ORIGINAL ARTICLE

# Molecular diversity analysis of rumen methanogenic *Archaea* from goat in eastern China by DGGE methods using different primer pairs

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## Abstract

**Aims:** To screen a pair of primers suitable for denaturing gradient gel electrophoretic (DGGE) analysis of ruminal methanogenic *Archaea* and to detect the archaeal communities in the rumen of goat.

**Methods and Results:** Nine primer pairs for 16S rDNA of methanogenic *Archaea*, including six for directed polymerase chain reaction (PCR) and three for nested PCR were first evaluated by PCR amplification of the total DNA from rumen fluids and bacteria. The DGGE analysis of rumen fluids was then conducted with three primer sets (344fGC/915r, 1106fGC/1378r and 519f/915rGC) of the nine pairs tested. Good separation and quality of patterns were obtained in DGGE analysis with primer pairs 1106fGC/1378r and 519f/915rGC. A total of 40 DNA fragments were excised from the DGGE gels and their sequences were determined. All fragments belonged to methanogenic *Archaea* while primer pair 519f/915rGC had better amplification ranges than the other two primer pairs.

**Conclusions:** The procedure of DGGE analysis with primer pair 519f/915rGC was more suitable for investigating methanogenic archaeal community in the rumen. The dominant methanogenic *Archaea* in the rumen of goat was *Methanobrevibacter* sp. and an unidentified methanogenic *Archaea*.

**Significance and Impact of the Study:** One pair of primers suitable for DGGE analysis of ruminal methanogenic *Archaea* was obtained and the molecular diversity of ruminal methanogenic *Archaea* in goat was investigated by PCR-DGGE.

## Introduction

Methane production from ruminants has been identified as the single largest source of anthropogenic methane (Mathison *et al.* 1998). Livestock emit methane as part of their natural digestive processes. Methane is formed in the rumen when hydrogen released by other microbes during fermentation of forage is used by methanogenic *Archaea* to reduce carbon dioxide. This loss of energy for the ruminant has been estimated to be between 2% and 12% of the animal's gross energy intake (Johnson and Johnson 1995). Moreover, methane is the second key green house gas and its ability to retain heat is 21 times more than carbon dioxide. Therefore, it is important to understand the mechanism of methanogenesis and in particular the ecology of methanogenic *Archaea* in the rumen.

In recent years, molecular ecological methods have been widely used to analyse the microbial community in various environments. The denaturing gradient gel electrophoresis (DGGE) of small subunit rRNA gene is one of these methods, which has the advantages that it permits rapid and simple monitoring of the spatial-temporal variability of microbial populations and is easy to obtain an overview of the dominant species. As for archaeal communities, DGGE analysis was first applied to analyse lake water samples (Ovreas *et al.* 1997) and then has been used in many studies on archaeal communities in various environments. For these studies, a number of primer pairs

 

have been developed and applied. Primer pair 344fGC/915r was used to analyse the methanogenic archaeal communities in the sediment of an acidic bog lake (Chan *et al.* 2002). Primer pair 519f/915rGC was used to analyse the archaeal communities in the anoxic water from Ace Lake (Coolen *et al.* 2004) and mud volcanoes of Black Sea (Stadnitskaia *et al.* 2005). Watanabe *et al.* (2004, 2006) reported that primer pairs 357fGC/691r and 1106fGC/1378r were suitable for analysing the community of methanogenic *Archaea* in paddy field soil.

Although different primers have been widely employed to describe methanogenic archaeal communities, information allowing comparison of quality and amplification range of the primers in ruminal archaeal community analysis is scare. Only a few studies have used two or more primer pairs in parallel or assessed the quantitative reliability of a primer set. Yu *et al.* (2008) showed that different methanogenic *Archaea* might be detected when different regions were targeted by polymerase chain reaction (PCR)-DGGE. Different regions of the bacterial 16S rRNA genes can produce different DGGE patterns (Yu and Morrison 2004). In the present study, nine reported specific primer pairs for 16S rRNA gene of *Archaea* were selected and evaluated by PCR amplification of bacteria and pooled rumen fluid samples from four local goats that is popular in eastern China. The selected primer pairs were further used for DGGE analysis of the archaeal community in the rumen.

## Materials and methods

### Animals and samples

Four fistulated local goats were individually accommodated in separate pens at Nanjing Agricultural University' animal house facility. The goats were on a 4 × 4 Latin square treatments with 15 day periods, fed one of four diets, which were 100% hay (I), 30% concentrate and 70% hay (II), 50% concentrate and 50% hay (III) and 70% concentrate and 30% hay (IV) respectively, and all had free access to water. Rumen fluids from the same treatment were collected, mixed and stored at −20°C for further analysis. The ruminal bacterial strain used as reference alongside with rumen methanogenic *Archaea* was *Escherichia coli* 131, which had been maintained in our laboratory.

### DNA extraction and PCR amplification

A portion of 1·5 ml of mixed rumen fluids from each treatment were subjected for DNA extraction according to a bead-beating method and followed by phenol-chloroform extraction (Zoetendal *et al.* 1998). DNA extracts (three replicates for each sample) were diluted 10 times in $ddH_2O$ prior to PCR reactions and 1 $\mu$l of the diluted DNA solutions (*c.* 20 ng) were used as templates. For bacteria, a portion of 1·5 ml of bacterial culture was collected and centrifuged at 9500***g*** and the pellets were suspended in 100 $\mu$l of TE for 15 min at 95°C. Clones and strains of ruminal methanogens obtained from goats by Pei (2008; GenBank accession nos: DQ985539, EF653448, EF584763, EU544027, EU544028, EF584758 and DQ985540, respectively) were selected as positive controls.

Two PCR protocols were used to amplify partial 16S rRNA gene sequences with primers shown in Table 1. For directed PCR, six reported primer pairs (344fGC/915r, 348fGC/691r, 357fGC/691r, 109f/515rGC, 519f/915rGC and 1106fGC/1378r) were used. For nested PCR, three reported primers (46f/1100r, 46f/1017r and 112f/1100r) were tested in the first round and the primer pair 344fGC/519r was used in the second round. All PCR reactions were done according to their corresponding references (Table 1). PCR amplification was performed in a T1 Thermal Cylcer (Biometra, Germany). Aliquots of 5 $\mu$L PCR products were analysed by electrophoresis on 1·2% agarose gel (w/v) to check the sizes and amounts of the amplicons.

### DGGE analysis

PCR amplicons were used for sequence-specific separation by DGGE according to Zoetendal *et al.* (1998) using a Dcode DGGE System (Bio-Rad, USA). For primer pair 519f/915rGC, DGGE was performed in 6% (w/v) polyacrylamide gels (acrylamide/*N,N*′-methylene bisacrylamide ratio, 37 : 1 [w/w]) in 0·5 × TAE buffer. The denaturant gradient range of the gel, in which 100% denaturant contained 7 mol $l^{-1}$ urea and 40% (v/v) formamide, was from 30% to 75%. For primer pair 1106fGC/1378r and 344fGC/915r, DGGE was performed in 8% (w/v) polyacrylamide gels and the denaturant gradient range of the gel was 32–62% and 20–45%, respectively. The electrophoresis was initiated by prerunning for 10 min at 200 V and subsequently ran at 85 V for 16 h at 60°C. The gel was stained with $AgNO_3$ according to Sanguinetti *et al.* (1994) and scanned using GS-800 scanner (Bio-Rad). The DGGE profile was analysed by Molecular Analyst 1·61 software (Bio-Rad).

### Cloning and sequencing

Interesting bands on DGGE gels were excised and used for sequence PCR according to Watanabe *et al.* (2004, 2006). Primers 519f, 1378r, 915r were used for sequencing the fragments obtained from primer pairs 519f/915rGC, 1106fGC/1378r, 344fGC/915r respectively. The sequences were determined in Invitrogen (Shanghai, China). Close

**Table 1** Primer pairs for *Archaea*-specific polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) targeting *rrs* genes

| Primer | Sequence (5′-3′) | Annealing temp (°C) | $MgCl_2$ (mmol $l^{-1}$) | Reference |
|---|---|---|---|---|
| 344fGC/915r | CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGC CCCCGCCCGACGGGGTGCAGCAGGCGCGA GTGCTCCCCCGCCAATTCCT | 71–61* | 1·5 | Chan *et al.* (2002) |
| 348fGC/691r | CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGTCCAGGCCCTACGGG GGATTACAAGATTTCAC | 49 | 2·0 | Watanabe *et al.* (2004) |
| 357fGC/691r | CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGCCCTACGGGGCGCAGCAG GGATTACAAGATTTCAC | 53 | 2·0 | Watanabe *et al.* (2004) |
| 109f/515rGC | ACTGCTCAGTAACAGTAACACGT CGCCCGGGGCGCGCCCCGGGCGGGGCGGG GGCACGGGCTGCTGGCAC | 52 | 2·0 | Roest *et al.* (2005) |
| 519f/915rGC | CAGCCGCCGCGGTAA CGCCCGCCGCGCCCCGCGCCCGGCCCGCCG CCCCCGCCCCGTGCTCCCCCGCCAATTCCT | 57 | 1·5 | Coolen *et al.* (2004) |
| 1106fGC/1378r | CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGTTAAGTCAGGCAACGAGC TGTGCAAGGAGCAGGGAC | 55 | 2·0 | Watanabe et al. (2006) |
| 46f/1100r | TTAAGCCATGCAAGT TGGGTCTCGCTCGTTGCC | 53·5 | 2·0 | Tang *et al.* (2005) |
| 46f/1017r | TTAAGCCATGCAAGT GGCCATGCACCTCCTCTC | 40 | 1·5 | Yoshida *et al.* (2005) |
| 112f/1100r | GCTCAGTAACACGTGG TGGGTCTCGCTCGTTGCC | 51 | 1·5 | Hoj *et al.* (2005) |
| 344fGC/519r | CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG CCCCCGCCCGACGGGGTGCAGCAGGCGCGA TTACCGCGGCGGCTG | 53·5 | 1·5 | Tang *et al.* (2005) |

Primers for directed PCR were: 344fGC/915r, 348fGC/691r, 357fGC/691r, 109f/515rGC, 519f/915rGC and 1106fGC/1378r. For the first round of nested PCR, the primers were: 46f/1100r, 46f/1017r and 112f/1100r. While for the second round of nested PCR, the only primer was 344fGC/519r. PCR mixture (50 $\mu$l) contained 2·5 U of Taq polymerase, 5 $\mu$l of Taq buffer, corresponding $Mg^{2+}$ (Shenneng, China) and final concentration of 0·2 $\mu$mol $l^{-1}$ of forward and reverse primers and 0·2 mmol $l^{-1}$ of dNTP.

*The annealing temperature in 20 cycles was reduced 0·5°C for each cycle from 71 to 61°C and then followed by 15 additional cycles at an annealing temperature of 61°C.

relatives and phylogenetic affiliations of the obtained sequences were determined by using the BLAST search programme at the NCBI website. A phylogenetic tree was constructed by 1000-fold bootstrap analysis using MEGA software. All sequences determined in the present study have been deposited in GenBank under accession numbers EU520263–EU520301, EU544031.

## Results

### PCR amplification of DNA from rumen fluids and bacteria

As shown in Fig. 1, the PCR products from primer pairs 109f/515rGC, 357fGC/691r were faint for the rumen fluids, which implied that it was difficult for these primers to amplify rumen samples. The primer pair 348fGC/691r generated three length-different PCR products when amplifying the rumen fluid sample I. This indicated that this primer pair could bind to more than one position of the total genomic DNA. Primer pair 1106fGC/1378r could amplify all the rumen samples as well as rumen bacteria. Primer pairs 344fGC/915r and 519f/915rGC generated clear PCR products from rumen *Archaea* samples, but did not amplify bacterial DNA.

For nested PCR, all three primers 46f/1100r, 46f/1017r and 112f/1100r nonspecifically amplified rumen content samples, which showed several DNA fragments with different lengths on agarose gel (data not shown). All three primer pairs also amplified bacterial DNA. Primer

 

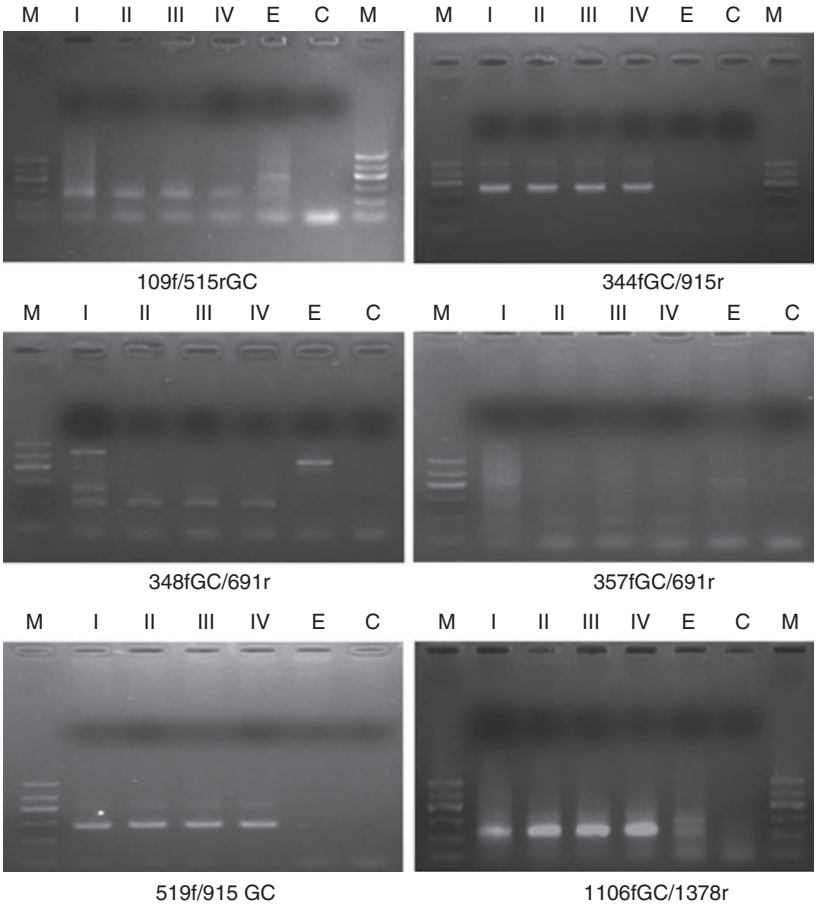


**Figure 1** Polymerase chain reaction (PCR) products amplified with directed PCR primer pairs: 109f/515rGC, 344fGC/915r, 348fGC/691r, 357fGC/691r, 519f/915rGC and 1106fGC/1378r. M, DNA ladder. The bands were 100, 300, 500, 700, 900 and 1200 bp, respectively; I, rumen fluids of local goats fed with 100% hay; II, rumen fluids of local goats fed with 70% hay and 30% concentrate; III, rumen fluids of local goats fed with 50% hay and 50% concentrate; IV, rumen fluids of local goats fed with 30% hay and 70% concentrate; E, *Escherichia coli* 131; C, negative control (PCR products without DNA template).

pair 344fGC/519r was used for the second round of nested PCR and produced faint bands on the agarose gel.

To further determine the amplification efficiency of the primers, methanogen clones and strains from the rumen of goat were used as templates and results showed that primer pairs 519f/915rGC, 344fGC/915r and 1106fGC/1378r could amplify all clones and strains (data not shown).

### Analysis of ruminal *Archaea* by DGGE

PCR fragments generated with primer pairs 519f/915rGC, 344fGC/915r and 1106fGC/1378r were analysed by DGGE (Fig. 2). DGGE fingerprints from three replicates of each sample were much similar (data not shown) and thus only one set was presented. DGGE band patterns with primer pairs 519f/915rGC and 1106fGC/1378r showed good resolution and separation. Only one clear band was obtained from PCR products with primer pair 344fGC/915r although a range of acrylamide concentrations and denaturant gradients were tested (data not shown).

Significant shift in archaeal community structure during the change of diet was observed. The intensity of some bands increased with increase in the amount of the concentrate (band 6); while some decreased (band 14). Also, some of the detectable bands in sample I (100% hay) became undetectable when the percentage of hay was decreased to 30% (band 15).

A total of 40 fragments were obtained from DGGE gels. All fragments belonged to methanogenic *Archaea*. Seventeen of the 33 fragments obtained with primer 519f/915rGC was 98% affiliated to *Methanobacterium* sp. GH (band 9), 100% similar to *Methanobrevibacter* sp. Z8 (band 12), 100% similar to *Methanobrevibacter* sp. 1Y (bands 18, 20, 22, 23, 25, 26, 27, 33, 34 and 35), 99% similar to *Methanobrevibacter* sp. 1Y (bands 19 and 21), 100% affiliated to *Methanobrevibacter* sp. ZA-10 (band 24), 99% affiliated to *Methanobrevibacter* sp. 110 (band 28) and 99% affiliated to *Methanobrevibacter smithii* ATCC 35061 (band 32). The remaining 16 fragments were separated in an unidentified cluster and the similarity among them was between 94·4% (bands 17 and 30) and 100% (bands 6 and 8). With primer 1106fGC/1378r, bands A, B, D and G had their sequences 98% affiliated to *Methanobrevibacter* sp. 110, band F 98% affiliated to *Methanobrevibacter ruminantium* and band H 100%

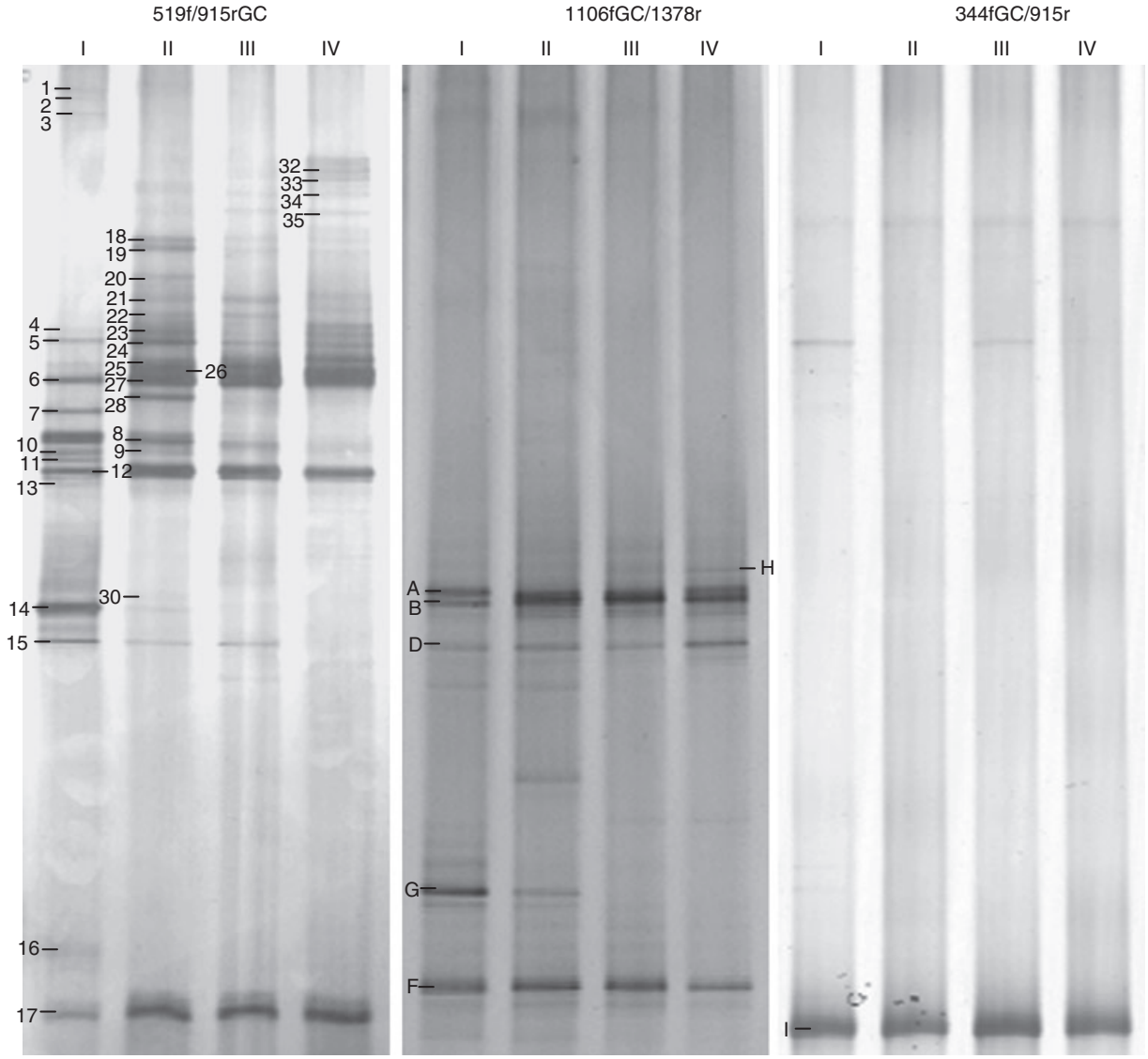


**Figure 2** Denaturing gradient gel electrophoresis band patterns obtained from rumen fluid samples of local goats in eastern China with primers 519f/915rGC, 1106fGC/1378r and 344fGC/915r. I, rumen fluids of local goats fed with 100% hay; II, rumen fluids of local goats fed with 70% hay and 30% concentrate; III, rumen fluids of local goats fed with 50% hay and 50% concentrate; IV, rumen fluids of local goats fed with 30% hay and 70% concentrate. The numbers and letters indicated show the bands whose sequences were determined and correspond to the ones on the phylogenetic tree.

similar to *Methanobrevibacter* sp. SM9. With primer 344fGC/915r, band I had its sequence 98% similar to *Methanosphaera stadtmanae*.

### Phylogenetic analysis

Phylogenetic analysis of the 33 fragments obtained with primer 519f/915rGC was shown in Fig. 3. All the sequences belonged to two clusters. Sixteen of the sequences belonged to an unidentified cluster. The remaining 17 sequences belonged to Methanobacteriales.

## Discussion

Many studies on molecular diversity of ruminal methanogens have been investigated by the cloning of 16S rRNA gene fragments. By this approach, Wright *et al.* (2006) found that of the total 78 rumen methanogen clones from the sheep in Queensland, Australia, 15 clones were similar to Methanobacteriales and Methanomicrobiales, and the remaining 63 were slightly similar to *Thermoplasma acidophilum* and *Thermoplasma volcanium*. Shin *et al.* (2004) showed that most of the

 

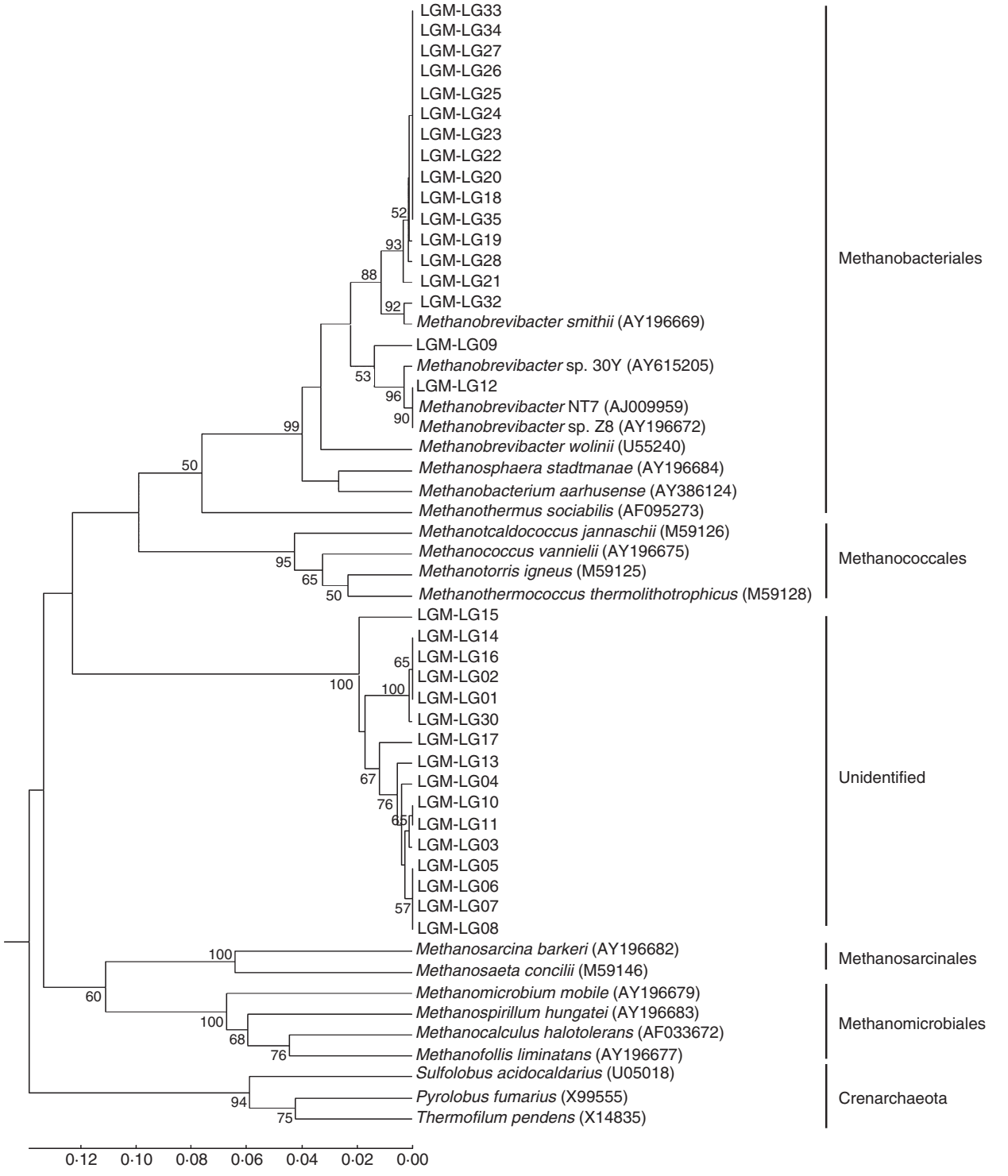


**Figure 3** Phylogenetic analysis of 16S rDNA sequences retrieved by denaturing gradient gel electrophoresis from rumen fluids of local goats with primer pair 519f/915rGC. The sequences obtained in the present study and their close relatives are indicated in bold. The root was determined by using Crenarchaeota 16S rRNA gene sequences as out-group. The topology of the tree was estimated by bootstraps based on 1000 replications. Numbers at the nodes are percentages supported by bootstrap evaluation.


 

clones from the rumen fluid, solid and epithelium were affiliated with Methanomicrobiaceae and Methanobacteriaceae while some belonged to unidentified methanogens.

Although clone library approach has been proved powerful in the identification of ruminal methanogens, it can be time-consuming, particularly for diversity analysis of many spatial or temporal samples. DGGE can offer simultaneous analysis of spatial or temporal samples, which could reflect an overall structure of the microbial community change over time in the environment. DGGE method has been widely used to analyse archaeal diversity in many environments and a number of primer pairs have been developed and applied. The present study investigated the applicability of the available PCR-DGGE primers in ruminal *Archaea* analysis. The results surprisingly demonstrated that some of the reported specific primer pairs for 16S rRNA gene of *Archaea* in other environments simply also amplified the bacterial DNA. Only primer sets 344fGC/915r, 1106fGC/1378r and 519f/915rGC could specifically amplify 16S rRNA gene of *Archaea*. However, only primer set 519f/915rGC could provide good separation on DGGE gel and reveal a wide diversity of methanogenic *Archaea*, suggesting its suitability for rumen methanogen diversity analysis.

Sequence analysis of the specific DGGE bands suggested that the predominated methanogenic *Archaea* in the rumen of goat belonged to *Methanobrevibacter* sp. and a cluster of unidentified methanogens. The 16 sequences of unidentified methanogens clustered within a strongly supported (100%) phylogenetic group. This unique group includes some complete 16S rRNA gene sequences from ruminants (Wright *et al.* 2006) and other environments (Godon *et al.* 1997), and some other partial 16S rRNA gene sequences (Irbis and Ushida 2004). This unique group may represent a new order of methanogens (Wright *et al.* 2004).

The specific bands on DGGE were manually excised and sequenced. This approach could directly identify the sequence of the specific bands. However, lengths of sequences generated from the DGGE gels are usually short (200–500 bp), and thus the sequence information does not always allow reliable phylogenetic analyses. Furthermore, co-migration of several different 16S rRNA gene sequences, which have the same melting behaviour, leads to overlapping DGGE bands that cannot be sequenced directly (Rolleke *et al.* 1999). Fragment length may also affect the DGGE performance. In our study, even after several attempts, we failed to get a good DGGE profile from PCR products of 600 bp amplicon with primer pair 344fGC/915r. Although some literature reported the DGGE profiles obtained from 600 bp PCR products (Chan *et al.* 2002), other reports demonstrated that PCR products of less than 500 bp would give a better DGGE performance (Ercolini 2004).

The rumen microbial complement is affected by dietary changes. This study showed that many methanogens seemed to have become nonpredominant in the rumen of animal fed low-fibre diets as revealed by the disappearance of some DGGE bands. Methanogens are the only recognized ruminal microbes belonging to the *Archaea* and are an integral part of the rumen microbial ecosystem (Joblin 2005). By scavenging hydrogen gas, methanogens play a key ecological role in keeping the partial pressure of hydrogen low so that fermentation can proceed efficiently in the rumen (Wolin *et al.* 1997). When the ruminants were fed with high-fibre diets, the cellulolytic microbes (anaerobic fungi, cellulolytic bacteria and ciliates) produced a large amount of hydrogen, which needs high activity of methanogens to reduce the gas pressure in the rumen.

In conclusion, nine sets of PCR primers used for other environment analysis were compared with rumen samples and primer pair 519f/915rGC was found to be more suitable for PCR-DGGE analysis of ruminal methanogens.

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