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1 Molecular detection of *Sarcocystis lutrae* in the European badger (*Meles meles*) in Scotland

2

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4

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10 SUMMARY

11 Neck samples from 54 badgers and 32 tongue samples of the same badgers (*Meles meles*), collected
12 in the Lothians and Borders regions of Scotland, were tested using PCRs directed against the 18S
13 ribosomal DNA and the internal transcribed spacer (ITS1) region of protozoan parasites of the
14 family Sarcocystidae. Positive results were obtained from 36 / 54 (67%) neck and 24 / 32 (75%)
15 tongue samples using an 18S rDNA PCR. A 468 base pair consensus sequence that was generated
16 from the 18S rDNA PCR amplicons (KX229728) showed 100% identity to *Sarcocystis lutrae*. The
17 ITS1 PCR results revealed that 12 / 20 (60%) neck and 10 / 20 (50%) tongue samples were positive
18 for Sarcocystidae DNA. A 1074 bp consensus sequence was generated from the ITS1 PCR
19 amplicons (KX431307) and showed 100% identity to *S. lutrae*. Multiple sequence alignments and
20 phylogenetic analysis support the finding that the rDNA found in badgers is identical to that of *S.*
21 *lutrae*. This parasite has not been previously reported in badgers or in the UK. *Sarcocystis lutrae*
22 has previously only been detected in tongue, skeletal muscle and diaphragm samples of the
23 Eurasian otter (*Lutra lutra*) in Norway and potentially in the arctic fox (*Vulpes lagopus*).

24

25 Keywords

26 *Sarcocystis lutrae*, rDNA, Scotland, European Badgers (*Meles meles*), 18S PCR, ITS1 PCR,
27 Phylogenetic analysis

28 KEY FINDINGS

- 29 • A 468 bp consensus sequence generated from the 18S rDNA of badger samples showed
- 30 100% identity to the 18S sequence for *Sarcocystis lutrae*.
- 31 • A 1074 bp consensus sequence generated from the ITS1 region of badger samples showed
- 32 100% identity to the ITS1 sequence of *S. lutrae*.
- 33 • 40 / 54 (74%) of badgers had at least one positive sample for *S. lutrae* DNA.
- 34 • This is the first reported detection of *S. lutrae* DNA (18S rDNA and ITS1 region) in badgers
- 35 in the United Kingdom.

36 INTRODUCTION

37 Current knowledge suggests that Sarcocystosis is caused by 200 currently identified species of
38 single-cell coccidian parasites in the phylum Apicomplexa, and genus *Sarcocystis*. These parasites
39 infect a wide range of definitive and intermediate hosts, including carnivorous animals, domestic
40 animals and humans (Dubey *et al.*, 2015; Dubey & Lindsay, 2006; Kaltungo & Musa, 2013). All
41 *Sarcocystis* parasites have an obligatory two host life cycle (some exception such as *S. neurona*
42 exist); asexual reproduction takes place in the intermediate host and sexual reproduction occurs in
43 the intestine of the definitive host (Dubey & Lindsay, 2006). *Sarcocystis neurona* has a very broad
44 intermediate host range and for example sporocysts from opossums can infect many hosts, of which
45 some are natural intermediate hosts (in which sarcocysts are formed), while others are aberrant
46 hosts (in which only schizonts are formed) (Dubey *et al.*, 2001a). Transmission from definitive to
47 intermediate host occur via the ingestion of oocysts/sporocysts from faeces via contaminated food
48 or water, and transmission from intermediate to definitive host occur via the ingestion of sarcocysts
49 which are found in muscle tissue (Dubey & Lindsay, 2006; Gjerde & Josefsen, 2015). However,
50 knowledge and understanding of all the life cycle stages of *Sarcocystis* species in wild carnivores is
51 incomplete and needs to be researched in more detail to help us to better understand disease
52 pathogenesis, symptomatology and impact of parasite diversity. More research is needed to
53 determine the range of clinical manifestations of *Sarcocystis* infections in wild carnivores, as there
54 is little information available about the signs and symptoms in these host species.

55 Only a few *Sarcocystis* species have been identified in wild carnivores of the family
56 Mustelidae (Dubey *et al.*, 2010). For example *S. neurona* has previously been identified in
57 (Eurasian) otters (*Enhydra lutris*) (Dubey *et al.*, 2003; Dubey *et al.*, 2001b; Miller *et al.*, 2009;
58 Wendte *et al.*, 2010), while *S. lutrae* has been found in the Eurasian otter (*Lutra lutra*) in Norway
59 (Gjerde & Josefsen, 2015). *Sarcocystis lutrae* has not been confirmed in another host species other
60 than the Eurasian otter and potentially arctic foxes (Gjerde & Josefsen, 2015; Gjerde & Schulze,
61 2014). Various *Sarcocystis* spp. including *S. hofmanni*, *S. melis*, *S. cf. sebeki* and *S. cf. gracilis*,

have previously been recorded by light microscopy (LM) and transmission electron microscopy (TEM) in heart, thigh, loin, thorax and tongue samples in European badgers (*Meles meles*) from Berlin (Odening *et al.*, 1994a; Odening *et al.*, 1994b). None of these *Sarcocystis* species in badgers have been identified in the United Kingdom. More recently, an unnamed species of *Sarcocystis* was recorded in the tongue, diaphragm, and masseter muscle of Japanese badgers (*Meles anakuma*) using haematoxylin and eosin (H&E) staining (Kubo *et al.*, 2009). To date LM of fresh muscle tissue and TEM have been examined to identify the *Sarcocystis* spp. found in badgers, meaning no DNA sequences are available for the *Sarcocystis* species previously identified in badgers. Only a few *Sarcocystis* species found in wild carnivores have been examined using molecular methods these include species, such as *S. arctica*, *S. lutrae*, *S. kalvikus*, and *S. kitikmeotensis* (Dubey *et al.*, 2015). Techniques, such as PCR and sequence analysis are more frequently used to identify *Sarcocystis* species (Gjerde & Josefsen, 2015). Polymorphisms in the 18S rDNA and ITS1 region may help with the speciation and discrimination of the different species within the *Sarcocystis* genus. The aim of this study was to determine the prevalence and species of *Sarcocystis* in muscle samples from European badgers (*Meles meles*).

MATERIALS AND METHODS

Collection of samples

Fifty-four European badger (*M. meles*) carcasses were collected from around the Lothians and Borders regions of Scotland, following fatal collisions with vehicles (badgers were collected with the knowledge and permission of Scottish Natural Heritage) (Bartley *et al.*, 2013). Carcasses were stored at -20°C prior to processing, full necropsies were performed when possible where samples of neck muscle, tongue, spleen, submandibular lymph node, liver, lung, brain, heart, blood and spinal cord were collected.

87 *DNA extraction*

88 DNA was extracted from muscle samples of 54 badgers. From those badgers, 54 neck samples and
89 32 tongue samples derived from the same animals were extracted. Approximately 1g of each
90 thawed tissue was transferred into a separate CK22 Precellys tissue homogenizer tube (Cepheid,
91 Stretton Derbyshire, UK), containing 1ml Nuclei Lysis Solution (Promega, Madison, WI, USA).
92 Samples were homogenized for 2 x 50s at 6500rpm using a Precellys 24 tissue homogenizer
93 (Depheid, Stretton Derbyshire, UK). 400µl of each homogenised tissues were added to a further
94 900µl of nuclei lysis solution and incubated at 55°C overnight. Samples were then processed using
95 the Wizard[®] genomic DNA (Promega, Madison WI, USA) purification protocol, which was adapted
96 to use 0.4g of starting material (Bartley *et al.*, 2013).

97

98 *Detection of protozoan DNA by 18S PCR and ITS1 PCR*

99 Parasite DNA was detected using a nested PCR, targeting the multi-copy 18S rDNA of the
100 ribosomal RNA gene family. The first round PCR used external primers that recognised various
101 apicomplexan parasites including *Neospora caninum*, *Toxoplasma gondii* and *Sarcocystis spp*
102 (Supplemental Table 1). Briefly, each 20µl reaction contained 2µl of 10x custom PCR mix- (45mM
103 Tris-HCl, 11mM (NH₄)₂SO₄, 4.5mM MgCl₂, 0.113mg/ml BSA, 4.4µM EDTA and 1.0mM each of
104 dATP, dCTP, dGTP and dTTP) (ABgene, Epsom, Surrey, UK), 0.25pM of each primer (Eurofins
105 MWG Operon), 0.75 units of BioTaq (Bioline, London, UK), 13.85µl of water and 2µl of sample
106 DNA (Burrells *et al.*, 2016). The PCR conditions for the first round were 95°C for 5 mins followed
107 by 35 cycles at 95°C for 1 min, 56°C for 1 min and 72°C for 1 min, with the final extension period
108 at 72°C for 5 mins. The primary PCR amplicons were diluted with 100µl DNase/RNase free water
109 and 2µl of the diluted primary amplification product was added as template DNA for the second
110 round amplification. Second round primers were designed to amplify only *Sarcocystis spp*.
111 (Supplemental Table 1). The specificity of the primers were tested using *S. neurona*, *S. lutrae*, *S.*

112 *gigantea*, *S. tenella*, *S. rileyi*, *S. fayeri*, *N. caninum* and *T. gondii* DNA samples (Data not shown).
113 The reaction conditions for the second round PCR were identical to the first round, with the
114 exception that internal forward and reverse primers were used. Each batch of samples analysed
115 contained a positive control: *S. lutrae* (obtained from this study) and negative controls: *N. caninum*,
116 *T. gondii* and water and were tested in duplicates. With each batch of badger samples extracted, a
117 negative (water) ‘extraction control’ was tested (Bartley *et al.*, 2013). Badgers that showed strong
118 positive bands for both neck and tongue samples in the 18S rDNA PCR, were tested further using
119 the ITS1 PCR. Here, Sarcocystidae were detected using the primers ‘SU1F’ and ‘5.8SR2’ that
120 amplify the ITS1 region (~1000 bp) and targets the adjacent 18S and 5.8S rDNA genes,
121 respectively (Gjerde, 2014) (Supplemental Table 1). The reaction conditions for the ITS1 PCR were
122 identical to those of the 18S rDNA PCR. The ITS1 region was selected to differentiate members of
123 the group Sarcocystidae as it is highly polymorphic compared to the 18S rDNA. PCR products
124 (6µl) were analysed by 2% agarose gel electrophoresis, stained with gel red (1:10,000) (Biotonium,
125 Hayward, USA) and visualised using UV light. Each batch of samples analysed by ITS1 PCR
126 contained a positive control: *S. lutrae* and negative control water.

127

128 *Cloning, DNA sequencing and sequence assembly*

129 The PCR products from twelve animals (both positives for tongue (n = 12) and neck muscle (n =
130 12)) using the 18S external primers and six positive PCR products from four animals (positives
131 tongue (n =4) and neck (n = 2)) using the external ITS1 PCR, were purified using the commercially
132 available Wizard[®] SV Gel and PCR Clean-up System (Promega, Madison WI, USA). The PCR
133 products were eluted in 50µl of DNase / RNase free water and the nucleic acid concentration was
134 determined by spectrophotometer (Nanodrop, ND1000). For each sample, 100ng of DNA was sent
135 for sequencing (Eurofins MWG Operon). The 18S amplicons were sequenced with the 18S primers
136 and the ITS1 amplicons were sequenced with the ITS1 primers (Supplemental Table 1).

137 Three first round PCR amplicons from the 18S rDNA PCR (tongue n= 1, neck n = 2) and a
138 further 3 PCR amplicons from the ITS1 PCR (tongue n= 2, neck n = 1) were cloned using the
139 pGEM®-T Easy Vector System (Promega, Madison WI, USA) as previously described (Bartley *et*
140 *al.*, 2016) with the following alterations. Two microliter (64ng) of the purified product were ligated
141 into the pGEM®-T Easy Vector (1µl at 50ng/µl) (Promega, Madison WI, USA) according to the
142 manufacturer's instructions. Following ligation, 1µl (8ng) of ligated vector/insert was used to
143 transform 40µl of high-efficiency competent JM109 cells ($\geq 1 \times 10^8$ cfu/µg DNA) (Promega,
144 Madison WI, USA) using manufacturer's instructions. A successful transformation was confirmed
145 using LB agar plates containing 100µg/ml ampicillin, spread with 100µl of IPTG (Isopropyl β-D-1-
146 thiogalactopyranoside) (100mM) and 20µl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-
147 galactopyranoside) (50mg/ml). White colonies were screened by PCR using the 18S external
148 primers and the SU1F and 5.8SR2 primers to confirm the presence of the *Sarcocystis* 18S rDNA
149 and ITS1 region insert. Three clones from each of the three badger samples from each of the 18S (n
150 = 9 clones) and ITS1 (n = 9 clones) PCR were sequenced (Eurofins, MWG Operon) using T7 and
151 SP6 primers. Additional internal S-ITS1-F and S-ITS1-R primers (Supplemental Table 1) were used
152 for the ITS1 clones to ensure a double stranded consensus sequence of over 1000 bp was generated.
153 Overall consensus sequences were generated for the 18S and ITS1 amplicons from each badger.

154 A Basic Local Alignment Search Tool (BLAST) search was completed to determine
155 percentage identity of the generated sequences against previously published sequences. Multiple
156 sequence alignments were performed using the BioEdit sequence alignment editor 7.1.3.0. to show
157 the difference between the closely related *Sarcocystis spp* . Phylogenetic analyses were performed
158 on both the 18S rDNA and ITS1 consensus sequences using MEGA6 software (Tamura *et al.*,
159 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on
160 the Tamura-Nei model (Tamura & Nei, 1993). Initial tree(s) for the heuristic search were obtained
161 by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the
162 Maximum Composite Likelihood. The tree is drawn to scale, with branch lengths measured in the

163 number of substitutions per site and all positions containing gaps and missing data were eliminated.
164 The phylogeny was tested with the bootstrap method, using 1000bootstrap replications.

165

166 *Statistical analysis*

167 The proportion of positive samples (prevalence), with confidence intervals (95% CI) was calculated
168 for the presence of *Sarcocystis* DNA in the tongue and neck muscle samples from badgers. The
169 numbers of badgers where either tongue, or neck muscle sample were positive and those animals
170 where both samples were positive were also calculated. All of the calculations were carried out
171 using the Minitab 17 software (v17.1.0.0).

172

173 RESULTS

174 *Screening of samples for the presence of protozoan DNA using the 18S rDNA PCR*

175 Five badgers were initially screened and DNA samples from leg, neck muscle, tongue, sub-
176 mandibular lymph node, liver, lung, brain, heart and spleen, were tested using the 18S external
177 primers in a single round PCR. Positive PCR amplicons were observed for 1/5 leg muscle, brain
178 and lung sample, 2/5 neck muscle samples and 3/5 tongue and spinal cord samples. Sequencing
179 PCR amplicons from one neck muscle and two tongue samples showed identity to *S. lutrae*
180 (accession KM657770). The PCR products from tongue and neck were the only samples to produce
181 identifiable sequences, and thus these organs were selected for further testing. Spinal cord was not
182 selected for further analysis, due to the limited numbers of samples available (n= 12).

183

184 *Verification of PCR specificity and sequencing*

185 The *Sarcocystis* specific 18S rDNA nested PCR was used to screen all muscle samples available,
186 tongue (n=32) and neck muscle samples (n=54) from 54 badgers. The results showed that 36 / 54
187 (67%) (95% CI: 52.5%, 78.9%) neck samples and 24 / 32 (75%) (95% CI: 56.5%, 88.5%) tongue

188 samples tested positive for *Sarcocystis* DNA. Twenty badger samples showed positive PCR results
189 for *Sarcocystis* DNA in both neck and tongue samples using the 18S rDNA PCR (20 / 32) (95% CI:
190 43.6%, 78.9%). Forty badgers tested positive with the *Sarcocystis* specific 18S rDNA PCR with at
191 least one tissue (40 / 54, 74%) (95% CI: 60.3%, 85.0%). No amplified products were observed for
192 the negative controls: water, *T. gondii* and *N. caninum*. Badgers (n=20) that showed positive results
193 for both tongue and neck in the 18S rDNA PCR were tested using the ITS1 PCR (Gjerde, 2014).
194 The ITS1 PCR revealed positive results for 12 / 20 (60%) (95% CI: 36.0%, 80.8%) neck and 10 / 20
195 (50%) (95% CI: 27.1%, 72.8%) tongue samples for Sarcocystidae DNA. No PCR amplicons were
196 generated for 4 / 20 badgers tested using the ITS1 primers.

197 Consensus sequences were generated for the 18S rDNA from 9 clones: tongue (n=3) and
198 neck (n=6) and for the ITS1 region from 9 clones: neck (n=3); and tongue (n=6). These clones were
199 used to create consensus sequences for the 18S rDNA and ITS1 amplicons for each animal. The 3
200 consensus sequences, each for the 18S rDNA and the ITS1 amplicons, were identical to each other
201 and were used to create a general consensus sequence for both the 18S rDNA and ITS1 regions. The
202 general consensus sequences for the 18S rDNA (468 bp) and the ITS1 region (1074 bp) were
203 submitted to Genbank (KX229728 and KX431307, respectively). When the 18S rDNA
204 (KX229728) and ITS1 (KX431307) sequences generated during this study, were compared on
205 NCBI BLAST against published DNA sequences, it was found that the 18S rDNA fragments
206 showed 100% identity to isolates of *S. lutrae* (18S rDNA: KM657770). The ITS1 sequence showed
207 99.2-100% identity to the 22 ITS1 sequences of *S. lutrae* found in the Eurasian otter (*Lutra lutra*)
208 (Gjerde & Josefsen, 2015).

209

210 *Phylogenetic relationship and multiple sequence alignments of S. lutrae and related species*

211 Phylogenetic analysis revealed that the *S. lutrae* rDNA found in badgers appears in the same clade
212 as the *S. lutrae* found in otters, as well as the closely related species *S. rileyi* and *S. turdusi* (Figure 1
213 A). The multiple sequence comparison demonstrated that the 18S rDNA fragment found in badgers

214 (KX229728) is identical to *S. lutrae* found in otters (KM657775) (Figure 2 A). The sequence
215 alignment of the 18S rDNA (Figure 2 A) shows polymorphic and conserved regions for the closely
216 related *Sarcocystis* species. The alignment shows that our sequence and *S. lutrae* are identical to
217 each other but are distinct from the other closely related species sequences by one additional ‘T’
218 base in comparison to *S. corvusi* and *S. arctica* and *S. turdusi*, and multiple base pair differences
219 from *S. rileyi*, *S. lacerate*, *S. mucosa* and *S. neurona* (Figure 2 A). The ITS1 region was also used
220 for sequence alignments and phylogenetic analysis, since the 18S rDNA gave poor discrimination
221 of closely related species. The ITS1 phylogenetic analysis showed a clearer differentiation from the
222 closely related *Sarcocystis* spp (*S. corvusi*, *S. arctica*, *S. neurona*, *S. turdusi* and *S. rileyi*), however
223 *S. kalvikus* was found in the same clade as *S. lutrae* from badgers and *S. lutrae* from otters (Figure 1
224 B). Yet, when using the ITS1 multiple sequence analysis, it can be seen that *S. kalvikus* can be
225 distinguished from the *S. lutrae* found in badgers and *S. lutrae* found in otters. The ITS1 region is
226 more polymorphic compared to the 18S rDNA and the ITS1 sequence comparison showed a clear
227 differentiation of *S. lutrae*, *S. kalvikus*, *S. turdusi*, *S. corvusi* and *S. arctica* (Figure 2 B). From the
228 multiple sequence analysis of both the 18S rDNA and ITS1 regions, it can be clearly seen that the
229 *Sarcocystis* spp rDNA fragments found in the sample of badgers in this study are identical to the *S.*
230 *lutrae* found in otters.

231

232 DISCUSSION

233 In this paper we report the detection of 18S rDNA and ITS1 region in tongue and neck muscles of
234 European badgers (*M. meles*) collected from around the Lothians and Borders regions of Scotland,
235 that show 100% sequence identity to DNA from *S. lutrae* (KM657770 and KM657775). *Sarcocystis*
236 *lutrae* has previously been identified in tongue, skeletal muscle and diaphragm in the Eurasian otter
237 (*Lutra lutra*) (Gjerde & Josefsen, 2015). Moreover, Gjerde & Schulze (2014), found that the cox1
238 sequence of *S. lutrae* from otters were identical with one of the cox1 sequence from an arctic fox

239 harbouring *S. arctica*. One of those *cox1* sequences was initially assigned to *S. arctica* even though
240 it differed slightly from the other *cox1* sequences obtained and was later re-assigned to *S. lutrae* and
241 thus the presence of *S. lutrae* in Arctic foxes can be disputed (Gjerde & Josefsen, 2015). This study
242 used the 18S rDNA and ITS1 region, to verify and identify the 18S rDNA fragments found in
243 badger samples. Phylogenetic and multiple sequence alignments have shown that the ITS1 region is
244 more polymorphic, compared to the 18S rDNA gene. Using multiple loci, such as the 18S rDNA
245 gene and the ITS1 region will help species identification and is more reliable than using one locus
246 alone. Few polymorphic regions in the 18S rDNA gene have previously been shown in especially
247 closely related *Sarcocystis* species (i.e. *S. lutrae*, *S. turdusi*, *S. arctica*, *S. wobeseri*) and little
248 sequence data has been generated. It has been shown that the ITS1 region gives a clearer
249 differentiation for these species (Gjerde & Josefsen, 2015; Gjerde & Schulze, 2014). Since the ITS1
250 region is not a gene, higher mutation densities are tolerated, making this region highly variable
251 among species and thus a useful marker for species identification for some, however, not all
252 *Sarcocystis* spp.

253 From this study it can be confirmed that the 18S rDNA and ITS1 region identified in
254 badgers showed 100% sequence identity to *Sarcocystis lutrae* (KM657770 and KM657775),
255 indicating that the *Sarcocystis* species detected is likely to be *S. lutrae*. Active infections may have
256 been detected if LM of fresh muscle tissue, such as tongue muscle, were analysed. *Sarcocystis*
257 *hofmanni*, *S. melis*, *S. cf. sebeki*, *S. cf. gracilis* and an unnamed *Sarcocystis* species have previously
258 been recorded in European badgers (*M. meles*) and Japanese badgers (*M. anakuma*) using TEM and,
259 LM of fresh muscle tissue (Kubo *et al.*, 2009; Odening *et al.*, 1994a; Odening *et al.*, 1994b). Since
260 those studies were conducted before molecular techniques were used in such research, no 18S or
261 ITS1 sequences were generated for these species. Testing both neck muscle and tongue for the
262 detection of *Sarcocystis* DNA proved advisable, as both these tissues showed a high presence of
263 *Sarcocystis* DNA and if only one tissue was tested the overall prevalence would have been lower.
264 The density of sarcocysts may vary in different types of muscle tissues, such as the diaphragm,

265 oesophagus, tongue and heart. These tissues are commonly used to demonstrate the presence of
266 sarcocysts in hosts (Dubey *et al.*, 2015).

267 Identification of *Sarcocystis* species based on morphology employs looking at structural
268 characteristics, such as sarcocyst wall and morphology; however, more than one *Sarcocystis* spp
269 may have the same sarcocysts morphology and the same species can occur in different hosts (Dubey
270 *et al.*, 2015; Dubey *et al.*, 1989). More recently molecular methods of *Sarcocystis* spp. have been
271 particular useful to distinguish between morphologically indistinguishable species in closely related
272 intermediate hosts, such as water buffaloes and cattle, and different cervids. Ideally, individual
273 sarcocysts should be excised from fresh muscle tissue, examined in wet mounts by LM, used to
274 extract DNA for molecular characterisation and fixed to study them using TEM. Using both
275 identification methods would allow phenotypic and genotypic data to be combined and be linked to
276 the species description. Using morphological characteristics alone for the identification may prove
277 difficult as size and shape is subject to change depending on the age of the Sarcocysts but this
278 observation can be strengthened by sequencing DNA amplicons from different regions of the
279 parasite genome (Dubey *et al.*, 2015).

280 The data presented in this study shows that the DNA detected in European badgers showed
281 sequence identity at two different loci to *S. lutrae* found in otters. This shows that badgers from the
282 Lothians and Borders regions of Scotland are frequently infected with *S. lutrae*. Badgers are
283 omnivores, and it is likely that they become infected through the ingestion of sporocysts shed by
284 definitive host (predator/scavenger) (Dubey & Lindsay, 2006). Birds, such as the white-tailed (sea)
285 eagle (*Haliaeetus albicilla*) suggested by (Gjerde & Josefsen, 2015) or birds of the family
286 *Corvidae*, as well as other badgers and foxes (*Vulpes vulpes*) may act as a definitive host of *S.*
287 *lutrae*. Further research, involving microscopic analysis, as well as multiple locus sequence typing,
288 is needed to confirm whether *S. lutrae* is widely distributed across Great Britain and whether *S.*
289 *lutrae* is only found in badgers, (Eurasian) otters and potentially arctic foxes.

290

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Table 1. Sequences and specificity of primers used for the detection of *Sarcocystis* spp DNA in badger samples.

Region	Primer	Forward	Primer	Species amplified	Reference
18S	External	Forward	NTS-18S-F1 5'-GCC ATG CAT GTC TAA GTA TAA G -'3	<i>N. caninum</i> , <i>T. gondii</i> and	This study
		Reverse	NTS-18S-R1 5'-CCT ATC ATT CCA ATC ACT AGA AAT -'3	<i>Sarcocystis</i> spp.	
	Internal	Forward	NTS-18S-F2 5'- GGA TAA CCG TGG TAA TTC TAT G -'3	<i>S. lutrae</i> , <i>S. neurona</i> and	
		Reverse	S-18S-G9 5'- CAT CGC CGA CCA AAA AGG -'3	<i>S. lacertae</i>	
ITS1	External	Forward	SU1F 5'- GAT TGA GTG TTC CGG TGA ATT ATT -'3	<i>Sarcocystidae</i>	Gjerde, 2014
		Reverse	5.8SR2 5'- AAG GTG CCA TTT GCG TTC AGA A -'3		
	Internal	Forward	S-ITS1-F 5'- TTT CTG TAG CGT TGA GAG GAG T -'3	<i>Sarcocystidae</i>	This study
		Reverse	S-ITS1-R 5'- CGC CTC GCT CAA CAT CAT CAT AAA -'3		

Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood method for selected members of the Sarcocystidae. A) 18S rDNA with the highest log likelihood (-1280.9024) and B) ITS1 spacer region with the highest log likelihood (-2396.9991). The percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Figure 2. Multiple sequence alignment of the polymorphic sections of the 18S and the ITS1 region amplified in this study: A) 18S rRNA gene region, B) ITS1 region. Boxing shows identical sequences of *S. lutrae* (18S KM657769 and ITS1 KM657775) and the *Sarcocystis* sequence detected in the badger samples (18S KX229728 and ITS1 KX431307). Dots represent identical base pairs and dashed lines represent gaps in the alignments. Numbers given above each alignment correspond to the nucleotide position in the sequence A) KM657769 and B) KM657775, respectively.

203x248mm (300 x 300 DPI)

A)

	260	270	280	290	300	310	320
Sarcocystis lutrae KM657769	CATTATGACC	TTT-TT---	---GGTCGGC	GATGGATCAT	TCAAGTTTCT	GACCTATCAG	CTTTGACGG
Sarcocystis sp. (S. lutrae Badger) KX229728							
Sarcocystis turdusi JF975681							
Sarcocystis corvusi JN256117							
Sarcocystis arctica KF601305							
Sarcocystis neurona U07812	G.						
Sarcocystis lacerate AY015113	G.						
Sarcocystis rileyi KJ396583	-CG-						
Sarcocystis mucosa AF109679	-CACG-						
Sarcocystis tenella L24383	GT.	A..C..TTGT	AAT..CT..	..A.....			

B)

	900	910	920	930	940	950	960
Sarcocystis lutrae KM657775	GT----	ATT ATATTA--	C TGCAA---	T CATATATTGC	T----CTGAT	TTCTATCAIC	TTGCT--TGC
Sarcocystis sp. (S. lutrae Badger) KX431307							
Sarcocystis sp. (S. kalvikus) -GU200661		G.			C.	A--C.	
Sarcocystis arctica KF601308		..G.C----	..A.....	..GC.C...	..TCT..	G..	..TG---CA
Sarcocystis turdusi JF975683	A..CGTATG.A	..CCG----	..A..GGTTC.	..CG..C..T	..---T...	GC.	..-----A
Sarcocystis corvusi JN256119	A.....	..CC----G	..A.....	..G..C...	..---T...	CCG	..-----A
Sarcocystis neurona AF204230	A..GACAGCCG	CAC...--G	ACAGC---G	G..CG..C..CT	..---TGAG.	A..GCGGTG.G	..GCGGTGG.T
Sarcocystis rileyi KJ396584	..CTACTGCCG	..CGA...--G	CA..TG---A	AGC..TC.A.	A----TGA..	GGTG..GTG.G	..GTGG--G..T

80x38mm (300 x 300 DPI)