Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 5 (7): 581-594, 2010
ISSN 1816-4935

Morphological and Molecular Characterization of Polycentric Rumen Fungi Belonging to the Genus *Orpinomyces* Isolated from Indian Cattle and Buffaloes

M. Sridhar, D. Kumar, S. Anandan, C.S. Prasad and K.T. Sampath
National Institute of Animal Nutrition and Physiology,
Adugodi, Bangalore-560030 Karnataka, India

Abstract: Considering the importance of polycentric anaerobic rumen fungi in fiber digestibility and in the paucity of any reports in Indian cattle an attempt was made to isolate and characterize *Orpinomyces* species employing a combination of both morphological and molecular methods. Around six hundred rumen liquor/fecal samples of cows and buffaloes were collected from various locations within the country in order to characterize *Opinomyces* species predominant in Indian cattle and buffaloes using medium 10X and hungate roll tubes. *Orpinomyces* genera were characterized by the presence of numerous coralloid sporangiophore complexes with large bulbous sporangia and polyflagellated zoospores similar to those of *Neocallimastix* sp., *Orpinomyces joyonii* and *Orpinomyces intercalaris* were the two predominant species obtained with the former being characterized by the spherical sporangia developing on branched or simple sporangiophore complexes with the hypae having light constrictions, while the latter had globose sporangia developing from expansion of hyphae or as lateral outgrowths but rarely terminal. The hydrolytic enzyme activities of the two species of *Orpinomyces* showed that *O. joyonii* was characterized by high endoglucanase, xylanase and β-glucosidase activity while *Orpinomyces intercalaris* showed higher activities of cellobiohydrolase and β-xylosidase showing different strategy for fiber degradation. The DNA-based methodologies were used to identify the isolated *Orpinomyces* using Internal transcribed spacer region (ITS1) analysis of the fungal rDNA genes. Three of the Indian isolates (NIANP 49, NIANP 58 and NIANP 60) had motifs 5, 8, 7 and 5 corresponding to Variable region I, II, III and IV, respectively and morphologically were identified as *Orpinomyces joyonii*. The sequence of two isolates (NIANP 57 and 59) however, was different from the rest of the isolates in having motif 8 in Variable region III and morphologically were identified as *Orpinomyces intercalaris*. Multiple alignments of obtained sequences showed all the isolates to fall within a single group together with the *Piromyces* II group. By the combination of morphological and phylogenetic analysis we showed that gut of Indian cattle and buffaloes was predominated by only two species viz.*Orpinomyces joyonii* and *Orpinomyces intercalaris*.

Key words: Anaerobic fungi, fiber digestion, hydrolytic enzymes, characterization, sequences, phylogenetic diversity, ribosomal DNA

Corresponding Author: M. Sridhar, National Institute of Animal Nutrition and Physiology, Adugodi, Bangalore-560030 Karnataka, India
Tel: 91-080-25711164 Fax: 91-080-2571420

INTRODUCTION

The anaerobic fungi are an unusual group of zoosporic fungi occupying a unique niche in the digestive tract (rumen, omasum, abomasum, small intestine, caecum, large intestine) and faeces of wild and domesticated ruminants and large monogastric herbivores (Theodorou *et al*., 1996). Until their discovery, the microbial population in the rumen was believed to be made up of bacteria and protozoa only. Among the rumen microbes, anaerobic fungi are important as they possess highly active enzymes which very efficiently break down cellulose and have a unique ability to break and penetrate the fibrous feed particles through fungal mycelium, providing more surface area for the action of other microbes.

All the anaerobic fungi studied so far are cellulolytic and are able to degrade structural carbohydrates of plant cell walls playing a vital role in the digestion of high fiber poor-quality forage. Thus in tropical regions where forages are generally fibrous and of poor quality, the development of methods to manipulate superior strains of fungi, naturally selected or genetically engineered, in the rumen would afford a means of improving the digestion of poor-quality fodder by large ruminants particular lactating cows and buffaloes. These anaerobic gut fungi are the primary invaders of the ingested fibrous feed particles and are classified into six genera : those with a monocentric thallus are *Neocallimastix*, *Piromyces* and *Caecomyces* while the fungi with a polycentric thallus are *Orpinomyces*, *Anaeromyces* and *Cyllamyces* (Ozkose *et al*., 2001).

Polycentric fungi are considered to be more important as they efficiently penetrate the fiber particles. DNA-based techniques have facilitated the understanding of the phylogenetic relationships and diversity of micro-organisms in natural ecosystems: they introduce considerably fewer biases in sampling than culture-based methodologies, can be generated directly from DNA and are considered more representative of the entire community than culture-derived data alone (Ward *et al*., 1990). Favored indicators of genetic diversity are the rRNA encoding gene sequences, particularly the internal transcribed spacers ITS1 and ITS2 and the intervening 5-8S rDNA as these can be used both to identify micro-organisms and to determine phylogenetic relationships within communities (Hausner *et al*., 2000; Vainio and Hantula, 2000; Ranjard *et al*., 2001), including the gut fungi (Brookman *et al*., 2000). Molecular characterization based on the variable region motif sequence of internal transcribed spacer region 1 (ITS1) was proposed by Tuckwell *et al.* (2005).

Considering the importance of anaerobic rumen fungi in fiber digestibility and in the paucity of any reports of the morphological and molecular characterization of anaerobic gut fungus in Indian cattle the current work was carried out to study the phylogenetic relationships and diversity of *Orpinomyces* isolated from cattle and buffaloes based on a combination of both.

MATERIALS AND METHODS

Isolates and Culture Conditions

Over six hundred fresh rumen liquor (cannulated bulls) and fecal samples (cow and buffaloes) collected from the Experimental Livestock Unit of the Institute and various places and farms from within the state as well as outside starting June 2005 and extending for a period of over two and a half years were transported to the laboratory maintaining the temperature at 39°C. Muslin cloth strained rumen liquor and fecal samples diluted 10 times in Anaerobic Dilution Solution (ADS) were used as inoculums (0.2 mL). The composition of the anaerobic culture medium used in the present study was based on the description of

medium 10 by Caldwell and Bryant (1966), as modified by Akin (1980) and Phillips and Gordon (1988, 1989). Inoculated Hungate roll tubes (Belco Germany) were kept at 39°C for 72 h. The individual fungal colonies were then transferred into broth tubes under gentle steam of CO_2 and the inoculated broth tubes re-incubated and sub cultured weekly. Stock cultures of the isolates were maintained in 10% glycerol stored under liquid nitrogen. Aliquots taken with the help of sterile syringes from the broths were observed under a Nikon Eclipse 50i microscope to identify the isolated anaerobic fungi. Further culture of *Orpinomyces* was carried out inside on anaerobic chamber (Coy Laboratory, USA) having an atmosphere of 90-95% carbon-di-oxide and 5-10% hydrogen.

Morphological Characterization

For the examination of zoospores and polycentric vegetative growth a small amount of culture medium was removed from the culture tube by the help of a syringe and placed on a clean microscope slide. For examining the vegetative growth of polycentric fungi fully grown biomass was removed and teased apart with a sterile loop before placing suitably small pieces on the slide for observation. Once the sample was placed on the slide it was overlaid with a cover slip before being observed by phase contrast microscopy. The overall appearance of the fungi was observed at 100-200X while details of structure, such as the numbers of flagella on zoospores, were observed at 400 X magnification.

Enzyme Assays

Endoglucanase, cellobiohydrolase and xylanase activities were estimated as described (Paul *et al*., 2003). The reaction mixture used for the estimation of these enzymes contained: 1.0 mL phosphate buffer (pH 6.8, 0.1 M), 0.5 mL substrate (1% carboxymethylcellulose, Sigma, low viscosity), 2% Avicel (Sigma) and 0.25% xylan from oat spelt (Himedia) and 0.5 mL enzyme; incubated at 39°C for 1 h for CMCase and 30 min for xylanase. The released reducing sugars were measured by the DNS method (Whealan, 1969). The β-Glucosidase and β-xylosidase activities were determined by measuring the increase in absorbance at 410 nm, following 10 min incubation of enzyme with 1 mM p-nitrophenyl-b-D-glucopyranoside and p-nitrophenyl-b-D-xylopyranoside. The reaction was stopped by the addition of sodium carbonate solution (Puls and Poutanen, 1989; Christakopoulos *et al*., 1995). The activities of endoglucanase, cellobiohydrolase and xylanase were expressed as ìmole of reducing sugar released per min under the assay conditions. The activities of β-glucosidase and β-xylosidase were expressed as μmole of p-nitrophenol released per min under the assay conditions. One International Unit (IU) of the enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar (expressed as glucose or xylose) and 1 μmol of p-nitrophenol per min. Protein was estimated as per the method of Lowry *et al.* (1951). All enzyme assays were carried out in three replicates.

DNA Extraction and Sequencing

Genomic DNA was extracted from the biomass of each of the individual genera obtained after repeated subculturing, using hexa decyl trimethyl ammonium bromide (CTAB) method described by Chen *et al.* (2006). The DNA concentrations of high purity ranging from 1.8- 2.0 (Absorbance ratio 260/280) was obtained. The PCR amplification of the genomic DNA was carried out using the ribosomal JB206 and ITS2 primer set: 18S forward primer JB206 (GGA AGT AAA AGT CGT AAC AAG G) and the 28S reverse primer JB205 (TCC TCC GCT TAT TAA TAT GC). The amplification of the cDNA was carried out in 50 μL of final volume. The PCR mix consisted of 5 μL of 10XPCR buffer, 2.5 mM $MgCl_2$, 2 mM dNTPs, 10 pmol of

primers JB206 and JB205, 2.5U Taq DNA polymerase and 300 ng of cDNA. The hot start procedure was adapted for thermal cycling in thermal cycler (icycler, Bio-rad, USA). The thermal profile of the PCR reaction was initial denaturation at 94°C for 5 min, 1 cycle; denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 50 sec, 35 cycles and final extension at 72°C for 10 min 1 cycle. The amplified products obtained were visualized by agarose gel electrophoresis and purified by gel extraction method. The purified amplicons were ligated with pGEMT-Easy vector and *E. coli* JM109 competent cells were transformed as per the manufacturer's protocol. The cells were inoculated into SOC broth and incubated for 1 h at 37°C, before being spread on Luria Bertani (LB) agar plates containing ampicillin (100 μg mL^{-1}), X gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) -80 μg mL^{-1} and IPTG (0.5 mM) and again incubated for 12 h at 37°C. The transformed colonies (white) were isolated and re-inoculated in fresh LB agar plates and colony PCR was done using the same set of primer for detection of specific product.

The colonies showing positive results were grown in 4 mL of LB broth (with Ampicillin) for 12 h at 37°C and plasmid extraction was done by Qiagen miniprep kit method as per manufacturer's protocol. The extracted plasmids were reconfirmed by Eco R I digestion for 3 h at 37°C and visualized for the released insert on agarose gel. The samples were then sent for sequencing. Multiple alignments of sequences were generated using CLUSTAL (Thompson *et al*., 1997) and manually edited using Align (http://science.do-mix.de/).These sequences were clustered to identify different motifs with differing levels of degeneracy within each of the four variable regions of the ITS1 sequences. Motifs were drawn from sequence 62-96 for variable region I; 130-196 for variable region II; 198-266 for variable region III and 264-318 for variable region IV. The DNA sequences of thirty anaerobic gut fungus were retrieved from Genbank and the evolutionary relationships plotted using the Neighbor-Joining method. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007).

Statistical Analysis

Data obtained from enzyme studies were analyzed by one way ANOVA and the differences between the means were compared by the Duncan multiple range test (Steel and Torrie, 1980).

RESULTS

Morphological Characterization

In present study under the *Orpinomyces* genera's *O. joyonii* and *O. intercalaris* were the two species isolated. The growth was thick and cottony in broth and quite profuse in petri-plates (Fig. 2 a, b). *Orpinomyces* sp., were characterized by the presence of numerous coralloid sporangiophore complexes and large bulbous sporangia and on agar plates produced a diffuse mycelium that grew to the edge of the plate within 5-7 days. Sporangia of *Orpinomyces* sp., were spherical with mostly lateral outgrowths of hyphae. *Orpinomyces joyonii* in particular was characterized by the presence of spherical sporangia developing either on branched or simple sporangiophore complexes and the hypae having mild constrictions (Fig. 1a). *Orpinomyces* intercalaris on the other hand had globose sporangia developing from hyphae as lateral outgrowths (Fig. 1b). A predominance of both the species of *Orpinomyces* was observed in cattle where they comprised 39.89% of the total fungal isolates as compared to 7.14% in buffaloes (Fig. 3a, b).

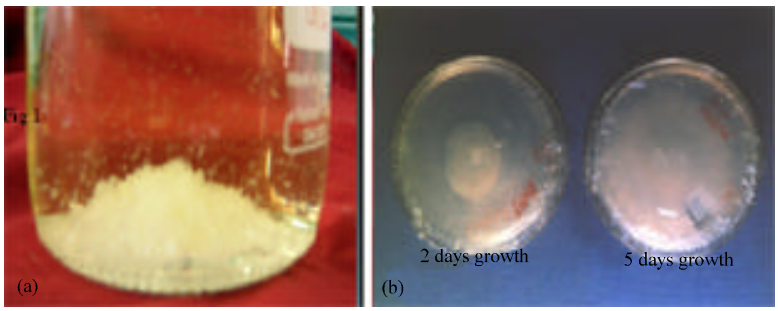


Fig. 1: Growth of Orpinomyces (a) in broth and (b) in petriplates

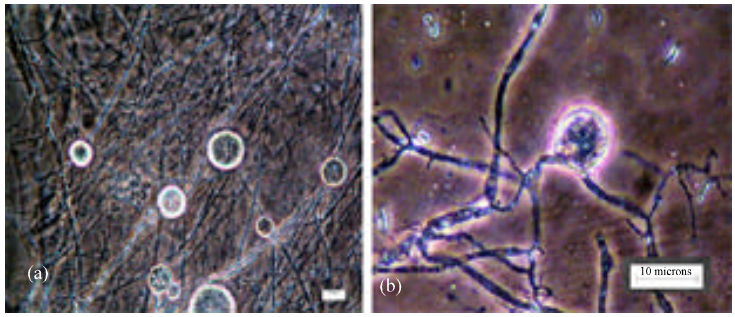


Fig. 2: The two different species. (a) *Orpinomyces* joyonii and (b) *Orpinomyces* intercalaris isolated from the Indian cattle and buffaloes

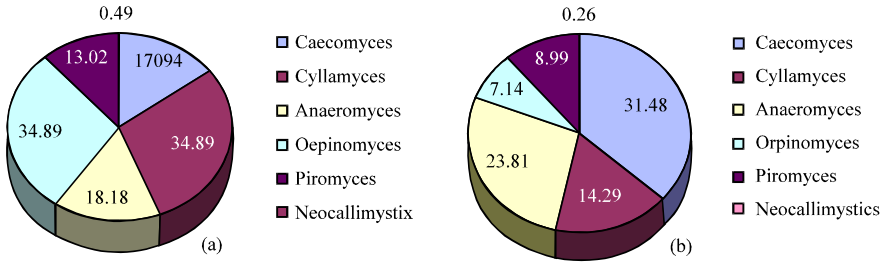


Fig. 3: Isolates of *Orpinomyces* as compared to total fungal isolates obtained (a) from cattle and (b) from buffaloes

Enzyme Studies

The anaerobic fungi produce a wide range of polysaccharide degrading enzymes and are known to have high cellulolytic and xylanolytic activities which have been found associated with the rhizomycelium and are also secreted into the surrounding environment. The enzymes estimated in the present experiment are the major enzymes responsible for the degradation of lignocellulosic feed. *Orpinomyces joyonii* and *Orpinomyces intercalaris* were tested for their fibrolytic enzyme activities to see if there were any major variations in the patterns of their enzyme secretion. The hydrolytic enzyme profile of some of the sequenced Indian isolates of Orpinomyces as observed from Table 1, showed *O. joyonii* to be characterized by high endoglucanase (range 198.6 to 286.0 I.U), xylanase (166.3 to 237.4 IU)

Table 1: The hydrolytic enzyme profile of some of the sequenced Indian isolates of *Orpinomyces* (values are the means from triplicate cultures)

	Isolates of orpinomyces			
Enzyme	*O. joyonii* NIANP49	*O. joyonii* NIANP58	*O. intercalaris* NIANP57	*O. intercalaris* NIANP59
Endoglucanase				
Activity *	286.00±72.8	198.60±65.4	32.600±1.2	94.330±12.34
Specific activity**	357.50±105.5	248.25±123.2	40.750±3.4	117.91±34.62
Xylanase				
Activity *	166.30±41.8	237.40±83.7	28.220±7.13	37.000±2.2
Specific activity**	207.88±97.3	296.75±97.5	35.280±9.8	46.250±3.1
β-glucosidase				
Activity***	140.70±54.6	107.40±65.2	3.4000±0.6	1.0000±0.6
Specific activity**	175.88±72.6	134.25±71.8	4.2500±1.8	1.2500±1.0
β-xylosidase				
Activity***	12.570±21.2	8.0000±2.5	135.10±25.1	180.90±253.3
Specific activity**	15.710±2.8	10.000±3.2	168.88±69.7	226.13±72.2
Cellobiohydrolase				
Activity*	10.300±0.8	14.900±1.8	29.600±2.9	43.500±6.8
Specific activity**	12.880±2.2	18.630±2.3	37.000±4.6	54.380±10.2

*One IU of enzyme activity expressed as 1 μmol of reducing sugar / ***1 μmol of p-nitrophenol per min liberated mL^{-1}. **Specific activity expressed as enzyme units/mg of protein

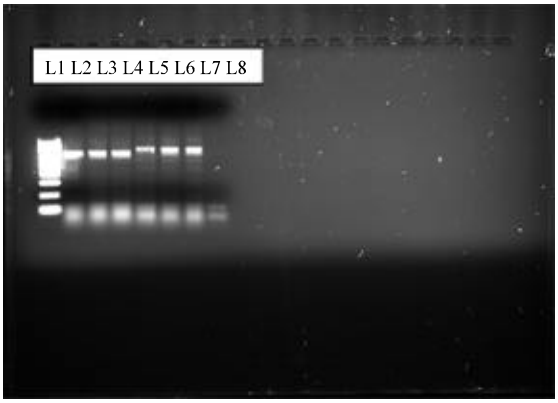
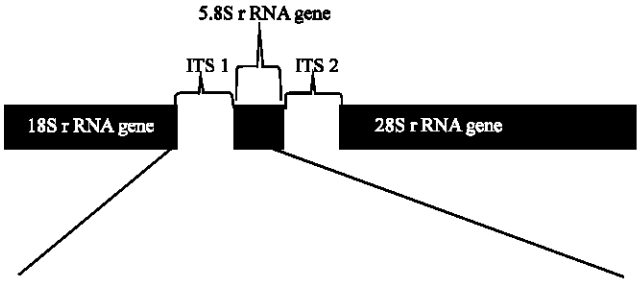


Fig. 4: Agarose gel electrophoresis of PCR product after amplification with JB 205 and JB 206. L1 = 100 bp DNA molecular wt., Marker, L2-L4 = *Orinomyces* isolate 1, L5-L7 = *Orinomyces* isolate 1, L8 = Negative control (without DNA)

and β-glucosidase activity (107.4 to 140.7 IU) while *Orpinomyces intercalaris* showed higher activities of cellobiohydrolase (29.6 to 43.5 IU) and β-xylosidase (135.1 to 180.9 IU).

Molecular Characterization

The DNA was isolated from all pure isolates of *Orpinomyces* by use of CTAB and as the extracted DNA was sufficiently pure as quantified by absorption spectroscopy as well as good 260 and 280 nm ratios (ratios of 1.8 to 1.9 and 1.9 to 2.0 indicating highly purified preparations of DNA) it required no further cleanup and was used to carry out PCR. Amplification products of the expected size (780 bp) were obtained from DNA extracts of *Orpinomyces* (Fig. 4). The amplified DNA was purified with a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions. The DNA was then ligated into the pGEM-T vector system and transformed into competent *Escherichia coli* (JM109) cells, before plasmid isolation using a QIAprep spin miniprep kit (QIAGEN). The samples were then



Variable region motif sequences of internal transcribed spacre region 1 (ITS 1) for *Orpinomyces* (Tuckwell *et al.*, 2005)

Region	Motif	Motif sequences	Group
I (82-96)	5	AATCAC(A|T)GT(T|A)TTGTATTTT	
	5a	ATCAC(A|T)TTT(T|)GT(A|T)TGTGATTT	
II (130-195)	8	TTG(T|)TTTTGT((AAA)|(TT))|(TAA))GAAA(A|)TTTATGT(T|C)GTCTAT	
III (199-266)	5	CCCTGTTGAATAGAGTGTTTTCG((AA)|(TT))CA((TGA)|(GGG))AATTCGCAGGG	
	6	CCCTG(G|A|A|(TA)|(AT)|TG(A|G)AAGAAA(A|)GTCGT(T)AAGATTTTTTTT-(T|)CATT(T)AGGG	*Opinomyces*/ Piromyces II
	7	CCCT(T)GGATT(T|)ATTTGGAAAAGTTGAAAGACTTTTTCATTT(A|)ACCA(A|)GGG	
	8	CCCTGGTGGTTT(T|)(T|)GGAAAAGTTGAAAAACTTTTTCGAATCC(C|(CT)|(TC))AGGG	
IV (264-318)	5	GG(G|)A(A|C)TAGGCTTTCATAGAC(A|C)TT	

Fig. 5: Variable region motif sequences of internal transcribed spacer region 1 (ITS1) for *Orpinomyces*

Table 2: The variable region motif sequences of the ITS 1 region of some of the sequenced Indian isolates of *Orpinomyces.*

		ITS 1 Variable regions				
Isolate	Genbank accession no.	I	II	III	IV	Sequence group
NIANP49	EU150189	5	8	7	5	*Orpinomyces*
NIANP57	EU150191	5	8	8	5	*Orpinomyces*
NIANP59	EU150190	5	8	8	5	*Orpinomyces*
NIANP58	EU150192	5	8	7	5	*Orpinomyces*
NIANP60	EU150188	5	8	7	5	*Orpinomyces*

sent for sequencing. Multiple alignments of sequences were generated using CLUSTAL and manually edited using Align. These sequences were clustered to identify different motifs with differing levels of degeneracy within each of the four variable regions of the ITS1 sequences (Fig. 5). Motifs were drawn from sequence 62-96 for variable region I; 130-196 for variable region II; 198-266 for variable region III and 264-318 for variable region IV.

The results of the sequencing of the Indian *Orpinomyces* isolates summarized in Table 2 shows the isolates were grouped under Orpinomyces sequence group based on Tuckwell *et al.* (2005). Of all the isolates sequenced the sequences of three of the Indian isolates of *Orpinomyces* (NIANP 49, NIANP 58 and NIANP 60) had motifs 5, 8, 7 and 5 corresponding to Variable region I, II, III and IV, respectively and morphologically were identified as *Orpinomyces joyonii*. The sequence of two isolates (NIANP 57 and 59) however, was different from the rest of the isolates in having motif 8 in Variable region III and morphologically were identified as *Orpinomyces intercalaris*. The DNA sequences of thirty anaerobic gut fungus were retrieved from Genbank and the evolutionary relationships plotted using the Neighbor -Joining method. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

As per the phylogenetic analysis, the published *Orpinomyces* and the five Indian isolates are clearly grouped together (Fig. 6). The isolates NIANP 60 and 58 are different from

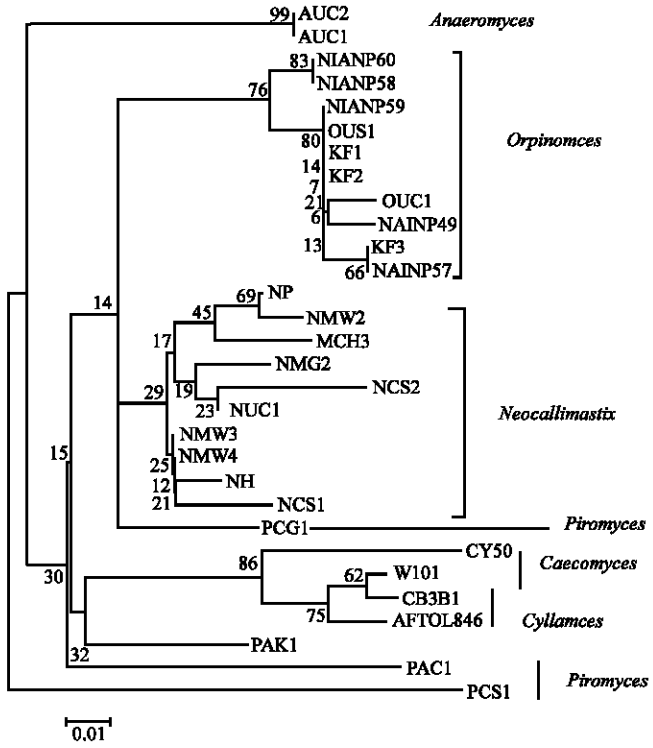


Fig. 6: Evolutionary relationships of 30 DNA sequences of anaerobic gut fungus retrieved from Genbank using the Neighbor -Joining method. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007)

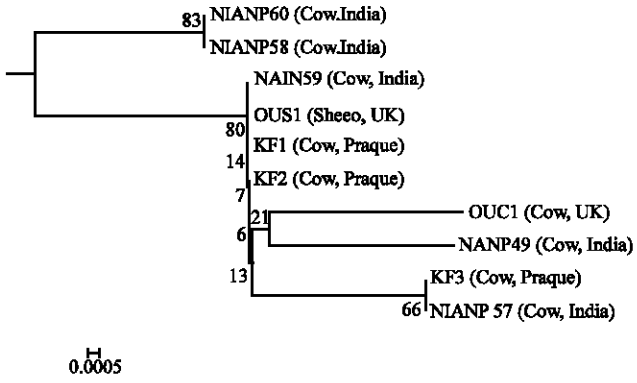


Fig. 7: Phylogenetic relationship within the *Orpinomyces* isolates with their source and Country of origin

the rest of the isolates. The isolate NIANP 59(Genbank Acc. No. EU150190) is similar to the OUS1 isolate of Sheep from UK (Fig. 7). The isolates NIANP 49 (Genbank Acc. No. EU150189) and 57 (Genbank Acc. No. EU150191) are similar to OUC1 (Cow, UK) and KF3 (Cow, Prague) isolates, respectively.

DISCUSSION

Morphological Characteristics

Several groups of workers simultaneously recognized the existence of polycentric types of anaerobic fungi in the rumen (Barr *et al*., 1989; Borneman *et al*., 1989; Breton *et al*., 1989; Phillips, 1989) which produce a very extensive branched rhizoid which contains nuclei and develops multiple sporangia at various intervals along the same rhizoid. *Orpinomyces* has polyflagellated zoospores similar to those of *Neocallimastix* sp. with polycentric thalli and metabolically and enzymatically also it appears similar to *Neocallimastix*. The genus is distinguished by the presence of many sporangiophore initials (swollen outgrowths from the hyphae) and globose sporangia formed either intercalarily in the hyphae or terminally on mature sporangiophores which can be single or branched complexes (Ho *et al*., 2000). Ho and Barr (1995) described two species from Malaysia viz., *Orpinomyces joyonii* and *Orpinomyces* intercalaris the former being characterized by spherical sporangia developing on branched or simple sporangiophore complexes and the hypae having light constrictions, while the latter had globose sporangia developing from expansion of hyphae or as lateral outgrowths but rarely terminal. Several workers have classified *Orpinomyces joyonii* based on zoospore morphology, polycentric nature and extensive rhizomycelia (Ho and Bauchop, 1991). Both *Orpinomyces joyonii* and *Orpinomyces* intercalaris were isolated in large numbers from the rumen as well as dung of Indian cattle.

Enzyme Studies

The principal cellulose degrading components are a mixture of several endoglucanases and cellobiohydrolases which work in combination with β-glucosidase. The presence and activity of some surface associated enzymes have been shown to fluctuate according to the stage of the life cycle (Breton *et al*., 1995; Gerbi *et al*., 1996). Comparisons have been difficult to make because of the different experimental conditions employed by different workers. Williams *et al*. (1994) reported no differences among genera in cellulolytic and xylanolytic enzyme profiles as measured by activity assay, although the level and regulation of activity differed among strains. Zuhainis *et al*. (2007) in studying the effects of phenolic monomers on the degradation of ^{14}C-cellulose by rumen fungi isolated from rumens of cattle, buffalo and goat reported the highest cellulolytic activity in *Neocallimastix frontalis* B9 followed by *Piromyces mae* B6 and *O. joyonii* C3. They further reported that the cellulolytic activities of *N. frontalis* isolates from buffalo, cattle and goat were not significantly different. The results of our study contribute to enzyme characterization of polycentric anaerobic fungi reported by Ho *et al*. (1994) and open a scope for reflection of probability that both groups of *Orpinomyces* isolates had different strategy for fiber degradation a finding not in agreement with aforementioned reports. *Orpinomyces intercalaris* showed higher activities of cellobiohydrolase and β-xylosidase and definitely would act on oligosaccharides while *O. joyonii* was characterized by high endoglucanase, xylanase and β-glucosidase activity which act on plant structural polysaccharides. Both groups cooperate in fiber degradation in the rumen environment. Paul *et al*. (2004) evaluated the effect of the inclusion of different strains of anaerobic fungi to mixed rumen micro flora of buffalo on *in vitro* feed digestion, fermentation characteristics and the fibrolytic enzyme profile and reported the production of carboxymethyl cellulase, xylanase, acetyl esterase and β-glucosidase to be significantly ($p<0.05$) higher in the FNG5 (*Piromyces* sp., isolated from faeces of nilgai) inoculated incubation medium. The FNG5 isolate also showed the highest ($p<0.05$) level of CMCase

(13.88 *vs.*, 9.75 mIU•mL^{-1}), xylanase (30.73 *vs.*, 23.29 mIU•mL^{-1}), acetyl esterase (308.8 *vs.*, 234.6 mIU•mL^{-1}) and -$\tilde{\beta}$ glucosidase (390.3 *vs.*, 316.3 mIU•mL^{-1}) as compared to the control group.

Tripathi *et al.* (2007) reported isolate WNG-12 (*Piromyces* sp.), to show maximum filter paper cellulase (23 mIU mL^{-1}) and xylanase (127 mIU mL^{-1}) activity, while WNG-5 (*Piromyces* sp.) showed maximum carboxymethyl cellulase activity (231 mIU mL^{-1}). Yanke *et al.* (1996) compared the cellulolytic and xylanolytic activities among genera of anaerobic fungi when grown on glucose, xylan and the cellulosic substrates, filter paper and Avicel. All the fungi had basal extra cellular fibrolytic activities that could be enhanced by growth on xylan or the cellulosic substrates. However, *Piromyces communis* strain 22 and *Neocallimastix patriciarum* strain 27 had substantially greater levels of fibrolytic activity than *Orpinomyces joyonii* strain 19-2 or *Neocallimastix frontalis* strain RE1. Zymogram analysis suggested both structural and regulatory differences amongst the enzyme systems of the fungi. Numerous and varied enzyme bands were evidenced for all the fungi, with substantial substrate influences seen in the xylanase activities. Their results suggest that while the enzymatic activities are functionally similar, there are likely significant variations in the enzyme systems of the anaerobic fungi a finding in agreement with that of the present study.

Molecular Characterization

The DNA-based techniques have been adopted for understanding the phylogenetic relationships and diversity of micro-organisms in natural ecosystems as they introduce considerably fewer biases in sampling than culture-based methodologies. They can be generated directly from DNA and are considered more representative of the entire community than culture-derived data alone (Ward *et al*., 1990). Molecular data has been used to clarify the classification of the anaerobic gut fungi. Dore and Stahl (1991) and Bowman *et al.* (1992) used partial 18S rRNA sequence data to support the assignment of these fungi to the chytridiomycetes. 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.

Favored indicators of genetic diversity are the rRNA encoding gene sequences, particularly the internal transcribed spacers ITS1 and ITS2 and the intervening 5-8S rDNA; these can be used both to identify micro-organisms and to determine phylogenetic relationships within communities (Hausner *et al*., 2000; Vainio and Hantula, 2000; Ranjard *et al*., 2001), including the gut fungi (Brookman *et al*., 2000). There is a high degree of conservation in 18S rRNA gene sequences across the *Neocallimastigales*, for which morphological criteria have been used as the principal means of classifying the six genera and their species that constitute the order. There has, however been more recent progress on the use of internally transcribed spacer region sequences as a reliable means of identifying anaerobic fungi to the genus level (Brookman *et al*., 2000; Tuckwell *et al*., 2005). Lockhart *et al*. (2006) designed oligonucleotide primers for the 18S rRNA genes of members of the *Neocallimastigales* and used it in a nested PCR protocol to amplify 787-bp fragments of DNA from landfill site samples and demonstrated the occurrence of members of the *Neocallimastigales* outside the mammalian gut for the first time.

Dore and Stahl (1991) and Li and Heath (1992) assessed the use of 18S rRNA sequences for the phylogenetic analysis of gut fungi. While the former found very little difference between the 18S rRNA sequences of *Neocallimastix* and *Piromyces* (97% identity) a shorter 18S rRNA segment studied by the latter could not distinguish between the genera *Orpinomyces*, *Neocallimastix* and *Piromyces*. Thus, while these studies were able to

produce trees showing the relationships between the gut fungi and other fungi, the 18S rRNA sequence was clearly unsuitable for determining relationships within the group of gut fungi. Li and Heath (1992) further used sequence data from a less well conserved ribosomal sequence, the internal transcribed spacer 1 region (ITS1) to compare and discriminate gut fungi and showed that the genera *Orpinomyces*, *Neocallimastix* and *Piromyces* were closely related to each other and more distantly related to the genera *Anaeromyces* and *Caecomyces*. However, they failed to determine the relationships within the two cluster groups. Thus they concluded that sequence data alone could not resolve the inter-relationships between these closely related genera. In a subsequent study, Li *et al*. (1993) used cladistic analysis of 42 morphological, ultrastructural and mitotic characters to attempt to determine the phylogenetic relationships of the anaerobic gut fungi.

Isozymes of twenty three cultures of the anaerobic rumen fungi and seven cultures of aerobic chytridiomycete fungi were analysed by PAGE by Ho *et al*. (1994) and *Orpinomyces* was found to be more related to *Piromyces* and *Caecomyces* than to *Neocallimastix*. *Orpinomyces intercalaris* C 70 from cattle showed large genetic variation from *O. joyonii*, indicating that it was a different species.

Kopecni (1995) in studies on rumen fungi in domestic and wild herbivores stated that systematics was based on spore and mycelium morphology which was dependent on growth conditions and hence they concluded that analysis of genetic information was the only reliable approach to rumen fungal systematics. The studies of Brookman *et al*. (2000) also showed, on average, pair wise identity of 97±4% for the 18S rRNA region which contrasts with the data from ITS1 sequences, which, as they show greater variation, are suitable for determining the relationships between two genera of gut fungi and thus has considerable potential for use in ecological studies of these organisms. The *Orpinomyces* isolates obtained in Indian cattle and buffaloes were similar to those reported from other parts of the world as ascertained from both morphological characteristics as well as sequence data and are in agreement with Hausner *et al*. (2000) and conclusively prove that sequence analysis of ITS1 region by variable region motif sequences or the traditional phylogenetic analysis can serve as a powerful tool for characterization and classification of anaerobic gut fungi to the generic level in large ecological studies but sequence data alone cannot resolve the inter-relationships between these closely related genera and has to be supplemented with data from morphological studies as ITS sequences alone are unlikely to be of use in understanding the relationships between fungi at the species level.

CONCLUSION

The study of anaerobic gut fungi is hampered by the lack of reliable methods for their accurate identification, differentiation and enumeration. Morphology of gut fungi coupled with enzyme assays is extremely important and cannot be eliminated from strain identification procedures because some genera of gut fungi exhibit a high genetic heterogeneity. If carried out with precision and expertise it definitely is inexpensive and reliable. Molecular biology on the other hand provides tools and approaches which aid in the challenges faced by rumen ecologists and nutritionists. A combination of both of the afore said approaches can definitely help in solving problems of systemic classification of anaerobic fungi.

ACKNOWLEDGMENTS

The authors are grateful to the Indian Council of Agricultural Research, New Delhi, India (ICAR) and the Australian Council for International Agricultural Research, Queensland,

Australia (ACIAR) for the bilateral collaboration and financial support received under the project, Increasing productivity of cattle in India and Australia by rumen fungal treatments under the aegis of which this work was carried out. The work was carried out at the National Institute of Animal Nutrition and Physiology, Adugodi, Bangalore, India from January 2005 to June 2008.

REFERENCES

Akin, D.E., 1980. Evaluation by electron microscopy and anaerobic culture of types of rumen bacteria associated with digestion of forage cell walls. Environ. Microbiol., 39: 242-252.

Barr, D.J.S., H. Kudo, K.D. Jakober and K.J. Cheng, 1989. Morphology and development of rumen fungi: *Neocallimastix* sp., *Piromyces communis* and *Orpinomyces bovis* gen. nov., sp. nov. J. Bot., 67: 2815-2824.

Borneman, W.S., D.E. Akin and L.G. Ljungdahl, 1989. Fermentation products and plant cell wall-degrading enzymes produced by monocentric and polycentric anaerobic ruminal fungi. Applied Environ. Microbiol., 55: 1066-1073.

Bowman, B.H., J.W. Taylor, A.G. Brownlee, J. Lee, S.D. Lu and T.J. White, 1992. Molecular evolution of the fungi: Relationships of the Basidiomycetes, Ascomycetes and Chytridiomycetes. Mol. Biol. Evol., 9: 285-296.

Breton, A., A. Bernalier, F. Bonnemoy, G. Fonty, B. Gaillard and P.H. Gouet, 1989. Morphological and metabolic characterization of a new species of strictly anaerobic rumen fungus: *Neocallimastix joyoni*. FEMS Microbiol. Lett., 58: 309-314.

Breton, A., B. Gaillard-Martinie, C. Gerbi, B.G. de Segura, R. Durand and B. Kherratia, 1995. Location by fluorescence microscopy of glycosidases and a xylanase in the anaerobic gut fungi *Caecomyces communis*, *Neocallimastix frontalis* and *Piromyces rhizinfita*. Curr. Microbiol., 31: 224-227.

Brookman, J.L., G. Mennim, A.P.J. Trinci, M.K. Theodorou and D.S. Tuckwell, 2000. Identification and characterization of anaerobic gut fungi using molecular methodologies based on ribosomal ITS1 and 18S rRNA. Microbiology, 146: 393-403.

Caldwell, D.R. and M.P. Bryant, 1966. Medium without rumen fluid for non-selective enumeration and isolation of rumen bacteria. Applied Microbiol., 14: 794-801.

Chen, H., S.L. Hopper, X. Li, L.G. Ljungdahl and C.E. Cerniglia, 2006. Isolation of extremely AT-rich genomic DNA and analysis of genes encoding carbohydrate-degrading enzymes from *Orpinomyces* sp. strain PC-2. Curr. Microbiol., 53: 396-400.

Christakopoulos, P., D. Kekos, B.J. Macris, M. Claeyssens and M.K. Bhat, 1995. Purification and mode of action of a low molecular mass endo-1,4-b-D-glucanase from *Fusarium oxysporum*. J. Biotechnol., 39: 85-93.

Dore, J. and D.A. Stahl, 1991. Phylogeny of anaerobic rumen Chytridiomycetes inferred from small subunit ribosomal RNA sequence comparison. Can. J. Bot., 69: 1964-1971.

Gerbi, C., J. Bata, A. Breton and G. Premier, 1996. Glycoside and polysaccharide hydrolase activity of the rumen anaerobic fungus *Caecomyces communis* (*Sphaeromoms communis* sensu Orpin) at early and final stages of the developmental cycle. Curr. Microbiol., 32: 256-259.

Hausner, G., G. Inglis, L.J. Yanke, L.M. Kawchuk and T.A. McAllister, 2000. Analysis of restriction fragment length polymorphisms in the ribosomal DNA of a selection of anaerobic chytrids. Can. J. Bot., 78: 917-927.

Ho, Y.W. and D.J.S. Barr, 1995. Classification of anaerobic gut fungi from herbivores with emphasis on rumen fungi from Malaysia. Mycologia, 87: 655-677.

Ho, Y.W. and T. Bauchop, 1991. Morphology of three polycentric rumen fungi and description of a procedure for the induction of zoosporogenesis and release of zoospores in cultures. J. Gen. Microbiol., 137: 213-217.

Ho, Y.W., I.Y. Khoo, S.G. Tan, N. Abdulah, S. Jalaludin and H. Kudo, 1994. Isoenzyme analysis of anaerobic rumen fungi and relationship to aerobic chytrids. Microbiology, 140: 1495-1504.

Ho, Y.W., N. Abdullah and S. Jalaludin, 2000. The diversity and taxonomy of anaerobic gut fungi. Fungal Diversity, 4: 37-51.

Kopecni, J., 1995. Rumen Fungi in Domestic and Wild Herbivores. In: Rumen Ecology Research Planning, Wallace, R.J. and A. Lahlou-Kassi (Eds.). ILRI, Ethiopia, pp: 103-120.

Li, J. and I.B. Heath, 1992. The phylogenetic relationships of the anaerobic chytridiomycetous gut fungi (*Neocallimasticaceae*) and the *Chytridiomycota*. I. Cladistic analysis of rRNA sequences. Can. J. Bot., 70: 1738-1746.

Li, J., I.B. Heath and L. Packer, 1993. The phylogenetic relationships of the anaerobic chytridiomycetous gut fungi (Neocallimasticaceae) and the *Chytridiomycota*. II. *Cladistic* analysis of structural data and description of *Neocallimasticales ord.* nov. Can. J. Bot., 71: 393-407.

Lockhart, R.J., M.I. van Dyke, I.R. Beadle, P. Humphreys and A.J. McCarthy, 2006. Molecular biological detection of anaerobic gut fungi (*Neocallimastigales*) from Landfill Sites. Applied Environ. Microbiol., 72: 5659-5661.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.

Ozkose, E., B.J. Thomas, D.R. Davies, G.W. Griffith and M.K. Theodorou, 2001. *Cyllamyces aberensis* gen. nov., sp. nov., a new anaerobic gut fungus with branched sporangiophores isolated from cattle. Can. J. Bot., 79: 666-673.

Paul, S.S., D.N. Kamra, V.R.B. Sastry, N.P. Sahu and A. Kumar, 2003. Effect of phenolic monomers on growth and hydrolytic enzyme activities of an anaerobic fungus isolated from wild nilgai (*Boselaphus tragocamelus*). Lett. Applied Microbiol., 36: 377-381.

Paul, S.S., D.N. Kamra, V.R.B. Sastry, N.P. Sahu and N. Agarwal, 2004. Effect of anaerobic fungi on *in vitro* feed digestion by mixed rumen microflora of buffalo. Reprod. Nutr. Dev., 44: 313-319.

Phillips M.W. and G.L.R. Gordon, 1989. Growth characteristics on cellobiose of three different anaerobic fungi isolated from the ovine rumen. Applied Environ. Microbiol., 55: 1695-1702.

Phillips, M.W. and G.L.R. Gordon, 1988. Sugar and polysaccharide fermentation by rumen anaerobic fungi from Australia, Britain and New Zealand. Biosystems, 21: 377-383.

Phillips, M.W., 1989. Unusual Rumen Fungi Isolated from Northern Australian Cattle and Water Buffalo. In: The Roles of Protozoa and Fungi in Ruminant Digestion, Nolan, J.V., R.A. Leng and D.I. Demeyer (Eds.). Penambul Books, Armidale, Australia pp: 247-249.

Puls, J. and K. Poutanen, 1989. Mechanisms of Enzymic Hydrolysis of Hemicelluloses (Xylans) and Procedures for Determinationof the Enzymic Activities Involved. In: Enzyme System for Lignocellulose Degradation, Coughlan, M.P. (Ed.). Elsevier Applied Science, London, pp: 151-165.

Ranjard, L., F. Poly, J.C. Lata, C. Mougel, J. Thioulouse and S. Nazaret, 2001. Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: Biological and methodological variability. Applied Environ. Microbiol., 67: 4479-4487.

Steel, R.G.D. and J.H. Torrie, 1980. Principles and Procedures of Statistics: A Biometrical Approach. 2nd Edn., McGraw Hill, New York, USA., ISBN-13: 978-0070609259.

Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24: 1596-1599.

Theodorou, M.K., G. Mennim, D. Davies, W.Y. Zhu, A.P.J. Trinci and J. Brookman, 1996. Anaerobic fungi in the digestive tract of mammalian herbivores and their potential for exploitation. Proc. Nutr. Soc., 55: 913-926.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins, 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res., 24: 4876-4882.

Tripathi,V.K., J.P. Sehgal, A.K. Puniya and K. Singh, 2007. Hydrolytic activities of anaerobic fungi from wild blue bull (*Boselaphus tragocamelus*) Anaerobe, 13: 36-39.

Tuckwell, D.S., M.J. Nicholson, C.S. McSweeney, M.K. Theodorou and J.L. Brookman, 2005. The rapid assignment of ruminal fungi to presumptive genera using ITS1 and ITS2 RNA secondary structures to produce group-specific fingerprints. Microbiology, 151: 1557-1567.

Vainio, E.J. and J. Hantula, 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol. Res., 104: 927-936.

Ward, D.M., R. Weller and M.M. Bateson, 1990. 16S ribosomal-RNA sequences reveal numerous uncultured microorganisms in a natural community. Nature, 345: 63-65.

Whealan, W.J., 1969. Hydrolysis with a-amylase. Meth. Carbohydr. Chem., 4: 252-260.

Williams, A.G., S.E. Withers and C.G. Orpin, 1994. Effect of the carbohydrate growth substrate on polysaccharolytic enzyme formation by anaerobic fungi isolated from the foregut and hindgut of nonruminant herbivores and the forestomach of ruminants. Lett. Applied Microbiol., 18: 147-151.

Yanke, L.J., L.B. Selinger, J.R. Lynn and K.J. Cheng, 1996. Comparison of the influence of carbon substrates on the fibrolytic activities of anaerobic rumen fungi. Anaerobe, 2: 373-378.

Zuhainis, S.W., N. Abdullah, A.R. Alimon and Y.W. Ho, 2007. Effects of phenolic Effects of phenolic monomers on the degradation of ^{14} C-Cellulose by rumen fungi. Res. J. Microbiol., 2: 918-925.