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ORIGINAL ARTICLE

Fungal diversity in sheep (*Ovis aries*) and cattle (*Bos taurus*) feces assessed by comparison of 18S, 28S and ITS ribosomal regions

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Abstract To explore the fungal diversity in ruminant feces for bioenergy, libraries based on internal transcribed spacer (ITS), 18S rRNA, and 28S rRNA regions were constructed, respectively. Although the libraries were constructed from the same DNA extracts, the fungal taxa analyses based on these libraries are different. The ITS and 28S libraries comprised higher proportions of fungal clones than 18S libraries, and the ITS libraries converged into the lower diversities. The ITS libraries could be used to analyze the fungal community. The 18S libraries were suitable for the fungi and protozoa community. However, the 28S are suitable for analysis of *Ascomycota* fungi. The major fungal taxa in cattle feces analyzed by ITS, 18S, and 28S libraries are similar to those of sheep feces, respectively. The fungal taxa detected by the ITS library comprised only 20 % fungal taxa detected by the three libraries. The 18S library comprised 30 % fungal taxa; the 28S library comprised about 50 % fungal taxa. The results indicated that primer sets toward different DNA regions lead to the difference in structures of fungal community. So the selection of primer sets may influence the fungal communities, and libraries based on single primer sets may underestimate the fungal diversity. The comparison of ITS, 18S, and 28S libraries could fid more diverse fungi than that based on only one library.

Keywords ITS · 18S rRNA · 28S rRNA · Cattle · Sheep

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Introduction

Fungi represent an essential functional component of ecosystems as decomposers, mutualists, and pathogens and are one of the most diverse groups of the Eukarya (Buée et al. 2009). In the 21st century, fungi increasingly will be studied as reserviors for sustainable solutions to energy problems, which will harness process-powered fungal enzymatic and biochemical machinery to yield new liquid fuels and chemicals (Baker et al. 2008). The fungi with enzymatic hydrolysis of lignocellulosic biomass showed potential to convert cheap lignocellulosic biomass into biofuel. Ruminant animals have the ability to convert low quality feeds into high quality protein (Varga and Kolver 1997). Anaerobic gut fungi produce potent fibrolytic enzymes, and it is thought that they are early colonisers of plant biomass, making them important contributors to fibre digestion (Nicholson et al. 2010). As the waste products of the digetive process, herbivore feces are predominantly composed of the cell wall polymers cellulose, hemicellulose, and lignin (Peterson et al. 2011). The novel gut and coprophilous fungi with fibrolytic enzymes can be found from fresh feces of ruminants. Current knowledge of gut fungal population size and diversity in the feces of herbivores is largely based upon morphological classifications after isolation and cultivation of fungi from their environment. This research has not been exhaustive, and it is recognised that there are many species yet to be described (Nicholson et al. 2010). The development of moleculer techniques has broadened the understanding of fungal diversity and enabled a more complete detection and comprehensive description of fungal communities.

As a rule, molecular diversity surveys targeting microbial eukaryotes have used domain-specific PCR primers to amplify 18S rRNA gene fragments from environmental samples (Jebaraj et al. 2010). Assessment of fungal diversity using ITS regions generated a greater richness than that based on

conserved 18S rRNA gene (Lord et al. 2002). In mycology, the ITS region is the most commonly sequenced region for queries of systematics and taxonomy at and below the genus level (Nilsson et al. 2009). As such, primers target most eukaryotic organisms and only a small fraction of amplicon diversity present in a sample; it was assumed that many fungi were not detected by these primer sets. As a favored phylogenetic marker among many mycologists, the fungal 28S rRNA gene had not been widely adopted to survey fungal diversity (Schoch et al. 2012). In order to explore the fungal communities in ruminant (sheep and cattle) feces comprehensively, the fungi were detected using molecular methodologies based on ribosomal ITS1-5.8S-ITS2, 18S rRNA, and 28S rRNA genes.

Materials and methods

Feces collection

Five sheep (*Ovis aries*) and five cattle (*Bos taurus*) were used as donors of the feces deposited on the pastures in Runan Town, Henan Province, China. These sheep were kept in total confinement and fed a diet based on hay and a feed supplement containing maize grain and mineral salt for 2 weeks. At the time of feces collection, sterilized bags were fitted to the animals for a few minutes to ensure the collection of a sufficient amount of feces. The cattle were fed on a standard diet containing green fodder maize and concentrate mixture. Fresh feces samples were collected by observing focal individuals until they defecated. Samples were collected in a clean resealable bag immediately after they were deposited, using sterile gloves. These bags were kept in an iced cooler and transported back to the lab within 24 h, then stored at 4 °C for isolation or -80 °C for DNA extraction.

DNA extraction from ruminant feces

The total DNA was extracted from the ruminant feces using a modified E.Z.N.A.™ Stool DNA kit (Omega Bio-tek. Inc. USA) according to the manufacturer's instructions. About 0.5 g of feces were frozen in liquid nitrogen and ground into fine powder in a sterilized and precooled mortar. The dry powder was then gently dispersed in Sp1 solution, and DNA was extracted according to the manufacturer's instructions. Finally, the DNA was resuspended in 20 μl of TE buffer for further studies.

PCR amplification of the fungal 18S, 28S, and ITS ribosome regions

The 18S rRNA genes were amplified with the Euk1A and Euk516R primers (Table 1), the ITS1-5.8S-ITS2 region amplified with ITS1F and ITS4B primers, and the 28S rRNA genes amplified with the Nl209 and Nl912 primers. The PCR reaction mixture (25 μL) contained 1×PCR buffer (Takara), 200 μM dNTP, 0.2 μM of each primer, 3 mM $MgCl_2$, and 2.5 U Taq DNA polymerase (Takara). After initial denaturation at 94 °C for 5 min, each thermal cycling was as follows: denaturation at 94 °C for 30 s, annealing at 56 °C for 40 s, and elongation at 72 °C for 1 min. At the end of 30 cycles, the last extension step was performed at 72 °C for 10 min.

The PCR products of five reactions from the same sample were pooled together to minimize PCR bias (Lai et al. 2007). The pooled PCR products were electrophoretically separated, and the band of approximate size in the electrophoresis pattern was excised and purified by the E.Z.N.A. gel extraction Kit according to the instructions of the manufacturer (Omega Bio-tek. Inc. USA).

Construction of 18S rDNA, 28S rDNA, and ITS region clone library

The purified PCR products were ligated into the pMD20-T Vector (Takara) and then transformed into competent *E. coli* DH5α cells. Colonies were screened on LB agar supplemented with ampicillin (100 μg ml^{-1}; Sigma, St. Louis, USA), X-gal (100 μg ml^{-1}, Takara), and IPTG (0.5 mM; Takara). The white clones were picked randomly for further studies. The 18S rDNA, 28S rDNA, and ITS region clone libraries of cattle and sheep feces were constructed separately. A total of 6 clone libraries were constructed.

Plasmid DNA was isolated from 250 randomly chosen clones by standard miniprep procedures from cattle and sheep fecal clone libraries, respectively. The 18S rDNA, 28S rDNA, and ITS region genes were reamplified using the above

Table 1 Fungal primer sets used in the study

Library type	Primer sets	Sequences
ITS Library (cattle)	ITS1F	5′-CTT GGT CAT TTA GAG GAA GTA A-3′
ITS Library (sheep)	ITS4B	5′-TCC TCC GCT TAT TGA TAT GC-3′
18S Library (cattle)	Euk1A	5′-CTG GTT GAT CCT GCC AG-3′
18S Library (sheep)	Euk516R	5′-ACC AGA CTT GCC CTC C-3′
28S Library (cattle)	Nl209	5′- AAG CGC AGG AAA AGA AAC CAA CAG-3′
28S Library (sheep)	Nl912	5′-TCA AAT CCA TCC GAG AAC ATC AG-3′

primers, and the correct size was verified using agarose gel electrophoresis. PCR aliquots (10 μl) were characterized by single digestion with the restriction endonucleases *Taq* I and *Msp* I. Restriction fragment length polymorphism (RFLP) patterns were classified based on the agrose gel electrophoresis patterns. Clones with similar restriction patterns were grouped together, and randomly chosen clones from each RFLP group were chosen for sequencing. Sequencing was performed by Shenyou Biotechnology, Ltd. (Shanghai, China). The sequences were examined for possible chimeras by programs UCHIME (http://drive5.com/uchime/uchime_download.html) to remove chimeric sequences, then deposited in the GenBank database and were assigned accession numbers KC922104-KC922411.

Phylogenetic analysis and diversity analysis

Environmental gene sequences were compared initially with those in GenBank using BLAST analysis to determine their approximate phylogenetic affiliation. Phylogenetic and diversity analysis were inferred by the neighbor-joining method. Evolutionary distances were calculated with the Kimura two parameter model (transition/transversion ratio=2.0). The diversity calculation analyses and rarefaction curves to number of observed operational taxonomic units (OTUs) were carried out using the program EstimatesS v.8.0 (http://viceroy.eeb.uconn.edu/estimates/). The library coverage values were calculated using the formula [1-(n/N)], in which n is the number of OTUs represented only by a single clone and N is the total number of clones in the library. Rarefaction curves to numbers of observed OTUs were calculated within the fungal assemblage at each dataset.

Results

Characteristics of libraries

By using three different fungal-specific PCR primer sets towards ITS1-5.8S-ITS2, 18S rRNA, and 28S rRNA regions for each DNA extract, six libraries were prepared. The libraries were named as ITS (cattle), 18S (cattle), 28S (cattle), ITS (sheep), 18S (sheep), 28S (sheep). From six libraries, we sequenced a total of 308 clones, estimating about 7 were affiliated to plants, 73 affiliated to protozoa and 228 affiliated to fungi. Twelve fungal phylotypes under the 100 % match criterion, 156 under the 99 % match criterion, 30 under the 98 % match criterion, and 51 under the 97 % match criterion were recognized (Table 2). Although the libraries were constructed from the same DNA extracts, the ITS and 28S libraries comprised higher proportions of fungal clones than 18S libraries; however, the ITS libraries converged into the lower diversities (Table 3). The rarefaction curves indicated that the number of sequences analyzed in 28S (cattle) and 18S (sheep) libraries might be insufficient to represent the whole fungal diversity in samples. However, the number of sequences analyzed in ITS (cattle) and ITS (sheep) are sufficient (Fig. 1).

Composition of libraries

In the study, the operational taxonomic units (OTUs) were defined by>97 % sequence similarity. Although the ITS (cattle), 18S (cattle), and 28S (cattle) liberaries were constructed from the same DNA extracts, the fungal taxa analysis based on these libraries are different (Fig. 2). The ITS (cattle) is comprised of *Ascomycota* (47 %), *Basidiomycota* (13 %), *Chytridiomycota* (33 %), and protozoa (0 %). The 18S (cattle) is comprised of *Ascomycota* (13 %), *Basidiomycota* (1 %), *Chytridiomycota* (13 %), and protozoa (70 %). The 28S (cattle) library is comprised of *Ascomycota* (86 %). Only mitosporic fungi were detected in the three libraries. Similar results were obtained from ITS (sheep), 18S (sheep), and 28S (sheep) libraries (Fig. 3). It was considered that primer sets toward different DNA regions lead to the difference in structures of fungal communities. The ITS libraries could be adopted to analyze the fungal community. The 18S libraries could be used to analyze the fungi and protozoa communities. However, the 28S are suitable for analysis of *Ascomycota* fungi. The major fungal taxa in cattle feces analyzed by ITS, 18S, and 28S libraries are similar to those of sheep feces,

Table 2 Characteristics of phylotypes of the ITS, 18S, and 28S libraries

Library type	Primer Sets	The proportion of (%) clones showed the different highest similarity						
		100 %	99 %	98 %	97 %	96 %	95 %	< 94 %
ITS Library (cattle)	ITS1F, ITS4B	5 %	40 %	5 %	25 %	0 %	0 %	25 %
18S Library (cattle)	Euk1A, Euk516R	4 %	45 %	21 %	19 %	0 %	6 %	5 %
28S Library (cattle)	Nl209, Nl912	0 %	33 %	7 %	22 %	15 %	10 %	13 %
ITS Library (sheep)	ITS1F, ITS4B	8 %	76 %	4 %	4 %	0 %	0 %	8 %
18S Library (sheep)	Euk1A, Euk516R	2 %	72 %	10 %	10 %	3 %	0 %	3 %
28S Library (sheep)	Nl209, Nl912	0 %	40 %	2 %	23 %	18 %	8 %	9 %

Table 3 The Shannon Weinner indexes and library coverages of the ITS, 18S, and 28S libraries

Library	Shannon Weinner index	Library coverage
ITS (cattle)	2.207	0.65
ITS (sheep)	2.179	0.72
18S (cattle)	2.699	0.85
18S (sheep)	3.069	0.75
28S (cattle)	3.159	0.7
28S (sheep)	2.487	0.9

respectively. However, the major fungal taxa in cattle and sheep feces obtained from ITS libraries are different from those of 18S and 28S libraries.

Fungal diversity

At the genus level, 10, 13, and 21 OTUs were detected in cattle feces by ITS, 18S and 28S libraries, respectively. A total of 43 fungal and protozoa genera were detected in cattle feces. The *Ascobolus* was detected in the cattle feces by ITS, 18S, and 28S libraries. The *Candida* were detected by ITS and 28S libraries, not by 18S libraries. In total, 11, 20, and 13 OTUs were detected in sheep feces by ITS, 18S, and 28S libraries, respectively. A total of 40 fungal and protozoa genera were detected in sheep feces by the three libraries. *Ascobolus* was detected in all three libraries. The *Preussia* genus was detected in the ITS and 28S libraries. The *Mortierella* genus was detected by the ITS and 18S libraries. The detection of *Ascobolus* genus by the six libraries indicates the inherent fungi in ruminant feces. Other fungal genera were detected by only one library or two libraries. In the study on fecal fungal diversity of ruminants, the fungal taxa detected by the ITS library comprised only 20 % fungal taxa detected by the three libraries; the 18S library comprised 30 % fungal taxa, and the 28S library comprised about 50 % fungal taxa. The comparison of ITS, 18S, and 28S libraries could find more diverse fungi than that based on only one library.

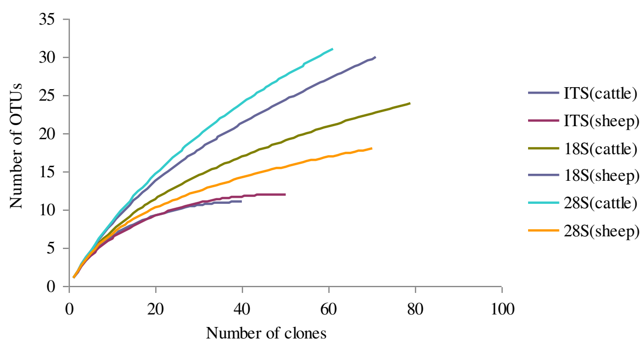

Fig. 1 Rarefaction curves of OTUs to sequencing effort in ITS, 18S, and 28S libraries

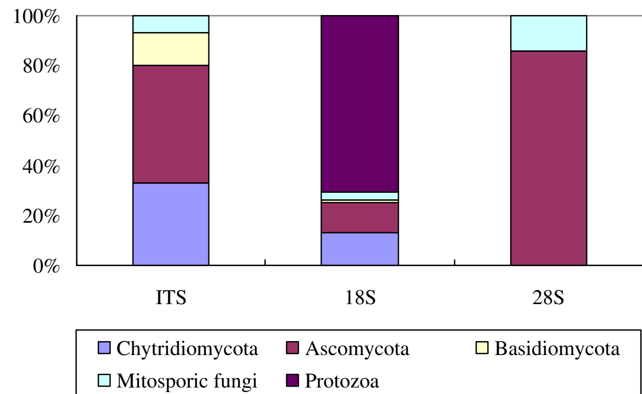

Fig. 2 Phylum or the higher-level taxonomic composition of fungi in cattle feces

Discussion

The sequential fruiting of coprophilous fungi on herbivore dung after deposition is a well known phenomenon (Abranches et al. 1998). Many species of fungi are specialised to live on dung. Diverse yeasts (such as *Debaryomyces*, *Pichia*, and *Candida*) and filament fungi (such as *Ascobolus*) have been isolated from herbivores (Abranches et al. 1998; Nyberg and Persson 2002). The yeasts *Candida*, *Rhodotorula,* and *Sporobolomyces,* and filament fungus *Ascobolus* were all detected in cattle and sheep feces in the studies. Anaerobic fungi belonging to the order *Neocallimastigales* play a key role in plant fiber degradation in the rumen (Griffith et al. 2009; Nagpal et al. 2011), and they have been detected in the previous studies (Liggenstoffer et al. 2010; Nicholson et al. 2010). The anaerobic fungi were detected in ITS (cattle), 18S (cattle), and 18S (sheep) libraries; however, they were not detected in ITS (sheep), 28S (cattle), and 28S (sheep) libraries. Compared with previous studies, it was believed that the fungal community analysis results of ITS, 18S, and 28S libraries are reliable. However, the fungal taxa detected by ITS, 18S, and 28S libraries are different. *Ascomycota* had low diversity (4 OTUs) in the ITS (cattle) library, but more diverse *Ascomycota* (19 OTUs) were

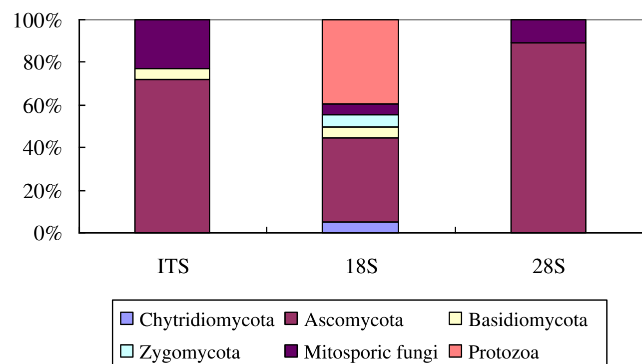

Fig. 3 Phylum or the higher-level taxonomic composition of fungi in sheep feces

detected in the 28S (cattle) library. The libraries were constructed by PCR from the same DNA template extracted with different primer sets. The differences might be due to the primer bias.

In mycology, the ITS region of the nuclear ribosomal repeat unit is by far the most commonly sequenced region for queries of systematics and taxonomy at and below the genus level (Nilsson et al. 2009). The ITS was generally superior to 28S in species discrimination and had a more clearly defined barcode gap (Schoch et al. 2012). However, all primer sets have a range of biases, and an appropriate solution will be to used more than one primer combination (Bellemain et al. 2010). The ITS region provided a more accurate and extensive assessment of community richness and species identity than the 18S region (Lord et al. 2002). In the study, the 18S libraries contained diverse protozoa in cattle and sheep feces; the 18S region is more suitable for protozoa diversity compared with the ITS region and the 28S region.

The fungal taxa detected by the ITS library comprised only 20 % fungal taxa detected by the three libraries; the 18S library comprised 30 % fungal taxa, and the 28S library comprised about 50 % fungal taxa. The fungal community analysis based on one primer set may underestimate the fungal diversity. A comprehensive fungal diversity analysis should be based on multiple primer sets.

Coprophilous fungi secrete enzymes to degrade the most recalcitrant parts of plant biomass that have resisted the digestive process; the secretomes of coprophilous fungi have high potential to contain novel and efficient plant cell wall-degrading enzymes of biotechnological interest (Peterson et al. 2011). The heat-tolerant, alkaline pH, or cold-tolerant extracellular enzymes have been found from fungal strains isolated from koala feces; the enzymes showed potential for industrial processes such as the manufacture of papers, detergents, food products, and bioenergy. The comprehensive analysis of fungal communities from herbivore feces would be helpful for exploration of the fungal resources with biotechnological applications.

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