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Database establishment for the secondary fungal DNA barcode *translational elongation factor 1 α* (TEF1 α)¹

Wieland Meyer, Laszlo Irinyi, Minh Thuy Vi Hoang, Vincent Robert, Dea Garcia-Hermoso, Marie Desnos-Ollivier, Chompoonek Yurayart, Chi-Ching Tsang, Chun-Yi Lee, Patrick C.Y. Woo, Ivan Mikhailovich Pchelin, Silke Uhrlaß, Pietro Nenoff, Ariya Chindamporn, Sharon Chen, Paul D.N. Hebert, Tania C. Sorrell, and the ISHAM barcoding of pathogenic fungi working group²

Abstract: With new or emerging fungal infections, human and animal fungal pathogens are a growing threat worldwide. Current diagnostic tools are slow, non-specific at the species and subspecies levels, and require specific morphological expertise to accurately identify pathogens from pure cultures. DNA barcodes are easily amplified, universal, short species-specific DNA sequences, which enable rapid identification by comparison with a well-curated reference sequence collection. The primary fungal DNA barcode, ITS region, was introduced in 2012 and is now routinely used in diagnostic laboratories. However, the ITS region only accurately identifies around 75% of all medically relevant fungal species, which has prompted the development of a secondary barcode to increase the resolution power and suitability of DNA barcoding for fungal disease diagnostics. The translational elongation factor 1 α (TEF1 α) was selected in 2015 as a secondary fungal DNA barcode, but it has not been implemented into practice, due to the absence of a reference database. Here, we have established a quality-controlled reference database for the secondary barcode that together with the ISHAM-ITS database, forms the ISHAM barcode database, available online at <http://its.mycologylab.org/>. We encourage the mycology community for active contributions.

Key words: fungal DNA barcoding, secondary fungal DNA barcode database, translational elongation factor 1 α .

Résumé : En raison d'infections fongiques nouvelles ou émergentes, les champignons pathogènes humains et animaux constituent une menace croissante mondialement. Les outils diagnostiques actuels sont lents, non-discriminants au niveau spécifique ou sous-spécifique, et ils nécessitent une expertise morphologique pour identifier avec justesse les agents pathogènes en cultures pures. Les codes à barres de l'ADN sont des séquences

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d'ADN courtes, spécifiques, faciles à amplifier et universelles, ce qui rend possible une identification rapide par simple comparaison avec une collection de séquences de référence bien constituée. Le principal code à barres de l'ADN chez les champignons est la région ITS, laquelle a été employée initialement en 2012 et est maintenant employée de manière routinière dans les laboratoires diagnostiques. Cependant, la région ITS ne permet d'identifier avec justesse qu'environ 75 % de tous les spécimens fongiques d'intérêt médical, ce qui a créé la nécessité de développer un code à barres secondaire de manière à augmenter la résolution et la pertinence du codage à barres de l'ADN pour le diagnostic des maladies fongiques. Le facteur d'élongation de la traduction 1α (*TEF1 α*) a été choisi en 2015 comme code à barres secondaire chez les champignons, mais n'a pas été tellement employé en raison de l'absence d'une base de données de séquences de référence. Dans ce travail, les auteurs ont établi une telle base de référence dont la qualité a été vérifiée pour le code à barres secondaire qui, conjointement à la base de données ISHAM-ITS, forme la base de données « ISHAM barcode database », disponible en ligne à <http://its.mycologylab.org/>. Les auteurs encouragent la communauté des mycologues à y contribuer activement. L'utilisation d'un système de codage à barres double facilitera l'identification juste de tous les champignons pathogènes d'intérêt clinique.

Mots-clés : codage à barres de l'ADN fongique, base de données d'un code à barres fongique secondaire, facteur d'élongation de la traduction 1α .

Introduction

The identification and delimitation of fungal species are often challenging using traditional methods, either because of the lack of distinct morphological and biochemical characters or the absence of sexual reproduction. Complex life cycles, such as yeast-mycelial transitions or the existence of synanamorphs of certain species further hamper correct morphological identification (Seifert and Samuels 2000). Conventional fungal identification methods using culture and microscopic analyses are often insensitive, slow (7–14 days), and heavily dependent on the level of mycological expertise (Alexander and Pfaller 2006; Balajee et al. 2007). Further morphological and biochemical traits are subjective, error prone, and frequently result in misidentifications (Sangoi et al. 2009). In addition, the fact that numerous fungal species cannot grow under laboratory conditions precludes their identification (Begerow et al. 2010; Nilsson et al. 2009). Phenotypic traits also fail to differentiate closely related species or species complexes with near-identical morphological characters but distinguishable genetic traits, such as, e.g., the members of the genera *Fusarium*, *Aspergillus*, and *Scedosporium* (Balajee et al. 2005; Gilgado et al. 2005; O'Donnell 2000).

Rapid and accurate identification is essential in clinical diagnostics, outbreak investigation, or other epidemiological studies, as well as in agricultural, quarantine, and industrial settings. Culture and “expert-free” methods capable of identifying fungi directly from biological specimens are needed. The early diagnosis of pathogens is critical in fungal disease management, public health, animal welfare, and plant protection, for quarantine purposes and in other industrial applications. To counter these difficulties in identification, many molecular techniques have been developed over the last 20 years. These include PCR-RFLP analysis (Dendis et al. 2003; Diguta et al. 2011), RAPD (Baires-Varguez et al. 2007; Brandt et al. 1998), hybridisation with genus/species specific DNA or RNA probes (Lindsley et al. 2001; Sandhu et al. 1995), PCR

fingerprinting (Hierro et al. 2004; Lieckfeldt et al. 1993), species-specific PCR assays (Kulik et al. 2004; Martin et al. 2000), real time PCR (Bergman et al. 2007; Klingspor and Jalal 2006), and, increasingly, DNA sequencing (Pryce et al. 2003; Romanelli et al. 2010). Sequence-based identification methods have proven to be more accurate than conventional methods in diagnostic clinical mycology (Balajee et al. 2007; Ciardo et al. 2006). Developments in molecular-based technologies are expected to improve further and simplify species or even strain identification, making it faster and more accurate in the future.

Among the applied molecular techniques, DNA barcoding is one of the most promising and efficient methods, as it enables rapid identification of species and recognition of cryptic species across all kingdoms of eukaryotic life. The concept was first proposed by Hebert and colleagues in 2003 (Hebert et al. 2003a). Barcodes are standardized, easily amplified, universal short DNA sequences (500–800 bp), which exhibit a high divergence at species level and allow rapid identification by comparison with a reference sequence collection of accurately identified species (Hajibabaei et al. 2007). Characteristics of barcodes necessary for their routine use include the following: ease of attainment from any specimen, irrespective of morphology or stage of the fungal life cycle; high taxonomic coverage; and a high resolution power (Hebert et al. 2003a). Ideally, barcodes must be unique to a single species, and to be constant within each species to ensure consistency of identification (Hebert et al. 2003a, 2003b; Letourneau et al. 2010). Additionally, interspecies variation should exceed the intraspecies variation, generating a “break” in the distribution of distances that is referred to as the barcoding gap (Meyer and Paulay 2005). This gap is generally evaluated and represented by the Kimura 2-parameter distance model (Kimura 1980).

Numerous genetic loci have been evaluated as a barcode for fungi, with varying success rates. Protein-coding genes show high taxonomic resolution and identification success in Ascomycota. The most commonly used

genes are *RPB1* (Crespo et al. 2007; Hofstetter et al. 2007; McLaughlin et al. 2009; O'Donnell et al. 2013; Tanabe et al. 2005), β -tubulin (Frisvad and Samson 2004), and partial translational elongation factor 1 α (*TEF1 α*) (James et al. 2006; O'Donnell et al. 2010; Schoch et al. 2009). The D1/D2 region of the large subunit (LSU) of the rDNA gene cluster has been used successfully for species identification and strain recognition in yeasts (Fell et al. 2000; Kurtzman and Robnett 1998; Scorzetti et al. 2002). However, the applicability of these genetic loci as a potential barcode is hampered by the lack of standardization of the amplification process, differences in primer selection, their species/genera-dependent resolution power, and the lack of quality-controlled reference databases.

The primary fungal barcode

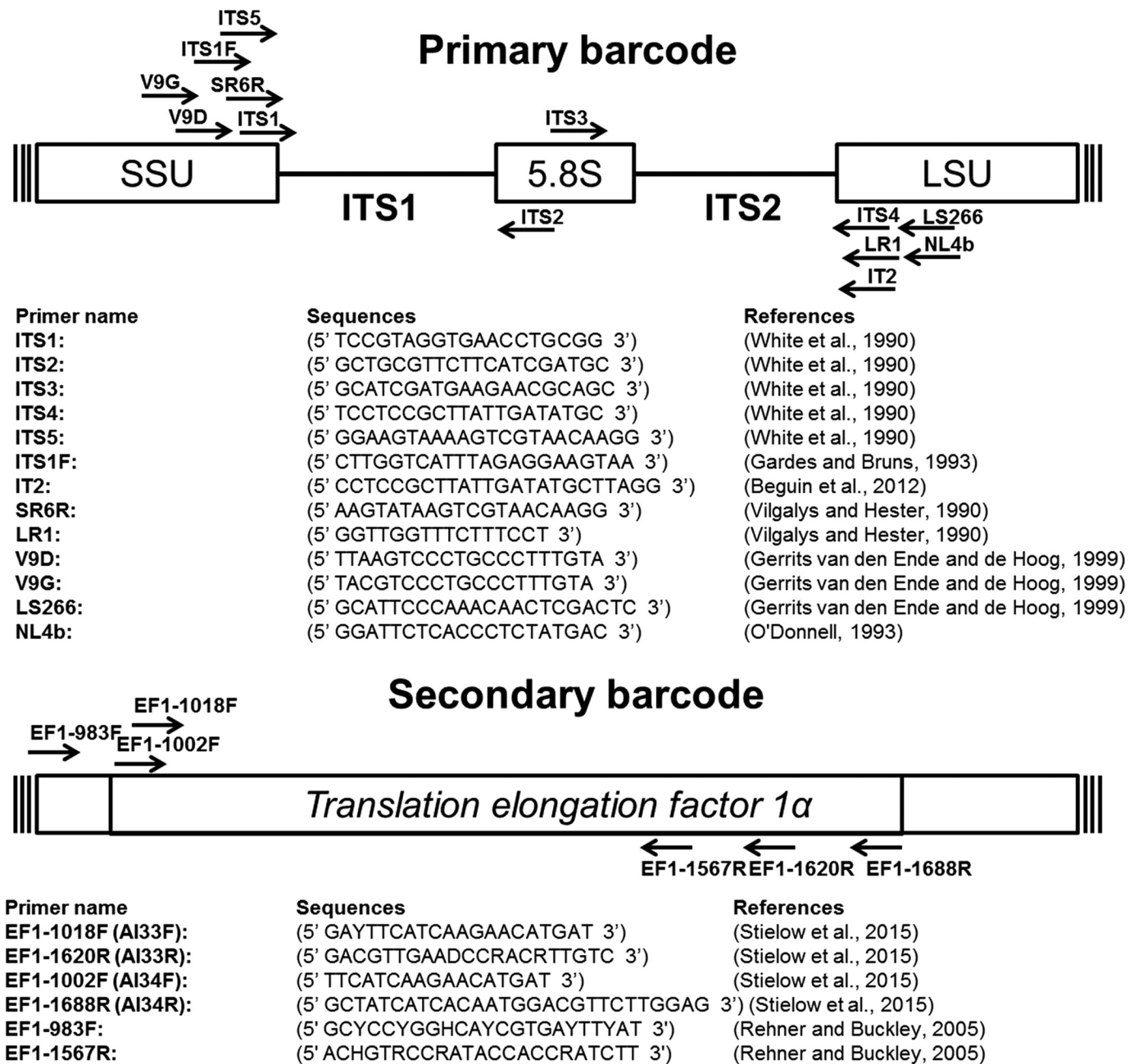
The internal transcribed spacer (ITS) region is comprised of three units, two non-coding, variable, ITS1 and ITS2 regions, separated by the highly conserved 5.8S gene, which is located between the 18S (small subunit (SSU)) and 28S (LSU) genes in the nrDNA repeat unit (White et al. 1990). The ITS region has long been used for species identification and phylogenetic studies in fungi (Bruns et al. 1991; Gardes and Bruns 1993; Hillis and Dixon 1991; Leaw et al. 2006; Meyer and Gams 2003). Its widespread popularity relies on several facts. There are many universal primers available to bind to DNA of most of the fungal taxa, with the most commonly used being the ITS1, ITS1F, ITS2, ITS3, ITS4, and ITS5 (Gardes and Bruns 1993; White et al. 1990). To avoid cross reactivity with plant or animal DNA, fungus-specific primers were also designed, such as SR6R and LR1 (Vilgalys and Hester 1990), V9D, V9G, and LS266 (Gerrits van den Ende and de Hoog 1999), IT2 (Beguin et al. 2012), ITS1F (Gardes and Bruns 1993), and NL4b (O'Donnell 1993) (Fig. 1). Multiple copies of the ITS are present in the genome which increases significantly the amplification efficiency, even from samples where the initial amount of DNA is low, such as environmental or clinical specimens (Vilgalys and Gonzalez 1990). The relatively short length of the ITS region allows easy amplification and subsequent Sanger sequencing (Seifert 2009). Finally, the ITS region has proven to have a good resolution power leading to species discrimination in most fungal taxa. A significant drawback is its limited resolution in closely related species, or species complexes containing cryptic species (Nilsson et al. 2008). Recognising the many advantages and relatively few limitations of the ITS region for use in identification across the fungal kingdom, it was proposed as a standard fungal DNA barcode in 2012 by Schoch and colleagues (Schoch et al. 2012) — although there were strong criticisms of this proposal by some mycologists (Kiss 2012). The main shortcoming of barcoding for microorganisms, not limited to fungi, has been the lack of adequate and validated reference libraries of DNA barcodes (Seifert 2009). In response to this, the

ISHAM-ITS reference database for human and animal pathogenic fungi was established in 2015 (Irinnyi et al. 2015). Currently (as of 21 September 2018) this database is in global use and contains 4200 complete ITS sequences, representing 645 fungal species. The ITS locus proved to be sufficient for correct identification of most medically relevant fungal species (Irinnyi et al. 2015). However, an exact cut-off point for identification could not be defined, in agreement with other studies (Nilsson et al. 2008), due to a high number of polymorphisms (up to 2.5% of the ITS region) in certain species. For these taxa, it is essential to consider alternative genetic regions to obtain superior resolution power and separation. Correct identification of these taxa cannot be guaranteed by using only ITS sequences, as has been documented in the global project “Assembling the Fungal Tree of Life” (<https://aftol.umn.edu>) (James et al. 2006). This is particularly relevant to species complexes and sibling species delineation. Preferably, an alternative barcode should contain a single, probably protein-coding gene sequence with high interspecies and low intraspecies divergence and high taxonomic coverage, hence meeting the requirements of a standard barcode, namely, amplified by universal primers with high PCR efficiencies.

The secondary fungal barcode

Despite the advantages of the ITS locus, such as robust PCR amplification and a high taxonomic coverage, it can only accurately identify around 75% of all medically relevant fungal species using a cut-off of 98.5% sequence identity (Irinnyi et al. 2015). It has also been shown that the resolution power of ITS at a higher taxonomic level is inferior to that of many protein coding genes (Nilsson et al. 2006; Seifert 2009). To enhance the selectivity of fungal DNA barcoding and enable the correct identification of all fungal species, the most pragmatic solution was to establish a secondary fungal barcode, recognising that it would be difficult to identify only one DNA region that fulfilled all requirements of a barcode (Stielow et al. 2015). Over the years, many loci have been tested as alternatives to the ITS region but no consensus had been reached among mycologists mainly due to the lack of standardization and the non-availability of universal primers (Frisvad and Samson 2004; McLaughlin et al. 2009; O'Donnell et al. 2010). Whole genome sequencing (WGS) has recently made it possible to carry out full genome comparisons of evolutionarily distinct fungi to identify potential secondary barcode markers and design universal primers for robust PCR amplification (Robert et al. 2011). The proposal was to identify genetic markers with high interspecies and low intraspecies sequence divergence, which accurately reflect higher-level taxonomic affiliations. In a recent study, 14 (partially) universal primer pairs targeting eight genetic markers were tested across more than 1500 species (1931 strains or specimens) to select the most optimal secondary barcode

Fig. 1. Schematic structure of the primary (ITS) and secondary (*TEF1α*) fungal DNA barcode regions indicating universal primers for their amplification.



or the Quick-DNA Fungal/Bacterial Kit (D6007, Zymo Research), according to the manufacturer's instructions. Laboratories contributing to the current study carried out the DNA extractions by using the methods routinely used in their laboratories.

PCR amplification of the secondary barcode

All PCR reactions were performed in a final volume of 50 μ L, made of 5 μ L dNTP mix, 5 μ L 10 $\times$ reaction buffer, 4 μ L 10 ng/ μ L forward and reverse primers, 3 μ L 50 mM MgCl₂, 15 μ L 10 ng/ μ L genomic DNA, 0.5 μ L Taq polymerase, and nuclease-free water up to 50 μ L. Partial amplification of the secondary fungal DNA barcode, *TEF1 α* region, was done using two sets of primers: Al33F (5'-GAYTTCATCAAGAACATGAT-3') and Al33R (5'-GACGTTGAADCCRACRTTGTC-3') or Al34F (5'-TTCATCAAGAACATGAT-3') and Al34R (5'-GCTATCATCACAAATGGACGTTCTTGGAG-3') with the following amplification protocol: 5 min initial denaturing at 94 $^{\circ}$ C, followed by 40 cycles of 50 min at 94 $^{\circ}$ C, 50 s annealing at 48 $^{\circ}$ C, 50 s at 72 $^{\circ}$ C, and 7 min final extension at 72 $^{\circ}$ C (Stielow et al. 2015). First, the primer set Al33F-Al33R was used to amplify the *TEF1 α* region, and if there was no amplification achieved the second primer set Al34F-Al34R was applied. PCR products were subjected to electrophoresis in a 1.5% agarose gel containing EtBr and visualised using UV illumination. Successfully amplified PCR products were sent for commercial sequencing, e.g., to Macrogen Inc., Korea, in both forward and reverse directions.

Data analysis

Bidirectional sequences were assembled and edited using Sequencher® ver. 5.3. (Gene Codes Corporation, Ann Arbor, MI USA). Sequences were manually checked, and ambiguous bases were corrected based on the forward and reverse trace files, considering the PHRED scores received. The sequences for each taxon were aligned with the program CLUSTALW (Thompson et al. 1994), which is part of the software MEGA ver. 5.2.2 (Tamura et al. 2011). 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) within species, which had sequences from more than three strains, giving the proportion of nucleotide differences in all haplotypes in the studied sample using the software DnaSP ver. 5.10.01 (Librado and Rozas 2009).

Results and discussion

Establishment of the secondary fungal barcode database

With the *TEF1 α* locus being selected as secondary barcode, a dedicated quality-controlled database covering all medically relevant fungal species was established to complement the ISHAM-ITS database.

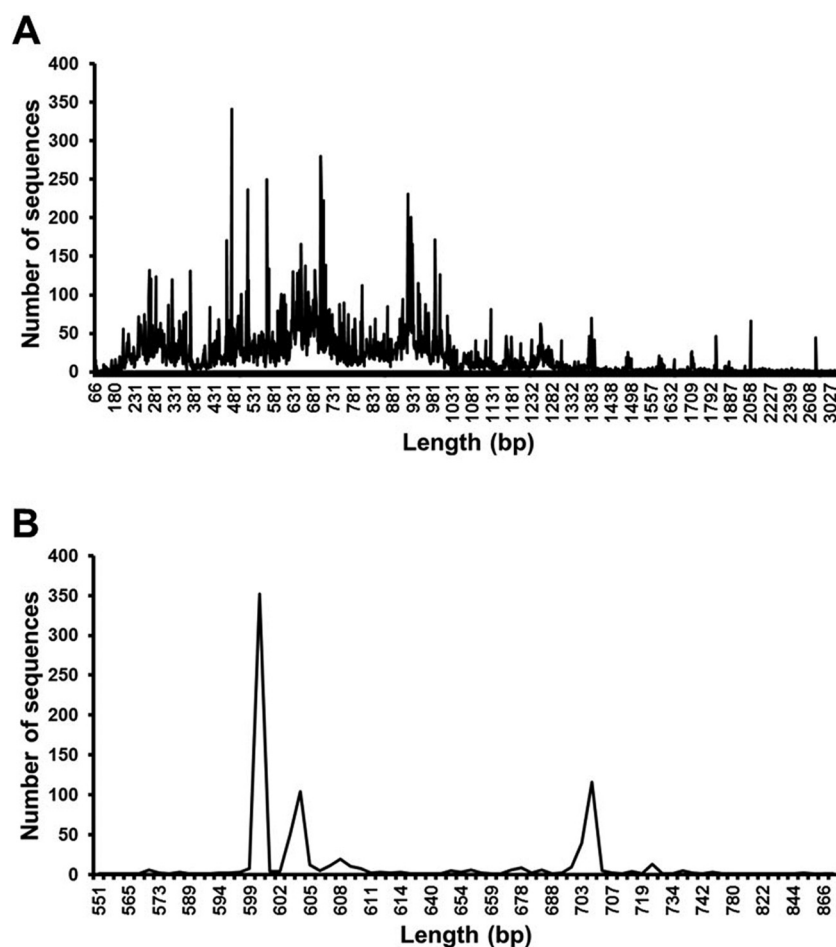
Currently there are 37 773 *TEF1 α* sequences available in GenBank (as of 15 March 2018), representing 6283 taxa, of which 670 are only classified to the genus level. However, there are only 6539 sequences from 269 clinically relevant species in 108 fungal genera. The majority of them belong to the genera *Fusarium* (2647), *Cryptococcus* (694), and *Sporothrix* (154). Of these, 114 species are represented by less than two sequences. The length of the *TEF1 α* sequences in GenBank ranges from 66 to ~3000 bp (Fig. 2A), indicating that some of the sequences might be too short for correct species identification. In addition, these sequences are not quality controlled and have not been verified at species level, which may lead to an incorrect identification.

The secondary fungal DNA barcode database was established using only quality-controlled *TEF1 α* sequences obtained from taxonomically verified fungal cultures. The newly established database contains 908 quality-controlled *TEF1 α* sequences, representing 186 pathogenic fungal species, was launched at the recent 7th International DNA Barcoding Conference at the Kruger National Park in South Africa (20–24 November 2017). Sequences from the genera *Candida*, *Cryptococcus*, and *Scedosporium* are most abundant in the new database. In total, 143 species are represented by three strains or less. The length of the partial *TEF1 α* sequences in the database ranges from 550–900 bp (Fig. 2B). In a few cases, shorter amplification products of the *TEF1 α* were obtained (e.g., for *Trichosporon* spp.).

Overall, the PCR success rate was very good, e.g., in the Molecular Mycology Research Laboratory, Westmead Institute for Medical Research, Westmead, NSW, Australia, 270 *TEF1 α* sequences were generated. From those, 220 were produced using the Al33F-Al33R primer set and 50 were amplified with the Al34F-Al34R primer set. There was no trend in amplification success for different fungal species; however, all strains for the species *Aspergillus niger*, *Candida albicans*, *Candida dubliniensis*, *Cluyveromyces marxianus*, and *Pichia kudriavzevii* required the Al34F-Al34R primer set for amplification. However, in some cases no amplification products for some strains of *Cladosporium* spp., *Rhodotorula* spp., or *Trichosporon* spp. were obtained. In those cases, modifications of the amplification conditions, like touchdown PCR, may be appropriate.

Comparison of the nucleotide diversity (π) and number of polymorphic sites (S) of 43 fungal species, with more than three strains per species in the new secondary fungal DNA barcode database (Fig. 3), showed that the *TEF1 α* locus is less diverse than the ITS locus for most of the species, with the intraspecies variation being below 1.5%. The pilot data reported herein showed a further reduction in the intraspecies diversity using the *TEF1 α* locus compared with the ITS region for 12 fungal species, including *Candida albicans*, *C. glabrata*, *C. metapsilosis*, *C. pararugosa*, *C. tropicalis*, *Clavispora lusitaniae*, *C. gattii*,

Fig. 2. Length distribution of secondary barcode (*TEF1 α*) sequences in (A) GenBank and (B) ISHAM barcode database. Only *TEF1 α* sequences longer than 550 bp were included.



Histoplasma capsulatum, *Kodamaea ohmeri*, *Scedosporium apiospermum*, *S. aurantiacum*, and *Yarrowia lipolytica*. The combination of the increased number of fungal species, which had less than 1.5% intraspecies variation, with the further reduction in intraspecies variation compared to the ITS confirms the secondary DNA barcode locus (*TEF1 α*) as a more discriminatory marker. Further studies, including more strains and covering all human and animal pathogenic fungi, are needed to enable specific indications for the use of the dual fungal DNA barcode.

As a result of this study, the ITS and the *TEF1 α* databases were combined together to form the new ISHAM barcode database, which currently contains 4200 ITS and 908 *TEF1 α* sequences. The ISHAM barcode database is available online at the following websites: <http://its.mycologylab.org/> or the ISHAM website (<http://isham.org>). This new database allows single locus or polyphasic identification of human and animal pathogenic fungi based on sequence alignments of either the ITS or the *TEF1 α* locus sequences, or both, against the reference sequences maintained in the ISHAM barcode database.

Call for data submission

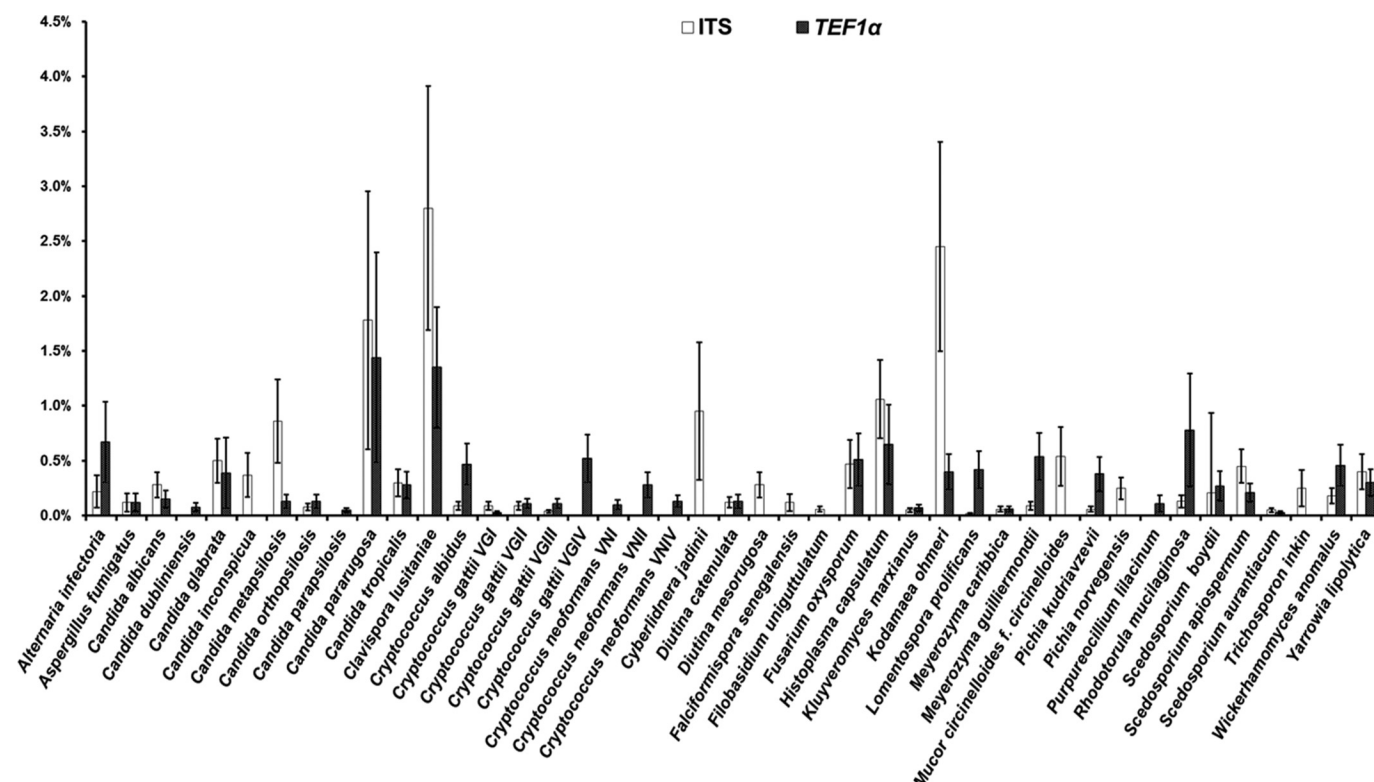
The database is open for further sequence submissions with the goal of eventually containing sequences from mul-

tiplex strains of all medically relevant species. Data obtained from taxonomically validated strains can be submitted directly into the database or via contacting the curators of the database: DNA-barcode.database@sydney.edu.au. After validation of the taxonomic metadata and verification of the submitted sequences by the database curator(s), they will be added to the database.

Value of the dual barcoding system for fungal identification

In summary, our data confirm that most medically relevant fungal species can be identified from their ITS sequence, confirming its status as a primary universal fungal DNA barcode. However, some species may not be distinguishable using the ITS region. This can be the case for the following two reasons: either the taxa are not adequately represented in the database, or the ITS region is unable to distinguish between biologically consistent groups. Under these circumstances, the application of a dual DNA barcoding system that incorporates a secondary barcode, *TEF1 α* , supported by a quality-controlled reference database, increases the ability to accurately identify all clinically important fungal pathogens. The herein established secondary fungal DNA barcode

Fig. 3. Intraspecies variation for species, which are represented by more than three strains, in the ITS regions (white bars) — primary DNA barcode — compared with the translation elongation factor 1 α (black bars) — secondary DNA barcode.



database offers the basis for a comprehensive database of the fungal kingdom.

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