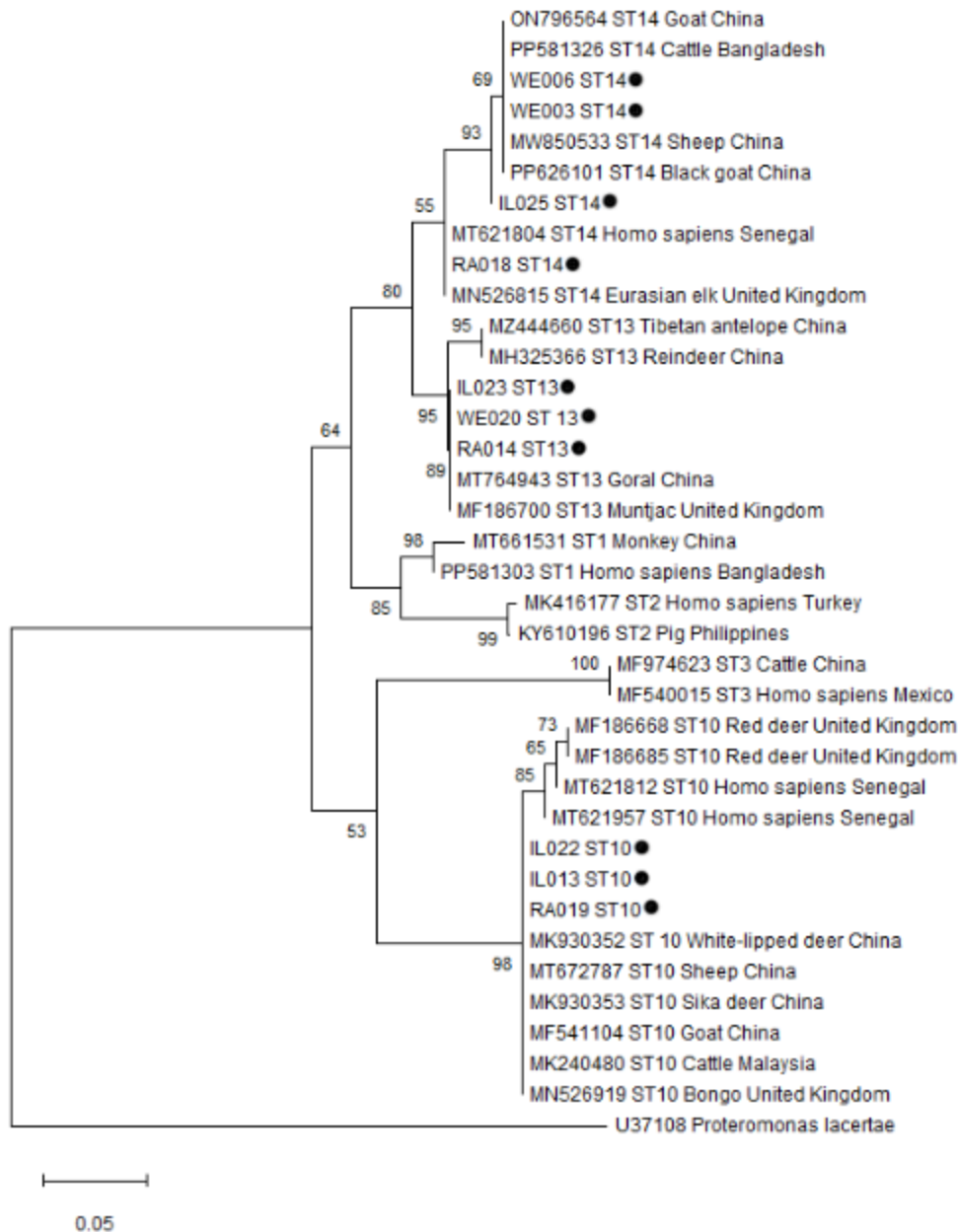


Figure 1

Phylogenetic tree based on sequences of the partial small subunit ribosomal RNA (SSU rRNA) of *Blastocystis* sp. The phylogenetic tree was constructed using the maximum likelihood method and K2+G substitution model. Sequence of *Proteromonas lacertae* served as outgroups. This analysis involved 37 nucleotide sequences and there were a total of 190 positions in the final dataset. The numbers on the branches are percent bootstrap support values from 1000 replicates. The scale shows the number of nucleotide substitutions per site between DNA sequences. Representative sequences identified in this study are marked with a dot.

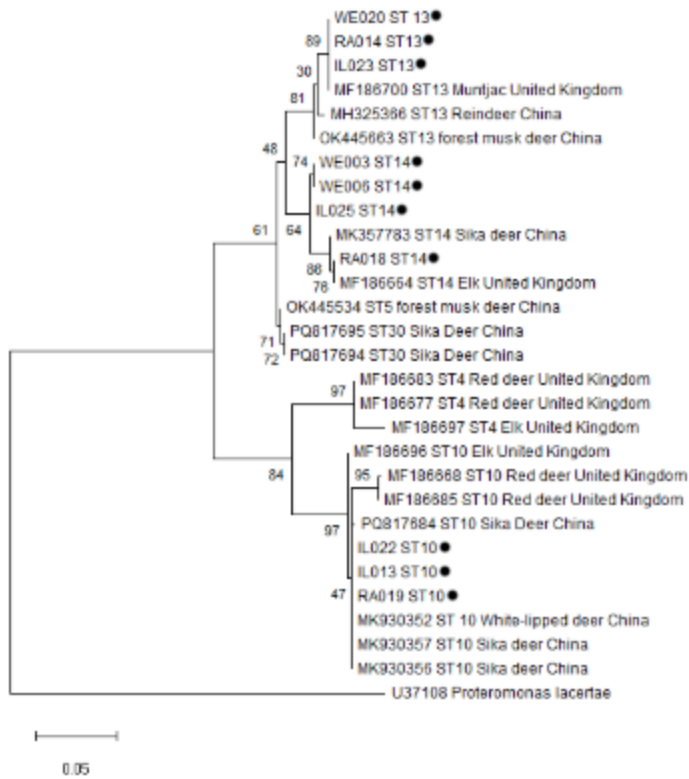


Figure 2

Phylogenetic tree based on sequences of the partial small subunit ribosomal RNA (SSU rRNA) of *Blastocystis* sp. The phylogenetic tree was constructed using the maximum likelihood method and K2+G substitution model. Sequence of *Proteromonas lacertae* served as outgroups. This analysis involved 29 nucleotide sequences and there were a total of 460 positions in the final dataset. The numbers on the branches are percent bootstrap support values from 1000 replicates. The scale shows the number of nucleotide substitutions per site between DNA sequences. Representative sequences identified in this study are marked with a dot.

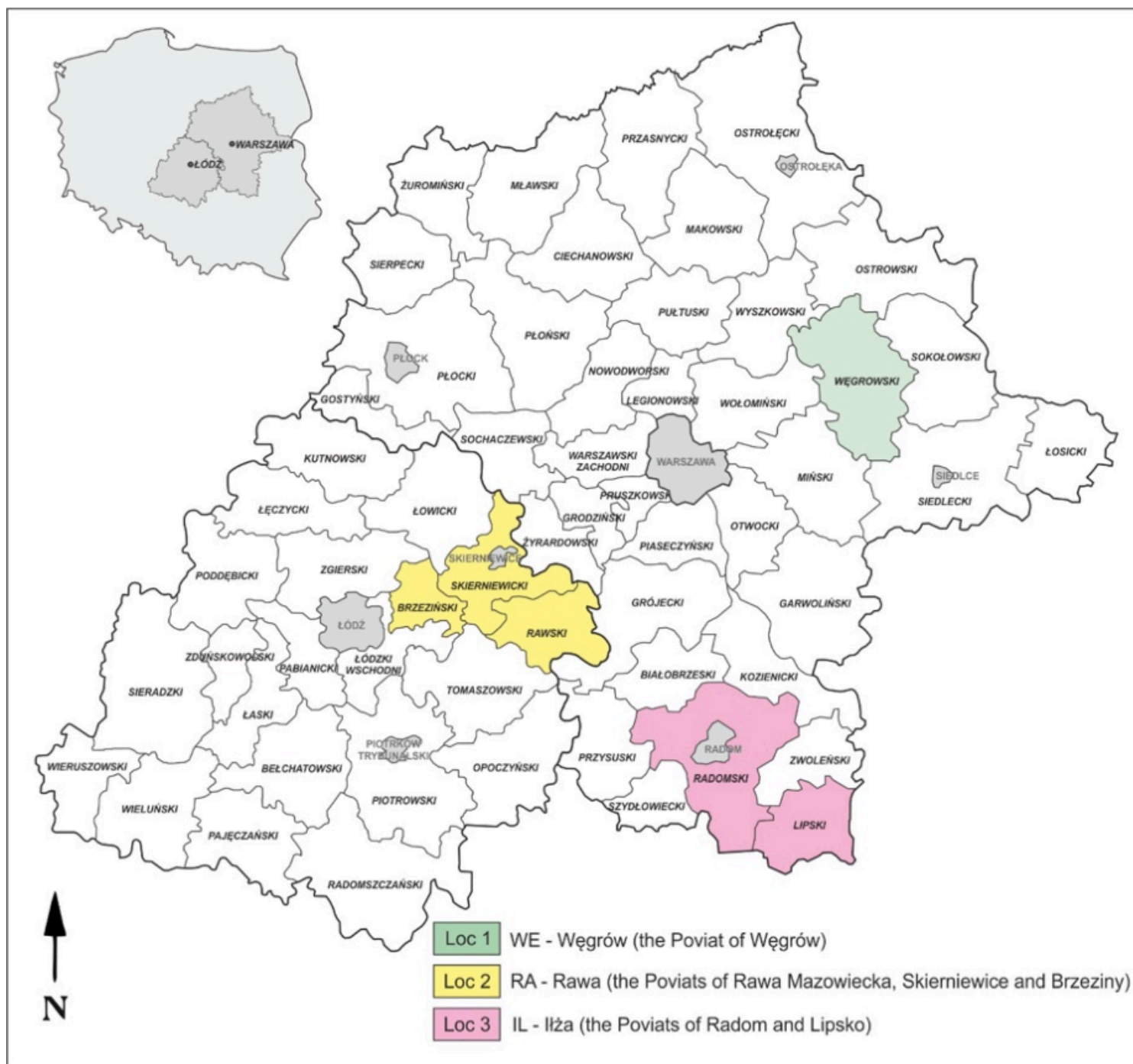


Figure 3

Geographic map of Poland, showing three localities of sampling sites.