Genetic diversity and diet specificity of ruminal Prevotella revealed by 16S rRNA gene-based analysis

Aschalew Z. Bekele, Satoshi Koike* and Yasuo Kobayashi

Graduate School of Agriculture, Hokkaido University, Sapporo, Japan

*__Correspondence:__ Satoshi Koike, Graduate School of Agriculture,

Hokkaido University, Sapporo 060-8589, Japan. Tel/Fax: +81 (11) 706-2812.

E-mail: skoike7@anim.agr.hokudai.ac.jp

Key words: rumen, *Prevotella*, diversity, diet specificity, 16S rDNA

Running title: Genetic diversity and diet specificity of rumen *Prevotella*

Abstract

16S rDNA-based analysis of rumen *Prevotella* was carried out to estimate the diversity and diet specificity of bacteria belonging to this genus. Total DNA was extracted from rumen digesta of three sheep fed two diets with different hay-to-concentrate ratios (10:1 and 1:2). Real-time PCR quantification of *Prevotella* revealed that the relative abundance of this genus in total rumen bacteria was up to 19.7%, while the representative species *Prevotella bryantii* and *Prevotella ruminicola* accounted for only 0.6% and 3.8%, respectively. Denaturing gradient gel electrophoresis analysis for *Prevotella* revealed shifts in community composition with diet. Analysis of 16S rDNA clone libraries showed significant differences (P=0.001) between clones detected from the sheep on the diets with different hay-to-concentrate ratios. The majority (87.8%) of *Prevotella* clones had <97% sequence similarity with known rumen *Prevotella*. These data suggest that uncultured *Prevotella* is more abundant than known *Prevotella* and that members of this genus appear to have specific metabolic niches.

Introduction

Ruminant animals harbor a diverse and dense microbial population in the rumen, which is essential for the bioconversion of feeds that otherwise are indigestible for the host digestive system. This complex microbial community comprises bacteria, protozoa, fungi (Hespell *et al.*, 1997; McSweeney *et al.*, 2005), methanogenic archaea (Morvan *et al.*, 1996) and bacteriophages (Klieve & Bauchop, 1998). The rumen bacteria are most abundant and carry out a considerable part of the biological degradation of plant fiber (Koike & Kobayashi, 2009). Comparative sequence analysis of rumen bacterial 16S rDNA clone libraries consistently has shown the dominance of two phyla in the rumen,

low G+C Gram-positive (LGCGP) bacteria and the *Cytophaga-Flavobacter-Bacteroides* (CFB) group (Whitford *et al.*, 1998; Tajima *et al.*, 1999; Koike *et al.*, 2003). Within the CFB group, *Prevotella*-related sequences were found to be predominant in the total 16S rDNA sequences retrieved from the particle-associated community in the rumen (Koike *et al.*, 2003; Whitford *et al.*, 1998). In a comprehensive 16S rDNA clone library-based analysis of rumen bacterial diversity, *Prevotella ruminicola*-related sequences were found as the single most abundant operational taxonomic units (OTUs) (Edwards *et al.*, 2004).

The genus *Prevotella* was proposed to distinguish certain former *Bacteroides* species (e.g. *Bacteroides melaninogenicus* and *Bacteroides oralis*, which were later reclassified as *Prevotella melaninogenicus* and *Prevotella oralis*, respectively) from "true" *Bacteroides* species more closely related to *Bacteroides fragilis* (Shah & Collins, 1990). There are four characterized rumen *Prevotella* spp: *P. ruminicola* (formerly known as *Bacteroides ruminicola*), *P. bryantii*, *P. albensis* and *P. brevis* (Avgustin *et al.*, 1997). Cultivated rumen *Prevotella* strains exhibit a higher degree of genetic divergence (Mannarelli *et al.*, 1991; Ramsak *et al.*, 2000), and differences in polysaccharide degrading abilities of the four characterized species have been demonstrated (Matsui *et al.*, 2000). In a phylogenetic analysis of a fiber-associated rumen bacterial community, large clusters of *Prevotella*-related sequences were retrieved from *in situ* incubated fiber in the rumen of sheep, implying the possible involvement of *Prevotella* in fiber breakdown (Koike *et al.*, 2003). Furthermore, *P. ruminicola* contribute to plant cell wall degradation by acting synergistically with cellulolytic bacteria (Osborne & Dehority, 1989).

In previous studies, attempts have been made to quantitatively describe rumen *Prevotella*. Culture-based studies showed that *Prevotella* strains account for 60% of total cultivable bacteria from the rumen of cows (Gylswyk *et al.*, 1990). Based on restriction enzyme profiling of PCR-amplified 16S rDNA sequences from rumen samples, Wood *et al.* (1998) reported that the relative abundance of rumen *Prevotella/Bacteroides* ribotypes in the total eubacterial 16S rDNA could range from 12 to 62%. They also demonstrated that the most abundant *Prevotella* ribotype was related to very few cultured strains, suggesting underrepresentation of certain members of the genus by cultured strains. Recent real-time PCR relative quantification studies showed that *Prevotella* comprised 42 to 60% of the total bacteria in the rumen, while the known *Prevotella* species accounted for only 2 to 4% of the total bacterial 16S rRNA gene copies, which indicates that the majority of *Prevotella* in the rumen are uncultured (Stevenson & Weimer, 2007).

Based on the genetic and phenotypic diversity of cultured *Prevotella* spp. it is likely that functional differences among the uncultured *Prevotella* occur. In this study, attempts were made to explore the genetic diversity and diet specificity of uncultured *Prevotella* in sheep fed two diets with different hay-to-concentrate ratios (10:1 or 1:2) using real-time PCR, denaturing gradient gel electrophoresis (DGGE) and 16S rDNA clone library analysis.

Materials and methods

Animals and sampling

Three rumen fistulated sheep (average body weight 96.7 ± 8.96 kg) were used in a crossover experimental design. In the first period, each animal was given a hay-diet containing orchardgrass hay (2.0 kg per day) and a commercial formula feed for sheep

(0.2 kg per day, Ram 76ME, Mercian, Tokyo, Japan), while in the second period each animal was fed a concentrate-diet containing 1.0 kg of the commercial formula feed and 0.5 kg of the orchardgrass hay. The orchardgrass hay contained 16% crude protein (CP), 47% neutral detergent fiber (NDF) and 63% total digestible nutrients (TDN), while the commercial formula feed contained 13% CP and 76% TDN on dry matter basis, respectively. Each diet was given for 3 weeks and rumen contents were sampled from individual animals prior to feeding on the last day of the experimental period. The samples were stored at -30°C until DNA was extracted. Throughout the experimental period, animals were kept in individual pens and fed once daily at 09:00 h. Water and a mineral block was available *ad libitum*. All procedures were approved by the Animal Care and Welfare Committee of Hokkaido University.

DNA extraction

Total DNA was extracted from 0.25 g wet rumen content samples following the RBB+C method according to Yu and Morrison (2004). Briefly, cells were lysed by repeated beating with glass beads (mini bead beater, BioSpec Products, Bartlesville, OK, USA) in the presence of 4% (w/v) sodium dodecyl sulfate, 500 mM NaCl, 50 mM Tris-HCl (pH 8.0) and 50 mM EDTA. Two different sized (0.1 mm and 0.5 mm) glass beads were used for disrupting the cells. After incubation of the lysate at 70°C for 15 min, nucleic acids were recovered by isopropanol precipitation. DNA was treated with DNase-free RNase and proteinase K, and purified by a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The quantity and quality of DNA was checked spectrophotometrically (Gene Quant spectrophotometer, Pharmacia Biotech, Cambridge, England) and the final concentration of DNA extracts adjusted to 10 ng/µl for all downstream applications.

Primer validation

Since the *Prevotella* genus-level (g-Prevo) primers used in the present study were originally developed for human gut *Prevotella* (Matsuki *et al.*, 2002), the specificity and coverage for rumen *Prevotella* was confirmed by *in silico* analysis. Forty sequences of rumen *Prevotella* 16S rDNA including the four characterized species and 26 rumen *Bacteroides* sequences were obtained from the GenBank database. The coverage and specificity of two sets of *Prevotella* genus-level primers, g-Prevo and PreGen4 (Stevenson and Weimer 2007) were tested *in silico*. The sequences were subjected to multiple alignments with the program Clustal X to identify sequence identities with the primer sets. In addition to the exact match of the primer sequences with the *Prevotella* and *Bacteroides* sequences, the presence of consecutive matching sequences at the 3' ends of the primer were considered to estimate the specificity.

Real-time PCR quantification of the 16S rRNA gene of target rumen bacteria

Plasmid DNA to be used as the standard in real-time PCR was obtained by cloning of 16S rDNA PCR products into *Escherichia coli* JM109 cells, as previously described (Koike *et al.*, 2007). For the species-specific PCR, the respective target species (Table 1) 16S rDNA were used to prepare the plasmid DNA, while plasmid DNA prepared from *P. ruminicola* 16S rDNA was used for the genus-specific and total bacterial quantification. The PCR primers used are shown in Table 1. PCR amplification for the quantification of target bacterial 16S rRNA gene was performed with a LightCycler 2.0 system (Roche Applied Science, Penzberg, Germany). The FastStart DNA Master SYBR Green I was used for PCR. The optimal amplification conditions for each primer pair were achieved with 3.5 mM (final concentration) $MgCl_2$. The reaction mixture in 20 µl of the final volume contained 2.5 mM $MgCl_2$,, 2 µl 10× Mastermix (containing

FastStart *Taq* DNA polymerase, reaction buffer, dNTP mixture, 1mM $MgCl_2$ and SYBR Green I dye), 0.5 pmol of each primer and 10 ng template DNA. The thermal profile consisted of denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, annealing at the temperature indicated for the primer pair (Table 1) for 5 s and 72°C for an appropriate extension time (Table 1). A 10-fold dilution series of the plasmid DNA standard for the respective target bacterial 16S rRNA gene was run along with the samples. Using standard curves obtained from the amplification profile of known concentrations of the plasmid DNA standard, the respective genes were quantified. To obtain the relative abundance of target bacteria in the rumen, the assay values for 16S rRNA gene copies of target bacteria were normalized to the total number of copies of rumen bacterial 16S rRNA genes.

DGGE for *Prevotella*

DGGE was used to observe shifts in the *Prevotella* community as a result of diet change. The analysis was carried out in a Bio-Rad DCode universal mutation detection system **(**Hercules, CA, USA). The g-Prevo primers employed for real-time PCR were used to amplify the V5-V8 regions of the 16S rRNA gene of *Prevotella*. An amplicon of around 530 bp for DGGE analysis was obtained by modifying the forward primer by addition of a 40 bp GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG CACGGGGGG-3'). PCR was conducted with a GeneAmp PCR 2400 thermal cycler (Perkin-Elmer, Yokohama, Japan). A reaction mixture containing 20 pmol of each primer, 5 µl of 10× ExTaq buffer, 10 pmol of each dNTP, 1.25 U polymerase (ExTaq, Takara, Otsu, Japan), and 10 ng of template DNA in a total volume of 50 µl was prepared. The temperature program for cycling consisted of an initial denaturation at

94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 55 °C for 30 s, and extension at 72°C for 30 s with a final extension at 72°C for 5 min.

PCR-amplified 16S rDNA fragments were separated using an 8% polyacrylamide gel with 0.5× TAE buffer (20 mM Tris-acetate, 10 mM sodium acetate, 0.5 mM EDTA, pH 8.0), and a 35% to 60% linear gradient of denaturant (100 % denaturant corresponded to 40% (v/v) deionised formamide and 7 M urea). The gel was run at 60°C, 80 V for 16 h, and then placed in fixing solution (10% ethanol and 0.5% acetic acid) for 2 h, stained in 0.1% (w/v) silver nitrate solution for 20 min and developed in 1.5% sodium hydroxide (w/v), 0.1% sodium borohydride (w/v) and 0.4% formaldehyde (v/v) for 8 min. Thereafter, the gel was rinsed and kept in distilled water till the image was scanned. Gel images were analyzed by BioNumerics software version 4.5 (Applied Maths, Kortrijk, Belgium). Normalized banding patterns were used to generate dendrograms by calculating Dice's similarity coefficient and by an unweighted pair group method with arithmetic averages clustering algorithm.

16S rDNA clone library analysis

Two clone libraries were constructed for the respective feeding conditions from composite samples; the samples were obtained from rumen content DNA from three animals under the same dietary conditions. PCR products were generated by g-Prevo primers with the same reaction and amplification conditions as described for DGGE with the exception of the forward primer without GC clamp. PCR products were cloned with a pGEM-T Easy Vector System (Promega, San Luis Obispo, CA, USA) according to the manufacturer's instructions. Clones containing the correct insert were sequenced at Takara Bio (Yokkaichi, Japan). Clone nomenclature was as follows: for the hay-associated *Prevotella* library, clone names begin with "HAPC" followed by the clone

number. Clone names in the concentrate-associated *Prevotella* library begin with "CAPC" followed by the clone number. All the sequences were deposited into the GenBank database with the following accession numbers (AB519308 - AB519446).

Phylogenetic analysis

Target sequences were automatically aligned using the multiple sequence alignment software ClustalX ver.1.81 (Thompson *et al.*, 1997). The alignment was checked manually for alignment errors and corrected. Phylogenetic analysis was performed using the neighbor-joining method (Saito & Nei, 1987) with a Kimura-2 correction in the software MEGA v.3.1. In order to statistically evaluate the branching of the tree, bootstrap analysis (Felsenstein, 1985) was carried out with 1000 resamplings of the data. Partial 16S rRNA gene sequences from the *Prevotella* clone libraries were compared with 16S rRNA gene sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST program, Altschul *et al.*, 1997) to obtain similarity values.

Diversity analysis

Clones generated from the respective feeding conditions were assigned to OTU based on a 97% sequence identity criterion (Stackebrandt & Goebel, 1994). Analysis of the diversity for the individual and combined libraries was carried out using the nonparametric estimator Chao1 (Chao, 1984) and the Shannon Index (Shannon & Weaver, 1949) through the FastGroupII web-based bioinformatics platform (http://biome.sdsu.edu/fastgroup/fg_tools.htm). Chao1 estimates the minimum richness (i.e., number of ribotypes) in a sample and is used to predict total number of OTU present (species richness). Shannon index combines richness (total number of ribotypes) and evenness (relative abundance of each ribotype), and it can be used as an overall

indicator of the level of diversity in a sample. The coverage of the clone libraries was calculated as [1 - (n/N)] × 100 by Good's method, where n is the number of singletons and N is the total number of sequences (Good, 1953). Comparison of the composition of the two clone libraries was performed with the web-based Library Shuffling (LIBSHUFF) program version 0.96 (http/libshuff.mib.uga.edu) (Henriksen, 2004) by calculating the homologous and heterologous coverage between libraries from the two different samples. The sequences were initially aligned by ClustalX and distance matrices were generated in the DNADIST program of the PHYLIP package (v. 3.66 using the Juke-Cantor model (Felsenstein, 1989) before submitting them to LIBSHUFF.

Results

Primer coverage and specificity

The forward g-Prevo primer showed exact match with 39 of the *Prevotella* sequences tested (Table 2). The remaining one *Prevotella* sequence had two nucleotide mismatches each at the 5' and 3' ends of the forward primer. The reverse primer had exact match with all the sequences. Therefore, the coverage of the g-Prevo primers was estimated to be at least 98% of rumen *Prevotella* sequences tested. Similarly, both the forward and reverse PreGen4 primers had exact sequence match with all the *Prevotella* sequences (Table 2). Both the forward and reverse g-Prevo primers had 3-7 and 2-3 nucleotide mismatches with all the *Bacteroides*, respectively. The mismatches were at both the 3' and 5' ends of the primers. On the other hand, the forward PreGen4 primer had exact match with 21 (80%) of 26 tested *Bacteroides* sequences. Although, the reverse PreGen4 primer had sequence mismatches with all the *Bacteroides* sequences, 6 sequences had 9-11 consecutive matching sequences at the 3' end (data not shown). Thus, the PreGen4 primers may potentially result in the non specific amplification of

Bacteroides sequences described above. Therefore, from the *in silico* analysis it was concluded that g-Prevo primers are more specific to ruminal *Prevotella* than PreGen4 primers. Based on their valid coverage and high specificity to ruminal *Prevotella*, the g-Prevo primers were selected to be used in this study.

Abundance of rumen *Prevotella*

Real-time PCR quantification of *Prevotella* revealed that the relative abundance of this genus in the total rumen bacteria of sheep was as high as 19.7% (Table 3). On the other hand, the commonly cultivated ruminal *Prevotella* species, *P. bryantii* and *P. ruminicola*, accounted for only 0.6% and 3.8%, respectively (Table 3). The relative abundance of *Prevotella* tended to increase when the animals were switched to a concentrate diet, although one animal showed no difference in the proportion of *Prevotella* in either diet (data not shown).

In order to demonstrate the proportion of classical ruminal bacterial species, the relative abundance of individual species was aggregated (Table 3). The sum of the relative abundance of 12 representative rumen bacterial species in the two dietary conditions accounted for 2.4 to 4.9% of the total rumen bacteria. The relative abundance of the rumen fibrolytic species (*Fibrobacter succinogenes, Ruminococcus albus and Ruminococcus flavefaciens*) tended to decrease in concentrate-fed sheep. In particular, the abundance of *F. succinogenes* decreased significantly ($P < 0.001$) when the sheep were fed a high-concentrate diet.

DGGE profile of *Prevotella* on hay and concentrate diets

The DGGE fingerprints of rumen *Prevotella* from the same diet showed a similar banding pattern and tended to cluster according to the diet, although a certain degree of animal-to-animal variation was observed (Fig. 1). The DGGE fingerprints revealed

unique bands for either the hay or concentrate diet, although there were common banding positions for the two dietary conditions. Comparative analysis of the DGGE profile across diet showed consistently more bands in samples from hay-fed animals (Fig. 1).

Sequence analysis

A total of 139 16S rRNA gene sequences, 60 from sheep on a hay diet and 79 from sheep on a concentrate diet, were subjected to sequence analysis after discarding those suspected to be chimeras. Good's coverage of the hay and concentrate libraries were 43.3% and 65.8%, respectively. Although the libraries were not comprehensive, we obtained diverse sequences of *Prevotella*, and diet-specificity was supported by both DGGE and library analysis.

Based on a 97% sequence similarity criterion (Stackebrandt & Goebel, 1994), only 17 clones (12.2%) from the two libraries were considered to represent the characterized rumen *Prevotella* species (*P. ruminicola* or *P. bryantii*) and the remaining 122 clones (87.8%) fell into the uncultured *Prevotella* (Table 4). Among the uncultured *Prevotella*, 60 clones (43.2%) had 92-96% similarity with previously reported sequences (Table 4). The Chao1 and Shannon indices predicted more diversity in the hay library (Table 4), and LIBSHUFF comparison showed significant (P=0.001) differences in the composition of the two libraries (data not shown).

Of the 17 clones that showed ≥ 97% sequence similarity with known *Prevotella* species, 16 clones were retrieved from concentrate-fed sheep (Table 4) and 11 clones were related to *P. ruminicola*, while 5 were related to *P. bryantii*. Only a single clone

from the hay diet was related to *P. ruminicola* at 97% sequence similarity. No sequences having ≥97% similarity with *P. brevis* and *P. albensis* were found.

The results of phylogenetic analysis of 16S rDNA sequences from the two libraries are shown in Fig. 2. Although the bootstrap values were < 50%, we divided the phylogenetic tree into 7 sections to show the distribution of the clones. Sixty-six out of 79 clones from the concentrate library were found in sections 1 and 3, meanwhile sections 4-7 contained 42 clones from the hay library. Hay clones were distributed in all sections of the tree.

Discussion

Application of molecular biological tools in the analysis of several environmental microbial communities revealed that only a small fraction of the microbiota is represented by cultured species (Janssen, 2006) and the rumen microbial community is no exception. A previous study indicated that only 11% of OTU detected in the rumen contain cultured representatives (Edwards *et al.*, 2004). We focused on the population dynamics, ecology and diversity of *Prevotella* in order to estimate the contribution of this genus to digestion of feed in the rumen. Real-time PCR quantification revealed that the proportion of two representative *Prevotella* species (*P. ruminicola* and *P. bryantii*) was one-quarter of that of the genus (4.4% vs. 19.7% for concentrate-fed sheep). This result indicates that *Prevotella* is abundant in the rumen and the majority of members of this genus are yet to be cultured. It was reported that the abundance of the other two ruminal *Prevotella* spp. (*P. brevis* and *P. albensis*) was negligible (Stevenson and Weimer, 2007). Similar to the other reports on rumen bacterial clone library analysis (Whitford *et al.*, 1998; Tajima *et al.*, 1999; Koike *et al.*, 2003), we did not find the

sequences of these two species in our clone libraries. Therefore, *P. brevis* and *P. albensis* seemed to be minor in the rumen, and they were not quantified.

The high proportion of *Prevotella* observed in the present study agrees with the report of Wood *et al.* (1998), who estimated the combined *Prevotella/Bacteroides* ribotypes in the rumen in the range of 12 to 62%. The numerical dominance of *Prevotella* spp. reported in different experiments (Gylswyk *et al.*, 1990; Wood *et al.* 1998; Stevenson and Weimer, 2007) suggests their importance in the ruminal digestion of feed.

Our results did not show numerical equivalence with those of Stevenson and Weimer (2007), who reported 42 to 60% *Prevotella* population in the total rumen bacteria; the disagreement in numerical values could be partially due to differences in the source of samples (sheep or cattle), DNA extraction and quantification methods, and the PCR primers used. We confirmed primer coverage and specificity *in silico*. The primer sequences (Matsuki *et al.*, 2002) used in the present study matched with almost all rumen *Prevotella* sequences retrieved from the database and were specific for *Prevotella*, while the primers used by Stevenson and Weimer (2007) could anneal both *Prevotella* and *Bacteroides*. Therefore, their primer set might have amplified ruminal *Bacteroides*, which have been frequently detected in previous analyses (Edwards *et al.*, 2004; Koike *et al.*, 2003), leading to overestimation of *Prevotella*. The RBB+C DNA extraction method that we used in this study gives not only high DNA yield but it also produces superior results in PCR-based studies of diversity (Yu and Morrison, 2004), which is indicative of a more complete lysis and representation of microbial community present in such samples. However, due to the animal species difference, it is likely that

the relative abundance as well as distribution of different *Prevotella* could be different in cattle and sheep.

Our phylogenetic analysis of *Prevotella* 16S rDNA sequences supports the findings of the quantification studies that indicated the predominance of uncultured strains. The majority (87.8%) of *Prevotella* clones had <97% sequence similarity with characterized rumen *Prevotella* which suggests that uncultured *Prevotella* are more abundant than cultured ones. Interestingly, the uncultured *Prevotella* clones were detected in similar proportions in both diets, suggesting their importance in ruminal fermentation of hay as well as concentrate diets. From the DGGE analysis, the common banding positions for both dietary conditions partially explain the versatile nature of *Prevotella* spp. reported previously (Avgustin *et al.*, 1994 & 1997; Matsui *et al.*, 2000). In the phylogenetic tree, OTU37 and OTU51 which are composed of clones from both libraries probably represent those rumen *Prevotella* involved in the breakdown of both hay and concentrate based diets. However, findings from DGGE and clone library analyses suggested the existence of diet-specific members of *Prevotella*. DGGE profiles tended to cluster according to the diet given, and this result provided molecular evidence for the presence of diet-specific subpopulations of *Prevotella* that might be involved in the degradation of either a hay or concentrate diet.

The phylogenetic relationship of sequences of the libraries for each dietary condition supported the DGGE observation. LIBSHUFF comparison of the two libraries confirmed significant differences (P=0.001) between the *Prevotella* community in hay- and concentrate-fed sheep, showing that members of *Prevotella* that were associated with the hay diet differed from those associated with the concentrate diet. The majority of clones in sections 1 and 3 of the phylogenetic tree are likely to be specific to the

concentrate diet as are those clones in sections 4-7 to the hay diet. The trend toward closer phylogenetic relationship of clones retrieved from the specific dietary conditions implies the presence of diet-specific phylotypes of *Prevotella.* However, more direct evidence is needed in order to link the proposed diet specific *Prevotella* lineages to their role in the ruminal fermentation of feed.

Our DGGE data further showed a consistently higher number of bands in samples from hay-fed animals. This finding corresponded with diversity analysis from clone libraries that showed higher diversity values (Chao1 and Shannon index) and a greater number of OTUs for clones generated from the hay diet. These results suggest the possible involvement of more diverse members of *Prevotella* in the degradation of a hay diet than that of concentrate.

In conclusion, *Prevotella* is a major member of the rumen bacterial community, and uncultured *Prevotella* constitute a large proportion of ruminal *Prevotella.* The diet-specific association of *Prevotella* clones observed suggests significant functional diversity of members of this genus in the rumen. This study provides evidence for the potential involvement of diverse groups of *Prevotella* in the degradation of feed in the rumen, particularly hay.

References

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**:403-410.

Avgustin G, Wright F & Flint H **(**1994) Genetic diversity and phylogenetic relationships among strains of *Prevotella* (*Bacteroides*) *ruminicola* from the rumen. *Int J Syst Bacteriol* **44**: 246-255.

Avgustin G., Wallace RJ & Flint H **(**1997) Phenotypic diversity among ruminal isolates of *Prevotella ruminicola*: proposal of *Prevotella brevis* sp. nov., *Prevotella bryantii* sp. nov., and *Prevotella albensis* sp. nov. and redefinition of *Prevotella ruminicola*. *Int J Syst Bacteriol* **47**:284-288.

Chao A (1984) Non-parametric estimation of the number of classes in a population. *Scand J Stat* **11:**265-270.

Edwards JE, McEwan NR, Anthony JT & Wallace RJ (2004) 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie van Leeuwenhoek* **86**:263-281.

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**:783-791.

Felsenstein J (1989) PHYLIP—phylogeny inference package (version 3.2). *Cladistics* **5:**164-166.

Flint HJ (1997) The rumen microbial ecosystem-some recent developments. *Trends Microbiol* **5**:483-488.

Good IJ (1953) The population frequencies of species and the estimation of population parameters. *Biometrika* **40**: 237–264.

Henriksen JR (2004) webLIBSHUFF (http://libshuff.mib.uga.edu).

Hespell RB, Akin DE & Dehority BA (1997) Bacteria, fungi and protozoa of the rumen. *Gastrointestinal Microbiology*,Vol. 2 (Mackie RI, White BA & Isaacson RE, eds), pp 59-141. Chapman and Hall, New York.

Janssen PH (2006) Identifying the dominant soil bacterial taxa in libraries of 16S rDNA and 16S rRNA genes. *Appl Environ Microbiol* **72**:1719-1728.

Klieve AV & Bauchop T (1988) Morphological diversity of ruminal bacteriophages from sheep and cattle. *Appl Environ Microbiol* **54**: 1637-1641.

Koike S, Yoshitani S, Kobayashi Y & Tanaka K (2003) Phylogenetic analysis of fiber-associated rumen bacterial community and PCR detection of uncultured bacteria. *FEMS Microbiol Lett* **229:** 23–30.

Koike S, Yabuki H & Kobayashi Y (2007) Validation and application of real-time polymerase chain reaction assays for representative rumen bacteria. *Animal Science Journal* **78,** 135–141.

Koike S & Kobayashi Y (2009) Fibrolytic rumen bacteria: Their ecology and function. *Asian-Aust J Anim Sci* **22**:131-138.

Mannarelli BM, Ericsson LD & Stack RJ (1991) Taxonomic relationships among strains of the anaerobic bacterium *Bacteroides ruminicola* determined by DNA and extracellular polysaccharide analysis *Appl Environ Microbiol* **57**:2975-2980.

Matsui H, Ogata K, Tajima K, Nakamura M, Nagamine T, Aminov RI & Benno Y **(**2000) Phenotypic characterization of polysaccharidases produced by four *Prevotella* type strains. *Curr Microbiol* **41**:45-49.

Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, Oyaizu H & Tanaka R **(**2002) Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Appl Environ Microbiol* **68:**5445-5451.

McSweeney CS, Denman ES & Mackie RI (2005) Rumen Bacteria. *Methods in Gut Microbial Ecology for Ruminants* (Makkar HPS & McSweeney CS, eds), pp 23-37. FAO/IAEA.

Morvan B, Bonnemoy F, . Fonty G & Gouet P (1996) Quantitative determination of H2-utilizing acetogenic and sulfate-reducing bacteria and methanogenic archaea from digestive tract of different mammals. *Curr Microbiol* **32**:129–133.

Osborne JM & Dehority BA (1989) Synergism in degradation and utilization of intact forage cellulose, hemicellulose and pectin by three pure cultures of ruminal bacteria. *Appl Environ Microbiol* **55**:2247-2250.

Ramsak A, Peterka M, Tajima K, Martin JC, Wood J, Johnston EA, Aminov RI, Flint JH & Avgustin G (2000) Unraveling the genetic diversity of ruminal bacteria belonging to the CFB phylum. *FEMS Microbiol Ecol* **33**: 69–79.

Saito N & Nei M (1987) The neighbour-joining method: a new method for constructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.

Shah HN & Collins DM (1990) *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. *Int J Syst Bacteriol* **40**: 205-208.

Shannon CE & Weaver W (1949) The mathematical theory of communication Urbana IL University of Illinois Press.

Stackebrandt E & Goebel EM (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bactereriol* **44**:846-849.

Stevenson DM & Weimer PJ (2007) Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl Microbiol Biotechnol* **75**: 165–174.

Tajima K, Aminov RI, Nagamine T, Ogata K, Nakamura M & Benno Y (1999) Rumen bacterial diversity as determined by sequence analysis of 16s rDNA libraries. *FEMS Microbiol Ecol* **29**: 159–169.

Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M & Benno Y (2001) Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Appl Environ Microbiol* **67**: 2766–2774.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG **(**1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Res* **25**:4876–4882

Van Gylswyk NO (1990) Enumeration and presumptive identification of some functional groups of bacteria in the rumen of dairy cows fed grass-silage based diets. *FEMS Microbiol Ecol* **73**:243–254.

Whitford MF, Forster RJ, Beard CE, Gong JH & Teather RM (1998) Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. *Anaerobe* **4**: 153–163.

Wood J, Scott KP, Avgustin G, Newbold CJ & Flint HJ (1998) Estimation of the relative abundance of different *Bacteroides* and *Prevotella* ribotypes in gut samples by restriction enzyme profiling of PCR-amplified 16S rRNA gene sequences. *Appl Environ Microbiol* **64:**3683-3689.

Yu Z & Morrison **(**2004) Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques* **36:**808-812.

Table 1 PCR primers used in this study

Target	Primer sequences (5'-3')	Annealing Temperature (°C)	Extension time (sec)	Product Size (bp)	Efficiency	Reference
Total bacteria	[F][1] ACTCCTACGGGAGGCAG [R][2]GACTACCAGGGTATCTAATCC	57	19	465	2.01	[a]
Genus *Prevotella*	[F] CACRGTAAACGATGGATGCC [R] GGTCGGGTTGCAGACC	55	22	534	1.87	[b]
Ruminococcus flavefaciens	[F] TCTGGAAACGGATGGTA [R] CCTTTAAGACAGGAGTTTACAA	55	15	295	1.84	[c]
Ruminococcus albus	[F] CCCTAAAAAGCAGTCTTAGTTCG [R] CCTCCTTGCGGTTAGAACA	55	10	175	1.84	[c]
Prevotella ruminicola	[F] GGTTATCTTGAGTGAGTT [R] CTGATGGCAACTAAAGAA	53	20	485	1.78	[d]
Prevotella bryantii	[F] ACTGCAGCGCGAACTGTCAGA [R] ACCTTACGGTGGCAGTGTCTC	68	22	540	1.86	[d]
Fibrobacter succinogenes	[F] GGTATGGGATGAGCTTGC [R] GCCTGCCCCTGAACTATC	60	18	446	1.93	[d]
Streptococcus bovis	[F] CTAATACCGCATAACAGCAT [R] AGAAACTTCCTATCTCTAGG	57	35	869	1.85	[d]
Treponema bryantii	[F] AGTCGAGCGGTAAGATTG [R] CAAAGCGTTTCTCTCACT	57	18	421	1.92	[d]
Selenomonas ruminantium	[F] TGCTAATACCGAATGTTG [R] TCCTGCACTCAAGAAAGA	57	22	513	1.94	[d]
Anaerovibrio lipolytica	[F] TGGGTGTTAGAAATGGATTC [R] CTCTCCTGCACTCAAGAATT	57	25	597	1.82	[d]
Ruminobacter amylophilus	[F] CAACCAGTCGCATTCAGA [R] CACTACTCATGGCAACAT	57	27	642	1.94	[d]
Succinivibrio dextrinosolvens	[F] TGGGAAGCTACCTGATAGAG [R] CCTTCAGAGAGGTTCTCACT	57	35	854	2.51	[d]
Megasphaera elsdenii	[F] GACCGAAACTGCGATGCTAGA [R] CGCCTCAGCGTCAGTTGTC	58	5	130	1.91	[e]

[a] Stevenson and Weimer (2007), [b] Matsuki *et al.* (2002), [c] Koike and Kobayashi (2001), [d] Tajima *et al.* (2001), [e] Ouwerker *et al.* (2002)
1- Forward primer, 2- Reverse primer.

Table 2 Sequence matches of *in silico* analysis of primers

Primer	Primer sequences (5'-3')	Exact sequence matches to ruminal	
		Prevotella (n=40)	*Bacteroides* (n=26)
g-Prevo	[F][1] CACRGTAAACGATGGATGCC	39/40	0/26
	[R][2] GGTCGGGTTGCAGACC	40/40	0/26
PreGen4	[F] GGTTCTGAGAGGAAGGTCCCC	40/40	21/26
	[R] TCCTGCACGCTACTTGGCTG	40/40	0*/26

*Six sequences had only 2-3 nucleotide mismatches in the middle and near the 5' end but had 9-11 consecutive sequence matches at the 3' end. 1-Forward primer, 2-Reverse primer

Table 3 Percentages of target bacteria relative to total bacteria in the rumen of sheep fed hay or concentrate diet as determined by real-time PCR

	Hay diet	Concentrate diet	
Target rumen bacteria	Mean±SD	Mean±SD	*P* value
Genus *Prevotella*	**13.5** ± 6.1	**19.7** ± 3.0	0.190
Prevotella bryantii	0.01 ± 0.01	0.6 ± 0.6	0.197
Prevotella ruminicola	0.8 ± 0.52	3.8 ± 3.8	0.300
Other representative species			
Fibrobacter succinogenes	0.20 ± 0.02	0.01 ± 0.01	**0.001**
Ruminococcus albus	0.44 ± 0.45	0.09 ± 0.05	0.249
Ruminococcus flavefaciens	0.41 ± 0.60	0.01 ± 0.01	0.369
Treponema bryantii	0.38 ± 0.35	0.02 ± 0.04	0.219
Selenomonas ruminantium	0.08 ± 0.12	0.05 ± 0.06	0.726
Streptococcus bovis	0.02 ± 0.01	0.01 ± 0.01	0.207
Succinivibrio dextrinosolvens	<0.01 ± 0.01	<0.01 ± 0.01	0.749
Megasphaera elsdenii	ND	0.02 ± 0.01	-
Ruminobacter amylophilus	0.08 ± 0.05	0.03 ± 0.04	0.277
Anaerovibrio lipolytica	ND	0.21 ± 0.11	-
Sum of 12 representative species	**2.4**	**4.9**	

ND**,** Not detected.

Table 4 Diversity of rumen *Prevotella* in hay or concentrate fed sheep determined by 16S rDNA sequencing

Library	No. of clones	OTU	Chao1	Shannon Index	Known *Prevotella*		Uncultured *Prevotella**	
					P. ruminicola	*P. bryantii*	Previously reported	Not reported
Concentrate	79	36	108	3.20	11 (1 OTU)[J]	5 (1 OTU)	34 (16 OTUs)	29 (19 OTUs)
Hay	60	43	158	3.61	1 (1 OTU)	0	28 (21 OTUs)	31 (20 OTUs)
Combined	139	75	262	3.87	12 (1 OTU)	5 (1 OTU)	62 (37 OTUs)	60 (39 OTUs)

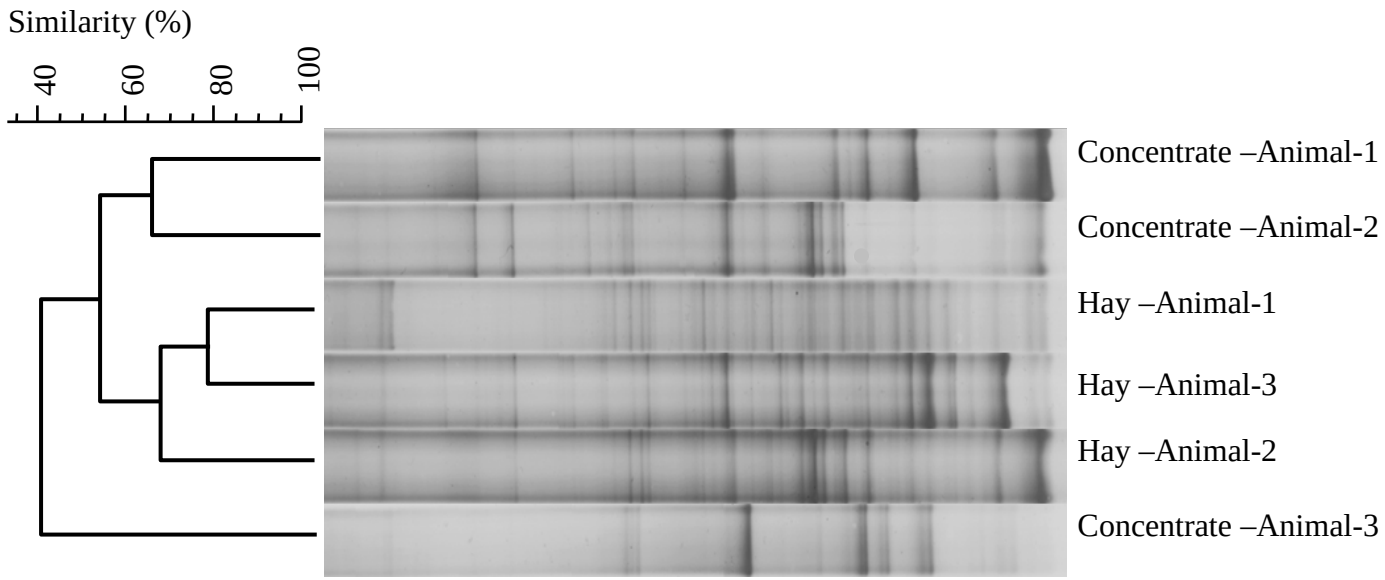
*Clones showing 92-96% sequence similarity with any sequence in the NCBI database were considered as not reported, while those having ≥97% similarity with uncultured clones were grouped as uncultured but previously reported.

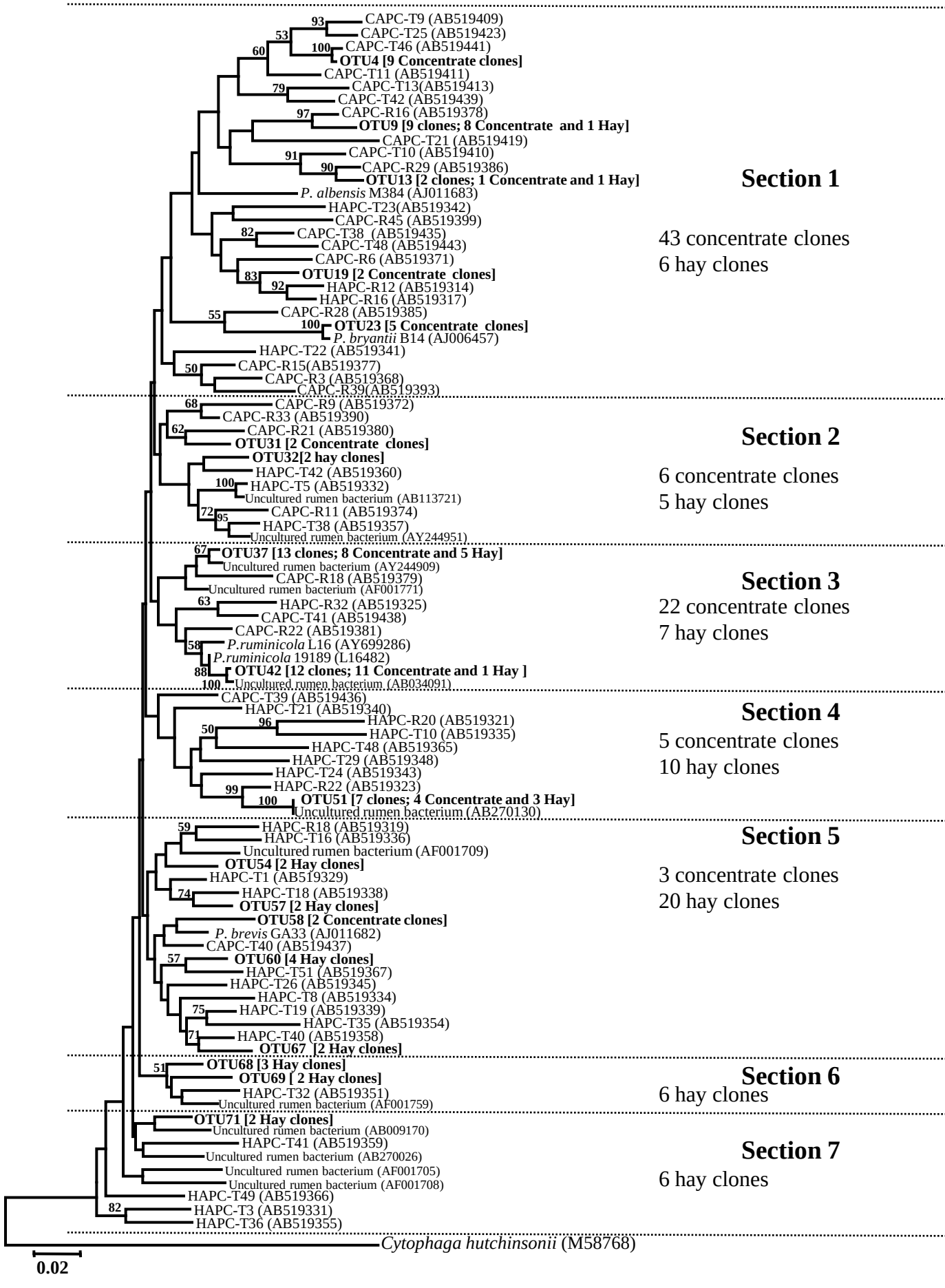
[J]Numbers in parentheses indicate predicted OTUs from the clones. Sequences related to *P. albensis* and *P. brevis* were not found.

Figure legends

Fig. 1 DGGE profiles of rumen *Prevotella* 16S rRNA genes with a dendrogram obtained by the unweighted pair group method with arithmetic mean clustering analysis.

Fig. 2 Phylogenetic placement of rumen *Prevotella* 16S rDNA clone sequences retrieved from sheep fed on hay (beginning with HAPC followed by clone number) or concentrate (beginning with CAPC followed by clone number). Clones having <97% sequence similarity were considered to belong to a distinct OTU. OTUs containing more than one clone are indicated in bold, and the number of clones in each OTU is indicated in brackets. Sequences of known *Prevotella* species and reported uncultured clones are included in the tree. Bootstrap values for 1000 trees are shown at branch points. Only values $\geq$50% are shown. The horizontal bar represents nucleotide substitutions per sequence positions.


Similarity (%)
40
60
80
100
Concentrate –Animal-1
Concentrate –Animal-2
Hay –Animal-1
Hay –Animal-3
Hay –Animal-2
Concentrate –Animal-3


CAPC-T9 (AB519409)
CAPC-T25 (AB519423)
CAPC-T46 (AB519441)
OTU4 [9 Concentrate clones]
CAPC-T11 (AB519411)
CAPC-T13(AB519413)
CAPC-T42 (AB519439)
CAPC-R16 (AB519378)
OTU9 [9 clones; 8 Concentrate and 1 Hay]
CAPC-T21 (AB519419)
CAPC-T10 (AB519410)
CAPC-R29 (AB519386)
OTU13 [2 clones; 1 Concentrate and 1 Hay]
P. albensis M384 (AJ011683)
HAPC-T23(AB519342)
CAPC-R45 (AB519399)
CAPC-T38 (AB519435)
CAPC-T48 (AB519443)
CAPC-R6 (AB519371)
OTU19 [2 Concentrate clones]
HAPC-R12 (AB519314)
HAPC-R16 (AB519317)
CAPC-R28 (AB519385)
OTU23 [5 Concentrate clones]
P. bryantii B14 (AJ006457)
HAPC-T22 (AB519341)
CAPC-R15(AB519377)
CAPC-R3 (AB519368)
CAPC-R39(AB519393)
CAPC-R9 (AB519372)
CAPC-R33 (AB519390)
CAPC-R21 (AB519380)
OTU31 [2 Concentrate clones]
OTU32[2 hay clones]
HAPC-T42 (AB519360)
HAPC-T5 (AB519332)
Uncultured rumen bacterium (AB113721)
CAPC-R11 (AB519374)
HAPC-T38 (AB519357)
Uncultured rumen bacterium (AY244951)
OTU37 [13 clones; 8 Concentrate and 5 Hay]
Uncultured rumen bacterium (AY244909)
CAPC-R18 (AB519379)
Uncultured rumen bacterium (AF001771)
HAPC-R32 (AB519325)
CAPC-T41 (AB519438)
CAPC-R22 (AB519381)
P.ruminicola L16 (AY699286)
P.ruminicola 19189 (L16482)
OTU42 [12 clones; 11 Concentrate and 1 Hay]
Uncultured rumen bacterium (AB034091)
CAPC-T39 (AB519436)
HAPC-T21 (AB519340)
HAPC-R20 (AB519321)
HAPC-T10 (AB519335)
HAPC-T48 (AB519365)
HAPC-T29 (AB519348)
HAPC-T24 (AB519343)
HAPC-R22 (AB519323)
OTU51 [7 clones; 4 Concentrate and 3 Hay]
Uncultured rumen bacterium (AB270130)
HAPC-R18 (AB519319)
HAPC-T16 (AB519336)
Uncultured rumen bacterium (AF001709)
OTU54 [2 Hay clones]
HAPC-T1 (AB519329)
HAPC-T18 (AB519338)
OTU57 [2 Hay clones]
OTU58 [2 Concentrate clones]
P. brevis GA33 (AJ011682)
CAPC-T40 (AB519437)
OTU60 [4 Hay clones]
HAPC-T51 (AB519367)
HAPC-T26 (AB519345)
HAPC-T8 (AB519334)
HAPC-T19 (AB519339)
HAPC-T35 (AB519354)
HAPC-T40 (AB519358)
OTU67 [2 Hay clones]
OTU68 [3 Hay clones]
OTU69 [2 Hay clones]
HAPC-T32 (AB519351)
Uncultured rumen bacterium (AF001759)
OTU71 [2 Hay clones]
Uncultured rumen bacterium (AB009170)
HAPC-T41 (AB519359)
Uncultured rumen bacterium (AB270026)
Uncultured rumen bacterium (AF001705)
Uncultured rumen bacterium (AF001708)
HAPC-T49 (AB519366)
HAPC-T3 (AB519331)
HAPC-T36 (AB519355)
Cytophaga hutchinsonii (M58768)
0.02
Section 1
43 concentrate clones
6 hay clones
Section 2
6 concentrate clones
5 hay clones
Section 3
22 concentrate clones
7 hay clones
Section 4
5 concentrate clones
10 hay clones
Section 5
3 concentrate clones
20 hay clones
Section 6
6 hay clones
Section 7
6 hay clones