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Full Length Research Paper

Culture-independent analysis of microflora in Gayals (*Bos frontalis*) feces

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Comparative DNA sequence analysis of 16S/18S rRNA genes (rDNA) were undertaken to further our understanding of the make-up of micro-organisms communities in the feces of Gayals. Total DNA were extracted from the feces of 5 Gayals. Two rDNA libraries (16S/18S rDNA) were constructed. In the 16S rDNA library, phylogenetic and sequence similarity analyses of the resultant 71 clone sequences revealed the presence of 67 operational taxonomic units (OTUs) or phylotypes and defined as having more than 97% of sequence similarity. The sequences were affiliated with the following phyla: Firmicutes (34.3%), Bacteroidetes (6.0%), Proteobacteria (4.5%), and uncultured bacteria (55.2%). A set of 58 sequences were analyzed in the 18S rDNA library, which were classified into 27 OTUs. They were mainly affiliated with the following phyla: Protozoa (25.9%), Basidiomycota (3.7%), Ascomycota (11.1%), and uncultured eukaryotes (59.3%). The sequence analysis indicated that more than half of the species, harbored in Gayals fecal belonged to the not-yet-cultured groups at 90% 16S/18S similarity levels with cultured species. In addition, micro-organisms of Chytridiomycetes was one of the most significant cellulose producing species obtained from the Gayal feces as well.

Key words: Gayal, culture-independent, fecal, phylogenetic analysis, 16S/18S rDNA.

INTRODUCTION

The Gayal or Mithun (*Bos frontalis*) is a rare semi-wild and semi-domestic bovine species which is distributed in the Dulong River and Nujiang River region of China, India, Bangladesh, Myanmar and Bhutan (Mao et al., 2005). Although they are distributed in different countries, they are at the edge of extinction in small numbers. Gayal has a very wide range of adaptation and activities, which overlap the Yak ecological zones (cold belt) and the Zebu ecological zones (tropical) (Zhao et al., 2003). They also have wide range of recipes and good feeding ability.

Gayal browse tree leaves and graze grasses, such as bamboo, reeds and other plant species. According to previous research, the animals thrive in adverse environments and attain mature live weights greater than other cattle maintained in similar environments (Deng et al., 2007). Actually, because of their rapid growth, characteristic body form, very good meat and strong productivity, they are not only very important in academic research but for exploitation and utilization. However, due to remoteness of their habitats and other ecological and sociopolitical factors, Gayals remain one of the least studied ungulates (Mohan et al., 2007).

The microbial ecology of gastrointestinal tract is composed of a large number of bacteria and a complex community (Yuhei et al., 2005). The relation between the host and most of its gastrointestinal tract symbionts is a form of obligatory mutualism. Those bacteria not only play an important role in health through their effects on gut morphology, nutrition, pathogenesis of intestinal disease,

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Abbreviations: VFA, Volatile fatty acids; **OTUs,** operational taxonomic units; **PCR,** polymerase chain reaction; **IPTG,** isopropyl–β–D-thiogalactopyranoside; **X-Gal,** 5–bromo–4–chloro–3–indolylb–D-galactopyranoside; **RFLP,** restriction fragment length polymorphism; **LB,** Luria-Bertani; BLAST, basic local alignment search tool.

and immune responses, they were also believed to protect against colonization of the intestines by pathogens and to stimulate the immune response. In return, the host intestinal tract provides the microorganisms an optimal environment for growth and reproduction. However, many factors can affect the composition of the avian bacterial community, such as diet, host, age, and environment etc.

Microbial populations were studied before basing on cultivation methods (Krause and Russell, 1996), considering that only a small fraction of the total microbial diversity can be recovered. These could not really reflect the microbial populations in the intestinal tract ecosystems because they require strict environmental conditions for survival and production, which resulted in more than 99% of environmental microorganism's non growth, at the artificial medium. With the rapid development of molecular techniques, cultured-independent methods provide an effective opportunity for the study of the microbes both quantitatively and qualitatively.

In spite of the great success of small subunit ribosomal RNA (SSU rRNA) based studies for the analysis of environmental prokaryotic diversity, this molecular approach has seldom been applied to microbial eukaryotes (David and López-García, 2002). Over the last several years, an unexpectedly high diversity of microbial eukaryotes (protozoa) was revealed with molecular (cultivation-independent) techniques (Amaral Zettler et al., 2002; Dı´ez et al., 2001; López-García et al., 2001; Moon-van der Staay et al., 2001). Most of them play an important role in the digestion of host (Deng et al., 2007).

Previous report (Orpin et al., 1988) has shown that Chytridiomycetes, including the anaerobic fungal species *Neocallimastix patricianum* and anaerobic bacterial degradation of cellulose have co -evolved with the most significant cellulose-producing bacteria of the herbivore gastrointestinal tract. The enzymes which come from these micro-organisms are able to efficiently degrade the polysaccharides of the plant cell walls by releasing into the rumen volatile fatty acids (VFA) and alcohol which are the end products of their fermentation. They play a role in the nutrition of the host animal through this way.

In this research, the molecular techniques now available were used to construct libraries of 16S rDNA clones and 18S rDNA clones of Gayals feces. This is the first analysis of the diversity of prokaryotic and microbial eukaryotes in the wild Gayals feces. To detect whether there is Chytridiomycetes in the Gayals feces, we have designed the special primer pairs Chyt-719/Chyt-1553 of it for PCR amplification. We aimed to get insight into the microbial diversity and the survival strategy of Gayal on fibrous diet, and further deduced the possible relations between microflora and host.

MATERIALS AND METHODS

Experimental materials

Simple random sampling was applied in selecting typical colonies of Gayals in their major area of habitat (the Yaping Gayal breeding farm in Fugong County, Yunnan Province). Five samples of fresh feces materials were collected from Gayals and mixed with 100% ethanol (Li et al., 2006). The samples were transported to the laboratory in an icebox and stored at -70℃.

Total DNA extraction

To minimize animal to animal variations, the aliquots of feces from the five animals were mixed before DNA extraction. Total DNA was extracted using an agarose gel DNA extraction kit (TaKara Co., Dalian, China). The DNA concentration and its integrity (size > 15 kb) were estimated by agarose gel electrophoresis with 0.8% (wt/vol) agarose^{-1}×Tris-borate-EDTA^{-1}ng of Gelview ml^{-1}(Antonia et al., 1999).

16S/18S rDNA amplification

Polymerase chain reaction (PCR) amplification of bacterial 16S rDNA was performed using the universal primers F27 (5′-AGATTGATCMTGGCTAGGGA-3′) and R1492 (5′-TACGGYTACC TTGTTACGACTT-3′) (Weisburg et al., 1991); 18S rDNA was amplified using the primers EF3 (5′-TCCTCTAAATGACCAAGTTT G-3′) and EF4 (5′-GGAAGGGRTGTATTTATTA G-3′) (Wu et al., 2006), and the dedicated primers for Chytridiomycetes Chyt-719 (GCACTTCATTGTGTGTACTG) and Chyt-1553 (GGATGAAACT CGTTGACTTC) (Hang and Yang, 2001). Samples were amplified in five replicate reactions to minimize stochastic PCR bias (Polz and Cavanaugh, 1998). The PCR was set up in 50 µL volumes containing 1.5 µL fecal DNA, 5 µL 10 × PCR buffer (containing 25 mmol/L Mg^{2+}), 4 µL dNTP (2.5 mmol/µL), 1.5 µL (10 pmol/µL) each primer, 0.5 µL Taq DNA polymerase and 36 µL ddH_2O. The amplification conditions were as follows: 4 min of initial denaturation at 94℃, followed by 30 cycles of denaturation at 94℃ for 1 min, annealing at 50℃ for 1 min 30 sec, extension at 72℃ for 2 min 30 sec with the last cycle followed by a 20 min extension step at 72℃. The PCR products were combined and visualized on an agarose gel, the bands were excised and DNA was purified from the gel slices using a TaKara DNA purification kit (TaKara Inc., Dalian, China). All purified DNA samples were then mixed in an Eppendorf tube.

Construction of 16S/18S rDNA clone libraries

The purified DNA products were ligated into pMD18T-vectors using a rapid ligation kit according to the instructions of the manufacturer (TaKara Inc., Dalian, China), and then transformed into *Escherichia coli* DH5α, which was prepared by the $CaCl_2$ method. Recombinant cells were selected on Luria-Bertani medium with ampicillin (100 µg/mL) and also with IPTG (isopropyl–β–D-thiogalactopyranoside) and X-Gal (5–bromo–4–chloro–3–indolylb–D-galactopyranoside). Colonies that contained a plasmid with an insert could not produce β - galactosidase and degrade X-Gal; consequently, they were white (Antonia et al., 1999; Sambrook et al., 1989).

RFLP analysis of 16S/18S rDNA

Possible redundancy among 100 clones with 1.5 and 1.6 kb 18S rDNA inserts was studied using restriction fragment length polymorphism (RFLP) analysis. Two vector primers: M13-47 (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′), and RV-M (5′-AGC GGATAACAATTTCACACAGG-3′) (Ke et al., 2007) were used to amplify each insert, by 30-cycle colony PCR. The products were digested directly by restriction nucleases *Afa I* and *Msp I*. The digested fragments were visualized on a 2.5% agarose gel, and

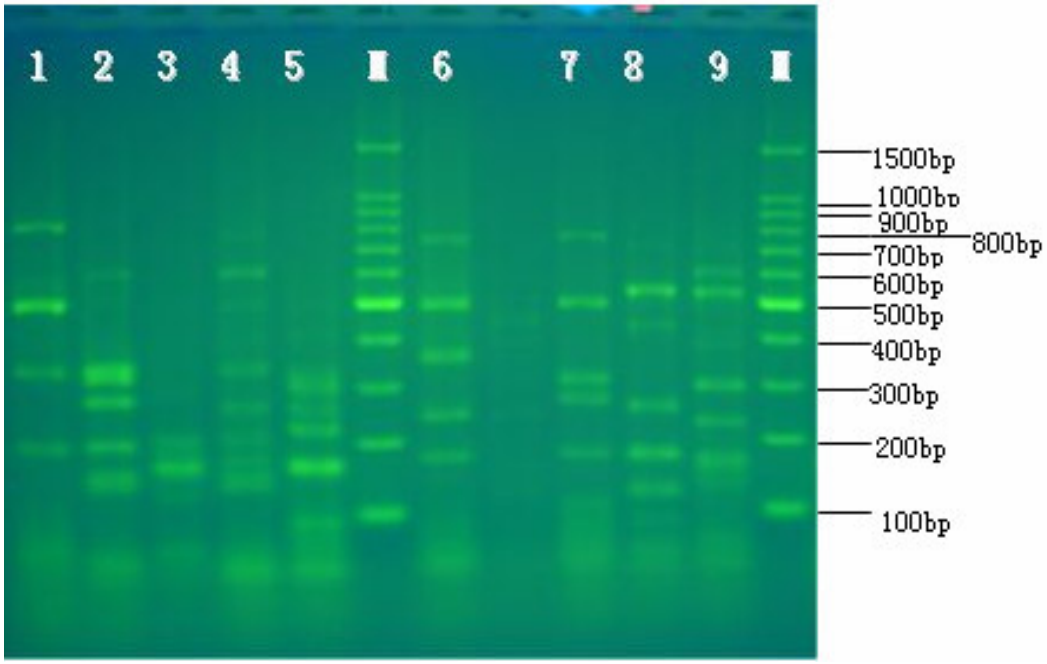

Figure 1. *Afa I* and *Msp I* restriction patterns of rDNA.

different clones were distinguished according to the RFLP patterns. One clone of each RFLP type was sequenced. The genetic diversity was subjected to statistical analysis by the percentage of coverage,which was calculated using the following formula:

Coverage = [1−(*n*/*N*)]×100% (Kemp and Aller, 2004).

Here, *n* is the number of phylotypes represented by one clone (16S rDNA sequence similarity of < 97%), *N* is the total number of clones.

Sequencing and sequence analysis

Selected cells were grown overnight in ampicillin-selective Luria-Bertani (LB) broth. The clones were then sequenced at the Huada Gene Sequencing facility. The resulting rDNA partial sequences were compared with GenBank entries using BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1900) to select reference sequences and obtain a preliminary phylogenetic affiliation of the clones. All the sequences obtained were checked for chimeric artifacts using the CHIMERA-CHECK program (Cole et al., 2005). The nearest neighbors were retrieved from the NCBI (http://www.ncbi. nih.gov/BLAST) through a BLAST search. The most similar sequences were retrieved and aligned with the sequences obtained in this work using CLUSTAL W version1.6. Gaps and regions of the alignments for which homology of residues could not be reasonably assumed, were excluded from the phylogenetic analysis. The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with the two-parameter model of Kimura (Kimura, 1980). The statistical significance of the tree branches was evaluated by bootstrap analysis (Felsenstein et al., 1985) of 1000 trees. The sequences were assigned to individual operational taxonomic units (OTUs; phylotypes) based on their phylogenetic positions and the 97% sequence similarity criterion. The nucleotide sequences have been submitted to the GenBank databases under accession numbers FJ848383 - FJ848511.

RESULTS

Total DNA extraction and 16S/18S rDNA amplification

A total of 50 µL DNA was extracted from the pooled fecal sample from five Gayals. This DNA was used directly as a template to amplify bacterial 16S rDNA and 18S rDNA. The PCR products were visualized on an agarose gel. The size of the amplification product conformed to the expected size, which is about 1500 and 1600 bp, respectively.

Construction of 16S/18S rDNA clone libraries

The purified DNA products were ligated into pMD18T-vectors and then used to construct clone libraries. We used blue-white screening to select for cells containing plasmids with inserts. Positive clones were picked using a toothpick and tested and verified by colony PCR using T-vector general primers. The size of the amplification product was conformed to the expected insert fragment size. Statistical analysis showed that the proportion of positive clones was about 95%.

RFLP analysis of 16S/18S rDNA

The colony PCR products showed different types of bands following digestion by the restrictive nucleases *Afa I* and *Msp I* (Figure 1). Although the RFLP results cannot accurately reflect all intestinal microbial groups present in Gayals, they do reflect the richness of the library. One

clone of each RFLP type was sequenced. The results of the check for chimeras showed that all of the sequences obtained were normal.

Bacterial diversity

In the 16S rDNA library, 71 clones, which had different RFLP types, were sequenced. No obvious chimeric artifacts of the 71 clone sequences were detected using the Check-Chimera Program. Therefore, all 71 sequences were phylogenetically analyzed. During the BLAST search, it was noteworthy that of the 71 sequences analyzed in this study, 5 sequences (DLN-23, DLN-104, DLN-109, DLN-124 and DLN-187) shared < 90% similarity to sequences found in GenBank. It was possible that these 5 sequences might represent undescribed bacterial genera and have not yet been reported. Clone DLN-43 shared 100% similarity with bacterium *gir* (EU464093). 26 sequences (37.68%) shared a sequence similarity of < 94% to sequences in GenBank database. 21 sequences (30.43%) shared ≥ 97% sequence similarity with other sequences. Only 3 sequences (DLN-16, DLN-150, and DLN-151) share similarities with cultivated species, which included *Bacillus* species and *Paenibacillus* species. All of the remainder matched uncultured bacteria that were derived from a previous research. Therefore, the majority of the sequences that were obtained in this study represented previously uncharacterized microbial species. Interestingly, 21 clones were close matched to the 16S rDNA sequences of unclassified bacteria from the bovine feces, which might be a reflection of sharing dietary patterns and exposure to similar microbial species as a result of their similar feeding patterns. In addition, 29 clones were matched to the unclassified bacteria from mammal gastrointestinal tract, which indicated that they were stable microflora in the process of animal evolution. We could pre-assess the evolutionary relation between mammals according to the microbial populations harbored in their gastrointestinal tract.

Based on the 97% sequence similarity criterion, a total of 71 clones were classified into 67 OTUs. In this study, 16S rDNA library included 187 clones in total. 71 different RFLP types were obtained, which were classified into 67 OTUs. According to the calculated formula: Coverage = $[1-(n/N)]\times100\%$, the result of calculation for the library coverage was 64.2%, which suggested that the study identified the dominant bacteria in the Gayals feces and the analysis was significant.

Because the similarity for most of the sequences with those of known gastrointestinal tract bacteria was too low to identify the sequence as representing a particular taxon, a phylogenetic tree was constructed to investigate the taxonomic placement. Based on sequence similarity, phylogenetic analysis was performed on the sequences obtained in this study and some of the reference sequences obtained from the database. The result of the BLAST search showed that 71 sequences were mainly affiliated to the following phyla: Firmicutes, Bacteroidetes, Proteobacteria, Lentisphaerae, and uncultured bacteria. The majority of the sequences shared similarity with the Firmicutes (53 sequences or 51 OTUs), particularly with the Ruminococcaceae (38 sequences or 36 OTUs). However, there are only 23 OTUs clustered in the Firmicutes. The remainder 29 OTUs were clustered at individual clade (Figure 2). Figure 3 shows the phylogenetic inferences among one of the taxonomic units affiliated with the Firmicutes, which included five subgroups as follows: Clostridiaceae (10 OTUs; 43.5%), Lachnospiraceae (1 OTU; 4.3%), Ruminococcaceae (6 OTUs; 26.1%), Bacillaceae (4 OTUs; 17.4%), and Catabacteraceae (2 OTUs; 8.7%). The vast majority of these sequences were clustered with the Clostridiaceae (10 OTUs), which was considered to be the dominant microflora of Firmicutes in many previous researches. The secondary dominant microflora was Ruminococcaceae, which included 6 OTUs in the phylogenetic tree. 2 OTUs (DLN-20 and DLN-152) belonged to Catabacteraceae. Similar sequences were also obtained from hindgut microbiota of a wood - feeding higher termite, which indicated that they played an important role in the digestive process. They could decompose the dietary fiber into nutrients that animals need. However, only one OTU (DLN-36) cluster in the Lachnospiraceae clade, which was often detected in the rumen. DLN-36 shared 100% bootstrap value with uncultured Lachnospiraceae bacterium (EU794281), which came from other bovine feces. Therefore, DLN-36 and uncultured Lachnospiraceae bacterium may share the same genetic status.

Bacteria of the Bacillaceae are parasite that always harbor in soil rot. Sometimes, they are parasitic in insects or animals and as pathogens, they inhabit insects or animals. In this study, 4 OTUs (DLN-187, DLN-16, DLN-150 and DLN- 125) of Bacillaceae were obtained in the Gayals feces as well. Clone DLN-150 shared 97% similarity with *Bacillus* species and 85% bootstrap value with it, indicating that the original strain of clone DLN-150 has similar function with *Bacillus* sp. If the strain *Bacillus* sp. is a pathogen harbored in the gastrointestinal tract, Gayals will be in danger.

29 OTUs (43.3%) could not cluster with any reference sequence in the phylogenetic tree, although they shared similarity with the bacteria from the Ruminococcaceae during the BLAST search. Therefore, we proposed that those sequences made the Gayal unique (Figure 3). They might play an important role in the health of animal and affect its physiology.

According to previous reports, some bacteria of Ruminococcaceae were the well-known rumen fibrotic bacteria, such as *Ruminococcus albus* and *Ruminococcus flavefaciens*. However, we could not identify those bacterial functions in this study because they shared low similarity with those cultured species. On the other hand,

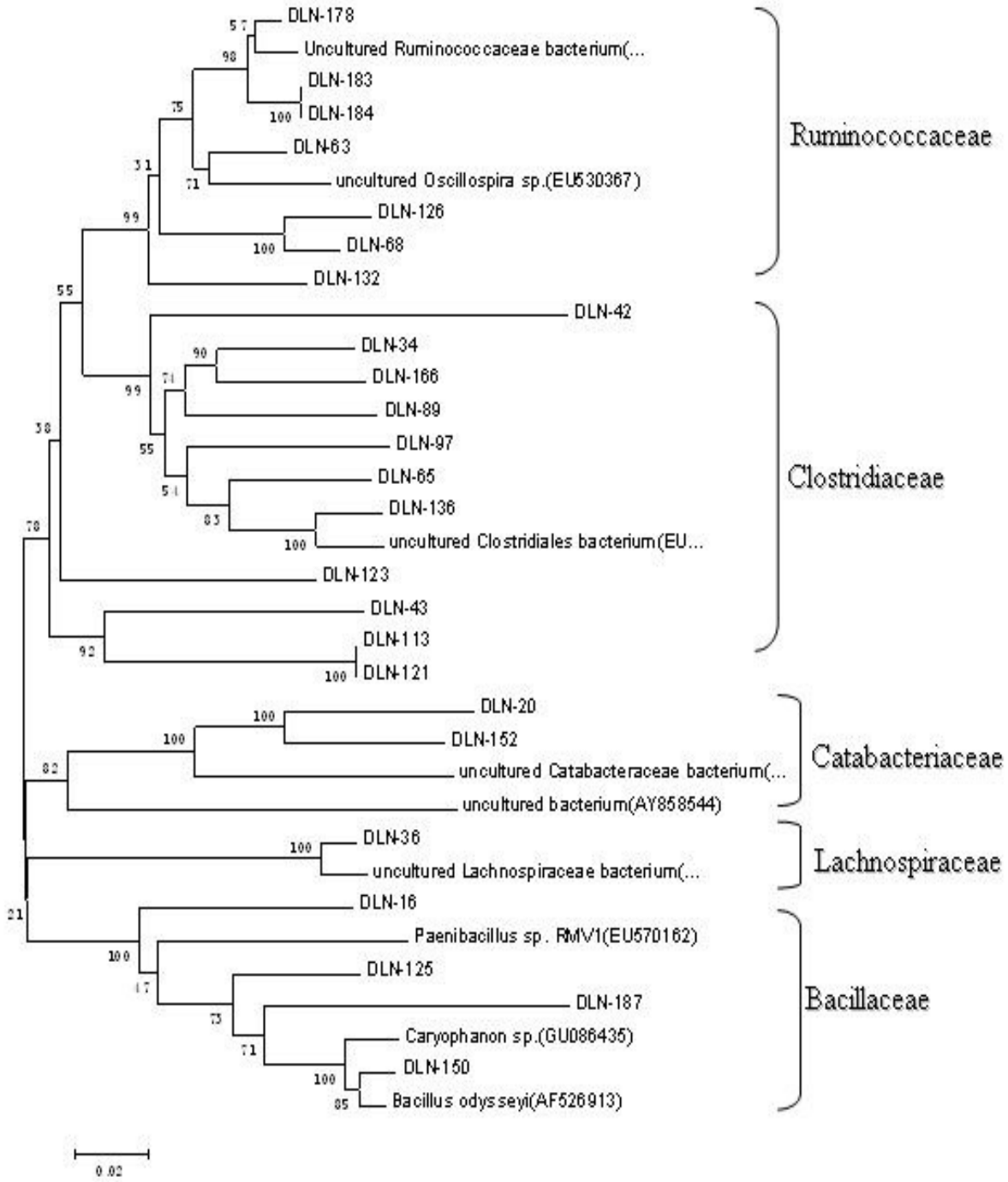

Figure 2. Phylogenetic tree based on 16S rDNA sequences for members of the firmicutes. Bar represents 0.02 sequence divergence.

there was no doubt that the bacteria of Ruminococcacea were dominant microflora in the Gayal intestinal tract. They might affect the processes of digestion and absorption of host and was the reason why Gayals attained greater mature live weights than those of cattle, maintained in the same harsh environment (Weidong et al., 2007).

The proportion, 29 out of 67 OTUs, occupied a very large part of the 16S rDNA library. Not only would this confirm that we had limited knowledge about the microorganisms that inhabit in gastrointestinal tract, but also demonstrated that the gastrointestinal tract microflora is an intricate ecosystem.

Among the remaining 15 OTUs, only 4 OTUs (DLN-82, DLN-66, DLN-114 and DLN-75) clustered in Bacteroidetes clade in the phylogenetic tree (Figure 3).

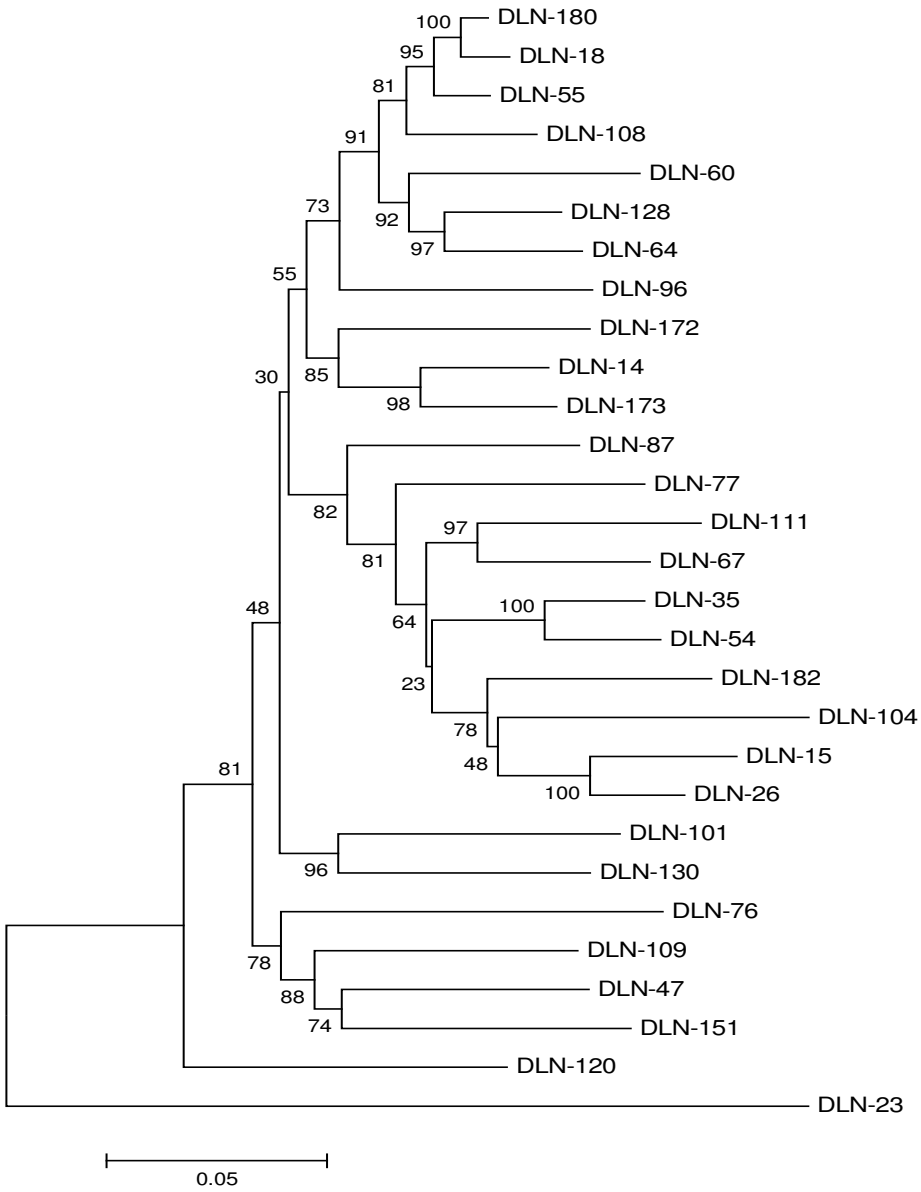

Figure 3. Phylogenetic tree based on 16S rDNA sequences for members of the Ruminococcaceae. Bar represents 0.05 sequence divergence.

This was not similar with Holstein cattle's fecal bacterial community (Yuhei et al., 2005). It might be relevant that the animals in our study were free - grazing, while animals were fed on artificial grain based diets in Yuhei Ozutsumi's study. 3 OTUs (DLN-124, DLN-137, and DLN-53) clustered with Proteobacteria. The bacteria of the Proteobacteria were obtained from many animals gastrointestinal tract. However, the number of them was different. There are more bacteria of the Proteobacteria in the animal's gastrointestinal tract fed on starch than those fed on dietary fiber, suggesting that they might function for starch digestion (Dengdi et al., 2005). 8 OTUs (11.9%) clustered with uncultured rumen bacteria. Indeed, the microbial ecology of gastrointestinal tract ecosystems is composed of rumen bacteria and indigenous intestinal bacteria. The rumen bacteria could not play a major function yet. Curiously, none of the OTU distributed in the Lentisphaerae. Although clone DLN-28 shared 93% similarity with *Victivallis vadensis*, they did not cluster with each other in the phylogenetic tree.

Eukaryotic diversity

The analysis of eukaryotic diversity of the Gayal feces was operated as in the case of bacteria analysis. The clone sequences, obtained from the 18S rDNA library, were digested by the restrictive nucleases *Afa I* and *Msp*

I. A total of 58 RFLP types were obtained and sequenced. No obvious chimeric artifacts were detected using the Check-Chimera Program. Therefore, all 58 sequences were subjected to sequence analysis with a sequence similarities search through the GenBank database and were phylogenetically analyzed. Through the BLAST search, 11 sequences (19.0%) shared < 90% sequence similarities to sequences found in GenBank. It is possible that these 11 sequences might represent undescribed bacterial genera. 38 sequences (65.5%) of the 18S rDNA clone library only had 90 - 94% similarities with database sequences. In total of analysed clone sequences, only two sequences (DLN-J-47 and DLN-J-50) share up to 99% similarities with *Coprinopsis atramentaria* and *Lasiobolus ciliatus*, respectively. Four sequences (DLN-J-24, DLN-J-75, DLN-J-79 and DLN-J-82) shared similarities of only 88% with database sequences. 22 sequences (37.9%) share similarities with cultivated species, which included *Oxyporus sp.*, *L. ciliatus*, *C. atramentaria*, *Melastiza cornubiensis*, *Basidiobolus haptosporus*, *Panaeolus sphinctrinus*, *Mythicomyces corneipes*, *Cheilymenia coprinaria*, and *Campanella* sp.

Comparing analysis of sequences similarities obtained from the 16S rDNA library and the 18S rDNA library, it is easy to find that there were two significant differences between them. Firstly, there were lower similarities in the 18S rDNA library, between the sequences obtained in this study and those in the database, than the BLAST search results of 16S rDNA clones library. Secondly, more sequences shared similarities with cultivated species, though most of them shared low similarities. The reason for these differences was that less research were taken on the microbial eukaryotes than on the prokaryotic diversity. Based on the 97% sequence similarity criterion, a total of 58 clones were classified into 27 OTUs, indicating that the sequences from eukaryotes were more complex than the sequences from prokaryotes. In this study, 18S rDNA library included 95 clones in total. 58 different RFLP types were obtained, which were classified into 27 OTUs.

According to the calculated formula: Coverage = [1–(n/N)] ×100%, the result of the calculation for the library coverage was 71.6%, which suggested that the study identified the dominant eukaryotes in the Gayal feces and the analysis was significant.

The BLAST search showed that all sequences mainly affiliated to the following phyla: Protozoa, Basidiomycota, Ascomycota, uncultured eukaryote. Because of less research on the eukaryote diversity of animal, the similarities between most of the obtained sequences and those of known fecal organisms were too low to identify the sequence as representing a particular taxon, it was necessary to build a phylogenetic tree for investigation of the taxonomic placement. The duplicate sequences of the same OTU were discarded when a phylogenetic tree was built. Phylogenetic distribution is shown in Figure 4. The majority of OTUs clustered in Group 2 (16 OTUs; 59.3%), which share similarities with uncultured eukaryotes, and could not cluster with any reference sequence in the phylogenetic tree. Therefore, we proposed that those sequences made the Gayal unique (Figure 5). They might play an important role in the health of animal and affect its physiology. However, because they shared low similarities with cultured species and less research on the eukaryotic diversity in ruminant, we could not identify their functions. There were 7 OTUs (25.9%) distribution in the clade of Protozoa. Clone DLN-J-70 shared 100% bootstrap value with uncultured Cercozoan, which indicated the stability of the genetic relationship. The remaining 6 OTUs clustered a clade. They were close to the Choanozoa in the phylogenetic tree. We can deduce they represented a group of eukaryotic organisms which shared similar evolutionary status with Choanozoa.

Interestingly, we obtained some sequences which shared similarities with Basidiomycota and Ascomycota. Those could not inhabit the gastrointestinal tract of Gayal. In other words, they might be a part of food source for the host. The phylogenetic tree shows 3 OTUs distributed in the Ascomycota clade. Clone DLN-J-68 shared 99% bootstrap value with uncultured rhizosphere ascomycete. However, only one OTU (DLN-J-28) cluster in Basidiomycota. It was noteworthy that we found that three clones shared similarities with Basidiobolus during the BLAST search. According to the previous report, Basidiobolus could cause a disease called Basidiobolomycosis or subcutaneous phycomycosis (also known as zygomycosis), which was manifested by eosinophilic granulomas in subcutaneous tissues. At present, it is unknown whether similar organism could trigger this disease on the Gayal and threaten the survival of the species. Therefore, further researches are required.

Anaerobic fungi

Using the special primer pairs Chyt-719/Chyt-1553 of Chytridiomycete for PCR amplification, the size of about 900 bp of the amplification product was obtained. The purified DNA products were ligated into pMD18T-vectors and then a clone library was constructed. The sequences were compared with GenBank entries using BLAST, and the results showed high similarity (more than 98%) with the phylum Neocallimastix, which contains the main cellulose producing bacteria in the rumen of herbivores. This indicated that these original stains might play an important role in the process of digestive dietary fiber and produce the nutrition for the host animals, such as volatile fatty acids.

After analysis the percent identity and divergence between these sequences using DNASTAR showed that these sequences have high percent of identity with each other except sequence DLN-Z-26. It only has 37% identity with others sequences obtained this experiment.

During the BLAST search, sequence DLN-Z-18 could not

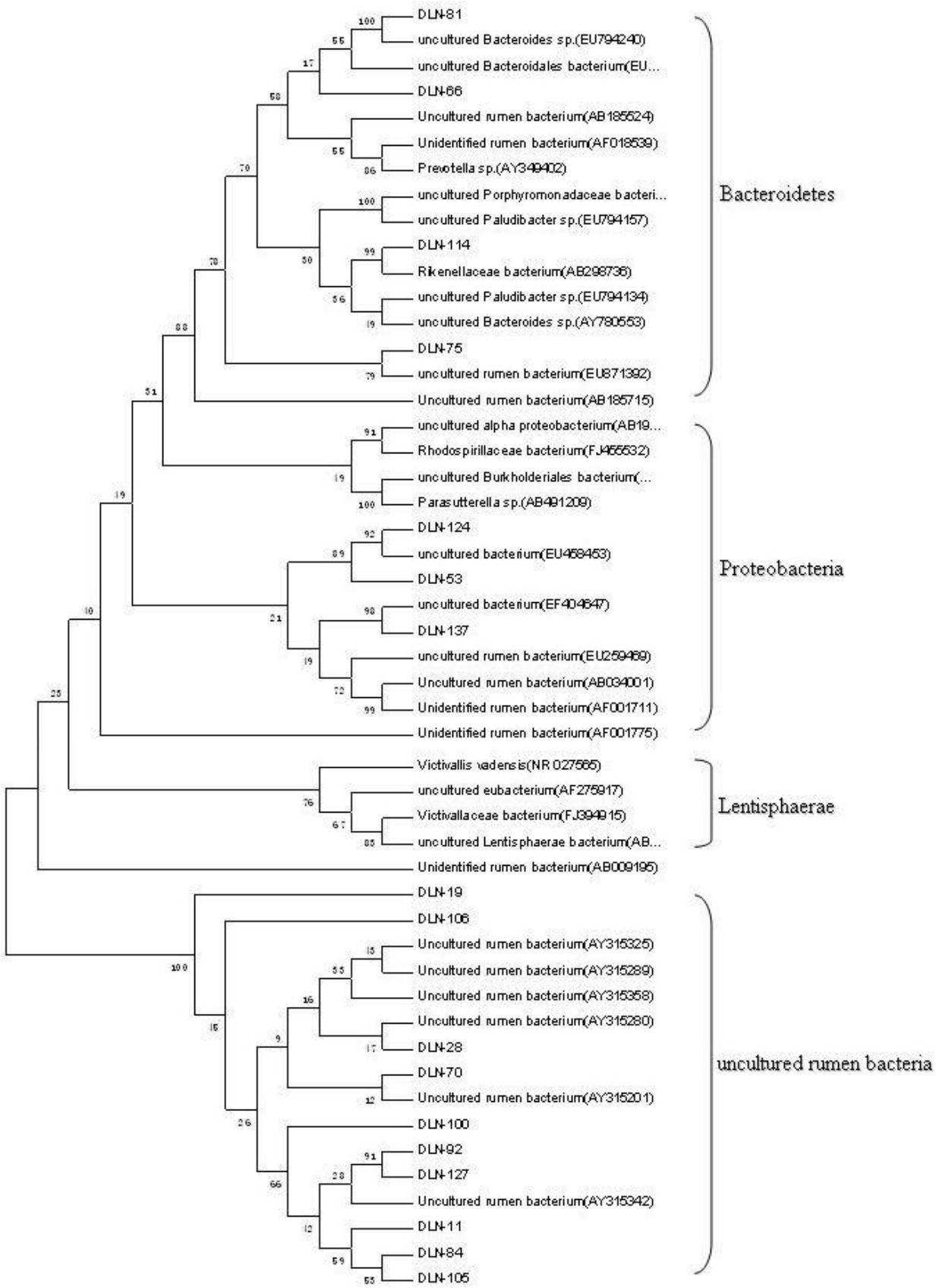

Figure 4. Phylogenetic tree based on 16S rDNA sequences for members of the Bacteroidetes, Porphyromonadaceae and uncultured rumen bacteria.

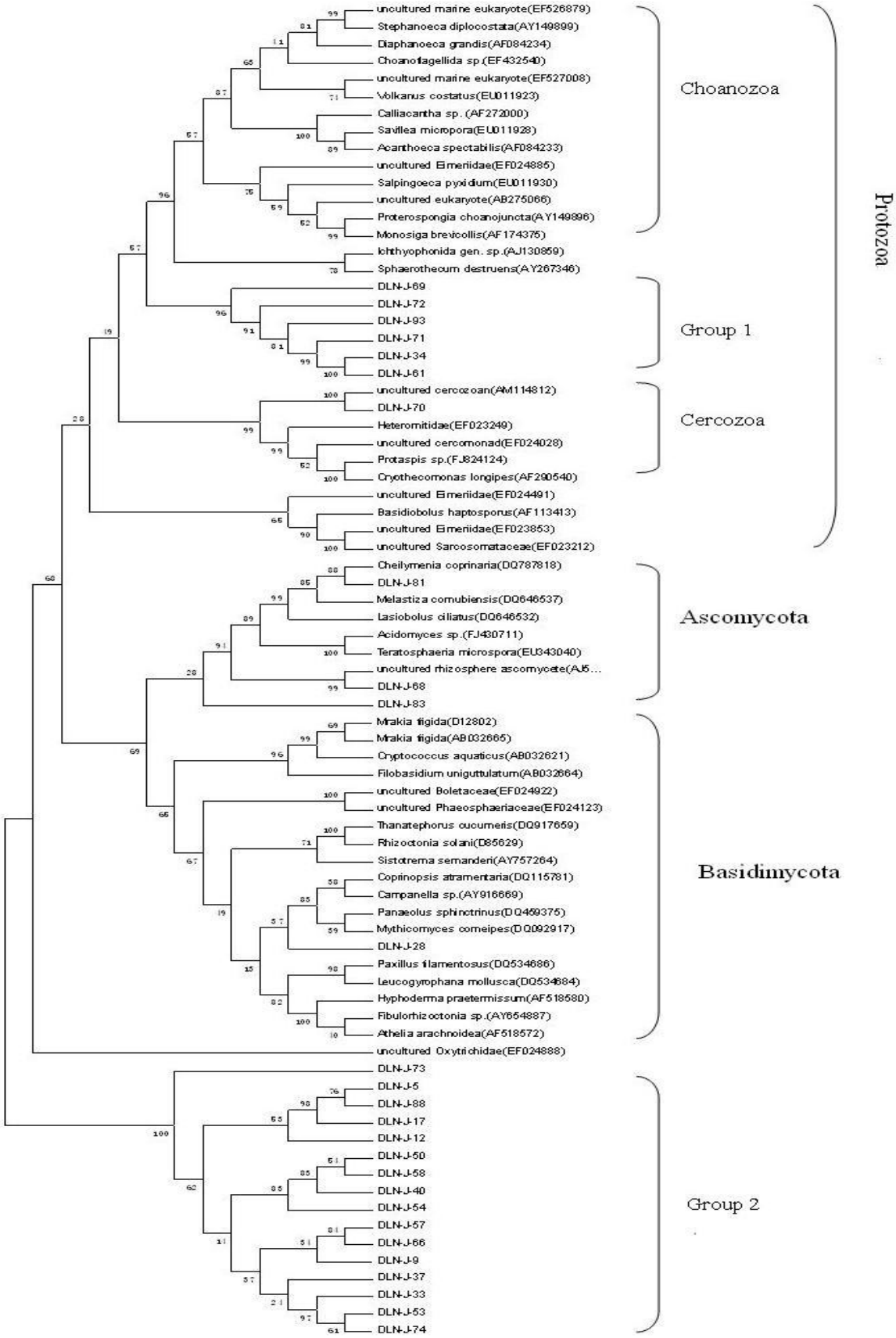

Figure 5. Phylogenetic tree based on 18S rDNA sequences.

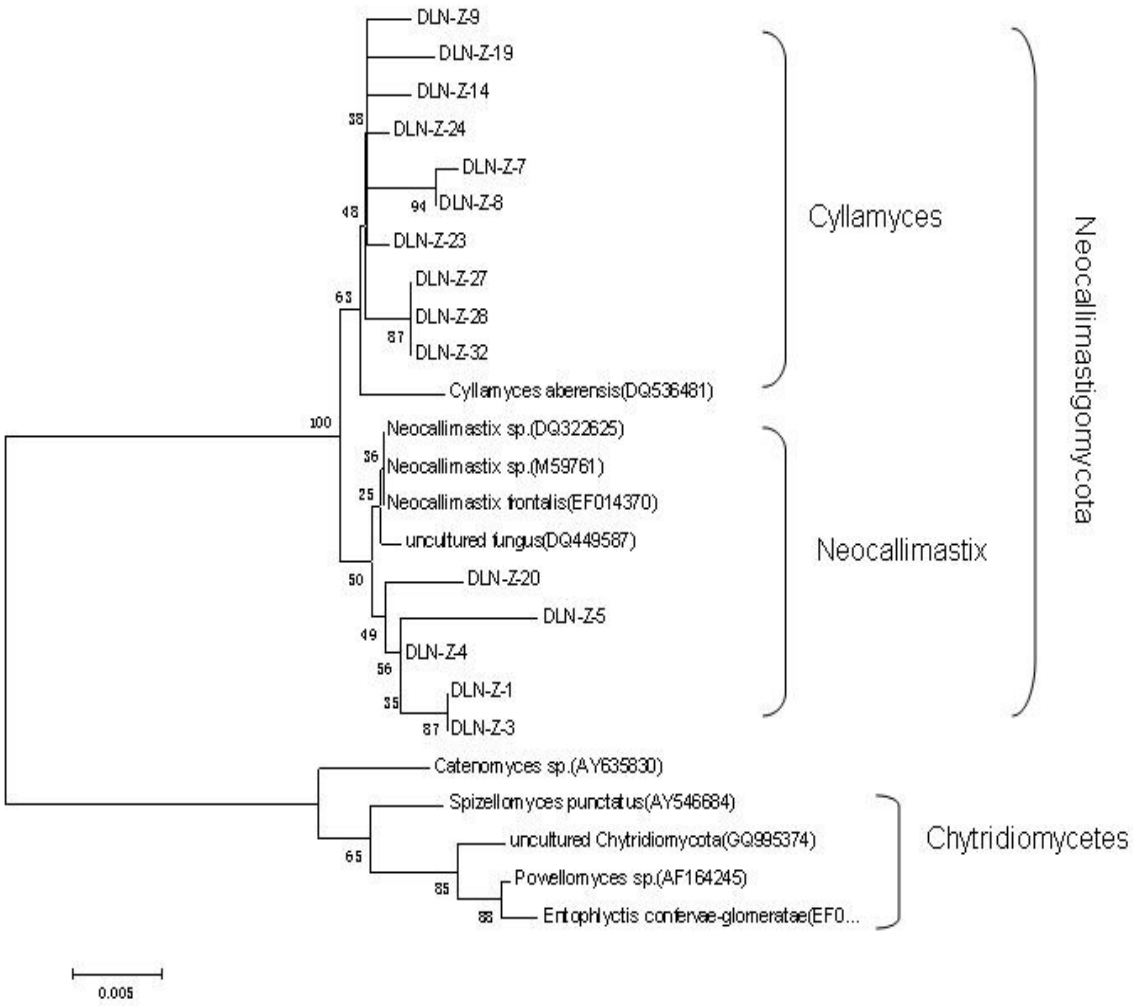

Figure 6. Phylogenetic tree based on the anaerobic fungi. Bar represents 0.005 sequence divergence.

match with any sequence in the GenBank. It indicated that DLN-Z-26 and DLN-Z-18 may be chimera sequences and were discarded when building the phylogenetic tree. 15 clones obtained in this study and reference sequences obtained from GenBank database were used in building the phylogenetic tree (Figure 6). The phylogenetic distribution shows that all of the sequences obtained form this study clustered with Neocallimastigomycota. They were classified into two clades, the clade of *Cyllamyces* and *Neocallimastix*, although sequences shared high similarities with each other during the analysis of the percent of identity and divergence using DNASTAR, and showed different evolutionary distance with each sequences. This suggested that they represented different order of taxonomic structures. The clade of *Cyllamyces* share 100% bootstrap value with the clade of *Neocallimastix*, indicating that their evolution is stable and parallel. The evolutionary position of Chytridiomycete and Neocallimastigomycota are not identified. Therefore, there are some different ideas about their position. Some researches classify the genus Neocallimastix in Chytridiomycete by analysis and comparison of 18S rDNA sequences. However, building a phylogenetic tree is one of the most accurate methods for identifying the evolutionary relations among organisms. Therefore, in this study, the phylogenetic tree also provided an evidence for identifying the evolutionary relationship between Chytridiomycetes and Neocallimastigomycota according to the evolutionary distances they shared.

DISCUSSION

Molecular scatology

The Gayal is rare and valuable endangered specie; it is therefore impossible to collect samples by conventional methods that involve destructive or invasive sampling. Therefore research on the genetic structure and variability of this species has been insufficient, owing to their vulnerability and difficulties of sampling. This problem, however, might be solved to a large extent by the use of

Table 1. Variation of diet affects harbor populations of bacteria of animal.

Day 0 (hay diet)	Day 3 (high-grain diet)	Day 28 (high-grain diet)
Total clones 51	Total clones 58	Total clones 41
LGCGPB 90.2%	LGCGPB 74.2%	LGCGPB 95.0%
CFB 3.9%	CFB 22.4%	CFB one clone
HGCGPB 2.0%	HGCGPB 3.4%	HGCGPB 0
Proteobacteria 3.9%		

fecal analysis based on molecular scatology, which has been developed in recent years. With the combination of traditional fecal analysis and molecular technology, biologists could study free-ranging endangered species without disturbing or even observing them (Wei et al., 2001). Fecal samples can be collected easily, and they contain genetic material that can provide much useful information about the animals, which can be used in studies involving micro-genetics, population ecology and behavioral ecology.

Bacteria diversity

Dynamical analysis

The microbial ecology of the intestinal tract and rumen of Gayal are complex communities. There are a lot of bacteria, fungi, protozoa, which play an important role in the health of the host. However, the populations of microbiota harbored in intestinal or rumen are not static. It is known that they can be influenced significantly by diet (Diyoshi et al., 2000) (Table 1), the host, and the environment (Table 2).

The data in Table 1 is a well documented fact that the rumen bacterial community structure depends on an animal's diet. There are some significant differences among three libraries: 1) more bacterial populations were obtained in the first library (day 0, hay diet). In the third library (day 28 on high-grain diet), only two communities were obtained. 2) The dominant microbial community of cattle derived from the three libraries was the low G+C Gram-positive bacterial (LGCGPB) phylum. 3) The number of the Cytophaga-Flexibacter-Bacteroides (CFB) phylum is relatively volatile among libraries. This is a dynamic system undergoing transition from one steady-state phase to another, which is different from other static snapshots of the system (Table 2). We can dynamically analyze the succession of predominant rumen bacterial populations during the switch from roughage to high grain diets.

Static analysis

Table 2 shows bacterial diversity of the gastrointestinal tract concerning *Bos grunniens* (Dengdi et al., 2005), *Bos taurus* (Dengdi et al., 2005; Yuhei et al., 2005; Wang et al., 2005; Kiyoshi et al., 1999), *Bos indicus* (Karen et al., 2003) and *B. frontalis* (Weidong et al., 2007). It is easy to find that different bacterial communities harbored in the cattle's gastrointestinal tract. For instance, five major phylum microbiota harbored in the *B. grunniens* rumen, nine phylum bacteria obtained in the *B. taurus* gastro-intestinal tract, three phylum bacteria appear in the *B. taurus* digestive tract, and five phylum microbiota exist in the *B. frontalis* gastrointestinal tract. These data only reflect a static snapshots ecology system of the bovine gastrointestinal tract. However, this steady state phase will be unstable if only some conditions changed, such as different host, different diets, different fractions of gastrointestinal tract content, and so on. From the data in the Table 2, we can also obtain evidences supporting these deductions. For example, there are different bacterial communities that were obtained from those Holstein cattle gastrointestinal tracts, even though the samples collected were from the same species of cattle.

Not only are there significant differences between individuals, but also within an individual. The majority of bacterial communities in rumen or feces of Gayals are different. According to this research, the sequences obtained from Gayals feces (16S rDNA) were affiliated with the following phyla: Firmicutes (34.3%), Bacteroidetes (6.0%), Proteobacteria (4.5%), and uncultured bacteria (55.2%). However, sequences from Gayal rumen (Weidong Deng et al., 2007), the majority of the clones identified (57.1%) were located in the low G+C subdivising, with most of the remainder (42.2% of clones) located in the Cytophaga-Flexibacter-Bacteroides (CFB) phylum and one clone (0.7%) was identified as a Spirochaete. This variation appears among the different fractions of the animal digestive tract. It may be significant on their function in the special digestive phase, such as the major function of rumen bacteria are to digest, while intestinal bacteria support absorption.

In addition, the bacterial community structure was also revealed by phylogenetic placement of sequences in relation to different fractions of rumen content (Kiyoshi et al., 1999). In the library from the rumen fluid, the sequences were affiliated with the following major phyla: low G+C Gram-positive bacteria (52.4%), Cytophaga-Flexibacter-Bacteroides (38.1%), Proteobacteria (4.7%) and Spiro-chaetes (2.4%). 2.4% had an uncertain affiliation. The vast majority of sequences from the rumen solids were

Table 2. Major microbial populations harbored in the bovine gastrointestinal tract.

Species	Sample source	management	Total clones and major microbiota (%)	PCR primers	animal diet
[a]Yak (*Bos grunniens*)*	Rumen	Grazing	194 clones in total LGCGPB (54.12) *Bacteroidetes* (30.93) *Archaea* (7.22) *Spirochaetes* (4.64) *Fibrobacteres* (3.09)	530f and 1492r	Grasses
[a]Jinnan cattle (*Bos taurus*)	Rumen	Housed farmed	197 clones in total *Bacteroidetes* (39.59) *Proteobacteria* (26.90) LGCGPB (22.34) *Cytophhaga* (5.59) *Fibrobacteres* (3.55) *Archaea* (1.52) *Spirochaetes* (0.51)	530f and 1492r	High-grain
[b]Holstein cattle *(Bos taurus*) (Japan)	Feces	Farmed	284 clones in total *Firmicutes* (81.3) *Bacteroidetes* (14.4) *Actinobacteria* (2.5) *Proteobacteria* (1.4)	27f and 1492r	Sudangrass and concentrate
[c]Zebu cattle (*Bos indicus*)	Digestive tract	Wild	252 clones in total *Firmicutes* (83.34) *Bacteroidetes* (13.33) *Verrucomicrobia* (3.33)	8f and 1492r	High fiber and taxio content
[d]Holstein cattle (China)	Rumen	Farmed	45 clones in total LGCGPB (40.0) *Bacteroidetes* (5.0) *Prevotella* (2.5) Other (52.5)	27f and 1492r	Fiber and grain
[e]Holstein cattle (Ibaraki, Japan)	Rumen fluid	Farmed	84 clones in total LGCGPB (52.4) CFB (38.1) *Proteobacteria* (4.7) *Spirochaetes* (2.4) Other (2.4)	27f and 1544r	Alfalfa-timothy hay and concentrate
[e]Holstein cattle (Ibaraki, Japan)	Rumen solids	Farmed	84 clones in total LGCGPB (71.4) CFB (26.2) Spirochaetes (2.4)	27f and 1544r	Alfalfa-timothy hay and concentrate
[f]Gayal (*Bos frontalis*)	Feces	Grazing	71 clones in total Firmicutes (34.3) Bacteroidetes (6.0) *Proteobacteria* (4.5) Other (55.2)	27f and 1492r	Wild conditions
[g]Gayal (*Bos frontalis*)	Rumen	Farmed	147 clones in total LGCGPB (57.1) CFB (42.2) Spirochaetes (0.7)	27f and 1492r	Bamboo leaves and twigs

Letters represent literature that the data comes from. a: Dengdi et al. (2005); b: Yuhei et al. (2005); c: Karen et al. (2003); d: Wang et al. (2005); e: Kiyoshi et al. (1999); f: This context; g: Weidong et al. (200)7.

found to be related to low G+C Gram-positive bacteria (71.4%) and the remaining sequences were placed within the Cytophaga-Flexibacter - Bacteroides (26.2%) and Spirochaetes (2.4%) phyla. Comparing the data with the studies of 16S rDNA sequence demonstrated that, gastrointestinal tract microbial communities of cattle appear to be dominated by Firmicutes, particularly with the genera *Clostridium, Ruminococcus* and *Eubacterium*. Firmicutes are prevalent in the gastrointestinal tracts of ruminants, herbivores, mammals and humans as well. However, we could not determine whether Firmicutes are truly dominant components of the gastrointestinal ecosystem because we used PCR analysis and there are many factors that can bias the results. The reason was not clear, but some physiological factors might have affected the composition of bacteria species.

A contrast among the surveys of gastrointestinal tract communities (Table 2) shows that the Bacteroidales is relatively volatile. It may be relevant that the animals were free-grazing or fed on artificial grain based diets. This variation is also shown in Table 1. Studies of 16S rDNA sequence information demonstrate that different microbiota appear in the different bovine gastrointestinal tract. Although the experiments were operated under similar conditions, these differences were inevitable. Maybe some factors can bias the results obtained when using PCR amplification and cloning for recovery of 16S rDNA from a mixed population. These factors include different PCR primers, lysis of bacteria, preferential cloning of PCR products, the number of PCR cycles, different methods of extraction of total DNA and the coverage value of the library and so on. As we all know that a library contains all possible microbial species when the coverage value is equal to 100%. However, it is actually impossible to attain a coverage value of 100%. In addition, the GenBank database cannot contain all of the microbial 16S rDNA sequences in nature. One of these factors change will lead to different result. Therefore, it is necessary to perform phylogenetic analysis by the culture method in addition to using 16S rDNA sequence analysis to be used in the analysis of this population.

The bacterial communities in the animal's gastrointestinal tract play an important role in the health of host. In this study, we obtained 38 sequences (36 OTUs) shared similarities with Ruminococcaceae in this study, which is one of the well-known rumen fibrotic bacteria families. Clone DLN-76 shared similarity with bacteria which can break down hemi-cellulose into mannose oligosaccharides. There was 92% homology between clone DLN-151 and *Paenibacillus* specie RMV1, which can break down lignin. These bacteria may cause the Gayals rapid growth, characteristic body form, very good meat and strong productivity. They influence the ability of the animal's absorption and improve the growth performance and immune status and allow the animals to subsist on digestion and absorption of cellulose. To sum up, there is a great library which can obtain many enzymes, particularly cellulase and hemi-cellulase, from the bacteria harbored in the gastrointestinal tract. If we can explore those enzymes well, they will bring great benefits to our society.

In addition, we can identify the animal's health according to the bacterial communities. We obtained 4 OTUs which shared similarities with Bacillaceae, particular the clone DLN-150 shared 97% similarity with *Bacillus* sp. and 85% bootstrap value with it. This indicated that the original strain of clone DLN-150 has similar function with *Bacillus* species. If the strain *Bacillus* species is a pathogen harbored in the gastrointestinal tract, Gayals will be in danger.

Although there are many studies which research on the prokaryotic diversity, we are not yet close to a complete catalogue of bacterial diversity in the gastrointestinal ecosystems. In view of the very high level of diversity encountered in these reports, it is clear that a far more extensive 16S rDNA sequence and analysis study from a diverse array of bovine species is necessary to provide a complete inventory of the prokaryotic diversity present in gastrointestinal communities.

Microbial eukaryotes (protozoa) diversity

Microbial eukaryotes are another majority component which harbors in the animals gastrointestinal tract and influence the capacities to digest forages of host. However, there is less study about it than the analysis of environmental prokaryotic diversity. Actually, not only do prokaryotes play a role in the digestion, but also eukaryotes, in particular Protozoa, play a more important role in it. In this study, more than a quarter sequences (7 OTUs 25.9%) were distributed with protozoa clade. According to the report of Deng et al. (2007) there are significant differences between the microbial populations in the rumen of Gayal and cattle, whereas there was no difference for protozoa. In addition, the research found an interesting phenomenon that the digestibility of fiber by Gayal was not enhanced in spite of the considerable increase in the number of cellulolytic bacteria. It was speculated that this may have been due to the role of rumen protozoa in the digestion of dietary fiber, because the number of protozoa was similar for both Gayal and cattle. In this research, we also obtained many clones which were similar with protozoa in the Gayal feces. This may be another reason that explains the greater live weights of Gayal, compared to cattle, grazing in the harsh natural environments in which Gayal are located naturally.

In the 18S rDNA library, some sequences which shared similarities with Basidiomycota and Ascomycota were obtained. They could not inhabit in the gastrointestinal tract of Gayal. In other words, they might be a part of food source for the host. Worth noting is the fact that three clones shared similarities with Basidiobolus were obtained in this library. Basidiobolus are pathogens, which could cause a disease called Basidiobolomycosis or sub-

cutaneous phycomycosis. At present, it is unknown whether similar organism could trigger this disease on the Gayal and threaten the survival of the species.

Anaerobic or anoxic-tolerant (intrinsically aerobic) eukaryotes are ecologically important, but have been poorly investigated using traditional approaches (e.g. culturing and microscopic observation), except in the case of human or livestock parasites such as Giardia and Trichomonas (Kiyotaka et al., 2007). Molecular studies of anoxic environments are now starting to unveil the ecology and biogeography of anaerobic or anoxic-tolerant eukaryotes. Around deep-sea hydrothermal vents, many sequences closely related to those of eukaryotic parasites (such as Kinetoplastida and Apicomplexa) have been detected (López-García et al., 2003), suggesting that parasitic protists inhabiting these areas are possibly hosted by dense animal populations and may be responsible for sudden massive mortality of those animals (Moreira and Lo´ pez-Garcı´a, 2003).

Anaerobic fungi (Chytridiomycetes) exist in Gayal feces

Since anaerobic fungi were observed by Orpin in 1975, several studies have shown that they abound colonize plant particles in the rumen. Fungi can digest the entire plant cell wall unaided and are more effective than bacteria in the degradation of sclerenchyma. Chytridiomycetes, including the anaerobic fungal species *N. patricianum* and anaerobic bacterial degradation of cellulose have co-evolved with the most significant cellulase-producing bacteria of the herbivore gastrointestinal tract. They have enzymes capable of weakening the structure of the plant and degrade cellulose and hemicellulose, as well as degrading the polysaccharides by releasing the rumen volatile fatty acids (VFA) and alcohol, the end products of their fermentation. They play a role in the nutrition of the host animal (Grenet et al., 1989). This indicated that the intestinal anaerobic fungi play an important role in herbivores to a large extent. However, the fungal population varies greatly according to the diet of the animal. Certain diets, such as those rich in sugar, considerably reduce the fungal population, while others, for example a diet of straw, eliminated the fungi completely.

Conclusion

After analysis of microbial populations that inhabit gastrointestinal tract of *B. frontalis*, it is clear that there are lots of bacteria, eukaryotes, protozoa, Chytridiomycetes, and anaerobic fungal species *N. patricianum*. Comparative sequence analysis revealed that only a little part of the molecular species corresponded to known organisms, indicating that the vary majority of the dominant organisms of the bovine gastrointestinal tract microflora have so far eluded scientific description. Indeed, the composition and activity of these floras have profound influences on health and disease through their involvement in the nutrition, pathogenesis, and immune function of the host. For instance, some of them can efficiently decompose the dietary fiber through producing cellulase or cooperate with each other to decompose cellulose and hemicellulose. This maybe one reason for *B. frontalis* to have rapid growth and high productivity under poor conditions. On the other hand, some other microorganisms, as a part of animal nutrition themselves, affect animal growth, such as rumen protozoa. They can not only provide efficient protozoa protein, but also play an important role in protein transformation of the rumen.

This study has provided an inventory of phylotypes in the gastrointestinal tract of cattles (Table 2). The results document a hitherto unknown microbial diversity and indicate that the majority of gastrointestinal tract microflora was uncharacterized.

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REFERENCES

Amaral Zettler LA, Go´ mez F, Zettler E, Keenan BG, Amils R, Sogin ML (2002) Eukaryotic diversity in Spain's River of Fire. Nature, pp. 137-417.

Altschul SF, Gish W, Miller W, Lipman DJ (1900). Basic local alignment search tool. J. Mol. Biol. 215(3): 403-410.

Antonia Suau, Re Gis Bonnet, Male`Ne Sutren, Jean-Jacques Godon, Glenn R, Gibson, Matthew D, Collins, Joel D (1999). Direct Analysis of Genes Encoding 16S rRNA from Complex Communities Reveals Many Novel Molecular Species within the Human Gut. Appl. Environ. Microbiol. 65(11): 4799-4807.

Cole JR, Chai B, Farris RJ (2005). The Ribosomal Database Project (RDP-II): Sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. 33(1): 294-296.

Deng W, Metha W, Ma S (2007). Phylogenetic analysis of 16S rDNA sequences manifest rumen bacterial diversity in Gayals fed fresh bamboo leaves and twigs (sinarundinaria). Asian-Aust. J. Anim. Sci. 20(7): 1057- 1066.

David M, López-García P (2002) The molecular ecology of microbial eukaryotes unveils a hidden world. Trends Microbiol. 10(1): 31-38.

Dengdi AN, Xiuzhu D, Zhiyang D (2005). Prokaryote diversity in the rumen of yak (*Bos grunniens*) and Jinnan cattle (*Bos taurus*) estimated by 16S rDNA homology analyses. Ecol. Environ. Microbiol. Anaerobe, 11: 207-215.

Dı´ez B, Pedro´s-Alio´ C, Massana R (2001). Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. Appl. Environ. Microbiol. 67: 2932-2941.

Grenet E, Fonty G, Jamot J (1989). Influence of diet and monensin on development of anaerobic fungi in the rumen, duodenum, cecum, and feces of cows. Appl. Environ. Microbiol. 55(9): 2360-2364.

Felsenstein J (1985).Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39: 91-783.
Hang X-M, Yang H (2001). Whitby Corne. PCR Amplif ication of Anaerobic Fungal 18S rDNA from Landf ill Sites. Chinese. J. Biotechnol. 17(5): 515-519.
Karen NE, Stepen ZH, Loanna H (2003). Phylogenetic analysis of the microbial populations in the wild herbivore gastrointestinal tract: insights into an unexplored niche, Environ. Microbiol. 5(11): 1212-1220.
Ke LI, Tianling Z, Yun T (2007). Bacterial community structure in intestine of the white shrimp, Litopenaeus vannamei. Acta Microbiol. Sinica, 47(4): 649-953.
Kemp PF, Aller JY (2004). Bacterial diversity in aquatic and other environments:What 16S rDNA libraries can tell us. FEMS Microbiol. Ecol. 47(2): 161-177.
Kimura M (1980). A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 20-111.
Kiyotaka T, Masashi T, Masaru K (2007). Genetic Diversity of Microbial Eukaryotes in Anoxic Sediment of the Saline Meromictic Lake Namako-ike (Japan): On the Detection of Anaerobic or Anoxic-tolerant Lineages of Eukaryotes. Protist, 158: 51-64.
Kiyoshi T, Roustam IA, Takafumi N, Koretsugu O, Mutsumi N, Hiroki M and Yoshimi B (1999). Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. FEMS Microbiol. Ecol. 29: 159-169.
Krause DO, Russell JB (1996). How many ruminal bacteria are there? J. Dairy Sci. 79: 1467-1475.
Li N, Li D-Q, Wang X-L, Liu Y (2006). The Effect of Different Preservation Methods of Faeces on Genomic DNA Extraction from Amimal. Int. J. Genet. 29(5): 341-345.
Lo´ pez-Garcı´a P, Rodriguez-Valera F, Pedros-Alio C, Moreira D (2001). Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature, 409: 603-607.
Lo´ pez-Garcı´a P, Philippe H, Gail F, Moreira D (2003). Autochthonous eukaryotic diversity in hydrothermal sediment and experimental microcolonizers at the Mid-Atlantic Ridge USA. Proc. Natl. Acad. Sci. 100: 697-702.
Mao H-M, Deng W-D, Wen J-K (2005). The Biology Characteristics of Gayal (*Bos frontalis*) and Potential Exploitation and Utilization. J. Yunnan Agric. Univ. 20(2): 258-261.
Mohan M, Chandan R, Prakash BS (2007). Development and validation of a highly sensitive economic enzymeimmunoassay for prolactin determination in blood plasma of mithun (*Bos frontalis*) and its application during milk let down and cyclicity. Anim. Reprod. Sci. 99: 182-195.
Moon-van der Staay SY, De Wachter R, Vaulot D (2001). Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature, 409: 607-610.
Moreira D, Lo´ pez-Garcı´a P (2003). Are hydrothermal vents oases for parasitic protists? Trends Parasitol. 19: 556-558.
Polz MF, Cavanaugh CM (1998). Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. 64(10): 3724-3730.
Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: a Laboratory Manual 2nd edn.Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.
Wang Y-L, Yang R-H, Mao A-J (2005). Phylogenetic diversity analyse of rumen bacteria using culture independent method. Acta Microbiol. Sinica, 45(6): 915-919.
Weidong D, Liping W, Songcheng M (2007). Comparison of Gayal (Bos frontalis) and Yunnan Yellow Cattle (*Bos taurus*): Rumen function, digestibilities and nitrogen balance during feeding of pelleted lucerne (Medicago sativum), Asian-Australasian. J. Anim. Sci. 26(6): 900-907.
Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991). 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol, 173: 697-703.
Wu F, Chen H, Shen C (2006). Systematic analysis of 18S rDNA total sequence of cultivable yeast in Luzhou-flavor fermented grains. J. Liquor-Making Sci. Technol. 142(4): 23-25.
Yuhei O, Hidenori H, Mitsuo S, Hisao I, Yoshimi B (2005). Culture-independent analysis of fecal microbiota in cattle. Biosci. Biotechnol. Biochem. 69(9): 1793-1797.
Zhao K-D, Ou C-H, Huang Y-L, He T-B (2003). Rare Animal Germpla sm Resources in Yunnan Province: Present Situation and Countermea sures of Preservation and Research on Dulong Cattle (*Bos frontalis*). J. Yellow Cattle Sci. 29(2): 71-74.