

## The Composition of Fungal Communities in the Rumen of Gayals (*Bos frontalis*), Yaks (*Bos grunniens*), and Yunnan and Tibetan Yellow Cattle (*Bos taurus*)

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Submitted 30 May 2019, revised 17 October 2019, accepted 17 October 2019

### Abstract

The rumen is a microbial-rich ecosystem in which rumen fungi play an important role in the feed digestion of ruminants. The composition of rumen fungi in free-range ruminants such as gayals, yaks, Tibetan yellow cattle, and the domesticated Yunnan yellow cattle was investigated by sequencing an internal transcribed spacer region 1 (ITS1) using Illumina MiSeq. A total of 285 092 optimized sequences and 904 operational taxonomic units (OTUs) were obtained from the four cattle breeds. The rumen fungi abundance and Chao and Simpson indexes were all higher in free-range ruminants than in domesticated ruminants. Three fungal phyla were identified by sequence comparison: Neocallimastigomycota, Basidiomycota, and Ascomycota. Basidiomycota and Ascomycota have very low abundance in the rumen of four breeds cattle but anaerobic fungi (AF) Neocallimastigomycota occurred in a high abundance. In Neocallimastigomycota, the dominant genera were *Piromyces*, *Anaeromyces*, *Cyllamyces*, *Neocallimastix*, and *Orpionmyces* in four cattle breeds. The composition of the major genera of Neocallimastigaceae varied greatly among the four cattle breeds. The unclassified genera were unequally distributed in gayals, yaks, Tibetan and Yunnan yellow cattle, accounting for 90.63%, 98.52%, 97.79%, and 27.01% respectively. It appears that free-range ruminants have more unknown rumen fungi than domesticated ruminants and the cattle breeds and animal diets had an impact on the diversity of rumen fungi.

**Key words:** gayals, yaks, Yunnan yellow cattle, Tibetan yellow cattle, rumen fungi, ITS-sequencing

### Introduction

Ruminant animals lack the carbohydrate-active enzyme encoding genes, so feed (carbohydrate) metabolism is completely dependent on the microorganisms residing in their rumen (Kameshwar and Qin 2018). Current research on rumen fungi has focused on anaerobic rumen fungi. Anaerobic rumen fungi play a very important role in the digestion and metabolism of carbohydrates in the rumen (Gruninger et al. 2018; Kameshwar and Qin 2018). Anaerobic rumen fungi can secrete large amounts of cellulolytic enzymes. Their hyphae can destroy the cell wall structure of

plant feed owing to the combination of enzymes and degradable cellulose and this improves the degradation and utilization rates of plant feed (Lee et al. 2000; Gruninger et al. 2018; Kameshwar et al. 2018). Currently, the rumen AF (anaerobic fungi) are classified into phylum Neocallimastigomycota (Gruninger et al. 2014) and Neocallimasticaceae (Hibbett et al. 2007). Neocallimasticaceae was divided into eleven genera, containing a large number of monocentric rumen AF: *Neocallimastix*, *Piromyces*, *Caecomyces*, *Oontomyces* (Dagar et al. 2015a), *Pecoramyces* (Hanafy et al. 2017), *Feramyces*, *Liebetanzomyces* (Hanafy et al. 2018), and *Buwchfawromyces* (Griffith et al. 2015), as well as three

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polycentric genera: *Orpinomyces*, *Anaeromyces* (Breton et al. 1990), and *Cyllumyces* (Emin et al. 2001). The studies have shown that these AF are important in the rumen microbial system (Orpin 1975; Ho and Barr 1995). However, recent studies have shown that Basidiomycota and Ascomycota phyla play an important role in the rumen digestion of Holstein cows and cashmere goats (Zhang et al. 2017; Han et al. 2019).

Little is known about rumen AF and most of them were identified by microscopic conventional cultivation techniques, which provides important information for rumen AF (Breton et al. 1990; Ho and Barr 1995). The limitations of these methods are mainly due to the strict growing requirements and the low survival rate but molecular biology techniques can overcome these problems (Pryce et al. 2006). The fungal ribosomal RNA gene includes a gene encoding 28S ribosomal DNA, 18S ribosomal DNA, and 5.8S ribosomal DNA, which in the internal transcribed spacer Region1 evolves rapidly (Sirohi et al. 2013; Elekwachi et al. 2017). The last-mentioned gene has the interspecies specificity and intraspecies conservation, and the length of the sequence is moderate enough to get sufficient information (Pryce et al. 2006; Campa et al. 2008; Bellemain et al. 2010). ITS sequencing is used to study the diversity of the community of rumen AF and provide genetic information for the classification and identification of fungi (Liggenstoffer et al. 2010; Koljalg et al. 2013).

The free-range gayals are mainly distributed in the Nujiang River and Dulong River areas of Yunnan Province, China. Yaks live exclusively on the Qinghai-Tibetan Plateau, China (An et al. 2005) and are well adapted to harsh environmental conditions. Yunnan yellow cattle and Tibetan yellow cattle are common, wide-ranging cattle. Yunnan yellow cattle live in the same region as gayals and Tibetan yellow cattle in the same region as yaks (Deng et al. 2007; Leng et al. 2012). Rumen bacteria in gayals, Yunnan yellow cattle and yak have been already studied (Deng et al. 2007), but there is no research on their rumen fungi. Rumen anaerobic fungi are the first microorganisms attached to fibers during rumen microbial degradation (Bauchop 1979) and play an important role in the degradation process (Dagar et al. 2015b). Anaerobic fungi degrade lignocellulose using a large portfolio of Carbohydrate-Active enZymes (CAZymes) and penetrating hyphae that physically disrupt the ultrastructure of the plant cell wall; such action may help to increase the surface area for bacterial colonization and further enzymatic digestion (Lee et al. 2000; Gruninger et al. 2018; Kameshwar et al. 2018). Rumen anaerobic fungi have great application potential in industrial production. The AF cellulose degradation ability shows that it can increase biogas production in co-culture with methanogens (Cheng 2018). Studies have also shown that

rumen AF can reduce animal energy loss by reducing CH<sub>4</sub> (greenhouse gas) production during digestion, and it can also be used to improve the straw lignocellulosic structure in biofuels and biochemical production (Andrea et al. 2018; Oliver and Schilling 2018). These four cattle breeds are very important cattle species in China, and there are very few studies on their rumen fungi. Therefore, this paper conducted a comprehensive analysis of rumen fungi from four breeds of cattle to help us understand their rumen fungi function.

## Experimental

### Materials and methods

**Animals and sampling.** Sixteen male samples ( $3 \pm 0.25$  years old) were used in this study, including four cattle breeds and each breed comprised four cattle. Gayals and Yunnan yellow cattle were from the Nujiang Region, Yunnan Province, China ( $27^{\circ} 46' 55.15''$  N,  $98^{\circ} 39' 49.99''$  E above sea level 2260 m). Yaks and Tibetan yellow cattle were from the Diqing Region, Yunnan Province, China ( $27^{\circ} 51' 30.61''$  N,  $99^{\circ} 41' 42.82''$  E, above sea level 3280 m). Gayals, yaks, and Tibetan yellow cattle lived outside, ate mainly wild grass, without any supervision. Yunnan yellow cattle lived in cattle lairs and were fed rice bran and corn (Table II). Rumen contents were collected by gastric tube, filtered through four layers of cheesecloth and stored at  $-80^{\circ}\text{C}$  before DNA extraction.

**DNA extraction, PCR amplification, and sequencing.** DNA was extracted from 0.5 g of rumen contents per sample after thawing and mixing well, with the E.Z.N.A DNA kits for soil, following the manufacturer's introduction. PCR was conducted using universal fungal primers for ITS1, which are as follows: forward primer ITS1 5'-GGAAGTAAAAGTCGTAACAAGG-3' and reverse primer ITS2 5'-GCTGCGTTCTTCATC-GATGC-3' (Man et al. 2018). Each tube for amplification contained 4  $\mu\text{l}$  5  $\times$  FastPfu Buffer, 2  $\mu\text{l}$  dNTPs at a concentration of 2.5 mM, 0.8  $\mu\text{l}$  each primer at a concentration of 5  $\mu\text{M}$ , 0.4  $\mu\text{l}$  FastPu polymerase, and 10 ng DNA template with double-distilled H<sub>2</sub>O (ddH<sub>2</sub>O) added to 20  $\mu\text{l}$ . PCR was performed at  $95^{\circ}\text{C}$  for 2 min, and 33 cycles of  $95^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 30 s followed by incubation at  $72^{\circ}\text{C}$  for 5 min. The PCR product was purified using AxyPrep DNA gel extraction kits and eluted with Tris-HCl buffer. PCR products were quantified using the PicoGreen<sup>®</sup> dsDNA Assay Kit and QuantiFluor<sup>™</sup>-ST Blue Fluorescence System (Promega).

**Phylogenetic analysis.** Amplicons of ITS-1 sequences, which were moderately conserved regions of the 18S rRNA gene, were used for the sequence-specific

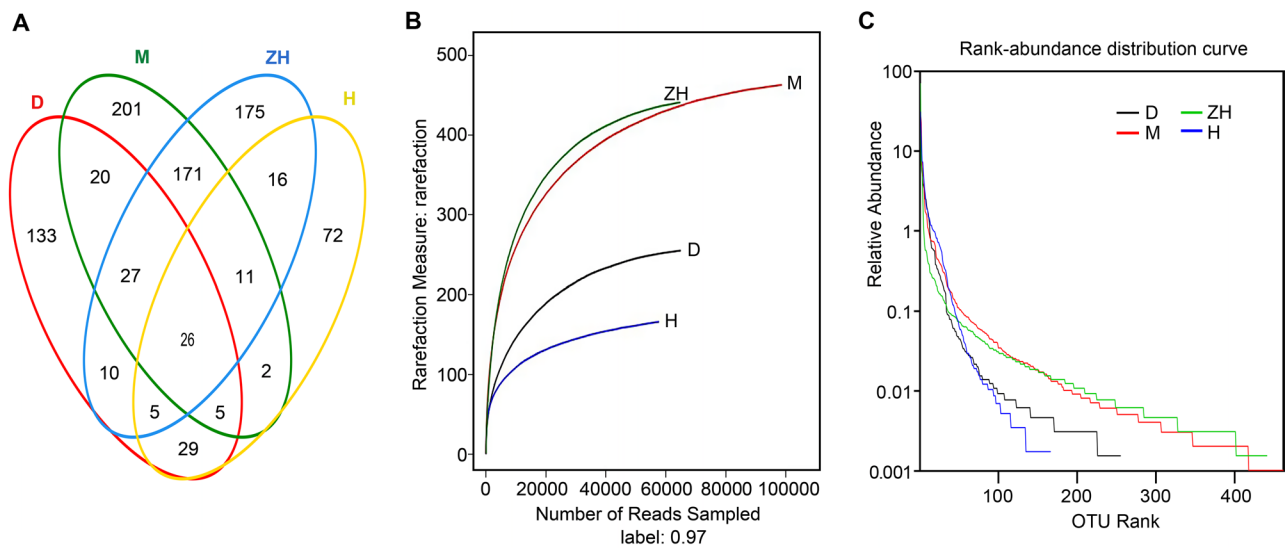


Fig. 1. Venn diagram, rarefaction index, and abundance distribution curves for D, H, M and ZH based on OTUs from cattle breeds, which had  $\geq 97\%$  similarity.

D – gayals; H – Yunnan yellow cattle; M – yaks; ZH – Tibetan yellow cattle. Red line represents D. Blue line represents ZH. Green line represents M. Yellow line represents H.

separation. ITS-1 sequencing was regarded as an effective method for recognizing the diversity and structure of rumen fungi. The PE reads obtained by Miseq sequencing are first spliced according to the overlapping relationship, and the quality and quality of the sequence were quality-controlled and filtered. The operational taxonomic units (OTUs) cluster analysis and species taxonomic analysis were performed after the samples were distinguished. The various diversity index analysis could be performed based on OTUs. Cluster analysis results could be used to analyze the diversity index of OTUs and the establishment of the sequencing depth. Based on the taxonomic information, the statistical analysis of community structure could be performed at each classification level. Based on 97% similarity of OTUs the successful identification of species from *Piromyces* and *Neocallimastix* in the goat and buffalo rumen was performed (Brookman et al. 2000). The 97% similarity level to partition OTUs was used in this work. Basic diversity estimates and rarefaction curves were computed as  $OTU_{0.05}$  outputs using Mothur (Schloss et al. 2011), including analyses of diversity indexes ACE, Chao, Shannon and Simpson. In addition to the diversity curves, rarefaction and rank-abundance curves were also generated to describe the sequencing depth using Mothur. The Unite database (Koljalg et al. 2013) was used to determine taxonomic information on OTUs. Based on taxonomic information such as abundance and the genera of all OTUs, phylogenetic trees were built that included abundance and structure of rumen fungi using MEGAN and NCBI databases. Venn and Heatmap diagrams were built using the R language (Jami et al. 2013).

## Results

A total of 622 176 original sequences were obtained from four cattle breeds by Illumina sequencing of which 297 745 sequences remained after quality control measures, and 285 092 sequences were used for phylogenetic analyses. The average length of trim sequences used for analyses was 317.6 bp. The average number of sequences from the different cattle breeds is shown in Table I. Rarefaction curve analysis (Fig. 1B) indicated that sequences collected in this study comprised the majority of rumen fungi sequences from the four cattle breeds. The taxonomic analysis was reflected in the cluster analysis of OTUs, with 904 OTUs from the four cattle breeds: 255 OTUs from gayals, 166 from Yunnan yellow cattle, 463 from yaks, and 441 from Tibetan yellow cattle (Table I). The four cattle breeds had unique OTUs of rumen AF and shared OTUs across rumen AF (Fig. 1A).

**Diversity analysis.** ACE, Chao, Shannon and Simpson diversity indexes were calculated to determine the diversity of rumen fungi in the cattle breeds. In this study, the largest ACE and Chao indexes were for yaks, followed by Tibetan yellow cattle, gayals, and Yunnan yellow cattle, which indicated that yaks had a larger number of rumen fungi than the other three species. Although the abundance of rumen fungi in gayals and Yunnan yellow cattle was lower than in yaks and Tibetan yellow cattle, their diversity was close to yaks and higher than Tibetan yellow cattle as demonstrated by the Shannon and Simpson indexes (Table I).

With increasing sequencing depth, the number of OTUs was unchanged (Fig. 1B). A rank-abundance

Table I  
Diversity index of anaerobic fungal communities among the cattle breeds.

Cattle breeds	Feed	Reads	0.97 (level)				
			OTU <sub>0.05</sub>	Ace	Chao	Shannon	Simpson
D	weeds	64818	255	<sup>a</sup> 272 ( <sup>b</sup> 263, <sup>c</sup> 288)	<sup>a</sup> 263 ( <sup>b</sup> 258, <sup>c</sup> 276)	<sup>a</sup> 2.17 ( <sup>b</sup> 2.15, <sup>c</sup> 2.18)	<sup>a</sup> 0.3102 ( <sup>b</sup> 0.3063, <sup>c</sup> 0.3142)
H	feed	57567	166	<sup>a</sup> 193 ( <sup>b</sup> 180, <sup>c</sup> 220)	<sup>a</sup> 191 ( <sup>b</sup> 176, <sup>c</sup> 225)	<sup>a</sup> 2.78 ( <sup>b</sup> 2.76, <sup>c</sup> 2.79)	<sup>a</sup> 0.1352 ( <sup>b</sup> 0.1332, <sup>c</sup> 0.1372)
M	weeds	98550	463	<sup>a</sup> 487 ( <sup>b</sup> 476, <sup>c</sup> 505)	<sup>a</sup> 477 ( <sup>b</sup> 470, <sup>c</sup> 494)	<sup>a</sup> 2.82 ( <sup>b</sup> 2.80, <sup>c</sup> 2.83)	<sup>a</sup> 0.1622 ( <sup>b</sup> 0.1606, <sup>c</sup> 0.1639)
ZH	weeds	64694	441	<sup>a</sup> 461 ( <sup>b</sup> 452, <sup>c</sup> 477)	<sup>a</sup> 451 ( <sup>b</sup> 445, <sup>c</sup> 466)	<sup>a</sup> 1.74 ( <sup>b</sup> 1.72, <sup>c</sup> 1.76)	<sup>a</sup> 0.4905 ( <sup>b</sup> 0.4858, <sup>c</sup> 0.4953)

Weeds: bamboo or other wild grass; feed: rice bran and corn

a – average; b – minimum number; c – maximum number;

D – gayals; H – Yunnan yellow cattle; M – yaks; ZH – Tibetan yellow cattle

distribution curve was constructed to reflect the abundance and uniformity of rumen fungi. The width of the curve indicated the highest abundance of rumen fungi for yaks and the least abundance for Yunnan yellow cattle; the abundance of Tibetan yellow cattle was close to yaks (Fig. 1C). According to the sequence abundance of the top 50 OTUs, the relative abundance of rumen fungi was similar. However, abundance differed among the different cattle breeds with increasing OTU ranking (Fig. 1C). The uniformity of rumen fungi was similar when the relative abundance was under 0.01, indicating that yaks, Tibetan yellow cattle, and gayals had a similar abundance and uniformity of fungi (Fig. 1C).

**Phylogenetic analysis.** A phylogenetic tree based on OTUs was constructed (Fig. 3). Rumen fungi from the

ITS-1 phylogenetic tree were mainly divided into three subdivisions. Three fungal phyla were identified by sequence comparison: Neocallimastigomycota, Basidiomycota, and Ascomycota (Table III), the remaining sequences were unclassified. We detected 62 dominant genera from Ascomycota, but the abundance was very low of each genus. The most abundant genus was *Cladosporium*, accounting for 306 sequences, mainly distributed in yaks. *Udeniomyces* was the most abundant among 29 genera from Basidiomycota, accounting for 2021 sequences, and was mainly distributed in Yunnan yellow cattle (Fig. 3). Further analysis showed large differences in the composition of the primary genera of Neocallimastigaceae between different cattle breeds. The dominant genera were *Piromyces*, *Anaeromyces*,

Table II  
Cattle diet and nutrient levels.

Feeds	Dietary nutrients (%)				
	DM	CP	EE	NDF	ADF
Bamboo diet	48.10 ± 9.85	13.06 ± 1.20	3.08 ± 0.69	72.13 ± 1.54	42.76 ± 3.02
Wild grass	70.24 ± 0.56	1.44 ± 0.10	0.04 ± 0.02	30.12 ± 0.17	18.83 ± 0.10
Rice bran	87.03 ± 0.22	12.82 ± 0.16	16.53 ± 0.18	22.91 ± 0.21	13.44 ± 0.52
Corn	86.15 ± 0.14	8.38 ± 0.13	3.01 ± 0.24	8.59 ± 0.62	3.27 ± 0.54

DM – Dry matter; CP – Crude protein; EE – Ether extract; NDF – Neutral detergent fiber;

ADF – Acid detergent fiber

Table III  
Fungal classification and the percentage statistics.

Total sequence	Phylum	Sequences	Percents	Dominant genus
285092	Neocallimastigomycota	63 535	22.28%	<i>Piromyces</i> , <i>Anaeromyces</i> , <i>Cyllumyces</i> , <i>Neocallimastix</i> and <i>Orpionmyces</i>
	Basidiomycota	6 030	2.11%	<i>Udeniomyces</i>
	Ascomycota	2 740	0.96%	<i>Cladosporium</i>

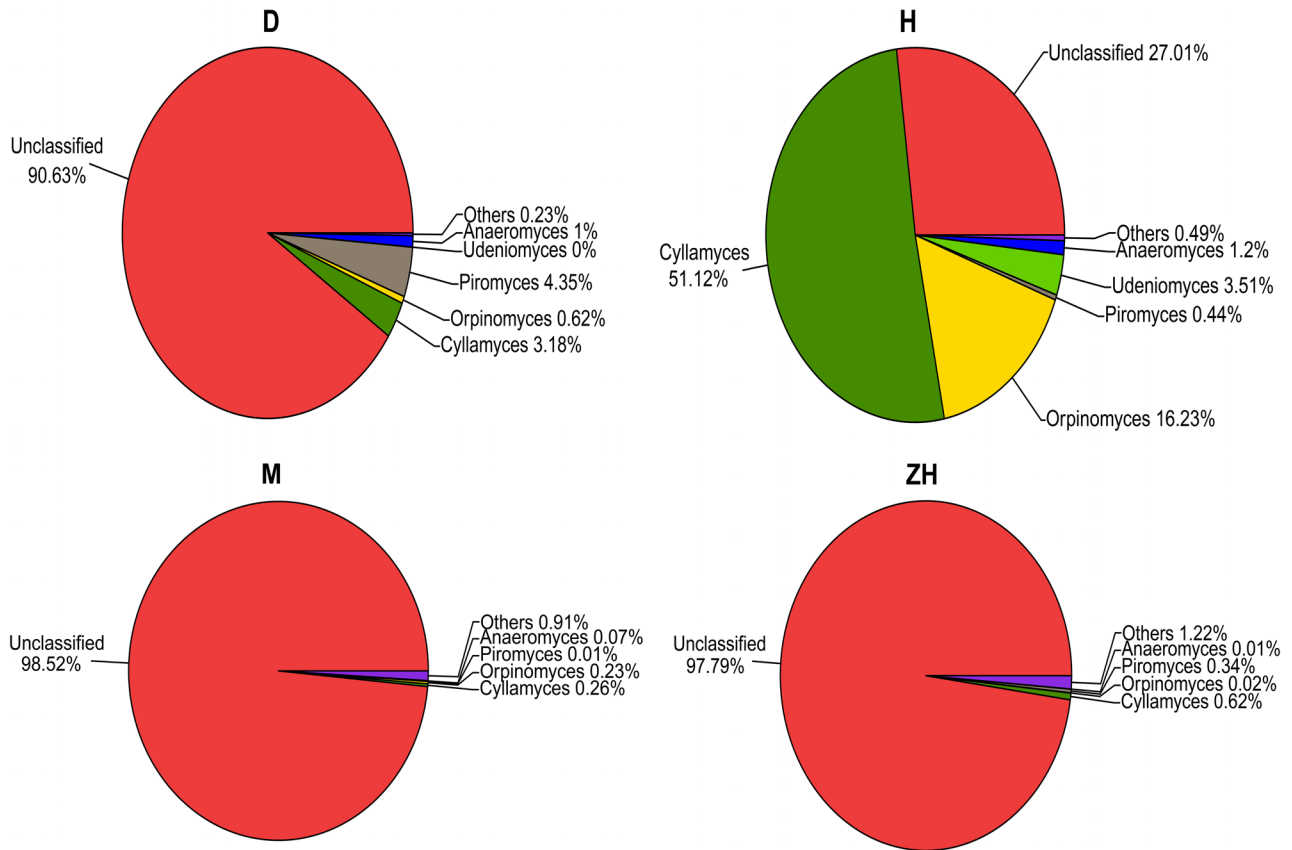


Fig. 2. Composition of rumen fungi genera. Others represent the abundance of rumen fungi lower than 1%.

D – gayals; H – Yunnan yellow cattle; M – yaks; ZH – Tibetan yellow cattle.

*Cyllamyces*, *Neocallimastix*, and *Orpinomyces*. In gayals, *Piromyces*, *Cyllamyces*, and *Anaeromyces* were the classified dominant rumen AF, accounting for 4.35%, 3.18%, and 1% of total sequences. *Cyllamyces* and *Orpinomyces* dominated in Yunnan yellow cattle, accounting for 51.12%, and 16.23% respectively. *Piromyces*, *Anaeromyces*, *Cyllamyces*, and *Orpinomyces* accounted for less than 1% of the total sequences in yaks and Tibetan yellow cattle. *Neocallimastix* was detected only in yaks and Tibetan yellow cattle and accounted for less than 0.01% of the total sequences. The most dominant genus was *Cyllamyces*, accounting for 32 146 sequences and 50.64% of the total *Neocallimastigaceae* sequences (Fig. 2). *Cyllamyces* accounted for 51.12% of the total sequences in Yunnan yellow cattle, was the second-most abundant (3.18%) in gayals, and the least abundant (0.26%) in yaks. *Piromyces* accounted for 4.35% of the total sequences in gayals, was the second-most abundant (0.44%) in Yunnan yellow cattle, and the least abundant (0.01%) in yaks. *Orpinomyces* accounted for 16.23% of the total sequences in Yunnan yellow cattle, was the second-most abundant (0.62%) in gayals, and the least abundant (0.02%) in Tibetan yellow cattle. *Anaeromyces* accounted for less than 1% of all species. *Udeniomyces* from Basidiomycota were detected only in Yunnan yellow cattle and comprised 3.51% of total sequences.

The hierarchical clustering heatmap analysis was performed at the class level based on the top 95 most abundant communities across the four cattle breeds (Fig. 4). Results were separated into five clusters. The abundance of *Anaeromyces*, *Orpinomyces*, *Piromyces*, and *Cyllamyces* was higher than for the other genera in the first cluster. In the second cluster, five fungal genera were more abundant in Yunnan yellow cattle compared to gayals, yaks, and Tibetan yellow cattle. In the third cluster, the unidentified class was the most abundant in gayals and yaks, with eight genera from Tibetan yellow cattle, which were more abundant than in gayals, yaks, and Yunnan yellow cattle. In yaks, 21 genera of fungi were more abundant than in gayals, Yunnan yellow cattle, and Tibetan yellow cattle in the fourth cluster. The 11 genera were the most dominant in gayals when compared to yaks, Tibetan yellow cattle, and Yunnan yellow cattle in the fifth cluster.

## Discussion

The previous studies have shown that Illumina sequencing has a higher capacity to explore rumen bacteria diversity than culture-dependent methods (Peng et al. 2015). PCR amplification of universal primers for

conserved regions within the rRNA genes, followed by DNA sequencing of the internal transcribed spacer (ITS) is widely used in fungal identification studies (Pryce et al. 2006). Primers using ITS1 can avoid bias in PCR amplification and reliably study the fungal abundance and species richness (Bellemain et al. 2010). This study used the second-generation sequencing technology to investigate the structure and diversity of rumen fungi communities in four cattle breeds. The results provide new information about rumen fungi communities. The analysis showed that the dominant rumen fungi clusters, distribution, and abundance present major differences among the cattle breeds, location, and feeds.

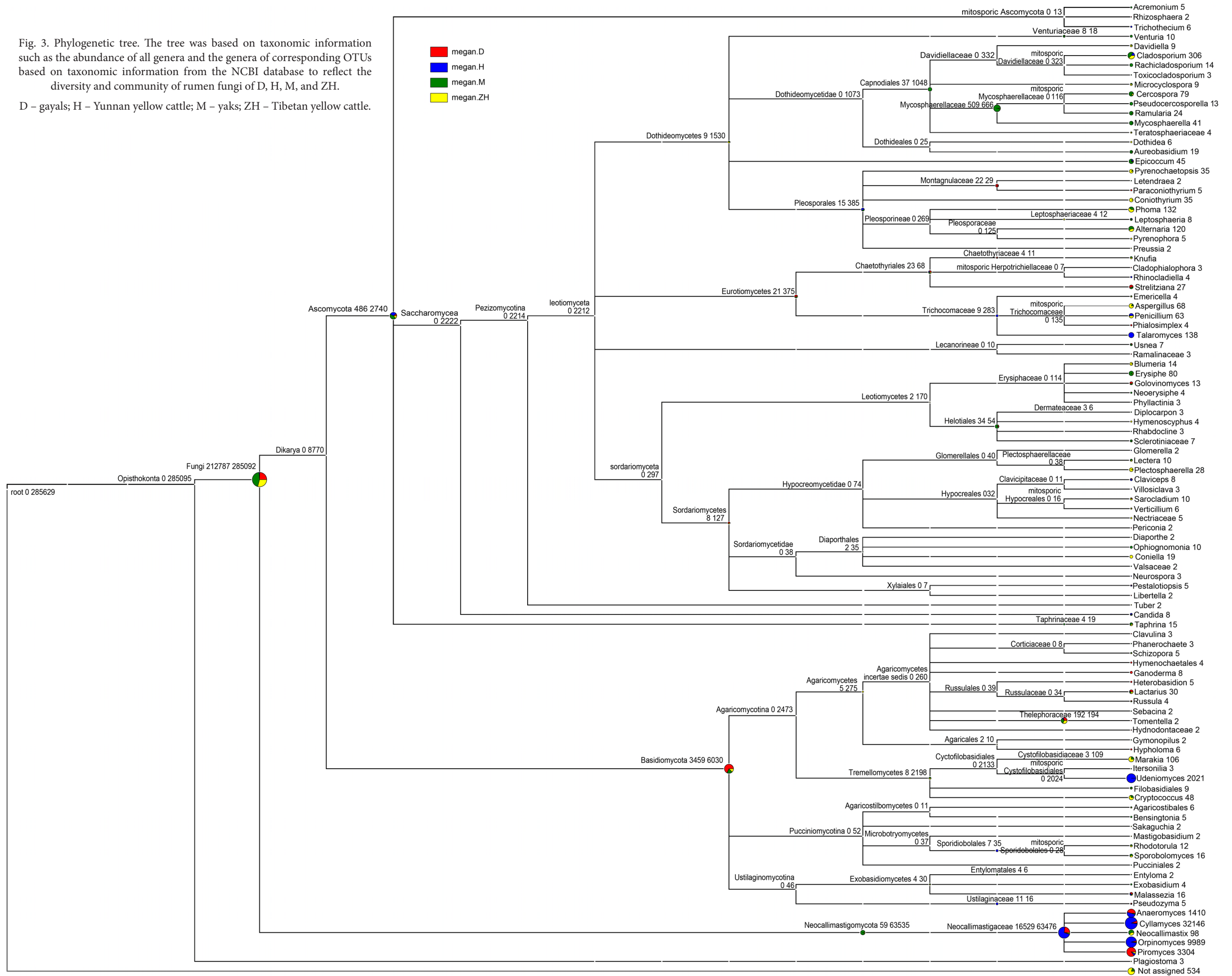
Free-range ruminants that use grass as food may require more anaerobic fungal cellulase to aid digestion than domesticated ruminants. When compared with Yunnan yellow cattle, gayals, yak, and Tibetan yellow cattle have abundant rumen fungi sequences (Table I) and more unique OTUs (Fig. 1A). Analysis of ACE, Chao, and Simpson indexes showed that gayals, yaks and Tibetan yellow cattle had higher indexes than Yunnan yellow cattle, but the Shannon index was smaller than for Yunnan yellow cattle (Table I). These results suggest that free-range gayals and Tibetan cattle can have higher rumen fungi diversity than domesticated Yunnan cattle. Unclassified sequences were 90.63% for gayals, 98.52% for yaks, 97.79% for Tibetan yellow cattle, and 27.01% for Yunnan yellow cattle (Fig. 2), which was consistent with the heatmap analysis (Fig. 4). These results showed that the class levels could be divided into five clusters based on the top 95 genera. Many unidentified genera were distributed in the third cluster and were dominant in gayals, yaks, and Tibetan yellow cattle. These results indicated that free-range ruminants were more likely to have unknown yet fungi.

Animal species and location may be the important factors influencing the distribution and abundance of dominant rumen fungi clusters. Analysis of phylogenetic trees detected three dominant phyla rumen fungi: Ascomycota, Basidiomycota, and Neocallimastigomycota in the four cattle breeds in this study (Fig. 3), similar to the results of Zhang and Han studies (Zhang et al. 2017; Han et al. 2019). But the abundance of Neocallimastigomycota is superior to Ascomycota and Basidiomycota in this study, contrary to the results of the study on the cashmere goats (Han et al. 2019). However, Neocallimastigomycota predominates in the rumen, which is similar to the results on most ruminant rumen anaerobic fungi (Youssef et al. 2013; Wei et al. 2016; Rabee et al. 2018). A previous study showed that the genera of Ascomycota and Basidiomycota efficiently produce beta-glucanase (Mintz-Cole et al. 2013), possibly promoting the digestibility of feed, even though their abundance is low.

Different cattle breeds have different dominant rumen fungi clusters (Fig. 4) and the abundance of rumen fungi (Table I). *Anaeromyces* and *Piromyces*, *Orpinomyces*, and *Cyllamyces* were most abundant in gayals and Yunnan yellow cattle in the first cluster. Five genera of rumen fungi in Yunnan yellow cattle were more numerous than was shown for gayals, yaks, and Tibetan yellow cattle. The eight genera of rumen fungi in Tibetan yellow cattle were more numerous than in gayals, yaks, and Yunnan yellow cattle. The 21 genera of rumen AF in yaks were more numerous than in gayals, Yunnan yellow cattle, and Tibetan yellow cattle. Finally, the 11 genera of rumen fungi in gayals were more numerous than in Yunnan yellow cattle, Tibetan yellow cattle, and yaks in the clusters 2–5. *Piromyces*, *Cyllamyces*, and *Anaeromyces* were the most often classified abundant rumen AF in gayals (Fig. 2 and 3). These three representative genera were more prevalent in 19 ruminant and nonruminant animals (Liggenstoffer et al. 2010). However, in the other three cattle breeds, *Piromyces* accounted for less than 0.44% of the total sequences. *Cyllamyces* was the most abundant rumen AF genus in a previous report, accounting for 67% of total sequences in domesticated ruminants (Fliegerova et al. 2010). In our study, *Cyllamyces* was mainly found in Yunnan yellow cattle, accounting for 51.12% of the total sequences, and were more numerous than in gayals (3.18%), Tibetan yellow cattle (0.62%), and yaks (0.26%) (Fig. 2). *Cyllamyces* was also detected in American bison by Liggenstoffer et al. (2010) but the abundance was less than 0.7% of the total sequences, suggesting that *Cyllamyces* is more likely to be present in domesticated ruminants than other genera, which is consistent with Ozkose et al. (2001). We found two *Anaeromyces* species, *A. elegans* and *A. mucronatus* (Breton et al. 1990) that have high cellulolytic, xylanolytic, and glycoside hydrolase activities in different ruminant hosts. The  $\beta$ -xylosidase activities of *A. mucronatus* are higher in buffalo and endo-1,4- $\beta$ -D-glucanohydrolase has the highest activity in alpaca (Fliegerova et al. 2002) and they are more effective at degrading the stem fragments of ryegrass than *Caecomyces* (Joblin et al. 2002). *Anaeromyces* occurred the least often in Tibetan yellow cattle, accounting for 0.01% of all sequences (Fig. 2). These findings were consistent with a previous study on the relationship of the abundance of *Piromyces* and *Anaeromyces* in crude feed and the host (Liggenstoffer et al. 2010). *Neocallimastix* accounted for less than 0.01% of the total sequences (Fig. 3) and was found only in yaks and Tibetan yellow cattle, which were selected from the Diqing Region. However, Kittelmann and coworkers have found that *Neocallimastix* is dominant in New Zealand cattle, accounting for 26.4% of all sequences (Kittelmann et al. 2012). This result indicates that the abundance of *Neocallimastix* may be related to cattle breeds or habitat.

Fig. 3. Phylogenetic tree. The tree was based on taxonomic information such as the abundance of all genera and the genera of corresponding OTUs based on taxonomic information from the NCBI database to reflect the diversity and community of rumen fungi of D, H, M, and ZH.

D – gayals; H – Yunnan yellow cattle; M – yaks; ZH – Tibetan yellow cattle.



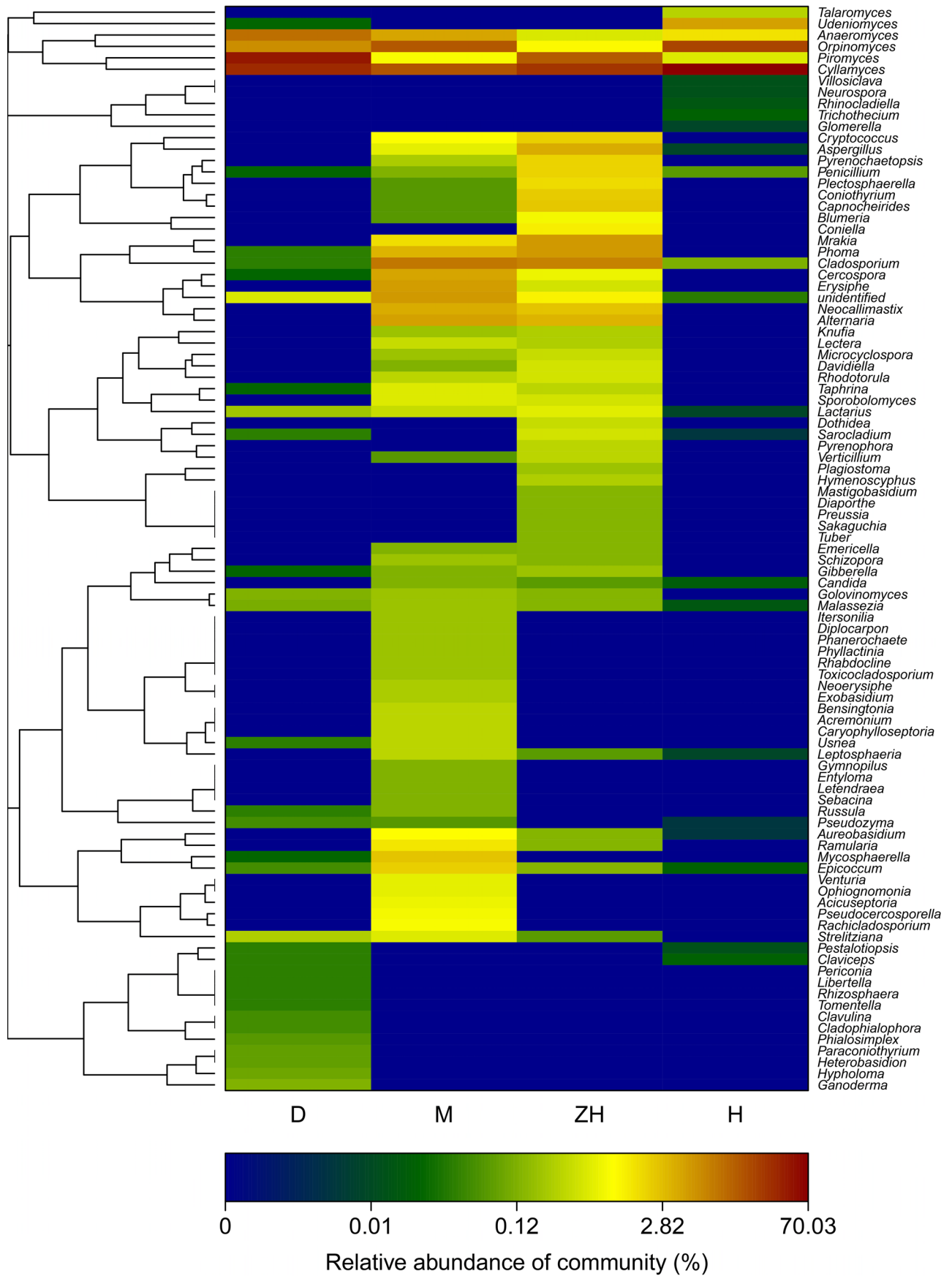


Fig. 4. Heatmap formed using the Bray-Curtis algorithm and the complete linkage method. The heatmap-plot describes the relative percentage of each fungal class within each cattle breed. Relative values for the fungal class are indicated by color intensity.

D – gayals; H – Yunnan yellow cattle; M – yaks; ZH – Tibetan yellow cattle.



Diet can also be an important factor. Han and coworkers research has shown that the concentrated feed has an important impact on the anaerobic fungal population of cashmere goats AF (Han et al. 2019). AF secrete a range of cell wall degrading enzymes such as free enzymes and cellulase multienzyme complexes (Cheng et al. 2018), which are effective degradation products of plant biomass (Haitjema et al. 2014). Screening of all rumen microbial CAZyme transcripts indicated that the AF of Neocallimastigaceae produced the largest share of cellulase transcripts (Söllinger et al. 2018). The studies have shown that *Orpinomyces* R001 and *Neocallimastix* M010 AF exhibit high digestion efficiency in the cell walls of straw silage and can cause the disappearance of *in situ* dry matter (Lee et al. 2015). The co-culture of *Neocallimastix frontalis* and *Methanobrevibacter ruminantium* showed high polysaccharide hydrolase (xylanase and FPase) and esterase activity (Wei et al. 2016). *Piromyces* sp. UH3-1 recognizes the secretion of lignin-regulating enzymes by the fungal pathway and is capable of producing a more efficient enzyme mixture (Hooker et al. 2018). Denman and coworkers have found that *Orpinomyces* had higher abundance with grain feed compared to fiber diets (Denman et al. 2008). However, another study found that the activity of cellulases and xylanases of *Orpinomyces* are related to the presence of *Neocallimastix* genus (Li et al. 1997). The biofortification of *Orpinomyces* sp. can significantly increase methane production (Akyol et al. 2019). In our study, *Orpinomyces* accounted for 16.23% of the total sequences in Yunnan yellow cattle and were more numerous than in gayals (0.62%), yaks (0.23%), and Tibetan yellow cattle (0.02%) (Fig. 2). This study suggested that *Orpinomyces* tended to be found in domesticated ruminants with easily digested diets.

This study found a large difference in rumen fungi abundance among four cattle breeds. Rumen fungi diversity and composition were mainly related to diet, and the use of its components depends on enzyme activity and quantity produced by these fungi. To better understand the relationship between fungal composition and function and the ruminant growth, as well as to extract cellulases from rumen fungi, the metagenomic and metatranscriptomic analyses should be used in future studies.

#### Ethical approval

All animal care procedures were approved and authorized by the animal ethics committee of Yunnan Agricultural University.

#### Acknowledgments

This study was financed by the National Natural Science Foundation of China (Project No: 31672452), the Key Research and Development Plan Project of Yunnan province (Project No: 2018BB001-2), and the Foundation of Yunnan Provincial Key

Laboratory of Animal and Feed Science (Project No: DYCX2015001) are acknowledged with gratitude.

#### Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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