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20 years since the introduction of DNA barcoding: from theory to application

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Abstract Traditionally, taxonomic identification has relied upon morphological characters. In the last two decades, molecular tools based on DNA sequences of short standardised gene fragments, termed DNA barcodes, have been developed for species discrimination. The most common DNA barcode used in animals is a fragment of the cytochrome c oxidase (*COI*) mitochondrial gene, while for plants, two chloroplast gene fragments from the RuBisCo large subunit (*rbcL*) and maturase K (*matK*) genes are widely used. Information gathered from DNA barcodes can be used beyond taxonomic studies and will have far-reaching implications across many fields of biology, including ecology (rapid biodiversity assessment and food chain analysis), conservation biology (monitoring of protected species), biosecurity (early identification of invasive pest species), medicine (identification of medically important pathogens and their vectors) and pharmacology (identification of active compounds). However, it is important that the limitations of DNA barcoding are understood and techniques continually adapted and improved as this young science matures.

Keywords Molecular tools · Identification of organisms · Gene fragments · *COI*

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Introduction

The identification of organisms on the basis of morphological characters often represents a challenging task requiring experienced taxonomists. These morphology-based procedures are usually time consuming and may not always provide resolution to the species level (Ingrisch 1995; Cywinska et al. 2006; Rindi et al. 2008; Packer et al. 2009). Moreover, even under the gaze of an experienced taxonomist, the phenotypic plasticity of taxa may lead to misidentifications (e.g. Gutiérrez-Gutiérrez et al. 2013; Nekola and Barthel 2002).

Molecular studies have revealed the existence of numerous biological species that have accumulated genetic divergence without accompanying morphological disparities and, thus, cannot be identified using the traditional morphological species concept—the recognition of such morphologically cryptic species is a major challenge to modern taxonomy (Heinrichs et al. 2011). Additionally, some animals undergo complex developmental life cycles consisting of several morphologically distinct stages; it is not uncommon for these species to have morphological keys that describe adult stages with reference to a single gender. In some cases, specimens may be damaged or incomplete, with only a small section of tissue available for identification, rendering morphological determination unlikely. The same is true for many plants, where vegetative states (periods lacking flowers or fruits) compromise taxonomic resolution.

In the last two decades, molecular short standardised DNA fragments, termed DNA barcodes, have been developed for species discrimination. Hebert et al. (2003a, b, 2004) argued that the integration of DNA barcoding into traditional taxonomic tools could efficiently disclose hidden biodiversity more rapidly and more reliably than traditional methods alone. They further argue that using identification keys is time consuming, and more and more people lack the taxonomic expertise to use them correctly.

The ability of DNA barcoding to distinguish species from a range of taxa and to reveal cryptic species has, nowadays, been well documented. DNA barcoding has proved useful in the study of taxonomically difficult taxa such as blackflys (fam. Simuliidae), where identification is hampered due to cryptic species or phenotypic plasticity (Rivera and Currie 2009). Moreover, this technique helped to recognise different developmental life stages of a single species, which was impossible by using morphological characters alone. Miller et al. (2005) used DNA sequence data to distinguish between previously unidentifiable larval stages of some diving beetles (Coleoptera: Dytiscidae), Paquin and Hedin (2004) did the same for immature stages of species of *Cicuria* (Araneae: Dictynidae), Jousson et al. (1998, 1999) for trematodes (Digenea) and Dezfuli et al. (2002) for tapeworms (Cestoda).

Species-level identification is crucial in many applications of economic and social importance. In such cases, fast identification is highly desirable. Implication of DNA barcoding has proven successful in rapid biodiversity assessment studies (as reviewed by Valentini et al. 2009), biomonitoring (Hajibabaei et al. 2011; Sweeney et al. 2011) including the monitoring of pathogen spread and their associated vectors (Azpurua et al. 2010), in forensics (Dawnay et al. 2007), in the investigation of the illegal trade of endangered species and their products (Muellner et al. 2011; Baker et al. 2000), in studies on feeding ecology (e.g. Rollo et al. 2002), medicinal and poisonous plants (Baker et al. 2012; Phua et al. 2008) and conservation initiatives (e.g. Smith et al. 2005).

DNA barcoding addresses many of the problems inherent to morphological taxonomy. With the number of taxonomists decreasing and the number of named species increasing, molecular tools have become a mainstay of modern taxonomic analysis. Only a small amount of tissue (one single cell at best) is needed for species determination, the analyses can be performed without prior knowledge of the specimen and can be applied to all stages of development (Hebert et al. 2003a; Savolainen et al. 2005; Floyd et al. 2002). Barcoding is now routinely used for multi-cellular organisms, such as aquatic hyphomycetes (Seena et al. 2010), butterflies (Burns et al. 2008) and birds (Hebert et al. 2004).

An important aspect of DNA barcoding is the opportunity to connect DNA barcoding programs to museum and herbarium specimens, allowing this new science to take advantage of hundreds of years of investment into verifiable taxonomic samples. Large natural history museums and herbaria across the world hold enormous amounts of specimens and are often supported by equipped laboratories with sequencing facilities. Moreover, they also employ experienced taxonomists whose main task is to identify the stored specimens. Natural history museums are, thus, organizations where DNA barcoding practices should be developed. Although there are still many requirements which need to be ensured (such as adequate facilities and a developed workflow of specimen processing), the most important advantage of museums is that DNA barcodes are backed up by real specimens which were first identified by experienced taxonomists by morphology. As descriptions of new species are based on specimens kept in public collections, it is highly encouraged that, also, DNA barcodes should be coupled with voucher material (Ellis 2008; Puillandre et al. 2012).

Nevertheless, these molecular approaches also have limitations. The selection of a barcode locus is complicated by the trade-off that arises between the need for universal application in a wide range of taxa and sequence substitution saturation (Kress et al. 2005a, b). In some cases, identical chloroplast or mitochondrial sequences are present in related species due to introgression, rendering these sources of DNA less useful or redundant for species discrimination. The heteroplasmy in the mtDNA genome (Rubinoff 2006) and the presence of nuclear pseudogenes of mitochondrial origin (NUMTs; non-functional copies of mtDNA in the nucleus) may also lead to misidentifications (Song et al. 2008). Although barcoding can serve as an important aid for taxonomic workflow, it cannot replace comprehensive taxonomic analyses and molecular phylogenetics. The primary role of this technique is not to build phylogenetic trees, but to provide rapid and accurate identifications of unidentified organisms with the use of verified reference material (Erickson and Kress 2012).

Global DNA barcoding efforts have resulted in the formation of the Consortium for the Barcode of Life (CBOL). In January 2013, the Barcode of Life Database (BOLD) contained more than 2.7 million specimen records, with 2 million having barcodes belonging to over 170,000 species (Ratnasingham and Hebert 2007; BOLD Systems 2013). Smaller databases, containing sequences of specialised groups, also exist [for example, Fungal Database (Crous et al. 2004), Genome Database for Rosaceae, GDR (Jung et al. 2008)].

Properties of DNA barcodes

The term DNA barcode was first used in 1993, when Arnot et al. (1993) published a paper describing the possibility of discriminating isolates of *Plasmodium falciparum* on the basis of a circumsporozoite gene. However, the idea of determining organisms using molecular tools is even older (e.g. McAndrew and Majumdar 1983; Anderson et al. 1985). Among the first molecular tools used to determinate speciational processes and species differences were allozymes, which were in use since the mid-1960s (e.g. Hubby and Lewontin 1966). The idea behind DNA barcodes is to find a single segment of DNA which is useful for the identification of all living taxa. The identification of specimens via barcodes relies on the target species having enough genetic differentiation

to allow for species separation, even where morphological similarities exist.

Researchers are still trying to find a single segment of DNA suitable for the identification of all taxa. Despite several years of work in this area, such a region has not been identified and a single universal DNA barcoding marker is unlikely to exist. The desired properties of DNA barcodes are clearly defined:

1. The DNA fragment must be nearly identical in specimens of the same species but different between individuals of different species,
2. The section must be standardised (the same section should be used in different taxonomic groups),
3. The marker must be robust, with conservative primer binding sites that allow it to be readily amplified and sequenced.

Species identification using DNA barcodes has been successful in algae (e.g. Saunders 2008), fungi (Seena et al. 2010), plants (Kress et al. 2005a, b; Chase et al. 2005; Fazekas et al. 2012) and many animal groups, such as spiders (Barrett and Hebert 2005), fish (Ward et al. 2005), birds (Hebert et al. 2004) and rodents (Robins et al. 2007). However, finding suitable markers for the identification of unicellular organisms has been difficult (Kuksa et al. 2009), and several taxonomic groups still require the use of several different DNA markers, which are described in more detail in the following sections.

Animals

In animals, the use of sequences from the mitochondrial genome is preferred over the nuclear genome because recombination is rare and mtDNA is haploid preventing sequencing errors due to heteroplasmy (Hebert et al. 2003b).

In vertebrates and some other animal groups, the system of barcodes relies on the region of the mitochondrial gene encoding the cytochrome c oxidase (*COI*). *COI* is the component of the respiratory chain that catalyses the reduction of oxygen to water. Subunits 1–3 form the functional core of the enzyme complex. The gene *COI* codes the catalytic subunit of the enzyme (GeneCards 2013).

In *COI* sequences, existing mutations are mostly substitutions; insertions and deletions only occur at the level of codon, which makes this marker easy to align. The *COI* sequence enables discrimination for more than 98 % of animal species (Ward et al. 2005; Hajibabaei et al. 2006). Hebert et al. (2003b) compared 13,320 congeneric species pairs in 11 animal phyla and found that p-distances at *COI* range from 1.0 % (Cnidaria) to 15.7 % (Annelida). As the protein coded by this gene is necessary for cellular respiration, the amino acid composition of this gene is highly constrained and, consequently, very slow to evolve (Lynch and Jarrell 1993). The use of mtDNA for species identification has been claimed to have high rates of success; most studies have shown error rates of less than 5 % (Waugh 2007).

Some authors have also reported problems with the mtDNA approach due to introgression. Introgression is the introduction of genes from one species into the gene pool of another species through repeated backcrossing of an interspecific hybrid with one of its parents. Introgression creates confusion about species boundaries between evolutionary lineages (phylogenies) that would normally be distinct (Rubinoff 2006). In a meta-analysis of phylogenetic studies, it was found that over 20 % of the studied lineages present problems due to mtDNA introgression (Funk and Omland 2003), suggesting that this may be a significant limitation of barcodes based on mtDNA.

In most eukaryotes, mtDNA is inherited uniparentally from the maternal parent (for a review, see Birky 2001; Gyawali and Lin 2013). However, exceptional forms of mtDNA inheritance also exist in some eukaryotic organisms (e.g. "doubly uniparental inheritance"; Śmietanka et al. 2010 and references therein; Doucet-Beaupré et al. 2012), which might bring inconsistences in DNA barcoding. In addition, the presence of mtDNA recombination may occur in some species (e.g. in molluscs; Burzyński et al. 2003), although this does not represent an obstacle to DNA-based species classification. The presence of heteroplasmy in mtDNA could mean that the mitochondria of an individual could represent a sample of the alleles within a population, like any other gene, therefore, requiring additional genetic markers for comparison (Rubinoff 2006). Another potential problem for mtDNA barcoding is NUMTs that are common in major clades of eukaryotes, and that can be easily amplified. Finally, some groups of eukaryotes lack mitochondria (amitochondriate eukaryotes), which makes mtDNA not suitable for studying these organisms (Scicluna et al. 2006). The above-mentioned limitations can be largely overcome by finding an alternative target or by adding molecular markers to provide additional information to more accurately discriminate species.

The *COI* region is currently used in several vertebrate (e.g. birds, fish) and invertebrate (e.g. insects) groups. However, in some taxa, such as nematodes, poriferans (Moura et al. 2008a), cnidarians (Wörheide and Erpenbeck 2007; Shearer and Coffroth 2008; Chen et al. 2009; McFadden et al. 2011), ctenophores (Ortman et al. 2010) and placozoans (Signorovitch et al. 2006, 2007), the *COI* region is replaced by ribosomal genes (Floyd et al. 2002; Holterman et al. 2008; Virgilio et al. 2010), due to the lack of variability in their mitochondrial genome (Hebert et al. 2003a, b; Shearer and Coffroth 2008). DNA barcoding in these basal phyla is moving toward multiple gene region approaches. Also, in amphibians and reptiles, 16S rDNA is used more frequently than *COI* in many taxa. Because those taxa are typically old, strongly divergent and contain deep conspecific lineages, species assignment may be problematic if reference databases are incomplete. Until a more comprehensive

COI reference database becomes available, 16S rRNA may act as a suitable complementary marker (Vences et al. 2005). However, some recent studies showed that *COI* is still a better marker for certain groups, such as hynobiid salamanders (Xia et al. 2012).

Plants

Searching for suitable plant DNA barcodes has proved to be more problematic than in animals. Due to lower heterogeneity in the mitochondrial *COI* gene of plants, this region is not suitable for distinguishing plant species. Botanists have spent a large amount of time searching for a DNA sequence outside the mitochondrial genome which could serve as a replacement for the *COI* gene. The nuclear internal transcribed spacer (ITS) region and the chloroplast intergenic spacer *trnH-psbA* (Kress et al. 2005a, b) have been discussed previously. With increased knowledge about plant genomes and with the increasing amount of universal primers available, the range of potential regions for the barcoding of plants has expanded. The search for the corresponding DNA barcode focused on the plant chloroplast genome, which is an alternative to the animal mitochondrial genome. The chloroplast genome could contain suitable barcoding markers because it is present in each plant cell in a high number of copies and consists of conserved gene sequences. The downside of the chloroplast genome is its relatively low rate of evolution. Focus has been placed upon identifying those regions that evolve quite rapidly, but still slowly enough to be present in all land plants and that are good candidates for robust, universal primers. As no marker that exhibits all of the desired characteristics required for plant barcoding has been found, researchers have proposed the simultaneous use of more than one region. The first proposals consisted of a combination of three regions, for example, a combination of three chloroplast genes (*rpoC1*, *rpoB* and *matK*), or a combination of two chloroplast genes (*rpoC1* and *matK*), and one intergenic spacer (*psbA-trnH*) (Chase et al. 2005). The nuclear ITS sequence has also been proposed. A turning point in the field of plant barcoding was reached with the publication of an article by the CBOL Plant Working Group (2009). This short article considered potential candidates for plant DNA barcodes. CBOL considered all the criteria for barcodes when evaluating the seven potential candidates, including four coding regions (*matK*, *rbcL*, *rpoC1* and *rpoB*) and three non-coding regions (*psbA-trnH*, *atpF-atpH* and *psbK-psbI*). Three remaining regions emerged as likely candidates (*rbcL*, *psbA-trnH* and *matK*), although none of them completely satisfy all of the barcoding marker criteria. As a consequence, some CBOL researchers working on plants proposed the combined use of all three markers (*rbcL*, *psbA-trnH* and *matK*). The need to amplify additional markers increases the expense and time required for taxonomic identification; therefore, some investigators have chosen a combination of two regions (*matK* and *rbcL*) as a satisfactory compromise that best meets the DNA barcoding criteria. In combination, these genes could identify species in 72 % of the cases and to the genus level in all cases. Additional knowledge about a sample's geographic origin and number of collocated members of the genus can help increase the likelihood of making a positive species identification. The regions *rbcL* and *matK* are, today, identified as core barcoding regions, while *psbA-trnH* was designated an important supplementary marker to be used in appropriate cases (Fazekas et al. 2012; Table 1).

Fungi

Although often invisible to the naked eye, fungi play an enormous role in terrestrial ecosystems. Morphologically and physiologically, they represent a very diverse group of organisms, ranging from unicellular microorganisms to macroorganisms. Species identification is often difficult, as they only occasionally display morphological characters suitable for identification (Eberhardt 2012). With the development of molecular techniques, species level taxonomy has been greatly altered and many cryptic species have been identified. However, identification based on molecular markers should always be coupled with appropriate voucher material. As a result, an increasing number of mycologists are accepting taxa delimited only by molecular markers, excluding the morphological and physiological characters and reproductive strategies (Taylor et al. 2007).

The Fungal Barcoding Database, managed by the International Fungal Working Group (http://www.fungalbarcoding.org) lists a number of regions used for fungal DNA barcoding (Table 1). The ITS region and the *D1/D2* region of the nuclear large subunit (LSU), both belonging to the group of nuclear ribosomal RNA genes, are amongst the most commonly sequenced and can be used in all fungal taxonomic groups. These two regions can be amplified easily using universal primers and similar protocols can be used in different fungi groups. Additionally, the amount of reference data for these two regions is the largest. However, even those two regions are often incapable of discriminating to the species level (Eberhardt 2012). Other regions are applied in selected taxonomic groups, such as the nuclear small subunit (SSU), and three low-copy protein markers, *RPB1*, *RPB2* or *MCM7* (see Table 1 for details). *COI* is also being used (Dentinger et al. 2011; Gilmore et al. 2009) and, for now, it is the only fungal marker that will (if all other requirements are met) obtain the "barcode flag" in GenBank (Eberhardt 2012).

CBOL is currently in the process of approving appropriate DNA markers for barcoding fungi. The BOLD (Ratnasingham and Hebert 2007) currently offers identification based only on ITS.

Table 1 Primary molecular markers currently used for DNA barcoding in plants, animals, fungi, algae and protists

Marker	Region (genome)	Used in which group	CBOL approved?
rbcL	Chloroplast	Plant, diatoms	CBL for plants. CBL for diatoms, and any other lineages of algae for which it has universality, provides species-level resolution and for which *COI*-5P is not a viable marker
matK	Chloroplast	Plant	CBL for plants
trnH-psbA	Chloroplast	Plant	SBL for plants
ITS	Nuclear	Plant, fungi	SBL for plants
COI	Mitochondrial	Animal, fungi, brown (Phaeophyceae) and red (Rhodophyta) algae	CBL for animals and fungi. CBL for brown and red algae and any other lineages for which it has universality and provides species-level resolution
LSU *D1*/*D2*	Nuclear	Fungi	SBL for plants
SSU	Nuclear	Fungi (Chytrids/Zygos, Ascomycota, Basidiomycota)	SBL for plants
RPB1	Nuclear	Fungi (for all groups)	SBL for plants
RPB2	Nuclear	Fungi (for all groups and for Basidiomycota)	SBL for plants
MCM7	Nuclear	Fungi (for all groups)	SBL for plants
LSU *D2*/*D3*	Nuclear		CBL in fungal lineages for which it provides species-level resolution; or SBL in all other fungal lineages to facilitate eukaryote-wide environmental surveys
tufA	Chloroplast	Chlorophytan green algae (Chlorophyta)	CBL for chlorophytan green algae

CBL core barcoding locus, *SBL* supplementary barcoding locus

Algae and protists

In this section, we describe the use of barcoding markers in lineages excluding animals, plants and true fungi. As previously stated, many organisms are difficult to determine in the vegetative state. This is particularly true for marine macroalgae, which often show very simple morphology and anatomy, extreme convergence, extensive phenotypic plasticity due to environmental factors and poorly understood life histories (Saunders and McDevit 2012). Similarly, studying microalgae poses the same difficulties as studying macroalgae, with the additional drawback of being extremely small and, thus, difficult to observe (Saunders and McDevit 2012). The lack of specialised taxonomists and, consequently, the lack of appropriate determination keys additionally hinders the research of these organisms. Molecular tools are a pragmatic option for the identification of these organisms and are becoming increasingly important in the study of these groups.

Saunders and McDevit (2012) list four DNA barcode markers (Table 1) used to study brown (Phaeophyceae), red (Rhodophyta), green (Chlorophyta) algae and microscopic diatoms (Bacillariophyta). The eukaryote-wide marker LSU *D2*/*D3* is used in broad ecological and environmental surveys, the standard *COI* region as a primary marker for the determination of brown and red algae, and the 3′ end region of the *rbcL* gene in diatoms. For chlorophytan green algae, a plastid elongation factor Tu gene *tufA* is used.

Practical uses of DNA barcodes

At first sight, taxonomy is the field that can benefit most from DNA barcoding. However, the development of new, faster and simpler molecular genetic methods has made DNA sequences more accessible and, thus, more useful to other branches of biology. Researchers predict an increasing use of DNA barcodes in conservation biology, ecological studies, medicine, pharmaceuticals and systems biology. Importantly, the application of DNA barcodes will be increasingly deployed for the identification of medically important pathogens and their invertebrate vectors, where morphological identification is often very difficult or impossible. Another promising aspect is the identification of species that are used in the manufacture of drugs of natural origin.

Biosecurity and public health

Illnesses and mortality resulting from infection with parasites borne by widespread vectors are of grave importance to human health (Besansky et al. 2003). The taxonomic identification of parasites is challenging, as they may go through different stages of development during their life cycle (larvae and/or pupae), may include multiple hosts and sometimes live deep in the host tissues (Besansky et al. 2003). A host can bear a whole community of parasites composed of different species that may be taxonomically cryptic. Taxonomic determination is crucial to the understanding of interactions between the host

and the parasite; it is the basis for understanding parasitic diseases affecting humans, domestic and wild animals (Leung et al. 2009). To determine parameters such as specialisation to the host, virulence and transmission, it is very important to understand the ecological–evolutionary relationship between the parasite and the host. The correct taxonomic identification of the parasite also provides further recognition of its major reservoirs and enables the differentiation between morphologically similar species that cause different diseases.

DNA barcoding proved to be successful in determining the vectors of Leishmaniasis, a disease affecting the skin, mucous membranes and visceral organs, which is transmitted by sand flies (Azpurua et al. 2010) and is caused by the flagellate *Leishmania*. In the analysis of 20 species of the genus *Lutzomyia*, researchers discovered that two species belonging to distinct phylogenetic clades transmit Leishmaniasis in high concentration and serve as vectors for the disease in humans and other mammals. Scientists fear that climate change could alter the geographical distribution of the host species and disease.

In tropical areas, mosquitoes represent important pathogen vectors. There are 41 genera of mosquitoes, containing approximately 3,500 species, but only a handful of species are medically important because they act as vectors for the transmission of viruses, nematodes and protozoans. Some species of mosquitoes spread malaria, dengue fever, chikungunya fever, Japanese encephalitis, yellow fever and other diseases, directly affecting the health of millions of people (Virgilio et al. 2010). In Africa, DNA barcoding is used to identify the mosquitoes that spread lymphatic filariasis, which has infected more than 120 million people in 80 countries (Becker et al. 2010).

Identification of the species by DNA barcodes is now commonly used in the ingredients disclosure of herbal mixtures or preparations. Active substances from natural sources are the basis of Western medicine, and remain the precursor to many pharmaceutical drugs. In recent years, treatments using medicinal plants for natural healing have gained popularity in the Western world. Because the effectiveness of such therapies depends upon the use of the correct species (Lou et al. 2010; Sucher and Carles 2008), misidentification can lead to the ingestion of unwanted active compounds that can interfere with the therapeutic effects of mixtures, potentially leading to life-threatening poisoning. For example, in 1989, two patients from Hong Kong suffered severe neuropathy and encephalopathy after eating a soup prepared from the poisonous roots of *Podophyllum hexandrum*; the species had been misidentified as *Gentiana rigescens*. In 2008, a woman in Singapore reported poisoning due to the consumption of a product which contained *Datura metel* instead of *Rhododendron molle* (Phua et al. 2008).

The usefulness of DNA barcodes can be illustrated also by the study of herbal preparations from *Actaea racemosa*. Many US female post-menopausal patients use herbal preparations from this species as a substitute for hormone replacement therapy; the plant contains active ingredients that bind to oestrogen receptors to alleviate menopausal symptoms. After testing more than 36 commercially available dietary supplements that should contain *A. racemosa* extract, Baker et al. (2012) found out that nine of them (25 %) contained the extract of three other *Actaea* species, which is alarming, since some of the these species are known to be toxic to humans.

Invasive alien species also represent a threat to ecosystem stability and human livelihood. With the spread of tourism and trade, the risk of movement of exotic species around the world is increasing and is even accelerated by changes in climate and land use. It is estimated that 1 % of species introduced to novel environments will become invasive and have serious economic impacts (Williamson 1996). The determination of invertebrate pests poses very similar problems as the identification of parasites; since pests can be practically impossible to identify in egg and larval stages, several countries have adopted DNA barcoding as a diagnostic tool. The United States Department of Agriculture (USDA) and the California Department of Food and Agriculture (CDFA) are using barcoding to track a specific new pest species in California, the light brown apple moth, *Epiphyas postvittana*, an invasive species originating from Australia (Floyd et al. 2010).

Biodiversity assessment

Assessing biodiversity using DNA barcodes provides advantages in ecosystems that are species-rich, difficult to access and poorly catalogued. The biodiversity loss that is evident now is most prominent in ecosystem-rich environments; it is given that many species will become extinct before they are taxonomically recorded (Mora et al. 2011). A recent assessment by Mora et al. (2011) predicts that some 86 % of the species on Earth, and 91 % in the ocean, still await description. Since most of the Earth's biodiversity is concentrated in developing countries, where resources for such assessments are inadequate, DNA barcodes could facilitate biodiversity assessments and lower the cost and time requirements of traditional taxonomic biodiversity research (Gaston and O'Neill 2004).

Obtaining samples can be done in a traditional way by sampling separate organisms in the ecosystem or by analysing environmental samples, namely, samples from soil, water and even the air that can contain a mixture of live individuals and traces of other organisms' DNA that were present near the sample. This approach is often referred to as environmental metagenomics. Although environmental metagenomics usually refers to the study of microbial communities through several DNA markers, there is still no consensus as to whether these two approaches—DNA barcoding and environmental

metagenomics—are really different approaches or just two sides of the same coin.

Bittner et al. (2010) explain that differences in the assessment of biodiversity by DNA barcoding and microbial metagenomics may be reasoned by contrasting their biological scope: microbial metagenomics mostly studies prokaryotes (and, thus, takes into account lateral gene transfer, LGT), while DNA barcoding has been used in eukaryotes (not affected by LGT). Thus, the main difference between the two approaches is mostly due to their use, taking into account the species problem in microbes.

The usefulness of DNA barcoding is not restricted to the research of recent biodiversity; it can also help reconstruct ecological conditions on Earth in the past. The paleoenvironment can be reconstructed by analysing sediments or ice and through remnant biological remains. Scientists have analysed the 700-year-old residues accumulated by rodents in the Atacama Desert in Chile, which include plant macrofossils, pollen, excrements, bones and bugs, impregnated with uric salts. Genetic analysis of the residues showed that, 700 years ago, the environment was much more humid, productive and diverse in this region (Kuch et al. 2002). Similarly, by analysing "the last supper" of the Neolithic mummy Otzi, the presence of deer and ibex DNA was identified from gastrointestinal samples (Rollo et al. 2002).

Two different approaches can be used for the analysis of food samples using either group-specific or universal primers (Nyström et al. 2006). If researchers are not familiar with the target animal's diet, universal primers are most suitable. For the analysis of degraded plant DNA (from the gastrointestinal tract or faeces), a 10–140 base pairs long stretch of intron *trnL* (UAA) proved useful in determining gymno- and angiosperms.

Barcoding and conservation

Phylogenetic diversity, measuring the taxonomic divergence between species, can predict biodiversity patterns in areas of interest. According to this method, conservation areas are designed or prioritised according to their phylogenetic diversity and not according to the number of species within the area. In contrast to the relatively fast and cheap assessment of biodiversity using DNA barcoding, assessments based on morphology are more time consuming, expensive and require skilled labour that takes decades of training. One of the potential uses of DNA barcodes in biodiversity conservation is rapid assessments of biodiversity from metagenomic studies to establish focal conservation hotspots (Hebert et al. 2003a).

One of the most important applied aspects of using DNA barcoding is the conservation of rare species involved in international (illegal) trade. For example, Moura et al. (2008b) used mtDNA sequences, including *COI*, to identify commercially fished shark species when morphological characters (e.g. fins, heads) were equivocal in discerning among and within genera.

Trade of important timber species, such as mahogany (fam. Meliaceae, of which 147 are listed as being threatened), is also being monitored using DNA barcoding. DNA barcoding with ITS alone revealed cryptic species and proved useful in identifying species listed in the Convention on International Trade of Endangered Species (CITES) appendices (Muellner et al. 2011).

Conclusions

There is no doubt that the DNA barcoding of animals as well as plants and other organisms will improve with advances in polymerase chain reaction (PCR) amplification and DNA sequencing. The technology of DNA sequencing in the last 25 years has greatly improved and, most recently, next-generation sequencing systems have become available, enabling the production of large amounts of DNA sequences in a very short time. These techniques are very suitable for the DNA barcoding of environmental samples composed of a mixture of several species, such as soil samples or samples from animal intestines.

However, the application of DNA barcoding will be limited until the discrimination thresholds between species yields 100 % accuracy. Krishnamurthy and Francis (2012) point out that establishing robust thresholds for species delimitation is a key component of the barcoding process, because only after a threshold in a target group is established it is possible to identify potential cryptic and overlooked species.

What we know today is that no single classification technique can be applied universally for species identification. However, in those cases where a single DNA region is not enough for the purpose of barcoding, a combination of two or more regions should be applied, such as in the case of plants. Researchers also predict that the development of new sequencing technologies will enable faster and cheaper analyses of DNA barcodes, which will consequently become available in other branches of science, such as medicine and pharmacy. On the other hand, the increasing amount of readily available DNA sequences poses a new problem, namely, how to efficiently analyse enormous numbers of sequences.

The rise of DNA barcoding has helped to raise the profile of taxonomic research and poses challenges as it is integrated into the wider context of scientific, social, economic and political arenas, especially nowadays when sequencing costs decrease and DNA-based species identification is becoming available to an increasingly wider community.

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