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DNA Barcoding: A Significant Molecular Approach for Identification Upto Species Level

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Abstract

DNA barcoding is a combination of biotechnological techniques in which DNA extraction, PCR amplification and DNA sequencing are mainly used, with the help of DNA barcoding organisms are identified up to species level specially the species which are cryptic and are not identified by traditional methods of taxonomy. In DNA barcoding organisms are differentiated on the basis of the variation in their specific gene sequences which are specific for specific species and these specific sequences are knows as Barcodes. Through DNA barcoding barcodes are generated and are stored in databases for future studies. In this article background of identification of different taxonomic groups through DNA barcoding and its importance were discussed briefly. One can use this technique at any stage of life, of an organism and on a wide range of organisms. With the help of this technique the organisms are identified easily, correctly and comparatively in much less time.

Keywords: DNA Barcoding, Molecular Identification, DNA Sequencing, Barcode.

Introduction

The term ‘DNA barcoding’ is of recent use in the literature (Floyd *et* al., 2002). It relies on the use of a standardized DNA region as a tag for rapid and accurate species identification (Hebert *et* al., 2005). Nevertheless, DNA barcoding is not a new concept. The term ‘DNA barcodes’ was first used in 1993 (Arnot *et* al., 1993), in a paper that did not receive very much attention from the scientific community. The concept of species identification using molecular tools is older still (Kangethe *et* al., 1982). However, the golden age of DNA barcoding began in 2003 (Hebert *et* al., 2003). The now well-established consortium for the Barcode of Life (CBOL, http:// barcoding.si.edu), an international initiative supporting the development of DNA barcoding, aims to both promote global standards and coordinate research in DNA barcoding. In 2003, (Paul Hebert *et* al. from the University of Guelph, Ontario, Canada, published a paper in the Proceedings of the Royal Society stating that the mitochondrial gene COI could serve as a genetic barcode for all animal life (Hebert *et* al., 2003). However, proposing a single gene barcode locus as a silver bullet to identify species across the whole animal kingdom.

DNA barcoding is a diagnostic technique for species identification, using a short, standardized DNA region, i.e., the ‘‘DNA barcode’’ (www.barcoding.si.edu). This technique uses PCR to amplify a fragment of a specific gene, which is then sequenced and compared to a database of known organisms. Over the last decade the field of DNA barcoding has emerged as a molecular method for species identification. DNA barcoding relies on a uniform region of the mitochondrial gene being amplified, sequenced, and analyzed by comparison to an open access database. 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 (Kerr *et al.*, 2007). 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 (Hollingsworth *et al.*, 2011). 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 (Stoeckle *et al.*, 2004). The precise

identification of organisms has been the realm of taxonomic experts who use specialized language and literature to describe and identify an organism; 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 (Seifert *et al*., 2007). The goal of scientists who perform DNA barcoding is to create a library of every organism on earth (Stoeckle *et al*., 2004).

Criteria for Ideal DNA Barcoding

The ideal DNA barcoding system should meet the following criteria (Taberlet *et* al., 2007)

1. The gene region sequenced should be nearly identical among individuals of the same species, but different between species.
2. It should be standardized, with the same DNA region used for different taxonomic groups.
3. The target DNA region should contain enough phylogenetic information to easily assign unknown or not yet 'barcoded' species to their taxonomic group (genus, family, etc.).
4. It should be extremely robust, with highly conserved priming sites and highly reliable DNA amplifications and sequencing. This is particularly important when using environmental samples, where each extract contains a mixture of many species to be identified at the same time.
5. The target DNA region should be short enough to allow amplification of degraded DNA. Usually, DNA regions longer than 150 bp are difficult to amplify from degraded DNA.

Thus, the ideal DNA barcoding marker should be variable, standardized, phylogenetically informative, extremely robust and short. Unfortunately, such an ideal DNA marker has not yet been found or, perhaps, does not even exist (Nielsen *et* al., 2006).

For DNA barcoding, it is important to have a suitable marker DNA sequence that satisfies a number of conditions. It should be easily and reliably amplified (in other words, it is necessary to have universal primers suitable for a wide range of species). It must be sufficiently variable in order to distinguish closely related species, but, at the same time, it should not have not too high of a substitution speed inside the species (Shneer, 2009).

Procedure of DNA Barcoding

DNA barcoding has three main steps: DNA extraction, PCR amplification, and DNA sequencing and analysis. DNA isolation is a key step because, without high quality DNA, the PCR amplification will not be optimal. The PCR amplification has to work so that there is DNA for sequencing. And finally, the sequencing analysis has to be successful for there to be an identification of the organism. Ensuring that these three steps are optimal is important for successful DNA barcoding. It is important to note that modifications to the DNA extraction process can sometimes be necessary.

Universal primer pairs are used to amplify a known region of the gene. By amplifying the same gene from diverse organisms it is possible to build a peer-reviewed library of gene sequences. It is important to know the taxonomic group (fish, bird, mammal, etc.) of the organism of interest because the PCR primers are specific to taxonomic group. For different taxonomic groups different genes are used for DNA barcoding.

It is necessary to check the amplification of PCR product; it is analyzed on an agarose gel to confirm that amplification has occurred. If there is a band, the PCR product can be sent for DNA sequencing. If there is no amplification, it will be necessary to troubleshoot the issue. This might require repeating the DNA extraction, trying out a different primer pair, or changing the master mix.

Once a PCR product has been obtained it is sent to a sequencing company to determine the identity of the organism. The sequencing company provides a ~700 base pair DNA sequence that without bioinformatics has no meaning. There are two programs that can be used to analyze the DNA sequence: Barcode of Life Data Systems (BOLD) and National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). Each program has positive and negative attributes.

DNA BOLD is a free program that compares the DNA sequence to samples that have been identified by a taxonomist and include additional data about the sample. This website is a hub for DNA barcoding information and analysis. NCBI BLAST is also a free program available online, were researchers can submit non-vouchered DNA sequences to the database. This program will compare a sequence to both vouchered and non-vouchered samples. Both of these programs use alignment programs to determine the identity of the unknown sequence. It is helpful to use both independent databases to identify the organism because it can increase the certainty of the identification. It is possible that the two programs will not agree on the identification it and not all organisms are in both the databases.

Specific Taxonomic Groups- Background and Methods

Prokaryotes

The term "DNA barcoding" almost never applies to prokaryotes (bacteria and archaea). However, we cannot help to mention that the identification and description of new prokaryote species and strains is at present largely built on 16S rRNA, which is part of a small ribosome subunit and is homologous to eukaryotic 18S rRNA (Stackebrandt and Goebel, 1994). In other words, DNA barcoding of protists and prokaryotes has the same basis. However, due to the small size of the latter genome, such works have been increasingly replaced with metagenomic studies and lately with the sequencing of complete genomes from single prokaryotic individuals (Rinke *et* al., 2013).

Fungi

DNA barcoding became an even more relevant method for fungi than for animals and plants. The number of fungal species is large. To date, about 1,00,000 species have been described, while the total number of fungal species ranges from 7,00,000 to several million according to different estimates

(Hawksworth, 1991; Schmit and Mueller, 2007; Begerow *et* al., 2010), and much less attention is paid to the taxonomy of fungi than to animals and plants. At the same time, most fungal species are characterized by relative morphological simplicity. It is not surprising that many DNA studies detected significant genetic variability in different fungal groups (Weiss *et* al., 2004; Crespo and Lumbsch, 2010). As in plants, the *cox1* mitochondrial gene sequence in fungi did not become a major marker for DNA barcoding. There are several reasons for this: the absence of conservative sequences suitable for primer selection; small number of nucleotide differences between closely related species; the presence of introns (up to 18 in the *Agaricus bisporus* (Férandon *et* al., 2013)); and the absence of mitochondria in some groups (Bullerwell and Lang, 2005; Gilmore *et* al., 2009). This place was occupied by the transcribed spacer rRNA region (ITS): ITS1 and ITS2 spacers separated by 5.8S rRNA gene (Fig. 1). The advantage of this sequence is that it is flanked by conservative 18S and 28S rRNA genes, for which it is easy to select universal primers. The ITS sequence has the highest amplification success as compared with other markers (Schoch *et* al., 2014; Xu, 2016). However, this marker has some disadvantages: it does not provide sufficient resolution in some cases (Xu *et* al., 2000); heterogeneity is sometimes found between copies in the genome. However, the International Fungal Barcoding Consortium acknowledged ITS as a main marker for fungal DNA barcoding (Schoch *et* al., 2014).

Protists

The information above about the prospects for fungal DNA barcoding equally applies to protists. According to different estimations, the number of described species varies from 40,000 (Hoef-Emden *et* al., 2007) to 75,000 (Pawlowski *et* al., 2012). The total estimated number of species is still difficult to assess and can be approximately from 1,50,000 to 1.5 million (Adl *et* al., 2005). As in the case of fungi, the rRNA cluster is the most suitable target for DNA barcoding. However, the protists are a paraphyletic and much more diverse genetically group. Thus, the 18S rRNA gene (more precisely, its V4 domain), not the ITS region, was taken as the main marker (Pawlowski *et* al., 2012). In addition, other markers are also frequently used, e.g., ITS or variable domains of 28S rRNA 5'-end; the mitochondrial *cox1* gene for protists with mitochondria; and plastid genes (e.g., 23S rRNA and *rbcL*) for protest photosynthesis.

Invertebrates

Folmer *et al.*, (1994) described “universal” DNA primers (named LCO1490 and HCO2198) for a 710 bp fragment of the mitochondrial cytochrome c oxidase I (COI) gene from eleven invertebrate phyla. These phyla include: Echinodermata, Mollusca, Annelida, Pogonophora, Arthropoda, Nemertinea, Echiura, Sipuncula, Platyhelminthes, Tardigrada and Coelenterata, and Vestimentifera. This publication helped to initiate the field of DNA barcoding. The original molecular technique was developed for phylogenetic studies of organisms from deep sea hydrothermal vents and cold water sulfide or methane seep communities. The PCR primers that these researchers designed have since been used for a wide range of studies and have been used to amplify DNA from more than 80 species. Whole cell DNA was isolated using a conventional hexadecyl-trimethyl-ammonium bromide (CTAB) protocol. The PCR was done in a 50 µL reaction mixture with 1 µL of DNA. Following amplification, the PCR product was analyzed on an agarose gel. Once the PCR amplification was performed, sequencing was done to verify that the sequence was COI and to build phylogenic trees.

Fish

There have been multiple papers that use DNA barcoding to identify fish. The Food and Drug Administration (FDA) is performing these analyses because it is important to ensure that fish available on the food market are labeled correctly by industry.

In 2007, Ivanova *et al.*, proposed the use of a primer cocktail (three forward and three reverse primers with M13 DNA fragments in the PCR primers) to amplify COI from representatives of 94 fish families. In this publication, M13-tailed primers were used to facilitate the sequencing and it was found that by incorporating the M13 tail into the forward and reverse primers it is possible to perform high throughput barcoding on taxonomically diverse samples. Each primer in the cocktail had M13-tails present; this enabled the researchers to use M13 sequencing primers to sequence the PCR products without having to use three different forward and reverse primer pairs.

The FDA has published a detailed SOP online for generating DNA barcodes suitable for species identification of an unknown fish tissue sample based on the publication (Handy *et al.*, 2011). Handy *et* al. were able to build on the work of Ivanova *et al.*, 2007 to create a single laboratory validated method for the generation of DNA barcodes that would meet regulatory compliance. The FDA’s SOP is robust and easy to follow. This SOP has been used at RDLES for the DNA barcoding of fish (Keele *et al.*, 2014).

Additional publications have surveyed the diversity of North American fish. April *et al.*, 2011 obtained the barcodes for 5,674 fish species (50 families, 178 genera, and 752 species) and was able to obtain sequences for more than 80% of the 902 Canadian and American species listed in the book “Common and Scientific Names of Fishes from the United Stated, Canada, and Mexico” (Nelson, 2004). The researchers were able to demonstrate that 90% of the fish sequences could be used to identify the organism by DNA barcoding. These authors also showed that the current fish taxonomy concealed diversity in some of the groups. For example, of the 752 expertly identified museum specimens analyzed, the researchers found 138 samples that needed to be reassessed by taxonomists. This research may help discover increased species diversity of fresh water fish in North America. The authors estimate that as many as 28% of the fresh water fish in Canada and America needed formal taxonomic descriptions. In the

future, the use of DNA barcoding will expand as fish populations are threatened and change.

Birds

Extensive DNA barcoding research has also been completed for birds. Hebert *et al*., 2004 was able to determine the DNA barcodes for 260 species of North American birds. All 260 species of birds had different COI sequences, and the differences between the closely related species was higher (18X) than the differences within a species. The researchers proposed that a 10-fold difference between DNA sequences could be used as a standard screening threshold to determine a new species. By using this threshold Herbert *et al*., 2004 was able to identity four new species of birds in North America.

Kerr *et al*., 2007 analyzed 643 species of North American birds primarily using the BirdF1, and BirdR1 primers. If the amplification was not successful then additional primers (FalcoFa, BirdR2, or VertebrateR1) were used. One reason the BirdF1/R1 primers did not always amplify the DNA was the significant difference between the DNA sequence and primer sequence prevented annealing. Most (94%) of the species analyzed had distinct barcodes. In the remaining 6%, the barcode clusters corresponded to small sets of closely related species that are known to hybridize.

Plants

Plants represent a more complex barcoding problem than other eukaryotes (such as animals) because plant mitochondrial genomes have a low nucleotide substitution rate (Hollingsworth *et* al., 2011). Plant scientists had much less luck with markers appropriate for DNA barcoding. Despite enormous efforts, no truly suitable marker could be selected. Unlike animals, plant mtDNA has a very low substitution rate as compared with nuclear and plastid genomes with fairly high rearrangement rates (Wolfe *et* al., 1987; Drouin *et* al., 2008); thus, mitochondrial genes are not applicable for plant DNA barcoding. Nuclear genes have (on average) higher substitution rate as compared with the plastid genes; however, their use is hampered by the complexity of the selection of universal primers and by the fact that most plants are paleopolyploids (Jiao *et* al., 2011; Li *et* al., 2015b). This especially concerns angiosperms, for which all families (except for several basal branches) passed at least two events of genome doubling (Soltis *et* al., 2015). The plastid genomes were a compromise choice: the substitution rate in them is slightly lower than in nuclear genomes; however, the set of the genes is more or less constant, and universal primers can be selected for many of them. Thus, more than a dozen plastid genes and nuclear ribosomal cluster sequences are currently used as markers for plant DNA barcoding. However, with the use of individual markers, it was found that the number of species successfully delimited based on DNA barcoding within individual genera and families sometimes does not exceed 50% (Fazekas *et* al., 2008; Hollingsworth *et* al., 2011). Obviously, such a low resolution cannot be acceptable; thus, several markers are usually simultaneously used. Independent attempts were made to standardize the set of markers for plant DNA barcoding (Chase *et* al., 2007; Kress and Erickson, 2007; Ford *et* al., 2009). Finally, the working group for plant DNA barcoding (Hollingsworth *et* al., 2009) accepted fragments of two plastid genes as a standard: a large subunit of ribulose- 1, 5-biphosphate carboxylase (*rbcL*) and maturase K (*matK*). This is a compromise solution: a marker for DNA barcoding must be easily amplified by universal primers and characterized by a high substitution rate. In the suggested combination, *rbcL* is easily amplified but has the lowest rate of evolution among the plastid genes, while the *matK* sequence evolves rapidly but amplifies poorly (Li *et* al., 2015a). Thus, the data on these markers are usually supplemented with auxiliary markers. Of them, the most frequently used are different plastid sequences (spacers between the *trnH* and *psbA*, *atpF* and *atpH*, *psbK* and *psbI* genes; fragments of *rpoC1*, *rpoB*, *accD*, *ndhJ*, *ycf1* genes), different fragments of the nuclear ribosomal cluster, and taxon-specific nuclear genes (Wang *et* al., 2011; Shekhovtsov *et* al., 2012). It is also suggested that the entire plastid genome as a whole be used as a super barcode (Kane and Cronk, 2008; Li *et* al., 2015a). With next-generation sequencing technologies, this approach becomes quite affordable; the need to isolate the organelles is an obstacle (it is necessary to have living plants for this). However, it is also possible to assemble plastid genomes according to the results of total DNA sequencing (Nock *et* al., 2011).

Mammals

Amplification of the COI gene for DNA barcoding can be difficult for mammals. This has led to the development of primer cocktails that contain multiple forward and reverse primers that contain the M13 sequence to ensure coverage of the COI gene. DNA barcoding was used to study Neotropical bats from Guyana (Clare *et al*., 2007). In this publication, the authors used the glass fiber protocol to isolate DNA from a 1-mm^3 piece of frozen tissue (liver, heart or kidney). The target COI was amplified using two different mammalian barcoding cocktails: C_VF1di and C_VR1di. In addition, an improved primer cocktail that contained M13 tailed versions of the primers (C_VF/C_VR) and an additional primer pair (LepF1_t1 and LepR1_t1) was used to determine the relationships between multiple species of bats.

Mini-barcodes that are approximately 100 bp in size have been designed and used with next generation sequencing to amplify thousands of DNA sequences at once. This approach was used for the analysis of rodent samples (Galan *et al*., 2012). Researchers designed primers to a 136 base pair fragment of the cytochrome b gene by aligning 9,071 rodent sequences and looking at the conserved region of the gene. The next generation sequencing was able to tag, multiplex, and sequence 1,140 amplicons in a single run. The researchers were able to validate the method on 265 identified rodent tissues that were from 103 different species. Mini-barcodes are short, ~150 base pair fragments, of the COI gene. Because of their size, it is possible to use next generation sequencing which allows for high throughput screening when all of the sample are

analyzed in parallel. This research showed the potential of next generation sequencing for obtaining accurate species identification using mini-barcodes. This technology could be applied to a broad range of organisms. This method will enable scientists to increase accuracy and decrease the cost and time need to perform DNA barcoding. The rapid development of next generation sequencing (NGS) has the potential to render DNA barcoding irrelevant because of the speed with which it generates large volumes of genomic data. To avoid obsolescence, the DNA barcoding movement must adapt to use this new technology (Taylor and Harris, 2012).

Benefits of DNA Barcoding in Research

Over the last ten years, DNA barcoding has increased researchers ability to identify organisms by molecular methods. Barcoding can help in identifying all life stages of an organism, which is often difficult to do using traditional taxonomic methods. In addition, the DNA barcoding primers can be used as a starting place in the design of species specific PCR primers. It is possible for researchers to use DNA barcoding for the detection of invasive and endangered species. DNA barcoding is performed on monotypic samples. A monotypic sample consists of either the whole, or part of a single organism body. It is easier to extract DNA and get a PCR product from a monotypic sample than from a complex, mixed environmental sample. DNA barcoding can also be used as a starting place in the design of species specific primers that can be used for raw water and other environmental samples. For example, the COI primers were used by Folmar *et al*., 1994 and Claxton *et al*., 1998 as a starting place to design primers that were specific to quagga and zebra mussels. Therefore, a monotypic sample can be analyzed with DNA barcoding primers to produce a DNA sequence that can be used to design new species specific primers, which can in turn be used to analyze a complex environmental sample to detect an organism of interest. This method of primer design is useful because some organisms do not have transcripts available in any database to use as a starting place for designing a molecular assay. DNA barcoding can also be used for a wide range of purposes: to support ownership or intellectual property rights (Stewart, 2005) to reveal cryptic species (Hebert *et al*., 2004); in forensics to link biological samples to crime scenes (Yoon, 1993; Coyle *et al*., 2005; Mildenhall, 2006) to support food safety and authenticity of labeling by confirming identity or purity (Galimberti *et al*., 2012; Huxley-Jones *et al*., 2012) and in ecological and environmental genomic studies (Valentini *et al*., 2009).

The Main DNA Barcoding Bodies And Resources

1. BOLD (The barcode of life data system) was created and is maintained by the University of Guelph in Ontario (Ratnasingham, Sujeevan, and Hebert, 2007) (http:// www.barcodinglife.com). It offers researchers a way to collect, manage, and analyze DNA barcode data. The goal is, over the next 20 years, to provide a barcode library for all eukaryotic life.
2. CBOL (Consortium for the barcode of life) (http:// www.barcodeoflife.org/) is a public reference library of species identifiers which could be used to assign unknown specimens to known species. CBOL was founded in 2004 and promotes barcoding through working groups, networks, workshops, conferences, outreach, and training. CBOL has 200 member organizations from 50 countries and operates from a Secretariat Office located in the Smithsonian Institution's National Museum of Natural History in Washington, DC.
3. iBOL (International Barcode of Life project) (http:// www.ibol.org/) consists of a group of hundreds of scientists from 25 nations working together to construct a DNA barcode reference library that will be the foundation for a DNA-based identification system for all multi-cellular life. Their five year (2010–2015) goal is to barcode five million specimens representing 500,000 species.
4. The GenBank online genetic sequence database (http://www.ncbi.nlm.nih.gov/genbank/) (Benson *et* al., 2013) is possibly one of the most important repositories of genetic information. GenBank contains over 108 million entries for over 260,000 named organisms and is one of the most frequently used databases for genomic authentication (Hennell *et* al., 2012). With the BLAST program (Altschul *et* al., 1990) an unknown DNA sequence can be rapidly and accurately compared to known and well characterized sequences.
5. MMDBD (Medicinal Materials DNA Barcode Database) (http://137.189.42.34/mherbsdb/index.php) is a website that includes DNA sequences and information and key references of the medicinal materials recorded in the Pharmacopoeia of the People's Republic of China, American Herbal Pharmacopoeia and other related references. This database, updated in May 2012 with 1658 species and 31,468 sequences available, provides information material for distinguishing medicinal materials (plant, animal, and fungi) from their common substitutes and adulterants (Lou *et* al., 2010).
6. The GDR (Genome Database for Rosaceae), founded in 2009, provides genetic markers and ESTs of Rosaceae. A large number of species in Rosaceae or rose family have a medicinal value (http://www. rosaceae.org/).

Conclusion

DNA barcoding is a useful molecular technique for the identification of unknown organisms at any life stage. This technology can be used on any life stage of an organism and on a wide range of organisms. With this molecular technique it is possible to analyze tissue from seeds or embryos to the fully grown adult organism. This technology decreases the number of organisms that have to be collected in the field and reduces the amount of time between

collection and identification. Barcoding can be used as a starting place in the design of species specific primers and assays from environmental samples. Overcoming and understanding the issues associated with barcoding will be an ongoing process as more samples are analyzed by DNA barcoding. Next generation sequencing is a more advance technique which is able to sequence large number of samples at the same time and give accurate species identification using mini-barcodes. This technology could be applied to a broad range of organisms. This method will enable scientists to increase accuracy and decrease the cost and time need to perform DNA barcoding.

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