Assessing anaerobic gut fungal (Neocalliamstigomycota) diversity using PacBio D1/D2 LSU rRNA amplicon sequencing and multi-year isolation

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Abstract

The anaerobic gut fungi (AGF, Neocallimastigomycota) reside in the alimentary tracts of herbivores where they play a central role in the breakdown of ingested plant material. Accurate assessment of AGF diversity has been hampered by inherent deficiencies of the internal transcribed spacer1 (ITS1) region as a phylogenetic marker. Here, we report on the development and implementation of the D1/D2 region of the large ribosomal subunit (D1/D2 LSU) as a novel marker for assessing AGF diversity in culture-independent surveys. Sequencing a 1.4-1.5 Kbp amplicon encompassing the ITS1-5.8S rRNA-ITS2-D1/D2 LSU region in the ribosomal RNA locus from fungal strains and environmental samples generated a reference D1/D2 LSU database for all cultured AGF genera, as well as the majority of candidate genera encountered in prior ITS1-based diversity surveys. Subsequently, a D1/D2 LSU-based diversity survey using long read PacBio SMRT sequencing technology was conducted on fecal samples from 21 wild and domesticated herbivores. Twenty-eight genera and candidate genera were identified in the 17.7 K sequences obtained, including multiple novel lineages that were predominantly, but not exclusively, identified in wild herbivores. Association between certain AGF genera and animal lifestyles, or animal host family was observed. Finally, to address the current paucity of AGF isolates, concurrent isolation efforts utilizing multiple approaches to maximize recovery yielded 216 isolates belonging to twelve different genera, several of which have no prior cultured-representatives. Our results establish the utility of D1/D2 LSU and PacBio sequencing for AGF diversity surveys, and the culturability of a wide range of AGF taxa, and demonstrate that wild herbivores represent a yet-untapped reservoir of AGF diversity.

Introduction

Members of the anaerobic gut fungi (AGF) are strict anaerobes that inhabit the rumen and alimentary tract of a wide range of foregut and hindgut herbivores. The AGF play an important role in the breakdown of ingested plant biomass via enzymatic and physical disruption in the herbivorous gut [1]. AGF represent a distinct basal fungal phylum (Neocallimastigomycota) that evolved 66 (±10) million years ago coinciding, and possibly enabling, mammalian transition from insectivory to herbivory [2].

Culture independent amplicon-based diversity surveys have been widely utilized to gauge anaerobic fungal diversity and community structure in herbivores [3, 4, 5, 6]. The internal transcribed spacer1 (ITS1) region within the ribosomal operon has been almost exclusively utilized as the phylogenetic marker of choice in culture-independent sequence-based phylogenetic assessments of AGF diversity [7]. Such choice is a reflection of its wider popularity as a marker within the kingdom Mycota [8, 9], the high sequence similarity and limited discriminatory power of the 18S rRNA gene between various AGF taxa [10], and its relatively shorter length, allowing high throughput pyrosequencing- and Illumina-based diversity assessments [3, 6]. However, concerns for the use of ITS1 in diversity assessment for the Mycota [11], basal fungi [12], and the Neocallimastigomycota [7] have been voiced. The ITS1 region is polymorphic, exhibiting considerable secondary structure (number and organization of helices [13]), and length [14] variability. Such polymorphism renders automated alignments, reproducible sequence divergence estimates, and classification of sequence data unreliable and highly dependent on alignment strategies and parameters specified. In addition, significant sequence divergence between copies of the ITS1 region within a single strain have been reported (up to 12.9% in [15]), values that exceed cutoffs utilized for species (even genus in some instances) level delineation

from sequence data [3, 16, 17, 18]. Such limitations often necessitate laborious subjective manual curation and secondary structure incorporation into alignment strategies [13], although it is well recognized that these efforts only partially alleviate, rather than completely address, such fundamental limitations.

The 28S large ribosomal subunit (LSU) is one of the original genes proposed for fungal barcoding [12]. Hypervariable domains 1 and 2 [19] within the LSU molecule (D1/D2 LSU) have previously been utilized for differentiating strains of AGF via molecular typing [20, 21, 22], or sequencing [23, 24]. Compared to ITS1, D1/D2 LSU region exhibits much lower levels of length heterogeneity and intra-strain sequence divergence in fungi [25], including the AGF [20]. Identification and taxonomic assignment of AGF strains based on D1/D2 LSU have gathered momentum; and D1/D2 LSU-based phylogenetic analysis has been reported in all manuscripts describing novel taxa since 2015 [15, 26, 27, 28, 29, 30, 50]. The potential use of D1/D2 LSU as a marker in culture-independent AGF diversity surveys has been proposed as a logical alternative for ITS1 [7, 14]. The lack of specific AGF primers and the relatively large size of the region (approximately 750 bp) has been viewed as a barrier to the wide utilization of short read, high-throughput, Illumina-based amplicon sequencing in such surveys. However, the recent development of AGF LSU-specific primers [24, 31], as well as the standardization and adoption of PacBio long-read sequencing for amplicon-based diversity surveys [32, 33] could enable this process.

Theoretically, a comprehensive assessment of diversity and community structure of a host-associated lineage necessitates sampling all (or the majority) of hosts reported to harbor such lineage. However, to date, the majority of AGF diversity surveys conducted have targeted a few domesticated herbivores, e.g. cows, sheep, and goats [4, 5, 34]. “Exotic” animals have been sampled from zoo settings only sporadically, and on an opportunistic basis [3, 35].

Isolation of AGF taxa enables taxonomic, metabolic, physiological, and ultrastructural characterization of individual taxa. As well, cultures availability enables subsequent –omics, synthetic and system-biology, and biogeography-based investigations [36, 37, 38, 39, 40, 41, 42], as well as evaluation of evolutionary processes underpinning speciation in the AGF [2, 43]. However, efforts to isolate and maintain AGF strains have lagged behind their aerobic counterparts mainly due to their strict anaerobic nature and the lack of reliable long-term storage procedures. Due to these difficulties, many historic isolates are no longer available, and most culture-based studies report on the isolation of a single or few strains using a single substrate/enrichment condition from one or few hosts [29, 44]. Indeed, a gap currently exists between the rate of discovery (via amplicon-based diversity surveys) and the rate of isolation of new taxa of AGF, and several yet-uncultured AGF lineages have been identified in culture-independent diversity surveys [17]. Whether yet-uncultured AGF taxa are refractory to isolation, or simply not yet cultured due to inadequate sampling and isolation efforts remains to be seen.

The current study aims to expand our understanding of the diversity of AGF while addressing all three impediments described above. First, we sought to develop D1/D2 LSU as a more robust marker for AGF diversity assessment by building a reference sequence database correlating ITS1 and D1/D2 LSU sequence data from cultured strains and environmental samples. Second, we sought to expand on AGF diversity by examining a wide range of animal hosts, including multiple previously unsampled wild herbivores. Third, we sought to demonstrate the utility of intensive sampling and utilization of various isolation strategies in recovering AGF strains and testing the hypothesis that many yet-uncultured AGF lineages are indeed amenable to cultivation. Collectively, these efforts provide an established framework for future utilization of

98 D1/D2 LSU amplification and PacBio sequencing for AGF community assessment, highlight the
99 value of sampling wild herbivores, and establish the culturability of a wide range of AGF taxa.

Results

A reference D1/D2-LSU dataset for the Neocallimastigomycota. A 1.4-1.5 Kbp amplicon product encompassing the ITS1-5.8S-ITS2-D1/D2 LSU region was amplified and sequenced from AGF pure cultures and environmental samples to correlate the D1/D2 LSU region to the corresponding ITS1 region, and to provide a reference D1/D2 database for future utilization in high-throughput diversity surveys. Using this approach, representative D1/D2 LSU of all the previously cultured AGF genera *Agriosomyces*, *Aklioshbomyces*, *Anaeromyces*, *Buwchfawromyces*, *Caecomyces*, *Capellomyces*, *Cyllamyces*, *Ghazallomyces*, *Joblinomyces*, *Feramyces*, *Khyollomyces*, *Liebetanzomyces*, *Neocallimastix*, *Orpinomyces*, *Pecoramyces*, *Piromyces*, and *Tahromyces* were obtained (Table 1). Representatives of the genus *Oontomyces* were not encountered in this study, but reference LSU and ITS1 sequences were obtained from prior publication [26]. In addition, representative sequences of D1/D2 LSU of candidate genera AL3, AL4, AL8, MN3, MN4, SK3, and SK4, previously identified in ITS1 culture-independent datasets were also obtained (Table 1, Datasets 1-3). Finally, representatives of six completely novel AGF candidate genera (RH1-RH6) were also identified (Table 1, Datasets 1-3) and confirmed as novel independent clades in ITS1 and D1/D2 LSU-based phylogenetic analysis. It should be noted that multiple previously reported yet-uncultured (candidate) genera have recently been successfully isolated, e.g. AL1 (*Khyollomyces*), AL5 (*Joblinomyces)*, AL6 (*Feramyces)*, AL7 (*Piromyces finnis)*, MN1 (*Cyllamyces)*, SP4 (*Liebetanzomyces)*, and SK2 (*Buwchfawromyces*). In addition, some previously proposed candidate genera clustered as members of already existing genera in our analysis, e.g. SP8 with *Cyllamyces,* and SP6 with *Neocallimastix* (Table 1). As such, we estimate that only representatives of candidate genera BlackRhino, SP1, SP2 [17], and the relatively rare AL2, DA1, DT1, JH1/SP5 (ITS1 sequence

representatives of these two candidate genera are 99.6% similar and so they should be considered as one candidate genus), KF1, MN2, SK1, SP3, and SP7 [3, 5, 13, 17, 45, 46, 47, 48] were not encountered in this study, and hence no reference LSU sequence data for these candidate genera are currently available (Table 1).

D1/D2 LSU versus ITS1 as a taxonomic marker.

Intra-genus length variability.

The ITS1 and D1/D2 LSU regions were bioinformatically extracted from the 116 Sanger-generated clone sequences from this study and previous studies [23, 28, 29, 49] (accession numbers in Table 1), the rRNA loci from two Neocallimastigomycota genomes (*Pecoramyces ruminantium* strain C1A, and *Neocallimastix californiae* strain G1) [36, 43] in which the entire rrn operon sequence data is available, and from the PacBio-generated environmental amplicons in this study. The ITS1 region displayed a high level of length heterogeneity, ranging in size between 141 and 250 bp (median 191 bp, Figure 1a), with 75% of sequences ranging between 182-208 bp in length. Some genera had shorter than median ITS1 region length, e.g. *Cyllamyces* (range 141-173 bp), *Buwchfawromyces* (range 155-169 bp), and candidate genus AL3 (range 145-148 bp), while others exhibited a longer than median ITS1 region length, e.g. *Liebetanzomyces* (range 198-225 bp), and candidate genus RH5 (range 191-224 bp) (Figure 1a). A third group of genera displayed a wide range of length heterogeneity, e.g. *Neocallimastix* (range 160-244 bp), *Caecomyces* (range 192-250), and *Piromyces* (range 173-225 bp). Few genera and candidate genera displayed a fairly narrow range of ITS1 length, e.g. AL3 (141-148 bp), but this is potentially a reflection of the paucity of sequences belonging to these genera obtained in this study (Figure 1a).

On the other hand, a much lower level of length heterogeneity was identified in the D1/D2 LSU (Figure 1b), ranging in size between 740-767 bp (median 760 bp), and where 75% of the sequences ranged between 757-761 bp, with all genera consistently displaying a much narrower D1/D2-LSU length heterogeneity, ranging between 11 bp in RH4 and 26 bp in the genera *Neocallimastix* and *Aklioshbomyces*.

Intra-genus sequence divergence.

The ITS1 region displayed intra-genus sequence divergence ranging from 0.4 to 21% (median 3.2%), with 75% of the pairwise divergence values ranging between 1.7-6%. Genera displaying the highest level of divergence were *Caecomyces* (1-18.9%, median 8.3%), *Cyllamyces* (0.6-19.6%, median 5.5%), and *Neocallimastix* (0.4-19.3%, median 5.5%) (Figure 1c). On the other hand, intra-genus sequence divergence of the D1/D2 LSU ranged between 0.1-9.2% (median 1.4%), with 75% of the pairwise divergence values ranging between 0.8-2.1%. Genera displaying highest level of divergence were *Feramyces* (0.1-7.8%), *Joblinomyces* (0.1-8.7%, *Caecomyces* (0.1-9%), and *Piromyces* (0.1-9.2%) (Figure 1d).

Within strain length variability.

Within strain length heterogenicity examined in 19 strains with 2 or more sequenced clones ranged between 0-5 bp (Figure 2a) for ITS1 region and 0-1 bp for the LSU region (Figure 2b).

Within strain sequence divergence.

Examining the 19 strains with more than two sequenced clones, the full ITS1 region showed intra-strain sequence divergence ranging from 0.1-10.01% (Figure 2c). Similar, and even higher levels of intra-strain ITS1 variability was previously reported e.g. up to 12.9% in *Buwchfawromyces eastonii* strain GE09 [15]. On the other hand, within strain D1/D2 LSU rRNA region showed a much lower sequence divergence, ranging from 0.13-1.84% (Figure 2d).

Neocallimastigomycota diversity assessment using D1/D2 LSU as a phylogenetic marker.

Phylogenetic diversity and Novelty.

A total of 17,697 high-quality long-read amplicons were obtained. Phylogenetic analysis using the D1/D2 LSU amplicons assigned all sequences into 28 different genera/candidate genera (Figure 3a, Figure 4a, Figure S1) and 298 species level $OTUs_{0.02}$. AGF genera identified in this study included members of the previously described genera *Anaeromyces*, *Buwchfawromyces*, *Caecomyces*, *Cyllamyces*, *Liebetanzomyces*, *Neocallimastix*, *Orpinomyces*, *Pecoramyces*, and *Piromyces.* In addition, sequences representing multiple novel genera were also identified (Figure 3a, Figure 4a, Figure S1), some of which have been subsequently isolated, named, and characterized in separate publications, e.g. *Feramyces* [28], *Aklioshbomyces*, *Agriosomyces*, *Ghazallomyces*, and *Khyollomyces* [50]. Finally, six novel candidate genera were identified and designated RH1-RH6 (Figure 3a, Figure 4a, Figure S1). All of these six novel genera were encountered in extremely low abundance in a few samples (Figure 3a), with the notable exception of RH5, which was present in high relative abundance in multiple animals e.g. domesticated sheep (96.22%), blackbuck deer (52.41%), axis deer (20.71%), and an aoudad sheep sample (11.75%).

Diversity estimates, and distribution patterns.

The number of AGF genera encountered per sample varied widely from 5 (in Pere David's deer, and Longhorn cattle) to 16 (in one Aoudad sheep sample) (Table 2, Figure 3a, Figure 4a). However, in each of these samples a distribution pattern was observed in which a few genera represent the absolute majority of the sequences obtained. Excluding genera present in less than 1% abundance would lower the number of genera encountered per animal to 1 (in white-tail deer

and dwarf goat) -10 (domesticated goat). Usually, 1-5 taxa were present in >10% abundance per animal (Figure 3b).

Using empirical cutoffs for ubiquity (presence in at least 50% of the animals studied) and abundance (above 1%), we identify five different distribution patterns for AGF genera encountered in this study (Figure 4b); 1. Ubiquitous mostly abundant genera: These are the genera identified in at least 50% of the animals studied and where their relative abundances exceed 1% in at least 50% of their hosts: This group includes *Piromyces*, *Feramyces*, *Khyollomyces*, RH5, *Neocallimastix*, *Cyllamyces*, and *Caecomyces*. 2. Ubiquitous mostly rare genera: These are the genera identified in at least 50% of the animals studied and where their relative abundances were lower than 1% in at least 50% of their hosts. This group includes *Orpinomyces*, and *Pecoramyces*. 3. Less ubiquitous but mostly abundant genera: These are the genera identified in < 50% of the animals studied but where their relative abundances exceed 1% in at least 50% of their hosts. This group includes *Ghazallomyces*, RH4, MN4, *Joblinomyces*, SK4, *Buwchfawromyces*, AL3, RH1, and RH3. 4. Less ubiquitous mostly rare genera: These are the genera identified in < 50% of the animals and where their relative abundances were lower than 1% in at least 50% of their hosts. This group includes *Liebetanzomyces*, *Anaeromyces*, AL8, *Aklioshbomyces*, RH2, and *Agriosomyces*. 5. Less ubiquitous consistently rare genera: These are the genera identified in < 50% of the animals and where their relative abundances never exceeded 1% in any of their hosts. This group includes RH6, AL4, MN3, and SK3.

Multiple diversity estimates (number of observed genera, Chao and Ace richness estimates, Shannon diversity index, Simpson evenness, as well as diversity rankings) were computed for each sample (Table 2). The highest genus-level richness was observed in aoudad sheep, dwarf goat, oryx, domesticated cow, domesticated goat, miniature donkey, zebra, and

blackbuck deer samples, while the highest genus-level diversity (based on diversity ranking and Shannon index) was observed in domesticated goat, alpaca, axis deer, blackbuck deer, mouflon ram, miniature donkey, oryx, and domesticated horse. On the other hand, the lowest genus-level richness was observed in longhorn cattle, Pere David's deer, Boer goat, domesticated horse, domesticated sheep, and alpaca, while the lowest genus-level diversity was observed in Fallow deer, zebra, domesticated sheep, dwarf goat, and white-tail deer.

When correlated to animal host phylogeny or animal lifestyle (24 possible combinations), all diversity estimates showed low correlation coefficients (Cramer's V statistic <0.49) at both the genus and the species equivalent levels (Table S1). Student t-tests were used to examine the significance of the difference in diversity estimates at the genus and species equivalent levels between animal host families (families Bovidae, Cervidae, and Equidae) as well as animal lifestyle (zoo-housed, wild, and domesticated). Only three of these showed a significant difference (Student t-test p-value <0.05): Family Bovidae had a significantly higher observed number of genera and significantly higher Chao estimate at the genus level, and zoo-housed animals had significantly lower Shannon diversity at the species equivalent level (Table S1).

Community structure.

We used a combination of ordination methods and Student t tests to identify associations between AGF genera and host factors. Non-metric multidimensional scaling based on the genus-level Bray-Curtis indices (Figure 5a-b) identified a few patterns. The genera *Aklioshbomyces*, *Ghazallomyces*, *Joblinomyces*, *Feramyces*, *Buwchfawromyces*, and *Pecoramyces* seem to be more prevalent in some wild animals (e.g. black buck deer, mouflon, oryx, axis deer, and white tailed deer; filled squares in Figure 5a), while some zoo-housed animals (e.g. elk, dwarf goat, and miniature donkey; grey squares in Figure 5a) clustered together based on the abundance of

Neocallimastix, *Caecomyces*, and *Liebetanzomyces*. Few domesticated animals (e.g. domesticated goat, longhorn, alpaca, and domesticated cow; open squares in Figure 5a) clustered together based on the abundance of *Cyllamyces*, AL8, MN3, MN4, RH1, RH3, RH4, and RH6. Animal host family had a slightly less apparent effect on AGF community structure (Figure 5b) with the exception of the importance of *Aklioshbomyces* and *Ghazallomyces* in family Cervidae, and AL3 and *Khyollomyces* in family Equidae.

To test the significance of these observed patterns, Student t-tests were used to identify significant associations between specific AGF taxa and host phylogeny (families Bovidae, Equidae, Cervidae), or animal lifestyle (zoo-housed, domesticated, wild). From all possible associations (168 total; 28 genera x 3 host families and 3 lifestyles), significant differences were observed only in the following cases. The AGF genera AL3, *Khyollomyces*, and *Piromyces* were significantly more abundant in family Equidae (p-value=0.014, 0.018, and 0.034 respectively), while the genera *Aklioshbomyces*, *Ghazallomyces*, and *Joblinomyces* were significantly more abundant in family Cervidae (p-value=0.074, 0.072, and 0.075 respectively). On the other hand, the animal lifestyle had slightly more significant effect on AGF community structure as follows: The genus *Neocallimastix* was significantly more abundant in zoo-housed animals (p-value=0.007), the genera *Aklioshbomyces*, *Buwchfawromyces*, and *Pecoramyces* were significantly more abundant in wild animals (p-value=0.047, 0.028, and 0.014 respectively), and the genera *Cyllamyces*, AL8, RH1, RH4, and RH6 were significantly more abundant in domesticated animals (p-value=0.001, 0.001, 0.011, 0.018, and 0.054 respectively). Finally, for individual animals species with enough replication in our study, the genera *Cyllamyces,* AL8, and RH1 were significantly more abundant in *Bos taurus* (p-values=1.86E-11, 3E-5, and 2.27E-9, respectively), the genera *Caecomyces* and RH5 were significantly more abundant in *Ovis aries*

(p-values=0.006, and 0.004 respectively), and the genera *Feramyces* and SK4 were significantly more abundant in *Ammotragus lervia* (p-values=0.002, and 0.0006, respectively). Further, some genera were only encountered in one animal, demonstrating a probable strong AGF genus-host preference. These genera include *Ghazallomyces* only encountered in axis deer, AL4 only encountered in domesticated sheep, MN3 only encountered in domesticated cow, and MN4 only encountered in domesticated goat.

Neocallimastigomycota isolation

A total of 216 AGF isolates were obtained from 21 animals (Table 3). Success in isolation and maintenance of that large number of isolates was enabled by the implementation of various techniques for isolation, and the development of a reliable storage procedure [51]. Isolates obtained belonged to 12 different genera (Table 3), six of which were exclusively isolated in this study, and characterized in separate publications (*Akhlioshbomyces*, *Ghazallomyces*, *Capellomyces, Agriosomyces*, *Khoyollomyces* (AL1), and *Feramyces* (AL6) [28, 50]. In general, 1-3 AGF genera were isolated per sample. Isolation efforts captured anywhere between 6.3% (1 of 16 genera) to 27.3% (3 of 11 genera) of AGF genera identified in a single sample using culture-independent D1/D2 LSU gene-based analysis. However, these values are highly affected by the fact that sequencing efforts are capable of identification of AGF genera present in extremely low levels of relative abundance. Indeed, excluding rare taxa (those present at <1% abundance), the culturability goes up to 10% (1 of 10 genera)-100% (2 of 2 genera).

We sought to determine how community structure and isolation efforts correlate, and whether obtaining isolates belonging to a specific genus could be predicted from the community structure of the sample. We observed a strong Pearson correlation (r=0.79) between the abundance of a certain genus in a sample and the frequency of its isolation. On the other hand,

the success of isolation of the most dominant member of the community was negatively affected by the sample evenness (Pearson correlation coefficient = -0.87). Indeed, our ability to isolate the novel genera *Aklioshbmyces, Ghazallomycota*, and *Khyollomyces* could be attributed to their presence in high relative abundance in samples from which they were recovered (Table 3), as opposed to their rarity/absence in other samples (Figure 3, 4). Within ubiquitous genera, we observed that the abundance-success of isolation correlation described above is stronger for monocentric taxa (Pearson correlation coefficients= 0.83, 0.96, 0.92, and 1 for *Pecoramyces*, *Feramyces*, *Neocallimastix*, and *Agriosomyces,* respectively), while such relationship was much weaker in polycentric taxa (Pearson correlation coefficients= 0.31, and 0.58 for *Orpinomyces,* and *Anaeromyces,* respectively). However, the polycentric nature of these genera (ability to propagate even in the absence of zoospore production, and the larger colony size on roll tubes) enabled their isolation even when they constituted a minor fraction of the total community.

Discussion

LSU as a phylogenetic marker for AGF diversity surveys. We highlight and quantify the advantages associated with the utilization of D1/D2 LSU as a phylomarker for the AGF when compared to the currently utilized ITS1 region (Figures 1, 2). We also report on overcoming the three main hurdles (lack of reference sequences from uncultured genera, correlating D1/D2 LSU data to currently available ITS1 datasets, and amplicon length precluding utilization of Illumina platform) associated with D1/D2 LSU use as a phylomarker. To address the lack of reference LSU sequence data, we undertook a multi-year isolation effort to provide a comprehensive D1/D2-LSU database from a wide range of AGF taxa, a necessary approach given the lack of LSU sequence data from multiple historic taxa, unavailability of AGF in culture collections, and difficulties in maintenance of this fastidious group of organisms. To correlate D1/D2 LSU data to currently available ITS1 datasets and overcome amplicon length constrains, we utilized a SMRT-PacBio sequencing approach to obtain sequences comprising the region spanning from the start of the ITS1 region to the end of the D1/D2-LSU region in the rRNA locus (1400-1500 bp). In the process, we not only increased the representation of D1/D2-LSU sequences from all cultured taxa, but also identified D1/D2-LSU sequences of yet-uncultured taxa previously defined only by their ITS1 sequences (e.g. AL3, AL4, AL8, MN3, MN4, SK3, and SK4), as well as defined 6 completely novel AGF candidate genera (RH1-RH6). Collectively, this dataset (17,697 sequences of environmental D1/D2 LSU annotated by their taxonomy (Dataset 3), plus 116 Sanger-generated clone sequences and genomic rRNA loci sequences (Table 1 with accession numbers) could pave the way for future D1/D2 LSU-based AGF diversity surveys. We anticipate that additional sampling and culture-independent studies using the whole region, as

well as future isolation efforts will identify the corresponding D1/D2-LSU region for those few yet-uncultured ITS1-defined lineages that we failed to capture in our study.

Single molecule real-time (SMRT) PacBio sequencing technology enables long read sequencing by a single uninterrupted DNA polymerase molecule. The SMRT sequencing protocol involves ligating hairpin adaptors to the ends of double-stranded DNA (PCR products in the case of culture-independent studies), leading to the circularization of the DNA. This subsequently allows the sequencing polymerase to pass around the molecule multiple times. The re-sequencing by multiple passages increases sequence coverage thereby significantly reducing error rates from initial values of up to 15%, to levels lower than 1%. Culture-independent studies in bacteria, archaea, and fungi [33, 52, 53, 54] have successfully applied the technology. We, here, provide the basis for its application to culture-independent studies in anaerobic gut fungi. We applied rigorous control to ensure the high quality of reads utilized to build the single molecule consensus read sequences (by using a minimum threshold of 5 full passes and 99.95% predicted accuracy), followed by pre-processing in Mothur to remove sequences with ambiguities or an average quality score below 25. Also, we anticipate that future AGF diversity studies employing PacBio sequencing of the D1/D2-LSU region (rather than the full ITS1-5.8S-ITS2-D1/D2 LSU region) would be further enabled by the shorter amplicon length (~700 as opposed to ~1300-1400 bp), as well as recent (e.g. Sequel II) and future anticipated improvements in SMRT sequencing technology.

Discovery and characterization of novel AGF lineages. D1/D2 LSU-based diversity assessment of 21 fecal samples identified multiple novel AGF candidate genera (Figure 3, 4), five of which were subsequently isolated and described in separate publications (*Feramyces* [28], *Aklioshbomyces*, *Agriosomyces*, *Ghazallomyces*, and *Khyollomyces* [50]). These results clearly

demonstrate that the scope of AGF diversity is much broader than implied from prior studies. This conclusion is in apparent disagreement with the recent work of Paul et al. [17], where the authors utilized a rarefaction-based approach on publicly available ITS1 AGF sequence data to suggest that AGF sampling efforts have reached saturation. However, we argue that using a rarefaction curve approach on publicly available datasets only elucidates coverage within samples already in the database, and not the broader AGF diversity in nature. Many prior studies have used relatively low throughput sequencing technologies, and repeatedly sampled few domesticated animals, and such pattern would result in encountering highly similar populations between different studies. We attribute the discovery and characterization of a wide range of novel AGF taxa within our dataset to sampling previously unsampled animal hosts, and the use of high-throughput sequencing that enabled access to rare members of the AGF community. Multiple novel AGF genera were isolated from animals previously unsampled for AGF diversity, e.g. *Aklioshbomyces* from white-tailed deer where it represented 98.5% of the community, *Ghazallomyces* from axis deer where it represented 27.8% of the community, and *Feramyces* from an aoudad sheep sample where it represented 55.3% of the community. It is notable that many of these novel taxa were only encountered in wild herbivores. Whether this novelty is a reflection of a lifestyle selecting for specific taxa, or a reflection of simply lack of prior sampling of wild animals due to logistic difficulties remains to be seen. This clearly demonstrates that novel AGF taxa remain to be discovered by sampling hitherto unsampled/poorly sampled animal hosts.

Further, a significant fraction of novel AGF candidate genera identified were present in extremely low relative abundance. Such pattern suggests the presence of numerous novel AGF taxa that appear to predominantly exist in relatively low abundance possibly as dormant

362 members of the AGF community in the herbivorous gut. The discovery and characterization of
363 the rare members of AGF community could significantly expand the scope of AGF diversity in
364 nature. The dynamics, rationale for occurrence, mechanisms of maintenance, putative role in
365 ecosystems, and evolutionary history of rare members of the community are currently unclear. It
366 has been suggested that a fraction of the rare biosphere could act as a seed bank of functional
367 redundancy that aids in ecosystem response to periodic (e.g. occurring as part of growth of the
368 animal host, or due to seasonal changes in feed types) or occasional (i.e. due to unexpected
369 disturbances) changes in the gut *in-situ* conditions. Regardless, such pattern highlights the value
370 of deeper sampling (to capture rare biosphere), as well as more extensive time-series, rather than
371 snapshot, sampling to capture patterns of promotion and demotion of members of the AGF
372 community within the lifespan of an animal.
373 **The value of AGF isolation efforts.** The strict anaerobic nature of AGF necessitates the
374 implementation of special techniques for their isolation and maintenance [55, 56]. Further, while
375 several storage methods based on cryopreservation have been proposed [57], the decrease in
376 temperature to the ultra-low values and the incidental O_2 exposure during revival of the
377 cryopreserved strains were shown before to be detrimental for some isolates. The lack of reliable
378 long-term storage procedures often necessitates frequent subculturing of strains (every 3-4 days),
379 which often leads to either the production of sporangia that do not differentiate to zoospores, or
380 the outright failure to produce sporangia [58].
381 Through a multi-year effort, we were successful in obtaining 216 isolates representing
382 twelve AGF genera. We attribute our success to using multiple strategies (enrichment on
383 multiple carbon sources, and paying special attention to picking colonies of different shapes and
384 sizes, and to picking several colonies of the same shape, as representatives of different genera are

385 known to produce colonies with very similar macroscopic features), but, more importantly, to
386 using a wide range of samples (with varying host lifestyle, gut type, and animal phylogeny). The
387 success of isolation of a certain genus was, in general, attributed to its abundance in the sample
388 (Pearson correlation coefficient=0.79), especially for monocentric genera (e.g. *Pecoramyces*,
389 *Feramyces*, *Neocallimastix*, and *Agriosomyces*), and was negatively correlated to the sample
390 evenness (Pearson correlation coefficient= -0.87). It remains to be seen if this is true and
391 reproducible for all samples and across laboratories. More efforts are certainly needed to develop
392 targeted isolation strategies for specific taxa that we failed to obtain in pure cultures despite our
393 best effort and despite their abundance in their respective sample (e.g. SK4 in one of the aoudad
394 sheep samples, and RH5 in the domesticated sheep and the axis deer samples).
395 In conclusion, our results establish the utility of D1/D2 LSU and PacBio sequencing for
396 AGF diversity surveys, and the culturability of a wide range of AGF taxa, and demonstrate that
397 wild herbivores represent a yet-untapped reservoir of AGF diversity.

Experimental Procedures

Samples. Fecal Samples were obtained from six domesticated, six zoo-housed, and nine wild animals (Table 2). The host animals belonged to the families *Bovidae* (11), *Cervidae* (6), *Equidae* (3), and *Camelidae* (1). The dataset encompassed some replicates from few animal species sometimes with lifestyle variations within a single animal species: *Bos taurus* (n=2; domesticated cow, and domesticated longhorn cattle), *Ovis aries* (n=2; domesticated sheep and wild mouflon ram), *Capra aegagrus* (n=3; domesticated goat, wild Boer goat, and zoo-housed dwarf goat), and *Ammotragus lervia* (n=2; Aoudad Sheep) (Table 2). Samples from domesticated animals were obtained from Oklahoma State University and surrounding farms between September 2016 and May 2018. Samples from the Oklahoma City Zoo were obtained in April 2019. For samples from wild herbivores, we enlisted the help of hunters in four separate hunting expeditions in Sutton, Val Verde, and Coke counties, Texas (April 2017, July 2017, and April 2018), and Payne County, Oklahoma (October 2017). Appropriate hunting licenses were obtained and the animals were shot either on private land with the owner's consent or on public land during the hunting season. All samples were stored on ice and promptly (within 20 minutes for domesticated samples, within 1 hour for zoo samples, and within 24 hours for samples obtained during hunting trips) transferred to the laboratory. Upon arrival, a portion of the sample was immediately used for setting enrichments for isolation efforts, while the rest was stored at -20ºC for DNA extraction.

Development of D1/D2 LSU locus as a phylogenetic marker.

A. Amplification of the ITS1-5.8S rRNA-ITS2-D1/D2 LSU from Neocallimastigomycota isolates. Biomass was harvested from 10 ml of 2-4-day old cultures and crushed in liquid nitrogen. DNA was extracted from the ground fungal biomass using DNeasy PowerPlant Pro Kit (Qiagen,

Germantown, Maryland) according to the manufacturer's instructions (Youssef et al. 2013). A PCR reaction targeting the region encompassing ITS1, 5.8S rRNA, ITS2, and D1/D2 region of the LSU rRNA' (Figure 6) was conducted using the primers ITS5-NL4 [23]. The PCR protocol consisted of an initial denaturation for 5 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 2 min, and a final extension of 72 °C for 20 min. PCR amplicons were purified using PureLink® PCR cleanup kit (Life Technologies, Carlsbad, California), followed by cloning into a TOPO-TA cloning vector according to the manufacturer's instructions (Life Technologies, Carlsbad, California). Clones (n=1-12 per isolate) were Sanger sequenced at the Oklahoma State University DNA sequencing core facility.

B. Amplification of the ITS1-5.8S-ITS2-D1/D2 LSU from environmental samples. Fecal material from different animals (0.25-0.5 g) were crushed in liquid nitrogen and total DNA was extracted from the ground sample using DNeasy PowerPlant Pro Kit (Qiagen, Germantown, Maryland) according to the manufacturer's instructions (Youssef et al. 2013). Extracted DNA was then used as a template for ITS1-5.8S-ITS2-D1/D2 LSU PCR amplification using ITS5 forward primer and the AGF-specific reverse primer GG-NL4 [24]. Primers were barcoded to allow PacBio sequencing and multiplexing (Table S2). Amplicons were purified using PureLink® PCR cleanup kit (Life Technologies, Carlsbad, California), quantified using Qubit® (Life Technologies, Carlsbad, California), pooled, and sequenced at Washington State University core facility using one cell of the single molecular real time (SMRT) Pacific Biosciences (PacBio) RSII system.

C. Environmental PacBio-generated sequences quality control. We performed a two-tier quality control protocol on the generated sequences. First, the raw reads were processed according to PacBio published protocols to obtain single molecule consensus reads. Second, we used rigorous

444 sequence quality control in Mothur [59] to remove any sequences with low quality from subsequent
445 analysis.
446 For Raw reads processing, the official PacBio pipeline (RS_Subreads.1)
447 (http://files.pacb.com/software/smrtanalysis/2.2.0/doc/smrtportal/help/!SSL!/Webhelp/CS_Prot_
448 RS_Subreads.htm) was utilized. Raw reads were filtered based on the minimum read length and
449 minimum read quality. The passing reads were then processed with the PacBio
450 RS_ReadsOfInsert protocol
451 (http://files.pacb.com/software/smrtanalysis/2.2.0/doc/smrtportal/help/!SSL!/Webhelp/CS_Prot_
452 RS_ReadsOfInsert.htm) for generating single-molecule consensus reads from the insert template.
453 Consensus reads had a minimum of 5 full passes, 99.95% predicted accuracy, and 1000 bp insert
454 length. The resulting consensus reads had a mean number of passes of 20, mean read length of
455 insert of 1429 bp, and mean polymerase read quality of 0.99.
456 Sequence quality control procedures were subsequently conducted in Mothur [59] to assess
457 the quality of the generated consensus reads utilizing stringent protocols previously suggested for
458 assessing bacterial, archaeal, and fungal diversity for similar sized amplicons [33, 52, 53, 54]. Reads
459 were filtered in Mothur using trim.seqs to remove reads longer than 2000 bp, reads with average
460 quality score below 25, reads with ambiguous bases, reads not containing the correct barcode
461 sequence, reads with more than 2 bp difference in the primer sequence, and reads with
462 homopolymer stretches longer than 12 bp. Reads with the primer sequence in the middle were
463 identified by performing a standalone Blastn-short using the primer sequence as the query, and
464 were subsequently removed using the remove.seqs command in Mothur.
465 A mock community (constituted of equal concentration of PCR products of 5 different
466 strains (*Aklioshbomyces papillarum* strain WT2, *Feramyces austinii* isolate DS10,

Liebetanzomyces sp. isolate Cel1A, *Piromyces* sp. isolate A1, and *Piromyces* sp. isolate Jen1) from our culture collection and for which we have obtained at least 5 Sanger clone sequences) was also sequenced. To establish whether the above approaches for overall read- and sequence-based quality control are adequate, we compared PacBio-generated mock sequences to the corresponding Sanger-generated clone sequences. The median percentage similarity between PacBio-generated sequences affiliated with a certain strain and the Sanger-generated clone sequences obtained for that strain (99.05±0.6 to 99.64±0.47) were not significantly different from the median percentage similarities between different clones of the same strain (98.91±0.6 to 99.72±0.47) (Student t-test p-value>0.1) attesting to the adequacy of the above quality control measures in removing low quality sequences.

D. A D1/D2 LSU reference database for cultured and yet-uncultured AGF taxa. A reference D1/D2-LSU sequence database for all Neocallimastigomycota cultured genera present in our culture collection was created via amplification, cloning, and sequencing of the ITS1-5.8S-ITS2-D1/D2 LSU allowing for a direct correlation and cross-referencing of both regions. To obtain D1/D2 LSU sequences representing yet-uncultured candidate genera previously defined by ITS1 sequence data [3, 5, 13, 17, 46], the ITS1 region from the PacBio-generated ITS1-5.8S-ITS2-D1/D2 LSU environmental amplicons was extracted in Mothur using the pcr.seqs command with the sequence of the MNGM2 reverse primer and the flag rdiffs=2 to allow for 2 differences in primer sequence. The trimmed sequences corresponding to the ITS1 region were compared, using blastn, to a manually curated Neocallimastigomycota ITS1 database encompassing all known cultured genera, as well as yet-uncultured taxa previously identified in culture-independent studies [3, 5, 13, 17, 46] (Figure 6). Sequences were classified as their first hit taxonomy if the percentage similarity to the first hit was >96% and the two sequences were aligned over >70% of

the query sequence length. A taxonomy file was then created that contained the name of each sequence in the PacBio-generated environmental dataset and its corresponding taxonomy and was used for assigning taxonomy to the D1/D2 LSU sequence data.

E. Comparison of D1/D2-LSU versus ITS1 as phylogenetic markers. We used the dataset of full length PacBio-generated sequences described above, in addition to 116 Sanger-generated clone sequences from this study and previous studies [23, 28, 29, 49], as well as genomic rRNA loci from two Neocallimastigomycota genomes (*Pecoramyces ruminantium* strain C1A, and *Neocallimastix californiae* strain G1) [36, 43] in which the entire rrn operon sequence data is available to compare the ITS1 and D1/D2-LSU regions with respect to heterogeneity in length and intra-genus sequence divergence. For every sequence, the ITS1, and the D1/D2-LSU regions were bioinformatically extracted in Mothur using the pcr.seqs command (with the reverse primer MNGM2, and the forward primer NL1, for the ITS1, and the D1/D2-LSU regions, respectively) and allowing for two differences in the primer sequence. The trimmed sequences (both ITS1 and D1/D2-LSU) were then sorted into files based on their taxonomy such that for each genus/taxon two fasta files were created, an ITS1 and a D1/D2-LSU. These fasta files were then used to compare length heterogeneity, and intra-genus sequence divergence as follows. Sequences lengths in each fasta file were obtained using the summary.seqs command in Mothur. Intra-genus sequence divergence values were obtained by first creating a multiple sequence alignment using the MAFFT aligner [60], followed by generating a sequence divergence distance matrix using the dist.seqs command in Mothur. Box plots for the distribution of length and sequence divergence were created in R.

AGF Diversity assessment using D1/D2 LSU locus.

A. Phylogenetic placement. The majority of the D1/D2-LSU sequences bioinformatically extracted from environmental sequences were assigned to an AGF genus as described above. D1/D2-LSU sequences that could not be confidently assigned to an AGF genus were sequentially inserted into a reference LSU tree to assess novelty. Further, the associated ITS1 sequences (obtained from the same amplicon) were similarly inserted into a reference ITS1 tree for confirmation. Sequences were assigned to a novel candidate genus when both loci (LSU and ITS1) cluster as novel, independent genus-level clades with high (>70%) bootstrap support in both trees.

B. Genus and species level delineation. Genus level assignments were conducted via a combination of similarity search and phylogenetic placement as described above. We chose not to depend on sequence divergence cutoffs for OTU delineation at the genus level since some genera exhibit high sequence similarity between their D1/D2-LSU sequences (e.g. *Liebetanzomyces*, *Capellomyces*, and *Anaeromyces* D1/D2-LSU sequence divergence ranges between 1.8-2.5%), while other genera are highly divergent (e.g. *Piromyces* intra-genus sequence divergence cutoff of the D1/D2-LSU region ranges between 0-5.7%), and as such "a one size fits all" approach should not be applied. On the other hand, a similar approach for OTUs delineation at the species equivalent level is problematic due to uncertainties in circumscribing species boundaries, and inadequate numbers of species representatives in many genera. Therefore, for OTU delineation at the species equivalent level, we reverted to using a sequence divergence cutoff. Historically, cutoffs of 3% [16] to 5% [3] were used for ITS1-based species equivalent delineation. However, D1/D2-LSU sequence data are more conserved when compared to LSU data in the Neocallimastigomycota [15, 26, 27, 28, 29, 30, 50], as well as other fungi [19]. To obtain an appropriate species equivalent cutoff, we used the 116 Sanger-generated clone sequences from

this study and previous studies [23, 28, 29, 49], as well as genomic rRNA loci from two Neocallimastigomycota genomes where the entire ribosomal operon sequence is available (*Pecoramyces ruminantium* strain C1A, and *Neocallimastix californiae* strain G1) [36, 43]. The ITS1 and D1/D2-LSU regions were bioinformatically extracted and sorted to separate fasta files. Sequences in each file were then aligned using MAFFT [60] and the alignment was used to create a distance matrix for every possible pairwise comparison using dist.seqs command in Mothur. The obtained pairwise distances for the ITS1, and the D1/D2-LSU regions were then correlated to obtain values of D1/D2-LSU sequence divergence cutoffs corresponding to the 3-5% range in ITS1. This was equivalent to 2.0-2.2%, and hence, for this study, a cutoff of 2% was used for OTU generation at the species equivalent level using the D1/D2-LSU region.

C. Diversity and community structure assessments. Genus and species equivalent OTUs generated as described above were used to calculate alpha diversity indices (Chao and Ace richness estimates, Shannon diversity index, Simpson evenness index) for the different samples studied using the summary.seqs command in Mothur. A shared OTUs file created in Mothur using the make.shared command was used to calculate Bray-Curtis beta diversity indices between different samples (using the summary.shared command in Mothur). The shared OTUs file was also used as a starting point for ranking the samples based on their diversity using both an information-related diversity ordering method (Renyi generalized entropy), and an expected number of species-related diversity ordering method (Hulbert family of diversity indices) (Table 2). For community structure visualization, Bray Curtis indices at the genus level were also used to perform non-metric multidimensional scaling using the metaMDS function in the Vegan package in R. Also, the percentage abundance of different genera across the samples studied were used in principal components analysis using the prcomp function in the labdsv package in

R. Ordination plots were generated from the two analyses (NMDS and PCA) using the ordiplot function.

D. Statistical analysis: Correlations of the diversity estimates to animal host factors including the animal lifestyle (domesticated, zoo-housed, wild), and the animal host families (Bovidae, Cervidea, Equidae, Camelidae) were calculated using χ^2-contingency tables followed by measuring the degree of association using Cramer's V statistics as detailed before [3]. In addition, to identify factors impacting AGF diversity, Student t-tests were used to identify significant differences in the above alpha diversity estimates based on animal lifestyle (zoo-housed, domesticated, wild), and host phylogeny (families Bovidae, Equidae, Cervidae). To test the effect of the above host factors on the AGF community structure, Student t-tests were used to identify significant associations between specific AGF taxa and animal lifestyle (zoo-housed, domesticated, wild) or host family (families Bovidae, Equidae, Cervidae).

Isolation efforts.

Fecal samples (either freshly obtained, or stored at -20ºC in sterile, air-tight plastic tubes) were used for isolation. Care was taken to avoid sample repeated freezing and thawing. Samples were first enriched by incubation for 24 h at 39ºC in rumen-fluid-cellobiose (RFC) medium supplemented with antibiotics (50 µg/mL kanamycin, 50 µg/mL penicillin, 20 µg/mL streptomycin, and 50 µg/ mL chloramphenicol) [27, 28, 29, 50, 51]. Subsequently, enrichments were serially diluted by adding approximately 1 ml of enriched samples to 9 mL of RF medium supplemented with 1% cellulose or a mixture of 0.5% switchgrass and 0.5% cellobiose. Since fungal hyphae and zoospores are usually attached to the coarse particulates in the enrichment, serial dilutions were conducted using Pasteur pipettes rather than syringes and needles, as the narrow bore of the needle prevented the fecal clumps from being transferred. Serial dilutions up

581 to a 10^{-5} dilution were incubated at 39^0C for 24–48 h. Dilutions showing visible signs of growth
582 (clumping or floating plant materials, a change in the color of cellulose, and production of gas
583 bubbles) were then used for the preparation of roll tubes [55, 56] on RFC agar medium. At the same
584 time, and as a backup strategy in case the roll tubes failed to produce visible colonies, the
585 dilution tubes themselves were subcultured and transferred to fresh medium with the same
586 carbon source. Single colonies on roll tubes were then picked into liquid RFC medium, and at
587 least three rounds of tube rolling and colony picking were conducted to ensure purity of the
588 obtained colonies. To maximize the chances of obtaining isolates belonging to different genera,
589 special attention was given, not only to picking colonies of different shapes and sizes, but also to
590 picking several colonies of the same shape, as representatives of different genera could produce
591 colonies with very similar macroscopic features. Isolates were maintained by biweekly
592 subculturing into RFC medium. For long-term storage, cultures were stored on agar medium
593 according to the procedure described by Calkins et al. [51].
594 **Data accession**. Sanger-generated clone sequences from pure cultures were deposited in
595 GenBank under accession numbers MT085665 - MT085741. SMRT-generated sequences were
596 deposited at DDBJ/EMBL/GenBank under the Bioproject accession number PRJNA609702,
597 Biosample accession numbers SAMN14258225, and Targeted Locus Study project accession
598 KDVX00000000. The version described in this paper is the first version, KDVX01000000.
599
600 **Acknowledgements:** This work has been funded by the NSF-DEB grant 1557102 to N.H.Y. and
601 M.S.E.
602
603 Competing interests: The authors declare no competing interest

Table 1. Representatives full-length sequences spanning the region "ITS1-5.8S rRNA-ITS2-D1/D2 LSU". GenBank accession numbers are shown for all clone sequences obtained from representative AGF isolates in our culture collection. For yet-uncultured AGF taxa, accession numbers refer to the SMRT generated sequence name in Datasets 1-3. Start and end positions of ITS1, 5.8S rRNA gene, ITS2, and the D1/D2 region of the LSU are shown.

Table 1. Representatives full length sequences spanning the region "ITS1-5.8S rRNA-ITS2-D1/D2 LSU". GenBank accession numbers are shown for all clone sequences obtained from representative AGF isolates in our culture collection. For yet-uncultured AGF taxa, accession numbers refer to the SMRT generated sequence name in Datasets 1-3. Start and end positions of ITS1, 5.8S rRNA gene, ITS2, and the D1/D2 region of the LSU are shown.

Name	GenBank Accession number*	Number of ITS1-5.8S-ITS2-D1/D2 LSU sequences		Position (number refers to the position within the accession where the region starts)				Allternate names	Reference
		# of isolates (# of clone sequences)	# of SMRT-generated environmental sequences	ITS1	5.8S	ITS2	LSU		
Cultured genera									
Agriosomyces		1 (2 clones)	222						
Agriosomyces longus strain MS2, clone B	MT085709			1-226	227-406	407-587	588 -1372		This study
Agriosomyces longus strain MS2, clone C	MT085708			1-219	220-401	402-582	583-1367		This study
Aklioshbomyces		1 (5 clones)	1009						
Aklioshbomyces papillarum strain WT2, clone 7	MT085737			1-182	183-357	358-540	541-1326		This study
Aklioshbomyces papillarum strain WT2, clone 8	MT085738			1-182	183-357	358-540	541-1326		This study
Aklioshbomyces papillarum strain WT2, clone 9	MT085739			1-182	183-360	361-538	539-1323		This study
Aklioshbomyces papillarum strain WT2, clone 10	MT085740			1-182	183-357	358-540	541-1325		This study
Aklioshbomyces papillarum strain WT2, clone 12	MT085741			1-184	185-359	360-538	539-1324		This study
Anaeromyces		7 (15 clones)	76						
Anaeromyces contortus isolate C3G Clone 10	MG605705.1			1-222	223-401	402-572	573-1356		29
Anaeromyces contortus isolate C3J Clone 2	MG605699.1			1-226	227-405	406-576	577-1362		29
Anaeromyces contortus isolate G3A Clone 1	MG605688.1			1-221	222-400	401-572	573-1358		29
Anaeromyces contortus isolate G3A clone 2	MG605684.1			1-219	220-397	398-569	570-1353		29
Anaeromyces contortus isolate G3A Clone 3	MG605681.1			1-221	222-399	400-571	572-1355		29
Anaeromyces contortus isolate G3A Clone 5	MG605697.1			1-223	224-402	403-573	574-1357		29
Anaeromyces contortus isolate G3C Clone 4	MG605685.1			1-217	218-395	396-567	568-1353		29
Anaeromyces contortus isolate G3C Clone 5	MG605679.1			1-221	222-399	400-572	573-1358		29
Anaeromyces contortus isolate G3C Clone 6	MG605683.1			1-221	222-399	400-571	572-1355		29
Anaeromyces contortus isolate G3G Clone 10	MG605690.1			1-220	221-398	399-571	572-1357		29
Anaeromyces contortus isolate G3G Clone 8	MG605686.1			1-216	217-394	395-566	567-1350		29
Anaeromyces contortus isolate G3G Clone 9	MG605691.1			1-221	222-399	400-572	573-1358		29
Anaeromyces contortus isolate Na Clone 5	MG605704.1			1-223	224-402	403-573	574-1357		29
Anaeromyces contortus isolate Na Clone 6	MG605701.1			1-226	227-406	407-578	579-1362		29
Anaeromyces contortus isolate X4 Clone 2	MG605706.1			1-223	224-402	403-573	574-1357		29
Buwchfawromyces		0	55					SK2	
Buwchfawromyces eastonii	AoudOld_160513			1-168	169-326	326-536	537-1235		This study
Caecomyces		3 (3 clones)	879						
Caecomyces sp. isolate DS1 Clone C3	MT085702			1-205	206-381	382-583	584-1366		This study
Caecomyces sp. isolate CYF	JQ782554.1			65-280	281-456	457-654	655-1379		23
Caecomyces sp. isolate CYR	JQ782555.1			65-274	275-450	451-646	647-1371		23
Capellomyces		2 (5 clones)	0						
Capellomyces foraminis isolate BGB11 Clone C1	MT085700			1-220	221-400	401-577	578-1360		This study
Capellomyces foraminis isolate BGB11 Clone C2	MT085697			1-220	221-401	402-578	579-1362		This study
Capellomyces foraminis isolate BGB11 Clone C3	MT085698			1-220	221-401	402-579	580-1363		This study
Capellomyces foraminis isolate BGB11 Clone C4	MT085699			1-220	221-401	402-578	579-1363		This study
Capellomyces elongatus	MT085701			1-250	251-432	433-609	610-1393		This study
Cyllamyces		1 (clones 5)	704					MN1, SP8	
Cyllamyces sp. isolate TSB2 Clone B10	MT085707			1-170	171-347	348-537	538-1320		This study
Cyllamyces sp. isolate TSB2 Clone B11	MT085705			1-170	171-347	348-538	539-1321		This study
Cyllamyces sp. isolate TSB2 Clone B12	MT085703			1-170	171-347	348-537	538-1320		This study
Cyllamyces sp. isolate TSB2 Clone B8	MT085704			1-168	169-344	345-536	537-1319		This study
Cyllamyces sp. isolate TSB2 Clone B9	MT085706			1-168	169-345	346-535	536-1318		This study
Ghazallomyces		1 (4 clones)	102						
Ghazallomyces constrictus isolate AXS31 Clone B1	MT085693			1-189	190-370	371-564	565-1348		This study
Ghazallomyces constrictus isolate AXS31 Clone B2	MT085695			1-186	187-364	365-556	557-1339		This study
Ghazallomyces constrictus isolate AXS31 Clone B3	MT085694			1-186	187-364	365-556	557-1339		This study
Ghazallomyces constrictus isolate AXS31 Clone B5	MT085696			1-189	190-367	368-552	553-1335		This study
Joblinomyces		2 (10 clones)	1076					AL5	
Joblinomyces apicalis isolate GFH681 Clone1	MT085665			1-213	214-388	389-561	562-1344		This study
Joblinomyces apicalis isolate GFH681 Clone2	MT085666			1-213	214-390	391-564	565-1347		This study
Joblinomyces apicalis isolate GFH681 Clone4	MT085667			1-215	216-393	394-568	569-1351		This study
Joblinomyces apicalis isolate GFH681 Clone5	MT085668			1-215	216-393	394-568	569-1351		This study
Joblinomyces apicalis isolate GFH681 Clone6	MT085669			1-213	214-389	390-563	564-1346		This study
Joblinomyces apicalis isolate GFH683 Clone1	MT085670			1-213	214-389	390-563	564-1346		This study

Joblinomyces apicalis isolate GFH683 Clone2	MT085671			1-213	214-389	390-563	564-1346		This study
Joblinomyces apicalis isolate GFH683 Clone3	MT085672			1-212	213-388	389-562	563-1345		This study
Joblinomyces apicalis isolate GFH683 Clone4	MT085673			1-213	214-389	390-563	564-1346		This study
Joblinomyces apicalis isolate GFH683 Clone5	MT085674			1-213	214-389	390-563	564-1346		This study
Feramyces		4 (11 clones)	2373					AL6	
Feramyces austinii isolate DS10 Clone 11	MG584196.1			1-192	193-368	369-570	571-1353		28
Feramyces austinii isolate DS10 Clone 12	MG584194.1			1-192	193-368	369-570	571-1353		28
Feramyces austinii isolate DS10 Clone 7	MG584192.1			1-192	193-368	369-571	572-1354		28
Feramyces austinii isolate DS10 Clone 8	MG584200.1			1-192	193-368	369-570	571-1352		28
Feramyces austinii isolate DS10 Clone 9	MG584197.1			1-192	193-368	369-570	571-1353		28
Feramyces austinii isolate F3A Clone 3	MG584193.1			1-192	193-368	369-570	571-1353		28
Feramyces austinii isolate F3B Clone 10	MG584190.1			1-192	193-368	369-570	571-1352		28
Feramyces austinii isolate R4A Clone 1	MG584191.1			1-192	193-368	369-570	571-1353		28
Feramyces austinii isolate R4A Clone 2	MG584199.1			1-192	193-368	369-570	571-1353		28
Feramyces austinii isolate R4A Clone 3	MG584198.1			1-192	193-368	369-570	571-1353		28
Feramyces austinii isolate R4A Clone 5	MG584195.1			1-192	193-368	369-570	571-1353		28
Khyollomyces		1 (1 clone)	2553					AL1	
Khyollomyces ramosus isolate ZS33 Clone 8	MT085710			1-193	194-369	370-543	544-1327		This study
Liebetanzomyces		1 (7 clones)	31					SP4	
Liebetanzomyces sp. isolate Cel1A Clone 2	MT085726			1-225	226-403	404-577	578-1361		This study
Liebetanzomyces sp. isolate Cel1A Clone 3	MT085727			1-225	226-403	404-577	578-1361		This study
Liebetanzomyces sp. isolate Cel1A Clone 4	MT085728			1-225	226-403	404-577	578-1361		This study
Liebetanzomyces sp. isolate Cel1A Clone 6	MT085729			1-225	226-403	404-577	578-1362		This study
Liebetanzomyces sp. isolate Cel1A Clone 7	MT085730			1-223	224-401	402-576	577-1362		This study
Liebetanzomyces sp. isolate Cel1A Clone 8	MT085731			1-225	226-403	404-577	578-1361		This study
Liebetanzomyces sp. isolate Cel1A Clone 9	MT085732			1-223	224-401	402-575	576-1359		This study
Neocallimastix		14 (14 clones)	794					SP6	
Neocallimastix californiae strain G1**	MCOG01000947.1			12566-12742	12743-12917	12918-13113	13114-13895		36
Neocallimastix californiae strain G1**	MCOG01000947.1			2806-2982	2983-3157	3158-3354	3355-4136		36
Neocallimastix californiae strain G1**	MCOG01001752.1			2193-2369	2018-2192	1821-2017	1820-1036		36
Neocallimastix cf. *cameroonii* isolate G3	MT085722			1-178	179-356	357-554	555-1338		This study
Neocallimastix sp isolate Hef5 Clone 1	MT085723			1-229	230-408	409-588	589-1371		This study
Neocallimastix sp isolate Hef6 Clone 6	MT085724			1-240	241-419	420-602	603-1385		This study
Neocallimastix sp isolate Hef7 Clone 3	MT085725			1-229	230-407	408-585	586-1368		This study
Neocallimastix cf. *frontalis* isolate NYF1	JQ782542.1			67-308	309-486	487-665	666-1390		23
Neocallimastix cf. *frontalis* isolate NYF2	JQ782543.1			67-307	308-485	486-672	673-1397		23
Neocallimastix cf. *frontalis* isolate NYF3	JQ782544.1			67-296	297-474	475-654	655-1379		23
Neocallimastix cf. *frontalis* isolate NYF4	JQ782545.1			67-310	311-488	489-676	677-1401		23
Neocallimastix cf. *frontalis* isolate NYR1	JQ782546.1			67-307	308-486	487-669	670-1394		23
Neocallimastix cf. *frontalis* isolate NYR2	JQ782547.1			67-317	318-496	497-676	677-1401		23
Neocallimastix cf. *frontalis* isolate NYR3	JQ782548.1			67-317	318-495	496-678	679-1403		23
Neocallimastix cf. *frontalis* isolate NYR4	JQ782549.1			67-295	296-473	474-654	655-1380		23
Neocallimastix cf. *frontalis* isolate NYR5	JQ782550.1			67-309	310-488	489-667	668-1392		23
Oontomyces		0	0						
Oontomyces anksri strain SSD-CIB1	JX017310.1			60-291	292-467	468-642	643-695		26
Oontomyces anksri strain SSD-CIB1	JX017314.1						1-772		26
Orpinomyces		23 clones	349						
Orpinomyces sp. OUS1	AJ864475.1			842-1056	1057-1241	1242-1418	1419-2201		49
Orpinomyces cf. *joyonii* isolate D3A Clone 3	MT085735			1-183	184-360	361-539	540-1322		This study
Orpinomyces cf. *joyonii* isolate D3A Clone F11	MT085736			1-182	183-357	358-537	538-1320		This study
Orpinomyces cf. *joyonii* isolate D3A Clone G09	MT085733			1-184	185-359	360-538	539-1321		This study
Orpinomyces cf. *joyonii* isolate D3A Clone H09	MT085734			1-187	188-362	363-540	541-1323		This study
Orpinomyces sp. OYF	JQ782551.1			67-267	268-447	448-630	631-1356		23
Orpinomyces sp. OYR2	JQ782553.1			65-253	254-431	432-610	611-1335		23
Pecoramyces		2 (4 clones)	248						
Pecoramyces ruminatium isolate C1A**	ASRE01020932.1			909-1095	1096-1271	1272-1452	1453-2235		43
Pecoramyces ruminatium isolate C1A**	ASRE01007038.1			790-976	977-1152	1153-1333	1334-2116		43
Pecoramyces ruminatium isolate C1A**	ASRE01022884.1			2760-2946	2584-2759	2403-2583	1620-2402		43
Pecoramyces ruminatium isolate S4B	MT085711			1-184	185-360	361-542	543-1325		This study
Piromyces		5 (26 clones)	3818					AL7, UC1	
Piromyces finnis strain finn***	MCFH01000027.1			1034-1105	1106-1285	1286-1470	1471-2253		36
Piromyces finnis strain finn***	MCFH01000027.1			9568-9639	9640-9819	9820-10004	10005-10787		36
Piromyces finnis strain finn***	MCFH01000027.1			18102-18173	18174-18353	18354-18538	18539-19321		36
Piromyces sp. isolate A1 Clone A1	MT085682			1-199	200-375	376-548	549-1333		This study
Piromyces sp. isolate A1 Clone A12	MT085684			1-198	199-374	375-547	548-1330		This study

Piromyces sp. isolate A1 Clone A2	MT085679			1-199	200-375	376-548	549-1331		This study
Piromyces sp. isolate A1 Clone A3	MT085683			1-199	200-375	376-548	549-1331		This study
Piromyces sp. isolate A1 Clone A4	MT085685			1-199	200-375	376-548	549-1331		This study
Piromyces sp. isolate A1 Clone A5	MT085688			1-199	200-375	376-548	549-1335		This study
Piromyces sp. isolate A1 Clone A6	MT085687			1-199	200-375	376-548	549-1331		This study
Piromyces sp. isolate A1 Clone A7	MT085686			1-199	200-375	376-548	549-1334		This study
Piromyces sp. isolate A1 Clone A8	MT085681			1-199	200-375	376-548	549-1331		This study
Piromyces sp. isolate A1 Clone A9	MT085680			1-199	200-375	376-548	549-1331		This study
Piromyces sp. isolate Cel1B Clone 1	MT085717			1-198	199-375	376-548	549-1331		This study
Piromyces sp. isolate Cel1B Clone 10	MT085721			1-198	199-374	375-547	548-1332		This study
Piromyces sp. isolate Cel1B Clone 2	MT085718			1-198	199-375	376-548	549-1331		This study
Piromyces sp. isolate Cel1B Clone 3	MT085719			1-198	199-375	376-549	550-1334		This study
Piromyces sp. isolate Cel1B Clone 6	MT085720			1-198	199-374	375-547	548-1332		This study
Piromyces sp. isolate DB3 Clone B2	MT085690			1-226	227-404	405-592	593-1350		This study
Piromyces sp. isolate DB3 Clone B3	MT085691			1-227	228-405	406-592	593-1359		This study
Piromyces sp. isolate DB3 Clone B4	MT085689			1-225	226-403	404-588	589-1351		This study
Piromyces sp. isolate Jen1 Clone 1	MT085712			1-201	202-378	379-552	553-1335		This study
Piromyces sp. isolate Jen1 Clone 2	MT085713			1-201	202-378	379-552	553-1335		This study
Piromyces sp. isolate Jen1 Clone 3	MT085714			1-201	202-377	378-550	551-1333		This study
Piromyces sp. isolate Jen1 Clone 4	MT085715			1-201	202-377	378-550	551-1333		This study
Piromyces sp. isolate Jen1 Clone 5	MT085716			1-201	202-378	379-552	553-1335		This study
Tahromyces		4 (4 clones)							
Tahromyces munnarensis isolate TDFKJa1924	MT085677			1-178	179-358	359-537	538-1316		This study
Tahromyces munnarensis isolate TDFKJa1926	MT085676			1-178	179-358	359-537	538-1307		This study
Tahromyces munnarensis isolate TDFKJa1927	MT085678			1-178	179-358	359-537	538-1320		This study
Tahromyces munnarensis isolate TDFKJa193	MT085675			1-178	179-358	359-537	538-1313		This study
Uncultured lineages									
Identified in this study (accession number is the sequence name in Supplementary datasets 1-3)									
AL3	DwGoat_61688		86	57-200	201-352	353-604	605-1358		This study
AL4	Sheep_129918		1	67-268	269-425	426-630	631-1394		This study
AL8	Cow_130070		151	88-273	274-432	433-653	654-1424		This study
MN3	Cow_90808		3	76-280	281-438	439-640	641-1423		This study
MN4	OSUGoat_119881		3	69-276	277-434	435-666	667-1444		This study
SK3	Aoud18_104764		3	67-279	280-436	437-676	677-1444		This study
SK4	Aoud18_141177		1387	71-264	265-421	422-644	645-1421		This study
Not identified in this study (GenBank accession number of a representative ITS1 sequence)									
AL2	GQ826457								3
BlackRhino	JF423850								5
DA1	JX184822								13
DT1	GQ850291								48
JH1/ SP5	GU911240								17, 46
KF1	GQ850345								45
MN2	AM690075								47
SK1	JF423570								5
SP1	GQ678747								17
SP2	GQ698377								17
SP3	GQ657498								17
SP7	GU910219.1								17
Novel lineages (accession number is the sequence name in Supplementary datasets 1-3)									
RH1	Cow_130696		13	66-222	223-378	379-593	594-1369		This study
RH2	Oryx_79099		74	67-270	271-425	426-660	661-1440		This study
RH3	AmBis_130671		3	71-246	247-404	405-613	614-1374		This study
RH4	Cow_156860		13	68-238	239-393	394-597	598-1379		This study
RH5	Sheep_119174		1681	69-280	281-438	439-646	647-1426		This study
RH6	Cow_144271		2	78-289	290-446	447-653	654-1433		This study

*GenBank Accession numbers are provided for Sanger sequenced clones from all fungal isolates. PacBio-generated datasets are present in GenBank in the Bioproject accession number PRJNA609702, Biosample accession numbers SAMN14258225, and Targeted Locus Study project accession KDVX00000000. FASTA files of ITS1-5.8S-ITS2-D1/D2 LSU region, as well as bioinformatically extracted ITS1 region and D1/D2 LSU regions are provided as supplementary documents (Datasets 1-3)

** Sequences extracted from a genomic assembly

610 Table 2. Animals sampled in this study, numbers of sequences obtained (N), number of observed OTUs, and various diversity indices both at the species
611 equivalent (0.02) and the genus levels

Sample	Host description		N*	Observed number of OTUs		Chao		Ace		Simpson evenness		Shannon		Diversity ranking**		Coverage***	
	Family	Lifestyle		Sp. Eq.	Genus	Sp. Eq.	Genus	Sp. Eq.	Genus	Sp. Eq.	Genus	Sp. Eq.	Genus	Sp. Eq.	Genus	Sp. Eq.	Genus
Alpaca	Camelidae	Domestic	240	19	9	26.2	9.5	30.5	10.8	0.27	0.50	1.99	1.64	17.3	18.8	0.94	0.99
American bison	Bovidae	Zoo	183	17	11	22.6	11.3	41.6	12.2	0.13	0.14	1.45	0.90	9.8	7.5	0.93	0.99
American elk	Cervidae	Zoo	99	11	9	16	11	28.2	15.3	0.17	0.21	1.12	1.01	6.3	10.5	0.92	0.96
Aoudad sheep (1)	Bovidae	Wild	3381	80	17	111	23	159.4	27	0.03	0.15	1.54	1.17	13.5	7	0.99	1
Aoudad sheep (2)	Bovidae	Wild	1779	55	13	57	13	59.9	13.4	0.05	0.13	1.78	0.91	11.2	11.8	0.99	1
Axis deer	Cervidae	Wild	367	18	9	18.6	9.5	19.6	11.6	0.36	0.49	2.17	1.61	19	19.5	0.99	0.99
Blackbuck deer	Bovidae	Wild	145	21	13	34.8	16	67.7	17	0.18	0.24	1.93	1.59	16	16.7	0.91	0.97
Boer goat	Bovidae	Wild	2503	41	9	49.1	9	58.8	9	0.05	0.21	1.12	0.88	6.5	8.3	0.99	1
Domestic cow	Bovidae	Domestic	727	45	13	46.7	16	48.5	15.4	0.06	0.14	1.95	1.03	13.5	10.2	0.98	1
Domestic goat	Bovidae	Domestic	162	23	15	39.5	16	70.7	17.2	0.40	0.43	2.52	2.10	20.3	21	0.87	1
Domestic horse	Equidae	Domestic	498	15	8	15.5	8.3	17.7	10	0.11	0.35	0.94	1.18	5.3	13.7	0.99	1
Domestic sheep	Bovidae	Domestic	1349	33	9	33.7	9	34.5	9.5	0.04	0.12	0.72	0.25	2.5	3	1	1
Dwarf goat	Bovidae	Zoo	519	15	8	20.3	18	36.7	17.5	0.08	0.13	0.49	0.14	1	2	0.98	0.99
Fallow deer	Cervidae	Wild	1368	43	12	46.7	12.3	48.7	13.2	0.08	0.13	1.67	0.78	14.5	5.3	0.99	1
Longhorn cattle	Bovidae	Domestic	62	9	5	16.5	5	47.2	5.8	0.28	0.41	1.37	0.96	11	10	0.82	0.98
Miniature donkey	Equidae	Zoo	56	12	8	30	11	191.6	18	0.23	0.46	1.50	1.46	11	17.2	0.80	0.93
Mouflon ram	Bovidae	Wild	297	17	11	35	11.3	111	14.3	0.23	0.31	1.80	1.55	16	17.2	0.95	0.99
Oryx	Bovidae	Wild	780	34	15	36.2	16	41.9	17.7	0.26	0.16	2.52	1.35	20.7	13.8	0.98	0.98

Pere David's deer	Cervidae	Zoo	169	7	6	10	6.5	17	7.8	0.32	0.36	0.96	0.88	6.7	10.5	0.96	0.99
White-tail deer	Cervidae	Wild	946	23	6	23	7.5	23.4	14.7	0.06	0.17	0.75	0.07	2.8	1	1	1
Zebra	Equidae	Zoo	2067	55	11	73	13	85.4	15	0.03	0.15	1.10	0.76	6	6	0.99	1

*: N refers to the number of sequences obtained for each of the animals sampled.
**: Diversity ranking is the average rank obtained using both an information-related diversity ordering method (Renyi generalized entropy), and an expected number of species-related diversity ordering method (Hulbert family of diversity indices). Samples are ranked from the least diverse (rank 1) to the most diverse (rank 21).
***: Coverage refers to the Good's coverage index.

629 Table 3. Number and sources of isolates obtained in thus study.

AGF genus	Source	Number of isolates	% Abundance in that animal
Agriosomyces	Mouflon	4	3.28
	Boer goat	1	8.5
Aklioshbomyces	White-tail deer	9	98.95
Anaeromyces	Domesticated cow	4	0.68
	domesticated goat	12	2.44
	American bison	4	1.63
	Alpaca	4	17.01
Caecomyces	Fallow deer	1	0.44
Capellamyces	Boer goat	5	ND
Feramyces	Aoudad sheep (1)	5	55.31
	Fallow deer	1	4.46
Ghazallomyces	Axis deer	11	27.79
Khyollomyces	Zebra	16	74.5
Neocallimastix	Dwarf goat	7	97.88
	Fallow deer	10	1.24
	Pere David's deer	10	54.44
	American elk	12	72
Orpinomyces	Domesticated cow	8	1.09
	Longhorn	3	3.03
	American bison	6	80.43
	Alpaca	6	33.2
Pecoramyces	Domesticated sheep	10	0.37
	Mouflon	3	12.79
	Oryx	11	13.78
	Aoudad sheep (2)	13	0.39
Piromyces	Domesticated cow	5	1.64
	domesticated sheep	3	1.6
	Mouflon ram	2	23.61
	Axis deer	1	28.88
	Blackbuck deer	7	6.21
	Domesticated horse	6	80.12
	Miniature donkey	16	69.64

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Figures Legends

Figure 1. Box and whisker plots showing the variability in intra-genus length (A-B) and sequence divergence cutoff (C-D) for the ITS1 (A, C) and D1/D2 LSU (B, D) regions. A cartoon of the rRNA locus is shown on top. Genera and candidate genera with at least 5 sequences encompassing the full region "ITS1-5.8S-ITS2-D1/D2 LSU" were used to construct this plot as detailed in the methods section. The candidate genera AL4, MN3, MN4, RH3, RH6, and SK3 had only a few sequence representatives (1-3) and so are not included in the plot.

Figure 2. Box and whisker plots showing the variability in intra-strain length (A-B) and sequence divergence cutoff (C-D) for the ITS1 (A, C) and D1/D2 LSU (B, D) regions.

Figure 3. AGF genera distribution across the animal studied. (A) Percentage abundance of AGF genera in the animals studied. The tree is intended to show the relationship between the animals and is not drawn to scale. Host phylogeny (family), lifestyle, and gut type are shown for each animal. The X-axis shows the percentage abundance of AGF genera. (B) Rank abundance curves are displayed for each animal showing a distribution pattern in which a few genera (1-5) represent the majority (>10%) of the sequences obtained.

Figure 4. (A) Phylogenetic tree constructed using the D1/D2 LSU sequences of representatives of each of the 28 genera/candidate genera identified in this study. Sequences were aligned using the MAFFT aligner and maximum likelihood tree was constructed in FastTree [61, 62]. Bootstrap values are based on 100 replicates and are shown for branches with >50% bootstrap support. Genera with cultured representatives are shown in black, uncultured candidate genera identified in previous ITS1-based studies are shown in green, while the 6 novel genera identified in the current study are shown in red. The distribution of each of these genera/candidate genera in the animals studied is shown as a heatmap on the right. (B) AGF genera distribution patterns. The

655 total number of animals harboring each of the genera identified in this study is shown on the Y-
656 axis, with the different colored stacked bars reflecting the number of animals where the genus
657 was the most abundant member, occurred with high (>5%) abundance, occurred with medium
658 (1-5%) abundance, or occurred with low (<1%) abundance. AGF genera are classified into one
659 of the five distribution patterns shown on top of the graph using empirical cutoffs for ubiquity
660 (presence in at least 50% of the animals studied, shown as the dotted line across the bottom bar
661 chart), as well as the fraction of animals where the genus abundance was above 1% (shown as
662 the top bar chart).

663 **Figure 5.** Nonmetric multidimensional scaling based on pairwise Bray-Curtis dissimilarity
664 indices at the genus level. Samples are shown as symbols and displayed in black text while AGF
665 genera are shown as '+' and displayed in red text. (A) Symbols reflect lifestyle with domesticated
666 animals shown as white squares, zoo-housed animals shown as grey squares, and wild animals
667 shown as black squares. (B) Symbols reflect animal host phylogeny with family Bovidae shown
668 as squares, family Cervidae shown as circles, family Equidae shown as hexagons, and family
669 Camelidae shown as a star. Abbreviations: Am Bison, American bison; Ax deer, Axis deer; B
670 goat, Boer goat; Bb deer, Blackbuck deer; Dw Goat, Dwarf goat; Fa deer, Fallow deer; Min Don,
671 Miniature donkey; PD deer, Pere David's deer; WT deer, White-tail deer.

672 **Figure 6.** Workflow diagram describing the methods employed in this study.

Figure 1

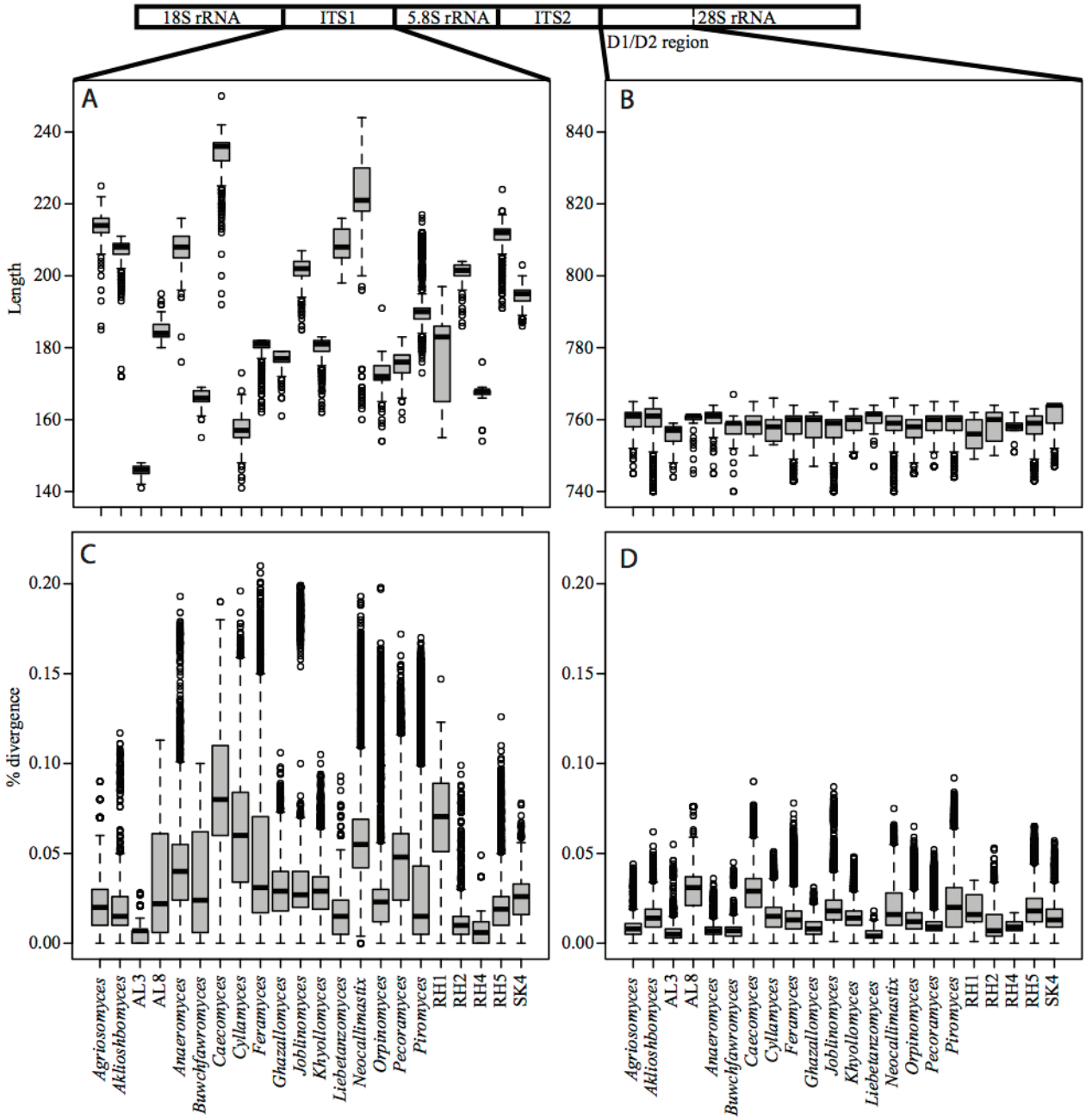


Figure 2

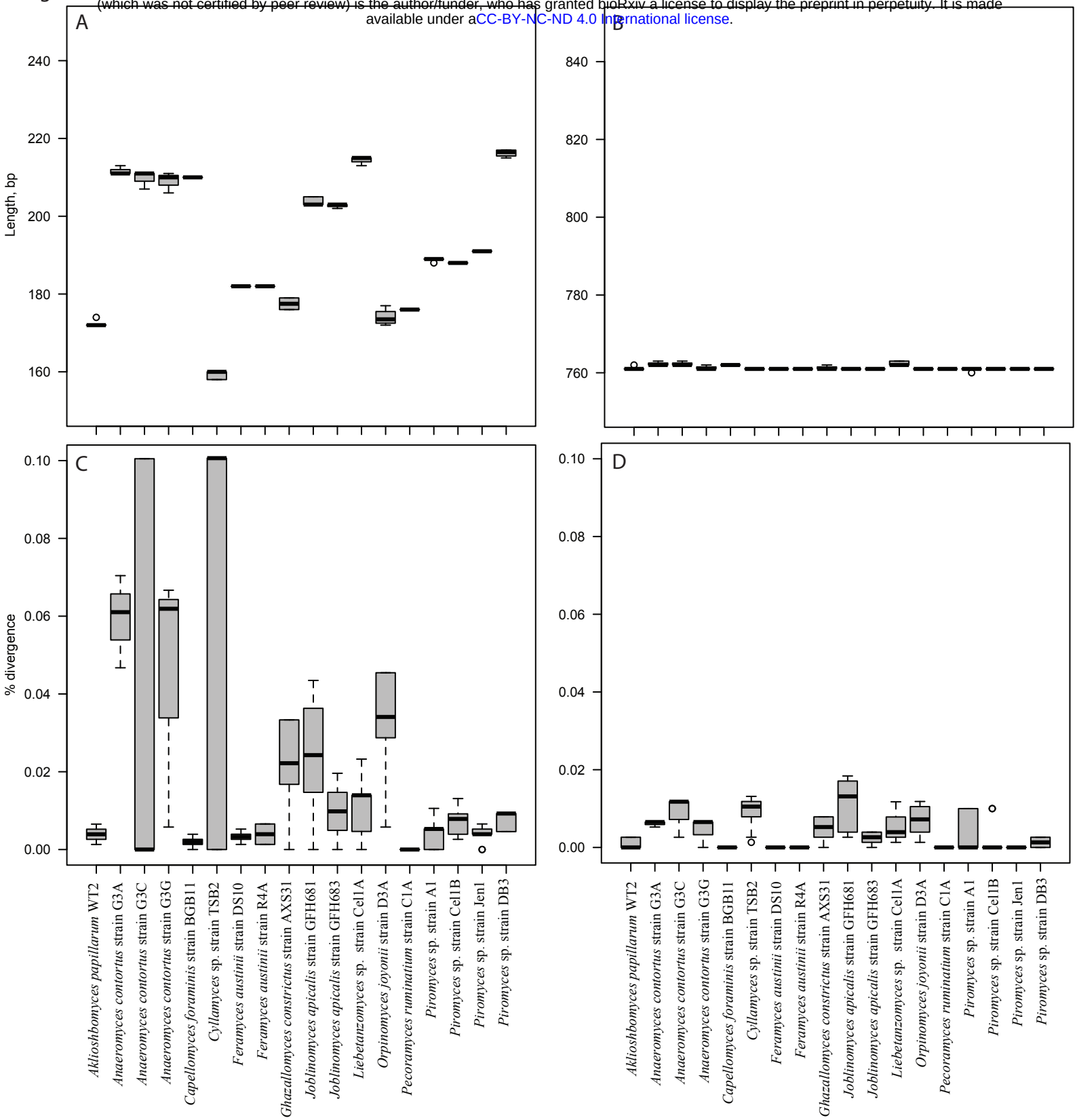
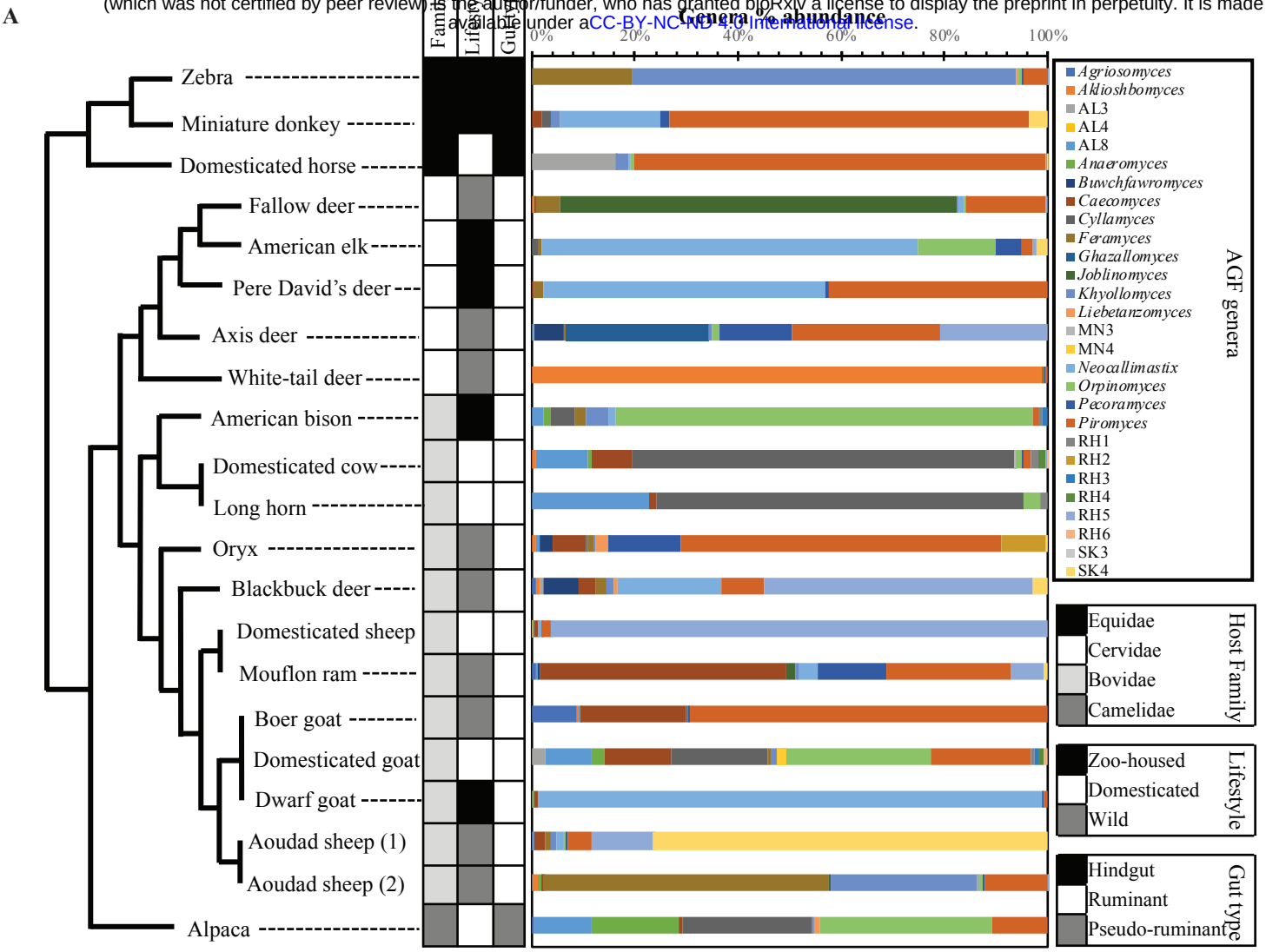


Figure 3



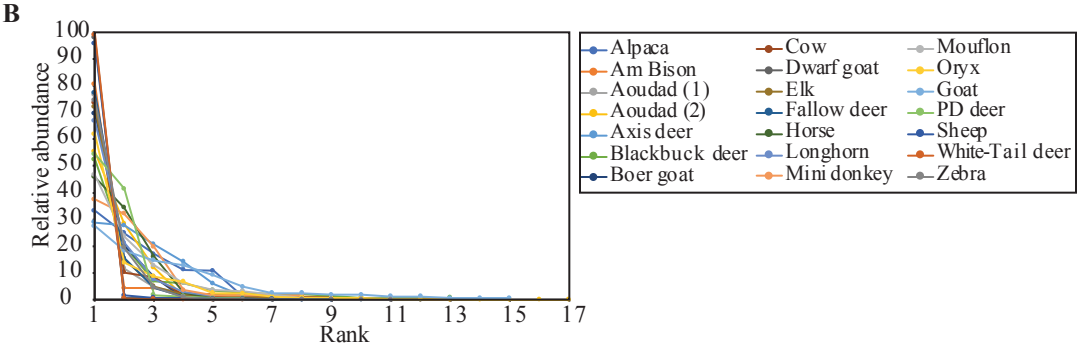


Figure 4

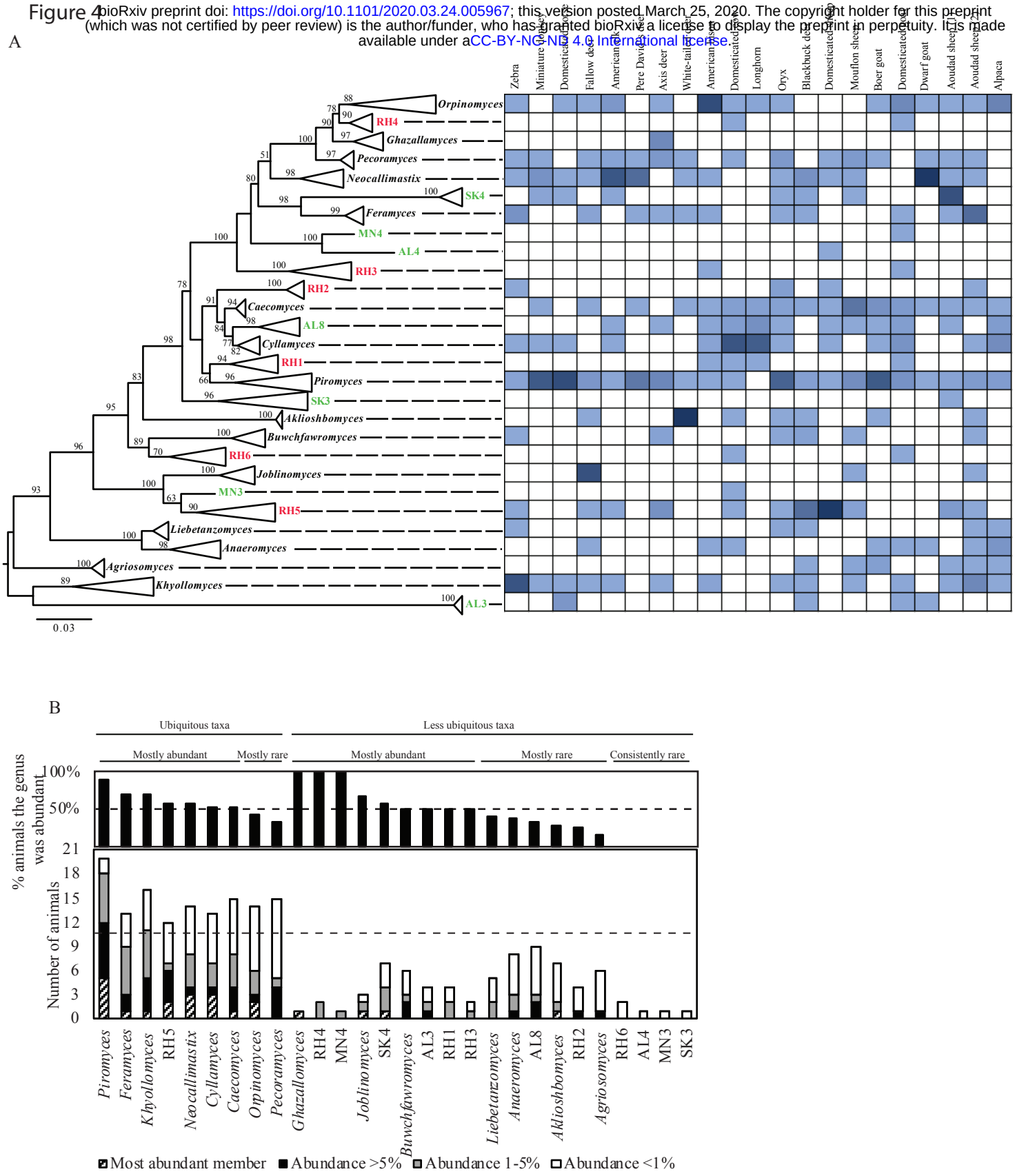


Figure 5

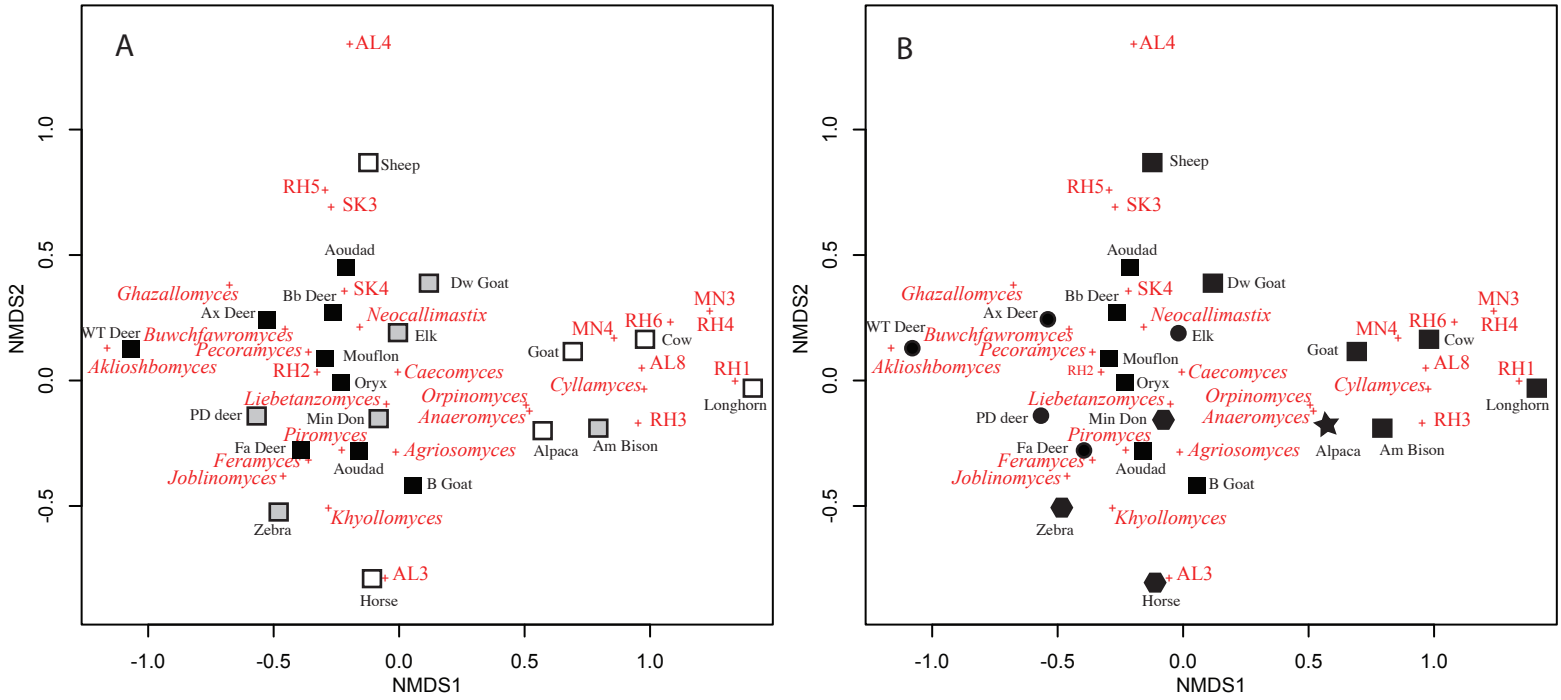
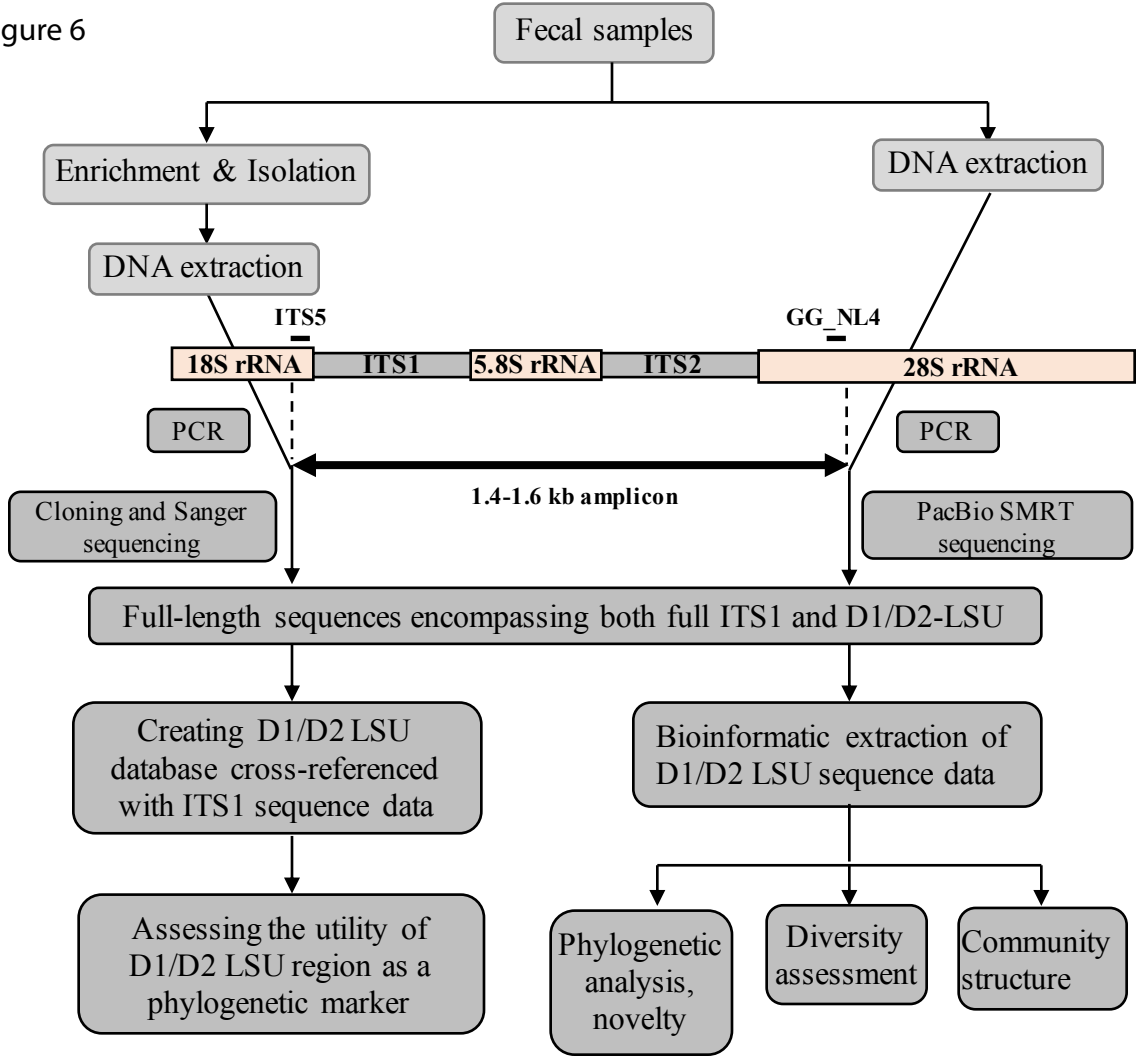


Figure 6


Fecal samples
Enrichment & Isolation
DNA extraction
DNA extraction
ITS5
GG_NL4
18S rRNA
ITS1
5.8S rRNA
ITS2
28S rRNA
PCR
PCR
1.4-1.6 kb amplicon
Cloning and Sanger sequencing
PacBio SMRT sequencing
Full-length sequences encompassing both full ITS1 and D1/D2-LSU
Creating D1/D2 LSU database cross-referenced with ITS1 sequence data
Bioinformatic extraction of D1/D2 LSU sequence data
Assessing the utility of D1/D2 LSU region as a phylogenetic marker
Phylogenetic analysis, novelty
Diversity assessment
Community structure

673 **References**

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