REVIEW ARTICLE

Applications of DNA bar coding in molecular systematics of fungi: A review

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ABSTRACT

Fungi are a large, diverse and economically important group of organisms. Estimates of the actual number of fungal species vary widely from 1.5 million to 13.5 million, with fewer than 100,000 now known. Some fungi have relative complex and conspicuous morphologies, but others have very simple morphologies. Identification of fungi isolated from samples in laboratories is mainly based on observing morphology under the microscope and use of various cultural techniques. These conventional methods are very time-consuming and laborious. It usually requires several weeks for the fungi to grow sufficiently on culture media, and the identification processes on fungal phenotypic structure. DNA barcoding techniques have provided standardized, reliable and cost-effective methods for fungal species identification. Gene sequencing and phylogenetic analysis targeting the internal transcribed spacer (*ITS*) region in the fungal genomes are the most commonly used molecular methods for fungal identification. The *ITS* region has been heavily used in both molecular methods and ecological studies of fungi, due to its high degree of interspecific variability, conserved primer sites and multiple copy nature in the genome. It has been recently accepted as a suitable marker for barcoding fungi. This approach has immensely being used currently for both phylogenetic reconstruction and species recognition.

Key Words: DNA bar coding, Molecular systematics of fungi, rDNA, *ITS.*

INTRODUCTION

Fungal taxonomy is an essential part of biological research especially in the context of its ecological and economic implications (Shenoy *et al*., 2007). Traditional methods for fungal species identification require diagnostic morphological characters and are often limited by the availability of fresh fruiting bodies and local identification resources. About 100,000 fungal species have been identified yet, but it has been estimated that there may be from 1.5 to 13.1 million species. According to Hibbett *et al.*, 2011, over the last decade, about 1200 new species of fungi have been described in each year. At that rate, it may take up to 4000 years to describe all species of fungi using current specimen-based approaches. The use of morphology is extremely useful in several cases to

assign organisms to well-defined categories but reliable diagnostic procedure can be time-consuming and require the expertise of different taxonomists (Pereira *et al*., 2008).

DNA barcoding techniques have provided standardized, reliable and cost-effective methods for fungal species identification. Gene sequencing and phylogenetic analysis targeting the internal transcribed spacer (*ITS*) region in the fungal genomes are the most commonly used molecular methods for fungal identification (Das & Deb, 2015). The *ITS* region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species. Because of its higher degree of variation than other genic regions of rDNA that allow selective amplification of fungal sequences (Bruns & Gardes, 1993).

DNA Barcoding:

Taxonomy is the science of classifying living organisms according to their morphology. Today identification of organisms has become highly critical as effects of global warming and accelerating habitat destruction. Classical taxonomy falls short in this race to catalog biological diversity before it disappears http://www.dnabarcoding101.org/introduction.html.

DNA barcoding is a method for identifying living organisms to species. It makes use of a short, highly variably regions of the genome that evolves fast enough to differ between closely related species. DNA barcodes function as molecular identifiers for each species, in the same way as the machine-readable black-and-white barcodes are used in the retail industry to identify commercial products. Using Polymerase Chain Reaction (PCR) to amplify a targeted genetic marker and DNA sequencing to determine the content of that sequence, DNA barcoding allows for the comparison of an informative sequence from an unknown fungal sample against a database of identified sequences (Harrington *et al*., 2014).

In effect, DNA barcoding in its modern form was popularized in a paper by Hebert *et al.* (2003), who proposed to use the mitochondrial gene *COI* (cytochrome c oxidase subunit) as the standard barcode for all animals.

A region of the chloroplast gene *rbc*L – RuBisCo large subunit – is used for barcoding plants (Chase *et al.,* 2005). The most abundant protein on earth, RuBisCo (Ribulose-1,5-bisphosphate carboxylase oxygenase) catalyzes the first step of carbon fixation. A region of the mitochondrial gene *COI* is used for barcoding animals. Cytochrome c oxidase is involved in the electron transport phase of respiration. *COI* in fungi is difficult to amplify, insufficiently variable, and some fungal groups lack mitochondria. Instead, the nuclear internal transcribed spacer (*ITS*), a variable region that surrounds the 5.8s ribosomal RNA gene, is targeted. Like organelle genes, there are many copies of *ITS* per genome, and the variability in fungi allows for their identification. During the last 15 years the *ITS* of nuclear DNA has been used as a target for analyzing fungal diversity in environmental samples, and has recently been selected as the standard marker for fungal DNA barcoding (Bellemain *et al*., 2010).

Internal transcribed spacer (ITS) in DNA barcoding of Fungi:

The nuclear ribosomal coding cistron (rDNA) has been widely utilized for detecting variation among isolates of a fungus. The rDNA is composed of tandemely repeated units, each unit being composed of 5S, 25S and 18S rDNA. Two noncoding regions exits in each repeat: The internal transcribed spacer (*ITS* 1 and *ITS* 2) and and intergenic non-transcribed spacer (*IGS*). It has typically been most useful for molecular systematics at the species level, and even within species. Because of its higher degree of variation than other genic regions of rDNA (for small- and large-subunit rRNA), variation among individual rDNA repeats can sometimes be observed within both the *ITS* and *IGS* regions. There are two *ITS's* in eukaryotes; *ITS1* is located between 18S and 5.8S rRNA genes, while *ITS2* is between 5.8S and 25S rRNA genes (*Lafontaine & Tollervey, 2001*). *ITS* has proven especially useful for elucidating relationships among species and closely related genera in clinically important yeast species (Chen et al., 2011). The *ITS* region is the most widely sequenced DNA region in molecular ecology of fungi (*Peay et al., 2008). Schoch et al. (2012), proposed that* among the regions of the ribosomal cistron, *ITS* region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation. The *ITS* region has been proposed as the prime fungal barcode species identification (http://www.allfungi.com/its-barcode.php). The *ITS* region of fungi varies in length from 450bp to 750bp. Approximately 172,000 full-

length fungal *ITS* sequences are available in Genbank. *ITS* marker exists in multiple copies in most fungal cells and is retrievable by relatively strong primers with an established record of reliability. From some latest review papers on fungal barcoding, it is observed that in ascomycetes, *ITS* had the most resolving power for species discrimination (Das & Deb, 2015).

DNA barcoding procedure

The process from field samples to species abundance data involves a long series of steps, from sampling via laboratory handling to bioinformatics treatment. At each step, there is a risk of losing and distorting information.

Lindahl *et al*., (2013) has reported a standard procedure for DNA Barcoding using *ITS* region in fungal identification. It involves following steps.

1. Collection of Samples:

Collection of fungal samples vary according to season, mycelia, growth pattern and fruiting bodies For fungi, use of fruiting bodies is always best since it is easier to obtain DNA from fruiting bodies than mycelia (http://www.dnabarcoding101.org). Multiple samples from same should be collected that appears similar, avoiding the contamination by other fungi. Fresh samples work well for DNA isolation (Kelly *et al*., 2011).

2. Handling of samples:

Freeze-drying at -20° C enables long-term storage at room temperature, and may also aid later sample homogenization. It also restricts sporulation and rapid growth of opportunists (Lindahl *et al*., 2013).

3. Homogenization and sub sampling:

Fungal tissue ~10–20 mg should be obtained from the sample. If working with more than one sample, care should be taken not to cross contaminate specimens (Lindahl *et al*., 2013 & http://www.dnabarcoding101.org). The samples are normally homogenized using appropriate lysis mixtures followed by storing at -20° C.

4. Extraction and purification of DNA:

DNA is extracted from the fungi using standard protocols (http://www.dnabarcoding101.org). Extraction should yield high and uniform amounts of DNA. To achieve this, same extraction protocol should ideally be used for all samples (Tedersoo *et al*., 2010). The DNA precipitation may have to be further purified by binding of DNA to a silica matrix (Lindahl *et al*., 2013).

5. Markers and primers: The ideal marker for fungal community studies should have primer sites that are shared by all fungi, be of appropriate length for efficient amplification and sequencing, have high interspecific variation but low intraspecific variation, and be possible to align across all fungi. This is the main reason why the *ITS* region has been particularly attractive for mycologists (Gazis *et al*., 2011).

6. Multiplying DNA by PCR: DNA is extracted from the fungi, and the barcode portion of the ITS gene is amplified by PCR. After thermal cycling, the amplified DNA is stored on ice or at -20 °C. Before sequencing, PCR products from different samples are mixed in equimolar proportion, so that the DNA sequence output is evenly distributed across all samples. The PCR products have to be purified, to remove primers and short DNA fragments (http://www.dnabarcoding101.org).

7. Analysing PCR Products by Gel Electrophoresis & Sequence alignment: The amplified sequence (amplicon) is submitted for sequencing in one or both directions (Das & Deb, 2015). The PCR samples are subjected to gel electrophoresis and the bands in each lane of the gel are interpreted (http://www.dnabarcoding101.org). *ITS* primers amplify differently sized products that migrate to different positions on the gel. DNA sequencing of *ITS* amplicon is required to determine the nucleotide sequence that constitutes the DNA barcode. A single, good-quality barcode from the forward strand is sufficient to identify an organism.

8. Bioinformatics analysis: The sequencing results are then used to search a DNA database. A close match quickly identifies a species that is already represented in the database (Das & Deb, 2015). Novel DNA barcodes can be submitted to GenBank (www.ncbi.nlm.nih.gov). For the fungi primers, the hits should all be to the nuclear internal transcribed spacer of the 5.8s ribosomal RNA gene.

9. Data interpretation: Computer based analysis are applicable for DNA barcoding (Das & Deb, 2015). Barcode Sequences were deposited in GenBank and

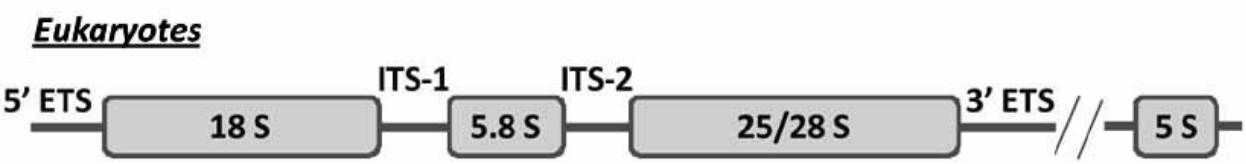

Fig: Ribosomal RNA genes

The genomic organization of ribosomal RNA (rRNA) genes - responsible for the synthesis of RNA species (the core of ribosomes). Almost all eukaryotes have several copies of each rDNA cluster organized in tandem repeats. In this case, each cluster contains the 18S, 5.8S and 25/28S rRNAs, while the 5S gene is present in separate repeat arrays in the majority of eukaryotes. Where ITS=internal transcribed spacers, ETS=external transcribed spacers.

Cited from: Pereira *et al*,. 2008. "Identification of Species with DNA-Based Technology: Current Progress and Challenges" *Recent Patents on DNA & Gene Sequences* 2008, Vol. 2, No. 3, P. 187-200.

compared against those sequences already found in the databases using the Basic BLAST search option of BLAST 2.0 (http://www.ncbi.nlm.nih.gov/BLAST & Photita *et al*., 2005). *ITS1* and *ITS2* including 5.8S sequences were aligned using the multiple sequence alignment program CLUSTAL W (http://www.dnabarcoding101.org).

Reports of identification of fungal species using DNA barcoding:

Robideau *et al*., (2011) had reported use of DNA barcoding in species identification of oomycetes especially in case of genera like *Phytopthora* and *Pythium*, they tried DNA barcoding for 1205 isolates representing 23 genera.

Seena, *et al*., (2010) examined the suitability of *ITS1*-5.8S-*ITS2* rRNA gene region to identify aquatic hyphomycetes, by sequencing and comparing these regions in 94 fungal isolates belonging to 19 species collected in Portuguese streams with different environmental conditions.

Khaund & Joshi (2014) had characterized 10 species of wild mushrooms of Meghalaya, viz. *Gomphus floccosus*, *Lactarius deliciosus*, *Lactarius volemus*, *Cantharellus cibarius*, *Tricholoma viridiolivaceum*, *Inocybe* aff. *sphaerospora*, *Laccaria vinaceoavellanea*, *Albatrellus ellisii*, *Ramaria maculatipes* and *Clavulina cristata*. They also reported that the final species identity generated by the *ITS* marker matched more accurately with the morphological characteristics/appearance of the specimens indicating the *ITS* region as a reliable barcode for identifying wild edible mushrooms. Irinyi *et al*. (2016) depicted standardization of *ITS* sequence based identification of fungi causing infections in humans and animals.

Khodadadi *et al*. (2014), in their studies tried to identify the clinically rare yeast isolates. 49 out of 855 (5.7%) yeast isolates which formerly remained unidentified by PCR-RFLP method were subjected to sequence analysis of *ITS* regions. These species include: *Hanseniaspora uvarum, Saccharomyces cerevisiae, Sporidiobolus salmonicolor, Pichia fabianii, Pichia fermentans, Candida famata, Candida inconspicua, Candida maqnoliae, Candida guilliermondii, Candida kefyr, Candida rugosa, Candida lusitaniae, Candida orthopsilsis*, and *Candida viswanathii*.

Geiser *et al*. (2007) published complete genome sequences of eight *Aspergillus* species (*Em. nidulans*, *A. oryzae, A. fumigatus, N. fischeri*, *A. terreus, A. clavatus, A. niger, A. flavus*) based on DNA barcoding.

Wang *et al*. (2016), evaluated six different loci in their study as potential barcodes in *Chaetomium* indicated that the 28S large subunit (LSU) nrDNA and the internal transcribed spacer regions and intervening 5.8S nrRNA (*ITS*) gene regions were unreliable to resolve species, whereas β-tubulin (*tub2*) and RNA polymerase II second largest subunit (*rpb2*) showed the greatest promise as DNA barcodes for differentiating *Chaetomium* species.

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