DNA Barcoding and Phylogenetic relationship of *Catharanthus roseus* indigenous specie; a rich source of phytochemicals and pharmacological potentials

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Abstract

Background: *Catharanthus roseus,* commonly known as Madagascar periwinkle, vinca rosea or sadabahar, is a plant belonging to Apocynaceae family. This plant is recognized for its medicinal potentials against various diseases such as, cancer, diabetes, inflammation, ulcers, bacterial infections, viral infections, tumurogenesis etc. Unless the correct identification of such medicinal gems, their potentials cannot be explored and utilized. For this purpose, DNA barcoding using reliable markers, such as rbcL gene plays a demanding role.

Methology: In this study, rbcL was used as a potential identification marker. DNA was extracted from fresh leaves, selected region i.e. rbcL was amplified, and amplicon was sequenced. The resulting sequence was compared with the sequence available in NCBI Genbank. Phylogenetic linkage was developed to characterize and confirm taxonomic position of *Catharanthus roseus.*

Results: The rbcL barcode employed in this study provided sufficient resolution to authenticate *C. roseus* and to distinguish it from its close relatives. The resulting rbcL sequence showed high similarity with corresponding sequences obtained from NCBI, placing *C. roseus* in a clade within Apocynaceae family. Gene was published in NCBI database Genbank with accession number PP706114.

Conclusion: To the best of our knowledge, this study is the first evidence of DNA barcoding of *Catharanthus roseus* in Pakistani specie. Our findings in this paper authenticate and demonstrate that rbcL is an effective marker for identification of *Catharanthus roseus.* It facilitates and enhances our ability to correctly identify plant species that hold prominent position in herbal medicine and ensure their integrity in medicinal research.

Keywords: *Catharanthus roseus,* DNA Barcoding, Madagascar periwinkle, Phylogenetic analysis, rbcL, Specie identification

1. Introduction

Demand for therapeutic herbs derived from plants is steadily increasing [1]. Because of their many biological and medicinal properties as well as their traditional uses in ethnobotany, plants have long been the center of interest for medical and business samples [2]. Unlike synthetic medicines, herbal medicines do not have any negative effects. This may be the cause of scientists' increased attention to the identification of potential medical benefits of numerous plant. The value of medicinal plants is enormous, and their application is growing daily [3]. One of the well-known medicinal herbs that is crucial to the treatment of cancer is *Catharanthus roseus*. Several lethal diseases are treated with the Apocynaceae plant *Catharanthus roseus* (L.). There are roughly two common cultivars of *C. roseus*, called "Rosea" (pink flowers) and "Alba" (white flowers), named for the color of their flowers [4]. *C*ommon names of *C. roseus* are "Sadabahar" or "Nayantara". The Greek word *Catharanthus* means "pure flower" and r*oseus*, which means red, rosy, or rose, is a word from Latin [5].

Catharanthus roseus is indigenous to the island of Madagascar in the Indian Ocean. Despite being common in many tropical and subtropical regions across the world today, including the South of the United States, this plant is endangered in the wild [5].

Numerous alkaloids, which are highly significant to medical science, are included in this plant extract. More than 200 alkaloids are found in various plant components, such as the root, leaf, stem, and shoot. Among the prominent alkaloids are rubacine, vinblastine, vincristine, vincamine, ajmalacine, ajmaline, serpentine and rubacine. These alkaloids exhibit a variety of pharmacological properties, including anti-oxidant, antimicrobial, anti-diabetic, anti-ulcer, hypotensive, antidiarrheal, antiarrhythmic, hypolipidemic, and memory-enhancing effects [6].

Catharanthus roseus's contribution to anticancer drugs is among the most prominent instances of its therapeutic value. Vinblastine and vincristine, two of the plant's special indole alkaloids, are used to treat a variety of cancers, including juvenile leukemia [7]. The American Society of Health-System Pharmacists' 2015 publications state that vincristine and vinblastin are well-known for their ability to suppress mitosis or to stop the division of the cells, which leads to the death of cells [8]. Hodgkin's disease and choriocarcinoma are recommended treatments for vinblastine, which is also used to treat neoplasms in experimental settings. Velban or Vincristine (oncovin) are two brands of vinblastine [5].

The roots yield the medicinally important indol alkaloids ajmalicine (antihypertensive effect) and its oxidized derivative, serpentine (tranquilizer) [9]. The hypoglycemic action in a dichloromethane : methanol (1:1) extract of *C. roseus* leaves and twigs was identified by Singh et al. using a streptozotocin-induced diabetic rat model [10].

C.roseus have alkaloid vincamie which have function to enhance memory and brain activity and for the treatment of Alzheimer and vascular dementia [11]. Various secondary metabolites, including alkaloids, flavonoids, and phenolic chemicals, have been linked to *C. roseus's* antibacterial action [12], According to reports, the alkaloids vincamine and vindoline found in the plant *Catharanthus roseus* demonstrated antiulcer potential [13].

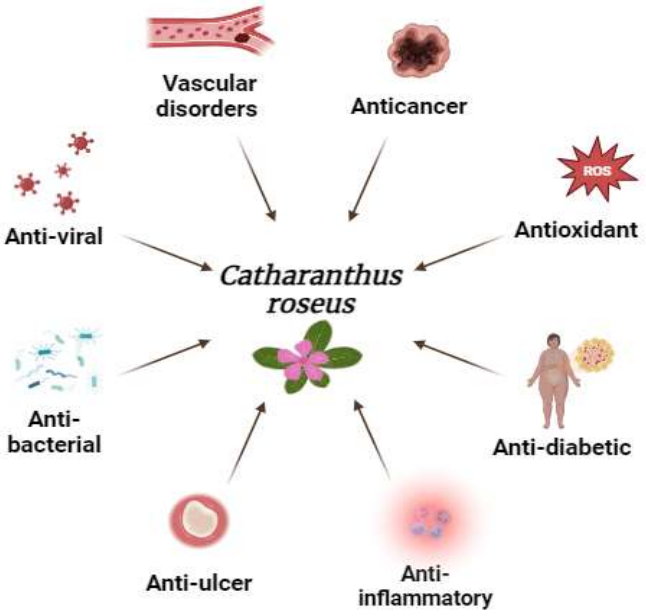

Figure 1: Pharmacological activities of *Catharanthus roseus*

Although many different diseases are treated with *Catharanthus roseus* and other therapeutic herbs in traditional medicine, the herbal business faces problems with the adulteration and substitution of medicinal herbs with closely related species. When a medication is tampered with or replaced with hazardous adulterants, its effectiveness declines and in certain situations, it can even be fatal [14]. Therefore, the effectiveness of the therapeutic herb depends on its proper identification through macroscopic and microscpic methods (i.e., appearance, color, surface texture) [15], chemical profiling (e.g. the technique of TLC, HPLC-Mass spectrometry) [16], and molecular identification (i.e., DNA barcoding)

DNA authentication offers greater dependability because it is a stable macromolecule that is present in every organ and is not impacted by outside influences [17]. Thus, the usage of DNA-based identifiers is crucial to the verification of medicinal plant identity. DNA barcoding is a revolutionary method for recognizing biological specimens by utilizing small DNA sequences from the genomes of either nuclei or organelle. It has become an efficient, quick, and precise method for verifying plant species, especially in the field of traditional Chinese medicine (TCM), to identify plant species through the analysis of short, standardized gene sections using DNA barcoding [18]. Plant DNA barcoding is more intricate and frequently calls for multiple sets of DNA markers [19]. The chloroplast genes matK and rbcL, and nuclear ribosomal DNA marker ITS , either alone or in combination, are the most widely used plant DNA barcodes [20].

The Consortium for Barcode of Life (CBOL), is presenting multiple working groups to discover the universal barcode gene, such as COI in metazoans, rbcL, matK, and ITS in plants, ITS in fungi, and 16S rRNA gene in both archaea and bacteria. Effective species identification has been achieved by the use of DNA barcodes in forensic biology, food distribution management, and disease research, among other fields [21]**.** With the advent of new sequencing techniques as ONT MinION nanopore, Next-generation sequencing (NGS), and Pac Bio, DNA barcoding has become more accurate, fast, and consistent.

[19].

The rationale behind choosing rbcL region in this study is that it not only has sufficient conserved areas for the production of universal primers, but also sufficient heterogeneity to enable species identification. Furthermore, accurate identification and documenting of the medicinal plants through rbcL may contribute to their sustainable use and conservation. The main concerns of our study is on the significance of accurately identifying species, interpreting traditional and indigenous knowledge about the use of therapeutic plants, and transferring this information to advance biodiversity prospecting in human health care [22]**.**

2. Materials and Method

2.1. Sample collection

The current research was conducted at the Centre for Applied Molecular Biology (CAMB), University of the Punjab, Lahore, Punjab-Pakistan. Young leaves of *Catharanthus roseus* plant were collected from the premises of CAMB.

2.2. Taxonomic Identification

The plant sample was taxonomically identified by Dr. Amin Ullah Shah from Botany Department, University of Sargodha, Sargodha, Punjab Pakistan.

2.3. DNA extraction and PCR amplification

The genomic DNA was extracted from the leaves of *Catharanthus roseus* using standard Qiagen DNA extraction Spin protocol using the DNA easy mini-DNA Etraction kit (QIAamp DNA mini kit Cat # 51306) according to the manufacturer's instructions. The quality and concentration of extracted DNA was determined through NanoDrop spectrophotometer and 0.8% (w/v) agarose gel electrophoresis. The isolated DNA was stored at -20°C for further use.

High quality template DNA was used for PCR amplification of sequence of interest (rbcL), using DreamTaq Green PCR Master Mix (2X) (Cat # K1081). The PCR reaction contained PCR master mix, 1µL each forward and reverse primers, and 2µL template DNA. The primer details are given as follows.

Details of primer sequence

Rubisco	**PCR Primer F**	**ATGTCACCACAAACAGAGACTAAAGC**
	PCR Primer R	**GAAACGGTCTCTCCAACGCAT**

Reaction was set to 35 cycles in Thermocycler using conditions outlined below given in Figure :

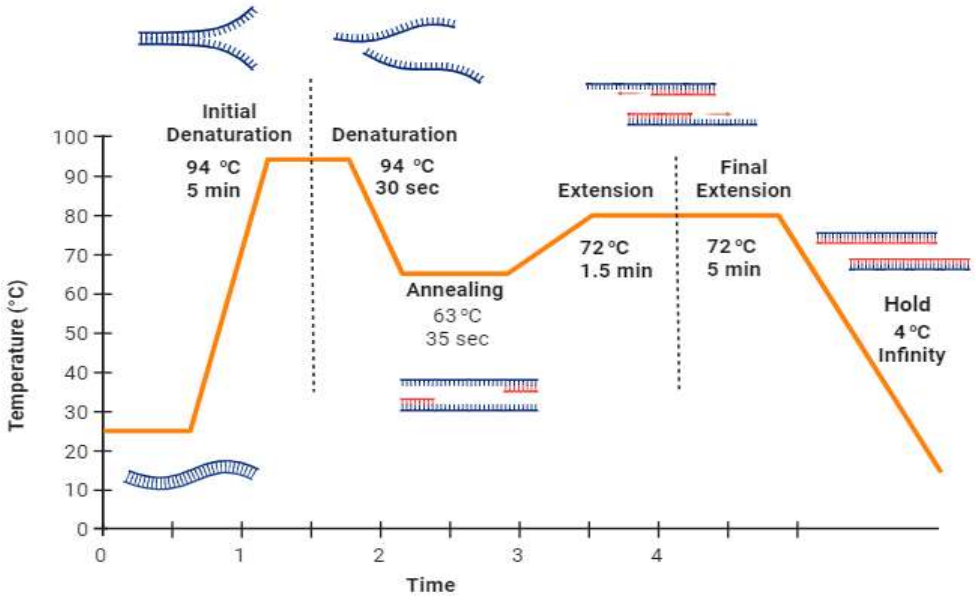

Figure 2: PCR cycling conditions

Amplified PCR product was resolved on 2 % agarose gel and amplicon size was confirmed by comparing it with 1Kb DNA Ladder.

2.4. Nucleotide sequencing

PCR product or amplicon observed on gel was cut using sterile blade and was purified by using Gene Jet Gel Extraction Kit (Cat # no K0691) according to manufacturer's instructions. The purified sample was sent to APICAL SCIENTIFIC SDN. BHD. (Formerly known as First BASE Laboratories Sdn Bhd), Malysia for sequencing.

2.5. Data Analysis and Multiple Sequence Alignment

After sequencing, the resultant sequence was edited and trimmed manually. For identification of species, we used two strategies i.e. Basic Local Alignment Tool (BLAST) and Maximum Likelihood method of phylogenetic tree. BLASTN tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) from NCBI was used to authenticate the taxonomic identity of our sequence and to identify the sequence on basis of percent identity and E-value. Molecular Evolutionary Genetics Analysis (MEGA) software was used for the statistical analysis of sequence data. Top ten highly similar sequences from BLAST were selected and their multiple and pairwise alignment was performed using ClustalW multiple alignment tool in MEGA X software. The evolutionary history was analyzed using Neighbour-joining method [23] and evolutionary distance was calculated using maximum-likelihood method based on Tamura-Nei model [24]. Furthermore, the sequencing result was used to count percent AT and percent GC content. The nucleotide sequence was submitted to Genbank with the accession number PP706114(https://www.ncbi.nlm.nih.gov/nuccore/PP706114.1/). Furthermore, DNA barcode was generated shown in figure 8.

3. Results

3.1. DNA Extraction and Sequencing

Fine quality DNA was extracted from the leaves of *Catharanthus roseus* plant, whereby its quality was checked on 2% agarose gel (shown in Figure 3). The rbcL loci was successfully amplified with a distinct signal band of expected size (0.6-0.6kb) depicted in

Figure . The PCR product, sequenced after purification, produced strong chromatogram signals with high-quality sequence results as shown in figure. The success rate of PCR amplification and sequencing, being the most valuable criteria to evaluate identification through barcoding, using rbcL primers was 100%. Furthermore, some base pairs were trimmed manually from the ends to enhance the quality of sequence results are shown in

Figure . The nucleotide composition of amplicon from selected plant species revealed 45% GC and 55% AT content.

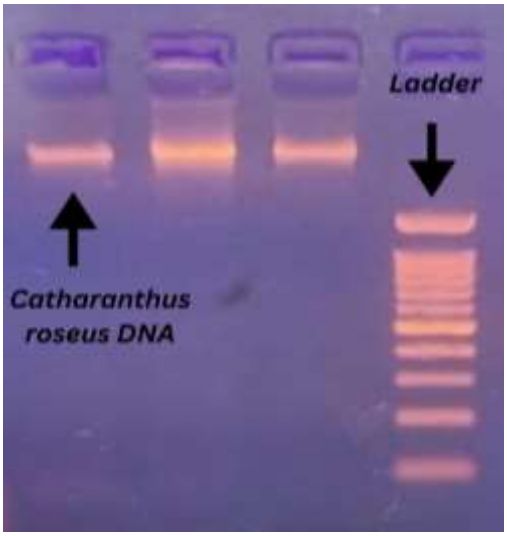

Figure 3: Isolated genomic DNA of *Catharanthus roseus*

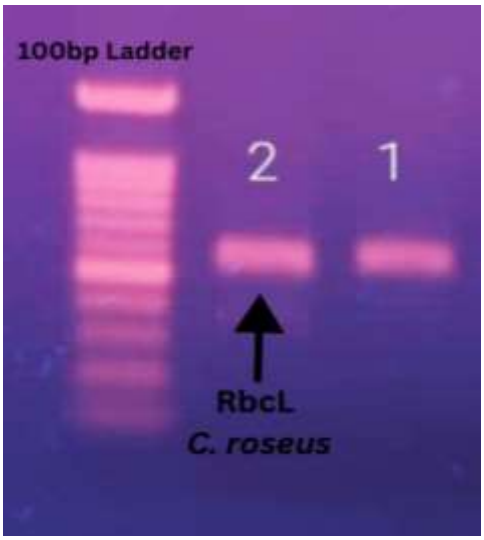

Figure 4: RbcL amplicon of *Catharanthus roseus*

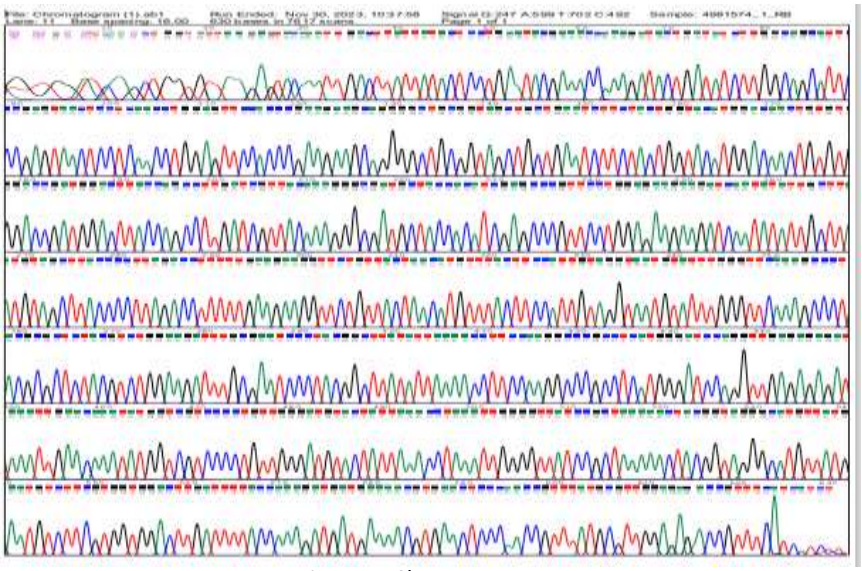

Figure 5: Chromatogram

3.2. Evolutionary relationship analysis

The obtained sequence was subjected to Basic Local Alignment Tool (BLAST) in NCBI database for sequence data analysis and top ten highly similar sequences with 98 to 99% similarity were selected (Figure). Evolutionary analyses was conducted in MEGA11 software [25]. The selected sequences were subsequently aligned with multiple sequence alignment by ClustalW algorithm in MEGA11 software.

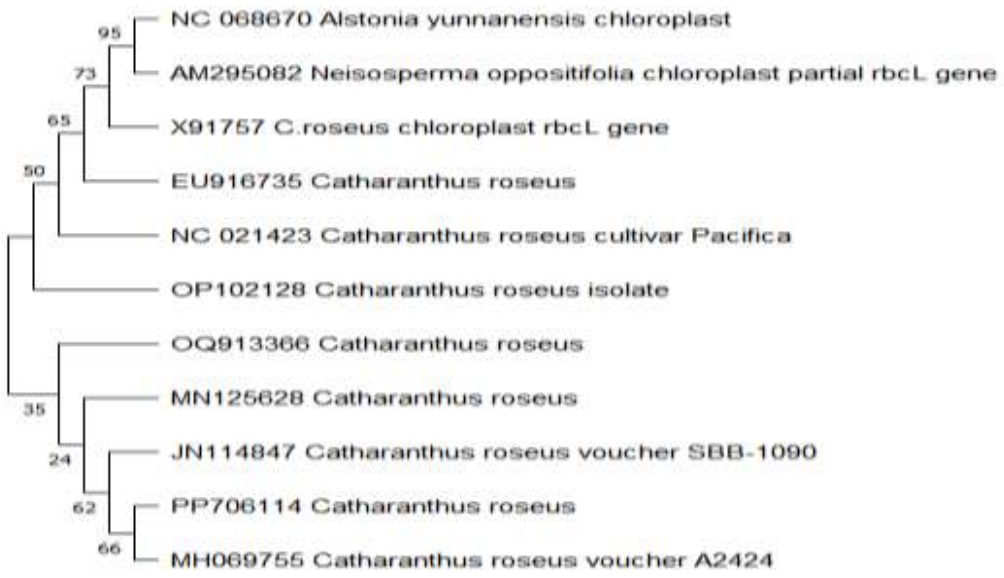

Figure 6: Bootstrap consensus tree.

The evolutionary history of taxa being analyzed was deduced using Neighbor-joining method [23]. It is represented by bootstrap consensus tree inferred from 1000 replicates [26]. Any branches on a phylogenetic tree that were supported by less than 50% of the bootstrap replicates have been collapsed, a common practice to avoid over-interpreting the poorly supported branches, which might be due to insufficient data or random noise. The percentage of bootstrap replicate trees (from a total of 1000 replicates) is displayed alongside each branch on the tree. Branches with high bootstrap values are regarded as more reliable, whereas those with low values are treated with more caution. The evolutionary distances between sequences were computed by Maximum Composite Likelihood method [24], whereby the distances are expressed as the number of base substitutions per site, indicating the average number of changes per nucleotide position during the evolution of sequences. The analysis involved 11 sequences. All the ambiguous position were removed by pairwise deletion. A total of 573 position were there in the final dataset.

Based on Rubisco Protein Partial Sanger sequence analysis, the tested subject has 99 percent evolutionary relations to *Catharanthus roseus.*

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Total score	E-Value	Match/ Total	Pct. (%)
MN125628.1	***Catharanthus roseus***	663	1.00	663	100	1059	0.00	573/663	100%

The QR code of *Catharanthus roseus* based on its rbcL sequence is given in figure 7.

Figure 7: QR code of *Catharanthus roseus* based on rbcL submitted in GenBank

4. Discussion

In this intended study, we successfully used rbcL gene as a marker for DNA barcoding of *Catharanthus roseus.* The rbcL has been found to provide sufficient phylogenetic signals involved in resolving relationships at various taxonomic levels [27]. The results of our study show that *Catharanthus roseus* has unique identification profile, consistent with the already known utility and specificity of rbcL in differentiating closely related species within family Apocynaceae [28]. The significant genetic distance was observed between specimens of *Catharanthus roseus* and other related species, reinforcing the efficiency of rbcL in creating lineages. Pei et al., (2015) successfully developed rbcL barcode of various forest plants with success rate of 90-100% [29]. Our result aligns with the findings of Ibrahim et al., (2019) who successfully utilized rbcL barcode for identification of different genotypes of Quinoa [30]. Furthermore, our study adds to the ongoing discourse regarding standardizing the markers for DNA barcoding. RbcL has proven a reliable barcoding candidate for identification in numerous cases, still it is recommended by Consortium for the Barcode of Life (CBOL) to use multiple markers for specie identification and to get more efficient and robust discriminatory power. RbcL in combination with other markers, can aid in more efficiently distinguishing closely related species, complementing mating, morphological and genetic data [31].

Additionally, this intended study underscore the utilization of DNA barcoding in identification and authentication of vital medicinal plants, an application crucial to prevent misidentification and adulteration that often plagues the market of herbal medicine [32]. Safety of the consumers and efficacy of herbal

products, all rely upon accurate identification of plant material and health implications of misidentified herbal supplements [33,34].

In the nutshell, the present study enhances our understanding of genetic landscape of *Catharanthus roseus* plant and validates the use of rbcL marker as effective tool for identification of medicinally important or otherwise all types of plants. Continued efforts for barcoding of plant species using region-specific and standardized markers will be efficient for the study, conservation and sustainable use of biodiversity.

Conclusion

DNA barcoding using rbcL gene has proven to be a reliable method for precise identification of *Catharanthus roseus.* The standardized protocol involving DNA extraction, PCR amplification, sequencing and phylogenetic analysis using bioinformatics tools allows for precise species verification, which is crucial for both pharmacological research and conservation efforts. The high resolution of rbcL marker in differentiating *C.roseus* from closely related species ensures its effectiveness as a molecular identification tool. It not only justifies the authenticity of *C.roseus* in medicinal applications but also facilitates the monitoring and preservation of this valuable plant species in its natural habitat. Future research can expand on these findings by incorporating additional genetic markers to enhance resolution and reliability of identification of plant species.

Conflict of interest

The authors have no conflict of interest regarding the information contained in this paper, it means that the authors do not have any personal, professional or financial interests that could influence the interpretations of research findings.

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Ethical Statements:

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