RESEARCH ARTICLE

Characterization of rumen ciliate community composition in domestic sheep, deer, and cattle, feeding on varying diets, by means of PCR-DGGE and clone libraries

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Abstract

The structure and variability of ciliate protozoal communities in the rumens of domestic New Zealand ruminants feeding on different diets was investigated. The relative abundance of ciliates compared with bacteria was similar across all samples. However, molecular fingerprinting of communities showed ruminant-specific differences in species composition. Community compositions of cattle were significantly influenced by diet. In contrast, diet effects in deer and sheep were weaker than the animal-to-animal variation. Cloning and sequencing of almost-full-length 18S rRNA genes from representative samples revealed that New Zealand ruminants were colonized by at least nine genera of ciliates and allowed the assignment of samples to two distinct community types. Cattle contained A-type communities, with most sequences closely related to those of the genera *Polyplastron* and *Ostracodinium*. Deer and sheep (with one exception) harboured B-type communities, with the majority of sequences belonging to the genera *Epidinium* and *Eudiplodinium*. It has been suggested that species composition of ciliate communities may impact methane formation in ruminants, with the B-type producing more methane. Therefore, manipulation of ciliate communities may be a means of mitigating methane emissions from grazing sheep and deer in New Zealand.

Introduction

The rumen harbours a complex microbiota of bacteria, archaea, fungi, and ciliate protozoa that act together to ferment plant material ingested by ruminant animals. Bacteria, fungi, and some ciliate species carry out the initial attack on the plant material, breaking down polymers, fermenting the resulting monomers and oligomers, and producing volatile fatty acids that are taken up by the ruminant as major carbon and energy sources. An additional product of this fermentation is hydrogen (H_2), which serves as an electron donor in the energy metabolism of methanogenic archaea and is converted to methane (CH_4). While the presence of ciliates in the rumen is not considered essential for the survival of the ruminant animal (Williams & Coleman, 1997), it benefits the remainder of the microbial community and thus the ruminant itself for several reasons. Rumen ciliates add degradative complexity to the rumen fermentation (Coleman, 1986), and so may help improve community resilience. Some rumen ciliates scavenge oxygen entering the rumen, which benefits the anaerobic degradation process (Ellis *et al.*, 1989). Furthermore, rumen ciliates may be important regulators of prokaryotic populations in the rumen and act to transfer bacterial nitrogen to higher trophic levels and to the ruminant host (Coleman, 1989; Bonhomme, 1990; Williams & Coleman, 1997). During fermentation of ingested plant material, large amounts of H_2 are produced in the hydrogenosomes of the anaerobic ciliates (Lindmark & Müller, 1973). This H_2 will be used by hydrogenotrophic methanogens. Indeed, some ciliates have been found to live in close ecto- or endosymbiotic relationships with methanogenic archaea (Vogels *et al.*, 1980; Stumm *et al.*, 1982), and so up to 37% of rumen-derived CH_4 can be produced by ciliate-associated methanogens (Finlay *et al.*, 1994; Newbold *et al.*, 1995).

CH_4 is implicated as a driver of global climate change (Smith *et al.*, 2007). Globally, domesticated ruminants are the source of 4.3% of total anthropogenic greenhouse gas

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emissions (data from the year 2000; Scheehle *et al*., 2006). However, in New Zealand, a country with a significant agricultural sector, ruminants account for 32% of total anthropogenic greenhouse gas emissions (Smith *et al*., 2007). Apart from being considered a major threat to the global climate, the production of CH_4 by free-living and ciliate-associated methanogens represents a feed energy loss for the ruminant of up to 10% of its intake (Nollet *et al*., 1997). Both the potential loss in animal productivity and the possible effect on the Earth's climate have fuelled the search for strategies to reduce CH_4 emissions from livestock. These strategies include the targeted knock-out of methanogens by vaccination or antimethanogen feed supplements, the substitution of methanogens with alternative H_2 users such as acetogenic bacteria, and defaunation, i.e. elimination of ciliates (Kreuzer, 1986; Hegarty, 1999; McAllister & Newbold, 2008).

The rumen ciliates are subdivided into the orders *Entodiniomorphida* and *Vestibuliferida*. These two orders comprise at least 25 genera that have been classified and are identified by their morphological features (Kamra, 2005). Factors that shape the ciliate fauna of ruminant animals are still largely unknown. Eadie (1962) identified four different ciliate community types, each characterized by one or more key species. The A-type community is characterized by the presence of *Polyplastron multivesiculatum*, the B-type community by the presence of either *Epidinium ecaudatum* or *Eudiplodinium maggii*, the O-type community is exclusively composed of *Entodinium* spp. and the vestibuliferid genera *Dasytricha* and *Isotricha*, and the cattle-specific K-type community is easily distinguished by the key species *Elytroplastron bubali*.

Rumen fluid from defaunated sheep that were reinoculated with an A-type ciliate community produced less CH_4 than fluid from sheep reinoculated with a B-type ciliate community (Newbold *et al*., 1995). These results are supported by the finding of Lloyd *et al*. (1996) that *P. multivesiculatum*, the key species of A-type ciliate communities, harbours a large number of intracellular bacteria but no methanogens. In contrast, *Epidinium* and possibly also *Eudiplodinium*, both key genera of the B-type community, harbour intra- and extracellular methanogenic archaea (Lloyd *et al*., 1996). Other studies detected archaeal 16S rRNA genes in single cells of B- as well as A-type ciliate species; however, the molecular techniques applied did not allow differentiation between true endosymbionts and ingested microorganisms (Tokura *et al*., 1997; Chagan *et al*., 1999; Irbis & Ushida, 2004). If A-type ciliate communities were shown to be linked to significantly lower methane emissions in large-scale animal experiments over a reasonable period of time, targeted selection against B-type communities may offer an effective strategy to mitigate methane. Selecting for ciliate communities that are dominated by species that do not form close associations with methanogens would maintain at least some of the functions and benefits of ciliates in the rumen, while potentially decreasing CH_4 emissions.

Analyses of ciliate communities in New Zealand ruminants date back to the middle of the last century, when Oxford (1958) and Clarke (1964) observed only B-type ciliate communities in the rumens of sheep and cattle. These analyses and most of our knowledge on rumen ciliates have emerged from microscopy-based studies, and have been reliant on tedious and time-consuming counting. Here, we combined molecular fingerprinting via denaturing gradient gel electrophoresis (DGGE) with quantitative PCR (qPCR) and phylogenetic analysis of almost-full-length 18S rRNA gene clone libraries to shed light on the structure and variation of ciliate communities in New Zealand ruminants. We wanted to know whether the community types proposed by Eadie (1962) were detectable using DNA-based methods. We also wanted to produce more full-length 18S rRNA gene sequence data to expand the phylogenetic framework for future large-scale gene-sequencing studies, and as a baseline for further studies relating protozoa community structure to CH_4 emissions.

Materials and methods

Collection of samples from ruminant animals

Samples of whole rumen contents consisting of fluid and solids (approximately 200 g) were collected via rumen fistulae from four mature wether sheep (Romney), five mature nonlactating dairy cows (Friesian–Jersey cross), and four mature castrated red deer kept in separate groups at AgResearch Ltd, Palmerston North (Table 1). These groups were fed with lucerne (*Medicago sativa*) silage (Chaffhage; The Great Hage Company, Reporoa, New Zealand) in the winter. The animals grazed outdoors *ad libitum* on a perennial rye grass (*Lolium perenne*) and white clover (*Trifolium repens*) pasture during the following summer and winter periods. Pasture-fed animals were on that diet throughout the whole season. The silage-fed animals were housed indoors during the experimental period, adapted to their feed for at least 15 days before sample collection, and were fed twice daily, at 08:00 and 16:00 hours, at 1.2 times their estimated energy requirements for maintenance. Samples of whole rumen contents were collected approximately 2 h after morning feeding. Whole rumen contents were also collected once, at slaughter, from another two, different flocks of sheep (Romney cross). Sheep of flock 2 were fed with a concentrate-based diet at AgResearch Ltd, Palmerston North (four animals; Table 1). Animals of flock 3 were kept at Massey University's Riverside farm, near Masterton, and separated into two groups: group 3A was fed perennial rye

Table 1. Identification of ruminant animals and overview on the variety of diets they were fed

Ruminant group	Animal identifiers	Diets in chronological order	Sampling method
Cattle	C1–C5	Silage, summer and winter pasture	Fistula
Deer	D1–D4	Silage, winter and summer pasture	Fistula
Sheep flock 1	S1–S4	Silage, winter and summer pasture	Fistula
Sheep flock 2	S5–S8	Concentrate-based diet	Slaughter
Sheep flock 3A	S9–S13	Autumn pasture	Slaughter
Sheep flock 3B	S14–S18	Willow	Slaughter

Samples were taken from cattle, deer, and three different flocks of sheep. Sheep of flock 3 were separated into groups A and B for the course of the experiment. Samples were either obtained via rumen fistulae or at slaughter.

Table 2. Primer sets tested and selected for qualitative (Phylogeny, DGGE) and quantitative assessment of ciliates (CqPCR) and bacteria (BqPCR) in the rumen of New Zealand ruminants

Primer pair	Amplicon size (bp)	Annealing temperature (°C)	Primer name	Primer sequence (5′–3′)	Database sequences with a mismatch to primer*	Reference for primer
CqPCR	232	54	Syl316F	GCT TTC GWT GGT AGT GTA TT	1/49	Sylvester *et al*. (2004)
			Syl539R	CTT GCC CTC YAA TCG TWC T	0/49	Sylvester *et al*. (2004)
DGGE-1†	285	54	Reg1062F	GGT GGT GCA TGG CCG	0/49	Elwood *et al*. (1985)
			Reg1302R	AAT TGC AAA GAT CTA TCC C	1/49	Rhind *et al*. (2002)
DGGE-2†	624	54	RP841F	GAC TAG GGA TTG GAG TGG	9/49	This study
			RP1416R	ACC GGA TCA CCC GGT ATC	0/49	This study
DGGE-3†	511	54	RP841F	GAC TAG GGA TTG GAG TGG	9/49	This study
			Reg1302R	AAT TGC AAA GAT CTA TCC C	1/49	Rhind *et al*. (2002)
Phylogeny	1535	55	P.SSU54F	CAY GTC TAA GTA TAA ATA ACT AC	2/49	Sylvester *et al*. (2004)
			P.SSU1747R	CTC TAG GTG ATW WGR TTT AC	0/49	Sylvester *et al*. (2004)
BqPCR	375	52	Ba519F	CAG CMG CCG CGG TAA NWC	NA	Stubner (2004)
			Ba907R	CCG TCA ATT CMT TTR AGT TT	NA	Muyzer *et al*. (1995)

Positions where primer mismatches can occur are underlined.

*Accession numbers of all analysed sequences of isolated ciliate species are given in Fig. 5.

†One primer in each pair was tagged at the 5′-end with a 40-bp-long GC-rich sequence segment (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G). For the majority of analyses, this was the 'R'-primer (see text for details).

NA, not analysed.

grass/white clover pasture during the autumn period (five animals) and group 3B was fed willow (*Salix* sp.; five animals; Table 1; for further details, see Ramirez-Restrepo *et al*., 2010). All animals had unlimited access to water at all times. Only one sample was collected from each animal-diet combination, to give 53 samples, which were immediately frozen at −80 °C and subsequently freeze-dried. The freeze-dried samples were homogenized by grinding in a 100 W household coffee grinder (Russell Hobbs, Mordialloc, Vic., Australia) and stored at −80 °C until further use.

Primer design and validation

A literature search was performed for published primer pairs able to amplify 18S rRNA genes from rumen ciliates. Published primers were checked for sequence identity with available 18S rRNA gene sequence data from rumen ciliates isolated to date and, where necessary, novel primers were designed from available sequence information (Table 2). All primer pairs listed in Table 2 were validated for specificity by constructing clone libraries ($n \approx 50$) using DNA extracted from a sample from sheep S4 fed summer pasture.

Extraction of total DNA

DNA was extracted from freeze-dried rumen samples (50 mg) using the method of Lueders *et al*. (2004). Briefly, cells were disrupted by combined bead-beating (FastPrep FP120; Qbiogene, Carlsbad, CA; 45 s at 6.5 m s^{-1}) and phenol–chloroform–isoamyl alcohol treatment. DNA was then precipitated by polyethylene glycol (30%) precipitation, washed with 70% (v/v) ice-cold ethanol, and eluted in molecular biology-grade water. DNA was stored at −20 °C.

Assessment of rumen ciliates and bacteria by real-time qPCR

Abundances of ciliates in ruminant samples were quantified using a Rotor-Gene 6000 real-time rotary analyzer (Corbett

Life Science, Concorde, NSW, Australia) and amplicon detection by SYBR Green I fluorescence (LightCycler Fast-Start DNA Master SYBR Green I Kit; Roche, Auckland, New Zealand). Primers for real-time amplification of bacteria and ciliates are listed in Table 2. Plasmids containing bacterial 16S rRNA or ciliate 18S rRNA gene inserts were constructed, quantified with the Quant-iT dsDNA BR Assay Kit on a Qubit fluorometer (Invitrogen, Carlsbad, CA), and diluted 10-fold in series to produce five standards from 2×10^3 to 2×10^7 copies per reaction for bacteria and 7×10^2 to 7×10^6 copies per reaction for ciliates, each in duplicate for use in the qPCR. Reactions were set up in a Gene-Disc 100 (Corbett Life Science) and sealed with permanent adhesive film (Corbett Life Science). Each reaction contained, in a total volume of 20 μL, 2 μL of Light Cycler Mix, 1 μM of each primer (Table 1), $MgCl_2$ to final concentrations of 2 mM (ciliates), or 4 mM (bacteria), 4 μg bovine serum albumin (Invitrogen), and 2 μL of standard or DNA template. Each template DNA was measured at four different dilutions for ciliates (1 : 75, 1 : 100, 1 : 250, and 1 : 500). Bacterial template DNA was amplified at three different DNA dilutions (1 : 500, 1 : 1000, and 1 : 5000), each in duplicate. The thermal protocol for qPCR amplification and detection was 10 min of initial denaturation (94 °C), followed by 50 amplification cycles [30 s at 94 °C; 5 s at 54 °C (for ciliates) or 52 °C (for bacteria); 10 s at 72 °C]. After each run, melting curves between 72 and 95 °C were evaluated to confirm the absence of unspecific signals.

Amplification of partial and full-length 18S rRNA genes

PCR amplification of 18S rRNA genes was carried out in a Hybaid Px2 Thermal Cycler (ThermoElectron, Milford, MA). Each 50-μL PCR contained 1 × Taq buffer (Roche), 1.5 mM $MgCl_2$, 0.75 U Taq DNA polymerase (Roche), 50 μM of each dNTP, 10 μg bovine serum albumin (Invitrogen), 0.5 μM of each primer (Table 2), and 1 μL of template DNA (10-fold diluted). Unspecific primer binding was minimized with a semi-hot start by transferring the reactions already containing the polymerase from 4 °C straight into the preheated thermal cycler (94 °C), and the amplification was performed as follows: initial denaturation at 94 °C for 3 min, 35 cycles of denaturing (94 °C, 30 s), annealing (see Table 2 for temperatures, 45 s) and elongation (72 °C, 1 min), and a final 7-min (or 30 min for DGGE; Janse *et al.*, 2004) extension at 72 °C. Correct sizes of PCR products were verified by agarose gel electrophoresis and subsequent visualization of bands under UV light. Gene amplicons were purified using the MinElute clean-up system (Qiagen, Hilden, Germany) and subsequently quantified on the NanoDrop (NanoDrop Technologies, Wilmington, DE).

DGGE fingerprinting of ciliate communities

PCR amplicons were digested with Mung Bean Nuclease for 15 min at 37 °C to remove single-stranded residues. A total volume of 12 μL contained 1 × Mung Bean buffer (Promega. Alexandria, NSW, Australia), 0.1 U Mung Bean Nuclease (Promega), and 300 ng of purified PCR product. Digests were spiked with 3 μL of DGGE loading dye [0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 70% (w/v) glycerol, in water, pH 8.0]. An optimal separation of amplicons was achieved by a gradient of 20–40% denaturants [100% denaturant was 7 M urea and 40% (v/v) formamide] in a 6% polyacrylamide gel prepared from a stock of 40% (w/v) acrylamide plus *N*,*N*′-methylenebisacrylamide (37.5 : 1 w/w). Selected PCR samples were loaded onto multiple gels to act as controls and to allow comparison of different gels. Marker IV (Nippongene, Tokyo, Japan) was used as a set of constant position markers in all gels. DGGE was performed with the Ingeny PhorU System (Ingeny, Goes, the Netherlands) in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8 with NaOH) at 60 °C for 7 h at 125 V. Gels were rinsed with water, stained for 20 min in 10 000 times diluted SYBR Gold nucleic acid stain (Invitrogen), and destained for at least 2 h in water, before they were photographed under UV transillumination.

Construction of partial and full-length 18S rRNA gene libraries

Ciliate 18S rRNA genes were cloned using the TA Cloning Kit (Invitrogen). DNA of randomly selected clones was subjected to vector-targeting PCR with primers Gem2987F (5′-CCC AGT CAC GAC GTT GTA AAA CG-3′) and Top168R (5′-ATG TTG TGT GGA ATT GTG AGC GG -3′), purified, quantified, and sequenced at either the Allan Wilson Centre Genome Sequencing Service (Massey University, Palmerston North, New Zealand) or Macrogen Inc. (Seoul, Republic of Korea). In total, we determined 364 sequences from sheep S4 (CqPCR primer set, 47 sequences, sample from animal when fed summer pasture, clone names prefixed with S4-SG-CqPCR, GenBank accessions HM212293–HM212324 and HM212395–HM212409; DGGE-1 primer set, 21 sequences, summer pasture, prefix S4-SG-DGGE1, HM212325–HM212345; DGGE-2 primer set, 32 sequences, summer pasture, prefix S4-SG-DGGE2, HM212261–HM212292; DGGE-3 primer set, 49 sequences, summer pasture, prefix S4-SG-DGGE3, HM212346–HM212394; Phylogeny primer set, 53 sequences, summer pasture, prefix S4-SG-PSSU, HM211855–HM211886 and HM212086–HM212106; Phylogeny primer set, 79 sequences, winter pasture, prefix S4-WG-PSSU, HM211887–HM211925 and HM212150–HM212189; Phylogeny primer set, 83 sequences, silage, prefix S4-SI-PSSU, HM211926–HM211965 and HM212107–HM212149), 148 sequences from sheep S2 (Phylogeny primer

set, 49 sequences, summer pasture, prefix S2-SG-PSSU, HM211966–HM212014; Phylogeny primer set, 46 sequences, winter pasture, prefix S2-WG-PSSU, HM212068–HM212085 and HM212233–HM212260; Phylogeny primer set, 53 sequences, silage, prefix S2-SI-PSSU, HM212015–HM212067), and 92 sequences from cow C5 (Phylogeny primer set, 43, summer pasture, prefix C5-SG-PSSU, HM212190–HM212232; Phylogeny primer set, 49, silage, prefix C5-SI-PSSU, HQ162053–HQ162101). A further 71 potentially chimeric sequences were identified in almost-full-length 18S rRNA gene libraries by fractional treeing (Ludwig *et al.*, 1997) with two individual filters covering either the first third (*Escherichia coli* positions 1125–14 988) or the final third (*E. coli* positions 31 168–43 102) of the sequences. These chimeric sequences were excluded from our analyses and not deposited in GenBank. The phylogenetic affiliations of almost-full-length ciliate 18S rRNA gene sequences were determined with the ARB software package (http://www.arb-home.de; version 2.5b; Ludwig *et al.*, 2004). A core tree consisting of *E. coli* positions 1029–43 275 was constructed with sequence data from isolated ciliate species, using the neighbor-joining method with Felsenstein's correction (Felsenstein, 1981). *Didinium nasutum* (Order *Haptorida*; GenBank accession number U57771) served as an outgroup sequence. Sequences amplified with the Phylogeny primer pair were added to the tree by the fast parsimony tool for tree construction using *E. coli* positions 1463–43 014. Shorter fragments amplified with the CqPCR and the three DGGE primer pairs were temporarily added to the tree with the fast parsimony tool for phylogenetic assignment of sequences.

Statistical analyses

Compositional differences in the libraries constructed with different primer combinations were tested for statistical significance using the χ^2 test in EXCEL (Microsoft Corp., Redmond, WA).

qPCR data were analysed using the ROTOR-GENE 6000 series software version 1.7 (Corbett Life Science) and subsequently exported to EXCEL for further evaluation. The mean abundance of ciliates in the rumen samples was calculated as ciliate 18S rRNA gene copies per bacterial 16S rRNA gene copy by log-transformation of the ratios, calculation of the mean, and subsequent back transformation. Ruminant and diet effects on the ratios, including their interaction, were estimated with F-statistics via a linear mixed model using the residual maximum likelihood (REML) algorithm in GENSTAT (Payne *et al.*, 2007).

DGGE banding patterns were analysed with the BIONUMERICS software v4.0 (Applied Maths Inc., Sint-Martens-Latem, Belgium). Cluster analysis was performed using the unweighted pair group method with arithmetic mean and the Pearson correlation. In order to test for statistical significance between treatment groups, similarity matrices were exported into EXCEL and subject to *t*-tests (two-tailed, unequal variance), comparing the Pearson correlations between samples that were within and between groups or treatments.

Results and discussion

Methods for general application

The choice of appropriate primers is crucial for the application of molecular monitoring techniques. Ideally, a primer should detect the entire diversity of the targeted group of microorganisms (coverage), while binding only to target DNA (specificity). We searched the literature for suitable primer pairs, tested them, and optimized their use to quantitatively and qualitatively characterize ciliate communities in ruminant animals. Primers Reg1062F and Reg1302R for DGGE fingerprinting of partial ciliate protozoal 18S rRNA genes were originally described by Elwood *et al.* (1985) and Rhind *et al.* (2002). This primer pair, designated DGGE-1 (Table 2), has since been used by several authors to compare rumen ciliate communities in sheep, goats, reindeer, and ibexes (Regensbogenova *et al.*, 2004; de la Fuente *et al.*, 2006, 2009; Shi *et al.*, 2008; Sundset *et al.*, 2009). To extend the 18S rRNA gene sequence information obtained from primer pair DGGE-1, we designed two new rumen ciliate-specific primers: RP841F and RP1416R. These primers were tested as a pair (DGGE-2; Table 2). In addition, RP841F was used in combination with Reg1302R (DGGE-3; Table 2). These two new primer combinations should allow us to almost double the 18S rRNA gene sequence information obtained from DGGE bands, from 251 bp for DGGE-1 to 588 bp for DGGE-2 and 474 bp for DGGE-3, excluding primer bases. To test the practical specificity and coverage of these primers for DGGE fingerprinting, we constructed amplicon libraries generated with each of the three primer sets (DGGE-1, DGGE-2, and DGGE-3) from a sample from the rumen of a pasture-fed sheep (animal S4 on summer pasture; Fig. 1). We also constructed amplicon libraries from the same rumen sample with a primer pair spanning almost the full-length of the 18S rRNA gene (Phylogeny; Table 2), and a primer pair targeting a small, ~232-bp-long region of the 18S rRNA gene (CqPCR; Table 2).

We found that the primer pair DGGE-1, consisting of the universal eukaryote primer Reg1062F (Elwood *et al.*, 1985) routinely used for DGGE of rumen ciliates in earlier studies, in combination with Reg1302R (Rhind *et al.*, 2002), amplified nontarget DNA sequences that were highly related to 18S rRNA genes of red fescue (*Festuca rubra*; GenBank accession number AF168844). These probably originated from pasture plants fed to the animal. The other primer combinations tested, including those with the new rumen ciliate-specific forward primer RP841F, did not yield any

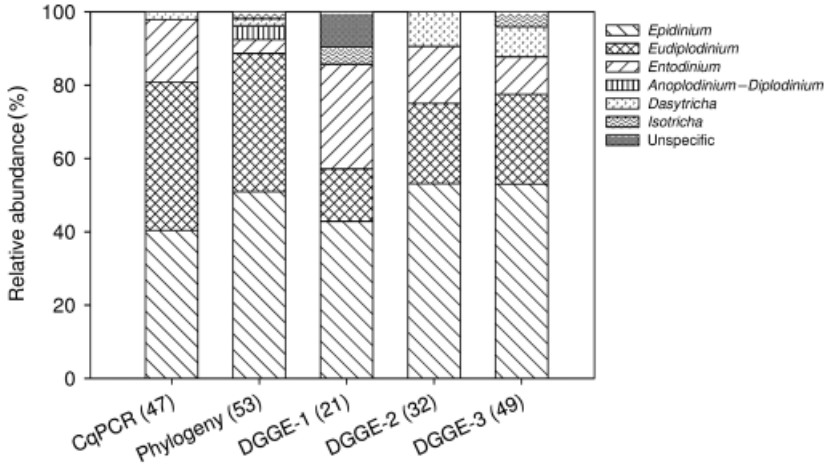


Fig. 1. Relative abundances of sequences assigned to different ciliate groups in clone libraries from sheep S4 feeding on summer pasture. The libraries were constructed with different primer pairs targeting rumen ciliates: Syl316F/Syl539R (CqPCR), PSSU54F/PSSU1747R (Phylogeny), Reg1062F/Reg1302R (DGGE-1), RP841F/RP1416R (DGGE-2), and RP841F/Reg1302R (DGGE-3).

products from nontarget DNA (Fig. 1). Excluding the nonspecific sequences obtained with primer combination DGGE-1, there was no difference in coverage between the different primer pairs ($P = 0.33$ in χ^2 test for difference). The dominant genera, which were *Epidinium*, *Eudiplodinium*, and *Entodinium*, were found in all libraries. The highest coverage of diversity was retrieved from the library generated with the Phylogeny primer pair (Fig. 1). Some genera, such as *Anoplodinium–Diplodinium*, *Isotricha*, and *Dasytricha*, were rare and therefore not consistently collected. The libraries generated using primer pairs CqPCR, DGGE-1, and DGGE-2 were the least diverse, lacking sequences of the *Anoplodinium–Diplodinium* cluster and either *Dasytricha* or *Isotricha*. We did not retrieve any sequences belonging to *Anoplodinium–Diplodinium* in the library generated with primer pair DGGE-3. However, *in silico* analysis showed that all tested primer pairs matched target regions in published sequences of the *Anoplodinium–Diplodinium* group, and also in most cases with the remaining 46 18S rRNA gene sequences of isolated species of rumen ciliates that have been deposited in the NCBI database to date (Table 2). It is likely that their absence was due to the small sample size. Primer RP841F, used in DGGE-2 and DGGE-3, has a single mismatch at primer nucleotide 15 (Table 2) with 18S rRNA genes from members of the vestibuliferid genus *Isotricha*. Introduction of a degeneracy into the primer sequence, however, is not advisable, because this may result in a double band formation during DGGE separation (Kowalchuk *et al*., 1997). Despite this single mismatch with sequences from *Isotricha* spp., primer RP841F successfully amplified 18S rRNA genes from members of this group, as confirmed by *Isotricha*-related sequences found in the DGGE-3 library (Fig. 1). Comparing the three primer pair options for DGGE fingerprinting, we observed the best phylogenetic coverage and no unspecific products with primer pair DGGE-3, and recommend this primer pair for application in DGGE.

Incorporation of the GC-clamp in the reverse primer of all the three different DGGE primer combinations resulted in better band resolution and more bands being detected, indicating a greater apparent diversity, than if the GC-clamp was part of the forward primer (data not shown). We therefore chose to perform DGGE with the GC-clamp as part of the reverse primer.

Next-generation, massively parallel sequencing methods are starting to take over from 'traditional' library-based or fingerprinting-based analyses of prokaryotic communities. However, for three cogent reasons, we preferred to use the DGGE-fingerprinting technique and a traditional clone library approach over next-generation sequencing methods to analyse rumen ciliates in this study. First, publicly available databases of 18S rRNA gene sequences contain very few almost-full-length entries that can serve as references for phylogenetic identification of sequencing reads. Second, the high degree of sequence similarity of ciliate 18S rRNA genes from different genera together with the relatively short read lengths obtained with pyrosequencing make an accurate phylogenetic assignment difficult. Third, our experimental set-up suggested that several samples would be highly similar to each other. Therefore, DGGE allowed for a cheap and easy prescreening of ciliate communities and for identifying interesting samples that were then subjected to a more thorough phylogenetic analysis via clone libraries.

Animal-to-animal variation of ciliate communities

We used qPCR to estimate the abundance of ciliate protozoal 18S rRNA genes in rumen samples from sheep ($n = 4$), red deer ($n = 4$), and cattle ($n = 4$) feeding on three different diets (summer pasture, winter pasture, and silage). Three additional groups of sheep from two different flocks were

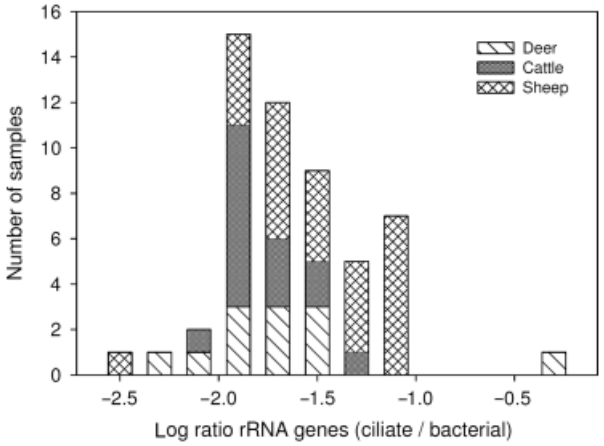


Fig. 2. Distribution of ratios (ciliate 18S rRNA gene copies per bacterial 18S rRNA gene copy) after quantification of ciliate and bacterial copy numbers via qPCR. Each bar describes the number of samples that possess a ratio falling within the range of 0.2 log-units.

also analysed; these had been feeding on autumn pasture ($n = 5$), willow fodder ($n = 5$), or a concentrate-based diet ($n = 4$). Ciliate abundances in all analysed samples averaged 0.02 (range: 0.007–0.5) ciliate 18S rRNA genes per bacterial 16S rRNA gene (Fig. 2). Statistical evaluation of qPCR data did not suggest that ciliate abundances were ruminant-specific ($F = 1.41$; d.f. = 2, 13; $P = 0.28$), diet-specific ($F = 0.82$; d.f. = 5, 36; $P = 0.55$) or influenced by a ruminant-diet interaction ($F = 1.42$; d.f. = 4, 28; $P = 0.25$). These findings indicated that differences due to ruminant or diet-related factors might, if at all, occur on the level of ciliate community composition.

DGGE fingerprinting was used to analyse the diversity of ciliates in the same samples. At least 29 distinct operational taxonomic units (OTUs), defined as bands that migrated to distinctly different positions in the DGGE gels, were present across all of the DGGE profiles of rumen ciliates generated from these 53 different rumen samples (e.g. Fig. 3). Each individual community profile contained a mean ($\pm$SD) of 8.9 ± 2.7 different OTUs (range 3–14). Fingerprints of sheep, deer, and cattle were composed of 8.3 ± 2.6 (3–14), 9.8 ± 2.3 (6–14), and 9.2 ± 3.1 (4–13) OTUs, respectively.

The degree of variation between animals belonging to one treatment group is a decisive factor as to whether statistically significant changes in microbial communities will be detected when the rumen is manipulated in experiments. Based on DGGE fingerprinting analysis, animal-to-animal variation between animals on the same diet, measured as within diet dissimilarities, was highest in sheep, slightly lower in red deer, and lowest in cattle (Table 3). Similarly, Regensbogenova *et al.* (2004) and Sundset *et al.* (2009) reported a large degree of variation in ciliate DGGE fingerprints generated from rumen samples of sheep and reindeer, respectively. In the samples of grazing sheep in this study (summer and winter pasture), animal-to-animal variation may be explained by the ability of sheep to feed selectively and *ad libitum* on certain plants as opposed to cattle, which do not possess similarly precise mouthparts (Hofmann, 1989). However, animal-to-animal variation in sheep was also high when feed was prepared and served to the animals (silage and concentrate-based feed). Purser & Moir (1966) hypothesized that differences in rumen physiology and function between individual sheep may be attributed to differences in their rumen volumes. These differences may select for different ciliate communities of individual sheep.

Ciliate community variations between ruminant species and diet

Cluster analysis and statistical evaluation of underlying similarity matrices were performed to examine potential effects of ruminant species and diet on the ciliate community structure in sheep, red deer, and cattle. Ruminant-specific differences in ciliate community composition were observed on the basis of specific clustering in dendrograms illustrating the degree of similarity of DGGE banding patterns (Fig. 4). We tested this by comparing the within-species dissimilarities (i.e. variation between individuals of the same species) with the between-species dissimilarities (i.e. differences between individuals of different species). If the ruminant species has little effect on community structure differences, the dissimilarities between samples from the same species will not be significantly different from the dissimilarities found between different individuals of different species. Ruminant-specific differences between ciliate communities in samples obtained from cattle and sheep from flock 1 fed on the same three diets were highly significant, with a mean similarity of $36.3 \pm 22.7\%$ between cattle and sheep (Fig. 4a; $P = 2 \times 10^{-56}$). Sheep S2 fed on silage was the only sample to contradict this ruminant-specific clustering and instead grouped with the cattle samples. DGGE profiles of ciliate communities from sheep and red deer did not cluster by ruminant species, but subclusters were identified in which samples seemed to group ruminant-specific (Fig. 4b and e). Sheep were more similar to deer than they were to cattle (Fig. 4b and e), with mean similarities of $89.0 \pm 6.0\%$ (between deer and sheep of flock 1) and $63.5 \pm 17.9\%$ (between deer and sheep of flocks 2 and 3). Although not immediately apparent from the dendrograms, the sheep profiles were significantly different to the red deer profiles [$P = 0.026$ (comparing the deer and the sheep of flock 1) and $P = 0.001$ (comparing the deer and the sheep of flocks 2 and 3)]. Comparison between red deer and cattle resulted in highly significant ruminant-specific clustering (mean similarity $62.0 \pm 6.4\%$; $P = 4 \times 10^{-91}$; Fig. 4c). The analysis of sheep samples from the three different

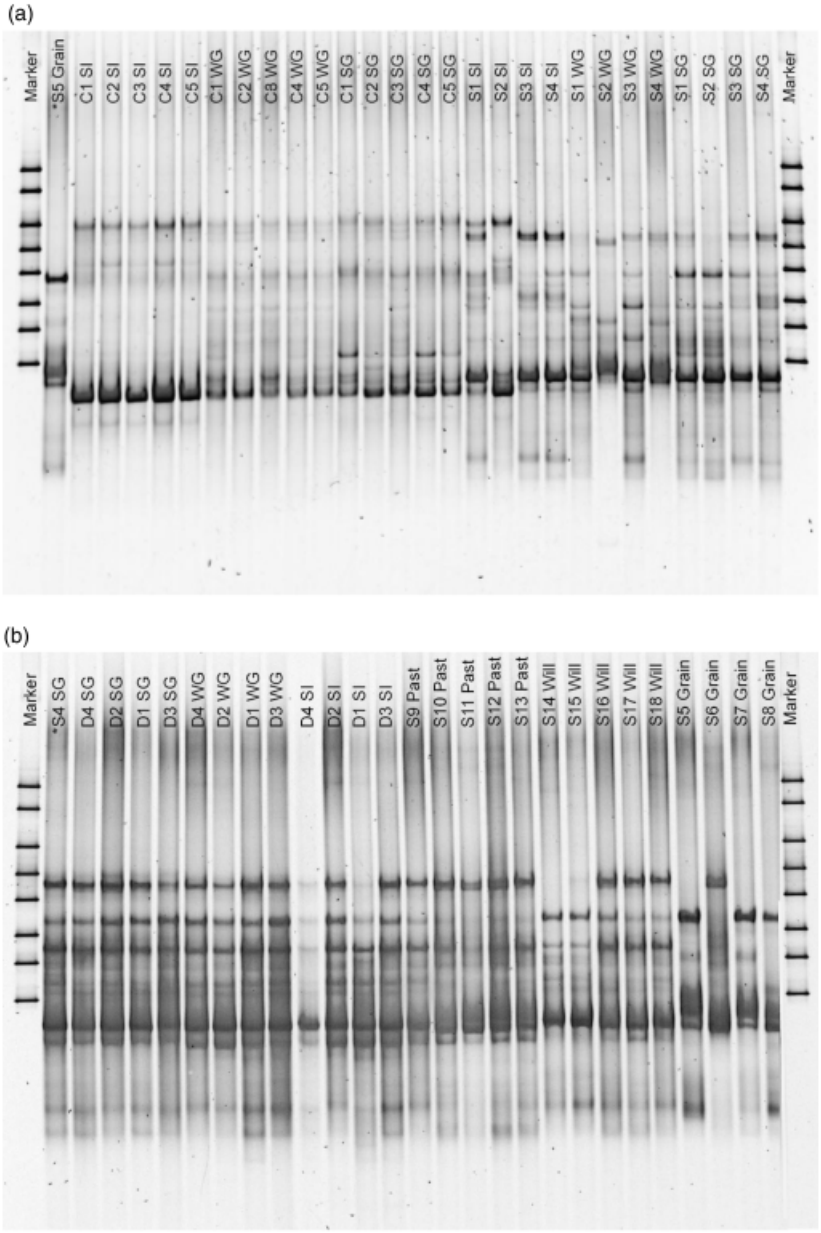


Fig. 3. DGGE gels of partial 18S rRNA genes amplified from ciliate communities in rumen samples of (a) four sheep and five cows and (b) four deer and 14 sheep feeding on summer pasture (SG), winter pasture (WG), silage (SI), autumn pasture (Past), willow (Will), or a concentrate-based diet (Grain). The outer two lanes were loaded with an external standard (Marker IV; Nippongene). Additionally, samples from a sheep on a concentrate-based diet (*S5 Grain) and a sheep on summer pasture (*S4 SG) were used as internal standards for intergel comparisons.

flocks revealed a mean similarity of all sheep samples of $64.8 \pm 12.1\%$ (Fig. 4d).

We also examined the effect of diet on rumen ciliate community structure. This was carried out by comparing the within-diet dissimilarities (i.e. variation inherent when multiple animals are fed the same diet) with the between-diet dissimilarities (i.e. differences when the same animals are fed different diets). If diet has little effect on community structure, the dissimilarities between samples from the same individuals on different diets will not be significantly different from the dissimilarities found between different individuals on the same diet. Two independent cluster analyses of DGGE profiles indicated that there were highly significant diet-specific differences in the ciliate communities of the cattle [$P = 4 \times 10^{-16}$ (Fig. 4a, Table 3) and $P = 1 \times 10^{-14}$ (Fig. 4c, Table 3)]. These differences may also reflect changes in rumen ciliate communities due to season, because the different diets were administered over the course of a year. In contrast, diet-based differences in deer appeared to be subtle, if present at all (Table 3; $0.0046 < P < 0.28$). It cannot be completely ruled out that minor shifts due to diet occurred, but this would have been masked by the animal-to-animal variation. At this stage, no conclusions can be drawn regarding a possible diet specificity in sheep. Even though the ciliate community composition of individual sheep changed during the experiment, no clear clustering by

diet was detectable for sheep of flock 1 ($7 \times 10^{-5} < P < 0.14$; Fig. 4d, Table 3). When comparing the three different flocks of sheep, the *t*-test indicates significance between treatment groups ($P = 2 \times 10^{-12}$). Because of the different locations of the sheep, however, it is not easy to distinguish between a diet and a potential flock effect. In order to confirm whether the administered diets had a significant effect on sheep and red deer, the number of animals for this experiment would have to be increased. Our results showed that cattle possess stable ciliate communities that consistently alter with diet, whereas ciliate community composition in sheep and red deer is variable. An example for the low stability of ciliate communities in sheep is seen in animal S2. DGGE cluster analysis revealed that the ciliate community of this particular sheep switched between 'sheep'- and 'cattle'-type community profiles within the course of the sampling period (~12 months; Fig. 4a). These shifts may be related to time, diet or other factors influencing ciliate communities in the rumen.

Table 3. Animal-to-animal variation in ciliate community structure, comparing all animals of a ruminant species feeding on the same diet (within-diet comparison) or the same animal on different diets (between-diet comparison)

Ruminant group	Data from figure	Dissimilarity (%)		Significance of difference of diet effect (*P*-value)
		Within diet	Between diet	
Sheep flock 1	4A	22.7 ± 18.8	30.6 ± 19.2	0.14
	4B	9.1 ± 6.2	13.7 ± 7.2	0.0142
	4D	21.7 ± 8.9	33.2 ± 9.5	7×10^{-5}
Cattle	4A	8.1 ± 5.2	24.0 ± 11.6	4×10^{-16}
	4C	8.7 ± 4.4	18.9 ± 6.2	1×10^{-14}
Deer	4B	4.6 ± 2.6	7.0 ± 3.3	0.0046
	4C	12.9 ± 10.6	16.6 ± 8.0	0.18
	4E	21.7 ± 11.9	25.5 ± 14.2	0.28

The data shown represent the results from multiple inter-ruminant comparisons on five different DGGE gels.

Phylogenetic placement of ciliate 18S rRNA genes from selected rumen samples

One of our aims was to increase the amount of 18S rRNA gene sequence information available for rumen ciliate protozoa. To do this, and to verify the cluster analyses based on DGGE profiles and to find out which ciliates underlay the observed differences in community structure, libraries of

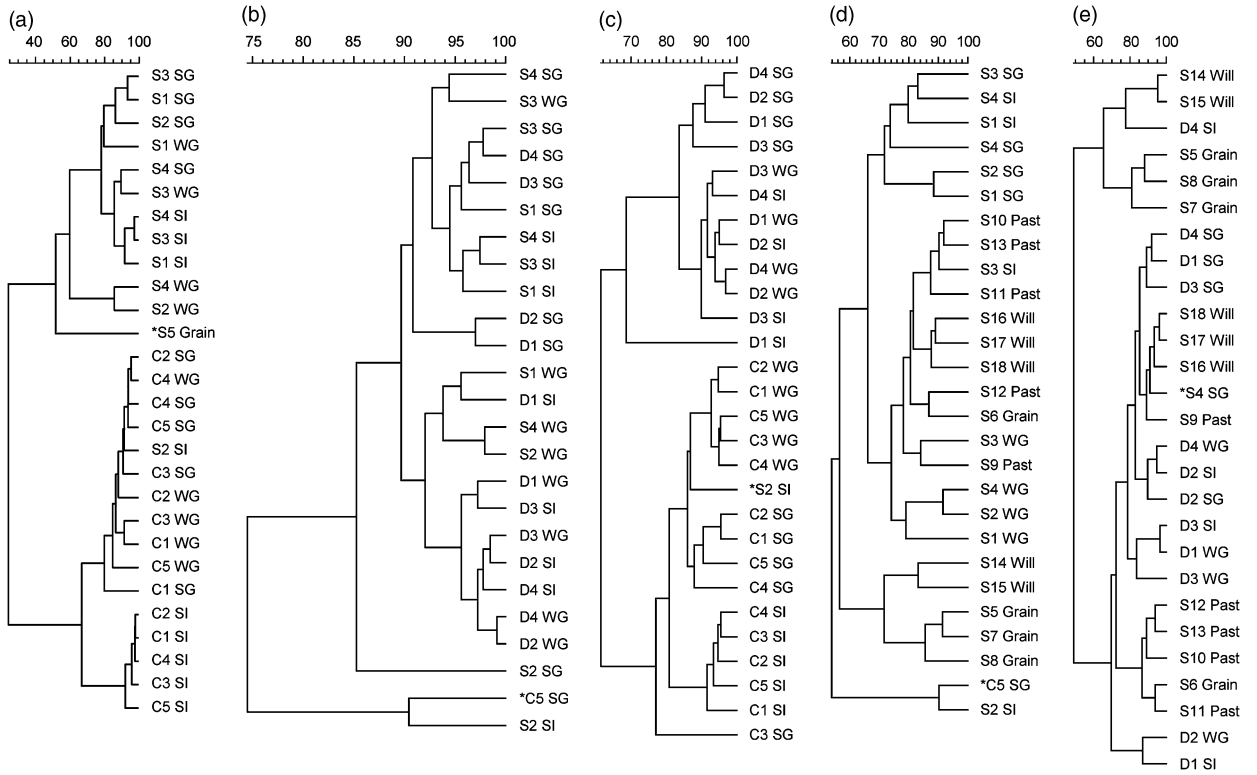


Fig. 4. UPGMA cluster analyses constructed with Pearson correlation from five DGGE gels. Comparisons of (a) sheep flock 1 with cattle, (b) sheep flock 1 with deer, (c) deer with cattle, (d) sheep from all flocks, and (e) sheep from flocks 2 and 3 with deer. *Samples that served as intergel standards. Diets are indicated by SG (summer pasture), WG (winter pasture), SI (silage), Grain (concentrate-based diet), Past (autumn pasture), and Will (willow fodder). The scale bar indicates the similarity between the DGGE profiles of any pair of samples at the right-hand-most common node between the samples given at the termini of the branches.

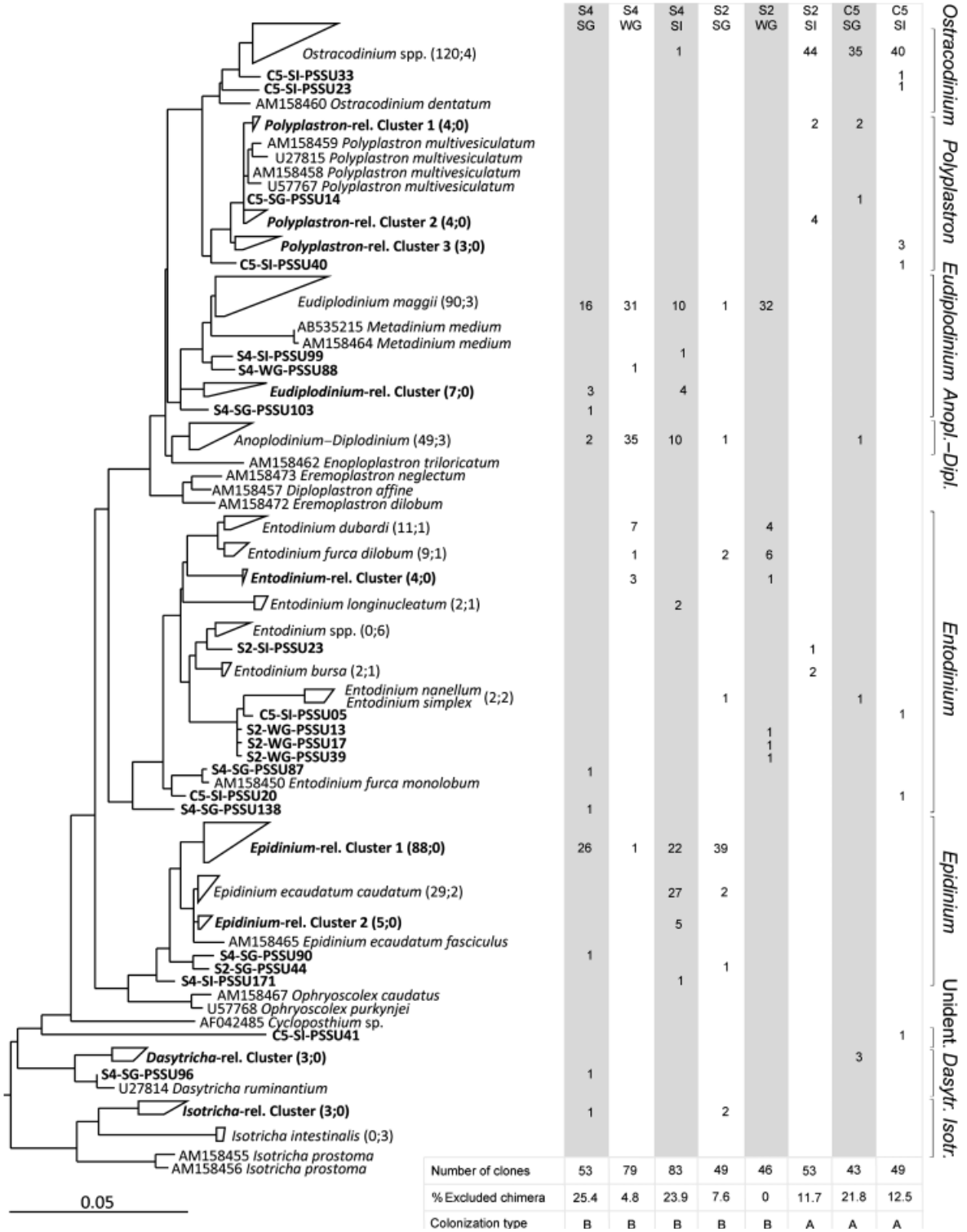
almost-full-length 18S rRNA genes with the Phylogeny primer pair were constructed from selected rumen samples. Three samples from sheep S2, on three different diets, were selected because DGGE profiles from these three samples were very dissimilar to each other. The samples from this animal clustered with those from other sheep when fed pasture, but the rumen sample from this animal when fed silage clustered with the samples from the cattle. We also selected the rumen samples from sheep S4, which clustered close to those from sheep S2 on summer and winter pasture in the DGGE analysis, and the samples from cow C5 on summer pasture and silage, which clustered closely to the sample from sheep S2 on silage or slightly outside in a separate group within the cluster of cattle-derived samples, respectively. The samples selected yielded a good representation of the overall diversity of ciliates in our set of rumen samples, and will form a solid basis for the construction of a rumen ciliate 18S rRNA gene reference database for future projects involving large-scale pyrosequencing.

Most of the libraries constructed from amplicons generated using the Phylogeny primer pair contained potentially chimeric sequences. These artefacts were likely formed during PCR due to the extraordinarily high similarity of ciliate 18S rRNA genes as compared, for example, with prokaryotic 16S rRNA genes. Chimeric sequences were detected by fractional treeing of all sequences (Ludwig *et al.*, 1997) and were excluded from further analyses. With only one exception (sheep S2 on silage), the number of chimera increased with an increase in community diversity at the genus level, as found by Qiu *et al.* (2001) previously. No chimeras were detected in the library produced from sheep S2 on winter pasture (two genera), whereas a total of 25.4% of sequences were chimeric in the library produced from sheep S4 on summer pasture (six genera). Especially now that the collection of large amounts of sequence data has become cheaper and easier, particularly when using next-generation sequencing, thorough phylogenetic analysis is essential to avoid the accumulation of artificial sequences in databases (Ashelford *et al.*, 2006).

Sequences retrieved from the eight clone libraries constructed were assigned to the following genera: *Epidinium*, *Eudiplodinium*, *Ostracodinium*, *Anoplodinium–Diplodinium*, *Entodinium*, *Polyplastron*, *Dasytricha*, and *Isotricha* (Fig. 5). These genera could represent the dominant ciliate protozoa in domestic ruminants in New Zealand. Interestingly, the dominance of *Epidinium* in some samples mirrors similar findings in New Zealand ruminants reported by Oxford (1958) who used microscopy to investigate protozoal communities. Sequence similarities between 18S rRNA genes from isolated species within the *Entodiniomorphida* vary widely, ranging from 96.9% to 100% between morphologically different species, while similarities between different entodiniomorphid genera range from 94.0% to 98.6%. The genus *Entodinium* contained the highest species diversity, with clone sequences closely related to *Entodinium bursa*, *Entodinium dubardi*, *Entodinium furca*, *Entodinium nanellum*, and *Entodinium simplex*. The distinct '*Entodinium*-related' cluster contained only sequences from sheep S2 and S4 on the winter pasture diet (Fig. 5). These sequences had 97.9–98.0% similarity to the 18S rRNA gene from *E. dubardi* (GenBank accession number AM158443). We retrieved a large number of sequences from four of the libraries that were 98.4–99.3% similar to the 18S rRNA gene sequence from the isolated species *Epidinium ecaudatum caudatum* (GenBank accession number AM158474). These distinct clusters of sequences may stem from so far undetected, novel ciliate species of the genera *Entodinium* and *Epidinium*. However, they may also represent named ciliate species that have not yet been obtained in pure culture and for which 18S rRNA gene sequence data are not yet available, or they may result from microheterogeneity of different 18S rRNA gene copies from the same organism. In order to precisely place phylogenetic sequence data of ciliates collected with molecular tools, efforts to isolate and cultivate these yet uncultured microorganisms undoubtedly have to be made. Overall, however, there was virtually no new genus-level diversity. Only one sequence (CS-SI-PSSU41; Fig. 5) of the total of 604 obtained using different primer sets (Fig. 1) and different samples (Fig. 5) could represent a new genus of rumen ciliates. This is in contrast to findings from studies of rumen bacteria and archaea, where novel genus-level groups are usually reported (Edwards *et al.*, 2004; Janssen & Kirs, 2008).

Sheep S4 on the summer pasture diet was mainly colonized by ciliates of the genera *Epidinium* and *Eudiplodinium*, representing 51% and 38% of the sequences obtained from this sample, respectively (Fig. 5). *Epidinium* was also the dominant genus in sheep S2 feeding on summer pasture (86% of all sequences). In contrast, in the samples of the same two sheep feeding on winter pasture, the major groups identified belonged to the genera *Eudiplodinium* (41% of the sequences from sheep S4, 70% from sheep S2) and *Anoplodinium–Diplodinium* (44% from sheep S4). These gross differences were in agreement with the clustering of these two samples based on their DGGE profiles ($\leq$ 87.4% similarity to the majority of sheep samples; Fig. 4a and d). Sequences affiliated with the genus *Epidinium* were scarcely represented in these two samples. However, *Epidinium* was the dominant ciliate genus among all sequences retrieved from the rumen sample of silage-fed sheep S4 (66%), while *Eudiplodinium* and *Anoplodinium–Diplodinium* related sequences accounted for 18% and 12%, respectively. Sequences belonging to the vestibuliferid genera *Isotricha* and *Dasytricha* were obtained only from the samples of sheep S2 and S4 fed on summer pasture (Fig. 5). A totally different ciliate community composition was observed in the samples of sheep S2 fed on silage and cow C5 on summer pasture and

silage, supporting the results of the cluster analysis from DGGE profiles (Fig. 4a and d). Strikingly, in these three samples, the vast majority of sequences clustered within the genus *Ostracodinium* (83% of the sequences obtained from sheep S2 on silage, and 81% and 86% from cow C5 on summer pasture and silage, respectively). Most of the remaining sequences were closely related to *P. multivesiculatum* (11%, 7%, and 8% of the sequences obtained from the sheep and two



cow samples, respectively). Our library data show that the differences detected using DGGE are based on underlying differences in community structure, and so confirm that DGGE is useful for preliminary screening of samples.

Distinct ciliate community patterns in domestic ruminants

Eadie (1962) described four distinct types of ciliate communities that establish in ruminant animals. A-type ciliate communities are characterized by the key species *P. multivesiculatum*, whereas B-type ciliate communities contain one or more of the three key species *Epidinium caudatum*, *Eudiplodinium maggii*, and *Metadinium medium*. *Elytroplastron bubali* represents the key species of the cattle-specific K-type community, whereas *Entodinium* spp. as well as the holotrichs *Dasytricha* and *Isotricha* are sole members of the O-type ciliate community. New Zealand sheep and cows have previously been found to harbour only B-type ciliate communities (Oxford, 1958; Bailey & Clarke, 1963; Clarke, 1964; Bauchop & Clarke, 1976; Bauchop, 1979). Our findings corroborate only partly these earlier results. All sheep samples, with the exception of sheep S2 fed silage, contained B-type ciliate communities, with either *Epidinium* or *Eudiplodinium* as predominant genera. The red deer samples showed subtle differences to the sheep samples, but further analysis of these samples using a multiplex PCR confirmed that the analysed red deer harboured B-type ciliate communities similar to those in the sheep (S. Kittelmann & P.H. Janssen, unpublished data). In contrast, libraries in combination with DGGE fingerprints suggested that the cattle samples as well as sheep S2 on silage contained A-type ciliate communities. A-type ciliate communities in our study were largely dominated by *Ostracodinium* spp. (81–86% of total clones) and *P. multivesiculatum* (7–11% of total clones). *Ostracodinium* was originally classified as a member of B-type ciliate communities (Eadie, 1967). However, since then, it has also been described to coexist with *P. multivesiculatum* in A- and mixed AB-type communities (Towne *et al.*, 1986, 1988). Our analyses show that the community types originally proposed by Eadie (1962), using microscopy, are also detectable using DNA-based methods.

Eadie (1962) suggested that ciliate communities possess varying stabilities, depending on the ruminant host. For sheep, it has been found that once a B-type community is 'invaded' by an A-type community, it will not change back to the B-type. For cattle, however, a community switch back to the B-type is apparently possible (Eadie, 1962). Based on the chronological order of samplings in our experiment, sheep S2 switched from the A-type (silage) to the B-type (winter pasture) and stayed with the B-type (summer pasture), implying that shifts from A- to B-type communities also occur in sheep.

Opportunities for methane mitigation

In this study we showed that different ruminants in New Zealand are colonized by distinct ciliate communities. Whereas sheep and deer harboured B-type communities (with one exception), cattle were colonized by A-type communities, regardless of the types of diet tested in this study. Previously, only B-type communities have been detected in sheep and cattle in New Zealand, using microscopy. To date, only little is known about the impact of distinct ciliate populations on CH_4 production. However, several lines of evidence suggest that type-A ciliate communities, in particular communities dominated by *P. multivesiculatum*, produce less CH_4 than B-type communities. Newbold *et al.* (1995) observed that rumen fluid from sheep with A-type ciliate communities resulted in less ciliate-associated CH_4 production than rumen fluid containing mixed B- or O-type communities. Ushida & Jouany (1996) examined *in vitro* CH_4 production by several single A-type ciliate species in comparison with that of a mixed A-type ciliate community, and found that *Isotricha prostoma* had a CH_4 emission rate similar to that of the total mixed A-type community, whereas *P. multivesiculatum* produced only trace amounts of CH_4. These results concur with the failure of *Methanobrevibacter* spp. to establish interspecies hydrogen transfer with *P. multivesiculatum* demonstrated by

Fig. 5. Neighbour-joining tree showing the clustering of clone sequences from sheep S2 and S4 and cow C5 feeding on different diets (SG, summer pasture; WG, winter pasture; SI, silage) based on their almost complete 18S rRNA genes (Phylogeny primer pair) with reference sequences. The scale bar indicates 0.05 nucleotide substitutions per nucleotide position. For clarity, some coherent groups of sequences are indicated only as triangles or trapezia. Numbers of clones from each library that grouped in the different lineages are given on the right. Numbers of total clone sequences obtained for a cluster (first number) and numbers of reference sequences belonging to a cluster (second number) are given in brackets. Lineages in bold font do not contain reference sequences from known isolates. Genus groupings are shown on the extreme right. Abbreviations are used as follows: *Anopl.–Dipl.*, *Anoplodinium–Diplodinium* group; *Dasytr.*, *Dasytricha*; *Isotr.*, *Isotricha*; Unident., unidentified sequence. Names and GenBank accession numbers of reference sequences in the schematic groupings are as follows: *Ostracodinium clipeolum* (AB536717), *Ostracodinium trivesiculatum* (AB536718), *Ostracodinium gracile* (AB535662, AM158468), *Eudiplodinium maggii* (U57766, AM158451, AM158452), *Diplodinium dentatum* (U57764), *Anoplodinium denticulatum monacanthum* (AM158440), *A. denticulatum denticulatum* (AM158470), *Entodinium dubardi* (AM158443), *Entodinium furca dilobum* (AM158442), *Entodinium longinucleatum* (AB481099), *Entodinium caudatum* (U57765, AM158444, AM158445, AM158446, AM158447), *E. furca monolobum* (AM158471), *Entodinium bursa* (AM158448), *Entodinium nanellum* (AM158449), *Entodinium simplex* (AM158466), *E. caudatum* (U57763), *Epidinium ecaudatum caudatum* (AM158474), and *Isotricha intestinalis* (U57770, AM158441, AM158453).

Ushida *et al.* (1995). *Polyplastron multivesiculatum*, the key species of the potentially low-CH_4 emitting A-type ciliate community, is heavily colonized by intracellular bacteria, but only associates with few methanogenic archaea (Finlay *et al.*, 1994; Lloyd *et al.*, 1996; Irbis & Ushida, 2004). This is in contrast to most other rumen ciliates belonging to all four community types, which harbour large numbers of ecto- and endosymbiotic methanogens. These interesting observations may offer new strategies for CH_4 reduction. A controlled shift of ciliate communities in favour of the A-type, or species that do not form close associations with methanogens, may represent a relatively cheap and easy means to reduce CH_4 emissions from grazing sheep and deer. Manipulation of ciliate communities may be realized simply by cohousing of sheep and deer with domesticated cattle colonized with an A-type community, by inoculation of A-type communities into the rumens of sheep and deer, or by vaccination against key species of B-type communities. It is important that the hypothesis that A-type ciliate communities are linked to significantly lower CH_4 emissions than B-type communities is verified in large-scale trials combining CH_4 measurements with a characterization of ciliate communities.

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