

1 Running Title: *Blastocystis* genetic diversity

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3 Genetic diversity of *Blastocystis* in livestock and zoo animals.

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21 Note: Supplementary data associated with this article

22

23 **Abstract**

24 *Blastocystis* is a common unicellular anaerobic eukaryote that inhabits the large intestine of
25 many animals worldwide, including humans. The finding of *Blastocystis* in faeces in
26 mammals and birds has led to proposals of zoonotic potential and that these hosts may be the
27 source of many human infections. *Blastocystis* is, however, a genetically diverse complex of
28 many distinct organisms (termed subtypes; STs), and sampling to date has been limited, both
29 geographically and in the range of hosts studied. In order to expand our understanding of host
30 specificity of *Blastocystis* STs, 557 samples were examined from various non-primate animal
31 hosts and from a variety of different countries in Africa, Asia and Europe. STs were
32 identified using ‘barcoding’ of the small subunit rRNA gene using DNA extracted either
33 from culture or directly from faeces. The host and geographic range of several STs has
34 thereby been greatly expanded and the evidence suggests that livestock is not a major
35 contributor to human infection. Two new STs were detected among the barcode sequences
36 obtained; for these, and for three others where the data were incomplete, the corresponding
37 genes were fully sequenced and phylogenetic analysis was undertaken.

38

39 **Keywords**

40 *Blastocystis*; epidemiology; livestock; phylogeny; subtype

41

42 **Introduction**

43 *Blastocystis* is an intestinal eukaryote belonging to the protist group ‘Stramenopiles’ and is
44 known to infect amphibians, reptiles, cockroaches and a wide range of birds and mammals,
45 including humans (Abe 2004; Abe et al. 2002; Abe et al. 2003a, b; Fayer et al. 2012; Parkar
46 et al. 2007; Parkar et al. 2010; Petrášová et al. 2011; Santín et al. 2011; Stensvold et al. 2009;
47 Tan 2004; Tan et al. 2013; Teow et al. 1991; Teow et al. 1992; Yamada et al. 1987;
48 Yoshikawa et al. 2003a, b, c; Yoshikawa et al. 2004b, c; Yoshikawa et al. 2007). *Blastocystis*
49 has been reported in many parasite surveys of animals in zoological gardens, especially in
50 non-human primates (Abe et al. 2003b; Abe 2004; Alfellani et al. in press; Stensvold et al.
51 2009), while studies of *Blastocystis* in domestic animals have also revealed high frequency of
52 infection (Abe et al. 2002; Nagel et al. 2012; Tan et al. 2013; Yamada et al. 1987).

53 *Blastocystis* exhibits extensive genetic heterogeneity in conserved genes, such as the
54 small-subunit rRNA gene (SSU rDNA) and the elongation factor-1 α gene (Abe 2004; Arisue
55 et al. 2003; Ho et al. 2000; Noël et al. 2003; Stensvold et al. 2012; Yoshikawa et al. 2003a,
56 c). These genetic variants have been grouped into discrete clades or subtypes (STs) based on
57 sequence similarity (Stensvold et al. 2007). At present 14 STs (ST1—ST14) have been
58 identified on the basis of SSU rRNA gene analysis from mammalian and avian hosts alone
59 (Fayer et al. 2012; Noël et al. 2003; Noël et al. 2005; Parkar et al. 2007; Stensvold et al.
60 2009; Yoshikawa et al. 2004a) of which nine have been identified in humans to date.

61 Many *Blastocystis* isolates from other mammals and birds belong to the same STs seen
62 in humans and so would seem to have zoonotic potential (Clark 1997; Parkar et al. 2007;
63 Parkar et al. 2010; Salim et al. 1999; Stensvold et al. 2008; Stensvold et al. 2009; Yoshikawa
64 et al. 2009). Therefore it has been proposed that human infections may result from zoonotic
65 transmission of the parasite, but the contribution of animal sources to human infection
66 remains to be confirmed, since the direction of transmission cannot be established with any

67 certainty. In fact Stensvold et al. (2012) have shown using multi-locus sequence typing that
68 variation within ST3 is much wider among non-human primates than it is in humans
69 suggesting that a shared ST alone may be too crude a criterion to make a link between human
70 and non-human infections.

71 The aims of this study were to determine the STs of *Blastocystis* present in livestock
72 from several different countries, primarily the UK and Libya, and to investigate the degree of
73 host specificity among these STs. In addition a number of wild and zoo mammals were
74 sampled to expand our understanding of the host range and genetic diversity of *Blastocystis*.

75

76 **Results**

77 *Sample screening*

78 Samples were obtained from a wide range of hosts, by targeted sampling of livestock at
79 cooperating farms, random trapping of wild rodents, collecting stool samples from zoo
80 animals, and using archival DNA samples from other studies. From the total number of 557
81 samples that were examined from 53 host species, 118 (21.2 %) were positive by sequencing
82 (Table 1). However sample positivity across groups was not evenly distributed. Only 6/68
83 rodents (8.8%) were positive while 110/416 artiodactyls were positive (26.4%), the two
84 biggest groups sampled. It is important to note, however, that these cannot be taken as
85 prevalence values as the samples are not equivalent across all hosts, with some DNAs being
86 from culture, others direct from stool, and the presence of inhibitors was not always tested in
87 the same way. Nevertheless, a positive result shows that *Blastocystis* is present in the host
88 and ST identification by sequencing strongly suggests that a host is susceptible to infection
89 with that subtype. Although the possibility cannot be completely excluded, it seems unlikely
90 that an animal would ingest sufficient infected faeces from another host to produce a positive

91 result after passage through its intestine given the dilution effect and the small size of the
92 faecal sample used for culture inoculation and DNA extraction.

93

94 *Subtype identification*

95 Among the 118 samples sequence-positive for *Blastocystis*, nine STs were detected
96 with two considered to be new STs, in samples collected from a camel and cattle (ST15) and
97 from a gundi (ST17) (Table 2; Figure 1). As part of a separate study, ST15 was also found in
98 several non-human primates (Alfellani et al. in press). ST5 was detected with the highest
99 frequency (33%) with ST10 being the second most common at 23%. However, as these STs
100 are most common in artiodactyls and the latter hosts provided most of the samples screened
101 as well as being the most frequently found to be *Blastocystis* positive, this may give an
102 unbalanced picture of overall ST distribution. Camels were the host infected with the widest
103 range of STs, but again this may be influenced by the number of samples analysed. Among
104 the hosts that were not found to contain *Blastocystis*, members of the genus *Equus* (zebra,
105 donkeys and horses) were the most sampled, with all of the 36 samples being negative,
106 whether the samples were from Libya or the UK and from culture or faecal DNA; note that
107 *Blastocystis* has been reported in a horse previously (Thathaisong et al. 2003). Among the
108 hosts that were positive are a number of new species in which *Blastocystis* has not previously
109 been reported, including Barbary sheep, gazelle, gundi, mouflon, anoa and mouse deer, and
110 additional STs have been found in previously studied hosts, e.g. ST3 in a giraffe.

111 Similarly, since no molecular data on *Blastocystis* from non-primates in Africa have
112 been published previously, the data from Libyan animals represents a geographic range
113 extension for several STs. Although ST1 and ST3 have been found in human samples from
114 Libya and elsewhere in Africa (Alfellani et al. 2013), ST10 has not previously been reported
115 from this continent.

116

117 *Host specificity*

118 Of the individual hosts studied, cattle have been the most widely sampled around the
119 world. In Table 3, the STs infecting 71 cattle in five countries worldwide are presented. With
120 the exception of Japan (where the technique used would not have detected it) the most
121 common ST detected in each location is ST10, indicating that there is no geographic
122 restriction to the distribution of this ST. Five STs were detected in total and 2-3 STs were
123 detected in cattle in each of the countries sampled.

124 Sampling of camels in Libya was sufficiently extensive for us to observe some
125 interesting differences between individual farms. Six farms with camels were sampled, five in
126 the area around Sebha in the south and one near Zawia in the north of the country. There
127 were six *Blastocystis* STs detected. However, ST10 and ST15 infections were detected on
128 one Sebha farm only, whereas ST1, ST3, ST5 and ST14 were each found on more than one
129 farm (Table 4) suggesting there is limited cross-infection occurring between farms even in
130 the same region. Camels are normally housed in a pen separate from other livestock, but all
131 farms kept other animals, often in close proximity to the camels, so we cannot exclude cross
132 infection occurring between host species.

133 A recent study of genetic diversity of *Blastocystis* in goats on five farms in Malaysia
134 revealed that on four farms only ST1 was detected, while the final farm showed the presence
135 of ST1, ST3, ST6 and ST7 (Tan et al. 2013). Together these two studies highlight the
136 epidemiological importance of sampling not only multiple individual animals but also
137 animals at multiple locations, even when the sites are geographically quite close together.

138 Seven complete SSU rDNA gene sequences were obtained. These include examples of
139 three previously published STs for which complete sequences were not yet available (ST10,
140 ST13 and ST14) as well as two new STs detected during this study (ST15 and ST17).

141 Comparison of ST10 barcode regions from this study confirmed the presence of two
142 clades within this ST previously detected in the original description (Stensvold et al. 2009).
143 In the barcode region these differ by about 1% which we consider to be intra-ST variation
144 and so only one example was sequenced. Likewise the two sequences for ST15, from a
145 gibbon and a camel, differed by less than 1%. For ST14, two genes were sequenced from
146 different hosts (a mouflon and a cow) and differ by 2.8%. This divergence is at the boundary
147 of what might be considered distinct STs but until further sampling is performed we have
148 decided to retain them within ST14. The problem of ST boundaries is discussed further
149 below.

150 The original host of *Blastocystis* ST13 was a quokka, a marsupial (*Setonix brachyurus*;
151 Parkar et al. 2010), whereas our example was obtained from a mouse deer. While this work
152 was ongoing a sequence identified as ST5 by Petrášová et al. (2011) was obtained from a
153 Tanzanian colobus monkey. However, comparison with our mouse deer-derived sequence
154 proved it to be ST13; the reason for the misattribution by Petrášová et al. (2011) is because
155 the quokka ST13 sequence lacks the barcode region, which was the SSU rDNA region
156 sequenced in that study. With representation among primates, marsupials and artiodactyls,
157 ST13 clearly has a very wide host range.

158

159 *Phylogenetic analysis*

160 Phylogenetic analysis showed that ST15 and ST17 are only distantly related to other
161 *Blastocystis* STs found in mammals to date, branching basal to the clades formed by STs 1-14
162 (Fig. 1). Recalculation of the *Blastocystis* tree using outgroup sequences from among the
163 stramenopiles (see Methods section) identified the branch leading to a clade that contains
164 ST15 as the location of the root. ST15 branches within a clade otherwise made up solely of
165 reptilian *Blastocystis* sequences, while ST17 clusters specifically with isolates from

166 cockroaches. The designation ST16 has been assigned to as yet unpublished sequences from
167 kangaroos obtained as part of a survey of marsupials (Yoshikawa et al. in preparation). ST16
168 also appears to lack a specific related mammalian lineage, with a reptilian *Blastocystis*
169 sequence as its closest relative (Fig. 1). As predicted from previous analyses, ST10 clusters
170 with STs 4 and 8 (Stensvold et al. 2009) and STs 13 and 14 with STs 5 and 12 (Fayer et al.
171 2012; Parkar et al. 2010).

172

173 **Discussion**

174 Mammals and birds have been proposed to be reservoirs for human infection with
175 *Blastocystis* since certain STs of this organism have been found in both humans and a wide
176 range of other animals (Abe et al. 2003b, c; Arisue et al. 2003b; Fayer et al. 2012; Parkar et
177 al. 2007; Parkar et al. 2010; Roberts et al. in press; Santín et al. 2011; Stensvold et al. 2008;
178 Stensvold et al. 2009; Yoshikawa et al. 2004). Additionally, people with close animal contact
179 were found to have a higher prevalence of *Blastocystis* infection (Salim et al. 1999) and some
180 zoo primate keepers have been found to be infected with STs or ST alleles that are otherwise
181 rare in humans but common in the monkeys they work with (Alfellani et al. in press; Scicluna
182 et al. 2006; Stensvold et al. 2012).

183 As in earlier studies, we have shown here that many domestic animals are infected with
184 *Blastocystis*. Relatively few authors have screened non-primate zoo animals for *Blastocystis*
185 (Abe et al. 2002; Lim et al. 2008; Roberts et al. in press), but the results of Parkar et al.
186 (2010) suggested that additional STs existed in such hosts and indeed we have found this to
187 be the case. The full host range of STs is as yet unclear and will require further screening of
188 samples from different hosts, and indeed the same hosts in different localities, in order to
189 obtain a clearer picture. Our results from screening camels in Libya highlight the potential
190 consequences of limiting sampling to one locality. Likewise, conclusions regarding host

191 restriction need to be made cautiously since, for example, ST13 has gone from appearing
192 perhaps to be a marsupial-specific ST to one found in diverse hosts and multiple continents. It
193 is therefore very likely that continued sampling will uncover additional novel STs and new
194 hosts for existing STs even among mammals since many regions of the world and animal
195 groups are yet to be sampled.

196 Despite the fact that the current picture is incomplete, some general conclusions can
197 nevertheless be made. The first is that grouping host species into higher taxa can be a useful
198 way of assessing specificity (Table 5). For example, artiodactyls tend to show a
199 preponderance of STs 5 and 10 no matter which species or region of the world they come
200 from. Rodents were earlier proposed to be a reservoir of ST4 for human infection, but the
201 present study did not find any examples of ST4 in the rodent hosts screened. This may mean
202 that only some rodent species carry this ST – further study is needed – but our results do
203 show that other STs are found in this host group; only ST4 had been reported previously.

204 The potential for livestock having a role as a major reservoir for zoonotic transmission
205 of *Blastocystis* infection is diminishing. In Libya, ST5 and ST10 were the most dominant STs
206 in livestock (50 % of samples) yet were not found in humans (Alfellani et al. 2013). In fact,
207 ST10 has never been found in humans and ST5 only very rarely, which means either that
208 those hosts do not contribute to human infections or that humans are not susceptible to
209 infection with these STs. In Libya none of the animals screened carried ST2 (Table 2) but this
210 ST was found in 8 % of human infections (Alfellani et al. 2013). ST1 and ST3 are the
211 dominant STs in Libyan humans (89 % of infections; Alfellani et al. 2013) but are relatively
212 rare in Libyan animals - 7 % and 9 % of samples, respectively, mostly in camels. It seems
213 unlikely from this that human *Blastocystis* is primarily of zoonotic origin in Libya. However,
214 the animals with which the population has the most contact are sheep and these have not been
215 sampled in Libya. Contact occurs during Eid al-Adha, the Muslim feast of sacrifice, and

216 sheep to human virus transmission has been linked to this ritual (Nougairede et al. 2013) so
217 potentially *Blastocystis* could be transmitted then too. To date sampling of sheep has only
218 been reported from Europe, where ST10 was found to dominate (7/14 samples); the situation
219 in Libyan sheep should be examined.

220 Interpretation of mixed infections is problematic, especially in artiodactyls. However,
221 not all mixed infections are the same. In our experience, some sequence traces show clear
222 evidence of a mixed infection but one ST is sufficiently ‘dominant’ to allow its identification.
223 In our data we have not counted these as mixed infections, and it is unclear how other
224 researchers have reported them. The degree of ‘dominance’ is certainly a continuum and so
225 mixed infections are likely to be greatly underestimated in most datasets derived from
226 sequence data. This is more of an issue in livestock and some zoo mammal samples than in
227 humans in our experience (although see the report by Meloni et al. (2012) for a clear multi-
228 ST mixed human infection).

229 A number of researchers have used the ST-specific PCR amplification approach of
230 Sequence-Tagged Site analysis (STS). This method allows the identification of STs within
231 mixed infections, but only when ST1—ST7 are involved. No STS primers are available for
232 ST8-ST17 and so when present in mixed infections these STs will not be detected. This is a
233 particular concern when livestock animals are being studied, given the finding that ST10 is so
234 common. For example, Tan et al. (2013) used STS to study goats in Malaysia and found ST1,
235 ST3, ST6 and ST7. We similarly found ST3 and ST7, but also ST10 in Libyan goats. It is not
236 possible to know whether ST10 is also present in Malaysian goats using the STS approach.
237 Similarly, Lee et al. (2012) found 6 *Blastocystis* infections in Nepalese artiodactyls that could
238 not be typed using STS primers; from our results it seems likely that these untyped infections
239 will include ST10. Interestingly, Petrášová et al. (2011) found evidence of mixed subtype
240 infections in their primate samples when sequencing the barcode region, but no evidence of

241 mixed infection in the same samples when using STS primers. This suggests that the primates
242 were co-infected with a subtype not amplified by STS primers. The ST range restriction and
243 other factors limit the utility of STS in certain types of analysis (Stensvold 2013).

244 During our work two new STs were discovered that are divergent compared to other
245 STs from mammals. We have based their definition as new subtypes on comparison of
246 complete SSU rDNA sequences and we feel that this should be a requirement before
247 assigning new ST numbers to novel sequences. The misattribution resulting from the
248 definition of ST13 based on a partial SSU rRNA gene (Parkar et al. 2010) would be avoided
249 in future if this guideline is adopted. As it is, it seems likely that the report by Petrášová et al.
250 (2011) of ST5 in a colobus monkey will be re-reported in future survey papers when the
251 availability of a complete gene sequence for ST13 at the time it was reported in 2010 would
252 have avoided this problem. Likewise, ST14 was also defined based on partial sequences
253 (Fayer et al. 2012); by chance we had independently identified the same organism in several
254 hosts and obtained the complete gene sequence. ST11 and ST12 still are not available as
255 complete sequences (Parkar et al. 2010) so similar problems involving these STs may still
256 occur.

257 Nevertheless, obtaining a complete gene sequence is not enough, as criteria for defining
258 the degree of divergence required to designate a novel sequence as a new ST are needed. This
259 is not straightforward, since in some STs extensive intra-ST diversity exists, up to 3 % in ST3
260 for example. For this reason we have been conservative in not designating the sequence
261 obtained from the mouflon as a new ST, preferring to keep it within ST14 until further
262 information on intra-ST14 variation becomes available. A consensus will be needed on the
263 degree of divergence that constitutes a novel ST (Clark et al. 2013) but it may be premature at
264 present as intra-ST diversity levels are unclear for several STs. At present, we are using 5 %

265 divergence as a benchmark for a new ST with less than 3 % representing intra-ST variation
266 pending additional data.

267 STs 15 and 17 are surprising in that they are not specifically related to any other
268 mammalian STs, but to reptilian and insect *Blastocystis*, respectively. Both, however, were
269 grown in culture at 37 °C and so contamination with reptilian and insect faeces seems
270 unlikely to be the source of the material, especially for ST15 where several hosts and
271 locations were involved – reptilian *Blastocystis* have been shown to be unable to grow at 37
272 °C (e.g. Teow et al. 1991). Nevertheless, the near ubiquity of *Blastocystis* means that the
273 potential for environmental contamination is real and, especially when culture is used, care
274 needs to be taken when sampling faeces to ensure that the source material is indeed from the
275 desired host.

276 In conclusion, the universe of mammalian *Blastocystis* is still expanding with 8 new
277 STs detected since 2007, thereby almost doubling their number. Further sampling from
278 diverse hosts and various geographical areas may on the one hand improve our ability to
279 define ST boundaries, but may on the other hand lead to the merging of certain STs if clear
280 boundaries cannot be identified. It will be interesting to see to what extent *Blastocystis* ST
281 distribution is linked to host groups rather than geography. Finally, the higher resolution data
282 generated by allele analysis within STs found in humans and other animals will provide
283 additional evidence for evaluating the potential for zoonotic transmission of *Blastocystis* in
284 various parts of the world. Allele analysis is necessary in view of our finding of cryptic host
285 specificity within subtypes (Alfellani et al. in press; Stensvold et al. 2012). As yet, only a few
286 cases of zoonotic transmission have been clearly documented by showing that the same strain
287 is present in both hosts and not just the same subtype. Hence, although *Blastocystis* appears
288 to be an extremely common parasite colonising probably more than 1 billion people, in our

289 opinion it seems most likely that *Blastocystis* in humans results primarily from anthroponotic
290 transmission.

291

292 **Methods**

293

294 *Source of specimens*

295 Faecal samples were collected from various animal hosts and from different countries
296 (Table 1). Many of the livestock and native animal samples from the UK were collected by
297 MSc students on parasitology field-trips in the Dartmoor/Exmoor region of the South-West of
298 England. Libyan samples were primarily collected from farms in the vicinity of Sebha in
299 South-Western Libya. The latter samples were screened by microscopy and culture in
300 modified Jones' medium (Leelayoova et al. 2002) or Robinson's medium (Clark and
301 Diamond 2002) whereas DNA was extracted directly from faeces in most of the other
302 samples. Cultures were incubated at 37 °C and examined every 2 days. Microscopy-positive
303 cultures were passaged into fresh medium for another 3–4 days, then *Blastocystis* was
304 harvested and DNA purified as previously described (Alfellani et al. 2013). In our
305 experience, this short-term cultivation approach permits mixed infections to be identified as it
306 prevents differential outgrowth of STs affecting the results (as seen by the fact that mixed
307 infections are readily detected). Cultures were discontinued after harvesting, which
308 unfortunately means that reference cultures of the new subtypes do not exist at present.
309 Faecal DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden,
310 Germany) according to the manufacturer's protocol.

311

312 *PCR and sequencing*

313 PCR and sequencing of the SSU rDNA barcode region were carried out as described by
314 Scicluna et al. (2006). Additionally, seven almost complete SSU rDNA sequences from either
315 novel STs or STs for which no complete sequence was previously available, were obtained
316 by sequencing of whole or partial gene PCR products with a mixture of pan-eukaryotic and
317 ST-specific primers, as described (Stensvold et al. 2012). Sequence files were edited and
318 assembled using the Staden software package (<http://staden.sourceforge.net/>) and have been
319 deposited in the NCBI nucleotide database with accession numbers KC148205-KC148211.

320

321 *Phylogenetic analysis*

322 Complete sequences were aligned with reference sequences for all previously named
323 STs and one novel ST from kangaroos (here designated ST16; accession numbers: EU427512
324 and EU427514 (Yoshikawa unpublished)), as well as non-mammal/bird sequences available
325 from GenBank, using the alignment tool MUSCLE as implemented in MEGA5 (Tamura et
326 al. 2011). The alignment was edited manually to remove regions of ambiguity, resulting in an
327 alignment of 1,462 positions for all 59 *Blastocystis* sequences included (supplementary file
328 1). Phylogenetic analyses were performed as described previously (Stensvold et al. 2012)
329 using distance (Neighbor-Joining) and maximum likelihood algorithms as implemented in
330 MEGA5 and Bayesian analysis (MrBayes 3.1.2; Huelsenbeck and Ronquist 2001). Bayesian
331 and maximum likelihood analysis used a General Time Reversible (GTR) model of
332 nucleotide substitution with four categories of among-site rate variation and the proportion of
333 invariant sites, the best model selected by ModelTest, implemented in MEGA5. Statistical
334 support for distance and maximum likelihood trees was evaluated using bootstrapping (1,000
335 replicates). Bayesian analysis used four Markov chain Monte Carlo (MCMC) strands,
336 1,000,000 generations, with trees sampled every 100 generations. The resulting average
337 standard deviation of split frequencies was less than 0.01. A consensus tree was produced

338 after excluding an initial burn-in of 25% of the samples, as recommended. A second
339 alignment was produced to place the root on the resulting trees. This contained a subset of the
340 *Blastocystis* reference sequences including all the established STs plus the new sequences.
341 Outgroups included two members of the Stramenopiles that are closely related to *Blastocystis*
342 (*Karotomorpha* and *Proteromonas*) plus three additional members of that group
343 (*Saprolegnia*, *Cafeteria* and *Wobblia*). This alignment contained 48 sequences in total.
344 Phylogenies were calculated as above; all three methods resulted in the same root placement.
345

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356

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489

490 Table 1. Animal samples collected from various hosts in different countries

Host	Scientific name	Location	Sample numbers	Sequence positive samples
Monotremata				
Echidna	<i>Tachyglossus aculeatus</i>	UK	1	0
Marsupialia				
Western gray kangaroo	<i>Macropus fuliginosus</i>	UK	1	0
Rodentia				
Bank vole	<i>Clethrionomys glareolus</i>	Poland	6	0
		UK	32	1
Black-tailed prairie dog	<i>Cynomys ludovicianus</i>	UK	1	0
Capybara	<i>Hydrochoerus hydrochaeris</i>	UK	1	0
Chinchilla	<i>Chinchilla lonigera</i>	Belgium	5	2
		Croatia	1	0
Crested porcupine	<i>Hystrix cristata</i>	UK	1	0
Degu	<i>Octodon degus</i>	Croatia	1	0
Prevost's squirrel	<i>Callosciurus prevostii</i>	UK	1	0
Syrian hamster	<i>Mesocricetus auratus</i>	Croatia	1	0
Yellow necked mouse	<i>Apodemus flavicollis</i>	Poland	1	1
Wood mouse	<i>Apodemus sylvaticus</i>	UK	13	1
Gundi	<i>Ctenodactylus gundi</i>	Libya	4	1
Lagomorpha				
European rabbit	<i>Oryctolagus cuniculus</i>	Croatia	1	0
		UK	1	0
Artiodactyla				
Anoa	<i>Bubalus quarlesi</i>	UK	3	2
European bison	<i>Bison bonasus</i>	Poland	1	0
Giraffe	<i>Giraffa camelopardalis</i>	UK	4	1
Mouse deer	<i>Tragulus javanicus</i>	UK	2	2
Red deer	<i>Cervus elaphus</i>	UK	3	0
Roe deer	<i>Capreolus capreolus</i>	UK	2	1
Fallow deer	<i>Dama dama</i>	Mauritius	2	2
Pudu	<i>Pudu puda</i>	UK	1	0
Camel	<i>Camelus dromedarius</i>	Libya	196	47
Cattle	<i>Bos taurus</i>	Libya	36	15
		France	2	0
		Italy	3	0
		UK	31	7
Goat	<i>Capra aegagrus aegagrus</i>	Libya	38	4
	<i>Capra aegagrus hircus</i>	Italy	2	0
Barbary sheep	<i>Ammotragus lervia</i>	Libya	5	1
Sheep	<i>Ovis aries</i>	Italy	2	0
		UK	51	12

Red River Hog	<i>Potamochoerus porcus</i>	UK	1	0
Collared peccary	<i>Pecari tajacu</i>	UK	1	0
Domestic pig	<i>Sus scrofa domesticus</i>	UK	7	2
		Vietnam	12	12
Gazelle	<i>Gazella arabica</i>	Libya	9	1
Bongo	<i>Tragelaphus eurycerus</i>	UK	1	0
		Czech		
Mouflon	<i>Ovis orientalis musimon</i>	Republic	1	1
Perissodactyla				
Brazilian tapir	<i>Tapirus terrestris</i>	UK	1	0
Black rhinoceros	<i>Diceros bicornis</i>	UK	1	1
Donkey	<i>Equus africanus asinus</i>	Libya	16	0
		UK	2	0
Horse	<i>Equus ferus caballus</i>	Libya	2	0
		UK	14	0
Mountain zebra	<i>Equus zebra hartmannae</i>	UK	2	0
Proboscidea				
African elephant	<i>Loxodonta africana</i>	UK	1	0
		Belgium	4	0
Carnivora				
European badger	<i>Meles meles</i>	UK	1	0
Dog	<i>Canis familiaris</i>	Croatia	3	0
		France	2	0
Domestic cat	<i>Felis catus</i>	France	2	0
Meerkat	<i>Suricata suricatta</i>	UK	1	0
Red fox	<i>Vulpes vulpes</i>	UK	1	0
Serval	<i>Leptailurus serval</i>	Croatia	1	0
Stoat	<i>Mustela erminea</i>	UK	2	0
Aves				
Ostrich	<i>Struthio camelus</i>	UK	1	0
Southern cassowary	<i>Casuarius casuarius</i>	UK	1	0
Chicken	<i>Gallus gallus</i>	Libya	3	1
		UK	1	0
		Mauritius	1	0
Macaw	<i>Ara hybrid</i>	Mauritius	1	0
Black Swan	<i>Cygnus atratus</i>	Mauritius	2	0
Domestic duck	<i>Anas platyrhynchos</i>	Mauritius	1	0
Domestic goose	<i>Anser anser</i>	UK	2	0
Total			557	118

492 Table 2. Subtype results from sequence-positive samples

Host	Scientific name	Location	Sequences	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	ST9	ST10	ST11	ST12	ST13	ST14	ST15	ST16	ST17	Mixed
Rodentia																					
Bank vole	<i>Clethrionomys glareolus</i>	UK	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
Chinchilla	<i>Chinchilla lanigera</i>	Belgium	2	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Yellow necked mouse	<i>Apodemus flavicollis</i>	Poland	1	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Wood mouse	<i>Apodemus sylvaticus</i>	UK	1	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Gundi	<i>Ctenodactylus gundi</i>	Libya	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—
Artiodactyla																					
Anoa	<i>Bubalus quarlesi</i>	UK	2	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	1
Giraffe	<i>Giraffa camelopardalis</i>	UK	1	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Mouse deer	<i>Tragulus javanicus</i>	UK	2	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—
Fallow deer	<i>Dama dama</i>	Mauritius	2	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—
Roe deer	<i>Capreolus capreolus</i>	UK	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
Camel	<i>Camelus dromedarius</i>	Libya	47	5	—	5	—	20	—	—	—	—	6	—	—	—	3	1	—	—	7
Cattle	<i>Bos taurus</i>	Libya	15	—	—	—	—	2	—	—	—	—	6	—	—	—	2	—	—	—	5
		UK	7	1	—	—	—	1	—	—	—	—	3	—	—	—	—	—	—	—	2
Goat	<i>Capra aegagrus aegagrus</i>	Libya	4	—	—	1	—	—	—	1	—	—	1	—	—	—	—	—	—	—	1
Barbary sheep	<i>Ammotragus lervia</i>	Libya	1	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—
Sheep	<i>Ovis aries</i>	UK	12	—	—	—	—	—	—	—	—	—	7	—	—	—	—	1	—	—	4
Domestic pig	<i>Sus scrofa domesticus</i>	Vietnam	12	—	—	—	—	12	—	—	—	—	—	—	—	—	—	—	—	—	—
		UK	2	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
Gazelle	<i>Gazella arabica</i>	Libya	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
Mouflon	<i>Ovis orientalis musimon</i>	Czech Republic	1	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—
Perissodactyla																					
Black rhinoceros	<i>Diceros bicornis</i>	UK	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
Aves																					
Chicken	<i>Gallus gallus</i>	Libya	1	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—
Total			118	6 (5%)	—	11 (9%)	—	39 (33%)	—	2 (2%)	—	—	27 (23%)	—	—	2 (2%)	6 (5%)	2 (2%)	—	1 (1%)	22 (19%)

493 Table 3. Comparison of *Blastocystis* STs in cattle in various countries

Country	<i>Blastocystis</i> ST						Total
	ST1	ST3	ST5	ST10	ST14	MIX	
Denmark	—	—	3 (12%)	22 (88%)	—	—	25
Libya	—	—	2 (13%)	6 (40%)	2 (13%)	5 (33%)	15
UK	1 (14%)	—	1 (14%)	3 (43%)	—	2 (29%)	7
USA	—	—	—	13 (81%)	1 (6%)	2 (13%)	16
Japan	1 (12.5%)	1 (12.5%)	6 (75%)	? ^a	? ^a	—	8
							71

a - This study (Yoshikawa et al. 2004a) used STS for subtyping and would not have detected infections with subtypes ST8-ST17

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495

496 Table 4. *Blastocystis* subtypes detected in camels from various farms in Libya

Subtype	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	ST9	ST10	ST11	ST12	ST13	ST14	ST15	ST16	ST17	Mix	Total
Farm 1 Sebha	—	—	1 (5%)	—	15 (75%)	—	—	—	—	—	—	—	—	1 (5%)	—	—	—	3 (15%)	20
Farm 2 Sebha	—	—	—	—	—	—	—	—	—	6 (60%)	—	—	—	1 (10%)	1 (10%)	—	—	2 (20%)	10
Farm 3 Sebha	2 (50%)	—	2 (50%)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4
Farm 4 Sebha	1 (17%)	—	2 (33%)	—	1 (17%)	—	—	—	—	—	—	—	—	—	—	—	—	2 (33%)	6
Farm 5 Sebha	—	—	—	—	1 (100%)	—	—	—	—	—	—	—	—	—	—	—	—	—	1
Farm 7 Zawia	2 (33%)	—	—	—	3 (50%)	—	—	—	—	—	—	—	—	1 (17%)	—	—	—	—	6
Total	5	—	5	—	20	—	—	—	—	6	—	—	—	3	1	—	—	7	47

497

498

499 Table 5. *Blastocystis* subtypes identified in different animal groups worldwide

Animal host group	Subtype																			Reference	
	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	ST9	ST10	ST11	ST12	ST13	ST14	ST15	ST16	ST17	Mixed	Untypable		
Placental Mammals																					
<i>Homo sapiens</i> (total)	882	343	1399	318	9	89	118	10	3	—	—	—	—	—	—	—	—	191	34	Alfellani et al. (2013)	
Non-human primates (total)	105	79	76	4	19	—	—	30	—	2	—	—	1	—	3	—	—	15	4	Alfellani et al. (in press)	
Artiodactyla	151	7	10	—	64	—	—	—	—	24	—	—	—	—	—	—	—	1	—	Stensvold et al. (2009) ^a	
	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	—	Parkar et al. (2010)
	—	—	—	—	7	—	—	—	—	7	—	—	—	—	—	—	—	—	—	—	Santín et al. (2011)
	43	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	29	—	Tan et al. (2013)
	—	—	—	—	—	—	—	—	—	6	—	—	—	1	—	—	—	—	2	—	Fayer et al. (2012)
	—	—	—	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—	1	6	Lee et al. (2012)
	3	—	2	1	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Roberts et al. (in press)
6	—	7	—	37	—	1	—	—	27	—	—	2	6	2	—	—	—	22	—	Present study	
Total	203	7	19	1	116	3	1	0	0	64	0	4	2	7	2	0	0	55	6		
Perissodactyla	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Stensvold et al. (2009)
—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Present study
Total	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Proboscidea	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—	Parkar et al. (2010)
—	—	—	—	—	—	—	—	—	—	—	11	—	—	—	—	—	—	—	—	—	Roberts et al. (in press)
Total	0	0	0	0	0	0	0	0	0	0	15	0	0	0	0	0	0	0	0	0	
Carnivora	1	3	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Stensvold et al. (2009)
	1	—	—	—	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	Eroglu et al. (2010)
	4	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	Nagel et al. (2012)
	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Roberts et al. (in press)

Total	6	3	3	1	0	0	3	0	0	0	0	0	0	0	0	0	0	2	0	
Rodentia	–	–	–	7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Stensvold et al. (2009)
	–	–	4	–	1	–	–	–	–	–	–	–	–	–	–	–	–	1	–	Present study
Total	0	0	4	7	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
<u>Marsupials</u>																				
Didelphimorphia	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Stensvold et al. (2009)
Diprotodontia	–	–	–	–	–	–	–	–	–	–	–	2	2	–	–	–	–	–	–	Parkar et al. (2010)
	–	–	–	9	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	Roberts et al. (in press)
	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	Yoshikawa et al. (unpublished)
Total	0	0	0	10	0	0	0	0	0	0	0	2	3	0	0	2	0	0	0	0
<u>Birds</u>																				
Anseriformes	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	Stensvold et al. (2009)
Galliformes	3	1	–	–	–	10	11	1	–	–	–	–	–	–	–	–	–	2	5	Stensvold et al. (2009)
	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	Santín et al. (2011)
	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	Present study
	–	1	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	Roberts et al. (in press)
Ratites	–	2	–	6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Roberts et al. (in press)
Unidentified	2	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	Eroglu et al. (2010)
Total	5	4	0	6	0	10	19	1	0	0	0	0	0	0	0	0	0	0	3	5
Mammals and birds total (excluding primates)																				
	215	14	26	25	118	13	23	1	0	64	15	6	5	7	2	2	1	60	11	608

500 ^a The numbers do not match those in this publication because of some double counting identified later, resulting from the same samples being

501 typed by different methods in two publications.

502 Figure 1. Phylogenetic relationships among SSU rDNA sequences of *Blastocystis*. The tree shown
503 is the one inferred using the Maximum-Likelihood method. The trees were computed as described
504 in the methods section and the bootstrap support values and posterior probabilities are shown next
505 to each node in the order Maximum Likelihood/Bayesian Analysis/Neighbor-Joining. An asterisk
506 indicates lower than 50 % bootstrap support or a posterior probability value of less than 0.5.
507 Accession numbers for the sequences used are listed parentheses with the common name in
508 English of the host. New subtypes are identified in a larger size bold font. Bar = estimated number
509 of substitutions per site.
510

