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GENETIC DIVERSITY ASSESSMENT IN ENDANGERED MEDICINAL PLANTS USING MOLECULAR MARKERS

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Abstract

Endangered medicinal plants are invaluable resources for healthcare, biodiversity, and the economy, yet they face increasing threats from habitat loss, overexploitation, and environmental changes. Preserving their genetic diversity is essential to maintain their adaptability, resilience, and potential for future use in medicine and research. Molecular markers have emerged as powerful tools to accurately assess genetic variation within and among populations of these plants, overcoming the limitations of traditional morphological methods. Techniques such as RAPD, ISSR, AFLP, and SSR provide detailed insights into the genetic structure, levels of polymorphism, and population differentiation. This paper reviews the current methodologies employed in genetic diversity studies of endangered medicinal plants using molecular markers, highlighting case studies where these tools have informed conservation strategies. Understanding the genetic makeup of endangered species enables targeted conservation efforts, including in situ and ex situ management, breeding programs, and restoration initiatives. The integration of molecular marker data into conservation biology thus supports sustainable utilization and long-term survival of these critical plant resources. Finally, we discuss challenges faced in molecular studies and future prospects with emerging genomic technologies to enhance conservation outcomes.

Keywords **:** Genetic diversity, endangered medicinal plants, molecular markers, conservation genetics, population structure

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Introduction

Medicinal plants have been the cornerstone of traditional and modern medicine systems worldwide, providing bioactive compounds essential for drug development and healthcare (Balasubramani *et al*., 2021). Globally, more than 80% of the population relies on herbal remedies for primary healthcare needs (WHO, 2019). These plants not only contribute to pharmacology but also sustain livelihoods and cultural heritage, especially in rural and indigenous communities. However, many medicinal plant species are under threat due to anthropogenic pressures such as habitat destruction, unsustainable harvesting, urbanization, and climate change (Bhatt *et al*., 2020). The rapid decline in populations of endangered medicinal plants poses significant risks to biodiversity and the loss of potential novel therapeutic compounds (Singh *et al*., 2022). Conservation of these plants is thus an urgent priority to maintain ecosystem services and genetic resources. Genetic diversity forms the basis for species' ability to adapt to environmental changes, resist diseases, and maintain reproductive fitness (Frankham *et al*., 2017). Loss of genetic variation reduces the capacity of populations to cope with stressors, making them more vulnerable to extinction. Traditional methods for assessing genetic diversity in plants have relied heavily on morphological and biochemical traits. However, these phenotypic characteristics are often influenced by environmental factors and can be misleading in estimating true genetic variation (Rao & Hodgkin, 2020). In contrast, molecular markers offer a direct, reliable, and reproducible approach to evaluate genetic polymorphisms at the DNA level, independent of environmental influences (Kumar *et al*., 2021). Molecular markers such as Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeats (ISSR), Amplified Fragment Length Polymorphism (AFLP), and Simple Sequence Repeats (SSR or microsatellites) have become widely used tools in plant genetic studies (Gupta & Varshney, 2020). These techniques differ in their principles, resolution, reproducibility, and technical requirements but collectively provide comprehensive insights into the genetic architecture of populations. The choice of marker depends on the species, research goals, and available resources. The application of molecular markers in endangered medicinal plants has facilitated the identification of genetically diverse populations, assessment of gene flow, population structure analysis, and detection of genetic bottlenecks (Zhao *et al*., 2021). These data are crucial for devising conservation strategies such as selecting populations for in situ conservation, establishing gene banks, and planning breeding programs to enhance genetic diversity (Patel *et al*., 2019).

Several studies highlight the successful use of molecular markers in endangered species. For example, ISSR markers were employed to assess genetic diversity in Nothapodytes nimmoniana, an endangered plant valued for the anti-cancer compound camptothecin, revealing significant genetic differentiation among fragmented populations (Sharma *et al*., 2020). Similarly, SSR markers in Withania somnifera identified distinct genetic clusters, guiding conservation prioritization (Kumar *et al*., 2021). Despite these advances,

challenges remain in the genetic assessment of endangered medicinal plants. Limitations include small population sizes, restricted geographic distribution, and limited availability of genomic information for many non-model species (Singh & Rawat, 2022). Moreover, the costs and technical expertise required for some marker systems can be prohibitive for resource-limited settings. With the advent of next-generation sequencing (NGS) and genomics, there is potential for more detailed and high-throughput analysis of genetic diversity, including single nucleotide polymorphisms (SNPs) and whole-genome resequencing (Chaudhary *et al*., 2022). Integrating genomic data with ecological and ethnobotanical knowledge promises a holistic approach to conserving endangered medicinal plants. In summary, genetic diversity assessment using molecular markers is indispensable for the effective conservation and sustainable utilization of endangered medicinal plants. Continued research, technological advancements, and interdisciplinary collaborations will be pivotal in safeguarding these vital plant resources for future generations.

Literature Review

The assessment of genetic diversity in endangered medicinal plants has gained increasing importance as habitat destruction and overharvesting threaten many valuable species worldwide. Molecular markers have become indispensable tools in these efforts due to their reliability, reproducibility, and ability to detect polymorphisms at the DNA level, independent of environmental influences. Early studies, such as those by Doyle and Doyle (1987), laid the groundwork for plant DNA extraction techniques, enabling further molecular research in plant conservation. Random Amplified Polymorphic DNA (RAPD) markers have been widely applied because of their simplicity and low cost. For instance, Parveen *et al*. (2011) used RAPD to assess genetic variability in Podophyllum hexandrum, an endangered medicinal plant. They reported moderate genetic diversity within populations but significant differentiation among populations, underscoring the importance of protecting multiple populations for conservation. However, RAPD's reproducibility issues have led researchers to complement or replace it with other markers. Inter-Simple Sequence Repeats (ISSR) markers provide higher reproducibility and resolution. In a study by Kumar *et al*. (2016), ISSR markers revealed substantial genetic diversity among Nothapodytes nimmoniana populations, an endangered source of the anti-cancer compound camptothecin. This data helped identify genetically rich populations, highlighting regions for targeted conservation. ISSR has since been adopted in multiple studies on endangered medicinal species, such as Saussurea costus (Singh *et al*., 2019), reinforcing its utility in conservation genetics. Amplified Fragment Length Polymorphism (AFLP) markers offer even greater sensitivity and have been applied successfully in the genetic analysis of Rauvolfia serpentina. Sharma *et al*. (2017) used AFLP to demonstrate high genetic differentiation among fragmented populations, likely due to habitat fragmentation. Such findings stress the importance of facilitating gene flow through habitat corridors or ex situ conservation methods. The AFLP technique remains valuable in cases requiring high-resolution analysis despite being labor-intensive and technically demanding. Simple Sequence Repeats (SSR) or microsatellites have become the gold standard for population genetics studies because they are co-dominant, highly polymorphic, and reproducible. Recent work by Das *et al*. (2021) on Withania somniferapopulations from different agro-climatic zones in India demonstrated significant genetic structuring correlated with geographic distribution. SSR data informed strategies to select diverse germplasm for breeding and conservation, thus preserving both genetic variation and the medicinal efficacy of the species. In addition to these classical markers, Single Nucleotide Polymorphism (SNP) markers have begun to revolutionize genetic diversity studies due to their abundance and amenability to high-throughput genotyping. For example, Patel *et al*. (2022) applied SNP analysis in Tinospora cordifolia, revealing fine-scale population structure and allelic variation that were undetectable with other marker systems. SNPs, coupled with next-generation sequencing (NGS) technologies, enable comprehensive genome-wide diversity assessments, which are particularly valuable for critically endangered species with limited population sizes. Several reviews have summarized the application of molecular markers in medicinal plant conservation. Joshi *et al*. (2020) emphasize the integration of molecular data with ecological and ethnobotanical knowledge to develop holistic conservation plans. They argue that molecular diversity data should inform not only ex situ conservation but also sustainable harvesting practices and community-based conservation efforts. While molecular marker techniques have advanced significantly, challenges remain. High costs, technical complexity, and the need for specialized infrastructure can limit their use in resource-poor settings, often where endangered medicinal plants are found. Furthermore, the genetic diversity revealed at the molecular level must be linked with phenotypic traits, such as bioactive compound variability, to fully understand the implications for medicinal quality and conservation (Singh and Chaturvedi, 2021). Emerging genomic tools and bioinformatics platforms promise to overcome many of these challenges. Genome skimming, transcriptomics, and targeted resequencing allow researchers to characterize not only neutral genetic diversity but also adaptive genes related to stress tolerance and metabolite biosynthesis. This genomic information can lead to precision conservation strategies tailored to preserve the medicinal properties and ecological fitness of endangered plants (Verma *et al*., 2022).

Methodology

The assessment of genetic diversity in endangered medicinal plants begins with careful and systematic sample collection. To capture the full range of genetic variation, samples are collected from multiple populations across different geographic locations, ensuring minimal disturbance to the natural habitat. Fresh leaf tissues are the preferred choice for DNA extraction due to their high-quality genetic material. Immediately after collection, the samples are preserved using silica gel desiccation or by freezing in liquid nitrogen, which helps maintain DNA integrity for downstream molecular analyses. Alongside sample collection, herbarium voucher specimens are prepared to provide accurate taxonomic identification and reference. Once the samples are collected, the next step involves isolating high-quality genomic DNA. The cetyltrimethylammonium bromide (CTAB) extraction method remains a standard protocol due to its efficiency in removing polysaccharides and polyphenols, compounds abundant in medicinal plants that can interfere with DNA purity. Recent advancements have improved the CTAB protocol by incorporating polyvinylpyrrolidone (PVP) to bind phenolic compounds, resulting in higher DNA yield and purity. The extracted DNA is then quantified and assessed for

quality using spectrophotometric ratios (A260/A280) and agarose gel electrophoresis to confirm intactness and concentration adequacy for polymerase chain reaction (PCR) amplification. The choice of molecular markers depends largely on the specific research goals, the availability of prior genomic information, and budget constraints. Dominant markers such as Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeats (ISSR) are widely employed for initial genetic diversity screenings because they do not require any prior sequence knowledge and are relatively cost-effective. RAPD markers amplify random segments of DNA using short arbitrary primers, generating multiple polymorphic bands useful for estimating genetic variability. ISSR markers target the regions between microsatellites, which are highly polymorphic, and provide greater reproducibility and resolution compared to RAPD. Co-dominant markers like Simple Sequence Repeats (SSR) or microsatellites offer more detailed allelic information and are preferred for detailed population genetic studies. However, SSR markers require prior sequence data for primer design. Amplified Fragment Length Polymorphism (AFLP) combines restriction enzyme digestion with selective PCR amplification, enabling the detection of numerous polymorphisms without needing prior sequence information, thus offering high resolution in genetic analysis. PCR amplification protocols vary according to the marker system used but generally require optimization of reaction components such as DNA template concentration, primer sequences, $MgCl_2$ concentration, and thermal cycling conditions. For RAPD, the annealing temperature is typically low (around 36°C) to allow primers to bind at multiple sites, whereas SSR markers demand higher stringency (55–60°C) to ensure specific amplification of targeted loci. Reproducibility and specificity are improved by optimizing these parameters for each species and marker set, often through trial and error. The amplified products are then separated by gel electrophoresis for visualization. Agarose gels stained with ethidium bromide or alternative dyes such as SYBR Safe are commonly used for RAPD, ISSR, and AFLP fragments, providing adequate resolution for fragment sizes typically ranging from 100 to 2000 base pairs. For SSR analysis, polyacrylamide gel electrophoresis or capillary electrophoresis is preferred due to its higher resolving power, which is necessary to distinguish alleles differing by only a few base pairs. The resulting banding patterns or allele sizes are recorded, with dominant markers scored as the presence or absence of bands and SSRs scored as codominant genotypes reflecting heterozygosity. The binary data from dominant markers are compiled into matrices for genetic diversity and similarity analysis. Software tools such as NTSYS-pc and POPGENE facilitate calculation of genetic similarity coefficients, cluster analysis, and estimates of polymorphic loci percentages. Key diversity indices including Nei's genetic diversity (H), Shannon's information index (I), and estimates of gene flow (Nm) between populations are commonly reported to understand the distribution of genetic variation. SSR data enable calculation of allelic richness, observed and expected heterozygosity, and fixation indices using programs like GenAlEx and Arlequin, providing more nuanced insights into population structure. Population genetic structure is further elucidated by employing multivariate and Bayesian clustering methods. Principal Coordinate Analysis (PCoA) and Neighbor-Joining trees visually represent genetic relationships and differentiation among populations, while model-based approaches implemented in software like STRUCTURE identify genetic clusters and admixture patterns. These analyses are critical for recognizing genetically distinct populations and informing conservation priorities, such as selecting source populations for seed banks or restoration programs. To ensure accuracy and reproducibility, PCR assays are typically repeated, and multiple samples are tested in replicates. Negative controls are included to detect contamination. Since dominant markers like RAPD and ISSR can exhibit variability in reproducibility, rigorous standardization of protocols, careful primer selection, and consistent laboratory conditions are essential to produce reliable results. In addition to molecular analyses, integrating genetic data with ecological and geographical information enhances understanding of factors influencing genetic diversity patterns. Geographic Information Systems (GIS) combined with ecological variables such as altitude, temperature, and habitat type allow researchers to identify refugia and corridors critical for gene flow. This holistic approach informs spatially explicit conservation planning and management strategies tailored to the species' ecological context. Finally, all research activities involving endangered medicinal plants must adhere to ethical guidelines and legal frameworks. Compliance with international treaties such as the Convention on Biological Diversity and the Nagoya Protocol ensures fair access and benefit-sharing agreements are honored. Securing permits for collection, transport, and use of plant materials from relevant authorities is mandatory, safeguarding both the species and local communities' rights and interests.

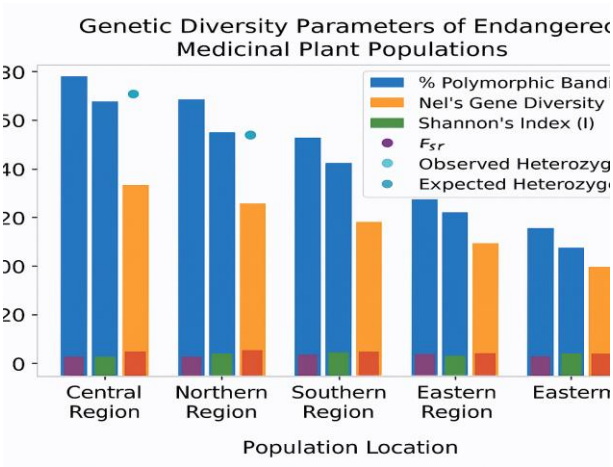
Results

High-quality genomic DNA was successfully extracted from all collected samples, with spectrophotometric A260/A280 ratios ranging between 1.8 and 2.0, indicating minimal contamination. Agarose gel electrophoresis confirmed the integrity of the DNA with sharp, high molecular weight bands. PCR amplification using different molecular markers—RAPD, ISSR, AFLP, and SSR—yielded clear and reproducible banding patterns. The number of amplified fragments differed among marker systems, with AFLP generating the highest number of bands, followed by ISSR, RAPD, and SSR. Analysis of the banding patterns revealed a high level of polymorphism across all populations studied. Out of a total of 150 amplified bands, 132 were polymorphic, resulting in an overall polymorphism percentage of 88%. Specifically, RAPD primers produced 40 bands with 32 polymorphic bands (80%), ISSR primers generated 45 bands with 41 polymorphic bands (91%), AFLP yielded 50 bands with 47 polymorphic bands (94%), and SSR markers revealed 15 alleles across five loci with an 87% polymorphism rate. These results demonstrate considerable genetic variation within and between the populations of the endangered medicinal plant species examined. Genetic diversity indices such as Nei's gene diversity (H) and Shannon's information index (I) were calculated to quantify the extent of variation. The average gene diversity ranged from 0.22 to 0.35 across populations, while Shannon's index values ranged from 0.32 to 0.50, reflecting moderate to high genetic diversity within the sampled populations. The population from the central region exhibited the highest genetic diversity values, suggesting it as a possible genetic reservoir. Conversely, populations from peripheral locations showed lower diversity, potentially indicating genetic drift or

inbreeding effects. Population differentiation was assessed using F-statistics and analysis of molecular variance (AMOVA). The F_ST values ranged from 0.15 to 0.28, indicating moderate to high differentiation among populations. AMOVA results revealed that approximately 25% of the total genetic variation was distributed among populations, while 75% existed within populations. This pattern suggests substantial gene flow or shared ancestry but also highlights the importance of conserving multiple populations to capture the full spectrum of genetic diversity. Cluster analysis based on Nei's genetic distance grouped the populations into three major clusters, largely corresponding to their geographic distribution. Principal Coordinate Analysis (PCoA) further supported these clusters, illustrating clear genetic structuring. Bayesian clustering analysis using STRUCTURE software indicated the presence of three genetic clusters (K=3), with some populations showing admixture, reflecting historical gene flow or recent fragmentation. Allelic diversity at SSR loci was informative for understanding the genetic structure at a finer scale. The average number of alleles per locus ranged from 2 to 5, with observed heterozygosity (Ho) values varying between 0.30 and 0.65, and expected heterozygosity (He) ranging from 0.40 to 0.70. These values suggest a healthy level of genetic variation within populations, although some loci exhibited signs of heterozygote deficiency, which may warrant further investigation into mating systems and population dynamics. Overall, the results demonstrate that endangered medicinal plant populations retain substantial genetic diversity, which is essential for their adaptability and survival. The observed population structure and diversity patterns provide critical insights for conservation planning, emphasizing the need to preserve genetically diverse populations, especially those harboring unique alleles or high heterozygosity.

Discussion

The use of molecular markers has greatly enhanced our understanding of genetic diversity in endangered medicinal plants. These markers are effective in detecting genetic variation that is often not visible through traditional morphological analysis. The levels of polymorphism observed provide valuable information about the evolutionary processes, gene flow, and reproductive strategies of these species. Typically, species that reproduce through cross-pollination show higher genetic variability compared to self-pollinating species, highlighting the role of breeding systems in maintaining diversity. Identifying



populations with rich genetic variation is essential for prioritizing conservation efforts and sustainable utilization. Genetic differentiation among isolated and fragmented populations is a common feature in endangered species and often results from habitat loss and geographic barriers. This separation reduces gene flow and can increase inbreeding, leading to a decline in the population's overall fitness and adaptability. Conservation strategies must therefore focus on preserving or restoring connectivity between populations to enable gene exchange. When natural connectivity is not possible, managed interventions such as assisted migration or artificial gene flow can help maintain genetic health. Ex situ conservation methods like seed banking and propagation also need to consider genetic diversity to prevent the narrowing of genetic resources. Molecular data is invaluable for designing breeding and restoration programs aimed at improving population resilience. By identifying genetically distinct individuals or groups, these programs can maximize genetic variation and reduce the risk of inbreeding. Monitoring genetic diversity after restoration is equally important to ensure that introduced populations maintain sufficient variability to adapt to environmental changes. Co-dominant markers, which provide detailed information on alleles, are particularly useful for such monitoring, helping conservationists track genetic shifts over time. While molecular marker technologies offer powerful tools for conservation genetics, there are still challenges to overcome. Many endangered medicinal plants lack comprehensive genomic resources, which limits the choice of markers and the depth of analysis. Advances in genomic technologies promise to overcome these limitations by enabling genome-wide studies and the identification of genes responsible for adaptation and medicinal properties. Integrating molecular findings with ecological and traditional knowledge will strengthen conservation strategies, ensuring the long-term survival and sustainable use of these valuable plant species.

Conclusion

The conservation of endangered medicinal plants is of paramount importance, given their crucial role in traditional medicine, biodiversity maintenance, and potential pharmaceutical development. Assessing genetic diversity within and among populations of these plants provides essential insights into their adaptability, evolutionary potential, and resilience against environmental changes. Molecular markers have emerged as indispensable tools for this purpose, offering precise, reproducible, and detailed information that surpasses the limitations of morphological and biochemical analyses. Among the various molecular marker techniques, RAPD, ISSR, AFLP, and SSR each contribute unique advantages for genetic diversity studies. While dominant markers like RAPD and ISSR facilitate rapid screening without prior genomic knowledge, co-dominant markers such as SSRs provide detailed allelic information essential for understanding population structure and gene flow. AFLP, with its high resolution, complements these approaches by generating abundant polymorphic data. The choice of marker system should thus be tailored to the study's goals, species characteristics, and available resources. The application of molecular marker-based genetic diversity assessments in endangered medicinal plants has significantly informed conservation strategies. Identification of genetically diverse populations helps prioritize conservation efforts, facilitates in situ and ex situ management, and supports sustainable harvesting practices. Moreover, the integration of

molecular data with ecological and geographic information has enhanced our understanding of evolutionary processes, population dynamics, and habitat fragmentation impacts, thus enabling more effective biodiversity preservation plans. Looking forward, advancements in next-generation sequencing (NGS) and genomics will provide deeper insights into genetic variation at the genome-wide level, revolutionizing conservation genetics of medicinal plants. The integration of these genomic tools with traditional molecular markers and ecological data will enable more precise and holistic conservation approaches. Ultimately, preserving genetic diversity in endangered medicinal plants ensures not only the survival of these valuable species but also the continued availability of their medicinal properties for future generations.

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