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Dual DNA barcoding using the primary (ITS1/2 region) and the secondary fungal DNA barcode *(translational elongation factor 1a (TEF1a))* – the basis of fast and accurate identification of mycotic agents

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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. 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. Further morphological and biochemical traits are subjective, error prone and frequently result in misidentifications. In addition, the fact that numerous fungal species cannot grow under laboratory conditions precludes their identification. Phenotypic traits also fail to differentiate closely related species or species complexes with near-identical morphological characteristics 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).

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Dual DNA barcoding using the primary (ITS1/2 region) and the secondary fungal DNA barcode *(translational elongation factor 1a (TEF1a))* – the basis of fast and accurate identification of mycotic agents

PROF WIELAND MEYER, DR LASZLO IRINYI, MINH THUY VI HIANG, PROF TANIA C SORRELL, A/PROF SHARON CHEN

Early and accurate identification is essential in clinical diagnostics. Culture and "expert-free" methods capable of identifying fungi directly from biological specimens are needed. 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). 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. 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. Ideally, barcodes must be unique to a single species, and stable within each species to ensure consistency of identification (Hebert et al. 2003a). 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).

Numerous genetic loci have been evaluated as a barcode for fungi, with varying success rates. *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). 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 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) (Figure 1). The ITS region was proposed as a standard fungal DNA barcode in 2012 (Schoch et al. 2012) alongside the development of a validated reference library, the ISHAM-ITS database for human and animal pathogenic fungi, established in 2015 (Irinyi et al. 2015). Currently (as per 21.09.2018) this database is in global use and contains 4,200 complete ITS sequences, representing 645 fungal species. The ITS locus proved to be sufficient for correct identification of most medically relevant fungal species (Irinyi et al. 2015). For the remaining taxa, it is essential to consider alternative genetic regions to obtain superior resolution power and separation. This is particularly relevant to species complexes and sibling species delineation.

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 (Irinyi et al. 2015). 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). Whole genome sequencing (WGS) has recently made it possible to carry out full genome comparisons of evolutionarily distinct fungi in order to identify potential secondary barcode markers and design universal primers for robust PCR amplification (Robert et al. 2011). Fourteen (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 marker (Stielow et al. 2015). As a result of this study the *TEF1α* gene proved to be the most promising candidate, and as such was proposed as the universal secondary fungal DNA barcode, based on its universal taxon applicability and the availability of universal primers, such as EF1-1018F (Al33F)/EF1-1620R (Al33R) or EF1-1002F (Al34F)/EF1-1688R (Al34R) (Figure 1 – *(Page 3)*).

Dual DNA barcoding using the primary (ITS1/2 region) and the secondary fungal DNA barcode *(translational elongation factor 1a (TEF1a))* – the basis of fast and accurate identification of mycotic agents

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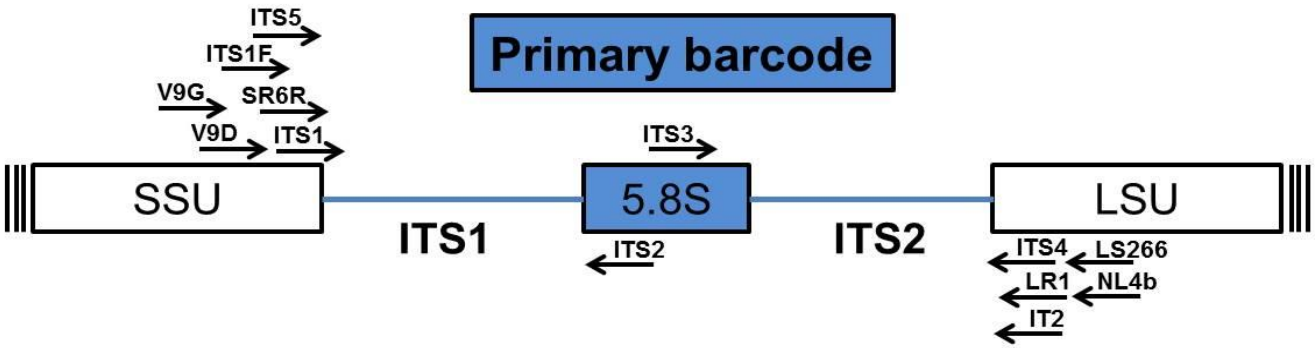
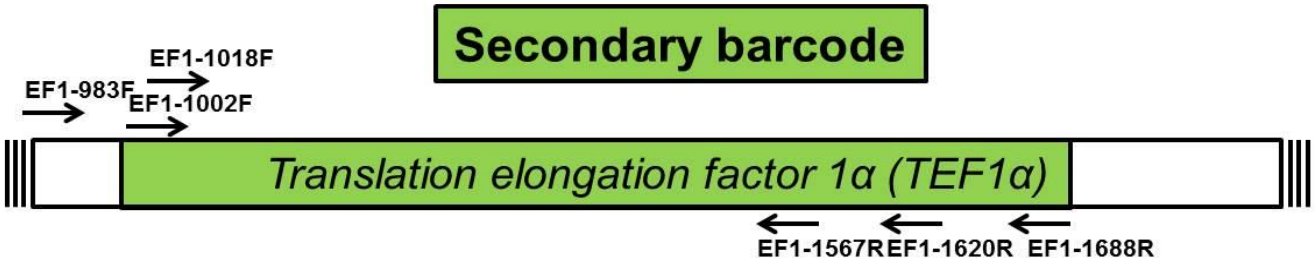


Primer name	Sequences	References
ITS1:	(5' TCCGTAGGTGAACCTGCGG 3')	(White et al., 1990)
ITS2:	(5' GCTGCGTTCTTCATCGATGC 3')	(White et al., 1990)
ITS3:	(5' GCATCGATGAAGAACGCAGC 3')	(White et al., 1990)
ITS4:	(5' TCCTCCGCTTATTGATATGC 3')	(White et al., 1990)
ITS5:	(5' GGAAGTAAAAGTCGTAACAAGG 3')	(White et al., 1990)
ITS1F:	(5' CTTGGTCATTTAGAGGAAGTAA 3')	(Gardes and Bruns, 1993)
IT2:	(5' CCTCCGCTTATTGATATGCTTAGG 3')	(Beguin et al., 2012)
SR6R:	**(5' AAGTATAAGTCGTAACAAGG 3')**	(Vilgalys and Hester, 1990)
LR1:	**(5' GGTTGGTTTCTTTCCT 3')**	(Vilgalys and Hester, 1990)
V9D:	(5' TTAAGTCCCTGCCCTTTGTA 3')	(Gerrits van den Ende and de Hoog, 1999)
V9G:	(5' TACGTCCCTGCCCTTTGTA 3')	(Gerrits van den Ende and de Hoog, 1999)
LS266:	(5' GCATTCCCAAACAACTCGACTC 3')	(Gerrits van den Ende and de Hoog, 1999)
NL4b:	(5' GGATTCTCACCCTCTATGAC 3')	(O'Donnell, 1993)



Primer name	Sequences	References
EF1-1018F (AI33F):	(5' GAYTTCATCAAGAACATGAT 3')	(Stielow et al., 2015)
EF1-1620R (AI33R):	(5' GACGTTGAADCCRACRTTGTC 3')	(Stielow et al., 2015)
EF1-1002F (AI34F):	(5' TTCATCAAGAACATGAT 3')	(Stielow et al., 2015)
EF1-1688R (AI34R):	(5' GCTATCATCACAATGGACGTTCTTGGAG 3')	(Stielow et al., 2015)
EF1-983F:	(5' GCYCCYGGHCAYCGTGAYTTYAT 3')	(Rehner and Buckley, 2005)
EF1-1567R:	(5' ACHGTRCCRATACCACCRATCTT 3')	(Rehner and Buckley, 2005)

Figure 1. Schematic structure of the primary (ITS) and secondary (TEF1α) fungal DNA barcode regions indicating universal primers for their amplification. Highlighted primers are most fungal specific.

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Establishment of the secondary fungal barcode database: With the *TEF1α* 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 using only quality-controlled *TEF1α* sequences obtained from taxonomically verified fungal cultures. The newly established database contains 908 quality-controlled *TEF1α* sequences, representing 186 pathogenic fungal species, and was launched at the recent 7th International DNA barcoding conference at the Kruger National Park in South Africa (20-24th November 2017). Sequences from the genera *Candida*, *Cryptococcus* and *Scedosporium* are most abundant in the new database. 143 species are represented by three strains or less. The length of the partial *TEF1α* sequences in the database ranges from 550-900 bp. In a few cases, shorter amplification products of the *TEF1α* were obtained (e.g., for *Trichosporon* spp.).

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 (Figure 2), showed that the *TEF1α* locus is less diverse than the ITS locus for most of the species, with the intraspecies variation being below 1.5 %, confirming the secondary DNA barcode locus (*TEF1α*) as a more discriminatory marker.

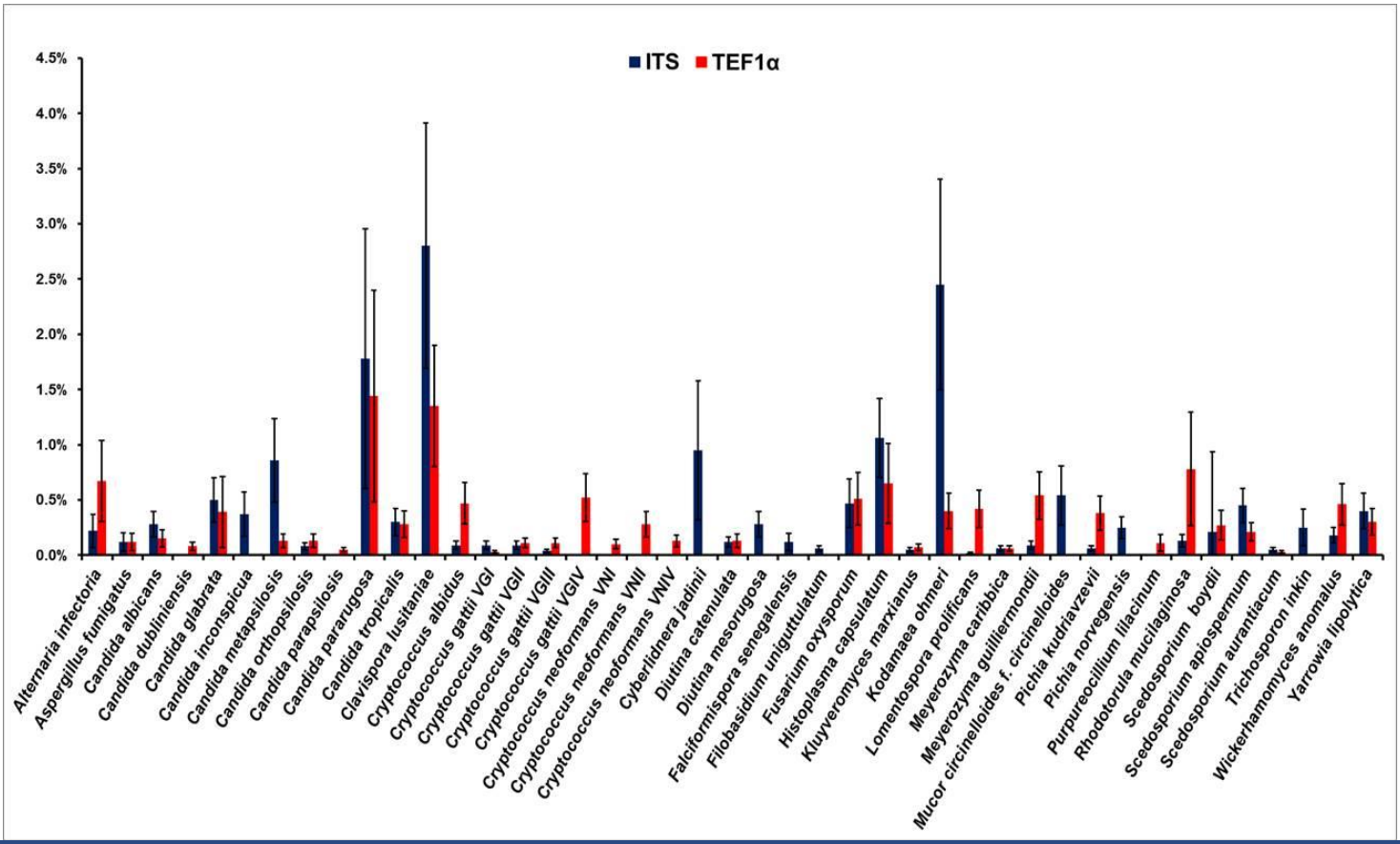

Figure 2. *Intraspecies variation for species, which are represented by more than three strains, in the primary (ITS) (blue bars) fungal DNA barcode compared with the translation elongation factor 1α (TEF1α) (red bars) secondary fungal DNA barcode.*

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New fungal DNA barcode database: The ITS and the *TEF1α* databases have now been combined to form the new "ISHAM BARCODING DATABASE", which currently contains 4200 ITS and 908 *TEF1α* sequences. The 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 *TEF1α* locus sequences, or both, against the reference sequences maintained in the "ISHAM BARCODING DATABASE".

Value of the dual barcoding system for fungal identification: In summary, 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 are not be distinguishable using the ITS region, due to the fact that 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, *TEF1α*, supported by a quality-controlled reference database, increases the ability to accurate identification of all clinically important fungal pathogens. Figure 3 shows the suggested workflow within a routine clinical diagnostic laboratory.

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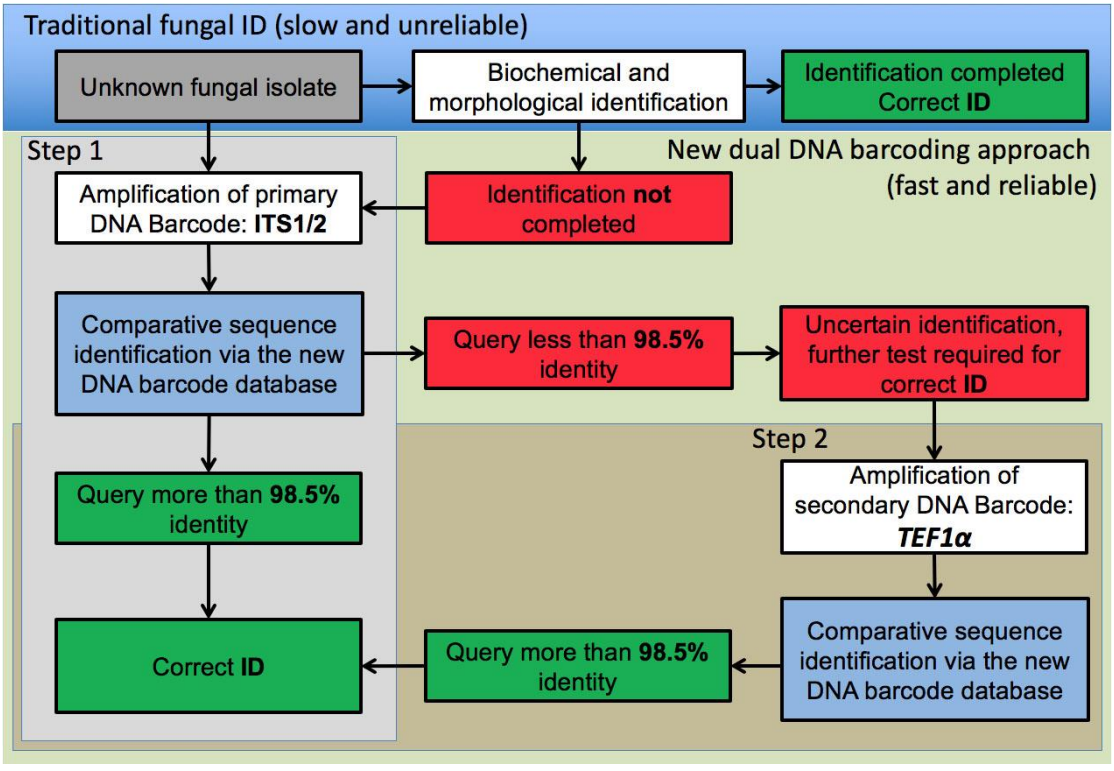

Figure 3. *Proposed workflow for the identification of mycotic agents in the routine clinical diagnostic laboratory.*

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Staff Profile

DR ELENA MARTINEZ

Dr Elena Martinez graduated as a clinical biochemist from the University of Uruguay, where she developed particular interest in mechanisms of antibiotic resistance in gram negative bacteria. This passion led her to post-graduate studies at the University of Technology Sydney, where she investigated mobile elements associated with antibiotic resistance in the opportunistic bacteria *Pseudomonas aeruginosa*. After earning her PhD at UTS in 2013, she joined the NHMRC Centre for Research Excellence in Tuberculosis Control and Prevention as a Postdoctoral researcher and later on moved as Postdoctoral Fellow to the Centre for Infectious Diseases and Microbiology – Public Health (CIDM-PH) Westmead. She is based at the NSW Mycobacterium Tuberculosis Reference Laboratory at Centre for Infectious Diseases and Microbiology (CIDM), Westmead Hospital, working on implementation of translational genomics for diagnosis and epidemiology.

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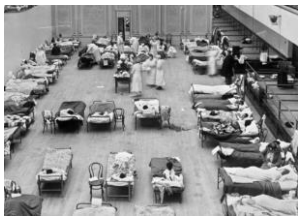
News & Events

The Centenary of the 1918 Pandemic: Advances in Diagnosis and Control of Influenza and Respiratory Viruses Symposium was co-hosted by CIDM-PH, MBI and NSW Health Pathology ICPMR at Westmead Hospital on 14th September 2018. The symposium was officially opened by Dr Kerry Chant, Chief Health Officer, Population and Public Health, Ministry of Health, followed by local and national key speakers. The symposium also featured an exhibition of memorabilia from the "Pandemic flu 1918" CSL archives.



Professor Dominic Dwyer, Professor Tania Sorrell, Dr Kerry Chant and Professor Vitali

Congratulations

New Funding

As a result of the successful Translational Research Grant titled 'Translating pathogen genomics into improved public health outcomes: Prospective evaluation of the effectiveness of genome sequencing-guided investigation of outbreaks' led by Professor Vitali Sintchenko, Professor Jon Iredell and CIDM-PH staff at Westmead, NSW Health have initiated continued funding for the state-wide public health pathogen genomics services at NSWHP ICPMR, Westmead.

NATA Accreditation

The CIDM-PH team has played a key role in the development and implementation of standard operating procedures and laboratory protocols for bacterial genome sequencing and staff training which enabled the accreditation of NSWHP ICPMR for microbial genomics surveillance and drug resistance testing.

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UPCOMING EVENTS

11-15 February 2019:

Sydney Summer School in Pathogen Genomics and Global Health

University of Sydney, Camperdown

2 August 2019:

Medical Entomology & Health Symposium

'Meeting the challenges of emerging mosquito-borne disease threats'

Westmead Education & Conference Centre, Westmead Hospital

Registrations opening soon

22 November 2019:

CIDM-PH Colloquium

Westmead Hospital, Sydney

Event Enquiries:

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