Running Title: *Blastocystis* genetic diversity

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

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**Abstract**

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

**Keywords**

*Blastocystis*; epidemiology; livestock; phylogeny; subtype

## Introduction

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

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

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

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

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

## Results

### *Sample screening*

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

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

*Subtype identification*

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

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

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

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

*Phylogenetic analysis*

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

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

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

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

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

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

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

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

## Methods

### *Source of specimens*

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

### *PCR and sequencing*

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

*Phylogenetic analysis*

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

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

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

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

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

491

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 | *Clethrionomys glareolus* | UK | 1 | — | — | — | — | 1 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Chinchilla | *Chinchilla lonigera* | Belgium | 2 | — | — | 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Yellow necked mouse | *Apodemus flavicollis* | Poland | 1 | — | — | 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Wood mouse | *Apodemus sylvaticus* | UK | 1 | — | — | 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Gundi | *Ctenodactylus gundi* | Libya | 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 1 | — |
| **Artiodactyla** | | | | | | | | | | | | | | | | | | | | | |
| Anoa | *Bubalus quarlesi* | UK | 2 | — | — | — | — | — | — | — | — | — | 1 | — | — | — | — | — | — | — | 1 |
| Giraffe | *Giraffa cameloparadalis* | UK | 1 | — | — | 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Mouse deer | *Tragulus javanicus* | UK | 2 | — | — | — | — | — | — | — | — | — | — | — | — | 2 | — | — | — | — | — |
| Fallow deer | *Dama dama* | Mauritius | 2 | — | — | — | — | — | — | — | — | — | 2 | — | — | — | — | — | — | — | — |
| Roe deer | *Capreolus capreolus* | UK | 1 | — | — | — | — | 1 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Camel | *Camelus dromedarius* | Libya | 47 | 5 | — | 5 | — | 20 | — | — | — | — | 6 | — | — | — | 3 | 1 | — | — | 7 |
| Cattle | *Bos taurus* | Libya | 15 | — | — | — | — | 2 | — | — | — | — | 6 | — | — | — | 2 | — | — | — | 5 |
| | | UK | 7 | 1 | — | — | — | 1 | — | — | — | — | 3 | — | — | — | — | — | — | — | 2 |
| Goat | *Capra aegagrus aegagrus* | Libya | 4 | — | — | 1 | — | — | — | 1 | — | — | 1 | — | — | — | — | — | — | — | 1 |
| Barbary sheep | *Ammotragus lervia* | Libya | 1 | — | — | — | — | — | — | — | — | — | 1 | — | — | — | — | — | — | — | — |
| Sheep | *Ovis aries* | UK | 12 | — | — | — | — | — | — | — | — | — | 7 | — | — | — | — | 1 | — | — | 4 |
| Domestic pig | *Sus scrofa domesticus* | Vietnam | 12 | — | — | — | — | 12 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | | UK | 2 | — | — | — | — | 1 | — | — | — | — | — | — | — | — | — | — | — | — | 1 |
| Gazelle | *Gazella arabica* | Libya | 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 1 |
| Mouflon | *Ovis orientalis musimon* | Czech Republic | 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | 1 | — | — | — | — |
| **Perissodactyla** | | | | | | | | | | | | | | | | | | | | | |
| Black rhinoceros | *Diceros bicornis* | UK | 1 | — | — | — | — | 1 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| **Aves** | | | | | | | | | | | | | | | | | | | | — | — |
| Chicken | *Gallus gallus* | 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 | *Blastocystis* 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

494

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

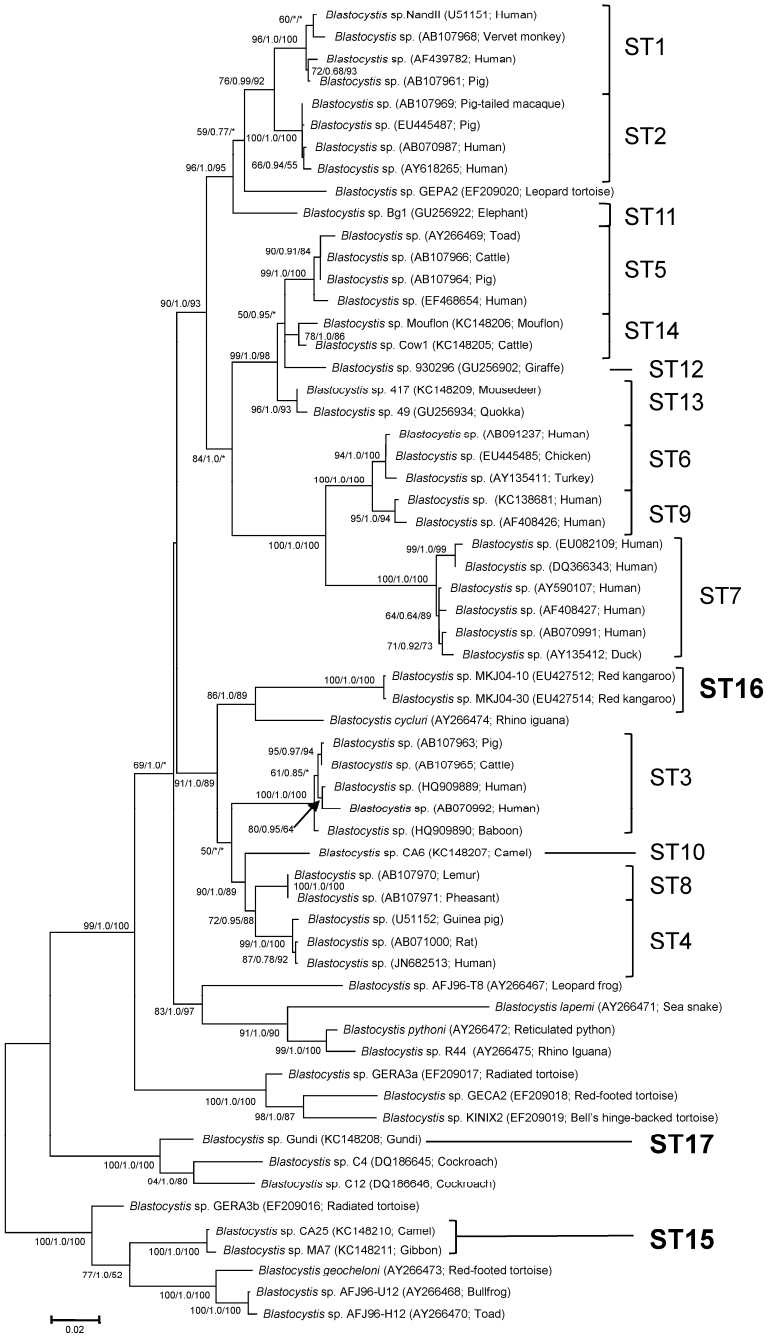
499 Table 5. *Blastocystis* subtypes identified in different animal groups worldwide

| Animal host group | Subtype | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ST1 | ST2 | ST3 | ST4 | ST5 | ST6 | ST7 | ST8 | ST9 | ST10 | ST11 | ST12 | ST13 | ST14 | ST15 | ST16 | ST17 | Mixed | Untypable | **Reference** |
| **Placental Mammals** | | | | | | | | | | | | | | | | | | | | |
| *Homo sapiens* (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** | |
| 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** | |
| 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** | **1** | **0** | **0** | |
| **Marsupials** | | | | | | | | | | | | | | | | | | | | |
| 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** | |
| **Birds** | | | | | | | | | | | | | | | | | | | | |
| 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** | **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** |

[a] The numbers do not match those in this publication because of some double counting identified later, resulting from the same samples being typed by different methods in two publications.

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


Blastocystis sp.NandII (U51151; Human)
Blastocystis sp. (AB107968; Vervet monkey)
Blastocystis sp. (AF439782; Human)
Blastocystis sp. (AB107961; Pig)
Blastocystis sp. (AB107969; Pig-tailed macaque)
Blastocystis sp. (EU445487; Pig)
Blastocystis sp. (AB070987; Human)
Blastocystis sp. (AY618265; Human)
Blastocystis sp. GEPA2 (EF209020; Leopard tortoise)
Blastocystis sp. Bg1 (GU256922; Elephant)
Blastocystis sp. (AY266469; Toad)
Blastocystis sp. (AB107966; Cattle)
Blastocystis sp. (AB107964; Pig)
Blastocystis sp. (EF468654; Human)
Blastocystis sp. Mouflon (KC148206; Mouflon)
Blastocystis sp. Cow1 (KC148205; Cattle)
Blastocystis sp. 930296 (GU256902; Giraffe)
Blastocystis sp. 417 (KC148209; Mousedeer)
Blastocystis sp. 49 (GU256934; Quokka)
Blastocystis sp. (AB091237; Human)
Blastocystis sp. (EU445485; Chicken)
Blastocystis sp. (AY135411; Turkey)
Blastocystis sp. (KC138681; Human)
Blastocystis sp. (AF408426; Human)
Blastocystis sp. (EU082109; Human)
Blastocystis sp. (DQ366343; Human)
Blastocystis sp. (AY590107; Human)
Blastocystis sp. (AF408427; Human)
Blastocystis sp. (AB070991; Human)
Blastocystis sp. (AY135412; Duck)
Blastocystis sp. MKJ04-10 (EU427512; Red kangaroo)
Blastocystis sp. MKJ04-30 (EU427514; Red kangaroo)
Blastocystis cycluri (AY266474; Rhino iguana)
Blastocystis sp. (AB107963; Pig)
Blastocystis sp. (AB107965; Cattle)
Blastocystis sp. (HQ909889; Human)
Blastocystis sp. (AB070992; Human)
Blastocystis sp. (HQ909890; Baboon)
Blastocystis sp. CA6 (KC148207; Camel)
Blastocystis sp. (AB107970; Lemur)
Blastocystis sp. (AB107971; Pheasant)
Blastocystis sp. (U51152; Guinea pig)
Blastocystis sp. (AB071000; Rat)
Blastocystis sp. (JN682513; Human)
Blastocystis sp. AFJ96-T8 (AY266467; Leopard frog)
Blastocystis lapemi (AY266471; Sea snake)
Blastocystis pythoni (AY266472; Reticulated python)
Blastocystis sp. R44 (AY266475; Rhino Iguana)
Blastocystis sp. GERA3a (EF209017; Radiated tortoise)
Blastocystis sp. GECA2 (EF209018; Red-footed tortoise)
Blastocystis sp. KINIX2 (EF209019; Bell's hinge-backed tortoise)
Blastocystis sp. Gundi (KC148208; Gundi)
Blastocystis sp. C4 (DQ186645; Cockroach)
Blastocystis sp. C12 (DQ186646; Cockroach)
Blastocystis sp. GERA3b (EF209016; Radiated tortoise)
Blastocystis sp. CA25 (KC148210; Camel)
Blastocystis sp. MA7 (KC148211; Gibbon)
Blastocystis geocheloni (AY266473; Red-footed tortoise)
Blastocystis sp. AFJ96-U12 (AY266468; Bullfrog)
Blastocystis sp. AFJ96-H12 (AY266470; Toad)
ST1
ST2
ST11
ST5
ST14
ST12
ST13
ST6
ST9
ST7
ST16
ST3
ST10
ST8
ST4
ST17
ST15
0.02

511