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Sequence-Based Identification of *Aspergillus, Fusarium*, and *Mucorales* Species in the Clinical Mycology Laboratory: Where Are We and Where Should We Go from Here?▽

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The identification of fungal species and determination of their significance in the clinical laboratory are complex practices that help establish or exclude a fungal cause of disease. In the past, the clinical mycologist utilized a limited array of phenotypic measurements for categorizing isolates to the species level. This scenario is shifting in favor of molecular identification strategies largely due to a combination of several factors: (i) the changing landscape of epidemiology of medically important fungi, in which novel organisms never before implicated in human infection are being reported from clinical samples (10, 41); (ii) reports of species-specific differences in antifungal susceptibilities of these newly recognized fungi (4, 10, 41); (iii) numerous studies demonstrating that morphology alone may not be a sufficiently objective method for species determination (7, 8, 10, 23, 41); and (iv) a growing scarcity of bench scientists and microbiologists trained in traditional mycology. With the increasing incidence of fungal infections and reports of invasive fungal infections in nontraditional populations, such as patients with critical illnesses, the onus is on the clinical microbiologist/mycologist to return a timely and accurate identification. Molecular methods are rapid with a turnaround time of about 24 h from the time of DNA extraction, yield results that are objective with data portable between labs, and could be more economical in the long run.

Few topics are more controversial or evoke such a passionate response as the term “species” to a mycologist. Molecular studies have demonstrated that a strategy where multiple genes (or portions thereof) are sequenced and the resultant data are analyzed by phylogenetic methods is a robust strategy for fungal species recognition. This concept, known as phylogenetic species recognition (PSR) (40), has been used successfully to define species in the genera *Fusarium* and *Aspergillus* (8, 23, 29, 31, 32). The advent of PSR has greatly clarified the taxonomy of these genera and as such is a powerful tool for fungal species delimitation. However, this methodology is expensive and requires phylogenetic expertise, which may be limiting factors in clinical microbiology laboratories. In reality, once a species has been delimited by PSR using several robust loci, sequence diversity within the species is known, and on the basis of this knowledge, comparative sequence analyses from a single locus can be used for rapid species identification. “Cutoff scores,” which are dependent on genetic diversity within and between sibling species, can then be provided.

Thus, it is important to clarify that our intent in this editorial is to address the practice of species “identification” as applied to a clinical setting and not species “classification” necessary for taxonomic

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categorization. Although the two terms can be overlapping, the purpose of an "identification" method in a clinical microbiology laboratory is the ability to provide a specific name or epithet to an organism rapidly and with precision, without the complex experimental research or detailed phylogenetic analyses vital for a taxonomic "classification" scheme. Such specific information can then be used by the physician in a decision-making algorithm that can guide patient management.

The field of medical mycology has embraced molecular methods of identification, resulting in the exploration of numerous potential targets, an explosion in the number of sequences from these loci, and recognition of previously unknown fungal species adding to the already staggering fungal diversity. On the other hand, this practice may have opened up a number of possibilities, at least from the perspective of a mycologist in a routine microbiology laboratory, resulting in considerable uncertainty about the best possible molecular method to obtain a species identification. Realizing this, a consortium of international experts was assembled as an International Society for Human and Animal Mycology (ISHAM; www.isham.org) working group on fungal molecular identification. With the goal of supporting clinical laboratories in their efforts to identify fungal species from culture by using molecular methods, the ISHAM working group agreed to begin by focusing on molecular strategies available for medically important fungi of the genera *Aspergillus* and *Fusarium* and the order *Mucorales* (Zygomycota). The advantages and limitations of these methods are discussed, and the recommendations of this working group are presented in this editorial.

COMPARATIVE SEQUENCE IDENTIFICATION STRATEGY

Today, comparative sequence-based identification strategies can be considered the new "gold standard" for fungal species identification (39). This method is based on PCR amplification of a selected region of genomic DNA (target locus), followed by sequencing of the resulting amplicon(s). Once a consensus sequence is obtained, it can be queried against a database library and evaluation for species identification can be performed by generating dendrograms, examining percent similarity/percent dissimilarity, or executing more sophisticated phylogenetic analyses. The current approach in clinical laboratory practice is to interpret sequence comparison results by generating a percent identity score, which is a single numeric score determined for each pair of aligned sequences and which measures the number of identical nucleotide matches in relation to the length of the alignment. Cutoff scores for species identification are arbitrary, and the scores can vary depending on numerous factors including the quality of the sequence, the number and accuracy of existing database records from the same species and locus, the length of the sequence fragment, and the software program employed. At present, there is no definitive study describing an absolute cutoff for same-species identity across the fungal kingdom and no consensus definition exists on how to define a species using such comparative sequence methodologies.

The success of a comparative sequencing strategy for the identification of a wide range of clinical fungi lies mainly in the choice of the appropriate locus. The gene target should be orthologous (i.e., evolved by common descent), having a high level of interspecies variation combined with low levels of intraspecific variation, and ideally should not undergo recombination. In addition, the target must be easy to amplify and sequence using standardized "universal" primer sets. Finally, the amplified DNA fragment should be within the size range obtainable with the most commonly used automated DNA sequencers (~600 to 800 bp) and easily aligned with a sequence database for comparison. Does such a utopian locus exist?

Multiple studies have demonstrated that comparative sequence-based identification using the nuclear ribosomal internal transcribed spacer (ITS) region (ITS1, 5.8S rRNA, and ITS2) located between the nuclear small- and large-subunit rRNA genes (43) could be employed for species complex-level identification of *Aspergillus* (21) and most *Mucorales* (37) species and for identification within some species complexes of *Fusarium* (Fig. 1) (31, 44). The ITS region satisfies most of the aforementioned requirements of a "universal" marker since this region can be reliably amplified for most fungi, is conserved, is present as multiple copies in the fungal genome, yields sufficient taxonomic resolution for most fungi, and has the additional advantage that the GenBank(http://www.ncbi.nlm.nih.gov), European Molecular Biology Laboratory nucleotide sequence database (http://www.ebi.ac.uk/embl/), and DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/) contain a large number of sequences from this locus, enabling a ready comparison of the sequence from an unknown isolate.

FIG. 1.

RNA polymerase II second largest subunit (*RPB2*) phylogeny of *Fusarium* (modified from Fig. 1 in reference 30), showing the utility of

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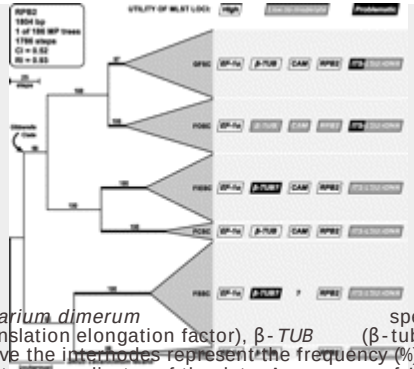

DNA sequence data from various loci for resolving at or near the species level within six medically important species complexes. GFSC, *Gibberella fujikuroi* species complex; FOSC, *Fusarium oxysporum* species complex; FIESC, *Fusarium incarnatum-equiseti* species complex; FCSC, *Fusarium chlamydosporum* species complex; FSSC, *Fusarium solani* species complex; FDSC, *Fusarium dimerum* species complex. Loci include *EF-1* α (translation elongation factor), β-*TUB* (β-tubulin), and *CAM* (calmodulin). Numbers above the internodes represent the frequency (%) with which they were recovered from 1,000 bootstrap replicates of the data. A sequence of *Lecanicillium lecanii* was used to root the phylogeny.

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There is considerable consensus regarding the use of ITS sequencing as the initial step in mold identification. An international *Aspergillus* working group recently recommended the use of the ITS region for subgenus/section-level identification for the genus *Aspergillus* (9). Also, the International Subcommission on Fungal Barcoding has proposed the ITS region as the prime fungal barcode or the default region for species identification (http://www.allfungi.com/its-barcode.php).

Significant disadvantages of the ITS region include (i) insufficient hypervariability to distinguish the various species in the *Aspergillus* sections and *Fusarium* species complexes; (ii) its failure to distinguish between closely related species (sibling species) because of insufficient nucleotide differences, for example, *Aspergillus lentulus* and *Aspergillus fumigatus*; and (iii) problems with the reliability of the ITS sequences deposited in the reference databases (e.g., GenBank/EMBL/DDBJ) (26).

Comparative sequence-based identification strategies can be meaningful only with the availability of well-curated, robust, and reliable databases that are populated with sequence data from type or reference strains (where possible), have been rigorously validated in terms of their nomenclature, and include sequences from a wide variety of target species. The most widely used database is GenBank, which contains a huge number of sequences, but these are combined with unedited and nonvalidated information, which may be updated and corrected only by the original submitter. Errors in fungal sequences within GenBank have been found to be as high as 20% (26). Despite calls for the process to be changed, to allow for third-party revision (11), there seems little prospect of this in the near future (34). On the other hand, smaller databases, such as those provided with commercial sequence-based identification systems, are often inadequate because of their lack of breadth (omitting many, often important species) and depth (containing few representatives of the same species) (19). To overcome these problems, specific sequence databases for particular groups of fungi, based on quality-controlled sequences, have been created mainly for plant-pathogenic, industrially important, and ectomycorrhizal ascomycete and basidiomycete fungi, e.g., *Fusarium* spp. (FUSARIUM-ID v. 1.0 [17]; http://fusarium.cbio.psu.edu), *Phaeoacremonium* spp. (http://www.cbs.knaw.nl/phaeoacremonium/biolomics.aspx), and *Trichoderma* spp. (http://www.isth.info/morphology.php), and mycorrhizal fungi (UNITE; http://unite.ut.ee/). Two ITS databases for medical fungi are available through the CBS Fungal Biodiversity Centre and at the Westmead Millennium Institute, University of Sydney (curated database; http://www.mycologylab.org/biolomicsid.aspx) (W. Meyer et al., unpublished data). Ideally, the mycology community needs to find a way of combining high-quality sequence and available species data present in numerous reference and research laboratories around the world.

SPECIES IDENTIFICATION IN *ASPERGILLUS, FUSARIUM*, AND THE *MUCORALES*

Aspergillus species.Taxonomically, the genus *Aspergillus* is divided into seven subgenera, which are further divided into several "sections"—for example, subgenus *Fumigati* encompasses two sections: *Fumigati* and *Cervini* (24).

Clinically relevant aspergilli are represented within several sections of the seven subgenera. For instance, the medically important species *A. fumigatus* and other relatives less commonly implicated in human infections such as *Neosartorya fischeri* and *A. lentulus* fall within section *Fumigati* of the genus

Aspergillus . Given that this classification scheme is unique to the genus *Aspergillus* , it is important to recognize that there can be two levels of identification: (i) identification to a given species complex, e.g., discrimination of *A. fumigatus* complex (subgenus *Fumigati* , section *Fumigati*) from *Aspergillus flavus* complex (subgenus *Circumdati* , section *Flavi*), and (ii) identification of species within a section, e.g., discrimination of *A. fumigatus* from *A. lentulus* (both members of the section *Fumigati* , subgenus *Fumigati*). Employing comparative sequence analysis of the ITS region, one can rapidly and unquestionably place an *Aspergillus* isolate within the respective sections, for instance, *Aspergillus ustus* (section *Usti*) or *Aspergillus terreus* (section *Terrei*) (21). In contrast, species identification within a given *Aspergillus* section, for instance, identification of the various species within section *Usti* (i.e., *Aspergillus calidoustus, A. ustus* , and *Aspergillus pseudodeflectus*), can be challenging given that the ITS region has few sites that are variable enough for this degree of resolution. In addition, several aspergilli have overlapping morphological features rendering phenotypic identification methods inadequate. Numerous studies have demonstrated that comparative sequence analyses of protein coding regions such as those for β-tubulin, calmodulin, and rodlet A can identify species within sections *Fumigati, Usti, Nigri* , and *Terrei* (4, 7, 8, 23, 36, 41).

Recognizing the growing role of molecular methods in *Aspergillus* species identification, an international *Aspergillus* working group (9) proposed the following recommendations: (i) the term “species complex” as an alternative to “section,” (ii) use of sequences from the ITS region for identification of *Aspergillus* isolates to the species complex level, and (iii) comparative sequence analyses of the β-tubulin region for species identification within a complex. This recommendation can be advantageous to clinical laboratories that rely on comparative sequence analyses of the ITS region (which are not variable enough for species identification within a section) and/or morphology for species identification (where overlapping morphologies can hinder resolution of species within the sections) as they can report the identification of an unknown organism to species complex, for instance, *A. terreus* complex. Thus, the term “complex” in such an identification scheme would indicate the placement of the isolate within a species complex but does not identify the isolate to a species within the complex.

Fusarium species.*Fusarium* species have emerged over the past 3 decades as an important genus of filamentous molds causing opportunistic infections in humans (27). Detailed molecular studies employing sequences of multiple loci such as elongation factor 1α (*EF-1* α) (17), β-tubulin (β-*TUB*), calmodulin (*CAM*), and RNA polymerase II second largest subunit (*RPB2*) (Fig. 1) and subsequent phylogenetic analyses of medically important fusaria have revealed the presence of multiple cryptic species within each morphologically recognized “morphospecies.” For instance, *Fusarium solani* actually represents a complex (i.e., *F. solani* species complex) (Fig. 1) of over 45 phylogenetically distinct species of which at least 20 are associated with human infections (31, 44). Similarly, members of the *Fusarium oxysporum* species complex are phylogenetically diverse (31, 44), as are members of the *Fusarium incarnatum-equiseti* species complex and *Fusarium chlamydosporum* species complex (Fig. 1) (30; K. O'Donnell, unpublished data). Cases involving the latter two complexes are typically reported as the polytypic morphospecies *F. incarnatum* /*Fusarium semitectum*/*F. equiseti* and *F. chlamydosporum* , respectively (38). Available data clearly demonstrate that sequences from the nuclear ribosomal ITS region and domains D1 and D2 of the 28S ribosomal DNA (rDNA) large subunit are too conserved to resolve most clinically important fusaria at the species level (31, 44), despite reports to the contrary (16, 20). Moreover, use of the ITS rDNA within the *Gibberella fujikuroi* species complex and *F. oxysporum* species complex (29) and β-tubulin within the *F. incarnatum-equiseti* species complex and *F. solani* species complex should be avoided due to paralogous or duplicated divergent alleles (32; O'Donnell, unpublished).

Mucorales .Evolutionary relationships of species within the order *Mucorales* (of the division Zygomycota) have been investigated by phylogenetic analyses of nuclear ribosomal 18S and 28S rDNA and translation elongation factor (*EF-1* α) gene sequences and have revealed that species within medically important genera such as *Absidia* and *Mucor* appear to be polyphyletic (i.e., from multiple evolutionary origins) (33, 42). Indeed, a recent taxonomic revision of *Absidia* based on physiological, phylogenetic, and morphological characters has been proposed (22), with reclassification of the human pathogen *Absidia corymbifera* as *Mycocladus corymbiferus* in a new family. Similarly, molecular and physiological data were used to distinguish two species within the morphospecies *Rhizopus oryzae* (1, 2, 35), with the proposal that the fumaric-malic acid-producing species be named *Rhizopus delemar* (2).

Several recent studies have demonstrated the utility of comparative sequence analyses of the nuclear 28S rDNA D1/D2 domains, the ITS region, actin, and partial translation elongation factor (*EF-1* α) gene sequences for resolving at or near the species level within the *Mucorales* (1, 2, 33, 37, 42). Analyses of intra- and interspecies variability of ITS sequences from 54 isolates of *Mucorales* belonging to 16 different species were evaluated recently, and the results support ITS sequencing as a reliable method for the accurate identification of most medically important *Mucorales* to the species level (37). However, it is important to note that some closely related species could not be resolved using ITS sequence data. Similarly, while ITS sequence data can be used for the identification of several *Rhizopus* species (1, 25), they lack sufficient variability to resolve *Rhizopus azygosporus* from *Rhizopus microsporus* . In addition the D1/D2 domains of the 28S rDNA and the high-affinity iron permease 1 gene (*FTR1*) appear to be useful targets for species identification, although the *FTR1* locus could not resolve all of the clinically relevant species within the genera *Rhizomucor* and *Mucor* (28). Thus, it is readily apparent that sequencing of more phylogenetically informative gene targets will be required for certain *Mucorales* and that phylogenetic analyses of several loci will be needed to fully assess species limits within the *Mucorales* .

RELEVANCE OF SPECIES IDENTIFICATION IN THE CLINICAL MICROBIOLOGY LABORATORY

An important issue to be considered when deciding the choice of loci and/or number of loci is the relevance of identifying every unknown isolate to the smallest taxonomic unit. In other words, should a clinical microbiology laboratory strive to identify every isolate to the species level, or is it sufficient to identify isolates to the genus or species complex level? Species-level identification of a fungal isolate recovered from a clinical specimen (especially from a sterile site from an immunocompromised patient) could be important given that species identification of appropriate isolates in high-risk populations may reveal the etiological agent of disease, aid selection and monitoring of antifungal therapy, and support epidemiological investigations leading to effective infection control measures. On the other hand, many sporadic isolates do not represent clinically important disease and it may be wasteful to devote resources to identifying such isolates without an understanding of their role in disease.

After identifying the unknown fungal isolate to the level of a species complex, should the laboratory go further to achieve species identification within a section or complex? This is difficult to answer; nevertheless, each one of us in the clinical microbiology laboratory faces this question every time we recover a fungus from a high-risk patient and/or read a manuscript describing yet another species within the complexes. The clinical significance of identifying isolates to species level, for example, *A. terreus* versus *A. fumigatus* in the genus *Aspergillus* , is evident given the different susceptibilities to the antifungal drug amphotericin B; however, the significance of identifying individual species within the complexes of aspergilli and fusaria and to the species level within the *Mucorales* is not fully apparent at this time. Studies have shown species-specific differences in antifungal susceptibilities within *Aspergillus* section *Fumigati* (4), while other studies have shown little or no difference in antifungal susceptibilities of species within the sections *Usti* (41) and *Terrei* (S. A. Balajee et al., unpublished data). Likewise, there appear to be limited species-specific differences in antifungal susceptibilities within the genus *Fusarium* (3, 6, 31). On the other hand, considerable interspecific variation in antifungal susceptibility of the *Mucorales* to polyenes and azoles has been observed in vitro (14, 15) and in in vivo animal models of zygomycosis (18), suggesting that species identification might be clinically relevant in the future as more active antifungal agents against these organisms become available and as in vitro breakpoints are defined.

Taken together, data regarding differences in pathogenicity and in vivo drug susceptibilities of the various species within *Aspergillus* and *Fusarium* complex do not categorically suggest that identification within these taxa will impact clinical and therapeutic decision making, at least at the present time. However, identification to species/strain level could inform the epidemiology of fungal infections and can be critical in outbreak investigations (12, 30). Accordingly, the decision to identify an unknown isolate to species level within a given section/complex of these genera will be based on the need of the clients whom the microbiology laboratory is serving (high-risk versus low-risk populations), site of isolation of the fungus (sterile versus nonsterile sites), funds, and personnel available.

CLSI RECOMMENDATIONS FOR FUNGAL SPECIES IDENTIFICATION

At present, DNA target sequencing can provide a quantitative metric to classify fungi; however, sequencing results can create laboratory uncertainty when assigning microorganisms to their appropriate taxonomical groups. Realizing this, in May 2008, the Clinical and Laboratory Standards Institute (formerly NCCLS) published a document, *Interpretive Criteria for*

Identification of Bacteria and Fungi by DNA Target Sequencing, to address the challenges of sequence analyses in general clinical laboratory practice (13). Specifically, the CLSI guideline provides a systematic and uniform approach to identify fungi by broad-range DNA target sequencing in the clinical laboratory. The document establishes guidelines for primer design, quality control parameters for amplification and sequencing, measurement of sequence quality, and assessment of reference databases. Since consensus has not yet been achieved in multilocus DNA sequencing and since most clinical laboratories do not have the resources to perform such analyses, the guideline focuses on the most commonly used target, the ITS region. For specific taxonomic groups, tables are provided to describe the relative strengths and limitations of individual DNA targets and list alternative DNA targets for those laboratories pursuing further phylogenetic resolution. Finally, the document discusses reporting strategies that are clinically relevant for specific groups of microorganisms. Since our current understanding of the diversity of clinically important phylogenetic species within *Aspergillus* and *Fusarium* is in flux and the biological importance of drawing finer phylogenetic distinctions remains to be determined, the guideline recommends that for certain taxa, clinical laboratories report sequence results for isolates only to the level of genus or species complex.

The CLSI document is largely centered around the ITS region as a target because of the general applicability, research backing, and literature validation of this target. Similarly to CLSI, the ISHAM working group details differences between species complexes and individual species and presents alternative targets that might offer the user more specific species identification in *Aspergillus* and *Fusarium* if such information is needed.

RECOMMENDATIONS OF THE WORKING GROUP

If the goal is to identify an unknown organism with no a priori knowledge, then the ITS region is a reasonable and extensively used choice for species complex identification within the genera *Aspergillus* and *Fusarium* and most species within the *Mucorales*.

Such a consensus on the employment of the ITS region as the default locus for use in the clinical laboratory setting would achieve international consistency in the way that other collaborative initiatives, such as the EORTC/MSG diagnostic criteria for invasive fungal infection (5), have been successful. This consensus should have the effect of enhancing the publication of ITS sequences and focusing commercial efforts on this strategy. Clinical laboratories that have been reluctant to adopt molecular technology in an atmosphere of conflicting opinions and evidence are more likely to implement methodology that has international backing. In addition, the quality of clinical and other research publications would be improved and harmonized based on the use of a universal locus.

This working group acknowledges the known shortcomings of the ITS locus and therefore recommends a staged sequence-based identification strategy (Fig. 2) for identification of aspergilli, fusaria, and the *Mucorales* in a clinical microbiology laboratory. Based on this proposed algorithm, when an unknown fungal isolate is received in a clinical microbiology laboratory, after initial morphological examination the laboratory can pursue morphological or molecular identification methods or choose a combination of the two methods (Fig. 2). When further resolution is required, comparative sequence analyses of one or several protein coding regions can be performed for species-level identification within *Aspergillus* and *Fusarium* complexes.

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FIG. 2.

An algorithm for identification of an unknown filamentous fungal species in a clinical microbiology laboratory. *, many factors affect percent identity scores including quality and length of query sequence and the number and accuracy of existing GenBank records for same species and locus. +, identification to species level within the *Aspergillus* and *Fusarium* complex can be achieved by comparative sequence analyses of protein coding regions.

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There is no universal agreement on the identity cutoff values that should be applied for same-species identity, and thus, a certain degree of interpretation will be required, at least until the issues already discussed have been resolved. At this time, the CLSI guidelines do not provide cutoff values because, at the time of writing, the available data did not support such cutoff values for fungi. Nevertheless, it will be important for the mycological community to refine guidelines in this difficult area for users in the clinical setting, to ensure consistency of interpretation. Thus far, analyzing ITS sequence data from >600 *Aspergillus* isolates from three different laboratories (S. A. Balajee, W. Meyer, and A. Velegraki, unpublished data) and employing both "in-house" sequence databases and the GenBank/EMBL/DDBJ database for sequence comparison, an identity of 94 to 100% to the respective type/validated strain is proposed for species complex-level identification within the genus *Aspergillus*. For the genus *Fusarium* (Fig. 1) and within most species within the *Mucorales*, we propose that if the ITS sequence of an unknown fungal isolate yields an identity of ≥99% to a type/reference strain, the isolate can be placed within one of six clinically relevant species complexes. When ITS comparative sequence analyses yield ambiguous data, the clinical laboratory may consider sending the isolate to a reference laboratory for identification.

When performing comparative sequence analyses (as outlined in Fig. 2), it is imperative to understand that the percent identity scores generated using GenBank/EMBL/DDBJ are influenced by numerous factors including the quality of the sequence, the number and accuracy of existing GenBank/EMBL/DDBJ records for the same species and locus, and the completeness of the sequence (double-stranded sequence). Importantly, because outputs can be ranked by maximum score, total score, or percent identity and searches can be customized for parameter preferences (i.e., gap penalties and BLAST algorithm), users should take advantage of some of the tutorials and background information prior to performing searches.

In order to improve the accuracy of sequence data, the working group further emphasizes the importance of completing database record fields (especially those of GenBank/EMBL/DDBJ) correctly when submitting sequences for inclusion in these databases. The teleomorph name should be included if known and available for the organism, and species names should follow guidelines established by the International Code of Botanical Nomenclature. Species identity and sequence accuracy can be confirmed with reference to other sources such as the Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl), the UK National Collection of Pathogenic Fungi (http://www.hpacultures.org.uk), and Mycobank (http://www.mycobank.org). It must be remembered, however, that isolates in these collections were mostly identified by morphology alone.

Comparative sequence-based identification is an evolving area of research with the constant addition of new sequences at novel and traditional loci to many different databases. Future studies will be needed to assess the validity of the proposal made in this editorial and to examine its utility in the clinical setting. As noted, the CLSI document is intended to be updated periodically, so that additional research-based evidence can be translated into better-defined algorithms and guidelines of practical benefit. It is an important beginning, and together with the efforts of the ISHAM working group, it should help guide and inform development of this clinical mycology laboratory methodology.

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FOOTNOTES

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