Review Article

International Society of Human and Animal Mycology (ISHAM)-ITS reference DNA barcoding database—the quality controlled standard tool for routine identification of human and animal pathogenic fungi

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Abstract

Human and animal fungal pathogens are a growing threat worldwide leading to emerging infections and creating new risks for established ones. There is a growing need for a rapid and accurate identification of pathogens to enable early diagnosis and targeted antifungal therapy. Morphological and biochemical identification methods are time-consuming and require trained experts. Alternatively, molecular methods, such as DNA barcoding, a powerful and easy tool for rapid monophasic identification, offer a practical approach for species identification and less demanding in terms of taxonomical expertise. However, its wide-spread use is still limited by a lack of quality-controlled reference databases and the evolving recognition and definition of new fungal species/complexes. An international consortium of medical mycology laboratories was formed aiming to establish a quality controlled ITS database under the umbrella of the ISHAM working group on "DNA barcoding of human and animal pathogenic fungi." A new database, containing 2800 ITS sequences representing 421 fungal species, providing the medical community with a freely accessible tool at http://www.isham.org/ and http://its.mycologylab.org/ to rapidly and reliably identify most agents of mycoses, was established. The generated sequences included in the new database were used to evaluate the variation and overall utility of the ITS region for the identification of pathogenic fungi at intra-and interspecies level. The average intraspecies variation ranged from 0 to

2.25%. This highlighted selected pathogenic fungal species, such as the dermatophytes and emerging yeast, for which additional molecular methods/genetic markers are required for their reliable identification from clinical and veterinary specimens.

Introduction

The number of human and animal fungal infections, ranging from superficial infections of the nails and skin, through mucocutaneous candidiasis to invasive fungal infections, have significantly increased over the last three decades, causing serious public health burdens and increased risk of biodiversity loss among animal species [1,2]. In humans, superficial infections affect an estimated 25% (= 1.7 billion) individuals world-wide. Oropharyngeal or genital mucosal infections are also common and can be disabling. For example, an estimated 75% of women of childbearing age suffering from vulvovaginitis, mainly caused by *Candida* species [3], which are the third most common opportunistic fungal disease agents after *Aspergillus* spp. worldwide [1]. Invasive fungal diseases are of great concern, due to their high mortality that can exceed 50%. More than 90% of fungal-related deaths are caused by four fungal genera: *Aspergillus, Candida, Cryptococcus,* and *Pneumocystis* [1,4,5]. Delays in diagnosis are not only associated with high mortality but also severe organ dysfunction, for example, respiratory failure (endemic fungal infections and chronic pulmonary aspergillosis), neurologic deficits (endemic fungal infections and cryptococcosis) [6], blindness and visual impairment (fungal keratitis) [7]. To better understand, control, and treat these diseases, more rapid and accurate identification of the causal agents is essential.

DNA barcoding, first proposed by Hebert et al. [8], utilizes DNA sequences to standardize the identification of organisms from all kingdoms to the species level by comparison to a reference collection of well-identified species. The principle behind barcoding is that species identification must be accurate, fast, cost-effective, culture independent, universally accessible, and feasible for nonexperts [9]. As a consequence, its popularity as a species identification tool has drastically increased. Barcodes are short diverse genetic sequences (500–800 bp) that are flanked by conserved regions allowing for the design of universal primers. From a pragmatic perspective, a universal sequence suitable for all kingdoms would be ideal, but the identification of a universal genetic region for a wide range of taxa remains elusive. The key concepts underlying barcoding are that the interspecies distances should exceed intraspecies distances, creating a barcoding gap [10], and that identifi-

cation is straightforward when a sequence is unique to a single species and constant within each species [8,11,12]. The most important question in barcoding is: How accurate and reliable are the delineation and identification of a species using a single gene?

The correct identification of fungi is essential for many biological purposes, such as the assessment of biodiversity, taxonomy and species conservation [9,13]. It is mandatory for clinical diagnosis and early initiation of appropriate antifungal therapy. Traditional identification based on morphology and biochemistry of pathogenic fungi is time-consuming and requires a certain level of morphological and taxonomical expertise. To overcome these limitations, DNA barcoding was evaluated in fungi, targeting numerous genetic loci, including *COX1* [14], protein-coding genes like RNA polymerase I and II [15–19], partial translation elongation factor 1-α [20–22], β-tubulin [23], and the internal transcribed spacer (ITS) regions [24,25]. The protein coding genes have proven to be a powerful tool for species delimitation, providing a high level of phylogenetic resolution and information [21,26,27]. However, the primers used to amplify these regions are usually restricted to specific taxa and amplification can often be problematic [16]. In contrast, the ITS regions are easily amplified with universal primers that are compatible among most fungal species. It has shown sufficient genetic variability for identification at interspecies level, and has been adopted as the official standard barcoding region for fungi [28]. However, use of the ITS region as a barcode has been criticized by Kiss [29] because of its inability to distinguish many closely related fungal species. In addition, for some fungi, the ITS regions alone do not provide accurate identification to species level [30]. In some groups of fungi (*Aspergillus*, *Colletotrichum*) the interspecies variation is insignificant [31,32] and in other groups (*Glomeromycota*, *Chytridiomycota*) the diversity within species is too high [33,34] Fungal genomes may contain more than 200 copies of the ribosomal region [35,36] dispersed over one or more chromosomal locations [37]. This results in polymorphism within a genome of one individual [38,39]. Intragenomic diversity is mainly explained by concerted evolutionary processes, for example, unequal crossing over between repeat units, gene conversion or gene amplification [39,40].

Despite these limitations the ITS region has been used in molecular identification and phylogenetic studies of human pathogenic fungi [41–48] long before its selection as the official fungal DNA barcode. The ITS sequences in publicly accessible databases are used routinely by the medical community to identify fungi at the species level on the basis of matching sequences. However, its widespread application has been compromised by the deposition of incorrectly identified or incomplete sequences in the commonly used public databases of the International Nucleotide Sequence Database Collaboration (INSDC) [49]. This includes GenBank [50], at the National Center for Biotechnology Information (NCBI), which is the major nucleotide sequence depository and is widely utilised by clinical microbiologists and the scientific community [51,52]. Because GenBank acts primarily as an archive, many sequences submitted have been annotated with incorrect or poorly defined species names. It has also been shown that more than 10% of the publicly available fungal ITS sequences were annotated incorrectly at species level [53]. As a consequence, a number of curated ITS databases have been created to ensure the correct identification of fungal species, for example, within the Barcode of Life Data System (BOLD) [54] and UNITE [55]. Partially in response to requests to allow third party annotation of GenBank records NCBI has also initiated a curated database RefSeq Targeted Loci (RTL) [56] that will provide a limited set of curated sequences obtained from type and verified material [57]. In a second, broader approach NCBI is currently annotating the type material associated with taxonomic names. This will allow type related searches to be conducted across multiple sequence markers or whole genomes [58]. Other reference databases are available for specific taxonomic groups, for example, *Fusarium* [59] and *Aspergillus* [60]. The deficiency of these reference databases with respect to human pathogenic fungi is the limited number of medically important fungal species contained within them. The demand for curated, reliable reference databases has increased significantly due to diminishing expertise in fungal morphology and its increasing replacement by the use of sequencing in fungal diagnostic laboratories.

To address these issues, a working group of the International Society for Human and Animal Mycology (ISHAM) on "Barcoding of Medical Fungi" was established in 2011 [61]. The working group identified the need to: (a) generate a medical barcode database by incorporating existing fungal group-specific databases; (b) extend the number of quality-controlled ITS sequences to cover all medically important fungal species; (c) evaluate the value of ITS as a barcode at intra-and interspecies level; (d) eventually incorporate these sequences into the BOLD database; (e) UNITE;

and (f) achieve a species status as "quality controlled reference sequences" for those sequences within RTL at NCBI.

The main objective of this study was to generate a publicly available, quality-controlled, ITS reference database for human and animal pathogenic fungal species and to evaluate the applicability of ITS sequences (the official barcode for fungi) as a genetic marker for species identification. The secondary aim was to highlight fungal taxa where additional genetic sequence information is recommended beyond the ITS for a more accurate identification.

Materials and methods

Generating the database

The ISHAM-ITS reference database is a result of an international collaboration between 14 medical mycology laboratories representing three continents (Table 1). The contributors provided a total of 2945 ITS sequences. Species were identified based on polyphasic identification including morphology, biochemical and physiological tests when appropriate and sequencing. After collecting all the data, the overall identity of sequences obtained from more than two strains per species was determined, including available type strains. In the case of species with less than two strains, trace files were checked for the quality and integrity of sequences. A total of 145 sequences that did not meet the inclusion criteria were discarded, as well as sequences that were misidentified or not identified to species level. Each taxon was provided with the taxonomic name, taking into account the "One Name = One Fungus" concept of the International Code of Nomenclature for algae, fungi and plants (ICN) [62]. The current taxonomical names were provided by using online nomenclature data resources such as MycoBank [63,64], Index Fungorum [65], the latest edition of The Yeasts [66], as well as the latest publications and consulting taxonomical experts of specific taxa. Where possible, former anamorph or teleomorph names and the most-used synonyms were also listed to facilitate reading for clinicians.

DNA isolation, amplification and sequencing

DNA was isolated and purified from cultures using the methods routinely used in the contributing laboratories. A number of fungal-specific universal primers (Table 2) were used to amplify the ITS region, polymerase chain reaction (PCR), and sequencing protocols varied from laboratory to laboratory according to the primers, chemical reagents, and thermocyclers used. Primers used differed depending on the fungal species investigated or starting material used.

Table 1. Institutions, number of quality controlled ITS sequences, and represented number of species contributed to the ISHAM-ITS reference database.

Institutions	Number of strains	Number of species
Molecular Mycology Research Laboratory, CIDM, Sydney Medical School-Westmead Hospital, The University of Sydney, WMI, Australia	663	173
Mycology Research Laboratory, Department of Microbiology, Medical School, the University of Athens Hellenic Collection of Pathogenic Fungi (UOA/HCPF), National and Kapodistrian University of Athens, Athens, Greece	417	117
Unitat de Microbiologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Spain	360	52
CBS-KNAW, Fungal Biodiversity Centre, Utrecht, The Netherlands	352	33
BCCM/IHEM, Biomedical fungi and yeasts collection, Scientific Institute of Public Health, Brussels, Belgium	289	92
Institut Pasteur, National Reference Center of Invasive Mycosis and Antifungals, Molecular Mycology Unit, CNRS URA 3012, Paris, France	223	106
Parasitology - Mycology, APHM, CHU Timone-Adultes, Marseille, France; Aix-Marseille University, UMR MD3 IP-TPT, Marseille, France	146	55
Mycology Laboratory, Department of Microbiology and Infectious Diseases, PathWest Laboratory Medicine WA, QEII Medical Centre, Nedlands, Western Australia, Australia	99	31
BDEEP-EA4547, CIIL, Institut Pasteur de Lille, CHU de Lille, Université de Lille2, Lille, France	73	18
Laboratório Especial de Micologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil	58	18
Instituto de Pesquisa Clínica Evandro Chagas (IPEC) - Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil	50	1
Facultad de Medicina, Departamento de Microbiología y Parasitología (Unidad de Micología), Universidad Nacional Autónoma de México, Ciudad de México, México	39	3
Centre of Molecular and Environmental Biology (CBMA), Biology Department, School of Sciences, University of Minho, Braga, Portugal	22	10
Universidade Federal de Goiás, Instituto de Ciências Biológicas, Laboratório de Biologia Molecular, Goiânia, Goiás, Brazil	9	2

In general ITS1, ITS3, ITS4 and ITS5 [67] are universal ribosomal primers, which are recommended being used if the amplification is based on pure fungal cultures. The primers SR6R and LR1 [68], V9D, V9G and LS266 [69] and ITS1F [70] have subsequently been designed to be fungal specific, they can be used for amplification based on pure culture as well as directly form clinical specimens, as they will avoid co-amplification of human DNA. The general PCR amplification conditions are given for each of the primer pairs in Table 2 [67–72]. All PCR products were sequenced in both the forward and reverse directions. Bidirectional sequences were assembled and edited using Sequencher® [73]. Trace files were manually checked and ambiguous bases were corrected based on the forward and reverse sequences taking into account the PHRED scores received with the sequence trace files.

Data analysis

The length, continuity and annotation of the ITS sequences were checked using ITSx 1.0.7. [74] and membership in one species was verified by centrality analysis [75] using the software BioloMICS ver. 7.5.44 [76]. Briefly, sequences of each species were aligned to find the "central sequence", which is the one having the highest average similarity to other members of the group. Questionable sequences that were very divergent from their central sequence, therefore doubtful as clear members of a species, were removed from further analyses. The sequences for each taxon were aligned using the program CLUSTALW [77] that is part of the software MEGA ver. 5.2.2 [78]. Resulting multiple alignments were then checked visually and edited when needed. For further analyses, the sequences were truncated at conserved sites to obtain equal 3′- and 5′-endings.

The intraspecies diversity was estimated by calculating the average nucleotide diversity (π), which gives the proportion of nucleotide differences in all haplotypes in the studied sample, the number of segregating polymorphic sites (S), and the proportion of polymorphic sites on base pair basis in a sample (Theta, Θ) of each species with sequences from more than two strains, using the software DnaSP ver. 5.10.01 [79].

Table 2. Primers and amplification conditions used to amplify ITS sequences maintained in the ISHAM-ITS reference database.

Primers	Amplification conditions
SR6R (5′ AAGTATAAGTCGTAACAAGG 3′) and LR1 (5′ GGTTGGTTTCTTTTCCT 3′)[68]	97ºC for 3 min; 30 cycles of denaturation (94ºC for 35 s), annealing (50ºC for 45 s), and extension (72ºC for 45 s); and a final extension step at 72ºC for 7 min
ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′)[67]	94ºC for 3 min; 35 cycles of denaturation (94ºC for 60 s), annealing (56ºC for 60 s), and extension (72ºC for 2 min); and a final extension step at 72ºC for 7 min
ITS5 (5′ GGAAGTAAAAGTCGTAACAAGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′)[67]	94ºC for 5 min.; 35 cycles of denaturation (94ºC for 30 s), annealing (55ºC for 1 min), and extension (72ºC 1 min and 20 s); and a final extension step at 72ºC for 7 min
ITS5 (5′ GGAAGTAAAAGTCGTAACAAGG 3′) and NL4b (5′ GGATTCTCACCCTCTATGAC 3′)[67,71]	94ºC for 5 min.; 35 cycles of denaturation (94ºC for 30 s), annealing (53ºC for 1 min), and extension (72ºC 1 min and 30 s); and a final extension step at 72ºC for 7 min
V9D (5′ TTAAGTCCCTGCCCTTTGTA 3′) and LS266 (5′ GCATTCCCAAACAACTCGACTC 3′)[69]	95ºC for 10 min; 30 cycles of denaturation (94ºC for 30 s), annealing (58ºC for 30 s), and extension (72ºC for 30 s); and a final extension step at 72ºC for 10 min
V9G (5′ TTACGTCCCTGCCCTTTGTA 3′) and LS266 (5′ GCATTCCCAAACAACTCGACTC 3′)[69]	94ºC for 5 min; 35 cycles of denaturation (94ºC for 60 min), annealing (56ºC for 30 s), and extension (72ºC for 2 min); and a final extension step at 72ºC for 10 min
ITS1F (5′ CTTGGTCATTTAGAGGAAGTAA 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′)[67,70]	95ºC for 5 min; 30 cycles of denaturation (95ºC for 30 s), annealing (58ºC for 30 s), and extension (72ºC for 1 min); and a final extension (72ºC for 10 min).
ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and IT2 (5′ CCTCCGCTTATTGATATGCTTAGG 3′)[67,72]	94ºC for 3 min; 35 cycles of denaturation (94ºC for 45 s), annealing (52ºC for 45 s), and extension (72ºC for 60 s); and a final extension at 72ºC for 7 min
ITS3 (5′ GCATCGATGAAGAACGCAGC 3′) and LS266 (5′ GCATTCCCAAACAACTCGACTC 3′)[67,69]	95ºC for 10 min; 30 cycles of denaturation (94ºC for 30 s), annealing (58ºC for 30 s), and extension (72ºC for 30 s); and a final extension step at 72ºC for 10 min

For interspecies analyses, all taxa were subjected to pair-wise sequence divergence calculations using the Kimura 2-parameter distance model (K2P) [80] using MEGA ver. 5.2.2. [78]. This model provides the best metric when genetic distances are low [81].

Barcoding gaps were evaluated by comparing the distribution of interspecies to intraspecies divergence within taxa sharing the same phylogenetic lineage [10]. In total, 17 barcoding gap analyses (of genera and phylogenetic clades), including two variants of the analysis for *Cryptococcus neoformans/Cryptococcus gattii* and *Arthrodermataceae/Trichophyton,* were performed (Table 3).

Sequence data were stored in BioloMICS ver. 7.5.44 [76] and statistical analyses were carried out in the statistical environment R [82].

Definitions

Species = a well-defined organism with a proven clinical relevance. Species complex = are organisms which form a cryptic species for which currently no proven evidence of individual medical relevance is known [83].

Results

Establishment of the quality controlled ISHAM-ITS reference database

A quality-controlled ITS reference database for human and animal pathogenic fungi was established as the result of the collaboration between 14 mycology laboratories from three continents. Altogether, the participating laboratories generated complete ITS (ITS1-5.8S-ITS2) sequences representing most of the pathogenic fungi. The number of ITS sequences and species contributed are shown in Table 1. According to the most recent taxonomic nomenclature, many species with different synonyms proved to be identical. Each sequence was associated with the current taxonomic species name, as well as with the most commonly used scientific names, used in a clinical setting. The sequences are freely accessible at http://www.isham.org/, directly from http://its.mycologylab.org/ or as specifically labelled ISHAM-ITS sequences in GenBank and UNITE. Of the 421 fungal species contained in the ISHAM-ITS sequences 71 representing the type culture of the species have also been submitted RTL at NCBI, following the principles laid out in Schoch et al. [57].

Table 3. Intraspecies diversity of the 176 fungal species with more than two strains in the ISHAM-ITS reference database.

Species	Number of strains	Number of nucleotide sites	Nucleotide diversity (π)	Number of polymorphic sites (S)	Proportion of polymorphic sites in a sample (Θ)	ITS is sufficient for identification
Acremonium fusidioides	3	520	0.00641	5	0.00641	yes
Acremonium implicatum	6	498	0.00375	5	0.000887	yes
Acremonium persicinum	6	494	0.00067	1	0.000887	yes
Alternaria alternata	7	475	0	0	0	yes
Alternaria infectoria	7	475	0	0	0	yes
Arthrographis kalrae	21	480	0.00091	2	0.001158	yes
Arthropsis hispanica	4	598	0.00251	3	0.002736	yes
Aspergillus calidoustus	5	482	0	0	0	yes
Aspergillus flavus	36	499	0.00071	1	0.000483	yes
Aspergillus fumigatiaffinis	4	505	0	0	0	yes
Aspergillus fumigatus	83	463	0.00094	6	0.002597	yes
Aspergillus hiratsukae	3	502	0.00531	4	0.005312	yes
Aspergillus nidulans	17	473	0.00047	1	0.000625	yes
Aspergillus niger	19	392	0	0	0	yes
Aspergillus ochraceus	3	491	0.00272	2	0.002716	yes
Aspergillus sydowii	3	480	0.00417	3	0.004167	yes
Aspergillus terreus	27	464	0.00061	2	0.001118	yes
Aspergillus tubingensis	18	425	0	0	0	yes
Aspergillus versicolor	6	433	0.00631	5	0.005057	yes
Aureobasidium pullulans	20	459	0.00764	15	0.009083	yes
Bipolaris cynodontis	9	376	0.00059	1	0.000981	yes
Bipolaris micropus	3	455	0.00147	1	0.001465	yes
Blastobotrys adeninivorans	4	547	0.00146	2	0.001755	yes
Blastobotrys raffinosifermentans	3	517	0.00387	3	0.003868	yes
Candida albicans	44	440	0.00298	10	0.005225	yes
Candida blankii	7	459	0	0	0	yes
Candida carpophila	3	602	0.00337	4	0.003681	yes
Candida catenulata	13	378	0.00122	1	0.000853	yes
Candida deformans	14	320	0.0077	7	0.008244	yes
Candida diddensiae	3	541	0	0	0	yes
Candida dubliniensis	16	451	0.00111	4	0.002673	yes
Candida duobushaemulonis	4	295	0	0	0	yes
Candida glabrata	29	791	0.00485	22	0.007304	yes
Candida haemulonis	6	285	0	0	0	yes
Candida inconspicua	7	413	0.0063	7	0.007423	yes
Candida intermedia	6	299	0.01672	12	0.017577	yes
Candida mesorugosa	13	314	0.00449	5	0.005131	yes
Candida metapsilosis	14	410	0.00397	4	0.003068	yes
Candida orthopsilosis	28	413	0.00255	5	0.005907	yes
Candida palmioleophila	3	632	0.00422	4	0.004219	yes
Candida parapsilosis	109	408	0.00014	2	0.000933	yes
Candida pararugosa	7	412	0.01133	11	0.010898	yes
Candida tropicalis	27	432	0.00352	13	0.007807	yes
Candida zeylanoides	4	579	0	0	0	yes
Cladophialophora bantiana	3	626	0	0	0	yes
Cladophialophora boppii	4	543	0.00184	2	0.002009	yes
Cladophialophora carrionii	6	538	0.00372	6	0.004884	yes
Clavispora lusitaniae	45	293	0.02248	22	0.018258	no
Cryptococcus albidus	18	583	0.00577	21	0.010472	yes
Cryptococcus carnescens	6	485	0	0	0	yes
Cryptococcus diffluens	3	612	0.00109	1	0.001089	yes
Cryptococcus gattii VGI	33	463	0.00108	1	0.000536	yes
Cryptococcus gattii VGII	41	463	0	0	0	yes

Table 3. *continued*

Species	Number of strains	Number of nucleotide sites	Nucleotide diversity (π)	Number of polymorphic sites (S)	Proportion of polymorphic sites in a sample (Θ)	ITS is sufficient for identification
Cryptococcus gattii VGIII	24	463	0	0	0	yes
Cryptococcus gattii VGIV	13	463	0	0	0	yes
Cryptococcus laurentii	6	444	0.00495	4	0.003946	yes
Cryptococcus magnus	6	522	0	0	0	yes
Cryptococcus neoformans var. *grubii* VNI	22	452	0	0	0	no
Cryptococcus neoformans var. *grubii* VNII	13	460	0	0	0	no
Cryptococcus neoformans var. *neoformans* VNIV	17	463	0	0	0	yes
Curvularia aeria	27	442	0.00311	11	0.006457	yes
Curvularia borreriae	4	572	0.00322	3	0.002861	yes
Curvularia geniculata	15	503	0.00101	2	0.00125	yes
Curvularia hawaiiensis	20	379	0.00136	1	0.000755	yes
Curvularia inaequalis	6	518	0.00129	2	0.001691	yes
Curvularia lunata	10	467	0.00107	1	0.000788	yes
Curvularia protuberata	3	562	0	0	0	yes
Curvularia sorghina	4	490	0.00102	1	0.001113	yes
Curvularia spicifera	37	367	0.00044	3	0.001958	yes
Curvularia verruculosa	6	524	0	0	0	yes
Cyberlindnera jadinii	7	520	0.00769	10	0.007849	yes
Debaryomyces hansenii	15	540	0.00187	3	0.001709	yes
Epidermophyton floccosum	5	692	0.00058	1	0.000694	yes
Exophiala bergeri	9	495	0.01016	12	0.00892	yes
Exophiala dermatitidis	22	539	0.00347	9	0.004777	yes
Exophiala exophialae	3	538	0.00124	1	0.001239	yes
Exophiala jeanselmei	26	470	0.00349	10	0.005576	yes
Exophiala oligosperma	62	460	0.00165	3	0.001389	yes
Exophiala spinifera	23	501	0.00841	16	0.008653	yes
Exophiala xenobiotica	39	476	0.00458	18	0.008838	yes
Exserohilum rostratum	37	411	0.00197	10	0.00532	yes
Filobasidium uniguttulatum	4	616	0.00081	1	0.000885	yes
Fonsecaea monophora	22	528	0.00634	17	0.008832	yes
Fonsecaea nubica	3	512	0.00586	6	0.006392	yes
Fonsecaea pedrosoi	32	483	0.00132	5	0.00257	yes
Fusarium delphinoides	3	526	0	0	0	yes
Fusarium falciforme	7	458	0	0	0	no
Fusarium keratoplasticum	8	469	0.00213	6	0.004236	no
Fusarium oxysporum	14	455	0.00128	2	0.001382	yes
Fusarium petroliphilum	6	481	0.00091	1	0.00071	no
Fusarium proliferatum	11	451	0.00073	1	0.000757	yes
Fusarium solani	9	466	0.01788	21	0.016581	no
Fusarium verticillioides	17	455	0	0	0	yes
Galactomyces candidus	6	333	0.01782	10	0.013152	yes
Hanseniaspora uvarum	3	633	0.00316	3	0.00316	yes
Histoplasma capsulatum	83	416	0.01126	38	0.018351	yes
Hormographiella aspergillata	4	566	0.00088	1	0.000964	yes
Hyphopichia burtonii	5	359	0.00501	4	0.005348	yes
Hypocrea orientalis	7	438	0.00065	1	0.000932	yes
Kazachstania pintolopesii	3	650	0.00513	5	0.005128	yes
Kluyveromyces lactis var. *lactis*	11	618	0	0	0	yes
Kluyveromyces marxianus	26	603	0.00165	5	0.002173	yes
Kodamaea ohmeri	23	341	0.01954	23	0.018275	no
Leptosphaeria senegalensis	3	573	0.00116	1	0.001163	yes
Lichtheimia corymbifera	5	650	0.00677	11	0.008123	yes
Lichtheimia ramosa	10	770	0.02214	55	0.025054	yes

Table 3. *continued*

Species	Number of strains	Number of nucleotide sites	Nucleotide diversity (π)	Number of polymorphic sites (S)	Proportion of polymorphic sites in a sample (Θ)	ITS is sufficient for identification
Lomentospora prolificans	35	475	0.00024	2	0.001022	yes
Magnusiomyces capitatus	4	365	0	0	0	yes
Medicopsis romeroi	3	467	0.00714	5	0.007138	yes
Meyerozyma caribbica	17	516	0.00155	3	0.001985	yes
Meyerozyma guilliermondii	34	516	0.00134	3	0.001444	yes
Microascus cirrosus	3	502	0	0	0	yes
Microsporum audouinii	7	666	0	0	0	yes
Microsporum canis	8	632	0	0	0	yes
Microsporum fulvum	6	617	0.00648	10	0.007098	yes
Microsporum gypseum	5	619	0	0	0	yes
Microsporum racemosum	3	556	0.00959	8	0.009592	yes
Millerozyma farinosa	3	626	0.01065	10	0.01065	yes
Mucor circinelloides	9	547	0.00792	11	0.007399	yes
Neoscytalidium dimidiatum	9	464	0.00048	1	0.000793	yes
Paracoccidioides brasiliensis	8	468	0.0148	17	0.01401	yes
Penicillium brevicompactum	3	539	0	0	0	yes
Phialemonium atrogriseum	3	524	0.00509	4	0.005089	yes
Pichia kudriavzevii	22	404	0.00206	4	0.002716	yes
Pichia manshurica	3	434	0	0	0	yes
Pichia norvegensis	14	398	0.00303	4	0.003239	yes
Pithomyces chartarum	7	568	0.00469	8	0.006168	yes
Pithomyces sacchari	6	549	0.00231	3	0.002393	yes
Purpureocillium lilacinum	5	501	0.0008	1	0.000958	yes
Rasamsonia aegroticola	10	467	0.0019	4	0.003151	yes
Rhinocladiella similis	18	497	0.00285	11	0.006435	yes
Rhizomucor pusillus	3	586	0.00341	3	0.003413	yes
Rhizopus microsporus	6	587	0.00693	8	0.005969	yes
Rhizopus oryzae	4	538	0.00217	2	0.002028	yes
Rhodotorula mucilaginosa	16	527	0.001	2	0.001144	yes
Saccharomyces cerevisiae	27	664	0.00098	7	0.002735	yes
Sarocladium kiliense	23	483	0.00546	16	0.009208	yes
Sarocladium strictum	8	484	0.00221	2	0.001594	yes
Scedosporium angustum	3	523	0.00382	3	0.003824	yes
Scedosporium apiospermum	46	497	0.00442	11	0.004587	yes
Scedosporium aurantiacum	45	497	0.00052	4	0.001841	yes
Scedosporium boydii	23	480	0.00287	9	0.005021	yes
Scedosporium dehoogii	27	518	0.0037	6	0.003005	yes
Scedosporium ellipsoideum	5	523	0.00191	2	0.001836	yes
Scedosporium minutisporum	7	520	0.00275	5	0.003925	yes
Scopulariopsis brevicaulis	17	459	0.00343	4	0.002578	yes
Scopulariopsis brumptii	7	416	0.00343	4	0.003925	yes
Scopulariopsis cinerea	5	502	0.00159	2	0.001912	yes
Scopulariopsis gracilis	12	533	0.00034	1	0.000621	yes
Scytalidium cuboideum	4	516	0.00129	1	0.001057	yes
Sporothrix schenckii	11	484	0.00255	4	0.002822	yes
Torulaspora delbrueckii	4	711	0.00563	8	0.006137	yes
Trichoderma atroviride	5	567	0.00212	3	0.00254	yes
Trichoderma citrinoviride	11	493	0.00074	2	0.001385	yes
Trichoderma harzianum	12	526	0.00599	9	0.005666	yes
Trichoderma koningiopsis	3	549	0	0	0	yes
Trichoderma longibrachiatum	20	521	0.00213	6	0.003246	yes
Trichophyton ajelloi	6	594	0.00112	2	0.001475	yes
Trichophyton erinacei	25	579	0.00541	16	0.007318	yes

Table 3. *continued*

Species	Number of strains	Number of nucleotide sites	Nucleotide diversity (π)	Number of polymorphic sites (S)	Proportion of polymorphic sites in a sample (Θ)	ITS is sufficient for identification
Trichophyton interdigitale	68	525	0.00189	4	0.001591	yes
Trichophyton mentagrophytes = T. quinckeanum	5	603	0	0	0	yes
Trichophyton persicolor	3	601	0.00111	1	0.001109	yes
Trichophyton rubrum	30	540	0.00228	4	0.00187	yes
Trichophyton schoenleinii	4	623	0	0	0	yes
Trichophyton simii	7	608	0.00157	2	0.001343	yes
Trichophyton terrestre	4	615	0	0	0	yes
Trichophyton tonsurans	6	597	0.00112	2	0.001467	yes
Trichophyton verrucosum	4	534	0	0	0	yes
Trichosporon asahii	7	447	0.00107	1	0.000913	yes
Trichosporon dermatis	4	440	0	0	0	yes
Trichosporon inkin	4	539	0.00371	4	0.004048	yes
Trichosporon montevideense	4	528	0	0	0	yes
Wickerhamomyces anomalus	37	522	0.00131	7	0.003212	yes
Yamadazyma mexicana	3	561	0.00119	1	0.001188	yes
Yamadazyma scolyti	3	622	0.00536	5	0.005359	yes
Yarrowia lipolytica	24	347	0.0062	15	0.011576	yes
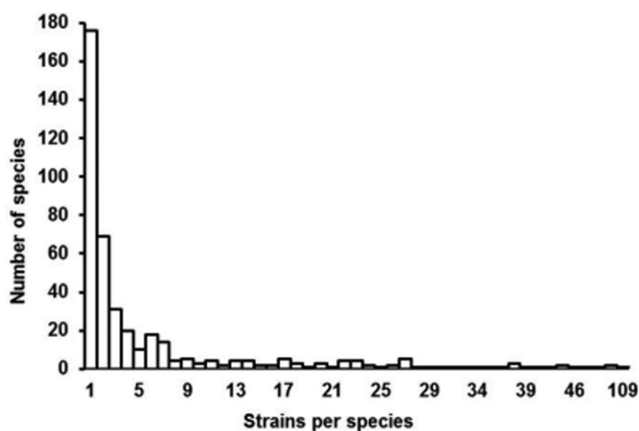
Figure 1. Distribution of the number of strains per species in the ISHAM-ITS reference database.

Figure 2. Length distribution of ITS sequences in the ISHAM-ITS reference database.

Number of sequences

At present, the quality-controlled ISHAM-ITS reference database contains 2800 complete ITS sequences representing 421 human/animal pathogenic fungal species. It contains 176 species represented by one strain, 69 species by two strains, and 176 species by a minimum of three to a maximum of 109 sequences. The distribution of strains per species was hyperbolic, meaning that the species with few strains were more frequent than those with many (Fig. 1).

Lengths of the ITS

The lengths of complete ITS sequences in the ISHAM-ITS reference database varied between 285 and 791 bp. The distribution of the number of nucleotides per se-

quence is given in Figure 2. The shortest complete ITS sequences were assigned to *Candida haemulonis* (285 bp), *Clavispora lusitaniae* (293 bp), and the longest ones to *Candida glabrata* (791 bp) and *Lichtheimia ramosa* (770 bp). The mean nucleotide length of ITS sequences in the database was 503 bp, while the median was 500 bp, indicating that the distribution of the sequence lengths was almost normal, with 0.08 skewness and 0.71 kurtosis (Fig. 2). These two metrics indicate that the population of sequences is centered around the average
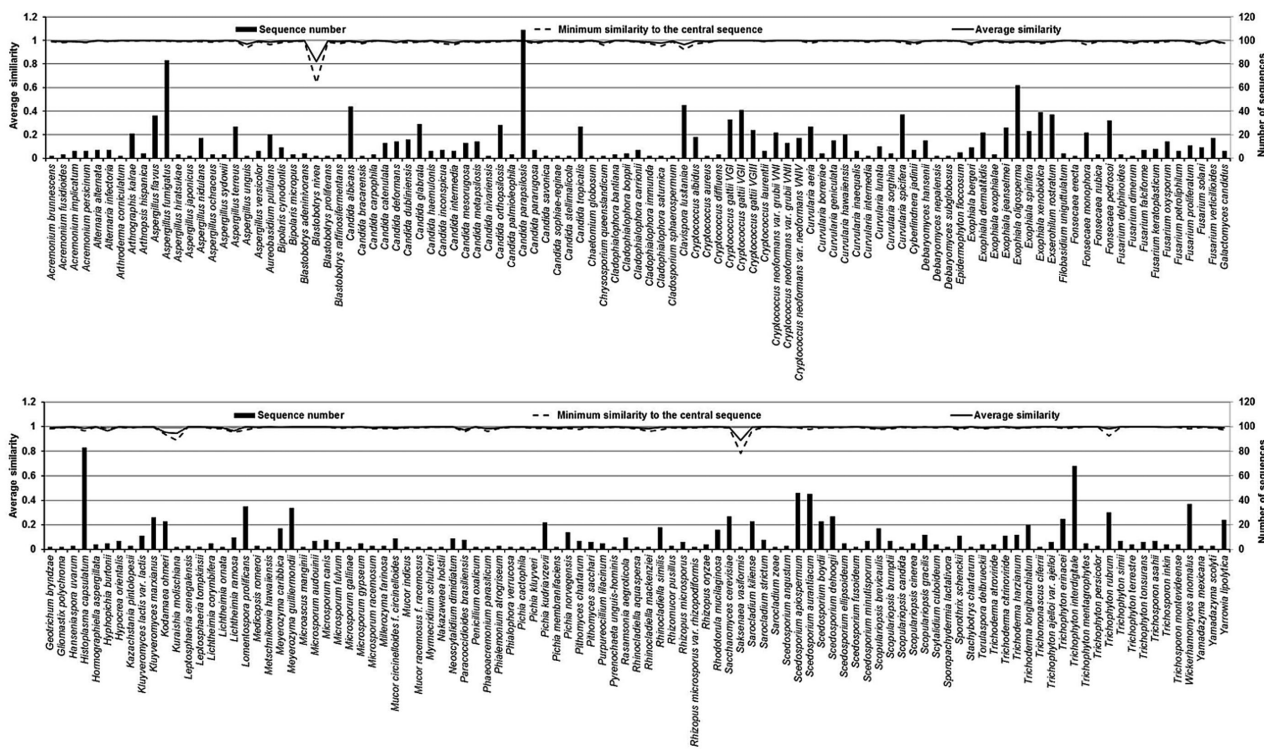
Figure 3. Average and the minimum similarity of the sequences to their central sequence as well as the number of the sequences within these species.

(skewness close to 0) and displays a more acute peak (kurtosis >0) than expected in a normal distribution. Altogether these metrics indicate that the sequences can be described rather well by a normal distribution very dense around the mean.

Quality of the database

There were 206 species, including 69 represented by only two strains, whose sequences showed diversity from the "central sequence" of the species. Figure 3 shows the average and the minimum similarity of the sequences to their central sequence as well as the number of the sequences within these species. The minimum similarity to the central sequence was less than 0.95% in the case of seven species, between 0.95–0.98% in 32 species and 0.98–0.998% in 167 species.

The average nucleotide diversity (π) was compared with the number of strains to test the hypothesis that the number of strain influences the variability. The nucleotide diversity and the number of strains did not show significant correlation, indicating that it is unlikely that the number of strains influences the variability. According to these two parameters, 160 out of the 176 species with more than two strains, were placed within a region spanning from 0 to 40 strains per species and from 0 to 1.1% variability within the species (Fig. 4). Six species (*Lichtheimia ramosa, Fusarium solani, Kodamaea ohmeri, Galactomyces*

candidus, Candida intermedia, and *Clavispora lusitaniae*) showed a high intraspecies variability of up to 2.25% based on the value of π. Nine species (*Histoplasma capsulatum, Scedosporium apiospermum, Scedosporium aurantiacum, Cryptococcus gattii* VGII, *Exophiala oligosperma, Trichophyton interdigitale, Aspergillus fumigatus, Candida parapsilosis,* and *Candida albicans*) were in a region with less than 1.1% intraspecies variability, although the number of strains per species ranged from 40 to 109. Interestingly, this group of taxa with relatively low variability includes some of the more important pathogenic fungi namely *A. fumigatus, C. parapsilosis,* and *C. albicans*.

Intraspecies genetic diversity of pathogenic fungal species in the ISHAM-ITS reference database

The two metrics of nucleotide diversity (π and Θ) generated very similar values (Table 3). The nucleotide diversity (π) estimated the proportion of nucleotide differences in all haplotypes and Θ measured the proportion of all segregating sites in a sample, thus being strongly influenced by rare haplotypes. The average nucleotide diversity per species was expressed as a percentage based on the value of π (Fig. 5).

In the ISHAM-ITS reference database, the average nucleotide diversity was less than 0.5% for 138 species, between 0.5–1.0% in 27 species, 1.01–1.5% in five species (*Exophiala bergeri, Millerozyma farinosa, H. capsulatum,*
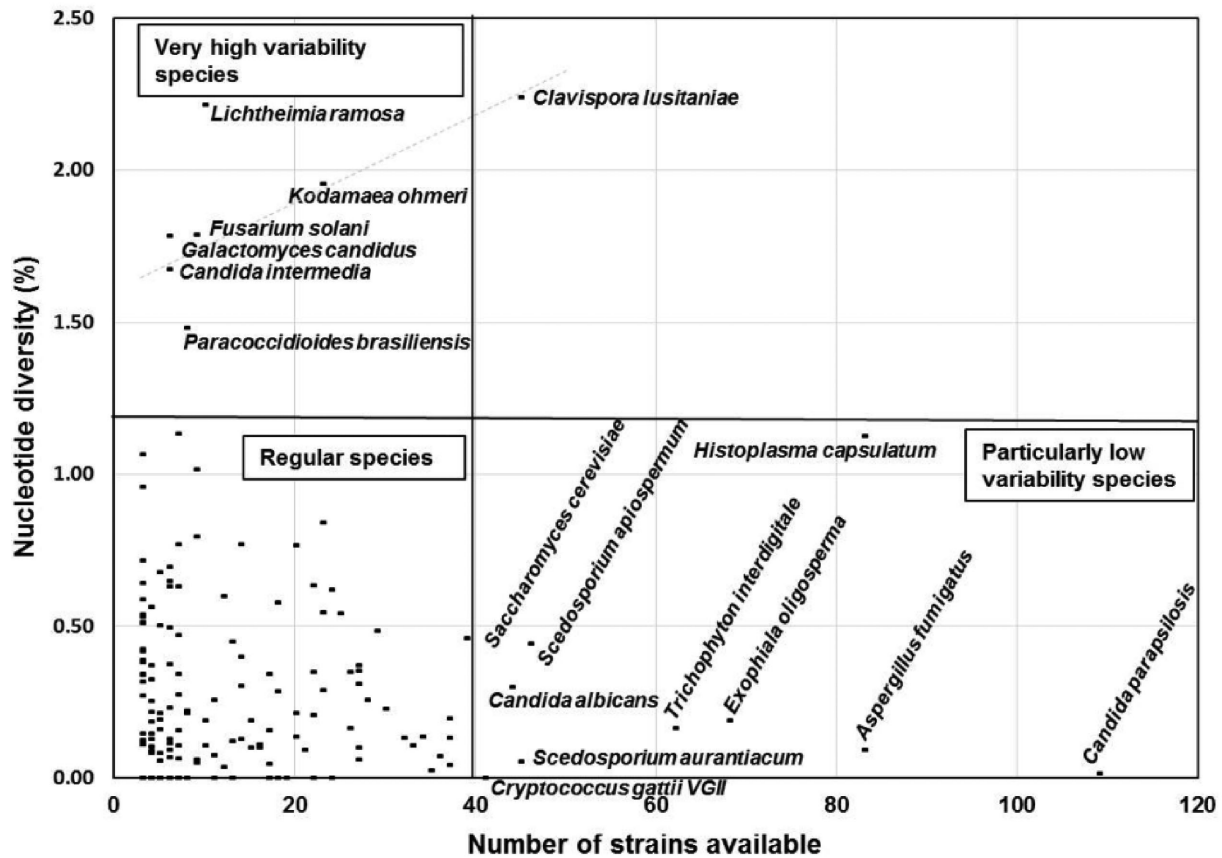
Figure 4. Nucleotide diversity (π) compared to the number of sequences by species in the ISHAM-ITS reference database.

Candida pararugosa, and *Paracoccidioides brasiliensis*), 1.5–2.0% in four species (*C. intermedia, G. candidus, F. solani,* and *K. ohmeri*), and more than 2% in two species (*Lichtheimia ramosa* and *C. lusitaniae*) (Table 3, Fig. 5).

The distribution of the distances from the "central sequence" of a species was hyperbolic, with the most frequent class, containing 63 species, representing more than one third of the species with more than two strains in the database, showing intraspecies variability ranging from 0 to 0.1%. More than half of the species with more than two strains in the database (97 species) were represented by species with less than 0.4% distance (Fig. 6).

The polymorphic site distribution showed a similar result. In 117 species, the number of polymorphic sites was less than five, in 35 species it was between five and ten, in 11 species between 11 and 15, in six species between 16 and 20 and finally more than 20 in seven species. The species with the highest number of segregating sites were *Cryptococcus albidus* (21 sites), the complex of *F. solani* (21 sites), *C. lusitaniae* (22 sites), *C. glabrata* (22 sites), *K. ohmeri* (23 sites), *H. capsulatum* (38 sites), and *L. ramosa* (55 sites) (Table 3). The value of Θ showed a strong correlation with the average nucleotide diversity

and the number of segregating sites. The proportion of rare haplotypes in a given sample was the highest in *F. solani*, *C. lusitaniae*, *K. ohmeri*, *H. capsulatum*, and *L. ramosa* (Table 3).

The intraspecies genetic analyses showed that the majority of medically important species had a low variability in ITS regions. Thus ITS sequencing can be used for the identification of most medical relevant fungal species (Table 3). The species with high intraspecies diversity within the ITS region require analysis of additional molecular markers to be reliably identified (see Table 4).

Barcoding gap analysis of the species represented in the ISHAM-ITS reference database

For the estimation of the barcoding gap the distribution of the Kimura 2-parameter (K2P) genetic distances within species and between species was calculated. In the ISHAM-ITS reference database, 17 taxonomical groups with more than two species sharing the same phylogenetic clade were identified based on previous data in MycoBank [63,64], Index Fungorum [65], and The Yeasts [66] (Table 5). The barcoding gap analysis was performed in all 17 taxa,
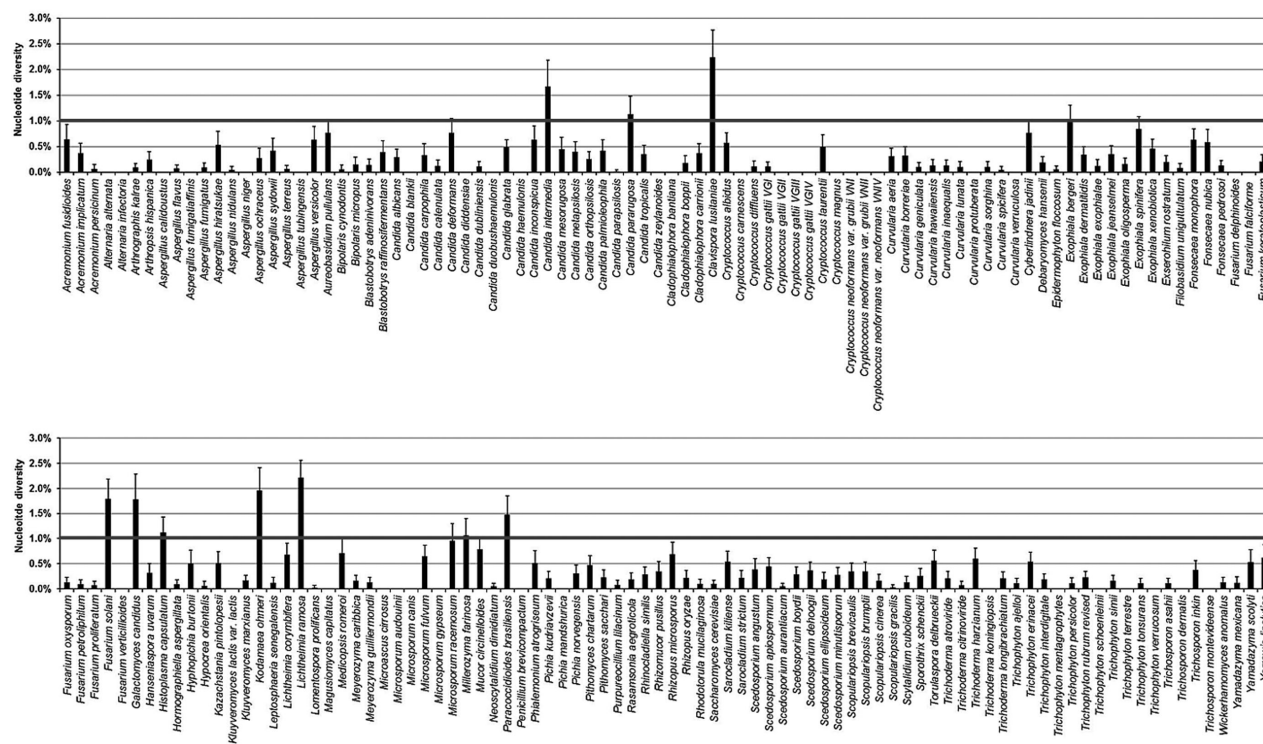
Figure 5. Average nucleotide diversity per species expressed as a percentage based on the value of π of the 176 fungal species with more than three strains in the ISHAM-ITS reference database. The error bars indicate the standard deviation of nucleotide differences.
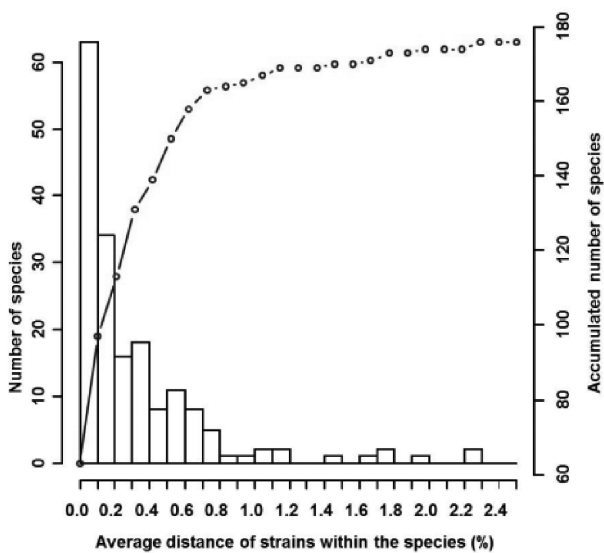

Figure 6. Distribution of average distance of s within species compared to the number of species in the ISHAM-ITS reference database.

including two versions of analysis for *C. neoformans/C. gattii* and *Arthrodermataceae/Trichophyton* (see Table 5). The distribution of genetic distances (intra- and inter-species) in each taxon is shown in Figures 7–10 and Supplementary Figures S1–S13. In 13 taxa (phylogenetic clades), a clear barcoding gap (K2P distance) was found (Table 5).

The smallest barcoding gap (0.0002) was found in the *Microsporum* spp., while the largest one was found in the *Cladophialophora* spp. (0.09). In these cases, the highest intraspecies distances were smaller than the lowest genetic distances between species, creating a barcoding gap. For the remaining four taxa *Cryptococcus* (Fig. 7), *Fusarium* (Fig. 8), *Scedosporium* (Fig. 9), and *Trichophyton* (Fig. 10), it was not possible to define a clear barcoding gap, meaning that the distributions of genetic distances within and between species overlapped.

Most of the studied taxa could be identified with the ITS barcode, although in some cases a clear discrimination could not be observed. There are two possible reasons for this: either the taxa is insufficiently studied or the ITS region is simply an inappropriate marker for discrimination between biologically consistent groups. Alternative loci and/or molecular methods are required for correct identification of these species (Table 5).

Discussion

ISHAM-ITS reference database

With a significant rise in the diversity of etiological agents of fungal infections in human and animal populations [1,2], rapid and accurate identification of pathogenic

Table 4. Taxa with high ITS diversity and alternative methods to be used for their reliable identification.

Taxa	Proposed alternatives
Clavispora lusitaniae	Morphological identification by mating unknowns with a strain of known mating type[89,90]
Fusarium solani species complex (FSSC)	MLST[116]; translation elongation factor 1-α (*TEF-1α*), RNA polymerase II gene (*RPB2*), secondary metabolite profiles[94]
Kodamaea ohmeri	Further taxonomic studies needed
Lichtheimia spp.	D1/D2 region, translation elongation factor 1-α (*TEF-1α*)[102]; MALDI-TOF[105]
Cryptococcus	AFLP[110]; PCR fingerprinting, RFLP of orotidine monophosphate pyrophosphorylase gene (*URA5*)[111]; MLST[114]
Scedosporium	β -tubulin (*BT2*)[122], AFLP[121]; LSU[124]
Arthrodermataceae	RAPD, PCR fingerprinting, AFLP, microsatellite markers[102]

Table 5. Barcoding gap based on Kimura 2-parameter genetic distances in 17 studied phylogenetic clades represented by more than two species, with two variants of analysis for *Cryptococcus neoformans/Cryptococcus gattii*, and *Arthrodermataceae/Trichophyton* in the ISHAM-ITS reference database.

| Taxa	Barcoding gap	Species included in the analyses represented with more than two strains by species
Acremonium	0.055	*Acremonium fusidioides; A. implicatum; A. persicinum; Phialemonium atrogriseum; Sarocladium kiliense; S. strictum;*
Arthrodermataceae	0.002	*Arthroderma benhamiae; A. fulvum; A. gypseum; A. insingulare; A. otae; A. persicolor; A. simii; A. uncinatum; A. vanbreuseghemii*
Aspergillus	0.002	*Aspergillus calidoustus; A. flavus; A. fumigatiaffinis; A. fumigatus; A. hiratsukae; A. nidulans; A. niger; A. ochraceus; A. sydowii; A. terreus; A. tubingensis*
Cladophialophora	0.09	*Cladophialophora bantiana; C. boppii; C. carrionii*
Cryptococcus (*Filobasidiella* clade divided into three taxa)	–	*Cryptococcus gattii; C. neoformans* var. *grubii; C. neoformans* var. *neoformans*
Cryptococcus (*Filobasidiella* clade divided into seven taxa)	–	*Cryptococcus gattii* VGI; *C. gattii* VGII; *C. gattii* VGIII; *C. gattii* VGIV; *C. neoformans* var. *grubii* VNI; *C. neoformans* var. *grubii* VNII; *C. neoformans* var. *neoformans* VNIV
Curvularia	0.001	*Curvularia aeria; C. borreriae; C. inaequalis; C. geniculata; C. hawaiiensis; C. inaequalis; C. lunata; C. protuberata; C. spicifera; C. sorghina; C. verruculosa*
Debaryomycetaceae (*Lodderomyces* clade)	0.001	*Candida albicans; C. dubliniensis; C. metapsilosis; C. orthopsilosis; C. parapsilosis; C. tropicalis; Debaryomyces hansenii*
Exophiala	0.015	*Exophiala bergeri; E. dermatitidis; E. exophialae; E. jeanselmei; E. oligosperma; E. spinifera; E. xenobiotica*
Fusarium	–	*Fusarium delphinoides; F. falciforme; F. oxysporum; F. proliferatum; F. solani; F. keratoplasticum; F. petroliphilum; F. verticillioides*
Metschnikowiaceae	0.0603	*Candida duobushaemulonis; C. haemulonis; C. intermedia; C. lusitaniae; Kodamaea ohmeri*
Microsporum	0.0002	*Microsporum audouinii; M. canis; M. fulvum; M. gypseum*
Pichiaceae	0.005	*Pichia kudriavzevii; P. norvegensis; P. manshurica*
Saccharomycetaceae	0.009	*Kluyveromyces marxianus; K. lactis* var. *lactis; Saccharomyces cerevisiae; Torulaspora delbrueckii*
Scedosporium	–	*Scedosporium angustum; S. apiospermum; S. aurantiacum; S. boydii; S. dehoogii; S. ellipsoideum; S. minutisporum*
Scopulariopsis	0.0034	*Scopulariopsis brevicaulis; S. brumptii; S. cinerea; S. gracilis*
Trichophyton	–	*Trichophyton ajelloi; T. erinacei; T. interdigitale; T. mentagrophytes (= T. quinckeanum); T. rubrum; T. schoenleinii; T. simii; T. terrestre; T. verrucosum*
Trichosporon	0.004	*Trichosporon asahii; T. dermatis; T. inkin; T. montevideense*
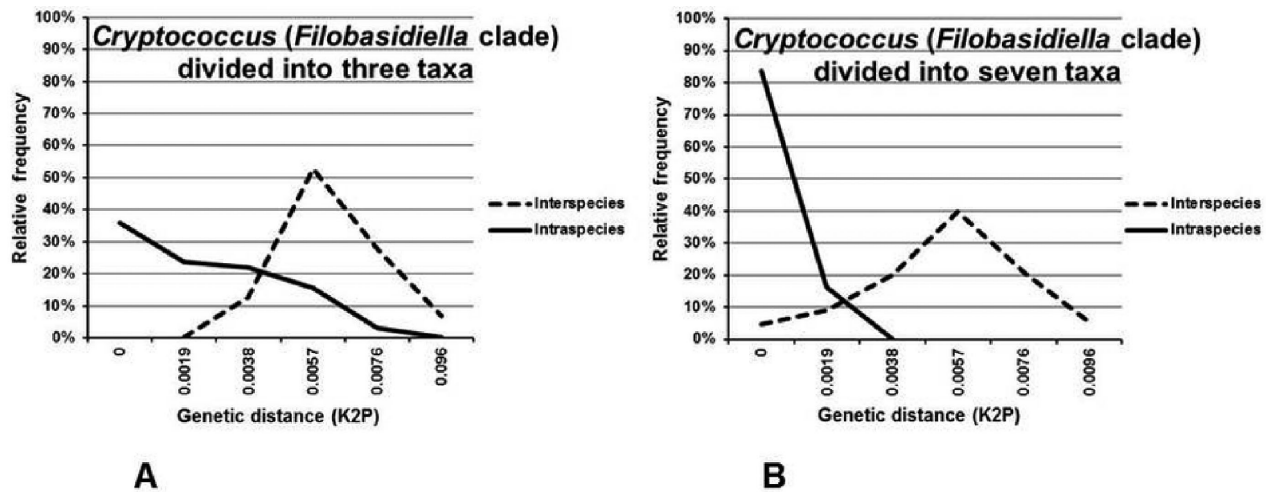
Figure 7. A) Distribution of interspecies (broken line) and intraspecies (solid line) pairwise Kimura 2-parameter genetic distances in *Cryptococcus* (*Filobasidiella* clade divided into three taxa) including *C. gattii; C. neoformans* var. *grubii; C. neoformans* var. *neoformans*. **B)** Distribution of interspecies (broken line) and intraspecies (solid line) pairwise Kimura 2-parameter genetic distances in *Cryptococcus* (*Filobasidiella* clade divided into seven taxa) including *C. gattii* VGI; *C. gattii* VGII; *C. gattii* VGIII; *C. gattii* VGIV; *C. neoformans* var. *grubii* VNI; *C. neoformans* var. *grubii* VNII; *C. neoformans* var. *neoformans* VNIV.
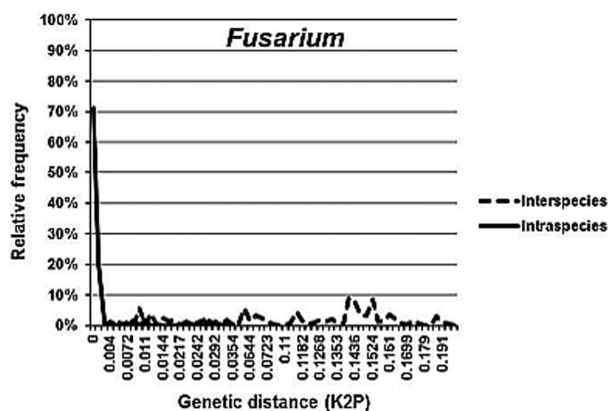
Figure 8. Distribution of interspecies (broken line) and intraspecies (solid line) pairwise Kimura 2-parameter genetic distances in *Fusarium* including *F. delphinoides; F. falciforme; F. oxysporum; F. proliferatum; F. solani; F. keratoplasticum; F. petroliphilum; F. verticillioides*.
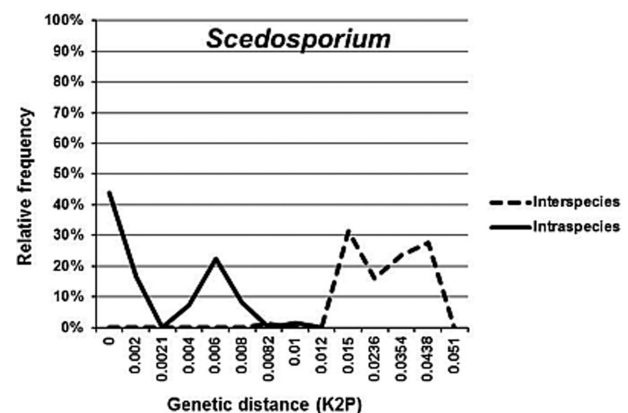
Figure 9. Distribution of interspecies (broken line) and intraspecies (solid line) pairwise Kimura 2-parameter genetic distances in *Scedosporium* including *S. angustum; S. apiospermum; S. aurantiacum; S. boydii; S. dehoogii; S. ellipsoideum; S. minutisporum*.

fungal species is one of the most important requirements for early and successful clinical treatment. As such, molecular information is expected to become a reliable tool for the identification of fungal species in medical diagnostic laboratories.

DNA barcoding represents a recent attempt to obtain rapid and accurate species identification based on comparative analysis of short but taxonomically significant sequences that has already found broad application in biology. However, the widespread application of fungal barcoding is hindered by a lack of reference databases. We herein report the establishment of the ISHAM-ITS reference database, containing 2800 quality controlled sequences,

covering 421 human/animal pathogenic fungal species, which is publicly accessible at http://its.mycologylab.org/ and http://www.isham.org/. The principal roles of this reference database are to provide a reliable source for diagnostic medical and veterinary mycology laboratories, to enable correct identification of the causal agents of fungal infections, rapid diagnosis of mycoses, and early initiation of appropriate antifungal therapy (Fig. 11).

Intraspecies variation

The intraspecies genetic diversity of the ITS region varied between 0 and 2.25% but in 170 species it was less
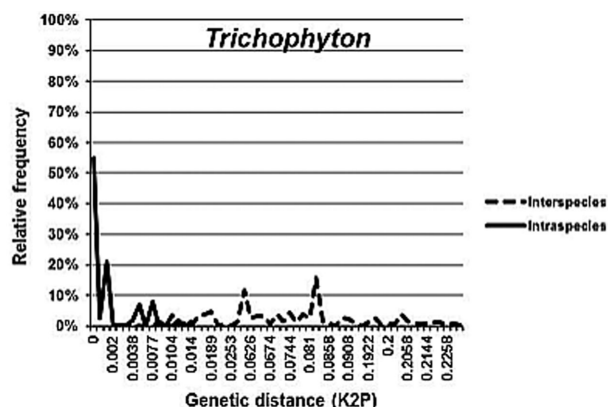
Figure 10. Distribution of interspecies (broken line) and intraspecies (solid line) pairwise Kimura 2-parameter genetic distances in *Trichophyton* including *T. ajelloi*; *T. erinacei*; *T. interdigitale*; *T. mentagrophytes* (=*T. quinckeanum*); *T. rubrum*; *T. schoenleinii*; *T. simii*; *T. terrestre*; *T. verrucosum*.

than 1.5%. The data generated in the present study are in agreement with previous studies stating that the genetic diversity of the ITS regions in fungi varies between taxa and that a single cut-off value cannot be established [33,84]. One could hypothesize that highly invasive fungal species show little variability because they are fully adapted to the host environment. However, further analyses are necessary to determine whether or not the variability calculated within the ITS regions is representative of the general genotypic and phenotypic variability within these species. Notably, the intraspecies diversity is more complex, with intragenomic polymorphism of rDNA repeats documented

in a number of fungal species [36,85]. Observed intraspecies diversity in medical fungi may partly be due to the intragenomic polymorphism. Although we were not able to address this issue, its impact on the functionality of the database is mitigated because the ITS sequences contained in the ISHAM-ITS reference database are the result of direct sequencing which leads to the amplification of the most abundant sequence in the sample.

Taxa with high intraspecies variation for which identification based solely on the ITS region could be problematic

In the ISHAM-ITS reference database, only six fungal species (*C. intermedia*, *C. lusitaniae*, *F. solani*, *G. candidus*, *K. ohmeri*, and *L. ramosa*) revealed an intraspecies diversity of more than 1.5%.

Clavispora lusitaniae

Among these six species, *C. lusitaniae* (the teleomorph of *Candida lusitaniae*) causes approximately 1–2% of episodes of candidemia, including nosocomial outbreaks [86]. The species is exceptionally polymorphic in the ITS region and the D2 domain of the large-subunit rDNA gene, containing more than 30 substitutions [87,88]. In the ISHAM-ITS reference database, the average nucleotide diversity for this species was 2.19%, with 22 polymorphic sites, which may be a problem for identification of strains with sequences that are currently not represented in the database. In this
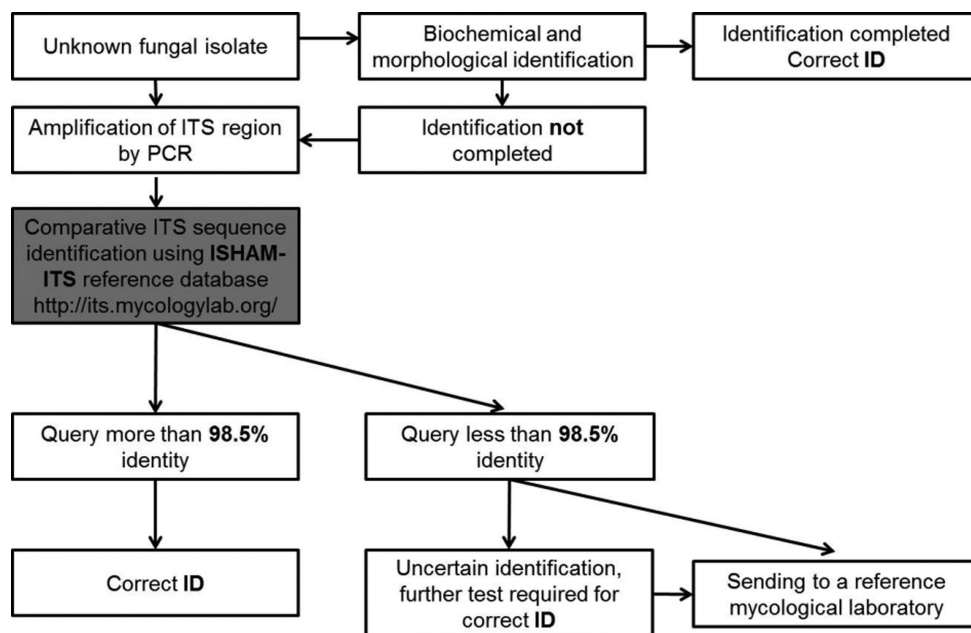

Figure 11. Proposed working flow to identify human and animal pathogenic fungi.

case, correct species identification, may be determined by mating type for sexual reproduction [89,90] (see Table 4). The polyphyletic nature of the genus *Clavispora* was recently confirmed by multigene sequence analysis [91]. Further taxonomic studies are required for a better delimitation of this species.

Fusarium solani species complex (FSSC)

The second highest intraspecies variation was found amongst *Fusarium* species, which are primarily saprobes, plant pathogens, often linked with pathological infections, mainly keratitis, in both humans and animals. The *F. solani* species complex is the most common group of fusaria responsible for human infections, primarily in immunocompromised individuals [92,93]. Before taxonomical reanalysis the ISHAM-ITS database contained ten different *Fusarium* species including the highly polyphyletic FSSC. Seven of these species showed below 0.5% intraspecies variability suggesting a good taxonomic delimitation which can in turn allow easy identification with the ITS. However, within the FSSC the average nucleotide diversity was 3.76%, indicating that this complex has remained unresolved and contains multiple other cryptic species. According to the latest taxonomic studies [94], *F. keratoplasticum, F. petroliphilum,* and *F. falciforme* have been separated from the FSSC as new taxa, reducing the average nucleotide diversity to 1.65% in the ISHAM-ITS reference database. This variation still represents a significantly high degree of sequence diversity, making it necessary to employ different markers for correct identification at the species level (Table 4 and see below).

Galactomyces candidus

G. candidus (anamorph *Geotrichum candidum*) is a ubiquitous and dimorphic yeast, which occurs commonly on moist substrates rich in nutrients. Occasionally it is found as an opportunistic pathogen in the human respiratory and gastro-intestinal tracts [92,95]. The taxonomic classification of the species was revised in 2004 by de Hoog and Smith [96]. A standardized protocol was proposed for the identification of *G. candidus* at species and strain level in 2006 [97]. According to a recent study [38], the ITS region, especially the ITS1 region of *G. candidus,* proved to be highly polymorphic at intraspecies and intragenomic levels. In the ISHAM-ITS database, the species was represented by five strains with 1.78% genetic diversity, mainly in the ITS1 region. Although the 18S-ITS1-5.8S-ITS2-26S as a whole provides an improved phylogenetic resolution for the different phylotypes, use of the ITS region alone is not suitable for rapid identification of the species [38].

Kodamaea ohmeri

Using the ISHAM-ITS reference database, *K. ohmeri* (syn.: *Pichia ohmeri*, the teleomorph of *Candida guilliermondii* var. *membranifaciens*) has been found to contain high intraspecies diversity. This is an ascosporogenic yeast, mainly used in the food industry for fermentation, but has recently emerged as a fungal pathogen, particularly in immunocompromised patients [98,99]. However, few studies on this species have been done. Recently a number of species have been found with characteristics similar to those of *K. ohmeri* raising the possibility of cryptic species and the potential misidentification of previously described isolates [100]. Phylogenetic analyses of the ITS sequences contained in the ISHAM-ITS reference database supported two clades not previously identified. Further studies are needed to taxonomically resolve possible cryptic species.

Lichtheimia spp

The next group of fungi with marked ITS intraspecies variation was *Lichtheimia* species, which causes life-threating rhinocerebral and bronchorespiratory mucormycoses [101]. Multigene sequence analysis (ITS, 28S, EF-1α) of 38 isolates identified morphologically as *L. corymbifera* revealed a new species, named *L. ramosa*, which differed in morphology and nucleotide sequences from *L. corymbifera* [102]. To date, from the five recognized species of the genus *Lichtheimia*, only three *L. corymbifera*, *L. ornata,* and *L. ramosa* are of clinical relevance [103]. *L. ramosa* proved to be more polymorphic than *L. corymbifera*, with more than 2% diversity in the ITS sequences. Similar values for the ITS region of *L. ramosa* have been reported by Walther et al. in 2013 [104], suggesting that different groups among *L. ramosa* should be considered as a separate species. If so, the ITS region would be an appropriate marker for identification of these species. In view of the high diversity observed among ITS sequences within *Lichtheimia*, currently it is recommended to use either a multiple gene approach [102] or MALDI-TOF [105] for a reliable identification (see Table 4).

Barcoding gap analysis

At interspecies level, clear barcoding gaps, ranging from 0.0002 to 0.09, were found in 13 of 17 taxonomical clades, containing at least three species with more than two strains. These included the taxa *Acremonium, Arthrodermataceae, Aspergillus, Cladophialophora, Curvularia, Debaryomycetaceae (Lodderomyces* clade), *Exophiala, Metschnikowiaceae, Microsporum, Pichiaceae, Saccharomycetaceae, Scopulariopsis,* and *Trichosporon.* Thus, the identification of these species based on ITS sequences is reliable, the taxonomy of the groups is well

defined and all the species in the current dataset are well delimited. However, four taxa showed no clear barcoding gap: *Cryptococcus*, *Fusarium*, *Scedosporium,* and *Trichophyton*. The species of these four clades require more insight to fully understand if and why the ITS barcoding fails to dissect this specific group or if these species are not yet well isolated from a taxonomic point of view. Additional molecular methods or genetic markers are required to accurately identify the species in this group (Table 4). The barcoding gap analyses presented herein are based on the current dataset in the ISHAM-ITS database, which may not reflect all known cryptic species of all studied taxa, for example, it is well known that *A. fumigatus* is species complex, and ITS will only enable an identification to the species complex, with additional sequencing of either *β*-tubulin [106] and calmodulin [107] being needed to identify the actual species.

Overall, ITS barcoding can be used as a screening system to evaluate and indicate to specialists which species require more attention at the taxonomic level.

Cryptococcus neoformans/*C. gattii* species complex

The *C. neoformans*/*C. gattii* species complex is a good example of how the delimitation of a species can be improved by molecular characterization. Cryptococcosis is a life-threatening systemic mycosis in a broad range of animals and humans. Most cases are due two species belonging to the family *Tremellaceae*. The causal agent of cryptococcosis was originally considered as one species until four serotypes were identified based on antigenic properties of the polysaccharide capsule [108]. Currently, the etiologic agents of cryptococcosis are divided into two species, *C. neoformans* (serotypes A, D, and AD) and *C. gattii* (serotypes B and C) [109]. Molecular genotyping methods have more recently revealed seven major haplotypes among the two species [110–113]. These include three lineages in *C. neoformans* (VNI/AFLP1, VNII/AFLP1A/1B, and VNIV/AFLP3) and four in *C. gattii* (VGI/AFLP4, VGII/AFLP6, VGIII/AFLP5 and VGIV/AFLP7) [114]. As with other species complexes, the *C. neoformans*/*C. gattii* species complex is a controversial topic and there is no agreement amongst taxonomists regarding the delimitation of the species. This is likely due to the absence of a consensus species definition for fungi. It has been suggested that every molecular type should be considered as a different variety or even as separate species [113]. The ISHAM-ITS reference database contains a large set of ITS sequences representing all seven major haploid molecular types of the *C. neoformans*/*C. gattii* species complex. In order to determine the effect of accurate taxonomic recognition, the genetic diversity within and between species was calculated in two different ways: (a) considering only *C.*

neoformans and *C. gattii* as species and (b) considering the seven major haplotypes as "species." In the first case, the average intraspecies diversity was 0.35% for *C. gattii* and 0.19% for *C. neoformans*. These values are consistent with genetic diversity within species. However, in the barcoding gap analyses the K2P genetic distances overlapped significantly (Table 5, Fig. 7A). In the second analysis based on the seven species assumption, the average genetic diversity among molecular types was 0–0.1%, which was significantly less variation than in the analysis based on the two-species assumption (Table 5, Fig. 7B). However, a clear barcoding gap was still absent, but the overlap was considerably less than in the first set. The only reason for the absence of a barcoding gap was that the VNI and VNII molecular types of *C. neoformans* could not be separated by ITS sequencing, which confirmed previous findings (43). Alternative methods are therefore needed to fully resolve this species complex. Currently AFLP analysis [110], *URA5*-RFLP analysis [111], MLMT/SCAR analysis [115] and MLST analysis using the ISHAM consensus MLST scheme for the *C. neoformans*/*C. gattii* species complex, which includes the following genetic loci: *CAP59, GPD1, LAC1, PLB1, SOD1, URA5,* and *IGS1* [114] are recommended to separate all major molecular types/potential species in this species complex.

Fusarium solani species complex (FSSC)

The second group of fungi lacking a clear barcoding gap comprised the FSSC. No clear barcoding gap was identified amongst *Fusarium* species in the ISHAM-ITS database (Fig. 8). The overlap of the K2P genetic distance within and between species was undeniably due to the poorly resolved *F. solani* species complex. For correct species identification, the following additional genetic loci are recommended: translation elongation factor 1-*α* (*TEF-1α*) and the RNA polymerase II gene (*RPB2*) [94]. An MLST method, including eight protein-coding genes was also developed to identify species in FSSC [116] (Table 4).

Scedosporium

The third group that lacked a barcoding gap was the ascomycetous fungal species of the genus *Scedosporium* (*Microascaceae*) (Fig. 9). They are well known emerging pathogens, which are associated with important human diseases [117–119] and animal infections [120]. In this group, important taxonomic changes have been made in recent years using different molecular methodologies [121]. Based on several genetic markers including the ITS region, *S. apiospermum* and *S. boydii* have been re-evaluated, resulting in the definition of *S. apiospermum* (heterothallic teleomorph *Pseudallescheria apiosperma*), *S. boydii*

(homothallic teleomorph *Pseudallescheria boydii*), *S. dehoogii, S. minutisporum* and *S. aurantiacum* [122,123]. The routine identification of species within the genus *Scedosporium* is complicated due to a high intraspecies but little constant interspecies variability in morphological characters mixed within the various synanamorphs and teleomorphs [123]. The ITS regions are a widely used molecular marker for the identification of these species, possibly in association with other markers. According to a new molecular study, these species can be reliably identified by ITS sequencing, although the distances between certain species (*S. boydii* and *S. apiospermum*) remain very small [121]. The identification of newly described species within the genus, *S. ellipsoideum, S. fusoideum,* and *S. angustum* is also questionable if only ITS sequences are used, as they cluster within *S. boydii*, with limited statistical support [121]. In the ISHAM-ITS reference database, the intraspecies diversity of *Scedosporium* species was low, indicating that they are all well-delineated taxa. The highest divergence was observed in *S. apiospermum, S. boydii,* and *S. dehoogii*. However, at interspecies level, no clear barcoding gap has been found since the smallest interspecies distances (*S. boydii – S. apiospermum* and *S. boydii – S. ellipsoideum*) were smaller than the biggest intraspecies distances found in *S. apiospermum, S. boydii,* and *S. dehoogii*. As such, to obtain a clear differentiation among all *Scedosporium* species, the amplification of the large subunit rRNA (LSU) [124], *β*-tubulin (*BT2*) [122], or AFLP [121] are recommended (Table 4).

Dermatophytes

The last group of species, which did not show a defined barcoding gap was the dermatophytes (Fig. 10). They comprise a highly polyphyletic group of fungi that attack keratinized tissue of humans and animals, causing dermatophytoses [125]. The anamorphic stages of dermatophyte species belong mainly to the genera *Microsporum, Trichophyton,* and *Epidermophyton*, while their teleomorphic stages belonged to *Arthroderma* [125]. The taxonomy of dermatophyte species has been changed and revised several times [126,127]. The nomenclature has recently become more unsettled because separate names are no longer used for the anamorph/teleomorph stages of fungi [62]. The application of different molecular and biochemical methods has largely contributed to the description, delineation and taxonomical re-evaluation of these species. However, many taxonomic questions still remain unresolved in these taxa. According to a recent phylogenetic study using four genetic markers, including the ITS region, many anamorph species in *Trichophyton* share the same teleomorph genus *Arthroderma* [128]. The most recent taxonomy, nomenclature and

phylogeny of the family are summarized in a review by Cafarchia et al. [127].

Currently, two opposing concepts exist for the medically well-known species *Trichophyton mentagrophytes*. In a phylogenetic study of the *T. mentagrophytes* complex by Gräser et al. [129], three clades containing *T. mentagrophytes* varieties were recovered. Based on clinical and morphological data, most varieties were reduced to synonym species, whereas two were elevated to species level [126,130,131]. This resulted in three clades assigned to *T. erinacei, T. interdigitale* and to *T. mentagrophytes*. The third clade was composed of two strains: CBS 318.56, originally identified as *T. mentagrophytes* var. *mentagrophytes*, and CBS 106.67, originally identified as *T. mentagrophytes* var. *quinckeanum*. The latter strain was considered incorrectly identified, and CBS 318.56 was designated by Gräser et al. [129] as the neotype for *T. mentagrophytes*. The choice of this neotype has been under debate ever since, as *T. mentagrophytes* in this sense are now encountered rarely in clinical surveys that use DNA sequencing for identification. At the same time, an unnamed zoophilic species closely related to *T. interdigitale* was detected which appeared to be quite common and seemed to fit the original concept of *T. mentagrophytes* [132,133]. In an article verifying the new dermatophyte taxonomy using mating results and phylogenetic analyses, Kawasaki [128] states that the selected neotype only corresponds to strains of *T. mentagrophytes* var. *quinckeanum*, a rather rare dermatophyte causing favus predominantly in rodents. Beguin et al. [72] found that the neotype strain CBS 318.56 was included in a clade consisting exclusively of strains originally identified as *T. (mentagrophytes* var.) *quinckeanum*. They also provided arguments on why this epithet should not be disposed of as a *nomen nudum*. Although part of the medical mycological community disagrees with the current neotype for *T. mentagrophytes*, no alternative neotype for *T. mentagrophytes* has been proposed so far.

In the ISHAM-ITS reference database, the three major genera of the dermatophytes are present with a number of species, including six *Microsporum*, 15 *Trichophyton*, four *Arthroderma* and one *Epidermophyton* species. These species showed a high similarity at the intraspecies level, except *T. erinacei,* which had still less than 1% ITS sequence variation. To evaluate the interspecies diversity and estimate the existence of a barcoding gap, the distribution of interspecies/intraspecies divergence in the genera *Trichophyton* and *Microsporum* was compared. The results indicated that there was a clear, though very small barcoding gap in the genus *Microsporum* but not in the genus *Trichophyton*, where the two overlapped. There were species, for example, *T. erinacei,* where the intraspecies K2P genetic

distance exceeded the interspecies K2P distances between two species. The difference in the ITS region was only a few nucleotides, e.g., between *T. mentagrophytes* (= *T. quinckeanum* strains) and *T. schoenleinii* or between *T. tonsurans* and *T. interdigitale*. However, evaluation of the former teleomorph stages of the species revealed that there was a clear barcoding gap (Supplementary Fig. S2) in the family *Arthrodermataceae*, since the different former anamorph species have a common former teleomorph genus. Based on the results of this study and the complex taxonomy of the dermatophytes it is strongly recommended that other molecular or biochemical features, for example, *BT2*, AFLP, PCR fingerprinting, or microsatellite analysis, be used to accurately identify the closely related species (*T. schoenleinii* – *T. mentagrophytes* (= *T. quinckeanum*), *T. tonsurans* – *T. interdigitale* and *T. verrucosum* – *T. erinacei*) of this group [72,134] (Table 4).

Algorithm consideration

The occurrence of taxa without a barcoding gap can be explained by the fact that the algorithms which have long been used by the barcoding community to calculate the genetic distances (K2P) [80] or the algorithm used in BLAST [135] for sequence matching between the query sequence and reference sequences represent different approaches from those commonly used for phylogenetic analyses. Both K2P and BLAST approaches are based on simple sequence similarities. The most commonly applied method for species delimitation using phylogenetic approaches in mycology is the genealogical concordance phylogenetic species recognition (GCPSR), first proposed by Taylor et al. [136]. This relies on the concordant discrimination of characters from three or more unlinked loci. Phylogenetic analysis can be performed using a variety of algorithms relying on complex, computationally intensive evolutionary models based on "phylogenetic signals." These methods are more robust and require more computational power and expertise. In exchange they give a more reliable summary of the evolutionary relatedness of the members of a specific taxonomic group. A common question often arises in the barcoding community whether a phylogenetic model is necessary for DNA barcode sequence analyses. In this study, we tested the discriminatory power of the official fungal barcode, the ITS regions [28], to identify human and animal pathogenic fungi and showed that it is efficient, using a simple sequence similarity based algorithm, for the identification of an unknown fungal disease agent in the majority of species. However, in sibling/cryptic species with only 1–2 bp differences, identification based only on ITS sequencing may be unreliable. Many articles have been published discriminating species

by only one or two polymorphic sites in the ITS region [43,47,121]. However, the majority of these studies used phylogenetic approaches, e.g., maximum likelihood, parsimony or Bayesian analysis [137–139]. It should be noted that, in contrast to phylogenetic methods, the DNA barcoding approach focuses on the use of a universal marker that maximises the number of specimens to be examined, whilst lowering the time spent on processing and analysis. This approach can be simplified in two major indications, namely specimen identification and species discovery [140,141]. The method popularly used in DNA barcoding approaches, K2P genetic distances, does not capture the same level of species distinctiveness with limited genetic variation. [142]. This is especially true when only one marker is used in the barcoding analyses. Specimen identification works best in concert with a well-annotated reference database that incorporates species boundaries delimited with phylogenetic multi-gene analyses. However, due to the paucity of sequence data in many fungi DNA databases barcoding will provide a first sweep of species discovery that should eventually be verified with more robust phylogenetic methods.

A basic step in phylogenetic analysis is the global alignment of all sequences. Beyond causing excessive gap opening and extension when divergent sequences are compared, this approach requires all sequences to be of the same length. It is questionable whether in the hectic practice of diagnostic labs this level of sequence quality and analytical care can be obtained, when the presence of life threatening pathogens has to be determined. Distance based algorithms seem to better fit these situations, maybe with upgrades in terms of taxonomic and bioinformatics conception [75,143,144], and with flexible distance algorithms [76].

The lack of interspecies gaps paves the way to three basic questions: (i) Is this relevant in the diagnostic practice? (ii) Is it due to unresolved taxonomy or to the intrinsic low power of the ITS barcode? and (iii) Are there taxonomic approaches and bioinformatics pipelines to reduce or resolve this problem?

The first question is a trivial one, but as long as the therapies for the unresolved species are similar, the lack of specific gaps is more a biological than a clinical problem. An attentive analysis from this point of view should accompany the purely taxonomic search, in order to pay particular attention to unresolved groups requiring different drug treatments. The second question is more complex. Many fungal species are not easily resolved for an exceeding number of taxonomic questions no matter of the single marker used. More insight on this point is necessary, maybe to develop easy to read indexes describing the ratio between the single marker vs. multiparameter species delimitation. This type of analysis seems to be necessary for further

development of molecular markers in order to define their effective "taxonomic resolution power". The evidence that many species presented a large variability does not impair the validity of ITS as a barcoding gene but suggest that particular attention must be paid in delimiting large species at the taxonomic level. Finally, the third question calls for a more attentive analysis of the species structure and of the algorithms necessary to discriminate them in fungi.

As a result of this study a quality–controlled reference ITS database, containing 2800 strains covering 421 species has been established and is publically accessible at http://its.mycologylab.org/ and http://www.isham.org/. The sequences selected in this study expand the number of medical species represented in the RTL ITS reference database at NCBI. There are several sequences with type information shared between the ISHAM-ITS database and RTL. Curators at NCBI will continuously verify additional single ITS accessions representing species where type information is currently unavailable. After a series of verifications these will serve as "verified" reference sequences [57] until a sequence obtained from type material is available. ISHAM-ITS database records are linked with their appropriate records at NCBI, similarly to the existing link between GenBank records and the UNITE and BOLD databases using Linkout (http://www.ncbi.nlm.nih.gov/projects/linkout/) and db_xref (http://www.ncbi.nlm.nih.gov/genbank/collab/db_xref) links. The results of the analysis of the sequences maintained in the database showed that ITS works well as a barcode for the majority of species. However, it has limitations in resolving species within species complexes and in sibling species delineation, where the difference of only one or a few nucleotide positions exist at the ITS locus. This study does not intend to challenge the current taxonomy of any fungal taxon. The goal was to highlight those taxa for the scientific community where additional genetic markers or molecular algorithms should be used for the reliable species identification.

Call for participation

The database is intended to cover all clinically relevant fungal species. It is open for further sequence submission to cover all medially relevant species with a sufficient number of strains, either via direct submission through the database (http://its.mycologylab.org/) or contacting the curators of the database (Prof. Wieland Meyer, wieland.meyer@sydney.edu.au or Laszlo Irinyi, laszlo.irinyi@sydney.edu.au).

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary material

Supplementary material is available at *Medical Mycology* online (http://www.mmy.oxfordjournals.org/).

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