Genetic diversity of Blastocystis in non-primate animals

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Genetic diversity of *Blastocystis* in non-primate animals

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KEY FINDINGS

- Distribution of *Blastocystis* subtypes across in a wide range of hosts
- Co-colonization of elk, goat, red deer, water vole with more than one subtype
- Differences in *Blastocystis* subtype distribution between wild and captive species
- Genetic divergence of *Blastocystis* subtypes within the park
- Long-term culture method of *Blastocystis* at 21oC in TYM with FBS

SUMMARY

Blastocystis is an anaerobic protist, commonly inhabiting the intestinal tract of both humans and animals. *Blastocystis* is extremely diverse comprising 17 genetically distinct subtypes. Pathogenicity of this enteric microbe is currently disputed and knowledge regarding its distribution, diversity and zoonotic potential is fragmentary. Most research has focused on *Blastocystis* from primates, while sampling from other animals remains limited. Herein, we investigated the prevalence and distribution of *Blastocystis* in animals held within a conservation park in South East England. A total of 118 samples were collected from 27 vertebrate species. The barcoding region of the small-subunit ribosomal RNA was used for molecular identification and subtyping. Sixty per cent of the samples were sequence positive for *Blastocystis* indicating a high prevalence and wide distribution among the animals in the park. Six subtypes were identified, one of which is potentially novel. Moreover, the majority of animals positively identified as carriers, suggesting that *Blastocystis* is not pathogenic in animals. This study provides a thorough investigation of *Blastocystis* prevalence within a wildlife park in the UK and can be used as a platform for further investigations on the distribution of other eukaryotic gut microbes.

Keywords: *Blastocystis*, subtype, prevalence, phylogeny, genetic diversity

INTRODUCTION

Blastocystis is a microbial eukaryote that inhabits the gastrointestinal tract of a variety of animals including humans, other primates, amphibians, reptiles, and even insects (Abe 2004; Parkar et al. 2010; Roberts et al. 2013; Stensvold et al. 2009; Yoshikawa et al. 2016). After fungi, *Blastocystis* is one of the most prevalent microbial eukaryotes in metazoans (Scanlan et al. 2014).

Until recently, *Blastocystis* was overlooked due to its small size and lack of distinct morphological features. Nonetheless, the advent of molecular methods has revealed an astounding genetic heterogeneity of *Blastocystis*. To date, 17 genetically diverse lineages have been reported (subtypes; ST), based on differences of the small subunit ribosomal RNA (SSU rRNA) (Stensvold and Clark 2016). *Blastocystis* has wide host range, with the same subtype found in several animal genera. Emerging data however, suggests that host specificity should be assessed based on lower than genus level taxonomy (Alfellani et al. 2013c). Of the 17 STs, only the first nine (ST1-ST9) and recently, ST12 have been found in humans (Ramirez et al. 2016; Stensvold and Clark 2016). *Blastocystis* has been reported in wild animals, pets and domesticated animals and those that are housed in zoos (Amenu et al. 2015; Figueroa 2015; Parkar et al. 2010; Puebla et al. 2017; Ruaux and Stang 2014; Schar et al. 2014; Wang et al. 2014). Nonetheless, the comprehensive range of non-primate hosts of the various STs remains unclear, since only a limited number of studies focus on screening such animals (Abe et al. 2002; Lim et al. 2008; Parkar et al. 2010; Perez Cordon et al. 2008; Roberts et al. 2013).

The presence of *Blastocystis* isolates in various animals that belong to the same STs as those in humans has led to the speculation that the organism has zoonotic potential (Parkar et al. 2010; Rajah Salim et al. 1999; Ramirez et al. 2014). Nonetheless, this scenario has come under scrutiny in recent years, since cases where the direction of transmission has been established conclusively are absent. Moreover, most molecular investigations of *Blastocystis* isolates from domesticated animals and their keepers have not revealed any shared subtypes, though there are notable exceptions (Alfellani et al. 2013b; Wang et al. 2014). Due to this controversy, there is an urgent need for further investigations on the distribution of *Blastocystis* in animals in captivity, since prevalence data and molecular characterization of *Blastocystis* in such animals remain sparse.

Herein, we examine *Blastocystis* isolates from Wildwood Trust, a wildlife park in East Kent, UK. The park's collection consists mostly of UK native and previously native wildlife, meaning that the chance of the identified isolates being local is very high. The aim of this study

was to investigate the prevalence, distribution, genetic diversity and host range of *Blastocystis* STs in animals at Wildwood Trust.

MATERIALS AND METHODS

Study site - Source of specimens:

A total of 118 faecal samples were collected from 27 different host species (Table 1) located at Wildwood Trust (Herne Bay, Kent, UK). Sampling covered a range of mammalian species, four bird species and one reptile species (Table 1).

Sample collection and storage

A licensed veterinarian visits the park on a monthly basis to monitor the animals' health, during the week of sampling no animals exhibit diarrhea. Faecal samples were collected between the months of July 2016 to January 2017. Wildwood Trust staff collected samples under the guidance of laboratory members; A minimum of one sample was collected per animal species, where possible (Table 1). In the cases where multiple animals of the same species were enclosed together, several samples were collected.

Once collected, samples were placed in sterile tubes and stored at 4 ^{o}C in sealed falcon tubes until DNA extraction. The faecal samples were subdivided shortly after collection to be used for microscopy, culturing and DNA extraction. Heat-fixed slides were made from all samples collected within an hour of collection.

Culturing

At Wildwood Trust, a total of 118 fresh samples were collected during July 2016 and October 2016 from 27 different species. Within half an hour of sampling, a small amount of water vole faecal sample was used to separately inoculate tubes containing media as follows: two tubes containing modified LYSGM (16.07 mM potassium phosphate dibasic, 2.94 mM potassium phosphate monobasic, 128.34 mM sodium chloride, 2.5 g/L yeast extract, 0.5 g/L liver extract, 5 ml adult bovine/horse serum) [modified TYSGM-9, (Diamond 1982), http://entamoeba.lshtm.ac.uk/xenic.htm], two tubes of TYM (22.2 g/L trypticase peptone, 11.1 g/L yeast extract, 16.23 mM maltose, 9.17 mM L-cysteine, 1.26 mM L-ascorbic acid, 5.1 mM potassium phosphate dibasic, 6.53 mM potassium phosphate monobasic) (DIAMOND 1957; Diamond 1983) enriched with fetal bovine serum (FBS) and 2 tubes with 0.5% Liver Digest (LD) media (0.5 g/L Oxoid liver extract). One tube from each media was incubated at 35^{o}C and the rest were left at room temperature. Samples were examined every three days under light

microscope with neutral red staining (see below). Cultures positive for *Blastocystis,* were subcultured every 10 days.

Staining and microscopy:

For the identification of live cells within cultures, a neutral red staining technique was employed (DeRenzis and Schechtman 1973). Ninety-four µl of cultured samples were mixed with equal volumes of freshly prepared 0.04% neutral red staining (Sigma, N2889) in 0.5 ml tubes and incubated for 10 min at 36°C. The samples were then centrifuged at 5000 x g for 30 seconds. The supernatant was removed and the pellet was re-suspended in 20 µl of 1 X PBS (pH 7.2) by vortexing. Ten µl of the mixture was placed on a glass slide under a 22-mm square coverslip and individual cells were observed under 200 x and 400 x magnification.

DNA extraction, PCR, cloning and sequencing:

DNA from faeces and cultures were extracted using the Microbiome DNA Purification Kit Purelink (Fisher, UK), following the manufacturer's specifications and protocols. The extracted DNA was stored at -20 ^{o}C for long-term usage. To amplify the fragment of interest, polymerase chain reaction (PCR) was carried out using the extracted DNA. DNA extracted from an axenic *Blastocystis* NandII culture was used as positive control in every PCR application. The conditions of amplification were as follows: 2 µl of the extracted DNA was used for amplification of a *Blastocystis* sp SSUrRNA fragment. 10 µl 5X buffer (Promega), 1 mM $MgCl_2$, 0.4 µM forward primer, 0.4 µM reverse primer, 0.2 mM dNTPs (Promega), 0.25 µl Taq polymerase, 30.75 µl HPLC grade water 2 µl DNA. The fragment was amplified in a total of 50 µl reaction, according to the standard conditions of for HiFI Taq polymerase (Promega). The broad specificity primers RD3 5´- GGGATCCTGATCCTTCCGCAGGTTCACCTAC-3´ and RD5 5´-GGAAGCTTATCTGGTTGATCCTGCCAGTA-3´(Clark 1997) were used for the first PCR. Cycling conditions were as follows: 95^{o}C 5 minutes, 35 cycles of denaturation at 95 ^{o}C for 30 seconds, annealing 55 ^{o}C for 30 seconds, extension at 72 ^{o} C for 1 minute 40 seconds and final extension at 72 ^{o} C for 5 minutes.

A second nested PCR was performed using the forward RD5F 5´-ATCTGGTTGATCCTGCCAGT-3´ and reverse BhRDr 5´-GAGCTTTTTAACTGCAACAACG -3´ (Scicluna et al. 2006) primers giving a fragment at approximately 650 bp. This fragment is considered the barcoding region for *Blastocystis* identification. Concentration of reagents in each reaction and PCR conditions were the same as above. One µl from the PCR mentioned above was used as template.

Positive PCR reactions from the nested-PCR were gel-extracted using the Thermo Scientific GeneJET Gel Extraction Kit (following manufacturer's instructions) and subsequently cloned in the pGEM-T vector (Promega) using the manufacturer's protocol. Five to ten colonies from each transformation were selected for sub culturing and plasmid purification using the GeneJET Plasmid Miniprep Kit. Positive plasmids were screened by digestion with EcoRI restriction enzyme, to confirm presence of the fragment of interest. Positive plasmids were bidirectionally sequenced using T7 and SP6 universal primers by Eurofins, UK.

Genetic distance and phylogenetic analysis

The obtained sequences were trimmed to eliminate vector fragments and forward and reverse sequences of each sample were joined using Sequencher. Blast searches of the obtained sequences against GenBank were performed to exclude bacterial contamination. A dataset including all new sequences identified as *Blastocystis* along with sequences spanning the breadth of diversity of *Blastocystis* subtypes was build and aligned using MAFFT v.7 (Katoh and Toh 2010). The alignment was further improved by visual check where necessary. Genetic distance was calculated using the Kimura2 parameter criterion. Gaps were considered as complete deletions. For this calculation, only the barcoding region of *Blastocystis* was used.

For the phylogenetic analysis, four additional outgroup taxa were included to the alignment and the entire sequence of SSUrRNA was used. The alignment contained a total of 90 taxa. Several sequences were represented only by their barcoding region in which case, the missing part of the sequence was considered as missing data. Following alignment with MAFFT, ambiguous positions were removed using trimAL (Capella-Gutierrez et al. 2009). After trimming the alignment contained 1163 sites. Phylogenetic trees were constructed by using maximum likelihood and Bayesian inference methods. Maximum likelihood trees were computed using the RAxML software (Stamatakis 2006). For each dataset bootstrap support was evaluated from 1000 bootstrap replicates. Bayesian inference tree was computed using MrBayes (Ronquist and Huelsenbeck 2003). Posterior probabilities were computed by running four chains with sampling occurring every 100th generation, whilst 25 % of trees were discarded as burn-in. Trees were run for 1,500,000 generations at which point all parameters converged at 0.01.

RESULTS

Culturing

Blastocystis grew in the tubes containing LYSGM and TYM+FBS at both 35 ^{o}C and room temperature. There was no *Blastocystis* growth in the 0.5 % LD media.

Screening of faecal samples

A total of 118 samples from 27 species were examined of which 71 (60 %) were sequence positive belonging to 11 species (41 %) (Table 1). Nonetheless, there was a notable difference in the presence of *Blastocystis* across hosts. With the exception of a single case, all sequence positive samples came from non-carnivorous animals. This was despite repeated sampling and sequencing attempts (Table 1). Specifically, 7/8 (87.5 %) of artiodactyls, 2/2 (100%) of rodents and 1/9 (11%) of carnivores were sequence positive for *Blastocystis*. No sequence positive samples were found in birds, snakes, and insectivores (Table 1).

Subtype identification and distribution in various hosts

Among the 71 *Blastocystis*-positive samples, six STs were detected (Table 2, Figure 1): ST1, ST4, ST5, ST10, ST14 and a potentially new subtype. Subtypes 4 and 10 colonized the most species (seven and six respectively) followed by ST14 (three), ST1 (two), ST5 (one) and an unidentified ST (one). We provide the first molecular data and subtyipng of *Blastocystis* from elk, water voles, pine martens and red squirrels. The Eurasian elk (artiodactyl) were the hosts harboring the widest range of subtypes, followed by pygmy goat (artiodactyl) and water vole (rodent). Most notably, four subtypes were found in the elk (ST4, ST10, ST14, unidentified), while goat and water vole harbored three (ST1, ST10 and ST14 in goat and ST1, ST4 and ST10 in water vole). The hosting of multiple subtypes within elk is of no doubt, as there is just a single elk in the park. The same cannot be verified for the goats and voles as the park houses several of them. Nonetheless, only two faecal samples were collected, which means that there are at least two subtypes present in a single goat. The three subtypes in water vole were identified only in the captive population of which three were sampled. The presence of all subtypes can be confirmed here, due to cloning being used rather than PCR purification of a single product.

Several samples were collected from two rodent species; the red squirrel and water vole. Subtype 4 was commonly detected in both species, while the range of subtypes previously reported within rodents can be expanded to include ST1. Several colonies were also screened from wallabies, diprotodontid marsupials. All samples from wallabies harbored ST10, which had not been reported previously from these marsupials.

Phylogenetic analysis

Though 71 clones were sequenced, only 20 of them were used in the phylogenetic analyses. In the cases where clusters contained identical clones, only a few representative sequences were kept. In total, the new sequences were subtyped as follows: ST4 (n=41); ST10

(n=22); ST14 (n=4); ST1 (n=2); ST5 (n=1) ST? (n=1). All *Blastocystis* sequences formed a strongly supported cluster (100BS/1.00BI). Most newly sequenced isolates grouped within clades formed by the officially accepted subtypes (Figure 1). The most basal sequences belonged to *Blastocystis* isolates from reptiles and cockroaches along with those from ST15, ST16 and ST17 in agreement with previous studies (Alfellani et al. 2013c; Yoshikawa et al. 2016). Subtype 3 sequences grouped together and sister to a clustered formed by ST10, ST8 and ST4. Subtypes 7, 9 and 6 clustered together, while ST11, ST2 and ST1 formed a separate clade. Subtypes 13 and 14 were not well resolved even when a subtree was constructed (data not shown). The ELB_WW Elk 1 clone 1 did not fall within any of the 17 STs and its position remains unresolved.

DISCUSSION

Approximately 61 animals from 27 species were examined. Forty one percent of all animals were sequence positive for *Blastocystis*. In select cases, we attempted to establish cultures of *Blastocystis*. The organism has been cultured in a wide range of media including egg media with Locke's solution, Iscove's modified Dulbecco's medium, Robinson's medium and Jones' media (Clark and Diamond 2002; Tan 2008). The latter was a widely-used formulation ideal for short term culturing of multiple subtypes (i.e. a few days). *Blastocystis* isolates originating from endothermic hosts are customarily cultured at 35 °C. Reported here was cultivation of *Blastocystis* from a water vole (*Arvicola amphibius*) in TYM media enriched with FBS. The culture had been maintained in the laboratory for at least 11 months. Although the origin of the isolate is an endothermic animal, it grew over abundantly at room temperature. This indicates that some isolates of *Blastocystis* can grow at lower temperatures given certain types of media. Whether all isolates of *Blastocystis* or only some can grow in TYM+FBS at room temperature needs further study.

Most of the animals that we examined harbored a single subtype of *Blastocystis*. Nonetheless, some animals carried more than one subtype. Mixed colonization was confirmed, because we employed cloning and screened multiple colonies from each sample, while previous studies only used direct sequencing from PCR products (Alfellani et al. 2013b; Alfellani et al. 2013c; Roberts et al. 2013; Stensvold et al. 2012; Stensvold 2013). Using this strategy, it was found that elk (*Alces alces*) harbored four subtypes. There has been no previous reporting of *Blastocystis* in elk; hence, to the best of our knowledge this is the first time the organism is being reported in this mammal. In cases where multiple subtypes are found within a single host, it is important to exclude contamination from other sources. The park has a single elk, which is

housed in an isolated enclosure on its own. Moreover, the faecal sample was collected at the moment of defecation precluding contamination from small, non-resident animals. More than one subtype was also detected in pygmy goats (ST=3), red deer (ST=2) and water voles (ST=3). Unlike in the case of the elk, we cannot definitively conclude that the detected subtypes in goats originated from a single individual per se, since enclosures housed multiple animals of the same species. While colonization with multiple subtypes is rare in humans, not much information is available for other animal species (Meloni et al. 2012). In light of our findings, it is tempting to speculate that the microbiota of at least some animals constitute of multiple *Blastocystis* subtypes. Sampling from more animals and use of methodologies similar to ours will shed further light as to whether presence of multiple subtypes is the norm within these and other animals.

Water voles also constitute an interesting case. There are two, temporary, populations of water vole being held within the park, together with permanent residents. These two groupings of water vole are temporarily brought in to captivity as part of a licensed, development mitigation programme and are subsequently to be introduced back into their natural environment locations; two separate sites in Essex, UK. This study can report that 'wild' water vole harbored ST4 only, whereas those in permanent captivity also harbored ST1. Wild water voles were sampled multiple times, while captive ones provided only a limited number of samples. Despite considerable effort (PCR, cloning and screening of clones) we were unable to detect ST1 in wild water voles. It is tempting to speculate that the 'captive' water vole acquired ST1 after their introduction in the park and that this is one of the many microbiota-related alterations associated with life in captivity (Kohl et al. 2017; Waite and Taylor 2014). However, since captive voles originated from two additional locations other than Essex, this hypothesis needs further testing involving surveys of all populations of origin.

ST10 and ST4 were the most widely distributed subtypes, each isolated from five species. As previously described, artiodactyls carried mostly ST10 (Alfellani et al. 2013c). It has been speculated that rodents are reservoirs of ST4 for human infection, though not all rodent species carry this specific ST (Alfellani et al., 2013b). Subtypes 3 and 17 were also found in rodents in previous investigations (Alfellani et al. 2013a; Stensvold et al. 2009). Herein, this study detected ST4 in all *Blastocystis* positive samples of rodents. Nonetheless, other subtypes were also found in the screened rodents: ST10 in red squirrels and ST1, ST5 and ST10 in water voles. Therefore, the study has been able to expand the number of subtypes recorded in rodents by identifying ST1 and ST10. It was also possible to expand the range of subtypes identified in goats to include ST14, along with the previously identified ST10, ST1, ST3, ST6 and ST7

(Alfellani et al. 2013b). The study also detected ST14 in four hosts, all of which belong to the artiodactyls.

To determine the monophyly and relationships among STs, phylogenetic analyses were performed. Traditionally, sampling of *Blastocystis* had focused on primates, especially humans. As a result, STs that were present in non-primates were reported infrequently and the clades of these STs remained sparsely populated. For instance, the resolution of the ST13 and ST14 has been problematic. Previously, Alfellani et al. (2013c) speculated that ST14 should be split into two subtypes, but refrained from doing so pending further sampling. The current study has shown that, when our isolates were added to the tree, ST14 splits into two distinct clades, with our samples populating both of these clades. Hence, supporting the idea that it should be considered as two STs. Moreover, one isolate from elk grouped independently of all other STs, suggesting that this might be a subtype. Genetic divergence analysis of the barcode region indicated that the genetic distance between our isolate and all other STs is over 5%, with the exception of ST13, with which it differed by 4.4%. The recommended threshold to define a new sequence is 5% divergence (Clark et al. 2013). Nonetheless, the full sequence and further samples are needed to confirm this finding since this is an individual partial sequence.

In summary, we present here a comprehensive study of *Blastocystis* prevalence focusing exclusively on non-primate animals in a zoo setting in the UK. Presented here has been the presence of six subtypes, with one potentially being novel. Through the use of cloning, it has been possible to conclusively record the presence of multiple STs within an individual animal. The sequences generated from this study have populated STs that were considered rare and for which not many sequences exist. Collectively, these highlight the need for sampling from a wide range of hosts.

CONFLICTS OF INTEREST

The authors declare no conflict of interest

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FIGURE LEGENDS

Figure 1. Maximum likelihood phylogenetic tree inferred from 90 SSUrRNA sequences and 1163 sites.
Newly generated sequences are shown in bold. Numerical values on the branches indicate bootstrap percentages and posterior probabilities in this order. Only bootstrap support values greater than 70 are shown. The accession numbers of all newly generated sequences are presented in Table S1.

Table 1: Animal samples collected from study hosts

Host	Scientific Name	Location	Sample Number	PCR Positive
Carnivora				
Badger	*Meles meles*	Wildwood	2	-
European Brown Bear	*Ursus arctos arctos*	Wildwood	2	-
Lynx	*Lynx lynx*	Wildwood	3	-
Otter	*Lutra lutra*	Wildwood	7	-
Pine Marten	*Martes martes*	Wildwood	2	1
Polecat	*Mustela putorius*	Wildwood	1	-
Red Fox	*Vulpes vulpes*	Wildwood	3	-
Scottish Wild Cat	*Felis silvestris*	Wildwood	11	-
Stoat	*Mustela erminea*	Wildwood	3	-
Anseriformes				
Barnacle Goose	*Branta leucopsis*	Wildwood	1	-
Pink Footed Goose	*Anser brachyrhynchus*	Wildwood	1	-
Artiodactyla				
Muntjac Deer	*Muntiacus reevesi*	Wildwood	1	1
European Bison	*Bison bonasus*	Wildwood	3	3
Eurasian Elk	*Alces alces*	Wildwood	2	1
Pygmy Goat	*Capra aegagrus hircus*	Wildwood	2	2
Red Deer	*Cervus elaphus*	Wildwood	1	1
Reindeer	*Rangifer tarandus*	Wildwood	1	-
Soay Sheep	*Ovis aries*	Wildwood	1	1
Wild Boar	*Sus scrofa*	Wildwood	2	1
Squamata				
Four-lined Snake	*Elaphe quatuorlineata*	Wildwood	1	-
Eulipotyphla				
Hedgehog	*Erinaceus europaeus*	Wildwood	1	-
Water Shrew	*Neomys fodiens*	Wildwood	6	-
Passeriformes				
Raven	*Corvus corax*	Wildwood	3	-
Red Billed Chough	*Pyrrhocorax pyrrhocorax*	Wildwood	1	-
Rodentia				
Red Squirrel	*Sciurus vulgaris*	Wildwood	3	2
Water Vole	*Arvicola amphibius*	Wildwood	2	2
Water Vole	*Arvicola amphibius*	Bulphan	(5) 30*	5
Water Vole	*Arvicola amphibius*	Tilbury	(3) 14*	3
Diprotodontia				
Wallaby	*Macropus rufogriseus*	Wildwood	3	2

*high sample number due to repetitive sampling from a small population

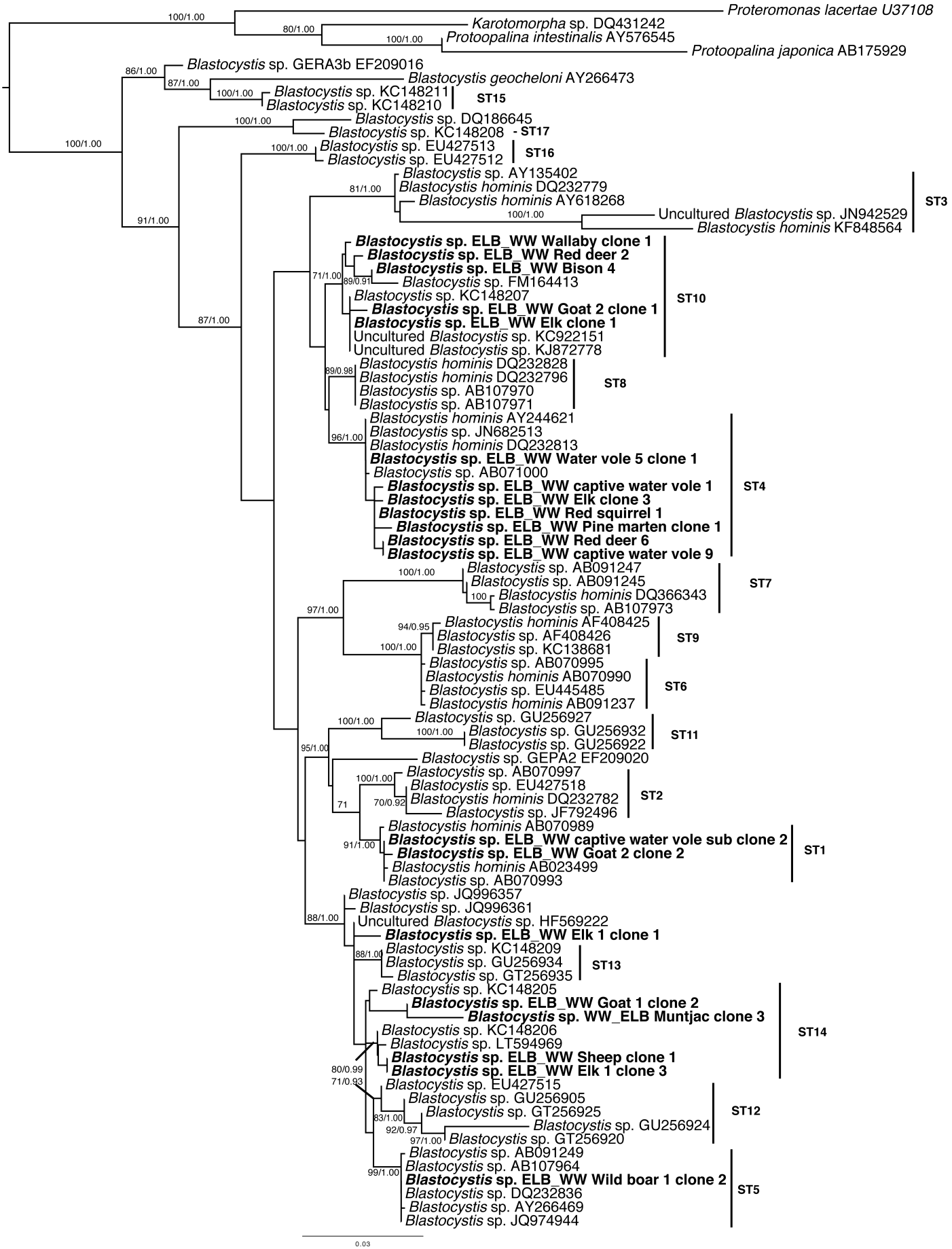
Table 2: Subtype results from sequencing positive samples

Host	Name	Location	PCR Positive Samples	Sequence Positive Clones	*Blastocystis* ST					
					ST1	ST4	ST5	ST10	ST 14	ST?
European Bison	*Bison bonasus*	Wildwood	3	*11*	-	-	-	**11/11**	-	-
Eurasian Elk	*Alces alces*	Wildwood	*1*	*4*	-	**1/4**	-	**1/4**	**1/4**	**1/4**
Muntjac Deer	*Muntiacus reevesi*	Wildwood	*1*	*1*	-	-	-	-	**1/1**	-
Pine Marten	*Martes martes*	Wildwood	*1*	*1*	-	**1/4**	-	-	-	-
Pygmy Goat	*Capra aegagrus hircus*	Wildwood	*2*	*3*	**1/3**	-	-	**1/3**	**1/3**	-
Red Deer	*Cervus elaphus*	Wildwood	*1*	*8*	-	**2/8**	-	**6/8**	-	-
Red Squirrel	*Sciurus vulgaris*	Wildwood	*2*	*1*	-	**1/1**	-	-	-	-
Soay Sheep	*Ovis aries*	Wildwood	*1*	*1*	-	-	-	-	**1/1**	-
Wallaby	*Macropus rufogriseus*	Wildwood	*2*	*2*	-	-	-	**2/2**	-	-
Water Vole	*Arvicola amphibius*	*Wildwood*	*2*	*12*	**1/12**	**10/12**	-	**1/12**	-	-
Water Vole PP	*Arvicola amphibius*	*Bulphan*	*5*	*17*	-	**17/17**	-	-	-	-
Water Vole TB	*Arvicola amphibius*	*Tilbury*	*3*	*9*	-	**9/9**	-	-	-	-
Wild Boar	*Sus scrofa*	Wildwood	*1*	*1*	-	-	**1/1**	-	-	-

Table S1: Corresponding accession numbers to the sequences shown in the phylogenetic tree (Figure 1).

Animal	Accession number
ELB_WW_Water vole 1_ clone 1	MF186640
ELB_WW_Water vole 3_clone 3	MF186641
ELB_WW_Water vole 2_clone 3	MF186642
ELB_WW_Water vole 2_clone 2	MF186643
ELB_WW_Water vole 1_ clone 2	MF186644
ELB_WW_Captive Water vole 3_Subculture_clone 4	MF186645
ELB_WW_Captive Water vole 1_Subculture_clone 3	MF186646
ELB_WW_Captive Water vole 1_Subculture_clone 2	MF186647
ELB_WW_Water vole 34_clone 3	MF186648
ELB_WW_Water vole 34_clone 1	MF186649
ELB_WW_Water vole 34_clone 2	MF186650
ELB_WW_Water vole 32_clone 3	MF186651
ELB_WW_Water vole 32_clone 2	MF186652
ELB_WW_Water vole 32_clone 1	MF186653
ELB_WW_Water vole 30_clone 3	MF186654
ELB_WW_Water vole 30_clone 2	MF186655
ELB_WW_Water vole 30_clone 1	MF186656
ELB_WW_Water vole 5_clone 2	MF186657
ELB_WW_Water vole 5_clone 3	MF186658
ELB_WW_Water vole 5_clone 1	MF186659
ELB_WW_Water vole 5_clone 5	MF186660
ELB_WW_Water vole 4_clone 3	MF186661
ELB_WW_Water vole 4_clone 3	MF186662
ELB_WW_Water vole 4_clone 1	MF186663
ELB_WW_Elk 1_clone 3	MF186664
ELB_WW_Elk 1_clone 1	MF186665
ELB_WW_Bison_1	MF186666
ELB_WW_Red squirrel_1	MF186667
ELB_WW_Red deer_1	MF186668
ELB_WW_Red deer_2	MF186669
ELB_WW_Captive Water vole_1	MF186670
ELB_WW_Red deer_3	MF186671
ELB_WW_Bison_2	MF186672
ELB_WW_Captive Water vole_2	MF186673
ELB_WW_Captive Water vole_3	MF186674
ELB_WW_Bison 2_1	MF186675
ELB_WW_Bison_3	MF186676
ELB_WW_Red deer_4	MF186677
ELB_WW_Bison_4	MF186678
ELB_WW_Red deer_5	MF186679
ELB_WW_Bison 2_2	MF186680
ELB_WW_Bison_5	MF186681
ELB_WW_Bison_6	MF186682
ELB_WW_Red deer_6	MF186683
ELB_WW_Captive Water vole_4	MF186684
ELB_WW_Red deer_7	MF186685
ELB_WW_Bison_7	MF186686
ELB_WW_Captive Water vole_5	MF186687

ELB_WW_Bison_8	MF186688
ELB_WW_Red deer_8	MF186689
ELB_WW_Captive Water vole_6	MF186690
ELB_WW_Captive Water vole_7	MF186691
ELB_WW_Captive Water vole_8	MF186692
ELB_WW_Captive Water vole_9	MF186693
ELB_WW_Wallaby_1	MF186694
ELB_WW_Bison_SP6_clone 1	MF186695
ELB_WW_Elk_clone 1	MF186696
ELB_WW_Elk_clone 3	MF186697
ELB_WW_Goat 2_clone 1	MF186698
ELB_WW_Goat 2_clone 2	MF186699
ELB_WW_Muntjac_clone 3	MF186700
ELB_WW_Pine marten_clone 1	MF186701
ELB_WW_Water vole 1_clone 3	MF186702
ELB_WW_Water vole 2_clone 1	MF186703
ELB_WW_Water vole 3_clone 2	MF186704
ELB_WW_Water vole 3_clone 3	MF186705
ELB_WW_Water vole 5_clone 3	MF186706
ELB_WW_Sheep_clone 1	MF186707
ELB_WW_Wallaby_clone 1	MF186708
ELB_WW_Wild boar_clone 1	MF186709
ELB_WW_Goat 1_clone 1	MF186709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Karotomorpha sp. DQ431242
Protoopalina intestinalis AY576545
Protoopalina japonica AB175929
Blastocystis sp. GERA3b EF209016
Blastocystis geocheloni AY266473
Blastocystis sp. KC148211
Blastocystis sp. KC148210
ST15
Blastocystis sp. DQ186645
Blastocystis sp. KC148208
ST17
Blastocystis sp. EU427513
Blastocystis sp. EU427512
ST16
Blastocystis sp. AY135402
Blastocystis hominis DQ232779
Blastocystis hominis AY618268
Uncultured Blastocystis sp. JN942529
Blastocystis hominis KF848564
ST3
Blastocystis sp. ELB_WW Wallaby clone 1
Blastocystis sp. ELB_WW Red deer 2
Blastocystis sp. ELB_WW Bison 4
Blastocystis sp. FM164413
Blastocystis sp. KC148207
Blastocystis sp. ELB_WW Goat 2 clone 1
Blastocystis sp. ELB_WW Elk clone 1
Uncultured Blastocystis sp. KC922151
Uncultured Blastocystis sp. KJ872778
ST10
Blastocystis hominis DQ232828
Blastocystis hominis DQ232796
Blastocystis sp. AB107970
Blastocystis sp. AB107971
ST8
Blastocystis hominis AY244621
Blastocystis sp. JN682513
Blastocystis hominis DQ232813
Blastocystis sp. ELB_WW Water vole 5 clone 1
Blastocystis sp. AB071000
Blastocystis sp. ELB_WW captive water vole 1
Blastocystis sp. ELB_WW Elk clone 3
Blastocystis sp. ELB_WW Red squirrel 1
Blastocystis sp. ELB_WW Pine marten clone 1
Blastocystis sp. ELB_WW Red deer 6
Blastocystis sp. ELB_WW captive water vole 9
ST4
Blastocystis sp. AB091247
Blastocystis sp. AB091245
Blastocystis hominis DQ366343
Blastocystis sp. AB107973
ST7
Blastocystis hominis AF408425
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Blastocystis sp. KC138681
ST9
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Blastocystis hominis AB091237
ST6
Blastocystis sp. GU256927
Blastocystis sp. GU256932
Blastocystis sp. GU256922
ST11
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Blastocystis sp. AB070997
Blastocystis sp. EU427518
Blastocystis hominis DQ232782
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ST2
Blastocystis hominis AB070989
Blastocystis sp. ELB_WW captive water vole sub clone 2
Blastocystis sp. ELB_WW Goat 2 clone 2
Blastocystis hominis AB023499
Blastocystis sp. AB070993
ST1
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Blastocystis sp. KC148209
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Blastocystis sp. GT256935
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Blastocystis sp. WW_ELB Muntjac clone 3
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Blastocystis sp. ELB_WW Sheep clone 1
Blastocystis sp. ELB_WW Elk 1 clone 3
ST14
Blastocystis sp. EU427515
Blastocystis sp. GU256905
Blastocystis sp. GT256925
Blastocystis sp. GU256924
Blastocystis sp. GT256920
ST12
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Blastocystis sp. AB107964
Blastocystis sp. ELB_WW Wild boar 1 clone 2
Blastocystis sp. DQ232836
Blastocystis sp. AY266469
Blastocystis sp. JQ974944
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