

Systematic Internal Transcribed Spacer Sequence Analysis for Identification of Clinical Mold Isolates in Diagnostic Mycology: a 5-Year Study^{∇†}

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The implementation of internal transcribed spacer (ITS) sequencing for routine identification of molds in the diagnostic mycology laboratory was analyzed in a 5-year study. All mold isolates ($n = 6,900$) recovered in our laboratory from 2005 to 2009 were included in this study. According to a defined work flow, which in addition to troublesome phenotypic identification takes clinical relevance into account, 233 isolates were subjected to ITS sequence analysis. Sequencing resulted in successful identification for 78.6% of the analyzed isolates (57.1% at species level, 21.5% at genus level). In comparison, extended in-depth phenotypic characterization of the isolates subjected to sequencing achieved taxonomic assignment for 47.6% of these, with a mere 13.3% at species level. Optimization of DNA extraction further improved the efficacy of molecular identification. This study is the first of its kind to testify to the systematic implementation of sequence-based identification procedures in the routine workup of mold isolates in the diagnostic mycology laboratory.

The diagnostic mycology laboratory is confronted with an increasing number of infections caused by an expanding spectrum of fungal species (9, 18, 19, 23, 28). An important reason for this development is the progress in medicine that results in prolonged life expectancy for patients with a compromised immune system, many of whom are particularly vulnerable to fungal infections.

Conventional identification of fungi is mainly based on phenotypic characteristics (11, 35). Micro- and macromorphologies have to be carefully evaluated for correct species assignment, requiring a great deal of expertise because of excessive morphological variability. In addition, some isolates do not form their typical structural characteristics required for identification under laboratory conditions (11). As a result, the diagnostic laboratory is often confronted with misidentifications and/or lack of identification. Genetic methods for identification and taxonomic classification of molds have been established in recent years (6, 38). In particular, the internal transcribed spacer (ITS) regions located between the 18S and 28S rRNA genes and including the ITS1 and ITS2 regions and the 5.8S rRNA gene have emerged as the most common target for molecularly based identification (4, 16, 37). The ITS regions occur in multiple copies in the fungal genome, and the number of ITS sequences available in public databases (such as GenBank, NCBI) is expanding continuously (31). Unfortunately, the quality of sequence entries is variable and taxo-

mic assignments in public databases are in part unreliable, hampering their use for identification (5, 27).

For a cost- and time-effective application of molecular identification procedures, we have previously defined selection criteria and assembled these in an algorithm. According to these criteria, mold isolates of clinical relevance that cannot readily be identified by standard conventional characteristics are subjected to ITS sequence-based identification (7). Analysis of the ITS regions is the most commonly used molecular method for identification of molds (3, 4, 29). Studies reporting on the use of molecularly based identification procedures in diagnostic mycology have mainly focused on method development (12, 25, 30, 40) and include anecdotal case reports (15, 17, 20). There are few studies addressing the use of systematic ITS sequencing implemented as a routine tool according to a defined work flow and its impact on diagnostic performance in a medical mycology laboratory (21). Here, we report on the results of a 5-year study on systematic implementation of ITS sequencing for identification of clinical mold isolates in the diagnostic laboratory.

MATERIALS AND METHODS

Conventional identification. Clinical specimens were cultured on general mycology media (Sabouraud dextrose agar containing gentamicin and chloramphenicol and brain heart infusion agar; Becton Dickinson AG, Allschwil, Switzerland) and on fungal selective media (Chromagar and Mycosel; Becton Dickinson AG) at 25°C for a maximum of 3 weeks and regularly examined for growth. Subcultures for identification were done as follows: (i) *Aspergillus* spp. on malt yeast agar (11) at 25°C, 35°C, and 42°C; (ii) *Mucorales* on potato carrot agar (9) at 25°C, 37°C, 40°C, 45°C, 50°C, and 56°C; (iii) all other molds on Sabouraud dextrose agar containing gentamicin and chloramphenicol at 25°C and 35°C and on Mycosel and potato carrot agar, both at 25°C. Phenotypic identification was based on macro- and micromorphological criteria, as described in reference 11.

DNA extraction. Mold isolates were subjected to DNA extraction using the InstaGene matrix (Bio-Rad, Reinach BL, Switzerland), as described previously (7). For DNA extraction, a fungal mycelium with a surface area of 2 to 4 cm² was obtained from Mycosel or Sabouraud dextrose agar plates. In case of PCR failure with InstaGene extract as the template, a freshly collected fungal mycelium was digested with lyticase (until 27 September 2007) or proteinase K (following 27

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September 2007). For lyticase digestion, the mycelium was incubated at 37°C for 2 h with 30 U of lyticase (Sigma-Aldrich Chemie GmbH, Schnellendorf, Germany) in 200 µl digestion buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0), followed by alkaline lysis with 10 µl 1 M NaOH and 10 µl 10% SDS, incubation for 10 min at 95°C, and neutralization with 10 µl 1 M HCl. For proteinase K digestion, the mycelium was vortexed for 3 min at the fastest setting with 80 µg proteinase K (recombinant PCR grade; Roche, Rotkreuz, Switzerland) and 50 mg glass beads (acid washed, <106 µm; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in 200 µl digestion buffer (50 mM Tris-HCl, 1 mM EDTA [pH 8.0], 0.5% SDS), incubated at 56°C for 3 h, and vortexed again for 3 min at the fastest setting. DNA was extracted and purified using the QIAamp DNA blood minikit (Qiagen, Hombrechtikon, Switzerland) according to the instructions of the manufacturer and eluted in 100 µl elution buffer.

Amplification and sequencing. PCR of the ITS regions was performed as described before using the LightCycler FastStart DNA Master SYBR green I kit (LightCycler reagents; Roche, Rotkreuz, Switzerland) and a PerkinElmer GeneAmp PCR System 9600 or 9700 (Applied Biosystems, Rotkreuz, Switzerland) (7). The amplified ITS regions were sequenced with primer ITS1 or, in case of failure, with primer ITS4, ITS3, or ITS2 (37) (Fig. 1) using the BigDye kit (Applied Biosystems, Rotkreuz, Switzerland) and an automated DNA sequencer (ABI Prism 3100-Avant genetic analyzer; Applied Biosystems). When the ITS1 and ITS2 regions were separately sequenced, a consensus sequence was created for homology analysis.

Sequence analysis. Sequences obtained (covering >90% of the ITS regions) were analyzed for homology using GenBank (NCBI) and the SmartGene ITS database (ITS validated database; SmartGene IDNS, Zug, Switzerland) in parallel. The SmartGene ITS database is a curated database which is designed to include most of the clinically relevant fungi and which contains some ITS sequences that are missing in GenBank. Sequence assignment to species and genus level was done according to guidelines published previously (7). A sequence was assigned to a species if the best matching reference sequence showed $\geq 98\%$ homology and the next best matching reference species showed at least 0.8% less sequence homology. A sequence was assigned to genus level on the basis of 95 to 98% homology to the best matching sequence or of $\geq 98\%$ homology with sequence entries for several species from the same genus. "No identification" was defined as <95% homology with the best matching reference sequence or as sequence homology of >95% with various genera present (7).

Prospective study. Over the study period of 5 years (from January 2005 to October 2009), clinical mold isolates were subjected to phenotypic and sequence-based identification measures following a defined work flow (7). Isolates for which (i) phenotypic identification by conventional characteristics did not result in species assignment within 5 days following primary subcultivation and (ii) which were judged to be clinically relevant were analyzed by ITS sequencing. Clinical relevance was defined as follows: (i) isolates from primarily sterile specimen sites growing at 35°C, (ii) isolates from nonsterile specimen sites with direct microscopy positive for hyphae, (iii) isolates from nonsterile specimen sites growing at 35°C and clinical evidence of infection, (iv) isolates from dermatological samples positive for hyphae in direct microscopy and growing at 35°C (dermatophytes were excluded from the study). In addition to being sequenced, the isolates included in this study were subjected to prolonged cultivations to allow for forming morphological characteristics and to facilitate taxonomic assignment based on phenotypic traits.

RESULTS

From January 2005 to October 2009, a total of 6,900 mold isolates were recovered in the diagnostic laboratory. These molds were found to represent 136 different species belonging to 81 different genera (see Table S1 in the supplemental material). Seven hundred twenty-six isolates could not be readily identified by standard phenotypic characteristics. Two hundred thirty-three (32.1%) of these isolates were categorized as potentially clinically relevant and subjected to ITS analysis. Sequences determined were analyzed using two databases: the GenBank database and the SmartGene validated ITS database.

During the 5-year study period, the number of sequenced isolates decreased from 88 in 2005 to 20 in the first 10 months of 2009 (data not shown). Using the two databases combined,

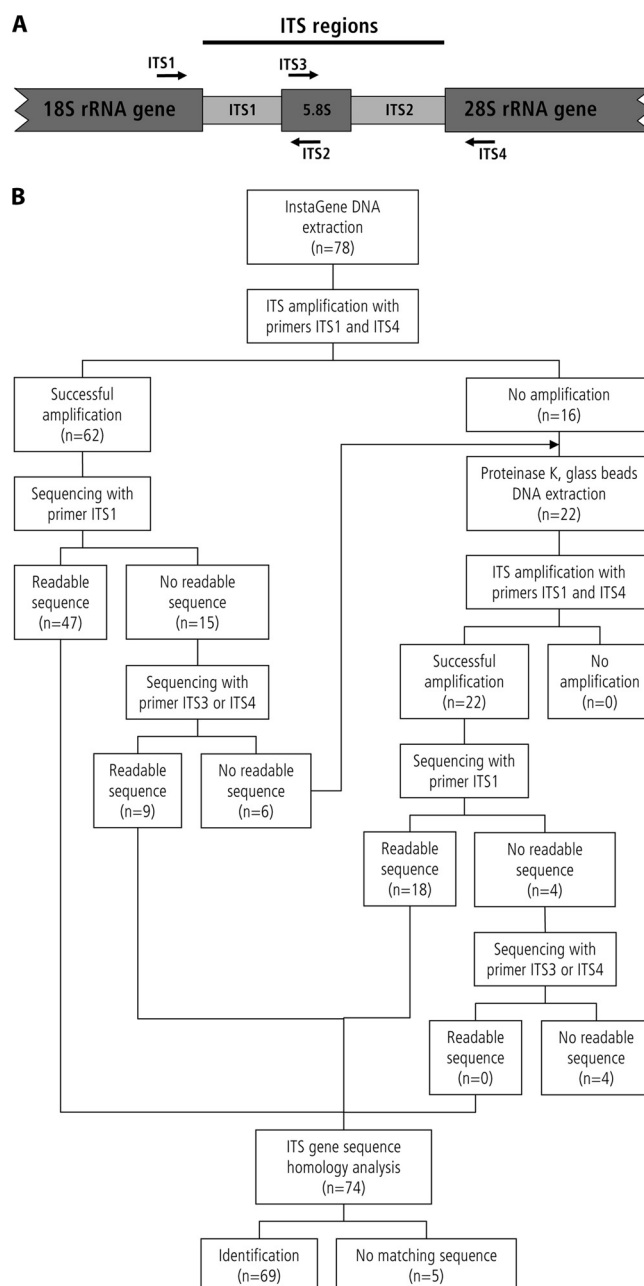


FIG. 1. Flow chart for DNA extraction and ITS sequencing of molds as implemented from 27 September 2007 onwards. (A) Schematic representation of the fungal ITS regions, consisting of ITS1, the 5.8 rRNA gene, and ITS2. Positions and directions of primers ITS1, ITS2, ITS3, and ITS4 (37) are indicated with arrows. (B) Flow chart of DNA extraction and ITS sequencing. n, number of isolates.

183 (78.6%) of the 233 isolates subjected to molecular identification were successfully identified by ITS sequence analysis (Table 1). Most of these isolates (57.1%) were assigned to species level. Genus level assignment was achieved for 21.5%; inability to differentiate at species level for these isolates was mostly due to high interspecies homology of the genera involved. Use of the SmartGene validated ITS database resulted in a higher percentage of identification at species level than

TABLE 1. Identification of fungal isolates selected for ITS sequence analysis based on defined criteria according a diagnostic work flow from January 2005 to October 2009

Identification method	Identification level or result	% of isolates (n)
Morphological characterization ^a	Species	13.3 (31)
	Genus	34.3 (80)
	No taxonomic assignment	52.4 (122)
ITS sequence analysis using SmartGene ^b and GenBank ^c	Species	57.1 (133)
	Genus	21.5 (50)
	No taxonomic assignment ^d	15.4 (36)
	Unsuccessful amplification	6.0 (14)
ITS sequence analysis using SmartGene ^b	Species	44.7 (104)
	Genus	19.3 (45)
	No taxonomic assignment ^d	30.0 (70)
	Unsuccessful amplification	6.0 (14)
ITS sequence analysis using GenBank ^c	Species	36.5 (85)
	Genus	28.3 (66)
	No taxonomic assignment ^d	29.0 (68)
	Unsuccessful amplification	6.0 (14)
Total		100 (233)

^a Taxonomic assignment based on extended in-depth phenotypic identification measures.

^b SmartGene, homology analysis using the SmartGene ITS database.

^c GenBank, homology analysis using the NCBI database.

^d No identification due to the lack of homologous sequences.

that obtained using the GenBank database (Table 1). Prolonged cultivation and morphological characterization of the 233 isolates enabled taxonomic assignment in 47.6% of the cases, with 13.3% at species level and 34.3% at genus level (Table 1). Successful identification by ITS sequencing increased from 77.3% in 2005 to 95.0% in 2009. This was associated with an increase in species assignment from 50.0% in 2005 to 80.0% in 2009 (data not shown). The isolates subjected to sequence analysis were assigned to 87 species belonging to 55 different genera (see Table S1 in the supplemental material), covering more than one-half of the spectrum of molds encountered in our laboratory.

During the study period, 36 (15.4%) of the 233 isolates could not be identified by sequencing because matching ITS reference sequences for these were lacking in the databases (Table 1). By phenotypic methods, 5 of these 36 isolates could be assigned to *Acremonium* spp., *Alternaria* spp., *Exophiala* spp., *Paecilomyces variotii*, and *Scytalidium* spp. ITS sequence analysis with no taxonomic assignment decreased from 18.2% in 2005 to 0.0% in 2009.

In the 5-year study period, 14 (6.0%) of the 233 isolates failed to yield an ITS amplicon (Table 1). On the basis of prolonged phenotypic identification procedures, 4 of these 14 isolates were identified as *Aspergillus fumigatus*, *Microascus cinereus*, *Onychocola canadensis*, and *Phialophora richardsiae*. Genus level assignment was achieved for 3 of the 14 isolates as *Aspergillus* spp., *Paecilomyces* spp., and *Syncephalastrum* spp. Seven of the 14 isolates could not be assigned at any taxonomic level.

An effort was made to improve DNA extraction when the initial InstaGene extraction was not successful. For efficient

DNA release from fungal cells, digestion with lyticase was replaced by proteinase K digestion combined with mechanical rupture of the cells by glass beads. Following implementation of this modification (from 27 September 2007 onwards), 82% of the isolates for which the initial InstaGene extraction failed to yield an amplicon were successfully amplified and sequenced (Fig. 1). The percentage of isolates for which ITS amplification failed was reduced from 12% in 2006 to 5% in 2008 to 2009.

DISCUSSION

From January 2005 to October 2009, a wide spectrum of fungal species were isolated in our diagnostic laboratory (see Table S1 in the supplemental material). This corroborates recent reports testifying to the growing number of potentially pathogenic fungal species (9, 10, 13, 14, 26, 36). Consequently, each laboratory isolate has to be evaluated carefully with regard to its pathogenic potential as well as its clinical relevance. The diagnostic laboratory initially has often limited or no access to clinical data on patients, which restricts available information mainly to the type of sample material, results of direct microscopy observation, and isolates' growth conditions. Isolates recovered from nonsterile sites may readily be categorized as contaminants or as common colonizers if essential pathogenicity characteristics are lacking, such as growth at 37°C (11). Identification by phenotypic traits is often challenging, since this requires growth on selected media at various temperatures and expertise in morphological characterization. For identification of clinically relevant fungi, molecular techniques based on sequencing of specific target regions are valuable alternatives to traditional phenotypic identification procedures, since sequence-based identification is independent of growth conditions and the formation of specific morphological structures (4, 6, 8).

Together with previously defined specific criteria along a diagnostic work flow that considers observations made in the laboratory as well as clinical information provided by the physician on request, ITS sequencing proved a reliable tool to enhance the rate of identification of clinically relevant fungi. Within the study period, 726 isolates could not be identified by phenotypic characteristics, of which 233 (32.1%) were selected on the basis of clinical relevance for further molecular identification (see Table S1 in the supplemental material). Sequence analysis of the ITS regions clearly improved identification in comparison with traditional morphology-based identification, assigning 78.6% of the analyzed 233 isolates to a defined taxon (Table 1). Identification at species level was possible for 57.1% of the sequenced isolates, and 21.5% were assigned to genus level. In comparison, further phenotypic characterization based on prolonged cultivations resulted in species assignment for 13.3% and in genus assignment for 34.3% of these isolates (Table 1). In comparison with GenBank a higher percentage of strains could be assigned to species level by using the SmartGene validated ITS database.

The data collected in the 5-year study period revealed a substantial increase in species identification by ITS sequence analysis from 50.0% in 2005 to 80.0% in 2009. Genus level assignment remained constant at around 20%, and the isolates which could not be assigned to a given taxon decreased from

18.2% to 0.0% in 2009. While this may reflect in part the continuous updating and enlargement of databases (27), we also note that a strict adherence to clinical relevance as a selection criterion for ITS sequence analysis and increased diagnostic expertise gained during the 5-year study period reduced the number of environmental isolates selected for sequencing and thus the total number of isolates subjected to sequencing. In contrast to established pathogenic species, environmental (and presumably nonpathogenic) isolates form a frequent source for ITS sequences which cannot be assigned to established taxa, since no corresponding sequence entries are available (7).

A systematic analysis comparing phenotypic identification and molecular identification revealed that some molds are particularly difficult to identify by phenotypic traits (see Table S1 in the supplemental material). Frequently isolated pathogenic molds such as *Beauveria* spp., *Cladosporium* spp., *Penicillium* spp. (at genus level), *Paecilomyces lilacinus*, *Scedosporium apiospermum*, *Scopulariopsis brevicaulis*, and *Aspergillus* spp. (at species level) are readily identified on the basis of morphological characteristics. In contrast, reliable identification of rarely encountered pathogens such as *Aureobasidium pullulans*, *Fusarium oxysporum*, *Fusarium solani*, *Penicillium marneffei*, *Phoma glomerata*, *Schizophyllum commune*, and *Scytalidium dimidiatum* was possible only by sequence analysis (see Table S1 in the supplemental material).

Efficient extraction of high-quality DNA is crucial for PCR amplification and sequence analysis. The use of sufficient fungal starting material is essential, since the amount of DNA per mycelial mass may vary in different growth stages. In most cases, the use of 2 to 4 cm² of mycelium taken from solid medium was found to be sufficient for successful DNA extraction. A wide spectrum of fungal species, which may show variations in cell wall composition and in genomic ITS copies, hampers the implementation of a universal DNA extraction method (2, 22, 33). In response, we applied a stepwise DNA extraction procedure using two different protocols (Fig. 1). Amplification of the ITS regions of some fungal isolates failed despite several attempts. This may reflect unsuccessful DNA extraction due to (i) low levels of DNA in the mycelial mass or (ii) inefficient disruption of the cell wall, possibly due to unknown modifications of cell wall composition. Alternatively, the PCR may have been inhibited by fungal components released during cell disruption. Few of the isolates for which amplification of the ITS regions was not successful were identified by further phenotypic investigations, indicating that these isolates belonged to various genera. Several isolates remained unidentified, since morphological characteristics did not form despite prolonged incubation. Interestingly, one out of the nine *Aspergillus fumigatus* isolates did not yield a successful amplification of the ITS regions (see Table S1 in the supplemental material). It was noted that this isolate showed only sparse sporulation after repeated UV induction. Presumably, the amount of extracted DNA obtained from the mycelium was not sufficient for proper gene amplification.

A literature survey indicates that this study is the first and most exhaustive report on systematic implementation of ITS sequencing for the purpose of mold identification in a diagnostic mycology laboratory. We defined a set of criteria and a diagnostic work flow for subjecting isolates to molecular iden-

tification. In comparison, available studies mainly reported on nonsystematic approaches analyzing smaller sets of isolates to address a more limited question (1, 34, 39). The initial phenotypic part of our identification strategy including standard morphological characteristics can be readily adapted to the individual laboratory expertise or could be complemented by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry in the near future (24, 32). ITS sequence analysis is a valuable diagnostic tool in the medical mycology laboratory, with a high percentage of species identification for molds which are difficult to identify by standard phenotypic characteristics. The number of isolates subjected to sequence analysis can be reduced significantly (in this study from 726 to 233 isolates) based on defined selection criteria, resulting in cost reduction without loss of clinical diagnostic quality. In conclusion, systematic and efficient combination of phenotypic and molecular procedures substantially improves identification of mold isolates in the diagnostic laboratory.

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