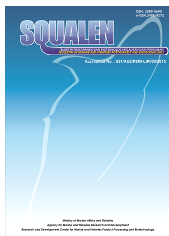

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INTERNAL TRANSCRIBED SPACER (ITS) as DNA BARCODING TO IDENTIFY FUNGAL SPECIES: a REVIEW

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Abstract

Despite the fact that fungi are important sources of both bioactive compounds and mycotoxins, and that they are very ubiquitous in our environment, their species identification is hampered by incomplete and often unclear literature. Fungi identification is primarily based on their phenotypic and physiological characteristics. Nowadays, many molecular methods to identify fungal species have been developed. One of the methods considered as a new concept to rapidly and accurately identify unknown fungal sample is DNA Barcoding. This literature review will outline the use of DNA barcoding approach to rapidly identify fungal species and the use of ITS region that recently has been designated as primary DNA barcode for fungal kingdom. “DNA barcode” is a short, highly variable and standardized DNA region with approximately 700 nucleotides in length, which is used as a unique pattern to identify living things. Internal Transcribed Spacer (ITS) region of nuclear DNA (rDNA) has become the most sequenced region to identify fungal taxonomy at species level, and even within species. ITS region is a highly polymorphic non-coding region with enough taxonomic units. Therefore, it is able to separate sequences into species level. Even though ribosomal ITS as a universal barcode marker for fungi is still hampered by few limitations, the ITS will remain as the key choice for fungal identification. The search for alternative regions as DNA marker to improve fungal identification, especially in specific heredities, has already started.

Keywords: DNA barcoding, fungal diversity, Internal Transcribed Spacer (ITS) , molecular identification, universal barcode

1. Introduction

Fungi denote the highest eukaryotic diversity on earth. According to Hibbett et al. (2011) and Das and Deb, (2015), approximately 1.5 million fungi species exist in our environment, while (O'Brien, Parrent, Jackson, Moncalvo & Vilgalys, 2005) estimated that the species' richness ranges from 3.5 to 5.1 million.

Numerous marine bioactive compounds had been isolated from fungi. Every year, approximately 200 new compounds, including polyketides, alkaloids, sesqui-terpenes, and aromatic compounds, are isolated and identified from marine derived fungi (Moghadamtousi, Nikzad, Kadir, Abubakar & Zandi, 2015). Many of them were reported to may have pharmacological potential as antifungal (Hong et al., 2015), antiviral (Moghadamtousi, Nikzad, Kadir, Abubakar & Zandi, 2015), and cytotoxic (Cao et al., 2015). On the other hand, fungi are also known for their pathogenic characteristics. Mycotoxins are fungal secondary metabolites that are toxic for humans, animals, and plants (Ismaiel and Papenbrock, 2015). As fungal contaminants of food, mycotoxins possess carcinogenic properties, life threatening toxicity, and other potential chronic effects (Ali, Xi & Coudray, 2016; Gong, Watson & Routledge, 2016). Some of the recognized genera of mycotoxigenic fungi are *Alternaria, Aspergillus, Claviceps, Fusarium, Penicillium,* and *Stachybotrys* (Reddy, Nurdijati & Salleh, 2010). Those genera are commonly associated with food during drying and storage (Pitt, 2000). Moreover, most of agricultural and fisheries commodities in developing countries are produced by traditional farmers who typically do not have proper facilities for pre and post harvest period (Rahayu, 2015). Dried salted fish, a traditional yet very famous fisheries product in Indonesia, has been reported to be contaminated by some fungal species

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(Indriati,Supriadi & Salasa, 2008; Rahayu, 2015; Wheeler, Hocking, Pitt & Anggawati,1986). Regardless of the fact that fungi are important sources of both bioactive compounds and mycotoxins, and that they are very ubiquitous in our environment, their species identification is hampered by incomplete and often unclear literature.

Fungi identification has been primarily based on their phenotypic and physiological characteristics. However, the unique characteristics of fungi create difficulties in morphology-based identification and classification. Thus, only well-trained experts are able to correctly identify fungi species solely based on fungal morphology (Samson, Houbraken,Thrane, Frisvad, & Andersen, 2010). Besides that, a well-trained technician may also be able to identify the specimens using step-by-step instructions from the morphological "keys" book. However, in many cases, experienced and professional taxonomists are needed. Therefore, an accurate and rapid identification approach on fungi (especially marine-derived fungi) is critical. Nowadays, many molecular methods to identify fungal species have been developed, including fluorescent in situ hybridization (FISH), DNA array hybridization, DNA sequencing, pulsed-field gel electrophoresis (PFGE), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and few other methods, but the most frequent technique used in fungal identification is DNA sequencing-based (Ali, Xi & Coudray, 2016; Pang & Mitchell, 2005; Samson, Houbraken,Thrane, Frisvad, & Andersen, 2010). One of the methods considered as a new concept for rapid and accurate identification of unknown fungal sample is DNA Barcoding (Chase & Fay, 2009; Das & Deb, 2015). This literature review will outline the use of DNA barcoding approach to rapidly identify fungal species and the use of ITS region that recently has been designated as the primary DNA barcode for fungal kingdom.

2. What Is DNA Barcoding?

"DNA barcode" is a short, highly variable and standardized DNA region, with about 700 nucleotides in length, which is used as a unique pattern to identify living things (Chase & Fay, 2009). DNA barcode is just like a unique pattern of bars that identifies each product in a supermarket. A supermarket scanner is able to distinguish items that for untrained eyes may look very alike, but have different barcodes. DNA barcodes can quickly and unambiguously analyze thousands of specimens in the database by computer program (Anon., 2014). Thus, DNA barcoding allows us to distinguish species and to identify both known and unknown species, even for non-taxonomist (Coissac, Hollingsworth, Lavergne, & Taberlet, 2016). DNA barcoding is an accurate, fast, universally accessible, and standardized method to identify species by using DNA sequences (Das & Deb, 2015). Moreover, it is also practical for non-expert taxonomist.

DNA barcoding concept was proposed for the first time by Hebert, Ball and Jeremy, (2003). In 2003, Hebert et al. proposed a 648 base pairs (bp) mitochondrial gene cytochrome c oxidase I (COX1) as the standard barcode for animals. Ever since, the use of COX1 has been very effective to identify animals' species. Currently, more than 4 million specimens and more than 398 thousands validated barcodes are listed in the Barcode of Life Data-system (BOLD) database (Anon., 2016). BOLD is an informatics workbench data system that gathers molecular, morphological, and distributional (geographical) data. BOLD serves the acquisition, analysis, storing, and publication of DNA barcode records (Ratnasingham & Hebert, 2007). Thus, DNA barcoding based identification systems enable the identification of both known and novel species (Hajibabaei, Janzen, Burns, Hallwachs & Hebert, 2006).

Regardless the success story of COX1 as the standard barcode for animals' species identification, the standardized barcodes for plants are more complex due to the lower variation of plants' mitochondrial DNA than animal mtDNA (Coissac, Hollingsworth, Lavergne, & Taberlet, 2016; Hollingsworth, 2011). In the data packages released by the International Barcode of Life project (iBOL) in quarterly basis, the number of COX1 barcode markers has always been much higher than the maturase K (MatK) and Ribulose-bisphosphate carboxylase (rbcl), which is plants barcodes. For example, in the data packages released in December 31, 2015, there are 163,325 COX1 barcode markers, but only 389 MatK and 1,523 rbcL barcode markers (Anon., 2015[b]). Even though alternative barcoding system for plants and fungi has been investigated, currently, many plant and fungal biological specimens are still identified based on their phenotypic and physiological characteristics, such as size, shape, color of body parts, etc.

The pipeline of DNA barcoding project is shown in Figure 1. Basically, the barcoding projects consist of four components, which are collecting specimens, laboratory analysis to obtain DNA barcode sequence of the specimens, constructing public DNA barcode reference library, and finding the closest matching species in the database (Anon., 2016).

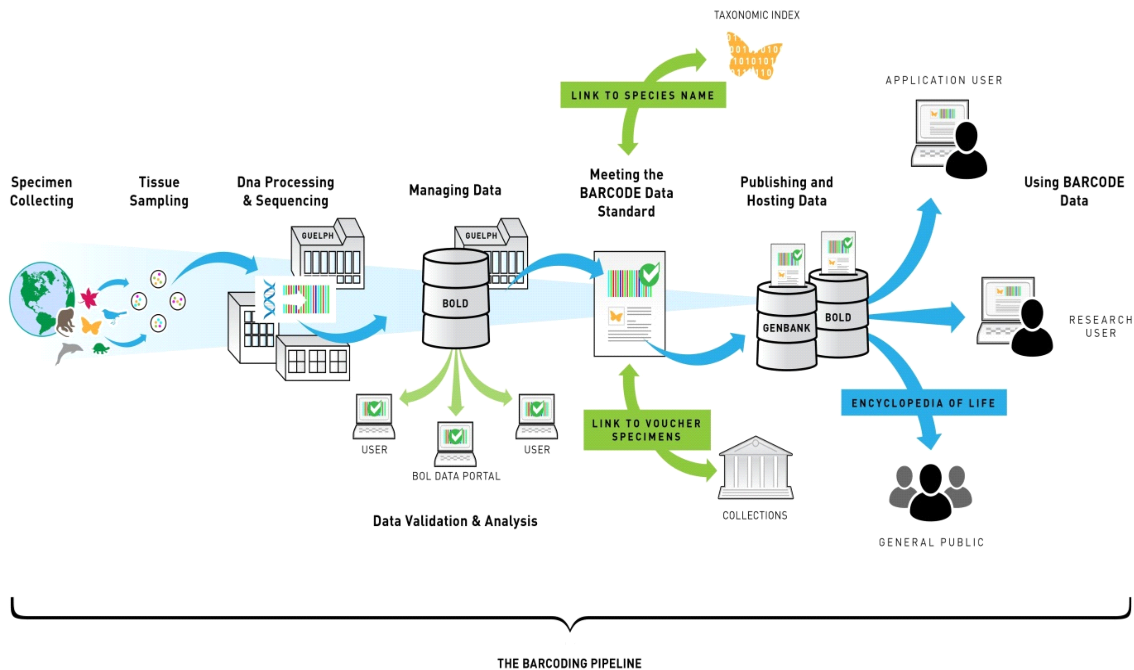

Figure 1. Workflow of The DNA barcoding (www.barcodeoflife.org).

3. Progress of DNA Barcoding Approach to Identify Fungi

Ideally, DNA barcode regions should be the same for all kingdoms (Schoch et al., 2012). In animals and algae, the variation between species (interspecific) in COX1 fragment is higher than within a species (intraspecific). Therefore, it is easy to distinguish even a closely related animal species. However, COX1 is not suitable to be used as the DNA barcode for most plants and fungi because the mitochondrial genes in the groups are evolving very slowly, which makes it difficult to distinguish the species (Chase & Fay, 2009). Due to COX1 drawback, there has been continuous effort to find DNA regions that will distinguish fungi species as a tool for both species identification and new species discovery.

Ideally, the interspecific variation of barcode locus should exceed the intraspecific variation (barcode gap) (Schoch et al., 2012). The barcoding locus will be optimal when the sequence is constant within species and is unique for each species (Hebert et al., 2003) to enable differentiation, even in closely related species. Moreover, a good reference datasheet has to be accessible to compare the obtained sequences (Samson et al., 2010). Several DNA loci of fungi have been used and studied extensively for identification, including large subunit RNA (25-28S), small-subunit (SSU) rDNA, and 5.8S and 18S ribosomal operons. However, all of those regions are too conserved for species identification. Until recently, only the Internal Transcribed Spacer (ITS) region of the nuclear DNA (rDNA) that becomes the most sequenced region to identify fungal taxonomy at species level, and even within species (Nilsson, Ryberg, Abarenkov, Sjökvist, & Kristiansson, 2009). ITS region shows higher degree of variations compared to the other regions of rDNA (SSU and LSU). Thus, the ITS region has recently been designated as the DNA barcode for fungal kingdom (Begerow, Nilsson, Unterseher & Maier, 2010; Bellemain et al., 2010).

ITS region is a highly polymorphic non-coding region with enough taxonomic units. Therefore, it is able to separate sequences into species level. It is located in the ribosomal RNA operon. The length ranges from 450 to 750 bp (Beeck et al., 2014). This region exists in two parts, ITS1 and ITS2, which are divided by the 5.8S rDNA. ITS region exists in numerous copies in the genome, and is easy to amplify. Another advantage of using ITS as DNA barcoding is that it had been used in many studies and that enormous reference sequences have existed in the NCBI sequence database, i.e. Genbank, EMBL, etc. (Samson et al., 2010). Moreover, as ITS region has been proposed as the primary fungal barcoding for fungi (Bellemain et al., 2010; Coissac et al., 2016; Das & Deb, 2015; Irinyi et al., 2015; Kõljalg et al., 2013), so the sequence available in the public database will be dramatically increased.

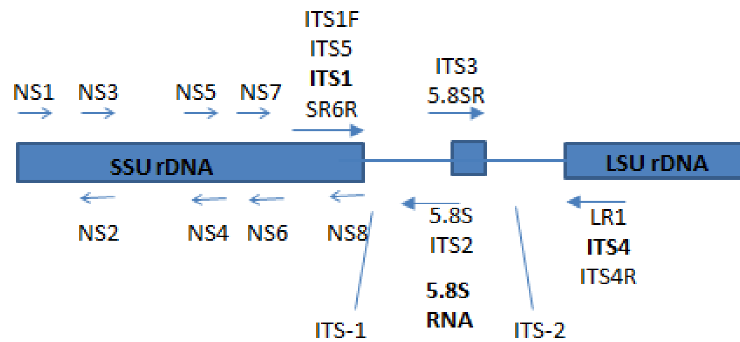

Figure 2. Diagram of primers location within the ribosomal cassette consisting of SSU, ITS1, 5.8S, ITS2, and LSU rDNA.

Table 1. Primers for amplification ITS region and annealing temperatures

Gene Primer	Primer Sequence	Tm (°C)	Reference
ITS1	TCCGTAGGTGAACCTGCGG	65	(White, Bruns, Lee & Taylor, 1990)
ITS2	GCTGCGTTCTTCATCGATGC	62	(White, Bruns, Lee & Taylor, 1990)
ITS3	GCATCGATGAAGAACGCAGC	62	(White, Bruns, Lee & Taylor, 1990)
ITS4	TCCTCCGCTTATTGATATGC	58	(White, Bruns, Lee & Taylor, 1990)
ITS5	GGAAGTAAAAGTCGTAACAAGG	63	(White, Bruns, Lee & Taylor, 1990)
ITS1-F	CTTGGTCATTTAGAGGAAGTAA	55	(Gardes & Bruns, 1993)
ITS4-B	CAGGAGACTTGTACACGGTCCAG	67	(Gardes & Bruns, 1993)
NS1	GTAGTCATATGCTTGTCTC	56	(White, Bruns, Lee & Taylor, 1990)
NS2	GGCTGCTGGCACCAGACTTGC	68	(White, Bruns, Lee & Taylor, 1990)
NS3	GCAAGTCTGGTGCCAGCAGCC	68	(White, Bruns, Lee & Taylor, 1990)
NS4	CTTCCGTTCAATTCCTTTAAG	56	(White, Bruns, Lee & Taylor, 1990)
NS5	AACTTAAAGGAATTGACGGAAG	57	(White, Bruns, Lee & Taylor, 1990)
NS6	GCATCACAGACCTGTTATTGCCTC	65	(White, Bruns, Lee & Taylor, 1990)
NS7	GAGGCAATAACAGGTCTGTGATGC	65	(White, Bruns, Lee & Taylor, 1990)
NS8	TCCGCAGGTTCACCTACGGA	65	(White, Bruns, Lee & Taylor, 1990)
NSA3	AAACTCTGTCGTGCTGGGGATA	67	(Martin & Rygiewicz, 2005)
NS11	GATTGAATGGCTTAGTGAGG	59	(Martin & Rygiewicz, 2005)

One of the important parts of DNA barcoding approach is the amplification of the targeted DNA barcode regions. However, any kind of sequencing technology is used. The key success of amplifying the targeted DNA barcode region is accurate specific primers. According to Beeck et al. (2014), the primers must be universal enough to cover a large group of taxa, while at the same time it should produce amplicons that are varied enough to powerfully distinguish the closely related species. Several taxon-specific primers have been designated to allow a selective amplification of fungal sequences (Figure 2 and Table 1). Among the ITS primers, ITS1 and ITS4 are the standard primers that are most frequently used by many laboratories.

Sequencing ITS regions is a good starting point for identifying fungal species, especially for the identification of completely unknown fungal species (Samson et al., 2010). However, as reported by Bellemain et al. (2010) ITS primers may produce amplification biases during the amplification of different parts of ITS region, especially in samples containing mixed templates (environmental samples/ metagenomic). For example, due to systematic length differences in ITS2 region, Bellemain et al. found that ascomycetes will be amplified easier than

Table 2. Overview of a typical PCR setup (Samson et al., 2010)

Component	Volume (µl)
DNA template (usually 20 ng/reaction)	1
MilliQ water	16.65
PCR buffer	2.5
dNTP (1 mM)	1.85
Dimethyl sulfoxide (DMSO)	1.25
Magnesium chloride, $MgCl_2$ (50 mM)	0.75
Forward Primer (10 µM)	0.45
Reverse Primer (10 µM)	0.45
Taq DNA polymerase (5 U/µl)	0.1
Final Volume	25

basidiomycetes, using those regions as targets. However, that bias can be avoided by using primers that only amplify ITS1. Thus, the use of ITS primers has to be selected carefully, especially when working with environmental samples as different ITS primer combinations should be analyzed in parallel (Bellemain et al.).

4. DNA Barcoding Procedure for Fungi Identification

The first step is growing the fungi in either solid agar media or liquid broth media. Whenever possible, collect all morphological characteristic data of the fungi, including macro-morphology (i.e. the size, the color, and the height of the fungal colonies) and micro-morphology (sporangiophores, sporangia, columellae, etc). The morphological characteristic data will be valuable to confirm correct identification based on DNA sequences present in the database.

The next step is genomic DNA extraction of the fungi sample. As fungal cells are not readily susceptible to lysis, a chemical, enzymatic, and/or chemical disruption approach is needed (Müller et al., 1998). There are many genomic DNA (gDNA) extraction protocols and many gDNA extraction kits available in the market. Basically, the steps in most fungal gDNA extraction protocol are: a). the lysis of the fungal cells by enzymatic, chemical or mechanical disruption, b). separating the DNA from the cells by centrifugation, c) removing the proteins, either by enzymatic or precipitation, d). the precipitation of the gDNA, e). removing salt by washing the gDNA, f). dissolving the gDNA with a suitable buffer.

After the gDNA is extracted, the next step is amplifying the ITS gene of interest by PCR. The general PCR set up is shown in Table 2. The annealing temperature depends on the primers used. An overview of commonly used ITS primers and their annealing temperature is given in Table 1. The PCR products are then analyzed by electrophoresis and are cleaned up from any unincorporated ddNTPs, unused primers, or Taq DNA polymerase. After the clean up, the amplicons are then used as a template for DNA sequencing. The sequencing results are compared to other sequences in the public database. After performing the database search, possible fungal species identity can be determined by examining the percent similarity and percent identity, or by generating a phylogenetic tree with near relative species (Balajee et al., 2009).

5. ITS Fungal Databases

Currently, there are several nucleotide sequence databases in the world. GenBank is one of the most important and the most influential nucleotide sequence database that can be accessed publicly. As GenBank is constructed by direct submissions from individual laboratories, some of the sequence data may be unreliable due to incorrect identification of the fungus from where the submitted sequence originates (Samson et al., 2010). However, that drawback has been managed since 2014 when National Center for Biotechnology Information (NCBI)'s fungi taxonomist, together with NCBI's RefSeq curator, in collaboration with mycology experts started curating fungal sequences from the ITS region (Anon., 2015[a]). All of the curated sequences have associated specimen data to ensure correct species identification and name change tracking. The re-annotated and verified fungi sequences are deposited in the RefSeq Targeted Loci (RTL) database, which can be accessed and searched using specialized BLAST (Schoch et al., 2014). Currently, several ITS fungal databases are also available, i.e. Barcode of Life Datasystem (BOLD), Canadian Centre for DNA Barcoding of Life (CBOL),

Table 3. Well-Established Databases for Fungal Identification

Genus	Databases	Locus	Reference
Penicillium and teleomorphs	GenBank; CBS Fungal Biodiversity Centre database	Calmodulin	(Das & Deb, 2015; Samson et al., 2010)
Aspergillus and teleomorphs	GenBank; CBS Fungal Biodiversity Centre database (http://www.cbs.knaw.nl/Aspergillus/ Biolomics.aspx)	β-tubulin	(Das & Deb, 2015; Samson et al., 2010)
Trichoderma	*TrichOKEY (http://www.isth.info/)*	ITS	(Samson et al., 2010)
	TrichoBLAST (http://www.isth.info/)	Elongation factor (EF-1α)	
Mycorrhizal fungi	Unified System for the DNA Based Fungal Species (https://unite.ut.ee/analysis.php)		(Das & Deb, 2015)
Fusarium	FUSARIUM-ID database (http://isolate.fusariumdb.org/)	TEF	(Geiser et al., 2004)
Phaeoacremonium	CBS Fungal Biodiversity Centre database (http://www.cbs.knaw.nl/phaeoacrem onium/)		(Das & Deb, 2015)

International Barcode of Life (iBOL), Mycobank (Das & Deb, 2015). Moreover, a number of specialized fungal sequence databases have also been built. The specialized fungal sequence databases (Table 3) may comprise DNA sequences that are not presented in public databases. However, as the specialized databases are established on different locus, initial morphological identification to genus level should be completed before applying the genes. For example, b-tubulin is the specialized locus for *Aspergillus* spp. That is why the ribosomal ITS as a universal barcode marker for fungi is still hampered by few limitations. However, the ITS will remain as the key choice for fungal identification. The search for alternative regions as DNA marker to improve fungal identification, especially in specific heredities, has already started (Schoch et al., 2014).

6. Conclusion

Many molecular methods to identify fungal species have been developed. One of the methods considered as a new concept to rapidly and accurately identify unknown fungal sample is DNA Barcoding. “DNA barcode” is a short, highly variable and standardized DNA region, with about 700 nucleotides in length, which is used as a unique pattern to identify living things. The Internal Transcribed Spacer (ITS) region of nuclear DNA (rDNA) has become the most sequenced region for fungal taxonomy identification at species level, and even within species. ITS region has recently been designated as DNA barcode for fungal kingdom. The region is a highly polymorphic non-coding region with enough taxonomic units- so it is able to separate sequences into species level. Even though ribosomal ITS as a universal barcode marker for fungi is still hampered by few limitations, the ITS will remain as the key choice for fungal identification.

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