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ORIGINAL ARTICLE

Ribosomal ITS1 sequence-based diversity analysis of anaerobic rumen fungi in cattle fed on high fiber diet

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Abstract Fiber degradation in the ruminant digestive process is a major activity accomplished by rumen microbes, a process in which the role of fungi is important. Therefore, the present study was conducted to establish the community structure of anaerobic rumen fungi in cattle fed on a high fiber diet using molecular approaches. Total community DNA was extracted, and the ribosomal internal transcribed spacer (ITS) 1 region was amplified, cloned, and sequenced. The resulting nucleotide sequences were used to construct a phylogenetic tree. A total of 52 clones were analyzed, revealing 31 different ITS1 gene phylotypes. Of these, 12 belonged to the genus *Orpinomyces* (48 % of clones), followed by uncultured *Neocallimastigale* clones (29 %), *Cyllamyces* spp. (9 %) and *Anaeromyces* spp. (8 %). Our results indicate that genus *Orpinomyces* dominates the rumen fungal community in Indian crossbred Karan Fries cattle.

Keywords Anaerobic rumen fungi · Fungal diversity · ITS1 · Cattle · *Orpinomyces*

Introduction

The huge population of 529.7 million livestock in India is mostly offered poor quality fibrous grasses, bulky crop-residues and agro-industrial by-products (Chhabra et al. 2009; MOA 2010). Ruminant animals harbor diverse microbial populations in the rumen where they carry out fermentation of the feed matter and fulfill the major nutritional needs of the host. Among different ruminal microbes, anaerobic fungi are known to be the most potent producers of fibrolytic, lignolytic, and other hydrolytic enzymes (Lee et al. 2000; Eckart et al. 2010; Dagar et al. 2011; Sirohi et al. 2012). In addition, through extensive mycelia penetration and expansion, anaerobic fungi facilitate other microbes to access more sites of otherwise refractory lignocellulosic feed stuffs (Grenet et al. 1989). Anaerobic fungi are obligate anaerobes and constitute nearly 20 % of the total ruminal microbial biomass (Sirohi et al. 2012). These are categorized under the order *Neocallimastigales*, class Neocallimastigomycetes in the phylum Neocallimastigomycota (Hibbet et al. 2007). A number of studies have suggested a positive relationship between the presence of fungi in the rumen and voluntary feed intake (Gordon and Phillips 1998; Edwards et al. 2008). Therefore, their role in ruminant nutrition is crucial and needs further investigation. Since the first description of anaerobic fungi *Neocallimastix frontalis* by Orpin (1975), only 6 genera and 20 species have been characterized up to now. Their isolation and maintenance are cumbersome and difficult due to their strict anaerobic and unculturable nature. Therefore, more novel species of rumen fungi are expected to exist in this ecosystem.

Culture-based approaches and traditional taxonomic classification systems rely on features like sugar utilization pattern, thallus morphology and the number of flagella per zoospores, which are highly pleomorphic and vary with cultural conditions (Joblin 1981; Munn 1994; Barr et al. 1995; Brookman et al. 2000; Griffith et al. 2009). Liggenstoffer et al. (2010) have shown the presence of eight novel anaerobic fungal lineages, indicating the presence of vast uncultured genera. In the last few decades, molecular approaches have gained greater impetus in studying the

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diversity and ecology of anaerobic fungi from different habitats as these allow the study of their abundance and species richness more accurately and reliably than culture-dependent methods. The internal transcribed spacer (ITS) region of nuclear DNA becomes an appealing target for analyzing fungi diversity in the rumen due to its large copy number (up to 250 per cell; Bellemain et al. 2010; Schoch et al. 2012; Kittelmann et al. 2012). The ITS region is comprised of ITS1 and ITS2 separated by the 5.8S gene, and is situated between the 18S (SSU) and 28S (LSU) genes in the nuclear DNA.

In order to design newer strategies towards enhanced fiber digestibility for increased animal productivity, it is necessary to understand the abundance and diversity of resident rumen fungi in ruminants with different diet conditions as well as during diet shift. This study was undertaken to study the structure of anaerobic fungal communities present in the rumen of Indian crossbred Karan Fries cattle fed on a high fiber diet. To the best of our knowledge, no report exists on the community structure of rumen fungi in crossbred Karan Fries cattle.

Materials and methods

Experimental animals and sample

Four rumen fistulated adult crossbred Karan Fries cattle (~3 years old), weighing 450±20 kg kand ept in the experimental shed of the Dairy Cattle Nutrition Division, National Dairy Research Institute, Karnal, India, were employed for this study. Animals were fed individually on a standard diet (concentrate/roughage ratio=40:60) with free access to water. The roughage part mainly consisted of wheat straw. The concentrate mixture was composed of 50 % maize, 30 % groundnut cake, 17 % wheat bran, 2 % mineral mixture and 1 % common salt. In vivo experiments were conducted with the approval of and following the guidelines of the Institutional Animal Ethics Committee of NDRI, Karnal (Haryana). A representative uniform rumen liquor sample was collected from each animal through a stomach tube attached to a vacuum pump, just after feeding. The samples were thoroughly mixed using a homogenizer and immediately processed for DNA extraction.

Extraction of community genomic DNA

Community genomic DNA was extracted as described previously (Brookman and Nicholson 2005), with little modification. Briefly, 500 mg of rumen content was finely ground in a pestle and mortar and 100 mg was transferred to a 1.5-ml microcentrifuge tube. Following the addition of 0.8 ml CTAB buffer (100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, 20 mM sodium EDTA, 2 % CTAB), the sample was incubated at 70 °C for 1 h with intermittent mixing. Chloroform (0.5 ml) was added and samples were centrifuged at 12,000 rpm for 20 min. The upper aqueous layer was precipitated with an equal volume of isopropanol and washed with 70 % chilled ethanol. The extracted DNA from all the animals was pooled on the basis of their concentration before PCR amplification.

PCR amplification of ITS1 region

ITS1 region was amplified by PCR using MN100 (forward) and MNGM2 (reverse) primers as reported earlier (Brookman et al. 2000; Edwards et al. 2008; Nicholson et al. 2010). PCR was carried out in a reaction mixture containing: 50 ng template DNA; 10× assay buffer, $MgCl_2$, 20 pmol of each primer; 100 μM dNTP mix; and *Taq* DNA polymerase (Fermentas, USA) using a Bio-Rad thermal cycler. PCR conditions includes initial denaturation at 95 °C for 5 min followed by 20 cycles of denaturation at 95 °C for 30 s, annealing at 68 °C for 30 s, extension at 72 °C for 30 s, and 15 cycles with denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. PCR products were analyzed by gel electrophoresis to confirm the amplification of the ITS1 region.

Clone library construction

PCR products were purified using a QIAquick gel purification kit according to the manufacturer's instructions (Qiagen, Germany) and subjected to cloning using a STRATA-Blunt end cloning kit (Stratagene, USA) as per the manufacturer's instructions. Positive clones were screened on the basis of blue/white screening. Plasmid DNA was extracted from the white clones and checked for the presence of inserts by PCR amplification. A total of 52 positive clones were selected and sent for sequencing at Xcelris Labs, Ahmedabad, India.

BLAST analysis

Sequences obtained after sequencing were aligned using ClustalW program in BioEdit ((Thompson et al. 1994; Hall 1999). The resulting sequences were compared with the Basic Local Alignment Search Tool (BLAST) in order to find out the sequence homology with the anaerobic rumen fungal sequences available in the GenBank database. Sequences obtained in this study were submitted to the GenBank database under the following

accession number JN227886–JN227892, JN205758–JN205789, and JN227894.

Phylogenetic analysis

Six reference sequences of ITS1 gene representing rumen fungal genera were included in the phylogenetic analyses. These included *Caecomyces communis* (AF492020), *Piromyces* sp. AF-CTS-CAP3 (GQ857641), *Orpinomyces* sp. AF-CTS-BTO1 (FJ501296), *Cyllamyces aberensis* isolate EO17 (FJ483845), *Neocallimastix frontalis* strain SR4 (AY429664), and *Anaeromyces* sp. AF-CTS-OAA2 (FJ501287). The evolutionary history of ITS1 gene sequences was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 58 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 147 positions in the final dataset. Evolutionary analyses were performed in MEGA5 (Tamura et al. 2011).

Results

The community structure of anaerobic fungi in the rumen of crossbred Karan Fries cattle fed on a high fiber diet was deciphered using ITS1 gene sequence-based phylogenetic analysis. PCR amplification of ITS1 region from community genomic DNA resulted in product size of 205–263 bp (Fig. 1). The phylogenetic tree constructed from the clone sequences showed 30 phylotypes (Table 1) arranged into five distinct groups (Fig. 2). Group I sequences were found to be closely related to *Cyllamyces aberensis* and represented by four phylotypes (NFG39, NFG40, NFG42, and NFG43). Group II did not match with any defined genus of anaerobic rumen fungi and was found to match with uncultured clones of order *Neocallimastigales*. This group comprised of one-third of the total phylotypes obtained in this study. Group III contained 10 phylotypes (NGF4, NGF5, NGF8, NGF17, NGF18, NGF24, NGF27, NGF32, NGF38, and NGF45) which clustered with *Orpinomyces* spp. with 97–100 % sequence similarity. Groups IV and V showed close resemblance to *Anaeromyces* spp. (phylotypes NFG11 and NFG15) and *Piromyces* spp. (phylotypes NFG19, NFG46, NFG49), respectively. Our results revealed the existence of novel fungal sequences which are not available in the GenBank database. These sequences were clustered with uncultured members of order *Neocallimastigales*. The rumen fungi community was composed of 48 % of clones related to genus *Orpinomyces* (25 of 52; Fig. 3) followed by uncultured *Neocallimastigales* clones (29 %), *Cyllamyces* spp. (9 %), *Piromyces* spp. (8 %), and *Anaeromyces* spp. (6 %). However, no defined clones of genus *Neocallimastix* and *Caecomyces* were obtained from the rumen of crossbred Karan Fries cattle.

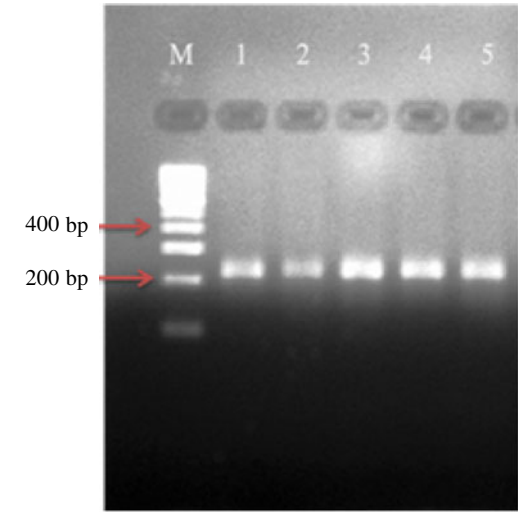

Fig. 1 PCR amplification of internal transcribed spacers (ITS1) region of anaerobic rumen fungi from Indian crossbred Karan Fries cattle. *Lanes 1–5* 210- to 250-bp products; *M* 100-bp DNA ladder

Discussion

In the present study, we determined the community composition of anaerobic rumen fungi in the widely domesticated crossbred Karan Fries cattle from the northern region of India. In this part of the country, farmers and dairymen rely on low-cost, poor quality roughage to meet the feeding requirements of their dairy animals. The role of rumen fungi in the digestion of these feed stuffs is considered crucial due to the high lignocellulose content in these diets.

Our results are in agreement with Sridhar et al. (2010), who found *Orpinomyces* spp. as the most dominant genus in Indian cattle on the basis of culture-based approaches. Similarly, *Orpinomyces* sp. F11 was found to exist in greater biomass in Brahman crossbred steers (Denman and McSweeney 2006). However, our findings are in contrast to (Liggenstoffer et al. 2010), who reported on the abundance of *Caecomyces* spp. and *Piromyces* spp., followed by *Neocallimastix* spp. and *Anaeromyces* spp. using culture-independent approaches. They concluded that *Orpinomyces* spp. were the least abundant while *Cyllamyces* spp. could not be detected. However, *Cyllamyces* was found to be the most

Table 1 Phylotypes of ITS1 gene sequences of anaerobic rumen fungi retrieved from the rumen samples of Indian crossbred Karan Fries cattle

OTU or phylotype	Number of clones	Size (bp)	GenBank accession no.	Nearest valid taxon	% sequence similarity
NFG1	1	208	JN205759	Uncultured *Neocallimastigales* clone	98
NFG4	2	223	JN205761	*Orpinomyces* sp.	98
NFG5	1	221	JN205762	*Orpinomyces* sp.	99
NFG8	1	225	JN205765	*Orpinomyces* sp.	98
NFG11	1	260	JN227886	*Anaeromyces* sp.	98
NFG12	1	263	JN205768	Uncultured *Neocallimastigales* clone	97
NFG15	2	258	JN205771	*Anaeromyces* sp.	100
NFG16	1	243	JN205772	Uncultured *Neocallimastigales* clone	97
NFG17	4	221	JN227887	*Orpinomyces* sp.	100
NFG18	1	224	JN205773	*Orpinomyces* sp.	99
NFG19	1	237	JN227888	*Piromyces* sp.	100
NFG20	5	242	JN205774	Uncultured *Neocallimastigales* clone	90
NFG22	1	207	JN205776	Uncultured *Neocallimastigales* clone	98
NFG23	1	242	JN205777	Uncultured *Neocallimastigales* clone	89
NFG24	1	225	JN205778	*Orpinomyces* sp.	98
NFG26	1	261	JN205780	Uncultured *Neocallimastigales* clone	99
NFG27	1	221	JN205781	*Orpinomyces* sp.	97
NFG31	2	208	JN205784	Uncultured *Neocallimastigales* clone	99
NFG32	1	222	JN205785	*Orpinomyces* sp.	98
NFG33	1	205	JN205786	Uncultured *Neocallimastigales* clone	99
NFG35	1	207	JN205788	Uncultured *Neocallimastigales* clone	96
NFG38	5	226	JN205790	*Orpinomyces* sp.	100
NFG39	1	210	JN205791	*Cyllamyces aberensis*	99
NFG40	1	208	JN205792	*Cyllamyces aberensis*	99
NFG42	1	208	JN227892	*Cyllamyces aberensis*	100
NFG43	2	208	JN227893	*Cyllamyces aberensis*	100
NFG45	1	226	JN227895	*Orpinomyces* sp.	100
NFG46	2	237	JN227896	*Piromyces* sp.	100
NFG49	1	240	JN227899	*Piromyces* sp.	99
NFG50	7	221	JN227900	*Orpinomyces* sp.	100

dominant fungal species in cow manure comprising 67 % of total sequences followed by *Piromyces* (24 %), *Anaeromyces* (7 %), *Neocallimastix* (2 %), and no sequences of *Orpinomyces* (Fliegerova et al. 2010). Due to the lack of a good reference strain for *Caecomyces*, the sequences related to it could not be conclusively distinguished from *Cyllamyces* and hence were grouped together. The phylogenetic categorization of the members of *Caecomyces* is considered a complicated task as no live culture nor ITS1 sequence information is available for *C. equi* (Kittelmann et al. 2012). These authors considered sequences related to *Caecomyces communis* CY50 as the true *Caecomyces* cluster, whereas sequences related to *Caecomyces sympodialis* W101 were grouped with *Cyllamyces* spp.

Recently, Kittelmann et al. (2012) found *Piromyces* and *Neocallimastix* spp. as the most abundant anaerobic fungi in 53 rumen samples collected from farmed sheep, cows and deer. However, *Anaeromyces*- and *Cyllamyces*-related sequences were not obtained in their study. Anaerobic fungal communities occupy distinct environmental niches influenced by diet and/or by the host animal. These results indicate the importance of diet and host in deciding the structure of ruminal fungal communities. Lee et al. (2000) found *Orpinomyces* strain KNGF-2 as the predominant fungus in Korean black goats. Paul et al. (2004) reported *Piromyces* sp. FNG5 as the best hydrolytic enzyme secretor in the wild blue bull (*Boselaphus tragocamelus*) from India. The presence of *Piromyces* in Karan Fries is supported by Sakurada et al. (1995) who isolated *Piromyces communis* from goats. *Anaeromyces* sp. was not found to colonize the sago waste, palm press fiber and rice straw in the rumen of goats (Ho and Barr 1995), whereas it readily colonize rice straw, palm press fiber and grass fragments in the rumen of cattle and buffaloes

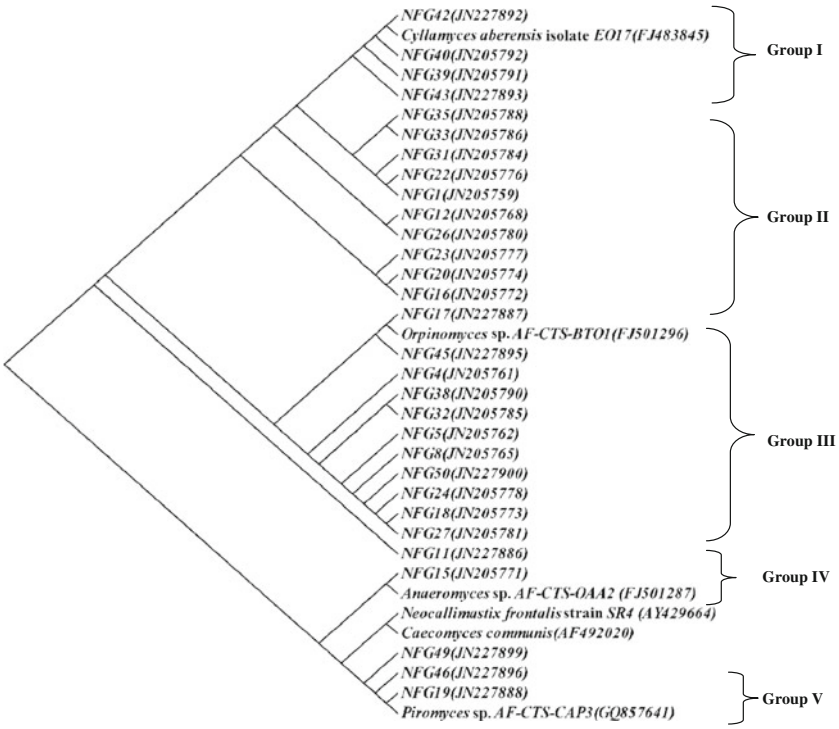

Fig. 2 Phylogenetic analysis of aligned ITS1 sequences of anaerobic rumen fungi from Indian crossbred Karan Fries cattle. The evolutionary history was inferred using the neighbor-joining method. The evolutionary distances were computed using the maximum composite likelihood method and the analyses were conducted in MEGA5

(Ho et al. 1991; Abdullah et al. 1992). Kittelmann et al. (2012) observed four novel anaerobic fungi sequence clusters that do not group with previously named genera. In addition, it was found that the diversity of rumen fungi in the rumens of domesticated ruminants is greater than previously presumed.

Although many studies have reported the presence of *Neocallimastix* (Phillips and Gordon 1988; Webb and Theodorou 1991; Ozkose et al. 2001) and *Caecomyces* (Nagpal et al. 2010) in different species of ruminants, we did not find any strains belonging to these genera in Karan Fries cattle by ITS1 gene sequencing. More interestingly, we were not able to confirm their presence in Karan Fries cattle even by culture-based and microscopical methods. A few researchers have evaluated the role of anaerobic fungi in enhancing fiber digestion in dairy animals through supplementation with live cultures of various fungal species. However, these studies met with mixed outcomes. Anaerobic fungi are believed to actively modulate the composition of bacteria, archaea, and ciliate protozoa in the rumen by virtue of their superior fiber penetration and degradation capabilities as well as the production of H_2. Rumen fungi are therefore implicated in CH_4 emissions by the host.

The present study concludes that *Orpinomyces* is the dominant anaerobic fungus present in the rumen of crossbred Karan Fries cattle fed on high fiber-based diet. These findings will certainly facilitate future studies on the relationship between the rumen fungal community structure and functioning in the rumen ecosystem. Deeper insights into the diversity of rumen fungi will offer new dietary manipulation options for enhancing the nutritive values of poor-quality fibrous feed materials and designing methane mitigation strategies.

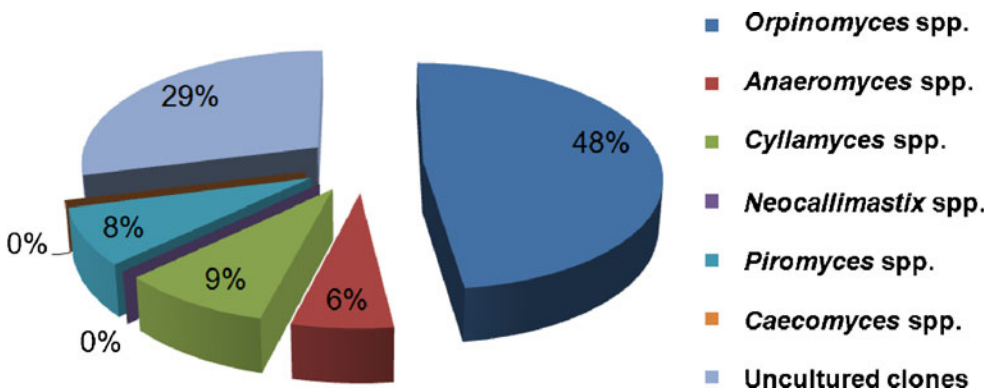

Fig. 3 Community composition and relative abundance of anaerobic rumen fungi in Indian crossbred Karan Fries cattle

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