Diversity Census of Fungi in the Ruminal Microbiome: A meta-analysis

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Abstract  This study was designed to examine the diversity census of fungi in rumen microbiome via meta-analysis of fungal 28S rDNA sequences. Both terms, "rumen" and "ruminal," were searched to retrieve the sequences of rumen fungi. As of September 2016, these sequences (n=165) of ruminal origin were retrieved from the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu), an archive of all 28S rDNA sequences and were assigned to the phyla Ascomycota, Neocallimastigomycota, and Basidiomycota, which accounted for 109, 48, and 8 of the 165 sequences, respectively. Ascomycota sequences were assigned to the genera Pseudonectria, Magnaporthe, Alternaria, Cochliobolus, Cladosporium, and Davidiella, including fungal plant pathogens or mycotoxigenic species. Moreover, Basidiomycota sequences were assigned to the genera Thanatephorus and Cryptococcus, including fungal plant pathogens. Furthermore, Neocallimastigomycota sequences were assigned to the genera Cyllamyces, Neocallimastix, Anaeromyces, Caecomyces, Orpinomyces, and Piromyces, which may degrade the major structural carbohydrates of the ingested plant material. This study provided a collective view of the rumen fungal diversity using a meta-analysis of 28S rDNA sequences. The present results will provide a direction for further studies on ruminal fungi and be applicable to the development of new analytic tools.

Keywords : 28S rDNA sequences, fungal diversity, meta-analysis, RDP, rumen microbiome
1. Introduction

Ruminants harbor diverse ruminal microbes, comprising bacteria, archaea, protozoans, and fungi, which are involved in ruminal fermentation of undigested feed [1-3]. Among these, fungi produce various fibrolytic and lignolytic enzymes, which contribute to ruminal digestion of fibers and penetration into plant material [4-8]. The majority of cultured ruminal fungi include those of genera Neocallimastix, Piromyces, Orpinomyces, Anaeromyces, and Caecomyces [9-11].

Because only a fraction of the total ruminal microbes can be identified using the culture-based method [12], the 16S rDNA locus has been used as a phylogenetic marker for taxonomic analysis of both bacteria and archaea [13]; the 28S rDNA locus, for fungi [14]. To investigate fungal diversity, fungal 28S rDNA sequences are generally obtained through traditional molecular cloning, followed by Sanger sequencing. Kim et al. [12] first conducted a meta-analysis of rDNA sequences retrieved from the RDP database to examine the collective bacterial diversity in the rumen. A similar meta-analysis was used to investigate the collective bacterial diversity in the feces of dogs [15].

The Ribosomal Database Project (RDP), Release 11 has provided fungal 28S rDNA sequences for taxonomic classification [14]. Although ruminal fungal 28S rDNA sequences retrieved from various studies are available in the RDP database, no meta-analysis of 28S rDNA sequences of ruminal fungi has been conducted.

In the current study, we retrieved all ruminal fungal 28S rDNA sequences from the RDP database and used them to investigate the diversity census of fungi in the ruminal microbiome.

2. Materials and Methods

As of September 2016, all 28S rDNA sequences retrieved from ruminal samples were obtained from the RDP database, Release 11, Update 5 (http://rdp.cme.msu.edu/), as described previously [15]. Both “rumen” and “ruminal” were search terms to obtain fungal sequences of only ruminal origin. From the retrieved 28S rDNA sequences, a taxonomy tree for ruminal fungi was generated using the ARB (from Latin arbor, tree) software package (Ludwig and Strunk, Munich, Germany) as described previously [12, 15]. A flowchart summarizing the methodology is shown in Fig. 1.

Fig. 1. A flowchart for the methodology followed for the present meta-analysis of ruminal fungal diversity

3. Results and Discussion

3.1 Data summary

A total of 165 fungal 28S rDNA sequences that were analyzed were obtained from 16 unpublished studies and accordingly, the fungi were classified into 3 phyla: Ascomycota (n=109), Neocallimastigomycota (n=48), and Basidiomycota (n=8). A taxonomic tree generated on the basis of these sequences is shown in Fig. 2.

3.2 Phylum Ascomycota

Ascomycota was the most abundant phylum and accounted for 66.5% of all the 165 sequences (Fig. 2). Fungi of this phylum were assigned to 4 classes: Sordariomycetes (n=10), Dothideomycetes (n=20), Leotiomycetes (n=1), and Saccharomycetes (n=73). The remaining 5 were designated as “unclassified Ascomycota.”

3.2.1 Class Sordariomycetes

Sequences of fungi in Class Sordariomycetes were
assigned to 3 orders: Xylariales (n=1), Hypocreales (n=3), and Magnaporthales (n=5) (Fig. 2). The remaining 1 sequence was designated as “unclassified Sordariomycetes.” Two of the 3 Hypocreales sequences were assigned to genus *Pseudonectria*, while all the 5 Magnaporthales sequences were assigned to genus *Magnaporthe*. Fungi of genus *Pseudonectria* have been reported to cause boxwood disease [16] and may therefore have originated from contaminated foliage fed to ruminants. Because genus *Magnaporthe* includes cereal pathogens [17], *Magnaporthe* may have been recovered from contaminated grains fed to ruminant animals.

### 3.2.2 Class Dothideomycetes

Sequences of fungi in class Dothideomycetes were assigned to 2 orders: Pleosporales (n=15) and Capnodiales (n=5) (Fig. 2). Pleosporales sequences were assigned to families Phaeosphaeriaceae (n=2) and Pleosporaceae (n=12), and the remaining 1 sequence was designated as “unclassified Pleosporales.” The 12 Pleosporaceae sequences were assigned to genera *Alternaria* (n=10) and *Cochliobolus* (n=2). Genus *Alternaria* includes mycotoxigenic fungal species, which may produce toxins that are detectable in animal feed [18-25]. Genus *Cochliobolus* includes cereal fungal pathogens [26], indicating its origin from contaminated grains fed to ruminants. Order Capnodiales sequences were assigned to family Davidiellaceae (n=4). Family Davidiellaceae included the genus *Cladosporium* complex (n=1) including mycotoxigenic fungal species [18-25] and genus *Davidiella* (n=1) including fungal plant pathogens [27]. 2 sequences were designated as “unclassified Davidiellaceae.” Both *Cladosporium* complex and *Davidiella* may have originated from contaminated feed of ruminants.

### 3.2.3 Class Leotiomycetes

Fungi of class Leotiomycetes comprised only 1 sequence that was assigned to order Helotiales (Fig. 2), which includes fungal plant pathogens [28]. Therefore, fungal species within order Helotiales may be present in contaminated feed for ruminants and do not constitute normal ruminal microbiota.

### 3.2.4 Class Saccharomycetes

Seventy-three sequences of fungi in class Saccharomycetes were assigned to family Saccharomycetaceae in order Saccharomycetales (Fig. 2). Saccharomycetaceae, a family of yeasts, included genera *Pichia* (n=15), *Lodderomyces* (n=3), and *Williopsis* (n=2). These yeasts seem common in silages and grains fed to ruminants but do not constitute normal ruminal microbiota [29].

### 3.3 Phylum Neocallimastigomyota

Forty-eight of 165 sequences of fungi of phylum Neocallimastigomycota (Fig. 2) were assigned to class Neocallimastigomycetes.

#### 3.3.1 Class Neocallimastigomycetes

All sequences were assigned to family Neocallimastigaceae, which included genera *Cyllamyces* (n=1), *Neocallimastix* (n=8), *Anaeromyces* (n=8), *Caecomyces* (n=2), *Orpinomyces* (n=16), and *Piromyces* (n=10) (Fig. 2). The remaining 3 sequences were designated as “unclassified Neocallimastigaceae.” *Orpinomyces* was the most dominant genus and accounted for 9.7% of all the 165 sequences and 32.7% of all Neocallimastigomycota sequences. It seems that fungi of genera *Neocallimastix* and *Caecomyces* constitute normal ruminal microbiota in different
Fig. 2. A taxonomy tree displaying rumen fungal diversity

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ruminants [32,33]. These genera of family Neocallimastigomycetes have been reported to degrade the major structural carbohydrates of ingested plant materials [9]. Therefore, further studies are required to isolate and characterize novel ruminal fungal species of family Neocallimastigomycetes.

3.4 Phylum Basidiomycota
Eight of 165 sequences of fungi of phylum Basidiomycota (Fig. 2) were assigned to classes Agaricomycetes (n=2), Tremellomycetes (n=1), Microbotryomycetes (n=1), Ustilaginomycetes (n=3), and Cystobasidiomycetes (n=1).

3.4.1 Agaricomycetes
Two sequences of fungi of class Agaricomycetes were assigned to family Ceratobasidiaceae, order Cantharellales (Fig. 2). These two sequences were assigned to genus Thanatephorus. Because Thanatephorus constitutes plant pathogens [34], they may have been recovered from contaminated feed for ruminants.

3.4.2 Class Tremellomycetes
One sequence of a fungus of class Tremellomycetes was assigned to family Tremellaceae, order Tremellales. This sequence was assigned to genus Cryptococcus, which includes animal pathogens. This genus may not constitute normal ruminal microbiota, since it seems to have originated from Cryptococcus infection in ruminants [35].

3.4.3 Class Microbotryomycetes
One sequence of a fungus of class Microbotryomycetes was assigned to family Sporidiobolales incertae sedis, order Sporidiobolales. This sequence was designated as the putative genus “Rhodotorula 4 - Sporidiobolales”, which includes yeast strains. Rhodotorula has been isolated from the rumen of musk oxen [36]. This yeast may be found in silages and grains fed to ruminant animals, but may not be considered an inhabitant of the rumen [29].

3.4.4 Class Cystobasidiomycetes
One sequence of a fungus of class Cystobasidiomycetes was designated as “unclassified Cystobasidiaceae,” family Cystobasidiaceae, order Cystobasidiales, but could not be assigned to any known genus. Because unclassified Cystobasidiaceae was represented by only 1 sequence, it may not constitute normal ruminal microbiota.

4. Conclusion
This study provides an overview of ruminal fungal diversity through a meta-analysis of 28S rDNA sequences of ruminal origin. Fungal species within phylum Neocallimastigomycota are thought to play important roles in digesting structural carbohydrates of plant material. However, fungal species of other phyla seem to be pathogens that originate from contaminated feed for ruminants. Although ruminal fungal diversity has been analyzed using the culture-independent method, limited information is available regarding functions of ruminal fungi. Further studies are required to culture novel ruminal fungal species and to increase the current knowledge of the physiological role of ruminal fungi.

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