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Assessing the Phylogenetic Relationships of *Baliospermum solanifolium* [*B. montanum* (Willd.) Müll. Arg.] in South India through DNA Barcoding

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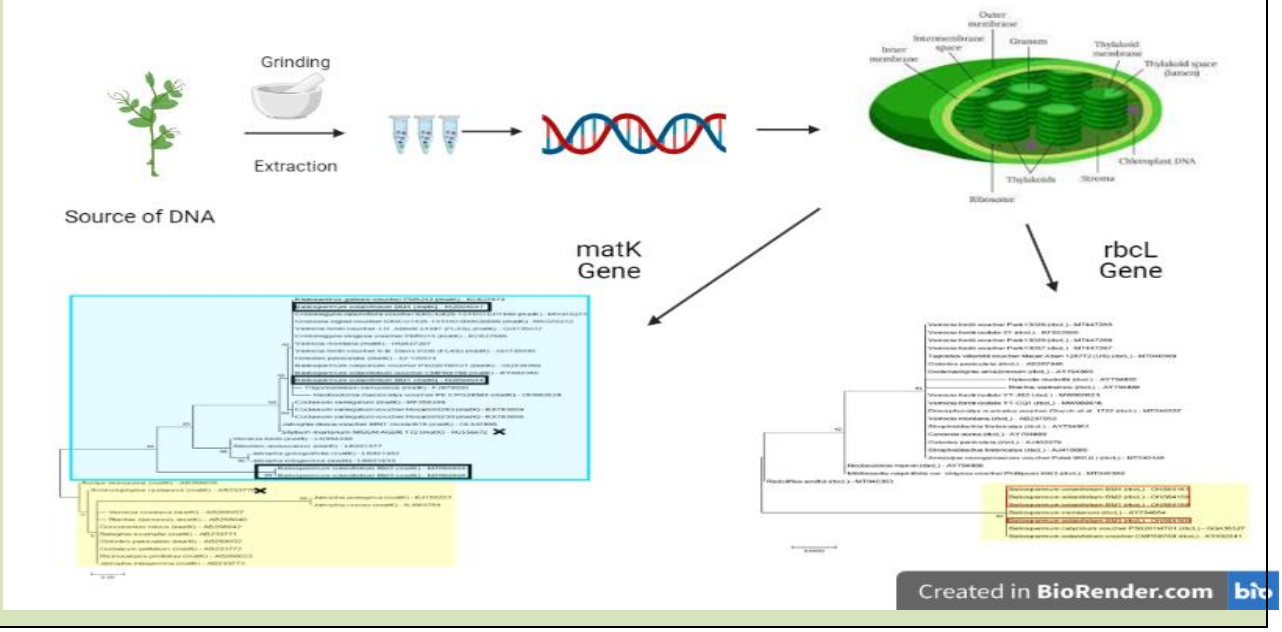
ABSTRACT: The near-threatened *Baliospermum solanifolium* [*B. montanum* (Willd.) Müll. Arg.] plant, found in several locations throughout India's sub-Himalayan region, from the Khasi Hills to Kashmir, was the subject of a DNA barcode investigation. The links between the many species in this genus have been established via morphological research, but there haven't been many attempts to study the evolutionary history at the molecular level. Plant DNA barcodes make the information accessible to the general public and aid in the understanding, preservation, and use of the world's biodiversity. Since a single universal barcode gene is not available, the main challenge is identifying the suitable barcoding gene for our sample. The chloroplast genes *matK* (maturase K) and *rbcL* (RuBisCo) were utilized in the study, with leaves DNA serving as the template for amplification. The samples of the *B. solanifolium* plant collected from four different geographic locations in South India were analyzed for their phylogenetic diversity. MEGA 7.0 was used for the phylogenetic analysis using maximum likelihood and neighbor-joining models with 1000 bootstraps. BLASTn search results indicated that the plastid *rbcL* and *matK* gene sequences were consistent with *B. solanifolium* as the source material and aligned using MUSCLE, in which Maximum Likelihood (ML) and Neighbor-Joining (NJ) trees were produced. The tree shows likely monophyletic for rbcL and divergence in the *matK* gene. This work suggests that the *rbcL* gene is a promising option for DNA barcoding of the *B. solanifolium* plant which helps in understanding the biodiversity.

Keywords: *Baliospermum solanifolium* (*B. montanum*), *matK*, *rbcL*, DNA barcoding, phylogenetic tree analysis.

Graphical Representation



INTRODUCTION

India's diverse ecosystem is home to many indigenous, rare, threatened, and endangered plant species. Many of the phytonutrients found in medicinal plants possess cytotoxic, phytotoxic, antibacterial, anti-inflammatory, antioxidant, and phytotoxic properties (Kotan *et al.,* 2013). Euphorbiaceae plants have been utilized by the world's population for their health and welfare. The spurge family (Euphorbiaceae) consists of 8000 species and 300 genera (Mwine and Van Damme 2011). Forty-seven genera of the Euphorbiaceae family have therapeutic potential, and southwest Asia is one of the most abundant regions and significant sites of variety for the Euphorbiaceae (Nisar *et al.,* 2011).

Baliospermum montanum (Euphorbiaceae), a leafy monoecious under-shrub found in scattered regions across India from the Khasi Hills to Kashmir, is most commonly found in wild areas of Bihar, West Bengal, the peninsula, and central India. Despite its wide distribution, the plant is classified as "Lower Risk near threatened" (LRnt) or "Near Threatened" (NT) by the IUCN observational status (Kumari *et al.,* 2012). This small tree or shrub can grow up to 4 meters tall and is characterized by its upright, rigid, and thickly branching growth habit. The root, leaves, and seeds of *B. montanum* have been used in Ayurvedic medicine to treat various health issues, such as skin problems, worm infections, gastrointestinal disorders, liver disorders, poisoning from snake bites, and stomach disorders (Johnson and Manickam 2003; Mali and Wadekar 2008). In addition, the root and its extracts have been found to have potential chemopreventive effects against colorectal cancer in combination with alcoholic extracts of *Annona squamosa* and *Bacopa monnieri* (Nandagaon and Kulkarni 2013). Animal studies have also shown that the alcoholic extract of the plant has hypotensive effects, reducing blood pressure (Johnson and Manickam 2003).

The identification of plants at the DNA level, known as molecular taxonomy, is a more reliable method than traditional techniques like macroscopic, microscopic, and chemical profiling (Schindel and Miller 2005). Genetic diversity is the foundation for species diversity and plays a crucial role in the study of a species. The abundance and distribution of a plant can impact its ability to evolve. The scientific community has made significant efforts to identify the best DNA regions for barcoding all plant species in a standardized manner across the globe (Kress *et al.,* 2005; Kress and Erickson 2007; CBOL, 2009; Yao *et al.,* 2009; Chen *et al.,* 2010). As a result, more herbs, and medicinal plants along with their products are being identified and validated using the plant DNA barcoding method (Hebert *et al*., 2003; China Plant BOL Group, 2011; Kool *et al*., 2012; Newmaster *et al.,* 2013; Raclariu *et al.,* 2017). According to numerous studies, barcoding has been found to accurately identify approximately 75–85% of species and over 90% of floras at the species level (Lahaye *et al.,* 2008; Kress *et al*., 2009 & 2010; Chen *et al*., 2010; Burgess *et al.,* 2011). Many studies have evaluated the effectiveness of DNA barcoding for cataloging species, monitoring biodiversity, authenticating traded medicinal herbs, and measuring the success of conservation efforts (Nithaniyal *et al.,* 2014).

Only a small portion of the 380,000 species of land plants, including ferns (13,000 species), angiosperms (352,000 species), gymnosperms (1,300 species), bryophytes (mosses, hornworts, liverworts), and other seed plants, have been identified and differentiated based on DNA sequences (Paton *et al.,* 2008). Nuclear, chloroplast, and mitochondrial genes have been extensively studied for variations in sequence at the genus level. The chloroplast's modest size, structure, and gene order are the product of uniparental inheritance and least recombination (Olmstead and Palmer 1994). Chloroplast genome sequencing is a useful tool for creating various DNA barcodes. Currently, the use of both coding and non-coding genes for systematics and evolution in flowering plants primarily relies on plastid DNA barcodes (Amar, 2020). DNA barcodes commonly used for plant DNA barcoding include *matK, rbcL* (ribulose-1,5-bisphosphate carboxylase oxygenase, large subunit), *ITS* (Internal Transcribed Spacer), *trnH-psbA* (Taberlet *et al*., 2007; Chase *et al.,* 2007; Fazekas *et al.,* 2008; CBOL, 2009; Naeem *et al.,* 2019; Vasa Dileep Reddy *et al.,* 2022) and a combination of plastid markers (such as *rbcL, matK*, and *trnH-psbA*) along with nuclear ribosomal DNA (*nrITS*). Furthermore, the plastid *trnH-psbA* intergenic spacer and the nuclear ribosomal RNA *ITS* have been widely used as supplemental loci (Alvarez and Wendel 2003; Bailey *et al.,* 2003; Razafimandimbison *et al*., 2004; Hao *et al*., 2004; Gonzalez *et al*., 2009). While amino acid sequences are acceptable for homologous genes from far-related organisms, DNA sequences are useful for comparing homologous genes from closely related organisms (Baldauf, 2003). Plants have highly conserved primordial organelles called chloroplasts (CP), which can be used to categorize and identify different types of plants (Zhang *et al*., 2020).

The *matK*(*orfK*) gene is located in the chloroplast within the *trnK* intron and has a length of 1500 bp (Selvaraj *et al.,* 2008), is involved in Group II intron splicing and is well conserved across the spectrum of plant systematics. The *matK* gene has desirable properties for DNA barcoding, including an optimal size, a high substitution rate, substantial variation at both the first and second codon positions, a low ratio of transitions to conversions, and the presence of a mutationally conserved region. These traits of the *matK* gene make it a useful tool for understanding plant relationships at the family and species levels. In plant systematic studies, the *rbcL* gene has been extensively analyzed for its potential (Ritland and Clegg 1987; Kim *et al*., 1992; Morgan and Soltis 1993; Olmstead *et al.,* 1993). Many researchers have also evaluated the *rbcL* gene sequence to understand plant systematics (Chase *et al.,* 1993). The use of two protein-coding genes, *matK*, and *rbcL*, as main plant DNA barcodes, has been

widely accepted in the past (Dong *et al.*, 2015). There have been very few studies on the phylogenetic relationships among species in the Euphorbiaceae family (Wurdack *et al.*, 2004; 2005). Earlier work on barcoding of a few Euphorbiaceae plants (*Jatropa curcas* L., *Jatropa gossypifolia* L., and *Ricinus communis* L.) through *ITS* gene had been reported (Pallavi Sahare and Srinivasu 2012). Arbitrary PCR-based DNA fingerprinting (RAPD) was employed for the DNA Fingerprinting analysis of *Baliospermum montanum* by Muazu *et al.*, (2016). As of now, no reports were recorded for *Baliospermum solanifolium* (*B. montanum*) on their phylogenetic analysis.

The current study aims to examine the genetic diversity and variation of *B. solanifolium* by collecting samples from diverse geographic locations in South India, comparing them with existing accessions, and demonstrating the phylogenetic relationship using the plastid *matK* and *rbcL* partial gene sequences.

MATERIALS AND METHODS

A. Collection of plant samples

The plant cuttings of *B. solanifolium* plants were collected from four different locations and maintained in the medicinal garden at Nandha Arts and Science College (NASC) in Erode, Tamil Nadu, India (Table 1 and Fig. 1). The plant species were identified and confirmed by the Botanical Survey of India (BSI) in Coimbatore Reginal Station, TNAU campus, Tamil Nadu, India before undergoing molecular analysis. The plant samples were designated as BM1 from Salem, Tamil Nadu (BSI/SRC/5/23/2021/Tech/189), BM2 from Palakkad, Kerala (BSI/SRC/5/23/2021/Tech/190), BM3 from Dindugal, Tamil Nadu (BSI/SRC/5/23/2021/Tech/191), and BM4 from Thrissur, Kerala (BSI/SRC/5/23/2021/Tech/192) (Table 2).

B. Plant DNA isolation

The DNA was extracted using the Nucleo Spin® Plant II Kit (Macherey-Nagel, GmbH & Co. KG, Germany) by adhering to the company's manual. The plant leaf samples weighing 100 mg were collected and homogenized using liquid nitrogen. Four hundred microliters of PL1 lysis buffer from the kit were added to the homogenized powdered sample and vortexed for 60 seconds. Ten microliters of RNase A solution were added and thoroughly mixed by inverting for 5 minutes. The homogenate was allowed to incubate at 65°C for 10 minutes. The sample was then transferred to a Nucleospin filter and centrifuged at 11000 × g for 2 minutes. The flow-through liquid was collected, and the filter was discarded. A total of 450 µl of PC buffer was added and thoroughly mixed. The solution was then fed into a Nucleospin Plant II column and centrifuged for 1 minute. The flow-through liquid was discarded. The column was filled with 400 µl of PW1 buffer and centrifuged at 11000 × g for 1 minute, with the flow-through liquid being discarded. Next, 700 µl of PW2 was added, centrifuged at 11000 × g, and the flow-through solution was discarded. Finally, 200 µl of PW2 buffer solution was added, and the column was centrifuged at 11000 × g for 2 minutes to dry the silica membrane. The column was transferred to a new 1.7-ml tube, and 50µl of PE buffer was added, followed by incubation for 5 minutes at 65 °C. The DNA was eluted from the column by centrifuging at 11000 × g for 1 minute. The recovered DNA was then stored at 4 °C for further examination.

C. Quantification of extracted DNA

The extracted DNA concentration was evaluated by the Nanodrop-2000 spectrophotometer. The quality of the isolated DNA was further analyzed using agarose gel electrophoresis (0.8%). To evaluate the DNA quality, 5 µl of it was mixed with 1 µl of a 6X gel-loading buffer, which included bromophenol blue (0.25%) and sucrose (30%) in TE buffer (pH 8.0). The 0.8% agarose gel was made using 0.5X TBE (Tris-Borate-EDTA) buffer, which contained ethidium bromide (0.5 g/ml). The DNA samples were loaded into the wells and subjected to electrophoresis using 0.5X TBE at 75V until the dye had reached the bottom of the gel. The gel was visualized under a UV transilluminator (Genei), and an image was captured and saved with the Gel Doc system (Bio-Rad) while illuminated by UV light.

D. Polymerase Chain Reaction (PCR)

(i) PCR Mixture. The PCR reaction was performed in a 50 µl volume. The reaction mixture consisted of 1µl of a mixture containing 50 ng of DNA, 5µl of 2X Phire Master Mix, 0.25 µl of each forward and reverse primer (10 picomoles), and 4 µl of distilled water. For BM1, BM3, and BM4, the *matK* forward primers MATK-XF (5'-TAATTTACGATCAATTCATTC-3') and reverse primer MATK-NR1 (5'-ACAAGAAAGGCGAAGTAT-3') were used (Sharma *et al.*, 2020). But for BM2, amplification was not achieved, so the MATK-ASWF (5'-CGATCTATTCATTCAATATTTC-3') and MATK-ASWR (5'- TCTAGCACACGAAAGTCGAAGT-3') were used instead. The RBCL-AF (5'-ATGTCACCACAAACAGAGACTAAAGC-3') and RBCL-724R (5'-TCGCATGTACCTGCAGTAGC-3') primers were tested for *rbcL* gene amplification.

(ii) PCR Amplification. The DNA amplification was carried out using a thermal cycler (Gene Amp PCR System 9700, Applied Biosystems). The PCR thermal profiles of *matK* and *rbcL* gene amplification were provided below.

***1. matK* (MATK-XF / MATK-NR1) primer:** 98°C for 30 s; 10 cycles of 98°C for 5 s, 45°C for 10 s,72°C for 15 s; 30 cycles of 98°C for 5 s, 50°C for 10 s,72°C for 15 s; and final extension of 72°C for 1 min.

***2. rbcL* (RBCL-AF/RBCL-724R) primer:** 98°C for 30 s;40 cycles of 98°C for 5 s, 58°C for 10 s, 72°C for 15 s; Final extension 72°C for 1 min.

(iii) Agarose gel electrophoresis of PCR products. The PCR products were evaluated using a 1.2% agarose gel prepared with 0.5X TBE buffer and 0.5 g/ml ethidium bromide. One microliter of the loading dye (6X) was mixed with 4 µl of the PCR products before loading. The sample was then run on an electrophoresis

system using 0.5X TBE buffer at 75V for 1–2 hours until the dye front reached the bottom of the gel. A 1 kb Plus DNA Ladder (2-log DNA ladder) (New England Biolabs® Inc) was used as a molecular reference. The gel was visualized using a UV transilluminator (Genei), and the image was captured using a gel documentation system (Bio-Rad) under UV light.

E. ExoSAP-IT Treatment

The PCR product mixture was treated with a combination of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP) ExoSAP-IT (GE Healthcare), to remove any unwanted primers and dNTPs without affecting any subsequent applications. 0.5 µL of ExoSAP-IT was added to 5 µL of the PCR product and then incubated at 37 °C for 15 minutes. The enzyme was then inactivated at 85°C for 5 minutes.

F. Sequencing using Big Dye Terminator v3.1 and Post-Sequencing PCR Cleanup

The sequencing reaction was performed in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing PCR mixture consisted of 1.9 µl of 5X sequencing buffer, 0.3 µl of each forward and reverse primer, 0.2 µl of the sequencing mix, 1 µl of the ExoSAP-treated PCR product, and 6.6 µl of distilled water. The procedure began with a 2-minute incubation at 96 °C, followed by 30 cycles of temperature ramping: 30 seconds at 96 °C, 40 seconds at 50 °C, and 4 minutes at 60 °C. The reaction was stored at 4 °C indefinitely after completion.

The post-sequencing PCR cleanup procedure involved combining 1 µl of 3M Sodium acetate, 0.1 µl of EDTA, 44 µl of 100% Ethanol, and 5 µl of distilled water. The following steps were taken to perform the cleanup: The ingredients of distilled water, 125 mM EDTA, 3M Sodium acetate (pH 4.6), and ethanol were combined and mixed thoroughly. The mixture (50 µl) was added to all wells of the sequencing plate containing the PCR product, and vortexed using a Mixmat vortex for thorough mixing. The mixture was incubated for 30 minutes at room temperature, then centrifuged at 3700 rpm for 30 minutes. The supernatant was discarded and 50 µl of 70% ethanol was added to all wells, followed by another centrifugation at 3700 rpm for 20 minutes. The process was repeated with a 70% ethanol wash and the supernatant was discarded. The pellet was then air-dried. The cleaned and air-dried product was then sequenced using the ABI 3500 DNA Analyzer (Applied Biosystems, USA).

G. DNA Sequence Alignment and Analysis

The quality of the obtained sequences was evaluated using the Sequence Scanner Software V1 (Applied Biosystems, USA). The raw sequences were edited and the low peak nucleotide signal was trimmed using the BioEdit tool 7.2. The partial *matK* and *rbcL* nucleotide sequences of all four plants were submitted to NCBI-GenBank and assigned accession numbers were provided in Table 2 .The *B. montanum* plants were searched in BOLD Systems for similar work (www.boldsystems.org/index.php/Public_SearchTerms) (Ratnasingham and Hebert 2007). The *matK* and *rbcL* sequences from four different locations of *B.solanifolium* were selected for further analysis and subjected to BLASTn analysis. The sequences were aligned and compared using the BioEdit Sequence Alignment Editor (Hall, 1999) and MUltiple Sequence Comparison by Log-Expectation (MUSCLE) (Thompson *et al.*, 1994) software programs since MUSCLE is claimed to achieve both better average accuracy and better speed than ClustalW2 or T-Coffee (Edgar, 2004).

H. Nucleotide composition study and A/T to G/C %

The *matK* and *rbcL* genes of the geographically different areas were subjected to nucleotide composition study(www.endmemo.com/bio/dnalength.php)and A/T to G/C % (www.biologicscorp.com/tools/GCContent/#.Yys203ZBzIW) for our original BM1, BM2, BM3, and BM4 datasets.

I. Phylogenetic Tree Generation

A phylogenetic tree was created to identify the relationships between the selected organisms' gene sequences. The results of the Neighbor-Joining (NJ) method were fast and quick, but the Maximum Likelihood (ML) approach is technically more accurate by a wide margin. In MEGA 7.0 software(www.megasofware.net), the ML and NJ methods were used for the dendrogram construction for both the *matK* and *rbcL* genes separately (Sneath and Sokal 1973; Saitou and Nei 1987; Tamura *et al.*, 2013).

J. General QR and Barcode

Generally used barcode patterns(www.biorad-ads.com/DNABarcodeWeb) and QR codes (www.the-qrcode-generator.com)were generated for the *matK* and *rbcL* genes for our samples. The QR codes can retrieve, and use the sequence data makingit relatively simple for basic users.

RESULTS AND DISCUSSION

The indigenous population in the Himalayan region utilizes Euphorbiaceae plants for various medicinal purposes, including their antibacterial, anticancer, antioxidant, antiplasmodial, antidiabetic, anthelmintic, and phytotoxic properties (Ali Hazrat *et al.,* 2020**).** These plant parts are a crucial component in many Traditional Thai Medicines (TTM), used to treat joint and muscle discomfort (Pinsornsak *et al.,* 2015**).** Research on *B. solanifolium* has shown that the solvents and aqueous extract of roots have antibacterial, free radical scavenging, immunomodulatory, hepatoprotective, anticancer, and anthelmintic properties (Ravindra and Raju 2008). Additionally, it has been reported that leaves are effective in treating abdominal tumors (Chopra *et al.,* 1994), bronchitis, and asthma (Nadkarni, 1988). Many domestic and international pharmaceutical companies utilize plant-

based formulations to treat a range of ailments and diseases globally (Johnson *et al.,* 2010). Research on the *B. solanifolium* (Euphorbiaceae) may be given a lot more relevance if species-level identification and phylogenetic analysis were conducted. The species-level identification of many Euphorbiaceae, however, is challenging based on herbarium specimens due to lacking fruit or floral material in all seasons. Moreover, due to adverse growth circumstances and conditions, individual plants only blossom perennial or at intervals of once in 2-3 years. DNA barcoding may be a great aid in identifying the species or clades as a supplement to other methods.

Plant products sold in the market as powders, roots, and barks can be identified by morphology, smell, color, and taste, the majority are not verifiable (Posthouwer *et al.,* 2018). Plant identification at the DNA level (Molecular taxonomy) is more reliable than more conventional identification techniques (macroscopic, microscopic, and chemical profiling). Genetic diversity serves as the basis for species diversity and is a crucial prelude to the study of a species. The abundance and distribution of any plant affect the capacity of a species or population to evolve (Futuyma, 1986). DNA barcoding allows for the quick identification of a plant or animal species using a minimum sample of tissue from any stage of growth (Lavin *et al.*, 2003; Steele and Wojciechowski 2003). DNA barcoding and high-throughput sequencing are substitutes that enable fast examination of several samples including diatoms at a cheaper cost than microscopy (Suvechha Kabiraj *et al.*, 2022).

Plant DNA barcodes are frequently derived from cpDNA (chloroplast DNA), a highly conserved region (Asif *et al.*, 2013). It has become possible to examine evolutionary connections between species using chloroplast DNA. For our work, the plastid region's DNA barcode genes *rbcL* and *matK* were selected as the basic DNA barcode genes based on the suggestion of CBOL (2009).

The plant samples from varied locations in the Southern part of India were collected and used (Table 1 and Fig. 1). The DNA was isolated and separated from the plant samples of *B. solanifolium* from different locations. The DNA samples were purified and analyzed at the RGCB in Trivandrum, Kerala, India. The PCR reaction was followed by sequencing using both the forward and reverse directions for the selected plants, using the Sanger Method. The most crucial factors in choosing the right barcode are the success rates of PCR and sequencing (Fernandes *et al.*, 2021). In *mat*K amplification, the primers MATK-XF and MATK-NR1 were used for BM1, BM3, and BM4. But in BM2, amplification was not achieved with these primers, so MATK-ASWF and MATK-ASWR were successful in amplification. The RBCL-AF and RBCL-724R primers were assessed for the *rbcL* gene amplification. There have been some reports discussing the PCR and sequencing issues with the *matK* gene (Saha *et al*., 2020). The *rbcL* gene has a high level of universality and high-quality bidirectional sequencing. There are no issues with *rbcL* gene amplification but a small inconsistency with *matK* primers and this does not appear to cause significant difficulties in our work samples. In the BOLD SYSTEMS search, only 6 hits of *B. montanum*, 4 of *B. calycinum*, and 1 of *B. solanifolium* were recorded. When seeking previous gene submissions in the BOLD system,10 specimen data were registered [*rbcL*-5; *matK*-2; *ITS*-1; *ITS2*-1; and combined **(***ITS, matK, rbcL*)-1] in the BOLD library(www.boldsystems.org/index.php/Public_SearchTerms).

The four plants' partial *mat*K and *rbc*L nucleotide sequences were submitted NCBI-GenBank, where accession numbers were assigned (Table 2). The nucleotide sequences of *matK* and *rbcL* from the collected *B. solanifolium* plants were compared with similar sequences retrieved from the NCBI nucleotide library through BLASTn. The *matK* and *rbcL* genes of the geographically different areas are subjected to nucleotide composition study and A/T to G/C % (Ismail *et al.,* 2020). The size percentage of the sequences of *matK* in all the main datasets were A (28-31%), T (38-39%), G (13-14%), and C (17-19%) whereas in *rbcL* sequence A (27%), T (30-31%), G (23%) and C (19-20%). The A/T (67-70%) varies to G/C (33-30%) in the *matK* gene and for the *rbcL* gene, it differs from 57-58 A/T % to 43-42 G/C % (Table 3). These results of the various investigations have not been entirely consistent, but they have still improved our understanding of the relationships between Euphorbiaceae plants. In our study, *matK* was deemed a suitable barcode for identifying the *B. solanifolium* plant based on the available diversity data. Although *rbcL* was effective in distinguishing *B. solanifolium*, it grouped into narrow clusters. The nucleotide and its percentage, along with the GC%, can predict the nucleotide variation that affects amplification based on the two barcode genes. This variation contributes to polymorphism and subsequent genetic divergence, resulting in the preservation of genetic diversity. Evolutionary processes, including selection, recombination, mutation, and population structures, all have an impact on species diversities.

There have been very few studies conducted on Euphorbiaceae plants (Wurdack *et al*., 2005; Park and Jansen 2007; van Ee *et al*., 2011; Wei *et al.*, 2021). DNA barcoding is a widely recognized solution to the challenges of plant identification. It is considered a fast and efficient method for identifying species and is used in a variety of applications such as accurate specimen identification, new species discovery authentication, combating illegal trade of endangered plants and animals, and forensic investigation to locate dangerous compounds in life-threatening situations (Soininen *et al*., 2009; Stech *et al.*, 2011). Different plant species may have varying abilities to distinguish from each other based on the chosen gene region.

The ability of *matK* and *rbcL* markers to distinguish jewel orchids had been reported by Ho *et al.* (2021). We evaluated two commonly used plant DNA barcodes, *matK*, and *rbcL*, to determine the best

barcode for better identification. The ML requires complicated computation and takes a long time to complete. However, the NJ technique can handle a lot of sequence data, and doing a bootstrap test is simple. Towards examining the phylogenetic relationships of the collected *B. solanifolium*, ML analysis, and NJ analysis were performed based on the similarity of partial chloroplast gene sequences of *matK* and *rbcL* gene were used. The NJ approach produces trees that are suitable for species identification. Lahaye *et al.* (2008) reported that the optimal topology was easily established. This history is illustrated in a phylogenetic tree that was estimated using 1000 bootstraps. Branches representing partitions that were repeated less than 50% of the time in the bootstrap test collapsed. The proportion of bootstrap replicates in which the related taxa were grouped is indicated next to the branches (1000 repetitions).

The NCBI-BLASTn website was used to conduct homology searches on the DNA sequence data with our own plant *mat*K and *rbc*L partial gene sequences. The general length of the *matK* gene is approximately 900 - 1500 base pairs, but our sample gene sequence had an average of 666bp after Bioediting. For the *matK* gene, the sequence from the nBLAST is taken for phylogenetic tree consideration with a minimum of 93.5% identity, and the complete chloroplast genome sequences were excluded. Overall, 37 sequences were considered for analysis, of which 35 belong to Euphorbiaceae, 1 Asteraceae, and 1 Malpghilaceae. Through the ML tree, it is distinct that the sequenced *matK* gene falls under two clades. Based on the analysis of the *matK* gene, the relationships between *B. solanifolium* (BM2) and *B. solanifolium* (BM3) were very close with 99% compared to other BM1 and BM4 (42%). A total of 12 *mat*K retrieved gene sequences were placed in an alternative clade (Fig. 2). The NJ tree also suggests BM2 and BM3 closeness (93%) but the BM1 and BM4 are not clear which were placed in different clades and it supports ML tree result (Fig. 4).

The *rbcL* gene, located at the plastid locus, can reveal evolutionary connections down to the genus level. *rbcL*gene provides a range of features for phylogenetic analysis, with a complete length of approximately 700 - 1400 bp (CBOL, 2009). But our sample sequence had an average value of 704 base pairs after Bioediting. It also has the advantage of high interspecies similarity and a low mutation rate compared to other cpDNA barcode genes (Kellogg and Juliano 1997). Considering the *rbcL* gene for analysis through BLASTn, the sequence percentage identity was increased to 98.5% since a lower percentage led to numeroushits. The sequences were reduced to 28 similar sequences and all belong to the Euphorbiaceae family for analysis. Through the ML tree, it's inferred that the samples BM1, BM2, BM3 and BM4 (*B. solanifolium*) were very close, and looked similar at 92%. *B. montanum* (AY794884), *B. calycinum* voucher (GQ436327), and *B. solanifolium* voucher (KY492341) also fallclose with 92%. The remaining 21 retrieved samples were well distant apart from the main core sequences and form a separate clade (Fig. 3) which is also supported by the NJ analysis (Fig. 5). Although the NJ approach is computationally efficient, simulation results demonstrate that the ML method is far more accurate than the NJ-MCL method (Som, 2009), which supports our results of better analysis through the ML along with NJ tree.

The phylogenetic trees produced from molecular sequences are frequently regarded as more trustworthy than those formed from morphological features because it is thought that molecular sequences are more likely to experience convergent evolution than morphologies are, which confuses phylogenetic reconstruction **(**Zou and Zhang 2016). DNA barcoding can be used through phylogenetic analysis to measure and monitor biodiversity and investigate the ecological and evolutionary factors that shape community composition (Heckenhauer *et al.*, 2017).

The uncertainty about the systematic placement and evolutionary connection of the three morphologically similar genera *Musa paradisiaca* L., *Ravenala madagascariensis* Sonn., and *Heliconia rostrata* Ruiz was clarified by using *matK* and *rbcL* genes (Liza Handique *et al*., 2013). At the moment, the use of *rbcL* genes for phylogenetic analysis within and among angiosperm families and seed plant groupings is a common practice.

However, *matK* evolves three times faster than *rbcL* in angiosperms, which may provide more detailed phylogenetic information and improved resolution for the taxa being studied (Chase *et al*., 2007; Hardig *et al*., 2010). Furthermore, the study revealed that the species of *Baliospermum* have unique relationships with other vouchered Euphorbiaceae plants. Through our output, there were minimal discrepancies in the relationships of some taxa when considered through *matK*, but the *rbcL* barcode demonstrated the best performance which is supported by Wu *et al*. (2019). But it was against consistent results for both the individual analysis of the *matK* and *rbcL* nucleotide sequence data by Ramesh *et al*. (2021) reports. Although morphological research has proven links between the several species, molecular phylogenetic investigations to understand the evolutionary concept in this genus are few.

Generally used barcode patterns and QR codes were generated for the *matK* and *rbcL* genes for our samples (Fig. 5 and 6). The QR code's ability to store and retrieve data as DNA sequences make data storage and retrieval relatively simple (Liu *et al*., 2012; Abdullah and Abdulhamid 2019). All samples were successfully code generated which makes them simple in retrieving and future use.

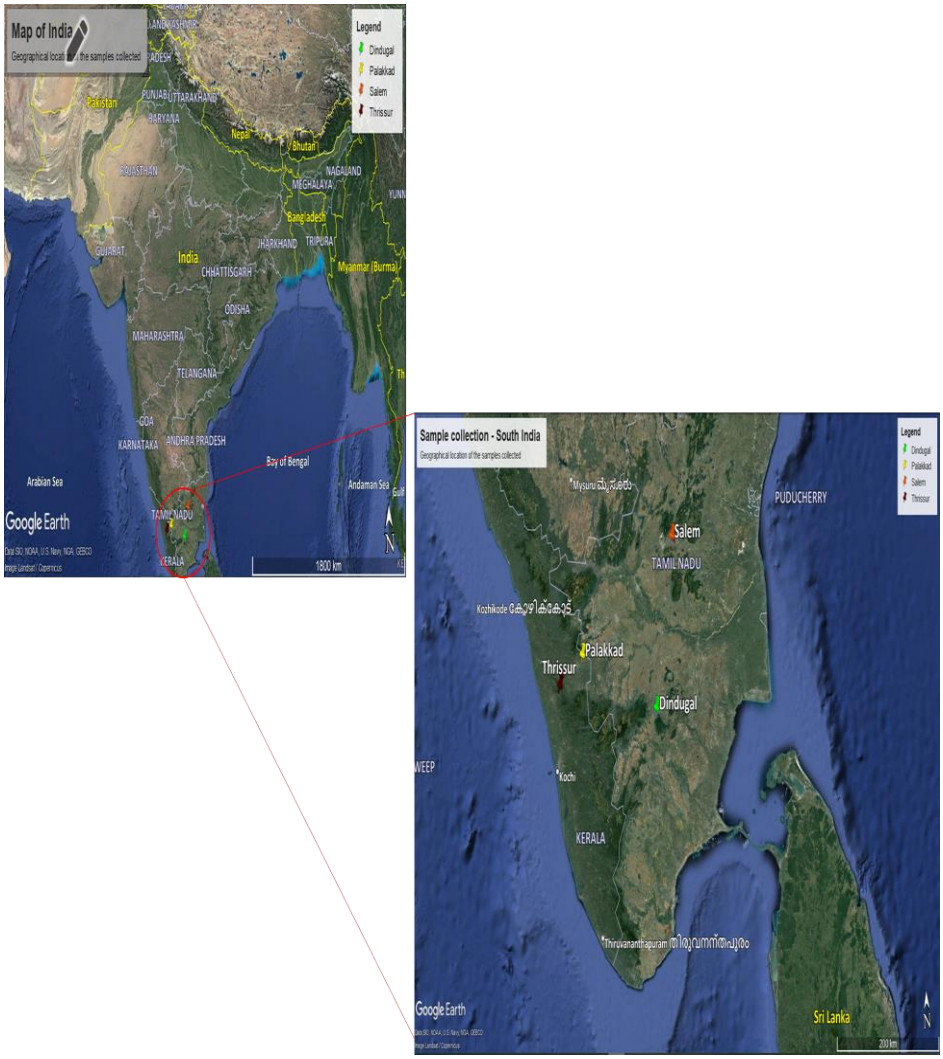


Fig. 1. *Baliospermum solanifolium* (*B. montanum*) collected from various geographical regions of Southern India (Orange-Salem, Tamil Nadu; Yellow-Palakkad, Kerala; Red-Thrissur, Kerala; Green-Dindugal, Tamil Nadu).

Table 1: Geographical location information for *Baliospermum solanifolium* samples collected for the study.

Samples	Place of Collection or Location	Collected by	Collection date	Latitude (N)	Longitude (E)
BM1	Salem, Tamil Nadu	Karthik S, Basker S and Shankar PD	13-07-2014	11.65822°N	78.28609°E
BM2	Palakkad, Kerala	Karthik S, Basker S and Shankar PD	20-06-2018	10.79069°N	76.72250°E
BM3	Dindugal, Tamil Nadu	Karthik S, Basker S and Shankar PD	19-01-2020	10.27949°N	77.93477°E
BM4	Thrissur, Kerala	Karthik S, Basker S and Shankar PD	26-01-2020	10.56473°N	76.32790°E

Table 2: The BSI voucher and accession numbers for the *Baliospermum solanifolium* genes submitted to GenBank (NCBI).

Taxon	Specimen Number	Voucher Number (BSI, India)	NCBI-Gen Bank Accession Number	
			***mat* K**	***rbc*L**
Baliospermum solanifolium1 (BM1)	BM1	BSI/SRC/5/23/2021/Tech/189	MZ694944	ON584158
Baliospermum solanifolium2 (BM2)	BM2	BSI/SRC/5/23/2021/Tech/190	MZ694945	ON584159
Baliospermum solanifolium3 (BM3)	BM3	BSI/SRC/5/23/2021/Tech/191	MZ694946	ON584160
Baliospermum solanifolium4 (BM4)	BM4	BSI/SRC/5/23/2021/Tech/192	MZ694947	ON584161

Table 3: *Baliospermum solanifolium* sequence size, nucleotide composition, A/T and G/C content of the *mat* K and *rbc*L genes.

Plant samples	Gene	Size (bp)	Composition								Content (%)	
			Adenine (A)		Thiamine (T)		Guanine (G)		Cytosine (C)			
			bp	%	bp	%	bp	%	bp	%	A/T	G/C
BM1	*matK*	844	256	**30**	311	**38**	124	**14**	153	**18**	**68**	**32**
BM2		708	224	**31**	258	**38**	103	**14**	123	**17**	**69**	**31**
BM3		514	161	**31**	194	**39**	70	**13**	89	**17**	**70**	**30**
BM4		597	172	**28**	227	**39**	84	**14**	114	**19**	**67**	**33**
BM1	*rbcL*	699	192	**27**	205	**30**	161	**23**	141	**20**	**57**	**43**
BM2		706	194	**27**	207	**31**	164	**23**	141	**19**	**58**	**42**
BM3		703	196	**27**	206	**31**	162	**23**	139	**19**	**58**	**42**
BM4		708	195	**27**	208	**30**	163	**23**	142	**20**	**57**	**43**

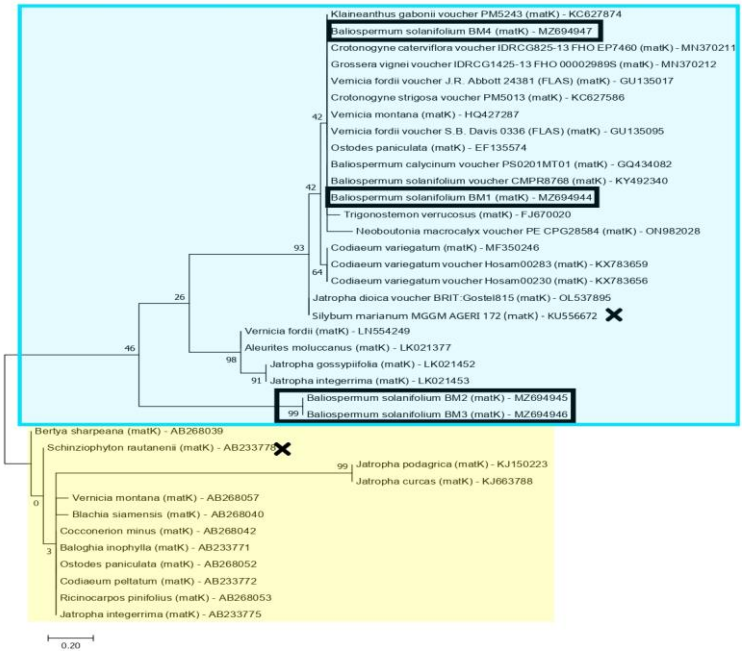
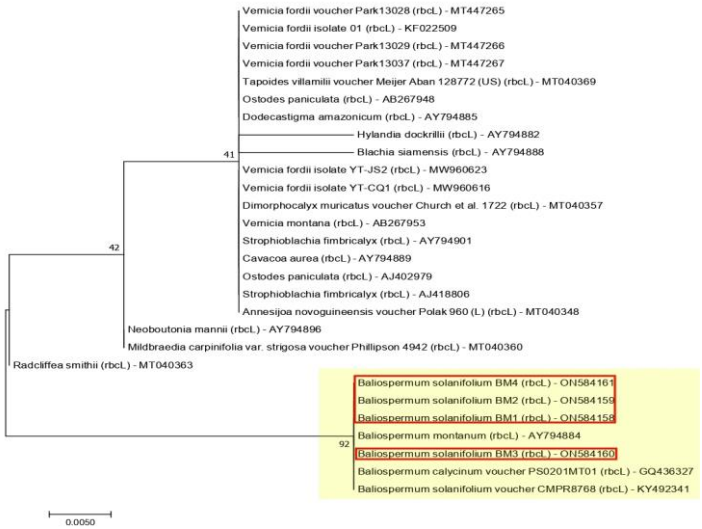


Fig. 2. Phylogenetic tree of *Baliospermum solanifolium* based on *mat* K gene sequences collected from various geographical regions, using closely related recorded plant sequences (37 Samples) analyzed with Maximum Likelihood (ML) method in MEGA 7, with 1000 bootstrap replicates with scale length 0.20 sequence divergence. The original sequences were boxed and the clade of interest is highlighted in blue. The distant species are highlighted in orange. The accessions belonging to other genera are marked with a cross mark.



(Scale length 0.005 sequence divergence). The original sequences were boxed and the clade of interest is highlighted with an orange shade. The distant species are not highlighted. All the accessions belong to Euphorbiaceae.

Fig. 3. Phylogenetic tree of *Baliospermum solanifolium* based on *rbc*L gene sequences collected from various geographical regions (28 samples) analyzed with Maximum Likelihood (ML) Method in MEGA 7, with 1000 Bootstrap Replicates.

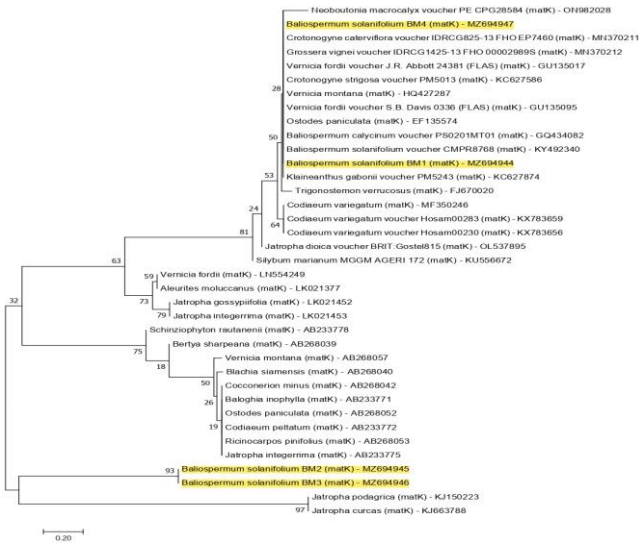


Fig. 4. Phylogenetic tree of *Baliospermum solanifolium* based on *mat*K gene sequences collected from various geographical regions, using closely related recorded plant sequences (37 Samples) analyzed with Neighbor-Joining (NJ) Method in MEGA 7, with 1000 bootstrap replicates. The original own samples were highlighted in yellow.

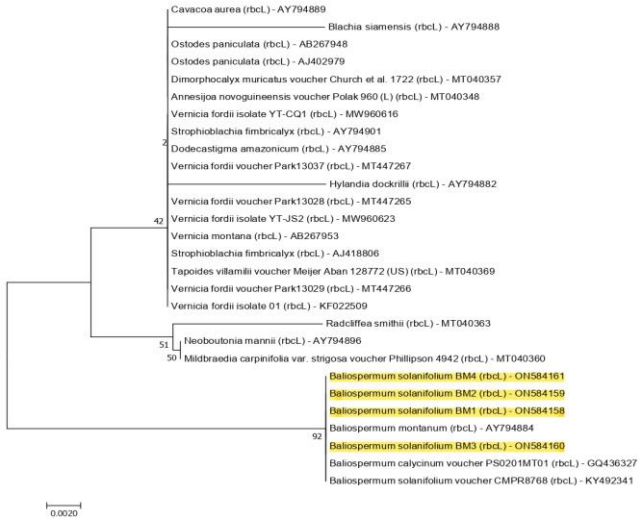


Fig. 5. Phylogenetic tree of *Baliospermum solanifolium* based on *rbc*L gene sequences collected from various geographical regions (28 samples) analyzed with Neighbor-Joining (NJ) Method in Mega 7, with 1000 Bootstrap Replicates. The original own samples were highlighted in yellow.

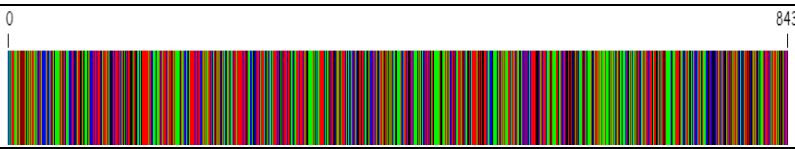
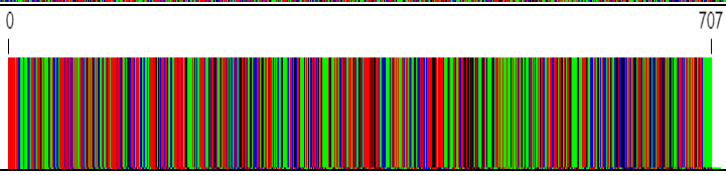
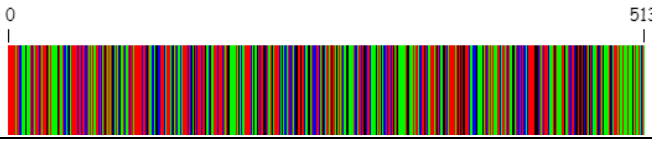
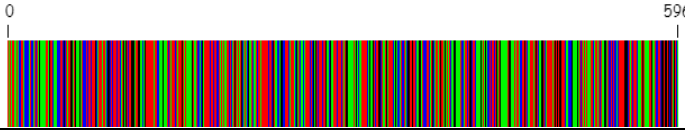
Accession No.	Barcode	QR code
BM1 *mat*K (MZ694944)		
BM2 *mat*K (MZ694945)		
BM3 *mat*K (MZ694946)		
BM4 *mat*K (MZ694947)		

Fig. 6. *Baliospermum solanifolium mat* K gene represented as a Barcode and QR Code illustratively.

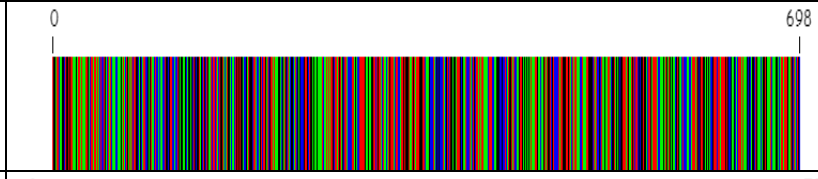
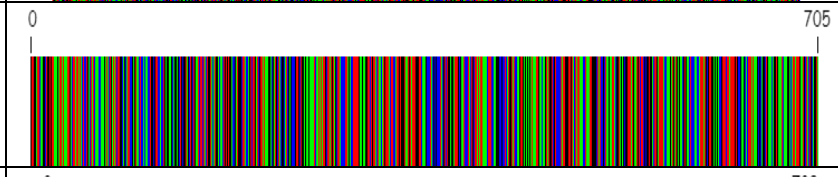
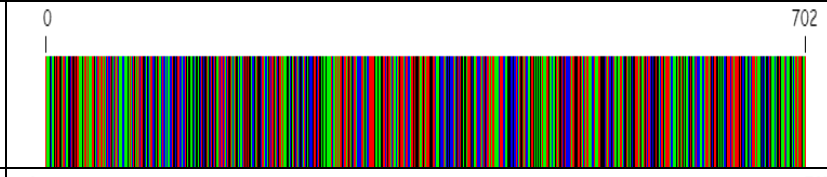
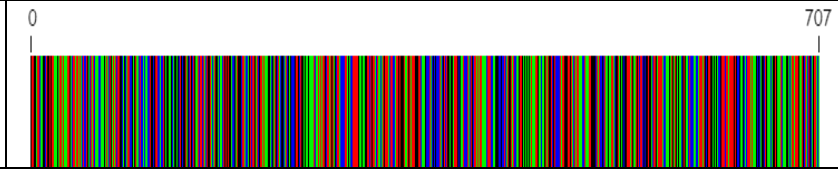
Accession No.	Barcode	QR code
BM1 *rbc*L (ON584158)		
BM2 *rbc*L (ON584159)		
BM3 *rbc*L (ON584160)		
BM4 *rbc*L (ON584161)		

Fig. 7. *Baliospermum solanifolium rbc* L gene represented as a Barcode and QR Code illustratively.

CONCLUSIONS

Our study demonstrates the potential of DNA barcoding in identifying the purity and authenticity of medicinal plant species, especially in the case of *B. solanifolium* (*B. montanum*). The phylogenetic tree based on plastid *matK* and *rbc* L sequences constructed by the NJ method using the MEGA program supports ML tree analysis. Furthermore, the tree topology evaluated using MEGA 7NJ was remarkably comparable to the ML tree. The *rbcL* gene was found to be effective in revealing the genetic diversity and variation within the species and in clustering the plants into a molecular phylogenetic tree compared to the *matK* gene. This highlights the importance of DNA barcoding in ensuring the quality and safety of plant-based medicines and in safeguarding the biodiversity of our planet. Our findings add to the growing body of evidence that DNA barcoding can play a critical role in plant systematics and conservation, as well as in the pharmaceutical industry. By providing a cost-effective and efficient method for species identification, DNA barcoding can help promote sustainable and responsible use of plant resources, ensuring the health and well-being of people and the environment. Although the discovery of phylogenetic biology has transformed the study of molecular and developmental evolution, its influence on ecology is still quite modest.

The *rbcL* and *matK* gene sequences obtained are only partial, meaning that there are still some variable regions in these genes that cannot be amplified due to the limitations of the primers. The variable regions are also the areas that distinguish one taxon from another. Our study contributes to the examination of the distant relationships among the sections of Euphorbiaceae plants through the use of *matK* and *rbcL*. This helps in the possible knowledge of the change in communities or distribution through time and also paves the way for future pharmacological research datasets.

FUTURE SCOPE

Rapid advances in DNA sequencing have enabled the cost-effective use of DNA sequences for species identification. Our work implies the usefulness of the *matK* and *rbcL* region in exploring the genetic diversity and variation within it among the different regions which can be influenced and adapted by our plants. Since *Baliospermum* (danti) roots have potent like Ayurveda and Siddha drugs, their purity can be currently analyzed, ensured, and authenticated through DNA barcoding genes. Also, the other barcoding individual barcoding gene and combinations can be assessed for betterment.

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Conflict of Interest: None.

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