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REVIEW

20 Years of DNA Barcoding – Achievements and Problems

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Abstract—Over 20 years of extensive studies on DNA barcoding of various types of multicellular organisms have resulted in the selection of specific markers for multiple taxonomic groups, development of primers for many selected markers, establishment of DNA barcodes for more than 400 thousand species, and creation of the BOLD database. Next-generation sequencing methods allow DNA barcodes to be obtained immediately for many samples, including those stored in museum collections. DNA barcode analysis has revealed many previously unknown and undescribed species in various animal groups. DNA barcoding has been successfully used in many practical applications. However, certain problems and controversial issues remain, primarily, regarding description of new species based on DNA barcodes and the accuracy of sample identification using reference libraries.

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INTRODUCTION

In 2007, the journal *Biochemistry (Moscow)* celebrated the 50th anniversary of the publication of the seminal article that laid the foundations for the concept of species-specificity of DNA [1]. That anniversary issue also presented our article [2] in which we described modern approaches to species identification based on DNA comparison. In particular, we discussed a new method based on the species specificity of DNA, that had been proposed in 2003 [3, 4], four years before the publication of the anniversary issue, and its first steps in science. The method was named DNA barcoding. In the following twenty years, DNA barcoding has been extensively tested in various groups of multicellular organisms. For some organisms, its application was very successfully, while other cases proved to be tough nuts to crack. Several thousand experimental papers and numerous reviews, both on this approach in general and its specific aspects, have been published [5-10]. The purpose of this article is to present examples of successful application of DNA barcoding, as well as briefly outline the problems of this method and emerging controversial issues.

DNA barcoding implies sequencing of a single small fragment of genomic DNA (DNA barcode) in all species of all taxonomic groups of animals and plants within a reasonable timeframe and creation of a database of these sequences as a tool for subsequent species identification. It was expected that this approach would be helpful in taxonomy studies, as well as facilitate the discovery of new species, particularly, cryptic ones (morphologically indistinguishable but differing in other, e.g., molecular characteristics), clarify the size of known species and relationships between them, and aide in the estimation of biodiversity in various regions and environments, especially those poorly studied. Beyond purely scientific issues, DNA barcoding can be useful in many other areas, such as legal practices related to the protection of endangered species, identification of poisonous, dangerous, and prohibited for cultivation and collection species, assessment of declared species composition of herbal materials in pharmacognosy, verification of declared species composition of animal and plant products on menus and shelves in markets and supermarkets,

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fight against harmful species, forensics, custom practices, etc. Scientists had predicted that in 15 years, a sequencer of a size of a mobile phone would be created and databases of barcode sequences would be complied, so it would be possible to collect a small fragment of a plant or animal right in the field, place it in the sequencer, obtain a sequence, connect to a database (DNA barcode library) over the internet, compare this sequence to those available in the database, and find out what species it is.

First, we should acknowledge that recent technological advances have indeed been enormous: miniature sequencers have appeared, and next-generation (second- and third-generation) sequencing (NGS) methods have been developed, allowing for simultaneous sequencing of a target region in multiple samples (the principle of metabarcoding) or identification of all its variants (in the case of highly repeated genome regions). Introduction of NGS has facilitated analysis of DNA from old specimens from museum collections and even damaged samples. Consequently, new bioinformatics methods have been developed to process massive amounts of sequencing data. However, creating a reference database, which is necessary for comparing the sequences of interest, has presented significant challenges, particularly for plants, but not only.

In addition to the long-existing NCBI database of DNA sequences (GenBank) (https://www.ncbi.nlm.nih.gov/), which contains many sequences of nuclear, mitochondrial, and chloroplast genomes, but often without information on the origin of biological material or the site where it was collected, in 2005, the Canadian Centre for Biodiversity Genomics (the world's largest scientific institution engaged in DNA barcoding) created a special DNA barcode database within the framework of the BOLD (Barcode of Life Data System) web platform (https://boldsystems.org/). The website states that the database accepts sequences of more than 150 markers, however, the main ones are COI (a fragment of the cytochrome *c* oxidase subunit I gene), *rbcL* (ribulose diphosphate carboxylase gene), *matK* (maturase gene), and ITSs (transcribed intergenic spacers of ribosomal genes). It was reported that by 2015, the International Barcode of Life Project would obtain reference sequences for 5 million samples representing approximately 60,000 plant species and 450,000 animal species, including those that had not yet been described [11]. As of 2019, the main databases contained sequences of ~300,000 described species (including 15% of all described animal species) [12, 13]. It was expected that by 2025, accumulated DNA barcodes would sufficiently represent the biotas of Europe and North America. It was predicted that the work on compiling a complete DNA barcode library (~100 million individual organisms) of the world flora and fauna could be finished within a few decades, provided sufficient funding [11]. So far, DNA barcodes have been obtained for 19.7 million specimens (as of June 27, 2025) (https://v4.boldsystems.org/index.php/TaxBrowser_Home).

To enter a barcode sequence into BOLD, it is necessary to provide a minimally necessary information about the specimen, such as the country/ocean where the specimen was collected and the date of collection. Other desirable information includes a photograph of the specimen, GPS coordinates of the collection site, taxonomic identification (up to the order or family level), and genetic information (gene region, PCR primer sequences and PCR conditions, chromatogram files used in contig assembly). The length of the final sequence should be more than 75% of the accepted barcode marker length (e.g., 500 bp for COI). The acceptable sequence quality is specified as less than 1% erroneous bases in the final trimmed contig. The sequence should correspond to the putative high-level taxonomic position (DNA barcode should cluster with related taxa).

Each DNA barcode is a sequence of a particular DNA fragment from a single individual. BOLD uses a special program (algorithm) that assigns barcode sequences to the clusters (operational taxonomic units, OTUs) with the extent of similarity selected by a researcher, here referred to as BINs (Barcode Index Numbers) [14, 15]. OTUs were proposed in numerical taxonomy for groups of organisms possessing morphological features that, to a certain extent, are characterized by similar numerical parameters and differ in these parameters from other groups [16]. If these features are DNA sequences of specific regions, such groups are sometimes referred to as mOTUs. OTUs/BINs may not correspond to species or genera, as they are "working" provisional (temporary) groupings.

The Table 1 shows the data retrieved from the BOLD website in July 2025, which might give an idea on the number of DNA sequences already obtained for the major groups of plants and animals. Of course, the overall estimate of the number of known species is very approximate and was taken by us from various sources. The studied species are those reported by the authors as included in the analysis, and not for all of them DNA sequences have been obtained. The depth of analysis in the groups varies widely – from 18% species (arachnids) to almost 100% species (fishes). The reported 100% study rate for vertebrates (which ranges from 33 to 68% in some major groups) can apparently be explained by different taxonomic interpretations used by different researchers. Clearly, the number of specimens examined per species varies widely, and not all studied species have been DNA barcoded, likely for various reasons, such as amplification failure or poor sequencing quality.

Table 1. Species, specimens, and DNA barcodes for some groups of multicellular organisms available in the BOLD database (July 2025)

Taxon	Approx. number of known species	Number of examined specimens	Number of examined species	% examined species of known species	Number of examined specimens per examined species	Number of species with DNA barcodes	% species with DNA barcodes of examined species	% species with DNA barcodes from known species	Number of BINs
Chordata (chordates)	>50,000	1,155,037	50,101	100	23	45,594	92	92	47,936
Mammalia (mammals)	>6500	226,969	4393	68	9	3993	77	61	5089
Aves (birds)	~11,000	103,007	7119	65	14	6248	87	57	6107
Reptilia (reptiles)	~12,000	36,934	3982	33	9	3562	89	30	5168
Actinopterygii (ray-finned fish)	~27,000	487,312	26,930	100	18	25,511	95	94	23,469
Primates (primates)	518	101,475	346	67	29	329	95	63	390
Arthropoda (arthropods)	>1,000,000	23,808,577	381,919	38	62	304,555	79	30	793,702
Insecta (insects)	>1,000,000	21,791,896	337,000	34	64	265,391	78	27	715,102
Lepidoptera (butterflies)	~160,000	2,968,511	127,519	80	23	107,521	84	67	157,100
Arachnida (arachnids)	~115,000	784,910	21,632	18	35	18,706	86	16	42,058
Echinodermata (echinoderms)	~7000	75,473	3451	50	22	2925	84	41	2873
Mollusca (mollusks)	~76,000	345,116	23,990	31	14	20,889	87	27	25,807
Nematoda (round worms)	>24,000	120,243	3718	15	40	1781	48	7	1919
Magnoliopsida (dicotyledons)	>200,000	369,713	97,749	51	4	49,879	51	26	0
Liliopsida (monocotyledons)	>60,000	108,318	28,216	48	4	17,903	63	30	0

This is especially typical for nematodes and plants (DNA barcodes were obtained for approximately half of the studied species). In most groups, the number of BINs exceeds the number of species with DNA barcodes, either slightly (vertebrates, primates, nematodes) or significantly (mammals, arthropods, insects, arachnids). In the latter case, it can be assumed that these groups contain many undescribed, possibly cryptic, species. In some groups (birds, fishes, echinoderms), the number of studied species with DNA barcodes roughly corresponds to or even exceeds the number of BINs. The number of BINs for plants has not been determined.

MARKERS USED IN DNA BARCODING

DNA barcoding was proposed by zoologists, who already had a good candidate for the barcoding region – a fragment of the mitochondrial COI gene that had been tested in numerous animal species. It exhibits sufficient and uniform variability and, therefore, can be used for species identification in important groups, such as birds, fish, insects, crustaceans, ciliates, and others. This DNA fragment contains a relatively conserved region, allowing the use of small primer sets for large groups. There are over 400 currently known primers for this marker listed in the BOLD database. However, for some groups, the COI fragment fails to yield good results, so other markers had to be used, either in combination with COI or alone. For many reptiles, amphibians, and gastropods, the mitochondrial 16S rDNA gene (typically, its fragment) is added to COI (amplified with degenerate primer sets), although it was reported that the use of complete gene sequences produced better results [17]. In coral polyps, for which a standard COI sequence proved to be insufficiently variable, the situation is complicated by the difficulty of species identification and a confusing taxonomy with many synonymous names. Other mitochondrial genome sequences selected for DNA barcoding of these organisms are ND6-ATP6, ND4-12S, COX3-COX2, ND5-ATP8 [18], mt-MutS (msh1), iGR1, and ND2 genes [19]. In species of sponges, which differ in a few morphological features that, at the same time, are highly variable within a species, the situation is even more complicated. For common sponges (class Demospongiae), the standard COI fragment is often suitable, especially with the addition of the 28S rDNA gene C region [20, 21]. However, in many calcareous sponges (class Calcarea), the mitochondrial genome has an increased level of mutability, a modified genetic code, and a number of other features [22, 23]. Therefore, it was proposed to use fragments of the 28S large (LSU) and 18S small (SSU) ribosomal subunit genes, internal transcribed spacers (ITSs), histone H3 gene, and some others as DNA barcodes [20, 24]. Hence, for many animal groups, DNA barcoding requires selection of specific DNA barcodes, as well as development of special primers.

For higher plants, the Consortium for the Barcode of Life (CBOL) initially approved the fragments of two regions – the chloroplast genes *rbcL* and *matK* – as DNA barcodes [25], although the percentage of resolution achieved with these barcodes was relatively low. In addition, both *rbcL* gene and, especially, evolutionarily labile *matK* gene usually require several primers per taxon, which complicates the study and significantly increases its cost. Soon, it was proposed [26] to use the ITS1 and ITS2 sequences, which are popular in phylogenetic studies and had been accumulated in large quantities by that time in the NCBI database. As a result, in 2011, the ITS regions, as well as the variable chloroplast spacer *trnH–psbA*, were recognized as plant DNA barcodes [27]. In practice, researchers are often dissatisfied with the results obtained with these markers, so other, usually chloroplast, sequences (*trnL*, *trnL-trnF*, *ycf1*, *nadF*, *rpoB*, *accD*, *clpP1*, etc.) are added. For some algae, the V domain of the plastid 23S rDNA gene (universal plasmid amplicon, UPA) was found to be a successful region [28]. So, different markers or their combinations proved to be best for different taxonomic groups. Examples of the use of these markers as plant DNA barcodes are presented in our earlier publication [29]. It was found to be especially difficult to select satisfactory regions for rapidly evolving groups, and attempts to do this are often unsuccessful.

The main accepted DNA barcodes for such large and complex group as fungi, are ITS1 and ITS2 (together or separately), sometimes with the addition of the translation elongation factor 1α (TEF1) gene. However, for a number of species, better results were obtained with the fungal intergenic spacer (IGS) and fragments of genes encoding β-tubulin II (TUB2), RNA polymerase II subunits, DNA topoisomerase I (TOPI), phosphoglycerate kinase (PGK), cytochrome c oxidase subunits (COI and COII), 28S and 18S nuclear genes of ribosomal RNA subunits, and others [30].

The standards adopted by the CBOL establish the optimal length of each DNA barcode (e.g., 648 bp for COI). When studying museum and damaged samples, only short sequences can be obtained sometimes [31, 32]; however, for many specimens, even such shorter fragments have allowed correct species identification [13, 33]. In some cases, DNA barcodes of 100 bp or less, called mini-barcodes, have yielded satisfactory results [21, 34]. Mini-barcodes are frequently used for solving applied problems, for example, when it is necessary to establish that the tested sample belongs (or, conversely, does not belong) to a particular

species rather than to exactly identify the species it belongs to. Thus, DNA mini-barcodes have been used to analyze food products for the presence of declared and undeclared impurities [35] and to facilitate measures to prevent illegal poaching and export of animals [36] or plants [34]. On the other hand, the use of super-barcodes, such as complete chloroplast genome sequences, has also been proposed, for example, for the precise identification of medicinal plants [37] or the fight against illegal logging of rosewood trees [38]. It was suggested that in the coming years and decades, DNA barcodes will undoubtedly include multiple markers, if not entire genomes [39].

SAMPLE SIZE

It has been repeatedly emphasized that for DNA barcoding libraries to reliably serve for species identification, each species must be represented by multiple samples covering its geographic distribution. Only then can the intraspecific variability be assessed and the interspecific boundaries established [12, 40]. Initially, the standards prescribed that in order to determine the intraspecific distances, DNA barcoding should be performed for 5-10 samples per species, and in the case of phylogeographic structure, this should be done for different locations. Later, it was proposed to increase this number to 20 [41] and even to 50 or more [42]. However, this is difficult to achieve, especially in large-scale studies and/or with multiple markers. Multiple specimens have been obtained for insects. Thus, in the study of Canadian insects (~30,000 species), DNA barcoding was performed for approximately one million samples, i.e., 30 samples per species [43]. For butterflies of the North America (814 species, representing 96% of all butterfly species), the sequences were obtained for 18 (on average) specimens per species, although 59 species were represented by singletons (single specimens) [44]. At the same time, the number of samples tested in large screening studies of regional floras rarely exceeds 2-4. In DNA barcoding of angiosperms and conifers of Wales (1143 species) [45] and vascular plants of Canada (more than 5000 species) [46], the number of samples per species was 3 (on average) and 4, respectively. *In silico* analysis of five marker sequences (*rbcL*, *matK*, *trnL-trnF*, *psbA-trnH*, ITS) of flowering plants (40,000-70,000 species from 547 families) available in the NCBI database showed that even an increase in the number of DNA barcodes for each species from one to 2-4 increased the reliability of species identification for each individual marker, so it was recommended to use at least three samples per species in the analysis [47]. It was shown that for insects, the presence of species with a small number of samples (less than 5) in the DNA barcode array can lead to a reduction in the detectable gap between the intraspecific and interspecific distances in a given group of species, since these distances overlap [48]. When analyzing a large dataset, it is preferable to exclude such taxa for better species resolution.

DNA BARCODING OF MUSEUM SAMPLES

From the very beginning, analysis of museum specimens – the foundation of taxonomy – has been considered an important goal of DNA barcoding. The term hDNA (historical DNA) was suggested for DNA isolated from museum specimens, as opposed to aDNA (ancient DNA) isolated from naturally preserved specimens over 1000 years old. Although museum specimens are typically younger (rarely over 200 years old), they have often been subjected to various treatments, including storage in alcohol or formalin, or treated for pest control, which can lead to DNA degradation. Advances in NGS technologies have enabled the use of an ever-wider range of museum specimens for DNA barcoding, thereby improving species identification. The ability to use very small amounts of tissue, even heavily damaged one, for DNA analysis allows to study the type specimens with virtually no disruption to their integrity, which is crucial. Many natural history museums and herbaria are establishing DNA banks. Herbarium DNA banks contain samples of isolated and purified genomic DNA from freshly collected or herbarium material and/or plant tissues dried in silica gel and intended for DNA extraction [49-52]. Plant DNA preparations are stored in freezers at −20°C (which usually guarantees the quality of the preparation for 3 years of storage) or at −80°C (guarantee for 10 years or longer), usually in small aliquots to avoid repeated thawing. Animal DNA banks contain DNA preparations, as well as tissue fragments, which are usually stored in liquid nitrogen (−190°C). DNA and tissue preparations are provided upon request to colleagues from other institutions (by exchange or for a fee). Such DNA samples should be of high molecular weight, with a concentration sufficient for multiple analysis procedures, without RNA and inhibitors of DNA polymerases.

The largest collections of plant DNA are at the Royal Kew Gardens (London, UK), with over 60,000 specimens (48,000 DNA samples and 12,000 tissue samples) and ~35,000 plant species (https://www.kew.org/science/collections-and-resources/collections/dna-and-tissue-bank), and the Botanic Garden and Botanical Museum (Berlin, Germany), with over 50,000 DNA samples of plants, algae, fungi, and protozoa (https://www.bgbm.org/en/dna-bank). The Museum für Naturkunde in Berlin has over 30,000 DNA and tissue

samples of vertebrates, mollusks, and arthropods (https://www.museumfuernaturkunde.berlin/en/research/dna-and-tissue-collection). If the DNA of a given species (or a specific sample in a collection of a particular institution) is not yet available in a DNA bank, some other institutions can offer the option of isolating it for a fee. The experience of the first years of operation of several DNA banks was used to create the protocols (workflows) for the optimal procedures [52], and the need for cooperation between DNA banks and maintenance of uniform standards has led to the creation of the Global Genome Biodiversity Network (GGBN) (https://www.ggbn.org/ggbn_portal/ [53], which includes 117 institutions (as of July 2025).

The study of herbarium specimens of 98% vascular plants (more than 5000 species) of the Canadian flora showed that the ability to successfully amplify DNA and to sequence the barcodes depends not only on the specimen's age and storage conditions, but also on its taxonomic (family) affiliation [54]. For specimens from large families, such as Apiaceae, Asteraceae, Brassicaceae, and Poaceae, the acceptable age was over 60 years. For plants of the Ericaceae, Rosaceae, and Pinaceae families, it was 10 years, after which the possibility of a successful procedure rapidly decreased. For Onagraceae, Polygonaceae, Saxifragaceae, and Dryopteridaceae, it was less than 10 years, while for representatives of Boraginaceae and Orchidaceae, DNA from even recent collections was poorly amplified, requiring development of specific protocols. This difference was explained by the insufficiently suitable primers, inhibitory effect of secondary metabolites present, and intragenomic polymorphism of ITS sequences [54].

The differences in the acceptable storage time of specimens in different taxonomic groups were also found for animal samples, although not within the framework of a single study. Thus, study of ~12,700 butterfly specimens (Lepidoptera) from the Australian National Insect Collection revealed a number of factors that affected the efficiency of detection of the standard-length COI amplicon by Sanger sequencing [55]. One of them was the specimen size, as the success of detection decreased rapidly with the increase in storage time in small specimens. In most groups, the probability of detection decreased during the first 30 years of specimen storage, followed by a plateau over the next 30 years, after which there was a further decrease, and only shorter amplicons were detected. Interestingly, the success of amplification sometimes varied in specimens collected by different collectors, even if they were collected at the same time. It was suggested that this may be related to the methods used to kill the animals and process the specimens [55]. In the study of specimens of saproxylic beetles from museum collections stored for 1 to 17 years, the success of obtaining DNA barcodes by NGS did not depend on the sample age [56].

Development of new technologies has made it possible to analyze new and unexpected DNA sources, such as bird nests constructed from plants, tens of thousands of which are stored in museum collections. Identification of plant species used in the construction of these nests can provide valuable information about changes in the landscape, bird ecology and biogeography, etc. [57]. Thus, a method has been developed for a more precise identification of bird species by analyzing DNA from museum specimens of bird eggshells by sampling very small fragments of eggshells without damaging the eggs [58].

METABARCODING

One of the first applications of metabarcoding (a technique that combines DNA barcoding with high-throughput sequencing to identify multiple species within a community of living organisms) was studying the intestinal content of vertebrates, including extinct ones. The DNA obtained from the gut is referred to as iDNA (ingested-derived DNA). [Note that the same abbreviation is used to denote invertebrate-derived DNA, i.e., DNA isolated from invertebrates, for example, bloodsucking and scavenging insects (fleas, mosquitoes, and flies), whose entire bodies are typically ground for analysis.] Analysis of iDNA often allows for non-invasive monitoring of animal biodiversity in a given area and is used for assessing the presence and relative abundance of various species, as well as for clarifying the biology of both insects and their food sources [59-61]. Insect species have often been evaluated and compared for solving specific scientific problems. For example, it was found that mammalian blood can remain in the intestines of fleas for up to several months, during which the flea can travel far from the location of the blood source [60].

Metabarcoding often uses the so-called eDNA (environmental, or ecological DNA) isolated from the environmental samples, such as soil, air, water, and atmospheric precipitation, that contain small remains and waste products of various animals (feathers, hairs, feces, mucus, etc.). In recent decades, particular attention has been focused on the studies of aquatic environments because of the constant decline in the number of marine and, especially, freshwater animal species. In these experiments, collected water samples are analyzed for all present DNA sequences. Insects for metabarcoding studies are often collected using Malaise traps, special devices that efficiently catch flying insects. Metabarcoding is also used in plant studies for the analysis of the herbivores' diet, plant sediments

in water bodies, interactions of pollinating insects with plants, pollen composition in the air, composition of food products and herbal materials, etc. [62-65].

Much attention in biodiversity studies is paid to comparing the accuracy of estimates and performance of metabarcoding methods vs. traditional methods. Many authors have reported that metabarcoding provided more accurate and rapid (with fewer analyses than traditional methods) estimate of species inhabiting a given habitat [66, 67]. Thus, eDNA metabarcoding was able to evaluate the local fish fauna within a season with the same degree of completeness as the long-term irregular collection [66]. However, it has been repeatedly emphasized that methods based on the use of eDNA require verification, calibration at each stage, and careful interpretation to the same extent that is needed when working with aDNA [5, 68].

DNA BARCODING DATABASES – SEQUENCE QUALITY AND ACCURACY OF SAMPLE IDENTIFICATION

It is commonly agreed that DNA libraries obtained by large-scale DNA barcoding of any major taxonomic group associated with a specific region or habitat with the use of specified DNA barcodes, rarely allow for the species-level identification. The high incidence of misidentifications in DNA sequence databases, including NCBI, has long been known. In the early days of molecular systematics, the studies had been conducted not by taxonomists or even botanists or zoologists, but by biochemists and geneticists, who had only a superficial understanding of the importance of accurate species identification and information about the sample provenance. For example, plants for analysis were ordered from catalogues provided by botanical gardens and not always properly verified. As DNA barcoding has become increasingly popular and DNA analysis started to be conducted by commercial firms, the situation became even worse. DNA barcoding is now performed not only by biologists, but also by specialists in agriculture, medicine, pharmacology, archaeology, bioinformatics, and other fields mostly unrelated to taxonomy. As a result, material for analysis can be purchased commercially with only the country or origin indicated. Even when the material is taken from a seed bank, sometimes only the bank's location (and not the catalogue's number) is provided. The name of the species may be given without the author, a voucher is missing, and it is unclear who, how, or even approximately by what characteristics identified the material. Nevertheless, a DNA barcode can be assigned to such sample, which can be entered into the reference database. The NBCI does not provide a mechanism for mandatory removal of sequences of incorrectly identified specimens, even when the error is detected.

From the very beginning of DNA barcoding, it has been stated that the created databases should be curated so that the quality of sequences meets the standards and the accuracy of species identification is verified by specialists, which was not (and is not) the case of NCBI database. It was assumed that these requirements would be implemented in the BOLD database, but, apparently, due to the declining number of taxonomists and biodiversity specialists worldwide, this could not be achieved [69, 70]. Although BOLD administrators check whether the submitted sequence meets all the requirements, including information about the specimen, they are likely unable to verify its correct taxonomic identification. Recently, an attempt was made to quantify the completeness of information (standard DNA barcodes) for metazoans of the central and eastern Pacific Ocean deposited in the NCBI and BOLD databases, and to compare these databases [70]. It was stated that NCBI leads in the data quantity, and BOLD leads in the sequence quality. However, both databases reported sequences that did not meet the standards – were too short, too long, or had a large number of ambiguous nucleotides –and were proposed for removal. Some datasets lacked taxonomic information. Uneven representation of different groups was noted, with a clear paucity of data for Porifera (sponges), Bryozoa (bryozoans), and Platyhelminthes (flatworms). However, it was also proposed to remove "sequences of overrepresented species" and "sequences with conflicting taxonomy" (i.e., which showed similarity to a group other than the one they were claimed to belong to, according to BLAST) and to "standardize taxonomic metadata to ensure taxonomic completeness" (when indicating species affiliation of a specimen). It is difficult to imagine how such actions could be implemented or who would undertake them (considering the data already entered).

Standard barcode sequences of plant, fungal, and insect specimens (a total of about a hundred species, including poisonous plants and forensic fungi and insects) from systematic collections of reputable scientific institutions were analyzed in [13]. Most of insect species were obtained from the Smithsonian National Museum of Natural History, USA. The comparison of DNA barcodes obtained in this study with the sequences available in the NCBI and BOLD databases showed a very high accuracy of plant (~81%) and fungal (~57%) species identifications. However, for insect species (including well-known and widespread ones), the accuracy of identification was less – 53% species in NCBI and 35% species in BOLD. The authors concluded that both databases contain many errors and that the curated BOLD database is not superior to the non-curated NCBI, although a significant proportion

of animal sequences in both databases represented insects. Researchers from the Canadian Centre for Biodiversity Genomics, which maintains and curates the BOLD database, decided to investigate [71] whether the source of the errors was in the databases or in the sequences obtained by Meiklejohn et al. [13]. They re-examined all samples, procedures, and results obtained for the insect species from the work of Meiklejohn et al. [13] and revealed several reasons why many identifications were incorrect. Along with a number of technical errors, it was found that during analysis, museum insect specimens could be contaminated with foreign DNA. Some discrepancies were related to the complexity and uncertainty of taxonomy of certain species [71].

It should be emphasized that ordering tissue and DNA samples from other institutions exacerbates the problems of species identification. These problems were examined in detail in the article by zoologists specializing in studying reptiles and DNA barcoding of these animals from the Smithsonian Institution (USA), which houses the largest collection of reptiles [72]. The authors compiled a DNA barcoding library for more than 500 reptile species. The barcodes were sequenced by the authors and employees of other institutions from 52 countries that had received materials from the museum collections. When possible, the correctness of identification of the provided specimens was verified. The three most significant problems were the following. First, the species names assigned to DNA barcodes were the names provided by the lending institution, without further verification and without considering recent taxonomic changes (which may not have been incorporated into the lending museum's database). Second, researchers did not conduct BLAST searches in the GenBank to verify the identification. Third, researchers submitted the data on a new species under the old name in the case of taxonomic splitting and failed to update the records after publication of the paper describing the new species. As a result, both NCBI and BOLD databases typically list correctly the genera of reptile specimens, but the accuracy of species identification is much lower. The authors noted that many institutions are experiencing funding and staff cuts or have to deal with additional tasks related to the database support, which increases the workload for the staff and makes it impossible to properly maintain the collections, or even to change the labels.

BIODIVERSITY DESCRIPTION AND NEW SPECIES

Traditional taxonomic analysis, which is necessary for identifying diagnostic traits and describing new species, often requires painstaking studies and a high level of professionalism. Analysis of various groups using molecular methods, in particular, DNA barcoding, has shown that animal species diversity is often higher than expected based on morphological analysis, and cryptic species are frequently discovered [73-75]. When the number of such species is high relative to the number of known species, it was been proposed to call them "dark" taxa [76, 77]. There are great concerns that many species, especially, in poorly studied regions, risk extinction without ever being discovered or described. Moreover, such species are still discovered in regions where the biodiversity has been studied for a long time and to a great extent, for example, in Germany and Sweden [78-81]. A DNA barcoding study of arthropods in Germany showed that the number of species of small arachnids Pseudoscorpionida (pseudoscorpions) should be increased by more than 40% [78] and even more for Diptera insects [79]. In Sweden, where the diversity of butterfly species has been studied better than anywhere else, with 2990 species described, DNA barcoding revealed more than 300 undescribed species [81]. The largest number of undescribed species has been found for arthropods, but also for many other invertebrates and vertebrates, as well as for fungi. The smallest number of undescribed species has been found in higher plants, which are characterized by a widespread hybridization and have a large number of hybrids (the so-called cryptic diversity) [82-85].

The proponents of DNA barcoding have persistently emphasized that one of its primary goals is to help in slowing down the loss of biodiversity, as monitoring of biodiversity is complicated by a large number of dark species, as well as species described so superficially that their specimens can only be identified by comparison with the type specimens, which is not always easy. Therefore, these researchers have adopted a new tactic. Two papers were recently published that described dark species in a large group of parasitoid wasps (ichneumon flies, order Hymenoptera) that lay eggs on or inside their hosts (other insects). After hatching, the larva consumes the host, eventually killing it. Ichneumon flies is an economically significant group, as many wasp species parasitize on agricultural pests, reducing their numbers.

The first paper [86] was entitled "A revolutionary protocol to describe understudied hyperdiverse taxa and overcome the taxonomic impediment." It examined parasitoid wasps of the Ichneumonoidea superfamily, which has ~44,000 described species (although it was suggested that this superfamily can include up to a million species). DNA barcodes were obtained for 336 wasps collected in Costa Rica. Based on BINs calculated by the BOLD system, 18 new species were identified that were assigned to two existing genera.

The species were given conventional binomial names and described with a minimum diagnosis consisting of the consensus DNA barcode (sequence) and a photo of the specimen (side view). For the second article [39], the same authors collaborated with a large team of scientists from different countries and institutions. The article's title began with the words: "Minimalist revision and description of 403 new species". The article contained descriptions of 403 new species of parasitoid wasps from other genera belonging to 11 subfamilies, also from Costa Rica. The descriptions were based on the same principles. And although both articles stated that the authors considered DNA-based descriptions as the first step in solving the taxonomic problems associated with megadiversity and lack of taxonomic resources (which standard approaches failed to resolve), both articles received critical responses [87-89].

The studies [39, 86] were criticized for the lack of adequate morphological descriptions and comparisons with previously described species, as well as for the fact that the identified species were unstable when the original data were analyzed using different species delimitation algorithms. Several other technical objections were raised against the use of the "minimalist approach" based on COI barcodes. According to the critics, such approach would only complicate subsequent taxonomic analysis of these groups rather than facilitate it. The authors of studies on parasitoid wasps immediately followed the criticism and presented their arguments against the critics' disappointing conclusions [90]. However, the discrepancies in the numbers of new species identified using different data processing methods have also been found in other studies [91, 92]. The debate still continues [93-95]. Since the description of new species with the possibility of their subsequent identification requires a great deal of work and studying the intra- and intergroup variability (at least at the morphological and molecular levels), such groups often remain, at best, in the OTU (BIN) status. Some of the most impressive arguments in favor of DNA barcoding were provided by the studies of the South American butterfly *Astraptes fulgerator*. The sequences of the COI fragment from hundreds of individuals of this species were divided into ten clusters, with some correlation between the cluster affiliation, type of caterpillar coloration, and caterpillar feeding on different plants. Hebert et al. [96] hypothesized that *Astraptes fulgerator* represents a complex including about ten undescribed species. This article has been cited more than 4,700 times, including in some reputable works, as an example of well-documented case of cryptic species in butterflies [97]. However, over the past 20 years, not a single new species based on the mentioned clusters has been described.

In their article, Meier et al. [95] noted that until recently, the calls for the integrative taxonomy, i.e., the use of both molecular and morphological characteristics in analysis, have remained largely declarations of intent. The authors estimated that the databases already contain 15 million DNA barcodes for insects, but only 10% taxonomic publications in 2018 included molecular data [95]. Note that 15 years ago, there were approximately 15,000 taxonomists worldwide that worked using traditional methods, and it is unlikely that their number has increased, if anything, by now. DNA barcoding has become cheaper, while qualified morphological expertise has not. We believe that DNA barcoding can at least help in identifying groups containing many unknown, undescribed species. The alternative for dark species is not a complete taxonomic analysis, but complete obscurity. Meanwhile, the knowledge of a DNA barcode sequence that distinguishes such species from a known related species, will lift them out of obscurity and make subjects of further investigations.

PRACTICAL APPLICATIONS OF DNA BARCODING

The use of DNA barcodes in some applied fields have become even more successful and advanced than in the biodiversity studies, as the range of subjects in the former is often limited and specific. The number of publications on this topic is so large that we will limit ourselves to referencing only recent reviews that discuss or mention such works.

DNA barcodes can be used to analyze and determine the composition of food products, both raw (fish and other seafood, meat, vegetables, spices, etc.) and cooked, to check for the presence of undeclared impurities, and to identify plants visited by bees in the production of honey [35, 98-100]. Other important areas include testing medicinal plants used as raw materials and in medicines [101-103] and species identification (especially insects) in forensic science and forensic examinations [33, 104, 105]. Increasingly relevant applications of DNA barcoding are control of agricultural pests and invasive species [106-108], environmental protection, and assistance in preventing illegal poaching and export of animals [36, 109] and plants [110, 111], in particular, with the participation of customs services.

FUTURE PROSPECTS

It has been suggested that compiling a complete DNA barcode library might require analysis of ~100 million samples and that this work could be completed by approximately 2040 [11]. Apparently, this

implied that one or few standard markers would be used. However, it has become clear that the initially stated goal of using one marker for all organisms is unlikely to be achieved. Many groups required application of specific markers or set of markers. However, the progress in sequencing technologies has been so significant that it is currently implied (or wished) that the sequencing of a complete genome of any organism will soon become a reality and even a routine procedure, and that such genomes will be used as DNA barcodes [39, 112]. Moreover, according to evolutionary genomics studies, all modern species of flowering plants have undergone one or more whole-genome duplications (polyploidizations), which usually accompanied interspecific, sometimes very distant, hybridization events [113]. According to very conservative estimates, at least 15% of angiosperm species are recent polyploids [114]. This complicates DNA barcoding of plants. The polyploid origin of a species might be indicated not by a single specific DNA marker sequence (DNA barcode), but by a combination of DNA barcodes obtained by the plant from its relatively recent ancestors. This combination of genomes of different origin can be identified by the locus-specific NGS of DNA barcodes, such as internal transcribed spacers ITS1 and ITS2 [115-117]. When combined with the establishment of close relationships, DNA barcoding provides new opportunities not only for the species identification but also for selection.

In conclusion, we would like to note that in the Russian Federation, DNA barcoding of different groups of organisms is one of the actively developing research fields. In addition to the reports cited above, we would like to mention the studies, mainly from recent years, on fishes [118-121], bats [122], rodents [123], reptiles [124], crustaceans [125], mollusks [126], insects [127-132], mites [133-134], annelids [74, 135-138], tardigrades [139], and other animal groups, which have been published in major journals. Plants [140-144], algae [145, 146], and fungi [147-150] have also been studied. Beside purely scientific investigations, there is also a number of applied studies [151-153]. DNA barcoding is entering our lives.

Abbreviations

COI	cytochrome *c* oxidase subunit I fragment
ITS	ribosomal gene intergenic transcribed spacer
matK	maturase gene
NGS	next-generation sequencing
rbcL	ribulose diphosphate carboxylase gene

Contributions

V.S.Sh. collected and analyzed published data and wrote the text of the article; A.V.R. discussed the topic and the structure of the review and edited the manuscript.

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Ethics approval and consent to participate

This work does not contain any studies involving human and animal subjects.

Conflict of interest

The authors of this work declare that they have no conflicts of interest.

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