Identification and characterization of anaerobic gut fungi using molecular methodologies based on ribosomal ITS1 and 18S rRNA

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The gut fungi are an unusual group of zoosporic fungi occupying a unique ecological niche, the anaerobic environment of the rumen. They exhibit two basic forms, with nuclear migration throughout the hyphal mass for polycentric species and with concentration of nuclear material in a zoosporangium for monocentric species. Differentiation between isolates of these fungi is difficult using conventional techniques. In this study, DNA-based methodologies were used to examine the relationships within and between two genera of monocentric gut fungi gathered from various geographical locations and host animals. The ribosomal ITS1 sequence from 16 mono- and 4 polycentric isolates was PCR-amplified and sequenced; the sequences obtained were aligned with published sequences and phylogenetic analyses were performed. These analyses clearly differentiate between the two genera and reflect the previously published physiological conclusions that Neocallimastix spp. constitute a more closely related genus than the relatively divergent genus Piromyces. The analyses place two type species N. frontalis and N. hurleyensis together but, contrary to a recent suggestion in the literature, place them apart from the other agreed species N. patriciarum. In situ hybridization and slot-blotting were investigated as potential methods for detection of and differentiation between monocentric gut fungi. DNA slot-blot analysis using ribosomal sequences is able to differentiate between gut fungal genera and thus has considerable potential for use in ecological studies of these organisms.

Keywords: anaerobic gut fungi, molecular phylogeny, in situ hybridization

INTRODUCTION

The study of anaerobic gut fungi is currently hampered by the lack of reliable methods for their accurate identification, differentiation and enumeration. Molecular biology may provide tools and approaches which will aid in the challenges faced by rumen ecologists and nutritionists. Since their recognition in 1975 (Orpin, 1975) anaerobic gut fungi have been classified as Chytridiomycetes, based on their thallus morphology (Orpin, 1975) and the presence of chitin in their cell walls (Orpin, 1976, 1977). There are five recognized genera: Neocallimastix, Piromyces, Orpinomyces, Anaeromyces and Caecomyces, which are delimited by thallus morphology (monocentric, polycentric and filamentous/bulbous) and the number of flagella per zoospore (uniflagellate or polyflagellate; Munn et al., 1988). These characters, visible using the light microscope, tend to be pleomorphic, varying with culture conditions, particularly carbon source. They are also difficult to use in some situations where gut fungi, particularly polycentric fungi, fail to produce abundant zoospores. Further classification of the gut fungi to the species level is at present contentious. Currently, the

Abbreviations: DAPI, 4,6-diamino-2-phenylindole; ITS, internal transcribed spacer.
The GenBank accession numbers for the sequences determined in this work are given in Methods.
species are defined according to taxonomic convention by zoospore ultrastructure (Heath et al., 1983; Orpin & Munn, 1986; Webb & Theodorou, 1991). However, Ho & Barr (1995) have questioned the validity of this method as it has been known for some time that the ultrastructural characters upon which the classification is based can alter with age, method and quality of preparation.

Molecular data have been used in an attempt to clarify the classification of the anaerobic gut fungi. Doré & 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. Li & Heath (1992) used sequence data from a less well conserved ribosomal sequence, the internal transcribed spacer 1 region (ITS1) to compare and discriminate gut fungi. These authors were able to show 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. Li & Heath (1992) 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. However, despite better resolution, the inter-relationship of the five gut fungal genera remains controversial.

The contentious nature of gut fungal classification is apparent from a review of the literature on their identification using classical methods. To date, three distinct species of Neocallimastix, N. frontalis (Heath et al., 1983), N. patriciarum (Orpin & Munn, 1986) and N. burleyensis (Webb & Theodorou, 1991), have been recognized. The first to be formally classified was N. frontalis (Heath et al., 1983). Apparently, this species differed in several respects from the organism originally isolated by Orpin (1975) and, as a consequence, the fungal isolate referred to as N. frontalis for a decade was subsequently reclassified as N. patriciarum (Orpin & Munn, 1986). The generic and specific names of some isolates of the polycentric fungi have also changed since they were first described. This included reassignment of Ruminomyces elegans to Anaeromyces elegans (Ho et al., 1990, 1993). The genera Sphaeromonas and Piromonas were also renamed as Caecomyces and Piromyces to take account of their fungal affinity (Gold et al., 1988). Ho & Barr (1995) recently proposed a radical reassessment of the classification of anaerobic gut fungal species. This involves recognition of the mode of zoospore release, measurement of the diameter of the mature zoosporangium, the size of the zoospore, the length of the flagella and the position of the zoosporangium on the thallus. Ho & Barr (1995) also proposed the reclassification of N. frontalis and N. patriciarum as a single species.

We present here a DNA-sequence-based phylogenetic analysis of Neocallimastix and Piromyces isolates, together with a smaller number of polycentric isolates, with the aim of determining the relationships within these genera. We also investigated the potential of in situ hybridization and slot-blotting with ITS1-based probes for detection and differentiation between the monocentric Piromyces and Neocallimastix isolates.

**METHODS**

**Isolation and maintenance of gut fungal cultures.** Twenty isolates were used in this study. The isolate code, animal hosts and countries of origin are listed in Table 1 together with the genus to which the isolates were assigned using light microscopical analysis. The isolates were obtained from distinctly different host animals and widely different geographical locations and were chosen for maximal apparent diversity by light microscopical analysis. Four polycentric isolates were designated as polycentric without assignation to a genus. N. frontalis MCH-3 was kindly supplied by Professor G. Fonty (Laboratoire de Microbiologie, INRA Centre de Recherches de Clermont-Ferrand Theix, 63122 Ceyrat, France). All isolates were obtained from faecal samples, except for N. burleyensis (Lowe et al., 1985), N. frontalis MCH-3 and Piromyces AUC1, which were isolated from the rumen, and the Piromyces isolate PAK1, which was obtained by M. K. Theodorou from the gut contents of a grey kangaroo.

Fungal cultures were maintained in Orpin’s medium (Orpin, 1975) with either cellobiose (2.5 g l⁻¹) or milled wheat straw (5 g l⁻¹) as the source of carbon, under anaerobic conditions, at 39 °C without shaking (Lowe et al., 1985).

**Genomic DNA extraction.** DNA was extracted from biomass harvested by centrifugation (2000 g, 5 min) from 60 h cultures grown on cellobiose using the method outlined below. Approximately 0.05 g biomass ground under liquid nitrogen was mixed thoroughly with 0.6 ml pre-warmed extraction buffer (0.7 M NaCl, 1 M Na₂SO₄, 0.1 M Tris/HCl pH 7.5, 0.05 M EDTA, 1% SDS). After incubation for 20 min at 65 °C an equal volume of chloroform/isoamyl alcohol was added and the mixture placed on ice for 30 min before centrifugation (13000 g, 30 min) in a microfuge. The uppermost (aqueous) layer was removed to a fresh tube and an equal volume of 2-propanol was added to precipitate the ‘dirty’ DNA. After 10 min at room temperature the tube was centrifuged briefly at low speed (4000 g, 2 min). The pellet was dried and then resuspended in 25 µl double-distilled H₂O. Remaining impurities were removed from the genomic DNA using a ‘Glass Max’ column (Gibco-BRL), following the manufacturer’s protocol.

**Amplification of the ribosomal ITS1 region.** The ribosomal ITS1 region defined by primers GM1 (5’-TGTACACCGCCGTCC-3’) and GM2 (5’-CTGGCTTCTTCTCATCGAT-3’) as described by Li & Heath (1992) was amplified from genomic DNA by PCR. To facilitate cloning of these amplified ITS1 regions into the pHBluescript vector (Stratagene) for subsequent storage and manipulations, they were amplified using GM1- and GM2-derived primers GM8 and GM9 with additional NotI linkers. The PCR reaction was performed in 100 µl reactions containing (final concentration): forward and reverse primers, 0.2 µM; dNTPs mixture, 200 µM; MgCl₂, 1.5 mM; KCl,
different ITS1 sequences; these are designated OUC1a and OUC1b.

The polycentric isolate OUC1 showed two

In situ hybridization studies. In situ hybridization of ITS1 DNA probes to zoospores of anaerobic fungi was performed using a modification of the method described by Amann et al. (1990). Five isolates were used, three Neocallimastix (NMW2,
NMW3 and *N. hurleyensis* and two *Piromyces* (PAC1 and PAK1). Zoospores were harvested from 2-d-old (mid-exponential phase) cultures by filtration through glass wool, washed in PBS and fixed in 4% (w/v) paraformaldehyde in PBS (3 h, 20 °C). The hybridization conditions were as follows. Zoospores (1–3 × 10⁷) were air-dried onto poly-l-lysine-coated slide wells and dehydrated with sequential 50/80/100% ethanol treatment. Hybridization took place with fluorescently labelled oligonucleotides in 10 × Denhardt’s solution (5 h, 40 °C) before washing with PBS plus 0.1% SDS (2 × 20 min, 40 °C). Nuclear material in the zoospores was then stained with 4,6-diamino-2-phenylindole (DAPI; 20 µg/ml, 1 µg/ml in H₂O, 2 min, 20 °C) and the slides air-dried before microscopical examination.

A *Neocallimastix*-specific probe (GM5) was designed by comparison of the available *Neocallimastix* and *Piromyces* ITS1 region sequence data. The target region chosen is completely conserved among the *Neocallimastix* isolates but is not present in the *Piromyces* isolates. The sequence of probe GM5 is 5′-CTCGAGTTAGGTAGTTGAT-3′; this corresponds to bases 162–178 of the published *Neocallimastix* ITS1 sequence (Li & Heath, 1992).

After initial experiments, modifications of the protocol were made in an attempt to reduce the level of apparently non-specific binding of labelled probe to the zoospore cell contents. The hybridization and wash temperatures were varied (30, 35, 40, 45 and 50 °C); the SDS concentration of the wash buffer was varied (0.1, 0.25 and 0.5%); and zoospores were also treated with specific reagents to reduce cytoplasmic contents. To avoid potential trapping of the labelled probe, membranes were permeabilized with Nonidet-90 (0.1% in PBS, 15 min, 20 °C). Cytoplasmic RNA was removed from the zoospore by RNase treatment (500 units in PBS) and protein was removed by Proteinase K treatment of zoospores (50 µg/ml in 0.005 M Tris/HCl, 0.005 M EDTA pH 7.8; 10 min, 37 °C)

**Detection and differentiation of fungal DNA by slot-blot hybridization.** Genomic DNA and PCR-amplified ITS1 rDNA from *N. hurleyensis*, the *N. hurleyensis*-like isolate NCS1, the *N. patriciarum*-like isolate NMW1, and *Piromyces* isolate PAC1 were used in these experiments. Genomic and PCR-amplified DNA was dried onto a nylon membrane using a slot-blot apparatus, following the manufacturer’s instructions (Schleicher and Schuell). The membrane was then probed either with a full-length ITS1 fragment generated by PCR amplification (primers GM1 and GM2; see above) of the plasmid copy of an isolate’s ITS1 sequence or with a truncated ITS1. The truncated form was generated by PCR amplification using primers JB200 (5′-CGGAAGGATCTATT-3′) and GM2, hence removing most of the domain I 18S rDNA sequence. The method for labelling the DNA probes, hybridizing and washing the membrane was the same as the standard method used for Southern blots and was performed using the manufacturer’s protocols.

Routinely, the membrane was pre-hybridized for 1 h at 68 °C and the radioactive probe was added directly to this solution. The hybridization was allowed to proceed overnight. Blots were washed three times for 15 min in wash solution (once with 3 × SSC, 0.1% SDS, then twice with 1 × SSC, 0.1% SDS) at 68 °C. Initial optimization experiments had shown that the maximum differentiation of signals was observed with these conditions; we also compared wash conditions of stringencies 3 × SSC, 0.1% SDS; 1 × SSC, 0.1% SDS; and 0.1 × SSC, 0.1% SDS. Intensities of signals produced on membranes were compared using phosphoimaging (Fuji-Bas).

**RESULTS**

**Alignment of the ribosomal sequences**

Ribosomal ITS1 sequence was generated from the genomic DNA of the 20 gut fungal isolates listed in Table 1. The ITS1 DNA sequences were aligned with the previously published sequences from the genera *Neocallimastix*, *Piromyces*, *Anaeromyces* and *Orpinomyces* (Li & Heath, 1992). The alignment (which is available at www.biomed.man.ac.uk/biochemweb/staffpages/jaynebrookman.htm) contains the entire ITS1 sequence plus flanking 18S and 5.8S rDNA regions. We divided the alignment into three domains: domain I (bases 1–150) represents the 3′ end of the 18S rDNA; domain II (bases 151–496) represents the true ITS1, which is spliced out as the ribosome matures (Musters et al., 1990); and domain III (bases 497–507) represents the 5′ end of the 5.8S rDNA sequence.

The 18S rDNA regions of the cloned fragments are very well conserved between the anaerobic gut fungal isolates, with a mean pairwise identity of 97.4% (domain I). However, one *Piromyces* isolate, PGC1, is unusual in that it has a large deletion between bases 107 and 130 and a further two-base deletion of residues 135/6.

The ITS1 sequence proper then begins with domain II, which shows greater variability than that seen for the 3′ end of the 18S rRNA gene, with 70.5% mean pairwise identity. A region between bases 154 and 185 shows a clear differentiation between the sequences from the *Neocallimastix* isolates and the other gut fungal sequences. Further downstream, a large region (bases 223–404) shows the greatest variability, with poor conservation of sequence amongst the total group of isolates, particularly bases 294–434. At the 3′ end of the anaerobic gut fungal ITS1 region (bases 405–496) sequence was generally well conserved between all the gut fungal isolates. Domain III, delineated by the 5′ end of the 5.8S rDNA, was short (11 bases, 497–507) and showed only a single basepair difference in the published *Anaeromyces* sequence, with all other isolates showing 100% identity between all the gut fungal sequences.

**Relatedness of the gut fungal isolates**

Fig. 1 shows the results of the phylogenetic analyses performed on the 25 gut fungal ITS1 sequences. Since positions 294–361 could not be accurately aligned, they were omitted from the analysis. The analysis methods used were maximum-likelihood, parsimony and distance matrix based (Table 2; Fig. 1a, b and c, respectively). The overall groupings of the isolates are the same for all three analyses presented, although the precise ordering within the different groupings varied between analyses. The trees could not be rooted, as alignment of the anaerobic gut fungal ITS1 region with any aerobic fungal sequence, such as those from *Saccharomyces cerevisiae* or *Aspergillus nidulans*, was not possible (data not shown).

The *Neocallimastix* isolates form a single clade by
Characterization of anaerobic gut fungi

parsimony analysis, and clearly cluster together by maximum-likelihood and distance-matrix analysis. This is supported by bootstrap values from all three methods of analysis. Although the precise relationships between Neocallimastix isolates vary with the analysis method used, there is a clear differentiation between N. hurleyensis/N. frontalis and N. patriciarum in all three dendrograms, which is supported by bootstrap values in the parsimony analysis (Fig. 1). N. hurleyensis and N. frontalis group together with NCS1, NMG2 and, depending on the analysis method used, NUC1.

The Piromyces isolates examined by us and designated as Piromyces by light microscopy criteria (PAC1, PAK1, PCG1, PCS1 and PLA1), are clearly separated from the Neocallimastix isolates in all three analyses; this is confirmed by bootstrap values from parsimony and distance analyses (Fig. 1). This separation is consistent with the light microscopical analysis of zoospores. The separation of the previously published Piromyces isolate sequence from the Neocallimastix isolates in these trees is less clear as it consistently clusters with the Neocallimastix/Orpinomyces set.

The published Orpinomyces and two polycentric isolates, OUC1 and OUS1, clearly group together (Fig. 1), suggesting that these isolates belong to the genus Orpinomyces. Two sequences, designated OUC1a and OUC1b, are given for the OUC1 isolate as this isolate consistently gave two distinct ITS1 sequences. The published Anaeromyces isolate and the polycentric isolates, AUC1 and AUC2, also group together in all three trees, suggesting that the AUC1 and AUC2 isolates are Anaeromyces. This correlates with a preliminary microscopical designation of these isolates as members of the genus Anaeromyces (data not shown). Interestingly, the Anaeromyces isolates split the Piromyces isolates in all dendrograms (Fig. 1), suggesting that the Anaeromyces are more similar to the monocentric genus Piromyces than to the other polycentric genus Orpinomyces.

Fig. 1. Phylogenetic analyses of the aligned ITS1 sequences. The geographical source and host animal of the isolates are shown in Table 1. (a) An unrooted tree generated using the maximum-likelihood algorithm (DNAML). (b) The consensus tree produced using maximum-parsimony analysis. Eight most-parsimonious trees were found, each requiring 568 steps; a summary of these trees is shown, with bootstrapped confidence values from 100 replicates superimposed where they are greater than 50%. (c) The tree produced using the distance-based algorithm DNADIST and FITCH. Note the clear separation of the Neocallimastix and our Piromyces isolates in all trees. Also note in (a) and (c) the relative distances separating the isolates within a genus; these are indicative of evolutionary distance. Analysis was carried out on bases 1–293 and 362–508, since the region 294–361 could not be accurately aligned. This region showed 81.5% mean pairwise identity across the alignment. In (a) and (c) the scale bars indicate expected number of base substitutions per site, with bar length corresponding to 5% difference. The dotted lines are not representative of evolutionary distance and are used to indicate the position of isolates where branch length is too short to allow placing of the branch label.
Table 2. Distances between fungal isolates as determined by PROTDIST

Analysis was carried out on bases 1–293 and 362–508 (see Fig. 1).

<table>
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<tr>
<th>AUCI</th>
<th>AUC2</th>
<th>OUC1B</th>
<th>Anaco</th>
<th>OUC1C</th>
<th>OR</th>
<th>OUC1A</th>
<th>Protac</th>
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The isolate codes used in the dendrograms indicate the original geographical location of the isolates (see Table 1). The *Neocallimastix* samples isolated from Malaysian water buffalo (NMW1–5) constitute the largest directly comparable set of isolates (Fig. 1). Three of these isolates, NMW3, 4 and 5, together with a *Neocallimastix* isolate from a Chinese sheep, NCS2, consistently group together in all dendrograms produced (Fig. 1). No other obvious groupings of geographically similar isolates were seen.

The treatment and assessment of insertions and deletions (indels) in non-coding sequence is complex. DNADIST treats indels by pairwise deletions; DNAPARS treats indels as a fifth state, as does DNAML. In an alternative approach to the analysis of indels, an alignment was analysed in which all positions across the alignment containing indels were deleted (in addition to positions 294–361). The resulting alignment of 238 bases was analysed by DNADIST/FITCH, DNAPARS and DNAML (data not shown). Although there was little variation across the alignment (minimum pairwise identity 90.7%), the topologies closely resembled those seen in Fig. 1 and supported the same conclusions as made from Fig. 1.

**Exploitation of ITS1 sequence data for in situ hybridization**

The aligned ITS1 sequences were used to design probes for assessing the potential of *in situ* hybridization, as a method for differentiation of anaerobic gut fungi in rumen samples. Zoospores from *Neocallimastix* and *Piromyces* cultures maintained in *vitro* were probed using a *Neocallimastix*-specific fluorescently labelled oligonucleotide. Initial hybridization using the method described by Amann et al. (1990) with the *Neocallimastix*-specific probe produced a signal of high intensity from the *Neocallimastix* zoospores and of lower intensity from the *Piromyces* zoospores. However, the signal for both types of zoospore had a uniform intensity over the entire zoospore and was not confined to the area of the nucleus, as would be expected from a rDNA targeted probe (Fig. 2).

Procedures for sequential optimization of the signals produced using changes in hybridization and wash stringency were used. Varying the intensity of hybridization and washes did not yield any significant changes in binding specificity so a median temperature of 40 °C was adopted for further experiments. Varying the detergent concentration in the wash buffer from 0–1% to 0–5% substantially disrupted the zoospore structure; at a concentration of 0–25% SDS some zoospore disruption was observed but the background signal was lowered slightly. Subsequent alterations to the method were made in an attempt to produce specific hybridization of the *Neocallimastix*-specific probe to the ITS1 rDNA within the nuclei of the *Neocallimastix* zoospores but not the *Piromyces* zoospores. Some improvement in signal specificity was seen with removal of cytoplasmic contents using membrane permeabilization with Nonidet-90, RNase treatment or Proteinase K treatment (data not shown). However, the improvement was at the expense of the observable cell structure, as after each of these treatments the zoospores were either damaged or, in the case of the detergent treatment, completely destroyed.
Characterization of anaerobic gut fungi

Fig. 2. In situ hybridization of a fluorescently labelled Neocallimastix-specific oligonucleotide probe (GMS5) to Neocallimastix and Piromyces zoospores. (a, b) Phase-contrast images; (c, d) zoospores labelled with the DNA dye DAPI; (e, f) fluorescent images of the labelled GM5 probe bound to zoospores from Neocallimastix isolate NMW1 (e) and Piromyces isolate PAK1 (f). Note that the intensity of specific fluorescence in (e) is considerably greater than the corresponding panel (f) for the Piromyces zoospores. However, also note that the area of signal from the fluorescently labelled probe is larger than the area of DNA outlined with DAPI staining (compare panels e and c), suggesting that the signal is not confined to the nucleus of the zoospore. Bar, 10 µm.

Fig. 3. Differentiation between fungal isolates by slot-blot hybridization using PCR-amplified ITS1 sequences. A membrane with a range of DNA loading (1–100 ng) from two isolates within the same clade [N. hurleyensis (lane 1) and NCS1 (lane 2)] and a Piromyces isolate, PAK1 (lane 3) was probed with a 32P-labelled ITS1 fragment from N. hurleyensis and the intensity of signal produced was quantified by phosphoimaging.

Slot-blotting methodologies for differentiating between fungal isolates

A membrane-based approach for the differentiation of the anaerobic gut fungi using PCR-amplified ITS1 fragments was also investigated. Fig. 3 shows a slot-blot containing amplified ITS1 DNA from N. hurleyensis and Neocallimastix NCS1 together with a Piromyces isolate (PAK1). Use of a 32P-labelled PCR-amplified full-length ITS1 region of N. hurleyensis as a probe enabled differentiation between Neocallimastix and Piromyces isolates. The N. hurleyensis ITS1 probe hybridized to the Neocallimastix DNA approximately 20 times more strongly than to the Piromyces DNA (Fig. 3). The probe could detect 5 ng DNA loadings for the N. hurleyensis and the Neocallimastix isolate NCS1 with similar intensity to the Piromyces (PAK1) signal for 100 ng loading.

Further slot-blot analyses of a variety of Neocallimastix and Piromyces isolates were performed using truncated ITS1 probes consisting of only the true variable ITS1 region with no 18S rDNA component; the results are shown in Fig. 4. For example, the Piromyces PLA1 truncated probe was able to detect 25 ng homologous rDNA from isolates of the other putative Piromyces species (PAK1 and PCS1; 13 and 21% maximum intensity, respectively) but gave no signal with Neocallimastix isolates at the same 25 ng loading. The PAK1 probe was able to detect 25 ng homologous
PAK1 DNA with this 2.5 ng loading, giving a comparable or greater signal (29% maximum intensity) than the 50 ng loading of the PLA1 *Piromyces* and all *Neocallimastix* isolates (10–28% maximum intensity). This probe showed greater binding to the *Piromyces* isolate, PCS1, but overall there was little differentiation between *Piromyces* and *Neocallimastix* isolates at the higher (50 ng) loading (Fig. 4). Similarly the *Neocallimastix* NMW1 truncated probe was efficient at detecting homologous DNA but only showed very slight differentiation between other *Neocallimastix* isolates (NMG2 and NCS1) and the *Piromyces* isolates (Fig. 4). The *N. hurleyensis* probe showed a similar pattern of conserved recognition of *Neocallimastix* isolates from the same groupings, NCS1 and NMG2 (Fig. 4; see also Fig. 1), as seen in Fig. 3 but little differentiation between *Piromyces* isolates and the more closely related *Neocallimastix* isolates from the *N. patriciarum*-type group.

**DISCUSSION**

**Molecular phylogeny**

Defining a species in the fungi is difficult, especially in those like the anaerobic gut fungi, which have no observed sexual stage. The analysis we report here sheds light on the relationships between isolates within the genera of monocentric gut fungi. The dendograms produced by all the algorithms used for the analysis of the ITS1 sequence data generated in this study reveal that the *Neocallimastix* isolates form a single clade. However, within this clade, it was clear that *N. hurleyensis*/*N. frontalis*, along with NCS1, NMG2 and NUC1, were separate from *N. patriciarum* and the other *Neocallimastix* isolates. This implies that the *Neocallimastix* fungi can be broadly divided into at least two separate groups (*N. patriciarum*-like and *N. frontalis*-like) and may justify designating the members of each clade as belonging to the species around which they have clustered. However, it is not possible to define a species on sequence data alone as there is no single definition of how many base changes are required to delimit a species rather than a subspecies or, conversely, a genus.

The grouping of *N. hurleyensis* with *N. frontalis* in this study suggests that there are at least two *Neocallimastix* species as exemplified by *N. patriciarum* and *N. frontalis* (as *N. frontalis* was defined before *N. hurleyensis*: Vavra & Joyon, 1966; Webb & Theodorou, 1991). This contradicts the findings of Ho & Barr (1995) and also the cladistic analysis of anaerobic gut fungal ultrastructural features by Li et al. (1993). These authors concluded that *N. hurleyensis* and *N. patriciarum* were more closely related to each other than to *N. frontalis*, but our conclusions are in agreement with comparisons of zoospore ultrastructural data (Webb & Theodorou, 1991) and fermentation characteristics (Theodorou et al., 1991).

The *Piromyces* isolates in this study appear as an extremely disparate set with no close groupings (Fig. 1). Ho & Barr (1995) concluded there were six recognizable species of *Piromyces*, each with unique characters, reiterating previous suggestions based on ultrastructure and morphology (Li et al., 1993) that *Piromyces* is a much more divergent genus than *Neocallimastix*. Our molecular data also strongly suggest that *Piromyces* is more divergent than *Neocallimastix*.

The grouping of the *Piromyces* isolates with the *Anaeomyces* sequences was consistently found in all dendrograms. Similarly, the separation of the *Neocallimastix* isolates was consistent for all methods of
analysis. These consistent groupings differ from the results presented by Li & Heath (1992), where ITS1 sequence data from single Neocallimastix, Piromyces, Anaeromyces and Orpinomyces isolates generated dendrograms with different branching orders depending on which algorithm was used, resulting in the pairing of different genera. Our approach, using a larger number of isolates within a more restricted range of genera, is therefore perhaps necessary for successful discrimination between relatively closely related but distinct organisms.

Doré & Stahl (1991) and Li & Heath (1992) both assessed the use of 18S rRNA sequences for the phylogenetic analysis of gut fungi. Doré & Stahl (1991) found very little difference between the 18S rRNA sequences of Neocallimastix and Piromyces (> 97% identity) while a shorter 18S rRNA segment studied by Li & Heath (1992) (covering the same region as that used here) 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/eukaryotes, the 18S rRNA sequence was clearly unsuitable for determining relationships within the group of gut fungi. Our data are consistent with this, showing, on average, pairwise identity of 97.4% for the 18S rRNA region (see Results). This contrasts with the data from ITS1 sequences, which, as they show greater variation, are suitable for determining the relationships between gut fungi.

**Effects of geographical and host animal origin on the type or species of anaerobic gut fungus isolated**

We found no clear evidence for an influence of geographical origin or animal host in determining which species of anaerobic gut fungi will be isolated. The Malaysian isolates were collected from different farms, and during the isolation procedure were selected for their differing morphologies in an attempt to create the most diverse group possible. One group of isolates, NMW3, 4 and 5, appeared to be closely related, which may suggest that that particular strain of Neocallimastix is favoured in Malaysian water buffalo. None of the isolates from Malaysian water buffalo were closely related to the *N. frontalis*-like isolates, perhaps suggesting a bias against these. The enrichment techniques used to isolate these samples were identical to the other samples and so any bias should reflect differences in the original faecal material.

Previous studies have indicated that herbivore host specificity in anaerobic gut fungi is minimal (Orpin, 1989; Marvin-Sikkema et al., 1992; Ho & Barr, 1995). Orpin (1989) showed that fungi from monogastric herbivores (horses) and other ruminants (reindeer) could establish in sheep. Thus, it seems that anaerobic gut fungi can pass between ungulates, although whether they are maintained or are able to out-compete the established gut fungal population is unknown.

The measurement of diversity of fungal species *in situ*, without isolation, would seem to be the best way to achieve a clear answer on the importance of host and geography in determining the fungal species maintained within the gut. We are at present investigating rDNA-based approaches complementary to those used here to assess the unbiased, uncultured populations of gut fungi within their ecosystem.

**In situ hybridization is not a useful tool for the evaluation of anaerobic gut fungi within their environment**

The use of *in situ* hybridization for the differentiation of fungal species/subspecies is an attractive proposition for the gut fungi. However, our study of *in situ* hybridization suggests that due to technical difficulties, at the present time the technology is of limited use with the gut fungi. We were unable to maintain the integrity of the zoospore and achieve specific, exclusive binding of the Neocallimastix probe to the nucleus of the Neocallimastix zoospore alone (see Fig. 2). There was also considerable variation in reactivity between zoospores from single laboratory-based axenic cultures; this was seen in all five isolates studied. The relatively low abundance of zoospores at any point within the rumen would also require large volumes of fluid to be screened to ensure measurement of representative samples of zoospores. In conclusion, the *in situ* hybridization approach does not appear to be of much value for ecological studies of fungal populations in the rumen.

**ITS1 slot-blot analysis shows potential for exploitation**

Slot-blot studies using ITS1 probes containing partial 18S rDNA fragments or truncated to remove these more conserved regions were performed on PCR-amplified ITS1 DNA to ascertain whether this approach would be a useful way of exploiting ITS1 sequences in environmental samples. Preliminary experiments using genomic DNA as a source of ITS1 for probing were not satisfactory as the labelling was inconsistent (data not shown). The feasibility of using a slot-blot detection system would require 25–100 ng target DNA to be loaded (see Figs 4 and 5). Using the method of DNA extraction from the zoospores of anaerobic gut fungi described by Tsai & Calza (1993), the yield of DNA per zoospore was reported to be 2.2 pg. The anaerobic gut fungal population is about 10^3 zoospores per ml of rumen contents. Therefore, the potential yield of fungal DNA extracted from rumen contents could be as high as 1 µg ml^-1. However, the yield of DNA in the rumen for an individual species of gut fungus is unlikely to be this high in practice, as it would represent DNA from a range of species of gut fungi. The amount of DNA extracted from an individual species may therefore be significantly below the detection limit of this protocol using genomic DNA, necessitating amplification of the target sequence by PCR prior to hybridization. By using a PCR-based
hybridization assay with full-length or truncated ITS1 probes from *N. hurleyensis*, we were able to demonstrate a 20-fold differentiation between DNA from *Neocallimastix* and *Piromyces* isolates (Figs 4 and 5).

Further characterization of the relative efficiencies of PCR amplification in different isolates would be required for the development of a PCR-based assay. The relatively narrow range of expected sizes for the ITS1 fragments from the isolates tested (412–479 bp) suggests that gross differences in amplification efficiency due to size are unlikely to occur. In samples derived directly from the environment, differences in extraction efficiency may pose a greater bias than relative efficiencies of PCR amplification, particularly when comparing the monocentric fungi such as *Neocallimastix* and *Piromyces* with the polycentric forms.

Many authors have found dot-blotting a very accurate method of quantifying micro-organisms in a mixed population (e.g. Stahl et al., 1988; Odenyo et al., 1994). Stahl et al. (1988) were able to use a signature oligonucleotide targeted to the 16S RNA to study perturbations in the numbers of rumen bacteria. The same method was used to study competition *in vitro* between three ruminal fibrolytic bacteria (Odenyo et al., 1994), using slot-blot quantification of relative 16S rRNA levels. In ecosystem samples genuine inconsistencies between the signal and the apparent total nucleic acid loaded can reveal undetected diversity. For example comparison of genus-specific probes and species-specific probes revealed previously unrecognized diversity within the genus *Fibrobacter* (Lin & Stahl, 1995). Similarly, comparison of probes in the gut ecosystem could be used to reveal the full extent of the diversity of the gut fungi. The data presented here strongly suggest that slot-blot approaches are likely to be useful within an environmental context and we will be optimizing their parameters for use with mixed population samples derived from the gastrointestinal tract and faeces.

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