

Review

The Expanding Role of DNA Barcodes: Indispensable Tools for Ecology, Evolution, and Conservation

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Abstract: DNA barcoding has transformed the fields of ecology, evolution, and conservation by providing a rapid and effective tool for species identification. The growth of DNA barcodes as a resource for biologists has followed advances in computational and sequencing technology that have enabled high-throughput barcoding applications. The global DNA barcode database is expanding to represent the diversity of species on Earth thanks to efforts by international consortia and expanding biological collections. Today, DNA barcoding is instrumental in advancing our understanding of how species evolve, how they interact, and how we can slow down their extirpation and extinction. This review focuses on current applications of DNA barcode sequences to address fundamental lines of research, as well as new and expanding applications of which DNA barcoding will play a central role.

Keywords: high-throughput sequencing; species interactions; metabarcoding; symbioses



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1. Introduction

The fields of ecology, evolution, and conservation are being transformed by novel resources and techniques in the biological sciences. One of these, DNA barcoding, has now realized its potential for the research community. Since the concept of DNA barcodes was first introduced in 2003 [1], tens of millions of barcode sequences have been made publicly available in reference databases for comparative research applications across the Tree of Life (Table 1). The growth of DNA barcode data in public repositories has been driven by several factors, including advances in sequencing technology, novel database management and other computational software, and the expansion of national and international consortia that support DNA barcode sequencing. Recent reviews have highlighted the growth of DNA barcode applications for phylogenetics and taxonomy (e.g., [2]). Other overviews suggest that DNA barcoding is a resilient field that will continue to grow as sequence databases are enriched, throughput expands, and automation provides an ever-expanding user-community with increased accessibility to DNA barcodes, as reported by [3]. This review highlights the advances and applications in DNA barcode sequencing that have been leveraged for novel research in ecology, evolution, and conservation.

1.1. Accurate and Reliable Identification of Species in Taxonomy, Ecology, Evolution, and Conservation

Hypothesis testing is at the heart of the biological sciences and is the standard for how we understand the complexity of the natural world. For most biodiversity research, the reliability and repeatability of hypothesis testing is dependent on accurate identifications of the species under investigation. Faulty identifications can result in faulty hypotheses. A fundamental challenge for any biologist, therefore, is to determine in a reliable and repeatable fashion the correct identification of any given biological sample. “DNA barcodes,” i.e., standardized short sequences of DNA between 400 and 800 base pairs long, which in

theory can be easily isolated and characterized for all species on the planet, were originally conceived to facilitate this task [1]. By combining the strengths of molecular biology, sequencing technology, and bioinformatics, DNA barcodes offer a quick and accurate means to recognize previously known, described, and named species and to retrieve information about them.

Table 1. Diversity and number of barcode sequences available in the Barcode of Life Data System (BOLD) database, taxon labels follow the BOLD format.

Taxon		Barcode Sequences Available ¹
Animals		11,607,692
	Acanthocephala	2302
	Acoelomorpha	20
	Annelida	112,010
	Arthropoda	11,486,730
	Brachiopoda	326
	Bryozoa	4529
	Chaetognatha	1775
	Chordata	877,866
	Cnidaria	32,680
	Ctenophora	649
	Echinodermata	326
	Entoprocta	76
	Gastrotricha	1351
	Gnathostomulida	24
	Hemichordata	263
	Kinorhyncha	720
	Mollusca	258,885
	Nematoda	36,513
	Nematomorpha	408
	Nemertea	6443
	Onychophora	1394
	Phoronida	172
	Placozoa	20
	Platyhelminthes	41,262
	Porifera	9668
	Priapulida	151
	Rhombozoa	48
	Rotifera	13,758
	Sipuncula	1367
	Tardigrada	3175
	Xenacoelomorpha	18
Fungi		178,231
	Ascomycota	99,779
	Basidiomycota	71,120
	Chytridiomycota	293
	Glomeromycota	3529
	Myxomycota	235
	Zygomycota	3275
Plants		572,154
	Bryophyta	22,675
	Chlorophyta	18,286
	Lycopodiophyta	1338
	Magnoliophyta	454,329
	Pinophyta	7661
	Pteridophyta	11,671
	Rhodophyta	56,194
Protists		10,463
	Chlorarachniophyta	67
	Ciliophora	819
	Heterokontophyta	7238
	Pyrrophytophyta	2339
Total		12,368,540

¹ Data accessed from https://www.boldsystems.org/index.php/TaxBrowser_Home, accessed on 26 January 2022.

For plants, DNA barcoding has truly become a universal tool for hypothesis testing by expanding the ability to identify a species at all stages of its life history (i.e., fruits, seeds, seedlings, mature individuals both fertile and sterile) from damaged or preserved

specimens, as well as environmental samples with multiple species. Accordingly, DNA barcodes have been applied to address fundamental questions in ecology, evolution, and conservation biology, such as: how are species assembled in communities; what is the extent and specificity of multispecies interactions in well-studied and previously poorly known environments; and where are the most evolutionarily rich habitats for priority conservation and natural area protection in this age of habitat degradation [4,5]. With regard to the applied users of taxonomy, DNA barcodes also serve as a means to identify regulated species, invasive species, and endangered species.

1.2. Generating, Applying, and Sharing DNA Barcodes

1.2.1. Sequencing Technology

Advances in sequencing technology have radically transformed the potential for DNA barcoding over the last decade by significantly reducing costs and time [6]. The current state-of-the-art sequencing platforms can rapidly sequence tens to hundreds of millions of short-length DNA fragments (50–300 base pairs with Illumina) or tens to hundreds of thousands of long DNA fragments (10,000–30,000 base pairs on PacBio® and Oxford Nanopore). The scale of targeted sequencing projects has expanded such that a single researcher can generate barcode sequences from hundreds or thousands of extracted DNA samples in a matter of hours [7–9]. The expanding scale of sequencing presents a great opportunity for the barcoding community, as it allows for rapid generation of a universal DNA barcode library across the Tree of Life. This is critical, as high-throughput sequencing leads to a better curated database of barcode sequences from known species, but also a greater representation of sequences from unidentified species (e.g., dark taxa, [10]). The universal library of DNA barcodes from known species is being populated at an increasing pace, but the global scientific community still lacks reference barcode data for a majority of species across all major lineages (Figure 1).

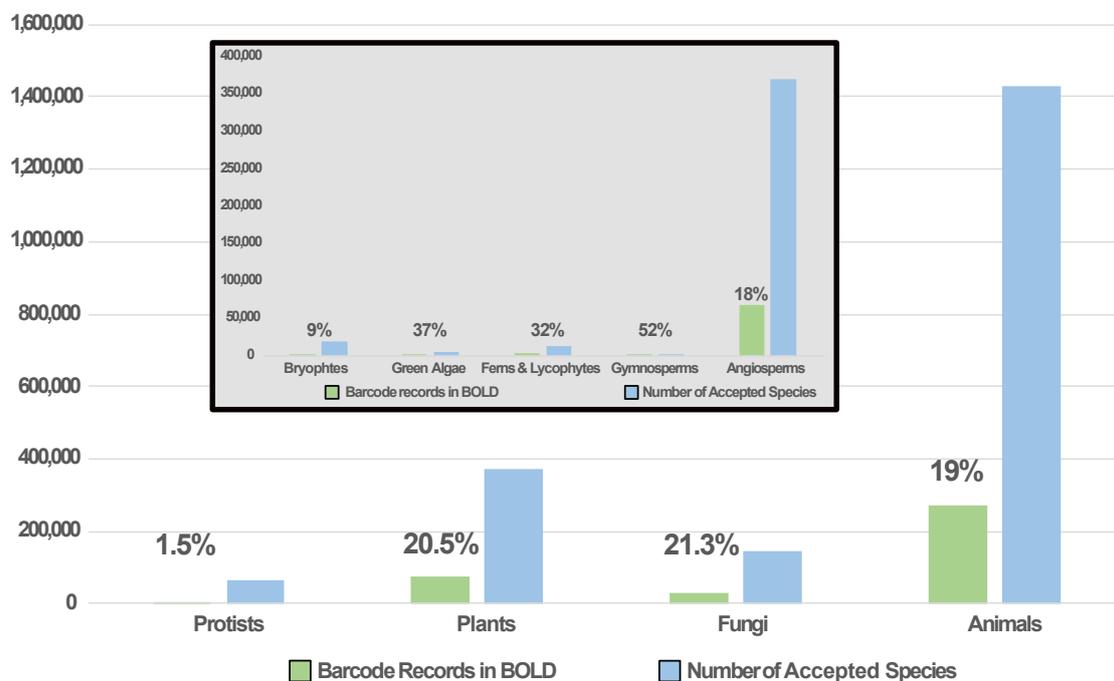


Figure 1. Numbers of species and DNA barcodes across the Tree of Life. The number of species in each of the four major groups of organisms on Earth (blue bars) according to the Catalog of Life are given along with the number of published barcode sequences in BOLD (green bars). Inset shows the major green plant clades (blue bars) with the number of barcode sequences in BOLD (green bars) adjacent to the number of accepted species (according to [11]). The estimated percentage of all species with DNA barcode sequences for that group is provided above the bars in this plot.

As the sequencing technology landscape continues to expand (also see Section 3 below), so does the traditional view of DNA barcodes. Longer sequence reads have led some researchers to consider longer barcode sequences with potentially greater discriminatory power for taxonomic identification. A number of recent studies have presented “super-barcodes” [12,13] or “ultra-barcodes” [14,15] as approaches that leverage whole organelle genomes (e.g., the chloroplast) or a combination of organellar and ribosomal DNA to provide significantly longer sequence data for barcoding. The super- or ultra-barcoding approach has been most commonly used for plants, which present a number of challenges to traditional DNA barcoding. Another alternative for traditional DNA barcoding leverages high-throughput sequencing technology to “skim” the genome (e.g., genome skimming, low-coverage sequence reads from a whole genome) as a universal barcode [16]. This approach circumvents the need for PCR, which can be problematic for preserved specimens with degraded DNA and also provides a method for less ambiguous reference databases for taxonomic identification [17]. Regardless of where the standard for DNA barcode technology is headed, barcode sequence databases will benefit from a growing number of sequences generated for known species.

1.2.2. Novel Computational Resources and Software

The Barcode of Life Data System (BOLD, <https://www.boldsystems.org/>, accessed on 26 January 2022 [18]) has been the core bioinformatics resource dedicated to hosting DNA barcode sequence data since it was launched in 2007. In addition, many computational resources and software have been developed to accommodate the expanding role of DNA barcodes. Some of these packages (e.g., MDOP [19]) help researchers to organize DNA barcoding data before uploading to databases, such as BOLD and NCBI’s Genbank, and still others are designed to assess the quality of data that have already been made publicly available (e.g., BAGS [20] and MACER [21]). The quality of DNA barcode data can be impacted by a number of factors, including poor sequence annotation, a lack of physical specimen vouchers, poor sequence quality, and incorrect consensus sequence building. The last of these factors is especially problematic for DNA barcoding methods based on high-throughput sequence reads. Fortunately, several recent software packages have been developed to address challenges with consensus sequence building, such as PIPEBAR, OverlapPER [22] and NGSpeciesID [23].

Taxonomic assignment is key for downstream applications of DNA barcode sequences and the accuracies of approaches, which assign sequences from unknown taxa to a recognized barcode sequence, are critical [24]. Despite the development of several tools to accurately assign sequences to taxa represented in barcode sequence databases, comparison across software has demonstrated that it remains challenging to accurately assign sequences to taxa at or below the level of genus [25]. Taxonomic assignment methods are being developed and refined rapidly, with several options published in just the last four years. Among these are the QIIME2 feature classifier [26], IDTAXA [27], MeTaxa2 [28], and Basta [29]. Although the methodology to perform taxonomic assessment is quickly evolving, older methods are accurate, still perform well, and continue to be used, such as Kraken2 [30], Protax [31], and the longstanding BLAST tool [32]. Beyond these methods, other options are optimized for clade-based metabarcoding reference databases (e.g., Fungi: funbarRF [33]) or have been developed as part of custom pipelines that have more specific user needs (e.g., the Anacapa Toolkit [34]). Ultimately, the ability of any computational method to accurately match a sequence from an unknown species is dependent upon well-curated, annotated, and comprehensive reference sequence databases. Focus should remain on populating DNA barcode reference databases with high-quality sequence data from accurately identified and vouchered collections.

1.2.3. National and International Sequencing Consortia

The effort to contribute DNA barcode sequence data is coordinated worldwide through both national and international organizations. Coordination of international barcoding

activities began in 2004 with the Consortium for the Barcode of Life, followed by the International Barcode of Life Project (iBOL, <https://ibol.org/>, accessed on 26 January 2022) in 2008. National efforts have also been launched in Austria (ABOL), Finland (FinBOL), Germany (GBOL), the Netherlands (NBOL), Norway (NorBOL), and Switzerland (SwissBOL) to name a few. Most recently, BIOSCAN [35], an international project organized by iBOL, was initiated and includes 1000 researchers in over 30 countries with the objective of generating DNA barcodes to discover species, to understand species interactions, and to monitor species in a global biological surveillance system. Once achieved, the collective goals of these organizations will result in a DNA barcode library for nearly all species on Earth.

In the nearly two decades since DNA barcodes were first proposed, other ambitious and sweeping networks have emerged that also reflect the fundamental goal of the DNA barcoding community: to leverage organismal DNA to understand life on Earth. One of these, the Global Genome Biodiversity Network (GGBN, https://www.ggbn.org/ggbn_portal/, accessed on 26 January 2022 [36]) represents a network of well-curated tissue collections that seeks to develop standards, share collection information, and facilitate biodiversity genomics research. More recently, the Earth BioGenome Project (EBP; <https://www.earthbiogenome.org/>, accessed on 26 January 2022) was launched [37] as a “moonshot” [38] for biology that aims to sequence whole genomes of all eukaryotic species on Earth in ten years. Although not specifically aimed at DNA barcode loci, EBP will indirectly provide a wealth of sequence data for the major DNA barcode loci of plants, animals, and fungi. DNA barcoding, which was originally considered to be at one end of the sequence spectrum, is now converging with entire genomes [39]. These global efforts, which have been described as “networks of networks,” build connections among more localized, often national endeavors.

The organization of DNA barcoding projects has often followed geopolitical boundaries and the most common denominator for large sequence programs reflects local, regional, or national funding structures. Some examples of these at a regional and national levels include the African Centre for DNA Barcoding (<https://www.acdb.co.za/>, accessed on 26 January 2022 [40]), the Canadian Centre for DNA Barcoding (<https://ccdb.ca/>, accessed on 26 January 2022), and the China Plant BOL (Barcode of Life) Group [41]. In a similar way, the United Kingdom’s Darwin Tree of Life Project (<https://www.darwintreeoflife.org/>, accessed on 26 January 2022 [42]) takes a geopolitical approach toward their goal to sequence the whole genomes of all eukaryotic species in Britain and Ireland. These focused, localized research networks contribute to international goals that help support the shared priority of advancing a global understanding of biodiversity and facilitate the use of DNA barcodes and other genetic tools for broader ecological, evolutionary, and conservation purposes.

1.2.4. Building the Plant DNA Barcode Library

With more than half a million plant DNA barcode sequences available today in the Barcode of Life Data Systems (BOLD, Figure 1), continuing to populate the global library is a major effort of botanists. In addition to the national and multinational projects described above, building the plant DNA barcode library can be enhanced by taking advantage of a number of diverse efforts, such as forest monitoring plots, individual lineage-based taxonomic studies, and regional floristic efforts. Forest monitoring plots, such as the Smithsonian’s Forest Global Earth Observatories (ForestGEO) and the National Science Foundation’s Long Term Ecological Research (LTER) sites, are rich resources because they have well-verified identifications, vouchered collections, and individually tagged trees that can be revisited by botanists if necessary [43–46]. Even if no specific monitoring plots have been established, many studies have generated DNA barcode libraries for specific habitats [47], plant communities [48], or regional taxa [49–51] and are thereby expanding the global plant genetic library. Individual taxonomists are also generating DNA barcodes for specific groups of plants as either standard markers (e.g., [52–55]) or as an offshoot of their basic molecular phylogenetic investigations aimed at understanding

evolutionary relationships. Preserved museum specimens can also be used to generate DNA barcodes [56]. It is significant that one recent study has encouraged a large-scale effort to sequence DNA barcodes from all types of specimens [57]. All of these DNA sequences add to the library of standard DNA barcode markers even if they do not carry the official GenBank DNA barcode designation.

Other efforts to generate DNA barcodes for entire regional floras are in some cases complete or just getting underway. One of the most impressive is the library that has been built for identifying the vascular plants of Canada [58], which includes sequence records (*rbcl*, *matK*, and ITS2) for 96% of the 5108 species known from that country. Another success story for plant DNA barcodes is the China Plant Barcode of Life [41]. This sixteen-year project has now generated and made available for use 120,000 DNA barcodes for 16,000 species, representing a significant sampling of the entire flora of China. Other examples are the recently completed DNA barcode library for the plants of the UK [59], and work that has started on the flora of the Arabian peninsula [60].

1.3. The Purpose and Structure of This Review

Today, more than ever, DNA barcodes are being used to advance our understanding of how species evolve, how they interact, and how we can slow down their extirpation and extinction (e.g., [61–63]). As sequencing technologies have improved and sequencing costs have declined, the use of DNA barcoding is skyrocketing and some of the most exciting prospects for using this new taxonomic tool are being realized. A number of comprehensive reviews of the application of plant DNA barcodes to the fields of ecology, evolution, and conservation have been provided in the past [5,64–68]. This review and the Special Issue of *Diversity* of which it is a part focus on current areas of research as well as new applications of DNA barcodes that are the direct result of the accumulation of barcode reference sequences, including past trials, experiments, and applications of this twenty-first century biological tool (Figure 2).

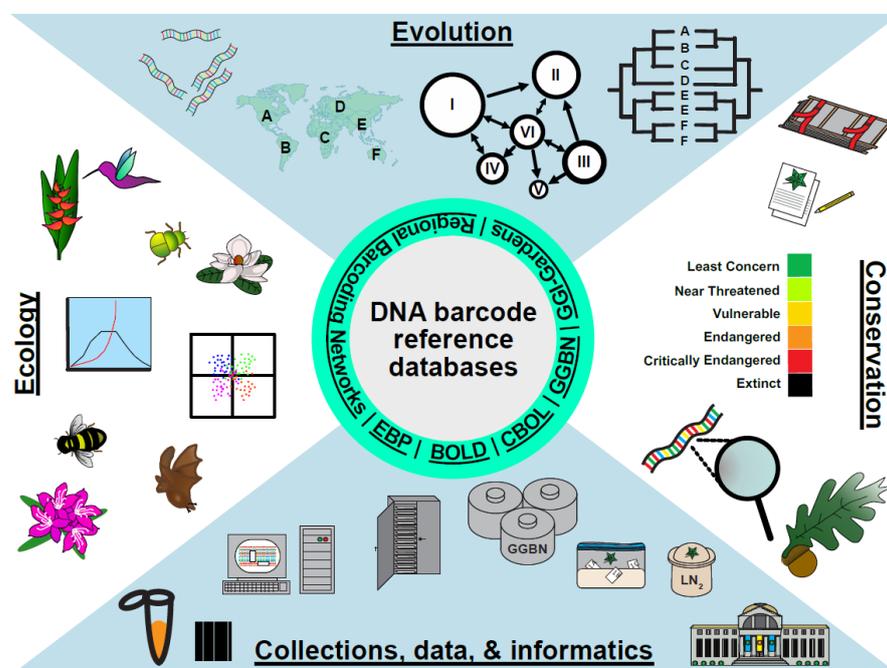


Figure 2. A graphical representation of DNA barcoding today. DNA barcode applications in ecology (left), evolution (top), and conservation (right) are supported by a foundation of collections, metadata, and informatics (bottom). These applications are facilitated by increasingly large DNA barcode reference databases (center circle) that are reciprocally built from and contribute to the major biological disciplines. National and international initiatives that support the growth of DNA barcode reference databases are core resources (green circle).

2. Current Applications

2.1. Improving Taxonomy and Species Identification

2.1.1. Defining Species Boundaries

Taxonomists have been using morphological features for the identification of both plants and animals since before the time of Carl von Linné. Yet, even after centuries of taxonomic work, perhaps only 20 percent of the species on Earth have been formally named [69]. Much work remains to be done. DNA barcoding provides a relatively new and significant tool to aid in the determination of species boundaries and discovery of new taxa. Entomologists have been pioneers in incorporating DNA barcode technologies for species discovery in the tropics, where the majority of biodiversity is found (e.g., [70–73]). Although the discriminatory power of barcode markers for plants is less than for insects, botanists have also used DNA barcodes as a taxonomic resource. Early studies, which have mostly focused on trees in tropical forest monitoring plots (e.g., [62,74,75]), demonstrated the difficulties of using DNA barcodes in plants (also see [76] for a recent study on African trees). However, the same studies also pointed out the advantages of being able to accurately identify sterile and juvenile specimens that lack morphological features required for identification. Costion et al. [77] applied a three-locus DNA barcode to estimate tree species diversity in a taxonomically poorly known tropical rain forest plot in Queensland, Australia, and concluded that DNA barcodes were a significant aid in rapid biodiversity assessment and determination of cryptic tree populations. A similar study in a central African rain forest plot recognized the high discriminatory power of barcode markers at the genus-level (95–100%), but somewhat lower species-level success (71–88%) in identification, especially in species-rich clades [78] or those with high rates of molecular evolution.

One of the major issues faced by plant taxonomists and ecologists attempting to use DNA barcodes in diverse forests, especially in the tropics, is that many species are new to science, therefore lack Latin binomials, and/or are members of poorly circumscribed species complexes that are difficult to identify even with traditional morphological data. Inventories and assessments of plant diversity in these habitats can be greatly enhanced by building DNA barcode libraries of these taxa [79]. Standardizing the DNA barcode markers and bioinformatics tools being used in different forest inventory projects (e.g., RAINFOR, <http://www.rainfor.org/>, accessed on 26 January 2022; the Amazon Tree Diversity Network [80], CForBio, <http://www.cfbiodiv.org/>, accessed on 26 January 2022; and ForestGEO [43]) will provide more confidence in identifications and maybe even allow rapid discovery and description of unknown taxa in these species-rich forests [79].

In addition to discovering new species, the introduction of integrative taxonomy has encouraged closer collaboration among biologists with different backgrounds, and in turn has promoted the use of DNA barcoding as a new tool in a broad taxonomic toolkit [81]. For very poorly documented regions or “understudied and hyperdiverse” taxa, DNA barcoding can be a key part of integrative workflows for species description and identification [82].

2.1.2. Regional Biodiversity Assessments

DNA barcode studies both benefit from and serve a key role in support of local and regional biodiversity assessments, including floristics. In many biodiverse regions, where species diversity is poorly known, collections-based exploration and inventory studies are vital for alpha taxonomy and conservation. Modern approaches to field expeditions employ a variety of strategies to collect and document species, which often include the collection of various data to inform biodiversity studies. These data incorporate traditional natural history specimens, photographs, ecological notes, and, more recently, vouchers intended for genetic and/or genomics research [83]. The collection of genetic vouchers and sequencing of DNA barcodes in standard species inventories help to build the global barcode reference database [84] as mentioned above, and often result in surprising discoveries of cryptic diversity (e.g., [85–87]).

2.2. Quantify Species Diversity

2.2.1. Species Richness and Phylogenetic Diversity

Fundamental to biodiversity research is the quantification of organismal diversity. Different approaches to this task may provide different interpretations by ecologists, evolutionary biologists, and conservation biologists regarding the role that biodiversity plays in ecosystem function, niche allocation, and species preservation. Phylogenetic diversity was proposed as a metric that quantifies diversity by summing the branch lengths of a given phylogenetic tree [88,89] and is arguably a more descriptive measure of biodiversity than alternative indices such as simple species richness and abundance [90]. DNA barcoding provides an efficient and rapid resource for generating phylogenies to measure phylogenetic diversity, particularly when combined with metabarcoding [91].

It should be noted however that despite the utility of DNA barcoding approaches in diversity assessment, limitations exist. Winter et al. [92] described some of the limitations of phylogenetic diversity insofar as the metric is applied to conservation applications. And although phylogenetic diversity has been lauded as an indicator of species interactions and ecosystem functions [93–95], caution has been urged against using this measure alone to conserve functional trait diversity in ecosystems. The growth of DNA barcode databases and new sequencing methods are facilitating the ability to analyze and understand phylogenetic diversity, but if these data are to be used as predictors for conservation and estimates of ecosystem function, they need to be carefully evaluated in combination with detailed trait databases. Among the earliest uses of DNA barcoding to quantify biodiversity were investigations of community assembly and function in long-term forest monitoring plots in Panama.

2.2.2. BCI as an Exemplar Tropical Field Site for DNA Barcoding

More than a decade ago the first community phylogeny based on DNA barcode sequence data was published for the trees in a forest dynamics plot on Barro Colorado Island (BCI) in Panama [62]. This publication set off a storm of new investigations that were able to add a well-supported evolutionary perspective to understanding species diversity and assembly in plant communities (e.g., [96–100]). The DNA barcode phylogeny generated for the approximately 300 species of trees on BCI also served as a template for a number of investigations of functional traits. The evolutionary context of such characteristics as soil associations [101], leaf toughness [102], wood nitrogen concentration and life-history strategies [103], foliar spectral traits [104], and anti-herbivore defense traits [105] was found to vary in each of these functional traits across the tree species in the BCI plot. Although some have concluded that phylogenetic indicators are not always tied to ecological determinants of community assembly [106], both phylogenetic- and trait-based approaches have greatly enhanced the understanding of community structure and function on BCI.

Belowground interactions among species have also been investigated at BCI using the DNA barcode library for trees. Jones et al. [107] mapped the belowground distribution of all trees and lianas greater than one centimeter in diameter using their genetic DNA barcode signature. Comparing underground species distributions with aboveground distributions showed that species interactions and spatial overlap was greater belowground than expected based on aboveground stem densities. Although this study raised several questions about methodology and analysis, it concluded that the potential for using DNA barcodes in this type of investigation was high.

The DNA barcode library for trees on BCI has now been expanded to include many of the shrubs and lianas as well as some epiphytes that occur in the forest on the island. Efforts to build DNA barcode libraries and apply DNA barcode methodologies to other groups of organisms (e.g., insects [108]) are underway. This rich genetic resource will greatly enhance future studies of ecological interactions and evolutionary signal in this tropical forest community in Panama.

2.3. Determining Community Structure and Species Interactions

2.3.1. Community Evolution and Assembly

DNA barcoding has played a significant role in expanding collaboration between systematists, who focus on species identification and evolutionary relationships, and ecologists, who investigate species interactions and patterns of associations [109]. As noted above for work conducted on Barro Colorado Island in Panama, plant DNA barcoding has been a boon to community ecologists seeking to understand the factors, such as species diversity pools and functional traits, that control the assembly of species into ecological communities [100,106]. Estimating a third component that may determine species assembly, namely evolutionary history, has always been hampered by the lack of well-resolved phylogenetic hypotheses on species relationships in communities. Determining if species in a community are more closely related than by chance (phylogenetic clustering), more distantly related than by chance (phylogenetic overdispersion), or randomly distributed across the plant tree of life is now readily ascertained by building a DNA barcode-based phylogenetic tree. The assumption follows that species in a community that are phylogenetically clustered are more likely to have similar ecological niches (i.e., phylogenetic niche conservatism) and have been assembled via abiotic filtering. The contrasting assumption is that phylogenetic overdispersion in a community is the result of biotic interactions among sympatric species. Based on these assumptions the impact of evolutionary history on community structure has been investigated using DNA barcodes across stages of forest succession [99], among habitats within a forest type [62,110,111], among forests across habitat gradients [112], and among communities across an entire country [113,114] and across the globe [45,115]. The generation of such community phylogenies has great promise for further testing the basic assumptions and rules governing species assemblies in plant communities (see [45]).

2.3.2. Herbivory and Food Webs

The accurate and repeatable identifications of species is imperative if we are to fully understand the ecology and evolution of interactions among partners in natural and human-altered environments. This requirement is especially true for specialized interactions, including mutualisms and antagonisms. The application of DNA barcodes as species-level markers has revolutionized our ability to track species interactions and the community networks they form, in boreal, temperate, and tropical habitats.

Food web interactions have been greatly clarified with the application of DNA barcodes. Smith et al. [116] using the CO1 DNA barcode marker were able to verify the food web structure of the spruce budworm and its numerous parasitoids to understand the population dynamics of this major pest of trees in boreal forests. The utility of DNA barcodes to identify the diversity of host plants for herbivorous beetles have been demonstrated in both neotropical [62,117] and Asian tropical forests [118]. These early studies used a limited number of molecular markers and were only able to identify the hosts at the generic or familial level.

The most comprehensive analyses between herbivorous beetles and their host plants have been conducted by García-Robledo and colleagues [72,73,119]. The host-specific relationships between rolled-leaf beetles in the genera *Cephaloleia* and *Chelobasis* (Chrysomelidae) and plants in the order Zingiberales have been well-studied by ecologists [120], but the application of DNA barcodes to both the beetles and the hosts have provided a much more detailed and quantitative measure of these interactions [74]. One of the advantages of using an easily extracted DNA barcode is that the beetles can be identified at any of their life stages and not only as adults as in most previous investigations using morphological features [119]. Once the basic network of food web interactions is established using DNA barcodes, comparisons can be made across habitats, elevations, and temperature gradients. Most recently Palmer et al. [121] have extended this methodology to the interactions among katydids and their host plants in a wet forest habitat in Panama. They found that, in general, these insects consumed a broad range of flowering plants and were rarely specialists on only a few species. It has been shown in numerous cases (e.g., Hebert et al. [70]) that

DNA barcodes can detect the presence of cryptic species, especially in insects. This power of DNA barcoding has greatly improved the understanding of species boundaries in the rolled-leaf beetles, allowing for more precise mapping of the insect–host networks. The detection of these cryptic species clearly demonstrated that the elevational distributions and thermal tolerances of the beetles was much narrower than previously thought, which will have an impact on the food web networks as climate change differentially impacts both host and herbivore migrations [73].

DNA barcodes have also altered our view of why tropical biomes are so diverse. It has long been held that specialized ecological interactions, which are common in tropical forests, will lead to reproductive isolation and speciation, and hence greater biotic diversity in the tropics. One such specialization is that between tropical flowers and the nectar-robbing floral mites that are carried from plant to plant on the bills of hummingbird pollinators [122]. This specialization allows floral mites to easily find mates and reproduce, because many conspecifics accumulate in the flowers of only a few species of plants. This “mating rendezvous hypothesis” [123] accounted for the host specialization in these mites. However, using DNA barcode markers to identify the mites, rather than morphological identification, has now shown that most floral mites are generalists and not specialists [124]. The mating rendezvous hypothesis is no longer supported, at least for mite diversification.

This detailed understanding of herbivore–host interactions using DNA barcodes has also been applied to large mammalian herbivores. In a semiarid African savannah, Kartzinel et al. [125] determined the extent that sympatric mammalian browsers and grazers could partition their diets. After building a library of plant DNA barcodes for the local flora, they quantified the diet breadth, composition, and overlap for seven co-occurring mammal species, ranging in size from dik-diks to elephants using DNA metabarcoding. Earlier conclusions on competition and coexistence in these habitats based on low-resolution analyses were shown to be misleading, according to the more high-resolution taxonomic data provided by the metabarcoding results. This work in Africa has now been extended to demonstrate that the abundance and diversity of food plants is negatively associated with their mammalian herbivores, apparently to avoid consumption [126]. The same type of DNA barcoding protocol has also been adapted to tracking and identifying the vectors of bird-dispersed fruits and seeds in the field [127] in order to build a quantifiable network of frugivores and seed dispersal interactions.

2.3.3. Symbiotic Relationships and Plant-Pollinator Interactions

Symbioses, perhaps the most characteristic of “species interactions,” entail very close relationships between two or more species living together, and DNA barcodes have facilitated researchers studying such close interactions [128]. In some groups (e.g., fungi, [129]), DNA barcoding has revolutionized the field, especially where symbiotic partners are very closely associated and interactions often exist at a cellular level (e.g., in lichens [130]). The use of DNA barcodes to understand symbioses is common in all major clades, including arthropods [131], vertebrates [132], green plants [133], and fungi [134]. An especially powerful tool for symbiosis-based research is metabarcoding [135], which allows for pooled sequencing from closely associated, symbiotic organisms that are otherwise difficult to isolate. The application of DNA barcodes to more closely track and untangle symbiotic relationships is still in its infancy (see below Section 3.1).

The interactions between plants and pollinators is a symbiotic mutualism that is critical for the survival of both partners. An understanding of the dynamics of these interactions is a priority for plant and insect ecologists to conserve biodiversity and to protect the agricultural crop supply chain. DNA barcodes have been explored for more than two decades as a means to identify plants from the insects that have visited them as pollinators [136]. Given the nature of pollination dynamics, samples removed either from plants or their pollinators can include a mixed community of pollen and, therefore, metabarcoding approaches provide a unique tool to identify the diversity contained in these mixed samples [137,138].

Clare et al. [139] were among the first to apply metabarcoding to study plant–pollinator interactions, extending the concepts earlier proposed by Valentini et al. [68] and Soininen et al. [140]. A key threshold for advancing these methods is a comprehensive DNA barcode sequence reference database. For example, the first national DNA barcode sequence reference database of Wales [141] has provided a benchmark for DNA metabarcoding studies of plant–pollinator interactions and this has recently grown into a comprehensive database for all of the United Kingdom [59]. Together, these databases have proven powerful for reconstructing bee foraging behavior [142–144]. These and other studies [145–147] have built a strong foundation for using DNA metabarcoding to study plant–pollinator interactions.

2.4. Protecting Species

2.4.1. Forensics and Monitoring Traffic in Endangered Plants

It is abundantly clear to all biologists that biodiversity is under severe threat across the globe due to natural resource overutilization and exploitation, major habitat degradation, and climate change caused by humans. Biodiversity conservation is imperative. DNA barcoding, as a tool primarily for species identification, can be used in three general ways to further biodiversity conservation: (1) to accurately monitor and thereby protect endangered species subject to illegal commercial trade (i.e., point-of-origin tracing [148,149]), (2) to track biological invasions, and (3) to provide data that will assist in the estimation of phylogenetic diversity for setting conservation priorities [150].

Although DNA barcode-based discrimination at the species-level is not possible in all groups of organisms, DNA barcodes have been utilized for forensic identification of algae [151], plants [152,153], invertebrates [154], and vertebrates [155]. A significant driving force in developing DNA barcode technology for plants has been the need for an accurate and inexpensive tool for the identification of illegal timber products, especially those listed in the Convention on International Trade of Endangered Species (CITES). For example, in tests of the commercially important mahogany family (Meliaceae), most of the standard DNA barcode markers fell short of expectations for discriminating species, although the nuclear ribosomal internal transcribed spacer (ITS) was able to identify some species in this family [156]. A higher level of discrimination using standard markers was demonstrated among commercially important and threatened species of trees collected at timber processing plants in the tropical dry forests of India [157]. This same success was demonstrated in timber species found in *Araucaria* rain forests of the southern Atlantic coast of Brazil [158], which contains many threatened species of trees, especially in the family Lauraceae. In Madagascar, a recognized biodiversity hotspot, Hassold et al. [159] used DNA barcodes in an effort to monitor illegal timber trade, especially in species of rosewood (*Dalbergia* in the Fabaceae). They demonstrated the limitations of the standard genetic markers in identifying closely related species of this genus, although some success was achieved. In addition to timber trees, DNA barcode libraries have been developed for other taxonomic groups of threatened and endangered plant taxa listed in CITES, e.g., orchids [160]. Currently no more effective tool than DNA barcoding exists for accurate identification of products sold in public markets [161–163] or as illegally harvested species intended for trade intercepted at ports [164,165]. As global DNA barcode reference libraries grow, so too does the capacity to enforce conservation laws and to monitor illegal trade in endangered plants.

Traditional medicines, teas, and herbal supplements are another important component of the commercial need for accurate plant species identifications by regulators and quality control specialists. It is estimated that medicinal plants account for billions of US dollars in annual revenues in the United States alone [166]. From the initial use of plant DNA barcodes, applications to monitor this market have been in development [167]. However, many of these trials to use DNA barcodes to identify commercial medicines and herbal supplements have shown limited success in discriminating among species. Some of the major obstacles have been the lack of comprehensive DNA barcode libraries required to make accurate comparisons among species of herbal teas and supplements, and the

absence of standardized taxonomy and common names listed in the herbal catalogs and pharmacopeias (e.g., Stoeckle et al. [168]; de Boer et al. [169]). Building the required DNA barcode libraries (see below) and unifying the taxonomy in the literature on traditional medicines are challenges for the future.

2.4.2. Tracking Biological Invasions

The field of conservation biology has also benefited from the accuracy of DNA barcoding methods to trace biological invasions. It has been estimated that the control of invasive species costs more than \$27 billion annually in the United States alone [170]. Fast detection can significantly reduce the cost of controlling biological invasions, and DNA barcodes and metabarcoding in particular have been demonstrated to provide the earliest invasive species detection methods available [171]. For example, one of the most widespread threats to marine ecosystems is the invasive zebra mussel, *Dreissena polymorpha*, and recent studies [172], using metabarcoding (or environmental DNA), have proven this method to be cost effective for early detection of this species in marine environments. Studies that quantify regional biodiversity using DNA barcodes have also proven effective for identifying biological invasions [173], where higher than expected phylogenetic diversity may result from the occurrence of non-native or invasive species.

In some groups of plants, invasive and weedy species are remarkably difficult to visually distinguish from non-invasive, endemic species, and several studies suggest DNA barcoding will facilitate proper identification and management by non-specialists. For example, current DNA barcodes in many plant taxa are unable to distinguish taxa at or below the genus-level, but new paradigms in barcode sequencing provide greater distinction of closely related species. Wang et al. [174] have advocated the use of super- or ultra-barcodes (e.g., whole chloroplast genomes) to monitor and detect flaxleaf fleabane, *Conyza bonariensis*, because, unlike traditional plant DNA barcodes, these super-barcodes are able to distinguish among closely related species in this diverse and difficult to identify genus.

2.4.3. Conservation Assessment

The taxonomic impediment [175] is also a significant problem in assessing species diversity and making accurate species determinations for conservation monitoring. This case is especially true in tropical biomes, where biodiversity is poorly known and a greater number of species lack verified scientific names. Species identification by non-taxonomists can be extremely difficult, especially when using non-fertile specimens often only labeled as “morphospecies” [176]. In such cases, DNA barcoding offers a solution for more uniform and accurate identifications, recognizing that some logistical hurdles may still impede the widespread use of DNA barcodes in this fashion [177].

In the relatively poorly known tropical forests of northern Queensland, Australia, it has been demonstrated that plant DNA barcodes can play a key role in estimating species richness and thereby determining conservation priorities [77]. Similarly, in the fragmented rain forest habitats in South Eastern Queensland, Shapcott et al. [61,169] generated plant DNA barcodes for 86% of the flora (770 species in 111 families) and calculated phylogenetic diversity (PD; see Owen [178]) measures for each of the 18 subregions in the area. For these forests, which have lately received renewed conservation attention and are taxonomically rich at the generic-level and less so at the species-level, species richness may not be the most appropriate measure for setting conservation priorities. The phylogenetic diversity estimates calculated from the DNA barcode data were used to prioritize subregions for conservation action and it was concluded that the local floristic patterns were consistent with both ancient ecological refugia and recent lineage range expansions [179].

Even though the Earth may be undergoing its sixth major extinction with extinction rates over one thousand times that of “normal” periods [69], observing an extinction event is rare. For plants the extinction of only 571 species over the last several hundred years has been carefully documented [180]. On the island of Palau in Micronesia, plant DNA barcodes were used to verify that a narrow range endemic tree described in the 1980s

known from only two mature individuals was *Timonius salsedoi* Fosberg and Sachet in the family Rubiaceae [181]. Subsequently, after a typhoon hit the area, a survey of the island revealed that both trees had perished. Although previously assessed as Critically Endangered by IUCN criteria, it is suspected that this species is now extinct [181].

DNA barcodes have significant potential as a tool for understanding and enhancing conservation efforts. Using standardized and comparable genetic information for species across broad geographic regions can have a substantial impact on basic biodiversity research (e.g., Mi et al. [112]; Erickson et al. [45]; Pei et al. [98]), as well as conservation monitoring and priority assessments in threatened habitats, in local communities, and across large geographic regions (e.g., Shapcott et al. [61]).

3. Looking Forward: The Expanding Technological Spectrum of DNA Barcodes

3.1. Metabarcoding

DNA metabarcoding [135] has emerged as a powerful technique to rapidly characterize species composition, species interactions, and—when combined with trait databases—functional aspects of biological diversity in communities. This method leverages high-throughput sequencing technology to sequence and/or extract DNA barcodes from pooled community or environmental samples. These samples represent DNA isolated from multiple species or other taxa that have been collected in bulk and targeted sequencing is performed on libraries enriched with (typically) DNA barcode amplicons [182]. Metabarcoding is an emergent field that leverages expansive DNA barcode sequence databases and the increasingly high-throughput capacity of DNA sequencing technology.

This technique allows ecologists to explore species interactions through a new lens and is illuminating species distribution and occurrence from ecosystems and habitats that have remained all but invisible. Metabarcoding is able to provide high-resolution inventories from the hidden worlds of below-ground microbial diversity [183], freshwater [184] and marine [185] benthic communities, and the movement and dispersal of airborne fungi [186] and plants [187]; however, this method is dependent upon well-curated reference collections and databases [188].

Beyond enhanced characterization of species communities, metabarcoding has been used to explore species interactions in a variety of contexts. Some of the earliest applications of DNA metabarcoding involved the analysis of vertebrate diets [189] and this method remains a powerful tool for understanding herbivory and predation (see [125,190–192]). More recently, metabarcoding has been used to reconstruct plant–pollinator networks [146,147] and identify economically important taxa [155] or those relevant to human health [193,194].

DNA metabarcoding was developed using short-read high-throughput sequencing platforms and while these are still the norm, they pose some limitations for the technique [8], especially for longer DNA barcode loci (e.g., *matK* for plants). As the technological standard moves toward long-read sequencing approaches, new sequencing platforms and software [195] are being developed. Some recent programs (e.g., Sahlin et al. [23]) have already been used to successfully extract DNA barcode sequences from mixed samples in previously published long-read data.

3.2. Super- and Ultrabarcoding

Much of the expanding role of barcodes in the past decade has been driven by the rapid growth of high-throughput sequencing technology. As opposed to traditional DNA barcodes, which target individual loci or a set of short loci with universal primers, “super-barcodes” and “ultra-barcodes” have been proposed as alternatives that compare information from entire organellar genomes and/or other long regions [12,13]. For plants, whole chloroplast sequencing has been common for over a decade [196]. Super- and ultra-barcoding provide some unique advantages over traditional barcoding. For example, in some large clades (e.g., the green plant tree of life), traditional DNA barcode loci are not present in all taxa [197] and universal PCR primers often don’t exist for some taxa in a given clade (see [8]). In these cases, ultra-barcoding provides a simple solution to chal-

lenges with traditional DNA barcodes, in which the entire chloroplast genome can serve as one single, long barcode locus or in combination with other loci (e.g., nuclear ribosomal DNA, [14]). Moreover, some traditional DNA barcode loci (e.g., *matK*, ca. 1000 bp) are simply too long for amplicon-based approaches using short-read sequencing platforms. Lastly, chloroplast genomes are abundant and typically easy to sequence even from recalcitrant (i.e., old and/or preserved) tissues and it's increasingly common to assemble whole organellar genomes from off-target reads even in targeted/capture-based sequencing applications [198].

As sequence databases grow, the concept of super- or ultra-barcodes is certain to follow. Rather than viewing alternative barcoding strategies as either/or choices, novel DNA barcoding strategies are complementary to locus-based markers, and each contributes to a growing, cumulative database of well-curated data for molecular species identification.

3.3. Macrogenetics

Computational science, international collaboration, and data accessibility are facilitating massive, integrative research across the biological sciences. Driven by the era of “big data” and increasingly interoperable datasets, new and emerging fields of research are making it possible to pursue “big questions” like never before. These expanding opportunities have led to the emergence of new fields of study and one of these, “macrogenetics” [199], has been facilitated by the growth of publicly available genetic and genomic datasets. The concept for this field is intended to echo that of “macroecology” and emphasizes the integration of large-scale datasets in genetics with other large, interoperable databases [200], such as the Global Biodiversity Information Facility (GBIF, [201]), WorldClim [202], DRYAD [203], the International Nucleotide Sequence Database Collaboration (INSDC [204]), and BOLD. DNA barcodes provide a vital source of information that can facilitate the emerging field of macrogenetics and indeed, the development of BOLD is credited as one of the key advances that underlies macrogenetics.

As a new and emerging field, macrogenetics is presented as the intersection of several biological foundations, united by large-scale genetic resources and including rich ecological data, collections science and museomics, biogeography, phylogeography, and evolutionary biology [200]. The promise of this new field is to synthesize big data across biological disciplines using genetic data to facilitate priorities for ecology, evolution, and conservation at global scale. Undoubtedly, the expanding role of DNA barcodes will play a central role in the development of macrogenetics. It is an exciting time to study ecology, evolution, and conservation.

4. Conclusions

In nearly two decades since DNA barcodes were first proposed, a remarkable increase has taken place in the representation, use, and integration of DNA barcodes across the biological sciences. Although sequence variation in traditional DNA barcodes is often insufficient for species-level discrimination in many large clades, the advances in computational and sequencing technology are changing the concept of DNA barcodes, from just a few loci to large, genome-scale sequences from organelles or genome-skim data. As technology expands and genome sequence representation increases across the Tree of Life, we envision a future in which the concept of DNA barcodes extends to a much larger interpretation of genome space. DNA barcoding continues to evolve with methodological and technological advances in conjunction with the increasing accessibility to high-throughput sequencing and the growing database of whole genome sequences fostered through international consortia, such as the Earth BioGenome Project [37,38]. A diversity of genetic tools is especially needed in clades, such as green plants, with highly complex genomes that require significant resources to assemble [205]. Until there is a corresponding breakthrough in computational capacity for the comparative analysis of large and highly complex genomes, DNA barcode sequences will play a vital role for species identification in community ecology, evolutionary biology, and conservation. DNA barcodes are a powerful resource

and the databases that maintain them continue to grow as they complement and benefit from the rapidly expanding frontiers of computational science and high-throughput sequencing technology.

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