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Phylogenetic Analysis of 16S rDNA Sequences Manifest Rumen Bacterial Diversity in Gayals (*Bos frontalis*) Fed Fresh Bamboo Leaves and Twigs (*Sinarumdinaria*)

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ABSTRACT : Six male Gayal (*Bos frontalis*), approximately two years of age and with a mean live weight of 203±17 kg (mean±standard deviation), were housed indoors in metabolism cages and fed bamboo (*Sinarumdinaria*) leaves and twigs. After an adjustment period of 24 days of feeding the diet, samples of rumen liquor were obtained for analyses of bacteria in the liquor. The diversity of rumen bacteria was investigated by constructing a 16S rDNA clone library. A total of 147 clones, comprising nearly full length sequences (with a mean length of 1.5 kb) were sequenced and submitted to an on-line similarity search and phylogenetic analysis. Using the criterion of 97% or greater similarity with the sequences of known bacteria, 17 clones were identified as *Ruminococcus albus*, *Butyrivibrio fibrosolvens*, *Quinella ovalis*, *Clostridium symbiosium*, *Succiniclasticum ruminis*, *Selenomonas ruminantium* and *Allisonella histaminiformans*, respectively. A further 22 clones shared similarity ranging from 90-97% with known bacteria but the similarity in sequences for the remaining 109 clones was less than 90% of those of known bacteria. Using a phylogenetic analysis it was found that the majority of the clones identified (57.1%) were located in the low G+C subdivision, with most of the remainder (42.2% of clones) located in the *Cytophage-Flexibacter-Bacteroides* (CFB) phylum and one clone (0.7%) was identified as a *Spirochaete*. It was apparent that Gayal have a large and diverse range of bacteria in the rumen liquor which differ from those of cattle and other ruminants. This may explain the greater live weights of Gayal, compared to cattle, grazing in the harsh natural environments in which Gayal are located naturally. (**Key Words** : Gayal, Rumen Bacteria, 16S rDNA, Phylogenetic Analysis)

INTRODUCTION

The Gayal or Mithun (*Bos frontalis*) is a rare semi-wild bovine species distributed throughout Bangladesh, Bhutan, China, India, Malaysia and Myanmar (Mondal et al., 2004; Rajkhowa et al., 2006). They belong to the family *Bovidae* (Giasuddin and Islam, 2003) and have adapted to harsh environmental conditions attaining higher live weights than cattle kept in similar environments (Cheng, 1984; Giasuddin et al., 2003; Mao et al., 2005). In these environments Gayal consume bamboo, reeds and woody plants which are not consumed by other local ruminants.

Ruminants are able to digest a range of plant materials by virtue of the extensive population of microbes, including bacteria, fungi and protozoa, which are found in the rumen, and provide the host with nutrients predominantly in the form of volatile fatty acids and microbial protein (Van Soest, 1994). The rumen bacteria have been shown by traditional procedures to belong to some 22 predominant species (Krause and Russell, 1996) but based on recent observations it is clear that this prior estimation represents only a small portion of the total diverse population that may colonise the rumen (Amann et al., 1995; Von Wintzingerode et al., 1997; Zoetendal et al., 1998; Krause et al., 2003; Shin et al., 2004a).

Molecular techniques based 16S/18S ribosomal RNA (rRNA) and their encoding genes (rDNA) have been used

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extensively to study rumen microbes both quantitatively (Stahl et al., 1988; Koike et al., 2003a; Sylvester et al., 2004) and quantitatively (Kocherginskaya et al., 2001; Shin et al., 2004b; Regensbogenova et al., 2004). These techniques have been used to construct a library of 16S rDNA clones of rumen microbes and to demonstrate the considerable diversity of rumen bacteria (White et al., 1999).

The present study was conducted to examine the diversity of rumen bacteria in lured Gayal offered a diet typical of that consumed under natural conditions. The molecular techniques now available were used to construct a library of 16S rDNA clones of rumen bacteria, and a phylogenetic tree for the clones isolated.

MATERIALS AND METHODS

Animals, diet and collection of rumen liquor

A herd of Gayal was lured into the Gayal Research Station (located at N 25°47'02.6", E 099°05'56.5" at an altitude of 2,260m above sea level) using salt. Six male Gayal approximately two years of age and with a live weight of 203±17.0 kg (mean±standard deviation) were selected from the herd and confined to metabolism cages in an open shed lit naturally. The animals were allowed to adjust to the environment for 24 days. They were fed fresh bamboo leaves and twigs (*Sinarundinaria* sp.) typical of the forage grazed naturally, which were harvested daily and offered *ad libitum* in equal portions at *c* 08:00 and 18:00 h.

At the end of the adjustment period representative portions of the diet offered were collected each day over four days for determination of chemical composition using the procedures published by Goering and Van Soest (1970) and the Association of Official Analytical Chemists (AOAC, 1990). The components measured were dry matter (DM), crude protein (CP), crude fibre (CF), ether extract (EE), ash, neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL).

Rumen liquor was collected on day 25 immediately before morning feeding. Approximately 500 ml of liquor was collected *via* a stomach tube located in the mid part of the rumen and connected to a vacuum pump (Yuangklang et al., 2005; Khampa et al., 2006a). The first 100 ml collected were discarded and the remaining liquor was passed through four layers of cheese cloth to remove particulate matter. The filtered liquor was transferred to previously sterilised tubes which were snap frozen in liquid nitrogen then stored at -80°C pending extraction of DNA.

Extraction of DNA

The frozen samples of rumen liquor were thawed and equal portions of the liquor from the six animals were pooled to obviate differences which may have arisen because of animal-to-animal variation. Total DNA in the pooled sample then was extracted as follows. An aliquot of 0.5 ml of homogenised rumen liquor was added to a tube (2 ml capacity) containing 0.5 g of sterilised glass beads (425-600 μm diameter; Sigma Chemical Company, USA) which then was centrifuged (10 minutes, 12,000 rpm, 4°C). The pellet was rinsed twice, with 0.5 ml saline-EDTA (0.15 M NaCl and 0.5 M EDTA, pH 8.0) followed by centrifugation (10 minutes, 12,000 rpm, 4°C), before re-suspension in 0.5 ml saline-EDTA. Mechanical disruption was effected using a Mini-Bead Beater™ Mill (Cole-Parmer International, USA) at maximum speed for 3 minutes on 3 occasions separated by periods of 2 minutes of chilling on ice. The bacterial DNA then was extracted using a Bacteria DNA Mini-Kit (Watson Biotechnologies Inc., Shanghai, P. R. China) in accordance with the manufacturer's guidelines. Finally, the pellet of DNA was dried then dissolved in TE buffer (pH 8.0) containing DNase-free RNase (100 μg/ml), prior to storage at -20°C.

PCR primers and amplification

Amplification of the 16S rDNA was achieved using:

- a forward primer F27
  (5'-AGAGTTTGATCMTGGCTCAG-3'); and
- a reverse primer R1492
  (5'-TAGGYTACCTTGTTACGACT-3').

A total of 20 μl of reaction mixture consisted of 20 pmol of each primer, *c* 100 ng of template DNA, 2 μl of 10× reaction buffer (Takara), 0.1 mM of each dNTP (Roche) and 2.5 U of *Taq* DNA polymerase (Promega). The PCR amplification was performed by Gene Thermal Cycle (Hangzhou, P. R. China) using the following program: denaturing at 95°C for 5 minutes, followed by 15 cycles of 30 seconds of denaturing at 95°C, 30 seconds of annealing at 55°C and 2 minutes of elongation at 72°C with a final extension at 72°C for 7 minutes. The PCR products were separated by electrophoresis in 1.0% (w:v) agarose gel and stained with ethidium bromide. Products were excised from the gel and purified with UNIQ-10 column DNA Gel Extraction Kits (Sangoo, Shanghai, P. R. China) in accordance with the directions of the manufacturer.

Cloning and sequencing

The purified PCR products were transferred directly into the TA Cloning® Kit with One Shot® TOP10 competent cells (Invitrogen, San Diego, USA) according to the protocol of the manufacturer. The recombinant plasmids then were extracted by the alkaline lysis mini-prep procedure (Birnboim and Doly, 1979) using a Mini-Plasmid Rapid Isolation Kit (BioDev-Tech, Beijing, P. R. China). The 16S rDNA was sequenced using an ABI PRISM Big

Dye Terminator Cycle Ready Reaction Kit and an ABI PRISM 3730 DNA Sequencer (Applied Biosystems) by Invitrogen Co. (Shanghai, P. R. China).

Sequence analyses and phylogenetic tree constructing

All reference sequences were obtained from the GenBank/EMBL/DDBJ/RDP. Sequences from the current study were analysed by the CHECK_CHIMERA program (Maidak et al., 2001) to remove any chimeric rDNA clone. Similarity with data base entries was assessed using an on-line BLAST search (Madden et al., 1996). Sequence alignment was achieved using multiple sequence alignment software CLUSTAL W Version 1.81 (Thompson et al., 1994). The criterion used to define a clone sequence as being that for a particular species of rumen bacteria was that the similarity of the sequence should be 97% or greater with that of the known species (Stackebrandt and Goebel, 1994). The phylogenetic tree was constructed by the neighbour-joining method (Saito and Nei, 1987) and the tree was evaluated using the bootstrap test based on 1,000 re-samplings (Felsenstein, 1985) based on the recommendation of Paster et al. (1991).

Nomenclature and nucleotide sequence accession numbers

The prefix GRC was used to denote clones identified. All nucleotide sequences have been deposited in GenBank under the accession numbers DQ673466-DQ673612.

Table 1. (i) Similarity values of 16S rDNA sequences retrieved from the rumen fluid of Gayal

OTU[a]	Phylum	Clones (entries)	Nearest relative (GenBank accession No)	Similarity (%)
GRC01	Low G+C subdivision	1	NA[b]	
GRC02	Low G+C subdivision	1	NA	
GRC03	Low G+C subdivision	1	NA	
GRC04	Low G+C subdivision	1	NA	
GRC05	CFB[c]	1	NA	
GRC06	CFB	1	NA	
GRC07	CFB	1	NA	
GRC08	Low G+C subdivision	3	*Ruminococcus albus* (AF030452)	99
GRC09	CFB	2	NA	
GRC10	Low G+C subdivision	1	NA	
GRC11	CFB	1	NA	
GRC12	Low G+C subdivision	2	NA	
GRC13	Low G+C subdivision	2	NA	
GRC14	Low G+C subdivision	1	NA	
GRC15	CFB	1	NA	
GRC16	CFB	1	NA	
GRC17	Low G+C subdivision	3	NA	
GRC18	Low G+C subdivision	1	NA	
GRC19	Low G+C subdivision	1	NA	
GRC20	Low G+C subdivision	1	NA	
GRC21	Low G+C subdivision	1	*Ruminococcus bromii* (X85099)	92
GRC22	Low G+C subdivision	1	NA	
GRC23	Low G+C subdivision	1	*Butyrivibrio fibrisolvens* (X89972)	97
GRC24	Low G+C subdivision	1	*Quinella ovalis* (M62701)	97
GRC25	Low G+C subdivision	1	NA	
GRC26	CFB	1	NA	
GRC27	Low G+C subdivision	1	NA	
GRC28	CFB	1	NA	
GRC29	Low G+C subdivision	1	NA	
GRC30	Low G+C subdivision	1	*Eubacterium cellulosolvens* (AY178842)	93
GRC31	Low G+C subdivision	1	NA	
GRC32	CFB	1	NA	
GRC33	CFB	1	NA	
GRC34	Low G+C subdivision	4	NA	
GRC35	Low G+C subdivision	2	NA	
GRC36	CFB	7	NA	
GRC37	CFB	1	NA	
GRC38	Low G+C subdivision	1	NA	
GRC39	Low G+C subdivision	1	*Clostridium symbiosum* (M59112)	98
GRC40	*Spirochaetes*	1	*Treponema bryantii* (M57737)	93

[a] OTU: Operational Taxonomy Units; [b] NA: Not available; [c] CFB: *Cytophaga-Flexibacter-Bacteroides*.

Table 1. (ii) Similarity values of 16S rDNA sequences retrieved from the rumen fluid of Gayal (continued)

OTU[a]	Phylum	Clones (entries)	Nearest relative (GenBank accession No)	Similarity (%)
GRC41	CFB[c]	1	NA[b]	
GRC42	Low G+C subdivision	1	*Succiniclasticum ruminis* (X81137)	97
GRC43	Low G+C subdivision	1	NA	
GRC44	Low G+C subdivision	1	NA	
GRC45	Low G+C subdivision	3	*Quinella ovalis* (M62701)	96
GRC46	Low G+C subdivision	1	*Eubacterium pyruvivorans* (AJ310135)	90
GRC47	Low G+C subdivision	1	NA	
GRC48	CFB	1	*Prevotella ruminicola* (L16482)	94
GRC49	Low G+C subdivision	1	NA	
GRC50	CFB	2	NA	
GRC51	CFB	1	*Prevotella ruminicola* (L16482)	95
GRC52	Low G+C subdivision	1	*Eubacterium cellulosolvens* (AY178842)	92
GRC53	Low G+C subdivision	2	*Selenomonas ruminantium* (DQ186901)	98
GRC54	Low G+C subdivision	1	NA	
GRC55	Low G+C subdivision	1	NA	
GRC56	Low G+C subdivision	1	NA	
GRC57	Low G+C subdivision	2	*Clostridium fimetarium* (AF126687)	93
GRC58	Low G+C subdivision	2	NA	
GRC59	Low G+C subdivision	3	*Pseudobutyrivibrio ruminis* (AF202262)	98
GRC60	Low G+C subdivision	1	NA	
GRC61	CFB	2	NA	
GRC62	Low G+C subdivision	2	NA	
GRC63	Low G+C subdivision	2	*Butyrivibrio fibrisolvens* (X89972)	99
GRC64	Low G+C subdivision	1	*Allisonella histaminiformans* (AF548373)	99
GRC65	Low G+C subdivision	1	NA	
GRC66	Low G+C subdivision	1	NA	
GRC67	CFB	1	NA	
GRC68	CFB	28	NA	
GRC69	Low G+C subdivision	1	NA	
GRC70	Low G+C subdivision	1	*Ruminococcus flavefaciens* (AY349157)	92
GRC71	Low G+C subdivision	2	NA	
GRC72	Low G+C subdivision	1	NA	
GRC73	Low G+C subdivision	2	*Ruminococcus albus* (AF030452)	95
GRC74	CFB	2	NA	
GRC75	Low G+C subdivision	1	*Ruminococcus bromii* (X85099)	95
GRC76	Low G+C subdivision	1	*Clostridium fusiformis* (AF028349)	96
GRC77	Low G+C subdivision	1	*Eubacterium cellulosolvens* (AY178842)	94
GRC78	Low G+C subdivision	2	NA	
GRC79	CFB[c]	1	NA[b]	
GRC80	Low G+C subdivision	1	*Ruminococcus gnavus* (X94967)	95
GRC81	CFB	2	NA	
GRC82	Low G+C subdivision	1	*Succiniclasticum ruminis* (X81137)	99
GRC83	Low G+C subdivision	1	*Acetitomaculum ruminis* (M59083)	92
GRC84	Low G+C subdivision	2	*Ruminococcus bromii* (X85099)	92
GRC85	CFB	1	NA	
GRC86	Low G+C subdivision	1	NA	

[a] OTU: Operational Taxonomy Units; [b] NA: Not available; [c] CFB: *Cytophaga-Flexibacter-Bacteroides*.

RESULTS

Chemical composition of the diet

The mean (n = 6) values±standard deviations of the means for the composition of the fresh bamboo leaves and twigs fed to the Gayal were: DM, 50.5±3.16; CP, 10.2±0.40; CF, 38.8±1.17; EE, 2.7±0.23; ash, 8.9±0.42; NDF, 75.3±0.72; ADF, 46.1±0.06; and ADL, 8.3±0.46.

Sequence similarity

A total of 147 16S rDNA clones of nearly full length and averaging 1,500 bp were isolated from the rumen liquor of the Gayal. These clones were classified into 86 Operational Taxonomy Units (OTU) (Table 1).

Of the 147 clones isolated, 16 (comprising ten OTU and representing 10.9% of the clones) had similarities of sequences with known species of rumen bacteria of 97% or

Table 2. Distributions of 16S rDNA clones and operational taxonomy units (OTU) retrieved from the rumen fluid of Gayal

Items	Clones[a]		OTU[b]	
	No. of clones	% total clones	No. of OTU	% total OTU
Similarity				
≥97%	16	10.9	10	11.3
90-97%	22	15.0	17	19.8
<90%	109	74.1	59	68.6
Phylum				
Low G+C subdivision	84	57.1	61	70.9
Ruminococcus albus	3		1	
Butyrivibrio fibrisolvens	3		2	
Allisonella histaminiformans	1		1	
Succiniclasticum ruminis	2		2	
Clostridium symbiosum	1		1	
Selenomonas ruminantium	2		1	
Pseudobutyrivibrio ruminis	3		1	
Quinella ovalis	1		1	
Uncultured groups[c]	49		37	
Cytophaga-Flexibacter-Bacteroides (CFB)	62	42.2	24	27.9
Uncultured groups	60	40.8	22	25.6
Spirochaetes	1	0.7	1	1.2
Uncultured groups	0		0	
Total	147		86	

[a] Clones having ≥97% similarity of 16S rDNA with a known species were referred to the bacterial names.
[b] Clones having >97% similarity of 16S rDNA among each other were defined as 1 operational taxonomy unit (OTU).
[c] Clones having <90% similarity of 16S rDNA with a known species and with >90% similarities among each other were defined as an uncultured group.

more. These corresponded to *Ruminococcus albus* (GRC08), *Butyrivibrio fibrisolvens* (GRC23 and GRC63), *Quinella ovalis* (GRC24), *Clostridium symbosium* (GRC39), *Succiniclasticum ruminis* (GRC42 and GRC82), *Selenomonas ruminantium* (GRC53), *Pseudobutyrivibrio ruminis* (GRC59) and *Allisonella histaminiformans* (GRC64). A further 22 clones (comprising 17 OTU and representing 15.0% of all clones isolated) shared similarities with the sequences for known rumen bacteria of between 90-97%. For the remaining 109 clones (comprising 59 OTU and 74.1% of isolated clones) the similarity with sequences of known rumen bacteria was less than 90% (Table 2).

Phylogenetic analysis

Because the similarity for most of the sequences with those of known rumen bacteria was too low to identify the sequence as representing a particular taxon, a phylogenetic tree was constructed to investigate the taxonomic placement. The results of this phylogenetic analysis are shown in Figure 1. The majority of the clones (57.1%) were positioned within the low G+C subdivision. Most of the remaining clones (42.2%) were classified as belonging within the *Cytophage-Flexibacter-Bacteroides* (CFB) phylum and only one clone (GRC40), comprising 0.7% of the total, was classified as belonging within the *Spirochaets*.

Within the low G+C phylum seven OTU formed a novel cluster in which *Q. ovalis* was located. The bootstrap value for the cluster was 92%. A further eight OTU clustered with *Ruminococcus bromii*, *R. flavifaciens* and *R. albus*, three OTU were related to *Eubacterium cellulosolvens*, nine were affiliated with *B. fibrisolvens*, and three were clustered with *R. gnavus* and *Clostridium fusiformis*. There were, however, groups of three, two, four, six and three OTU which formed five independent clusters which were not linked to any known rumen bacteria. A further two OTU (GRC25 and GRC66) constituted a new cluster deeply branched in the low G+C phylum not related to any known bacterium even though the bootstrap confidence level was 100% (Figure 1).

In the CFB phylum only two OTU clustered with *Prevotella* spp., sharing 94 and 95% similarity and having a bootstrap value of 100% indicating the stability of the group. Four and a separate single OTU constituted new clusters located between *Cytophaga hutchinsoni* and *C. fermentans*. The remaining 17 OTU constituted three separate clusters which were remotely associated with *Bacteroides* spp. and *Prevotella* spp. (Figure 1).

A single clone (GRC40) located within the *Spirochaetes* shared 93% similarity with *Treponema bryanti* and had a bootstrap value of 100%.

DISCUSSION

It is clear that the rumen of the Gayal contained an extensive and diverse array of bacterial species. The majority of the species (57.1%) were classified as belonging to the low G+C subdivision with most of the remaining species (42.2%) being located in the CFB subdivision. Only one organism classified as a *Spirochaete* was identified and

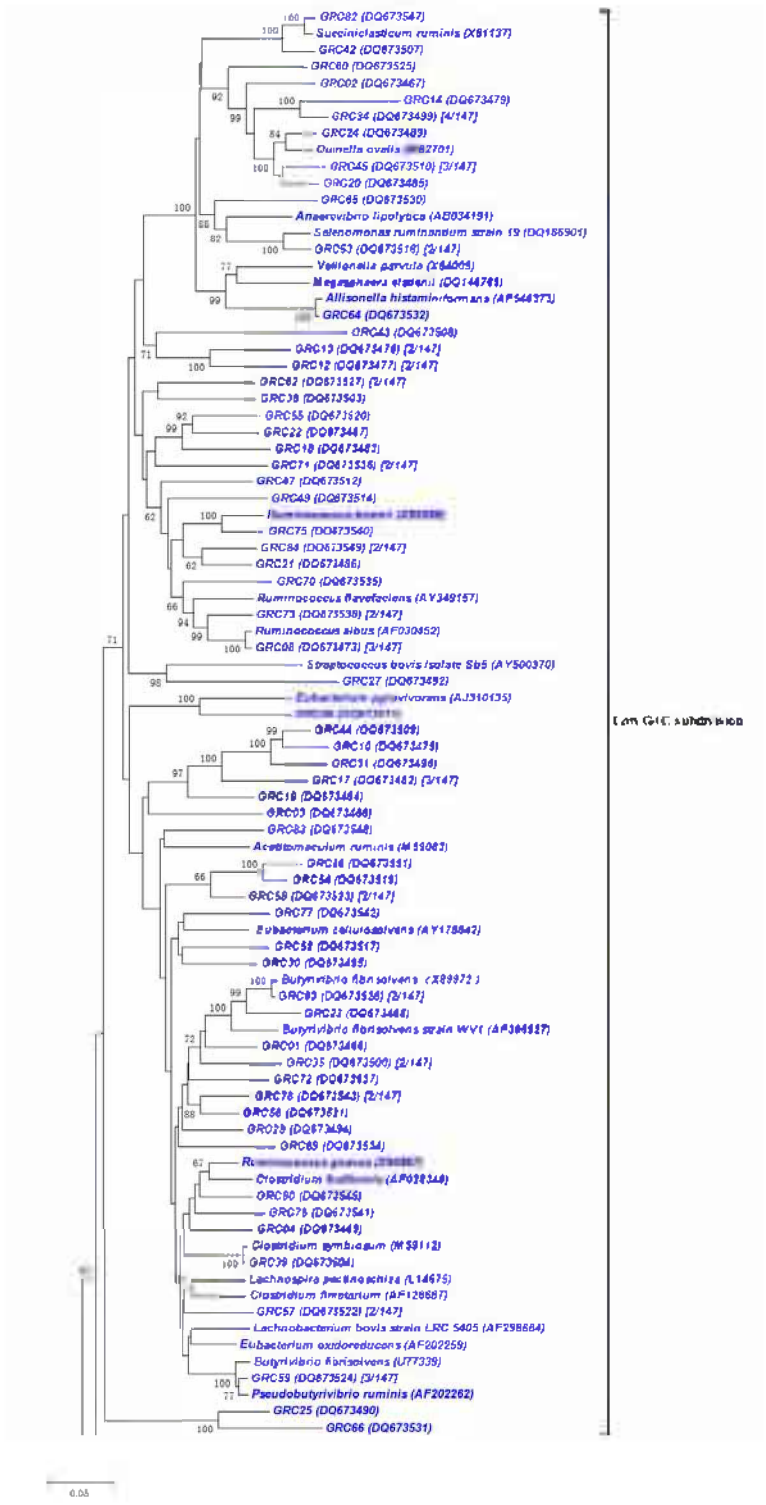

Figure 1. (i) Phylogenetic tree of 16S rDNA sequences from the Gayal rumen clones (begin with GRC followed by OTU number). The *Methanobacterium formicicum* (AY196659) and *Methanobrevibacter ruminantium* (AY196666) are used as the out-group for rooting the tree. Numbers in parentheses represent the sequence accession numbers in GenBank. Numbers in square brackets indicate the clone number out of the total clones. The number at each branch points is the percentage supported by 1000 bootstrap trees. Only values of 60% or above are shown. The bar represents 5% sequence divergence.

no *Proteobacteria* were identified. The proportions of bacteria located on the low G+C and CFB phyla for the Gayal, whilst exceeding those previously reported, approximated the proportions which have been reported.

Figure 1. (ii) Phylogenetic tree of 16S rDNA sequences from the Gayal rumen clones (begin with GRC followed by OTU number). The *Methanobacterium formicicum* (AY196659) and *Methanobrevibacter ruminantium* (AY196666) are used as the out-group for rooting the tree. Numbers in parentheses represent the sequence accession numbers in GenBank. Numbers in square brackets indicate the clone number out of the total clones. The number at each branch points is the percentage supported by 1000 bootstrap trees. Only values of 60% or above are shown. The bar represents 5% sequence divergence.

Thus Tajima et al. (1999) reported that 52.4% of 42 bacteria identified in the rumen liquor of Holstein cow (*Bos taurus*) fed a diet of hay belonged to the low G+C phylum and 38.1% to the CFB phylum. Similarly, An et al. (2005) reported that of the 194 clones isolated from the rumen liquor of yak (*Bos grunniens*) grazing on the Qinghai-Tibetan Plateau, 54.1% were low G+C organisms and 30.9% were CFB organisms. Moreover, Edwards et al. (2004) in summarising the published data for rumen bacteria reported that on average 54% of rumen bacteria were members of the low G+C phylum and 40% were from the CFB phylum.

Although the cellulolytic bacterium *Fibrobacter succinogenes* was not detected in the Gayal in the present study, other classical fibrolytic bacteria such as *Ruminococcus spp* and *B. fibrosolvens* (Hungate, 1966; Lila et al., 2005; Srinivas and Krishnamoorthy, 2005; Khampa et al., 2006a, 2006b) were recovered in three OTU. In total, 20 OTU (amounting to 23.3% of the OTU) identified in the present study were related to known fibrolytic bacteria. Briefly, eight OTU were identified as *Ruminococci* (*R. bromii*, *R. flavofaciens* and *R. albus* viz. GRC47, GRC49, GRC75, GRC84, GRC21, GRC70, GRC73 and GRC08) and a further OTU was located with *R. gnavus* (GRC80). Three OTU (GRC04, GRC76 and GRC80) were located between *Ruminococci* and *Clostridia* and an additional nine OTU (GRC63, GRC23, GRC01, GRC35, GRC72, GRC78, GRC56, GRC29 and GRC69) were associated with *B. fibrosiolvens*. It is of interest that *R. flavofaciens* (GRC70) was detected in the rumen liquor whereas others have reported this bacterium to be associated with the particulate material in the rumen (Tajima et al., 1999; An et al., 2005). Also of note was the detection of *Q. ovalis* (GRC24) and other related bacteria (GRC20 and GRC45) and *A. histaminiformans* (GRC64); the latter having been reported to be found in cattle and horses with laminitis (Garner et al., 2002). Neither *Q. ovalis* nor *A. histaminiformans* have been reported in the clone libraries of bacterial 16S rDNA isolated from the rumen in European cattle (*Bos taurus*: Whitford et al., 1998; Tajima et al., 1999, Ozutsumi et al., 2005), sheep (*Ovis aries*: Koike et al., 2003b), wild antelope except Thompson's gazelle (*Gazella rufifrons*) and Zebu cattle (*Bos indicus*: Nelson et al., 2003) and yak or Chinese Yellow Cattle, *Bos taurus* (An et al., 2005).

Amongst the bacteria comprising the CFB subdivision, only two OTU (GRC48 and GRC51) were closely related to *Prevotella ruminicola* which has been identified previously as the most numerous of the bacteria in the rumen (Stewart et al., 1997). The remaining 22 OTU located in the CFB subdivision was clustered loosely and their identification requires further investigation. Overall, the present results were consistent with previous reports that CFB bacteria were less numerous in the rumen in wild animals (Nelson et al., 2003; An et al., 2005) compared to domestic cattle. In

connection with the latter. Whitford et al. (1998) reported a predominance (101 of 133 clones) of 16S rDNA sequences similar to that for *P. ruminacola* in isolated material from domestic cattle.

The majority (59 of 86 or 68.6% of the OTU, comprising 109 of 147 or 74.1% of clones) of the bacteria identified in the present study had a similarity in sequence of 90% or less compared to known rumen bacterial species. Similar observations have been reported by Nelson et al. (2003) who found that only 24 of 279 clones isolated from the digestive tracts of Zebu cattle, zebra (Eq*uus quagga*) and three wild antelope (Eland, *Tauotragus oryx*; Thompson's gazelle; and Grant's gazelle, *Gazella granti*) were similar to known species of bacteria. An et al. (2005) reported that 49 of 91 (53.9%) OTU, comprising 106 of 194 (54.6%) clones, isolated from the rumen of the yak and 27 of 88 (30.7%) OTU consisting 68 of 197 (34.5%) clones isolated from Chinese Yellow Cattle were similar to identified species. On contrast, for domestic sheep fed orchardgrass or alfalfa hay diet (CP 11.6 or 16.5%, NDF 68.2 or 45.5%), Koike et al. (2003b) cloned 91 16S rDNA sequences from the fibre-associated bacteria in the rumen and found that 38 of these (41.8%) were less than 90% similar to known species. As a result, our finds further supported the result as shown by Kobayashi (2006), who pointed out that rumen samples of wild and semi-wild ruminants could contain novel bacteria to a greater extent than those of domestic counterpart.

It might be argued that the variations in the species of bacteria in the rumen are affected by diet. Indeed, Latham et al. (1972) and Tajima et al. (2001) have shown that diet affects the composition of the microbial population of the rumen. However, differences between the present observations and those published previously are considered to be related, at least in part, to animal species. For example, *Q. ovalis* and *A. histaminiformans*, which have been associated with sheep fed diets high in readily fermented carbohydrates (Krumholz et al., 1993) and cattle and horses (Garner et al., 2002), were retrieved from the rumen liquor of Gayal in the present study. However, these two kinds of bacteria were not emerged in bacterial 16S rDNA clone libraries of rumen fluid from cow fed hay diet, transition diet and high-grain diet (Tajima et al., 2000). Furthermore, another species commonly found in the rumen of ruminants fed grain based diets, *S. ruminatium* (Stewart et al., 1997), was identified in the rumen liquor of the Gayal, but the starch digesting bacterium *Ruminobacter amylophilus* (Stewart et al., 1997) was not identified in the present study. Moreover, in previous comparative studies between Gayal and Yunnan Yellow Cattle (*Bos taurus*) fed pelleted lucerne (*Medicago sativum*), Deng et al. (2007) reported that there was a significantly higher population of total as well as cellulolytic and amylolytic bacteria compared to Yunnan Yellow Cattle, which was associated with higher concentrations of volatile fatty acids in the rumen of the Gayal compared to the cattle. Further evidence that Gayal and Yunnan Yellow Cattle have different populations of rumen bacteria has been obtained *in vitro* where enhanced gas production and digestibility of DM have been measured for the Gayal under same condition (Xi et al., 2007).

In interpreting the results of the present study some caution needs to be expressed because of possible flaws in the analytical techniques utilised. As noted by Von Wintzergerode et al. (1997), technical biases during amplification of bacterial 16S rDNA from total DNA should be considered. First, bacteria for which genomic DNA has high G+C contents may be less readily amplified in the PCR process with universal primers (Reysenbach et al., 1992). Indeed Tajima et al. (2001) confirmed this by amplifying *F. succinogenes* (not identified in the present study) and showing a prolonged lag phase as compared to other bacteria under identical amplification conditions. Secondly, genome size and the copies of *rrn* genes also affect the amount of PCR amplification products which will be used as the target for cloning, even though the cloning step is random (Farrelly et al., 1995). Thirdly, the number of PCR cycles influences the bacterial diversity (Suzuki and Giovannoni, 1996) and to overcome this effect the number of PCR cycles employed was restricted to 15 with the view to improving the efficiency of cloning.

CONCLUSIONS AND RECOMMENDATIONS

In spite of these possible deficiencies in analytical techniques, it is concluded that Gayal have a large and diverse range of bacteria in the rumen. The present results are in conformity with the observations of Deng et al. (2007) that rumen liquor of Gayal contains greater numbers of total bacteria than similarly fed cattle. This would offer an explanation for the greater live weights of Gayal, compared to cattle, under field conditions. Clearly, further studies should be undertaken to confirm and extend the present observations.

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