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Identification of methanotrophs in the buffalo rumen by 16S rRNA sequence analysis

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ABSTRACT

In the present study, methanotrophs inhabiting the rumen of Murrah buffalo were detected by molecular technique. The methanotrophs specific primer sets (type l and type ll methanotrophs) were used to amplify 16S rDNA sequence from buffalo rumen. The PCR product of an expected DNA length was observed in the 1% agarose gel, which was purified and then cloned. Different gene clones were screened for inserts using the respective PCR primers. Out of 15 colonies, 10 colonies were positive for inserts of 16S rRNA gene sequences. One recombinant plasmid DNA from each group was sequenced and the nucleotide sequence (592bp and 486bp) obtained with type I and type II primer sets were deposited in the GenBank database under the accession number HQ699778 and HQ699779, respectively. The sequences obtained in present study showed ≤83% sequence similarity with known methanotrophs and most closely (96 to 99%) related to the 16S rRNA gene of uncultured rumen bacteria. The findings lead to a supposition that the novel methanotrophs with 16S rRNA gene similar to uncultured rumen bacteria are present in the Murrah buffalo rumen.

Key words: 16S rRNA gene, Buffalo, Rumen, Methanotrophs

Methane-oxidizing bacteria (methanotrophs) are detected in various environments like soil, lakes, sludge etc. and characterized (Auman *et al*. 2000, Bull *et al*. 2000), however, very scanty information is available on methane oxidation (Moss *et al*. 2001, Kajikawa *et al*. 2003, Jha 2011) and presence of methanotrophs in the rumen. In a variety of environments, cultivation-independent techniques are developed for detection of methanotrophs (Murrell and Radajewski 2000). The 16S rRNA gene is structurally and functionally the most conserved in prokaryotes and due to availability of the large database of sequences, the one obvious marker for the methanotrophs is 16S rRNA gene. Methane-oxidizing bacteria are classified into type I and type II methanotrophs on the basis of 16S rRNA phylogeny, morphology, the pathways of carbon assimilation utilized, membrane type and composition. Sequence analysis of 16S rRNA confirmed that type I and type II methanotrophs belong to gamma-Proteobacteria and alpha-Proteobacteria, respectively (Murrell and Radajewski 2000). Wise *et al*. (1999) and Gulledge *et al*. (2001) developed sets of 16S rRNA-targeted oligonucleotide primers for the genus of methanotrophs. Among the family or genus specific primers, MethT1dF-MethT1bR for type I and MethT2R for type II methanotrophs (Wise *et al*. 1999) are most commonly used and successfully applied to amplify 16S rRNA genes of methanotrophs from different environmental samples including landfill soils (Wise *et al*. 1999), coastal sediments (Carini *et al.* 2003), Mono Lake (Carini *et al*. 2005), wetlands (Horz *et al.* 2001, Bodelier *et al.* 2005) and the rumen (Mitsumori *et al*. 2002). The primers MethT1dF and MethT1bR were designed to target only 4 genera (*Methylomonas*, *Methylobacter*, *Methylomicrobium* and *Methylococcus*) and MethT2R was only for 2 genera (*Methylosinus* and *Methylocystis*). Jha *et al.* (2010) reviewed that additional genera of type I and type II methanotrophs exist. In order to cover the expanded diversity of extant methanotrophs, Chen *et al*. (2007) designed and validated a new group-specific degenerate primers of 16S rRNA gene for methanotrophs type I and type II. The clone library was generated from PCR products amplified from cDNA of landfill soil and found that from the 10 clones, 9 of the sequences corresponded to 16S rRNA genes of methanotrophs. The present study was undertaken with the intent to detect the presence of methanotrophs by using specific primers (Chen *et al*. 2007) to amplify partial sequence of 16S rRNA gene in the Murrah buffalo rumen fluid.

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MATERIALS AND METHODS

Animal and sample collection: A ruminally fistulated male Murrah buffalo (body weight, 650 kg) was given a maintenance ration containing 3 kg of wheat straw, 30 kg of berseem fodder and 2.5 kg concentrate mixture in 2 divided meals. Ruminal contents were collected through the fistula at 8.30 AM before feeding and watering and strained through 2 layers of muslin cloth to collect rumen liquor. Freshly collected rumen liquor was centrifuged at 3,000 rpm for 10 min at 4°C to remove feed particles and ruminal protozoa. Supernatant was collected in another tube and centrifuged at 12,000 rpm for 15 min at 4°C. Bacterial cells thus settled at the bottom in form of pellets.

Isolation of bacterial genomic DNA and PCR procedures: Isolation of DNA was carried out from the bacterial pellet (approximately 0.3 ml) using a mini kit. Obtained concentration of genomic DNA from DNA elute was 110 ng μl^{-1} with optical density 2.2. The isolated DNA was used as a template for PCR to amplify 16S rRNA gene. The first PCR was carried out to amplify almost full-length bacterial 16S rDNA fragments, using the bacteria-specific primers 27F (5'-GAGTTTGATCMTGGCTCAG-3') (Lane 1991) and 1529R (5'-CAKAAAGGAGGTG ATCC-3') (Snaidr *et al*. 1997). The amplified PCR products were purified by purification kit and used as a template for the second PCR. The type I and type II methanotrophs specific primer sets were used for

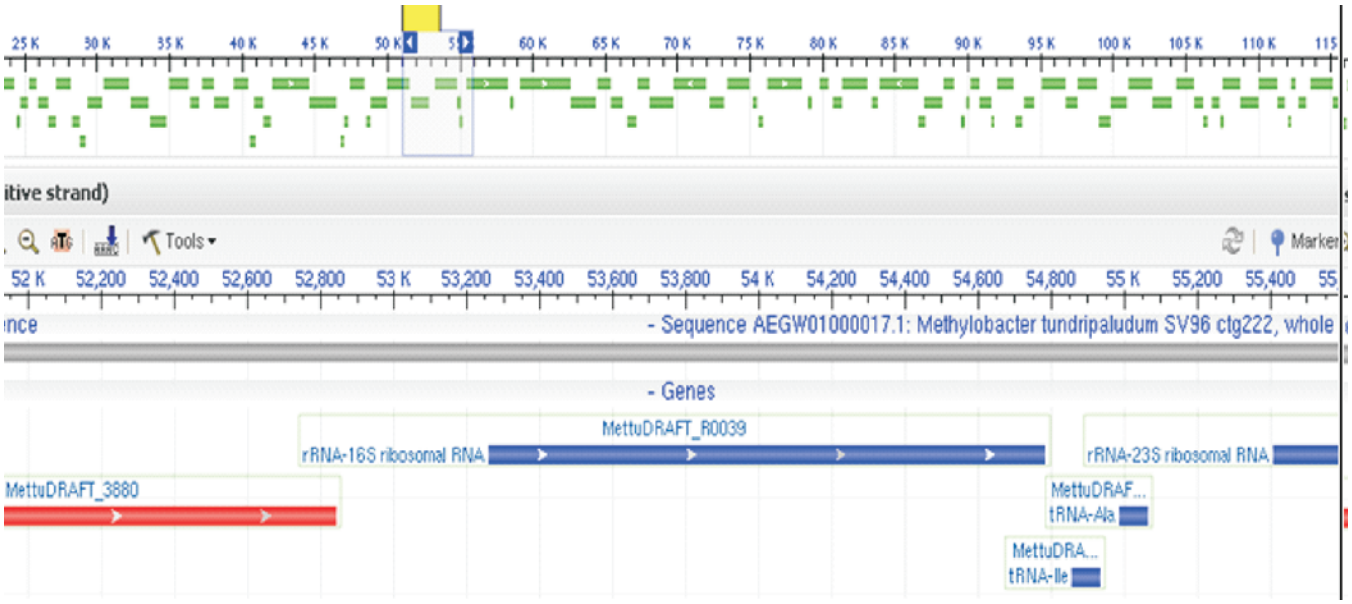

Fig. 1. Location of 16S rRNA gene sequence length 1523 bp (53260… 54782) in genomic DNA of *Methylobacter tundripaludum* SV96 ctg222 (Accession: AEGW01000017.1) from NCBI, used to locate primers to amplify 697 bp (53272 to 53968) with type I primer set.

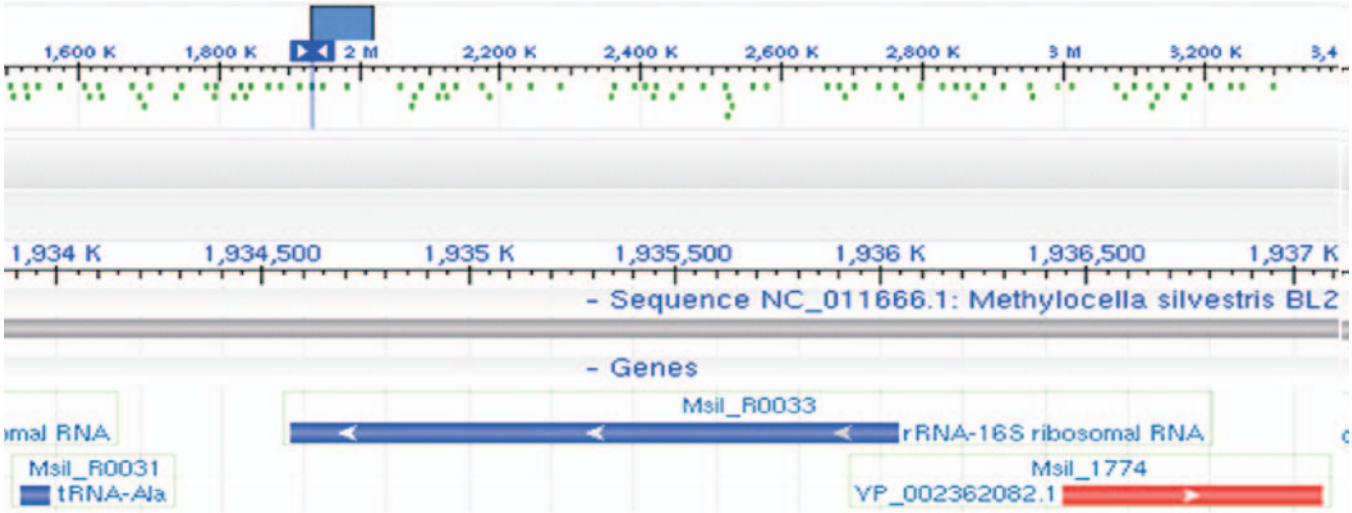

Fig. 2. Location of 16S rRNA gene sequence length 1475 bp (1934569… 1936043) in genomic DNA of *Methylocella silvestris* BL2 (Accession: NC_011666.1) from NCBI used to locate primers to amplify 548 bp (1935091to 1935638) with type II primer set.

Table 1. PCR cycling program for different primer combinations

Program	1st step	2nd step			Final step
	Initial denaturation	Denaturation	Annealing	Extension	Extension
First PCR	2 min 94°C 1 cycle	40 sec 94°C 35 cycles	35 sec 51°C	90 sec 72°C	10 min 72°C 1 cycle
Second PCR (Type I)	2 min 94°C 1 cycle	30 sec 94°C 35 cycles	20 sec 65°C	20 sec 72°C	5 min 72°C 1 cycle
Second PCR (Type II)	2 min 94°C 1 cycle	30 sec 94°C 35 cycles	25 sec 63°C	25 sec 72°C	5 min 72°C 1 cycle

second amplification (Chen *et al*. 2007). The type I set was of type IF (5'- ATGCTTAACACATGCAAGTC GAACG-3') and type IR (5'- CCACTGGTGTTCCTTCMGAT-3') and type II set comprised type IIF (5'- GGGAMGATAATGACGGTA CCWGGA-3') and type IIR (5'- GTCAARAGCT GGTAAGGTTC-3'). The 16S rRNA gene sequence information used to locate the forward primers and reverse primers for amplification of partial sequences of 16S rRNA gene from type I and type II primer sets are presented in Figs 1 and 2, respectively. The PCR reaction mixtures contained 1× PCR buffer, 1.5mM $MgCl_2$, 200 µM dNTP, 10 pmol of each primer, 1U of Taq polymerase and 110 ng of isolated DNA in first PCR and 1µl of 1: 100 dilutions of first PCR products in second PCR in a final volume of 25 µl. The PCR cycling programmes for the different primer combinations are listed in Table 1. The second PCR products were separated by electrophoresis on 1% agarose gel and stained with ethidium bromide and purified with purification kit.

Cloning, sequencing and sequence analysis: Purified PCR products of type I and type II primer sets were cloned in pDrive cloning vector (PCR product cloning kit) and transformants (*E. coli* XL-1) were randomly picked up. The gene clones (15 from each type I and type II clones) were screened for inserts using the respective PCR primers and got 10 positive insert in both type I and type II clones. Recombinant plasmid DNA was isolated from positive clones using the kit and out of 10 only 1 isolated plasmid from each group was custom sequenced from commercial laboratory. The obtained gene sequences were aligned by ClustalW (Thomson *et al*. 1994) version 1.83 and edited by using BioEdit software. Sequences which shared 77 to 83% identity of type I and type II methanotrophs nucleotide sequences and ≥91% similarity of uncultured rumen and non rumen bacterium to our query sequences were downloaded from GenBank following BLAST searches to know about the phylogenic position of sequences identified.

RESULTS AND DISCUSSION

Identification of methanotrophs in the rumen: Methanotrophs are divided into 2 taxonomic groups: type I and type II. Type I represents following 10 genera: *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylosarcina*, *Methylosphaera*, *Methylosoma*, *Methylohalobium*, *Methylococcus*, *Methylocaldum* and *Methylothermus*, which belong to the gamma subdivision of the *Proteobacteria*. The type II is represented by only 4 genera (*Methylosinus*, *Methylocystis*, *Methylocella* and *Methylocapsa*) of the alpha subdivision of the *Proteobacteria*. The present study was planned to amplify the partial sequence of 16S rRNA gene from the genomic DNA of rumen contents of buffalo by using the type I and type II methanotrophs specific primer sets. The PCR products of an expected DNA length (about 697 and 548 bp) were obtained with type I and type II primer sets, respectively (Figs 3 A, B). Therefore, the type I and type II methanotrophs were detected in this study. Cloning and sequencing of partial sequence of 16S rRNA amplicon could identify the rumen methanotrophs. The nucleotide sequences amplified with type I and type II sets of primers were found to be 592bp and 486bp, respectively and the determined nucleotide sequences were deposited in the GenBank database under accession number, HQ699778 (clone name, om10) and HQ699779 (clone name, nj10). Previously, Mitsumori *et al*. (2002) planned to detect the methanotrophic community from the rumen fluid and rumen epithelium of a Holstein cow by using methanotrophs specific primers (Wise *et al*. 1999). They could amplify an expected size of amplicon with type I primer set, however, after

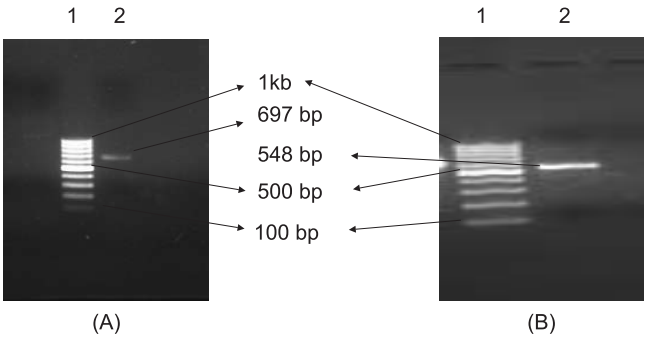

Fig. 3. Agarose gel (1%) showing PCR products obtained with methanotrophs-specific primers.A, Lane 1 shows DNA size-markers and lane 2 displays amplified product with the type I primer set. B, Lane 1 shows DNA size-markers and lane 2 displays amplified product with the type II primer set.

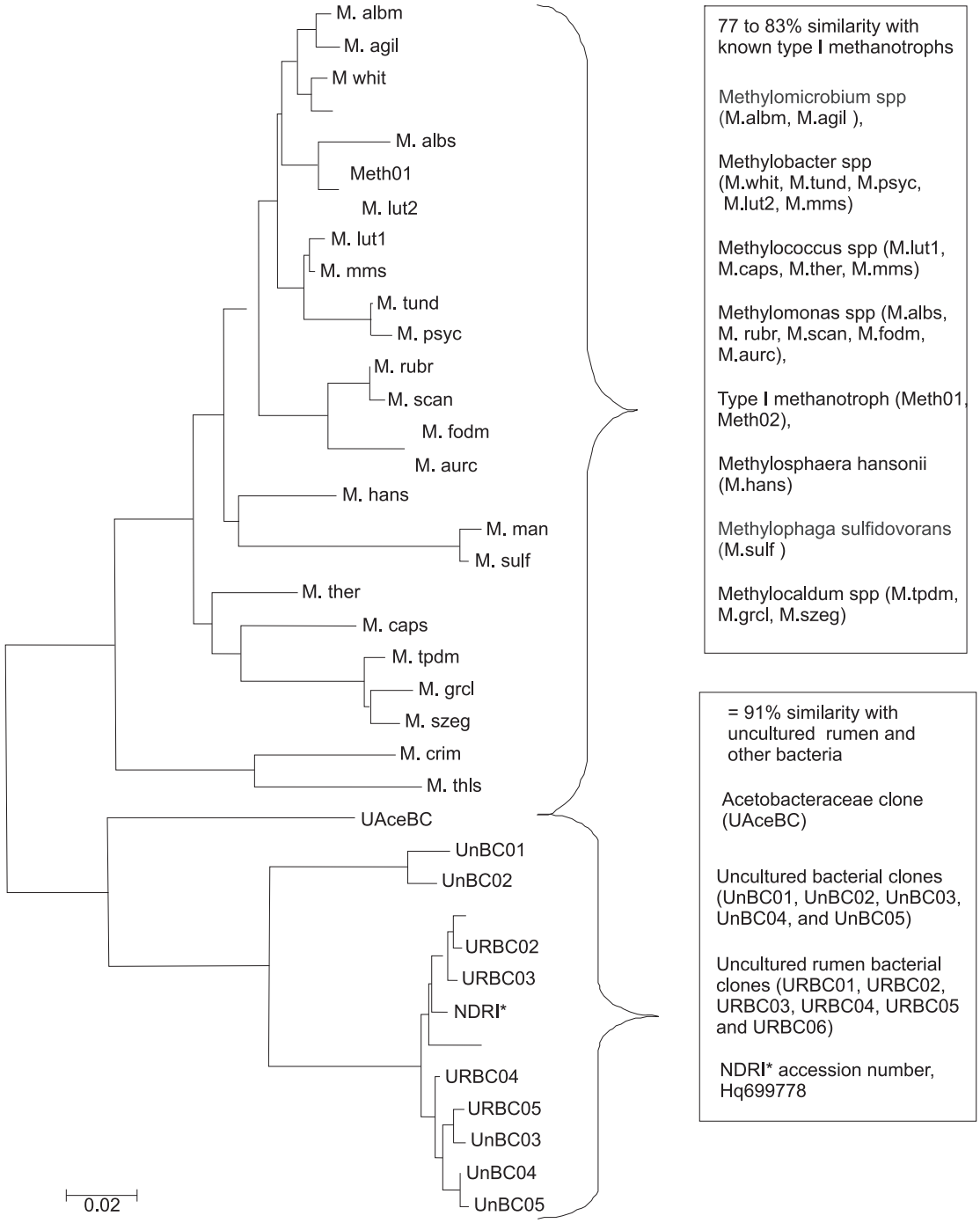

Fig. 4. Phylogenetic placement of 16S rRNA gene sequence (NDRI*) from buffalo rumen fluid showing relationship with similar sequences available in GenBank. Nucleotide sequence of 592bp of 16S rRNA gene sequence was used for constructing the N.J. tree using MEGA 4.1 software.

sequencing it was not found to be related with methanotrophs (Mitsumori *et al*. 2002).

Phylogenetic analysis of sequences: The phylogenetic placement of sequences under the accession numbers HQ699778 (NDRI*) and HQ699779 (NDRI2) of present study are Figs 4 and 5, respectively. The PCR product, amplified with the type I primer set was sequenced (592 bp) and found ≤83% similarity with known methanotrophs (type I). The sequence showed 83% identity with *Methylophaga thiooxydans* (ABXT01000015.1 and ABXT01000035.1), 81% with *Methylobacter tundripaludum* SV96 (AEGW01000017.1) and 77% with *Methylococcus*

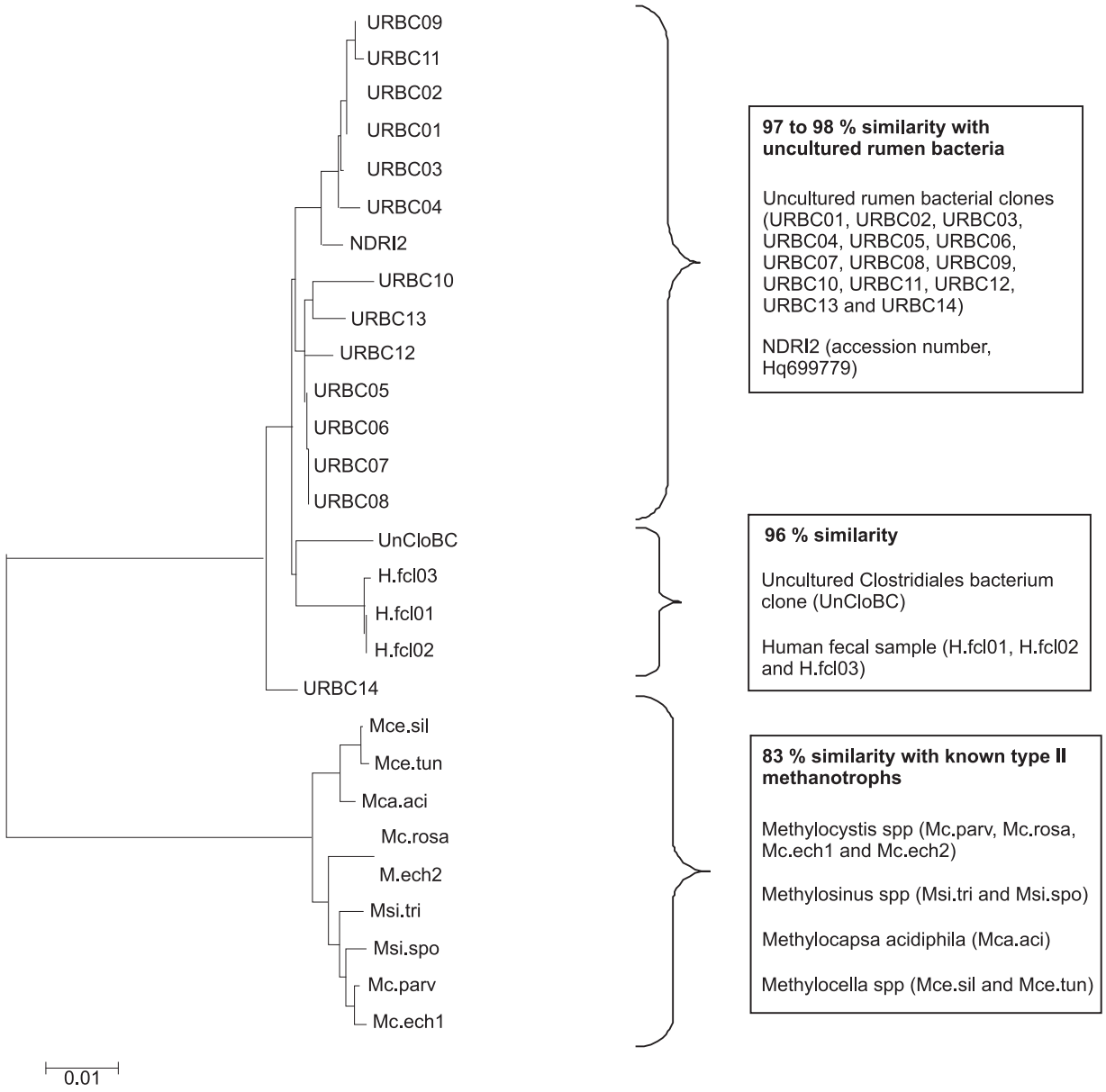

Fig. 5. Phylogenetic placement of 16S rRNA gene sequence (NDRI2) from buffalo rumen fluid showing relationship with similar sequences available in GenBank. Nucleotide sequence of 486bp of 16S rRNA gene sequence was used for constructing the N. J. Tree using MEGA 4.1 software.

capsulatus str. Bath (NC_002977.6). The sequence was found to correspond to 16S rRNA gene of type I methanotrophs most closely related to the 16S rRNA gene of uncultured rumen bacteria of accession numbers; GQ327821.1 (99%), GQ327690.1 (99%), GQ327810.1 (98%), EU850563.1 (98%), EU259443.1 (97%) and AB185652.1 (96%). The sequence demonstrated high (98%) identity to the 16S rRNA gene of uncultured bacterium from other sources under the accession numbers; EU843170.1, EU842727.1, EU842317.1, EU842326.1 and AB494952.1 and 90 to 91% similarity with FN377799.1, FN377792.1 and AY571491.1.

The amplified PCR products of type II primer set was cloned into a plasmid vector and sequenced. The obtained 16S rRNA gene sequence showed 83% sequence similarity with known methanotrophs (type II) as; *Methylocystis* sp. ATCC 49242 (AEVM01000005.1), *Methylosinus trichosporium* OB3b (ADVE01000118.1), *Methylocella silvestris* BL2 (NC_011666.1) and *Methylobacterium nodulans* ORS (NC_011894.1). The nucleotide sequence clustered with uncultured rumen bacterium, showed 97 to 98% identities with the accession numbers- GQ327189.1, GU302775.1, AB185700.1, EU381917.1, GQ327007.1 and GQ327709.1. The sequence also showed the high level of identities (96% similarity) with 16S rRNA sequence

amplified from human fecal sample, the accession numbers; FP077172.1, FP075640.1 and FP075204.1.

The nucleotide sequence of 16S rRNA gene obtained with type I and type II primer sets showed ≤83% identity with known methanotrophs and showed 96 to 99% similarity with 16S rRNA gene sequences of uncultured rumen bacteria. The findings of the present study lead to a supposition that the novel methanotrophs with 16S rRNA gene similar to uncultured rumen bacteria are present in buffalo rumen. Further studies are required in this direction to ascertain the biochemical properties as well as physiological and nutritional importance of methanotrophic bacteria in the buffalo rumen.

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