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

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SUMMARY

To address the molecular diversity and occurrence of pathogenic species of the genus *Entamoeba* spp. in wild non-human primates (NHP) we conducted molecular-phylogenetic analyses on *Entamoeba* from wild chimpanzees living in the Issa Valley, Tanzania. We compared the sensitivity of molecular [using a genus-specific polymerase chain reaction (PCR)] and coproscopic detection (merthiolate-iodine-formaldehyde concentration) of *Entamoeba* spp. We identified *Entamoeba* spp. in 72 chimpanzee fecal samples (79%) subjected to species-specific PCRs for six *Entamoeba* species/groups (*Entamoeba histolytica, Entamoeba nuttalli, Entamoeba dispar, Entamoeba moshkovskii, Entamoeba coli* and *Entamoeba polecki* ST2). We recorded three *Entamoeba* species: *E. coli* (47%), *E. dispar* (16%), *Entamoeba hartmanni* (51%). Coproscopically, we could only distinguish the cysts of complex *E. histolytica/dispar/moshkovskii/nuttalli* and *E. coli*. Molecular prevalence of entamoebas was higher than the prevalence based on the coproscopic examination. Our molecular phylogenies showed that sequences of *E. dispar* and *E. coli* from Issa chimpanzees are closely related to sequences from humans and other NHP from GenBank. The results showed that wild chimpanzees harbour *Entamoeba* species similar to those occurring in humans; however, no pathogenic species were detected. Molecular-phylogenetic methods are critical to improve diagnostics of entamoebas in wild NHP and for determining an accurate prevalence of *Entamoeba* species.

Key words: *Entamoeba*, molecular diversity, great apes, chimpanzee, savannah.

INTRODUCTION

Protists represent important model organisms for studying parasite transmission between non-human primates (NHP) and humans, primarily because of high genetic diversity, lower host specificity and life cycles that facilitate their transmission (Pedersen *et al*. 2005). Yet to date, few studies have been conducted on the molecular diversity of protists in populations of wild African great apes (Petrášová *et al*. 2011; Sak *et al*. 2013, 2014). Given that humans increasingly encroach upon wild primate habitats (Chapman and Lambert, 2000), understanding the biology and diversity of potentially zoonotic protists is an important part of One Health approach in conservation medicine (http://www.onehealthinitiative.com).

Cysts of the amoebas of the genus *Entamoeba* are commonly detected in fecal samples of wild NHPs, including chimpanzees, by light microscopy following concentration coproscopic methods (e.g. Ashford *et al*. 2000; Lilly *et al*. 2002; Muehlenbein, 2005; Howells *et al*. 2011; Kooriyama *et al*. 2012; Kalousová *et al*. 2014). However, it is difficult if not impossible to differentiate the pathogenic (*Entamoeba histolytica* and *Entamoeba nuttalli*) from the non-pathogenic species (specifically *Entamoeba*

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dispar and *Entamoeba moshkovskii*), as all the above mentioned species form morphologically indistinguishable cysts with four nuclei (Kebede *et al*. 2004; Visser *et al*. 2006). Only molecular techniques allow distinguishing commensal species from species with confirmed pathogenicity (Tachibana *et al*. 2000; Verweij *et al*. 2001; Stensvold *et al*. 2010). Moreover, the distribution of *E. histolytica* and *E. nutalli* remains poorly explored, as they are only recently separated from each other and known both from NHP and humans (Tachibana *et al*. 2007, 2009, 2015; Levecke *et al*. 2010, 2015; Stensvold *et al*. 2010).

Several studies have used molecular tools for studying *Entamoeba* spp. in captive NHP, where both pathogenic *Entamoeba* species and also commensal ones (*E. dispar, Entamoeba coli, Entamoeba hartmanni, E. moshkovskii* or *Entamoeba polecki* ST2) have been documented (Verweij *et al*. 2003; Tachibana *et al*. 2009; Levecke *et al*. 2010; Rivera *et al*. 2010; Regan *et al*. 2014). To date, such techniques have not yet been employed in wild-living NHP with the exception of Tachibana *et al*. (2015), who molecularly characterized *E. nuttalli* strains in wild toque macaques (*Macaca sinica*) in Sri Lanka. Levecke *et al*. (2010) molecularly detected four at that time undetermined *Entamoeba* lineages in captive NHPs, which Stensvold *et al*. (2011) later determined as the lineage *Entamoeba* RL3. However, this fact may indicate the presence of yet undescribed species in the wild counterparts.

We carried out a survey on the molecular diversity of *Entamoeba* spp. in a community of savanna chimpanzees living in the Issa Valley, Ugalla (Western Tanzania) with emphasis on species with zoonotic potential. We also focused on species that we assume to naturally occur in chimpanzees. A recent study proposed a new nomenclature of *Entamoeba* species for novel or undetermined lineages and based on the morphology (specifically nuclei numbers) also suggested a division of the *Entamoeba* spp. to the complexes of uni-, tetra- and octo-nucleated cysts, which are not monophyletic (Stensvold *et al*. 2011). Accordingly, we designed polymerase chain reaction (PCR) protocols to distinguish *E. polecki* ST2 (belonging to entamoebas producing uninucleated cysts), group of *E. coli* (octonucleated cysts) and, finally, we conducted species-specific PCR for *E. histolytica* complex (with tetranucleated cysts) to distinguish the pathogenic species (*E. histolytica* and *E. nuttalli*) from the commensal ones (*E. dispar* and *E. moshkovskii*).

MATERIAL AND METHODS

Study site and subjects

The Issa Valley research station is located in the Ugalla region, ~100 km east of Lake Tanganyika, in Western Tanzania. Ugalla covers ~3352 km^2 with an elevation range of 980–1712 m above sea level and consists of flat plateaus broken up broad valleys, steep hills and severe slopes (Moore, 1994). The Issa Valley is dominated by savanna (miombo) woodland vegetation, but also has very thin riverine evergreen forest strips, swamps and grassland (Moore, 1994). The climate of Issa includes a dry season from May to September and a rainy season from October/November to April/May (Hernandez-Aguilar *et al*. 2013). Average annual rainfall is around 1200 mm (range: 980–1350 from 2008 to 2014), and the temperature varies between 14 and 34 °C. The Issa chimpanzee community is estimated to have 67 individuals based on preliminary genetic analyses (Rudicell *et al*. 2011), and overall, the population density at Issa is estimated to be 0·25 individuals km^{-2} (Piel *et al*. 2015).

Sample collection

One hundred and seven fecal samples were non-invasively collected between February 2009 and February 2010 and between March and May 2012 by following chimpanzee parties and upon encountering fresh nest groups opportunistically; all samples originated from a single chimpanzee community (Rudicell *et al*. 2011). Issa chimpanzees are only partially habituated and therefore we could not attribute the fecal samples to specific individuals. Each fecal sample was stored in 20 mL vials in 96% ethanol and part of the samples ($n = 33$) was simultaneously stored also in 4% formaldehyde.

Coproscopic analyses

Thirty-three samples fixed in 4% formaldehyde were used for coproscopic analyses (Supplementary Table S1). The standard protocol for detection of *Entamoeba* cysts was followed and the merthiolate-iodine-formaldehyde concentration (MIFC) technique was used (Blagg *et al*. 1955). Two millilitres of sediment suspension was mixed with 5 mL of MIFC solution, 1 mL of Lugol's iodine and 6 mL of ether in a 15 mL Falcon tube. Subsequently, it was centrifuged at 280 ***g*** for 2 min, the supernatant was discarded and the residual sediment was examined by light microscopy using ×1000 magnification.

Molecular analyses and sequencing of Entamoeba spp.

All fecal samples ($n = 107$) preserved in 96% ethanol were molecularly analysed (Supplementary Table S1). Two hundred milligrams of each sample was dried overnight at 37 °C, and then the total DNA was extracted using the kit PSP® Spin Stool DNA kit (Stratec) following the manufacturer's protocol. First, the samples positive for *Entamoeba* were identified using semi-nested PCR, amplifying a conserved part of the small subunit ribosomal RNA (SSU rRNA)

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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

gene specific for the *Entamoeba* genus. PCR conditions are described below, and all primers are summarized in Table 1. In the first round of this semi-nested PCR, our designed reverse primer, Entam_5 (Table 1) with Entam_1 as a forward one were used. In the second PCR round, published primers, namely forward Entam_1 and reverse Entam_2 were used (for more details see Table 1). The size of amplicons was ~650 bp. Then, only samples positive for *Entamoeba* spp. were screened using PCRs specific for six *Entamoeba* species (based on the part of the SSU rRNA gene): (i) *E. histolytica* (size of product: 475 bp), (ii) *E. nuttalli* (size of product: 848 bp), (iii) *E. dispar* (size of product: 195 bp), (iv) *E. moshkovskii* (size of product: 580 bp), (v) *E. coli* (size of product: 290 bp) and (vi) *E. polecki* ST2 (size of product: 680 bp) (for more detail see Table 1 and Fig. 1). All species-specific PCRs were performed separately to prevent the competitive inhibition of *Entamoeba* spp. DNA.

For all PCRs, the published conditions listed in Table 1 were followed, except for the first round of semi-nested PCR: 5 min at 95 °C, 35 cycles of 1 min at 95, 60 and 72 °C, and final elongation for 10 min at 72 °C. For positive controls, isolates from *in vitro* cultures (*E. histolytica, E. dispar, E. moshkovskii* and *E. coli*) were used, whereas in the case of *E. nuttalli* an isolate obtained from feces of a Hamadryas baboon (*Papio hamadryas*) from a sanctuary for exotic animals was used (AAP, the Netherlands; see Levecke *et al*. 2010) and an isolate of *E. polecki* ST1 from feces of a domestic pig (*Sus scrofa domestica*) for the *E. polecki* was used. All amplicons from both types of PCR (species- and genus-specific) were used for sequencing. The PCR products were purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Each sample was bi-directionally sequenced (using primers from second round of semi-nested PCR and species specific primers for confirmation) and used for phylogenetic analyses (see below).

Phylogenetic and statistical analyses

A dataset consisting of 193 SSU rRNA gene sequences of the genus *Entamoeba* was created, including the sequences obtained in the present study. The sequences were aligned using the MAFFT method (Katoh *et al*. 2002) on the MAFFT 7 server (http://mafft.cbrc.jp/alignment/server/) with the G-INS-i algorithm at default settings. The alignment was manually edited in BioEdit 7·0·4·1. The final dataset contained 585 aligned characters and is available from the corresponding author upon request. Phylogenetic trees were constructed by maximum likelihood and Bayesian methods. Maximum likelihood analysis was performed in RAxML 7·0·3 (Stamatakis, 2006) under the GTRGAMMAI model, which was selected by Akaike Information Criterion implemented in Modeltest 3·7 (Posada and Crandall, 1998). Bootstrap support values were generated in RAxML from 1000 pseudoreplicate datasets. Bayesian analysis was carried out using MrBayes 3·2 (Ronquist *et al*. 2012) under the GTR + I + Γ + covarion model. Four Markov Chain Monte Carlo chains were run for 2 million generations until the average standard deviation (S.D.) of split frequencies based on last 75% of generations was lower than 0·01. The trees were sampled for every 500th generation. The first 25% of trees were removed as burn-in. The McNemar test was performed in GraphPad

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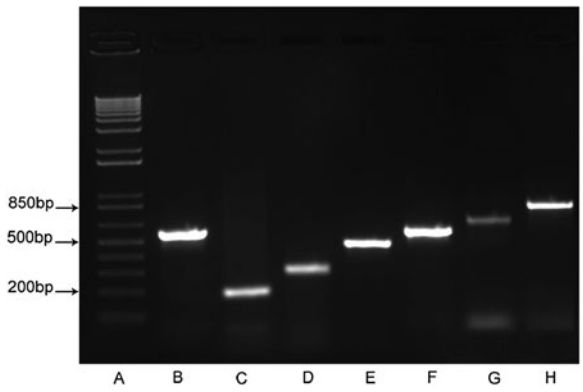

Fig. 1. The products of *Entamoeba* genus-specific PCR and several *Entamoeba* species-specific PCRs conducted on the samples used as positive controls (note: only for demonstration of the feasibility of the species-specific PCRs): (A) marker, (B) *Entamoeba*-genus specific PCR from culture of *Entamoeba* invades, (C) *Entamoeba dispar* (~195 bp), (D) *Entamoeba coli* (~290 bp), (E) *Entamoeba histolytica* (~475 bp), (F) *Entamoeba moshkovskii* (~600 bp), (G) *Entamoeba polecki* ST2 (~680 bp), (H) *Entamoeba nuttalli* (~848 bp).

(http://graphpad.com/) to compare the sensitivity of *Entamoeba* spp. detection using either the coproscopic or PCR method.

Prediction of the secondary structure of the SSU rRNA molecule

A secondary structure of the SSU rRNA of *E. histolytica* was obtained as inferred from GenBank sequence X65163 from Comparative RNA Web Site (www.rna.icmb.utexas.edu). The conservative elements of the secondary structure of other *Entamoeba* sequences were identified manually by inspecting the alignment used for the phylogenetic analysis (see above).

RESULTS

PCR and coproscopy based prevalence of Entamoeba spp.

The multinuclear thick-walled cysts of *Entamoeba* spp. were coproscopically detected in three out of 33 fecal samples (9%). The cysts of complex *E. histolytica/dispar/moshkovskii/nuttalli* and *E. coli* were identified. The prevalence of *Entamoeba* species using molecular tools in the same dataset of samples was higher and reached 58% (19/33). The PCR method was significantly more sensitive for the detection of *Entamoeba* spp. than the MIFC technique (McNemar: $\chi^2 = 14{\cdot}1$; D.F. = 1; $P = 0{\cdot}0002$).

Using genus-specific PCR targeting the SSU rRNA gene, 72 samples positive for *Entamoeba* spp. (72/107; 67%) was identified. Species-specific PCR assays revealed the presence of two *Entamoeba* species, namely 33 samples positive for *E. coli* (33/107; 31%), and 10 samples positive for *E. dispar* (10/107; 9%). Neither the pathogenic *Entamoeba* species such as *E. histolytica* or *E. nuttalli* nor commensal *E. moshkovskii* or *E. polecki* ST2 were detected in any sample. However, 36 samples that were positive for *Entamoeba* spp. using genus specific primers remained negative in all six protocols of species-specific PCRs. The amplicons were sequenced and BLAST was used to identify similar sequences in GenBank. All sequences obtained were highly similar to *E. hartmanni* (98% similarity). Finally, all samples that were positive using the genus-specific PCR were sequenced to allow diagnosis of *E. hartmanni*. *Entamoeba hartmanni* was documented in 34 samples (34/107; 32%). For details see the Supplementary Table S1. The newly determined sequences are available in GenBank under accession numbers KU320609–KU320612.

In some samples ($n = 12$), co-infections of detected *Entamoeba* species were found. The most represented species *E. hartmanni* (detected using genus-specific PCR) was detected in co-infection with both *E. coli* (in 10 samples) and also with *E. dispar* (only in one sample) (both detected using species-specific PCR). The co-infection of all three species was found in one sample only (again *E. hartmanni* was detected using genus-specific PCR and *E. coli* with *E. dispar* with species-specific PCRs). For details see Supplementary Table.

Molecular identification of Entamoeba spp.

The newly determined SSU rRNA gene sequences were subjected to BLAST search against the GenBank nr/nt database (megablast with default parameters). Twenty samples (for details see the Supplementary Table S1) contained identical SSU rRNA gene sequences, which shared 96–98% similarity with all GenBank sequences of *E. hartmanni*, for accession numbers see Fig. 2. SSU rRNA gene sequences of two further samples (T2041 and T3766) were identical and shared 98–99% similarity with sequences of *E. coli* subtype 2 (see Stensvold *et al.* 2011). The sequence of T2003 differed in a single nucleotide from sequences of T2041 and T3766. The sequence of T3403 shared 99% similarity with sequences of *E. dispar* and *E. nuttalli* (including sequences of *E. nuttalli* AB197936 and AB426549, which were deposited in GenBank as *E. histolytica*). To confirm phylogenetic position of the obtained *Entamoeba* samples, a phylogenetic analysis of the genus *Entamoeba* including the new sequences was performed. Topology of the resulting phylogenetic tree was consistent with results from BLAST searches (Fig. 2), with a few notable exceptions.

In the resulting tree, sequence T3403 appeared closely related to *E. dispar* sequences Z49256

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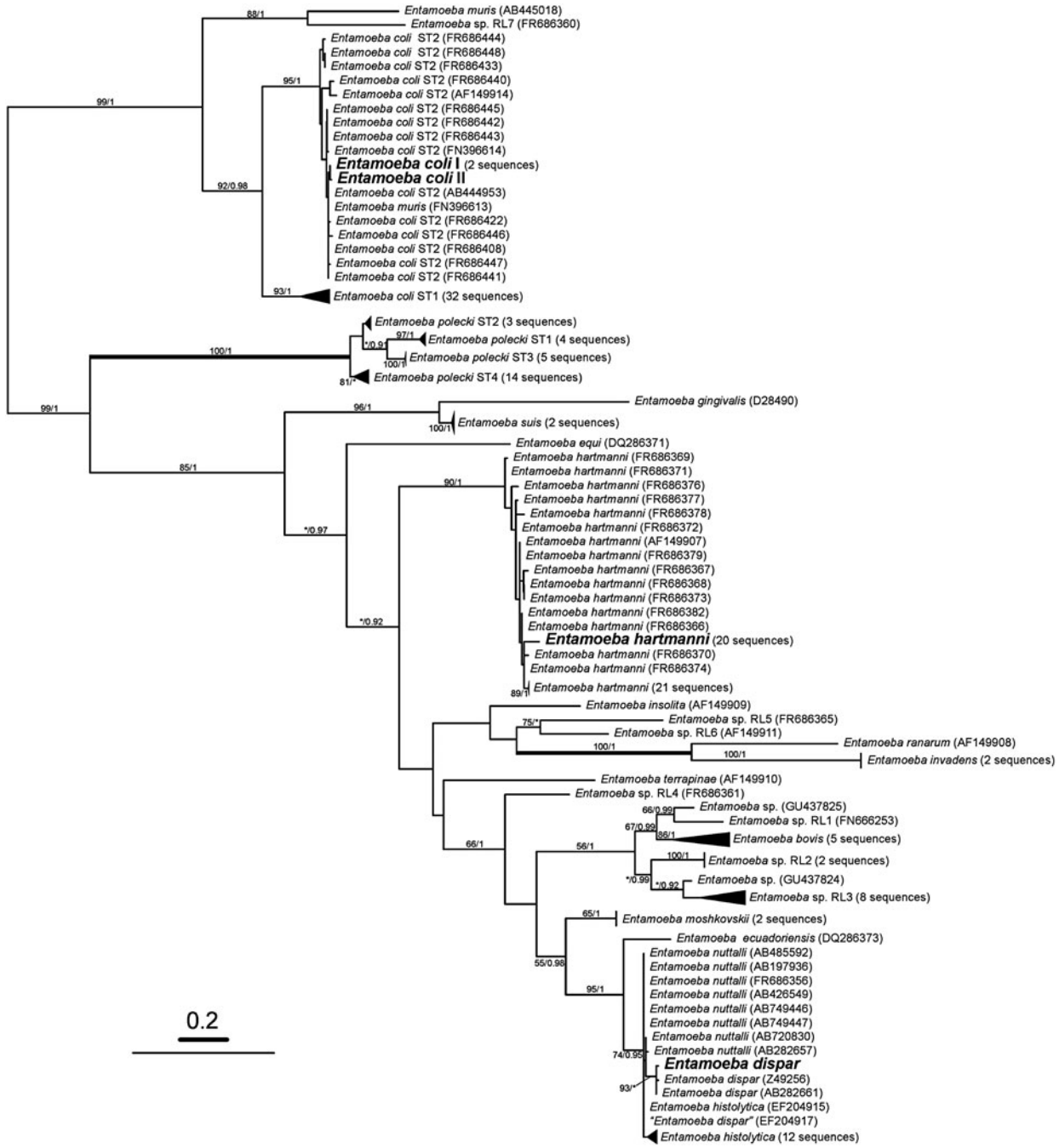

Fig. 2. Unrooted phylogenetic tree of the genus *Entamoeba* based on partial SSU rRNA gene sequences. The tree was constructed by the maximum likelihood method (GTRGAMMAI model). The values at the branches represent statistical support in maximum likelihood bootstrap values/Bayesian posterior probabilities. Support values below 50%/0.50 are not showed or are represented by an asterisk (*). New sequences are in bold.

(isolate from a human) and AB282661 (isolate from a rhesus monkey). The third GenBank sequence labelled as *E. dispar*, EF204917, occupied a different position within *E. histolytica*/*E. nuttalli*/*E. dispar* complex suggesting a possibility that it has been originally misidentified and belongs, in fact, to *E. histolytica*. To confirm the identity of T3403, the secondary structure of its SSU rRNA gene was examined and two features distinguishing *E. dispar* from the closely related *E. histolytica* (including the sequence EF204917) and *E. nuttalli* were identified: (i) A:G (instead of G:A) base pair within the stem of helix 10 (positions 181 and 198 in the sequence Z49256; for terminology of conservative elements see Wuyts *et al*. 2001) and (ii) GTAAG motif within helix E10_1 (positions 211–215 in the sequence Z49256).

Because our sequences affiliated with *E. hartmanni* were relatively divergent SSU rRNA gene sequences, the secondary structure of the corresponding SSU rRNA molecule was examined as well. They possessed a unique motif ACT in the

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loop in helix 17, which contrasted with all other *E. hartmanni* sequences, (including those obtained from NHP) having GTAA in the corresponding area (positions 438–441 in the sequence FR686371).

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 NHP. 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 amoebas 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 (Z49256; the clade of *E. dispar* isolates has 93% bootstrap support). Our *E. coli* sequence clustered with human-derived isolates and with one sequence from a captive western lowland gorilla (*Gorilla gorilla gorilla*). Moreover, the sequence fell into *E. coli* ST2 group, which has been identified from captive NHP and from humans who have recently travelled in tropical Africa or Asia (Stensvold *et al*. 2011). Based on present analyses, one can only speculate if *E. dispar* and *E. coli* ST2 found in Issa chimpanzees occur naturally in the Issa community or originate from humans. Given Issa chimpanzees do not regularly encounter humans aside from researchers; it is likely that both *E. dispar* and *E. coli* naturally occur in these apes. Future studies targeting the presence of amoebas in other Issa primate species can help to uncover the epidemiology of non-pathogenic amoeba infections.

We did not include the species-specific PCR for *E. hartmanni* into our protocol because we did not record the tetra-nucleated cysts typical for *E. hartmanni* in our previous study (for more details see Kalousová *et al*. 2014). Spherical cysts of *E. hartmanni* are smaller, ~5–10 μm, while the cysts of *E. histolytica*-complex are more than 10 μm in diameter (Ash and Orihel, 2007). However, using genus-specific PCR followed by sequencing, we identified many samples that were positive for *E. hartmanni* using genus-specific PCR followed by sequencing. Our sequences branched within the well-supported lineage of *E. hartmanni*, that included isolates from humans (FR686374–79; AF149907) and captive NHP such as the barbary macaque (*Macaca sylvanus*) (FR686369, FR686372), patas monkey (*Erythrocebus patas*) (FR686373), woolly monkey (*Lagothrix lagotricha*) (FR686366, FR686368), vervet monkey (*Chlorocebus pygerythrus*) (FR686373) and Bornean orangutan (*Pongo pygmaeus*) (FR686370). However, based on the SSU rRNA secondary structure, our sequence represents a novel sequence variant, different from other sequences of this species, as well as those obtained from NHP (see Stensvold *et al*. 2011). It is presently not possible to provide further details regarding the morphology and biology of this novel variant, because we have not obtained trophozoites for final taxonomical identification. Nevertheless, it is likely that *E. hartmanni* is non-pathogenic for chimpanzees similar to *E. hartmanni* in humans (Sard *et al*. 2011).

We have demonstrated that wild chimpanzees that do not live in close proximity to a large human population nonetheless harbour several *Entamoeba* species closely related to those occurring in humans. We found the microscopic detection to be unreliable for diagnostics of amoebas, due to low sensitivity and inability to distinguish between pathogenic and non-pathogenic species with similar cyst morphology. In summary, molecular-phylogenetic methods are fundamental for improving diagnostics of *Entamoeba* spp. in wild NHP and for understanding the epidemiology and zoonotic transmission of these parasites.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0031182016000263

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