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Genetic Diversity of Gastrointestinal tract Fungi in Buffalo by Molecular methods on the basis of Polymerase Chain Reaction

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ABSTRACT: Buffalo are able to utilize feed more efficiently than beef cattle where the feed supply is of low quantity and or quality. Therefore, the present study was conducted to establish the community structure of anaerobic rumen fungi in buffalo using molecular approaches. Polymerase chain reaction approach was used in this study to determine the population of major anaerobic rumen fungi in buffalo in digesta and rumen fluid. 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 12 clones were analyzed. Sequence analysis of ITS1 spacer seems a promising tool for comparing a variety of rumen fungal isolates.

Key words: Anaerobic rumen fungi, buffalo, ITS1, rRNA, PCR

INTRODUCTION

The anaerobic gut fungi are the only known obligately anaerobic fungi. For the majority of their life cycles, they are found tightly associated with solid digesta in the rumen and or hindgut. They produce potent fibrolytic enzymes and grow invasively on and into the plant material they are digesting making them important contributors to fibre digestion. This close association with intestinal digesta has made it difficult to accurately determine the amount of fungal biomass present in the rumen, Orpin (1984) suggesting fungal 8% contribution to the total microbial biomass. It is clear that the rumen microbial complement is affected by dietary changes, and that the fungi are more important in digestion in the rumens of animals fed with high-fibre diets (Bauchop, 1979 and Lee *et al.*, 2000). It seems likely that the gut fungi play an important role within the rumen as primary colonizers of plant fibre (Akin *et al.*, 1983).

Present knowledge of anaerobic gut fungal population diversity within the gastrointestinal tract is based upon isolation, cultivation and observations in vivo (Davies *et al.*, 1993). It is likely that there are many species yet to be described, some of which may be nonculturable. The development of molecular techniques has greatly broadened our view of microbial diversity and enabled a more complete detection and description of microbial communities (Bauchop, 1979 and Ranjard *et al.*, 2001). The development of molecular biological techniques in the last decade has enabled the new approach to the characterization of fungi. Li and Heath (1992) studied relationship of gut fungi based on ITS1 sequences and found that Anaeromyces isolates were more distant from other rumen fungi. The whole DNA sequences showed above 80% similarity among Piromyces, Neocallimastix and Orpinomyces, whereas the similarities between Anaeromyces and these three genera were only 70% (Fliegerova *et al.*, 2002). Molecular analyses have therefore been used in attempts to clarify the classification of anaerobic fungi. Ribosomal sequences of internal transcribed spacer regions ITS1 and ITS2 (Brookman *et al.*, 2000; Fliegerova *et al.*, 2004) have been applied successfully for discrimination between Orpinomyces and Anaeromyces. Sequencing and subsequent comparison of acquired results with gene-bank data is time consuming. In this research, we try to determine the genetic diversity of the gastrointestinal tract anaerobic fungi in Azarbayejan Iranian buffalos.

MATERIAL AND METHODS

This research was done in the Department of Animal Science, Shabestar Branch Islamic Azad University in Iran. For the sampling of buffalo rumen, the necessary coordination was carried out by the industrial slaughterhouse of Uromia. Buffalo were slaughtered and samples of rumen contents were taken.

Samples of rumen content were collected randomly from rumen in the slaughter house. Finally, after 24-48 hours, the samples were transferred to the laboratory for DNA extractions.

Total genomic DNA was extracted by using RBB+C method that described at follow (Yu and Morrison 2004).

A. Cell lysis:

(a) Transfer 0.25 g of sample into a fresh 2-mL screw-cap tube. Add 1 mL of lysis buffer [500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate (SDS)] and 0.4 g of sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm).

(b) Homogenize for 3 min at maximum speed on a Mini-Beadbeater™ (BioSpec Products, Bartlesville, OK, USA).

(c) Incubate at 70°C for 15 min, with gentle shaking by hand every 5 min.

(d) Centrifuge at 4°C for 5 min at 16,000× g. Transfer the supernatant to a fresh 2-mL Eppendorf® tube.

(e) Add 300 µL of fresh lysis buffer to the lysis tube and repeat steps 2–4, and then pool the supernatant.

B. Precipitation of nucleic acids:

(a) Add 260 µL of 10 M ammonium acetate to each lysate tube, mix well, and incubate on ice for 5 min.

(b) Centrifuge at 4°C for 10 min at 16,000× g.

(c) Transfer the supernatant to two 1.5-mL Eppendorf tubes, add one volume of isopropanol and mix well, and incubate on ice for 30 min.

(d) Centrifuge at 4°C for 15 min at 16,000× g, remove the supernatant using aspiration, wash the nucleic acids pellet with 70% ethanol, and dry the pellet under vacuum for 3 min.

(e) Dissolve the nucleic acid pellet in 100 µL of TE (Tris-EDTA) buffer and pool the two aliquots.

C. Removal of RNA, protein, and purification:

(a) Add 2 µL of DNase-free RNase (10 mg/mL) and incubate at 37°C for 15 min.

(a) Add 15 µL of proteinase K and 200 µL of Buffer AL (from the QIAamp DNA Stool Mini Kit), mix well, and incubate at 70°C for 10 min.

(a) Add 200 µL of ethanol and mix well. Transfer to a QIAamp column and centrifuge at 16,000× g for 1 min.

(b) Discard the flow through, add 500 µL of Buffer AW1 (Qiagen), and centrifuge for 1 min at room temperature.

(c) Discard the flow through, add 500 µL of Buffer AW2 (Qiagen), and centrifuge for 1 min at room temperature.

(d) Dry the column by centrifugation at room temperature for 1 min.

(e) Add 200 µL of Buffer AE (Qiagen) and incubate at room temperature for 2 min.

(f) Centrifuge at room temperature for 1 min to elute the DNA.

(g) Aliquot the DNA solution into four tubes. Run 2 µL on a 0.8% gel to check the DNA quality.

(h) Store the DNA solutions at -20°C.

The quality of the community DNA was assessed by 1% agarose gel electrophoresis. The ribosomal ITS1 region defined by primers Good92F GM1 (5′-TGTACACACCGCCCGTC-3′) and GM2 (5′-CTGCGTTCTTCATCGAT-3′) as described by Li and Heath (1992). The PCR reaction was performed in 100 µl reactions containing (final concentration): forward and reverse primers, 0.2 µM; dNTPs mixture, 200 µM; $MgCl_2$, 1.5 mM; KCl, 50 mM; Tris/HCl pH 8.4, 10 mM; and Taq polymerase, 0.25 Units. Approximately 50 ng genomic DNA were used as template for each amplification. The temperature conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 48 °C for 1 min and extension at 72°C for 1.5 min. Final step was carried out at 72°C for 10 min.The PCR products quality was assessed by 0.8% agarose gel electrophoresis and the amplified DNA was purified with a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions. The DNA was then ligated into the pTG19-T PCR cloning vector system and transformed into competent Escherichia coli (DH5α) cells, before plasmid isolation using a GF-1 Plasmid DNA Extraction Kit. After the plasmid extraction, 15µl of the extracted plasmid was sent to the ShineGene Company of China for sequencing with Universal M13 primers.

Sequences from the current study were trimmed manually and analysed by the CHECK_CHIMERA program (Maidak *et al.*, 2001). The similarity searches for sequences were carried out by BLAST (Madden *et al.*, 1996) and alignment was done using CLUSTAL W (Thompson *et al.*, 1997). The phylogenetic analysis was carried out using MEGA software version4 (Tamura *et al.*, 2007) and the phylogenetic relatedness was estimated using the neighbour-joining method and by using the MEGA4 program (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

Detection of microbes by their DNA requires a sensitive methodology and PCR is commonly used. The ribosomal genes are often chosen as a suitable amplicon for environmental studies as they are multicopy in eukaryotic genomes providing enhanced sensitivity, and can be used for phylogenetic analyses and delimitation of diverse groups of organisms across and between the kingdoms.

The rDNA genes are particularly well suited for this purpose as they are present in all forms of life and consist of alternating variable and conserved regions. The small subunit rRNA gene sequences, the ITS1 gene in eukaryotes, are conventionally used for phylogenetic comparisons. However, as this gene is more than 97% identical between genera of the anaerobic fungi (Dore and Stahl, 1991) the less conserved internal transcribed spacer (ITS) regions have proven to be more useful (Brookman *et.al*, 2000). Molecular data has been used to clarify the classification of the anaerobic rumen fungi. Favored indicators of genetic diversity are the rRNA encoding gene sequences, particularly the internal transcribed spacers ITS1, this can be used to identify micro-organisms and to determine pylogenetic relationship within communities, including the rumen fungi (Hausner *et. al*, 2000 and Vainio and Hantula, 2000). In this research, our purpose is to determine the genetic diversity of the rumen anaerobic fungi in buffalos of the Azerbayjan in Iran. PCR products quality was assessed by 0.8% agarose gel electrophoresis (Fig. 1).

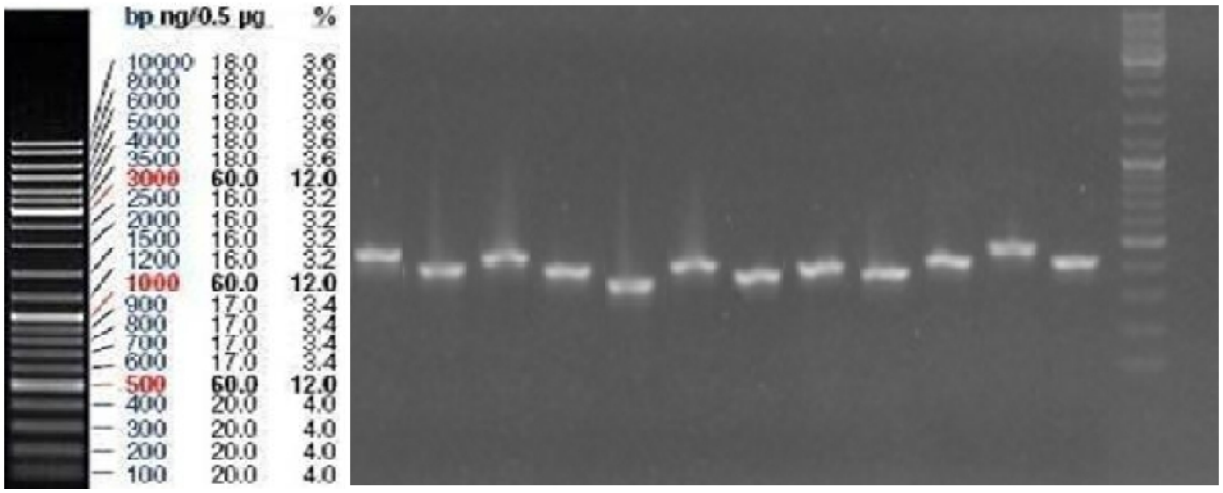

Fig. 1. Analysis of PCR products by agarose gel (0.8 %) electrophoresis.

The GenBank accession numbers for the sequences determined are: AIB01-1, KJ130471; AIB01-2, KJ130472; AIB01-3, KJ130473; AIB01-4, KJ130474; AIB01-5, KJ130475; AIB01-6, KJ130476; AIB01-7, KJ130477; AIB01-8, KJ130478; AIB01-9, KJ130479; AIB01-10, KJ130480, AIB01-11, KJ130481, AIB01-12, KJ130482. Table 1 showed Phylotypes of ITS1 gene sequences of anaerobic rumen fungi retrieved from the rumen samples of buffalo. The phylogenetic tree was drawn using the Neighbor-joining method and the MEGA4 software (Fig. 2). The results show that the ITSI sequence is less conserved in one genera and it can have a little differences in a genera or between the different genera. In this study the observed changes were in 1% of the number of ITS1 region nucleotides. Novel groups identified in the fungal ITS data may be assigned to newly defined genera as characterization of isolated strains progresses (Tuckwell *et al.*, 2005).

Table 2: Phylotypes of ITS1 gene sequences of anaerobic rumen fungi retrieved from the rumen samples of buffalo.

Phylotype	Accession no.	Size (bp) GenBank	Nearest valid taxon	% sequence similarity
AIB01-1	KJ130471	364	Caecomyce sp.	99
AIB01-2	KJ130472	375	Caecomyces sp.	100
AIB01-3	KJ130473	483	Orpinomyces sp.	99
AIB01-4	KJ130474	435	Orpinomyces sp.	99
AIB01-5	KJ130475	414	Piromyces sp.	98
AIB01-6	KJ130476	401	Neocallimastix sp.	98
AIB01-7	KJ130477	485	Neocallimastix sp.	97
AIB01-8	KJ130478	445	Neocallimastix sp.	96
AIB01-9	KJ130479	426	Piromyces sp.	97
AIB01-10	KJ130480	432	Orpinomyces sp.	99
AIB01-11	KJ130481	460	Piromyces sp.	98
AIB01-12	KJ130482	416	Anaeromyces sp.	99

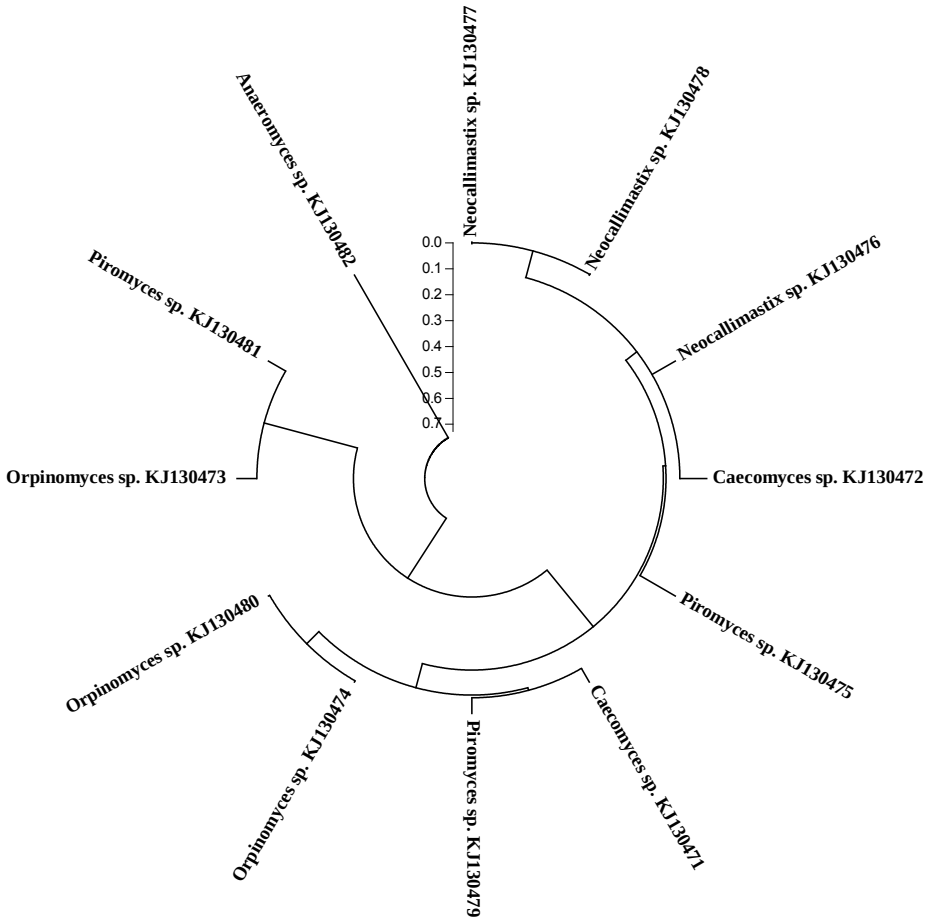

Fig. 2. Neighbor-joining phylogenetic tree of aligned ITS1 sequences of anaerobic rumen fungi.

The anaerobic gut fungi are eukaryotic organisms and therefore require a different genetic marker to the rumen bacteria for identification and differentiation. The use of 18S rDNA has not been found to be useful in differentiating between the members of the gut fungal family, Neocallimastigaceae, as they are too similar. We have used the more variable, ITS region 1 between the structural genes of the ribosomal repeat as sequences with suitable levels of variability for phylogenetic studies. The ITS regions from the anaerobic gut fungi show length polymorphisms, and there is an approximate relationship between the length of the ITS1 and genera. The paucity of morphological features presents a problem regarding the taxonomy of anaerobic fungi. While examining plant material from the digestive tract, fungi often appear as the complex cluster and this makes the classification even up to genus level difficult. At a time when there is little disagreement as to the status of the six genera, subgeneric classification is problematic since difficulties associated with exchange and long-term maintenance of cultures impeded direct morphological and physiological comparisons among isolates. With the advent of molecular taxonomy, it is hoped that DNA sequence comparisons and phylogenetic reconstruction will elucidate the relatedness of the various taxa.

Majority of the sequences deposited relate to the ribosomal RNA genes widely used in phylogenetic reconstruction. The small ribosomal (18S) subunit is highly conserved in different taxa and thus contains little phylogenetically useful information for subgeneric classification (Li and Heath, 1992). In contrast, the internal transcribed spacer (ITS) regions, widely used for study of closely related fungal taxa, show a high level of variability (Li and Heath, 1992; Brookman *et al.*, 2000; Fliegerova *et al.*, 2004), and has been used to differentiate the morphologically similar monocentric (Neocallimastix, Piromyces) and polycentric (Anaeromyces, Orpinomyces) genera. Brookman *et al.*, (2000) also reported that the two multi-flagellated taxa (Neocallimastix, Orpinomyces) were closely related based on the ultrastructure of the zoospores.

Unfortunately, various problems including the presence of divergent ITS sequences within individual isolates has hampered widespread use of this locus for taxonomic studies (Ozkose, 2001), though PCR amplification of DNA from environmental samples (rumen fluid, digesta etc.) using ITS primers may prove valuable for ecological studies (Tuckwell *et al.*, 2005).

In conclusion, it was well shown that the applicability of PCR techniques for the quantification of rumen anaerobic fungi in the digesta and rumen fluid of buffalo have provided additionally useful data. The most reliable method to detect genetic variation between fungal species is analysis of rDNA that contains highly conserved DNA sequences as well as more variable regions. Sequence analysis of ITS1 spacer seems a promising tool for comparing a variety of rumen fungal isolates. However, molecular techniques will become useful techniques for rumen ecology research to manipulate rumen fermentation to improve ruminant feeding efficiency especially under conditions of low-quality roughage.

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