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Understanding the drifts in DNA barcoding: a systematic review

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Abstract

Although DNA-based analytical methods had been around for a while, it wasn't until 2003 that the term "DNA barcoding" became popular. The research-analytical and application paradigms have continued to develop and diversify since then. Initially, it was only applicable to the animal kingdom and later the method was modified by scientists studying plant biology tailoring it to fit the needs. This document provides a meta-analysis of DNA barcoding research trends, specifically in plant sciences, examining its methodological advancements, application diversity and evolving research themes. By classifying and analyzing the current data trends, we offer insights into the ongoing transformations of DNA barcoding. Furthermore, actionable recommendations for future research are proposed, including the development of more reliable, cost-effective markers and exploring ecological and biodiversity applications. This analysis serves as a guide for both novice and experienced researchers to navigate the rapidly advancing field of DNA barcoding.

Keywords

DNA barcoding; meta-analysis; species identification; trends study

Introduction

DNA barcoding is a specifically designed technique that enables the identification of individuals or their parts, thereof at the species level using short, standardized sequences as identification tags (1). During the early years, the technique was foreseen as a quick solution to taxonomic identification. Over the years, the uses and applications of this technique expanded with technological advances and progress in various fields of research. Initially, the technique was mostly used for animals and Cytochrome c oxidase I (COI) was the only widely used marker. However, during the late 2000s, with the conquest for a comparable strategy in plants and other kingdoms various other barcodes were explored (2). This led to a plethora of different markers. They have been broadly categorized as core and supplementary markers, each suited to the specific needs of different kingdoms of organisms. As new requirements emerged, the technique found various applications, such as ensuring biosafety and developing strategies for the conservation of endemic and endangered species. The core markers are universal markers that work for a broad range of organisms whereas the supplementary markers are specific to a certain taxonomic group which are used when the core barcode marker is not sufficient to distinguish between closely related species. Consequently, metabarcoding came into the picture which has allowed for the bulk identification of species from environmental samples such as soil, water and air. This has made it possible to study ecological interactions, the

physiological effects of these interactions, metagenomic research, etc. (3). In the last 2 decades, DNA barcoding has undergone significant changes and has evolved into a crucial analytical technique for various molecular studies requiring identification such as forensics, physiology, ecology and environmental research. Saying that its potential applications are endless is no exaggeration. Therefore, we have analysed the trends since the technique's inception and explored its various aspects from a plant science perspective.

Our goal is to provide an overview of DNA barcoding and its technical applications in research. We have carefully examined the sub-themes and their complexities, discussed their limitations and highlighted their potential prospects.

Theoretical Foundations and Practical Applications of DNA Barcoding

To be able to use a technique, understanding the working principle and its potential applications of any technique is crucial from academic and professional standpoints. DNA barcoding is closely linked to alpha taxonomy due to its role in species identification. It is extensively used in molecular taxonomy to determine phylogenetic relationships and support clades across various taxonomic ranks (4). The term "phylogeny", which was previously used to refer to any tree-like depiction of evolutionary history, has also been broadened as a field due to DNA barcoding. With the increasing popularity of the technique, these short sequences have been used to find the phylogenetic relationships among different organisms, however, methodological disagreements persisted, leading to trees being evaluated more for the insights provided by DNA barcoding than for the methods used to produce them (5). Thus, it is equally important to use the technique of DNA barcoding with adept information on the various models of evolution and methods of generating the trees to reach a conclusion. The assumption that species should be adequately recorded and appear monophyletic is at least one broad result of this usage that directly affects DNA barcoding (6). When trees are seen as the arbiters of species boundaries, the mismatch between the graphical representation of a monophyletic group and the traits behind it is emphasized (7). Compared to conventional taxonomic techniques, DNA barcoding has several benefits, including speed, precision and objectivity (8). Additionally, it can be used to find cryptic species and distinguish specimens with flawed or incomplete morphology. DNA contamination, the requirement for high -quality DNA samples and the reliance on reference databases are some of the drawbacks of DNA barcoding. Dilemmas about DNA barcoding have been expressed regarding the lack of unanimity concerning the choice of a DNA marker, the likelihood of misidentification and misinterpretation and the moral concerns associated with using DNA from endangered species (7).

The barcoding techniques also have their shortfalls, depending on the nature of the technique selected. For example, Single-locus approaches often struggle with distinguishing closely related species due to low interspecific divergence and potential overlap in genetic variation. Challenges arise particularly with taxa where morphological differences are subtle, as seen in studies of North American birds, where misidentifications occurred due to intraspecific variability in mitochondrial DNA sequences (9). While multi-locus barcoding increases resolution, conflicts between loci and increased computational demands can complicate taxonomic assignments. This is especially true for polyploid species or hybrids, where individual loci may suggest different evolutionary histories. Genome-based approaches, despite their accuracy, face barriers such as high costs and computational demands, which limit their feasibility for large-scale biodiversity studies, particularly in resource-limited settings.

The rapid adoption of DNA barcoding has revolutionized species identification and biodiversity assessment. However, it also raises significant ethical and ecological questions, such as the implications of revealing genetic data of endangered species, which might inadvertently aid biopiracy or illegal wildlife trade. Similarly, privacy concerns related to biosafety and the unauthorized use of genetic information remain largely unaddressed. These considerations necessitate a balanced approach to harnessing the potential of DNA barcoding while mitigating associated risks.

Despite challenges like disagreements over DNA marker selection and ethical concerns about using DNA from endangered species, DNA barcoding continues to evolve. Future directions include developing new DNA markers, integrating morphological and molecular data and expanding reference databases to cover more species and geographical areas.

Materials and Methods

Data collection

We collected all the published research articles available on PubMed and Google Scholar from 2003, until December 2022, that had “DNA barcoding”, in their title, keyword, or abstract. We downloaded all the papers year-wise, along with their abstracts.

Data mining and sorting

Using our expertise in the field, we categorized the articles based on various strategies and themes. This primary and secondary classification process involved thoroughly reading the abstracts and methodologies to grasp the research themes, the number and types of loci used, and other pertinent details. The data was then systematically sorted and organized in MS Excel.

The categories were chosen primarily to ensure a comprehensive analysis and accurate representation of the methodologies and applications associated with DNA barcoding; a systematic categorization approach was adopted for the purpose. The rationale for the selected categories and terminologies is detailed below:

Approach and methodology adopted for DNA barcoding: Categories such as Single Locus (SL), Multiple

Locus (ML) and Tiered Approach (TT) were included to account for the diversity in molecular markers and their usage in DNA barcoding. These distinctions allow for assessing the effectiveness and adaptability of different marker strategies in species identification. Novel technologies like Bar-HRM (BH) and the Genome-based Approach (GW) were considered to capture advances in sequencing-independent and genome-wide methodologies.

Research paradigm: This category delineates the scope of DNA barcoding studies into specific research domains such as biodiversity (BE), plant taxonomy (PT), evolutionary studies (EP), routine applications (RA) and bioinformatics tools (BIF). This classification enables a nuanced understanding of how DNA barcoding is utilized across various scientific objectives and practical applications. Reviews (RVW) were separately analysed to synthesize overarching trends and insights.

Statistical Analysis

We collected data on the number of loci used and the technique employed each year from NCBI PubMed. For each year, we calculated the mean number of loci used and the standard deviation. We sorted the number of research articles by theme and calculated the Pearson correlation coefficient between each technique and theme, ensuring statistical significance with $p < 0.05$. To further enhance the reliability of the trends observed, additional statistical analyses were conducted, including the calculation of confidence intervals (95%) and effect sizes for key comparisons.

This structured approach allowed us to identify patterns and trends. The data categorized into different strategies and themes was further divided into subthemes (see Table 1). This secondary data was used to analyze trends, highlight advancements and interpret paradigm shifts.

In the last 2 decades, a sample size of 2100 peer reviewed research articles on DNA barcoding were mined. These papers showed a comparable and scalable changes in the trends of research indicating a paradigm shift in the purpose, efficacy and potential advantages of this technique. This analysis provides insights into the evolving landscape of DNA barcoding and its future potential. After mining and classifying the data as secondary data, we analysed the fields of research and the purposes of the studies to understand the diverse applications and evolution of the technique. Initially, DNA barcoding was primarily used for phylogenetic analysis. Over time, its application has significantly expanded to include environmental studies and biodiversity research, reflecting its growing versatility and importance.

Our analysis primarily categorized the data based on DNA barcoding techniques, revealing trends that indicate a shift from single locus identification to the use of multiple loci. This transition highlights the evolving complexity and precision in the application of DNA barcoding.

Table 1. Parameters used for secondary data categorizing and analysis. Here primary categorization represents the methodology used and the secondary data represents the further categorization of the data based on the research paradigm, regardless of the technique used

Category	Terminology	Determinant
Approach and methodology adopted for DNA barcoding	Single locus “SL”	A single locus represents a unique locus that is selected and amplified to identify the species and develop a barcode.
	Multiple Locus “ML”	Multiple locus represents the usage of a combination of 2 or more unique loci amplified to identify the species and develop a barcode
	Tiered Approach “TT”	The tiered Approach represents a special type of multi-locus approach where a combination of the core marker is used first followed by supplementary markers (As suggested by CBOL)
	Bar-HRM technology “BH”	Bar-HRM technology is DNA barcoding based on real-time PCR and high-resolution melting analysis. It is a sequencing-independent method and uses fluorescent dye for the detection of double-stranded DNA.
	Genome-based approach “GW”	Genome-wide approach refers to the usage of macro-barcodes as the entire organelles for subsequent barcoding
Research paradigm	Biodiversity and Ecological Studies “BE”	Studies were done for measuring parameters of biodiversity such as species richness, abundance, diversity, or any other ecological interactions, surveys, etc using DNA Barcoding
	Plant Taxonomy “PT”	Taxonomic studies were done for species identification and/ or delineation or molecular Taxonomy by using DNA barcoding
	Evolutionary Studies and Systematics “EP”	Phylogenetic analysis, species interrelationship, lineage, studies regarding monophyly and species delineation using DNA barcodes a s their primary set of data were included. They were further categorized depending on the model used.
	Routine Applications “RA”	Routine applications administering DNA barcoding as a tool or technique for adulteration studies, forensic studies, biosafety, and palaeobotanical studies were considered valid data points.
	Review “RVW”	Reviews that were related to DNA Barcoding and mentioned in either their titles, abstract, or keywords were considered valid data points
	Bioinformatics Tools and Techniques “BIF”	Bioinformatic tools implied or derived to use data of process data regarding DNA Barcoding, excluding those used for Evolutionary studies (to prevent redundancy) were implied as valid data and were considered.

After mining and classifying the data as secondary data, we analysed the fields of research and the purposes of the studies to understand the diverse applications and evolution of the technique. Initially, DNA barcoding was primarily used for phylogenetic analysis. Over time, its application has significantly expanded to include environmental studies and biodiversity research, reflecting its growing versatility and importance.

The shift from single locus to multiple loci methodologies represents a substantial advancement in the field. This transition allows for more accurate and comprehensive species identification, which is crucial for ecological and evolutionary studies. Moreover, the integration of DNA barcoding into environmental and biodiversity research underscores its utility in addressing contemporary scientific challenges, such as ecosystem monitoring, conservation efforts and understanding species interactions in various habitats.

The statistical analysis of publication trends revealed significant insights into the focus areas of DNA barcoding research. Biodiversity and ecological studies (BE) and plant taxonomy (PT) emerged as dominant research paradigms, with their mean publication counts supported by confidence intervals of 22.62–49.28 and 20.54–48.26, respectively. Evolutionary studies (EP), routine applications (RA), reviews (RVW) and bioinformatics tools (BIF) showed relatively lower yet consistent contributions (Table 2). Strong positive correlations were observed, notably between BE and PT ($r = 0.94$, $p < 0.001$), BE and RVW ($r = 0.88$, $p < 0.001$) and PT and RVW ($r = 0.92$, $p < 0.001$), underscoring interconnected growth across these fields (Table 3). These findings highlight the synergistic evolution of DNA barcoding research, driven by its versatile applicability across ecological, taxonomic and practical domains. The changes from the early 2000s to later years have been thoroughly discussed below:

Table 2. Confidence intervals for publication categories

Category	95% Confidence Interval (CI)
Biodiversity and Ecological Studies (BE)	(22.62, 49.28)
Plant Taxonomy (PT)	(20.54, 48.26)
Evolutionary Studies (EP)	(7.08, 17.22)
Routine Applications (RA)	(8.45, 23.35)
Reviews (RVW)	(5.65, 13.25)
Bioinformatics Tools (BIF)	(1.54, 4.66)

Table 3. Correlation analysis between publication trends in different categories

Pair of categories	Correlation coefficient	p-value
BE and PT	0.94	$p < 0.001$
BE and RVW	0.88	$p < 0.001$
PT and RVW	0.92	$p < 0.001$
EP and RVW	0.88	$p < 0.001$
RA and RVW	0.70	$p < 0.001$

DNA barcoding papers in the early 2000s: DNA barcoding debuted as a technique for rapid species identification (9, 10) and advanced in various research areas. Later on, the field significantly advanced and diversified into different approaches depending upon the species, locus of interest, etc. Until 2007, the growth in the field was low as depicted in Fig. 1. During the early 2000s the application of single locus and multi-locus barcodes were both equally more prevalent (Fig. 2). In the nascent years of development of this technique, researchers proposed many perspective marker loci that could help in the identification of species (11). The research areas were limited to either taxonomic studies or biodiversity studies, majorly dealing with species identification or delimitation of newly found species (12). This technique became important for contemporary evolutionary scientists as the field's comprehension and the interest of evolutionary biologists expanded. Depending on the desired paradigm, several sub-themes can be split for evolutionary studies as far as barcoding is concerned (Fig. 3A-E). One of the major drawbacks of the era was, how the application of DNA barcoding was used is that the biologists focused more on obtaining monophyly rather than including a cohesive character-based analysis at that time. Neighbor-joining (NJ) and Distance-based approaches (DA) were most common for phylogenetic studies during this phase. Therefore, it was rare to find biologists implying character-based or statistical approaches. It was not until 2009 that probabilistic methods for evolutionary studies were used in addition to barcoding. These techniques together have significant data support and bring about a truer evolutionary lineage of the species under study.

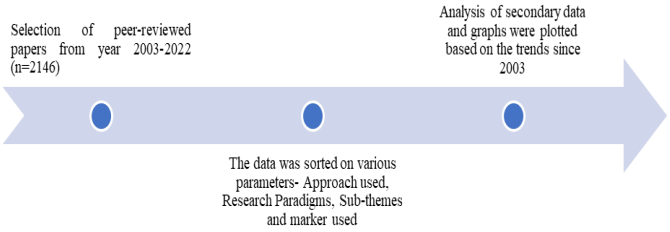

Fig. 1. Steps in the analysis of trends in DNA barcoding through the years.

***DNA barcoding papers since the 2010s*:** A decade after the development of this technique, researchers further diversified its applications to include routine uses such as forensic studies (13, 14), biosafety analysis (15) and quality control of food and herbal products (16, 17). Additionally, the technique was deployed for studies of biodiversity hotspots (18, 19) and enabled analysis of critically endangered species (11).

With advancements in molecular taxonomy, new species were continuously being explored (20). Ecological studies, including interaction studies like insect-pest dynamics (21), pollinator behaviour and niche delimitations were facilitated by these advancements (11). Markers specific for plant families, algae, bryophytes and other clades were also being explored (22–24).

Such diverse applications necessitated alternative methodologies and approaches to meet the specific

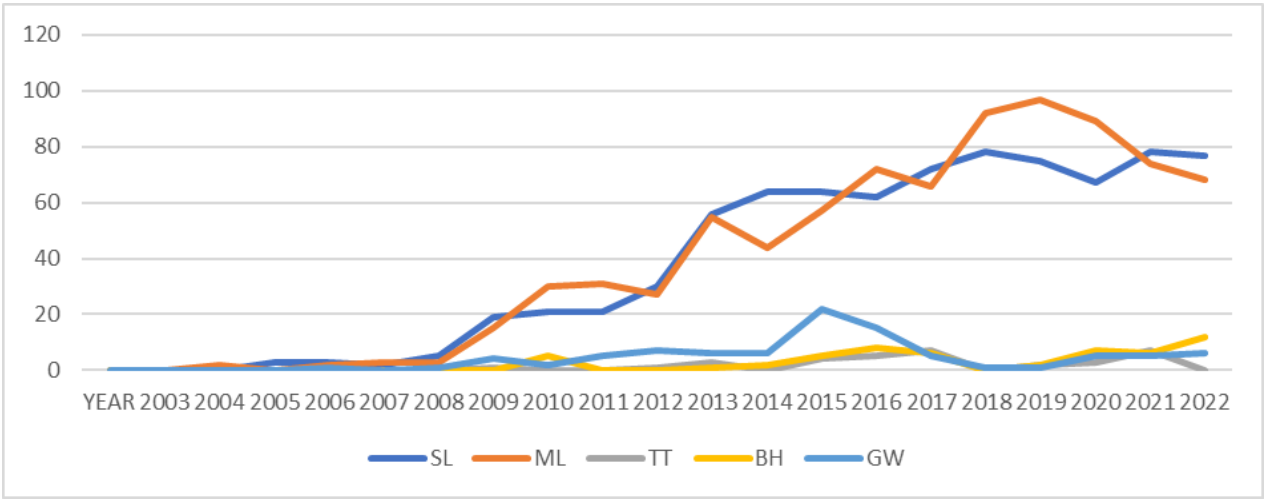

Fig. 2. Trends in DNA barcoding approach through the years 2003 to 2022 are depicted by a line graph. Here SL represents a single locus, ML represents Multiple Locus, TT represents a tiered approach, BH represents the Bar-HRM technique and GW represents the genome-wide technique. The x-axis showcases the number of publications that were considered for secondary data construction.

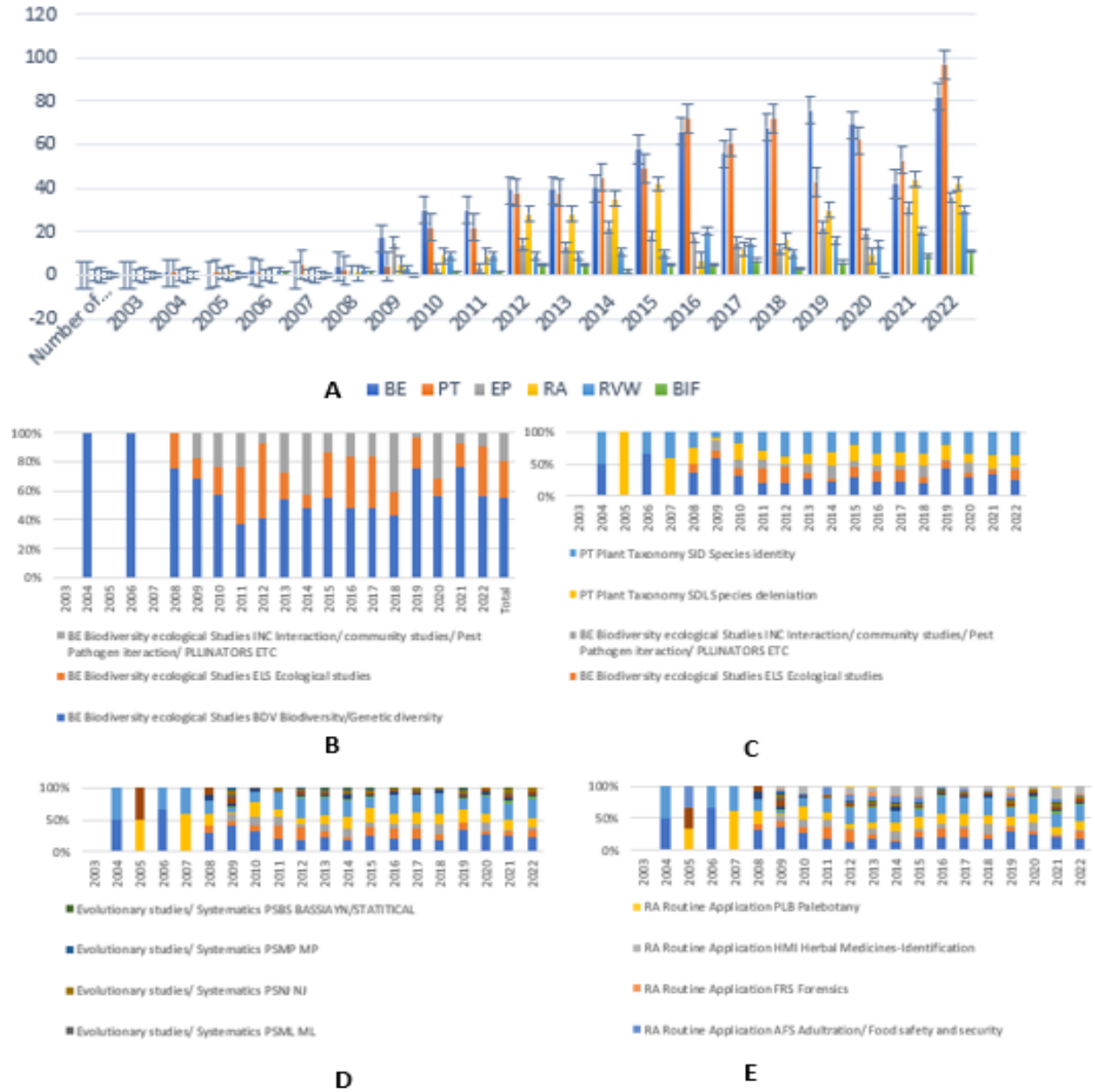

Fig. 3. Graphical representation trends of the themes (A) and the respective sub-theme (B-E) of research done in the field of "DNA barcoding" (includes papers using barcoding in the title or abstract or keywords) throughout the years 2003-2022. The x-axis represents the years that were considered whilst, the y-axis represents the percent publication, of the total publications taken under the study (B-E). Trends in the research paradigms of DNA barcoding through the years 2003 to 2022 are depicted by a bar graph. The vertical lines represent the standard deviation from the mean. The y-axis depicts variation in the number of publications through the years, whereas each year has various research paradigms that were used for categorical segregation of thedata. Here, the various research paradigms considered are, “BE” represents Biodiversity and Ecological Studies, “PT” represents Plant Taxonomy/Species identity, “EP” represents Evolutionary Studies and Systematics, “RA” represents Routine Applications, “RVW” represents Review and “BIF” represent Bioinformatics tools and techniques. The x-axis showcases the number of publications that were considered for secondary data construction.

needs, thereby leading to the adoption of a multi-locus approach (Fig. 2). Compared to the single locus technique, this approach had the advantage of robust data support due to the rapidly building DNA libraries and dedicated Barcoding Data bases initiated during the era (25). The multi-locus approach provided stronger support for species identification which increased its popularity manifold. Even though the cost of generating the multi-locus barcode data is higher, due to much stronger support, the technique saw a new dawn. However, several classical taxonomists expressed their concern and critically opposed the technique, drawing attention towards errors such as statistical errors, under-representation of diversity, etc. They also warned about the drawbacks of using the technique for species delineation and novel species discovery (26, 27).

To address these shortcomings and to achieve more statistically robust results, genome-wide approaches began to include “mega-barcodes”, which include entire organellar DNA as barcodes. This not only created more robust data but also allowed for a high degree of specificity. The establishment of a robust DNA library unquestionably initiated a rapid cascade of DNA identification utilisation across diverse fields. This significantly expanded the application in species delineation and catalysing several molecular and phylogenetic studies using various models as evidenced by the rise in the percentage of publications from 2010 onwards (Fig. 2, 3A). Throughout this period, researchers continued to explore this innovative technique across various dimensions and fields, significantly contributing to the field of science. One of the major constraints in the early years was the high cost of DNA sequencing associated with this technique. Nonetheless, this opened the door for Meta-Barcoding, which increased the technique's applicability and scope.

Research in DNA barcoding from 2015 onwards: As scientific advancements progressed and in response to the high cost of sequencing, a sequence-independent approach also evolved. This approach relied on high-resolution melting analysis of the barcode sequence, enabling rapid species identification without the need for sequencing. This method gained popularity from 2014 onwards. This technique is applicable for differentiating closely related species at a lower cost (28). The utilization of the technique experienced a significant decline from 2017 onwards (Fig. 2). It is presumed that this decline is due to limited analytical applications, leading to a decrease in the adoption of the approach. Additionally, the high cost associated with the genome-wide approach has contributed to this trend. Sequencing the entire organelle, particularly in cases where ambiguity for species identity is low or species identification can be unequivocally achieved with the multi-locus approach, seems rather unnecessary and impractical, considering a substantial investment of both cost and time involved. Therefore, the multi-locus techniques and single-locus techniques have remained prevalent even after a decade of advancements in the field. Tiered approaches proved most effective for routine samples where the cost-effectiveness was crucial for the stakeholders. Consequently, the multi-locus approach was modified to separate loci into core and supplementary markers, facilitating a tiered approach. Supplementary markers were used only when ambiguity arose with core markers in species identification, thereby reducing the overall cost associated with sequencing. Starting in 2015, the research field diversified significantly and the application of these techniques in taxonomic and phylogenetic studies also increased.

New Bioinformatic tools were especially being developed to run barcode-related programs and manage data (Fig. 3). A more inclusive approach to phylogenetics emerged, incorporating tree-based and tree-independent methods, including the character-based approach. Publication trends on the number of tree-independent methods have been on the rise since 2013, with NJ (Neighbor-Joining) and DA (Discriminant Analysis) methods being the most relevant and widely used. A range of ecological studies were due to this technique, broadly categorized as biodiversity studies including studies related to genetic diversity, ecological interactions including but not limited to plant-pollinator interaction, pest-pathogen identification, mobility of pollinators being tracked by pollen DNA, etc. This led to the development of a subdomain of meta-barcoding, which enables the identification of organisms from soil, air and water ushering in an unprecedented era of biodiversity identification.

The broadening scope of DNA barcoding, from phylogenetic analysis to diverse ecological applications, demonstrates its dynamic nature and adaptability. This expansion is likely to continue as new markers and technologies emerge, further enhancing the technique's relevance and impact in scientific research. While simplistic correlation studies of existing markers and techniques can indicate which methods to use for specific purposes (Fig. 4), the choice of markers should be made carefully depending on the research objective. Efforts should be made to reduce costs, even when using whole organellar genomes, by identifying effective regions of dissimilarity and proposing them as new markers. Developing new markers can ultimately help create a more meaningful database for routine use of the technique.

Overall, the evolution of DNA barcoding reflects its significant contributions to multiple scientific disciplines and its potential for future advancements in understanding and preserving biodiversity. While the technical advancements in DNA barcoding are evident, emerging concerns warrant attention. For instance, the disclosure of genetic data from rare or endangered species could expose them to exploitation or unethical use, challenging conservation efforts. Additionally, biosafety protocols for genetic data handling are underdeveloped, raising questions about privacy and the unintended consequences of mismanaged datasets. Addressing these issues is critical for ensuring the responsible application of

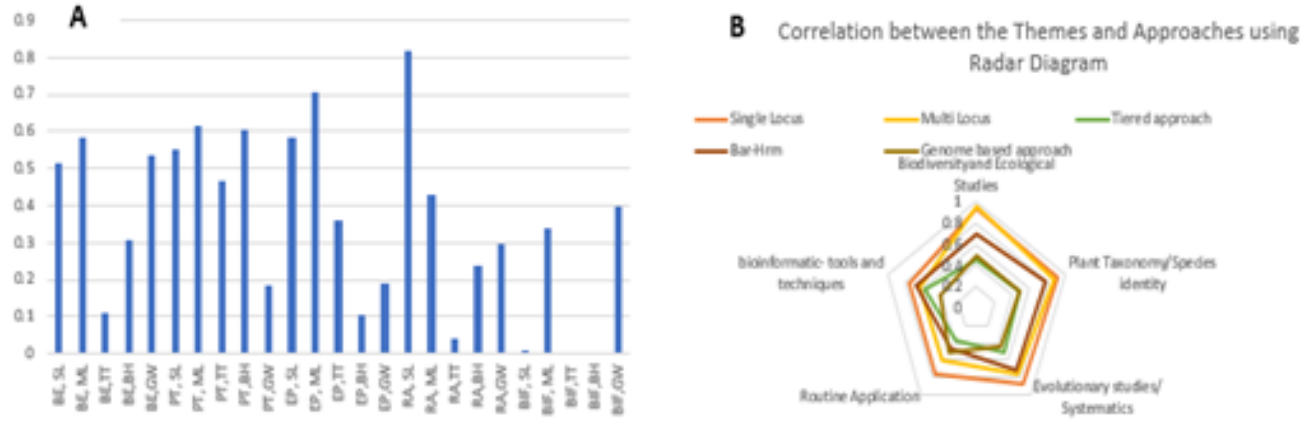

Fig. 4. A. A bar graph depicting correlation values of a particular theme vis-à-vis approach. The number of publications made per year and the technique in that particular research theme that appeared each year were taken to calculate the correlation. **B.** A radar diagram depicting the correlation values of different themes with the different approaches used. The coloured lines for each approach depict the value correlation with each themesuggesting which approach was most popular among which type of theme strictly depending upon the number of publications of a particular theme vs the numberof publications of a particular approach each year.

DNA barcoding technologies (29).

Conclusion

The technique, though straightforward, has limitations and requires species-specific adjustments, as genetically similar species may not show significant variation based on a single locus. The reliance on existing markers and the concept of the barcoding gap, which distinguishes between intra- and inter-specific genetic distances, needs careful consideration. A broader global representation of species in studies and a comprehensive approach, including statistical and character-based analyses, are recommended to avoid misleading conclusions. Additionally, the development of new, cost-effective markers is essential for accurate species identification, rather than defaulting to genome-based approaches.

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Author contribution

All the authors contributed equally to the conceptualisation of the work, interpretation, analysis, writing, reviewing and editing of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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