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# Bacterial diversity in the rumen of mithun (*Bos frontalis*) fed on mixed tree leaves and rice straw based diet

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**This work was done to study the bacterial diversity of mithun (*Bos frontalis*) fed on mixed tree leaves and rice straw based diet. Genomic DNA was extracted from the rumen liquor of mithun, 16S rDNA sequences were amplified, cloned and randomly selected for sequencing. The nearest neighbors were retrieved from the NCBI through a BLAST search and a phylogenetic tree was constructed. In our findings, 12% of clones showed similarity with known bacterial species (*Prevotella ruminicola*, *Butyrivibrio fibrisolvens*, *Pseudobutyrivibrio ruminis, Succinivibrio dextrinosolvens* and *Ruminococcus flavefaciens)* and 6% of clones showed similarity with known bacterial genus (*Butyrivibrio* species, *Streptococcus* species) of 97-100% similarities. Twenty-two percent of clones showed similarity with known bacteria (*P. ruminicola*, *Prevotella* species, *Sporanaerobacter acetigenes*, Clostridiales bacterium, Bacteroidetes bacterium) of 90-97% similarities. Sequences of all the clones were also classified by using taxonomic classifier software available at Ribosomal Database Project and classification showed that, all the clones were under four phyla, namely Bacteroidetes (54%), Firmicutes (36%), Proteobacteria (4%) and Tenericutes (2%). The experiment showed that, bacterial population in the rumen of mithun fed on mixed tree leaves and rice straw based diet harbor diversified species of bacteria responsible for lignocellulosic feedstuffs.**

**Key words:** Tree leaves, bacteria, clones, sequences.

## INTRODUCTION

The North-East India, being at the confluence of three major bio-geographical realms of the world, is extremely rich in floral and faunal biodiversity with several endemic species. Among these, mithun (*Bos frontalis*) is considered as the most important bovine species and the people not only use them as pride object of social sacrifice but as life currency in their local transactions (Annual Report, 2012). This unique bovine species is believed to be domesticated more than 8000 years ago and is mainly available in the four north-eastern hilly states of Arunachal Pradesh, Nagaland, Mizoram and Manipur. It plays an important role in economic, social

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**Table 1.** Chemical composition of feed and fodder (percentage on DM basis) fed to mithun during the experiment.

| Item | Concentrate mixture | *Ficus hirta* | D*ebrogesia longifolia* | Litsea sps | *Legroestromea spaciosa.* | Mixed tree leaves calculated | Paddy straw |
|---|---|---|---|---|---|---|---|
| DM | 89.25 | 23.6 | 28.0 | 35.14 | 36.81 | 30.88 | 87.30 |
| OM | 91.11 | 93.8 | 89.8 | 93.70 | 94.99 | 93.07 | 85.92 |
| CP | 17.71 | 15.6 | 14.0 | 14.60 | 14.32 | 14.63 | 4.36 |
| EE | 8.35 | 1.8 | 1.8 | 2.80 | 2.14 | 2.14 | 1.08 |
| CF | 9.70 | 18.3 | 12.8 | 16.30 | 26.53 | 18.48 | 33.79 |
| NFE | 55.35 | 58.1 | 61.2 | 60.0 | 52.0 | 57.82 | 46.69 |
| TA | 8.89 | 6.2 | 10.2 | 6.3 | 5.01 | 6.93 | 14.08 |

and cultural life of the tribal people of this region. It is primarily reared as a meat animal and is highly preferred among the tribal people of the region. This animal is reared exclusively under free grazing condition. Mithun is an extremely efficient grazer on steep hilly slopes as compared to other animals. It basically thrives on the jungle forages, tree fodders, shrubs, herbs and other natural vegetation. It prefers to browse and move around the forest in search of selective forages. Farmers do not provide any additional supplement except for occasional common salt feeding, especially at the time of restraining for some purposes (Moyong, 2012). The performance of this species of animal was also found to be satisfactory in confinement when reared on tree leaves based ration (Das et al., 2010).

Rumen microbes have been extensively studied in ruminants like cattle and buffaloes both qualitatively (Koike et al., 2003; Sylvester et al., 2004) and quantitatively (Shin et al., 2004) using DNA-based technologies (16s RNA/18s RNA gene). These techniques have been further used to construct a library of 16S rDNA clones of rumen microbes and to demonstrate considerable diversity of rumen bacteria. The microbes present in the rumen ecosystem of mithun convert the tree leaves and shrubs rich in lignocellulosic materials into volatile fatty acids and microbial protein for the animals (Das et al., 2010). The diversity study of rumen bacteria in mithun will provide sufficient information for rumen manipulation in future for improving growth and production. In the present experiment, the rumen bacterial diversity of mithun were studied, fed on mixed tree leaves and rice straw based diet by amplification, cloning and sequencing of 16S rRNA gene of bacteria, followed by sequence comparison and phylogenetic analysis.

## MATERIALS AND METHODS

### Experimental animals

The experiment was carried out on five adult mithun about 3 years of age at Research Farm of National Research Centre on Mithun, Jharnapani, Medziphema, Nagaland, India. The diet consisted of mixed tree leaves, paddy straw and concentrates mixture (Table 1). Approximately 50% of dry matter (DM) requirement was met through concentrate mixture and rest through mixed tree leaves and paddy straw (2:1 ratio on fresh basis) according to the standard developed in the institute. The tree leaves consisted of temechiedie (*Ficus hirta*), Pedu (*Debrogesia longifolia*), thenha (Litsea sps) and thumero (*Legroestromea spaciosa*). Leaves of these tree foliages were cut, carried daily, mixed in equal proportions and fed to the experimental animals. Fresh drinking water was offered *ad lib* two times a day. All the animals were maintained on uniform feeding regime for a period of one year.

### Rumen sample collection

Approximately 50 ml of rumen fluid from each animal was collected via a stomach tube located in the middle part of the rumen and connected to a vacuum pump at 3 h post feeding. Samples were pooled and filtered through four layers of muslin cloth to remove particulate matter. Strained samples were used in the laboratory for the total bacterial DNA extraction.

### DNA extraction, PCR amplification, cloning and sequencing

Genomic DNA was extracted from the mixed rumen liquor by using the standard kit manufactured by Bangalore Genei India Pvt. Ltd., Peenya, Bangalore, India. The DNA were checked by agarose gel electrophoresis and then polymerase chain reaction (PCR) amplification of bacterial 16S rDNA was performed using the universal primer of bacteria F27(5'-AGATTGATCMTGGCTAGGGA-3') and R1492 (5'-TACGGYTACCTTGTTACGACTT -3') as reported by Weisburg et al. (1991). The PCR reaction was set up in 25 µl volumes containing 1 µl template, 2.5 µl 10x buffer, 1.5 µl 25 mM $MgCl_2$, 1 µl of each primer, 0.5 µl of 25 mM dNTP mix, 0.5 µl Taq DNA polymerase and distilled water. The amplification conditions are standardized for universal primer. The amplification conditions were as follows: 3 min of initial denaturation at 95°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 2 min with the last cycle followed by a 10 min extension step at 72°C. The PCR product was visualized on an agarose gel (Figure 1), the bands were excised and DNA was purified from the gel slices using the standard kit manufactured by Bangalore Genei India Pvt. Ltd., Peenya, Bangalore, India. The purified PCR product was stored at -20°C for further processing.

The purified PCR products were ligated into T vector using ligation kit manufactured by Bangalore Genei (India) Pvt. Ltd according to the instruction and then transferred into *E. coli*, DH5.

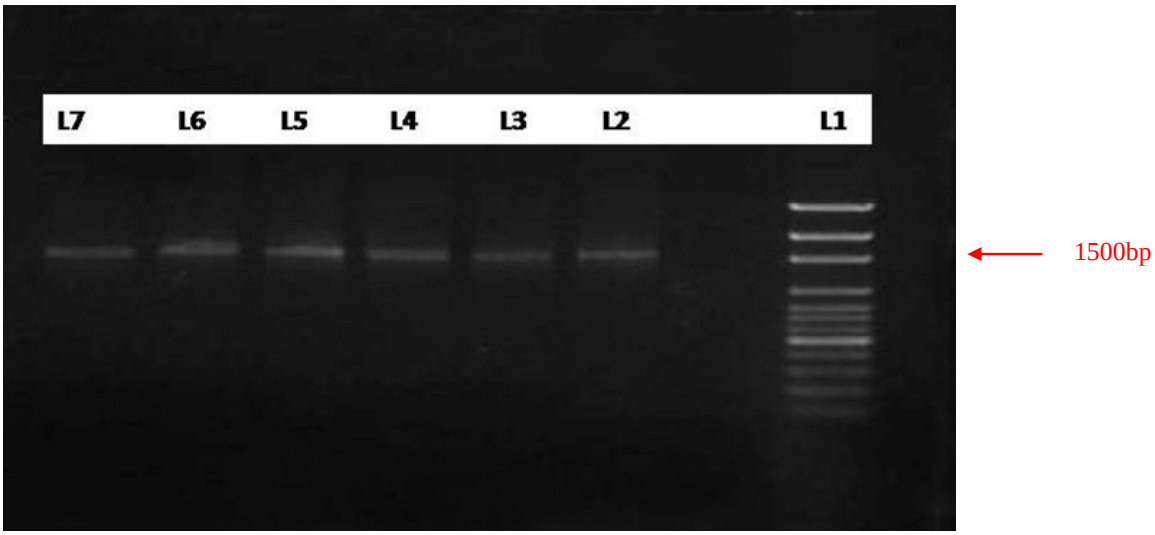


**Figure 1.** Agarose gel electrophoresis of PCR product after amplification with universal primer of bacteria (LI: 1 kb DNA marker, L2-L7: test samples).

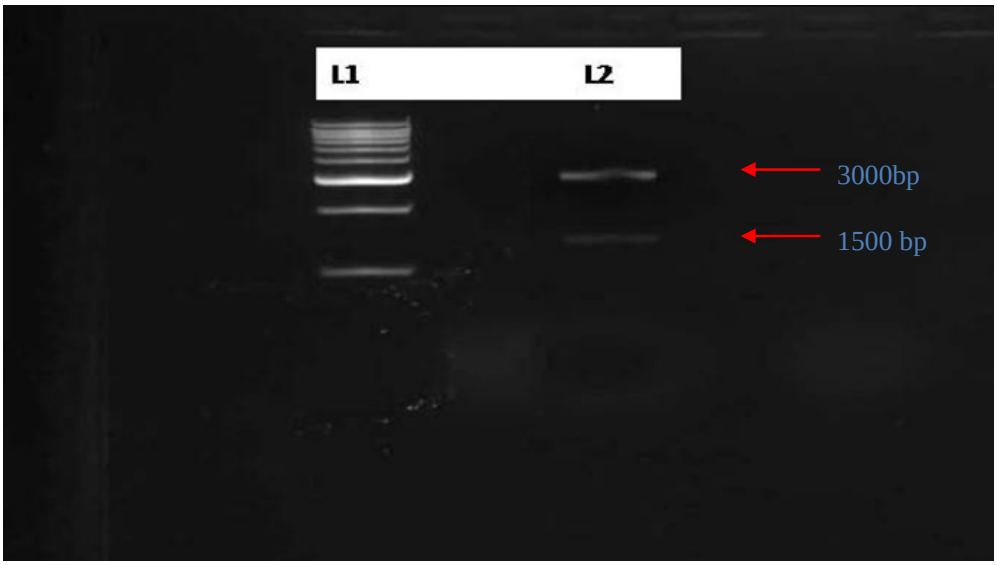


**Figure 2.** Agarose gel electrophoresis of PCR product showing restriction enzyme digestion of Plasmid DNA (LI: DNA marker, L2: test sample).

Recombinant cells were allowed to grow on LB medium containing ampicillin, IPTG and X gal for overnight and then the white colonies were picked up. The extraction of recombinant plasmid was carried out by using plasmid extraction kit manufactured by Himedia Laboratories, Mumbai (HiPura Plasmid DNA Minispin Purification Spin Kit). The restriction enzyme digestion of plasmid DNA was carried out to confirm the identity of PCR products (Figure 2). Sequencing of clones was performed with an ABI prism genetic Analyser by Bioserve, Hyderabad. The nearest neighbors were retrieved from the NCBI (http://www.ncbi. nih.gov/BLAST) through a BLAST search.

**Sequence analysis and construction of phylogenetic tree**

Sequences from the current study were analyzed by the CHECK_CHIMERA program (Maidak et al., 2001) to remove any chimeric rDNA clone. 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 sequences of the isolates were compared with those available

in the database. The obtained sequences were aligned using clustal V method of megAlign software (DNASTAR) and then phylogenetic tree was plotted.

## RESULTS AND DISCUSSION

### Chemical composition of ration

The chemical composition feed and fodder during the experimental period is given in Table 1. The crude protein content of concentrate mixture, mixed tree leaves and paddy straw was estimated to be 17.71, 12.01 and 4.36%, respectively. The crude protein content of tree leaves/shrubs reported in this experiment is comparatively higher than the green fodder of other parts of India. The mithun is reared on tree leaves available in the forest of north-east region of India whereas cattle and buffaloes are normally fed on cultivated green fodder. Hence, there is provision of excellent vegetation for the mithun in this area as compared to cattle and buffaloes in other parts of India.

### Similarity of sequences

A total of 100 clones were isolated from the mixed rumen liquor of mithun (*Bos frontalis*) and 72 of the clones were randomly selected for sequencing. All sequences were checked for vector sequence contamination and then submitted to GenBank in NCBI. The result of similarity values of clones (16S rDNA sequences) is presented in Table 2. In our findings, 9 clones (12% of clones) showed similarity with known bacterial species (*Prevotella ruminicola* 4, Butyrivibrio *fibrisolvens* 2, *PseudoButyrivibrio ruminis* 1, Succinivibrio *dextrinosolvens* 1 and Ruminococcus *flavefaciens* 1) and 4 clones (6% of clones) showed similarity with known bacterial genus (*Butyrivibrio* species 3, *Streptococcus* sp 1) of 97-100% similarities. Identification using sequence similarity demands sequences having similarity more than 97%. Many sequences showed similarity value of less than 97%, thus confirming their difference with known sequences at species level. Sixteen of the clones (22%) showed similarity with known bacteria of 90-97% similarities (*Prevotella ruminicola* 7, *Prevotella* species 4, *Sporanaerobacter acetignes* 1, Clostridiales bacterium 2, Bacteroidetes bacterium 2). Forty three (60%) of clones in this study were uncultured rumen bacterium.

Sequences of all the clones were also classified by using taxonomic classifier software available at Ribosomal Database Project. The sequences were submitted to the software and classification showed that, all the clones were under four phyla, namely Bacteroidetes (54%, 39 clones), Firmicutes (36%, 26 clones), Proteobacteria (4%, 3 clones) and Tenericutes (2%, 1 clones). Four percent (three clones) were unidentified bacteria in this study. The majority of bacteria were from the genus *Prevotella* (Phylum- Bacteroidetes) as they have a very significant role in the digestion of feed stuffs of rumen. Under the phylum Firmicutes, the bacteria of family Succiniclasticum, Ruminococcacae, Lachnospiraceae, Streptococcaceae and Enterococcaceae were identified. Succinivibrio and Vampirovibrio are the two types of bacteria (genus) identified under the phylum Proteobacteria. This work was similar to the findings of Patel (2011) who revealed through the Ribosomal Database Project (RDP) classification that, the clones of rumen in goat were mainly distributed into two phyla, namely Bacteroidetes (35.0%) and Firmicutes (33.0%). In contrast to these findings, Tajima et al. (1999) reported that 52.4% of clones identified in the rumen of Holstein cow fed a diet of hay belonged to the firmicutes, and 38.1% to the Cytophaga-Flexibacter-Bacteroides (CFB) phylum. Other studies (Edwards et al., 2004; Deng et al., 2007) reported almost the same experimental findings of Tajima et al. (1999) who worked on the microbes of ruminant animals.

### Phylogenetic analysis

The similarity for most of the sequences with those of known rumen bacteria was too low for accurate identification of the sequence. Therefore, a phylogenetic tree was constructed using MEGA version 5 software to investigate the taxonomic placement. The results of this phylogenetic analysis are shown in Figure 3. The phylogenetic tree was mainly divided into two clusters, cluster I and II. Cluster I is again divided into five sub-groups. In sub-group I, 13 clones are grouped separately out of which four clones are *Butyrivibrio* species (NRCMK61, NRCMK50, NRCMK46 and NRCMK49), one clone (NRCMK60) is *Pseudobutyrivibrio* species and the remainder are uncultured rumen bacteria. Sub-group II consisted of 12 clones out of which, 5 clones (NRCMB1, NRCMK31, NRCMK15, NRCMK17 and NRCMK23) are of *Prevotella* species, two clones of Bacteriodetes bacterium (NRCMK29 and NRCMK65) and the remaining 5 clones are of uncultured bacteria. In sub-group III, 14 clones are grouped together and most of the clones (10 ) in this group are of uncultured rumen bacteria, one clone is *Ruminococcus flavefaciens* (NRCMK70), one clone is *Streptococcus* sp. (NRCMK47) and two Clostridiales bacterium ( NRCMK10 and NRCMK13). In sub-group IV, 10 clones are grouped together and all the clones in this sub-group are uncultured rumen bacteria. One clone (NRCMK22) is separated from all the sub-groups of cluster I which may be termed as unidentified bacteria. Similarly, In Cluster II, 22 clones are grouped together out of which two clones (NRCMK64 and NRCMK57) are separated from others. Remaining 20 clones are grouped

**Table 2.** Similarity values of clones (16S rDNA sequences) retrieved from the rumen fluid of mithun.

| Clone no. | Accession no (query sequence) | Nearest valid relatives | Accession no (subject sequence) | Identity |
|---|---|---|---|---|
| NRCM K10 | JX855075 | Clostridiales bacterium | AB702858 | 95 |
| NRCM K18 | JX855076 | Uncultured rumen bacterium clone | GQ327497 | 93 |
| NRCM K7 | JX855077 | Uncultured rumen bacterium clone | HM008741 | 94 |
| NRCM K11 | JX855078 | Uncultured rumen bacterium clone | EF436438 | 94 |
| NRCM K28 | JX855079 | Uncultured rumen bacterium clone | GU302718 | 95 |
| NRCM K36 | JX855080 | Prevotella sp. clone | DQ308605 | 96 |
| NRCM K34 | JX855081 | Uncultured rumen bacterium clone | EU842835 | 98 |
| NRCM K17 | JX855082 | Prevotella ruminicola | AB501167 | 95 |
| NRCM K21 | JX855083 | Uncultured rumen bacterium clone | AB270090 | 99 |
| NRCM K4 | JX855084 | Uncultured rumen bacterium clone | EF445288 | 96 |
| NRCM K33 | JX855085 | Prevotella ruminicola | AB501171 | 97 |
| NRCM K31 | JX855086 | Prevotella ruminicola | AB501153 | 95 |
| NRCM K15 | JX855087 | Prevotella ruminicola | AB501173 | 94 |
| NRCM K13 | JX855088 | Clostridiales bacterium | GQ358492 | 91 |
| NRCM K24 | JX855089 | Prevotella ruminicola | AB501172 | 96 |
| NRCM K1 | JX855090 | Uncultured rumen bacterium clone | DQ673507 | 96 |
| NRCM K25 | JX855091 | Uncultured rumen bacterium clone | GQ327548 | 97 |
| NRCM K19 | JX855092 | Uncultured rumen bacterium clone | GQ327497 | 90 |
| NRCM K40 | JX855093 | Uncultured rumen bacterium clone | EF445285 | 97 |
| NRCM K20 | JX855094 | Uncultured rumen bacterium clone | AB034128 | 93 |
| NRCM K30 | JX855095 | Prevotella ruminicola | CP002006. | 99 |
| NRCM K9 | X855096 | Uncultured rumen bacterium clone | EF686573 | 95 |
| NRCM K23 | JX855097 | Prevotella ruminicola | AB501173 | 96 |
| NRCM K39 | JX855098 | Uncultured rumen bacterium clone | EU461449 | 98 |
| NRCM K5 | JX855099 | Uncultured rumen bacterium clone | EF445288 | 96 |
| NRCM K14 | JX855100 | Uncultured rumen bacterium clone | EU719218 | 90 |
| NRCM K37 | JX855101 | Prevotella ruminicola clone | AB501168 | 93 |
| NRCM K12 | JX855102 | Uncultured rumen bacterium gene | AB185591 | 95 |
| NRCM K3 | JX855103 | Uncultured rumen bacterium clone. | EU842595 | 99 |
| NRCM K26 | JX855104 | Uncultured rumen bacterium clone | EU259500 | 97 |
| NRCM K8 | X855105 | Uncultured rumen bacterium clone | EU778030 | 96 |
| NRCM K16 | JX855106 | Uncultured rumen bacterium clone | GU302850 | 96 |
| NRCMK 29 | JX855107 | Bacteroidetes bacterium clone | HM104872 | 93 |
| NRCM K6 | JX855108 | Uncultured rumen bacterium clone | FJ684957 | 90 |
| NRCM K2 | JX855109 | Uncultured rumen bacterium clone | EF436384 | 98 |
| NRCM K35 | JX855110 | Prevotella ruminicola | AB501163 | 98 |
| NRCM K22 | JX855111 | Uncultured rumen bacterium clone | AB270037 | 97 |
| NRCM K38 | JX855112 | Uncultured rumen bacterium clone | GQ327819 | 98 |
| NRCM K32 | JX855113 | Prevotella ruminicola | AY699286 | 98 |
| NRCM K27 | JX855114 | Uncultured rumen bacterium clone | AY838556 | 97 |
| NRCM K45 | KC020714 | Uncultured rumen bacterium clone | EU844417 | 96 |
| NRCM K53 | KC020715 | Uncultured rumen bacterium clone | JF633438 | 98 |
| NRCM K51 | KC020716 | Uncultured rumen bacterium clone | EU459560 | 94 |
| NRCM K46 | KC020717 | Butyrivibrio fibrisolvens | EU684229 | 99 |
| NRCM K52 | KC020718 | Uncultured rumen bacterium clone | HQ399710 | 93 |
| NRCM K44 | KC020719 | Uncultured rumen bacterium clone | AY854313 | 97 |
| NRCM K58 | KC020720 | Uncultur Uncultured rumen bacterium clone | DQ673473 | 95 |
| NRCM K55 | KC020721 | Uncultured rumen bacterium clone | JF635508 | 98 |

**Table 2.** Contd.

| | | | | |
|---|---|---|---|---|
| NRCM K42 | KC020722 | Uncultured rumen bacterium clone | HM816731 | 97 |
| NRCMK49 | KC020723 | Butyrivibrio species | EU714408 | 98 |
| NRCM K56 | KC020724 | Uncultured rumen bacterium clone | FJ032385 | 95 |
| NRCM K48 | KC020725 | Uncultured rumen bacterium clone | JX003956 | 93 |
| NRCM K47 | KC020726 | Streptococcus sp. | GQ139522 | 99 |
| NRCM K50 | KC020727 | Butyrivibrio species | AM039823 | 99 |
| NRCM K59 | KC020728 | Uncultured rumen bacterium clone | AB270006 | 94 |
| NRCM K41 | KC020729 | Uncultured organism clone | HQ746244 | 97 |
| NRCM K60 | KC171704 | PseudoButyrivibrio ruminis | NR026315 | 97 |
| NRCM K61 | KC171705 | Butyrivibrio sp. | EU714408 | 98 |
| NRCM K62 | KC171707 | Succinivibrio dextrinosolvens | NR026476 | 99 |
| NRCM k63 | KC171716 | Prevotella ruminicola | AB501169 | 94 |
| NRCM K64 | - | Uncultured rumen bacterium clone | GU302745 | 96 |
| NRCM k65 | KC171713 | Bacteroidetes bacterium clone | HM104836 | 98 |
| NRCM K66 | KC171715 | B.fibrisolvens | X89979 | 99 |
| NRCM k67 | KC171709 | Sporanaerobacter acetigenes | NR025151 | 96 |
| NRCM K43 | KC171712 | Uncultured rumen bacterium clone | AB270037 | 98 |
| NRCM K54 | KC171714 | Uncultured rumen bacterium clone | AB270090 | 97 |
| NRCM K57 | KC171708 | Prevotella species | JF893676 | 90 |
| NRCM K68 | KC171710 | Uncultured rumen bacterium clone | DQ673497 | 92 |
| NRCM K69 | KC171706 | Prevotella species | AB003385 | 96 |
| NRCM K70 | KC171711 | Ruminococcus flavefaciens | AM915269 | 98 |
| NRCM B1 | JQ290364 | Prevotella species clone | AB501166 | 93 |
| NRCM B2 | JQ290365 | Uncultured rumen bacterium clone | AB244120 | 98 |

together in three subgroups. Sub-group I contained two uncultured rumen bacteria. Sub-group II contained nine clones out of which six are of uncultured rumen bacteria and another three are of *Prevotella ruminicola* (NRCMK24), *Succinivibrio dextrinosolvens* (NRCMK62) and *Butyrivibrio fibrisolvens* (NRCMK66). Sub-group III, contained 9 clones, out of which, eight clones are of *Prevotella* species (NRCMK30, NRCMK32, NRCMK33, NRCMK35, NRCMK36, NRCMK37, NRCMK63, NRCMK69) and one is of uncultured rumen bacteria.

In this experiment, majority of bacteria were found to be of *Prevotella* species as they take part in digestion of feed stuffs in the rumen. *P. ruminicola* is a proteolytic bacterium and plays a key role in ruminal protein degradation (Wallace, 1996; McKain et al., 1992). Whitford et al. (1998) also reported that 16S rDNA sequences similar to those of *P. ruminicola* prevailed in isolated material from domestic cattle. Pandya et al. (2010) while studying the bacterial diversity in the rumen of Indian Surti buffalo (*Bubalus bubalis*), reported that, the CFB (Cytophaga-Flavobacteria-Bacteroides) bacteria were less numerous than Frimicutes in the rumen of buffaloes. This suggests that, the particular type of diet given to the animals can have a significant impact on the bacterial diversity of the rumen (Hungate, 1969). The microbial population in the mithun (*Bos frontalis*) in NE region of India fed on mixed tree leaves and rice straw based diet are different from other domestic animals.

The clones of fibrolytic bacteria like *B. fibrisolvens* and *R. flavefaciens* were isolated (Khampa et al., 2006; Leng et al., 2011), but *Fibrobacter succinogens* was not isolated similar to the findings of Deng et al. (2007). The starch degrading bacteria (*Succinivibrio dextrinosolvens)* were also isolated in the present study. *Streptococcus* species were isolated in the present experiments which are responsible for starch degradation (Cotta, 1988) and some strains of *Streptococcus* are also responsible for fibre degradation. Deng et al. (2007) found that the numbers of cellulolytic and amylolytic bacteria were increased in mithun as compared to cattle (*Bos taurus*) and dominant bacteria isolated in the study of Leng et al. (2011) were cellulolytic and amylolytic. In our study also, both cellulolytic and amylolytic bacteria were isolated from the rumen of mithun. Sulphate reducing bacteria (*Sporanaerobacter acetigenes*) was isolated in the present study. Though many bacteria reduce sulphate during their synthesis of sulphur containing amino acids, presence of sulphate reducing bacteria in rumen of mithun is benefit for growth of the animal.

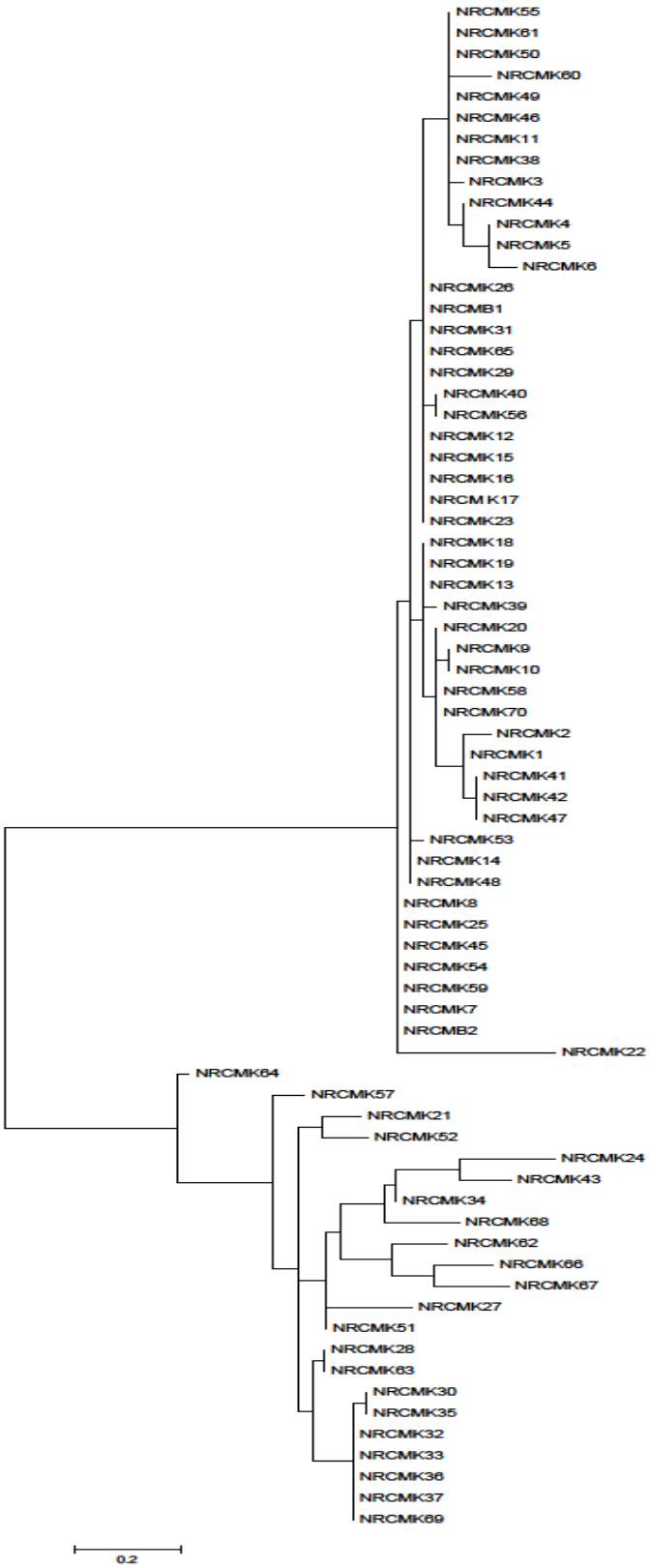


**Figure 3.** Phylogenetic tree of 16S rDNA sequences of clones recovered from rumen of mithun.

## Conclusion

The experiment showed that, feeding of mixed tree leaves and rice straw based diet harbor diversified species of bacteria, that is, *P. ruminicola*, *B. fibrisolvens*, *P. ruminis, S. dextrinosolvens, R. flavefaciens, Streptococcus* species etc. in the rumen of mithun for digestion of feed stuffs.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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