Applications of *ITS* region in fungal DNA barcoding:

A Current Perspective

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Abstract:
Traditionally identification of fungi is based on the collective information gathered from macroscopic and microscopic observations, staining, and/or any additional tests. Identification may range from a broad classification at the genus level to a more specific identification at the species level. This process is very tedious and time-consuming, as different fungi exhibit different morphological and cultural characteristics. Moreover, the accessibility of specialized media and mastery of fungal biology are crucial requirements for accurate identification. It is estimated that the estimate of the number of fungal species on Earth varies widely from 1.5 million to 13.5 million, but only 100,000 are characterized to date. With the advent of modern molecular techniques, new methods for the identification of fungal species have been developed. One such method is DNA Barcoding can be potentially used to identify the unknown fungal sample rapidly and explicitly. DNA barcoding has proven to be a vital resource and an innovative molecular diagnostic tool in this genomics era. DNA barcoding aims to identify the species using short and target gene sequences. These sequences vary sufficiently between the species and remain relatively conserved within the same species. The most used gene for fungal DNA barcoding is the internal transcribed spacer (*ITS*) region of the ribosomal RNA gene. Since it is the highly polymorphic noncoding region best suited for fungal identification. This is particularly valuable when dealing with complex or cryptic species where traditional morphological identification might be challenging.

Key Words: DNA barcoding, fungal biology, *ITS*, ribosomal RNA gene, genomics, identification.

Introduction:

Fungi are a unique group of organisms in their behaviour and cellular organization. In terms of biodiversity many fungal organisms are yet to be identified (Zhou & May, 2022). Compared with animals and plants, fungi are much less studied, and fungal taxonomy itself has a low profile. The fungal taxonomy plays a crucial role in biological research, and its significance extends to both ecological and economic domains. (Schmit & Mueller, 2007; Shenoy *et. al.,* 2007 and Wu *et. al.,* 2019).

Indeed, traditional methods of fungal identification often involve the observation of macroscopic and microscopic features of fungi in their natural habitat or after cultivation on specific media inside the laboratories. This approach to morphological identification is based on characteristics of specimens such as fruiting bodies, spores, and mycelium. However, this has its limitations, and not all fungi can be easily identified through this approach. Culturing the specific fungal organism can be challenging since seasonal, physiological, and nutritional requirements vary considerably(Gautam *et. al.,* 2022). In cases where traditional methods are insufficient, molecular techniques have become invaluable tools for fungal identification and classification.

It has been estimated that around 1.5 and 13.5 million fungal species exist, of which only 100,000 species are now identified and characterized (Lücking *et. al.,* 2021).

The estimate provided in the context of Hibbett *et. al.* (2011) underscores the substantial challenge of cataloguing and describing the immense diversity of fungal species on Earth. The pace of discovering and formally describing new fungal species, while significant, may still be insufficient to keep up with the rate at which new species are estimated to exist.

The fungal identification using DNA barcoding techniques has provided standardized, reliable, and cost-effective methods at the species level (Das & Deb, 2015). In comparison with other DNA sequences, the internal transcribed spacer (*ITS*) region is the most analysed for fungal identification through gene sequencing and phylogenetic analysis (Seena *et. al.,* 2010).

The *ITS* region is situated between the small subunit (18S) and large subunit (28S) of the rRNA genes in the fungal genome. It consists of two sub-regions, *ITS1* and *ITS2*, separated by the 5.8S rRNA gene. This region is most extensively used in DNA barcoding procedures as a standard DNA barcode (Eberhardt 2012). The ITS region exhibits a relatively high degree of sequence variability among fungal species, making it suitable for distinguishing closely related species. While the *ITS1* and *ITS2* regions show high variability, the flanking regions (18S and 28S) are relatively conserved. This allows for the design of universal primers that can amplify the *ITS* region across a wide range of fungal taxa.

DNA Barcoding:

Traditional taxonomy often relies on observable morphological features, but certain species may exhibit minimal morphological differences despite significant genetic diversity. Molecular tools, such as DNA barcoding provide a more accurate representation of evolutionary relationships and can uncover hidden or obscure diversity that might be failed to notice using morphological traits alone (http://www.dnabarcoding101.org/introduction.html.).

DNA barcoding is a molecular technique that involves analyzing short, standardized DNA sequences to identify and classify species. In the retail industry, barcodes are used to uniquely identify and track products. Similarly, DNA barcodes provide a unique genetic identifier for species. The idea is that by examining this specific DNA sequence, scientists can quickly and accurately identify the species of an organism. This is particularly useful in fields such as biodiversity research, ecology, and forensics.

DNA barcoding often involves the use of Polymerase Chain Reaction (PCR) to selectively amplify a targeted genetic marker from the DNA of an organism. Once the targeted DNA region is amplified, DNA sequencing is employed to determine the actual nucleotide sequence of that specific region(Harrington et al., 2014).

This sequence is then used as the "barcode" for the organism. In the case of fungal DNA barcoding, commonly used markers include regions of the ribosomal RNA gene (e.g., *ITS* region – Internal Transcribed Spacer) or other genomic regions that exhibit variability between different fungal species.

The choice of the gene or genomic region is critical in DNA barcoding as it needs to be conserved enough to allow for PCR amplification but variable enough to distinguish between different species.

A portion of the mitochondrial cytochrome c oxidase subunit I (COI) gene is commonly used for all animals. The use of the COI gene was proposed by Hebert *et al*. in 2003. The rationale behind this choice was that the COI gene is conserved within species but exhibits sufficient variation between species to serve as a reliable identifier.

The For plants, chloroplast gene rbcL – RuBisCo large subunit is a commonly used gene for barcoding. The rbcL gene was proposed for plant DNA barcoding by Chase *et al*. in 2005. The choice of rbcL is based on its universal presence in plants, ease of amplification, and the level of variation observed between different plant species.

The technique relies on a small region of DNA, typically a portion of the mitochondrial cytochrome c oxidase subunit I (COI) gene for all animals, or region of the chloroplast gene rbcL – RuBisCo large subunit – is used for barcoding in plants (Chase *et. al.,* 2005).

The target DNA sequence is selectively amplified using the Polymerase Chain Reaction (PCR) and then sequenced. The obtained DNA sequence is compared to a reference database containing DNA sequences from previously identified specimens. The reference database can be created using DNA sequences from voucher specimens that have been accurately identified using traditional taxonomy, or from public databases such as GenBank or BOLD (Barcode of Life Data Systems).

The COI gene, commonly used for animal DNA barcoding, faces challenges in fungi. This includes difficulties in amplification and insufficient variability for effective species discrimination. Some fungal groups lack mitochondria, or their mitochondrial genomes might have features that make COI less suitable as a barcode marker.

The Internal Transcribed Spacer (*ITS*) region, located in the nuclear genome, is chosen as an alternative for fungal DNA barcoding. The *ITS* region is situated between the small subunit (18S) and large subunit (28S) ribosomal RNA genes and includes the 5.8S ribosomal RNA gene. This region is known for its variability and is widely used for studying fungal diversity. Over the last two decades, the *ITS* region of nuclear DNA has been a popular target for analyzing fungal diversity. The selection of *ITS* as the standard marker for fungal DNA barcoding reflects its utility and effectiveness in identifying and characterizing fungal species (Bellemain *et. al.,* 2010).

DNA Barcoding in Fungi using Internal transcribed spacer (*ITS*):

For DNA barcoding in fungi, the internal transcribed spacer (*ITS*) region of the ribosomal DNA (rDNA) is commonly used as the DNA barcode. The ribosomal DNA (rDNA) in eukaryotic cells is organized as tandemly repeated units, often clustered in one or more chromosomal loci. Each repeating unit typically contains genes encoding the three main types of ribosomal RNA (rRNA): 5S rRNA, 18S, rRNA, and 25S (or 28S) rRNA. These rRNA genes play crucial roles in the synthesis of ribosomes, which are cellular structures responsible for protein synthesis.

The *ITS* region is situated between the small subunit (SSU) and large subunit (LSU) rDNA genes in the eukaryotic ribosomal RNA (rRNA) gene cluster (Lafontaine & Tollervey, 2001). In fungi, this region consists of two non-coding regions: *ITS1* (between SSU and 5.8S rRNA) and *ITS2* (between 5.8S rRNA and LSU) and intergenic non-transcribed spacer (*IGS*). The *ITS* and *IGS* regions of the rDNA are often treated as relatively conserved markers since they can exhibit great variability among individual rDNA repeats within a genome. This variability can manifest in terms of sequence divergence and rearrangements that make them effective molecular markers in studies such as DNA barcoding and phylogenetic analysis.

The use of *ITS* region in fungal DNA barcoding has been successful in identifying and differentiating species in many fungal groups, including Ascomycota, Basidiomycota, and Zygomycota (Tekpinar, & Kalmer, 2019). Due to the combination of high variability, ease of amplification, and the availability of extensive reference databases has made *ITS* as a cornerstone in fungal barcoding, taxonomy, and ecological studies (Antil *et. al.,* 2022).

Indeed, the *ITS* region has been particularly valuable in elucidating relationships among species and closely related genera, especially in clinically important yeast species. The study by Chen *et al.* in 2011 likely highlights the effectiveness of the *ITS* region in the context of molecular identification and phylogenetic analysis within the realm of medical mycology.

Schoch *et. al.* (2012), proposed the suitability of the *ITS* region as a prime fungal barcode for species identification and emphasized the distinct advantages of the *ITS* region for broad fungal identification (http://www.allfungi.com/itsbarcode.php).

The length variation of the *ITS* region in fungi ranging from 450bp to 750bp, highlights its dynamic nature. This variability can be advantageous in capturing diverse sequence information, contributing to the resolving power of the marker.

The GenBank records show the availability of approximately 172,000 full-length fungal *ITS* sequences (Gosavi, 2016). The presence of the ITS marker in multiple copies within most fungal cells is a key feature. The observation from recent review papers that in ascomycetes, the *ITS* region has the most resolving power for species discrimination (Das & Deb, 2015). This underscores the wealth of data and diversity captured by this region. Such a large dataset enhances the reference resources available for fungal identification and phylogenetic analysis and also has potential applications in fields such as ecology, medicine, and agriculture (Peay *et al.,* 2008).

The procedure of DNA barcoding:

Indeed, the process of moving from field samples to species abundance data in DNA barcoding involves multiple steps, each introducing potential challenges and sources of variation. The specific procedures may vary slightly depending on the laboratory, target organisms, and goals of the study. Lindahl *et al.,* (2013) have reported a standard procedure for fungal identification with DNA Barcoding using *ITS* region. It involves following steps.

The DNA barcoding procedure for fungi using the *ITS* region typically involves the steps given below:

1. **Sample Collection**: A fresh sample of the fungus being studied is collected, typically fruiting bodies, mycelium, or spores according to seasonal availability (Kelly *et. al.,* 2011). Fruiting bodies are often preferred over mycelial samples for DNA extraction due to ease of extraction, higher DNA yields, and distinct morphology (http://www.dnabarcoding101.org). Collecting multiple samples helps reduce the risk of contamination by other fungal species. Contamination can occur if environmental DNA is inadvertently mixed with the target sample during collection, handling, or processing (Gosavi, 2016).

2. **Storage of samples**: The samples are subjected to freeze-drying at -20°C. Freeze-drying is a process that involves freezing the samples and then removing the ice by sublimation, resulting in a dried product. It can restrict sporulation and also inhibit the rapid growth of opportunistic microorganisms that could potentially contaminate or overgrow the fungal sample (Lindahl *et. al.,* 2013).

3. **Homogenization and sub-sampling**: The samples are homogenized using appropriate lysis mixtures. Homogenization is a process that breaks down the biological material into a uniform and consistent mixture, facilitating subsequent analysis. Fungal tissue samples of approximately 10–20 mg are recommended for the analysis. It's crucial to be careful when working with multiple samples to prevent any unintended mixing or contamination between specimens. After homogenization, the samples are stored at -20°C. This low temperature helps preserve the integrity of the genetic material, such as DNA until further analysis is conducted (Lindahl *et. al.,* 2013 & http://www.dnabarcoding101.org).

4. **Extraction and purification of DNA from fungal samples**:

DNA can be extracted from the fungal tissue samples using a commercial kit designed for DNA extraction or a standard laboratory protocol. Commercial kits often provide standardized and efficient procedures for DNA extraction. High-quality DNA is essential for accurate genetic analysis (http://www.dnabarcoding101.org). To ensure consistency and comparability across samples, the same extraction protocol should ideally be used for all samples. This practice helps minimize variability in the DNA extraction process, leading to more reliable results (Tedersoo *et. al.,* 2010). Silica matrices, often in the form of columns or beads, are commonly used for DNA purification to remove impurities and ensure a higher level of purity in the final DNA sample (Lindahl *et. al.,* 2013).

5. **Markers and primers**: The ideal marker should have high interspecific variation and low intraspecific variations. It should have primer sites shared by all fungi, allowing for the amplification of a broad range of fungal species. The marker should be of an appropriate length for efficient amplification and sequencing, facilitating the analysis of fungal communities (Gazis *et. al.,* 2011; Gardes & Bruns, 1993). The application of these primers is specified for the identification of mycorrhizae and rusts, indicating the versatility of the ITS region for studying different fungal ecological roles and pathogenic processes.

6. **PCR form multiplication of DNA from sample**: The *ITS* region is amplified using PCR with specific primers, such as *ITS1* and *ITS4* primers. These primers are designed to target the ITS region of fungal ribosomal DNA, as specified by Gardes & Bruns (1993). The PCR reaction mixture should include the extracted DNA from samples, suitable PCR primers,

appropriate DNA polymerase enzyme, and other necessary reagents. This mixture is essential for DNA amplification during the PCR process (Gosavi, 2016). After thermal cycling, the amplified DNA is stored on ice at -20 °C. This step is important for preserving the integrity of the amplified DNA before further processing. (http://www.dnabarcoding101.org). It involves the PCR amplification of the *ITS* region using specific primers, followed by the storage of amplified DNA, equimolar mixing of PCR products from different samples, and purification of the PCR products before sequencing. This process is part of the larger workflow for studying fungal communities and is in line with best practices for generating reliable DNA sequence data.

7. **Analysis of PCR Products using Gel Electrophoresis & Sequence alignment**: The amplified sequence is submitted for sequencing, which is carried out in one or both directions. This sequencing step is crucial for obtaining the nucleotide sequence information of the amplified DNA fragments (Das & Deb, 2015). PCR samples are subjected to gel electrophoresis. Bands in each lane of the gel are interpreted to visualize the size and quantity of the amplified DNA products. Different-sized products from *ITS* primers may migrate to different positions on the gel (http://www.dnabarcoding101.org). DNA sequencing of the *ITS* amplicon is required to determine the nucleotide sequence that constitutes the DNA barcode. This sequence information is essential for identifying and characterizing the amplified DNA fragments. A single, good-quality barcode obtained from the forward strand is mentioned as sufficient to identify an organism.

8. **Bioinformatic analysis**: The sequencing results are employed to search a DNA database. This database likely contains reference sequences of known organisms. A close match in the database quickly identifies a species. This suggests that the bioinformatics analysis relies on sequence similarity searches to find the most similar sequences in the database (Das & Deb, 2015). Novel DNA barcodes, which may represent new or previously unidentified species, 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. For the fungi primers, the hits obtained during database searches should align to the nuclear internal transcribed spacer (ITS) of the 5.8S ribosomal RNA gene. This region is commonly used in fungal DNA barcoding due to its variability and taxonomic informativeness.

9. **Interpretation of data**: The interpretation of DNA barcoding data involves computer-based analysis (Das & Deb, 2015). Then developed barcode sequences are deposited in GenBank. The deposited sequences are then compared with those already present in the databases, presumably to identify known species or find close matches. The Basic BLAST search option of BLAST 2.0 is employed for comparing barcode sequences (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 DNA barcoding for identification of fungal species:

There have been many reports indicating the use of DNA barcoding techniques for the identification of fungal species, particularly with the *ITS* region as the DNA barcode. Here are a few examples:

1. A report of complete genome sequences for eight different species of *Aspergillus* based on DNA barcoding was published by Geiser *et. al.,* (2007).
2. Seena, *et. al.,* (2010) noted that *ITS1*- 5.8S-*ITS2* rRNA gene region is the most suitable in the identification of aquatic hyphomycetes. They sequenced and compared these regions from 94 different isolates from 19 fungal species collected in environmental conditions from Portuguese streams.
3. Application of DNA barcoding techniques in the identification of species of oomycetean fungi like *Phytopthora* and *Pythium* was reported by Robideau *et. al.,* (2011). The barcoding procedures were tried by them for 1205 isolates belonging to 23 different genera.
4. Khaund & Joshi (2014) used the technique of DNA barcoding towards the identification of 10 species of wild edible mushrooms found in Meghalaya. They reported that the species identification done by use of the *ITS* marker was more accurate than that of the morphological identification.
5. Khodadadi *et. al.,* (2014), tried an application of barcoding for the identification of rare isolates of yeast which were clinically significant. This method was found to be superior to the PCR-RFLP method used by them before and many more isolates were characterized up to the species level using the same.
6. Chen *et.al.,* (2014) used DNA barcoding to identify 103 species of fungi from a diverse range of habitats in China. The researchers amplified and sequenced the *ITS* region and compared the resulting sequences to a reference database of known fungal *ITS* sequences. They were able to identify the species of all 103 fungal specimens, including several new records for China.
7. Irinyi *et. al.,* (2016) delineated potential application of *ITS* base DNA barcoding techniques in animal and human fungal pathogens.

8. Wang *et. al.,* (2016), investigated six different loci in their study to assess their suitability as DNA barcodes for distinguishing *Chaetomium* species. They found out that the 28S large subunit (LSU), nuclear ribosomal DNA (nrDNA) and the *ITS* regions with the 5.8S nrRNA (ITS) gene regions were found to be unreliable for resolving species within *Chaetomium*. They also reported that β-tubulin (tub2) and RNA polymerase II second largest subunit (rpb2) were showing the greatest promise as DNA barcodes.
9. Badotti *et. al.,* (2017), identified 64 species of *Cortinarius* mushrooms using DNA barcoding from the Pacific Northwest region of North America. They amplified and sequenced the *ITS* region and compared the sequences to a reference database of *Cortinarius ITS* sequences. They were able to accurately identify all 64 *Cortinarius* species, including several that were previously unknown to the region.

10. Réblová *et. al.,* (2021) published a study in Molecular Ecology Resources in 2021 used DNA barcoding to identify 163 species of fungi from the Amazon rainforest in Peru. The researchers amplified and sequenced the *ITS* region and compared the sequences to a reference database of known fungal *ITS* sequences. They were able to identify the species of all 163 fungal specimens, including several that were previously unknown to the Amazon rainforest.
    Overall, these and other studies demonstrate the effectiveness of DNA barcoding for identifying fungal species, particularly when used in combination with morphological and ecological information. The use of DNA barcoding has greatly advanced the identification and study of fungal diversity and it has the potential to enhance our understanding of fungal biology and ecology.

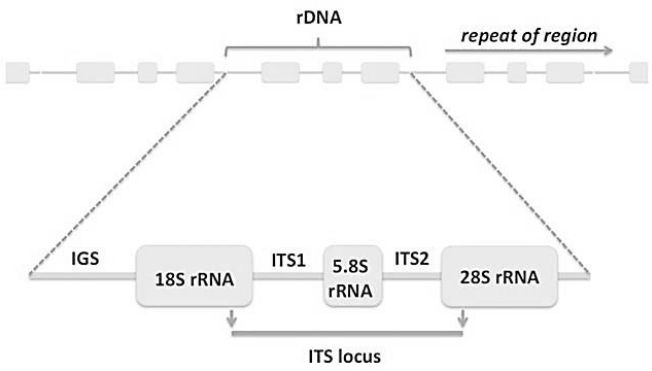

Figure: Structure of fungal nuclear ribosomal DNA: Showing regions coding for 18S rRNA, 5.8S rRNA, and 28s rRNA. Noncoding sequences at the intergenic spacer (*IGS*) and internal transcribed spacer regions (*ITS1* and *ITS2*) is presented. The position of the "ITS locus," an area commonly used for barcoding, is marked.
Cited from: Puvača, N., Budakov, D., Petrović, A., Vuković, G., Merkuri, J., Avantaggiato, G., ... & Cara, M. (2020). Molecular Characterization of Alternaria spp. and Presence of Toxin in Isolated Genes. *Journal of Agronomy, Technology and Engineering Management*. 3(6), 507-515.

Applications of DNA barcoding in fungi:

1. DNA barcoding has several applications in fungi, including species identification, detection of cryptic species, discovery of new species, and phylogenetic analysis. The identification of fungal species is essential for ecological studies, plant pathology, and medical mycology. DNA barcoding can accurately identify fungal species based on a short DNA sequence, even when traditional methods fail.

2. One of the main challenges in fungal taxonomy is the presence of cryptic species, which are morphologically similar but genetically distinct. DNA barcoding can distinguish between closely related species and identify cryptic species that would otherwise be overlooked. DNA

barcoding can also facilitate the discovery of new species by identifying sequences that do not match any known species in the reference database.

3.Phylogenetic analysis is another application of DNA barcoding in fungi. It can provide insights into the evolutionary relationships among fungal species and improve our understanding of fungal diversity and evolution. DNA barcoding can also be used to assess the genetic diversity of fungal populations and monitor changes in fungal communities over time.

Challenges of DNA barcoding in fungi:

1. Although DNA barcoding has many applications in fungi, there are also several challenges associated with this technique. One of the main challenges is the choice of DNA region to barcode. Unlike animals and plants, there is no consensus on which DNA region is the most suitable for barcoding fungi. Different regions have been proposed, including the internal transcribed spacer (*ITS*) region, the large subunit (LSU) region, and the mitochondrial cytochrome oxidase subunit 1 (*COI*) region. The choice of DNA region can affect the accuracy of species identification and the ability to distinguish between closely related species.
2. Another challenge is the presence of intraspecific variation in fungal DNA sequences. This can be due to several factors, including hybridization, horizontal gene transfer, and gene duplication. Intraspecific variation can lead to the misidentification of fungal species and reduce the accuracy of DNA barcoding.

Future directions for DNA barcoding in fungi:

DNA barcoding has already revolutionized our ability to identify and study fungal diversity, but there are still many potential future directions for this technology in the field of mycology. The following needs to be taken care in future:

1. **Expansion of reference databases**: As more fungal DNA sequences are generated and added to reference databases such as UNITE and NCBI, the accuracy and resolution of DNA barcoding for fungi will continue to improve. This will enable more accurate and precise identification of fungal species, especially for those that are difficult to identify based on morphology alone.

2. **Development of new barcode regions**: While the *ITS* region is currently the most widely used DNA barcode for fungi, there may be other regions of the fungal genome that could be more informative for certain taxonomic groups or research questions. Efforts are underway to evaluate the efficacy of alternative barcode regions such as the 18S and 28S rRNA genes, as well as other mitochondrial genes.

3. **Integration with metagenomic approaches**: DNA barcoding can be combined with metagenomic approaches, which analyse all DNA present in a given environmental sample, to gain a more comprehensive understanding of fungal diversity in natural communities. This approach has already been used in studies of fungal diversity in soil and plant-associated microbiomes.

4. **Application to ecological and evolutionary studies**: DNA barcoding can be used to study the ecological and evolutionary dynamics of fungal populations, including patterns of

geographic distribution, host associations, and diversification. As more fungal DNA sequence data becomes available, it will be possible to answer more complex questions about the biology and evolution of fungi.

Overall, the future of DNA barcoding in fungi is bright, with many exciting opportunities for new discoveries and advancements in the field of mycology.

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