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Review Article

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Advancements in DNA-based molecular markers to unravel the genetic diversity of endangered plants

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ABSTRACT

One of the primary requirements to develop effective and practical sustainable management strategies for endangered plant species, is the assessment of genetic diversity. DNA-based molecular markers have proven themselves to be an excellent tool to assess many parameters including elucidation of genetic diversity for the past few decades. During this period, DNA-based marker technology has undergone many changes. From the initial RFLP to the contemporary systems that use high-throughput sequencing or next-generation sequencing platforms that have revolutionized genetic diversity assessment. This review focuses on the application of the molecular markers and, a few of their variants, to evaluate the genetic diversity of endangered plants along with their advantages, limitations as well as future prospects and scope.

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INTRODUCTION

Genetic diversity assessment of plant species deemed endangered is extremely important to develop effective and practical conservation strategies, as it forms the basis for informed decision-making and sustainable management practice. Over the years, the landscape of molecular marker technologies has undergone a profound transformation, facilitating a deeper understanding of entire genomes. Historically, attempts to elucidate the genetic diversity of endangered plants relied on conventional markers which often had limited resolution. However, the surge in technological innovations has ushered in a new era. This shift is not merely a chronological progression but signifies a paradigm shift, enabling researchers to delve much deeper into the intricacies of genetic variation and diversity. Starting at Restriction Fragment Length Polymorphisms (RFLPs) (Botstein *et al.*, 1980) to the extremely advanced Genotyping-by-Sequencing (GBS) approaches (He *et al.*, 2014), discussing the usage, advantages, and limitations, providing a comprehensive understanding of their utility to unravel the genetic diversity of endangered flora. This is to further contribute to the ongoing discourse in genetic diversity and conservation genomics, fostering a deeper understanding of the intricate interaction between technology, biodiversity, and the sustainable management of endangered plant populations.

Among the early torch bearers of molecular marker technology, RFLPs carved a niche for themselves. The principle of detecting variations in DNA fragment lengths provided a rudimentary yet insightful glimpse into genetic differences. Random Amplified Polymorphic DNA (Williams *et al.*, 1990) and Amplified Fragment Length Polymorphisms (AFLP) (Vos *et al.*, 1995) added another layer to this narrative. Despite the challenges posed by dominant marker systems and limited transferability between species, AFLPs found their application in certain conservation contexts, contributing to the growing toolbox of molecular markers. The landscape underwent a major shift with the advent of Simple Sequence Repeats or SSR, initiating a phase characterized by increased robustness and applicability. SSRs, or microsatellites, with their high polymorphism levels, became a staple in genetic diversity assessments, offering a more dynamic and adaptable approach. This transition marked a turning point in the molecular marker narrative, with SSRs becoming a method of choice in studies focused on endangered plant populations.

The next major breakthrough was the introduction of Single Nucleotide Polymorphisms (SNPs), offering a leap in throughput and genotyping efficiency. The attractiveness of high-throughput sequencing (HTS) technologies gained momentum, reflecting a broader trend in the scientific community's inclination towards higher throughput, automated, and cost-effective methodologies. The advent of GBS and other next-generation sequencing approaches catapulted the field into an era of unparalleled

genomic exploration (He *et al.*, 2014). The E-utilities of NCBI were used to count the number of publications present in its database (Supplementary Data 1) having the keywords 'genetic diversity' and 'endangered' along with the different types of markers applied for the study (RFLP, RAPD, ISSR, AFLP, SSR, EST-SSR, SNP, GBS). The graphical representation of this data (Supplementary Data 2) shows a marked increase in the number of studies after the year 2010 (Figure 1). These technologies, marked by their ability to generate massive datasets at reduced costs, have redefined the boundaries of genetic diversity research, making them increasingly indispensable in the study of endangered plant populations (Table 1).

DNA-BASED MOLECULAR MARKERS

Restriction Fragment Length Polymorphisms (RFLPs)

At its core, the principle of RFLP analysis revolves around the detection of variations in DNA fragment lengths resulting from the differential presence of restriction enzyme recognition sites. This hybridization-based method provided early researchers with a window into the genomic diversity of plants (Kochert, 1991), albeit with certain limitations. These markers were co-dominant in nature and had the ability to identify a unique locus. However, the labor-intensive nature of the technique, coupled with the requirement for substantial DNA quantities (~8-18 µg), constrained its widespread application. As molecular marker methodologies evolved, the limitations of RFLPs became apparent, prompting the scientific community to seek more streamlined approaches.

While RFLPs may no longer be at the forefront of molecular marker technologies, their historical significance endures. Acknowledging the foundational role, they played in shaping our understanding, and assessing the genetic diversity of endangered plants sets the stage for the subsequent stages. A PCR-based modification of the RFLP procedure called Terminal Restriction Fragment Length Polymorphism (TRFLP) is now extensively utilized in microbial ecological studies to analyze the diversity and composition of microbial communities within various environmental samples (Chee-Sanford & Connor, 2023).

Random Amplified Polymorphic DNA (RAPD)

RAPDs present themselves as a noteworthy technique, offering insights into genetic diversity with its own set of attributes. The essence of RAPD methodology lies in its reliance on short, arbitrary DNA primers for the amplification of genomic regions. This simplicity, combined with the absence of prior genomic information, renders RAPDs an accessible tool for genetic diversity studies, especially in non-model plant species, making it particularly valuable in scenarios where genomic information is limited (Haque *et al.*, 2010). This analysis involves a few steps and reagents compared to some other molecular marker techniques, this makes the process very cost-effective and low resource-consuming. The rapid nature of RAPD assays allows for a relatively quick turnaround in obtaining results. This feature can be beneficial in time-sensitive studies or when dealing with a large number of samples. Also, the number of markers generated by this method is large and, which are distributed almost across the genome.

RAPDs did alleviate the methodological complexities of RFLP but it was a dominant marker system and there were issues with the results not being reproducible (Skroch & Nienhuis, 1995). Over the years a few modifications were introduced like DNA amplification fingerprinting (DAF) (Caetano-Anollés *et al.*, 1991), mini hairpin primer driven DAF (mhpDAF) (Caetano-Anollés & Gresshoff, 1994) but these methods required Polyacrylamide Gel Electrophoresis and silver staining. Amplicon hybridization-based methods were also introduced but these made the procedure complex (Penner *et al.*, 1996).

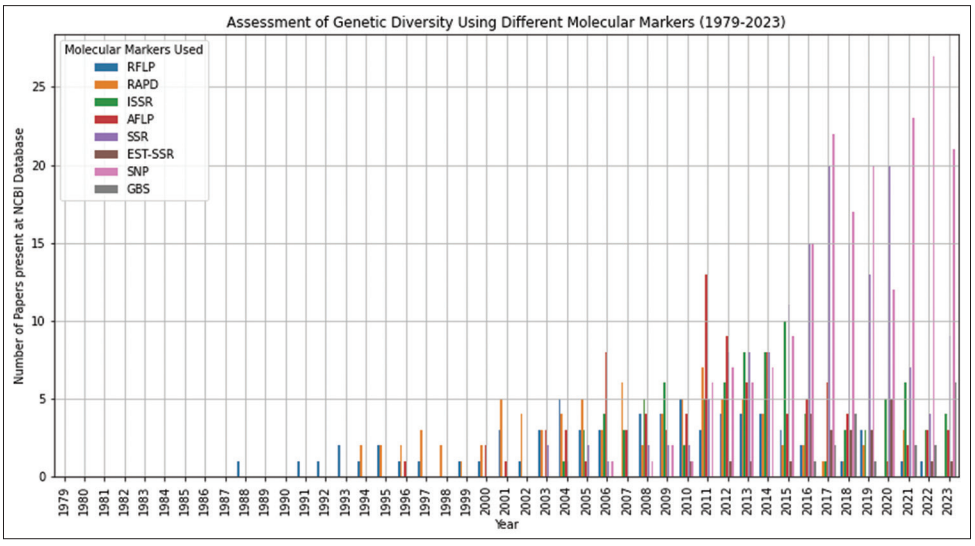

Figure 1: Number of publications present in NCBI database (1979-2023) with keywords 'genetic diversity' and 'endangered', and the type of molecular marker used

Inter-simple Sequence Repeats (ISSRs)

To address specificity and reproducibility issues present in RAPD, amplification of the microsatellite region with a single but long primer was developed (Meyer *et al.*, 1993). These were called inter simple sequence repeats (ISSRs). The primers being used were longer than those used in RAPD and thus required higher temperatures to anneal to template DNA.

Similar to RAPDs, ISSRs offer a distinct advantage in genetic analysis by circumventing the necessity for prior target sequence knowledge. The prevalence of microsatellites within genomes, coupled with their remarkable variability present in individuals within and among species, underscores the practicality and effectiveness of ISSRs. This inherent abundance and hypervariability ensure the robustness and applicability of ISSRs in diverse genetic studies, including population genetics, phylogenetics, and conservation biology (Li *et al.*, 2023).

AFLPs (Amplified Fragment Length Polymorphisms)

AFLP markers are particularly advantageous in endangered plant conservation due to their ability to capture genetic variation at multiple loci across the genome (Vos *et al.*, 1995). This comprehensive view of genetic diversity happens to be crucial for understanding the adaptive capability of a population, identifying unique genetic variants, and prioritizing conservation efforts. AFLP analysis allows researchers to unravel the intricate genetic structure of endangered plant populations, revealing patterns of gene flow, genetic drift, and demographic history (Zhao *et al.*, 2024). By quantifying levels of genetic diversity and detecting signs of inbreeding or genetic bottlenecks (Mafakheri *et al.*, 2022), AFLP analysis provides insights into the long-term viability of populations and informs management strategies aimed at maintaining genetic diversity and adaptive potential (Hosseini *et al.*, 2021). Moreover, AFLP markers can be used to identify individuals or populations with high genetic distinctiveness, which may serve as important reservoirs of genetic diversity for future conservation efforts (Kumar *et al.*, 2021a).

A key advantage of AFLP markers is their versatility and applicability to a wide range of plants, these include those plants which have limited genomic resources or taxonomic representation, as no prior DNA sequence information is required. This versatility enables researchers to study endangered plants with diverse evolutionary histories, ecological niches, and conservation statuses (Bobo-Pinilla *et al.*, 2018). AFLP analysis can uncover cryptic genetic diversity within species, identify evolutionary significant units for conservation prioritization, and inform reintroduction or translocation programs aimed at restoring populations in the wild. This is possible due to the generation of a comparatively large number of polymorphic loci.

However, it's important to acknowledge the challenges associated with AFLP analysis. The labor-intensive nature of AFLP protocols, including primer design, optimization, and fragment analysis, requires significant time, expertise, and resources. Additionally, the dominant nature of AFLP markers complicates data interpretation, as heterozygotes cannot be distinguished from homozygotes. This may lead to challenges in estimating allele frequencies, assessing population structure, and inferring demographic processes accurately. Though, AFLP markers are comparatively more reproducible than RAPD markers (Hu *et al.*, 2024), they may not be easily transferable across different plant species hindering the broader applicability of this technique.

Simple Sequence Repeat Markers (SSRs)

Microsatellites represent repetitive DNA sequences, typically comprising short nucleotide motifs (up to 5 nucleotides) that are repeated in tandem across the genome (Alves *et al.*, 2024). Simple sequence repeats is another name for these sequences. These sequences, characterized by their variability in repeat length among individuals, serve as vital genetic markers. Microsatellites or SSRs are omnipresent within plant genomes, residing in both coding and non-coding regions (Geethanjali *et al.*, 2024). Endangered plants, facing myriad threats such as habitat loss, fragmentation, climate change, and anthropogenic activities, require meticulous genetic assessments to inform conservation decisions and mitigate the risk of extinction (Van Rossum & Godé, 2022). SSR markers, characterized by their high variability, co-dominant inheritance (allowing for the discrimination of heterozygous and homozygous genotypes), and abundance across plant genomes, offer a powerful tool through which to scrutinize the genetic variability of endangered plant populations (Yu *et al.*, 2021). The fine-scale resolution afforded by SSR analysis enables the identification of subtle genetic variations within and among populations, even in taxa with limited population sizes or fragmented distributions. This precision is particularly sought after in the conservation strategy of endangered plants, where identifying unique genetic variants, delineating evolutionarily significant units, and prioritizing conservation actions are paramount (Malkócs *et al.*, 2020; Aldaba Núñez *et al.*, 2021).

SSR markers can be developed using relatively straightforward methodologies, such as DNA sequencing and PCR-based techniques. This accessibility facilitates the genetic characterization of non-model species and populations with restricted genetic resources, ensuring access to genetic tools for researchers (Wang *et al.*, 2020). In practice, SSR markers have been instrumental in elucidating the genetic diversity, evolutionary dynamics, and population structure of endangered plant species across diverse ecosystems and geographic regions. SSR analysis unveils patterns of genetic differentiation, gene flow, and demographic history critical for informed conservation decision-making. Additionally, SSR markers serve as indispensable tools in assessing the genetic health and viability of endangered plant populations, and identifying populations at risk of inbreeding depression, or reduced adaptive potential (Yousefzadeh *et al.*, 2021; Ul Islam *et al.*, 2023).

However, SSR marker application, with the aim of conservation of endangered plants is not devoid of challenges and

considerations. The development of SSR markers may entail considerable investments in terms of time, resources, and expertise, particularly for species or populations with complex genomic architectures (Alves *et al.*, 2024). Also, SSRs are generally transferable within closely related species but challenges may arise in their transferability to more distantly related taxa (Aiello *et al.*, 2020). This limitation needs to be considered when selecting markers for genetic diversity studies. Furthermore, the interpretation of SSR data requires careful consideration of factors such as null alleles, allelic stuttering, and scoring errors, which may affect the accuracy and reliability of genetic analyses, particularly in endangered plant populations characterized by high levels of inbreeding or low levels of genetic diversity.

A variation of the SSR markers is EST (Expressed Sequence Tag)-SSR markers (Cardle *et al.*, 2000). These markers are derived from expressed sequences in the genome and provide valuable insights into the functional aspects of genetic variation. The integration of EST-SSRs into the study of endangered plants not only enhances our understanding of population structure and genetic diversity but also furnishes valuable data on the adaptive traits essential for the survival of these species. EST-SSR markers are particularly advantageous due to their association with expressed genes. This linkage means that variations detected using EST-SSRs are more likely to be functionally relevant, reflecting traits that have been subject to natural selection. This functional relevance is critical in the context of endangered plants, where understanding the genetic basis of adaptive traits can inform conservation strategies aimed at enhancing the resilience of these species to environmental changes (Zhang *et al.*, 2022). One of the notable advantages of EST-SSR markers is their ability to be transferred among closer species. Since coding sequences tend to be more conserved than non-coding regions, EST-SSR markers developed for one species can often be applied to other species within the same genus or family (Jiang *et al.*, 2020). This transferability is particularly useful in the study of endangered plants, many of which are non-model species with limited genomic resources. By using EST-SSR markers, researchers can leverage existing genetic information from related species, facilitating the study of genetic diversity and adaptation in endangered plants without the need for extensive de novo marker development (Li *et al.*, 2020). The application of EST-SSRs in conservation genetics also extends to their use in marker-assisted selection. By associating EST-SSR markers with adaptive traits, such as drought tolerance, disease resistance, or reproductive success, conservationists can identify and prioritize individuals or populations that possess these beneficial traits (Zhou *et al.*, 2023b). This information is invaluable for breeding programs aimed at enhancing the adaptive potential of endangered plants, ensuring that conservation efforts are not only focused on preserving genetic diversity but also on maintaining the functional traits that are critical for the survival of the species (Vu *et al.*, 2020).

One of the primary limitations of EST-SSR is the potential for lower levels of polymorphism compared to genomic SSRs Coding regions are often subject to purifying selection, which maintains the integrity of these sequences and can reduce the variability observed in these regions (Paliwal *et al.*, 2022). This reduced variability can limit the effectiveness of EST-SSRs in distinguishing between closely related individuals or populations, particularly in species with already low genetic diversity. Another challenge associated with EST-SSR markers is the complexity and cost of their development. Constructing cDNA libraries, sequencing expressed sequences, and identifying SSR motifs within these sequences require significant technological infrastructure, expertise, and investment. Although advances in next-generation sequencing technologies have reduced some of these barriers, the initial setup and analysis remain resource-intensive (Palumbo *et al.*, 2018). This complexity can be a significant hurdle for conservation efforts focused on endangered plants, which often operate with limited funding and resources.

Despite these challenges, the benefits of EST-SSR markers in conservation genetics are substantial. Their ability to link genetic variation with functional traits provides a deeper discernment of the potential of endangered plants to adapt to changing conditions, either biotic or abiotic. By integrating EST-SSR markers with ecological and environmental data, researchers can develop holistic conservation plans that address the multifaceted challenges faced by endangered plants (Zhao *et al.*, 2019).

Single Nucleotide Polymorphisms (SNPs)

Single Nucleotide Polymorphisms (SNPs) offer in-depth insights into the genetic architecture of endangered plant species. These markers, which represent variations at single nucleotide positions in the genome, are abundant and evenly distributed, providing a rich source of genetic information (Gai *et al.*, 2021).

The most significant advantage of SNP markers is their ubiquity and high density across the genome. The extensive coverage provided allows for a detailed examination of genetic variation on a very fine scale. SNPs can be applied for the assessment of genetic diversity within and among populations, helping to identify genetic bottlenecks, inbreeding, and other factors that can compromise the viability of endangered plant species (Jacquemart *et al.*, 2021). The dense array of genetic data points provided by SNPs enable the detection of subtle population structures that might be overlooked with less informative markers. This capability is essential for understanding how endangered plants may adapt to changing environments, such as those induced by climate change (Wang *et al.*, 2023). By pinpointing adaptive genetic variation, conservationists can prioritize the protection of populations with high adaptive potential, thereby enhancing the resilience of species to future environmental challenges (Teixeira & Nazareno, 2021).

However, applying SNP markers for conservation genetics is not without its challenges. The initial discovery and validation of SNPs require significant investment in next-generation sequencing (NGS) technologies and bioinformatics expertise.

Table 1: Advantages and disadvantages of DNA-based markers

Marker Type	Advantages	Disadvantages
RFLP	Highly reproducible	Requires large amounts of high-quality DNA
	Co-dominant marker	Labor-intensive and time-consuming
	Provides high-resolution data	Costly
RAPD	Simple and quick	Low reproducibility
	No prior sequence information required	Dominant marker
	Inexpensive	Sensitivity to reaction conditions
ISSR	No prior sequence information needed	Dominant marker
	High reproducibility compared to RAPD	Limited polymorphism information
	Cost-effective	May require optimization for specific taxa
AFLP	High reproducibility	Requires high-quality DNA
	Detects multiple loci	Dominant marker
	No prior sequence information needed	Complex and costly protocol
SSR	High reproducibility	Development of primers is costly and time-
(Microsatellites)	Co-dominant marker	consuming
	High polymorphism	Requires prior sequence information
	Widely applicable in population studies	
EST-SSR	Derived from expressed regions	Limited to transcribed regions
	Co-dominant marker	Primer design dependent on sequence data
	Functional markers linked to traits	Moderate cost
SNP	High abundance in the genome	High cost for discovery
	Co-dominant marker	Requires prior sequence information
	Amenable to high-throughput genotyping	May yield low polymorphism
High	Generates genome-wide data	Expensive
Throughput	Detects rare alleles	Requires advanced bioinformatics expertise
Sequencing	High resolution and accuracy	Challenges in data handling and storage
(HTS)	Amenable to large-scale studies	

While the cost of conducting NGS has decreased over the years, the data analysis component still remains complex and resource-intensive. Developing a robust SNP genotyping platform involves designing and validating markers that are polymorphic within the target species, which can be particularly challenging for non-model organisms with little or no prior genomic information (Huang *et al.*, 2024). Moreover, while SNPs provide high-resolution genetic data, they represent single-point mutations and may not capture more complex genetic variations, such as copy number variations, deletions, and insertions. These structural variants can also play critical roles in adaptation and evolution, and their exclusion from SNP-based studies may lead to an incomplete understanding of genetic diversity. Therefore, SNPs should ideally be used in conjunction with other types of genetic markers to obtain a comprehensive understanding of the genome. The interpretation of SNP data also poses challenges, particularly in the context of endangered species with low levels of genetic diversity. In such populations, the differentiation between neutral genetic variation and selection-driven changes can be difficult. Also, the presence of null alleles, genotyping errors, and population-specific allele frequencies can complicate data analysis and interpretation.

Despite these challenges, the integration of SNP markers into conservation genetics holds immense promise. By combining SNP data with ecological, phenotypic, and environmental information, researchers can develop holistic conservation strategies. For instance, integrating SNP data with habitat modeling can help to identify genetically diverse populations that can be conserved and protected (Nygaard *et al.*, 2022). Similarly, SNP-based insights into gene flow and genetic connectivity can inform habitat restoration and corridor design, ensuring that endangered plant populations remain interconnected and genetically viable (Cheng *et al.*, 2020).

HIGH THROUGHPUT SEQUENCING ERA

The introduction of automated high throughput sequencing (HTS) technologies has induced a paradigm shift not only in molecular biology but in other disciplines as well (Sharma *et al.*, 2022). These advanced sequencing methods, which include NGS platforms for eg. Illumina, pyrosequencing, PacBio, Oxford Nanopore etc., have enabled the fast and cost-effective generation of vast amounts of genomic data. The application of HTS technologies in conservation genetics provides a detailed and comprehensive view of the genomic landscape of endangered plants (Mahdavikia *et al.*, 2024). By sequencing entire genomes or specific genomic regions, researchers can identify genetic markers, including SNPs, insertions, deletions, and microsatellites (Zhang *et al.*, 2017). This wealth of genetic information is a critical factor in the management and conservation of endangered species. It is well known that the higher levels of genetic diversity, the higher the chances of a species to adapt and survive in the long term, as diversity enhances the ability to respond to environmental and anthropological stresses.

The most powerful impact of HTS on the study of endangered plants is the ability to perform genome-wide association studies (GWAS) and genotyping by sequencing (GBS). GWAS approaches allow researchers to link genetic variation to phenotypic traits, thereby identifying genomic regions under selection and genes associated with adaptive traits (Lasky *et al.*, 2023). For endangered plants, this means that

conservation efforts can be more targeted and effective, focusing on individuals or populations with unique adaptive genetic variations that enhance survival in changing environments or specific habitats. This targeted approach is particularly valuable for developing breeding programs and reintroduction strategies that maximize the adaptive potential of endangered populations.

GBS, on the other hand, happens to be a modification of restriction site-associated DNA sequencing (RADseq) methods with the aim to perform quick, cost-effective library preparation in a high-throughput manner (Kumar *et al.*, 2021b). GBS can be applied to any plant species, regardless of the availability of a reference genome. This universality is advantageous for conservation efforts, as many endangered plants lack well-characterized genomes (Garcia *et al.*, 2024). By enabling the discovery of novel genetic variants and the genotyping of existing ones, GBS provides a robust framework for understanding genetic diversity in non-model species (Zhu *et al.*, 2023). This information is essential for identifying genetically distinct populations, assessing genetic bottlenecks, and guiding conservation strategies aimed at maximizing genetic variability (Zhou *et al.*, 2023a).

Apart from the numerous advantages of HTS technologies, the procedure has a few challenges and limitations associated with it too. One of the primary challenges is the complexity of data analysis, as HTS generates large volumes of data that require sophisticated bioinformatics tools and expertise for proper analysis and interpretation, especially in the case of polyploid species (Wang *et al.*, 2022). The processes are computationally intensive and require robust bioinformatics infrastructure. The quality of the sequencing data can vary, necessitating stringent quality control measures to ensure accurate and reliable results. Although the cost associated with HTS has significantly decreased over the years, in many projects, particularly those involving numerous individuals or populations, the cost can still be substantial. This financial barrier may limit the widespread adoption of HTS technologies in some conservation programs. But, with the increase in attention gained by biodiversity and conservation issues, a shift in policy formulations of institutions and authorities may happen which can result in more financial allocations and better scientific planning (Wiedenfeld *et al.*, 2021).

FUTURE SCOPE

Novel sequencing technologies, advanced bioinformatics tools, and innovative data integration methods are poised to reduce the complexity and technical challenges associated with genetic diversity assessment (Salgotra & Chauhan, 2023). Machine learning algorithms and artificial intelligence will play pivotal, if not defining roles, in extracting meaningful information from datasets derived from HTS/NGS. These intelligent systems hold the potential to guide conservation efforts with utmost precision. To provide a holistic understanding of conservation, a multi-omics approach is required (Kapoor *et al.*, 2021), this will not only help with fine-scale genetic assessment but also shed light on the functional dynamics governing the response of endangered plants to environmental stressors.

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