

Anaerobic fungi: *Neocallimastigomycota*

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Abstract: This contribution is based on the six oral presentations given at the Special Interest Group session on anaerobic fungi held during IMC9. These fungi, recently elevated to the status of a separate phylum (*Neocallimastigomycota*), distinct from the chytrid fungi, possess several unique traits that make their study both fascinating yet challenging to mycologists. There are several genome sequencing programs underway in the US but these are hampered by the highly AT-rich genomes. Next-generation sequencing has also allowed more detailed investigation of the ecology and diversity of these fungi, and it is apparent that several new taxa beyond the six genera already named exist within the digestive tracts of mammalian herbivores, with others potentially inhabiting other anaerobic niches. By increased collaboration between the various labs studying these fungi, it is hoped to develop a stable taxonomic backbone for these fungi and to facilitate exchange of both cultures and genetic data.

Key words:

chytrid
basal fungi
genome sequencing
phylogenetics
next-generation sequencing
hydrogenosome

Article info: Submitted: 27 October 2010; Accepted: 20 November 2010; Published: 23 November 2010.

INTRODUCTION

Since their discovery by Colin Orpin in the 1970s, the anaerobic fungi, now members of the recently erected phylum *Neocallimastigomycota*, have aroused the curiosity of mycologists, not only due to their distinctive physiology but also because of their biotechnological potential, for more efficient animal nutrition and also biomass conversion/biofuel production. This contribution, based on a Special Interest Group session held during IMC9, brought together mycologists interested in anaerobic fungi to share recent discoveries and to discuss how best to move forward research in this area. Six speakers kindly agreed to speak at the session, with a further seven non-speakers in attendance. Given the small numbers of mycologists now engaged in research on anaerobic fungi, this was a pleasing quorum, with expertise in genomic, phylogenetic, morphological, physiological and ecological aspects of the biology of these fungi being represented. This synopsis of the session amounts to a mini-review of the current state of research on these fascinating fungi.

CONTRIBUTIONS

The session began with a presentation from Scott Baker on the status of two genome sequencing projects (*Piromyces* E2 and *Orpinomyces* SR2). The AT richness of these genomes (approaching 80 % in non-coding regions) has caused significant technical problems for genomic sequencing and assembly. The Joint Genome Initiative (JGI) is working through technical issues with assembling the genome, but does plan to release the sequence in the near future. In contrast, the Expressed Sequence Tag (EST) library sequencing project did not encounter any technical issues and will be made available concurrent with the eventual release of the genome sequence. Until then, contact Scott for information on accessing ESTs (scott.baker@pnl.gov). Much of the interest in these fungi relates to the genes/enzymes important for biorefining and biofuel production, notably xylose isomerases and glycosyl hydrolases (xylanases, cellulases).

Kate Fliegerova, who also presented a poster (4.015) at the main IMC9 congress, further explored the biotechnological

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potential of these fungi. Some of the cellulases of anaerobic fungi originated via horizontal gene transfer from bacteria, so these are the only fungi known to possess cellulosomes, cell-wall associated multienzyme complexes (Garcia-Valve *et al.* 2000, Steenbakkers *et al.* 2001). Combined with their anaerobic metabolism and ability grow at elevated temperatures (39 °C), they have great biotechnological potential. In Prague, Kate and her colleagues have explored the use of anaerobic fungi to improve the hydrolytic phase of biogas production. They have also investigated which fungi are present in the cow manure used to prime the biogas fermentations (Fliegerova *et al.* 2010), finding members of the genus *Cyllumyces*, the sixth and most recently discovered group of anaerobic fungi (Ozkose *et al.* 2001) to be dominant, with the 'most famous' (and type) genus *Neocallimastix* comprising only a small proportion of the population. This imbalance is also reflected in the literature, possibly a result of the widespread use of wheat straw for the culture-based isolation of these organisms (Griffith *et al.* 2009).

The application of culture-independent approaches to assessing the diversity of anaerobic fungi was the subject of Audra Ligenstoffer's presentation. Her PhD project at the Oklahoma State University used barcoded 454 sequencing to determine the fungal symbionts present in the faeces of 30 species of larger herbivores, many from Oklahoma City Zoo. Her findings have recently been published (Ligenstoffer *et al.* 2010) and demonstrated not only confirmation of the occurrence of anaerobic fungi in a non-mammal host (green iguana) but also the existence of eight novel groups of fungi, with these new taxa (likely to represent new genera) comprising almost 40 % of the >250,000 ITS sequences obtained. Whilst it can be difficult to be certain that zoo animals have not acquired new symbionts whilst in captivity, some of these novel groups (NG) did show some host specificity, for example with NG6 comprising nearly all the fungi in kudu and NG8 being found only in Somali wild ass (Fig. 1).

With such a high rate of taxon discovery, the *Neocallimastigomycota*, the newest of the fungal phyla

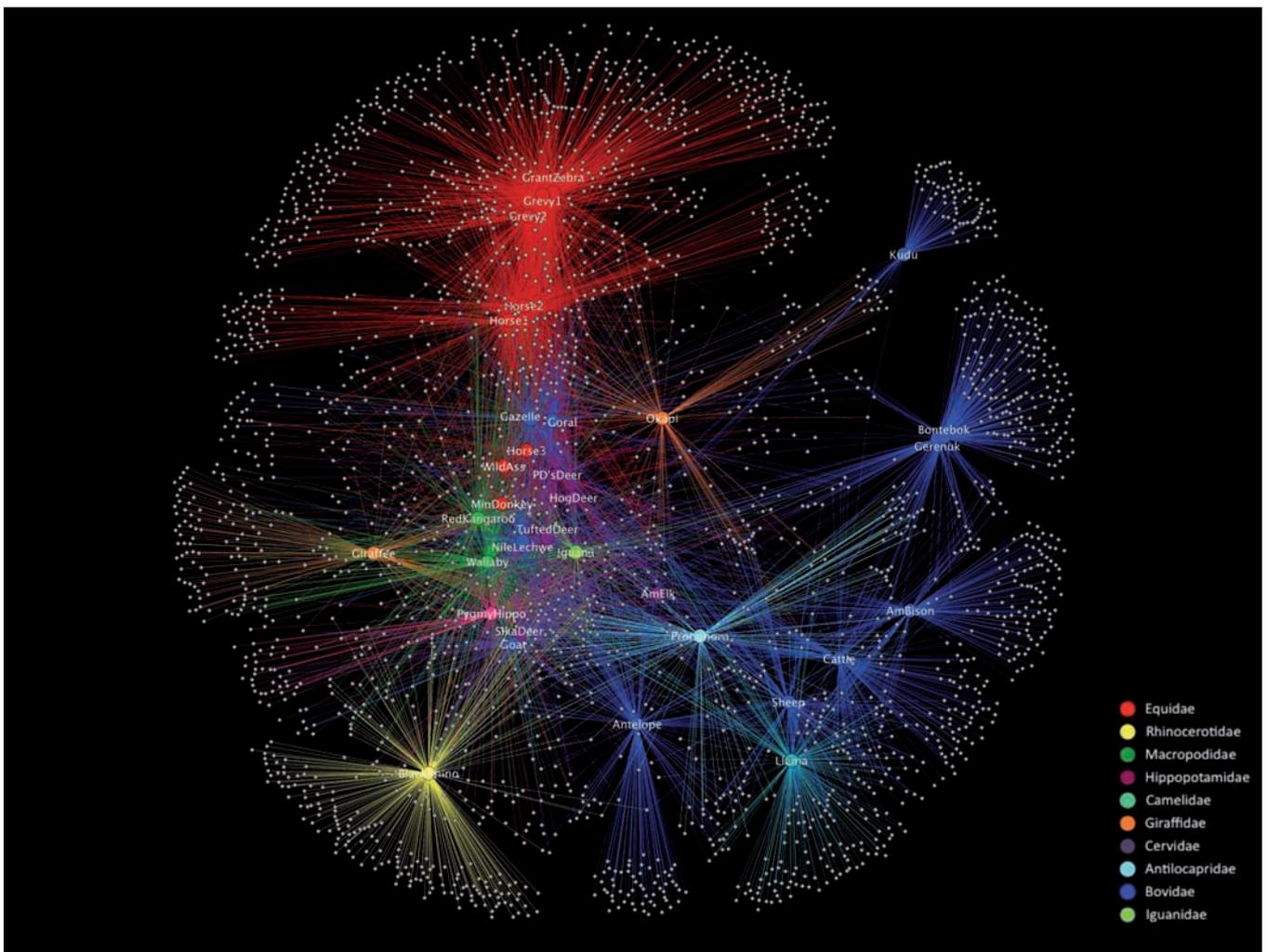


Fig. 1. Network graph highlighting shared OTUs between different anaerobic fungal communities in different animal hosts. The graph is colour-coded by animal host phylogeny (family). Circular nodes indicate animal data sets, whereas smaller square, grey nodes represent individual OTUs. Data sets with a higher proportion of shared OTUs are pulled to the middle, whereas data sets with a high proportion of unique OTUs remain on the periphery. The distance between any two data sets is a function of the number of shared OTUs between the two. Figure supplied by Audra Ligenstoffer.

(Hibbett *et al.* 2007) clearly has more taxonomic gems awaiting discovery, notably in habitats beyond the digestive tracts of vertebrates. Anaerobic fungal sequences do appear in environmental clone libraries (Lockhart *et al.* 2006) but this attests more to the resilience of their resting spores (Ozkose 2001) than to their active metabolism in these habitats. However, it is already clear that a robust taxonomic scheme based on gene sequence data rather than the meagre morphological traits is needed. Kerstin Voigt specialises in the phylogenetics of the lower fungi and questions the acceptability of the phylum *Neocallimastigomycota* (Ebersberger *et al.* 2010). In collaboration with Kate Fliegerova and Ingo Ebersberger from Vienna, Kerstin has taken a phylogenomic approach (concatenated supermatrices of data) to generate

more robust phylogenies. A key element of this approach is the use of orthologous genes for comparisons, requiring first the identification of the original member of any gene family within an organism, prior to any interspecific comparisons. Application of this more robust approach for the anaerobic fungi requires sequence data from genes other than the rRNA locus, however, Kerstin's initial analyses, like Audra's 454 data, revealed the presence of four novel genera (Fig. 2). It was also clear that some GenBank accessions are mis-labelled, highlighting the difficulty in morphological classification of these fungi.

If there is one feature of the *Neocallimastigomycota* that intrigues microbiologists more generally, it is their obligately anaerobic metabolism. Prior to their "official" discovery by Colin

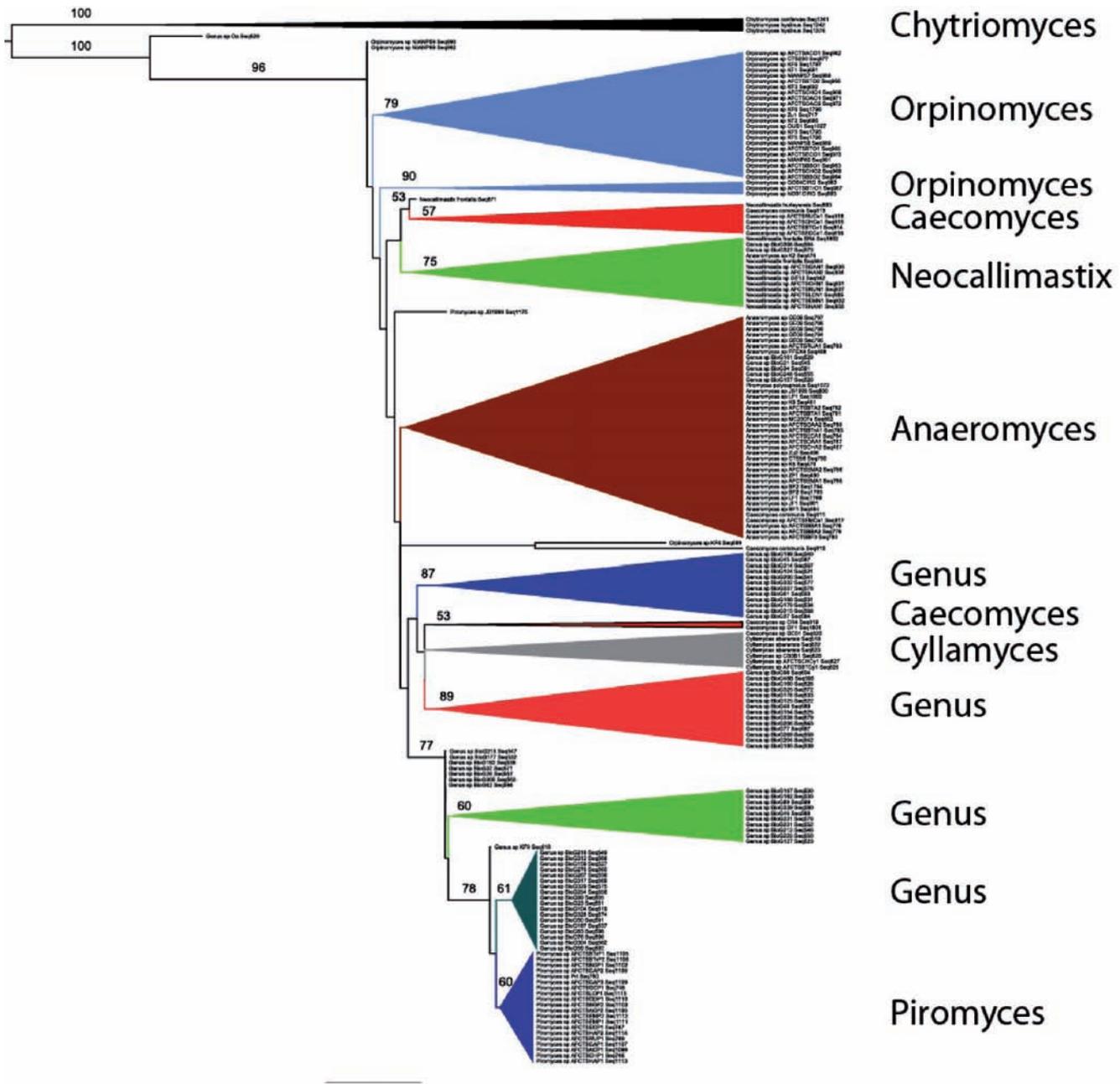


Fig. 2. Phylogenetic tree based on a maximum likelihood analysis using RAxML v. 7.2.6 (Stamatakis 2006) with the aligned ITS1-5.8SITS2 region out of a total of 186 chytrids.

Orpin (1974), these organism had been reported by several rumen microbiologists, and even named by Liebetanz (1910) as the flagellate protozoan *Callimastix*. During the 1960's, Hungate and Prins had also noted these organisms but had dismissed them as contaminants. Thus the dogma that there are no anaerobic fungi was not easily overturned. Despite the near-absence of any useful fossil record, increasingly accurate molecular clock approaches consistently show that the fungi diverged from the more primitive metazoans (animals) some 1000 million years ago. At that time, between the two great oxygenation events (at ca. 2400 and 600 Myr), primitive eukaryotes (including the earliest fungi) were exposed to low atmospheric oxygen levels (<10 %) and many potential niches (e.g. the sea) were highly anoxic (euxinic) (Dahl *et al.* 2010). Mark van der Giezen's research has focused on the metabolism of anaerobic eukaryotes (van der Giezen *et al.* 2005) and in particular how the mitochondrion, thought to have been acquired endosymbiotically to alleviate oxidative stress, evolved into the hydrogenosome, converting malate and pyruvate to hydrogen, CO₂ and acetate. Mark described the several lines of evidence that strongly suggest a mitochondrial origin also for the hydrogenosomes of the anaerobic fungi (van der Giezen *et al.* 1997, 2009).

Some mycologists interested in lower fungi attended the concurrent SIG session on *Evolution and biodiversity of basal lineages of fungi* organised by Satoshi Sekimoto and Tim James. We were, however, fortunate that Gordon Beakes had agreed to flit between sessions and to end our session with an overview of the *Neocallimastigomycota* within the broader panoply of flagellate fungi. Though he is foremost a microscopist, Gordon has made a valuable contribution to work on anaerobic fungi by providing hitherto the only quantitative assessment of the significance of anaerobic fungi to rumen metabolism. This study estimated that the fungi comprised some 20 % of the microbial biomass of sheep fed hay and pelleted lucerne diet (Rezaeian *et al.* 2004). Surprisingly many rumen microbiologists still do not consider the fungi to be a significant component of the rumen microbiota. This belief is fostered partly through ignorance (if you don't look you don't see- not dissimilar to the view of mycorrhizas by many plant physiologists) but also by the bias towards investigation of livestock being fed low fibre/high concentrate diets. With an enlarging human population and increasing demand for animal products, it is likely that animal production will have to become more reliant on high fibre feedstocks, with fungi being of greater importance in efficient rumen function.

A common theme from several speakers was the need for funding to catalyse research on anaerobic fungi. In addition to the biotechnological interest mentioned by Scott and Kate, it is important to explore the possibilities of funding in the area of rumen metabolism, as noted above. In addition to exploring the role of the fungi in fibre digestion, there is also the interaction between the fungi and the methanogenic *Archaea* of the rumen, since emission of methane from livestock production is now recognised to be a major source of this potent greenhouse gas.

Discussions at the end of the meeting and for the duration of IMC9 also addressed how we might promote future collaboration, for instance by establishment of a repository of important isolates. Since they are difficult to culture and require specialised equipment to exclude oxygen, it is unlikely that any of the major culture collections will take on this daunting task. However, it is hoped that a repository for frozen cultures may be found to ensure that representative cultures of the major taxa are made freely available. Furthermore, we agreed to collaborate by sharing of molecular data to work towards a stable taxonomy for these fungi, including the physiological, genetic and morphological classification of the novel taxa that have recently emerged

ACKNOWLEDGEMENTS

As organiser of this Special Interest Group, GWG would like to express his heartfelt thanks to all the participants, especially the speakers and hopes that our discussions both during and after the SIG will lead to a resurgence in work on these fungi. GWG is also grateful to the IMC9 for facilitating the organisation of this meeting.

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