



Review article

Beyond a single locus: Evolutionary constraints and genomic alternatives to plant DNA barcoding

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ABSTRACT: DNA barcoding has transformed species identification by using short, standardized genetic sequences for taxonomic assignment. While the mitochondrial *COI* gene is a universal barcode in animals, an equivalent marker for plants has not been established. The chloroplast genes *rbcl* and *matK* are international standards, but their limited discriminatory power has led to ongoing exploration of alternative loci and multilocus strategies. This review discusses the historical contexts, technical challenges, and biological factors, such as slow mitochondrial evolution, polyploidy, and hybridization, that have prevented the adoption of a single universal plant barcode. We examine emerging approaches that combine high-throughput sequencing, machine learning, and comprehensive reference databases, moving from a single-marker approach to a genomic toolkit. The lack of a universal barcode reflects the evolutionary complexity of plants, highlighting the need for integrative, scalable, and application-oriented frameworks in future plant barcoding research. We advocate for a practical genomic toolkit tailored to the unique challenges of plant evolution.

KEY WORDS: biodiversity monitoring, chloroplast and nuclear loci, genome-wide approaches, multilocus markers, species discrimination.

INTRODUCTION

Concept of DNA Barcoding and Its Applications in Animal Research

DNA barcoding is a molecular biology method that extracts and sequences representative DNA fragments from known and unknown samples for species identification (Antil *et al.*, 2023). These DNA barcodes are typically highly conserved, allowing the use of universal primers for PCR amplification, yet variable enough to distinguish species or taxonomic units (Hebert *et al.*, 2003; Ahmed *et al.*, 2022; Antil *et al.*, 2023). The resulting sequences are compared to genetic databases, such as the Barcode of Life Data System (BOLD) or GenBank, to identify the species or taxonomic unit (CBOL Plant Working Group *et al.*, 2009; Pawlowski *et al.*, 2012). Unlike phylogenetic studies, which aim to infer evolutionary relationships among taxa and reconstruct their evolutionary history, DNA barcoding primarily focuses on matching unknown sequences to reference databases for rapid species identification. Therefore, DNA barcoding is typically not intended to resolve deep evolutionary relationships or to revise taxonomy, although barcode data can sometimes provide auxiliary information for such purposes. This method is simple, fast, cost-effective, and reliable, and it is widely used in species identification, environmental DNA (eDNA) detection, biodiversity monitoring, forensic science, and related fields (Ahmed *et al.*, 2022; Antil *et al.*, 2023). It is most commonly applied to species classification and identification across animals, plants, fungi, and bacteria (Ahmed *et al.*, 2022; Antil *et al.*, 2023).

In animals, the cytochrome c oxidase subunit 1 (*COI*) gene is the standard DNA barcode (Hebert *et al.*, 2003). Located in the mitochondrial genome and approximately 650 bp long, the *COI* gene is essential for mitochondrial respiration. Mitochondrial DNA (mtDNA), including the *COI* gene, is preferred because it has limited recombination, fewer insertion or deletion events, and a higher mutation rate than nuclear genes. These characteristics provide enough genetic diversity to distinguish closely related species, making the *COI* gene effective for animal taxonomy (Hebert *et al.*, 2003).

Development of Plant DNA Barcoding and International Standards

Following the success of animal DNA barcoding, researchers have explored applying similar methods to other taxonomic groups, including plants. While several studies have sought a universal DNA barcode for plants, their genetic complexity has made it difficult to identify a single suitable region. To address this, an international consortium was formed to evaluate potential genetic markers for plant DNA barcoding.

The Consortium for the Barcode of Life (CBOL) Plant Working Group assessed candidate genetic regions for plant DNA barcoding. They proposed the combination of the *rbcl* (large subunit of ribulose 1,5-bisphosphate carboxylase) and *matK* (maturase K) as the international standard (CBOL Plant Working Group *et al.*, 2009). The study compared seven chloroplast regions, including *rbcl*, *matK*, the *psbA-trnH* (the intergenic spacer between the photosystem II protein D1 gene and the transfer RNA-histidine gene), *rpoCl* (RNA polymerase

**BOX: EVOLUTION OF DNA BARCODING****A. Animals**

Hebert *et al.* (2003) pioneered DNA barcoding in animals by proposing a 648-bp fragment of mitochondrial *COI* as a universal barcode (cf. Schmid *et al.*, 2025). Subsequent studies confirmed its effectiveness; for example, Hebert *et al.* (2004) found that *COI* sequences in 260 North American bird species showed interspecific differences ~18× greater than within species. The BOLD system, launched in 2007, compiles *COI* reference sequences (Ratnasingham and Hebert, 2007). Today, *COI* remains the core animal barcode; for example, it is available for >25,000 fish species (Schmid *et al.*, 2025). Barcoding now covers insects, vertebrates, and other metazoans, significantly expanding species libraries. Recent advances include next-generation sequencing (NGS) methods such as mini-barcodes, multiplexed *COI* assays, and eDNA/metabarcoding, which facilitate biodiversity surveys in bulk samples. In fish and vertebrate eDNA studies, *COI* is still widely used, although shorter rRNA markers (12S/16S) are sometimes preferred for degraded samples (Schmid *et al.*, 2025). Overall, animal barcoding has progressed from single-locus *COI* to comprehensive reference databases (BOLD) and high-throughput applications, with *COI* as the principal marker (Ratnasingham and Hebert, 2007; Schmid *et al.*, 2025).

B. Plants

Early plant barcoding in the mid-2000s tested various plastid genes, with *matK* emerging as a key marker in large-scale surveys (Lahaye *et al.*, 2008). In 2008–2009, the CBOL Plant Working Group recommended a two-locus barcode of *rbcL* + *matK* for land plants (CBOL Plant Working Group *et al.*, 2009). Researchers often add markers such as the chloroplast *trnH-psbA* spacer and nuclear ITS to improve species discrimination (Chen *et al.*, 2010; China Plant BOL Group *et al.*, 2011). With NGS, barcoding has shifted to “super-barcodes.” High-throughput approaches now use DNA metabarcoding and target enrichment. DNA metabarcoding applies universal primers to amplify DNA from complex environmental samples (eDNA), allowing simultaneous identification of multiple taxa. Target enrichment, such as Angiosperms353, uses customized biotinylated RNA or DNA probes to ‘capture’ hundreds of conserved nuclear orthologs (Letsiou *et al.*, 2024). This method is especially effective for resolving phylogenetic relationships in groups where hybridization or polyploidy complicates single-locus markers. These advances support plant metabarcoding and eDNA surveys, enabling detection of plant DNA from sources like pollen, soil, and water. In summary, plant barcoding has evolved from single genes (*rbcL*, *matK*) to multilocus combinations, and now to whole-plastome and enriched multi-gene approaches (CBOL Plant Working Group *et al.*, 2009; Li *et al.*, 2015).

C. Fungi

Early fungal barcoding explored several loci, but consensus soon favored ITS. In 2007, a fungal-barcode consortium formally proposed nuclear ribosomal ITS as the primary fungal barcode (Schoch *et al.*, 2012). Schoch *et al.* (2012) showed that ITS offered the best species-level resolution and recommended it for broad fungal coverage. By 2010, ITS was the preferred marker for both individual specimens and environmental samples (Bellemain *et al.*, 2010). ITS of ITS quickly became standard for fungal community studies (soil, root, air, gut, etc.), often referred to as metabarcoding. ITS remains the leading marker for fungal eDNA and metabarcoding today (Lücking *et al.*, 2020). In summary, fungal barcoding has shifted from multiple loci to ITS, now widely used in NGS community surveys and eDNA monitoring (Bellemain *et al.*, 2010; Lücking *et al.*, 2020).

The historical progression of DNA barcoding approaches, from single-gene markers to genomic-scale methods, is summarized in Fig. 1.

C1), *rpoB* (RNA polymerase B), *atpF-atpH* (the spacer between ATP synthase subunits CFO I and CFO III), and *psbK-psbI* (the spacer between polypeptides K and I of photosystem II). These sequences were obtained from 907 samples, representing 445 angiosperm species, 38 gymnosperms, and 67 cryptogams.

Statistical analysis showed that single-gene regions achieved species-resolution rates between 43% (*rpoC1*) and 69% (*psbK-psbI* and *trnH-psbA*). Two-gene combinations ranged from 59% to 75%, while three-gene combinations ranged from 65% to 76%. Considering primer universality, sequence structure, genetic variability, and sequencing costs, the CBOL Plant Working Group recommended *rbcL* + *matK* as the international standard for plant DNA barcoding (CBOL Plant Working Group *et al.*, 2009).

Why is There No Universal Plant DNA Barcode Like Animal *COI*?

Despite the adoption of *rbcL* + *matK* as a standard, this dual-locus system is not equivalent to a true universal

barcode like *COI* in animals, since no single DNA region in plants has proven both sufficiently variable and broadly amplifiable across taxa (Antil *et al.*, 2023). This raises the question of why plants lack a universal single-locus barcode comparable to the animal *COI* gene. This paper explores the challenges faced by plant DNA barcoding and the technical issues yet to be overcome.

Characteristics of Plant Genomes: Low Variability in Chloroplast Genes and Nuclear Gene Multiplicity

Compared to nDNA, cpDNA is typically maternally inherited in angiosperms, passing from mother to offspring without recombination, which makes it a stable and consistent genetic marker across generations (Pharmawati *et al.*, 2004; Tateishi *et al.*, 2007; Ellis *et al.*, 2008; Vrancken and Wesselingh, 2010; Bendich, 2013). Its lack of recombination and resistance to hybridization events increases its reliability for species identification (Nock *et al.*, 2011; Li *et al.*, 2015; Zhang *et al.*, 2017). Furthermore, the inverted repeat (IR) regions in cpDNA have very low substitution rates due to biased gene

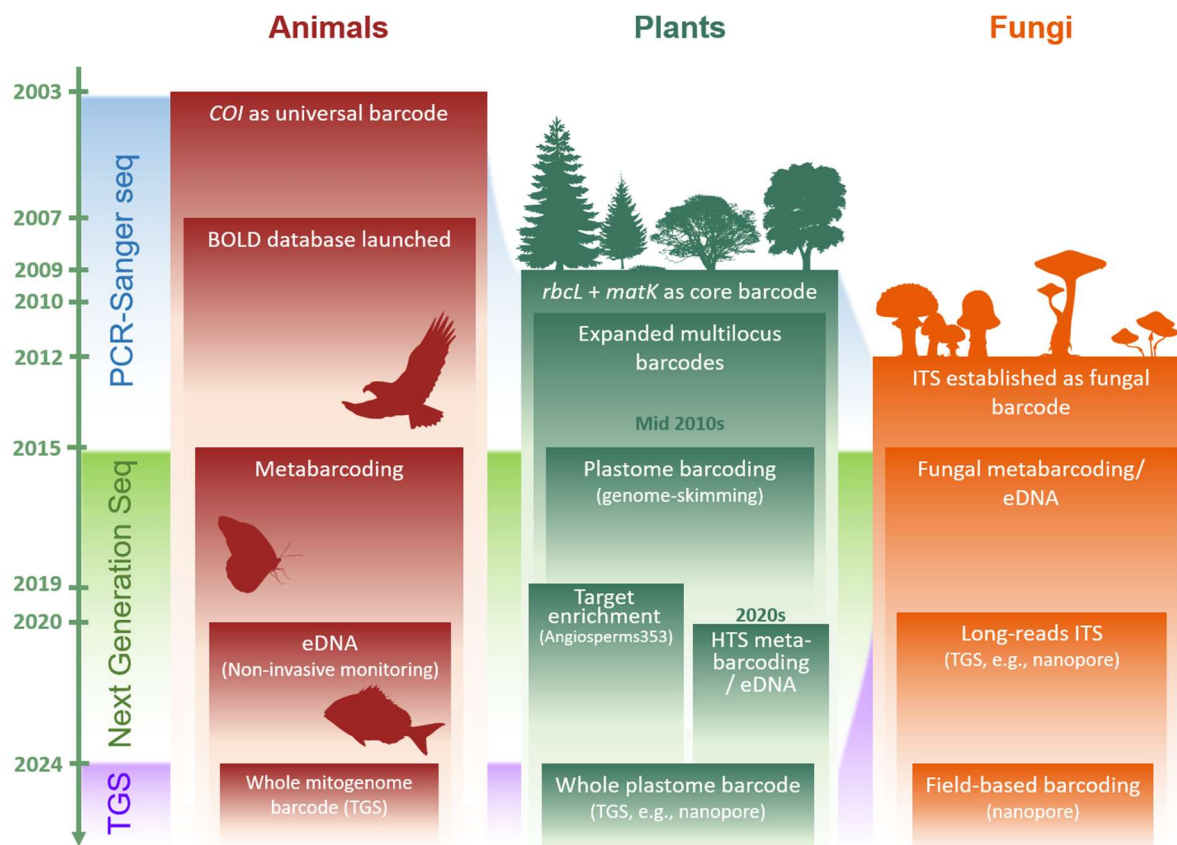


Fig. 1. Evolutionary timeline of DNA barcoding approaches across major taxonomic groups. The figure illustrates key milestones in the development of DNA barcoding from single-locus systems to multilocus and high-throughput genomic approaches. Animal barcoding began with the adoption of *COI* as a universal marker, followed by the development of metabarcoding and eDNA applications. Plant barcoding evolved from the CBOL-designated *rbcL* + *matK* core loci to multilocus, plastome, and target-enrichment (Angiosperms353) approaches. Fungal barcoding was established with ITS as the universal marker and later expanded to ITS-based metabarcoding. The timeline highlights the parallel yet distinct technological trajectories of barcoding across animals, plants, and fungi.

conversion, maintaining sequence homogeneity (Li *et al.*, 2016).

While animal mitochondrial DNA serves as a reliable barcode, plant mtDNA is less effective due to its varied genome size (ranging from 200 to 2,500 kb) and frequent structural rearrangements (Wolfe *et al.*, 1987), which complicate sequencing and alignment (Wang *et al.*, 2024). The generally slower evolutionary rate of plant mtDNA also limits its variability for species identification (Palmer and Herbon, 1988). However, it is important to note that these rates vary significantly across different plant lineages. For instance, substitution rates can be highly accelerated in some genera, such as *Silene* and *Pelargonium*, making a universal marker difficult to identify (Mower *et al.*, 2007; Weng *et al.*, 2017).

CpDNA has been the primary focus of plant DNA barcoding due to its abundance and structural stability. Chloroplast genes show low evolutionary rates and variability (Shaw *et al.*, 2007; Dong *et al.*, 2012; Krawczyk *et al.*, 2023), largely because strong selective pressures maintain gene integrity for photosynthesis (Kusumi and Iba, 2014; Yu *et al.*, 2019). Inverted Repeat

(IR) regions also help stabilize the genome through concerted evolution (Ping *et al.*, 2021).

The rate of plastid DNA evolution varies among species. In mammals, mtDNA evolves about five times faster than nuclear DNA (nDNA), while in angiosperms, it is five times slower (Brown *et al.*, 1982). While cpDNA evolves faster than plant mtDNA overall, gene evolution rates within the chloroplast differ. For instance, *matK* and *rbcL* evolve more slowly than other regions in Fagaceae (Pang *et al.*, 2019).

In complex groups like Lauraceae, standard plastid loci (*rbcL*, *matK*, *trnH-psbA*) and taxon-specific regions (*ycf1*, *ndhH-rps15*, *trnL-ycf2*) often provide limited resolution due to hybridization and chloroplast capture (Liu *et al.*, 2022). No single locus can reliably distinguish all plant species, though combining multiple loci (e.g., *matK*, *rpoB*, and *rpoCI*) can improve species discrimination, even if 100% identification remains elusive (Fazekas *et al.*, 2008; Singh *et al.*, 2012).

Unlike the conservative chloroplast genome, nuclear genes (especially low-copy nuclear genes) generally evolve rapidly and provide greater genetic diversity



(Small *et al.*, 2004). Frequent gene duplications lead to multiple copies (paralogs), offering redundancy or specialization (Naciri and Manen, 2010; Zarrei *et al.*, 2015; Saad and Cobb, 2016; Zhang *et al.*, 2019; Lloreta-Trull, 2025). While this variability enables high-resolution barcoding, it also complicates primer design and sequence alignment. Polyploidy and hybridization further challenge the process.

In summary, organellar genomes (cpDNA and mtDNA) offer advantages like uniparental inheritance and stability but are limited by low substitution rates and genome rearrangements (Wang *et al.*, 2024). Nuclear genes provide higher evolutionary rates and resolution, but issues with paralogy, polyploidy, and universal primer design remain. These limitations explain why no single locus serves as a universal plant barcode, highlighting the need for integrated genomic approaches.

The Impact of Hybridization and Polyploidy on Sequence Analysis

Polyploidization and hybridization frequently occur in plants, altering genome structure and creating significant challenges for DNA barcoding. These main challenges include:

- (1) **Increased Genetic Complexity:** The combination of divergent genomes in hybrids and polyploids creates complex genetic backgrounds, making it difficult to design primers that universally amplify target regions across species or ploidy levels (Brysting *et al.*, 2007; Renny-Byfield and Wendel, 2014). Hybridization can also cause introgression, where small genomic regions transfer between species, further obscuring species boundaries (Osuna-Mascaró *et al.*, 2023).
- (2) **Presence of Homoeologs:** Polyploids contain multiple gene copies (homoeologs) from different parental genomes, complicating primer design. Primers must often distinguish between these copies to prevent non-specific amplification (Fazekas *et al.*, 2012; Zhang *et al.*, 2019).
- (3) **Reduced Marker Resolution:** DNA barcodes may be less effective in polyploid species because of multiple gene copies and possible gene loss or sub- and neo-functionalization (Zarrei *et al.*, 2015; Zhang *et al.*, 2019). Careful selection and validation of barcode regions are needed to ensure adequate species identification.

Evolutionary Rate Heterogeneity and the Need for Multi-marker Integration

Differences in evolutionary rates among genetic loci further complicate DNA barcoding. Key barcode genes, such as *rbcL* and *matK*, evolve at different rates, leading to conflicting phylogenetic outcomes. For instance, in Lamiaceae and Verbenaceae, *matK* is effective for resolving deeper relationships, while *rbcL* is better for species delimitation (Oyebanji *et al.*, 2020). Other loci,

such as the chloroplast intergenic spacers *atpF-atpH* and *psbK-psbI*, also show variable evolutionary rates. Highly variable regions like *trnH-psbA* are important for addressing complex phylogenetic questions (Dong *et al.*, 2012). Regions such as ITS2 have high divergence rates, making them effective for species identification in some groups, like *Piper* (Piperaceae) (Egydio Brandão *et al.*, 2020), but less effective in others, such as *Eryngium* (Apiaceae), due to their polyphyletic patterns (Acharya *et al.*, 2022).

These differences in evolutionary rates can lead to inaccurate taxonomic assignments, as observed in closely related plant lineages such as the subfamilies Annonoideae and Malmeoideae of Annonaceae (Hoekstra *et al.*, 2017). Molecular data are also essential for correcting past morphological misidentifications. For example, studies have shown that *Macrotyloma sargarhwalensis* is conspecific with *Clitoria annua* (Fabaceae) (Pradheep *et al.*, 2022). Therefore, integrating data from multiple loci is necessary for robust phylogenetic analyses and accurate taxonomic resolution.

EXISTING SOLUTIONS AND LIMITATIONS

Advantages and Disadvantages of the *rbcL* + *matK* Barcode Standard

Although CBOL recommended *rbcL* + *matK* as a universal barcode, several studies suggest this combination is not effective for species identification. For instance, Huang *et al.* (2015) reported low species-level identification success (24.7–28.5% for *rbcL* and 31.6–35.3% for *matK*), though the markers were useful at the family and genus levels in Asian tropical trees.

The findings of Huang *et al.* (2015) are not unique; many studies report similar limitations with this marker combination. In *Calligonum* (Polygonaceae), *rbcL* and *matK* provided only 6.25% resolution (Li *et al.*, 2014), and in Bromeliaceae, only 43.48% discrimination (Maia *et al.*, 2012). Integrating hypervariable nuclear regions is essential for improved resolution. For instance, in Arecaceae, adding nrITS2 increased discrimination to 92% (Jeanson *et al.*, 2011). In Orchidaceae, nrDNA loci offered superior species resolution within *Dendrobium* (Chattopadhyay *et al.*, 2017).

Marker selection often requires balancing informativeness and amplification efficiency. 'Efficiency' is the technical success rate of PCR amplification and sequencing across taxa, while 'Resolution' or 'Discrimination Power' is the percentage of species successfully identified (CBOL Plant Working Group *et al.*, 2009). In Lamiaceae, *rbcL* had limited resolution, whereas *matK* and *trnH-psbA* offered better discrimination but lower efficiency (Hafez *et al.*, 2025). Similarly, *matK* was less effective for resolving taxonomic issues in *Polygonum* (Polygonaceae) (Yamskikh *et al.*, 2023). These results highlight the limitations of the dual-



Table 1 Summary of plastid and nuclear markers frequently applied in plant DNA barcoding, highlighting their genome origin, variability, amplification reliability, discriminatory power, strengths, and limitations.

Marker(s)	Genome source	Variability	Amplification reliability	Typical discrimination power	Strengths	Limitations	Key references
<i>rbcL</i>	Plastid (coding)	Low	Very high	Family/genus level	Easy to amplify, broad taxonomic coverage	Poor species resolution	(CBOL Plant Working Group <i>et al.</i> , 2009)
<i>matK</i>	Plastid (coding)	Moderate-high	Moderate	~70% species resolution	Higher variability than <i>rbcL</i>	Primer universality issues	(CBOL Plant Working Group <i>et al.</i> , 2009)
<i>rbcL + matK</i>	Plastid (coding)	Moderate	High	~70–75%	CBOL core barcode, standardized	Insufficient for closely related taxa	(Bafeel <i>et al.</i> , 2011; Ferri <i>et al.</i> , 2015)
<i>trnH-psbA</i>	Plastid (intergenic spacer)	High	Moderate	Improves species-level resolution	High variability	Alignment difficulties, indels	(Kress and Erickson, 2007)
<i>trnL-F</i>	Plastid (intergenic spacer)	Moderate	High	Genus/species level	Widely used, easy to amplify	Lower variability than <i>trnH-psbA</i>	(Taberlet <i>et al.</i> , 1991)
<i>ndhF</i>	Plastid (coding)	Moderate	Moderate	Lineage-dependent	Useful in some groups (e.g., Asteraceae)	Not universal	(Kim and Jansen, 1995)
ITS / ITS2	Nuclear (ribosomal DNA)	High	High	Often >80%	High resolution, useful in many families	Paralogs, pseudogenes, intragenomic variation	(Chen <i>et al.</i> , 2010; China Plant BOL Group <i>et al.</i> , 2011)
<i>ycf1</i>	Plastid (coding, large gene)	Very high	Moderate	High, even in close relatives	Strong species discrimination	Primer design, size	(Dong <i>et al.</i> , 2015)
Multilocus (e.g. <i>rbcL + matK + ITS + trnH-psbA</i>)	Plastid + nuclear	High	Variable	>80%	Combines strengths of different loci	Higher cost, complex workflows	(Zhang <i>et al.</i> , 2015a; Zhang <i>et al.</i> , 2025)
Plastome-wide ("super-barcoding")	Plastid (whole genome)	Very high	High with NGS	Near 100%	Genome-scale data, robust	Higher cost, more data-intensive	(Kane <i>et al.</i> , 2012; Coissac <i>et al.</i> , 2016)
Genome-wide SNPs	Nuclear + plastid	Very high	High with NGS	Near 100%	Best resolution, species-specific markers possible	High cost, bioinformatic demand	(Coissac <i>et al.</i> , 2016)

plastid approach and support the need for a multilocus barcoding strategy.

Performance of Other Candidate Genes in Plants

In addition to *rbcL + matK*, several other genes have been proposed for plant DNA barcoding. Xu *et al.* (2015) recommend ITS and *matK* for identifying *Dendrobium* (Orchidaceae) species, while *trnH-psbA* has demonstrated superior performance in temperate grasslands (Loera-Sánchez *et al.*, 2020). Highly variable plastid intergenic spacers such as *trnH-psbA* and nuclear markers like ITS provide greater sequence divergence, especially for groups like Orchidaceae and Theaceae (Chen *et al.*, 2010; China Plant BOL Group *et al.*, 2011). The plastid gene *ycf1* is also promising due to its high variability, performing well in challenging groups such as *Quercus* (Fagaceae) (Dong *et al.*, 2015). Multilocus approaches that combine these genes can achieve over 80% accuracy (Zhang *et al.*, 2015a; Zhang *et al.*, 2025). Table 1 summarizes the major plastid and nuclear loci tested as plant DNA barcodes, along with their combinations, strengths, and limitations.

Advances in high-throughput sequencing (HTS) have significantly improved genetic data generation compared to traditional methods. Wilkinson *et al.* (2017) demonstrated that integrating HTS with Sonication-MicroAssembly (SMA) techniques enhances DNA barcoding. HTS platforms such as Illumina MiSeq provide more comprehensive data (Kajtoch, 2014). Additionally, Microfluidic Enrichment Barcoding (MEBarcoding), which utilizes the Fluidigm Access Array to amplify targeted regions across multiple DNA samples, increases throughput and efficiency for constructing large reference libraries (Gostel *et al.*, 2020).

Combining barcoding with HTS via metabarcoding is essential for analyzing complex eDNA and providing a comprehensive view of plant biodiversity and species interactions (Yoccoz *et al.*, 2012; Liu, 2019; Leontidou *et al.*, 2021). This approach supports the development of extensive regional or global reference libraries, such as by sequencing six conventional barcode fragments (*ITS1*, *ITS2*, *matK1*, *matK2*, *rbcL1*, and *rbcL2*) from hundreds of flowering plants (Liu *et al.*, 2021a). HTS-supported DNA barcoding using nine loci like *trnL-F* and *ITS* has



been vital for identifying species in taxonomically challenging groups, including Eurasian yews (*Taxus*, Taxodiaceae), and directly aids conservation and monitoring of illegal trade (Liu *et al.*, 2011a). These technologies now allow for complete plastome barcoding and genome-wide SNP analyses, offering higher resolution (Kane *et al.*, 2012; Coissac *et al.*, 2016). In summary, while plastid loci are convenient for plant barcoding, nuclear markers and genome-scale approaches provide the discrimination needed to distinguish closely related or cryptic taxa, highlighting the difficulty of establishing a single universal plant barcode.

Genomic Toolkit Framework for Plant Identification and Evolutionary Complexity

Instead of using a single universal barcode, identifying plants and understanding their evolution requires a step-by-step approach that selects the right DNA markers based on how closely related the plants are, how complex their evolution is, and what the research aims to discover. Studies show that no single marker works best across all plant groups. Using a mix of plastid and nuclear markers provides a stronger, more flexible way to identify and classify plants.

At higher taxonomic ranks, such as order and family, conservative chloroplast coding genes, such as *rbcL* and *matK*, are the most widely used and cost-effective markers. Their high universality, ease of amplification, and standardized primer availability make them suitable for large-scale floristic surveys and broad taxonomic inventories, where amplification success and cross-taxon comparability are essential (Gao *et al.*, 2010; El-Atroush *et al.*, 2015; Srivastava *et al.*, 2016; Marchese *et al.*, 2021; Matiz-Ceron *et al.*, 2022; Setsuko *et al.*, 2023). However, *rbcL* exhibits relatively low interspecific variation, which limits its resolving power at the species level (Piredda *et al.*, 2011; Shi *et al.*, 2011; Dong *et al.*, 2015).

For genus- and species-level identification, faster-evolving nuclear and plastid regions are necessary to achieve sufficient discriminatory power. Nuclear ribosomal markers such as ITS and ITS2 are among the most effective loci for resolving closely related species due to their high evolutionary rates and substantial interspecific divergence (Gao *et al.*, 2010; China Plant BOL Group *et al.*, 2011; Liu *et al.*, 2011a; Yesson *et al.*, 2011; El-Atroush *et al.*, 2015; Zhang *et al.*, 2015b; Liu *et al.*, 2019). Multiple studies support the inclusion of ITS in plant barcoding pipelines to enhance species-level resolution, especially in taxonomically complex groups. Noncoding plastid regions such as *trnH-psbA* and the *trnL* intron also provide complementary resolution at the genus and species levels (Gao *et al.*, 2010; El-Atroush *et al.*, 2015; Matiz-Ceron *et al.*, 2022; Setsuko *et al.*, 2023) and are widely used in community phylogenetics and ecological network analyses (Gill *et al.*, 2019). However, the performance of these plastid spacers varies

substantially among taxonomic groups, highlighting the importance of marker combinations over single-locus approaches (Liu *et al.*, 2011b; Liu *et al.*, 2014).

Recent studies identify the plastid gene *ycf1* as a particularly effective barcode candidate in species-level discrimination due to its high sequence variability (Dong *et al.*, 2015; Liu *et al.*, 2022). In practice, multilocus strategies that combine *rbcL*, *matK*, and either ITS/ITS2 or *trnH-psbA* achieve high identification success, with species resolution rates exceeding 90% in local floras (Burgess *et al.*, 2011; Poovitha *et al.*, 2016; Jiang *et al.*, 2020). Furthermore, markers such as *rbcL* and ITS2 are used in conservation monitoring, endangered species identification, and forensic investigations, demonstrating their applied value beyond taxonomy (Gao *et al.*, 2010; Nithaniyal *et al.*, 2021).

Overall, these findings support a useful set of genetic tools, where choosing markers matches the level of classification and research goals. Stable plastid genes work well for identifying broad groups, while rapidly evolving nuclear and plastid regions help distinguish closely related species. This layered system provides a flexible, tested approach to identify plants, study evolution, and measure biodiversity, moving beyond the limitations of single-barcode methods toward a more integrated, adaptable use of genetic markers.

However, in plant groups characterized by hybridization, polyploidy, chloroplast capture, or incomplete lineage sorting (ILS), single-locus or few-loci DNA barcoding often fails to resolve evolutionary relationships (Li *et al.*, 2015). Under such circumstances, genomic-scale approaches become essential. Genome skimming, which recovers complete plastomes and high-copy nuclear regions, provides robust phylogenetic signal for both shallow and intermediate evolutionary scales (Fu *et al.*, 2022; Reginato, 2022; Thalén *et al.*, 2023). Target enrichment (Hyb-Seq) enables the simultaneous capture of hundreds to thousands of nuclear loci, allowing explicit testing of reticulate evolution and polyploid origins (Morales-Briones *et al.*, 2022; Španiel *et al.*, 2023). For fine-scale population differentiation and local adaptation, reduced-representation sequencing methods such as RAD-seq (Lowry *et al.*, 2017; Fan *et al.*, 2018; Pearman *et al.*, 2022) and GBS (Heffelfinger *et al.*, 2014; Vaux *et al.*, 2023), as well as whole-genome resequencing (Martin *et al.*, 2018; Lu *et al.*, 2025), provide dense SNP datasets suitable for demographic inference and genotype-environment association analyses.

Beyond the Angiosperms353 panel, the integration of Benchmarking Universal Single-Copy Orthologs (BUSCOs) has become a critical component of the genomic toolkit (Simão *et al.*, 2015). These markers provide a standardized set of near-universal orthologs that help resolve deep-level relationships and facilitate the identification of paralogous sequences that might otherwise confound species delimitation (Waterhouse *et al.*, 2017).



Table 2. A practical genomic toolkit for plant identification and evolutionary inference. The table summarizes commonly used genetic markers and sequencing methods across different taxonomic levels and research objectives, highlighting their primary advantages and limitations under varying degrees of evolutionary complexity.

Research Goal / Taxonomic Level	Recommended Markers	Sequencing Method	Main Advantages	Major Limitations
Order / Family identification	<i>rbcl</i> , <i>matK</i>	Sanger sequencing	High universality, easy amplification, and standardized	Low species resolution
Genus-level discrimination	<i>trnH-psbA</i> , <i>trnL</i> intron	Sanger sequencing	Moderate variation, useful for community phylogeny	Limited power in recent radiations
Species-level identification	ITS, ITS2, <i>ycf1</i>	Sanger sequencing	High resolution for closely related species	Sensitive to paralogy and contamination (ITS)
Deep & shallow phylogeny	Complete plastome	Genome skimming	Strong phylogenetic signal, low cost	Maternal inheritance, chloroplast capture
Reticulate evolution, polyploidy	Low-copy nuclear genes	Target enrichment (Hyb-Seq)	Resolves hybridization & ILS	Higher cost, bioinformatic complexity
Population structure & adaptation	Genome-wide SNPs	RAD-seq, GBS	High marker density, cost-efficient	Missing data, allele dropout
Demography & local adaptation	Whole genome SNPs	Whole-genome resequencing	Highest resolution	Expensive, computationally intensive

Accordingly, an effective plant genomic toolkit should follow a decision-based strategy, in which the choice of markers and sequencing methods is guided jointly by taxonomic rank, evolutionary complexity, and research objective, rather than by convenience or convention alone (Table 2).

PERSPECTIVES AND CONTROVERSIES

Transitioning to Genomic Toolkits: Multilocus and Bioinformatic Advances

Given the inherent limitations of single-gene markers in plants, research has shifted toward multilocus and genomic approaches to enhance species discrimination. This strategy balances the need for sequence variability with the use of conserved primer regions for reliable amplification (Rubinoff *et al.*, 2006). By leveraging multiple loci, researchers can achieve more accurate species delimitation even when utilizing short or degraded DNA fragments (Phillips *et al.*, 2022).

Recent technological and bioinformatic advancements have further strengthened this framework. Tools such as rCRUX, an open-source R package for building curated reference databases, significantly improve the accuracy of taxonomic assignment (Curd *et al.*, 2024). This transition toward genomic toolkits represents a logical progression in plant barcoding, offering the flexibility and robustness required to resolve complex biodiversity research questions that a single-locus approach cannot address (Faure and Joly, 2015; Phillips *et al.*, 2022).

Evolutionary and Biological Constraints of Plant DNA Barcodes

Furthermore, biological complexities such as ILS and frequent hybridization further undermine species discrimination. For instance, studies on *Salix* (Salicaceae) (Twyford, 2014) and British *Euphrasia* (Orobanchaceae) (Wang *et al.*, 2018) have demonstrated extensive haplotype sharing, while ancestral genetic variation

randomly distributed among descendant species has been observed to erroneously group taxa in *Crataegus* (Rosaceae) (Zarrei *et al.*, 2015) and *Carex* (Cyperaceae) (Clerc-Blain *et al.*, 2010). To overcome these limitations, combining multiple loci from both chloroplast and nuclear regions has proven more effective in addressing these evolutionary challenges and providing accurate species discrimination (Rubinoff *et al.*, 2006; Kress and Erickson, 2007; Wang *et al.*, 2011; Ojeda *et al.*, 2014). This integrated strategy leverages the complementary strengths of different loci to achieve higher resolution than single-locus barcoding.

Reflecting on the “Universal” Barcode Ideal: Should We Focus on Multilocus Approaches?

The concept of a single, universal DNA barcode for plants may be overly idealized and warrants reconsideration. Before pursuing a single solution, it is essential to clarify the intended use of DNA barcodes. For rapid screening, identification where phenotypic traits are insufficient (e.g., seed or seedling identification), detection of trace evidence (e.g., eDNA or metagenomics of environmental microbes), coarse assignments at the family or genus level, or quick identification of rare plant specimens at borders, a universal barcode is both practical and necessary (Valentini *et al.*, 2009). In forensic science or biosecurity, Sanger-based single-locus barcoding remains the 'gold standard' due to its cost-effectiveness and speed. However, for reconstructing interspecific phylogeny or conducting intraspecific population genetic studies, DNA barcoding often lacks sufficient genetic variation. In these cases, other molecular markers, such as SSR markers, are more appropriate (Selkoe and Toonen, 2006).

A universal DNA barcode for plants is fundamentally flawed, as it is effective in groups that are already easily identified morphologically and thus adds little identification value. Although single-locus barcoding is less expensive (Li *et al.*, 2015), its limited discriminatory

**Table 3** Comparing the cost, applicability, and resolution of different DNA barcoding technologies.

Method	Typical Cost per Sample (USD)	Data Scale	Resolution	Typical Use	Reference
Sanger (<i>rbcL</i> + <i>matK</i>)	5–10	1–2 loci	Moderate	Species ID	(CBOL Plant Working Group <i>et al.</i> , 2009)
Illumina MiSeq (amplicon)	20–40	10–100 loci	High	Metabarcoding	(Coissac <i>et al.</i> , 2016)
Target capture / plastome	50–80	Hundreds of loci	Very high	Phylogenomic studies	(Liu <i>et al.</i> , 2021b; Manzanilla <i>et al.</i> , 2022)
Oxford Nanopore (MinION)	30–50	Variable	High	Field ID / rapid test	(Srivathsan <i>et al.</i> , 2021)

power makes it a poor investment (Mahadani *et al.*, 2022; Kimta *et al.*, 2025). In contrast, genome-wide and multilocus strategies, while more costly, provide greater accuracy and enable the development of species-specific markers, making them a more effective solution (Hollingsworth *et al.*, 2016; Liu *et al.*, 2022).

Advances in sequencing technologies have made whole-chloroplast genome sequencing more accessible, providing a "super-barcode" for high-resolution identification (Yang *et al.*, 2014; Li *et al.*, 2015). However, this method is not yet cost-effective for routine use in general laboratories. Given the limitations of single-locus barcodes and the advantages of multilocus approaches, it is advisable to prioritize multilocus strategies for plant DNA barcoding. These methods offer higher resolution and flexibility, making them better suited to the complexity of plant species identification. Combining loci such as *rbcL*, *matK*, *trnH-psbA*, and ITS, along with advances in whole-genome sequencing and metabarcoding, provides a strong foundation for future plant barcoding efforts (Fazekas *et al.*, 2008; CBOL Plant Working Group *et al.*, 2009; Li *et al.*, 2015; Arulandhu *et al.*, 2017; Liu *et al.*, 2022).

COST AND PRACTICALITY OF GENOMIC BARCODING APPROACHES

HTS technologies provide exceptional resolution, though their cost-effectiveness compared to traditional methods remains a concern. Conventional Sanger sequencing of two plastid loci (*rbcL* and *matK*) typically costs USD 5 to 10 per sample. In contrast, amplicon-based Illumina MiSeq sequencing ranges from USD 20 to 40 per sample, depending on multiplexing. Shotgun or target-enrichment sequencing costs USD 50 to 80 per sample and enables recovery of hundreds of loci (Table 3). When accounting for labor in multiple PCRs and data curation, HTS approaches become competitive for studies involving hundreds of individuals (Dopheide *et al.*, 2025). Portable devices like the Oxford Nanopore MinION further reduce barriers to in-field species identification (Srivathsan *et al.*, 2021). Although initial costs are higher, genomic barcoding offers scalability and efficiency once established.

The effectiveness of plant barcoding also relies on the quality of reference databases. Platforms such as BOLD Systems, GenBank, and QIIME 2 support sequence comparison, but challenges remain, including mis-

annotation, geographic bias, and limited haplotype coverage (Phillips *et al.*, 2022; Chorlton, 2024). Current best practices recommend building local reference libraries from verified specimens and incorporating metadata to enhance traceability (Chorlton, 2024). Automated pipelines that use quality filters and probabilistic classifiers further improve accuracy in barcoding and metabarcoding workflows.

Recent advancements demonstrate a convergence of DNA barcoding, population genomics, and environmental monitoring. Metabarcoding and eDNA sequencing allow detection of entire plant communities from diverse samples, thereby transforming biodiversity assessments (Ruppert *et al.*, 2019). Integrating machine learning with barcode datasets enables automated species assignment (Nanni *et al.*, 2025). As reference libraries expand and sequencing costs decrease, plant DNA barcoding is becoming a multifunctional tool for ecological, evolutionary, and conservation research at unprecedented scales.

CONCLUSION

Future plant DNA barcoding should integrate multilocus and genomic data with advanced computational tools to achieve more precise and scalable species identification. NGS, eDNA, and machine learning will expand taxa coverage and enhance reliability. Maintaining robust and up-to-date databases such as GenBank is essential as genetic data grows. Effective plant biodiversity research requires collaboration among taxonomists, molecular biologists, and ecologists. Since no single DNA barcode captures the full diversity of plant genomes, research should prioritize integrative, genome-informed frameworks that use advanced sequencing and comprehensive reference libraries to support high-resolution, scalable identification and biodiversity monitoring.

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