Agric Res (December 2013) 2(4):360–366
DOI 10.1007/s40003-013-0071-9

FULL-LENGTH RESEARCH ARTICLE

Molecular diversity of protozoa in rumen of Indian buffalo (*Bubalus bubalis*)

K. M. Singh · P. R. Pandya · A. K. Tripathi · G. R. Patel · S. Parnerkar · R. K. Kothari · C. G. Joshi

Received: 4 August 2012 / Accepted: 17 July 2013 / Published online: 29 August 2013

Abstract Protozoa communities in buffalo rumen were characterized using 18S rRNA gene library prepared from the pooled DNA sample obtained from three adult animals. A total of 172 clones were sequenced, which were grouped into 53 operational taxonomic units (OTUs) based on unique 18S r DNA sequences with 95 % confidence intervals. Phylogenetic analyses showed that 40 OTUs (124 of 172 clones) belonged to uncultured protozoa group, indicating that this group is most dominant component of protozoa resident in rumen of Indian buffalo. 44 clones (12 OTUs) belonged to the class *Kinetofragminophorea*. Among *Kinetofragminophorea*, 44 clones fell into two species identified as *Dasytricha ruminantium*-like clone (27 clones) and *Isotricha prostoma*-like clone (17 clones). These include 11 single-clone OTUs, so Good's coverage (93.75 %) of 18S rRNA libraries indicated that the sequences identified in the libraries represent the majority of protozoa diversity present in rumen.

Keywords Buffalo rumen · Protozoa · 18S rDNA · Phylogenetic analysis

Introduction

Protozoa are unicellular eukaryotic microorganisms which are ubiquitous in nature and anthropogenic environments. The rumen ciliates are potentially an agriculturally important group of protozoa found in domestic and wild ruminants [30]. Several factors seem to influence the composition of the protozoan population in the rumen. These include type and amount of feed consumed, pH, turnover rate, and feed level. The rumen microorganisms utilize carbohydrates as a carbon source in energy-yielding processes, whereas hydrogen gas (H_2) is released as byproduct during ATP generation. In the protozoa, in turn, benefit from hydrogen removal by the methanogens because hydrogen is inhibitory to protozoan metabolism [32]. Earlier reports have provided evidence of strong relationships, such as endosymbiosis between ruminal protozoa and methanogens [8, 28]. Ciliate protozoa play a diverse role in the ruminal metabolism of nutrients. To improve the efficiency of feed crude protein utilization, considerable effort has been made to find a means of total elimination of protozoa from the rumen (defaunation) and a massive reduction in the rumen protozoan population (reduced fauna). Chemical drenching of experimental animals has been found to improve milk production. Detection and identification of protozoa have commonly been achieved through microscopic examination of morphological features. It remains difficult and time-consuming to reliably detect or identify many protozoan species by these

K. M. Singh (✉) · A. K. Tripathi · C. G. Joshi
Department of Animal Biotechnology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand 388 001, Gujarat, India
e-mail: kmsingh18@gmail.com

P. R. Pandya · G. R. Patel · S. Parnerkar
Animal Nutrition Research Station, AAU, Anand, Gujarat, India

R. K. Kothari
Department of Microbiology, Christ College, Rajkot, Gujarat, India

K. M. Singh
Department of Genetics, ARIBAS, New V V Nagar, Anand, India

methods, as protozoa may be fragile and inconspicuous and as it may be difficult to determine whether a given morphological feature can be regarded as distinct or not [3, 9, 23]. The anaerobic ruminal protozoa have been well studied [31], but much of this work is based on microscopic examination [4]. Difficulties in cultivating protozoa, and their polymorphic nature, have delayed effective assessment of protozoan ecology and taxonomy [5]. The small subunit ribosomal RNA (SSU-rRNA) gene called 16S rRNA in prokaryotes and 18S rRNA in eukaryotes is widely used as molecular marker to identify morphologically indistinguishable species, to infer their phylogenetic relationships, and to elucidate diversity. PCR-sequencing methods have been extensively used to examine the protozoal diversity in rumen samples [13]. PCR–DGGE has also been used in the profiling of protozoal communities in the rumen [20, 24].

India possesses more than 50 % of world's buffalo population; Indian buffalo produce more than 50 % milk in India [15]. Surti is a popular breed of buffalo found in central Gujarat state. The Surti buffaloes are of medium size having docile temperament and body weight 300–350 kg at maturity. Since our animals mainly sustain on crop residues, the protozoan population is expected much different than that of exotic cattle. The present study was conducted to examine the diversity of rumen protozoa in Surti buffalo offered diet, green fodder bajra (*Pennisetum purpureum*), mature pasture grass (*Dichanthium annulatum*), and compound concentrate mixture. The molecular techniques were used to construct a library of 18S rDNA clones of rumen protozoa, and a phylogenetic tree for the clones isolated.

Materials and Methods

Sampling and DNA Extraction

The permission of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) was obtained prior to initiation of the study. The experiments were carried out on three adult Surti buffaloes (*Bubalus bubalis*), approximately 3 years of age with a mean live weight of 201 ± 18 kg, which were reared at the Department of Animal Nutrition, College of Veterinary Science and A.H. Anand. All the animals were maintained under uniform feeding regime (ICAR 1998) for minimum 21 days for dietary adaptation. The diet comprised of green fodder bajra (*P. purpureum*), mature pasture grass (*D. annulatum*), and compound concentrate mixture (20 % CP, 65 % TDN). The animals were offered 10 kg green, ad-lib dry grass and 2.5 kg of concentrate mixture daily. Animals were let loose daily for 2 h in the morning and evening during which they had free access to drinking water. Approximately 500 ml of rumen fluid was collected after 21 days (dietary adaptation) using flexible tube at 0, 2, 4, and 6 h after feeding [14]. About 100 ml rumen fluid was passed through four layers of cheese cloth (autoclave) in laminar air flow to remove particulate matter. Remaining rumen fluid was stored at −80 °C for further study. Total DNA (0, 2, 4, 6 h × 3 animals) were extracted from pooled sample by using a commercially available kit according to the manufacturer's instructions (QIAGEN Stool kit; QIAGEN, CA). The total DNA mixture was used as a template in PCR to amplify 18S r DNA.

PCR Primers and Amplification

A protozoa-specific forward primer (5′-ACTTTCGATGGTAGTGTATTGGACTAC-3′) was used with a Eukarya-specific reverse primer (5′-ATGATCCTTCTGCAGGTTCACCTAC-3′) [19]. Subsequently, 18S r DNA fragment were amplified by PCR using the metagenomic DNA. A total of 25 µl of reaction mixture consisted of 10 pmol of each primer, 30 ng of template DNA, and 12.5 µl of master mix (Fermentas, UK). The PCR amplification was performed by Thermal Cycler (ABI, USA) and the PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 30 cycles of amplification consisting of a 1-min denaturation step at 94 °C, a 1-min annealing step at 37 °C, a 3-min extension step at 72 °C, and a final 10-min extension at 72 °C. The anticipated product of approximately 1.36 kb was cleaned using a Qiagen DNA Gel Extraction Kits (QIAGEN, CA) in accordance with the directions of the manufacturer.

Cloning and Sequencing

The purified PCR products were cloned in InstaT/A cloning kit (Fermentas, UK) as per the instructions of the manufacturer. The recombinant plasmids after were extracted by the Qiagen mini-prep plasmids extraction kit (QIAGEN, CA). Plasmid inserts were amplified with primers M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) and nucleotide sequences of cloned genes were determined by sequencing with M13F/M13R primer in ABI Prism 310 Genetic analyser (Applied Biosystems Inc., CA) using BigDye Terminator (version 3.1) at the Animal Biotechnology Laboratory, AAU, Anand, Gujarat, India. Finally a total of 172 clones were sequenced.

Sequence Analysis

All reference sequences were obtained from the GenBank/EMBL/DDBJ/ [2]. Sequences (∼550 bp) from the current

study were analysed by the CHECK_CHIMERA program [18] to remove any chimera rDNA clone. The similarity searches for sequences were carried out by BLAST (http://www.ncbi.nlm.nih.gov/ BLAST/Blast.cgi [17] and alignment was done using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html [26]. Ambiguously and incorrectly aligned positions were aligned manually. The distance matrix was calculated using the DNADIST program included in PHYLIP [7] and used to assign sequences in various operational taxonomic units (OTUs) or phylotypes by DOTUR [21] with 95 % confidence intervals to quantify the diversity of phylotypes and total of 53 OTUs were distinguished, based on unique 18S rDNA sequences. The percentage of Good's coverage was calculated as $[1 - (n/N)] \times 100$, where 'n' is the number of single-clone OTUs and 'N' is the library size. Phylogenetic tree was constructed by the neighbor-joining method using MEGA 4.0 [25]. Bootstrap re-sampling analysis for 1,000 replicates was performed to estimate the confidence of tree topologies [7].

Nomenclature and Nucleotide Sequence Accession Numbers

The prefix IBRP was used to denote OTU identified and nucleotide sequences have been deposited in the GenBank database under the accession numbers EU345005 to EU345176.

Results

Sequence Analysis

All the sequences (172) were subjected to online homology search in GenBank [2] which implements the BLAST algorithm [17]. The summary of Blast result are given in Table 1. The sequences generated in this study showed 79–96 % sequence similarities with the sequences of protozoa available in the GenBank. The clones sequenced in this study were grouped into 53 OTUs, based on rDNA sequences. Of the 53 OTUs, 12 had >90 % similarity with the 18S rDNA sequences available in the GenBank, 27 had 86–90 % similarity and 14 had <85 % similarity.

Phylogenetic Analysis

The phylogenetic analysis of the sequences (Fig. 1) showed that 40 OTUs, representing 124 clones, belonged to unidentified protozoa. Twelve OTUs (44 clones) belonged to the class *Kinetofragminophorea*. Within the *Kinetofragmi nophorea*, seven OTUs, representating 27 clones, grouped with *Dasytricha ruminantium*-like clones and five OYUs representing 17 clones grouped with *Isotricha prostoma*-like clones. The *phylogenetic analysis indicate that the OTU19 (4 Clones) belonged to haptorida*. However, Blast results hit with known protozoa i.e. *Troglodytella abrassarti*. This is due to very low similarity (Table 2). Thus, 18S rRNA sequences obtained from rumen formed tightly-clustered affiliated to the different groups. The total 11 single-clone OTUs, so Good's coverage (93.75 %) of 18S rDNA libraries indicated that the sequences identified in libraries represent the protozoan diversity in the rumen.

Discussion

The molecular inventory of protozoa revealed in present study showed the occurrence of complex protozoa communities in buffalo rumen ecosystem. The number and distribution of phylotypes indicates the protozoa diversity in rumen of Surti buffalo. Compared to other ecosystems, there is no previous information about the rumen protozoa of Indian buffalo. The relative lack of information on ruminal protozoa may be due to difficulties with isolation, culture, or maintenance. Rumen isolates often lose viability for unknown reasons during purification or sub culturing of pure isolates. More than 24 genera of ruminal protozoa have been described based on cultivation and morphological studies [4, 29]). Although, sequences of few genera are available in sequence databases. Most genera are representatives of typical bovine rumen populations viz *Entodinium*, *Diplodinium*, *Eudiplodinium*, *Ostracodinium*, *Metadinium*, *Enoploplastron*, *Polyplastron*, *Epidinium*, *Ophryoscolex*, *Isotricha*, and *Dasytricha* [13, 30]. The present study revealed the phylogenetic diversity of the protozoan community in the rumen fluid of Surti buffalo by analyzing protozoan 18S rDNA sequences. BLASTn searches showed that sequenced clones shared similarity (79–96 %) with ruminal protozoan sequences with GenBank database. The rumen fluid library was classified into three phylogenetic groups. The largest group was affiliated with the *unidentified* protozoa (40 OTUs, 124 clones), second group affiliated with the *Kinetofragminophorea* protozoa (12 OTUs, 44 clones), and third the group affiliated with the *Haptorida* protozoa (01 OTU, 04 clones). The predominant protozoa identified in this study were the unidentified group (Table 2). These results agree with the report of *Isotricha* and *Dasytricha* genera in the cattle rumen [13, 16]. High number of *Entodinium* sp. has been reported by Akbar et al. [1] in Ghizel Sheep fed in pasture and nourished by dried grape by-product and Leng et al. [16] in Yunnan Yellow Cattle fed malt meal. Interestingly,

Table 1 Similarity values of 18S rRNA sequences retrieved from the rumen fluid of Surti buffaloes

OTU	No. of clone	Nearest relative	Accession no.	Similarity (%)
IBRP1	02	*Ostracodinium gracile*	AM158468	87
IBRP2	04	Uncultured rumen protozoa	AF502929	89
IBRP3	03	*Ostracodinium gracile*	AM158468	89
IBRP4	12	Uncultured Canadian Arcott wether rumen protozoa	DQ832560	88
IBRP5	04	*Ostracodinium gracile*	AM158468	90
IBRP6	04	Uncultured Canadian Arcott wether rumen protozoa	DQ832560	87
IBRP7	03	*Cycloposthium ishikawai*	EF632076	86
IBRP8	04	*Cycloposthium edentatum*	EF632077	86
IBRP9	06	Uncultured rumen protozoa clone YCRPB55	EU163779	87
IBRP10	03	*Isotricha prostoma*	AM158455	88
IBRP11	05	Uncultured Canadian Arcott wether rumen protozoa	DQ832560	94
IBRP12	06	Uncultured Canadian Arcott wether rumen protozoa	DQ832564	92
IBRP13	01	Uncultured ciliate	AM158846	86
IBRP14	03	*Ostracodinium gracile*	AM158468	88
IBRP15	05	*Isotricha prostoma*	AM158455	92
IBRP16	02	Uncultured rumen protozoa	EU163783	81
IBRP17	02	*Ostracodinium gracile*	AM158468	89
IBRP18	10	*Isotricha prostoma*	AM158455	91
IBRP19	04	*Troglodytella abrassarti*	AB437347	86
IBRP20	02	Uncultured Canadian Arcott wether rumen protozoa	DQ832565	84
IBRP21	03	Uncultured rumen protozoa clone CRA9	AF502927	86
IBRP22	06	*Teuthophrys trisulca africana*	DQ411863	85
IBRP23	08	Uncultured rumen protozoa clone YCRPB55	EU163779	89
IBRP24	11	*Dasytricha ruminantium*	AM158463	91
IBRP25	02	Uncultured rumen protozoa	EU163779	84
IBRP26	02	Uncultured rumen protozoa clone CRA5	AF502923	83
IBRP27	06	Uncultured rumen protozoa clone YCRPB55	EU163779	89
IBRP28	02	Uncultured ciliate	AM158846	89
IBRP29	02	Uncultured ciliate	AM158873	91
IBRP30	07	*Dasytricha ruminantium*	AM158463	86
IBRP31	02	*Ostracodinium gracile*	AM158468	87
IBRP32	04	*Ostracodinium gracile*	AM158468	89
IBRP33	01	*Isotricha prostoma*	AM158454	84
IBRP34	02	Uncultured rumen protozoa clone YCRPB59	EU163783	85
IBRP35	04	*Dasytricha ruminantium*	AM158463	87
IBRP36	01	Uncultured rumen protozoa	EU163779	93
IBRP37	01	Uncultured rumen protozoa clone YCRPB55	EU163779	86
IBRP38	02	Uncultured rumen protozoa clone YCRPB55	EU163779	91
IBRP39	01	*Polyplastron multivesiculatum*	AM158458	92
IBRP40	03	Uncultured rumen protozoa clone YCRPB59	EU163783	91
IBRP41	01	*Ostracodinium gracile*	AM158468	89
IBRP42	02	Uncultured Canadian Arcott wether rumen protozoa	DQ832560	83
IBRP43	01	*Dasytricha ruminantium*	AM158463	86
IBRP44	01	*Isotricha prostoma*	AM158456	84
IBRP45	02	*Cycloposthium ishikawai*	EF632076	89
IBRP46	02	Uncultured rumen protozoa clone YCRPB59	EU163783	82
IBRP47	01	Uncultured rumen protozoa clone YCRPB1	EU163725	91
IBRP48	02	Uncultured ciliate	AM158846	96

Table 1 continued

OTU	No. of clone	Nearest relative	Accession no.	Similarity (%)
IBRP49	01	*Ostracodinium gracile*	AM158468	80
IBRP50	01	Uncultured rumen protozoa clone YCRPB65	EU163789	79
IBRP51	01	*Isotricha prostoma*	AM158454	86
IBRP52	01	*Ostracodinium gracile*	AM158468	80
IBRP53	01	Uncultured rumen protozoa clone YCRPB59	EU163783	82

Total = 172 clones

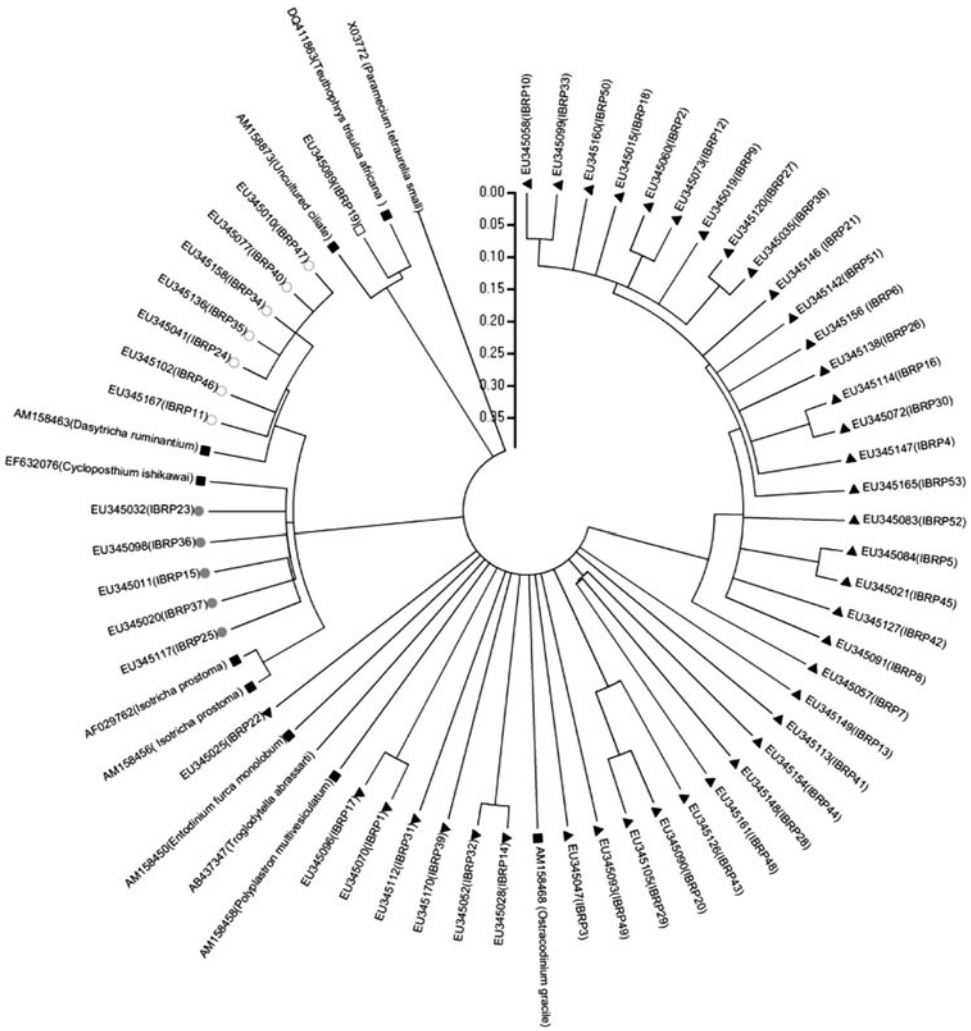

Fig. 1 Phylogenetic relationships of partial 18S rRNA sequences of clones recovered from Surti buffalo rumen samples. The rooted tree was inferred by the neighbor-joining method with 1,000 bootstrap replicates using the MEGA 4 tree building program. The *Paramecium tetraurelia* (X03772) are used as the out-group for rooting the tree. The scale bar represents 5 % sequence divergence. The symbol *filled square* indicates the reference sequences, *open circle* indicates *Dasytricha ruminantium*-like clone, *filled circle* indicates *Isotricha prostoma*-like clone, *open square* indicates haptorida protozoa, and *filled triangle* indicates unidentified protozoa; reference sequence (AB437347: *Troglodytella abrassarti*)

we could not detect the sequences related to *Entodinium* sp.; this may be due to the diet composition. Dehority and Odenyo [6] suggest that a selection for highly concentrated feed stuffs would lead to an *Entodinium* only fauna, as they observed in Grant's gazelle. Karnati et al. [13] reported that their protozoan-specific primers (used here) had a single mismatch with the 18S rDNA of *Entodinium* sp., but matched exactly with the 18S rDNA sequences of other protozoan species. They suggested that a PCR primer degenerates at the mismatched position would help minimize PCR bias, allowing for more representative retrieval of ruminal protozoan 18S rDNA from complex ruminal samples. The diversities of different clone libraries of rumen protozoa have been given in Table 3.

However, *Isotricha* sp. and *Polyplastron* sp. were identified in the washed ciliate suspension and many

Table 2 Analysis of 18S rDNA phylotypes diversity retrieved from the rumen fluid of Surti buffaloes

Taxon	No. of operational taxonomic units (OTUs)	No. of clones
1.Unidentified protozoa	40	124
2. Kinetofragminophorea	12	44
a. *Dasytricha ruminantium*-like clone	07	27
b. *Isotricha prostoma*-like clone	05	17
3. Haptorida protozoa	01	04
Total	53	172

Table 3 The diversity comparing of different clone libraries of rumen protozoa

Clone libraries	Karnati et al. [13]		Shin et al. [22]	Leng et al. [16]		Fuente et al. [10]	Our library
Animals	Cow		Cow	Yunnan yellow cattle		Spanishi bex	Surti buffalo
Clone numbers	12	11	37	66	55	8	172
Diets	Ration (1:1)[b]	Alfalfa	Ration (1:4)[c]	Malt meal	Straw	Pasture	Mix ration[d]
Clone distribution (%)							
Entodinium	97.3	77.2	81.1	72.7	60.0	>90	ND
Dipladenia[a]	1.7	4.6	ND	ND	ND	ND	>13.0
Epidinium	0.3	<0.5	18.9	ND	ND	ND	ND
Ophryoscolex	ND	0.9	ND	ND	ND	ND	ND
Isotricha	<0.3	6.0	ND	16.7	ND	ND	>13.0
Dasytricha	0.7	11.2	ND	10.6	ND	ND	>10.0

ND not detectable

[a] Subfamily, containing the genera *Diplodinium*, *Eudiplodinium*, *Ostracodinium*, *Metadinium*, *Enoploplastron*, and *Polyplastron*

[b] The diet contained 50 % forage (mixture of corn silage and haylage) and 50 % concentrate (corn grain and soybean meal) on a DM basis

[c] 4:1 rice hull to concentrated

[d] The diet contained: green fodder Napier bajra 21 (*Pennisetum purpureum*), mature pasture grass (*Dichanthium annulatum*), and concentrate mixture (20 % crude protein, 65 % total digestible nutrients)

methanogens were detected on ciliate cells by F420 auto fluorescence [27]. Similar results were also observed in our studies. Biochemical differences between *Isotricha* and *Dasytricha* have been examined by [11, 12]. *Dasytricha* is more versatile than *Isotricha*, fermenting cellobiose, galactose, and maltose. Fermentation products from galactose were the same as those formed from glucose. From glucose, the holotrich produced lactic, acetic, and butyric acids, carbon dioxide, hydrogen, and traces of propionic acid [12]. A single OTU (IBRP19) located within the haptorida protozoa and may represent a dietary transient.

Conclusions

In conclusion, Surti buffalo rumen harbors protozoal community in the rumen that is composed of several genera including, *Dasytricha*, *Isotricha*, *Ostracodinium*, *Polyplastron*, *Cycloposthium*), *Teuthophrys*, and *Troglodytella*. While majority of the sequences were unidentified. An advanced set of protozoan-specific phylogenetic probes and quantitative real time PCR assay are needed to their distribution throughout rumen microbial communities. Future studies to understand the effects of varying rumen protozoa on different animal feeding habits, digestion and methanogens will also be important.

Acknowledgments Financial support provided by the Department of Biotechnology, Govt. of India, New Delhi is gratefully acknowledged.

References

1. Akbar T et al (2009) The study of diversity of ciliate protozoa in Ghizel sheep fed in pasture and nourished by dried grape by-product. Am J Anim Vet Sci 4(2):37–41
2. Benson DA et al (2007) GenBank. Nucleic Acids Res 35:D1–D25
3. Caron DA et al (2004) The growing contribution of molecular biology and immunology to protistan ecology: molecular signatures as ecological tools. J Eukaryot Microbiol 51:38–48
4. Dehority BA (1993) Laboratory manual for classification and morphology of rumen ciliate protozoa. CRC Press, Boca Raton

5. Dehority BA (1994) Rumen ciliate protozoa of the blue duiker (*Cephalophus monticola*), with observations on morphological variation lines within the species *Entodinium dubardi*. J Eukaryot Microbiol 41:103–111
6. Dehority BA, Odenyo AA (2003) Influence of diet on the rumen protozoal fauna of indigenous African wild ruminants. J Eukaryot Microbiol 50:220–223
7. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
8. Finlay BJ et al (1994) Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiol Lett 117:57–162
9. Fried J et al (2002) Improvement of ciliate identification and quantification: a new protocol for fluorescence in situ hybridization (FISH) in combination with silver stain techniques. Syst Appl Microbiol 25:555–571
10. Fuente G et al (2009) Rumen protozoal diversity in the Spanish ibex (*Capra pyrenaica hispanica*) As compared with domestic goats (*Capra hircus*). Eur J Protistol 45:112–120
11. Gutierrez J (1955) Experiments on the culture and physiology of holotrichs from the bovine rumen. Biochem J 60(3):516–522
12. Howard BH (1959) The biochemistry of rumen protozoa: carbohydrate fermentation by *Dasytricha* and *Isotricha*. Biochem J 71(4):671–675
13. Karnati SKR et al (1994) Technical note: specific PCR amplification of protozoal 18S rDNA sequences from DNA extracted from ruminal samples of cows. J Anim Sci 81:812–815
14. Khamapa S et al (2006) Effects of urea level and sodium dimalate in concentrate containing high cassava chip on ruminal fermentation efficiency, microbial protein synthesis in lactating dairy cows raised under tropical condition. Asian Aust J Anim Sci 19:837–844
15. Kumar S et al (2007) Phylogeography and domestication of Indian river buffalo. BMC Evol Biol 7:186
16. Leng J et al (2011) Assessment of protozoa in Yunnan Yellow Cattle rumen based on the 18S rRNA sequences. Mol Biol Rep 38(1):577–585
17. Madden TL et al (1996) Application of network BLAST server. Methods Enzymol 266:131–141
18. Maidak BL et al (2001) The RDP-II (Ribosomal Database Project). Nucleic Acids Res 29:173–174
19. Medlin L et al (1998) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 1:491–499
20. Regensbogenova M et al (2004) A re-appraisal of the diversity of the methanogens associated with the rumen ciliates. FEMS Microbiol Lett 238:307
21. Schloss PD, Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol 71:1501–1506
22. Shin EC et al (2004) Phylogenetic analysis of protozoa in the rumen contents of cow based on the 18S rDNA sequences. J Appl Microbiol 97:378–383
23. Sims GP et al (2002) Identification and phylogenetic analysis of morphologically similar naked amoebae using small subunit ribosomal RNA. J Eukaryot Microbiol 49:478–484
24. Sylvester JT et al (2005) Evaluation of a real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal nitrogen. J Dairy Sci 88:2083–2095
25. Tamura K et al (2007) MEGA4: Molecular Evolutionary Genetics Analysis software version 4.0. Mol Biol Evol 24:1596–1599
26. Thompson JD et al (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
27. Tokura M et al (1999) Phylogenetic study of methanogens associated with rumen ciliates. Curr Microbiol 39:123–128
28. Ushida K et al (1997) Ciliate protozoa and ruminal methanogenesis. In: Onodera R, Itabashi H, Ushida K, Yano H, Sasaki Y (eds) Rumen microbes and digestive physiology in ruminants. Japan Sci Soc Press, Tokyo, pp 209–220
29. Williams AG (1986) Rumen holotrich ciliate protozoa. Microbiol Rev 50:25–49
30. Williams AG, Coleman GS (1992) In: Wright ADG (ed) The rumen protozoa. Springer, New York, pp 4–83
31. Williams AG, Coleman GS (1997) The rumen protozoa. In: Hobson PN, Stewart CS (eds) The rumen microbial ecosystem. Blackie Academic & Professional, New York
32. Wolin MJ (1974) Metabolic interactions among intestinal microorganisms. Am J Clin Nutr 27:1320–1328