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Characterization and rank assignment criteria for the anaerobic fungi (Neocallimastigomycota)

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Abstract

Establishing a solid taxonomic framework is crucial for enabling discovery and documentation efforts. This ensures effective communication between scientists as well as reproducibility of results between laboratories, and facilitates the exchange and preservation of biological material. Such framework can only be achieved by establishing clear criteria for taxa characterization and rank assignment. Within the anaerobic fungi (phylum Neocallimastigomycota), the need for such criteria is especially vital. Difficulties associated with their isolation, maintenance and long-term storage often result in limited availability and loss of previously described taxa. To this end, we provide here a list of morphological, microscopic, phylogenetic and phenotypic criteria for assessment and documentation when characterizing newly obtained Neocallimastigomycota isolates. We also recommend a polyphasic rank-assignment scheme for novel genus-, species- and strain-level designations for newly obtained Neocallimastigomycota isolates.

Members of the kingdom fungi colonize a wide range of terrestrial, aquatic, marine, animal- and plant-associated ecosystems; and collectively display a great capacity for growth and survival under a wide range of environmental conditions [1]. This remarkable ability for niche adaptation is reflected in the high level of morphotypic, microscopic, phenotypic and genomic traits exhibited by members of the kingdom. Fungal taxonomists investigate the relationships between and within fungal lineages by identifying, assessing and comparing such traits across taxa. The broad field of fungal taxonomy encompasses nomenclature (assigning names and establishing procedures for naming), characterization (defining and using a set of experimental procedures to document informative traits allowing discrimination between taxa) and classification (establishing a framework for assigning taxa into taxonomic ranks, and using such framework for assigning ranks to newly isolated strains). Fungal nomenclature is governed by the International Code of Nomenclature for algae, fungi, and plants (hereafter, the Code). The Code [2] sets formal requirements for nomenclature of novel taxa, including registration of nomenclature novelties in recognized repositories (MycoBank or Index Fungorum), type designation, type material deposition in appropriate repositories, and guidelines for publication of a valid name and ensuring its legitimacy. Criteria for characterization and classification are set by the International Committee of Taxonomy of Fungi (ICTF). The recent manuscript by Aime *et al.* [3] elaborates on the best practices for ensuring availability of descriptive data and preventing publication of taxonomically superfluous names. However, unlike rules of nomenclature that are applicable to all the Mycota, criteria for characterization and classification/rank assignment can differ greatly between fungal lineages. As such, lineage-specific guidelines for characterization and classification should be developed and formulated by the relevant scientific community [3].

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Keywords: anaerobic fungi; Nocallimastigomycota; characterization; rank assignment; large ribosomal subunit.
Abbreviations: ICTF, International Committee of Taxonomy of Fungi; ITS, internal transcribed spacer; LSU, large ribosomal subunit; OTU, operational taxonomic unit.
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The anaerobic fungi (phylum Neocallimastigomycota) inhabit the alimentary tract of herbivores, and display multiple adaptive strategies that enable them to survive and thrive in this permanently anoxic, prokaryote-dominated ecosystem. Multiple novel anaerobic fungal genera [4–10] and species [11–13] have recently been described. In spite of such progress, criteria for characterization and rank assignment for novel isolates in the Neocallimastigomycota have not been formulated, although discussions on the relative importance of specific traits as part of rank assignment justifications in some prior taxa description manuscripts [4, 7, 14] have been proposed. Therefore, the purpose of this manuscript is twofold. Firstly we provide a list of morphological, microscopic, phylogenetic and phenotypic criteria that should be assessed and documented when characterizing newly obtained Neocallimastigomycota isolates. Secondly, to suggest a rank assignment scheme for accommodating newly obtained Neocallimastigomycota isolates. This is especially important for the anaerobic gut fungi, because of difficulties associated with their isolation, maintenance and long-term storage.

Before undertaking detailed characterization efforts, ensuring the purity of isolates obtained is vital. Anaerobic fungi could be co-isolated or contaminated by bacteria and methanogenic archaea during the isolation and maintenance process. Alternatively, mixed cultures of anaerobic gut fungi could be obtained during the isolation process. To ensure purity, isolates should be derived from a single colony rather than by dilution to extinction procedures. We recommend multiple rounds of dilution, rolling tubes and colony picking of the culture. Various antibiotic cocktails to guard against bacterial contamination are commonly employed, and contamination is assessed by microscopic observation. Amplification and direct sequencing of genes known to exhibit minimal strain variability could be undertaken, with the quality of obtained sequencing data used as an additional confirmation of the purity of the sample.

PROPOSED CRITERIA FOR NEOCALLIMASTIGOMYCOTA CHARACTERIZATION

A list of morphotypic, microscopic, phenotypic and phylogenetic criteria recommended for describing novel Neocallimastigomycota isolates is provided in Table 1. The proposed criteria were formulated through in-depth discussions within the community of Neocallimastigomycota taxonomists, as well as by assessing arguments presented in prior taxa description papers [4–7, 14] and reviews [15] during the last four decades. The criteria are meant to be thorough and detailed to enable consistent and information-grounded assessments of novelty, and to preserve knowledge for future comparative purposes. However, they are not meant to be onerous to the point of discouraging characterization efforts or to dictate a specific formatting over another (e.g. full-length manuscripts over fungal diversity notes). The criteria are also not intended to constrain characterization efforts and reporting additional traits should certainly be encouraged (for example, documenting unique previously unreported microscopic structures, unique growth phenotypes, information on gene copy numbers for various loci, and levels of enzymatic activities). Criteria in Table 1 are divided to two categories: those that are indispensable for accurate assessment of the identity and taxonomy of obtained strains, and those that are recommended for complete description of new Neocallimastigomycota isolates. In addition, a list of additional criteria reported in prior taxa description manuscripts is provided in Table 2. While neither indispensable nor recommended for anaerobic gut fungi characterization, these additional criteria could be helpful for providing a complete description of new Neocallimastigomycota isolates.

The utilization of multi-locus based phylogeny [16], whole genome phylogenomic analysis [17], genome-wide synteny [18] and amino acid identity estimates [19] in Neocallimastigomycota taxonomy could provide extremely valuable additional information and thresholds for circumscribing ranks within the lineage. Such efforts for anaerobic fungi, however, have lagged behind other major fungal lineages. This is a reflection of the lack of adequate genome/transcriptome coverage for all representative genera, as well as the loss of multiple historic strains. The value and insights provided by comparative –omics approaches are undisputed; however, we do not propose such efforts is not proposed as a requirement for the taxa description process in the Neocallimastigomycota.

Although the International Committee on Systematics of Prokaryotes would set forth subcommittees to enact minimal and recommended standards for description of novel genera and species; no such requirements are set forth for by the ICTF for Fungi. The criteria proposed here are meant to establish an agreement in the community regarding the Neocallimastigomycota and to guide the expectations of authors, reviewers, and editors in the Neocallimastigomycota community. Details and illustrations of the listed morphotypic and microscopic features have been provided in prior reviews [15, 20]. The relative importance of various criteria (i.e. indispensable or recommended) is highlighted in Table 1.

SPECIES HYPOTHESIS AND RANK ASSIGNMENT IN THE NEOCALLIMASTIGOMYCOTA

What criteria govern the accommodation of an anaerobic fungal isolate into a specific rank, and how can boundaries be circumscribed? Analysis of prior taxa description papers demonstrates wide variations in arguments set forth for rank assignments. Many earlier authors were 'splitters', proposing a new species designation based on minor criteria, some of which could be a function of inter-laboratory variability and/or media composition (e.g. *Caecomyces communis* and *C. equi* [21], *Piromyces communis* and *P. dumbonicus* [22], *Neocallimastix frontalis* and *N. hurleyensis* [23]), see references [15] and

Table 1. Proposed reporting criteria for characterization of new Neocallimastigomycota taxa

Parameter	Information to provide
Indispensable criteria for accurate assessment	
I. Morphology/microscopic criteria*	
1. Colony morphology	• Shape, size, colour, and edge-centre differences.
2. Liquid growth patterns	• Thin/heavy biofilm, powdery/sand-like, cottony, ball or floc-like, attachment to the container's glass surface, colour.
3. Zoospores*	• Zoospore flagellation pattern: monoflagellated (n=1–4); polyflagellated (n>4) • Zoospore size • Length of flagella
4. Thallus development pattern	• Monocentric or polycentric
5. Sporangial development†	• Endogenous, pseudo-intercalary endogenous, and/or exogenous in monocentric taxa; terminal and/or intercalary in polycentric taxa.
6. Sporangiophores†	• Sporangiophore dimensions (length, width), branching (branched, unbranched), shape (eggcup shape, wide-flattened), and size (wide, narrow). • Occurrence of subsporangial swelling.
7. Sporangia	• Sporangial shapes (e.g., globose, ellipsoidal with or without constriction at the middle, ovoid, bowling pin-shaped, egg-shaped, pyriform, heart-shaped, triangular, mucronate with a pointed apex, elongated, rhomboidal), size, and arrangement. • Sporangial uniformity or pleomorphy, shape differences between various types of sporangia (e.g. exogenous versus endogenous versus intercalary). • Sporangial necks (the point between sporangia and sporangiophore or sporangia and rhizoid) (e.g., tightly constricted, broad), and neck ports (narrow or wide). • Occurrence of specific sporangial structures, e.g. papillae.
8. Rhizoidal growth pattern	• Filamentous or bulbous. • For filamentous growth: occurrence of narrow and wide hyphae, the level of branching and twisting, constriction patterns (regular or irregular intervals), and presence of rhizoidal swellings. • For bulbous growth: holdfast patterns (single, multiple), number of sporangiophore per holdfast, and number of sporangia.
II. Phylogenetic criteria	
1. Internal transcribed spacer-1 (ITS1)‡	• Sequences from a minimum of 12 clones are required. Alternatively, sequences from 12 distinct copies corresponding to the amplified fragment from a sequenced genome could be utilized. • Documenting within strain variability, and phylogenetic position and relation to currently described taxa.
2. D1/D2 variable region of the large ribosomal subunit (D1/D2 LSU)§	• Sequences from a minimum of 12 clones are required. Alternatively, sequences from 12 distinct copies corresponding to the amplified fragment from a sequenced genome could be utilized. • Documenting within strain variability, and phylogenetic position and relation to currently described taxa.
Recommended criteria for complete description of new Neocallimastigomycota isolates.	
I. Morphology/microscopic criteria	
1. Zoospore release mechanism	• How zoospores are released: through an apical pore, through rupture of the sporangial wall, or a combination of both. • Sporangial fate: dissolution, detachment, and/or remaining intact after spore release.
2. Additional structures	• Formation of additional specific structures during the life cycle, e.g. hyphal coils, resting stages (particularly in old cultures).
3. Stability of key traits‖	• Examining cultures grown under different conditions, substrates, as well as during different stages of growth to examine trait consistency and association with various growth stages or culturing conditions.
II. Phylogenetic criteria	
1. Ribosomal RNA operon¶,#	• A region covering ITS1-5.8S rRNA-ITS2-D1/D2 LSU

*Determining the number of flagella on some spores could be challenging, with visualization angles and aggregation of multiple flagella in a single locomotory organelle during swimming leading to uncertain counts. Observing and counting flagella from multiple (e.g. >50) zoospores is recommended when possible, as well as reporting average numbers for polyflagellated taxa, and frequency of oligo (bi-, tri-, and tetra-) flagellation in 'mono' flagellated taxa.
†Sporangia and sporangiophore pleomorphy has been observed in multiple genera (e.g. in genera *Feramyces* [7], and *Liebetanzomyces* [39]), and could be more pronounced when different media compositions are used (e.g. Fig. S1 in [20]). This could be avoided by the utilization of standard media (e.g. cellobiose-based media used in [40]). Further, the level of pleomorphy in itself should be reported and could be used as an informative trait for taxa delineation.
‡Recommended primers to use: for the ITS1 region only, primers MN100 (TCCTACCCTTTGTGAATTTG) and MNGM2 (CTGCGTTCTTCATCGTTGCG) [41]. In case of possible mismatches, as observed previously for *Buwchfawromyces* [4], amplifying longer regions and bioinformatically extracting the ITS1 region can also be achieved using primers Neo 18S For (AATCCTTCGGATTGGCT) and Neo 5.8S Rev (CGAGAACCAAGAGATCCA) for amplifying partial 18S rRNA gene, full ITS1 region and partial 5.8S rRNA gene [42], or ITS1F (TCCGTAGGTGAACCTGCGG) and ITS4R (TCCTCCGCTTATTGATATGC) for amplifying the whole ITS region encompassing ITS1-5.8S rRNA-ITS2 [43].
§Recommended primers to use for amplifying the D1/D2 domains within the LSU rRNA are the general fungal primers NL1F (GCATATCAATAAGCGGAGGAAAAG) and NL4R (GGTCCGTGTTTCAAGACGG) [44], or pairing the general fungal forward primer NL1F with the anaerobic fungal-specific reverse primer GGNL4 (TCAACATCCTAAGCGTAGGTA) [45].
‖Continuous subculturing and 'domestication' of isolates could lead to changes in an isolate's microscopic features. For example, polycentric taxa often cease to produce sporangia or zoospores with continuous subculturing [46], and hence microscopic characterization of such taxa should be undertaken promptly post isolation.
¶Recommended primers to use are ITS1F-NL4R [24, 47].
#Cloning and sequencing the entire operon and bioinformatically extracting ITS1 and LSU region for phylogenetic analysis could be a substitute for individual ITS1 and LSU amplification and sequencing described above. The same minimum number of clones [12] recommended for individual ITS1 and D1/D2 LSU amplicons should be observed.

[24] for additional details. New species have also been proposed based on the identification of specific microscopic trait(s), but such traits could have been overlooked in taxa description manuscripts of closest relatives. More recent studies have proposed new species for morphologically identical strains solely based on sequence divergence values (e.g. *P. irregularis* [11]). In contrast, some studies have been extremely conservative in rank assignment, proposing a new species in spite of clear differences justifying proposition of a new genus (e.g. *Neocallimastix joyonii* [25]). Furthermore, earlier studies relied solely on microscopic data, while newer taxa description manuscripts reported both microscopic as well as molecular data

Table 2. Additional criteria reported in prior taxa description manuscripts

Parameter	Information to provide
Spore ultrastructure	• TEM pictures for cross and longitudinal sections of the flagella. • Sections through the zoospore body to show the organelles (e.g. nucleus, ribosome-like particles, hydrogenosome, microtubules) • Sections through thalli and sporangia.
Substrate utilization pattern	• Ability to grow on a wide range of sugar monomers, dimers, oligomers, and plant polysaccharides (e.g. starch, cellulose, xylan, pectin/polygalacturonic acid). • Ability to grow on proteins, complex media, and/or fatty acids.
Oxygen sensitivity	• Viability after challenging with atmospheric air for various time intervals (e.g. 0.5, 1, 3, 12 h or 1 day)
Fermentation end-products	• Production and quantification of volatile fatty acids, dicarboxylic acids, alcohol, H_2, CO_2, and other metabolic end-products. • Variation in products nature/ratio when grown on different substrates.
Biogeography and ecological distribution	• Similarity search to identify closely related strains and species encountered in previous culture-independent surveys, whether the new isolates represent a previously reported yet-uncultured lineage, as well as possible host preferences and biogeographic patterns.

(e.g. ITS1, D1/D2 loci). Recent comparative studies have used a broader, whole-genome phylogenomic approach for taxa delineation [17]. However, as stated above, these efforts, while extremely promising, require the broad availability of genomic and transcriptomic data. Further, preliminary efforts have suggested that tree topologies recovered by whole genome phylogenomic analysis are often accurately reflected by those recovered by D1/D2 LSU phylogenetic analysis. As such, generation and assessment of whole genome or transcriptome data is not seen as absolutely required when formulating rank assignment decisions for new isolates within the Neocallimastigomycota.

Due to the lack of consistency in rank assignment approaches highlighted above, we propose guidelines for combining microscopic features and phylogenetic analysis for rank assignment decisions in the Neocallimastigomycota. Broadly, the scheme is guided by the following principles:

- Genus-defining criteria include: (1) zoospore flagellation pattern (monoflagellated or polyflagellated), (2) thallus developmental pattern (monocentric or polycentric, determined by 4′,6-diamidino-2-phenylindole or bisbenzimide staining of live cultures and visualization by fluorescence microscopy), and (3) rhizoidal growth pattern (filamentous or bulbous) are genus-defining criteria in the Neocallimastigomycota. All species within a genus should display the same phenotype with regard to these three criteria.
- Novel genera and species designations require clear documentation of phylogenetic novelty in both ITS1 and D1/D2 LSU loci at specific thresholds, as well as documenting clear and stable differences in macroscopic and/or microscopic features from the nearest described taxa.

Specifically, we propose that a novel genus designation should be conferred on new isolates forming a monophyletic clade that is distinct from all previously described genera in D1/D2 LSU and ITS1 phylogenetic trees with adequate statistical support (e.g. >70% bootstrap, >95% posterior probability) and exhibiting unambiguous morphological differences to their closest phylogenetic relatives in one or more of the three cardinal criteria of spore flagellation, thallus developmental and rhizoidal growth patterns. Detection of a difference in such criteria would justify proposing a new genus, regardless of percentage sequence divergence estimates to its closest cultured and described relative. In cases in which no such differences are observed; a new genus should only be proposed based on exhibiting a high level of sequence divergence in D1/D2 LSU and ITS1 loci that justifies accommodation as a new genus rather than a species. Sequence divergence values are clearly a function of the region amplified, alignment methods (pairwise versus multiple sequence alignments), and settings employed (e.g. gap opening and gap extension penalties). A minimum D1/D2 LSU sequence divergence threshold of 3% from the closest cultured, validly described taxa is proposed for genus level delineation (calculated using relative BLASTn with the default parameters of gap existence cost, 5; gap extension cost, 2; match score, 2; mismatch score, −3). A list of validly described taxa as defined by the Code has recently been compiled [20]. Utilizing this threshold value with existing genera yielded groupings consistent with the current ones with two exceptions: the genus *Piromyces* where the intra-genus sequence divergence cutoff of the D1/D2-LSU region ranges between 0 and 5.7%, and the *Anaeromyces–Liebetanzomyces–Capellomyces–Oontomyces* clade where the inter-genus sequence divergence ranges between 1.8 and 2.5%. However, within this clade, the propositions of different genera have been justified based on a clear difference in thallus developmental patterns (polycentric in *Anaeromyces* versus monocentric in the three other genera), a criterion that justifies proposing a new genus regardless of sequence divergence estimates (as stated earlier). Similarly, a minimum ITS1 sequence divergence threshold of 5% from the closest relative (calculated using BLASTn with the default parameters as outlined above) against cultured, validly described taxa is proposed

for genus level delineation. This threshold value is based on similar assessments of inter-genus sequence divergence estimates [26, 27], and has subsequently been used in culture-independent diversity surveys [28, 29].

We propose that a novel species designation within an existing genus should be used to accommodate isolate(s) forming a monophyletic branch that is distinct from all other species within the genus in both D1/D2 LSU and ITS1 phylogenetic trees. Multiple ITS1 and D1/D2 LSU sequences from strain(s), especially the type strain, should be examined (Table 1). There could potentially be occasional overlaps between one or more ITS1 sequences from a novel species and those from an existing species within the same genus due to the wide within-strain (i.e. intragenomic) ITS1 sequence divergence [24]. However, encountering overlaps in D1/D2 LSU rRNA sequence trees should preclude proposing a novel species.

Several factors should be taken into consideration when proposing minimum D1/D2 LSU or ITS1 sequence divergence thresholds for novel species assignment. Only seven out of the twenty currently described genera have more than one species. Within these genera, the majority of prior rank assignment decisions were primarily based on microscopic differences. Within genera with multiple species for which sequence information is available, D1/D2 LSU sequence divergence ranged between 1.8% (i.e. for *Capellomyces foraminis* versus *C. elongis*, and *Orpinomyces joyonii* versus *O. intercalaris*), and 2.2% (i.e. for *Neocallimastix frontalis* versus *N. cameroonii*, and *Anaeromyces mucronatus* versus *A. contortus*). As such, a threshold value of 2% is proposed, and such value has been used for species-level operational taxonomic unit (OTU) designation in culture-independent surveys [24]. Similarly, an ITS1 sequence divergence estimate of 2% is proposed as a general guide for species-level assignments, while taking into account within-strain sequence divergence, a phenomenon that could result in a range of values when utilizing various copies of the ITS1 locus. This value has also been suggested in [26] and used for species-level OTU designation in subsequent diversity surveys [28, 29].

In addition, assessment of all traits described in Table 1, and comparison to all other members of the genus should be undertaken. Distinct differences in one or more stable morphological feature(s) are commonly observed between new and existing species within the same genus, e.g. the formation of intercalary sporangia in *Orpinomyces intercalaris* differentiates it from *O. joyonii*. Reporting such characteristics represents a very important resource to enhance knowledge of the overall characteristics and capabilities of a genus.

Finally, we propose that a subspecies designation should be used to describe strains that show low sequence divergence when compared to previously reported and validly published Neocallimastigomycota species, and/or exhibit minor differences in microscopic and/or phenotypic traits, e.g. substrate utilization patterns, fermentation products, growth rate, size and organization of specific microscopic structures. For strains exhibiting exact morphological, microscopic, phylogenetic and phenotypic criteria, a conferre (*cf.*) strain designation should be used [20].

The requirements highlighted above are mostly concerned with procedures for validly describing and naming novel isolates, as well as for their assignment to a specific rank within the Neocallimastigomycota. Such procedures, however, should not impede progress in wider aspects of Neocallimastigomycota biology. Indeed, the use of alphanumeric designations to identify strains has been a long-standing and widely accepted approach in the fields of biotechnology [30–33], biochemistry [34–36], cell biology [37, 38] and beyond.

Finally, it is important to note that the proposed criteria for characterization and rank assignment in this manuscript are a reflection of the current state of knowledge and methodological feasibility. Future periodic evaluations should be undertaken for addition, removal, or modification of the proposed criteria to account for newer observations as well as experimental and bioinformatic advances.

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Conflicts of interest
The authors declare that there no conflict of interest.

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