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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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

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.

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).

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

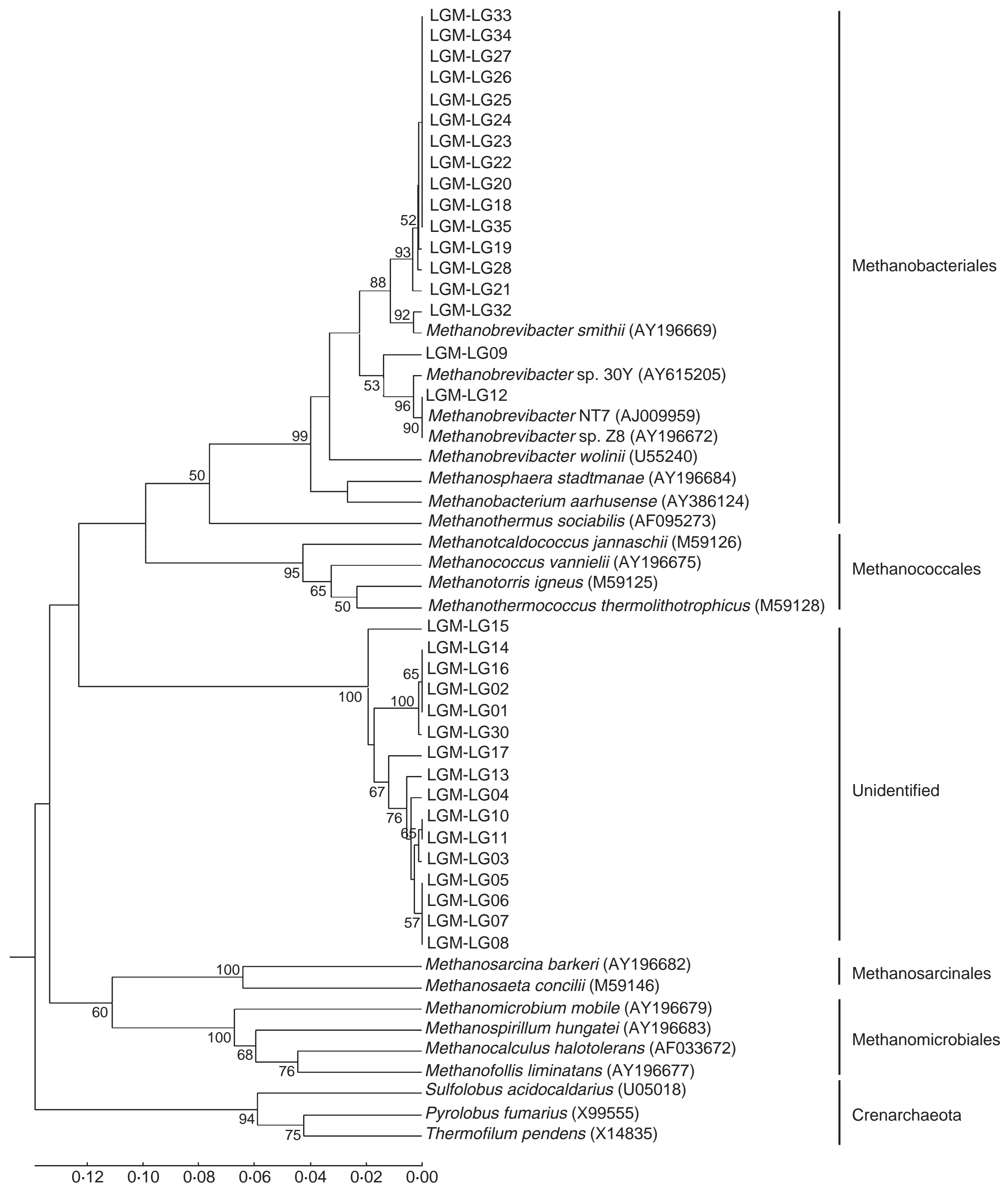

Figure 3 Phylogenetic analysis of 16S rDNA sequences retrieved by denaturing gradient gel electrophoresis from rumen fluids of local goats with primer pair 519f/915rGC. The sequences obtained in the present study and their close relatives are indicated in bold. The root was determined by using Crenarchaeota 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.

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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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