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Isozyme analysis of anaerobic rumen fungi and their relationship to aerobic chytrids

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Isozymes of 23 cultures of the anaerobic rumen fungi and seven cultures of aerobic chytridiomycete fungi were analysed by PAGE. A total of 14 isozyme loci were successfully typed by PAGE. They were peptidase A & C-1, peptidase A & C-2, peptidase D-1, peptidase D-2, malate dehydrogenase-1, malate dehydrogenase-2, esterase-1, esterase-2, malic enzyme-1, malic enzyme-2, isocitrate dehydrogenase, shikimate dehydrogenase, phosphoglucomutase and 6-phosphogluconate dehydrogenase. Isozyme analysis can be used for studying the genetic relationships among the different anaerobic rumen fungi and the aerobic chytridiomycete fungi and the isozyme characteristics can serve as additional taxonomic criteria in the classification of the anaerobic rumen fungi. A dendrogram based on the isozyme data demonstrated that the anaerobic rumen fungi formed a cluster, indicating a monophyletic group, distinctly separated from the aerobic chytridiomycete fungi. *Piromyces communis* and *P. minutus* showed a close relationship but *P. spiralis* showed a more distant relationship to both *P. communis* and *P. minutus*. *Piromyces* as a whole was more related to *Caecomyces* than to *Neocallimastix*. *Orpinomyces* was also 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 is a different species.

Keywords: Rumen fungi, anaerobic fungi, isozyme analysis, aerobic chytrids, *Neocallimastix*

INTRODUCTION

An anaerobic rumen fungus, *Neocallimastix frontalis*, (Braune) Vavra *et* Joyon ex Heath, was isolated in 1975 from the rumen contents of sheep (Orpin, 1975). However, its taxonomic status was not established until Heath *et al.* (1983) studied its ultrastructure, particularly the zoospore ultrastructure, and formally assigned it to the class Chytridiomycetes, order Spizellomycetales and a new family, Neocallimastigaceae [syn. Neocallimasticaceae,† Index of Fungi Supplement (Family Names), 1989] was established to accommodate it. Interest in the anaerobic gut fungi (fungi from the rumen as well as other parts of the gut of herbivores) in recent years has resulted in the discovery and description of many new species (Gold *et al.*, 1988; Barr *et al.*, 1989; Li *et al.*, 1990; Breton *et al.*, 1990, 1991; Ho *et al.*, 1990, 1993a, b, c, 1994).

† The original spelling (Neocallimasticaceae) was corrected to Neocallimastigaceae in the Index of Fungi Supplement (Family Names), 1989.

Many characteristics such as the life cycle, reproductive structures and vegetative thalli of the anaerobic fungi show that they are more closely related to the Chytridiomycetes than to any other group of fungi. Molecular data (18S rRNA sequences) of the fungi also show that they are indeed chytridiomycete fungi (Dore & Stahl, 1991; Bowman *et al.*, 1992; Li & Heath, 1992). The classification of the Chytridiomycetes based solely on morphological characteristics has been a matter of contention since many Chytridiomycete species exhibit extensive morphological variation, particularly on different substrates. In view of this, Barr (1980) proposed a new taxonomic system based on thallus development and zoospore ultrastructures which are considered more consistent and reliable. Under this new system, the former order Chytridiales was divided into two orders, namely, the Spizellomycetales Barr and Chytridiales *sensu* Barr. However, problems still exist for some chytridiomycete fungi which exhibit characteristics of both orders or of different families.

The classification of all the anaerobic gut fungi, studied so

Table 1. Isozyme allele frequencies in the anaerobic rumen fungi and aerobic chytrids

Locus	Allele	Fungus*																		
		1 (4)	2 (5)	3 (1)	4 (2)	5 (2)	6 (2)	7 (1)	8 (1)	9 (1)	10 (1)	11 (1)	12 (1)	13 (1)	14 (1)	15 (1)	16 (1)	17 (1)	18 (1)	19 (1)
PEP A & C-1	100	1·0	0·80	1·0	1·0	1·0	–	–	–	1·0	–	1·0	1·0	1·0	–	–	1·0	–	–	–
	102	–	0·20	–	–	–	–	–	–	–	–	–	–	–	1·0	1·0	–	1·0	–	–
	0	–	–	–	–	–	1·0	1·0	1·0	–	1·0	–	–	–	–	–	–	–	1·0	1·0
PEP A & C-2	100	–	–	–	–	–	1·0	1·0	–	1·0	–	–	–	–	–	–	–	–	–	–
	102	–	–	–	0·5	1·0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	72	–	–	–	–	–	–	–	–	–	–	1·0	1·0	–	–	–	–	–	–	–
	68	1·00	0·80	1·0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	64	–	0·20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	60	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0
	56	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–
	52	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	1·0	–	–	–
	50	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–
	46	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	1·0	–	–	–	–
	0	–	–	–	0·50	–	–	–	1·0	–	1·0	–	–	–	–	–	–	–	–	–
PEP D-1	100	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–	–	–	–	–
	106	0·25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	104	–	–	–	0·5	–	–	–	–	–	–	–	–	–	1·0	1·0	–	–	–	–
	102	0·75	0·80	1·0	–	–	–	–	–	–	–	–	–	1·0	–	–	1·0	–	1·0	1·0
	0	–	0·20	–	0·5	1·0	1·0	1·0	1·0	–	1·0	1·0	1·0	–	–	–	–	1·0	–	–
PEP D-2	100	–	–	–	0·5	–	–	–	–	1·0	1·0	–	–	–	–	–	–	1·0	1·0	1·0
	0	1·0	1·0	1·0	0·5	1·0	1·0	1·0	1·0	–	–	1·0	1·0	1·0	1·0	1·0	1·0	–	–	–
MDH-1	100	0·5	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	–	–	–	–	–	–
	106	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–
	98	0·5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	96	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	1·0	1·0	1·0
	0	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–
MDH-2	100	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–	–	–	–	–
	102	–	–	–	0·5	–	–	–	–	–	1·0	–	–	–	–	–	–	–	–	–
	96	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–	–	–
	94	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	1·0	–	–
	92	–	0·2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	90	1·0	0·8	1·0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	88	–	–	–	0·5	1·0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	86	–	–	–	–	–	1·0	1·0	1·0	–	–	–	–	–	–	–	–	–	–	–
	82	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–
	76	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–
	0	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	1·0	–	1·0	1·0
ES-1	100	1·0	1·0	1·0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	88	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–	–	–
	60	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–	–
	0	–	–	–	1·0	1·0	1·0	1·0	1·0	1·0	1·0	–	–	1·0	1·0	1·0	1·0	1·0	1·0	1·0
ES-2	100	1·0	1·0	1·0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	0	–	–	–	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
ME-1	100	–	0·2	1·0	0·5	–	–	–	–	1·0	1·0	1·0	1·0	–	–	–	–	–	–	–
	96	1·0	0·6	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–
	92	–	1·0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	88	–	–	–	0·5	1·0	1·0	1·0	1·0	–	–	–	–	–	–	–	–	–	–	–
	82	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–
	74	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0
	68	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–
	50	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	1·0	–	–
	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–

***Table 1** (cont.)*

Locus	Allele	Fungus*																		
		1 (4)	2 (5)	3 (1)	4 (2)	5 (2)	6 (2)	7 (1)	8 (1)	9 (1)	10 (1)	11 (1)	12 (1)	13 (1)	14 (1)	15 (1)	16 (1)	17 (1)	18 (1)	19 (1)
ME-2	100	0·4	1·0	–	–	–	–	–	–	1·0	–	–	1·0	–	–	–	–	1·0	–	–
	116	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–
	114	–	0·2	–	0·5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	106	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–	–	–
	92	1·0	0·4	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–	–	–	–
	90	–	–	–	0·5	1·0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	88	–	–	–	–	–	0·15	–	1·0	–	–	–	–	–	–	–	–	–	–	–
	86	–	–	–	–	–	0·5	1·0	–	–	–	–	–	–	–	–	–	–	–	–
	82	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–
	66	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–
	58	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–
	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	1·0
IDH	100	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–	–	–	–	–
	108	–	0·2	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–	–	–	–
	102	1·0	0·4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	98	–	0·2	1·0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	96	–	–	–	–	–	0·5	–	1·0	–	–	–	–	–	–	–	–	–	–	–
	92	–	0·2	–	0·5	0·5	–	1·0	–	–	–	1·0	–	–	–	–	–	–	–	–
	90	–	–	–	–	0·5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	0	–	–	–	0·5	–	0·5	–	–	–	–	–	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
SkDH	100	1·0	0·8	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	–	–	–	1·0	1·0	1·0	1·0	1·0	–
	112	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–
	98	–	0·2	–	–	–	–	–	–	–	–	1·0	1·0	–	–	–	–	–	–	–
PGM	100	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–	–	–	–	–
	96	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–	–	–	–	–
	94	–	–	–	–	–	0·5	–	–	–	–	–	–	–	–	–	–	–	–	–
	92	0·75	0·8	1·0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	74	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–
	70	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0
	0	0·25	0·2	–	1·0	1·0	0·5	1·0	1·0	–	1·0	–	1·0	1·0	1·0	1·0	–	1·0	1·0	–
6-PGDH	100	–	–	–	–	–	–	–	–	–	–	–	–	1·0	1·0	–	1·0	1·0	–	–
	110	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–
	102	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1·0	–	–	–	–
	0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	–	–	–	–	–	–	1·0

* 1, *N. variabilis* (buffalo); 2, *N. variabilis* (cattle); 3, *Neocallimastix* sp. C148; 4, *P. communis* (goat); 5, *P. communis* (sheep); 6, *P. minutus* (goat); 7, *P. minutus* (sheep); 8, *P. minutus* (deer); 9, *P. spiralis* (goat); 10, *Caecomyces* sp. (buffalo); 11, *Orpinomyces joyonii* (buffalo); 12, *Orpinomyces intercalaris* C70 (cattle); 13, *S. plurigibbosus* BR 33; 14, *Chytridium confervae* BR 97; 15, *S. punctatus* BR 117; 16, *R. rosea* BR 60; 17, *S. acuminatus* BR 62; 18, *R. rosea* BR 186; 19, *Catenaria anguillulae* BR 105. Numbers in parentheses indicate the number of isolates used.

far, is based mainly on the morphological and ultrastructural characteristics. As morphological characteristics may vary under different cultural conditions, other characteristics such as biochemical and genetic properties could be used as additional taxonomic criteria. Dore & Stahl (1991), Li & Heath (1992) and Li *et al.* (1993) assessed the phylogenetic relationships among the anaerobic gut fungi and their relationships with other eukaryotes using 18S rRNA sequences and cladistic analysis of structural data and showed that the anaerobic gut fungi are monophyletic and form a closely related group in the Chytridiomycetes. However, the gut fungi were found to be distinct from both the Spizellomycetales and the Chytridiales and a new order, the Neocallimastigales [syn. Neocallimasticales, Index of Fungi Supplement (Family Names), 1989] was established to accommodate them (Li *et al.*, 1993). At the generic level, the phylogenetic relationships among *Neocallimastix*, *Piromyces* and *Orpinomyces* are still controversial (Li & Heath, 1992; Li *et al.*, 1993) and need further analyses.

The genetic relationships of different taxa can also be assessed by comparing their isozymes. The objective of the present investigation was to determine the relationships among the anaerobic rumen fungi and their relationships to the aerobic chytridiomycete fungi based on their isozyme characteristics.

METHODS

Fungal isolates used. Twenty-three isolates of anaerobic rumen fungi and seven isolates of aerobic chytridiomycete fungi were studied. The anaerobic rumen fungal isolates, obtained from the rumen of fistulated animals, were *Neocallimastix variabilis* Ho [four isolates from buffalo (*Bubalus bubalis*), five isolates from cattle (*Bos indicus*), including isolate C 15 which is the type culture for *N. variabilis*], one isolate, C 148 of *Neocallimastix* sp. from cattle, five isolates of *Piromyces minutus* Ho [two isolates from goat (*Capra hircus*), two from sheep (*Ovis aries*) and the type culture, D 2, from deer (*Cervus nippon*)], four isolates of *P. communis* Gold (two cultures from sheep and two from goat), one isolate of *P. spiralis* Ho (the type culture, G 34, from goat), one isolate of *Caecomyces* sp. (from buffalo), one isolate of *Orpinomyces joyonii* (Breton *et al.*) Li *et al.* (B 48 from buffalo) and one isolate of *O. intercalaris* Ho sp. (C 70, from cattle). When the fungal species are represented by more than one isolate from a particular host, the isolates were obtained from separate animals on different occasions.

The seven aerobic chytridiomycete fungal isolates were obtained from Dr D. J. S. Barr, Centre for Land and Biological Resources Research, Central Experimental Farm, Research Branch, Agriculture Canada, Ottawa, Ontario, Canada. The isolates (one each) were *Spizellomyces plurigibbosus* Barr (BR 33), *S. acuminatus* Barr (BR 62), *S. punctatus* (Koch) Barr (BR 117), *Rhizophlyctis rosea* (de Bary & Woronin) Fischer (BR 60), *R. rosea* (BR 186), *Chytridium confervae* (Wille) Minden (BR 97) and *Catenaria anguillulae* Sorokin (BR 105). *Spizellomyces* and *Rhizophlyctis* belong to the order Spizellomycetales, *Chytridium confervae* to the order Chytridiales and *Catenaria anguillulae* to the order Blastocladiales.

Culturing fungal isolates for routine maintenance and for morphological studies. The media for culturing the anaerobic rumen fungal isolates for morphological studies were glucose sloppy medium, cellulose sloppy medium (Ho & Bauchop, 1991) and liquid medium (Barr *et al.*, 1989) with straw as the carbon source. The fungal isolates were grown anaerobically (Hungate, 1969) at 39 °C in roll tubes containing 7·0 ml medium.

The aerobic chytridiomycete fungi were cultured in petri dishes (9 cm diameter) containing 15 ml yeast–peptone–glucose agar. The medium was similar to that described by Barr (1984). The cultures were incubated at 28 ± 1 °C.

Morphological characteristics of both the anaerobic rumen fungi and the aerobic chytridiomycete fungi were observed daily for 3–4 d using bright-field and phase-contrast microscopy.

Culturing fungal isolates for electrophoresis. The medium used for culturing the anaerobic rumen fungi for electrophoresis was glucose broth medium which was glucose sloppy medium without agar. The medium was prepared under anaerobic conditions using techniques described by Hungate (1969), and 500 ml and 1 l of the medium were dispensed into 500 ml and 1 l Duran bottles (Schott), respectively. The bottles were closed tightly with screw caps. Ten replicates were made for each medium volume and the bottles were autoclaved for 30 min at 121 °C and 1·4 kg cm^{-1}. When cooled, the bottles were inoculated with 1 % (v/v) fungal inoculum and incubated at 39 °C for 3 d after which the fungal thalli were harvested.

The aerobic chytridiomycete fungi were cultured in 250 ml Erlenmeyer flasks containing 100 ml yeast–peptone–glucose liquid medium. The medium was similar to yeast–peptone–glucose agar except that agar was omitted. The medium was autoclaved and inoculated in the same manner as described above. The culture was incubated at 28 ± 1 °C for 3 d after which the fungal thalli were harvested for electrophoresis.

Harvesting of fungal thalli for electrophoresis. *Neocallimastix variabilis*, *O. joyonii* and *O. intercalaris* sp. which produced thalli that were quite large were harvested by filtering through a Buchner funnel lined with a nylon cloth (mesh size 20 μm). *Spizellomyces acuminatus* (BR 62) which produced small thalli of low density was harvested by microfiltration through a layer of cellulose nitrate filter paper with a pore size of 0·45 μm diameter. All the other fungal isolates were harvested by centrifuging twice at 30000 ***g*** for 40 min at 4 °C. All the harvested fungal thalli were washed three times with distilled water and stored immediately in liquid nitrogen at −196 °C. The stored fungal thalli were used for enzyme extraction within a week.

Extraction of enzyme. About 0·2 g of frozen fungal thalli taken from liquid nitrogen storage was ground into a fine powder using an ice-cold mortar and pestle and 0·3 ml extraction buffer, 0·1 M Tris/HCl, pH 7·1, was added. The extract was then transferred into ampoules and centrifuged at 20000 ***g*** for 40 min at 4 °C. The supernatant was used for electrophoresis.

PAGE. PAGE was carried out using the 7% horizontal gel procedure described by Tan & Teng (1979). Twenty-four enzyme systems were studied using CA-7 buffer (Steiner & Joslyn, 1979). They were alcohol dehydrogenase (ADH) EC 1.1.1.1, peptidase A & C (Leu-Ala) (PEP A & C) EC 3.4.11, peptidase D (Phe-Pro) (PEP D) EC 3.4.13.9, succinate dehydrogenase (SuDH) EC 1.3.99.1, lactate dehydrogenase (LDH) EC 1.1.1.27, aconitase (ACO) EC 4.2.1.3, β-hydroxybutyrate dehydrogenase (HBDH) EC 1.1.1.30, xanthine dehydrogenase (XDH) EC 1.1.1.204, sorbitol dehydrogenase (SORDH) EC 1.1.1.14, glutamate dehydrogenase (GLD) EC 1.4.1.2, glutamate oxaloacetate transaminase (GOT) EC 2.6.1.1, hexokinase (HK) EC 2.7.1.1, acid phosphatase (ACP) EC 3.1.3.2, glucose dehydrogenase (GDH) EC 1.1.1.47, glucose-6-phosphate dehydrogenase (G6PD) EC 1.1.1.49, shikimate dehydrogenase (SkDH) EC 1.1.1.25, isocitrate dehydrogenase (IDH) EC 1.1.1.42, phosphoglucoisomerase (PGI) EC 5.3.1.9, malic enzyme (ME) EC 1.1.1.40, mannose-6-phosphate isomerase (MPI) EC 5.3.1.8, malate dehydrogenase (MDH) EC 1.1.1.37, phosphoglucomutase (PGM) EC 5.4.2.2, 6-phosphogluconate dehydrogenase (6-PGDH) EC 1.1.1.44 and carboxylesterase (ES) EC 3.1.1.1.

The staining procedures for the various enzymes were adapted from Harris & Hopkinson (1976) and Vallejos (1983). The stained gels were rinsed with a wash solution containing 250 ml methanol, 100 ml glacial acetic acid and 650 ml distilled water. The isozyme banding patterns were observed directly from the gel which was placed on a light box and scored. The BIOSYS-1 computer package of Swofford & Selander (1989) was used to analyse the isozyme data. The experiments were done twice. All the chemicals used for enzyme extraction and PAGE were from Sigma.

Nei's unbiased genetic identity (*I*) (Nei, 1978) was used to estimate the proportion of genes that are identical in structure in any two populations of fungi. For fungal species with more than one isolate from an animal species, the isolates were analysed

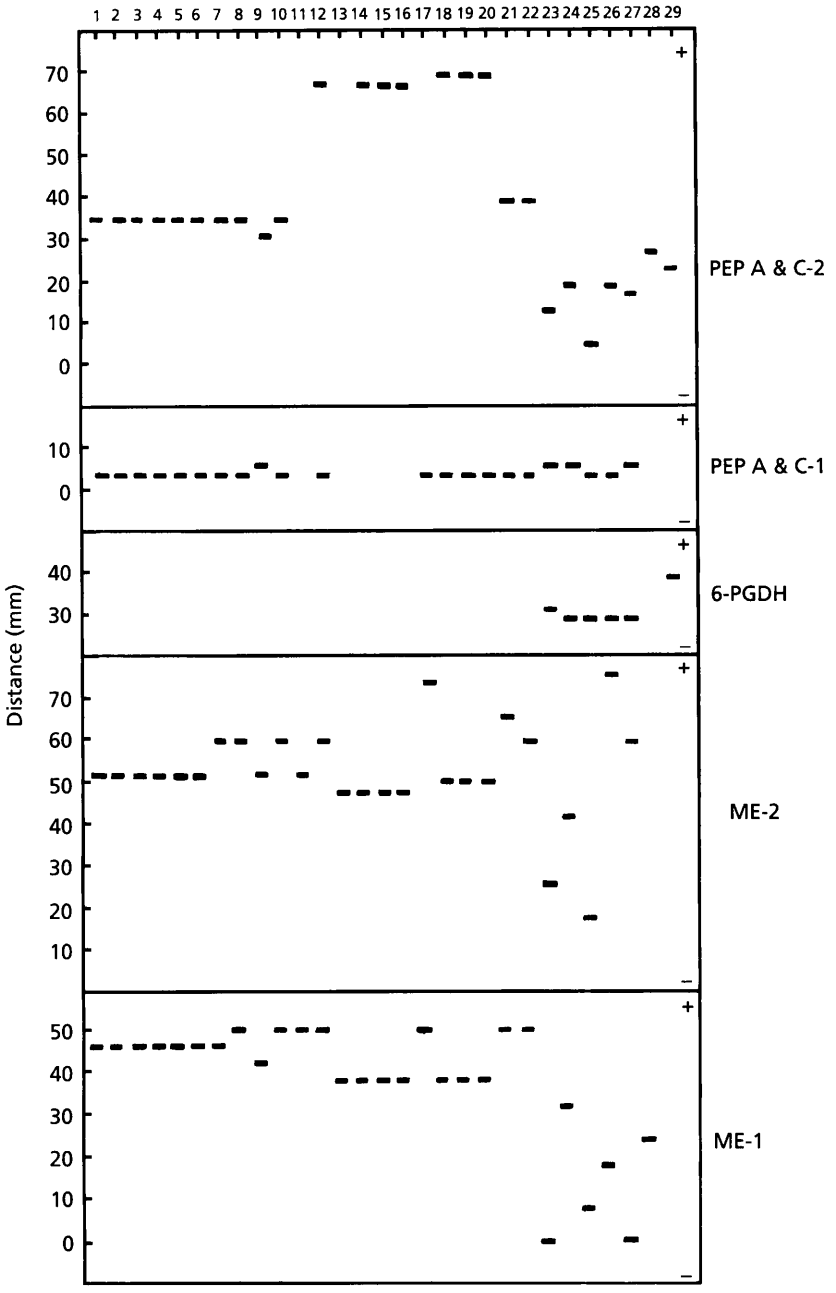
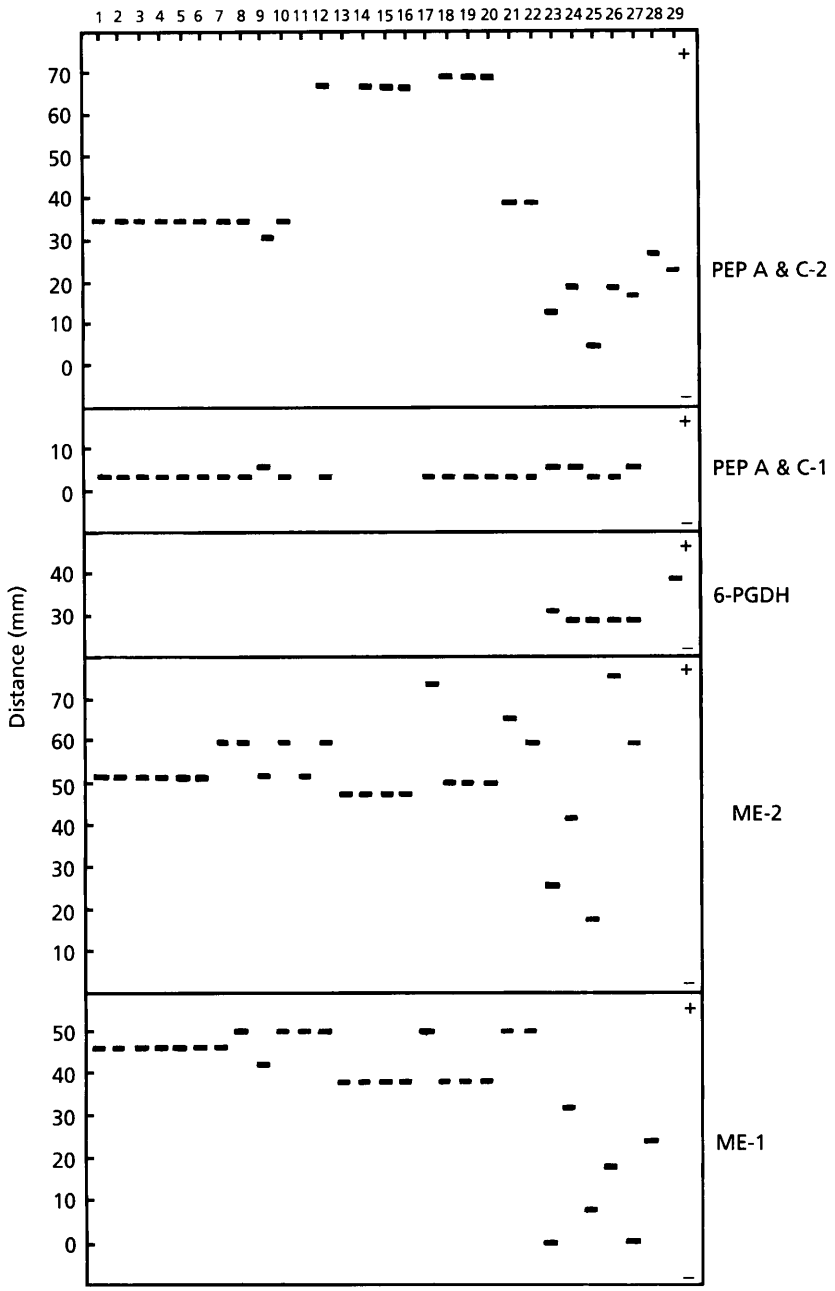


Fig. 2. Diagrammatic representation of isozyme banding patterns of PEP A & C-1 & -2, 6-PGDH and ME-1 & -2. The fungi used are as listed in the legend to Fig. 1.

The phenotypes observed for all the isozyme loci are shown diagramatically in Figs 1 and 2. Representative gels of isozyme banding patterns for MDH-1 and -2 are shown photographically in Figs 3 and 4.

Nei's genetic identity or similarity (I) and Nei's genetic distance (D) (Table 2) were calculated to give an overall view of the relationships among the anaerobic rumen fungi and their relatedness to the aerobic chytrids. The dendrogram based on Nei's genetic identities is shown in Fig. 5. The populations were divided into two major clusters: one group containing the anaerobic rumen fungi and the other group containing the aerobic chytridiomycete fungi. Generally, the genetic identity for a particular species of rumen fungus originating from different host animals was higher than 0·80. This indicates that the host environment did not influence the phenotype of the rumen fungus and future studies can be conducted using isolates from one host animal.

The isolate C 148, a *Neocallimastix* sp. originating from cattle, showed slight variation ($\bar{I} = 0{\cdot}845$, $\bar{D} = 0{\cdot}190$, Table 3) from the other *N. variabilis* isolates originating from cattle and buffalo. Although morphological characteristics of the rhizoidal systems of *Neocallimastix* sp. C 148 and *N. variabillis* showed some differences [rhizoids of *Neocallimastix* sp. C 148 were very short, compact and closely-branched below the sporangium but those of *N.*



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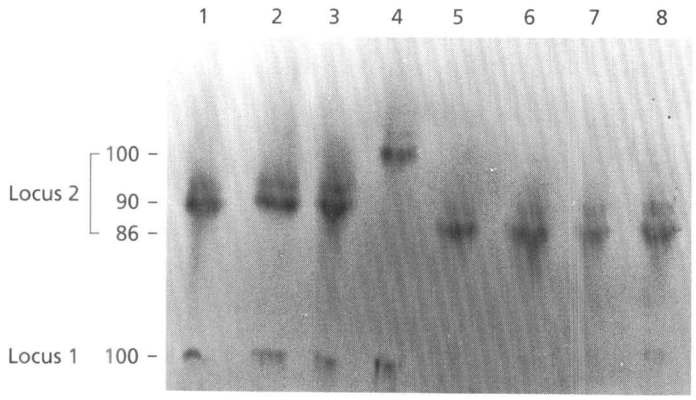

Fig. 3

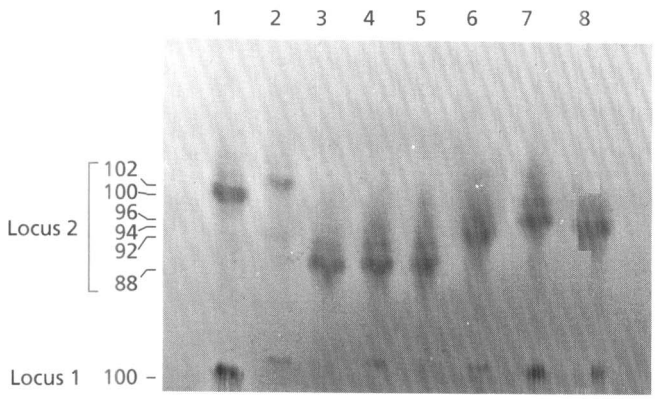

Fig. 4

Fig. 3. Typical gel with isozyme banding patterns for MDH-1 and -2 of anaerobic rumen fungi *N. variabilis* from cattle (1 and 2), *Neocalimastix* sp. C148 from cattle (3), *P. spiralis* (4), *P. minutus* from deer (5), *P. minutus* from goat (6 & 7) and *P. minutus* from sheep (8). Locus 1 shows allele 100 and locus 2 shows alleles 100, 90 and 86.

Fig. 4. Typical gel with isozyme banding patterns for MDH-1 and -2 of anaerobic rumen fungi *P. spiralis* (1), *P. communis* from goat (2 and 3), *P. communis* from sheep (4 and 5), *N. variabilis* from cattle (6), *O. intercalaris* (7) and *O. joyonii* (8). Locus 1 shows allele 100 and locus 2 shows alleles 102, 100, 96, 94, 92 and 88.

variabilis were longer, less dense with a coiled main rhizoid (Ho *et al.*, 1993a)], the genetic distance ($\bar{D}$ = 0·190) was too small to distinctly separate *Neocallimastix* sp. C 148 as a different species, but it may be a subspecies.

Piromyces communis and *P. minutus* fell into the same cluster (Fig. 5) indicating a close relationship ($\bar{I}$ = 0·663, $\bar{D}$ = 0·412, Table 3). This is reflected by some of their morphological characteristics such as sporangium shape, rhizoidal system and manner of zoospore release which are quite similar. *Piromyces spiralis*, however, showed a more distant relationship to both *P. communis* ($\bar{I}$ = 0·526, $\bar{D}$ = 0·657, Table 3) and *P. minutus* ($\bar{I}$ = 0·416, $\bar{D}$ = 0·882, Table 3) (Fig. 5). This is supported by morphological studies which showed that the rhizoidal system, thallus development and sporangial wall of *P. spiralis* were quite different from those of *P. communis* and *P. minutus* (Ho *et al.*, 1993b, c). The thallus development of *P. spiralis* is strictly endogenous, the rhizoidal system is extensive and the rhizoids are highly coiled. The neck and port of the main rhizoid of *P. spiralis* are also constricted and usually the portion below the neck is swollen and bulbous. The sporangial wall is highly evanescent and dissolution of the sporangial wall is so rapid that the zoospores are usually found in a cluster on the main rhizoid without a sporangial wall (Ho *et al.*, 1993c).

Piromyces as a whole, showed a closer relationship to *Caecomyces* ($\bar{I}$ = 0·629, $\bar{D}$ = 0·480, Table 3) than to *Neocallimastix* ($\bar{I}$ = 0·361, $\bar{D}$ = 1·039, Table 3). In contrast, Dore & Stahl (1991) using 18S rRNA sequences found *Piromyces* (*P. communis*) to be more closely related to *Neocallimastix* (*N. frontalis*) than to *Caecomyces* (*C. communis* syn. *Sphaeromonas communis*; Gold *et al.*, 1988). Recently, Li *et al.* (1993), using cladistic analysis of structural data, found that *P. dumbonica* and *C. equi* were closely related forming a cluster but *C. communis* was consistently isolated and was closer to *Neocallimastix*.

Orpinomyces joyonii (B 48) and *O. intercalaris* (C 70), although clustered together, showed a low genetic identity value (I) of 0·571 (D = 0·560). This supports the contention that they are two different species based on morphological differences (Ho *et al.*, 1994). The sporangia of *O. intercalaris* (C 70) are intercalary, produced within the hyphae in the rhizomycelium, but those of *O. joyonii* (B 48) are terminal, borne at the apex of sporangiophore complexes. The zoospore cyst of *O. intercalaris* (C 70) is also persistent and remains as a distinct structure attached to the rhizomycelium.

The dendrogram (Fig. 5) and the matrix of genetic similarity and distance coefficients (Tables 2 and 3) showed that *Orpinomyces* (as a whole) is more related to *Piromyces* ($\bar{I}$ = 0·469, $\bar{D}$ = 0·775, Table 3) and *Caecomyces* ($\bar{I}$ = 0·465, $\bar{D}$ = 0·770, Table 3) than to *Neocallimastix* ($\bar{I}$ = 0·344, $\bar{D}$ = 1·079, Table 3). This result concurs in part with the findings of Dore & Stahl (1991) where rRNA sequences showed *Orpinomyces* (*N. joyonii* syn. *O. joyonii*; Li *et al.*, 1991) and *Piromyces* (*P. communis*) to be closely related but they were closer to *Neocallimastix* (*N. frontalis*) than to *Caecomyces* (*C. communis* syn. *S. communis*). However, Li & Heath (1992), using cladistic analysis of rRNA sequences, found that the relationships between *Orpinomyces*, *Piromyces* and *Neocallimastix* were not clear and depended upon the algorithms used. *Orpinomyces* could be close to *Piromyces* and remote from *Neocallimastix* or close to *Neocallimastix* and remote from *Piromyces*. The majority of their data, however, seemed to favour, but did not strongly support, the close relationship between *Orpinomyces* and *Piromyces*. To clarify the relationships, Li *et al.* (1993) later used cladistic analysis of all available structural data which included morphological, ultrastructural and mitotic characteristics of the anaerobic gut fungi but again found the relationships between the three genera to be

Table 2. Matrix of genetic similarity (*I*) and distance (*D*) coefficients

Numbers below diagonal: Nei (1978) unbiased genetic distance (*D*); numbers above diagonal: Nei (1978) unbiased genetic identity or similarity (*I*).

Population*	Fungus*																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1		0·936	0·754	0·439	0·367	0·295	0·283	0·283	0·264	0·358	0·264	0·283	0·264	0·151	0·170	0·283	0·170	0·151	0·132
2	0·067		0·908	0·525	0·448	0·379	0·377	0·360	0·360	0·428	0·394	0·411	0·326	0·154	0·188	0·291	0·188	0·206	0·171
3	0·283	0·096		0·437	0·366	0·309	0·286	0·286	0·429	0·357	0·357	0·357	0·286	0·143	0·143	0·286	0·143	0·214	0·143
4	0·824	0·645	0·827		0·874	0·638	0·656	0·656	0·612	0·744	0·525	0·612	0·525	0·437	0·481	0·437	0·481	0·437	0·350
5	1·003	0·804	1·005	0·135		0·672	0·695	0·659	0·439	0·586	0·476	0·586	0·439	0·293	0·366	0·366	0·366	0·293	0·220
6	1·220	0·969	1·176	0·450	0·398		0·926	0·887	0·463	0·656	0·386	0·540	0·386	0·386	0·386	0·347	0·386	0·309	0·347
7	1·263	0·976	1·253	0·421	0·363	0·077		0·786	0·429	0·643	0·429	0·500	0·357	0·357	0·357	0·286	0·357	0·286	0·286
8	1·263	1·022	1·253	0·421	0·417	0·120	0·241		0·357	0·714	0·357	0·500	0·357	0·357	0·357	0·286	0·357	0·286	0·286
9	1·332	1·022	0·847	0·490	0·823	0·770	0·847	1·030		0·429	0·357	0·357	0·286	0·286	0·214	0·286	0·214	0·357	0·286
10	1·027	0·848	1·030	0·296	0·535	0·422	0·442	0·336	0·847		0·429	0·500	0·357	0·357	0·357	0·286	0·357	0·286	0·286
11	1·332	0·931	1·030	0·645	0·743	0·953	0·847	1·030	1·030	0·847		0·571	0·286	0·071	0·143	0·286	0·143	0·071	0·241
12	1·263	0·889	1·030	0·490	0·535	0·616	0·693	0·693	1·030	0·693	0·560		0·429	0·214	0·286	0·286	0·286	0·286	0·286
13	1·332	1·122	1·253	0·645	0·823	0·953	1·030	1·030	1·253	1·030	1·253	0·847		0·357	0·357	0·500	0·429	0·429	0·286
14	1·892	1·870	1·946	0·827	1·228	0·953	1·030	1·030	1·253	1·030	2·639	1·540	1·030		0·429	0·429	0·429	0·571	0·643
15	1·774	1·669	1·946	0·732	1·005	0·953	1·030	1·030	1·540	1·030	1·946	1·253	1·030	0·847		0·357	0·571	0·571	0·286
16	1·263	1·234	1·253	0·827	1·005	1·058	1·253	1·253	1·253	1·253	1·253	1·253	0·693	0·847	1·030		0·571	0·429	0·357
17	1·774	1·669	1·946	0·732	1·005	0·953	1·030	1·030	1·540	1·030	1·946	1·253	0·847	0·847	0·560	0·560		0·500	0·286
18	1·892	1·582	1·540	0·827	1·228	1·176	1·253	1·253	1·030	1·253	2·639	1·253	0·847	0·560	0·560	0·847	0·693		0·429
19	2·026	1·764	1·946	1·050	1·516	1·058	1·253	1·253	1·253	1·253	1·540	1·253	1·253	0·442	1·253	1·030	1·253	0·847	

* 1, *N. variabilis* (buffalo); 2, *N. variabilis* (cattle); 3, *Neocallimastix* sp. C148; 4, *P. communis* (goat); 5, *P. communis* (sheep); 6, *P. minutus* (goat); 7, *P. minutus* (sheep); 8, *P. minutus* (deer); 9, *P. spiralis* (goat); 10, *Caecomyces* sp. (buffalo); 11, *O. joyonii* (buffalo); 12, *O. intercalaris* C70 (cattle); 13, *S. plurigibbosus* BR 33; 14, *Chytridium confervae* BR 97; 15, *S. punctatus* BR 117; 16, R. *rosea* BR 60; 17, *S. acuminatus* BR 62; 18, R. *rosea* BR 186; 19, *Catenaria anguillulae* BR 105.

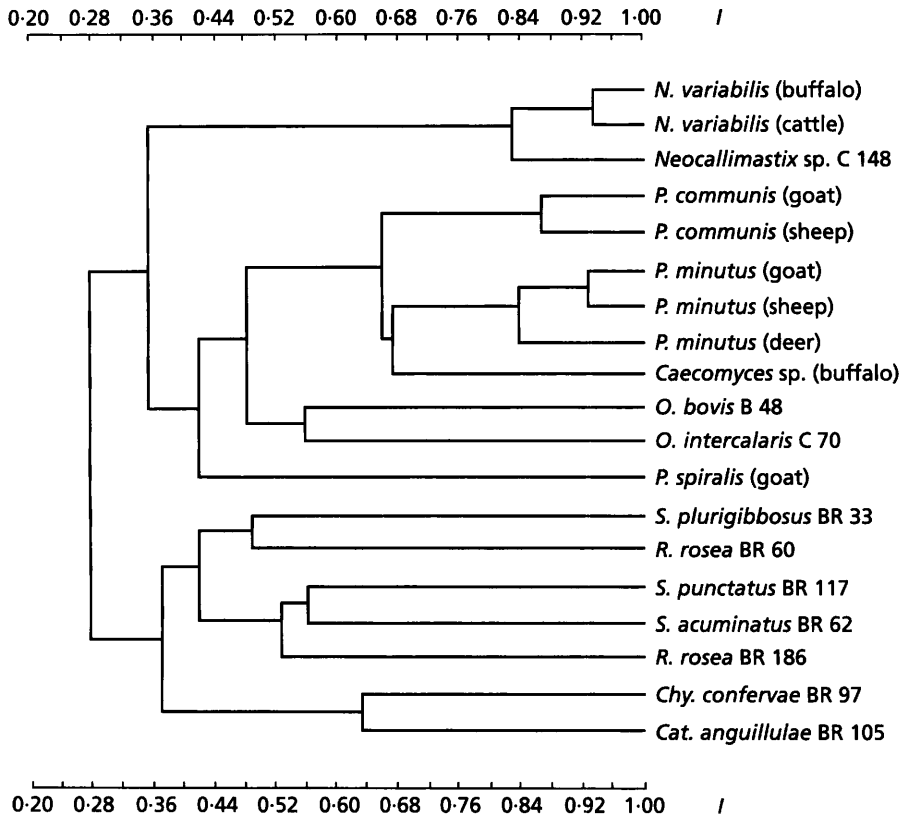

Fig. 5. Dendrogram showing the genetic relationships among the anaerobic rumen fungi and the aerobic chytridiomycete fungi.

inconsistent depending on the set of characteristics used or the outgroup chosen. *Orpinomyces* could be distant from *Piromyces* and close to or distant from *Neocallimastix*.

Based on the genetic identity data of all the anaerobic rumen fungi studied, *P. communis* seems to be most related to the aerobic chytrids, followed by *P. minutus*, *Caecomyces*

Table 3. **Relationships among the anaerobic rumen fungi based on the mean cumulative similarity ($\bar{I}$) or distance ($\bar{D}$) coefficients**

Relationship between fungi	$\bar{I}$*	$\bar{D}$†
N. variabilis and *Neocallimastix* sp. C148	0·845	0·190
P. minutus and *P. communis*	0·663	0·412
P. spiralis and *P. communis*	0·526	0·657
P. spiralis and *P. minutus*	0·416	0·882
Piromyces and *Caecomyces*	0·629	0·480
Piromyces and *Neocallimastix*	0·361	1·039
Caecomyces and *Neocallimastix*	0·381	0·968
O. joyonii and *O. intercalaris* C70	0·571	0·560
Orpinomyces and *Piromyces*	0·469	0·775
Orpinomyces and *Caecomyces*	0·465	0·770
Orpinomyces and *Neocallimastix*	0·344	1·0792

* Higher value indicates closer relationship.

† Higher value indicates greater genetic distance and more remote relationship.

Table 4. **The relatedness of different species of anaerobic rumen fungi to the aerobic chytridiomycete fungi based on the mean cumulative similarity coefficients ($\bar{I}$)**

Species	$\bar{I}$*
Piromyces communis	0·392
Piromyces minutus	0·339
Caecomyces sp.	0·327
Orpinomyces intercalaris C70	0·296
Piromyces spiralis	0·276
Neocallimastix variabilis	0·203
Neocallimastix sp. C148	0·194
Orpinomyces joyonii	0·177

* Higher value indicates closer relationship.

sp., *O. intercalaris* C 70, *P. spiralis*, *N. variabilis*, *Neocallimastix* sp. C 148 and finally *O. joyonii* C 48 (Table 4).

The dendrogram based on Nei's genetic identity (Fig. 5) shows that the anaerobic rumen fungi form a distinct cluster indicating a monophyletic group. They also form a separate cluster from the Spizellomycetales, indicating that as a group they are distinctive from the Spizellomycetales. This supports the establishment of a new order, the Neocallimastigales, by Li *et al.* (1993) to accommodate the anaerobic gut fungi. Other workers (Heath & Bauchop, 1985; Munn *et al.*, 1987) have also observed distinct differences in some of the mitotic and ultrastructural characteristics of the anaerobic gut fungi and have suggested the establishment of a new order for the anaerobic gut fungi.

In this study, isozymes were used to determine the genetic relationships among existing species of anaerobic rumen fungi and aerobic chytrids. For this purpose, Nei's genetic distances (Nei, 1978) based on gene frequencies and the UPGMA dendrogram (Sneath & Sokal, 1973) are suitable. Cladistic analysis has been used and recommended for the study of phylogenetic relationships among species of fungi but this approach is not used here as we are not proposing a phylogenetic tree, nor are we determining the evolutionary relationships between species. Phylogenetic systematics is a taxonomic system based on the theory of descent. When phylogenetic relationships are developed using the principle of parsimony (minimizing numbers of assumptions), a pattern of characters may be explained by a number of conflicting relationships or trees. In these cases, a further analytical tool is needed for rejecting some of them. It is in these cases that the cladistic analysis is frequently used. When the principle behind cladistic analysis is scrutinized, it has been noted that if the cladistic type of outgroup analysis can be applied unambiguously, it coincides with the encaptic pattern achieved by the parsimony analysis and is not required (Lorenzen, 1993). When there is a need for further analysis to accept the correct hypothesis(es) from varying results from parsimony analysis, cladistic analysis is useless or is misleading. Depending on the outgroup, different hypotheses are suggested (Lorenzen, 1993).

Nei's genetic distance gives the measure of the amount of genetic divergence between populations (species) or subpopulations (subspecies). It accomplishes a hierarchical grouping of populations based on normalized identity. The identity here refers to how 'indistinguishable' the populations are and not to 'identify by descent'.

In conclusion, this work demonstrates the usefulness of isozyme characteristics for studying the genetic relationships among the anaerobic rumen fungi and their relationships with other aerobic chytridiomycete fungi. Isozyme characteristics can be used as additional taxonomic criteria in the classification of the anaerobic rumen fungi. The genetic data from this study show that: (i) the anaerobic rumen fungi form a cluster, indicating a monophyletic group distinctly separated from the aerobic chytridiomycete fungi; (ii) they are separate from the Spizellomycetales; (iii) *P. communis* and *P. minutus* are more related to each other than to *P. spiralis*; (iv) *Piromyces* and *Caecomyces* are closer to each other than either is to *Neocallimastix*; (v) *Orpinomyces* is more related to *Piromyces* than to *Neocallimastix*; and (vi) *O. intercalaris* C 70 is distinctly different from *O. joyonii*, indicating that it is a new species.

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