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

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

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Running title: *Entamoeba* species in savanna chimpanzees

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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 PCR) and coproscopic detection (merthiolate-iodine-formaldehyde concentration) of *Entamoeba* spp. We identified *Entamoeba* spp. in 72 chimpanzee faecal samples (79 %) subjected to species-specific PCRs for six *Entamoeba* species/groups (*E. histolytica, E. nuttalli, E. dispar, E. moshkovskii, E. coli,* and *E. polecki* ST2). We recorded three *Entamoeba* species: *E. coli* (47 %), *E. dispar* (16 %), *E. 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, savanna

Key findings:

- first molecular survey of *Entamoeba* spp. in wild great apes
- *E. coli*, *E. dispar*, and *E. hartmanni* recorded in wild eastern chimpanzees
- *E. dispar* and *E. coli* closely related to other sequences from non-human primates and humans
- no pathogenic species of *Entamoeba* recorded
- molecular methods more sensitive than coproscopic ones for detection of entamoebas

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 faecal samples of wild NHPs, including chimpanzees, by light microscopy following concentration coproscopic methods (e.g. Ashford et al., 2000; Lilly et al., 2000; 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 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 of *Entamoeba* spp. in captive NHP, where both pathogenic *Entamoeba* species and also commensal ones (*E. dispar*, *E. coli*, *E. hartmanni, E. moshkovskii* or *E. 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 characterizated of *Entamoeba nuttalli* strains in wild toque macaques (*Macaca sinica*) in Sri Lanka. Levecke et al. (2010) molecularly detected four undetermined *Entamoeba* lineages in captive NHPs, which may also indicate the presence of yet undescribed species in their 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 (Stensvold et al., 2011) proposed a new nomenclature of *Entamoeba* species for novel or undetermined lineages and also suggested a division of the *Entamoeba* spp. to the complexes of uni-, tetra- and octo-nucleated cysts. Accordingly, we designed 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 approximately 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 slops (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 1,200 mm (range: 980–1,350 from 2008–2014), and the temperature varies between 14 and 34 °C. The Issa chimpanzee community is estimated to number 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 faecal 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 faecal samples to specific individuals. Each faecal sample was stored in 20 ml vials in 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 280g for two minutes, the supernatant was discarded and the residual sediment was examined by light microscopy using × 1,000 magnification.

Molecular analyses and sequencing of Entamoeba *spp.*

All faecal 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, we the samples positive for *Entamoeba* were identified using semi-nested PCR, amplifying the conservative part of SSU rRNA 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 approximately 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) *Entamoeba 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 Figure 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: five minutes at 95°C, 35 cycles of one minute at 95°C, 60°C and 72°C, and final elongation for 10 minutes 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 faeces 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 faeces of a domestic pig (*Sus scrofa domestica*) for the *E. polecki* was used. For sequencing, all amplicons from species-specific PCRs were used and amplicons from the genus-specific PCR; the PCR products were purified using the Qiagen extraction gel kit. 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 data set consisting of 193 SSU rRNA gene sequences of the genus *Entamoeba* was created, including the sequences obtained in the present study. The sequences using the MAFFT method (Katoh et al., 2002) were aligned with the help of 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 data set 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. Bootstrap support values were generated in RAxML from 1000 pseudoreplicate data sets. Bayesian analysis was carried out using MrBayes 3.2 (Ronquist et al., 2012) under the GTR + I + Γ + covarion model. Four MCMCs were run for two million generations until the average standard deviation of split frequencies based on last 75 % of generations was lower than 0.01. The trees were sampled every 500th generation. The first 25 % of trees were removed as burn-in. The McNemar test was performed in GraphPad (http://graphpad.com/) to compare the sensitivity of *Entamoeba* spp. detection using either the coproscopic or PCR method.

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

A secondary structure of the SSU rRNA of *Entamoeba 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).

3. Results

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

The multinuclear thick-walled cysts of *Entamoeba* spp. were coproscopically detected in three out of 33 faecal 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 = 14.1; d.f. = 1; p=0.0002).

Using genus-specific PCR targeting the SSU r DNA, 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 *Entamoeba hartmanni* (98% similarity). Finally, all samples that were positive using the genus-specific PCR were sequenced to prevent misdiagnosis of *E. hartmanni*. *E. hartmanni* was documented in 34 samples (34/107; 32%). For details see the Supplementary table S1.

The combination of genus- and species/group-specific PCR revealed co-infections of two or three species of entamoebas: *E. hartmanni* /*E. coli* (10 samples), *E. hartmanni* /*E. dispar* (1 sample), and *E. hartmanni* /*E. coli* /*E. dispar* (1 sample); for details see the Supplementary table S1.

3.2. Molecular identification of Entamoeba *spp.*

The newly determined SSU rRNA 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% to 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% to 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*, *E. nuttalli* and sequences AB197936 and AB426549 of *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 (Figure 2), with a few notable exceptions.

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

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

(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, approx. 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 currently not possible to provide further details regarding the morphology and biology of this novel variant, because we have not

obtained trophozoites, which are necessary for final 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.

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467 Figure legends:

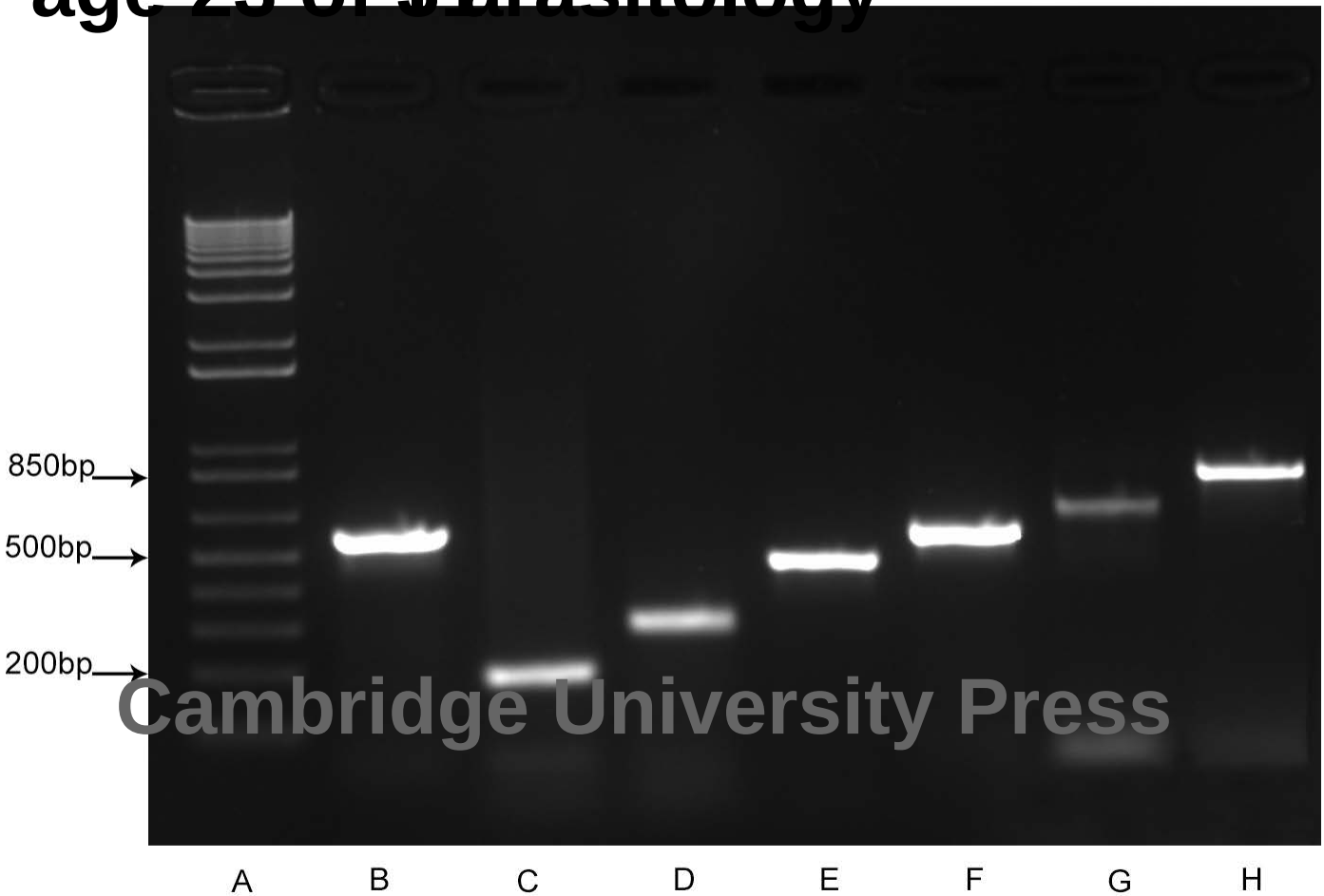
468 Figure 1. The products of *Entamoeba* genus-specific PCR and several *Entamoeba* species-
469 specific PCRs conducted on the samples used as positive controls (note: only for
470 demonstration of the feasibility of the species-specific PCRs): (A) marker, (B) *Entamoeba*-
471 genus specific PCR from 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).

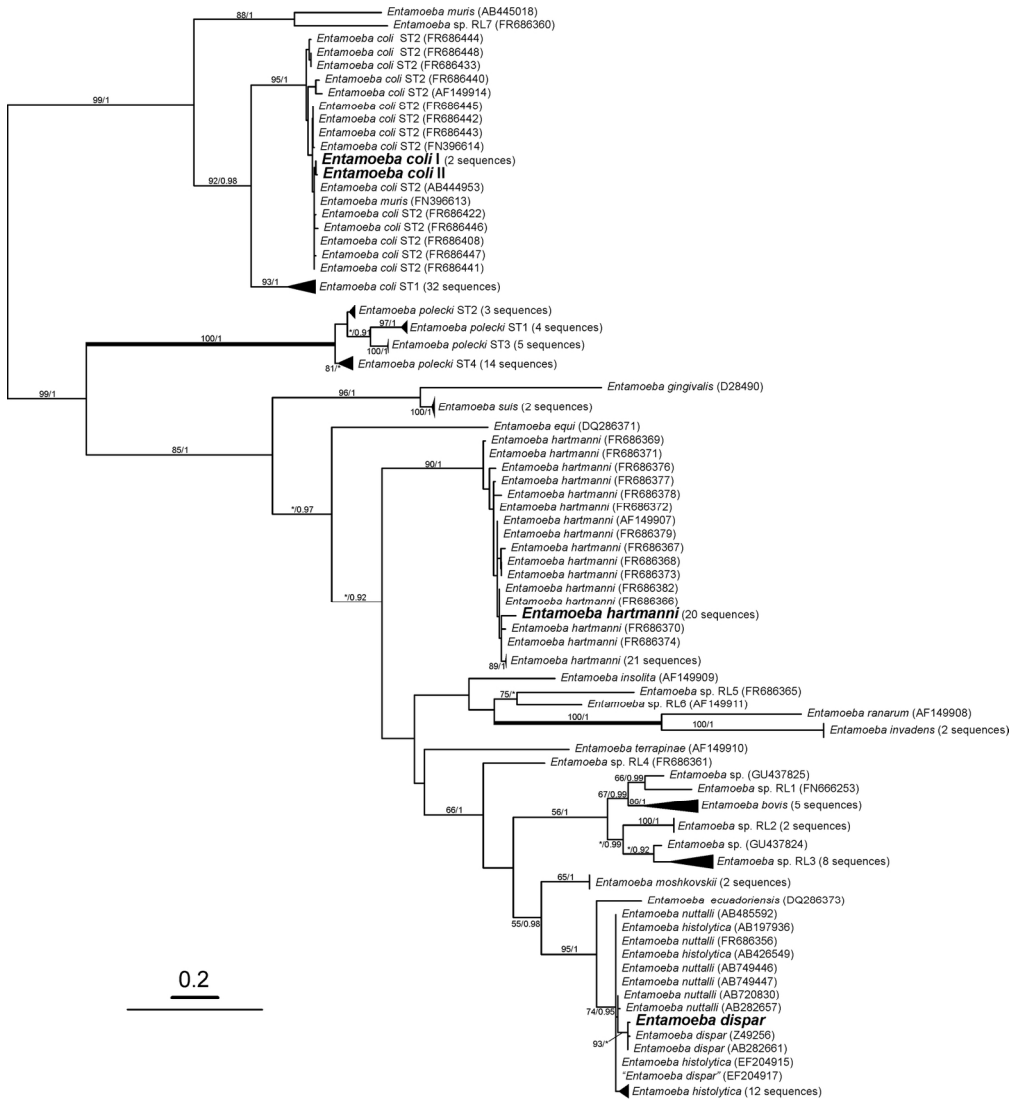
474

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