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Phylogenetic analysis of yeast in the rumen contents of cattle based on the 26S rDNA sequence

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SUMMARY

The composition of yeast communities in the rumen of cattle was investigated using comparative DNA sequence analysis of yeast 26S rDNA genes. 26S rDNA libraries were constructed from rumen fluid (FF), rumen solid (FS) and rumen epithelium (FE). A total of 97 clones, containing a partial 26S rDNA sequence of 0·6 kb length, were sequenced and subjected to an on-line similarity search.

The 41 FF clones could be divided into five classes. The largest class was affiliated with *Pezizomycotina* class (85·4% of clones), and the remaining classes were related with the *Urediniomycotina* (2·4%), *Hymenomycetes* (4·9%), *Ustilaginomycetes* (4·9%) and *Saccharomycotina* (2·4%) classes. The 26 FE clones could be divided into three classes and the *Saccharomycetes* class (92·4% of clones) was the largest group. The remaining classes were related with either *Pezizomycotina* (3·8%) or *Ustilaginomycetes* (3·8%). The 30 FS clones were all affiliated with *Saccharomycotina*. *Saccharomycotina* were predominant in rumen epithelium and rumen solid while *Pezizomycotina* were predominant in rumen fluid. Yeast belonging to the *Saccharomycotina* class was predominant in the rumen as a whole (57%). One clone (FF34) had less than 90% similarity to any sequence in the database and was thus apparently unrelated to any previously described yeast.

INTRODUCTION

Yeasts were observed in the rumen contents of ruminants by Klein & Müller (1941). Lund (1974) isolated yeasts belonging to *Candida* (including corresponding species of *Pichia*), *Trichosporon*, *Torulopsis*, *Kluyveromyces*, *Saccharomycopsis* and *Hansenula*. The predominating species were *Candida krusei*, *Trichosporon cutaneum* and *Trichosporon capitatum*. Clarke & di Menna (1961) found viable counts of between 80–13 000 yeast colonies/g rumen content of cows. Also, Smith (1965) found an average of about 300 yeast cells/g rumen content of oxen. Most recent studies of yeast in rumen were focused on determining the effects of direct-fed microbials (DFM) on ruminant performance (Lachance *et al*. 2001; Fujihara *et al*. 2003). Hession *et al*. (1992) suggest that yeasts have a limited ability to grow in the ruminal environment and are therefore unlikely to have important effects on the rumen ecosystem.

Modern approaches to yeast identification include sequence analysis of selected regions of DNA, namely the D1/D2 domain of the 26S rDNA, a fragment of approximately 600–650 bp. Strains of the same species typically have either identical D1/D2 sequences or less than two mismatches (Fell *et al*. 2000). To bring an overall perspective to species relationships, Kurtzman & Robnett (1998) compared sequences from the D1/D2 domain. PCR techniques, in conjunction with cloning and sequence analyses, are useful in examining uncultured yeast because they do not rely on the ability to culture the yeast (Hugenholtz *et al*. 1998). The goal of this study is to examine the phylogenetic diversity of the yeast community in the rumen fluid, rumen solid and rumen epithelium of cattle by direct retrieval and 26S rDNA sequences in a culture-independent manner. To the best of our knowledge, this is the first information on the yeast population in three kinds of bovine ruminant contents.

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The first two authors (E.C.S. & Y.K.K.) contributed equally to this work.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Escherichia coli DH5α cells and recombinant *E. coli* cells were cultured in LB containing ampicillin (50 μg/ml).

Sampling

Samples of rumen contents were obtained from a closed herd at the Chinju National University (Chinju, Korea). This study used rumen-fistulated Korean cattle (Hanwoo) with body weight of about 400 kg. The animal was fed twice daily with a mixed ration of rice hull and concentrated feed (purchased from Daehan Food, Ulsan, Korea) in a 4:1 ratio. Three replicate samples of total rumen contents were collected from one animal via the ruminal fistula before the morning feeding for 3 days (Prasanpanich *et al*. 2002). The samples on ice were immediately transferred into an anaerobic box and were separated into the rumen fluid for the first library construction and feed particle fractions for the second library construction. The rumen contents were divided into rumen fluid and feed particle fractions by squeezing through two layers of cheesecloth and were then stored at −80 °C. At the same time, microorganisms on the rumen epithelium of the dorsal sac were collected by scraping with a spatula and suspended in 1·5 ml of 0·85% NaCl–50 mM sodium phosphate buffer (pH 6·5).

Total DNA extraction and PCR

The collected rumen fluid, rumen solid, and rumen epithelium samples were centrifuged at 14 000 *g* for 5 min at 4 °C. The obtained pellet of approximately 0·2 g was subjected to DNA extraction using the G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology, Suwon, Korea). The extracted DNA was used as a template for PCR to amplify 26S rDNA. The PCR primers used to amplify 26S rDNA fragments were the yeast-specific primers, 5′-ACCCGCT GAAYTTAAGCATAT-3′ (NLF184/21 mer, forward primer based on the position number of *Saccharomyces cerevisiae* LSU rRNA) and 5′-CTCCTTG GTCGTGTTTCAAGACGG-3′ (NLR818/25 mer, reverse primer) (VanderAuwera *et al*. 1994). SuperTherm DNA polymerase (JMR, SidCup, Kent, UK), 1·5 mM $MgCl_2$, 2 mM dNTP, and the primers in a final volume of 50 μl. Fifteen cycles (denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min) were followed by a final incubation at 72 °C for 10 min. The anticipated product of approximately 0·6 kb was isolated after an agarose gel electrophoresis of the amplified mixture using a gel extraction kit (NucleoGen, Seoul, Korea).

Cloning, sequencing and sequence analysis

PCR products were directly cloned into the pGEM-T Easy vector (Promega) and recombinant colonies were randomly picked. Samples for nucleotide sequencing were prepared by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CN, USA). The samples were analysed with an automated DNA sequencer (model 3100; Applied Biosystems, Foster City, CA, USA). All reference sequences were obtained from the GenBank and Ribosomal Database Project (RDP) databases. Our sequences were analysed by the CHECK_CHIMERA program (Maidak *et al*. 2000) to exclude sequences from chimeric rDNA clones. Similarity search against database entries was done using online BLAST search. Sequences were aligned using the multiple sequence alignment program CLUSTAL W version 1.6 (Thompson *et al*. 1994). Gaps and positions with ambiguities were excluded from the phylogenetic analysis. Phylogenetic analysis was performed using the neighbour-joining methods (Saito & Nei 1987). Bootstrap analysis was performed with data resampled 1000 times using DNAMAN analysis system (Lynnon Biosoft, Canada). Outlying sequences from the results of blastN data using FS I and FS II to root the tree were used.

Nucleotide sequence accession numbers and nomenclature

Nucleotide sequences have been deposited in the GenBank database under the accession numbers AY464842 ~ AY464938. For the libraries of rumen fluid, solid, and epithelium, the clone names begin with the letters FF, FS and FE, respectively (e.g. FF1 for clone of the fluid library).

RESULTS

Similarity with database sequences

A total of 97 clones were obtained from three libraries, the first library from rumen fluid, the second from rumen solid, and the third from rumen epithelium of Korean cattle. All clones from the three libraries were subjected to sequence analysis followed by online homology searches using two databases, GenBank which implements the BLAST algorithm and the RDP database which implements the SIMILARITY_RANK program (Tables 1, 2 and 3).

There are no exact 26S rDNA similarity limits for defining specific taxa such as genus and species. Ninety-six sequences (0·99) from the 97 clones in our libraries can be identified as belonging to a particular group. Those groups follow (with the number of clones in that group in parentheses): *Setosphaeria monoceras* (14), *Raciborskiomyces longisetosum* (4),

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Table 1. *Similarity values of 26S rDNA sequences retrieved from the rumen fluid*

Group	Clone (Accession no.)	Class	Nearest relative* (Accession no.)	Similarity (%)
FF I	FF01 (AY464842), FF02 (AY464933), FF06 (AY464937), FF08 (AY464843), FF09 (AY464844), FF12 (AY464847), FF18 (AY464853), FF21 (AY464856), FF22 (AY464857), FF27 (AY464862), FF28 (AY464863), FF37 (AY464872), FF38 (AY464873), FF40 (AY464875)	*Pezizomycotina*	*Setosphaeria monoceras* (AY016368)	95–**99**†
FF II	FF03 (AY464934), FF04 (AY464935), FF10 (AY464845), FF20 (AY464855)	*Pezizomycotina*	*Raciborskiomyces longisetosum* (AY016367)	**99**
FF III	FF05 (AY464936), FF23 (AY464858), FF26 (AY464861), FF36 (AY464871)	*Pezizomycotina*	*Letendraea helminthicola* (AY016362)	91–94
FF IV	FF07 (AY464938), FF14 (AY464849), FF24 (AY464859), FF31 (AY464866), FF41 (AY464876)	*Pezizomycotina*	*Magnaporthe grisea* (AB026819)	**98–99**
FF V	FF35 (AY464870)	*Pezizomycotina*	*Monographella nivalis* (AF452030)	96
FF VI	FF15 (AY464850)	*Pezizomycotina*	*Antarctic yeast* CBS 8931 (AY040649)	94
FF VII	FF16 (AY464851)	*Pezizomycotina*	*Myrothecium verrucaria* (AJ301999)	**98**
FF VIII	FF17 (AY464852)	*Pezizomycotina*	*Arthrobotrys guizhouensis* (AY056598)	92
FF IX	FF19 (AY464854)	*Pezizomycotina*	*Epichloe glyceriae* (L07132)	93
FF X	FF29 (AY464864)	*Pezizomycotina*	*Mycosphaerella lateralis* (AF309583)	95
FF XI	FF32 (AY464867)	*Pezizomycotina*	*Hyponectria* sp. HKUCC 5553 (AF452040)	**98**
FF XII	FF11 (AY464846)	*Urediniomycetes*	*Occultifur externus* (AF189911)	97
FF XIII	FF25 (AY464860)	*Ustilaginomycetes*	*Ustilago affinis* (AF133581)	**99**
FF XVI	FF33 (AY464868)	*Ustilaginomycetes*	*Pseudozyma rugulosa* (AB089371)	**99**
FF XV	FF30 (AY464865)	*Saccharomycotina*	*Williopsis saturnus* (AJ507804)	97
FF XVI	FF13 (AY464848), FF39 (AY464874)	*Hymenomycetes*	*Thanatephorus cucumeris* (AF354058)	**98**
FF XVII	FF34 (AY464869)		NA‡	

* Accession number of the nearest relative. When more than one sequence had the same similarity, only the accession number of the first sequence is given.
† Database sequences with >97% similarity are shown in bold.
‡ NA: not available.

Magnaporthe grisea (5), *Occultifur externus* (1), *Thanatephorus cucumeris* (2), *Antarctic yeast* CBS 8931 (1), *Myrothecium verrucaria* (1), *Arthrobotrys guizhouensis* (1), *Epichloe glyceriae* (1), *Ustilago affinis* (1), *Letendraea helminthicola* (4), *Mycosphaerella lateralis* (1), *Williopsis saturnus* (1), *Hyponectria* sp. HKUCC 5553 (1), *Pseudozyma rugulosa* (2), *Monographella nivalis* (1), *Galactomyces* sp. NRRL Y-6418 (1), *Acremonium alternatum* (1), *Geotrichum silvicola* (50) and *Galactomyces* sp. (3). Because the similarity values for a majority of our sequences were too low (less than 98%) to allocate them with a reasonable degree of confidence to particular taxa, phylogenetic analysis was performed to clarify their taxonomic position. Phylogenetic clustering of yeast groups is proposed to be a more useful guide for defining yeast taxa than a specific similarity value (Fell *et al*. 2000).

Forty-one clones of the FF library were analysed for DNA similarity (Table 1). Most of our sequences had the similarity with a cultured isolate as follows: *S. monoceras* (FF01, FF02, FF06, FF08, FF09, FF12, FF18, FF21, FF22, FF27, FF28, FF37, FF38, and FF40), *R. longisetosum* (FF03, FF04, FF10, and

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Table 2. *Similarity values of 26S rDNA sequences retrieved from the rumen epithelium*

Group	Clone (Accession no.)	Class	Nearest relative* (Accession no.)	Similarity (%)
FE I	FE03 (AY464879), FE05 (AY464881), FE06 (AY464882), FE07 (AY464883), FE08 (AY464884), FE09 (AY464885), FE10 (AY464886), FE11 (AY464887), FE12 (AY464888), FE13 (AY464889), FE14 (AY464890), FE15 (AY464891), FE16 (AY464892), FE17 (AY464893), FE18 (AY464894), FE19 (AY464895), FE20 (AY464896), FE21 (AY464897), FE22 (AY464898), FE23 (AY464899), FE24 (AY464900), FE25 (AY464901), FE26 (AY464902)	*Saccharomycotina*	*Geotrichum silvicola* (AY158042)	**98–99**†
FE II	FE01 (AY464877)	*Saccharomycotina*	*Galactomyces* sp. NRRL Y-6418 (AF017396)	97
FE III	FE02 (AY464878)	*Pezizomycotina*	*Acremonium alternatum* (U57349)	**99**
FE IV	FE04 (AY464880)	*Ustilaginomycetes*	*Pseudozyma rugulosa* (AB089371)	**99**

* Accession number of the nearest relative. When more than one sequence had the same similarity, only the accession number of the first sequence is given.
† Database sequences with >97% similarity are shown in bold.

Table 3. *Similarity values of 26S rDNA sequences retrieved from the rumen solid*

Group	Clone (Accession no.)	Class	Nearest relative* (Accession no.)	Similarity (%)
FS I	FS01 (AY464903), FS02 (AY464904), FS03 (AY464905)	*Saccharomycotina*	*Galactomyces* sp. (AF017396)	97
FS II	FS04 (AY464906), FS05 (AY464907), FS06 (AY464908), FS07 (AY464909), FS08 (AY464910), FS09 (AY464911), FS10 (AY464912), FS11 (AY464913), FS12 (AY464914), FS13 (AY464915), FS14 (AY464916), FS15 (AY464917), FS16 (AY464918), FS17 (AY464919), FS18 (AY464920), FS19 (AY464921), FS20 (AY464922), FS21 (AY464923), FS22 (AY464924), FS23 (AY464925), FS24 (AY464926), FS25 (AY464927), FS26 (AY464928), FS27 (AY464929), FS28 (AY464930), FS29 (AY464931), FS30 (AY464932)	*Saccharomycotina*	*Geotrichum silvicola* (AY158042)	**98–99**†

* Accession number of the nearest relative. When more than one sequence had the same similarity, only the accession number of the first sequence is given.
† Database sequences with >97% similarity are shown in bold.

FF20), *M. grisea* (FF07, FF14, FF24, FF31, and FF41), *O. externus* (FF11), *T. cucumeris* (FF13 and FF39), *A. yeast* CBS 8931 (FF15), *M. verrucaria* (FF16), *A. guizhouensis* (FF17), *E. glyceriae* (FF19), *U. affinis* (FF25), *L. helminthicola* (FF05, FF23, FF26, and FF36), *M. lateralis* (FF29), *W. saturnus* (FF30), *Hyponectria* sp. HKUCC 5553 (FF32), *P. rugulosa* (FF33) and *M. nivalis* (FF35) (Table 1). The remaining sequence (FF34) in this library had less than 90% similarity with the database sequences.

In the FE library, all of the 26 clones analysed were similar to the cultured rumen yeast, *Galactomyces* sp.

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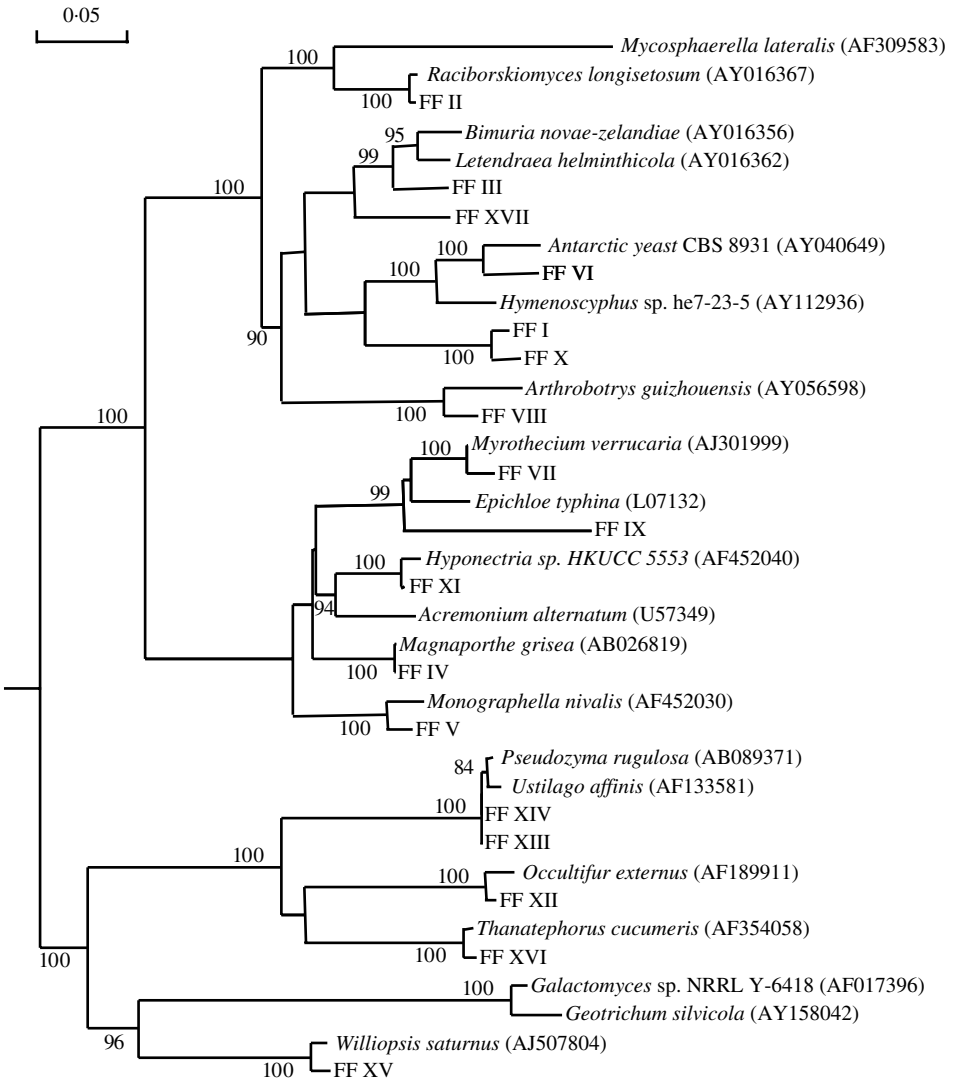

Fig. 1. Phylogenetic placement of 26S rDNA sequences from the rumen fluid. Numbers above each node are confidence levels (%) generated from 1000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position. Only bootstrap values of greater than 80 % are shown.

NRRL Y-6418 (FE01), *A. alternatum* (FE02), *P. rugulosa* (FE04), and *G. silvicola* (FE03 and FE05-FE26) (Table 2). Twenty-five sequences had more than 98 % similarity with database entries and only one clone had less than 98 % similarity range.

Thirty clones were analysed from the FS library. Twenty-seven clones in this library had sequence matches in the range of 98–100 % similarity with cultured rumen isolates of *G. silvicola* (FS04–FS30) (Table 3). Three clones (FS01–FS03) had less than 98 % similarity with database entries.

Phylogenetic placement of sequences from three libraries

The results of phylogenetic analysis of the FF library are shown in Fig. 1. In this library, the majority of sequences (85·4 %) were placed within the *Pezizomycotina* class. This may reflect the functional importance of the symbiotic relationship between this class and the host animal. Two sequences (FF25 and FF33) were related to the *Ustilaginomycetes* class. One sequence in the *Urediniomycetes* class (FF11) was

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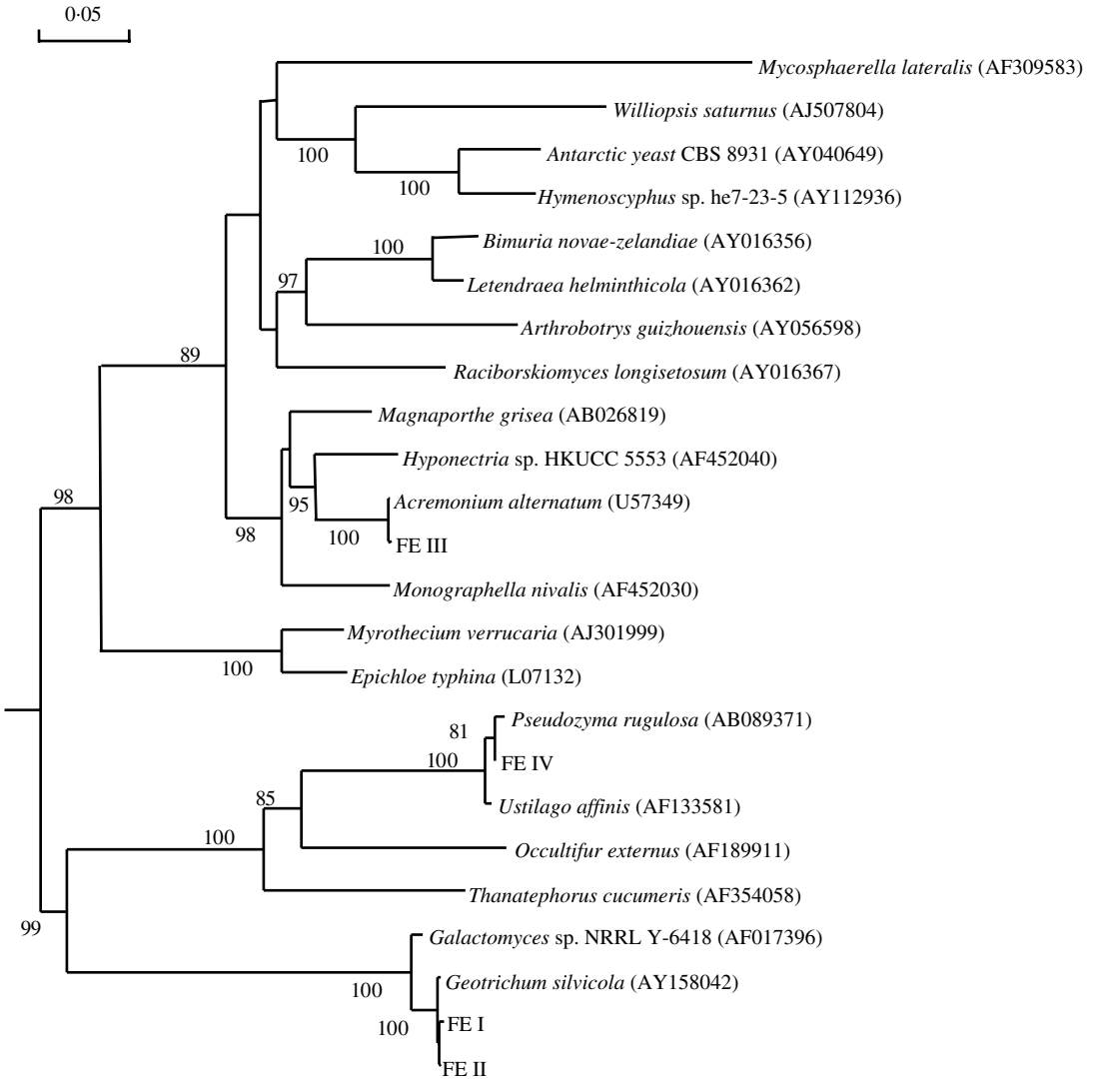

Fig. 2. Phylogenetic placement of 26S rDNA sequences from the rumen epithelium. Numbers above each node are confidence levels (%) generated from 1000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position. Only bootstrap values of greater than 80 % are shown.

related to the strain of *O. externus*. Two clones (FF13 and FF39) in the *Hymenomycetes* were clustered with the strain of *T. cucumeris*. One clone (FF30) was related to the *Saccharomycetes* class. But, unlike the FE library and FS library, none were related to the sequences of *G. silvicola* and *Galactomyces* sp.

The results of phylogenetic analysis of the FE library are shown in Fig. 2. The majority of sequences in this library belonged to the *Saccharomycetes* class. Most of the FE sequences (0·923) were phylogenetically placed within the *Saccharomycetes* class. This may reflect the functional importance of the symbiotic relationship between this class and the host animal, as with the FF library. Twenty-four sequences were related to the *G. silvicola* cluster. One sequence (FE02) was closely related to the type strain of *A. alternatum* while the remaining clone (FE04) was related to the type strain of *P. regulosa*.

The results of phylogenetic analysis of the FS library are shown in Fig. 3. The majority of sequences in this

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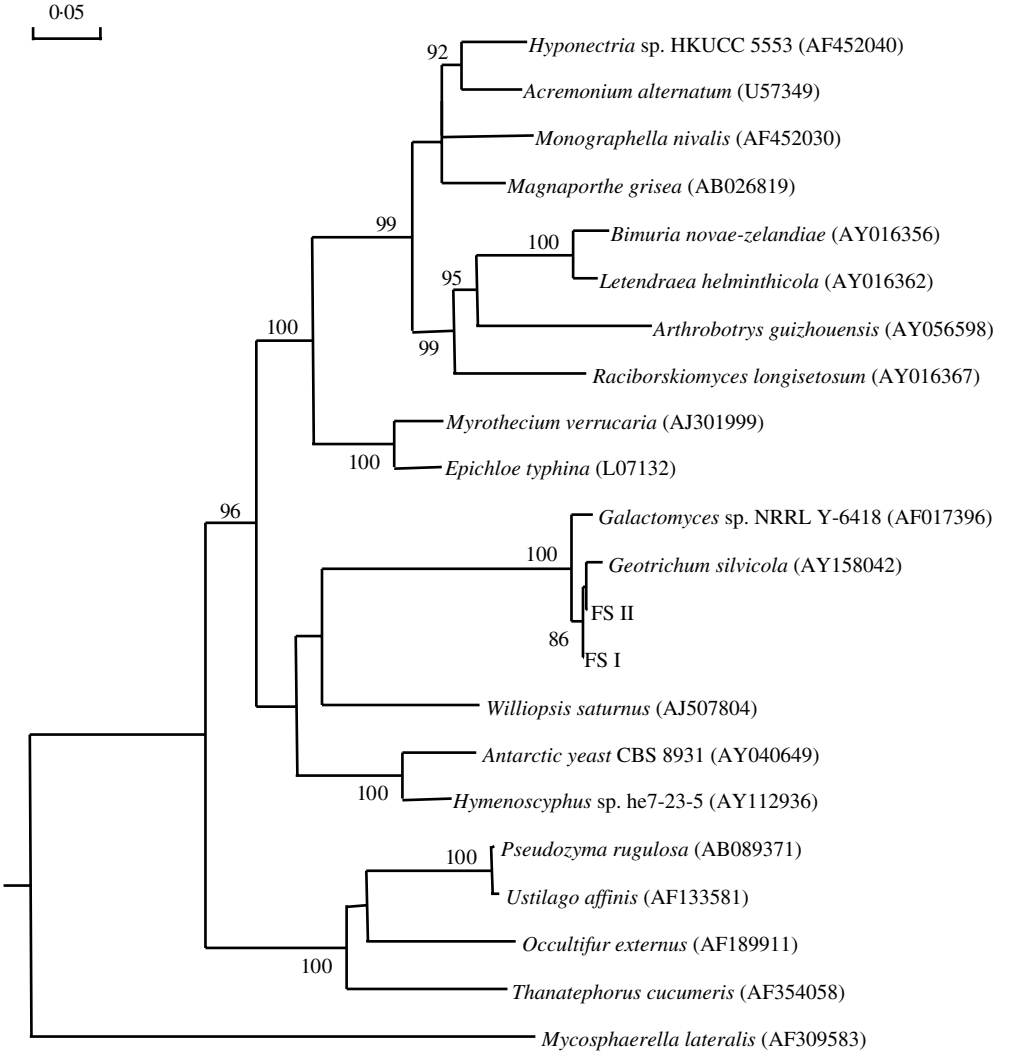

Fig. 3. Phylogenetic placement of 26S rDNA sequences from the rumen solid. Numbers above each node are confidence levels (%) generated from 1000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position. Only bootstrap values of greater than 80 % are shown.

library were placed within the *Saccharomycotina* class. All the FS sequences were phylogenetically placed within the *Saccharomycotina* class. All clones were related with *Geotrichum* with a high confidence. As with the FE library, there was a FS II group related to the type strain of *G. silvicola*.

DISCUSSION

Compared with other ecosystems, there have been only a few studies examining yeast in the rumen. However, yeasts are a critical component of the rumen as evidenced by the fact that yeast feed additives have been used as an alternative to antimicrobial feed additives for 10 years. Some of the benefits associated with the presence of yeast in the rumen include: increased dry matter digestion (Carro *et al*. 1992), increased initial rates of fibre digestion (Williams *et al*. 1991), and increased milk production in dairy cattle (Kung *et al*. 1997).

This study reveals the phylogenetic diversity of the yeast community in the rumen fluid, rumen solid and rumen epithelium of cattle by analysis of yeast 26S rDNA sequences in a culture-independent manner.

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Table 4. *Overview of relationship between phylum analysis results from each of library*

Phylum Class	FF* (%)	FE* (%)	FS* (%)	Number of total clones (%)
Ascomycota				
Pezizomycotina	35 (85·4)	1 (3·8)		36 (37·0)
Saccaromycotina	1 (2·4)	24 (92·4)	30 (100)	55 (57·0)
Basidiomycota				
Hymenomycetes	2 (4·9)			2 (2·0)
Ustilaginomycetes	2 (4·9)	1 (3·8)		3 (3·0)
Urediniomycetes	1 (2·4)			1 (1·0)
Number of total clones (%)	41 (100)	26 (100)	30 (100)	97 (100)

* FF, Rumen fluid yeast; FE, Rumen epithelium yeast; FS, Rumen solid yeast.

BLAST searches showed that 97 sequenced clones shared a various degree of similarity (89–100%) with the yeast sequences from GenBank (Tables 1, 2 and 3). Most of the sequences derived from the rumen solid and epithelium libraries of cattle were related to those of the *Saccharomycotina* class, whereas those of the rumen fluid library were related to the *Pezizomycotina* class (Table 4).

It is interesting that the 30 clones of the FS library were classified to only one class of *Saccharomycotina*, and also that the *Saccharomycotina* were predominant in rumen epithelium while *Pezizomycotina* were in rumen fluid. It may not be certain whether the difference in yeast distribution originated from diets containing the various microbial populations. It is also assumed that one animal used in the present study is not enough to cover the diversity of the yeast in rumen. In order to elucidate the exact population of yeasts in rumen contents, it will be necessary to compare the biodiversity of yeasts in various rumen samples collected from different cattle fed with the same kind of diets. Also, it is notable that the predominant family of whole rumen yeast was found to belong to the *Saccharomycotina* class (0·57). Considering that the rDNA sequences of many clones have the same similarity with yeast in the databases, it suggests that some kind of yeast may exist in rumen primarily. However, these studies demonstrate the usefulness of 26S rDNA-specific primers in assessing the biodiversity of yeast communities in rumen and indicate possible limitations of the approach that require consideration (Ian *et al*. 2003).

The work presented here, which compares the extent of rDNA relatedness with nucleotide divergence in domain D1/D2, indicates that nearly all currently recognized yeasts can be identified by their unique D1/D2 sequences. Consequently, use of the D1/D3 rDNA database has the potential to markedly increase the accuracy of yeast identifications, and the effort required is less than that needed for preparation of the less reliable standard fermentation and assimilation tests in rumen. Analysis of these data has also given an overview of phylogenetic relationships among the yeasts (Kurtzman & Robnett 1998).

The scope of functional roles and the extent of yeast diversity have yet to be understood, as most yeast of the rumen remain undescribed. The PCR primers described in this report provide unique tools to further characterize this important group of organisms (Borneman & Hartin 2000). Considering the variation of each position amounts to 5%, when eukaryotic nuclear large subunit (LSU) rRNA with *Saccharomyces cerevisiae* as reference organism was amplified by various universal primers (http://www.psb.ugent.be/rRNA/), the PCR primer to amplify 26S rDNA used in this study is not enough to cover most phyla of yeast in rumen. A more specific set of phylogenetic primers for the microorganism will be used in future studies to more fully resolve the character of microbial diversity. Because by-products of the rumen microbial community metabolism largely determine nutrition of the host animal, metabolic interactions that define that community and its collective activities also determine host nutrition and production (Sharp *et al*. 1998). The use of a more specific set of phylogenetic probes for the yeast and quantitative PCR techniques will help determine the quantitative significance of the newly identified groups and the distribution of the identified yeast clusters in rumen microbial communities as well as to determine the effect of variance of rumen yeast flora on animal diet.

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REFERENCES

Borneman, J. & Hartin, R. J. (2000). PCR primers that amplify fungal rRNA genes from environmental samples. *Applied and Environmental Microbiology* **66**, 4356–4360.

Carro, M. D., Lebzien, P. & Rohr, K. (1992). Effects of yeast culture on rumen fermentation, digestibility and duodenal flow in dairy cows fed a silage based diet. *Livestock Production Science* **32**, 219–229.

Clarke, R. T. J. & Di Menna, M. E. (1961). Yeast from the bovine rumen. *Journal of General Microbiology* **25**, 113–117.

Fell, J. W., Boekhout, T., Fonseca, A., Scorzetti, G. & Statzell-Tallman, A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *International Journal of Systematic and Evolutionary Microbiology* **50**, 1351–1371.

Fujihara, T., Todoroki, M. & Nakamura, K. (2003). The effect of rumen protozoa on the urinary excretion of purine derivatives in goats. *Journal of Agricultural Science, Cambridge* **140**, 101–105.

Hession, A. O., Tung, R. S., Kreck, E. M. & Kung, J. L. (1992). Effect of adding live yeast cultures on *in vitro* ruminal fermentation. *Journal of Animal Science* **70** (Suppl. 1), 309 (Abstr.).

Hugenholtz, P., Goebel, B. M. & Pace, N. R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology* **180**, 4765–4774.

Ian, C. A., Colin, D. C. & James, I. P. (2003). Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. *Environmental Microbiology* **5**, 36–47.

Klein, W. & Muller, R. (1941). Das Eiweissminimum, die zymogene Symbiose und die Erzeugung von Mikrobeneiweiss im Pansen aus Stickstoffverbindungen nicht eiweissartiger. *Natur. Zeitschrift für Tierzüchtung und Züchtungsbiologie* **48**, 255–276.

Kung, L., Kreck, E. M., Tung, R. S., Hession, A. O., Sheperd, A. C., Cohen, M. A., Swain, H. E. & Leedle, J. A. Z. (1997). Effects of a live yeast culture and enzymes on in vitro ruminal fermentation and milk production of dairy cows. *Journal of Dairy Science* **80**, 2045–2051.

Kurtzman, C. P. & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek* **73**, 331–371.

Lachance, M. A., Starmer, W. T., Rosa, C. A., Bowles, J. M., Barker, J. S. & Janzen, D. H. (2001). Biogeography of the yeasts of ephemeral flowers and their insects. *FEMS Yeast Research* **1**, 1–8.

Lund, A. (1974). Yeasts and moulds in the bovine rumen. *Journal of General Microbiology* **81**, 453–462.

Maidak, B. L., Cole, J. R., Lilburn, T. G., Parker, C. T., Saxman, P. R., Stredwick, J. M., Garrity, G. M., LI, B., Olsen, G. J., Pramanik, S., Schmidt, T. M. & Tiedje, J. M. (2000). The RDP (Ribosomal Database Project) continues. *Nucleic Acids Research* **28**, 173–174.

Prasanpanich, S., Siwichai, S., Tunsaringkarn, K., Thwaites, C. J. & Vajrabukka, C. (2002). Physiological responses of lactating cows under grazing and indoor feeding conditions in the tropics. *Journal of Agricultural Science, Cambridge* **138**, 341–344.

Saito, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology Evolution* **4**, 406–425.

Sharp, R., Ziemer, C. J., Stern, M. D. & Stahl, D. A. (1998). Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. *FEMS Microbiology Ecology* **26**, 71–78.

Smith, H. W. (1965). Observation on the flora of the alimentary tract of animals and factors affecting its composition. *Journal of Pathology and Bacteriology* **89**, 95–122.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680.

VanderAuwera, G., Chapelle, S. & De Wachter, R. (1994). Structure of the large ribosomal subunit RNA of *Phytophtora megasperma*, and phylogeny of the *Oomycetes*. *FEBS Letters* **338**, 133–136.

Williams, P. E. V., Tait, C. A. G., Innes, G. M. & Newbold, C. J. (1991). Effects of the inclusion of yeast culture (*Saccharomyces cerevisiae* plus growth medium) in the diet of dairy cows on milk yield and forage degradation and fermentation patterns in the rumen of steers. *Journal of Animal Science* **69**, 3016–3026.