IJHAS
International Journal of Health and Allied Sciences
Official Publication of JSS Academy of Higher Education and Research (JSS AHER), Mysuru

Volume 13 | Issue 1 Article 4

Curating And Validating Universally Applicable Primers For Efficient ITS2-Based DNA Barcoding Across Plant Taxa

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Recommended Citation
Bhat, Ajay R; Hegde, Subrahmanya; Kammar, Supriya S; Muthamma, MB; Mudgal, Gaurav; H, Prashanth Babu; Charukesi, Rajulu; and Mohan, Thotegowdanapalya C (2024) "Curating And Validating Universally Applicable Primers For Efficient ITS2-Based DNA Barcoding Across Plant Taxa," *International Journal of Health and Allied Sciences*: Vol. 13: Iss. 1, Article 4.
DOI: 10.55691/2278-344X.1087
Available at: https://rescon.jssuni.edu.in/ijhas/vol13/iss1/4

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Cover Page Footnote

Acknowledgments: This work is supported by K-FIST (L1) Vision Group on Science and Technology, Govt of Karnataka, SERB-Startup Research Grant, Science and Engineering Research Board, Department of Science and Technology, Government of India, Global Challenges Research Fund (GCRF), United Kingdom, in collaboration with the University of Warwick, United Kingdom and JSS Academy of Higher Education and Research, Mysuru. We thank the Centre of Excellence in Molecular Biology and Regenerative Medicine (CEMR) headed by Dr Subbarao, JSS Medical College for providing extended laboratory support.

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ORIGINAL STUDY

Curating and Validating Universally Applicable Primers for Efficient ITS2-based DNA Barcoding Across Plant Taxa

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Abstract

Rapid and accurate identification of plant species increasingly seeks to employ molecular techniques. The ITS2 region is highly regarded in DNA barcoding due to its short length and ease of sequencing, making it an ideal candidate for species identification. In this study, through meticulous analysis and comparison of primer sequences across a wide range of plant taxa, we curated a collection of primers with demonstrated universality, capable of efficiently amplifying the ITS2 region across diverse plant species. To validate the universality of the identified primers, we employed both *in silico* and *in vitro* approaches. *In silico* analysis involved bioinformatics tools to assess primer binding sites across a vast array of plant DNA sequences available in public databases. Subsequently, *in vitro* experiments were conducted to verify the amplification success of the primers using DNA samples extracted from various plant specimens. Through this comprehensive validation process, we ensured the reliability and applicability of the selected primers for DNA barcoding purposes. The significance of our findings lies in the establishment of a standardized approach for DNA barcoding using the ITS2 region, which facilitates accurate and efficient plant species identification. By providing researchers with a universally applicable set of primers, we aim to streamline the primer selection process, thereby reducing the time and effort involved in experimental design. This standardized protocol promotes consistency and reproducibility in DNA barcoding studies, ultimately advancing our understanding of plant biodiversity and aiding in conservation efforts.

Keywords: ITS2, DNA barcoding, DNA markers, Species characterization, Identification, Conservation, Taxonomy

1. Introduction

The identification and taxonomic classification of plant species is essential for their usage, and conservation of biodiversity. It has historically relied on morphology-based techniques, where taxonomists grouped plants primarily using observable traits like floral characteristics, fruit morphology, growth habits, and other structural features [1,2]. This formed the basis for taxonomy systems proposed by pioneering botanists including Carl Linnaeus, who established the binomial nomenclature for naming species. Well, in its inception, primitive major explorations exercised sampling and maintenance of voucher records in the herbarium. Advancements in quantitative approaches like morphometrics enabled more rigorous analysis of morphological and anatomical variation by measuring and analyzing characters like leaf shape, colour, venation patterns, palynology, phenology,

Received 22 July 2024; revised 9 August 2024; accepted 17 August 2024.
Available online 9 October 2024

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https://doi.org/10.55691/2278-344X.1087

and trichome distribution [3]. Later, naturalists also considered Information about plants' habitat preferences, geographic distribution, and ecological interactions to enable clues for taxonomic classification, especially when combined with other sources of data [4–7]. However, such classification systems built solely on visual assessment of morphology and/or developmental changes had limitations in accurately demarcating boundaries between closely related plant taxa. More often studies have shown that plants document phenotypic plasticity in response to environmental cues [8]. Therefore, for rapid and accurate identification of plant species, the usage of molecular techniques was becoming increasingly essential [9,10]. With time and involvement from core taxonomist communities, morphometrics-based plant identification and characterizations also had seen various chronological advancements with technological interventions and the new normal with the use of various biochemical and molecular markers such as the use of isozymes, cytology, pigments, phytoliths, digital imprints [11–21] With advancements in communication technologies, globalization and digitization and as well additionally the use of internet based cloud computing and archival and social networking platforms has also vastly aided in consistently solving various taxonomical issues [22]. Chemotaxonomy also emerged as an aid to traditional taxonomy, utilizing biochemical techniques to profile secondary metabolites and other chemical constituents in plants to elucidate evolutionary relationships [23–26]. However, like with plant morphology, phytochemical profiles can also demonstrate considerable plasticity in response to environmental factors, constraining the reliability of classification frameworks derived from these approaches alone [27–29].

Molecular techniques instigated a phylogenetics revolution in plant systematics by allowing direct examination of heritable genomic regions. Protein electrophoresis was one of the earliest molecular approaches for systematic analyses of genetic diversity [30,31]. The more modern normal links genes to related taxonomical profiles [32]. This paved the way for DNA-DNA hybridization studies which measured the degree of reassociation between DNA sequences from different specimens to assess genetic distance and relatedness. The advent of PCR and DNA sequencing enabled plant taxonomists to efficiently analyze specific chloroplast and nuclear gene regions and non-coding spacers to reconstruct evolutionary relationships [33–37]. This marked a pivotal shift in plant systematics from classification founded predominantly on morphology to frameworks reflecting true phylogenies.

DNA barcoding has now transformed and standardized workflows in plant taxonomy by utilizing sequence diversity in short standardized gene regions for species identification. For plants, the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (Fig. 1) has emerged as the optimal barcode marker compared to other widely used loci like rbcL, matK, and trnH-psbA [38,39]. The ITS is a spacer region situated between the genes encoding the smaller and larger subunits of rRNA. Specifically, ITS1 is positioned between the 18s rRNA and 5.8s rRNA genes, spanning an average length of 600 base pairs. This and the whole ITS region have previously been in prominent use for plant-specific barcoding. However, ambiguities were witnessed with the long lengths and more so because of that unintended amplification from fungal contaminants, expected with the sample in the event of PCR amplification. The latter is due to the substantial ITS sequence similarity between plants and fungi. In particular, the ITS2 spacer exhibits high interspecific divergence, permitting clear discrimination between even closely related plant species with up to 97% identification success rate [40,41], however, demonstrates conserved primer binding sites in regions with flanking 5.8S and 26S rRNA genes, enabling universal primers to amplify ITS2 across diverse taxa. The short sequence length (~400 bp) further enables easy and economical sequencing of the ITS2 barcode [42,43,45]. Public databases like BOLD and NCBI GenBank contain ITS2 barcodes for nearly 50% of cataloged plant species, highlighting the expanding adoption of ITS2 barcoding [44].

Equipped with PCR, sequencing, and molecular phylogenetics, plant taxonomists can now accurately reconstruct evolutionary histories and confidently assign unknown plant specimens to species

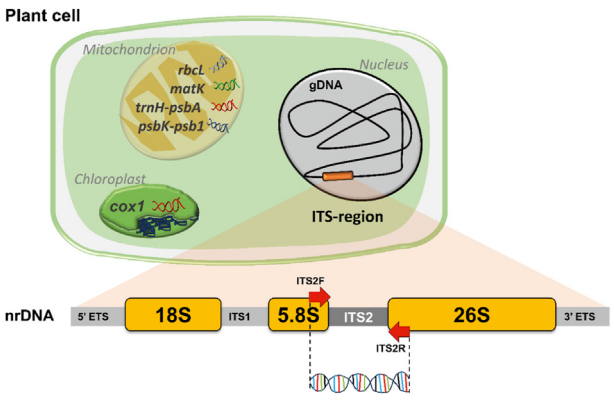

Fig. 1. Schematic representation of ITS region of the nuclear ribosomal RNA (nrRNA) genes. Abbreviated here are, gDNA, genomic DNA, ETS- External Transcribed spacer, ITS1 and ITS2 – Internal Transcribed spacers, ITS2F and ITS2R – Forward and Reverse primers to amplify ITS2 region.

or lineages within a rigorous phylogenetic framework. DNA barcoding using standardized universal ITS2 primers has streamlined and simplified workflows, providing efficient, reliable plant identification to build comprehensive inventories of global plant biodiversity [46]. Ascribed to this, in the recent decade, ITS2 has been accepted as the ideal DNA barcode by the plant scientist community and largely used to identify and classify a wide range of plants from weeds to medicinal plants [47–49].

To ensure the practicality of DNA barcoding, the availability of universal primers is paramount for users' convenience. Before the widespread acceptance of ITS2 as an optimal barcode, researchers employed various ITS2 primers for plant identification. Presently, the literature documents great variabilities in ITS2-based primer selections over 20 primer sets for ITS2 (Table 1), posing a challenge for novices in primer selection, leading to time constraints and ambiguity. Many different primer pairs

Table 1. List of primers used by different researchers to amplify ITS2.

Primer set number	Primer sequence	Reference
1.	ITS2 F - ATGCGATACTTGGTGTGAAT ITS2 R - GACGCTTCTCCAGACTACAAT	[39,40,42,43,50–61]
2.	ITS2 F - GGGGCGGATATTGGCCTCCCCTTG ITS2 R - GACGCTTCTCCAGACTACAAT	[62]
3.	ITS2 F - ATGCGATACTTGGTGTGAAT ITS2 R - TCCTCCGCTTATTGATATGC	[63]
4.	ITS2 F - ATGCGATACTTGGTGTGAAT ITS2 R - GACGCTTCTCCAGACTACAT	[64]
5.	ITS2 F - ATGCGATACTTGGTGTGAATTATAGAAT ITS2 R - GACGCTTCTCCAGACTACAAT	[65]
6.	ITS2 F - ATGCGATACTTGGTGTGAATTATAGAAT ITS2 R - GACGCTTCTCCAGACTACAAT	[66]
7.	ITS2 F - AATGATACGGCGACCACCGAGATCTACAC ITS2 R - ACACTCTTTCCCTACACGACGCTCTTCCGATCT	[67]
8.	ITS2 F - AACCTGCGGAAGGATCATTGTC ITS2 R - TGATATGCTTAAACTCAGCGGGTA	[68]
9.	ITS2 F - CCCGTGAACCATCGAGTCTTT ITS2 R - GACGGCTCGCCTCTCAAC	[69]
10.	ITS2 F - ATGCGATACTTGGTGTGAAT ITS2 R - TCCTCCGCTTATTGATATGC	[70]
11.	ITS2 F - GCGATACTTGGTGTGAAT ITS2 R - GACGCTTCCCAGACTAAAT	[47]
12.	ITS2 F - GCATCGATGAAGAACGCAGC ITS2 R - TCCTCCGCTTATTGATATGC	[71]
13.	ITS2 F - AGGAGAAGTCGTAACAAGGT ITS2 R - TCCTCCGCTTATTGATATGC	[72]
14.	ITS2 F - GGAAGTAAAAGTCGTAACAAGG ITS2 R - TCCTCCGCTTATTGATATGC	[73]
15.	ITS2 F - AGGAGAAGTCGTAACAAG ITS2 R - GTTTCTTTTCCTCCGCT	[74]
16.	ITS2 F - GGAAGTAAAAGTCGTAACAAGG ITS2 R - TCCTCCGCTTATTGATATGC	[75]
17.	ITS2 F - ATGCGATACTTGGTGTGAATTATAGAAT ITS2 R - GACGCTTCTCCAGACTACAAT	[76]
18.	ITS2 F - ATGCGATACTTGGTGTGAAT ITS2 R - TCCTCCGCTTATTGATATGC	[77]
19.	ITS2 F - CGTAGCGAAATGCGATACTTGGTG ITS2 R - TCCTCCGCTTATTGATATGC	[78]
20.	ITS2 F - TCTCGCATCGATGAAGAACG ITS2 R - CCATGCTTAAACTCAGCGGGT	[79]
21.	ITS2 F - CCTTATCATTTAGAGGAAGGA ITS2 R - TCCTCCGCTTATTGATATGC	[43]
22.	ITS2 F - ATGCGATACTTGGTGTGAAT ITS2 R - GACGCTTCTCCAGACTACAAT	[42]
23.	ITS2 F - GTAGGTGAACCTGCAGAAGGATCA ITS2 R - CCATGCTTAAACTCAGCGGGT	[80]
24.	ITS2 F - GCATCGATGAAGAACGCAGC ITS2 R - TCCTCCGCTTATTGATATGC	[81]

have been reported for amplifying the ITS2 region in plants. The high variability in ITS2 primer choice is largely due to the lack of standardized universal primers in early DNA barcoding studies. Different research groups often designed and optimized primers for their specific set of taxa. For example, Chen and coworkers tested 17 existing ITS2 primer pairs a decade or more ago and found their universality across diverse plant taxa was quite low, ranging from 6.4% to 53.6% of PCR success [40]. This highlighted the need for truly universal ITS2 primers. In the last 5 years or so, the plant barcode community has coalesced around a core set of universal ITS2 primers for seed plants: ITS-S2F, ITS-S3F, ITS4R, ITS-B, and ITS-u3. These have demonstrated high PCR and sequencing success rates across thousands of species in multiple studies. However, many researchers continue to use their custom ITS2 primers or some of the older sets or at the latest designed by Chen's group [40]. So, there are certainly there exist 20 or more different ITS2 primer pairs reported. Other than this, some later reviews published in the year 2015 have compiled some of the most used ITS2 primers. For example, Wang et al. listed 21 ITS2 primer pairs. So, the convergence on a core set of primers is only sparingly reported. Thus, our manuscript conducts an exhaustive literature review, identifying a universal primer set, and validates it through meticulous *in silico* and *in vitro* analyses.

2. Materials and methods

2.1. Finding ITS2 primers, retrieval of ITS sequences, and in silico validation

All the ITS2-specific primers were obtained from the available literature in Pubmed. The primers mentioned in the literature were collected and summarized as shown in Table 1. The ITS2 sequences for the different plant species of 20 different families were downloaded in the FASTA format from the ITS2 database (http://its2.bioapps.biozentrum.uni-wuerzburg.de) and NCBI (National Centre for Biotechnology Information) database. The respective ITS2-specific complete rRNA sequences were obtained from GenBank. Multiple alignments of retrieved rRNA sequence and the primers were done using the BioEdit tool.

2.2. Confirmation of universality of ITS2-specific primes using PCR

Genomic DNA isolation and quantification. Total genomic DNA (gDNA) was isolated from the fresh juvenile leaf tissues of the 3 plant species (Rice, Banana, and Almond) using a gDNA extraction kit (Qiagen DNeasy® Plant Mini Kit) by following the manufacturer's protocol. The isolated gDNAs were quantified using a nanodrop spectrophotometer (Denovix DS-11) and quality was assessed via agarose gel electrophoresis (Agarose concentration 0.8%) (Bio-Rad Laboratories, India). Total gDNA concentration adjusted to 100 ng μL−1 was used for PCR amplification with primer Set 1 ITS2 primers (Table 1).

3. Results

3.1. In silico analysis of ITS2-specific universal primers

The multiple sequence alignment of primer set number-1 against these rRNA sequences of various plant species using the Bioedit tool showed that both the forward (Fig. 2a) and reverse (Fig. 2b) primers have showed >90% and >95% sequence similarity respectively to the rest of the sequences. Moreover, the 3′ end of the primer set-1 shows no dissimilarity which is important for stringent primer binding during the PCR. Our *in-silico* analysis shows that the primer set-1 could be used as a universal primer for the ITS2 marker to perform barcoding of plant species.

3.2. In vitro validation of ITS2-specific universal primers using PCR

To experimentally prove the universality of the ITS2-specific universal primers, we have performed polymerase chain reaction (PCR) using primer set 1 for rice, banana, and almond plants. In all the 3 plant species we have observed good amplification at approximately 500 bp of ITS2 region (Fig. 3). Our results confirm that researchers can utilize the primer set-1 as a universal primer to perform DNA barcoding in diverse plant species.

4. Discussion

The development of an optimal DNA barcode is of utmost importance for its widespread applicability across a range of plant species. An ideal barcode should contain significant species-level genetic variability. It should exhibit good PCR amplification efficiency by using a universal primer, enabling the use of a single set of primers for barcoding across different species. Ideally, the chosen universal primers should amplify a short sequence of 600–700 bp for easy sequencing and *In-silico* analysis [82]. In

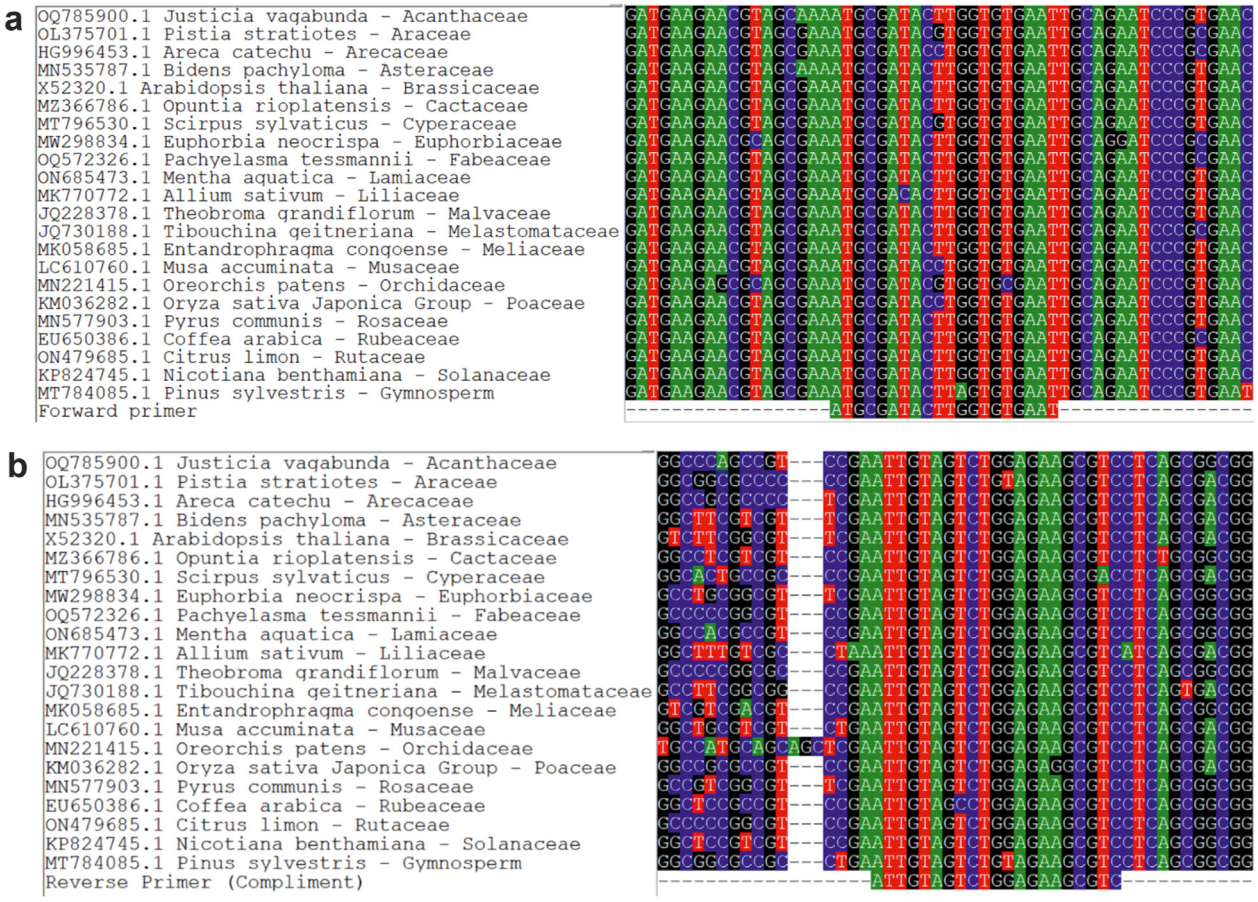

Fig. 2. ***Multiple sequence alignment of ITS2 region.*** *Specific alignments with* ***a****, forward primer; and* ***b****, reverse primer with the corresponding rRNA sequences of 20 different family plant species.*

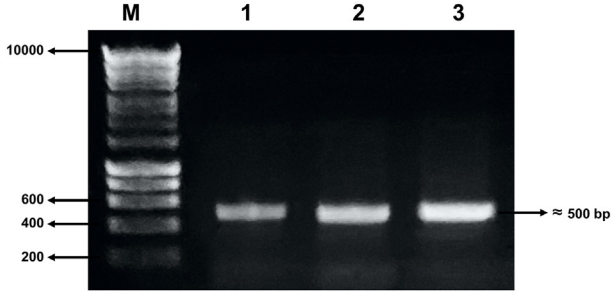

Fig. 3. ***PCR amplification of ITS2 region.*** *Using primer set-1. Total DNA extracted from Rice, Almond, and banana plants was used as the template. Amplicon length is ≈ 500 bp; M—10 kb Marker, For amplicons in Lane 1- Rice, Lane 2- Almond, and Lane 3- Banana.*

the recent past, the ITS2 marker has been specifically used as a core DNA barcode for plants. Nonetheless, to our knowledge, there is no such universal primer being adopted by the researchers for the ITS2 marker. To identify and suggest a universal set of primers for ITS2 sequence-based identification, we did an extensive survey of the literature with works published from 1990 to 2023. We have referred to more than 40 published research papers of these times and could ascertain as a consensus the use of some 34 distinct sets of primers for the ITS2 patterning (Table 1). Out of 42 researchers, 18 researchers have used the primers set number 1 (Table 1). The rest of the researchers have used different combinations of forward and reverse primers for ITS2 as shown in Table 1. Our analysis indicates that novice researchers may find it challenging to identify and select an appropriate primer set for their experiments Therefore, to find a universal set of primers we have performed *in silico* analysis and *in vitro* validation of the available set of primers. Our literature analysis found that 18 researchers have used the ITS2 primer set number 1 (Table 1) to perform barcoding in various plant species. Therefore, to analyze the universality of these primers, and to identify an ideal single set of universal primers for ITS2, we have compared the suitability of both reverse and forward primers across different species of plants. Utilizing the ITS2 database [83] and the NCBI database we have obtained rRNA sequences of representative plants

Table 2. List of families used for validation by multiple sequence alignment.

Sl No.	Family
1	Acanthaceae
2	Araceae
3	Arecaceae
4	Asteraceae
5	Brassicaceae
6	Cactaceae
7	Cyperaceae
8	Euphorbiaceae
9	Fabaceae
10	Lamiaceae
11	Liliaceae
12	Malvaceae
13	Melastomataceae
14	Meliaceae
15	Musaceae
16	Orchidaceae
17	Poaceae
18	Rosaceae
19	Rubeaceae
20	Rutaceae
21	Solanaceae
22	Pinaceae

from the top 22 major plant families, accounting for over 150,000 accepted species of plants, including 21 angiosperm (flowering plant) families and 1 gymnosperm (non-flowering seed plant) family (Table 2). These plant families represent a significant portion of the world's plant diversity [84].

5. Conclusions

The conventional taxonomic methods face considerable challenges, especially in differentiating closely related species. The advent of molecular techniques, notably DNA barcoding, has significantly advanced the field of taxonomy by providing a reliable, efficient, and quick method for species identification. The ITS2 region, identified as an ideal DNA barcode due to its shorter length and ease of sequencing, has gained widespread acceptance within the scientific community. One of the critical challenges in utilizing ITS2 for DNA barcoding has been the selection of appropriate universal primers. A major drawback in using ITS as a barcode is the occurrence of polymorphic ITS regions and pseudogenization is infrequently observed in a few species [85]. The extent to which these issues might significantly affect a substantial portion of barcoding studies remains uncertain [86,87]. In instances where this uncommon problem arises, Next Generation Sequencing (NGS) emerges as the viable solution [85]. With an extensive literature review, our study has not only identified a reliable set of universal primers but has also validated their universality through *in silico* and *in vitro* analyses. Our results demonstrate the practicality and efficacy of a specific primer set (primer set-1) (Table 1) for DNA barcoding across a diverse range of plant species. By providing a validated universal primer set, our research contributes to facilitating researchers, particularly novices, in streamlining their DNA barcoding experiments and contributing to plant biodiversity studies, conservation efforts, and broader ecological research. Our results contribute towards enhancing our understanding of plant diversity and ultimately support global biodiversity conservation initiatives.

Funding

This work is supported by K-FIST (L1) Vision Group on Science and Technology, Govt of Karnataka. SERB-Startup Research Grant, Science and Engineering Research Board, Government of India. Global Challenges Research Fund (GCRF), United Kingdom, in collaboration with the University of Warwick, United Kingdom. And JSS Academy of Higher Education & Research, Mysuru.

Ethics statements

Not applicable.

Acknowledgments

We thank the Centre of Excellence in Molecular Biology and Regenerative Medicine (CEMR) headed by Dr Subbarao, JSS Medical College for providing extended laboratory support.

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