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Diversity of anaerobic gut fungal populations analysed using ribosomal ITS1 sequences in faeces of wild and domesticated herbivores

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ABSTRACT

Gut fungal-specific PCR primers have been used to selectively amplify the ITS1 region of gut fungal rDNA recovered from faeces of domestic and wild animals to investigate population diversity. Two different gel-based methods are described for separating populations of gut fungal rDNA amplicons, namely (1) denaturing gradient gel electrophoresis (DGGE) and (2) separation according to small size differences using Spreadex, a proprietary matrix for electrophoresis. Gut fungal populations were characterised by analysis of rDNA in faeces of seventeen domesticated and ten wild herbivores. Sequences derived from these gel-based characterisations were analysed and classified using a hidden Markov model-based fingerprint matching algorithm. Faecal samples contained a broad spectrum of fungi and sequences from five of the six recognised genera were identified, including *Cyllamyces*, the most recently described gut fungal genus, which was found to be widely distributed in the samples. Furthermore, four other novel groupings of gut fungal sequences were identified that did not cluster with sequences from any of the previously described genera. Both gel- and sequence- based profiles for gut fungal populations suggested a lack of geographical restriction on occurrence of any individual fungal type.

1. Introduction

Chytrids belonging to the Neocallimastigales family are the only known obligately anaerobic fungi and are found primarily in the intestines of large herbivores. There are six recognised genera of gut fungi, namely *Neocallimastix*, *Piromyces*, *Orpinomyces*, *Anaeromyces*, *Caecomyces* and the recently described *Cyllamyces* [1,2]. During typical vegetative growth the fungus is intimately associated with digesta solids in the rumen and/or hindgut, and for reproductive purposes motile zoospores are produced to enable rapid colonisation of freshly ingested plant material [3]. Anaerobic chytrid fungi produce potent fibrolytic enzymes and it is thought that they are early colonisers of plant biomass, making them important contributors to fibre digestion [4,5]. It is clear that rumen microbial populations are affected by dietary change and that the fungi are more prevalent in the gut of animals on high-fibre diets [6,7].

A prerequisite to understanding some of the host and dietary factors affecting fungal populations in the intestinal tract ecosystem is the ability to identify and quantify the presence and diversity of gut fungi from environmental samples (digesta and faeces). Current knowledge of anaerobic gut fungal population size and diversity in the intestines of herbivores is largely based upon morphological classifications after isolation and cultivation of fungi from their environment and a small number of studies that have sought to enumerate gut fungal populations *in vivo* [8,9]. This research has not been exhaustive and it is recognised that there are many species yet to be described, some of which may not be cultivable or may be under represented in conventional, culture-based enumerations.

The development of molecular techniques has broadened the understanding of microbial diversity and enabled a more complete detection and comprehensive description of microbial communities [10]. The application of molecular techniques for the analysis of microbial communities provides a simple means of following temporal and spatial changes in community structure.

Phylogenetic analysis of members of the Neocallimastigales has been problematic and has hampered the development of molecular ecological techniques for monitoring anaerobic gut fungal populations.

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Due to the extremely high level of conservation seen in the rRNA-encoding genes (16S, 5.8S, and 28S), internal transcribed spacer (ITS) sequences have been successfully used to infer phylogenetic relationships [11–13]. The recent development of a hidden Markov model (HMM)-based fingerprinting algorithm for gut fungal ITS sequences [13] provides a useful tool for the rapid analysis of gut fungal ITS sequences and has paved the way for the development of molecular techniques for investigating the ecology of anaerobic gut fungi.

Rumen fistulation of domesticated animals enables direct access to microbial populations and the ability to make comparative and temporal evaluations thereof. However, this approach is not suitable for large-scale comparisons of many different animals or for investigation of microbial populations in wild animals. Conversely, faeces from these animals are readily available and can be used as endpoint samples as gut fungi present along the digestive tract are often isolated from faecal samples [8,14–17].

This investigation reports on the development of selective PCR-based amplifications of fungal populations from faeces together with gel-based systems for the visualisation of gut fungal population diversity from wild and domesticated herbivores. The HMM-based fingerprinting algorithm [13] has been implemented to identify members of the gut fungal populations, providing characterisation of population structures in a variety of animals.

2. Materials and methods

2.1. Sample collection and fungal cultures

Details of host animal and country of origin for each of the axenic gut fungal isolates used in this study are shown in Table 1. Faecal samples were collected immediately after deposition and stored on ice, then at −20 °C. Table 2 shows the country of origin and host animal with its respective feed type for each of the fungi isolated from faecal samples.

2.2. Extraction of genomic DNA

Genomic DNA was prepared from axenic cultures as described by Brookman et al. [11]. Faecal samples were extracted using a modified CTAB procedure as follows: 0.8 ml CTAB buffer (100 mM Tris–HCl (pH 8.0); 1.4 M NaCl; 20 mM EDTA (sodium salt); 2% hexadecyltrimethylammonium bromide) was added to a 0.1-ml volume of faecal sample finely ground in liquid nitrogen using a pestle and mortar. The mixture was vortexed thoroughly to mix and then incubated at 70 °C for 30 min. The sample was extracted by mixing with 0.5 ml chloroform and recovery of the aqueous layer (~0.5 ml) by centrifugation (13 000*g*, 10 min). The DNA was precipitated by addition of 0.3 ml isopropanol, mixed and incubated at room temperature for 10 min before centrifugation (13 000*g*, 10 min). The DNA was washed in 70% (v/v) ethanol at 70 °C for 10 min and then centrifuged as before. Ethanol was removed before dissolving in 50–100 μl 10 mM Tris–HCl (pH 8) at 37 °C. Samples were stored at −20 °C in molecular biology grade water.

Table 1
Host animal and country of origin for anaerobic gut fungal isolates.

Fungal isolate	Host animal	Country of origin	Genus	Reference
NMZ4	Zebu	Malaysia	*Neocallimastix*	Brookman et al. [3]
NMW5	Water buffalo	Malaysia		
NCS1	Sheep	China		
AUC1	Bovine cow	U.K.	Anaeromyces	
PLA1	Alpaca	Chile	Piromyces	
PCS1	Sheep	China		
PUC1	Bovine cow	U.K.		
OUC5	Bovine cow	U.K.	Orpinomyces	
OUS1	Sheep	U.K.		
TSB1	Sable	Quarantine Sanctuary, Northern Territory, Australia	*Neocallimastix*	Tuckwell et al. [35]
TBT3	Banteng			
TAP F8	Kudu		Orpinomyces	
PN1	Sheep		*Piromyces*	
TAX1	Addax			
TNL2	Nilgai			
TZB1	Zebra			

Table 2
Donor animal, country of origin, and respective feed type (where known) for faecal isolates.

Faecal sample	Donor animal	Geographical origin	Pasture type
J1	Brahman/Murray Grey cow	Jimboomba, Queensland, Australia	Rhodes, *Paspalum* (bahia grass) and Green Panic
J2	Suffolk/Merino sheep (ram)		
J3–J5	Merino sheep (ewes)		
T1–T6	Brahman cross cattle (male)	Townsville, Queensland, Australia	Tropical native pasture
R1–R3	Brahman cross cattle (male)	Rockhampton, Queensland, Australia	Green panic and Buffel
S1–S3	Belmont Red cross cattle (male)	Samford, Queensland, Australia	*P. notatum* (bahia grass)
Buffalo 1&2	African buffalo	Zimbabwe	Not known (samples obtained from wild animals)
Impala 1 &2	Impala		
Eland	Eland		
Elephant 1 & 2	Elephant		
Hippopotamus 1 & 2	Hippopotamus		
Zebra	Zebra		

2.3. PCR primers and amplification

Gut fungal-specific primers MN100 (TCCTACCCTTTGTGAATTTG) and MNGM2 (CTGCGTTCTTCATCGTTGCG) were designed from the 5′ end of the ITS1 and 5.8S rDNA regions respectively. For DGGE, primer MNGM2 was modified to contain a 40-bp GC-clamp sequence at its 5′ end according to Muyzer et al. [18] (CGCCCGC CGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG) to create primer MNGM2C. PCR reactions contained 2.5 μl 10× buffer, 2.5 μl 1/25 dilution genomic DNA, 0.5 μl dNTP mix (10 mM each), 0.5 μl. Advantage 2 polymerase mix (BD Biosciences, California), 10 picomoles of each primer and molecular biology grade water to make a final reaction volume of 25 μl. Thermal cycling consisted of 95 °C for 5 min followed by 20 cycles of: 95 °C for 30 s, 68 °C (−0.5 °C each cycle) for 30 s, 72 °C for 30 s; then 12 cycles of: 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; followed by 72 °C for 6 min. Successful amplification was verified by electrophoresis of the reaction mixture on a 1% (w/v) agarose gel. For amplification of both ITS regions from pure gut fungal cultures primers GM1 (TGTACACAC CGCCCGTC) and JB205 (TCCTCCGCTTATTAATATGC) were used to amplify approximately 850 bp of sequence which included ITS1, the 5.8S rDNA gene, ITS2, and flanking regions. Reactions were prepared as described above but were amplified using 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by an extension of 72 °C for 6 min on the final cycle.

2.4. Electrophoresis conditions

DGGE was performed using a D-Code Universal Mutation Detection System (Bio-Rad Laboratories Ltd., UK) with 16 cm × 16 cm glass plates separated by 1 mm spacers. DGGE was performed using a gradient of 15–30% denaturant in 10% acrylamide/bis-acrylamide (37.5:1) where 100% denaturant solution contained 7 M urea and 40% (v/v) deionised formamide. Optimal denaturant and electrophoretic conditions were established by perpendicular