Rapid Communication

Genetic characterisation of uninucleated cyst-producing *Entamoeba* spp. from ruminants ✯

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Abstract

Six ssrRNA gene sequences were obtained by PCR amplification of DNA from uninucleated *Entamoeba* cysts isolated from fresh faeces of sheep, cows, a roe deer and a reindeer. Phylogenetic analysis using sequences of non-, uni-, quadri- and octonucleate cyst-producing *Entamoeba* spp. for comparison showed that all six isolates formed a separate clade nested within the clade of quadrinucleate cyst producers. The data indicate that *Entamoeba bovis* can be isolated from ruminant hosts other than cattle, and we suggest that organisms clustering with the sheep and cattle isolates analysed in the present study be named *E. bovis*.

Keywords: *Entamoeba*, Parasite, Ruminants, Genetic diversity, PCR

Organisms of the genus *Entamoeba* can be isolated from a variety of vertebrates and invertebrates and comprise parasitic species of varying pathobiological significance. Species within the genus can all be assigned to either non-, uni-, quadri- or octonucleated cyst-producing morphological groups. Uninucleated cyst-producing entamoebae have been isolated from humans, non-human primates, other mammals and birds (e.g. Noble and Noble, 1952; Kingston and Stabler, 1978; Silberman et al., 1999; Verweij et al., 2001; Clark et al., 2006; Skirnisson and Hansson, 2006). Ruminants such as cattle and sheep appear to be common hosts of uninucleate cyst-producing entamoebae (Noble and Noble, 1952; Skirnisson and Hansson, 2006) (Table 1). The differentiation between species and the assignment of species names in this group have depended largely on morphological data and the host in which organisms were identified. However, since cyst morphology varies substantially within as well as between uninucleated cyst-producing species from different ruminant hosts (Table 1), morphological data alone are not enough to distinguish between some of the named *Entamoeba* spp. (Noble and Noble, 1952). To date species names have been used mainly to indicate the host from which a particular isolate was recovered, for instance *Entamoeba bovis* from cattle and *Entamoeba ovis* from sheep. Uninucleated cysts from goats, however, have been attributed to *Entamoeba debliecki* or *Entamoeba polecki* (Noble and Noble, 1952) (Table 1), both of which are also hosted by pigs. Genetic characterisation of *Entamoeba suis* separated this from *E. polecki* (Clark et al., 2006), showing that pigs can host at least two distantly related species of uninucleated entamoebae. Hence, molecular data are needed not only in order to establish definitive identification but also clarify the epidemiology and pathobiological significance – if any – of *Entamoeba* spp. isolated from ruminants, and to improve our understanding of the evolution in the genus *Entamoeba*.

Data from the genetic characterisation of *Entamoeba* spp. from ruminants have not yet been published. The aim of this study was to provide such data on *Entamoeba* isolated from sheep, cattle, roe deer and reindeer, and to identify their evolutionary and taxonomic status based on phylogenetic inferences using ssrRNA gene sequences.

Cysts were isolated from fresh faecal samples from sheep, cattle, a roe deer and a reindeer by sucrose gradient purification (Lebbad et al., 2008) (Table 2). Sheep samples were also positive by microscopy for *Giardia*. One hundred cysts from each of Sheep310, Cow349 and Roedeer352 were measured by light microscopy and frequency distributions of cyst diameter were compared by *t*-test analysis; *P*-values of < 0.01 were considered statistically significant.

Purified *Entamoeba* cysts from sheep, cattle and roe deer isolates were studied using IFAT and a monoclonal antibody (mAb) originally shown to react with *Entamoeba histolytica* and *Entamoeba dispar* cysts, but not with *Entamoeba hartmanni*, *Entamoeba coli*, or *E. polecki* (unpublished observations). All IFAT stainings were combined with DAPI staining and, in addition, some samples were also stained with 0.5% Calcofluor.

DNA was extracted from cyst suspensions using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) after an initial disruption of the purified cysts with a Mini-BeadBeater (Biospec Products Inc. , USA) (Lebbad et al., 2008). After PCR amplification and sequencing using the ENTAM1/ENTAM2 primers (Verweij et al., 2001) and others of more general eukaryotic specificity (Clark et al., 2006), specific primers were designed and combined with the general eukaryotic primers to fully sequence the ssrRNA gene (Table 3). PCR products were purified and sequenced directly in both directions using an ABI 3730 capillary sequencer. Sequences were edited manually by the use of CHROMAS (Technelysium Pty Ltd, Queensland, Australia) and entered into isolate-specific databases using the Staden

Package (http://staden.sourceforge.net/). The six sequences were deposited in the NCBI nucleotide database with accession nos. **FN666248-FN666253**.

Full ssrRNA gene sequences from each of the six isolates were incorporated into the alignment produced by Clark et al. (2006). After removal of ambiguously aligned bases the alignment included 1,572 positions. Phylogenetic analyses were performed using distance (Neighbor joining as implemented in MEGA 4.0; Kumar et al., 2008), maximum likelihood (PHYML 2.4.5; Guindon and Gascuel, 2003) and Bayesian (MrBayes 3.1.2; Huelsenbeck and Ronquist, 2001) methods. Bayesian and maximum likelihood analysis used a General Time Reversible (GTR) model of nucleotide substitution with four categories of among-site rate variation and the proportion of invariant sites. Statistical support for distance and maximum likelihood trees was evaluated using bootstrapping (1,000 replicates). Bayesian analysis used four Markov chain Monte Carlo (MCMC) strands, 1,000,000 generations, with trees sampled every 100 generations. A consensus tree was produced after excluding an initial burn-in of 25% of the samples, as recommended.

Cyst diameter frequency counts for all three samples analysed gave unimodal distributions, and cysts from the sheep and the cow exhibited very similar average cyst diameter ranges ($P > 0.01$). However, cysts from the roe deer had a narrower size range and were significantly larger on average than cysts from the other two hosts ($P < 0.0001$) (Table 2).

By DAPI staining the vast majority of the *Entamoeba* cysts screened by microscopy were found to be uninucleate; a very few were binucleate and none were quadri- or octonucleate. Calcofluor staining revealed that all organisms observed had a cyst wall and therefore that none of them could be trophozoites.

Uninucleate cysts from all samples reacted with the mAb, visualised by FITC-staining. However, since by microscopy 4-6% of the cysts from the sheep samples were seen

not to react with the mAb, PCR using specific primers (unpublished data) for identification of *E. polecki* (all subtypes) was performed but no amplification of the DNA was seen. Moreover, sequencing of PCR amplicons produced by the ENTAM 1/2 primers of broader specificity did not provide any evidence of mixed *Entamoeba* infection. It is possible that non-reacting cysts are immature and the antigen to which the mAb binds might only be produced late in the maturation process.

The analysis of *Entamoeba* sequences from sheep, reindeer and roe deer required very little manual editing, if any, whereas analysis of the sequences from cattle isolates was more complicated, since a few base calls were ambiguous despite multiple amplification and sequencing efforts. Therefore, a few base positions are annotated as degenerate bases in both of the sequences from cattle (accession nos. **FN666248** and **FN666249**), and may represent differences between individual gene copies in these isolates.

Phylogenetic analysis revealed that the six sequences from the four ruminant hosts belong to a separate clade, branching within the previously described group of quadrinucleate cyst-producing species (Fig. 1). High bootstrap values separated the group consisting of the ruminant isolates plus the quadrinucleate cyst-producing species from groups of non-, other uni- or octonucleate cyst-producing species, which implies that the ruminant entamoebae studied here are descended from a quadrinucleated cyst-producing ancestor. These amoebae are most closely related to organisms such as *E. histolytica*, and only remotely related to other uninucleated cyst producers such as *E. polecki* (Fig. 1). The roe deer sequence was the basal lineage within the ruminant clade, supported by a bootstrap value of 100% and a posterior probability value of 1.0. The cattle, sheep and reindeer isolates clustered together closely and, interestingly, the two sheep sequences were not specifically related within this clade, being separated by the reindeer sequence, high posterior probabilities and moderate bootstrap values.

For a long time, evidence supported the hypothesis that morphological characteristics of *Entamoeba* cysts mirrored the genetic diversity of the genus *Entamoeba* and that the placement of species into separate clusters could be predicted from the number of nuclei within the mature cyst (Clark et al., 2006). However, the uninucleate cyst-producing species *E. suis* was recently shown to branch at the base of the quadrinucleate cyst-producing clade, although it is most closely related to the non-cyst-producing species *Entamoeba gingivalis* (Clark et al., 2006). The sequences obtained in the present study were all derived from uninucleated amoebic cysts yet clearly emerge from within the quadrinucleate cyst-producing clade, which means that grouping species based on cyst nuclei number does not always reflect genetic relationships.

The ENTAM1/2 primers were designed to target all species of the genus *Entamoeba* and no other organisms (Verweij et al., 2001). Analysis of the chromatogram obtained from sequencing the ENTAM1/2 PCR product for Sheep310 apparently revealed a mixed infection as seen by the presence of double peaks. However, analysis of the underlying minor sequence disclosed the presence of a *Candida* PCR product in the sample and not another species of *Entamoeba*. This means that the cysts that did not react with the mAb most likely did not represent another *Entamoeba* sp. It also means that the ENTAM1/2 primers are not as specific for *Entamoeba* as originally thought.

The morphological data collected in this study resemble closely those reported by Noble and Noble (1952) on sheep and cattle entamoebae. In the present study, most cysts were found to be uninucleate. In very few instances, binucleate cysts were seen. However, so-called supranucleate cysts have also been reported for other parasites such as *E.coli, Endolimax nana, Iodamoeba bütschlii* (Dobell, 1919) and *E. polecki* (Levin and Armstrong, 1970).

Kingston and Stabler (1978) reported finding *E. bovis* and *E. coli* in white-tailed deer.

In the present study, it was observed that the *Entamoeba* isolate obtained from a roe deer was genetically distinct from the *Entamoeba* found in the cattle, and that the two sheep sequences, although different, were more related to the cattle *Entamoeba* than was the deer sequence. Examination of cysts from the roe deer revealed a narrower cyst diameter range than for the cysts from cattle and sheep, and although the maximum diameter recorded in the present study was only 12.0 µm, the cysts from the roe deer were larger on average than those found in cattle and sheep (Table 1). Unfortunately, cyst measurements were not available for the reindeer isolate. However, this study showed that the roe deer isolate differed significantly from the cattle and sheep isolates not only phylogenetically, but also morphologically, and may represent a distinct species.

The nomenclature of *Entamoeba* species found in ungulates (ruminants plus pigs) in general has been very confused. In the absence of molecular data, the assignment of species names to a given isolate has relied on parasite morphology and host species. There are probably several reasons why confusion has prevailed. Firstly, Noble and Noble (1952) stressed that various physical and chemical factors pertaining to the processing and analysis of cyst preparations affect cyst morphology. Secondly, we know that multiple *Entamoeba* spp. can be isolated from pigs (Clark et al., 2006) and cattle (unpublished observations). Moreover, we know that many *Entamoeba* spp. have been isolated from more than one host species, and it is likely that different species of ungulates may be infected by (variants of) the same *Entamoeba* species (Mackinnon and Dibbs, 1938). Finally, we know that there is a considerable range of cyst diameter within isolates and a considerable overlap among isolates from different hosts. The presence of bimodal cyst diameter frequency distributions of *Entamoeba* identified in pigs, as observed by for example Noble and Noble (1952), complicates interpretation of data, since this could be due to a true variation in size within a species or to the presence of a mixed species infection. Interestingly, Noble and Noble (1952)

concluded that uninucleate entamoebae isolated from the faeces of cattle, goats, sheep, and pigs were morphologically indistinguishable.

Although we did not obtain the same sequence from cysts in two different hosts, the phylogenetic analysis in the present study provides indirect evidence that the same host species can be infected with different variants of the same *Entamoeba* species, since the two sequences from sheep are interspersed with cattle and reindeer isolates. The segregation of the two sheep isolates (Fig. 1) renders *E. ovis* a paraphyletic taxon in the event that the species name *E. bovis* is retained for the other isolates. Since the genetic distance between the cattle, sheep and reindeer isolates is relatively small (2-3%), we believe that these isolates represent genotypes of the same species and that the cattle, sheep and reindeer isolates all belong to what should be termed *E. bovis* (Liebetanz, 1905). Whether roe deer are indeed infected with a separate species, as suggested here, remains to be established. This will require more extensive sampling of entamoebae in ruminants and other ungulates.

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208 **References**

209 Burrows, R. B., Klink, G. E., 1955. *Endamoeba polecki* infections in man. Am. J. Hyg. 62,
210 156—167.
211 Bray, R. S., 1964. A check-list of the parasitic protozoa of West Africa with some notes of
212 their classification. Bull. Inst. Fr. Afr. Noire 26, 238—315.
213 Clark, C. G., Kaffashian, F., Tawari, B., Windsor, J. J., Twigg-Flesner, A., Davies-Morel, M.
214 C. G., Blessmann, J., Ebert, F., Peschel, B., Van, A. L., Jackson, C. J.,
215 Macfarlane, L., Tannich, E., 2006. New insights into the phylogeny of
216 *Entamoeba* species provided by analysis of four new small-subunit rRNA
217 genes. Int. J. Syst. Evol. Microbiol. 56, 2235—2239.
218 Dobell, C., 1919. The Amoebae Living in Man. A Zoological Monograph. J. Bale, Sons, and
219 Danielson, London.
220 Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large
221 phylogenies by maximum likelihood. Syst. Biol. 52, 696—704.
222 Huelsenbeck, J. P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic
223 trees. Bioinformatics 17, 754—755.
224 Kingston, N., Stabler, R. M., 1978. Two species of *Entamoeba* from white-tailed deer,
225 *Odocoileus virginianus*, from Georgia. J. Parasitol. 64, 14—16.
226 Kumar, S., Nei, M., Dudley, J., Tamura, K., 2008. MEGA: a biologist-centric software for
227 evolutionary analysis of DNA and protein sequences. Brief. Bioinform. 9,
228 299—306.
229 Lebbad, M., Ankarklev, J., Tellez, A., Leiva, B., Andersson, J. O., Svärd, S., 2008.
230 Dominance of *Giardia* assemblage B in Léon, Nicaragua. Acta Trop. 106, 44—
231 53.

Levin, R. L., Amstrong, D. E., 1970. Human infection with *Entamoeba polecki*. Am. J. Clin. Pathol. 54, 611—614.

Levine, N. D., 1973. Protozoan Parasites of Domestic Animals and of Man. Burgess Publishing Company, Minneapolis, Minnesota.

Mackinnon, D. L., Dibb, M. J., 1938. Report on intestinal protozoa of some mammals in the Zoological Gardens at Regent's Park. Proc. Zool. Soc. London. B. 108, 323—346.

Noble, G. A., 1955. *Entamoeba bubalus*, n.sp., from Carabao. J. Eukaryot. Microbiol. 2, 19—20.

Noble, G. A., 1954. *Entamoeba dilimani* sp. nov. from Philippine goats. Phil. J. Sci. 83, 113—116.

Noble, G. A., Noble, E. R., 1952. Entamoebae in farm animals. J. Parasitol. 38, 571—595.

Prowazek, S. von, 1912. *Entamoeba.* Arch. Protistenk. 25, 273—274.

Silberman, J. D., Clark, C. G., Diamond, L. S., Sogin, M. L., 1999. Phylogeny of the genera *Entamoeba* and *Endolimax* as deduced from small subunit ribosomal RNA gene sequence analysis. Mol. Biol. Evol. 16, 1740—1751.

Skirnisson, K., Hansson, H., 2006. Causes of diarrhoea in lambs during autumn and early winter in an Icelandic flock of sheep. Icel. Agric. Sci. 19, 43—57.

Triffitt, M. J., 1926. Observations on amoebae found in the faeces of certain African ungulates. Protozoology 2, 27—30.

Verweij, J. J., Polderman, A. M., Clark, C. G., 2001. Genetic variation among human isolates of uninucleated cyst-producing *Entamoeba* species. J. Clin. Microbiol. 39, 1644—1646.

Figure legend

Fig. 1. Phylogenetic analysis of the *Entamoeba* ssrRNA gene sequences. The six new sequences from this study (Cow349, Cow351, Sheep297, Sheep310, Reindeer100 and Roedeer352) were incorporated into an existing alignment consisting of 1,572 unambiguously aligned positions (Clark et al., 2006). The tree from the Bayesian analysis is shown. Bootstrap support and posterior probabilities are shown at each node in the order: maximum-likelihood/distance/Bayesian analysis. An asterisk indicates that support for a node by one method was less than 50% and unlabelled nodes indicate that two or more analyses gave less than 50% support. Bar: 0.05 substitutions per site.

269 Table 1. Uninucleated cyst-producing *Entamoeba* spp. reported from ungulates. For a detailed morphological description of the various
270 cysts and species, please refer to works by Levine (1973) and Noble and Noble (1952).

Species/name[a]	Host	Cyst size (mean)[b]	Reference
Entamoeba bovis	Cattle (*Bos taurus*)	4—15 µm (8.8 µm)	Noble and Noble, 1952
	White-tailed deer (*Odocoileus virginianus*)	6—11 µm (8.2 µm)	Kingston and Stabler, 1978
	Gnu (*Connochaetes taurinus*)	6—13 µm (9.0 µm)	Mackinnon and Dibb, 1938
	Bay Duiker (*Cephalophus dorsalis*)	N/A	Bray, 1964
Entamoeba ovis	Sheep (*Ovis aries*)	4—13 µm (7.2 µm)	Noble and Noble, 1952
	Sable antelope (*Hippotragus niger*)	N/A	Triffitt, 1926
	Water buck (*Cobus ellipsiprymus*)	N/A	Triffitt, 1926
	Eland (*Oreas canna*)	5—12 µm (N/A)	Triffitt, 1926
Entamoeba debliecki[c]	Goat (*Capra hircus*)	4—12 µm (6.4 µm)	Noble and Noble, 1952
Entamoeba dilimani	Goat (*Capra hircus*)	5—16 µm (9.7 µm)	Noble, 1954
Entamoeba bubalus	Carabao (*Bubalus bubalis carabanensis*)	5—9 µm (8.0 µm)	Noble, 1955

Entamoeba suis	Pig (*Sus domesticus*)	9.5—15.5 µm (12.85 µm)	Clark et al., 2006
Entamoeba polecki	Pig (*Sus domesticus*)	10—12 µm (N/A)	Prowazek, 1912
	Pig (*Sus domesticus*)	4—17 µm (8.09 µm)	Noble and Noble, 1952
	Wild boar (*Sus scrofa*)	9—15 µm (N/A)	Mackinnon and Dibb, 1938
	Indian boar (*Sus cristatus*)	9—15 µm (N/A)	Mackinnon and Dibb, 1938
	Giant Forest hog (*Hylochoerus meinertzhageni*)	9—15 µm (N/A)	Mackinnon and Dibb, 1938

[a]*Entamoeba gedoelsti* and *Entamoeba caprae* have been found in horses and a goat, respectively, but since cysts have not been reported, these species are not included in the table.

[b]Rounded figures.

[c]*Entamoeba debliecki* has been considered a small variant of *E. suis* or synonymous with *E. polecki* (Noble and Noble, 1952; Burrows and Klink, 1955).

N/A, data not available.

278 Table 2. *Entamoeba* samples included in this study. See text for details.

Sample ID	Host	Country of origin	Cyst diameter of 100 cysts (mean) [S.D.]	Accession number of corresponding sequence
Cow349	*Bos taurus*	Sweden	3.9—14.4 µm (6.6 µm) [1.80]	**FN666248**
Cow351	*Bos taurus*	Sweden	N/A	**FN666249**
Sheep310	*Ovis aries*	Sweden	5.4—13.8 µm (7.2 µm) [1.36]	**FN666250**
Sheep297	*Ovis aries*	Sweden	N/A	**FN666251**
Reindeer100	*Rangifer tarandus*	Iceland	N/A	**FN666252**
Roe Deer352	*Capreolus capreolus*	Sweden	7.2—12.0 µm (9.3 µm) [0.77]	**FN666253**

279 N/A, data not available.

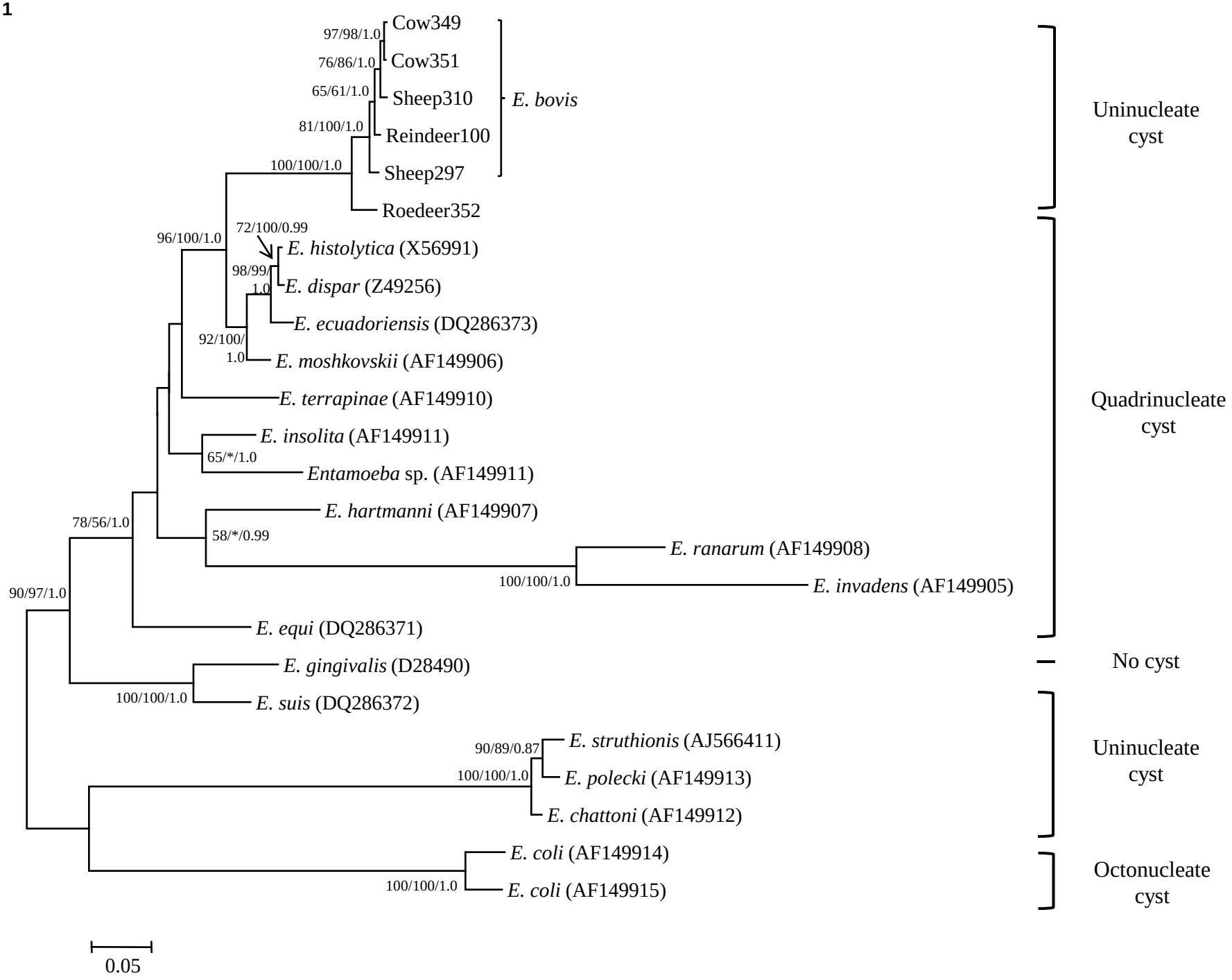
280 Table 3. Primers used to obtain the full sequence of the *Entamoeba* ssrRNA gene from all ruminant isolates.

Primer ID	Sequence (5'-3')	Reference
RD5	ATCTGGTTGATCCTGCCAGT	Clark et al., 2006
RD3	ATCCTTCCGCAGGTTCACCTAC	Clark et al., 2006
AEMH3.1	AAGGGCATCACGGACCTGTT	Clark et al., 2006
EntOv_430F	GTAGTGACGACAAATAACTCTTG	Present study
EntOv_1200F	GAAAACTTACCAAGACCGAACAG	Present study
EntUng_500R	CCTCCAATTGATTTCTTTAGAG	Present study
EntUng_900R	TTTCGTTCTTGATTAATGAACG	Present study

281

282

Figure 1


Cow349
Cow351
Sheep310
Reindeer100
Sheep297
Roedeer352
E. bovis
97/98/1.0
76/86/1.0
65/61/1.0
81/100/1.0
100/100/1.0
72/100/0.99
96/100/1.0
E. histolytica (X56991)
98/99/1.0
E. dispar (Z49256)
E. ecuadoriensis (DQ286373)
92/100/1.0
E. moshkovskii (AF149906)
E. terrapinae (AF149910)
E. insolita (AF149911)
65/*/1.0
Entamoeba sp. (AF149911)
E. hartmanni (AF149907)
78/56/1.0
58/*/0.99
E. ranarum (AF149908)
100/100/1.0
E. invadens (AF149905)
90/97/1.0
E. equi (DQ286371)
E. gingivalis (D28490)
100/100/1.0
E. suis (DQ286372)
90/89/0.87
E. struthionis (AJ566411)
100/100/1.0
E. polecki (AF149913)
E. chattoni (AF149912)
E. coli (AF149914)
100/100/1.0
E. coli (AF149915)
0.05
Uninucleate cyst
Quadrinucleate cyst
No cyst
Uninucleate cyst
Octonucleate cyst