Advances, Challenges, and the Future of Molecular Identification of Fungi

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ABSTRACT:

It is a very well-established fact that fungi play a crucial role ecologically and in agriculture, medicine, pharmaceutical and nutraceutical industries. Despite their significant role in varied sectors, fungi remain largely ignored when it comes to documenting their diversity. Traditional taxonomy has been a hurdle when it comes to cryptic species. DNA barcoding has arisen as one of the key tactics in identification of fungi. This review aims to survey these techniques which rely on the DNA as a source of information for the identification of fungi. It also aims to gauge its potential applications and limitations, finally proposing a consolidated approach. These approaches combine multi-gene phylogeny, metagenomics, and AI-based tools used to for taxonomic purposes. There remains a consistent challenge regarding molecular fungal identification, but recent advances in the field promise a thorough and straight-forward methodology for studying diversity of fungi.

KEYWORDS: Fungal DNA Barcoding, Molecular Fungal Identification, Mutli-gene Phylogeny.

INTRODUCTION:

The ever-elusive fungi are a vital part of the world's biodiversity. They have been known to help stabilise ecosystems, and aid in nutrient recycling thereby influencing plant health. Suffice to say, fungi are the internal cogs in the machine of biotic and abiotic interactions. Their significance is great, yet their diversity has been majorly ignored by the greatest minds in the world. Fungal biodiversity remains in its nascent stage in comparison to the diversity plants and animals. It is estimated that only 5-10%, of the approximately 2.2-3.8 million species of fungi, have been officially studied and described (Hawksworth & Lücking, 2017). As for plants, animals and algae, even for fungi, morphological identification remains insufficient to describe new species. Cryptic species and limited diagnostic features remain a hurdle in this case.

Traditional methods to identify fungi depend on morphological characters, such spore structure and ornamentation, hyphal structure and arrangements, and characteristics of fruiting bodies. The issue with these features is that they heavily get influenced by environmental conditions, and they also vary across the life cycle of a fungus. Many a times, researchers find it difficult to get a fungus which sporulates under laboratory conditions. These hurdles make correct fungal identification a challenge (Kerr and Leavitt, 2023). Hence, there was a much-needed shift in the approach to identify fungi, giving rise to molecular identification methods. Consequently, DNA barcoding surfaced as a means to overcome the limitations of morphological identification.

Molecular biology has become quite advanced in the sense that it aims to provide more hardy, reproducible and accurate results when it comes to DNA barcoding. DNA barcoding entails amplifying and sequencing a standardised short region of the fungal genome as a means of accurately identifying a fungal species. Internal Transcribed Spacer (ITS) region of the nuclear ribosomal RNA gene cluster has emerged as the most commonly used barcode sequence (Stielow et al., 2015). Technologies such as High Throughput Sequencing (HTS) and environmental DNA (eDNA) have allowed researchers to study fungi *in situ*, along with cryptic and unculturable species (Begerow et al., 2010).

This review aims to delve into recent developments in the field of fungal molecular identification highlight DNA barcoding and metabarcoding as an up-and-coming technology to address limitations of the current methodologies. The potential future of these technologies in ecology, medicine and industry has also been explored.

FUNGAL DNA BARCODING:

DNA barcoding entails identifying, amplifying and sequencing short, standard DNA sequences of an organisms' genome, ultimately helping in the identification of that organism till generic or specific level by employing a phylogenetic analysis of those sequences identified. Across various domains of life this technique has been employed and it has been successful in accurate identification of species that are difficult to morphologically distinguish.

Internal Transcribed Spacer (ITS) has become a standard barcode region since White et al. amplified the region for phylogenetic studies in 1990. The ITS region lies within the nuclear ribosomal RNA gene cluster (Schoch et al., 2012). The ITS region has two spacer regions ITS1 and ITS2, situated between the rRNA genes 18S, 5.8S, and 28S which are highly conserved regions. These characteristics have made the region ideal for taxonomic differentiation of fungal species.

The ITS region is a favoured as a fungal DNA barcode for several reasons such as (Begerow et al., 2010):

- **Universality**: ITS primers can amplify DNA across a broad range of fungal taxa.
- **Interspecific variability**: The sequences have enough variability to distinguish between closed related species.
- **Availability of Extensive Databases**: UNITE Database and GenBank house a large ITS dataset for comparison.

There are limitations to ITS as well. For the fungal groups such as Fusarium, Penicillium and members of Mucorales, it becomes difficult to distinguish between closely related species using only ITS region (Lücking et al., 2020; Xu, 2016). Resolution of cryptic species using only ITS has sometimes been not efficient because of nearly identical ITS sequences.

1. Multi-Locus Barcoding: It was immediately realised that use of a single gene locus for the identification of fungi was not going to be an efficient strategy in fungal taxonomy. Multi-locus sequence typing (MLST) was developed. Simply speaking a second DNA barcode region was needed for an effective species level identification of fungi. Some of the secondary DNA barcode regions are as listed below:

- **Translation Elongation Factor 1-α (TEF1α)**: It was used by Rehner and Buckley (2005) to establish and resolve the phylogenetic relationship between *Beauveria* and *Cordyceps*. It has been extensively used to identify *Trichoderma*, *Fusarium*, and other ascomycetes. Due to its high species discriminatory power, and primers availability make it an important gene locus for fungal identification (O'Donnell et al., 2008).
- **RNA Polymerase II Largest Subunit (RPB1)**: The gene locus helps to resolve phylogenetic relationships across Ascomycota and Basidiomycota. It is highly conserved locus which provides hardy information across taxonomic levels (Liu et al., 1999).
- **RNA Polymerase II Second Largest Subunit (RPB2)**: This gene locus is used in combination with RPB1 and TEF1 gene loci for species identification in genera like *Aspergillus*. It has higher variability than RPB1, hence making it effective in resolving species complexes (Lutzoni et al. 2004).
- **Beta-Tubulin (TUB2)**: The gene locus is useful in the identification of genera like *Penicillium*, *Aspergillus* and *Botrytis*. It complements ITS in cryptic species differentiation. It is a protein coding locus with variations in introns (Glass and Donaldson, 1995). Woudenberg et al., 2009 established a link between *Didymella* teleomorphs and *Phoma clematidina*.
- **18S rRNA (Small Subunit – SSU)**: This locus is used for environmental DNA surveys for a broader profiling of fungi from mixed samples. It has universal applicability (Banos et al., 2018).
- **Large Subunit (LSU or 28S rRNA)**: It is often paired with ITS and SSU gene loci for phylogenetic studies, only effective till genus or family level. It offers high-quality alignments for evolutionary relationship development among fungal groups (Vilgalys and Hester, 1990).
- **Actin (ACT1)**: This gene locus is useful in differentiating species in genera like *Colletotrichum* and *Aspergillus*. It is another protein coding gene locus and has moderate variability. It can be used on conjunction with ITS and TEF1 (Carbone and Kohn, 1999).
- **Calmodulin (CaM)**: This gene locus is widely applied in cases of *Aspergillus* and *Penicillium* species identification. It offers better resolution in species level identification (Hong et al., 2006).
- **Chitin Synthase (CHS1)**: This protein coding gene locus us useful for resolving complex plant pathogenic groups such as *Fusarium* and *Colletotrichum* (Carbone and Kohn, 1999).
- **Intergenic Spacer Region (IGS) of rDNA**: It is more variable that ITS and it is used for the intraspecific studies (Carbone and Kohn, 1999).

Multi-locus strategies are important in the field of medicine and agriculture where accurate and precise species level identification can help in making proper decisions of treatments (Vu et al., 2019). At the same time, the accurate identification of fungi brings in the much-needed respite in the field of fungal taxonomy. This ultimately contributes to fungal biodiversity around the world.

Table No. 1: Comparative uses of DNA Bardcode Sequences in Phylogenetic Analysis

Taxonomic Level	DNA Barcode Region (s)	Primary Use
Phylum and Class Level	SSU, LSU	Broad taxonomic identification
Genus Level	ITS, TEF1	Moderate resolution at genus level
Species & Strain Level	TEF1, TUB2, ACT, CaM, IGS	High-resolution species/strain ID

2. Role of DNA Barcoding: DNA barcoding has helped in various aspects of fungal diversity studies. It has made the discovery and description of fungal species jump leaps and bounds. Rapid and precise species identification from degraded samples can be done very easily now. Cryptic species can be decoded with precision. Researchers can now effectively establish and interpret, phylogenetic relationships leading to the formation of fungal tree of life. Olds et al., 2022 used cost effective metabarcoding method efficiently. They used two-step PCR approach and sequenced the nrITS2 region from 766 macrofungal specimens, achieving over 90% identification accuracy. They demonstrated the usefulness of metabarcoding techniques. Metabarcoding of environmental samples has led to revelation of dynamics of fungal communities. It does throw light on the association of fungal communities with other microbial communities in an ecosystem. Environmental samples from diverse ecosystems can be collected, such as from forests, agricultural soils, extreme environments like deserts, higher altitude regions and polar regions. Fungal DNA barcoding finds its application in all these aspects; the final aim being a better understanding of fungal diversity and functioning of ecosystems (Pham et al., 2024; Qi et al., 2024).

MOLECULAR FUNGAL IDENTIFICATION AND TECHNOLOGICAL ADVANCES IN THE FIELD:

The past few decades have led to a growth in sequencing techniques, bioinformatic tools, and development of molecular diagnostic studies. This has reshaped molecular identification in all phyla. Methods such as Next Generation Sequencing (NGS), digital PCR, Metagenomics have contributed to the strength of phylogenetic analyses. When aptly applied to fungal biology, they give us valuable insights, but they also come with limitations (Hibbett et al., 2016).

1. Polymerase Chain Reaction (PCR) and Sanger Sequencing: Polymerase Chain Reaction followed by Sanger's sequencing is the most common methodology adopted for fungal identification. Amplification of gene loci such as ITS using universal primers is effective for isolates which are pure cultured. Limitations include labour intensiveness, and reduced effectiveness if the samples are mixed (Schoch et al., 2012). PCR is followed by Sanger's sequencing which helps in clinical diagnostics and taxonomic revisions of fungi. Although, it becomes ineffective when dealing with large-scale samples (Nilsson et al., 2019).

2. Next-Generation Sequencing (NGS) and DNA Metabarcoding: 454 pyrosequencing was an expensive yet prime platform for fungal community studies. This high-throughput sequencing technology made a great contribution to the understanding of soil microbiota, but it was discontinued in 2016 with the advent of better methods. Next-Generation Sequencing Techniques such as Illumina MiSeq, HiSeq and NovaSeq and Ion Torrent PGM and GeneStudio platforms offer multiplicity in the sense of parallel sequencing. DNA metabarcoding has revolutionised the world of fungal diversity studies. The method has unveiled previously unrecognised fungi from soil, water, air and plants. Ecological interactions between fungal taxa can be established if this method is implemented effectively (Yu et al., 2023). The limitation lies in primer bias and less curated reference sequence databases (Nilsson et al., 2019).

3. Long-Read Sequencing Technologies: Oxford Nanopore MinION, GridION and PrometION, along with PacBio RSII and Sequel are long-read sequencing technologies where one continuous sequence read can span entire ITS regions or other multiple loci. This leads to higher resolution in phylogenetics with advantages over short-read platforms like Illumina (Nilsson et al., 2019). This aids in population genetics and identification of fungi from environmental samples. These sequencing techniques are costlier, but these they continue to grow with respect to resolution of complex taxa (Heeger et al., 2018; Wurzbacher et al., 2018).

Table No. 3: Comparative table showing the next generation sequencing techniques and long-read sequencing techniques (Nilsson et al., 2019).

Platform	Company	Generation	Read Length	Throughput	Accuracy	Strengths	Weaknesses	Use in Mycology
454 Pyrosequencing	Roche (Discontinued)	2nd Gen	800–1,000 bp	~1.2M reads/run	Moderate	Long reads, early driver of fungal HTS	Homopolymer errors, high cost, discontinued	Foundational, now obsolete
Ion Torrent	Thermo Fisher Scientific	2nd Gen	400–600 bp	5–12M reads/run	Moderate	Fast, affordable	Homopolymer bias, limited adoption in mycology	Rarely used
Illumina MiSeq/HiSeq/NovaSeq	Illumina	2nd Gen	2 × 300 bp (paired-end)	20–6,000 Gb	High	High accuracy, deep sequencing, standard for metabarcoding	Short reads, amplicon length limited (~550 bp)	Most widely used
PacBio RSII/Sequel	Pacific Biosciences	3rd Gen	3–25 kb (avg)	~400K reads/SMRT cell	High (with CCS)	Long reads, full-length ITS, high phylogenetic resolution	Lower throughput, higher cost	Advanced phylogenetics, full ITS
Oxford Nanopore	Oxford Nanopore Technologies	3rd Gen	Up to 2.4 Mb	Millions of reads	Low–Moderate	Portable, ultra-long reads, field sequencing	High error rate, under development for mycology	Experimental, in situ potential

4. Real-Time PCR and Droplet Digital PCR: Quantitative real-time PCR (qPCR) and droplet digital PCR (ddPCR) promise to give a speedy and sensitive fungal identification. Minute and degraded samples can also be assessed. These methods find application in the agriculture field for pathogen screenings. The methods provide for high specificity and quantitative assessment making them suitable for regular diagnostics. Lievens et al., 2006 demonstrated the viability of the technique for complex biological samples as well. Martinez-Diz et al., 2020 found ddPCR to be more sensitive over qPCR while assessing soil samples for *Ilyonectria liriodendra*, a soil borne pathogen known to cause black-foot disease in grapevine.

5. Metagenomics and Metatranscriptomics: Metagenomics aims to target all DNA sequences present in an environmental sample. This allows for an across-the-board identification of all organisms like fungi, bacteria, viruses, etc. Using metagenomics Donovan et al., 2018 showed that *Candida tropicalis* and *Candida loboi* were likely the same species. Metatranscriptomics, as the name suggests, focuses on RNA giving information of genetic expression and metabolic pathways. The composition and abundance of transcribed genes gives us a peak into the interactions of soil micro-ogranisms and plants (Kuske et al., 2015). This approach enables discovery of novel genes and biosynthetic pathways. The methodologies remain computationally demanding and expensive (Nilsson et al., 2019).

6. Bioinformatics and Fungal Databases: The triumph of molecular identification relies on hardy and accurate databases. UNITE database, GenBank, FungiDB, and MycoBank aim to revolutionise the fungal taxonomy world. They do have their own challenges when dealing with mislabelled sequences, conflicting taxonomical results, and gaps in metadata. The good thing is that these databases keep getting updated, and improvement have happened in the curation and integration of metadata to augment fungal barcoding studies. (Nilsson et al., 2019).

Table No. 3: The following table lists down the key features of a few fungal databases:

Database	Website	Primary Focus	Key Features	Data Type	Integration	Use in Mycology	References
UNITE	https://unite.ut.ee	Fungal ITS region; species hypotheses; DNA barcoding	~2.4M species hypotheses with DOIs; curated ITS data; community annotations; cross-kingdom links	Barcode and taxonomy-based ITS sequence data	Feeds into metabarcoding tools; integrates with GBIF and other resources	Species identification and barcoding, ecological and taxonomy research	Nilsson et al., 2018; Abarenkov et al. 2024
GenBank	https://www.ncbi.nlm.nih.gov/genbank/	General nucleotide sequence repository for all organisms	9.9 trillion bp from 2.1B sequences; daily sync with ENA/DDBJ; viral data integration	All nucleotide sequences and metadata	Core part of INSDC with ENA and DDBJ	Sequence archiving, reference data for BLAST and pipelines	Sayers et al., 2021
FungiDB	http://FungiDB.org	Functional genomics of fungi (genomes, RNA-seq, protein interactions)	18+ fungal genomes; RNA-seq, microarray, orthology tools; advanced query interface	Genomic and functional data (expression, annotations)	Part of EuPathDB infrastructure; cross-database querying	Gene function analysis, comparative genomics	Stajich et al., 2011
MycoBank	http://www.MycoBank.org	Fungal nomenclature and taxonomic registration	Mandatory name registration for fungi; LSIDs; typification events; curator access	Taxonomic names, metadata, linked sequence/culture data	Connected to Index Fungorum, GBIF, and others	Official registration of new fungal taxa and names	Crous et al., 2004; Robert et al., 2013

APPLICATIONS OF MOLECULAR IDENTIFICATION OF FUNGI:

Molecular identification of fungi finds applications in various field and sectors such as medicine, ecology, agriculture, taxonomy, etc. Rapid identification of fungal species has led to better ecological monitoring, disease diagnosis and fungal exploitation for betterment of humankind.

1. Clinical and Medical Mycology: Timely identification of fungal pathogens is vital for an effective and accurate diagnosis of a disease. This can bring about a proper treatment for a patient. Morphological identification is no doubt time consuming and may fail to identify non-sporulating fungi, that is where molecular identification comes into the picture.

- Real-time PCR and other sequencing tools can detect fungi within hours.
- Differentiation between pathogenic species can be easily done.
- DNA metabarcoding allows for analysis of rare and unculturable fungi in complex clinical samples, such as sputum, blood, or cerebrospinal fluid (Yu et al., 2023).

Molecular diagnostics have led to adapted and precise antifungal therapies, thereby, reducing mortality in invasive fungal infections (Wickes and Wiederhold, 2018).

2. Agricultural and Plant Pathology: Losses of crops due to fungal pathogens has been a major problem in the agriculture field, which even threaten global food security. Molecular tools in identification of fungi have led to early detection of plant pathogens. Before symptoms appear, plant tissue samples can be analysed for presence of fungal pathogens. MLST techniques have proven effective in monitoring the spread of pathogenic strains from one region to another. Even certain biocontrol agents can be identified giving a boost to the integrated pest management strategies (IPM) (Raja et al., 2025; Abbà et al., 2024; Das and Deb, 2015).

3. Environmental and Ecological Monitoring: Fungi can be employed for soil health assessment and management. As they are key nutrient cyclers, their presence in environmental samples is easily detectable using the current molecular identification techniques. Subsequently we can get an overall environmental impact of fungal populations. Swift identification of arbuscular and ectomycorrhizal fungi among plants also helps us understand symbiotic relationships. Identification of diversity of aquatic fungi in freshwater systems gives us an overview of water quality. Biodiversity surveys using eDNA is transforming biology with respect to conservation and restoration (Pham et al., 2024; Qi et al., 2024; Xu, 2016).

4. Industrial Applications, Food Safety and Mycotoxin Monitoring: Fungi have played an important role in industrial and biotechnological applications. Fungal enzymes, organic acids and biofuels have been utilised in various sectors of industries. Correct industrial strain identification is important for a proper fermentation and bioconversion process. Novel fungi from varied environments promise a wide array of applications in industries such has enzymes, myco-remediation against pesticides and heavy metals, antibiotic manufacturing etc. Prevention of contamination during industrial processes based in molecular identification of contaminant fungi ensures product quality. Contaminant fungi may produce mycotoxins such as aflatoxins, fumonisins, etc., which threaten food quality and lead to spoilage. qPCR assays can help quantify and identify toxic fungal contaminants. Environmental samples can be collected from air, surfaces and equipment to monitor production environments and keep they safe from fungal contaminants. Consumer safety becomes of utmost importance here. (Ezeonuegbu et al., 2022; Wickes and Wiederhold, 2018; Aslam et al., 2017).

FUTURE PROSPECTS AND CONCLUSION:

Molecular identification tools are becoming more influential in fungal biodiversity studies with the advancement of sequencing technologies, computational biology and other integrative approaches. Third generation sequencing technologies like Oxford Nanopore and PacBio are rapidly gaining credibility because of their ability to generate full-length barcodes and analysis of the same with improved species differentiation (Cai et al., 2025). Portable diagnostic tools, Artificial intelligence (AI) and machine learning are being developed to automate the classification of fungal sequences and predict their ecological roles. Tools such as neural networks can analyze complex DNA barcoding outputs and provide taxonomic assignments with increasing accuracy (Chinnasamy et al., 2023). Crowdsourced data collection, smartphone-based sequencing, and accessible bioinformatics tools are democratizing fungal research. Expansion of fungal databases such as UNITE, BOLD, MycoBank, FungiDB, etc., is bringing about accuracy and coverage of understudied fungal groups and encouraging scientists to contribute more (Abarenkov et al., 2024). Fungal systematics and its applications have come a long way.

Conclusively, fungal DNA barcoding has changed the landscape of fungal diversity studies in various sectors, and it is still evolving. Challenges remain, but a promising future stands tall for the fungal world in science, medicine, industrial prowess and sustainability.

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