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Blastocystis sp. Subtype Diversity in Wild Carnivore Species from Spain

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1 Running head: *Blastocystis* in Wild Carnivores from Spain
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3 ***Blastocystis* sp. Subtype Diversity in Wild Carnivore Species from Spain**
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41 **ABSTRACT**
42 The occurrence and molecular diversity of the Stramenopile eukaryote *Blastocystis* sp.
43 was investigated by PCR and sequencing (Sanger and NGS) methods in 380 faecal
44 specimens of free-living carnivores in Spain. *Blastocystis* sp. was confirmed in 1.6%
45 (6/380) of the specimens analysed. Two samples from a common genet and a fox were
46 successfully subtyped as ST7 by Sanger. Using NGS, ST14 was found in a fox and a
47 European polecat, ST7 in a fox, and two additional foxes presented mixed infections of
48 ST1/ST2/ST4 and ST1/ST2/ST7, respectively. Wild carnivore species could act as
49 carriers of zoonotic *Blastocystis* subtypes.
50
51 **Keywords**
52 Subtypes; Zoonotic transmission; Sanger; Next Generation Sequencing; Wildlife
53

54 **INTRODUCTION**
55 *Blastocystis* sp. (Stramenopiles, Blastocystidae), one of the most common enteric
56 parasites in humans globally (Scanlan and Stenvold 2013), possesses elusive
57 transmission pathways. Although the life cycle of *Blastocystis* is not fully understood,
58 faecal-oral transmission through ingestion of cyst-contaminated water or food is mostly
59 accepted. *Blastocystis* pathogenicity remains controversial. *Blastocystis* infections have
60 been linked with gastrointestinal symptoms, irritable bowel syndrome, and extra-
61 intestinal disorders (Casero et al. 2015; Nourrisson et al. 2014), but in most instances
62 *Blastocystis* carriage is asymptomatic (Zhang et al. 2016). Indeed, recent
63 metagenomics-based studies seem to suggest that *Blastocystis* colonization is associated
64 with a healthy gut microbiota (Audebert et al. 2016).
65 *Blastocystis* is frequently reported in a wide range of vertebrates including
66 human and non-human primates, other mammals and birds. Animal-to-human (or vice
67 versa) transmission has been suggested in several molecular epidemiological surveys
68 (e.g. Eroglu and Koltas 2010; Ramírez et al. 2014; Stensvold et al. 2009) but not in
69 others (Paulos et al. 2018). Taken together, these data seem to indicate that
70 zoonotic/anthroponotic transmission of *Blastocystis* sp. likely occurs under appropriate
71 conditions. A high degree of genetic diversity has been found within *Blastocystis* sp.
72 (Alfellani et al. 2013b; Stensvold et al. 2012;) leading to the description of at least 26
73 subtypes (ST), based on polymorphism at the small subunit (*ssu*) rDNA gene, with
74 marked differences in host specificities and even geographical distributions (Alfellani et
75 al. 2013a; Clark et al. 2013; Maloney et al. 2019a). Among them, ST1–9 and ST12 have
76 been reported in humans. Additionally, subtype-dependent variability in *Blastocystis*
77 pathogenicity has been proposed by some authors (Domínguez-Márquez et al. 2009;
78 Ramírez et al. 2014; Stensvold et al. 2011).
79 Little research has been conducted to investigate the occurrence of this
80 eukaryote in wild animal populations (Alfellani et al. 2013b; Betts et al. 2017; Roberts
81 et al., 2013) and most animal studies focused on livestock species or wild animals kept
82 in zoological gardens (Abe et al. 2002; Alfellani et al. 2013b, Cian et al. 2017; Maloney
83 et al. 2019a; Stensvold et al. 2009). Here we present novel data on the occurrence and
84 molecular diversity of *Blastocystis* sp. in free-living carnivore populations in Spain.
85
86
87 **MATERIALS AND METHODS**
88
89 **Sample collection**
90 A total of 380 faecal specimens from 13 wildlife mammalian species belonging to the
91 Canidae (*n* = 187), Erinaceidae (*n* = 2), Felidae (*n* = 25), Herpestidae (*n* = 11),
92 Mustelidae (*n* = 133), Procyonidae (*n* = 11), and Viverridae (*n* = 11) families were
93 collected in seven Spanish regions between December 2013-October 2017 (Table 1;
94 Data S1). Samples were obtained from road- and hunter-killed animals, from
95 accidentally found carcasses, camera-trap surveys, or animals entering rescue shelters.
96 Hunted animals had been legally shot during official hunting seasons.
97
98 **DNA extraction**
99 Total DNA was extracted from an aliquot of ~200 mg of fresh faecal material using the
100 QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) following the
101 manufacturer's instructions. Purified DNA samples (200 µl) were stored at –20 °C until
102 downstream PCR-based diagnostic and subtyping analyses were conducted.
103

104 **Molecular detection and characterization of *Blastocystis* sp. using Sanger**
105 **sequencing**
106 Identification of *Blastocystis* sp. was achieved by a PCR protocol targeting a fragment
107 of the *ssu* rDNA gene as previously described (Scicluna et al. 2006). Briefly, this
108 method uses the barcoding primers RD5 and BhRDr to generate a PCR product of
109 ~600-bp. Laboratory-confirmed *Blastocystis* positive, negative, and no-template
110 controls were included as controls in each PCR run. PCR amplicons were visualized on
111 2% D5 agarose gels (Conda, Madrid, Spain) stained with Pronasafe nucleic acid
112 staining solution (Conda). All PCR products were sequenced in both directions with the
113 same PCR primers using Big Dye™ chemistries and an ABI 3730xl sequencer analyser
114 (Applied Biosystems, Foster City, CA). Generated DNA consensus sequences were
115 aligned to appropriate reference sequences using the MEGA 7 software
116 (http://www.megasoftware.net/) to identify *Blastocystis* subtypes (Tamura et al. 2013).
117 *Blastocystis* sequences were submitted to the publicly available online *Blastocystis* 18S
118 database (http://pubmlst.org/blastocystis/) for subtype confirmation and allele
119 identification. These nucleotide sequences were deposited in the GenBank database
120 under accession numbers MK587503 and MK587504.
121
122 **Molecular detection, next generation amplicon sequencing library preparation,**
123 **and bioinformatic analysis**
124 Next generation amplicon sequencing (NGS) libraries were prepared and analysed as
125 previously described (Maloney et al. 2019b). Briefly, all samples were screened by PCR
126 using primers ILMN_Blast505_532F and ILMN_Blast998_1017R. These primers
127 amplify a fragment of the *ssu* rDNA gene and are identical to
128 Blast505_532F/Blast998_1017R (Santín et al. 2011), with the only exception of
129 containing the Illumina overhang adapter sequences on the 5' end. Final libraries were
130 quantified using the Quant-iT dsDNA Broad-Range Assay Kit (ThermoFisher,
131 Waltham, MA) on a SpectraMax iD5 (Molecular devices, San Jose, CA) prior to
132 normalization. A final pooled library concentration of 8 pM with 20% PhiX control was
133 sequenced using Illumina MiSeq 600 cycle v3 chemistry (Illumina, San Diego, CA,
134 USA). Paired end reads were processed and analysed with an in-house pipeline that uses
135 the BBTools package v38.22 (Bushnell 2014), VSEARCH v2.8.0 (Rognes et al. 2016),
136 and BLAST+ 2.7.1. After removing singletons, clustering and the assignment of
137 centroid sequences to operational taxonomic units (OTU) was performed within each
138 sample at a 98% identity threshold. All raw fastq files were deposited to the NCBI
139 sequence read archive under the accession number PRJNA523831. The nucleotide
140 sequences for unique OTUs obtained in this study have been deposited in GenBank
141 under the accession numbers MK587489-MK587502.
142
143
144 **RESULTS AND DISCUSSION**
145 *Blastocystis* was found and confirmed by Sanger or NGS in nearly 1.6% (6/380) of the
146 fecal samples analysed (Table 1). The protist was identified in four (2.2%) red foxes
147 (*Vulpes vulpes*) from Basque Country, Castile-La Mancha, and Extremadura, one
148 (9.0%) common genet (*Genetta genetta*) from Castile-La Mancha, and one (25.0%)
149 European polecat (*Mustela putorius*) from Extremadura (Table 1and Data S1). In the
150 only previous epidemiological survey assessing the presence of *Blastocystis* sp. in wild
151 carnivore species kept in captivity in Spain, an 8.3% prevalence was found by
152 microscopy in red foxes, Hudson Bay wolves, Iberian wolves, and brown bears (Pérez-
153 Cordón et al. 2008). This figure is over 5-fold higher than that (~1.6%) reported here in

154 free-living carnivores. This discrepancy is likely due either to differences in the animal
155 populations compared or to the higher infection pressure expected to occur in animals
156 living in crowded conditions. *Blastocystis* sp. has also been reported at prevalence rates
157 ranging from 0–7.5% in captive (Alfellani et al. 2013b; Cian et al. 2017) and free-living
158 (Betts et al. 2017) carnivore species, and from 0–2.6% in domestic dogs (Abe et al.
159 2002; Moura et al. 2018; Paulos et al. 2018) in different geographical areas of the
160 world. In contrast, the occurrence of the parasite has been identified at comparatively
161 higher rates (6.0–54.1%) in domestic ruminants, particularly cattle and sheep (Lee et al.,
162 2018; Li et al. 2018; Masuda et al. 2018). Taken together, these findings seem to
163 indicate that *Blastocystis* colonization/infection is less common in strict carnivore
164 species than in ruminants. These discrepancies may arise from specific differences in
165 gut microbiome profiles between carnivores and herbivores, or to differences in the
166 structure and functionality of the gastrointestinal tract, as obligatory carnivores have a
167 reduced caecum compared to that of herbivores. It is currently unknown if the presence
168 of *Blastocystis* in carnivores represents active infection or is the result of consumption
169 of an infected animal.
170 Out of the 380 samples tested using primers RD5 and BhRDr 12 samples were
171 PCR positive, but only two specimens, one from a young female genet (Sample #98)
172 and one from an adult vixen (Sample #113), were successfully subtyped by the Sanger
173 method as ST7 allele 140. Both animals were from the province of Ciudad Real in the
174 autonomous regions of Castile-La Mancha (Data S2). The high number of positives by
175 PCR that could not be successfully identified as *Blastocystis* when using primers RD5
176 and BhRDr could be related to their ability to amplify other eukaryotes (mostly fungi),
177 with no obvious differences in PCR product size, especially when screening DNA
178 extracted directly from faeces (Stensvold 2013). Therefore, barcoding primers are better
179 suited for molecular characterization of already known positive samples and not for
180 screening (Stensvold and Clark 2016a). The Santín et al. (2011) primers perform better
181 for screening faecal specimens for *Blastocystis* as they produce fewer false positives
182 without compromising specificity and sensitivity.
183 Analysis of all samples (*n* = 380) by NGS using the ILMN_Blast505_532F and
184 ILMN_Blast998_1017R primers allowed the identification of five *Blastocystis* positive.
185 A total of 2,611,628 read pairs were generated from those samples. After trimming,
186 quality filtering and pair merging, 444,911 merged read pairs were retained. 418,784
187 merged reads remained following chimera filtering. Clustering generated 16 OTUs
188 across those five samples. There were 13 OTUs that were unique *Blastocystis* sequences
189 in the study population (Data S3). NGS confirmed the presence of ST7 in fox #113 and
190 identified and subtyped *Blastocystis* in four additional samples including three foxes
191 and one European polecat (Data S2). NGS also revealed a much higher genetic diversity
192 with identification of three subtypes in two foxes (samples #127 and #156) as well as
193 intra-subtype variability in three foxes (samples #113, #127 and #156) for ST1, ST2,
194 and ST7 (Data S2). The relative abundance of *Blastocystis* subtypes identified in each
195 positive sample is shown in Data S4. ST7 displayed the greatest intra-subtype
196 variability with five unique OTUs, two of which were found in both ST7 positive
197 samples (#113 and #156) (Data S2). ST1 and ST2 also displayed intra-subtype
198 variability in this study with three unique OTUs observed for both subtypes. No intra-
199 subtype variability was observed for ST4 or ST14 (Data S2).
200 Combining Sanger and NGS sequencing, five *Blastocystis* subtypes were
201 identified in the surveyed population, namely ST1, ST2, ST4, ST7, and ST14. ST14
202 was found in a fox and a European polecat, ST7 in a fox and a common genet, and the
203 other two foxes presented mixed infections with ST1/ST2/ST4 and ST1/ST2/ST7,

204 respectively. Mixed subtype infections were observed in two of the five (40.0%)
205 *Blastocystis*-positive samples by NGS and included the identification of subtypes
206 present in low abundance (Data S2 and Data S4). No mixed subtype infections were
207 found using the traditional method, barcoding primers coupled with Sanger sequencing,
208 possibly due to the inherent nature of PCR, which preferentially amplifies the
209 predominant subtypes that will be identified by Sanger sequencing. The use of NGS
210 could aid in understanding host-parasite specific epidemiological cycling in nature
211 because of its capacity to resolve a higher level of genetic diversity identifying low
212 abundance of *Blastocystis* STs within the same host (Maloney et al. 2019b). ST7 was
213 the most common ST found causing single or mixed infections in combination with
214 other *Blastocystis* STs in three foxes and a common genet. Of interest, ST7 is a
215 relatively common finding in birds (Stensvold et al. 2009), suggesting that wild
216 carnivores may acquire *Blastocystis* by preying on birds carrying the protist. The fact
217 that ST7 has been reported previously in a variety of human populations in Angola,
218 Colombia or Thailand (Dacal et al. 2018; Ramirez et al. 2016; Yowang et al. 2018)
219 including Spanish symptomatic individuals (Carmena, unpublished data), indicates that
220 ST7 could be zoonotically transmitted. The finding of ST14 in a fox and a European
221 polecat is interesting, as this *Blastocystis* subtype has so far been primarily found in
222 production animals (Fayer et al. 2012; Li et al. 2018) and wildlife members of the
223 family Artiodactyla (Cian et al. 2017). There is no evidence of zoonotic transmission of
224 ST14 as it has not been yet detected in humans. This is, to the best of our knowledge,
225 the first description of ST14 in a carnivore species.
226 The identification of ST1, ST2, and ST4 in foxes expands the host range of these
227 potentially zoonotic subtypes and supports the potential role of wild carnivores as
228 carriers of zoonotic *Blastocystis* subtypes. There is limited information of *Blastocystis*
229 subtypes in captive or free-living carnivore species and only few studies have reported
230 molecular-based surveys. ST2 and ST3 were previously identified in a captive cheetah
231 and a grey wolf in France (Cian et al. 2017), ST4 in a pine marten from a conservation
232 park in the UK (Betts et al. 2017), and ST8 in omnivorous common wild opossums in
233 Colombia (Ramírez et al. 2014). Companion animal species, dogs and cats, have also
234 been investigated to assess their potential role as suitable reservoirs of *Blastocystis*
235 transmission to humans. Evidence of transmission between pets and their owners
236 involving ST1, ST2, and/or ST3 (the *Blastocystis* subtypes more prevalently found
237 circulating in humans) has been indicated by some surveys conducted in Australia
238 (Nagel et al. 2012), Philippines (Belleza et al. 2016), and Turkey (Eroglu and Koltas
239 2010). In contrast, a recent Spanish study showed that domestic dogs and cats had a
240 negligible role as source of human infections (Paulos et al. 2018).
241 Regarding the diversity and frequency of *Blastocystis* in Spain, ST1, ST2, ST3,
242 and ST4 have been identified in a general, asymptomatic population in the North of the
243 country (Paulos et al. 2018), whereas those same subtypes and to much lower extent
244 ST6 and ST7, have been described in clinical samples (Carmena, unpublished data).
245 Interestingly, ST4 has been previously documented as the most prevalent (94.1%)
246 *Blastocystis* ST in mono-infected patients with acute or chronic diarrhoea (Domínguez-
247 Márquez et al. 2009), but the least frequent ST circulating in asymptomatic subjects in
248 Spain (Paulos et al. 2018), raising the question of whether ST4 is more pathogenic than
249 other *Blastocystis* STs. Our results demonstrate that STs with zoonotic potential are
250 present in carnivores, corroborating their potential role as a source of *Blastocystis*
251 human infection and environmental contamination. In additions, this population was
252 examined for other intestinal parasites with zoonotic potential including several

253 *Cryptosporidium* species and *Enterocytozoon bieneusi* genotypes supporting free-living
254 carnivores as a source of zoonotic parasites (Mateo et al. 2017; Santín et al. 2018).
255 In conclusion, this is the first study that explored the occurrence and genetic
256 diversity of *Blastocystis* in wild carnivores in Spain. The host range for *Blastocystis* and
257 its subtypes was expanded with the identification of ST1, ST4, and ST2 in foxes; ST14
258 in a fox and a European polecat; and ST7 in a fox and a common genet. The application
259 of NGS provided higher resolution allowing the identification of mixed infections
260 (representing 40.0% among *Blastocystis* positives) as well as detection of low
261 abundance subtypes. Further studies using high resolution methods such NGS are
262 necessary to understand the dynamics of *Blastocystis* transmission in wild populations
263 and their role in the zoonotic transmission of this stramenopile.
264
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272
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413 **SUPPORTING INFORMATION**
414 **Data S1**. Map of Spain showing the geographical distribution of the main carnivore
415 families sampled in the present study. Black and dark red silhouettes represent
416 *Blastocystis*-negative and *Blastocystis*-positive results by molecular methods,
417 respectively.
418
419 **Data S2**. *Blastocystis* subtypes identified by Sanger- and NGS-based (including unique
420 OTUs information and percentage of reads) methods in each carnivore-positive sample
421 in Spain are presented.
422
423 **Data S3**. Unique operational taxonomic units (OTUs) obtained for *Blastocystis*
424 subtypes by next generation amplicon sequencing.
425
426 **Data S4**. Relative abundance of *Blastocystis* sp. subtypes observed in each positive
427 carnivore sample using next generation amplicon sequencing (NGS).