INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES
IJPAES
Volume-4, Issue-3, July-Sept-2014
ISSN 2231-4490

Coden : IJPAES www.ijpaes.com
Received: 5th May-2014 *Revised: 26th May-2014* *Accepted: 17th June-2014*
Research article

IDENTIFICATION OF ANAEROBIC RUMEN FUNGI USING MOLECULAR METHODOLOGIES BASED ON RIBOSOMAL ITS1

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ABSTRACT: Ruminal anaerobic fungi are important contributors to the utilization of poor-quality, high fiber pastures and crop residues by ruminants. Nucleic acid-based techniques which can be used to characterize complex microbial communities without incubation are now being employed regularly in ruminant nutrition studies. The foundation of the molecular ecology techniques is ITS1 rDNA sequence analysis which has provided a phylogenetically based classification scheme for identification of rumen fungi members. In this research, we try to determine the genetic diversity of the gastrointestinal tract anaerobic fungi in buffalos of Iran. After the sampling of the rumen contents, the genomic DNA was extracted from 10 fungal samples and then the electrophoresis was done to confirm the operations accuracy and after that, the PRC cultivation of ITSI region from the rRNA genes. The primers were GM1 and GM2. The phylogenetic tree was drawn using the Neighbor-joining method and the MEGA software. Sequence analysis of ITS1 spacer seems a promising tool for comparing a variety of rumen fungal isolates.
Key words: Anaerobic rumen fungi, ITS1, rRNA, PCR, Buffalo

INTRODUCTION

Anaerobic fungi have been isolated from many sites along the digestive tract of ruminants [1]. As well as being present in the more important species of domesticated ruminants (sheep, goats, cattle and water buffalo), anaerobic fungi occur widely among many different species of herbivorous mammal, including ruminant ruminant-like and other foregut fermenting non-ruminant animals as well as hindgut fermenting animals [2, 3]. The anaerobic fungi are thought to be the primary colonizers of plant material in the rumen, and together with rumen bacteria and protozoa they are responsible for the degradation of ingested plant biomass that would be otherwise indigestible to the host animal. Agroforestry has immense potential to support animal (buffalo) productivity [4]. Progress in understanding the ecology of the rumen ecosystem has been enhanced by the development of molecular phylogenetic approaches for the fungi, together with more advanced rumen bacterial methodologies [5, 6]. Therefore the specific role of rumen anaerobic rumen fungi is important as it has to compete with the other microbes of the ecosystem for its survival. The enumeration of a specific species of rumen anaerobic rumen fungi in the ecosystem is difficult. This is due to selection of the medium used for enumeration as the relative numbers of these fungi. In addition, very large proportion of rumen anaerobic fungi is non culturable, but is active in the rumen fermentation. Therefore, it is essential to search for some better technique of quantifying specific microbes in this ecosystem. Therefore, information is available on the culturable anaerobic fungi of the rumen is incomplete.

The classification of rumen anaerobic fungi based on phenotypic characteristics is not sufficient to study the diversity among the culturable rumen fungi. Therefore, variations in the numbers of rumen anaerobic fungi can be studied easily by using different oligonucleotide DNA probes, to some regions of rumen fungi ITS1 rRNA. There is a high degree of conservation in 18S rRNA gene sequences across the *Neocallimastigales*, for which morphological criteria have been used as the principal means of classifying the six genera and their species that constitute the order.

There has, however, been more recent progress on the use of internally transcribed spacer region sequences as a reliable means of identifying anaerobic fungi to the genus level [7, 8]. DNA-based techniques have facilitated the understanding of the phylogenetic relationships and diversity of micro-organisms in natural ecosystems: they introduce considerably fewer biases in sampling than culture-based methodologies, can be generated directly from DNA and are considered more representative of the entire community than culture-derived data alone [9]. Favoured indicators of genetic diversity are the rRNA encoding gene sequences, particularly the internal transcribed spacers ITS1 and ITS2 and the intervening 5.8S rDNA; these can be used both to identify micro-organisms and to determine phylogenetic relationships within communities [10, 11, 12].

MATERIAL AND METHODS

Animals and sampling

This research was done in the fall of 2013, 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. The slaughter house of Uromia Iran was selected for sample collection and samples were collected immediately after slaughtering of Buffalo in the slaughter house. 4 samples of rumen content were collected randomly from rumen of 10 Buffalo in the slaughter house and were poured to some twisted special bottles (the bottles were autoclave before) and were put in a flask which was containing 39 degrees water. For preventing the accumulation of gas in the bottles, only a 1/3 of their volume was filled with the rumen contents and this temperature was maintained.

DNA extraction and PCR

Total genomic DNA was extracted by using RBB+C method [13]. The detailed procedures of the RBB+C method are described in Table 1. Cell lysis is achieved by bead beating in the presence of 4% (w/v) sodium dodecyl sulfate (SDS), 500 mM NaCl, and 50 mM EDTA. The buffer should also protect the released DNA from degradation by DNases, which are very active in rumen and gastrointestinal samples [14]. After bead beating, most of the impurities and the SDS are removed by precipitation with ammonium acetate, and then the nucleic acids are recovered by precipitation with isopropanol. Genomic DNA can then be purified via sequential digestions with RNAase and proteinase K, followed by the use of QIAamp columns.

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 [14]. The primer synthesis was done by the ShineGene Company of China. 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 ^{o}C for 5 min, followed by 30 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 48 ^{o}C for 1 min and extension at 72 ^{o}C for 1.5 min. Final step was carried out at 72 ^{o}C for 10 min.The PCR products quality was assessed by 0.8% agarose gel electrophoresis (Figure 1) 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. The plasmid transfer to the E.coliDH5α bacterium was done by a heat shock.

The plasmid extraction kit was prepared by Malaysia Vivantis Company. 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 [16]. The similarity searches for sequences were carried out by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) [17], and alignment was done using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) [18]. The phylogenetic analysis was carried out using MEGA software version 4 [19]. The phylogenetic relatedness was estimated using the neighbour-joining method and by using the MEGA4 program [20].

Table 1. Protocol of the (RBB+C) Method

I. Cell lysis:

1. 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).
2. Homogenize for 3 min at maximum speed on a Mini-Beadbeater™ (BioSpec Products, Bartlesville, OK, USA).
3. Incubate at 70°C for 15 min, with gentle shaking by hand every 5 min.
4. Centrifuge at 4°C for 5 min at 16,000× g. Transfer the supernatant to a fresh 2-mL Eppendorf® tube.
5. Add 300 µL of fresh lysis buffer to the lysis tube and repeat steps 2–4, and then pool the supernatant.

II. Precipitation of nucleic acids:

6. Add 260 µL of 10 M ammonium acetate to each lysate tube, mix well, and incubate on ice for 5 min.
7. Centrifuge at 4°C for 10 min at 16,000× g.
8. 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.
9. 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.
10. Dissolve the nucleic acid pellet in 100 µL of TE (Tris-EDTA) buffer and pool the two aliquots.

III. Removal of RNA, protein, and purification:

11. Add 2 µL of DNase-free RNase (10 mg/mL) and incubate at 37°C for 15 min.
12. 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.
13. Add 200 µL of ethanol and mix well. Transfer to a QIAamp column and centrifuge at 16,000× g for 1 min.
14. Discard the flow through, add 500 µL of Buffer AW1 (Qiagen), and centrifuge for 1 min at room temperature.
15. Discard the flow through, add 500 µL of Buffer AW2 (Qiagen), and centrifuge for 1 min at room temperature.
16. Dry the column by centrifugation at room temperature for 1 min.
17. Add 200 µL of Buffer AE (Qiagen) and incubate at room temperature for 2 min.
18. Centrifuge at room temperature for 1 min to elute the DNA.
19. Aliquot the DNA solution into four tubes. Run 2 µL on a 0.8% gel to check the DNA quality.
20. Store the DNA solutions at -20°C.

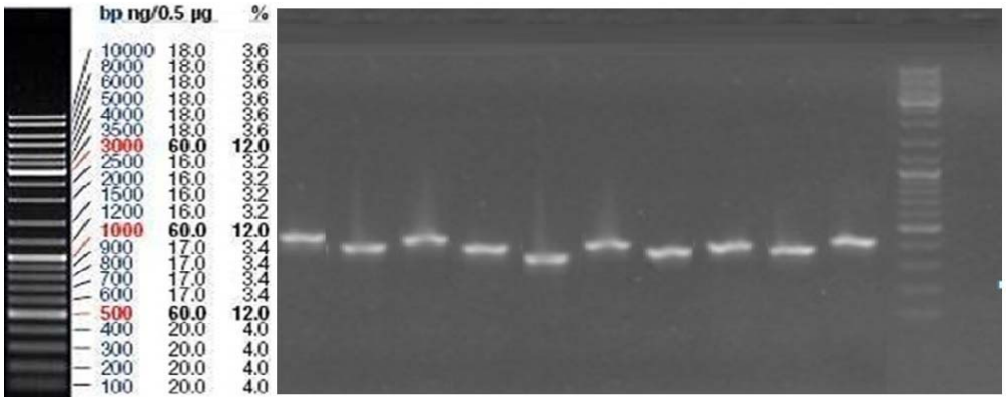

Figure1. Analysis of PCR products by agarose gel (0.8 %) electrophoresis.

RESULTS AND DISCUSSION

The DNA-based techniques have been adopted for understanding the phylogenetic relationship and diversity of micro-organism in natural ecosystems as they introduce considerably fewer biases in sampling the culture-based methodologies. They can be generated directly from DNA and are considered more representative of the entire community than culture-derived data alone [8]. 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 [10, 11].

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

18S rDNA fragment analyses have shown very few differences, indicating that this gene regions are too highly conserved [5] and not sufficiently variable for intra-specific studies on fungi [21]. However, short non-coding ribosomal ITS regions, which are a spacer extremely variable in both sequence and length, provide an excellent tool to separate amplication products, and sequence this hypervariable regions for discriminating OTU [22]. Therefore, it is more suitable to fungi for detecting differences between and within species than 18S rRNA/rDNA.

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	Caecomyces 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

Li and Heath [15] used ITS1 to compare and discriminate gut fungi. Brookman et al. [5] examined the relationships within and between two genera of monocentric gut fungi gathered from various geographical locations and host animals.

In this research, our purpose is to determine the genetic diversity of the gastrointestinal tract anaerobic rumen fungi in buffalos of the Azerbayjan in Iran. The goal was the PRC cultivation of ITSI region from the rRNA genes took place with the use of GM1 and GM2 primers of the anaerobic rumen fungi. The phylogenetic tree was drawn using the Neighbor-joining method and the MEGA4 software (Figure 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 [8].

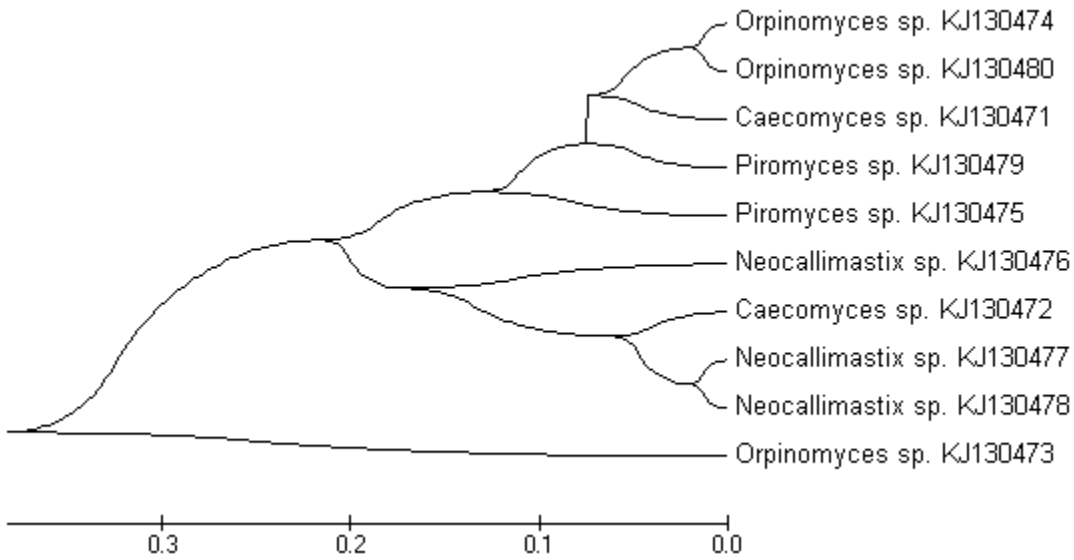

Figure. 2. Neighbor-joining phylogenetic tree of aligned ITS1 sequences of anaerobic rumen fungi

Interest in the genetics of rumen micro-organisms was first sparked by the prospect of creating manipulated strains that might be used to improve rumen function. While some progress has been achieved towards this end, it is now more widely recognized that molecular genetics has vital role to play in understanding the dynamics and diversity of rumen microbial communities, in understanding the functioning on enzyme systems and in unraveling the evolution of rumen micro-organism. in addition natural horizontal gene transfer is a potentially important, but little studied, factor in the adaptation and evolution of the rumen community and might also be involved in disseminating antibiotic resistance genes or possibly even transgenes derived from modified feed plants or microbial additives, to different gut micro-organism.

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 [6] 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.

ACKNOWLEDGEMENT

This article is part of Ph.D thesis (supervisors; Dr. Mohammad Chamani and Dr. Naser Maheri-Sis and Advisors; Dr. Mahdi Amin Afshar and Dr. Ramin Salamat Doust-Nobar) in animal nutrition, Islamic Azad University, Shabestar Branch. Authors thanks from Animal Research Centre and Animal Nutrition Laboratories of Islamic Azad University, Shabestar Branch, Shabestar, Iran.

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