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ORIGINAL ARTICLE

Molecular Biological Detection of Anaerobic Gut Fungi (*Neocallimastigales*) in ruminants

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ABSTRACT
The purpose of this study was carried out to evaluate the relationships within and between two genera of monocentric gut fungi gathered from various geographical locations and host animals. The phylogenetic diversity of members of the gut anaerobic fungi (phylum Neocallimastigomycota) was investigated in three different ruminants using the internal transcribed spacer region-1 (ITS-1) ribosomal RNA region as a phylogenetic marker. A total of 15 sequences representing all known anaerobic fungal genera were obtained in this study. The ribosomal ITS1 region defined by primers forward (5"-CTACCGATTGAATGGCTTAG-3") and reverse (5"-CTGCGTTCTTCATCGAT-3") was amplified from genomic DNA by PCR. Sequences affiliated with the genus Anaeromyces were the most abundant, being encountered in 9 different samples, and representing 60% of the sequences obtained. On the other hand, sequences affiliated with the genera Orpinomyces were the least abundant, being encountered in 1, and representing 6.6%, of the total sequences obtained. Further, 33.3% of the sequences obtained did not cluster with previously identified genera and formed eight phylogenetically distinct novel anaerobic fungal lineages.
Keywords: *PCR; anaerobic fungi; Neocallimastigales; ruminant*

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INTRODUCTION

It was only in 1975 that the anaerobic rumen fungi were identified and recognized as an integral part of the rumen microbiota [14]. The presence of chitin in their cell walls [15,16] and fungi make up 8% of the rumen microbial population, which indicates active degradation of cellulose. The significance and possible role of the rumen fungi in fiber digestion was recognized when extensive colonization of fibrous plant materials by the fungi was observed in the rumen of sheep and cattle [2,3]. The fungi preferentially colonise the thick-walled sclerenchyma and vascular tissues [1,7,8,9,12].

Anaerobic fungi in the digestive system of ruminants due to a unique ecosystem, are one of the unusual groups of anaerobic fungi that constituting Zoospore among fungi, also due to the lack of reliable and reproducible method; identification, differentiation and enumeration of anaerobic rumen fungi has defects, Molecular Biology is one of the best means to help you for confrontation the challenges to create.

There are six recognized genera: *Neocallimastix*, *Piromyces*, *Orpinomyces*, *Anaeromyces, Cyllamyces* and *Caecomyces*, which are delimited by thallus morphology (monocentric, polycentric and filamentous or bulbous) and the number of flagella per zoospore (uniflagellate or polyflagellate [13]. These characters, visible using the light microscope, tend to be pleomorphic, varying with culture conditions, particularly carbon source.

Molecular data have been used in an attempt to clarify the classification of the anaerobic gut fungi. Dore & Stahl [6] and Bowman *et al*. [4] used partial 18S rRNA sequence data to support the assignment of these fungi to the *Chytridiomycetes* [4,6]. However, these studies do not address the inter-relationships between the genera, probably because the 18S rRNA sequence is too highly conserved for their resolution within good limits of confidence. Li & Heath used sequence data from a less well conserved ribosomal sequence, the internal transcribed spacer region-1 (ITS1) to compare and discriminate gut fungi [10,11].

However, recent research dealing with diversity of animal gut fungi indicates that the number of 6 genera and 21 species of anaerobic fungi is neither terminal, nor most likely confined to ruminant and large herbivores only. We present here a DNA-sequence-based phylogenetic analysis of *phylum Neocallimastigomycota* with the aim of determining the relationships within these genera.

MATERIALS AND METHODS

Sampling

Fecal sample were obtain from slaughtered animals from farms surrounding the cities of Mazandaran and Lorestan province, Iran; in the month of July, august and September 2013. Fresh fecal samples were collected from animals in 50 ml sterile falcon Tube immediately after slaughtered. Sampling was performed in the distal colon and stored on ice on-site, transferred in a cold box and promptly transferred and stored in a -20˚C freezer. Care was taken to avoid cross-contamination between different samples (Table 1).

DNA extraction

Genomic extraction was performed on samples of digestive system of ruminant. 50 mg of gastrointestinal content was mixed with 500 µl TES Buffer in a 2 ml micro tube (100 mM Tris pH=8; EDTA 10mM and SDS 2%), TES Buffer will keep in the refrigerator and it should be brought to a temperature of 60˚ Celsius before use it. Then 5 µl Proteinase K added to the micro tube and incubated at 60˚C for 60 min. optimum pH of this enzyme (Proteinase K) is 7.5-12 but often ranges from 7.9-9 is used.

After going through time the above mentioned, 140 µl NaCl and 65 µl CTAB 10% added to the micro tube and incubated at 60˚C for 10 min. then added chloroform to micro tubes equal volume (710 µl) and then contents of micro tube were mixed with up and down.

After 10 min at room temperature the tubes was centrifuged at 10,000 rpm for 10 min and the supernatant layer was removed and transferred to a fresh filtered tube and twice centrifuged for 1 min at 10,000 rpm. 700 µl ethanol was added to the micro tube and centrifuged it for 1 min at 10,000 rpm, discharge alcohol ethanol with centrifuge completely.

The amount of 45 µl ddW, TE Buffer or wash solution, was added to the filter, DNA should be stored at 4 degree Celsius. Qualitative analysis of extracted DNA was performed on Agarose gel 1% (Figure 1). All materials were obtained from Merck.

Amplification of the ribosomal ITS1 region

The ribosomal ITS1 region defined by forward primer (5'-CTACCGATTGAATGGCTTAG-3') and reverse primer (5'-AGATCCATTGTCAAAAGTTGTT-3') was amplified from genomic DNA by PCR. Primer design was performed on the Ribosomal ITS1 gene. Were designed by Oligo 7 software and examined. Also by NCBI website was done in an alignment.

Primers were synthesized by Shine Gene The PCR reaction was performed in 10 µl reactions containing (final concentration): forward and reverse primers, 0.2 µl; dNTPs mixture (10 mM), 0.3 µl; $MgCl_2$, (1.5 mM) 0.3 µl; PCR Buffer (10X), 1 µl; Taq DNA Polymerase, 0.5 µl; DNA Template, 1 µl; Approximately 50 ng genomic DNA was used as the template for each amplification.

The temperature conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 40 seconds, annealing at 47 °C for 35 seconds and extension at 72 °C for 35 seconds. The presence of a single product between 378 and 477 bp was verified by Agarose gel electrophoresis (Figure 2). 15 microliters of the PCR products were sent for sequencing to Shine Gene.

RESULTS

Analysis of DNA sequence data

The anaerobic fungi sequences data produced were aligned to a published ITS1 AF using ClustalW. Phylogenetic analysis was carried out using the MEGA v5.05 program. The maximum-likelihood algorithm was also used to construct a phylogenetic tree. The anaerobic fungi sequences data produced were aligned to a published ITS1 AF using ClustalW. Phylogenetic analysis was carried out using the MEGA v5.05 program. The maximum-likelihood algorithm was also used to construct a phylogenetic tree.

Fig shows the results of the phylogenetic analyses performed on the 15 gut fungal ITS1 sequences. The analysis methods used were maximum-likelihood, parsimony and distance matrix based (Fig. a & b respectively). In this study, 10 sequences have a length of between 378 to 477 nucleotides were phylogenetic were, *Anaeromyces Sp* was the most frequent And the minimum frequency was *Orpinomyces Sp.*Distinguish between groups of anaerobic fungi tract using conventional techniques is difficult, costly and time consuming and in this study, DNA based molecular methods were used for this purpose.

The GenBank accession numbers for the sequences determined are

LS1,AF170187.1;LS2,AF170187.1;LS3,AF170187.1;LS4,AF170187.1;LS5,AF170187.1;MC1,AF170187.1;MC2,AF170188.1;MC3,AF170189.1; MC4,AF170195.1;MB1,AF170196.1; MB2 ,170187.1; MB3,170187.1; MC5, 170195.1; MC6, 170198.1; MC7, 170198.1.

Analysis of DNA sequence data

The anaerobic fungi sequences data produced were aligned to a published ITS1 AF using Clustal w. Phylogenetic analysis was carried out using the MEGA v5.05 program. The maximum-likelihood algorithm was also used to construct a phylogenetic tree.

Fig. 4 shows the results of the phylogenetic analyses performed on the 15 gut fungal ITS1 sequences. The analysis methods used were maximum-likelihood, parsimony and distance matrix based (Fig. a and b respectively).In this study, 15 sequences have a length of between 378 to 477 nucleotides were phylogenetic were, *Anaeromyces Sp* was the most frequent

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Distinguish between groups of anaerobic fungi tract using conventional techniques is difficult, costly and time consuming and in this study, DNA based molecular methods were used for this purpose.

Table 1. Description of Isolates used in this study.

Isolate Code	Origin	Host Animal
LS1	**L**	**S**
LS2	**L**	**S**
LS3	**L**	**S**
LS4	**L**	**S**
LS5	**L**	**S**
MC1	**M**	**C**
MC2	**M**	**C**
MC3	**M**	**C**
MC4	**M**	**C**
MB1	**M**	**B**
MB2	**M**	**B**
MB3	**M**	**B**
MC5	**M**	**C**
MC6	**M**	**C**
MC7	**M**	**C**

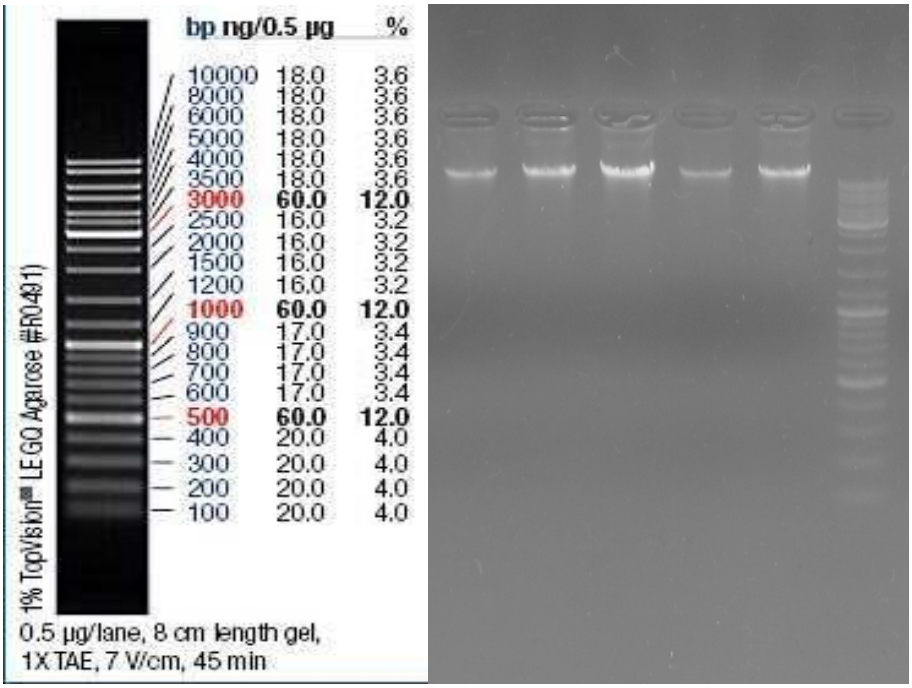

Figure 1. **Qualitative analysis of extracted DNA on Agarose gel 1% (left), pattern unlock Ladder Fermentas GeneRuler # SM0331 DNA Ladder Mix (right).**

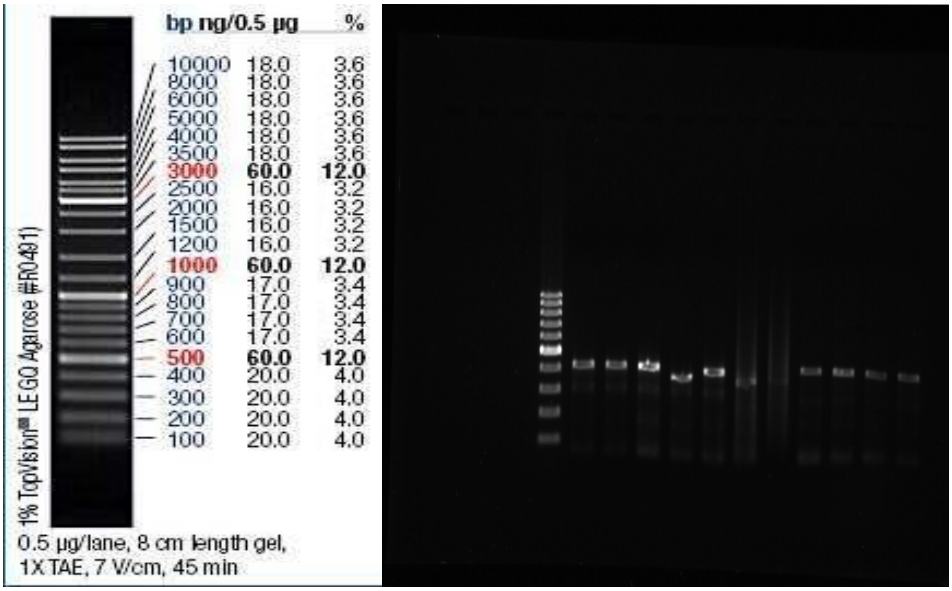

Figure 2. Qualitative analysis of PCR products with primers F & R on Agarose gel 1% (left), pattern unlock Ladder Fermentas GeneRuler # SM0331 DNA Ladder Mix (right).Ladder ؛ Fermentas GeneRuler # SM0331 DNA Ladder Mix

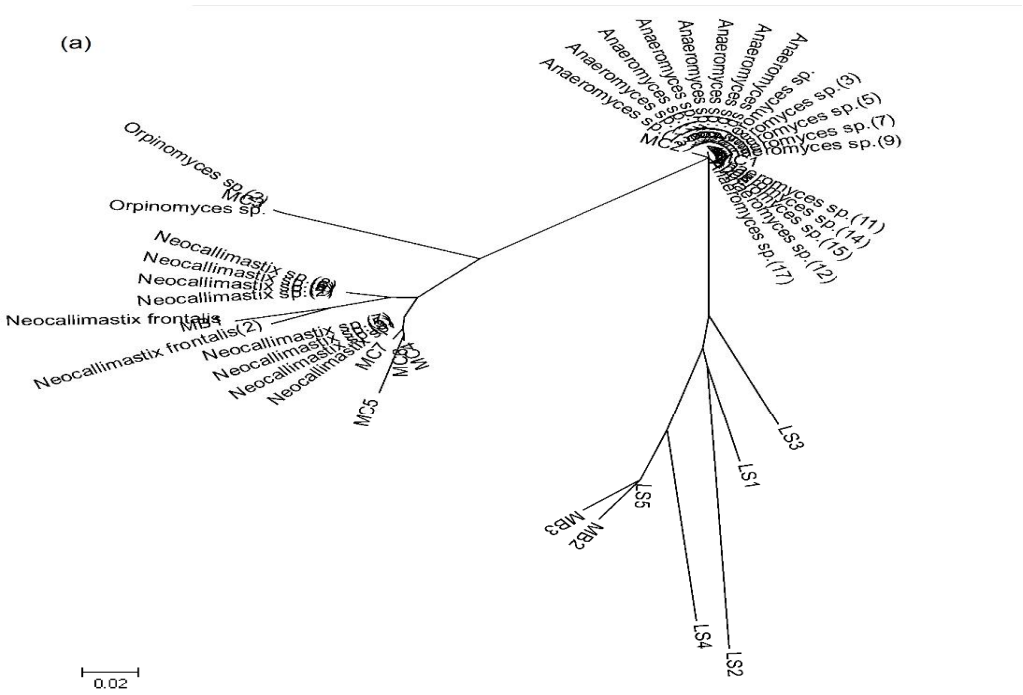

Figure 3 – a : Phylogenetic analyses of the aligned ITS1 sequences. The source and host animal of the isolates are shown in Table 1. (a); An unrooted tree generated using the maximum likelihood algorithm (DNAML). The consensus tree produced using maximum-parsimony analysis. The tree with the highest log likelihood (-1852.6998) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 45 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 312 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [35].

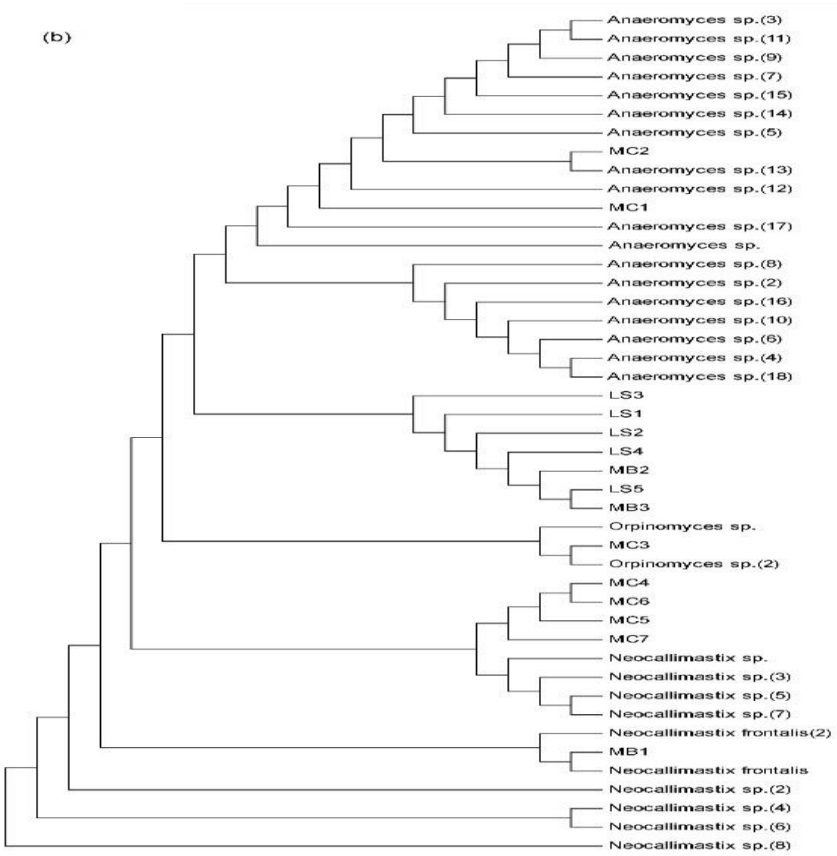

Figure 3 (b); Figure. Maximum Parsimony analysis of taxa. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 135 most parsimonious trees (length = 291) is shown. The consistency index is 0.828179 (0.785408), the retention index is 0.944568 (0.944568), and the composite index is 0.782271 (0.741871) for all sites and parsimony-informative sites (in parentheses). The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 0 in which the initial trees were obtained with the random addition of sequences (10 replicates). The analysis involved 45 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 312 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

DISCUSSION

Phylogenetic analysis

ITS-1 genes of the studied isolates, available on the Gene Bank were used for the phylogenetic analysis (Figure 3 a & b). A total of 10 sequences were obtained from 15 different samples. In all, 58.82% of the total sequences generated were kept after implementing quality control measures, yielding 10 sequences that were used for further phylogenetic analyses. The range of sequence lengths included in the analysis was between 378-477 nucleotides in length. Classification of anaerobic fungi is observed (Table 1). In this study Between *Monocentric genera* succeeded ITS-1 gene amplification *Neocallimastix* By means of the polymerase chain reaction, Also Anaeromyces and Orpinomyces genera were identified that are Polycentric.

Genus-level taxonomic placement

Polycentric genera. Sequences affiliated with the genus *Anaeromyces sp.* were the most abundant in the entire data set, being encountered in 9 different samples, and representing 60% of the total number of sequences obtained. *Anaeromyces Sp* was the most frequent and the minimum frequency was *Orpinomyces Sp.*

Monocentric genera. Sequences affiliated with the genus *Neocallimastix* were being encountered in 5 different samples.

Novel AF groups

In addition to the members of previously described *genera*, a significant fraction (50% of total sequences) could not be assigned to any of these six genera. Phylogenetic analysis suggested that these groups belonged to eight different novel lineages that were designated novel groups LSA1-LSA5 (Figure 1). These lineages remained *Polycentric* regardless of the two-building algorithm used (Parsimony, Maximum likelihood).

The high level of AF phylogenetic diversity observed in animal hosts surveyed is evident by the fact the average number of species per sample is higher than the total number of AF species currently described. We acknowledge that our estimates are solely based on sequence divergence values of a single amplicon, rather than a thorough microscopic, biochemical, and sequence analysis.

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