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Note

Methanogenic archaea diversity in Hanwoo (*Bos taurus coreanae*) rumen fluid, rectal dung, and barn floor manure using a culture-independent method based on *mcr*A gene sequences

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ABSTRACT

The diversity of methanogenic archaea associated with Korean Hanwoo cattle was analyzed using *mcr*A gene sequences from samples of rumen fluid (RF), rectal dung (RD), and barn floor manure (BFM). The predominant species were *Methanobrevibacter ruminantium* in RF and BFM(63.6% and 62.4%, respectively) and *Methanocorpusculum labreanum* in RD (53.2%).

The agricultural sector produces 50–60% of total global methane emissions, and livestock production operations, particularly ruminants, are known as the primary source of this methane [1]. Methane is produced by methanogens, which are obligate anaerobic microorganisms belonging to the archaeal phylum *Euryarchaeota* [2]. In the past decade, there has been an increasing interest in rumen methanogenic archaea because of their role in methane production and loss of energy from ingested feed [3]. From the rumen, food material ingested by the animal travels through the rest of the digestive system, the rectum, the anus, and then out into the environment. It is thought that some of the methanogens present in the rumen travel with the food material [4]. At present, information on the diversity and population of methanogens is limited to studies of the rumen. The species of methanogenic archaea most commonly isolated from the rumen are strains of *Methanobrevibacter*, *Methanomicrobium*, *Methanobacterium*, and *Methanosarcina*. Methanogenic archaea are difficult to study through culture-based methods; therefore, many researchers have instead used culture-independent techniques to study their populations [5]. In a recent study, Lenhart et al. [6] reported that saprotrophic fungi produce methane without the involvement of methanogenic archaea. In Korea, the number of Hanwoo reached approximately three million in 2012, which is the highest number of ruminant livestock to date. This particular breed is highly valued for its beef. This study aims to identify the methanogenic archaea present in Hanwoo rumen fluid, rectal dung, and barn floor manure using the *mcr*A gene as a biological marker.

This study used the three rumen-fistulated Hanwoo (*Bos taurus coreanae*) that were approximately three years old and 55 kg in weight. The cows were fed twice daily with concentrate and rice straw. Three independent samples were obtained at the same time, namely, the rumen fluid (RF), rectal dung (RD), and manure from the barn floor (BFM). The pH, carbon, and nitrogen content of the samples were determined as described previously [7]. Each sample (approximately 1 g) was mixed with 20 mL of phosphate buffered saline (pH 7.2) and vortexed for 30 min. The samples for DNA extraction were collected through 4 layers of cheesecloth and centrifuged at 14,000× *g* for 5 min at 4 °C. The pellets were then subjected to DNA extraction using the FastDNA SPIN Kit (MP

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Table 1
Physicochemical properties of the rumen fluid (RF), rectal dung (RD), and barn floor manure (BFM) of Hanwoo.

Sample[a]	pH	C (%)	N (%)	C:N ratio
RF	7.23	38.74	1.94	19.98
RD	7.43	36.26	1.63	22.27
BFM	9.16	36.29	2.00	18.15

[a] RF: rumen fluid; RD: rectal dung; and BFM: barn floor manure.

Biomedicals, CA, USA) according to the manufacturer's instructions. The PCR primers used to amplify the *mcr*A (methyl coenzyme-M reductase α subunit) fragments were the *mcr*A-specific primers, 5′-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3′ (forward) and 5′-TTCATTGCRTAGTTWGGRTAGTT-3′ (reverse) [8]. The *mcr*A (genes were amplified by PCR using the purified DNA), *Top* DNA polymerase, a dNTPs mixture, and 10× reaction buffer. Fifteen cycles (denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 45 s) were followed by a final incubation at 72 °C for 10 min. The anticipated product of approximately 500 bp was isolated after agarose gel electrophoresis of the amplified mixture using a Qiagen DNA gel extraction kit (Qiagen, CA, USA). Recombinant clones and the insert size in the purified plasmids were confirmed as described previously [6]. The *mcr*A gene sequences were confirmed using the Basic Local Alignment Search Tool (BLAST) in NCBI. The phylogenetic tree was calculated from *mcrA* genes and proteins using the neighbor-joining program MEGA 5.05 as described by Rastogi et al. [4]. Bootstrapping (1000 replicate reconstructions) was used to estimate the reliability of tree topologies. The gene sequences were deposited in GenBank under accession numbers KF279047 to KF279131.

The diversity of methanogenic archaea in Hanwoo RF, RD, and BFM was studied by analyzing PCR-amplified *mcr*A molecules. The properties of the samples are shown in Table 1. The pH of the BFM sample (9.16) was higher than those of the RF and RD samples, which had similar values (7.23 and 7.43, respectively). After excretion of the fecal matter from cow, low molecule fatty acids were volatilized and some proteins were mineralized to ammonia during its stay in the bottom. Hence, the pH of BFM sample is higher than that of the other samples. The carbon contents were similar in the three samples with 38.74%, 36.26%, and 36.29% for the RF, RD, and BFM samples, respectively. Studies have described the archaeal diversity in RF and RD [3–5,7,10–14] but a comparison between RF, RD, and BFM has not yet been reported.

A total of 433 *mcr*A clones were analyzed from the RF, RD, and BFM samples, and their phylotypes were classified into 6, 6, and 8 operational taxonomic units (OTUs), respectively. Archaea most closely related to *Methanobrevibacter millerae*, *Methanobrevibacter ruminantium*, and *Methanobrevibacter* sp. WBY1 were found in all three samples. *Methanobrevibacter gottschalkii*, Methanogenic archaeon, and *Methanomicrobium mobile* were unique to the RF sample; *Methanocorpusculum labreanum*, *Methanocorpusculum* sp. MSP, and *Methanoculleus bourgensis* were unique to the RD sample; and *Methanoregula boonei*, *Methanobrevibacter woesei*, *Methanolobus bombayensis*, *Methanosarcina thermophila*, and *Methanosphaera stadtmanae* were unique to the BFM sample (Table 2).

All of the clone sequences showed between 83 and 96% similarity to sequences listed in the searched databases. The predominant species were *M. ruminantium* in the RF and BFM samples (63.6% and 53.2%, respectively), and *M. labreanum* in the RD sample (62.4%). *M. gottschalkii* (1.4%), *Methanobrevibacter milerae* (21.2%), *Methanobrevibacter* sp. WBY1 (2.1%), Methanogenic archaeon (4.9%), and *M. mobile* (6.8%) were isolated from the RF sample; *M. millerae* (32.7%), *M. ruminantium*(9.9%), *Methanobrevibacter* sp. WBY1 (2.1%), *Methanocorpusculum* sp. MSP (1.4%), and *M. bourgensis* (0.7%) were isolated from the RD sample; and *M. boonei* (8.9%), *M. millerae* (13.0%), *Methanobrevibacter* sp. WBY1 (7.5%), *M. woesei* (2.7%), *M. bombayensis* (2.1%), *M. thermophila* (0.7%), and *M. stadtmanae* (2.7%) were isolated from the BFM sample (Table 2).

The phylogenetic relationships between the affiliated RF, RD, and BFM archaea were analyzed by clarifying the taxonomic position of clones allocated with reasonable confidence to particular taxa. The largest cluster (73.2% of the clones) contained five OTUs and grouped with the *Methanobacteriales*; this grouping was supported by high bootstrap values. Phylotypes within the *Methanomicrobiales* represented 21.0% of the clones and spanned seven OTUs. The remaining two OTUs represented four clones (0.9%) and seven clones (4.9%) were closely related to cultured *Methanosarcinales* species and unknown archaea, respectively (Fig. 1).

Table 2
Similarity values of *mcr*A gene sequences retrieved from the rumen fluid (RF), rectal dung (RD), and barn floor manure (BFM)of Hanwoo.

Sample[a]	Phylotype	Accession no.	No. of clone	Nearest valid relative	Similarity[b] (%)	Composition (%)	Functional group
RF	HANU1.01–HANU1.02	KF279047–KF279048	2	*Methanobrevibacter gottschalkii*	91–93	1.4	Hydrogenotrophs
	HANU1.03–HANU1.12	KF279049–KF279058	31	*Methanobrevibacter milerae*	90–96	21.2	Hydrogenotrophs
	HANU1.13–HANU1.28	KF279059–KF279074	93	*Methanobrevibacter ruminantium*	88–96	**63.6**[c]	Hydrogenotrophs
	HANU1.29	KF279075	3	*Methanobrevibacter* sp. WBY1	94	2.1	Hydrogenotrophs
	HANU1.30–HANU1.31	KF279076–KF279077	7	Methanogenic archaeon	85–90	4.9	Unknown
	HANU1.32	KF279078	10	*Methanomicrobium mobile*	99	6.8	Hydrogenotrophs
RD	HANU2.01–HANU2.10	KF279079–KF279088	46	*Methanobrevibacter milerae*	89–95	32.7	Hydrogenotrophs
	HANU2.11–HANU2.14	KF279089–KF279092	14	*Methanobrevibacter ruminantium*	88–95	9.9	Hydrogenotrophs
	HANU2.15–HANU2.16	KF279093–KF279094	3	*Methanobrevibacter* sp. WBY1	94–95	2.1	Hydrogenotrophs
	HANU2.17–HANU2.24	KF279095–KF279102	72	*Methanocorpusculum labreanum*	87–88	**53.2**	Hydrogenotrophs
	HANU2.25–HANU2.26	KF279103–KF279104	2	*Methanocorpusculum* sp. MSP	83–87	1.4	Hydrogenotrophs
	HANU2.27	KF279105	1	*Methanoculleus bourgensis*	93	0.7	Hydrogenotrophs
BFM	HANU3.01–HANU3.02	KF279106–KF279107	13	*Methanoregula boonei*	84–85	8.9	Hydrogenotrophs
	HANU3.03–HANU3.10	KF279108–KF279115	19	*Methanobrevibacter milerae*	90–95	13.0	Hydrogenotrophs
	HANU3.11–HANU3.17	KF279116–KF279122	91	*Methanobrevibacter ruminantium*	86–96	**62.4**	Hydrogenotrophs
	HANU3.18–HANU3.20	KF279123–KF279125	11	*Methanobrevibacter* sp. WBY1	90–96	7.5	Hydrogenotrophs
	HANU3.21	KF279126	4	*Methanobrevibacter woesei*	89	2.7	Hydrogenotrophs
	HANU3.22	KF279127	3	*Methanolobus bombayensis*	93	2.1	Acetotrophs
	HANU3.23	KF279128	1	*Methanosarcina thermophila*	95	0.7	Acetotrophs
	HANU3.24–HANU3.26	KF279129–KF279131	4	*Methanosphaera stadtmanae*	87–92	2.7	Hydrogenotrophs

[a] RF: rumen fluid; RD: rectal dung; and BFM: barn floor manure.
[b] Range of *mcr*A gene sequences is similarity values between phylotypes and type strain.
[c] Bold words indicate predominant in Hanwoo RF, RD, and BFM.