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DNA barcoding: A practical tool for taxonomic research

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Abstract
Species identification is fundamental for biodiversity recognition. Traditional morphometric taxonomic studies have described species for years but they have significant limitations. DNA barcoding uses short-standardized sequence from the suitable region of the genome to distinguish individuals at species level. A 648 bp of 5' cytochrome c oxidase gene (COI) of mitochondria is recognized as a universal standardised species level barcode for animals. Freely available and well-curated DNA barcode reference libraries are necessary for DNA barcoding. The goal of the Consortium for the Barcode of Life (CBOL) is to establish DNA barcoding as a global standard in taxonomy. BIOSCAN is an international project that involves 1000 researchers from 30 countries to accelerate species discovery and interactions. The DNA barcoding workflow consists of three main components-specimen collection and management, molecular analysis and bioinformatics. Role of DNA barcoding in wide range of purposes include species identification, identifying disease vectors, identifying invasive species, protecting endangered species, identifying animal parts in traditional medicine and food authenticity testing. Future prospects for DNA barcoding look bright thanks to advancements in bioinformatics and next-generation sequencing (NGS) technologies. Although an effective complement to conventional methods, DNA barcoding is not error-free. Nonetheless, given the immense potential shown by DNA barcoding in species identification it may become a standard tool.

Keywords: Biodiversity, taxonomy, DNA barcode markers

Introduction
Species identification is a fundamental part of recognizing and describing biodiversity which is important for developing a primary understanding of our biological world. Taxonomy has been performing the task of describing, naming and classifying species for over 250 years. However, the task of routine species identification has been plagued by significant limitations. Many individuals are left unidentified since morphological keys often remain effective only for a particular life stage or gender and despite some progress made in the field, a high level of expertise is required to use these keys which makes misdiagnoses common [19]. This approach overlooks morphologically cryptic taxa common in many groups further adding into the confusion [23]. In addition, biological effects of global climate also illustrate the importance of identification of organisms so as to preserve species due to increasing habitat destruction. The recent advances in sequencing and computational technologies have introduced the possibility of using variations in short DNA sequences for identification of species, known as DNA barcoding. The increased attention that DNA barcodes have received in recent years presents exciting opportunities to be used as a new tool for taxonomy. Additionally, researchers have explored how DNA barcodes might be used to answer relevant questions in evolution, ecology and conservation biology.

DNA barcoding
A DNA barcode is a short nucleotide sequence extracted from a suitable region of an organism's genome which can identify it at species level.

It is built upon the concept that a short-standardized sequence can distinguish individuals of a species because genetic variation that is present between species is greater than that which is present within species [4].

Characteristics of DNA barcodes

A gene region is considered as a DNA barcode if three criteria are satisfied [24]:

1. It should have short sequence length (400-800 bp) facilitating current capabilities of DNA extraction and amplification
2. It should have conserved flanking sites to develop universal PCR primers for broad taxonomic application, and
3. There should be significant species-level genetic variability and divergence

The comparison of intra- and interspecific genetic distances is done with the focus on existence or lack of a barcoding gap. Average genetic distance within a species (intraspecific) should be ten times less from that between species (interspecific) [29].

Standard DNA barcode markers

Various standard DNA barcode markers are reported in different group of organisms.

Animals

In animals, mitochondrial DNA occurs as a single double-helical circular molecule with 13 protein-coding genes, 2 ribosomal genes, a non-protein-coding control region, and several tRNAs in animals. Mitochondrial genes are haploid, lack introns and show limited recombination so they get preference over nuclear genes [16, 3]. A 648 bp long segment of the 5' end of cytochrome c oxidase gene (COI) is recognized as universal standardised species level barcode for animals [16]. It is single locus with protein-coding region, haploid and maternally inherited. The copy number being high per cell ensures that sequence can be recovered from poorly preserved samples [15]. The COI is prioritized above other mitochondrial genes since the primers are highly specific, robust and exhibit high level of accuracy to recover the 5' end of the target DNA [12]. Additionally, deletions and insertions are rare, and intraspecific variation in COI is often less than 10% of interspecific variation [3].

Plants

A combination of nuclear and chloroplast DNA barcodes for plants is recommended. Ribulose-1,5-biphosphate carboxylase/oxygenase (rbcL) combined with maturase K (matK) is recommended despite discrimination success in plant species being low compared to mitochondrial COI in animals. Other plant barcode markers include the nuclear ribosomal internal transcribed spacer (ITS) and the non-coding intergenic spacer psbA-trnH in the chloroplast genome [8].

Bacteria

The commonly utilized barcoding regions in bacteria are 16S rRNA and Tuf genes [26]. In *Wolbachia* 16S rRNA and type II chaperonin, the ortholog of cpn60 were found to be suitable for archeal detection [43].

Fungi

The International Fungal Barcode Consortium recommends the use of nuclear ribosomal ITS as the principal fungal barcode, which has emerged as a promising contender for fungal DNA barcoding. Advantages of ITS include its relatively short length, presence of universal primers and multicopy genome structure for easy amplification as well as high evolutionary rate for sufficient species resolving power [42].

Protists

A nested approach for selection of an appropriate barcode has been embraced by the protist barcoding community. A universal pre-barcode, the variable V4 region of 18S rDNA, has been supplanted by additional barcode markers that are taxon-specific [34]. These additional markers which are frequently used include, e.g., ITS or variable domains of 28S rRNA 5' end; the mitochondrial COI gene for protists with mitochondria; and plastid genes (e.g., 23S rRNA and rbcL) for protist photosynthesis.

DNA barcode data repositories and databases

Freely available and well-curated DNA barcode reference libraries are necessary for the effective application of DNA barcoding for species identification. Since its creation in 2007 by the Canadian Center for DNA Barcoding (CCDB), the Barcode of Life Data Systems (BOLD) has served as the core bioinformatics resource for holding DNA barcode sequence data, which comprises over nine million DNA barcodes [37]. It is an online workbench and database that supports the assembly and use of DNA barcode data. In May 2004, the Consortium for the Barcode of Life (CBOL) was created which is an international organisation devoted to developing DNA barcoding as a global standard in taxonomy. More than 120 member organisations from 45 countries are part of this organisation which includes museums, herbaria, zoos, research organisations, governmental and intergovernmental agencies as well as other organisations involved in taxonomic research and biodiversity issues. Later in 2008, the International Barcode of Life consortium (iBOL) was established which made significant efforts promoting biodiversity research. The iBOL is a huge international collaboration of 26 countries that aims to establish an automated identification system based on a DNA barcode library of all eukaryotes. The CBOL and iBOL have launched campaigns to build DNA barcode libraries of each animal group. The major targets are fish (Fish-BOL) [48], birds (ABBI) [17], mammals (Mammalia Barcode of Life), marine life (MarBOL) and insects. Trichoptera, Lepidoptera, bees and ants (Formicidae) are among the insects for which campaigns have also been launched. Barcodes for 500,000 species have been produced as part of the BARCODE 500K project (2010-2015), initiated by iBOL. Another international project called BIOSCAN (2019-2027) was launched by iBOL with the goals of understanding species dynamics, examining interactions between species and expediting the discovery of new species. It involves around 1000 researchers from 30 countries [18]. When the combined goals of all these organizations are achieved, a DNA barcode library covering nearly every species on Earth will be established.

DNA-barcoding workflow

The main components in the workflow of DNA barcoding includes specimen collection and management, molecular analysis and bioinformatics.

Specimen collection and management: Obtaining all

relevant permits from local and national authorities is necessary before planning fieldwork. In certain situations, preservation of one or more tissue samples combined with live specimen photographs is advised, particularly for large specimens for which it is not possible to collect the entire organism. A 96-100% concentration of ethanol is often utilized in the field for both killing and preserving organisms. Date of sampling, name of collector, method of sampling, GPS coordinates, location, habitat type and other relevant information are among the data gathered in the field. Throughout the barcoding workflow, specimens must be continuously connected to collection metadata by placing the appropriate labels in jars and vials used for collecting. DNA barcoding workflows typically require the sorting of specimens from bulk samples into individual specimens, which are placed in individual vials containing ethanol (96-100%), or pinned in case of insects and pressed in case of plants, then labelling, databasing and imaging is done followed by tissue sampling and DNA extraction. A major part of the reference DNA barcode database consists of specimen photographs, commonly known as e-vouchers. If entire specimens are utilized for DNA extraction, imaging specimens prior to molecular analysis is ideal for capturing reasonably intact morphology.

Molecular analysis

DNA extraction is the process of isolating DNA from other cellular components. The source of DNA can vary from whole specimens to fragments of skin, muscles, feathers, organs, gut contents, faeces, seeds, pollen, and even body swabs or cells shed into the environment. Any of the currently available methods such as phenol-chloroform, commercial DNA extraction kits, etc., can be used to extract DNA from collected samples. Following extraction, amplification of DNA samples using appropriate markers via PCR following the three stages-denaturation, annealing, and extension is performed. The barcode region cannot be successfully amplified without primers. While there are no universal primers, those having a wide range of applications are essential for the majority of barcoding studies as they can amplify the target region in many taxa. Various sequencing technologies are used for DNA sequencing which includes sanger sequencing, next-generation sequencing (NGS) and third-generation sequencing technologies, etc. Over the past ten years, cost and time associated with DNA barcoding have been drastically reduced thanks to advances in sequencing technology [49]. The current cutting-edge sequencing platforms such as Illumina can sequence tens to hundreds of millions of short-length DNA fragments around 50-300 bp and PacBio and Oxford Nanopore in which tens to hundreds of thousands of long DNA fragments around 10,000-30,000 bp can be sequenced [14]. High-throughput sequencing (HTS) platforms provide a new option as they can generate high fidelity reads for the amplicons employed for DNA barcoding.

Bioinformatics

For molecular data analysis and multiple sequence alignment commonly used software includes ClustalW, MEGA 10 [45], ABGD [36], Taxon DNA [28], Geneious vR6.1.6 and MAFFT v7.017 [21]. Similarity searches are carried out with the sequenced DNA data from collected samples by means of bioinformatic techniques, and databases and tools like NCBI, BLAST, etc. The sequence is submitted to GenBank to obtain the accession number if the similarity search yields statistically authentic results [7]. BOLD then uses data from NCBI for species identification [37]. Distance algorithms are frequently used to evaluate barcode sequences, and individual samples are taxonomically assigned based on how close they are to other samples, as indicated by the percent sequence divergence, or visualization on a tree, which is most frequently produced using the neighbour-joining (NJ) algorithm. Sequence divergences among organisms are measured using the Kimura 2 parameter (K2P) for genetic distance correction [15]. An online system called the Barcode Index Number System (BINs), developed by BOLD, automatically clusters animal COI barcode sequences generating web pages for each cluster. They are analogous to operational taxonomic units (OTUs), now routinely used as a species proxy having significantly improved the use of barcode data in groups with poor taxonomic knowledge [38].

Applications of DNA barcoding

Species identification

Discovery of three new species of frogs in the past three years namely, *Philautus nerostagona* from Wayanad (Kerala) and *Philautus anili* sp. nov. and *Philautus dubois* sp. nov. from Wayanad and Kodaikanal (Tamil Nadu), respectively. In addition, a new family of frog was created, Nasikabatrachidae from the Western Ghats, and a primate species *Macaca munzala* from the forests of Arunachal Pradesh, India[1]. DNA barcoding of 1630 specimens of an underestimated genus *Triplophysa,* a freshwater fish, was done in Qinghai-Tibet Plateau, a biodiversity hotspot, revealing the presence of 2 cryptic species namely *T. robusta* and *T. minxianensis* among 24 native species [47]. DNA barcoding was successfully applied in the identification of road-killed avian carcasses in which the barcode sequences of 6 different birds' species were reported that were not present in NCBI database from India [39].

Identifying disease vectors

Since the end of 20th century, it has been estimated that at least 75% of the emerging infection diseases for humans were zoonotic [46]. Thus, monitoring and control of zoonotic diseases is nowadays one of the most important concerns in global economies and human health [40]. The COI barcode sequences were used to recognize members of Sigmodontinae subfamily in Brazil which are reservoirs of zoonoses including hantaviruses, Chagas disease, leishmaniasis and arenaviruses [30]. The presence of 14 sand fly species (vectors for leishmaniasis, bartonellosis and sand fly fever) of two genera was recorded in eight Balkan countries by a large-scale field survey that thoroughly updated the knowledge of sand fly fauna in this important and yet understudied region [9].

Identifying invasive species

Armyworms were identified using DNA barcoding in Florida and this technique can be employed along with morphological methods to identify *Spodoptera* which are invasive pests found in North America [31]. In the U.S. wheat, potato, corn and other crops were being infested by the larvae of click beetle (Coleoptera: Elateridae) commonly known as wireworms. DNA barcoding was employed as a control method against these larvae, establishing a connection between the larvae and adult. Additionally, new relationships between the larval and adult forms of the *Hemicrepidius carbonatus*, *Metanomus insidiosus* and *Hadromorphus Callidus* species were developed [10].

Protecting endangered species

The unregulated hunting of wildlife is an emerging issue as it involves the harvesting of millions of tons of wild animals per year which consist of mostly mammals [13]. Although considered illegal, the bushmeat hunting is an increasing economic activity in many countries such as western and central Africa and other tropical regions [32]. Four mitochondrial gene fragments (including the barcode COI) were sequenced in more than 300 African bushmeat samples belonging to nine orders and 59 species. Subsequently these sequences were added as references in a query database called DNABUSHMEAT, which provides an efficient DNA typing decision pipeline to trace the origin of bushmeat items. This project also contributes in filling the existing gap of African mammals' representations in the international archives (i.e., NCBI and BOLD) [13]. A novel cocktail primer was used to amplify the COI gene in a forensic analysis about the unknown origin of 21 claws. Five samples were identified up to the family level, and the sixteen samples under study were able to be tracked to their species level (*Panthera leo* or *P. pardus*) [22].

Identifying animal parts in traditional medicine

Animal organs or parts used in traditional medicine can include many threatened or endangered species. The illegal hunting and trading of rhinoceros horn, saiga antelope horn, bear bile crystals, etc., are some of the commonly used ingredients in traditional Asian medicine [50]. Authentication of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) listed shark species from processed fins and even fin soups and skin-care products have also been done [5]. Utilisation of 12 DNA barcode in a multi-locus metabarcoding approach employing Illumina MiSeq amplicon sequencing revealed that Ma pak leung sea-dog hard capsules and Cobra performance enhancer hard capsules, which are used to treat sexual weakness, consist of DNA from nondeclared taxa such as *Bos taurus* and *Homo sapiens* instead of labelled species [2].

Food authenticity testing

Food products are frequently mislabelled in both domestic and international markets since many of them can no longer be identified on visuals alone after processing and freezing [11]. Evaluation of originality of processed food with standard methods is difficult at times. Testing the authenticity of food products even after processing can now be done effectively with DNA barcoding. Additionally, it is also being used to accurately identify natural health products. DNA barcoding using rbcL, ITS, psbA-trnH and matK identified contamination in the ultra-fine powder of roasted barley tea showing that 77% of the product was contaminated by other plant substances [20]. 44 herbal items from 12 companies, comprising nearly 30 species of herbs, were analyzed using DNA barcoding. Only two companies had pure products while the others contained contaminations, substitutes and fillers that not only were not disclosed on the products but also posed health risks to the general public. The study also developed a reference library containing barcodes belonging to 100 species [33].

Technical advances in DNA barcoding

DNA Mini-barcoding

DNA mini-barcoding use mini-barcodes of size ≤ 200 bp DNA sequences which can be amplified more quickly than conventional barcodes, thus overcoming the limitations of DNA barcoding [44].

DNA Metabarcoding

DNA barcodes can be extracted or sequenced from mixed population or environmental samples using NGS technology offering a quick way of biodiversity assessment. Identification of endangered plant and animal species in complex samples has been done using a multi-locus approach of DNA metabarcoding [2]. It has also made it possible to understand herbivory and predation and analysing the diets of vertebrates [35, 6].

Super- and Ultra-barcoding

Entire organellar genomes as well as other long regions is compared, in contrast to conventional DNA barcodes that use universal primers to target specific loci or a collection of small loci [25]. Chloroplast genomes are widely distributed and easy to sequence, even from old and preserved tissues. Further in targeted or capture-based sequencing applications, it is becoming more frequent to assemble the entire organellar genomes from off-target reads [27].

Bar-HRM technology

Bar-HRM technology which combines DNA barcoding with high-resolution melting (HRM) technology has substantially improved the identification of medicinal plants allowing a resolution accuracy to identify change in a single nucleotide. This technique requires no sequence-specific probes and sequencing and thus species identification can be done using rbcL, matK, psbA-trnH, ITS, etc [51].

Limitations of DNA barcoding

The inconsistent use of barcoding gap concept by authors is a significant challenge for DNA barcoding. While some studies define species in relative terms by distinguishing intra-specific from inter-specific genetic variation, a thresholds approach is recommended by other studies as a quantitative and objective method of defining species diversity. The use mitochondrial DNA (mtDNA) has been found to be significantly restricted by introgression due to hybridization[41]. Species boundaries that should be normally distinct between evolutionary lineages or phylogenies get blurred as a result of introgression. Nuclear mitochondrial DNA sequences (NUMTs), which are prevalent in major eukaryotic clades, can also lead to complications in DNA barcoding since they can be misinterpreted for the actual barcode sequence. The retrieval of accurate sequences may be hampered by Heteroplasmy which is the existence of two or more variations of the barcode region inside a single individual. The validity of DNA barcoding depends on establishing reference libraries of known sequences and their absence or the lack of control of the correct identification of the source specimens by expert taxonomists, can irremediably affect the assignment of newly generated query sequences. Furthermore, in certain organisms target region may not be amplified by the universal primers which are used for DNA barcoding. When DNA is degraded, which occurs with old museum specimens or tissue samples subjected to chemicals that damage DNA like high temperatures, the likelihood of successfully recovering a sequence is decreased.

Future prospects

Future prospects for DNA barcoding looks bright thanks to advancements in bioinformatics and NGS technologies. High-throughput sequencers will keep advancing, aiding barcoding

workflows. With BOLD recognized as one of the major breakthroughs that underpins the newly emerging field of macrogenetics, DNA barcodes have become an essential source of information for it. In order to support global priorities for ecology, evolution and conservation, it offers to integrate large data from several biological disciplines using genetic data. Moreover, DNA barcoding when combined with a large group of citizen scientists will surely quicken the inventory of life on Earth, promises to democratize taxonomic process.

Conclusion

A significant potential has shown by DNA barcoding in identifying species enabling users to connect these specimens to databases of taxonomic information and also species with currently no data available have been highlighted. Despite its shortcomings and limitations, it would be unwise to overlook the potential of DNA barcoding given the size of the work of cataloguing the world's biota with so many species not yet identified. It could be a helpful tool for taxonomists and numerous other organisations and/or people who are interested in species identification. Various repositories of DNA barcode data have accelerated the production of reference barcode libraries. Biodiversity research and equal opportunities in academics depend heavily on access to these reference databases. It will not be an exaggeration to say that DNA barcoding is benefiting society and science simultaneously.

Conflict of Interest

Not available.

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