

1 **Review**

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3 Novel *Entamoeba* findings in non-human primates

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6 Hany M. Elsheikha,¹ Carl S. Regan,^{1,2} and C. Graham Clark^{3,*}

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10 ¹ Faculty of Medicine and Health Sciences, School of Veterinary Medicine and Science,
11 University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK

12

13 ² Current address: Vets4pets Dover Whitfield, White Cliffs Retail Park, Whitfield, Dover,
14 CT16 3PS, UK

15

16 ³ Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical
17 Medicine, London, UK

18

19 *Correspondence:

20 graham.clark@lshtm.ac.uk

21 (C. Graham Clark)

22

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24 **Abstract**

25

26 In addition to well-known human-infecting species, *Entamoeba* species not found in humans
27 have been identified recently in non-human primates (NHPs). Importantly, it has become clear
28 that the organism identified as *Entamoeba histolytica* in NHPs is usually a distinct species,
29 *Entamoeba nuttalli*. Many DNA-based stool surveys use species-specific detection methods
30 and so may miss the full range of *Entamoeba* species present. In addition, authors may be using
31 the same species name to describe distinct organisms. These various shortcomings may not be
32 obvious to readers. In this review, we clarify the relationships between *Entamoeba* species'
33 names based on morphological and molecular data, and highlight gaps in recently published
34 data on *Entamoeba* species in wild NHPs resulting from the use of variable methodology.

35

36 **Humans and NHPs are both primates, but how similar are their *Entamoeba* species?**

37

38 Humans are primates, and therefore it would be logical to assume that the parasite fauna of
39 humans and **non-human primates** (NHPs; see Glossary) is likely to be similar. However, this
40 simplistic view ignores the huge range of life-styles, diets and ecological specialisations
41 exhibited by NHPs, and the millions of years of independent evolution that separate us from
42 even our closest NHP relatives, the great apes. Nevertheless, humans and NHPs do appear to
43 have many parasites in common, at least when identified via microscopy. Over recent decades,
44 molecular tools have allowed us to re-examine these similarities and to challenge the
45 assumption that apparent morphological identity equates to species identity. This review
46 discusses how molecular tools provide a clearer picture of the relationships between intestinal
47 amoebae of the genus *Entamoeba* in humans and NHPs and where gaps in our understanding
48 remain.

49

50 **What is causing invasive amoebiasis in humans and NHPs?**

51

52 The focus on *Entamoeba* is largely due to *Entamoeba histolytica* being a significant cause of
53 morbidity and mortality in humans. Published estimates suggest this organism is responsible
54 for millions of cases of disease and over 50,000 deaths in humans annually [1]. Although these
55 numbers are extrapolated from a limited number of studies, *E. histolytica* is certainly
56 responsible for a significant amount of disease in some locations. Captive NHPs occasionally
57 die from a disease that is, superficially, indistinguishable from that caused by *E. histolytica* in
58 humans (e.g. [2]). Several other *Entamoeba* species that resemble *E. histolytica*
59 morphologically have been described in both humans and NHPs, making microscopic
60 diagnosis problematic. Morphologically distinct, non-pathogenic species of *Entamoeba* also
61 appear to be shared by humans and NHPs, further complicating diagnosis (see below).

62

63 **The morphology era**

64

65 The existence of species of *Entamoeba* in humans and NHPs that appear identical by
66 microscopy has been known for over a century. At that time, organisms in new hosts were often
67 given new species names, whether morphologically distinguishable or not. A major work by
68 Dobell [3] concluded that all named intestinal species of *Entamoeba* in humans could be
69 assigned to either *E. histolytica* or *Entamoeba coli*, but he equivocated about *Entamoeba* from
70 NHPs on the grounds of insufficient data; he later concluded that intestinal *Entamoeba* species
71 in NHPs were also *E. histolytica* and *E. coli* [4]. His two-species nomenclature stayed
72 essentially intact for 35 years.

73

74 In the mid-1950s, Burrows [5] resurrected the name *Entamoeba hartmanni* for an organism
75 that parasitologists were referring to as ‘small race *E. histolytica*’. Dobell [3] had viewed *E.*
76 *hartmanni* as a synonym of *E. histolytica*; however, Burrows showed that the sizes of *E.*
77 *histolytica* ‘large race’ and ‘small race’ cysts were not a continuum but had a clear bimodal
78 distribution. This first ‘break’ with the Dobell nomenclature was quickly adopted, because
79 parasitologists were already primed to accept it.

80

81 **The molecular era**

82

83 *Entamoeba hartmanni* was the last change to Dobell’s nomenclature scheme based on
84 morphology alone. Additional changes followed but not for many years, as the changes were
85 primarily dependent on **small subunit ribosomal RNA gene** (SSU-rDNA) analyses. Emile
86 Brumpt [6] proposed the existence of *Entamoeba dispar*, a non-pathogenic species
87 morphologically identical to *E. histolytica*. This proposal was rejected by most parasitologists
88 at the time (see discussion following [7]) and the name *E. dispar* virtually disappeared from
89 the literature. Suspicion that Brumpt had been correct followed on from studies based on both
90 lectin agglutination [8] and **isoenzyme** patterns [9], in which two groups within *E. histolytica*
91 were identified, only one of which was found in patients with invasive disease. Subsequently,
92 studies (cited in [10]) using monoclonal antibodies, DNA hybridization, SSU-rDNA restriction
93 fragment length polymorphism, and eventually DNA sequencing all identified the same two
94 groups of strains, and this led to the formal redescription of *E. dispar* as a species distinct from
95 *E. histolytica* [10].

96

97 Other SSU-rDNA-based changes to the nomenclature of human *Entamoeba* species include
98 the reassignment of ‘*E. histolytica*-like’ amoebae to the species *Entamoeba moshkovskii* [11]

99 and the recognition that uninucleate cysts occasionally seen in humans were not always
100 immature *E. histolytica* but were in fact *Entamoeba polecki* [12]. Most recently, *Entamoeba*
101 *bangladeshi* was described as a new human species [13]; if it were not for SSU-rDNA
102 sequences this organism would have been identified as *E. moshkovskii* despite it being
103 genetically quite distinct.

104

105 The nomenclature for *Entamoeba* species in NHPs has followed suit, for the most part.
106 *Entamoeba hartmanni* is commonly found in NHPs. *Entamoeba dispar* is also widespread in
107 NHPs. *Entamoeba chattoni* had long been accepted as a NHP-specific species of *Entamoeba*
108 with uninucleate cysts. It was designated a **subtype** of *E. polecki* a few years ago [14], but this
109 change of nomenclature for *E. chattoni* has not been universally accepted; this will be discussed
110 further below.

111

112 Thus, for the most part, the NHP *Entamoeba* nomenclature changes simply mirrored those in
113 humans without any investigations to evaluate whether they were in fact the same organisms.
114 This was understandable initially because there was no reason to suspect there were differences
115 and the investigative tools were not readily available to many researchers. However, now that
116 molecular techniques are routine in most research laboratories and some diagnostic
117 laboratories, investigations into the diversity and identity of *Entamoeba* in NHPs have become
118 more common and are revealing some surprising and important findings.

119

120 The evidence for *Entamoeba* genetic diversity in NHPs is based almost exclusively on SSU-
121 rDNA analyses. Analyses of other markers are rarely possible because most studies use DNA
122 extracted directly from stool samples, but when available they show the same species
123 relationships. SSU-rDNA is a multicopy gene, which makes it relatively easy to amplify from

124 stool samples. In addition, and in contrast to some eukaryotes, the *Entamoeba* SSU-rDNA is
125 relatively fast evolving (as evidenced by long branches in phylogenetic trees) meaning that
126 sufficient resolution is obtained to allow differentiation of *Entamoeba* taxa using this gene
127 alone.

128

129 ***Entamoeba nuttalli***

130 *Entamoeba histolytica* causes disease of two main types: 1. amoebic dysentery/colitis, resulting
131 from trophozoite invasion of the colonic mucosa and leading to ulceration, bleeding and the
132 production of loose stool with blood and mucus; 2. amoebic liver abscess, resulting from
133 haematogenous spread of trophozoites from the colon via the portal system to the liver, where
134 tissue lysis leads to formation of a sterile pus-filled abscess [15]. Both types of disease have
135 been reported in NHPs, and there have been a number of reports over the years of spontaneous
136 invasive disease occurring in captive NHPs. Histologically, the diseases in humans and NHPs
137 appear identical, as do the amoebae under the microscope [e.g. 2]. *Entamoeba histolytica* of
138 human origin has been shown experimentally to be capable of infecting NHPs, where it can
139 cause indistinguishable pathology [e.g. 16]. The organism responsible was therefore presumed
140 to be *E. histolytica* in all cases of disease in NHPs.

141

142 In the last 10 years, however, molecular studies have been performed on amoebae from cases
143 of invasive amoebiasis occurring spontaneously in NHPs. The amoebae in NHPs are
144 consistently distinguishable from *E. histolytica* using a variety of DNA and protein markers:
145 isoenzymes, SSU-rDNA and short tandem-repeat-containing loci [17-20]. Although closely
146 related to *E. histolytica* – indeed it has been called “*E. histolytica*-like variant” [19] and “*E.*
147 *histolytica* NHP variant” [21] by some – this is clearly a distinct organism and the name *E.*
148 *nuttalli* has been revived for this amoeba [17]. *Entamoeba nuttalli* was originally described by

149 Castellani [22] in the liver abscess of a toque macaque (*Macaca sinica*) in Sri Lanka and is one
150 of the species considered synonymous with *E. histolytica* by Dobell [3, 23]. Although we
151 cannot prove after 110 years that the amoeba observed by Castellani is the same as the one now
152 being called *E. nuttalli*, this seems quite likely. A recent survey of wild toque macaques in Sri
153 Lanka detected asymptomatic carriage of *E. nuttalli* in 18.5% of the 227 animals studied [24].
154 *Entamoeba histolytica* was not detected in the population. *Entamoeba nuttalli* has been found
155 in a variety of other NHPs – guenon, baboon, colobus and chimpanzee – in addition to other
156 species of both captive and wild macaques [19, 25, 26].

157

158 The host and geographic ranges of *E. nuttalli* seem to be quite large, but so far it seems to be
159 found primarily in primates of the Old World. Invasive disease has been reported in captive
160 spider monkeys [25], but whether it infects wild New World NHPs is unknown. Only one
161 human infection with *E. nuttalli* has been reported to date, in a zookeeper [27]. This is despite
162 analyses of human samples that would have revealed its presence if it had been there.
163 Isoenzyme analysis, which was used widely for *Entamoeba* species differentiation in the 1980s
164 and early 1990s [e.g. 28], would have distinguished *E. nuttalli* from *E. histolytica* [17, 19], but
165 although many thousands of human samples were studied in order to differentiate *E. dispar*
166 and *E. histolytica*, no evidence of what is now being called *E. nuttalli* was reported. A second
167 human infection has apparently been identified in Iraq, based on a sequence in GenBank stated
168 to be of human origin [55; GenBank accession number: KP233837].

169

170 Note that most DNA-based diagnostic tools cannot distinguish *E. nuttalli* from *E. histolytica*,
171 unless combined with sequencing, and neither can some commercial antigen-based diagnostic
172 kits and monoclonal antibodies [17]. Therefore, although it seems unlikely that significant
173 numbers of humans will be found to be infected with *E. nuttalli*, such infections may occur

174 occasionally among those who have close contact with NHPs, and may go unrecognized
175 depending on the diagnostic method used. Primer pairs specific for *E. nuttalli* do now exist [17,
176 25] so that positive identification of this species without sequencing is possible.

177

178 NHPs can be infected experimentally with *E. histolytica* cysts of human origin [23, 29],
179 although no invasive disease has resulted from such experiments. Captive NHP infections
180 involving *E. histolytica* have been confirmed by DNA sequencing [30]. Therefore, it cannot be
181 ruled out that some natural *E. histolytica* infections will occur in wild NHPs – most likely
182 among those that come into contact regularly with humans or human waste – although there
183 are no sequence-confirmed infections to date [e.g. 56]. It is impossible retrospectively to know
184 which organism was responsible for the invasive amoebiasis cases in NHPs reported in the
185 literature. Indeed, it is not possible to be certain that the amoeba observed was responsible for
186 the disease in some cases – the presence of an *Entamoeba* and dysentery in the same host does
187 not necessarily imply cause and effect.

188

189 ***Entamoeba polecki***

190 *Entamoeba polecki* produces cysts with one nucleus, as does *E. chattoni*. Sequencing of their
191 SSU-rDNAs revealed them to be closely related organisms [31]. The former species is
192 traditionally associated with pigs and the latter with NHPs. Despite sporadic reports of *E.*
193 *polecki* infections in humans for many years [32], when uninucleated cysts were seen in
194 humans it was generally assumed that they represented immature cysts of *E. histolytica* rather
195 than of *E. polecki* or *E. chattoni*. Verweij et al. [12] studied human *Entamoeba* infections where
196 only uninucleated cysts were seen and found four distinct SSU-rDNA sequences. Two of these
197 sequences were essentially identical to those of *E. polecki* and *E. chattoni* isolated from a pig
198 and a monkey, respectively, while the other two sequences were related but distinct. This meant

199 that there were four closely-related organisms with two names between them and that *E.*
200 *polecki* and *E. chattoni* were not host-specific since all four organisms were found in humans.
201
202 Verweij et al. proposed [12] that the four should be viewed as variants of the same organism
203 and called ‘*E. polecki*-like’, as the name *E. polecki* has precedence. Later, Stensvold et al. [14]
204 proposed that they should be considered subtypes and numbered ST1-ST4, with the former *E.*
205 *polecki* becoming *E. polecki* ST1 and the former *E. chattoni* becoming *E. polecki* ST2. The
206 rationale for this approach is that there is no host specificity and no known difference except
207 for small amounts of sequence divergence. This subtype nomenclature has not been fully
208 accepted. One of the two ‘unnamed’ subtypes was in the interim named *Entamoeba struthionis*
209 [33] as it was isolated from an ostrich, but this subtype (ST3) has subsequently been found in
210 pigs [34] as well as humans. The fourth subtype has never had a species name and for a long
211 time was only known from humans, where it is the most common subtype. Recently, however,
212 ST4 was found to be the only *E. polecki* subtype in wild Celebes crested macaques (*Macaca*
213 *nigra*) [35], proving that *E. polecki* ST2 (*E. chattoni*) is not the only subtype found in NHPs.
214 It is possible that *E. polecki* ST1 and ST3 will also eventually be identified in NHP hosts. In
215 the absence of host-specificity, use of the ‘*E. polecki* subtype’ nomenclature seems appropriate.

216

217 ***Entamoeba dispar*, *Entamoeba hartmanni* and *Entamoeba coli***

218 For the most part, these three species meet the original expectation that human and NHP
219 *Entamoeba* species are the same. *Entamoeba dispar* is quite a homogeneous species and there
220 is no indication to date that *E. dispar* from humans is in any way distinct from that in NHPs.
221 Although *E. hartmanni* shows a greater degree of SSU-rDNA variation than *E. dispar*, there is
222 no obvious clustering of sequences that reflects human or NHP origin [14, 36], suggesting it is
223 a discrete species with moderate intraspecific variation.

224

225 The situation in *E. coli* is more complex and less clear-cut. *Entamoeba coli* samples from
226 humans group into two clusters, which have been named ST1 and ST2 [14]; ST1 appears to be
227 slightly more common than ST2 in humans. When NHP *E. coli* samples are examined, the
228 same two STs are identified, with ST2 being slightly more common, although this is based on
229 relatively few samples. ST2 was recently identified in wild mountain gorillas (*Gorilla beringei*)
230 [36]. The degree of divergence between the SSU-rDNAs of the two subtypes is substantial and
231 distinct species names could be justified. However, other than this sequence divergence, there
232 are no known differences between the two subtypes to date. *Entamoeba coli* cysts can vary
233 quite dramatically in size [37, 38]. Whether this size variation is a morphological reflection of
234 the underlying sequence divergence remains to be established.

235

236 Another *Entamoeba* that has been detected in NHPs is *Entamoeba* RL7 [14]. No species name
237 has been assigned to this organism – it is simply known by its **ribosomal lineage** (RL) number
238 [14]. *Entamoeba* RL7 was originally identified in a sample from a Phayre's leaf monkey
239 (*Trachypithecus phayrei*) [14], but it has subsequently been detected in humans in West Africa
240 [34]. Uniquely, this *Entamoeba* is most closely related to *Entamoeba muris* (Figure 1), which,
241 like *E. coli*, produces cysts with eight nuclei. Based on morphology, this organism previously
242 would have been reported as *E. coli*.

243

244 **NHP-restricted *Entamoeba* Species**

245

246 There are several NHP-restricted *Entamoeba* sequences worthy of discussion here. The first is
247 *Entamoeba* RL3, which to date has only been detected in langurs of various species and
248 produces cysts with a single nucleus. In the past it would likely have been reported as *E.*

249 *chattoni* based on microscopy. No infections with this organism have been reported in humans,
250 or indeed in any other NHP. It is closely related to, but distinct from, *Entamoeba bovis* and
251 related lineages that are confined to ungulates [14]. RL3 has only been found in a few samples
252 but it is notable that two lineages of *Entamoeba* (RL3 and RL7) have to date been detected
253 exclusively in langurs. Whether this is linked to their unusual foregut fermentative digestion is
254 unclear.

255

256 Villanueva-García et al. [39] recently reported SSU-rDNA sequences of an apparently novel
257 *Entamoeba* in two species of Howler monkey. Because these were only partial sequences they
258 were given a **conditional lineage** identifier [34] rather than a RL number. *Entamoeba* CL8 is
259 clearly distinct from previously sequenced *Entamoeba* SSU-rDNAs and, interestingly, the CL8
260 sequence branches within a cluster of sequences obtained from reptilian *Entamoeba* strains.
261 Villanueva-García et al. found a second *Entamoeba* sequence in their samples that is virtually
262 identical to *Entamoeba* RL6, which was originally described from the green iguana (*Iguana*
263 *iguana*) [14, 40]. The complete SSU-rDNA sequence of both these organisms would be helpful
264 in order to confirm their phylogenetic tree placement.

265

266 Finally, there has been one report of *Entamoeba suis* from a gorilla (*Gorilla gorilla*) [14], but
267 whether this is a natural host for this *Entamoeba* species remains to be established. This species
268 also produces cysts with a single nucleus.

269

270 **Missing *Entamoeba* Species?**

271

272 Perhaps surprisingly, there are to date no reports of *E. moshkovskii* from NHPs. This organism
273 is actually a species complex with substantial intra-specific sequence variation [40] and is

274 being reported from humans with increasing frequency now that PCR-based detection is being
275 employed [e.g. 41-43]. *Entamoeba moshkovskii* has also been detected in cattle, elephants [34],
276 reptiles [44] and insects [Silberman JD, personal communication], so it is likely only a matter
277 of time before it is also found in NHPs. Not all published molecular studies have tested for this
278 species and in those that did it is not clear whether the primers used would detect all variants
279 of this genetically diverse species complex. The most recently described *Entamoeba* of
280 humans, *E. bangladeshi* [13], is also yet to be reported from NHPs.

281

282 *Entamoeba gingivalis*, which colonises the gingival pockets in the mouth of humans, is listed
283 as having been found in NHPs [e.g. in 45]. No molecular data are available to know whether
284 the organisms reported in NHPs differ from those in humans. This may be important, as there
285 are at least two SSU-rDNA variants of *E. gingivalis* in humans [40] and additional diversity
286 could exist in other hosts.

287

288 A summary of the relationships between species names and identifiers can be found in Table
289 1 and an outline phylogenetic tree depicting the relationships between *Entamoeba* SSU-rDNA
290 sequences is depicted in Figure 1.

291

292 **Captive vs. Wild NHPs**

293

294 Data on the presence and prevalence of *Entamoeba* species in NHPs is patchy at best, and most
295 reports are based on animals in zoological parks. This is a problem when it comes to
296 interpreting the data. The first issue is how to interpret the presence of parasites in captive
297 NHPs. Animals in captivity may be exposed to organisms they would never encounter in the
298 wild. Therefore, the data only indicate that the NHP species is capable of becoming colonised

299 by the parasite identified, not that it is a natural host for this parasite. A second issue is the
300 impact of captivity on prevalence. It is likely that animals come in contact with faeces and
301 faecal contamination of food and water more frequently in captivity than they would in the
302 wild; this is especially true of species that are primarily or exclusively arboreal. Only by
303 studying wild NHPs can ‘natural’ infections be identified, although in the case of peri-urban
304 and urban NHPs the possibility of infection through contact with human faeces cannot be
305 excluded. It is, of course, also likely that wild NHPs will ingest faeces from other hosts,
306 accidentally or on purpose. If the ingested faeces contains *Entamoeba* cysts it is possible that
307 DNA of these organisms will be detected when the NHP faeces is screened by PCR. However,
308 unless the NHP species ingests faeces frequently and in significant amounts it would be
309 unlucky if the small amount of NHP faeces analysed contained detectable DNA of *Entamoeba*
310 cysts that were just passing through.

311

312 Relatively few studies of *Entamoeba* in wild NHPs have employed molecular diagnostics to
313 date, and microscopy does not differentiate most of the known *Entamoeba* species: only *E.*
314 *histolytica*, *E. coli* and *E. chattoni* are regularly reported in publications reliant on microscopy.
315 Each of these names actually represents a mixture of distinct organisms united only by the
316 number of nuclei in their mature cyst. *Entamoeba hartmanni* is the only additional species that
317 can be identified by morphology, but only if cyst diameters are measured; often this is either
318 not the case or the information is not given. As a result, only studies employing sequence-based
319 identification will be discussed below. We recognise that this excludes the vast majority of
320 studies, but if the data are not interpretable we feel they are better omitted.

321

322 Molecular studies in wild NHPs published to date (Table 2) are few in number, mostly involve
323 Old World NHPs, and vary in the methodology used. In some studies, species-specific PCR

324 has been used, but often not all known species were tested for despite primers being available,
325 leaving gaps in the data (Table 2, notes). When species-specific PCR has been used, this often
326 means subtypes were not identified and potentially interesting data on sequence variation and
327 host range have been lost. Several studies did not test for *E. hartmanni*, leading to a false
328 impression of the distribution of this *Entamoeba* species in NHPs. It is notable that *E.*
329 *histolytica* was not detected in any of these studies.

330

331 The use of only species-specific primers can mean that novel *Entamoeba* species are missed.
332 For example, if Villanueva-García et al. [39] had used species-specific primers for *Entamoeba*,
333 the two novel *Entamoeba* species found in Howler monkeys (CL8 and RL6) would not have
334 been identified – the samples would have appeared negative even though *Entamoeba*
335 organisms were present. Sequencing of products amplified using genus-specific primers may
336 seem the best way forward, but there is a catch. NHPs are often carriers of multiple *Entamoeba*
337 species and mixed PCR products give unreadable sequences with the standard DNA
338 sequencing. The approach of Jirků-Pomajbíková et al. [46] could be a good compromise –
339 genus-specific amplification coupled with nested species-specific PCR. This allows
340 identification of species in mixed infections yet does not miss mono-infections with novel
341 *Entamoeba* species, as these would be positive with genus-specific but negative with all the
342 species-specific primers used. Jirků-Pomajbíková et al. [46] did not initially test for *E.*
343 *hartmanni* but through sequencing discovered that it was the *Entamoeba* present in the samples
344 positive with the genus-specific primers but negative with the species-specific primer pairs
345 used. However, this method will only identify the presence of novel *Entamoeba* species if they
346 are present as a single infection unless it is combined with cloning of the PCR products.

347

348 It seems likely that identification of *Entamoeba* in NHPs in the future will be through
349 microbiome data, whether from targeted amplification and sequencing of a portion of
350 eukaryotic SSU-rDNA or by extraction of such sequences from metagenomic data. Both
351 approaches are in use in humans and have identified *Entamoeba* when present, but to date have
352 rarely been applied to NHP samples. In one example, Wegener Parfrey et al. [47] identified *E.*
353 *hartmanni* (among many other eukaryotes) in captive NHPs through eukaryote-targeted SSU-
354 rDNA amplification and 454 sequencing. Similarly, random sequencing of faecal DNA has the
355 potential to identify not only all the species present, but could enable assembly of partial or
356 complete genomes for the organisms identified [e.g. 48]. While such approaches are expensive
357 and likely to be available only to a few at present, the holistic information on the eukaryome
358 of NHPs likely to be obtained by such approaches makes them very attractive and we look
359 forward to seeing the data emerge in the next few years.

360

361 **Concluding Remarks**

362

363 Currently, at least six *Entamoeba* species with valid published names have been confirmed by
364 molecular analysis in NHPs: *E. coli*, *E. polecki*, *E. histolytica*, *E. nuttalli*, *E. dispar* and *E.*
365 *hartmanni*. However, in addition there are multiple subtypes within *E. coli* and *E. polecki*, plus
366 organisms with no name but distinct gene sequences (*Entamoeba* RL3, RL6, RL7 and CL8).
367 This remarkable expansion in known diversity has been driven largely by the use of molecular
368 techniques that have facilitated the identification of many novel and previously unrecognised
369 *Entamoeba* species in NHPs.

370

371 However, many points remain to be clarified (see “Outstanding Questions”). It is unclear
372 whether *E. moshkovskii*, *E. bangladeshi* and *E. gingivalis* colonise NHPs as well as humans.

373 Novel sequences with no linked species name are likely to continue to be detected in NHPs
374 around the world. This search for new types of *Entamoeba* in NHPs is essential as it remains
375 to be proven whether only *E. nuttalli* is responsible for morbidity and mortality in these hosts.
376 However, unless the correct approaches are used, such organisms will remain undiscovered.

377

378 We now know that NHPs are infected by both NHP-restricted and human-infective *Entamoeba*
379 species. Morphological diagnosis of *Entamoeba* species will always be problematic, but most
380 molecular approaches used to date may also be considerably underestimating the prevalence,
381 diversity, and distribution of *Entamoeba* in NHPs. At the same time, insufficient taxon
382 sampling and the heavy focus on humans may well have led us to inaccurate conclusions about
383 *Entamoeba* evolution. Fortunately, interest in the eukaryotic microbiome is growing in parallel
384 with improvements in technology, and it is likely that within the next few years a better
385 understanding of the evolution and host ranges of *Entamoeba* in NHPs will emerge.

386

387 Metagenomic analyses could allow the use of genes other than SSU-rDNA for phylogenetic
388 analyses. Obtaining sequence data for other genes is difficult - if not impossible - using
389 traditional molecular approaches and DNA from faecal samples. Multigene phylogenies may
390 well provide greater resolution that could confirm or refute our current views of relationships
391 within *Entamoeba*. Greater resolution is essential for evaluating the relative importance of
392 cospeciation and host-switching in the evolution of primate *Entamoeba* species. It seems likely
393 that these data will start to become available in the near future.

394

395 A recent study showed a significant reduction in the gut microbiome diversity of captive NHPs,
396 with a shift occurring from a wild NHP microbiome state toward a modern human microbiome
397 state [49]. Whether alterations in the lifestyle and diet of captive NHPs or the disruption of

398 normal hierarchical social behavior [50] has led to this perturbation of their gut microbiome,
399 the change may predispose captive NHPs to infection with certain *Entamoeba* spp, normally
400 confined to humans. Comparison of gut microbiomes across NHPs living in the wild,
401 semicaptivity and captivity using sequencing of both bacteria and *Entamoeba* SSU rDNA, is
402 already possible. Such data will allow us to investigate the correlation between microbiota
403 signatures and prevalence of specific *Entamoeba* species in NHPs.

404

405 There is much more to learn regarding both the microbiome and the eukaryome of NHPs,
406 especially those in the wild. There has been a strong focus on Old World primates, in particular
407 macaques, while New World primates are significantly underrepresented and prosimians have
408 not been studied. It is hoped that the range of species sampled will broaden, otherwise we will
409 continue to have a rather limited view of *Entamoeba* diversity in NHPs.

410

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603 **Glossary**

604

605 **Conditional lineage (CL):** an *Entamoeba* identified as likely to be distinct based on
606 sequencing of partial SSU-rDNA, but for which sufficient data are not yet available. See RL,
607 below.

608

609 **Isoenzymes:** each of two or more sequence variants of an enzyme that exhibit different
610 migration in electrophoresis gels due to charge differences.

611

612 **Non-human primates (NHPs):** all members of the order Primates other than humans; NHPs
613 share many similarities with humans in terms of physiology, anatomy, immunology, and
614 neurology, but are very diverse in their ecology, diet, etc. The split between humans and NHPs
615 is an artificial one, as humans are much more closely related to some NHPs than others.

616

617 **Ribosomal lineage (RL):** an *Entamoeba* taxon identified as distinct by sequencing of its
618 complete SSU-rDNA gene. Often no corresponding morphological data are available. In
619 other groups of organisms these are often called operational taxonomic units (OTUs) but in
620 this case, it is clear that they belong to the genus *Entamoeba*.

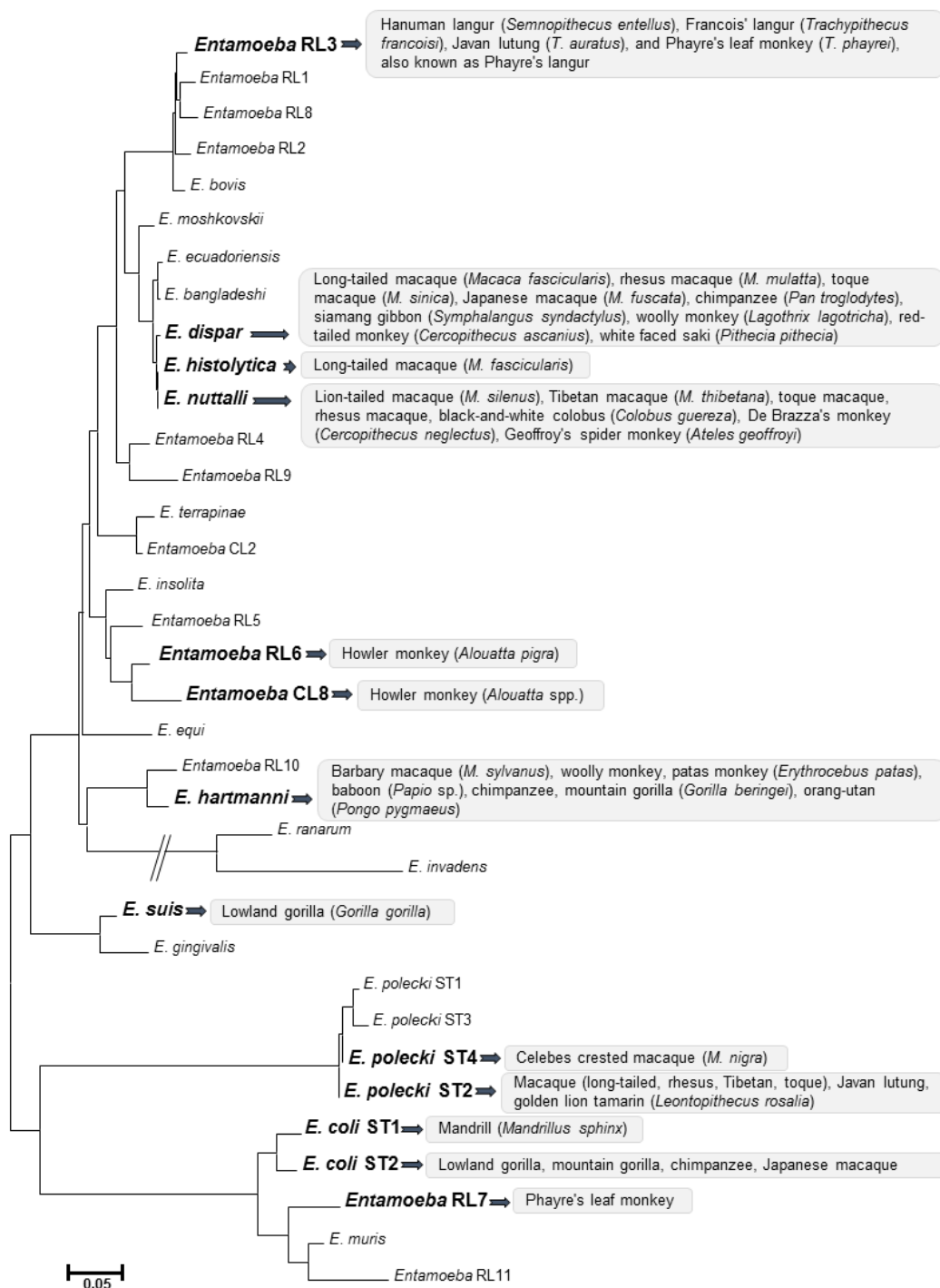
621

622 **Small subunit ribosomal RNA gene (SSU-rDNA):** the gene encoding the smaller of the two
623 major RNA components of the ribosome, also known as 18S rDNA. This gene is the most
624 widely used single locus for phylogenetic analyses in eukaryotes and bacteria. In *Entamoeba*,
625 the gene size generally falls between 1800 and 2200 bases.

626

627 **Subtype:** a discrete genetic clade within a named species.

628 **Figure 1: Phylogenetic relationships among *Entamoeba* species.** The phylogenetic tree
 629 shown is modified from Figure 1 in Jacob et al. [34]. Names in bold lettering are those that
 630 have been identified by sequencing of SSU-rDNA in NHPs. Adjacent to the *Entamoeba* names
 631 are those of the NHP species (wild or captive) in which the *Entamoeba* has been identified.
 632



633

Dobell nomenclature ^a	Current species names	Identified in primates (incl. humans)	Molecular identification in NHPs	
<i>E. histolytica</i>	<i>E. histolytica</i>	<i>E. histolytica</i>	Y ^b	
	<i>E. dispar</i>	<i>E. dispar</i>	Y	
	<i>E. hartmanni</i>	<i>E. hartmanni</i>	Y	
	<i>E. nuttalli</i>	<i>E. nuttalli</i>	Y	
	<i>E. moshkovskii</i>	<i>E. moshkovskii</i> (complex)	N	
	<i>E. polecki</i>		<i>E. polecki</i> ST1 ^c	N
			<i>E. polecki</i> ST4	Y
	<i>E. chattoni</i>		<i>E. polecki</i> ST2	Y
			<i>E. polecki</i> ST3	N
	<i>E. bangladeshi</i>	<i>E. bangladeshi</i>	<i>E. bangladeshi</i>	N
	<i>E. suis</i>	<i>E. suis</i>	<i>E. suis</i>	Y ^b
<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i> ST1	Y ^b	
		<i>E. coli</i> ST2	Y	
<i>E. gingivalis</i>	<i>E. gingivalis</i>	<i>E. gingivalis</i> ribodeme 1 ^d	N	
		<i>E. gingivalis</i> ribodeme 2	N	
	None		<i>Entamoeba</i> RL3 ^e	Y
			<i>Entamoeba</i> RL6	Y
			<i>Entamoeba</i> RL7	Y
		<i>Entamoeba</i> CL8 ^f	Y	

Table 1. Correspondence between historic, binomial, and sequence-based nomenclature for *Entamoeba* species in primates.

^aDobell's nomenclature is that proposed in his 1919 monograph [3].

^bIdentified in captive NHPs only, to date.

^c Subtypes (ST) are distinct small-subunit ribosomal DNA sequence variants that clearly fall within a named species.

^d Ribodemes are small-subunit ribosomal DNA variants detected by restriction enzymes.

^e Ribosomal (RL) [14]] lineages indicate complete small-subunit ribosomal DNA sequences that are clearly distinct from all named species.

^f Conditional (CL) [34] lineages indicate partial small-subunit ribosomal DNA sequences that are clearly distinct from all named species.

NHP species	Type of amplification	Total no. of samples	Species identified (no. of samples)	Reference	Notes
Rhesus macaques (<i>Macaca mulatta</i>)	Species-specific	715	<i>E. nuttalli</i> (440), <i>E. dispar</i> (16), <i>E. coli</i> (574), <i>E. polecki</i> ST2 (649)	51	a
Rhesus macaque (<i>Macaca mulatta</i>)	Species-specific	112	<i>E. nuttalli</i> (57), <i>E. dispar</i> (13), <i>E. coli</i> (83), <i>E. polecki</i> ST2 (96)	26	b
Tibetan macaque (<i>Macaca thibetana</i>)	Species-specific	89	<i>E. nuttalli</i> (15), <i>E. coli</i> (37), <i>E. polecki</i> ST2 (59)	52	c
Savannah woodland chimpanzee (<i>Pan troglodytes schweinfurthii</i>)	Genus- and species-specific	107	<i>E. hartmanni</i> (32), <i>E. dispar</i> (10), <i>E. coli</i> ST2 (33)	46	d
Celebes crested macaque (<i>Macaca nigra</i>)	Species/subtype-specific	77	<i>E. polecki</i> ST4 (75)	35	e
Toque macaque (<i>Macaca sinica</i>)	Species-specific	227	<i>E. nuttalli</i> (42), <i>E. dispar</i> (1), <i>E. coli</i> (40), <i>E. polecki</i> ST2 (197)	24	f
Rhesus macaque (<i>Macaca mulatta</i>)	Genus- and species-specific	128	<i>E. coli</i> (63), unidentified <i>Entamoeba</i> (65)	53	g
Mountain gorilla (<i>Gorilla beringei beringei</i>)	Genus-specific	68	<i>E. coli</i> ST2 (4), <i>E. hartmanni</i> (33)	36	h
Howler monkeys (<i>Alouatta palliata</i> and <i>A. pigra</i>)	Genus-specific	155	<i>Entamoeba</i> CL8 (6 from <i>A. pigra</i> , 1 from <i>A. palliata</i>), <i>Entamoeba</i> RL6 (1 from <i>A. pigra</i>)	39	i

Table 2. Summary of results from molecular screening of faecal samples from wild NHP populations*

* The publication by Dong et al. [54] includes data on several NHP species in China (mostly *Macaca mulatta* and *M. fascicularis*) but it is not possible to identify which results came from sampling wild populations. Samples were tested by species-specific amplification for *E. histolytica*,

E. nuttalli, *E. dispar*, *E. moshkovskii*, *E. coli*, and *E. polecki* ST2. Only *E. coli* and *E. dispar* were detected. No tests for *E. hartmanni* or other *E. polecki* subtypes were performed.

a: Authors also tested captive macaques; these are excluded from the table. Tested for *E. histolytica*, *E. dispar*, *E. nuttalli*, *E. coli*, and *E. polecki* ST2 only. No test for *E. hartmanni*, *E. moshkovskii* or other *E. polecki* subtypes.

b: Tested for *E. histolytica*, *E. dispar*, *E. nuttalli*, *E. moshkovskii*, *E. coli*, and *E. polecki* ST2 only. No test for *E. hartmanni* or other *E. polecki* subtypes.

c: Tested for *E. histolytica*, *E. dispar*, *E. nuttalli*, *E. coli*, and *E. polecki* ST2 only. No test for *E. hartmanni*, *E. moshkovskii* or other *E. polecki* subtypes.

d: Genus-PCR-positive samples were tested for *E. histolytica*, *E. nuttalli*, *E. dispar*, *E. moshkovskii*, *E. coli*, and *E. polecki* ST2. Genus-PCR positive, but species-specific PCR negative samples were sequenced and identified as *E. hartmanni*.

e: Tested for *E. histolytica*, *E. dispar*, *E. nuttalli*, *E. moshkovskii*, *E. coli*, and *E. polecki* ST1, ST2 and ST4. No test for *E. hartmanni* or *E. polecki* ST3.

f: Tested for *E. histolytica*, *E. dispar*, *E. nuttalli*, *E. moshkovskii*, *E. coli*, and *E. polecki* ST2. No test for *E. hartmanni* or other *E. polecki* subtypes.

g: Genus-PCR positive samples were tested for *E. coli*. Multiplex PCR for *E. histolytica*, *E. dispar* and *E. moshkovskii* on all samples. No test for *E. nuttalli*, *E. polecki*, or *E. hartmanni*.

h: Sequencing of Genus-PCR positive amplicons identified only these two species.

i: Sequencing of Genus-PCR positive amplicons identified only these two organisms.