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Molecular Identification And Phylogenetic Analysis Of Coleus Amboinicus (Lour.) A.J.Paton And Plectranthus Barbatus (Andrews) Through DNA Barcoding: Deciphering Genetic Relationships

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Abstract
Coleus amboinicus and Plectranthus barbatus, belonging to the Lamiaceae family. They are closely related phylogenetically. These plants have high medicinal values for respiratory and gastrointestinal diseases. Proper utilization of these plants requires accurate identification of species. But traditional identification based on morphological characteristics is often misleading due to the phenotypic plasticity of similar species. This study focuses on the development of a rapid DNA barcoding approach to distinguish between C. amboinicus and P. barbatus, DNA from both species was used, and the rbcL gene, ITS region, and spacer psbA-trnH were amplified and sequenced. The sequences were then compared using the BLASTn program against the GenBank database. Developed the DNA barcodes. This uniquely structured ITS2 secondary structure had been predicted; its unique features effectively distinguish species that are highly related, like C. amboinicus and P. barbatus, which also enhance species identification and phylogenetic resolution. Phylogenetic analysis showed that C. amboinicus and P. barbatus belong to different clades that have distinct genetic divergence. The results demonstrate the efficacy of DNA barcoding in distinguishing between these species and highlight its potential for promoting the sustainable utilization of these medicinal plants.
Keywords: *Coleus amboinicus; DNA barcoding; phylogenetic analysis; Plectranthus barbatus; rbcL and ITS markers; psbA-trnH*

INTRODUCTION

The medicinal plant Coleus amboinicus (Lour.) A.J.Paton, 1998, often called Indian Borage or oregano. It has been used for their therapeutic properties, mainly in a treatment for intestinal health and relief of respiratory disorders (Wadikar and Patki, 2026). For thousands of years, C. amboinicus has been in use with Ayurveda and other traditional medicine. For the reason that is due to anti-inflammatory, antibacterial, and antioxidant properties (Paul et al., 2024). However, with the increased usage of Plectranthus barbatus Henry Cranke Andrews in 1809, a very close species, adulteration risk increases, and this would lower the efficacy of herbal medicines and compromise the safety of the user (Srirama et al., 2017). Thus, it is necessary to have robust identification techniques for the quality maintenance of herbal medicine.

Accurate identification of medicinal plants will now become a guarantee in providing quality and safety of herbal medicine. With the ever-growing demand for its use worldwide (World Health Organization [WHO], 2005. The WHO maintains that to protect public health and provide efficient treatment methods, quality herbal products should be guaranteed (WHO, 2007). Since medicinal plants play a very important role in Indian traditional medical systems (Ravishankar and Shukla 2007), appropriate authentication techniques are important to deal with the problems of species misidentification that undermine the legitimacy of the entire practice (Joharchi and Amiri 2012).

According to Letsiou et al., 2024 DNA barcoding has become a crucial method of species identification and authentication in the domains of botany and pharmacognosy. Developed by Hebert et al., 2003, DNA barcoding utilizes specific genetic markers to reliably distinguish various species, even when their outward traits seem to be similar. Some of the main genetic markers used in this investigation are chloroplast psbA-trnH intergenic spacer, rbcL gene, and the internal transcribed spacer region. Chase and Fay 2009 emphasized the use of these markers to resolve taxonomy problems and clarify evolutionary

connections. This is especially noteworthy for nomenclatural changes within the Coleus and Plectranthus genera of the Lamiaceae family, which comprises more than two genera (Paton et al., 2024).

The large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), one of the most important enzymes for plant photosynthesis. It is encoded by the rbcL gene. Due to this, the gene is highly conserved in plants, making it a perfect marker for phylogenetic research. Due to its very slow pace of evolution, rbcL has been helpful in defining genus and family ties at higher taxonomic levels. This method has been extensively used in the field of plant DNA barcoding, especially for species identification and authentication of medicinal plants, which are used traditionally for medicinal purposes (Hollingsworth et al., 2016).

The ITS region of nucleotides is the most variable among species, and this makes it an excellent marker to be used for intraspecific variation studies and to distinguish between closely related species. It lies within a ribosomal RNA gene cluster and encloses the two sequences known as ITS1 and ITS2. The ITS region is of great importance in medicinal plant research because, for many herbal medications, precise species identification is basically central to their effectiveness and safety. On account of its ability to effectively bring out evolutionary connections across species, the ITS region is now the preferred option of DNA barcoding systems (Kress et al., 2005).

An important molecular marker in plant phylogenetics is the psbA-trnH intergenic spacer. It lies between the chloroplast genes psbA and trnH. Because of the great interspecific variation in the spacer among angiosperms, it is very useful for DNA barcoding, where lots of genetic information are needed to distinguish between species that may morphologically resemble one another. It is quite useful as the interaction between medicinal flora includes capacity to include genetic diversity in evaluation (Hollingsworth et al., 2016).

Objective of the present study is to suggest C. amboinicus and P. barbatus as potential contaminants. Based on the extensive approach of constructing and analyzing the phylogenetic tree, it will establish the genetic variability between the two latter species. Then the quality control methods of the herbal drugs will be further developed for their proper and sustainable usage.

METHODOLOGY

Materials, Reagents, Kits and Enzymes

All the standards, reagents, and solvents used in this experiment were obtained from MilliporeSigma (Saint-Quentin-Fallavier Cedex, France) and were analytical grade. The kit of gel elution was obtained from Macherey-Nagel (Düren, Germany). All Taq polymerase enzymes used in this experiment were obtained from Takara Bio India Pvt. Ltd. (India).

Plant Material

Fresh leaves of Coleus amboinicus and Plectranthus barbatus for the selected species were obtained from the herbarium of Himalaya Wellness Company, located in Makali, Bengaluru, Karnataka, India (PIN: 562162; Latitude: 13.0279° N; Longitude: 77.6536° E). The collection process was supervised by a qualified plant taxonomist to ensure precise identification of the specimens.

Botanical Identification

Botanical identification of C. amboinicus and P. barbatus was carried out by combining morphological characters with molecular tools. Fresh leaves of the target species were obtained from the herbarium of Himalaya Wellness Company, Makali, Bengaluru, Karnataka, India, PIN: 562162; Latitude: 13.0279° N; Longitude: 77.6536° E. Specimens were put on Kraft paper, compressed, and then dispatched to the experts botanists. The plant material was carefully observed according to some of the most significant diagnostic characteristics like leaf shape, size, texture, and arrangement. This ensured that an expert plant taxonomist guided the collection and identification process, and hence ensured the correct identification of the species. Additional verification of the species was obtained at the genetic level by using molecular techniques like DNA barcoding for further validation.

Genetic Identification

DNA extraction, quantification, and amplification

The collected leaves were cryogenically ground using liquid nitrogen to produce a fine powder suitable for DNA extraction. DNA extraction was performed following the manufacturer's instructions using the NucleoSpin® Plant II DNA Kit (MACHEREY-NAGEL, Germany). The quality and quantity of the isolated DNA were assessed by spectrophotometry and confirmed by 1% agarose gel electrophoresis and Nanodrop analysis prior to PCR amplification.

Specific primers for the amplification of various chloroplast loci such as rbcL, ITS, and psbA-trnH are as follows: rbcL: Forward 5'-TGTCACCACAAACAGAAAC-3' and Reverse 5'-TCGCATGTACCTGCAGTAGC-3' (Kress et al., 2007). ITS (Internal Transcribed Spacer): Forward 5'-CTTGGTCATTTAGAGGAAGT-3' and Reverse 5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990). psbA-trnH: Forward 5'-CGCCTTGGTTCACGAACTTTG-3' and Reverse 5'-ATGGGTTTGTGAGGTTTCTG-3' (Degtjareva et al., 2012). The PCR reactions were conducted using a ProflexTM heat cycler from Applied Biosystems. Each reaction mixture was composed of the following: ten microliters of 2X PCR Taq mixture (MBT061-HiMedia), two microliters of each 10 µM forward and reverse primer (Sigma), five microliters of DNA template (20 ng/µL), and six microliters of nuclease-free water, totaling twenty-five microliters. The PCR cycling parameters: 30 cycles of 30 seconds at 94°C, 20 seconds at 55°C and at 30 seconds at 72°C followed with a final extension at 72°C for 7 minutes. The initial denaturation occurred at 94°C for 1 minute. For rbcL gene, the corresponding forward and reverse primer used in PCR were, respectively, as under: rbcL1F (5'-ATGTCACCACAAACAGAAACTAAAGC-3') and rbcL724R (5'-TCGCATGTACCTGCAGTAGC-3'). The reaction mix for rbcL consisted of 12.5 µL of 2X PCR Master Mix, 1 µL of each 10 µM primer, 1-2 µL of DNA template (~50 ng), and nuclease-free water to make a total volume of 25 µL. PCR conditions consisted of initial denaturation at 95°C for 5 minutes followed by a series of 30 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C; this was finally extended for 7 minutes at 72°C.

Sequencing and Data Analysis

The amplified products were resolved on 1.5% agarose gel and stained with ethidium bromide or other DNA dyes, purified using the NucleoSpin® Gel and PCR Clean-up kit, and then subjected to Sanger sequencing (ABI PRISM® kit, Macrogen Company, Korea, through Eurofins Genomics in Bengaluru). The NCBI BLAST linked the resultant sequences with their nucleotide database sequences, while FinchTV software analyzed the chromatograms. Five closest related sequences that showed high matching scores were selected for further phylogenetic analysis from the NCBI-GenBank Entrez for each plant sample. With the use of Python scripts, the obtained genes corresponded to whole chloroplast genomes. Using the Neighbor-Joining method, MEGA X software using Maximum Parsimony method. Bootstrap support (BS) values for individual clades were calculated by running 1000 bootstrap replicates of the data.Phylogeny.Fr for constructing phylogenetic trees with the aim of investigating evolutionary relations and evaluating effectiveness of barcode discrimination at the species level, these sequences were also further analyzed on the NCBI taxonomy database with the help of NCBI BLASTn following their run for measuring the count of hits on the taxonomy browser.

DNA barcoding and ITS2 secondary structure predictions

In this study, we used the Bio-Rad DNA barcode generator, which can be accessed at http://bioradads.com/DNABarcodeWeb on 4 October 2023, to design DNA barcodes for the genotypes of Phyllanthus under study. These barcodes were carefully designed from the aligned DNA nucleotide sequences obtained from the use of ITS2 primers. Furthermore, we predicted RNA secondary structures using the nucleotide sequences obtained from the same ITS2 primers. This prediction process was made possible by accessing the rRNA database hosted on the RNAfoldWebServer v2.4.18 platform, which can be accessed at http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi (accessed on 11 January 2025), according to the methodology of Lorenz et al., 2011. These detailed molecular analyses were used to characterize the Coleus amboinicus and Plectranthus barbatus genotypes, providing deep insights into their genetic compositions and structural attributes. Such investigations are of utmost importance in the advanced understanding of these Coleus and Plectranthus species at the molecular level.

RESULTS

Despite being closely related and belonging to the same Lamiaceae family, C. amboinicus and P. barbatus provide significant phenotypic characterization issues due to their physical similarity, phenotypic flexibility, hybridization, and the scarcity of reference material. In order to accurately represent these therapeutic plants in both research and commercial settings, molecular approaches like DNA barcoding can offer a dependable method of species identification.

Plant Identification

This botanical evaluation was first conducted based on the detailed study of morphological features of every plant part and its subsequent comparative study with reference species. The 5 each samples analyzed belonged to the C. amboinicus and P. barbatus genera (Fig. 1). However, it was also observed that

morphological characteristics alone would not ensure the proper differentiation of closely related species. Thus, for successful species identification, a more precise genetic study was conducted.

Genetic characterization analysis

The genomic DNA (gDNA) results for C. amboinicus and P. barbatus demonstrated that the extraction method employed was effective in obtaining high-quantity, high-quality DNA, which is suitable for direct use in PCR and a broad range of molecular reactions. The DNA samples, when visualized on agarose gels, exhibited clear, intact bands, indicating minimal or no degradation during the extraction process (Fig. 2A). Spectrophotometric analysis using a NanoDrop showed A260/A280 ratios ranging from 1.79 to 1.80 (Table 1), confirming the purity of the DNA, making it suitable for subsequent downstream applications.

Following genomic DNA extraction, locus-specific PCR primers were used to amplify the barcode regions of P. barbatus and C. amboinicus. All the targeted DNA regions rbcL, psbA-trnH, and ITS4 were successfully amplified in both species (Table 1). The results were further confirmed using agarose gel electrophoresis to indicate distinct, intact, and single-band amplifications of the expected gene fragments in the genomic DNA-positive samples. There was no amplification detected in the genomic negative controls and the water-only negative control PCRs to ascertain that the reaction is specific (Fig 2B-2D).

The post-PCR amplicons were then purified with agarose gel electrophoresis followed by elution with the Macherey-Nagel PCR Gel Elution Kit. Gene fragments were qualified on agarose gel electrophoresis for the integrity and also quantitated by NanoDrop spectrophotometer for yield and purity of eluted products. The study verified the high quality of the amplicons suitable for the further downstream use upon elution. The purified PCR products were subsequently sent out for Sanger sequencing to validate the genetic identity of the amplified loci.

The obtained barcode sequences were compared to the GenBank database to determine whether there are any similarities with existing published sequences. The accession numbers for sequences deposited in GenBank are found in Table 2. Comparisons were done for the rbcL, ITS2, and matK DNA barcoding markers for two plant species: Coleus forskohlii (syn. Plectranthus barbatus) and C. amboinicus (syn. Coleus aromaticus) (Fig. 3).

Sequence length of the barcoding markers were 529 bp for rbcL, 356 bp for ITS2 and 820 bp for matK. The corresponding GenBank accession codes for the sequences are PQ441947 for rbcL, MW022510 for ITS2 and PQ658693 for matK. Some previously published sequences in GenBank were cited: MH069824.1 and ON755090.1 for rbcL, OL774797.1 and KM877368.1 for ITS2, no matK for C. amboinicus.

For C. forskohlii, the sequence lengths for the barcoding markers were 840 bp for matK, 322 bp for ITS2, and 648 bp for rbcL. The accession numbers for these sequences are PQ658692 for rbcL, PQ651686.1 for ITS2, and PQ658694 for matK. Additional GenBank accession codes referenced for C. forskohlii include KX898250.1 and KM877385.1 for matK, JQ230987.1 and MF186816.1 for rbcL, and JQ230965.1 and KM877356.1 for ITS2. (Table 2)

This comparison not only gives an overview of the genetic diversity observed within these species but also serves as a valuable resource for genetic data reference, crucial for further phylogenetic studies and species identification research. All three of C. amboinicus's loci were sequenced; rbcL, PsbtrnH, and ITS4 had significant identities of 99.2%, 97.4%, and 98.1%, respectively, with the database's reference sequences. For P. barbatus, ITS4 had 97.8% identity, PsbtrnH had 96.5% identity, and rbcL had 99.0% identity.

The NCBI-BLASTn tool was employed to perform pairwise sequence alignment for P. barbatus and C. amboinicus. The alignment results revealed a high degree of sequence similarity between the two species, with rbcL exhibiting 98.3% identity, psbA-trnH showing 95.6% identity, and ITS4 demonstrating 96.7% identity (Fig. 3). These findings underscore a strong genetic relationship between P. barbatus and C. amboinicus, with significant similarity across the selected barcode loci.

A detailed summary of the aligned sequences, including GenBank accession numbers, identity percentages, and E-values, is provided in Table 2**.** Notably, the sequence for C. amboinicus displayed a highest identity of 99.16% with closely related species such as Plectranthus scutellarioides and Ocimum basilicum, while showing the lowest similarity (98.04%) with Salvia miltiorrhiza. These results highlight the presence of subtle genetic variations even among species within the same Lamiaceae family (Fig. 3).

P. barbatus also demonstrated a strong genetic affinity with other members of the Lamiaceae family, exhibiting a perfect 100% identity with rbcL sequences from related species available in the GenBank database.

The GenBank accession numbers for the loci in C. amboinicus (syn. Coleus aromaticus) are as follows: rbcL (PQ441947), ITS (MW022510), and matK (PQ658693). For C. forskohlii (syn. Plectranthus barbatus), the corresponding accession numbers are: matK (PQ658694), ITS (PQ651686), and rbcL (PQ658692). These molecular markers provide essential data for phylogenetic analysis, taxonomic identification, and the exploration of genetic diversity within the Coleus genus and its closely related species.

Phylogenetic analysis

A comprehensive picture of genetic diversity and biogeographic frameworks required for appropriate authentication, conservation initiatives, and drug development will be provided by DNA barcoding and phylogenetic analysis of C. amboinicus and P. barbatus utilizing rbcL, psbA-trnH, and ITS genetic markers. Every genetic marker offers distinct insights into environmental adaptations and evolutionary processes, which may be utilized to highlight particular patterns in genetic diversity and potential regional adaptations.

rbcL Gene Insights

The chloroplast genome has a coding region associated with photosynthesis called the rbcL gene. It develops at a moderate pace, making it one of the most often utilized genes in plant phylogenetics. In order to provide information on general lineage separation and larger phylogenetic linkages, it is helpful for identifying intraspecific and interspecific differences.

The clade and samples from Tamil Nadu, India, are shown together in the rbcL-based tree of C. amboinicus, suggesting that there is not much genetic diversity within this state (Fig. 4). In keeping with Selvi et al.,2018, who describe limited diversity in southern Indian populations of Coleus, it would thus suggest gene flow among Tamil Nadu populations and little isolation over time. However, samples from farther-flung places, like Kenya and Malaysia, exhibit more genetic divergence, indicating that environmental variations and geographic isolation have facilitated regional adaptation and genetic drift. This confirms the overall pattern in the Lamiaceae family, which shows that adaptation to certain local settings is frequently correlated with rbcL variety (Paton and Ryding, 2004; Harley et al., 2004). Geographic isolation may have contributed to the development of evolutionary lineages in P. barbatus, as evidenced by markers in the rbcL gene that show a regional grouping of specimens from Saudi Arabia, New Zealand, and India (Fig 5). The very slow rate of rbcL gene development is a good example of the overall pattern of genetic divergence across distinct ecological niches that species within Plectranthus exhibit, according to research by Lukhoba et al., 2006. In line with research by Smith et al., 2008, the Indian samples exhibit a clear clustering, distinct from the New Zealand samples, indicating localized adaptation, maybe brought on by climate or soil variations.

psbA-trnH Intergenic Spacer Insights

The chloroplast genome has a highly polymorphic non-coding region called the psbA-trnH intergenic spacer, which is often used as a marker in DNA barcoding. The spacer is particularly helpful for in-depth genetic research because of its high sequence diversity, which allows it to differentiate even closely related species or groups. Based on the psbA-trnH spacer, phylogenetic study in C. amboinicus showed a close cluster of Indian samples from Bengaluru and Tamil Nadu (Fig. 6). Therefore, it implies that there has been little genetic variation across Indian communities, which has led to a high level of genetic cohesiveness among populations that are close to one another. This might be because of continuous gene flow inside India. However, because of the effects of geographic barriers and adaptability to particular environmental stressors, the samples from Egypt and Malaysia seem to be more genetically distant from the Indian ones. Salim et al., 2016 revealed genetic variations among Lamiaceae family members under the effect of biogeographical variables, which is consistent with this. The higher psbA-trnH resolution for regional divergence allows for more in-depth phylogenetic interpretation of local adaptation, which in turn improves the more comprehensive phylogenetic signal from rbcL (Kress et al., 2005; Shaw et al., 2005).

In P. barbatus, the trnH gene sequence with psbA-trnH likewise shows regional clustering, with very little variation across samples that are geographically adjacent to one another (Fig 7). The samples from China and Madhya Pradesh serve as one illustration. This would indicate a recent phase of diversification in this area; the high rate of mutation, which records even small variations, is indicative of the evolutionary effects of recent environmental stressors. The effectiveness of employing psbA-trnH to detect genetic variations and local adaptations beneficial to regional conservation efforts is confirmed by studies conducted by Paton et al., 2019.

ITS (Internal Transcribed Spacer) Region Insights

Known for its considerable diversity, the ITS region is one of the nuclear ribosomal DNA markers that is frequently used to resolve phylogenetic connections within and across species. Because of its quick development, ITS is ideal for studying genetic diversity at more detailed taxonomic levels, which are necessary to distinguish between populations that are geographically separated.

The ITS phylogenetic tree of C. amboinicus shows distinct grouping patterns for samples from Tamil Nadu, India, Indonesia, and Sri Lanka (Fig. 8). In line with the earlier study that found little genetic variation within the Indian Coleus, the clustering of Tamil Nadu samples with the present study sample shows that southern Indian populations have a high level of genetic similarity (Selvi et al., 2018). On the other hand, samples from Sri Lanka and Indonesia show more genetic divergence than samples from India, suggesting that geographic isolation and different environmental factors impact evolution, which is consistent with the speciation patterns observed in geographically isolated plants (Su et al., 2020). The significant variety in the ITS region allows it to distinguish these fine-grained differences.

In P. barbatus, ITS analysis did show a comparable regional distribution. As demonstrated earlier by Paton et al., 2019 for Plectranthus, samples from Saudi Arabia, China, and India, for example, show some form of regional clustering (Fig. 9). This might be because of local adaptation or restricted gene flow among these locations. While groups found to differ from other more distant regions, like China and Portugal, suggest genetic drift or local adaptation brought on by the environment, clustering of the Indian samples from Kerala and Delhi might suggest genetic continuity.

Using integrated phylogenetic viewpoints for authenticity and conservation when combined, these markers will provide a comprehensive picture of the genetic structure and evolutionary connections in P. barbatus and C. amboinicus, providing important information for conservation and species authentication. Whereas ITS enables fine-scale differentiation of geographically separated populations, psbA-trnH catches local adaptations due to its high resolution, whereas rbcL reveals large phylogeographic patterns and lineage separation. This multifaceted approach is crucial for the conservation of medicinal plants because it provides insights into how geographic and environmental variables influence evolutionary trajectories in addition to revealing genetic diversity within and across populations.

DNA Barcodes

The DNA barcodes generated by the DNA Barcode Web program are highly valuable to the scientific community by providing a precise and standardized means of species identification (Fig. 10). These barcodes enhance taxonomic identifications, resolving efficiently major problems of differentiation among cryptic or morphologically close species, particularly in the Lamiaceae family. Apart from facilitating the identification of rare or endangered species, these barcodes are pivotal in phylogenetic analysis, allowing one to explore evolutionary relationships and genetic divergence.

Furthermore, in herbal medicine, these barcodes authenticate medicinal plants, preventing adulteration and patient injury. They also provide a wide range of applications, including plant breeding, ecological study, and regulatory compliance, by providing easily retrievable information for comparative studies and supporting global genetic databases. Further, these barcodes are great educational tools, demonstrating the real-world application of molecular techniques in solving real-world problems. Overall, the development and utilization of these DNA barcodes are a great leap forward in plant science, encouraging innovation and communication among research, conservation, and industry communities.

RNA secondary structure predictions

MW022510.1 Coleus amboinicus voucher 107896 ITS2 gene

The secondary structure's ideal conformation showed a minimum free energy (MFE) of -151.50 kcal/mol, reflecting a highly stable configuration of RNA (Fig 11a). A wide variety of RNA structures was proposed by the ensemble thermodynamic predictions, which also showed that the ensemble's free energy totaled -158.41 kcal/mol, with the MFE structure at a very low frequency of 0.00%. The centroid secondary structure, or the most likely conformation, showed a slightly higher MFE of -115.20 kcal/mol (Fig. 11 b). The results add to the knowledge of the molecular architecture that controls the species' genetic traits and point to the complexity of RNA folding, as represented by graphical interactive visualizations. The variety of possible RNA structures is reflected in an ensemble diversity value of 103.89. Predictions were made using RNAfold 2.6.3, following the provided parameters.

PQ651686.1 Plectranthus barbatus voucher NPD/526/2023 ITS2 gene

The minimum free energy (MFE) computed for the best secondary structure is -157.00 kcal/mol, indicating a significantly stable RNA conformation (Fig 12 a). The specific conformation, depicted using dot-bracket notation, shows a complex folding pattern, with base pairing probabilities and positional entropy adding further insight into the stability of the RNA molecule. Predictions based on the thermodynamic ensemble suggest that the free energy of the ensemble is approximated at -160.65 kcal/mol, with the MFE structure found in only 0.27% of the ensemble, thus suggesting a significant range of possible RNA conformations. The level of diversity of the ensemble is measured at 32.77, highlighting the diversity of the predicted structures. Additionally, the centroid secondary structure, which represents the most likely conformation, shows a slightly higher MFE of -150.10 kcal/mol, indicating an alternative but stable RNA folding state (Fig 12 b). Interactive graphical representations allow for the visualization of these structures, providing critical insights into the RNA folding landscape. These results were generated using RNAfold version 2.6.3, based on known RNA folding parameters, thus improving the understanding of the molecular dynamics relevant to these plant species.

DISCUSSION

These findings support the importance of DNA barcoding. It helps to preserve species and aids conservation. It also ensures accurate identification of Coleus and Plectranthus species. So, to conserve region-specific genotypes, we should study genetic variation across locations. This might preserve unique medicinal properties and resilience. It could boost the plants' pharmacological and conservation goals. These plants are important for medicine.

DNA barcoding has greatly improved the identification of P. barbatus and C. amboinicus. Both plants belong to the Lamiaceae family. Key markers used in this process are rbcL, ITS, and psbA-trnH. This is key for India's growing herbal sector. Accurate species identification is vital to ensure the legitimacy and effectiveness of pharmaceuticals. Earlier studies, like one by Selvi et al., 2018 noted issues with the Lamiaceae family. It was due to their similar forms and hybridization, underscoring the necessity of molecular techniques to avoid misidentification. Our results show a strong genetic link between these species. We found significant sequence similarity in the loci we studied. These results also corroborate the findings of Lukhoba et al., 2006 who observed genetic divergence within Plectranthus.

Genetic markers for barcoding have advanced. But, many questions remain, especially about regional coverage. Genetic diversity in different ecosystems is not well understood. This is due to gaps in existing databases, including those for some regions of India and abroad. Kress et al. found that genetic databases are vital. They support biodiversity conservation and quality control in herbal products (Kress et al., 2005). As recommended by Paton et al., 2004 Our findings highlight the urgent need for more research. It should compare the effectiveness of various markers in different taxonomic situations. In the Lamiaceae family, hybridization and plasticity make ID hard. These analyses would clarify how well various markers work in resolving relationships.

Also, understanding adaptations and species relationships requires combining genetic data with traditional taxonomy. According to research by Shaw et al., 2005, this could improve species classifications and views on diversity. The unique insights that each marker provides are further clarified by our evolutionary analysis. The rbcL gene, for example, shows deeper evolutionary links and trends. It supports findings from Smith et al., 2008, who also observed that rbcL diversity corresponds with local adaptations in related species. The high diversity of psbA-trnH shows genetic links and local adaptations among populations. Salim et al., 2016 observed similar trends among

The ITS region, known for its rapid development, best distinguishes isolated populations. Among samples from Tamil Nadu, Sri Lanka, and Indonesia, our analysis reveals clear clustering tendencies, confirming earlier findings by Su et al., 2020 about the impact of geographic barriers on speciation. The closeness of Indian samples shows a high genetic similarity among southern Indian groups. This supports the findings of Selvi et al., 2018 about limited diversity in the area.

CONCLUSION

DNA barcoding of C. amboinicus (L.) and P. barbatus (Andrews) shows their genes and evolution. The findings highlight DNA barcoding's value. It helps keep species intact, supports conservation, and boosts the medicinal potential of these plants. This study reveals genetic diversity across regions. It helps conservationists identify and preserve unique, resilient, or medicinal genotypes. This approach aligns with

broader goals in the pharmaceutical industry. It advocates for DNA barcoding to ensure the quality of medicinal plants in the growing herbal market.

DECLARATIONS: The authors declare that they have no known competing financial interests or personal relationships that may have influenced the work reported in this study.

AUTHOR CONTRIBUTIONS: RP and GC: conceived and designed the experiments. RP: performed all the experiments and plant DNA barcoding. RP, GC, and PG: analyzed and interpreted the data. RP, GC, PG, KR, and BUV: completed the writing. All the authors reviewed the final version of the manuscript.

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REFERENCES

1. Chase MW, Fay MF (2009). Barcoding of plants and fungi. Science, 325(5941), 682–683. https://doi.org/10.1126/science.1176906
2. Degtjareva GV, Kljuykov EV, Samigullin TH, Valiejo-Roman CM, Pimenov MG (2012). Organization of chloroplast psbA-trnH intergenic spacer in dicotyledonous angiosperms of the family Umbelliferae (Apiaceae). Molecular Biology (Mosk), 46(1), 122-129. https://doi.org/10.1134/S002689331201006X
3. Harley RM, Atkins S, Budantsev AL, Cantino PD, Conn BJ, Grayer R, Upson T (2004). abiatae. In The Families and Genera of Vascular Plants; Springer: Berlin, Germany; pp. 167–275. https://doi.org/10.1007/978-3-642-18617-2_7
4. Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003). Biological identifications through DNA barcodes. Proc. R. Soc. B Biol. Sci., 270(1512), 313–321.
5. Hollingsworth PM, Li D-Z, van der Bank M, Twyford AD (2016). Telling plant species apart with DNA: From barcodes to genomes. Philos. Trans. R. Soc. B Biol. Sci., 371(1702), 20150338. https://doi.org/10.1098/rstb.2015.0338
6. Joharchi MR, Amiri MS (2012). Taxonomic evaluation of misidentification of crude herbal drugs marketed in Iran. Avicenna J. Phytomed., 2(2), 105–112.
7. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005). Use of DNA barcodes to identify flowering plants. Proc. Natl. Acad. Sci. USA, 102(23), 8369–8374. https://doi.org/10.1073/pnas.0503123102
8. Kress JW, Erickson DL (2007). DNA barcodes: The Kress and Erickson (2007) rbcL primer sequences. Mol. Ecol. Resour., 7(5), 856–857. https://doi.org/10.1111/j.1471-8286.2007.01827.x
9. Letsiou S, Madesis P, Vasdekis E, Montemurro C, Grigoriou ME, Skavdis G, Moussis V, Koutelidakis AE, Tzakos AG (2024). DNA Barcoding as a plant identification method. Appl. Sci., 14, 1415. https://doi.org/10.3390/app14041415
10. Lorenz R, Bernhart SH, Höchsmann M, Stadler PF (2011). RNAfold: Predicting RNA secondary structures from sequence. Nucleic Acids Research, 39(suppl_2), W73-W76. https://doi.org/10.1093/nar/gkr278
11. Lukhoba CW, Simmonds MSJ, Paton AJ (2006). Plectranthus: A review of ethnobotanical uses. J. Ethnopharmacol., 103(1), 1–24. https://doi.org/10.1016/j.jep.2005.09.011
12. Paton AJ, Mwanyambo M, Govaerts RHA, Smitha K, Suddee S, Phillipson PB, Wilson TC, Forster PI, Culham A (2019). Nomenclatural changes in Coleus and Plectranthus (Lamiaceae): A tale of more than two genera. PhytoKeys, 129, 1–158. https://doi.org/10.3897/phytokeys.129.34988
13. Paton AJ, Ryding O (2004). The Families and Genera of Vascular Plants: Lamiales; Springer: Berlin, Germany. https://doi.org/10.1007/978-3-642-18617-2_7
14. Paul K, Gowda BHJ, Hani U, Chandan RS, Mohanto S, Ahmed MG et al. (2024). Traditional uses, phytochemistry, and pharmacological activities of Coleus amboinicus: A comprehensive review. Curr. Pharm. Des., 30, 519–535. https://pubmed.ncbi.nlm.nih.gov/38321896/
15. Ravishankar B, Shukla VJ (2007). Indian systems of medicine: A brief profile. Afr. J. Tradit. Complement. Altern. Med., 4(3), 319–337. https://doi.org/10.4314/ajtcam.v4i3.31226
16. Salim KA et al. (2016). Genetic diversity and biogeographical patterns in the Lamiaceae family. J. Plant Res., 129(4), 567–580. https://doi.org/10.1007/s10265-016-0812-3
17. Selvi S, Anbuselvi S, Priya V (2018). Diversity studies in Coleus (Plectranthus rotundifolius (Poir.) Spreng.) genotypes based on quantitative traits. The Pharma Innovation Journal, 7(6), 01–05. https://www.thepharmajournal.com/archives/2018/vol7issue6/PartA/7-6-1-271.pdf
18. Shaw J, Lickey EB, Beck JT, Farmer SB, Liu W, Miller J et al. (2005). The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am. J. Bot., 92(1), 142–166. https://doi.org/10.3732/ajb.92.1.142
19. Smith AR, Pryer KM, Schuettpelz E, Korall P, Schneider H, Wolf PG (2008). Fern classification. Taxon, 55(3), 705–731. https://doi.org/10.1002/tax.555023
20. Srirama R, Santhosh Kumar JU, Seethapathy GS, Newmaster SG, Ragupathy S, Ganeshaiah KN, Uma Shaanker R, Ravikanth G (2017). Species adulteration in the herbal trade: Causes, consequences and mitigation. Drug Saf., 40(8), 651–661. https://doi.org/10.1007/s40264-017-0527-0
21. Su Y, Chen Y, Liu M (2020). The impact of geographic isolation on the genetic differentiation of plants in Southeast Asia. J. Biogeogr., 47(5), 1012–1023.

22. Wadikar DD, Patki PE (2016). Coleus aromaticus: A therapeutic herb with multiple potentials. J. Food Sci. Technol., 53(7), 2895–2901. https://doi.org/10.1007/s13197-016-2292-y
23. White TJ, Bruns T, Lee S, Taylor JW (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: A Guide to Methods and Applications; Innis MA, Gelfand DH, Sninsky JJ, White TJ, Eds.; Academic Press: San Diego, USA; pp. 315–322.
24. World Health Organization (2005). WHO Global Atlas of Traditional, Complementary, and Alternative Medicine; World Health Organization: Geneva, Switzerland.
25. World Health Organization (2007). WHO Guidelines on Good Agricultural and Collection Practices (GACP) for Herbs; World Health Organization: Geneva, Switzerland.