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DNA barcoding of fungi using Ribosomal ITS Marker for genetic diversity analysis: A Review

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ABSTRACT

DNA barcode is a very short, standardized DNA sequence in a well-known gene. It provides a way to identify the species to which a plant, animal or fungus belongs. DNA barcoding is a new concept of identifying unknown organisms based on species-specific DNA regions. fungi are a group of eukaryotic organisms that includes microorganisms and these organisms are belongs to kingdom Fungi, which is separated from plants, animals, protists, and bacteria. The nuclear ribosomal Internal Transcribed Spacer ITS region is widely used as a DNA barcoding marker to characterize the diversity and composition of fungal communities. Since the early 1990s, 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. Primers have long been available for the nuclear ITS (Internal transcribed spacer) rDNA region which are now commonly used for fungal identification.COX1 gene is of limited use as a barcode for true Fungi as the length of fungal COX1 is highly variable (1.6–22 kb). The ITS region of fungi varies in length from 450bp to 750bp. Due to some limitations in COX1 gene ITS is introduced for barcoding of fungal species identification. ITS is a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. Most mycologists suggested that the internal transcribed spacer (ITS) of the nuclear ribosomal RNA, the first barcode for the kingdom Fungi. 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. The International Sub-commission on Fungal barcoding has proposed the ITS region as the prime fungal barcode or the default region for species identification.

Key words: *DNA barcode, COX 1 gene, ITS rDNA, fungal identification, biodiversity.*

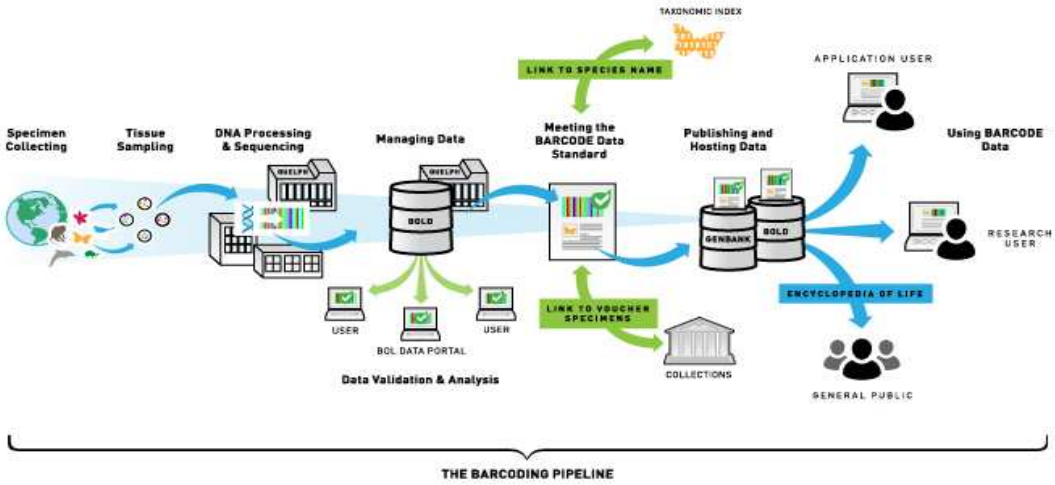
INTRODUCTION

Fungi represent the greatest eukaryotic diversity on earth and they become the primary decomposers in ecosystems. It was estimated that 1.5 million species of fungi exist in our environment[7]. Nowadays DNA barcoding has become a standardised tool for the assessment of global biodiversity patterns and it can allow diagnosis of known species as well as unknown species to non taxonomists[25]. DNA barcoding is a fast, accurate, and standardized method for species level identification, by using short DNA sequences. In 2003, Hebert from the University of Guelph, Ontario, Canada, proposed a new technique called DNA barcoding. He and his associates published a paper entitled "Biological identifications through DNA barcodes"[3].So he was called the father of DNA barcoding.

DNA bar coding technology: The concept of DNA barcoding proposes that effective, broad identification systems can be based on sequence based diversity in short, standardized gene regions[9, 2, 5].

In DNA barcoding, species are separated by standardized barcode gap analyses or phylogenetic tree-building methods. A barcode gap exists if the minimum interspecific variation is bigger than the maximum intraspecific variation. Alternatively, phylogenetic neighbour joining analysis based on Kimura two-parameter (K2P = K80) distances is a suggested standard method and in future more sophisticated phylogenetic methods will most likely be applied. The first official barcode accepted by the Consortium for the Barcode of Life (CBOL) consists of approx. 650 bp of the cytochrome oxidase subunit I (COX1) gene in the mitochondrial DNA, and it is mainly used for animal species identification[13].The mitochondrial DNA of plants has a low substitution rate, and a two-locus barcode from the chloroplast region, using the matK and rbcL genes consequently was proposed by the CBOL plant working group[10]. The two-locus barcode successfully separated different species with a success rate of approx. 72 %, which did not increase significantly when taking into account all seven markers (matK, rbcL, rpoC1, rpoB, psbK-psbI, trnH-psbA, psbK-psbI) analysed together. COX1 works well for most animal species [43, 27]; however some problems have been detected within the fungi.

Fig. 1: DNA Barcoding workflow (www.barcodeoflife.org)



Procedure of DNA bar coding: - DNA bar coding is used to identify plants, fungi, or animals—or products made from them. First, a sample of tissue is collected, preserving the specimen whenever possible and noting its geographical location and local environment. A small leaf disc, a whole insect, or samples of muscle are suitable sources. DNA is extracted from the fungi, and the barcode portion of the *ITS* gene is amplified by PCR. The amplified sequence (amplicon) is submitted for sequencing in one or both directions.

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. However, some barcodes will be entirely new, and identification may rely on placing the unknown species in a phylogenetic tree with near relatives. Novel DNA barcodes can be submitted to GenBank (www.ncbi.nlm.nih.gov). As of October 2013, the Barcode of Life Data System (BOLD: www.boldsystems.org; had a total of 192 350 species and 2 509 708 eukaryote specimens with DNA barcode data. The vast majority of barcodes in BOLD are for animals (193 664 species with CO1 sequences), followed by plants (54 974 species with rbcL sequences) and fungi (4266 species with ITS sequences) [30].

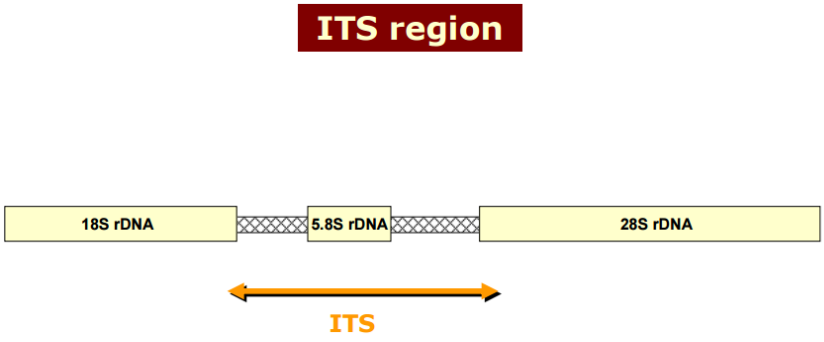
DNA bar coding for fungal species Identification: DNA bar coding is defined as the standardized analysis of an easily amplifiable PCR fragment for sequence-based identification of species. Identifications must be accurate, rapid, cost-effective, culture-independent, universally accessible and usable by non experts[3].

A part of the mitochondrial cytochrome c oxidase 1 (COX1) gene has become the first official animal DNA barcode[11] (http://www.barcoding.si.edu/) and for plants system is based on the plastid loci rbcL and matK[10], and in fungi, ITS is the official DNA barcode. A standardized DNA-based species identification system for fungi would be extremely useful. There are 1, 00 000 named fungi[15], and estimates suggest that as many as 1.5–3.5 million species exist[8, 26] in environment. Identification of many of these, particularly from their vegetative state, will only be possible by molecular methods. Primers have been available for the nuclear ITS rDNA region which are now commonly used for fungal identification[16, 35]. The ITS rDNA region will probably be proposed to the Consortium for the Barcode of Life (CBOL,http://www.barcoding.si.edu) as a fungal barcode[34]. As for many other organism groups, fungal sequence data derived from inaccurately identified material exist in the public databases[32].

Limitations of COX1 gene for fungal barcode: There are some limitations in COX1 gene. This region is unsuitable for easy PCR-amplification, sequencing and species identification for fungal barcoding according to the CBOL standards. Although this region showed effective for Penicillium spp. the length of fungal COX1 is highly variable (1.6–22 kb). The shortest barcoding region varies in length from 642 bp to > 12 kb. Moreover fungal species-level discrimination with COX1 genes may be inaccurate and in Fusarium and the Aspergillus niger other than ITS . The barcode region of COX1 contains several introns[21]. Moreover, the mtDNA of Glomus diaphanum contains a COX1 intron with high sequence similarity to a corresponding COX1 intron in plants and Rhizopus oryzae[22]. The plant intron is thought to have originated by horizontal gene transfer (HGT) from fungi. The COX1 genes have low copy no.

Emergence of Internal transcribed spacer (ITS) as a marker for DNA bar coding: *COX1* is of limited use as a barcode for true Fungi. Although there is interspecies variability in some groups. ITS is a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript from 5' to 3'. rDNA region consists of multiple copies (up to 200 copies per haploid genome) arranged in tandem repeats comprising the 18S small subunit, the 5.8S, and the 28S large subunit genes separated by internal transcribed spacer regions (ITS1 and ITS2)[1,23]. Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because it is easy to amplify even from small quantities of DNA (due to the high copy number of rRNA genes), and has a high degree of variation even between closely related species. Most mycologists expect that the internal transcribed spacer (ITS) of the nuclear ribosomal DNA will be established as the first barcode for the kingdom Fungi. This marker exists in multiple copies in most fungal cells and is retrievable by relatively strong primers with an established record of reliability. Because of this sensitivity and universality of the primers, a large reference database exists in Gen Bank.

Fig. 2: ITS region of ribosomal RNA[14]



Other well-established ITS databases e.g. is:

- CBS Fungal Biodiversity Centre database for medical fungi (http://www.cbs.knaw.nl/databases/)
- Fusarium species (FUSARIUM-ID v. 1.0; http://fusarium.cbio.psu.edu/index.html)
- Phaeoacremonium species (http://www.cbs.knaw.nl/phaeoacremonium/biolomics.aspx)
- Trichoderma species (http://www.isth.info/morphology.php)
- Mycorrhizal fungi (UNITE http://unite.ut.ee/).

Alternative barcode region for Fungal DNA barcoding: Other than ITS1/ITS2 other alternative barcode region for fungi are COX1 gene, D1/D2 LSU rDNA gene, Histone spacer, TUB, ACT, Elongation Factor, RNA polymerase genes such as RPB1, RPB. The D1/D2 LSU rDNA region was also considered for most groups of fungi, the D1/D2 region does not give sufficient signal at the species level. Considering that sequencing is becoming less expensive and the real cost is in the collection of the samples, sequencing this longer region might be desirable in the future. With so many fungi still not sequenced, the ITS often does not place an unknown with even a close match and thus the D1/D2 region works for placing unknown species especially when isolating fungi from the environment.

Table 1: Some ITS fungal databases and their description

S. No.	ITS Databases	Description
1.	Barcode of Life Datasystem (BOLD) http://www.barcodinglife.org)	BOLD is a web platform that provides an integrated environment for the assembly and use of DNA barcode data.
2.	Canadian Centre for DNA Barcoding (CCDB) www.dnabarcoding.ca	The CCDB is the birthplace of DNA Barcoding and offers unparalleled access to species identifications for over 180,000 animals, plants and molds.
3.	Consortium for the Barcode of Life (CBOL) (www.barcodeoflife.org)	Established in 2004 through support from the Alfred P. Sloan Foundation, CBOL promotes barcoding through Working Groups, networks, workshops, conferences, outreach, and training.
4.	European Consortium for the Barcode of Life (ECBOL) www.ecbol.org	(ECBOL) was established within EDIT Work package 3.4 "DNA barcoding". ECBOL functions as an information and coordination hub for taxonomists in Europe.
5.	International Barcode of Life (iBOL)	IBOL uses sequence diversity in short, standardized gene regions -- DNA barcodes. IBOL's main mission is extending the geographic and taxonomic coverage of the barcode reference library (BOLD).
6.	Quarantine Barcode of Life (QBOL) www.qbol.org	Developing DNA barcoding to identify quarantine organisms in support of plant health. to develop a database of DNA barcode sequences plus relevant taxonomic/geographic/host data.
7.	Assembling the Fungal Tree of Life (AFTOL)	The Assembling the Fungal Tree of Life (AFTOL) project is dedicated to significantly enhancing our understanding of the evolution of the Kingdom Fungi. AFTOL developed the first database to catalog fungal subcellular characters.
8.	ITS database for ectomycorrhizal fungi (UNITE) (http://unite.ut.ee)	UNITE is an rDNA sequence database to provide a stable platform for sequence identification of ectomycorrhizal and basidiomycetes. It is equipped with a BLAST interface for rapid similarity searches against the records of the database.
9.	Mycobank (www.mycobank.org)	MycoBank is an online database, documenting new mycological names and combinations.
10.	Maarj *AM*	Web-based database for studies on the diversity of arbuscular mycorrhizal fungi (Glomeromycota).

Qualifications of ITS region of rDNA: Within the true Fungi the ITS region varies from 527-700 bp and thus is easy to sequence using high throughput techniques. A huge database of ITS sequences already exists in GenBank. ITS has been sequenced for a sampling of all the major groups of true Fungi. In addition fungal-specific ITS primers exist, have been widely applied, and are known to work well across the major groups of fungi. A formal proposal to make ITS the barcode region for fungi will be submitted to CBOL. In addition, the ITS has been almost universally used by mycologists in sequencing fungi. The rest of the region can evolve without much selection, hence change is become rapid. While useful at the species or close to species level, alignment of the ITS region between major groups of fungi and even within order and families is usually not possible. Assuming the ITS sequences were alignable, the name of this gene as deposited in GenBank has varied with the over 65,000 accessions as has the length of the sequence submitted making extraction of all sequences of true Fungi difficult[31].

Requirements for DNA barcode for true fungi: selection of barcoding locus was based on the realization that the locus needed to be multicopy, have strong primer sites, and vary between species.

The multicopy requirement was based on the need to obtain sequences from old and degraded specimens limiting to rDNA segments or mtDNA loci. The robust primer requirement was based on the need to amplify from environmental samples with unknown contents, and only rDNA segments meet this requirement. The need for interspecies variation is obvious and it can say that no single locus would always meet this need[31].

Table 2: **Distribution of some fungal databases[29]**

S. No.	Name of the Fungal Species	No. of databases
1.	Penicillin spp.	430
2.	Monilinia spp.	54
3.	Aspergillus spp.	34
4.	Colletotrichum spp.	452
5.	Phytophtora spp.	77
6.	Fusarium spp.	1365
7.	Calonectria spp.	160
8.	Stenocarplella spp.	32
9.	Phoma spp.	309
10.	Mycosphaerella spp.	205
11.	Ceratocystis spp.	92

Bioinformatics analysis for the application of barcode sequences for fungi: Computer based analysis are applicable for DNA barcoding. Barcode Sequences were deposited in GenBank and 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)[28]. ITS1 and ITS2 including 5.8S sequences were aligned using the multiple sequence alignment program CLUSTAL W[41]. Phylogenetic trees were constructed from distance matrix values by the neighbor-joining (NJ) method[36]. In addition, sequence similarity search like BLAST should be applied and interpreted carefully, mainly due to the presence of conserved sequence region 5.8S rRNA inside the barcode locus and database population. Therefore it would be useful to discuss methods of barcode identifications currently used in different groups of true Fungi. The International Subcommission on *Trichoderma* and *Hypocrea* (ISTH) DNA barcode systems exemplifies this problem and one solution to it. Phylogenetic analysis were performed by GenBank (http://www.ncbi.nlm.nih.gov) were presented in FASTA format. Alignment of DNA sequences was done using ClustalW program. Phylogeny tree was constructed using *Phylogeny* in MEGA 5.03 program using Maximum Likelihood and evolutionary distance was analyzed and multiple sequence alignment (MSA) was analyzed using MEGA.

Over the last decade the field of DNA barcoding has emerged as a molecular method for species identification. Using molecular taxonomy to create a biological barcode that identifies organisms is the central goal of DNA barcoding, as well as creating a standardized reference library for the DNA based identification of target species[16]. There are two central principals of DNA barcoding: standardization of the PCR methods and protocols, and the ability to grow the data as the science progresses[11]. DNA barcoding can correct field misidentification, reduces ambiguity of species identification, makes species identification more exact, democratizes access by creating open access databases, and expands technical expertise of taxonomists[37]. DNA based identification systems use standardized molecular biology techniques (DNA extraction, PCR, and DNA sequencing) that can increase the speed of the identification of an unknown organism[38]. The goal of scientists who perform DNA barcoding is to create a library of every organism on earth [37]. In this paper the author studied that marine fungal richness in the shallow sediments off the Southeast coast of India. All morphologically identified fungal species were barcoded and digital records of the species were created in International DNA databases (NCBI) [42]. Similarly this paper showed that Six DNA regions were evaluated as potential DNA barcodes for Fungi, Three subunits from the nuclear ribosomal RNA cistron were compared together with regions of three representative proteincoding genes.

Among the regions of the ribosomal cistron, the internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap[39].

Comparative Study of some barcode region for fungal species: It was shown that several analyses to allow direct comparison of the barcoding use of the four barcode region under consideration (i.e., ITS, LSU, SSU, and RPB1). In basidiomycetes , ITS had the most resolving power for species discrimination. Overall, ribosomal markers had fewer problems with PCR amplification than protein-coding markers. ITS sequences shared among different species have already been documented in species-rich Pezizomycotina genera with shorter amplicons, such as the economically important genera Cladosporium , Penicillium, and Fusarium. In Aspergillus, ITS sequences are identical in mycotoxigenic, industrial, and medically important species, Although the ITS region is a potentially effective DNA barcode in several lichenized lineages.[18] In addition to Fungi, ITS may also be applicable as a barcode for other organisms. It is used in Chlorophyta and plants as well as in Oomycota. It was shown that some key aspects of the sequence variation in three gene regions (*BenA*, ITS, and *CO1*) that considered as the basis for a barcode system in *Penicillium*. These results indicate that both *BenA* and ITS show a high incidence of indels that complicate alignments within the subgenus, whereas no indels were detected in *CO1*. The ITS region has also been widely used in fungal taxonomy and it is known to show variation between species e.g., between Phytium ultimum and P. helicoides[19]; Peronospora arborescens and P. cristata[24]; Colletotrichum gloeosporioides and C. acutatum[20].

In the genus Penicillium, COX1 seems to be a likely candidate for species identification[38], although it has considerable size variations within the genus. The COX1 gene of fungi varied between 1, 548 bp to 22 kb, similar to the barcoding region within COX1 gene which ranged from 642 bp to 12.3 kb[33, 34]. In another case Only one COX1 gene was published for just one glomeromycotan species with a length of 2, 200 bp based on NC_12056[21]. However, the length could be varied as seen in other parts of the mitochondrial genome in the Glomeromycota. Multiple copies of the COX1 gene have been found in the genus Fusarium and proved inadequate for species level identification[5]. A similar situation was observed within the complex group of Aspergillus niger[6]. COX1 gene in Glomus diaphanum with a high sequence similarity to both a plant sequence and a Rhizopus oryzae COX1. This may result from a lateral gene transfer from fungi to plants[43, 22].

CONCLUSION

Designing and developing taxonomic specific DNA barcoding methods has been an ongoing process for researchers around the world. DNA barcoding methods are always improving and it is important to remain current with the identification of new primers and methods as new taxonomic groups are analyzed and methods are developed. In this review we have concluded that ITS region is very much effective for DNA barcoding of fungi groups and it is the major tools for identification of known as well as unknown organisms.

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