Dasytricha *Dominance in Surti Buffalo Rumen Revealed by 18S rRNA Sequences and Real-Time PCR Assay*

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Abstract The genetic diversity of protozoa in Surti buffalo rumen was studied by amplified ribosomal DNA restriction analysis, 18S rDNA sequence homology and phylogenetic and Real-time PCR analysis methods. Three animals were fed diet comprised green fodder Napier bajra 21 (*Pennisetum purpureum*), mature pasture grass (*Dicanthium annulatum*) and concentrate mixture (20% crude protein, 65% total digestible nutrients). A protozoa-specific primer (P-SSU-342f) and a eukarya-specific primer (Medlin B) were used to amplify a 1,360 bp fragment of DNA encoding protozoal small subunit (SSU) ribosomal RNA from rumen fluid. A total of 91 clones were examined and identified 14 different 18S RNA sequences based on PCR–RFLP pattern. These 14 phylotypes were distributed into four genera-based 18S rDNA database sequences and identified as *Dasytricha* (57 clones), *Isotricha* (14 clones), *Ostracodinium* (11 clones) and *Polyplastron* (9 clones). Phylogenetic analyses were also used to infer the makeup of protozoa communities in the rumen of Surti buffalo. Out of 14 sequences, 8 sequences (69 clones) clustered with the *Dasytricha ruminantium*-like clone and 4 sequences (13 clones) were also phylogenetically placed with the *Isotricha* prostoma-like clone. Moreover, 2 phylotypes (9 clones) were related to *Polyplastron multivesiculatum*-like clone. In addition, the number of 18S rDNA gene copies of *Dasytricha ruminantium* (0.05% to ciliate protozoa) was higher than *Entodinium sp.* (2.0×10^5 vs. 1.3×10^4) in per ml ruminal fluid.

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Introduction

Protozoa can be defined as motile unicellular eukaryotes possessing a capacity for phagotrophy and comprise an extremely diverse assemblage of organisms commonly grouped into amoebae, ciliates, flagellates and intracellular parasitic apicomplexans [4, 10]. The rumen ciliates are potentially an agriculturally important group of protozoa found in domestic and wild ruminants [41] and play a diverse role in the ruminal metabolism of nutrients. Several factors seem to influence the composition of the protozoan population in the rumen, which include type and amount of feed consume, pH and turnover rate. Moreover, rumen protozoa utilize hydrogen-generating fermentation to provide substrates for methanogens, and in turn, benefited from hydrogen removal by the methanogens as hydrogen is inhibitory to protozoan metabolism [43]. Earlier reports have provided evidence of strong relationships such as endosymbiosis between ruminal protozoa and methanogens [11, 37].

The species and population of rumen protozoa were based on the host's feeding conditions, diet composition, physiological factors, breeds and diurnal variation

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[7, 12–14, 24, 25]. Morphological classification of protozoal species and the known effects on bacterial populations and host nutrition have been well described [5, 38, 41], but much of these work were based on microscopic examination. Although protozoa contribute to fibre degradation and slow the fermentation rate by engulfing starch granules [38], they also probably are the main antagonists for efficient protein utilization in the rumen microbial ecosystem [39, 40]. Further, rumen protozoa have complex growth requirements and most of protozoa are, therefore, difficult to culture. So, the microscopic examination methods may underestimate protozoal population due to the tendency of some species to lysis or dissolving during sample collection and processing [30]. The small subunit ribosomal RNA (SSU-rRNA) gene called 16S rDNA in prokaryotes and 18S rDNA in eukaryotes is widely used as molecular marker to identify morphologically indistinguishable species, to infer their phylogenetic relationships and to elucidate diversity.

India possesses more than 50% of world's buffalo population contributing more than 60% milk production [18]. Surti is a popular breed of buffalo found in central Gujarat state, which are of medium size and docile temperament with body weight 350–375 kg at maturity. Since our animals mainly sustain on crop residues, the protozoa population is expected much different than that of exotic cattle. In recent years, molecular methods based on 16S/18S rDNA and their encoding genes (rDNA) have been used extensively to study bacteria and fungi in rumen microbial ecology [3, 17, 26, 33, 48, 49]. PCR-sequencing methods have been extensively used to examine protozoal diversity in rumen samples [15, 32]. PCR-DGGE and real-time PCR have also been used in the profiling of protozoal communities in the rumen [27, 28, 31].

In present study, we retrieved and analysed of 18S rDNA sequences to examine phylogenetic diversity and population load in Surti buffalo rumen offered green fodder bajra (*Pennisetum purpureum*), mature pasture grass (*Dicanthium annulatum*) and compound concentrate mixture in the diet.

Materials and Methods

Sampling and Total DNA Extraction

The experiment was carried out on 3 adult Surti buffaloes, at approximately 3 years of age and with a mean live weight of 201 ± 18 kg, reared at the Department of Animal Nutrition, College of Veterinary Science and Animal Husbandry, Anand. All the animals were maintained under a uniform feeding regime for at least 21 days. The diet comprised green fodder Napier bajra 21 (*Pennisetum purpureum*), mature pasture grass (*Dicanthium annulatum*) and concentrate mixture (20% crude protein, 65% total digestible nutrients). The animals were offered 10 kg green, ad-lib dry grass and 2.5 kg of concentrate mixture daily. All Animals were let loose daily for 2 h morning and evening, during which they had free access to drinking water. 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.

Approximately 500 ml of rumen fluid was collected before the morning feeding [16] and squeezed through two layers of sterilized cheesecloth and stored at −80°C. Total DNA was extracted separately by using a commercially available kit according to the manufacturer's instructions (QIAGEN Stool kit; QIAGEN, CA).We finally pooled all the DNA samples, which was used as a template in PCR to amplify 18S rDNA.

PCR Primer and Amplification

Approximately 1,360 bp of 18S rRNA gene were amplified using the protozoa-specific primer P-SSU-342f (5′-ACTT TCGATGGTAGTGTATTGGACTAC-3′) [15] and reverse primer Medlin B (5′-ATGATCCTTCTGCAGGTTCACC TAC-3′) [23]. A total of 25 µl of reaction mixture consisted of 10 pmol of each primer, 100–150 ng of template DNA and 12.5 µl of Master mix (Fermentas, UK). The PCR amplification was performed by Thermal Cycler (ABI, USA) using the following program: denaturing at 95°C for 5 min, followed by 35 cycles of 30 s of denaturing at 95°C, 30 s of annealing at 37°C, 2 min of elongation at 72°C and final extension at 72°C for 10 min. The PCR products were separated by electrophoresis in 1.20% (w/v) agarose gel and stained with ethidium bromide. The anticipated product of approximately 1.36 kb was separated by agarose gel electrophoresis and cleaned by using a QIAquick DNA Gel Extraction Kit (QIAGEN, CA) in accordance with the directions of the manufacturer.

Cloning, Screening and RFLP

The purified PCR products were cloned using InstaTA cloning kit (Fermentas, UK) according to the manufacturer's protocol into *E coli* DH5-α. The blue and white colonies were screened on Luria–Bertani plates with 100 µg ampicillin ml and X-gal (0.5 mM) which resulted into 91 positive clones. Plasmid DNA was extracted by lysis of the clones at 95°C for 5 min followed by centrifugation. The supernatant containing plasmid DNA was further amplified with plasmid-specific primers M13f (5′-GTAAAACGA CGGCCAG-3′) and M13r (5′-CAGGAAACAGCTAT GAC-3′). The total 25 µl reaction mixture volume consisted of 12.5 µl of Master mix (Fermentas, UK), 10 pmol of each

primer, 3.0 µl plasmid DNA and remaining nuclease-free water. PCR condition was denaturation for 5 min at 94°C, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min and the final extension at 72°C for 10 min. Aliquots (10 ul) of all successfully recovered cloned sequences were digested with 0.5 U of the restriction endonuclease HaeIII at 37°C for overnight as described by Wright et al. [44]. The digested DNA was visualized by electrophoresis on 1.5% agarose gels. Each different restriction profile was assumed to indicate a different sequence. At least one clone corresponding to each different RFLP profile was purified and used for sequencing.

Sequence and Phylogenetic Analysis

Sequencing was performed for all the clones in the library with an ABI Prism 310 Genetic Analyser (Applied Biosystems Inc., CA) using BigDye Terminator (version 3.1) at Animal Biotechnology Laboratory, AAU, Anand, Gujarat, India. The 18S rDNA sequences obtained were initially recognized and aligned against the known sequences in the GenBank/EMBL [2] database using the BLAST program of the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) search.

Sequences from the current study were trimmed (remove low-quality base calls from the start and end of DNA sequence) manually and analysed by the CHECK_CHIMERA program [21] to remove any chimeric rDNA clone. The similarity searches for sequences were carried out by BLAST (http://www.ncbi.nlm.nih.gov/ BLAST/Blast.cgi) [20], and alignment was done using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) [36].

The phylogenetic analysis was carried out using MEGA software version 4.0 [34]. The phylogenetic relatedness was estimated using the neighbour-joining method [28], whereas the evolutionary distance was computed using the maximum composite likelihood method [35]. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). One thousand bootstrap replications were performed to place the confidence estimates on the major groups resolved in the tree. The bootstrap consensus tree inferred from 1,000 replicates represents the evolutionary history of the sequences analysed [9].

Real-Time PCR Analysis

Plasmid DNA contains the ciliate protozoa, *Dasytricha ruminatium* and *Entodinium sp*. gene sequence, used as the standard DNA in real-time PCR, was obtained by PCR cloning with the specific primer sets [30]. After the confirmation of a single band of the correct size with respective pair of primers on an agarose gel, the PCR products were excised from the gel. The PCR products were purified using the Qiagen gel Purification Kit (Qiagen, CA) and cloned and sequenced as previously described. The concentration of the plasmid was determined with a Nanodrop spectrophotometer, and copy number of each standard plasmid was calculated using formula: Copy No/µl = Concentration of plasmids (gm/µl) $\times$ 6.022×10^{23}/length of recombinant plasmid (bp) $\times$ 660, (660 = Molecular weight of nucleotide base, 6.022×10^{23} = Avogadro's number). The optimal amplification conditions for each primer set were obtained with 5 pmol each primer with the combination of annealing temperature and extension time as described by Skillman et al. [30] using Quantifast 2X SYBR Green master mix (Qiagen) on ABI system (ABI 7500). The tenfold dilution series of the standard plasmid for the respective target was run along with the corresponding samples in duplicate.

The copy numbers of 18S rRNA genes of targeted protozoa per ml rumen fluid were calculated using the following equation: (QM $\times$ C $\times$ DV)/(S $\times$ V), where QM was the quantitative mean of the copy number, C was the DNA concentration of each sample, DV was dilution volume of extracted DNA, S was the DNA amount (ng) subjected to analysis, and V was rumen fluid volume subjected to DNA extraction. In the reaction, nearly perfect linear regressions (r^2 = 0.9930 to 0.9995 and slope (-3.3 to -4) were obtained between threshold cycle and quantities of standard for all targets, and data generated from the reaction were used for further analysis.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the 18S RNA gene from the representative clones (based on RFLP) have been deposited in NCBI under the accession numbers HM566249-HM566262.

Results

Similarity with Database Sequences

A total of 91 clones from sample were screened, and 14 distinct sequences (i.e. phylotypes) were generated based on PCR–RFLP pattern. Fourteen sequences were subjected to similarity analysis using BLAST search [1] and SIMILARIY_RANK program [21] (Table 1). In the library, 57 clones from the 91 clones were identified as belonging to *Dasytricha* with 94–98% identity, 14 clones were identified as belonging to Isotricha with 96–99% similarity, 11 clones had 96–98% similar to *Ostracodinium,* and 9 clones were identified as belonging to *Polyplastron* with 96–97% identity. (Table 1).

Table 1 Similarity values of 18S rRNA sequences retrieved from Surti buffalo rumen samples

Phylotypes	Clone no	Accession no	Genera	Nearest taxa (Accession no)	Identity (%)
ORFSBRP1	20	HM566249	*Dasytricha ruminantium*	*Dasytricha ruminantium* (AM158463)	98
ORFSBRP2	1	HM566250	*Isotricha prostoma*	*Isotricha prostoma* (AM158455)	98
ORFSBRP3	9	HM566251	*Ostracodinium*	Uncultured rumen protozoa clone C5-SI-PSSU-19 (HQ162101)	98
ORFSBRP4	7	HM566252	*Polyplastron multivesiculatum*	*Polyplastron multivesiculatum* (AM158458)	97
ORFSBRP5	18	HM566253	*Dasytricha*	Uncultured rumen protozoa clone AVCRPN23 (EU79611)	99
ORFSBRP6	4	HM566254	*Isotricha*	Uncultured rumen protozoa clone AVCRPN30 (EU796118)	99
ORFSBRP7	15	HM566255	*Dasytricha*	Uncultured rumen protozoa clone AVCRPN122 (EU796210)	97
ORFSBRP8	2	HM566256	*Polyplastron*	Uncultured rumen protozoa clone C5-SG-PSSU-57 (HM212231)	96
ORFSBRP9	3	HM566257	*Isotricha*	Uncultured rumen protozoa clone YCRPB55 (EU163779)	97
ORFSBRP10	4	HM566258	*Isotricha prostoma*	*Isotricha prostoma* (AM158456)	96
ORFSBRP11	3	HM566259	*Dasytricha*	Uncultured rumen protozoa clone AVCRPN63 (EU796151)	99
ORFSBRP12	2	HM566260	*Isotricha*	Uncultured ciliate partial 18S rRNA gene, clone C184 (AM158697)	99
ORFSBRP13	2	HM566261	*Ostracodinium*	Uncultured rumen protozoa clone AVCRPN71 (EU796159)	99
ORFSBRP14	1	HM566262	*Dasytricha*	Uncultured rumen protozoa clone S4-SG-PSSU-96 (HM211872)	94

Phylogenetic Analysis of Sequence and Quantitation of Protozoa

The results of phylogenetic analysis of sequences are shown in Fig. 1. From the figure, rumen protozoa from 18S rRNA phylogenetic tree mainly divided into three clusters. The first cluster of eight sequences (69 clones) was phylogenetically placed within genus *Dasytricha*, which has only one species *Dasytricha ruminantium*. The second cluster of 4 sequences (13 clones) was phylogenetically placed within genus *Isotricha*. There were two species *Isotricha prostoma* and *Isotricha intestinalis* within genus *Isotricha* according to morphological classification. All four sequences were shared 97–98% similarity with

Isotricha prostoma. The third cluster of 2 sequences (9 clones) was phylogenetically placed within genus *Polyplastron* and sequences shared 96–97% similarity with *Polyplastron multivesiculatum*. Interestingly, no any Entodinium sequences were observed in present study. Ciliate protozoa were detected 4.1×10^8 copies per ml ruminal fluid. The number of 18S rDNA gene copies of *Dasytricha ruminantium* (2.0×10^5 per ml ruminal fluid) was higher than *Entodinium sp*. (1.3×10^4 per ml ruminal fluid) and accounting 0.050% of ciliate protozoa.

Discussion

Method has been developed for molecular identification of rumen protozoa without the need for cultivation [15, 29]. Compared with the rumen bacteria and fungi, the available information on rumen protozoa was still relative limited. By classical morphological criteria, more than 42 genera of rumen protozoa have been described [6, 42]. But, sequences of few genera which generally represent the predominant genera have been deposited in NCBI database [45–47]. Microscopic examination revealed eleven typical genera of rumen protozoa: *Entodinium*, *Diplodinium*, *Eudiplodinium, Epidinium, Ophryoscolex, Polyplastron, Isotricha, Dasytricha, Ostracodinium, Metadinium* and *Enoploplastron* [15, 41]. Zhang [50] reported ciliate protozoa in rumen fluid sample of two local Yellow Cattle fed rice straw. They reported ten species of protozoa: *Diplodinium*, *Isotricha, Entodinium, ophryoscolex, Eodinium, Ostracodinium, Eudiplodinium, Elytroplaston, Epidinium* and *Caloscolex*. However, in present study, there were only a few genera occurred in our library, which may be due to diurnal variation of rumen protozoal types, and some protozoa were autolysis during the sample collection and processing [22]. Further, there were only a few methods of DNA extraction for rumen protozoa [2]. Thus, it might be possible that some protozoal DNA could not be extracted, or the extracted DNA of some species was too low for amplification, as well as the amount of impurities in extracted DNA.PCR amplification bias, primers and geological distribution could be other reasons for the true diversity of the composition of rumen protozoa [15].

This study reveals the phylogenetic diversity of the rumen protozoa in Surti rumen by analysing protozoal 18S rDNA sequences in a culture-independent manner. All of

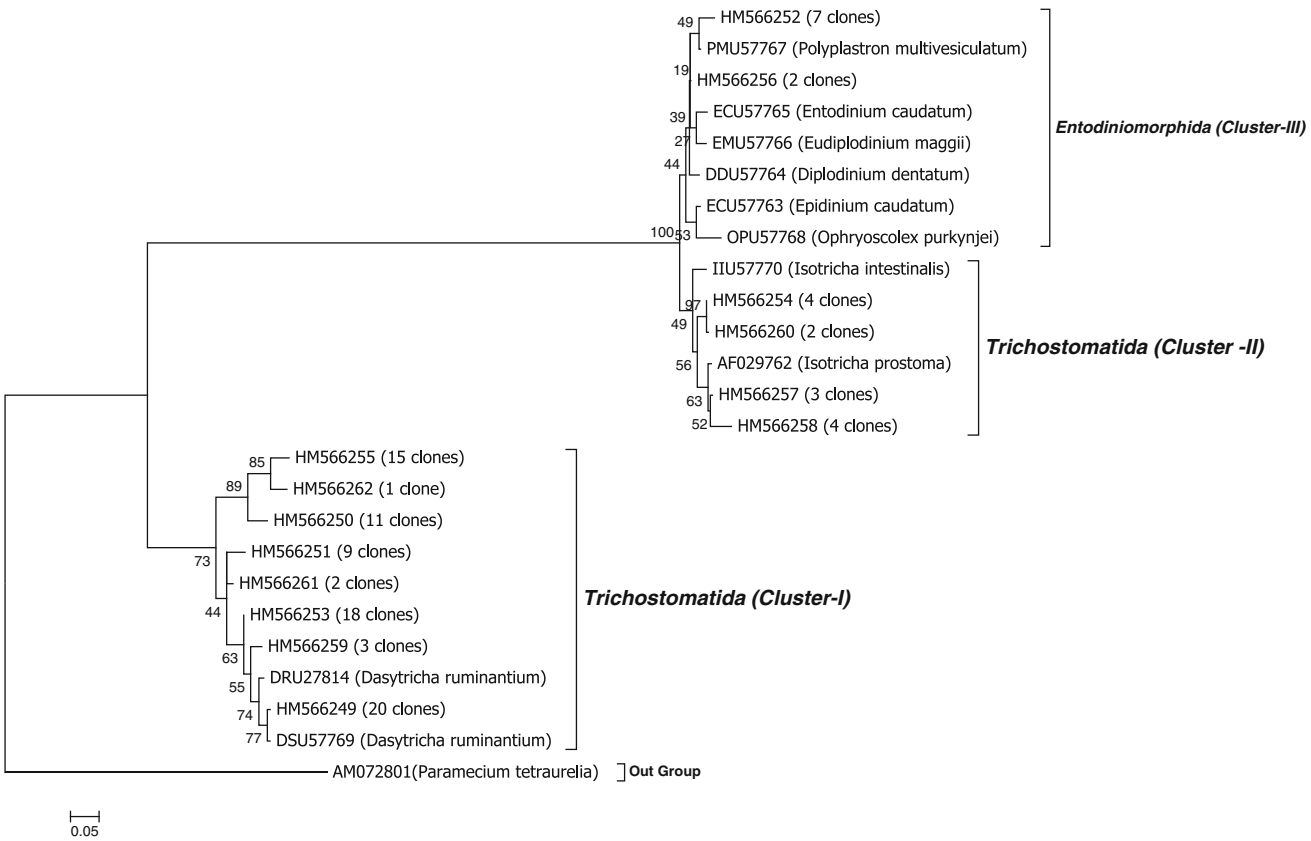

Fig. 1 Phylogenetic relationships of partial 18S rDNA sequences of clones recovered from Surti rumen samples. The rooted tree was inferred by the neighbour-joining method with 1,000 bootstrap replicates using the MEGA 4 tree building program. The Paramecium tetraurelia (AM072801) are used as the out-group for rooting the tree. The *scale bar* represents 5% sequence divergence

the cloned 18S rRNA sequences were associated with four protozoa genera (Table1): *Dasytricha* (8 sequences: HM566249, HM56650, HM56651, HM56653, HM56655, HM56659, HM56661 and HM56662), *Isotricha* (4 sequences: HM56654, HM56657, HM56658 and HM5 6660) and *Polyplastron* (2 sequences: HM56652 and HM56656) (Fig. 1). BLAST search showed that all sequenced shared high degree of similarity (94–99%) with ruminal protozoal 18S rDNA sequences with NCBI GenBank database (Table 1). Our observation compared with the libraries which are described by Karnati et al. [15], Shin et al. [29] and Leng el al. [49] (Table 2). It can be found that Karnati's library was associated with those of the genus *Entodinium* and *Dasytricha*, Shin's library belonged to *Entodinium* and *Epidinium*, whereas Leng's library was associated with *Entodinium*, *Isotricha*, *Dasytricha* and *Diplodinium*. However, our library was composed by *Dasytricha*, *Isotricha*, *Ostracodinium* and *Polyplastron*. The highest proportion was *Dasytricha* (62.63%) in Surti buffalo rumen fed green fodder Napier bajra 21 (*Pennisetum purpureum*), mature pasture grass (*Dicanthium annulatum*) and concentrate mixture (20% crude protein, 65% total digestible nutrients). Interestingly, in our library, no *Entodinium*, *Epidinium* and *Ophryoscolex*-related sequences were observed (Table 2). Compared with previous reports [15, 29], such discrepancy may be due to diets, breeds, areas and samples analysis. When *Isotricha* was less dominant, clone sequences might not reflect the true situation in complex ruminal samples because of PCR bias [15]. To assess true diversity, more sequences of different species are also needed. Moreover, considerable phylogenetic diversity was found from a limited number of sequenced clones, further study could be complemented present understanding. Karnati et al. [15] used PCR methods to examine protozoal diversity in rumen samples. They used protozoal-specific primers to achieve amplification of only protozoal 18S rDNA from community DNA, which was subsequently cloned, sequenced and compared to database sequences for identification. Alternatively, restriction fragment length polymorphism analysis of the PCR-amplified 18S rDNA gene could identify different protozoal species, including those with the same genera or among closely related genera [26]. Sylvester et al. [32] and Regensbogenova et al. [27] designed protozoa-specific primers suitable for PCR–DGGE analysis. They identified the major protozoal species recovered by PCR–DGGE by excision of the intense DGGE bands, re-amplification and DNA sequencing.

To enumerate *Dasytricha ruminantium* population in the Surti rumen, real-time PCR assay was applied and found to be the most abundant ciliate protozoa (2.0×10^5 per ml ruminal fluid) with the largest number of species. However, Skillman et al. [30] reported that *Entodinium sp*. was the major ciliate protozoa in sheep rumen fed hay diet and detected 2.45×10^5 per ml of ruminal fluid, particularly in relation to the inherent variability in protozoal populations between sheep fed the same diet. Facilitated by these molecular biology techniques, the distribution of different

Table 2 The diversity comparing of different clone libraries of rumen protozoa

Clone libraries	Karnati et al. [15]		Shin et al. [29]	Leng et al. [19]		Our library
Animals	Cow		Cow	Yunnan Yellow Cattle		Surti Buffalo
Clone numbers	12	11	37	66	55	91
Diets	Ration (1:1)[b]	Alfalfa	Ration (1:4)[c]	Malt meal	Straw	Mix ration[d]
Clone distribution (%)						
Entodinium	97.3	77.2	81.1	72.7	60.0	ND
Diplodiniina[a]	1.7	4.6	ND	ND	ND	21.97
Epidinium	0.3	<0.5	18.9	ND	ND	ND
Ophryoscolex	ND	0.9	ND	ND	ND	ND
Isotricha	<0.3	6.0	ND	16.7	ND	15.38
Dasytricha	0.7	11.2	ND	10.6	ND	62.63
Diplodinium	ND	ND	ND	ND	40.0	ND

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 concentrate

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

protozoal species or group in different phases or locations in the rumen of ruminants fed different diets can be examined more accurately [8, 22]. As the complexity and dynamic nature of rumen micro-organisms, 18S rRNA sequences analysis alone is not enough to be clear about the real situation of the composition of rumen protozoa. Therefore, it is necessary to understand the effects of varying rumen protozoa on different animal feeding habit and methanogenesis will also be important.

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