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Article

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Molecular identification of Entamoeba species in savanna woodland chimpanzees (Pan troglodytes schweinfurthii)

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1   **Molecular identification of *Entamoeba* species in savanna woodland chimpanzees (*Pan***

2   ***troglodytes schweinfurthii*)**

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26

27   **Running title:** *Entamoeba* species in savanna chimpanzees

28

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43   **Summary:** To address the molecular diversity and occurrence of pathogenic species of the

44   genus *Entamoeba* spp. in wild non-human primates (NHP) we conducted molecular-

45   phylogenetic analyses on *Entamoeba* from wild chimpanzees living in the Issa Valley,

46   Tanzania. We compared the sensitivity of molecular (using a genus-specific PCR) and

47   coproscopic detection (merthiolate-iodine-formaldehyde concentration) of *Entamoeba* spp.

48   We identified *Entamoeba* spp. in 72 chimpanzee faecal samples (79 %) subjected to species-

49   specific PCRs for six *Entamoeba* species/groups (*E. histolytica, E. nuttalli, E. dispar, E.*

50   *moshkovskii, E. coli,* and *E. polecki* ST2). We recorded three *Entamoeba* species: *E. coli* (47

51   %), *E. dispar* (16 %), *E. hartmanni* (51 %). Coproscopically we could only distinguish the

52   cysts of complex *E. histolytica/dispar/moshkovskii/nuttalli* and *E. coli*. Molecular prevalence

53   of entamoebas was higher than the prevalence based on the coproscopic examination. Our

54   molecular phylogenies showed that sequences of *E. dispar* and *E. coli* from Issa chimpanzees

55   are closely related to sequences from humans and other NHP from GenBank. The results

56   showed that wild chimpanzees harbour *Entamoeba* species similar to those occurring in

57   humans; however, no pathogenic species were detected. Molecular-phylogenetic methods are

58   critical to improve diagnostics of entamoebas in wild NHP and for determining an accurate

59   prevalence of *Entamoeba* species.

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61   **Key words:** *Entamoeba*, molecular diversity, great apes, chimpanzee, savanna

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66 **Key findings:**

67 ▪ first molecular survey of *Entamoeba* spp. in wild great apes

68 ▪ *E. coli*, *E. dispar*, and *E. hartmanni* recorded in wild eastern chimpanzees

69 ▪ *E. dispar* and *E. coli* closely related to other sequences from non-human primates and

70 humans

71 ▪ no pathogenic species of *Entamoeba* recorded

72 ▪ molecular methods more sensitive than coproscopic ones for detection of entamoebas

73

74 **Introduction**

75 Protists represent important model organisms for studying parasite transmission between

76 non-human primates (NHP) and humans, primarily because of high genetic diversity, lower

77 host specificity, and life cycles that facilitate their transmission (Pedersen et al., 2005). Yet to

78 date, few studies have been conducted on the molecular diversity of protists in populations of

79 wild African great apes (Petrášová et al., 2011; Sak et al., 2013, 2014). Given that humans

80 increasingly encroach upon wild primate habitats (Chapman and Lambert, 2000),

81 understanding the biology and diversity of potentially zoonotic protists is an important part of

82 One Health approach in conservation medicine (http://www.onehealthinitiative.com).

83 Cysts of the amoebas of the genus *Entamoeba* are commonly detected in faecal samples

84 of wild NHPs, including chimpanzees, by light microscopy following concentration

85 coproscopic methods (e.g. Ashford et al., 2000; Lilly et al., 2000; Muehlenbein, 2005;

86 Howells et al., 2011; Kooriyama et al., 2012; Kalousová et al., 2014). However, it is difficult

87 if not impossible to differentiate the pathogenic (*Entamoeba histolytica* and *Entamoeba*

88 *nuttalli*) from the non-pathogenic species (specifically *Entamoeba dispar* and *Entamoeba*

89 *moshkovskii*), as all the above mentioned species form morphologically indistinguishable

90   cysts with four nuclei (Kebede et al., 2004; Visser et al., 2006). Only molecular techniques

91   allow distinguishing commensal species from species with confirmed pathogenicity

92   (Tachibana et al., 2000; Verweij et al., 2001; Stensvold et al., 2010). Moreover, the

93   distribution of *E. histolytica* and *E. nutalli* remains poorly explored, as they are only recently

94   separated from each other and known both from NHP and humans (Tachibana et al., 2007,

95   2009, 2015; Levecke et al., 2010, 2015; Stensvold et al., 2010)

96   Several studies have used molecular tools for studying of *Entamoeba* spp. in captive

97   NHP, where both pathogenic *Entamoeba* species and also commensal ones (*E. dispar*, *E. coli*,

98   *E. hartmanni, E. moshkovskii* or *E. polecki* ST2) have been documented (Verweij et al., 2003;

99   Tachibana et al., 2009; Levecke et al., 2010; Rivera et al., 2010; Regan et al., 2014). To date,

100 such techniques have not yet been employed in wild-living NHP with the exception of

101 Tachibana et al. (2015), who molecularly characterizated of *Entamoeba nuttalli* strains in wild

102 toque macaques (*Macaca sinica*) in Sri Lanka. Levecke et al. (2010) molecularly detected

103 four undetermined *Entamoeba* lineages in captive NHPs, which may also indicate the

104 presence of yet undescribed species in their wild counterparts.

105 We carried out a survey on the molecular diversity of *Entamoeba* spp. in a community of

106 savanna chimpanzees living in the Issa Valley, Ugalla (western Tanzania) with emphasis on

107 species with zoonotic potential. We also focused on species that we assume to naturally occur

108 in chimpanzees. A recent study (Stensvold et al., 2011) proposed a new nomenclature of

109 *Entamoeba* species for novel or undetermined lineages and also suggested a division of the

110 *Entamoeba* spp. to the complexes of uni-, tetra- and octo-nucleated cysts. Accordingly, we

111 designed PCR protocols to distinguish *E. polecki* ST2 (belonging to entamoebas producing

112 uninucleated cysts), group of *E. coli* (octonucleated cysts) and, finally, we conducted species-

113 specific PCR for *E. histolytica* complex (with tetranucleated cysts) to distinguish the

114   pathogenic species (*E. histolytica* and *E. nuttalli*) from the commensal ones (*E. dispar* and *E.*

115   *moshkovskii*).

116

117   **Material and methods**

118   *Study site and subjects*

119   The Issa Valley research station is located in the Ugalla region, ~100 km east of Lake

120   Tanganyika, in western Tanzania. Ugalla covers approximately 3352 km^2 with an elevation

121   range of 980 – 1712 m above sea level and consists of flat plateaus broken up broad valleys,

122   steep hills, and severe slops (Moore, 1994). The Issa Valley is dominated by savanna

123   (miombo) woodland vegetation, but also has very thin riverine evergreen forest strips,

124   swamps and grassland (Moore, 1994). The climate of Issa includes a dry season from May to

125   September and a rainy season from October/November to April/May (Hernandez-Aguilar et

126   al., 2013). Average annual rainfall is around 1,200 mm (range: 980–1,350 from 2008–2014),

127   and the temperature varies between 14 and 34 °C. The Issa chimpanzee community is

128   estimated to number 67 individuals based on preliminary genetic analyses (Rudicell et al.,

129   2011), and overall, the population density at Issa is estimated to be 0.25 individuals/km^2 (Piel

130   et al., 2015).

131

132   *Sample collection*

133   One hundred and seven faecal samples were non-invasively collected between February

134   2009 and February 2010 and between March and May 2012 by following chimpanzee parties

135   and upon encountering fresh nest groups opportunistically; all samples originated from a

136   single chimpanzee community (Rudicell et al., 2011). Issa chimpanzees are only partially

137   habituated and therefore we could not attribute the faecal samples to specific individuals.

138   Each faecal sample was stored in 20 ml vials in in 96% ethanol and part of the samples (n=33)

139   was simultaneously stored also in 4% formaldehyde.

140   *Coproscopic analyses*

141   Thirty-three samples fixed in 4% formaldehyde were used for coproscopic analyses

142   (Supplementary table S1). The standard protocol for detection of *Entamoeba* cysts was

143   followed and the merthiolate-iodine-formaldehyde concentration (MIFC) technique was used

144   (Blagg et al., 1955). Two millilitres of sediment suspension was mixed with 5 ml of MIFC

145   solution, 1 ml of Lugol's iodine, and 6 ml of ether in a 15 ml Falcon tube. Subsequently, it

146   was centrifuged at 280g for two minutes, the supernatant was discarded and the residual

147   sediment was examined by light microscopy using × 1,000 magnification.

148   *Molecular analyses and sequencing of* Entamoeba *spp.*

149   All faecal samples (n=107) preserved in 96% ethanol were molecularly analysed

150   (Supplementary table S1). Two hundred milligrams of each sample was dried overnight at 37

151   °C, and then the total DNA was extracted using the kit PSP® Spin Stool DNA kit (Stratec)

152   following the manufacturer's protocol. First, we the samples positive for *Entamoeba* were

153   identified using semi-nested PCR, amplifying the conservative part of SSU rRNA gene

154   specific for the *Entamoeba* genus. PCR conditions are described below, and all primers are

155   summarized in Table 1. In the first round of this semi-nested PCR, our designed reverse

156   primer, Entam_5 (Table 1) with Entam_1 as a forward one were used. In the second PCR

157   round, published primers, namely forward Entam_1 and reverse Entam_2 were used (for more

158   details see Table 1). The size of amplicons was approximately 650 bp. Then, only samples

159   positive for *Entamoeba* spp. were screened using PCRs specific for six *Entamoeba* species

160   (based on the part of the SSU rRNA gene): (i) *Entamoeba histolytica* (size of product: 475

161   bp), (ii) *E. nuttalli* (size of product: 848 bp), (iii) *E. dispar* (size of product: 195 bp), (iv) *E.*

162   *moshkovskii* (size of product: 580 bp), (v) *E. coli* (size of product: 290 bp), and (vi) *E. polecki*

163   ST2 (size of product: 680 bp) (for more detail see Table 1 and Figure 1). All species-specific

164   PCRs were performed separately to prevent the competitive inhibition of *Entamoeba* spp.

165   DNA.

166   For all PCRs, the published conditions listed in Table 1 were followed, except for the first

167   round of semi-nested PCR: five minutes at 95°C, 35 cycles of one minute at 95°C, 60°C and

168   72°C, and final elongation for 10 minutes at 72°C. For positive controls, isolates from *in vitro*

169   cultures (*E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. coli*) were used, whereas in the case

170   of *E. nuttalli* an isolate obtained from faeces of a Hamadryas baboon (*Papio hamadryas*) from

171   a sanctuary for exotic animals was used (AAP, the Netherlands; see Levecke et al., 2010) and

172   an isolate of *E. polecki* ST1 from faeces of a domestic pig (*Sus scrofa domestica*) for the *E.*

173   *polecki* was used. For sequencing, all amplicons from species-specific PCRs were used and

174   amplicons from the genus-specific PCR; the PCR products were purified using the Qiagen

175   extraction gel kit. Each sample was bi-directionally sequenced (using primers from second

176   round of semi-nested PCR and species specific primers for confirmation) and used for

177   phylogenetic analyses (see below).

178

179   *Phylogenetic and statistical analyses*

180   A data set consisting of 193 SSU rRNA gene sequences of the genus *Entamoeba* was

181   created, including the sequences obtained in the present study. The sequences using the

182   MAFFT method (Katoh et al., 2002) were aligned with the help of the MAFFT 7 server

183   (http://mafft.cbrc.jp/alignment/server/) with the G-INS-i algorithm at default settings. The

184   alignment was manually edited in BioEdit 7.0.4.1. The final data set contained 585 aligned

185 characters and is available from the corresponding author upon request. Phylogenetic trees

186 were constructed by maximum likelihood and Bayesian methods. Maximum likelihood

187 analysis was performed in RAxML 7.0.3 (Stamatakis, 2006) under the GTRGAMMAI model.

188 Bootstrap support values were generated in RAxML from 1000 pseudoreplicate data sets.

189 Bayesian analysis was carried out using MrBayes 3.2 (Ronquist et al., 2012) under the GTR +

190 I + Γ + covarion model. Four MCMCs were run for two million generations until the average

191 standard deviation of split frequencies based on last 75 % of generations was lower than 0.01.

192 The trees were sampled every 500th generation. The first 25 % of trees were removed as

193 burn-in. The McNemar test was performed in GraphPad (http://graphpad.com/) to compare

194 the sensitivity of *Entamoeba* spp. detection using either the coproscopic or PCR method.

195 *2.6. Prediction of the secondary structure of the SSU rRNA molecule*

196   A secondary structure of the SSU rRNA of *Entamoeba histolytica* was obtained as inferred

197 from   GenBank   sequence   X65163   from   Comparative   RNA   Web   Site

198 (www.rna.icmb.utexas.edu). The conservative elements of the secondary structure of other

199 *Entamoeba* sequences were identified manually by inspecting the alignment used for the

200 phylogenetic analysis (see above).

201

202 **3. Results**

203 *3.1. PCR and coproscopy based prevalence of* Entamoeba *spp.*

204   The multinuclear thick-walled cysts of *Entamoeba* spp. were coproscopically detected in

205 three   out   of   33   faecal   samples   (9%).   The   cysts   of   complex

206 *E. histolytica/dispar/moshkovskii/nuttalli* and *E. coli* were identified. The prevalence of

207 *Entamoeba* species using molecular tools in the same dataset of samples was higher and

208 reached 58% (19/33). The PCR method was significantly more sensitive for the detection of

209 *Entamoeba* spp. than the MIFC technique (McNemar: chi = 14.1; d.f. = 1; p=0.0002).

210   Using genus-specific PCR targeting the SSU r DNA, 72 samples positive for *Entamoeba*

211 spp. (72/107; 67%) was identified. Species-specific PCR assays revealed the presence of two

212 *Entamoeba* species, namely 33 samples positive for *E. coli* (33/107; 31%), and 10 samples

213 positive for *E. dispar* (10/107; 9%). Neither the pathogenic *Entamoeba* species such as *E.*

214 *histolytica* or *E. nuttalli* nor commensal *E. moshkovskii* or *E. polecki* ST2 were detected in

215 any sample. However, 36 samples that were positive for *Entamoeba* spp. using genus specific

216 primers remained negative in all six protocols of species-specific PCRs. The amplicons were

217 sequenced and BLAST was used to identify similar sequences in GenBank. All sequences

218 obtained were highly similar to *Entamoeba hartmanni* (98% similarity). Finally, all samples

219 that were positive using the genus-specific PCR were sequenced to prevent misdiagnosis of *E.*

220 *hartmanni*. *E. hartmanni* was documented in 34 samples (34/107; 32%). For details see the

221 Supplementary table S1.

222   The combination of genus- and species/group-specific PCR revealed co-infections of two

223 or three species of entamoebas: *E. hartmanni* /*E. coli* (10 samples), *E. hartmanni* /*E. dispar*

224 (1 sample), and *E. hartmanni* /*E. coli* /*E. dispar* (1 sample); for details see the Supplementary

225 table S1.

226

227 *3.2. Molecular identification of* Entamoeba *spp.*

228   The newly determined SSU rRNA sequences were subjected to BLAST search against the

229 GenBank nr/nt database (megablast with default parameters). Twenty samples (for details see

230 the Supplementary table S1) contained identical SSU rRNA gene sequences, which shared

231 96% to 98% similarity with all GenBank sequences of *E. hartmanni*, for accession numbers

232　see Fig. 2. SSU rRNA gene sequences of two further samples (T2041 and T3766) were

233　identical and shared 98% to 99% similarity with sequences of *E. coli* subtype 2 (see Stensvold

234　et al., 2011). The sequence of T2003 differed in a single nucleotide from sequences of T2041

235　and T3766. The sequence of T3403 shared 99% similarity with sequences of *E. dispar*, *E.*

236　*nuttalli* and sequences AB197936 and AB426549 of *E. histolytica*. To confirm phylogenetic

237　position of the obtained *Entamoeba* samples, a phylogenetic analysis of the genus *Entamoeba*

238　including the new sequences was performed. Topology of the resulting phylogenetic tree was

239　consistent with results from BLAST searches (Figure 2), with a few notable exceptions.

240　In the resulting tree, sequence T3403 appeared closely related to *E. dispar* sequences

241　Z49256 (isolate from a human) and AB282661 (isolate from a rhesus monkey). The third

242　GenBank sequence labelled as *E. dispar*, EF204917, occupied a different position within

243　*E. histolytica*/*E. nuttalli*/*E. dispar* complex suggesting a possibility that it has been originally

244　misidentified and belongs, in fact, to *E. histolytica*. To confirm the identity of T3403, the

245　secondary structure of its SSU rRNA gene was examined and two features distinguishing *E.*

246　*dispar* from the closely related *E. histolytica* (including the sequence EF204917) and *E.*

247　*nuttalli* were identified: (i) A:G (instead of G:A) base pair within the stem of helix 10

248　(positions 181 and 198 in the sequence Z49256; for terminology of conservative elements see

249　Wuyts et al., 2001), and (ii) GTAAG motif within helix E10_1 (positions 211–215 in the

250　sequence Z49256).

251　Because our sequences affiliated with *E. hartmanni* were relatively divergent SSU rRNA

252　gene sequences, the secondary structure of the corresponding SSU rRNA molecule was

253　examined as well. They possessed a unique motif ACT in the loop in helix 17, which

254　contrasted with all other *E. hartmanni* sequences, (including those obtained from NHP)

255　having GTAA in the corresponding area (positions 438–441 in the sequence FR686371).

256

4. Discussion

The diversity of amoebas infecting great apes is poorly understood, despite the fact that their cysts are commonly reported in general parasitological studies on free ranging and captive NHP (e.g. Gillespie et al., 2010; Howells et al., 2011). Most of these studies suffer from methodological challenges in identification of amoebas to the species/lineage level. In the present study, we investigated the molecular diversity of amoebas of the genus *Entamoeba* in a community of wild eastern chimpanzees in Issa Valley, Tanzania.

Comparing the results of "classic" microscopy with PCR, we clearly showed the limitations of microscopic detection. The microscopy failed to detect the cysts of entamoebas in 16 out of 19 PCR positive samples. Low sensitivity of microscopy/MIF corresponds well with previous data in Kalousová et al. (2014) who reported only 6.7 % prevalence of *Entamoeba* spp. in Issa chimpanzees.

Using species specific diagnostic PCR assays, we identified three *Entamoeba* species, namely *E. dispar*, *E. coli*, and a new sequence variant of *E. hartmanni*. Among the *Entamoeba* species, those with tetranucleated cysts deserve more attention, as this group includes also pathogenic *E. histolytica* and *E. nuttalli*. Previous microscopy-based studies detecting the tetranucleated cysts have presumed occurrence of pathogenic entamoebas in chimpanzees (e.g. Sleeman et al., 2000; Lilly et al. 2002; Gillespie et al., 2010), implying possible cross-transmission between humans and PHP. Our data demonstrate that the presence of tetranucleated cysts does not necessarily mean the presence of pathogenic amoebas. Tetranucleated cysts could rather represent the commensal amoebaes such as *E. dispar* in case of our sample set.

Our phylogenetic analysis showed that the sequence of *E. dispar* is closely related to isolates from Nepalese rhesus macaques (*Macaca mulatta*) (AB282661) and from humans

281   (Z49256; the clade of *E. dispar* isolates has 93% bootstrap support). Our *E. coli* sequence

282   clustered with human-derived isolates and with one sequence from a captive western lowland

283   gorilla (*Gorilla gorilla gorilla*). Moreover, the sequence fell into *E. coli* ST2 group, which has

284   been identified from captive NHP and from humans who have recently travelled in tropical

285   Africa or Asia (Stensvold et al., 2011). Based on present analyses, one can only speculate if *E.*

286   *dispar* and *E. coli* ST2 found in Issa chimpanzees occur naturally in the Issa community or

287   originate from humans. Given Issa chimpanzees do not regularly encounter humans aside

288   from researchers; it is likely that both *E. dispar* and *E. coli* naturally occur in these apes.

289   Future studies targeting the presence of amoebas in other Issa primate species can help to

290   uncover the epidemiology of non-pathogenic amoeba infections.

291   We did not include the species-specific PCR for *E. hartmanni* into our protocol because we

292   did not record the tetra-nucleated cysts typical for *E. hartmanni* in our previous study (for

293   more details see Kalousová et al., 2014). Spherical cysts of *E. hartmanni* are smaller, approx.

294   5–10 μm, while the cysts of *E. histolytica*-complex are more than 10 μm in diameter (Ash and

295   Orihel, 2007). However, using genus-specific PCR followed by sequencing, we identified

296   many samples that were positive for *E. hartmanni* using genus-specific PCR followed by

297   sequencing. Our sequences branched within the well supported lineage of *E. hartmanni,* that

298   included isolates from humans (FR686374–79; AF149907) and captive NHP such as the

299   barbary macaque (*Macaca sylvanus*) (FR686369, FR686372), patas monkey (*Erythrocebus*

300   *patas*) (FR686373), woolly monkey (*Lagothrix lagotricha*) (FR686366, FR686368), vervet

301   monkey (*Chlorocebus pygerythrus*) (FR686373), and Bornean orangutan (*Pongo pygmaeus*)

302   (FR686370). However, based on the SSU rRNA secondary structure, our sequence represents

303   a novel sequence variant, different from other sequences of this species, as well as those

304   obtained from NHP (see Stensvold et al., 2011). It is currently not possible to provide further

305   details regarding the morphology and biology of this novel variant, because we have not

306   obtained trophozoites, which are necessary for final identification. Nevertheless, it is likely

307   that *E. hartmanni* is non-pathogenic for chimpanzees similar to *E. hartmanni* in humans (Sard

308   et al., 2011).

309   We have demonstrated that wild chimpanzees that do not live in close proximity to a large

310   human population nonetheless harbour several *Entamoeba* species closely related to those

311   occurring in humans. We found the microscopic detection to be unreliable for diagnostics of

312   amoebas, due to low sensitivity and inability to distinguish between pathogenic and non-

313   pathogenic species with similar cyst morphology.   In summary, molecular-phylogenetic

314   methods are fundamental for improving diagnostics of *Entamoeba* spp. in wild NHP and for

315   understanding the epidemiology and zoonotic transmission of these parasites.

316

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336   **References**

337   **Ash, L. R., Orihel, T. C.** (2007). *Atlas of Human Parasitology*, 5th Edn. American Society

338   for Clinical Parasitology, Singapore.

339   **Ashford, R. W., Reid, G. D. F., Wrangham, R. W.** (2000). Intestinal parasites of the

340   chimpanzee, *Pan troglodytes*, in Kibale Forest, Uganda. *Annals of Tropical Medicine and*

341   *Parasitology* **94**, 173–179.

342   **Blagg, W., Schloegel, E. L., Mansour, N. S., Khalaf, G. I.** (1955). A new concentration

343   technic for the demonstration of protozoa and helminth eggs in feces. *American Society of*

344   *Tropical Medicine and Hygiene* **4**, 23–28.

345   **Chapman, C. A., Lambert, J. E.** (2000). Habitat alteration and the conservation of African

346   primates: Case study of Kibale National Park, Uganda. *American Journal of Primatology* **50**,

347   169-185.

348   **Gillespie, T. R., Lonsdorf, E. V., Canfield, E. P., Meyer, D. J., Nadler, Y., Raphael, J.,**

349   **Pusey, A. E., Pond, J., Pauley, J., Mlengeya, T., Travis, D. A.** (2010). Demographic and

350   ecological effects on patterns of parasitism in eastern chimpanzees (*Pan troglodytes*

351   *schweinfurthii*) in Gombe National Park, Tanzania. *American Journal of Physical*

352   *Anthropolgy* **143**, 534–544.

353 **Hamzah, Z., Petmitr, S., Mungthin, M., Leelayoova, S., Chavalitshewinkoon-Petmitr, P.**

354 (2006). Differential detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba*

355 *moshkovskii* by a single-round PCR assay. *Journal of Clinical Microbiology* **44**, 3196–3200.

356 **Hernandez-Aguilar, R. A., Moore, J., Stanford, C. B.** (2013). Chimpanzee nesting patterns

357 in savanna habitat: Environmental influences and preferences. *American Journal of*

358 *Primatology* **75**, 979–994.

359 **Howells, M. E., Pruetz, J., Gillespie, T. R.** (2011). Patterns of gastro-intestinal parasites and

360 commensals as an index of population and ecosystem health: The case of sympatric western

361 chimpanzees (*Pan troglodytes verus*) and guinea baboons (*Papio hamadryas papio*) at

362 Fongoli, Senegal. *American Journal of Primatology* **73**, 173–179.

363 **Kalousová, B., Piel, A. K., Pomajbíková, K., Modrý, D., Stewart, F. A., Petrželková, K.**

364 **J.** (2014). Gastrointestinal parasites of savanna chimpanzees (*Pan troglodytes schweinfurthii*)

365 in Ugalla, Tanzania. *International Journal of Primatology* **35**, 463–475.

366 **Katoh, K., Misawa, K., Kuma, K., Miyata, T.** (2002). MAFFT: a novel method for rapid

367 multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**,

368 3059–3066.

369 **Kebede, A., Verweij, J. J., Petros, B., Polderman, A. M.** (2004). Short communication:

370 Misleading microscopy in amoebiasis. *Tropical Medicine & International Health* **9**, 651–652.

371 **Kooriyama, T., Hasegawa, H., Shimozuru, M., Tsubota, T., Nishida, T., Iwaki, T.** (2012).

372 Parasitology of five primates in Mahale Mountains National Park, Tanzania. *Primates* **53**,

373 365–375.

374 **Levecke, B., Dreesen, L., Dorny, P., Verweij, J. J., Vercammen, F., Casaert, S.,**

375 **Vercruysse, J., Geldhof, P.** (2010). Molecular identification of *Entamoeba* spp. in captive

376 nonhuman primates. *Journal of Clinical Microbiology* **48**, 2988–2990.

377 **Levecke, B., Dorny, P., Vercammen, F., Visser, L.G., Van Esbroeck, M., Vercruysse, J.,**

378 **Verweij J.J.** (2015). Transmission of *Entamoeba nuttalli* and *Trichuris trichiura* from

379 nonhuman primates to humans. *Emerging Infectious Diseases* **21**, 1871-1872.

380 **Lilly, A. A., Mehlman, P. T., Doran, D.** (2002). Intestinal parasites in gorillas, chimpanzees,

381 and humans at Mondika research site, Dzanga-Ndoki National Park, Central African

382 Republic. *International Journal of Primatology* **23**, 555–573.

383 **Moore, J.** (1994). Plants of the Tongwe East Forest Reserve (Ugalla), Tanzania. *Tropics* **3**,

384 333–340.

385 **Muehlenbein, M. P.** (2005). Parasitological analyses of the male chimpanzees (*Pan*

386 *troglodytes schweinfurthii*) at Ngogo, Kibale National Park, Uganda. *American Journal of*

387 *Primatology* **65**, 167–179.

388 **Pedersen, A. B., Altizer, S., Poss, M., Cunningham, A. A.,Nunn, C. L.** (2005). Patterns of

389 host specificity and transmission among parasites of wild primates. *International Journal For*

390 *Parasitology* **35**, 647-657.

391 **Petrášová, J., Uzlíková, M., Kostka, M., Petrželková, K. J., Huffman, M. A., Modrý, D.**

392 (2011). Diversity and host specificity of *Blastocystis* in syntopic primates on Rubondo Island,

393 Tanzania. *International Journal For Parasitology* **41**, 1113–1120.

394 **Piel, A. K., Lenoel, A., Johnson, C., Stewart, F.** (2015). Deterring poaching in western

395 Tanzania: The presence of wildlife researchers. *Global Ecology and Conservation* **15**, 188–

396 199.

397   **Regan, C. S., Yon, L., Hossain, M., Elsheikha, H. M.** (2014). Prevalence of *Entamoeba*

398   species in captive primates in zoological gardens in the UK. *Peer J* **2**, e492.

399   **Rivera, W. L., Yason, J., Adao, D. E. V**. (2010). *Entamoeba histolytica* and *E. dispar*

400   infections in captive macaques (*Macaca fascicularis*) in the Philippines. *Primates* **51**, 69–74.

401   **Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Hohna, S.,**

402   **Larget, B., Liu, L., Suchard, M. A., Huelsenbeck, J. P.** (2012). MrBayes 3.2: Efficient

403   Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. *Systematic*

404   *Biology* **61**, 539–542.

405   **Rudicell, R. S., Piel, A. K., Stewart, F., Moore, D. L., Learn, G. H., Li, Y., Takehisa, J.,**

406   **Pintea, L., Shaw, G. M., Moore, J., Sharp, P. M., Hahn, B. H.** (2011). High prevalence of

407   Simian Immunodeficiency Virus Infection in a community of savanna chimpanzees. *Journal*

408   *of Virology* **85**, 9918–9928.

409   **Sak, B., Petrželková, K. J., Květoňová, D., Mynarová, A., Shutt, K. A., Pomajbíková, K.,**

410   **Kalousová, B., Modrý, D., Benavides, J., Todd, A., Kváč, M.** (2013). Long-term

411   monitoring of microsporidia, *Cryptosporidium* and *Giardia* infections in western lowland

412   gorillas (*Gorilla gorilla gorilla*) at different stages of habituation in Dzanga Sangha Protected

413   Areas, Central African Republic. *Plos One* **8**, e71840.

414   **Sak, B., Petrželková, K. J., Květoňová, D., Mynářová, A., Pomajbíková, K., Kalousová,**

415   **B., Modrý, D., Cranfield, M. R., Kváč, M.** (2014). Microsporidia, *Cryptosporidium* and

416   *Giardia* infections in mountain gorillas (*Gorilla beringei beringei*) in National Park Virunga

417   Volcanoes, Rwanda. *Plos One* **9**, e109751.

418   **Sard, B. G., Navarro, R. T. , Sanchis, J. G. E.** (2011). Non-pathogenic intestinal amoebae: a

419   clinical-analytical overview. *Enfermedades Infecciosas y Microbiología Clínica* **29**, 20-28.

420   **Sleeman, J. M., Meader, L. L., Mudakikwa, A. B., Foster, J. W., Patton, S.** (2000).

421   Gastrointestinal parasites of mountain gorillas (*Gorilla gorilla beringei*) in the Parc National

422   des Volcans, Rwanda. *Journal of Zoo and Wildlife Medicine* **31**, 322–328.

423   **Stamatakis, A.** (2006). RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses

424   with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688–2690.

425   **Stensvold, C. R., Lebbad, M., Verweij, J. J., Jespersgaard, C., von Samson-**

426   **Himmelstjerna, G., Nielsen, S. S., Nielsen, H. V.** (2010). Identification and delineation of

427   members of the *Entamoeba* complex by pyrosequencing. *Molecular and Cellular Probes* **24**,

428   403–406.

429   **Stensvold, C. R., Lebbad, M., Victory, E. L., Verweij, J. J., Tannich, E., Alfellani, M.,**

430   **Legarraga, P., Clark, C. G.** (2011). Increased sampling reveals novel lineages of

431   *Entamoeba*: Consequences of genetic diversity and host specificity for taxonomy and

432   molecular detection. *Protist* **162**, 525–541.

433   **Suzuki, J., Kobayashi, S., Murata, R., Tajima, H., Hashizaki, F., Yanagawa, Y.,**

434   **Takeuchi, T.** (2008). A survey of amoebic infections and differentiation of an *Entamoeba*

435   *histolytica*-like variant (JSK2004) in nonhuman primates by a multiplex polymerase chain

436   reaction. *Journal of Zoo and Wildlife Medicine* **39**, 370–379.

437   **Tachibana, H., Cheng, X. J., Kobayashi, S., Fujita, Y., Udono, T.** (2000). *Entamoeba*

438   *dispar*, but not *E. histolytica*, detected in a colony of chimpanzees in Japan. *Parasitology*

439   *Research* **86**, 537–541.

440   **Tachibana, H., Yanagi, T., Pandey, K., Cheng, X. J., Kobayashi, S., Sherchand, J. B.,**

441   **Kanbara, H.** (2007). An *Entamoeba* sp. strain isolated from rhesus monkey is virulent but

442   genetically different from *Entamoeba histolytica*. *Molecular and Biochemical Parasitology*

443   **153**, 107–114.

444   **Tachibana, H., Yanagi, T., Akatsuka, A., Kobayashi, S., Kanbara, H., Tsutsumi, V.**

445   (2009). Isolation and characterization of a potentially virulent species *Entamoeba nuttalli*

446   from captive Japanese macaques. *Parasitology* **136**, 1169–1177.

447   **Tachibana, H., Yanagi, T., Feng, M., Bandara, K.B., Kobayashi, S., Cheng X.,**

448   **Hirayama, K., Rajapakse, R. P.** (2015). Isolation and molecular characterization of

449   *Entamoeba nuttalli* strains showing novel isoenzyme patterns from wild toque macaques in

450   Sri Lanka. *Journal of Eukaryotic Microbiology*, ahead of print

451   **Verweij, J. J., Polderman, A. M., Clark, C. G.** (2001). Genetic variation among human

452   isolates of uninucleated cyst-producing *Entamoeba* species. *Journal of Clinical Microbiology*

453   **39**, 1644–1646.

454   **Verweij, J. J., Vermeer, J., Brienen, E. A. T., Blotkamp, C., Laeijendecker, D., van**

455   **Lieshout, L., Polderman, A. M.** (2003). *Entamoeba histolytica* infections in captive

456   primates. *Parasitology Research* **90**, 100–103.

457   **Visser, L. G., Verweij, J. J., Van Esbroeck, M., Edeling, W. M., Clerinx, J., Polderman,**

458   **A. M.** (2006). Diagnostic methods for differentiation of *Entamoeba histolytica* and

459   *Entamoeba dispar* in carriers: performance and clinical implications in a non-endemic setting.

460   *International Journal of Medical Microbiology* **296**, 397–403.

461   **Wuyts, J., Van de Peer, Y., Wachter, R. D.** (2001). Distribution of substitution rates and

462   location of insertion sites in the tertiary structure of ribosomal RNA. *Nucleic Acids Research*

463   **29**, 5017–5028.

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466

467   Figure legends:

468   Figure 1. The products of *Entamoeba* genus-specific PCR and several *Entamoeba* species-

469   specific PCRs conducted on the samples used as positive controls (note: only for

470   demonstration of the feasibility of the species-specific PCRs): (A) marker, (B) *Entamoeba*-

471   genus specific PCR from culture of *Entamoeba* invades, (C) *Entamoeba dispar* (~195 bp), (D)

472   *Entamoeba coli* (~290 bp), (E) *Entamoeba histolytica* (~475 bp), (F) *Entamoeba moshkovskii*

473   (~600 bp), (G) *Entamoeba polecki* ST2 (~680 bp), (H) *Entamoeba nuttalli* (~848 bp).

474

475   Figure 2. Unrooted phylogenetic tree of the genus *Entamoeba* based on partial SSU rRNA

476   gene sequences. The tree was constructed by the maximum likelihood method

477   (GTRGAMMAI model). The values at the branches represent statistical support in maximum

478   likelihood bootstrap values/Bayesian posterior probabilities. Support values below 50%/.50

479   are not showed or are represented by an asterisk (*). New sequences are in bold.

Table 1. List of primers used in diagnostic PCR for detection of *Entamoeba* genus and in species-specific PCRs; univ – universal, fwd – forward, rev – reverse, SN-PCR – semi-nested PCR, SS-PCR – species/group-specific PCR.

| Primer name	Primer characterization	Primer sequence (5'--3')	Reference
Entam1	univ. fwd for SN-PCR (1.,2.rnd)	GTTGATCCTGCCAGTATTATATG	Verweij et al (2001)
Entam2	univ. rev. for SN-PCR (2.rnd)	CACTATTGGAGCTGGAATTAC	Verweij et al (2001)
Entam5	univ. rev. for SN-PCR (1.rnd)	CRACTACGAGCKTTTTAAWCAC	our designed
EnthF	fwd *E. histolytica* for SS-PCR	ATGGCCAATTCATTCAATGA	Suzuki et al. (2008)
EnthR	rev *E. histolytica* for SS-PCR	TACTTACATAAAGTCTTCAAAATGT	Suzuki et al. (2008)
EntnF	fwd *E. nuttalli* for SS-PCR	ATTTTATACATTTTGAAGACTTTGCA	Suzuki et al. (2008)
EntnR	rev *E. nuttalli* for SS-PCR	CTCTAACCGAAATTAGATAACTAC	Suzuki et al. (2008)
EntdF	fwd *E. dispar* for SS-PCR	GTTAGTTATCTAATTTCGATTAGAAC	Suzuki et al. (2008)
EntdR	rev *E. dispar* for SS-PCR	ACACCACTTACTATCCCTACCTA	Suzuki et al. (2008)
EntaF	fwd *E. moshkovskii* for SS-PCR	ATGCACGAGAGCGAAAGCAT	Hamzah et al. (2006)
EmR	rev *E. moshkovskii* for SS-PCR	TGACCGGAGCCAGAGACAT	Hamzah et al. (2006)
Entcoli_100F	rev *E. coli* for SS-PCR	GAAGCTGCGAACGGCTCATTAC	Stensvold et al. (2011)
Entcoli_390R	fwd *E. coli* for SS-PCR	CACCTTGGTAAGCCACTACC	Stensvold et al. (2011)
EpolF	rev *E. polecki* for SS-PCR	GGAAGGCTCATTATAACAGTTATAG	newly designed
EpolR	fwd *E. polecki* for SS-PCR	CCTCATTATTATCCTATGCTTC	newly designed
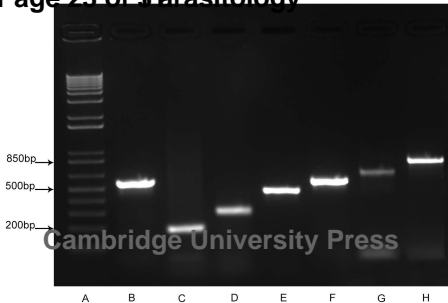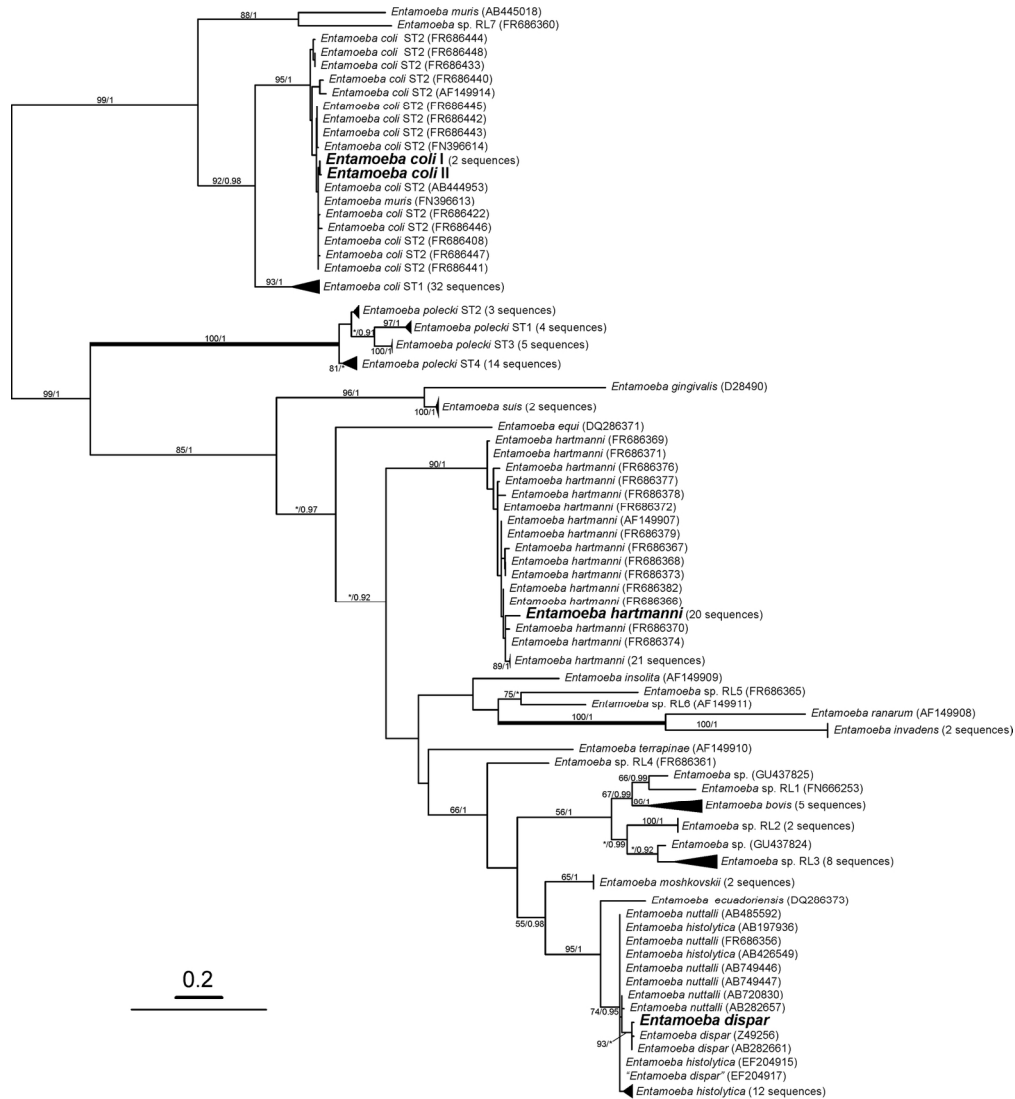
Unrooted phylogenetic tree of the genus Entamoeba based on partial SSU rRNA gene sequences. The tree was constructed by the maximum likelihood method (GTRGAMMAI model). The values at the branches represent statistical support in maximum likelihood bootstrap values/Bayesian posterior probabilities. Support values below 50%/.50 are not showed or are represented by an asterisk (*). New sequences are in bold.
156x170mm (300 x 300 DPI)

Supplementary table S1. Summary of all molecularly analyzed fecal samples from Issa chimpanzees together with overview of their molecular positivity (for *Entamoeba* spp., *E. hartmanni*, *E. dispar*, and *E. coli*) and the positivity based on the microscopy examination following MIF technique (performed only in 33 samples).

Evidence No	*Entamoeba* spp.	*E. hartmanni*	*E. dispar*	*E.coli*	positivity in microscopy
T1991/09	+	+	neg	neg	+
T1992/09	+	neg	neg	+	+
T1993/09	neg	neg	neg	neg	neg
T1994/09	neg	neg	neg	neg	neg
T1995/09	+	+	+	neg	neg
T2003/09	+	neg	neg	+	+
T2004/09	neg	neg	neg	neg	neg
T2005/09	neg	neg	neg	neg	neg
T2006/09	+	+	neg	neg	neg
T2011/09	+	neg	neg	neg	neg
T2037/09	+	neg	+	neg	neg
T2038/09	neg	neg	neg	neg	neg
T2039/09	+	+	neg	neg	neg
T2040/09	neg	neg	neg	neg	neg
T2041/09	+	neg	neg	+	neg

T2043/09	neg	neg	neg	neg	neg
T2044/09	neg	neg	neg	neg	neg
T2045/09	neg	neg	neg	neg	neg
T2057/09	neg	neg	neg	neg	neg
T2058/09	+	neg	neg	+	neg
T2059/09	+	neg	neg	+	neg
T2060/09	neg	neg	neg	neg	neg
T2061/09	+	neg	neg	+	neg
T2071/09	+	+	neg	neg	neg
T2072/09	neg	neg	neg	neg	neg
T2073/09	+	+	neg	neg	neg
T2074/09	+	neg	neg	+	neg
T2075/09	+	neg	neg	+	neg
T2076/09	neg	neg	neg	neg	neg
T2077/09	+	+	neg	+	neg
T2081/09	neg	neg	neg	neg	neg
T2082/09	+	+	neg	neg	neg
T2083/09	+	+	+	neg	neg
T3386/12	+	+	neg	neg	NA
T3387/12	neg	neg	neg	neg	NA
T3388/12	neg	neg	neg	neg	NA

T3389/12	neg	neg	neg	neg	NA
T3390/12	+	+	neg	neg	NA
T3391/12	neg	neg	neg	neg	NA
T3392/12	+	neg	+	+	NA
T3393/12	+	+	neg	neg	NA
T3394/12	neg	neg	+	neg	NA
T3395/12	+	neg	neg	+	NA
T3396/12	neg	neg	neg	neg	NA
T3397/12	neg	neg	neg	neg	NA
T3398/12	neg	neg	neg	neg	NA
T3399/12	neg	neg	neg	neg	NA
T3400/12	+	neg	+	neg	NA
T3401/12	neg	neg	neg	neg	NA
T3402/12	+	+	neg	neg	NA
T3403/12	+	neg	+	neg	NA
T3404/12	+	+	neg	neg	NA
T3405/12	+	+	neg	neg	NA
T3406/12	+	neg	neg	+	NA
T3407/12	+	neg	+	neg	NA
T3409/12	+	neg	neg	+	NA
T3410/12	+	neg	+	neg	NA

T3411/12	+	+	neg	neg	NA
T3412/12	+	neg	neg	+	NA
T3413/12	+	neg	+	neg	NA
T3414/12	+	+	neg	neg	NA
T3415/12	+	neg	neg	neg	NA
T3416/12	neg	neg	neg	neg	NA
T3417/12	+	neg	neg	neg	NA
T3418/12	+	neg	neg	neg	NA
T3419/12	+	+	neg	+	NA
T3738/12	+	neg	neg	+	NA
T3739/12	+	neg	neg	+	NA
T3740/12	+	+	neg	neg	NA
T3741/12	+	neg	neg	+	NA
T3742/12	+	neg	neg	+	NA
T3743/12	+	neg	neg	+	NA
T3744/12	neg	neg	neg	neg	NA
T3745/12	+	neg	neg	neg	NA
T3746/12	+	neg	neg	neg	NA
T3747/12	+	+	neg	+	NA
T3748/12	+	neg	neg	+	NA
T3749/12	neg	neg	neg	neg	NA

T3750/12	+	+	neg	neg	NA
T3751/12	+	+	neg	neg	NA
T3752/12	+	+	neg	+	NA
T3753/12	+	+	neg	neg	NA
T3754/12	neg	neg	neg	neg	NA
T3755/12	+	+	neg	+	NA
T3756/12	neg	neg	neg	neg	NA
T3757/12	+	+	neg	+	NA
T3758/12	+	+	neg	neg	NA
T3759/12	neg	neg	neg	neg	NA
T3760/12	+	neg	neg	+	NA
T3761/12	neg	neg	neg	neg	NA
T3762/12	+	+	neg	+	NA
T3763/12	+	neg	neg	neg	NA
T3764/12	+	+	neg	+	NA
T3765/12	+	+	neg	+	NA
T3766/12	+	neg	neg	+	NA
T3767/12	+	neg	neg	+	NA
T3768/12	+	+	neg	neg	NA
T3769/12	neg	neg	neg	neg	NA
T3770/12	+	+	neg	+	NA

T3771/12	neg	neg	neg	neg	NA
T3772/12	+	neg	neg	neg	NA
T3773/12	+	neg	neg	neg	NA
T3774/12	+	+	neg	neg	NA
T3775/12	+	neg	neg	+	NA
T3776/12	+	+	neg	neg	NA
T3777/12	neg	neg	neg	neg	NA