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Identification and Genetic diversity of Anaerobic Gut Fungi in Buffalo using Molecular Methodologies based on Ribosomal ITS1 rRNA

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ABSTRACT: Anaerobic fungi are the significant constituent of rumen microbiota in livestock that rely on poor-quality fibrous diets. Differentiation between isolates of these fungi is difficult using conventional techniques. In this study, DNA-based methodologies were used to study to determine the population of Anaeromyces anaerobic rumen fungi in buffalo indigesta and rumen fluid. The foundation of the molecular ecology techniques is ITS1 rDNA sequence analysis which has provided a phylogenetically based classification scheme for enumeration and identification of microbial community members. After the sampling of the rumen contents and the anaerobic fungi cultivation, the genomic DNA was extracted from 13 fungal samples. The PRC cultivation of ITS1 region from the rRNA genes took place with the use of Good92R and Good92F primers of the Anaeromyces anaerobic fungi. The phylogenetic tree was drawn using the Neighbor-joining method and the MEGA software. The results show that the ITS1 sequence is less conserved in one genera and it can have a little differences in a genera or between the genera. Therefore, the application of molecular approaches, especially those focused on ITS1 ribosomal RNA (rRNA) sequence diversity, is able to differentiate between gut fungal genera.

Keywords: Anaerobic rumen fungi, ribosomal RNA, ITS1, Anaeromyces, Phylogenetic

INTRODUCTION

Although the main diet of herbivorous mammals is plant material, they are totally dependent on the microorganisms in their digestive tracts to break complex polysaccharides into small molecules prior to absorption. Rumen bacteria, protozoa and fungi are the main contributors to the process of digestion (Durand *et al.*, 1995).Research into the microbiology of the rumen has been undertaken for many years with substantial contributions in bacteriology and protozoology being made during the 1940s, 1950s and 1960s. However, it was not until 1975 that Orpin (1975) identified the rumen anaerobic fungi. It is now generally accepted in microbial ecology that cultivation-based approaches provide an incomplete picture of microbial diversity in the gastrointestinal (GI) tract because only a minority of microbes can be obtained in culture. Therefore, the application of molecular approaches, especially those focused on ITS1 ribosomal RNA (rRNA) sequence diversity, has become popular as they enable researchers to bypass the cultivation step (Zoetendal *et al.*, 2004).

Microbial communities cannot be accurately described without the use of culture independent techniques, and sequencing of 16S rRNA genes has become a standard procedure in the identification of isolates. During the last decade, approaches based on sequence variability have frequently been applied to determine the microbial community structures in complex ecosystems, and developments are still ongoing. Most phylogenetic information from the GI tract has been gathered by sequencing of cloned ITS1 rRNA gene amplicons that have been obtained by polymerase chain reaction (PCR) of ITS1 rRNA genes present in the genomes of all rumen anaerobic fungi. With the advent of molecular taxonomy, it is hoped that DNA sequence comparisons and phylogenetic reconstruction will elucidate the relatedness of the various taxa. Indeed, a number of molecular phylogenetic papers are on record (Brookman *et al.* 2000; Fliegerova *et al.* 2004; Tuckwell *et al.* 2005), and over 100 nucleotide sequences deposited with Gene bank (http://www.ncbi.nlm.nih.gov).

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 multiflagellated 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 this research, we try to determine the genetic diversity of the gastrointestinal tract anaerobic fungi (Anaeromyces) 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 Town. 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).

The quality of the community DNA was assessed by 1% agarose gel electrophoresis. The ribosomal ITS1region defined by primers Good92F (5′-CTACCGATTGAATGGCTTAG-3′) and Good92R (5′-AGATCCATTGTCAAAAGTTGTT-3′) were designed base on the recorded sequence of NCBI site (http://www.ncbi.nlm.nih.gov). with the access code of AF170187.1. The PCR reaction was performed in 100 µl reactions containing (final concentration): forward and reverse primers, 0.2 µM; dNTPs mixture, 200 µM; MgCl2, 1.5 mM; KCl, 50mM; Tris/HCl pH 8.4, 10 mM; and Taq polymerase, 0.25 Units. Approximately 50 ng genomic DNA was 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 carriedout at 72°C for 10 min (Fig. 1).

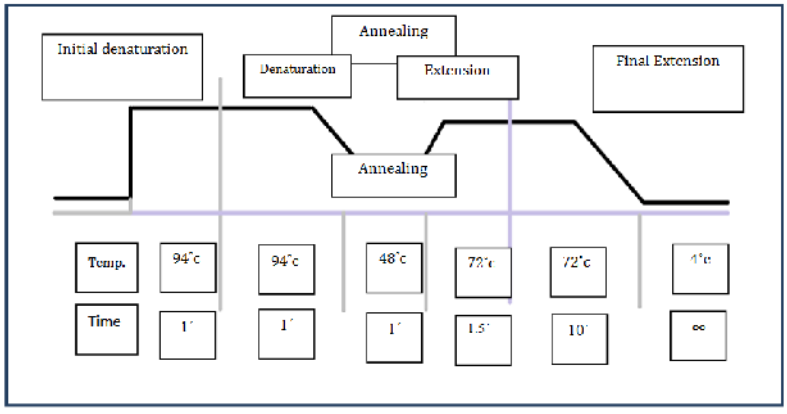

Fig. 1. The temperature program for PCR**.**

The PCR products quality was assessed by 0.8% agarose gel electrophoresis and the amplified DNA was purified with a QIA quick 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 Shine Gene Company of China for sequencing with Universal M13 primers. Diagrammatic steps show in figure 2 of the cloning vector system, transformed into competent Escherichia coli and plasmid isolation.

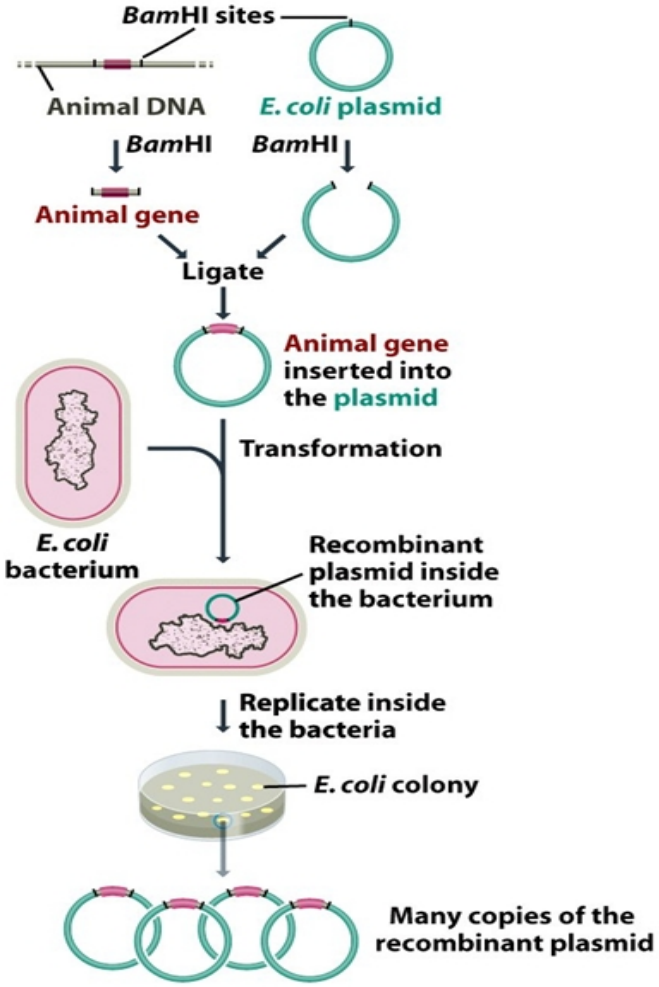

Fig. 2. Diagrammatic representation of the cloning vector system, transformed into competent Escherichia coli and plasmid isolation.

Sequences from the current study were 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 version4.0 (Tamura et al. 2007) and the phylogenetic relatedness was estimated using the neighbour-joining method and by using the MEGA 4.0 program (Saitou and Nei, 1987).

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 (Tuckwell *et al.,* 2005). 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. 3).

The Gen Bank 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 these gene regions are too highly conserved (Brookman *et al.,* 2000) and not sufficiently variable for intra-specific studies on fungi (Pelandakis *et al.,* 2000). 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 hyper variable regions for discriminating OTU (Garcia-Martinez et *al.,* 1999). Therefore, it is more suitable to fungi for detecting differences between and within species than 18S rRNA/rDNA. The Gen Bank accession numbers for the sequences determined are: AIB01, KF789494; AIB02, KF789495; AIB03, KF789496; AIB04, KF789497; AIB05, KF789498; AIB06, KF789499; AIB07, KF789500; AIB08, KF789501; AIB09, KF789502; AIB10, KF789503; AIB11, KF789504; AIB12, KF789505 and AIB13, KF789506. Table 1 showed Phylotypes of ITS1 gene sequences of Anaeromyces anaerobic rumen fungi retrieved from the rumen samples of buffalo.

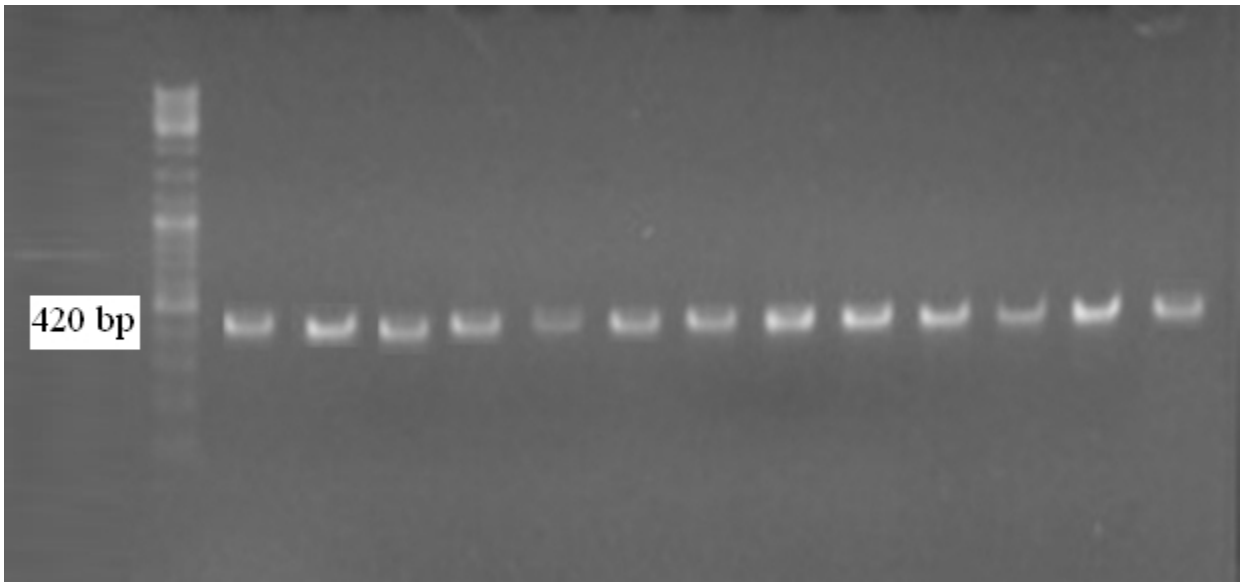

Fig. 3. Analysis of PCR products by agarose gel (0.8 %) electrophoresis.

Table 1: Phylotypes of ITS1 gene sequences of Anaeromyces anaerobic rumen fungi retrieved from the rumen samples of buffalo.

Phylotype	Accession no.	Size(bp)GenBank	Nearestvalidtaxon	% sequence similarity
AIB01	KF789494	420	Anaeromyces sp.	99
AIB02	KF789495	420	Anaeromyces sp.	99
AIB03	KF789496	420	Anaeromyces sp.	99
AIB04	KF789497	420	Anaeromyces sp.	99
AIB05	KF789498	420	Anaeromyces sp.	99
AIB06	KF789499	419	Anaeromyces sp.	99
AIB07	KF789500	420	Anaeromyces sp.	99
AIB08	KF789501	421	Anaeromyces sp.	99
AIB09	KF789502	418	Anaeromyces sp.	98
AIB10	KF789503	419	Anaeromyces sp.	98
AIB11	KF789504	420	Anaeromyces sp.	99
AIB12	KF789505	420	Anaeromyces sp.	98
AIB13	KF789506	421	Anaeromyces sp.	98

The phylogenetic tree was drawn using the Neighbor-joining method and the MEGA 4.0 software (Fig. 4). The results show that the ITS1 sequence is less conserved in one genera and it can have a little differences in a genera or between the different genera (Goudarzi *et al.*, 2015). Considerable advances have been made over recent years in our understanding of the physical characteristics of individual genomes. The advent of entire sequenced genomes from a variety of prokaryotic and eukaryotic organisms has required sophisticated methods of sequence and comparative genomic analysis (Matthew J. Nicholson 2005). 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 culturesimpeded direct morphological and physiological comparisons among isolates. With the advent ofmolecular taxonomy, it is hoped that DNA sequence comparisons and phylogenetic reconstruction will elucidate the relatedness of the various taxa.

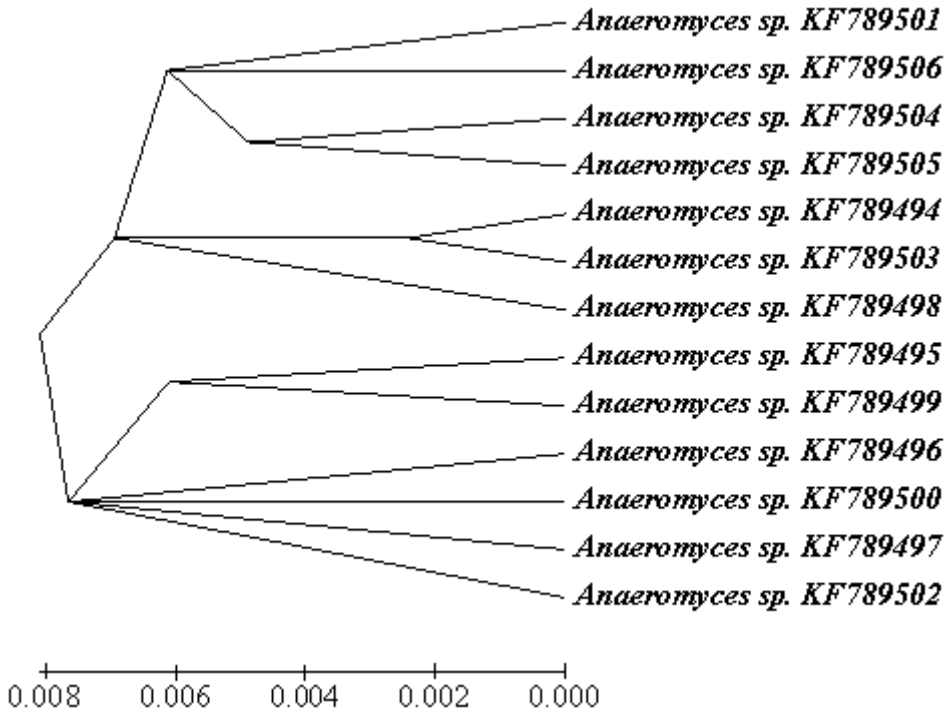

Fig. 4. Neighbor-joining phylogenetic tree of aligned ITS1 sequences of Anaeromyces anaerobic rumen fungi.

Majority of the sequences deposited relate to the ribosomal RNA genes widely used inphylogenetic reconstruction. The small ribosomal (18S) subunit is highly conserved in differenttaxa 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 studyof 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. The most reliable method to detect genetic variation between fungal species is analysis of rDNA that contains highlyconserved DNA sequences as well as more variable regions. Sequence analysis of ITS1 spacer seems apromising tool for comparing a variety of rumen fungal isolates.

Rumen microbiologists have constantly shown curiosity in manipulation of the rumen microbial ecosystem to boost feedstuff utilization and improved milk production. It is now a well established fact that anaerobic fungi participate in hydrolysis of plant biomass in ruminants, based on superior penetration of plant tissues over bacteria or protozoa, and thus leading to an enhanced degradation of forage in the rumen. Therefore, a substantial potential exists for the manipulation of fungal population and activity in the rumen to benefit even from poor quality herbages.

Currently, the focus of GI tract ecology is switching slowly from 16S rRNA approaches towards genomic and transcriptomic approaches, and perhaps our view of the impact of pre- and probiotics on the GI tract community should follow this example. In the future, there will be developments towards high-throughput approaches in microbial ecology. In addition, we expect to see major developments in 'lab-on-a-chip' technology. Despite rapid developments in modern molecular techniques and all their challenges, a potential danger is that scientists may easily be tempted to perform descriptive rather than hypothesis-driven research. 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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