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Genetic diversity investigation of some Darjeeling Himalayan *Rhododendron* species based on RAPD method

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ABSTRACT

Ten rare, threatened, and endangered *Rhododendron* species were collected from different places of altitudinal ranges starting from 2,247 to 3,580 masl. Of Darjeeling Hill. The random amplified polymorphic DNA (RAPD) method was used to measure the degree of the Genetic variation and the interconnection within the species. Out of 19, 6 oligonucleotide primers were chosen based on their performance, for the study. Genetic Similarity Coefficient ranged from 0.45455 to 0.93827 with an average of 79.43% polymorphism. This present study shows a low average level of genetic diversity [Ho = 0.986, Hs = 0.979, Ht = 0.985, I = 3.973, polymorphic information content = 0.2096] in the population of *Rhododendron*. As well as, a negative value of inbreeding coefficients. The mean inbreeding coefficient (Fst) within subpopulations is 0.006. A RAPD-based dendrogram constructed from Jaccard's estimates in Free Tree, through the use of neighbor-joining cluster analysis, divided nine (out of ten) *Rhododendron* species into two main sister groups. The study found that *Rhododendron* species are at risk of out-crossing depression and require conservation.

1. INTRODUCTION

In India, the Himalayas are the primary habitat for the 85 species of *Rhododendron* (Ericaceae) [1]. Of these, 36 species are unique to the Himalayan regions of Darjeeling and Sikkim [2].

Even though *Rhododendron* sp. plants have economic and ethno-medical significance, they are of the least studied plants in India [3,4]. Flowers of this plant have been used to make pickles, juice, jam, syrup, honey, squash, dried items, and treat diarrhea, headache, inflammation, viral, bacterial, and fungal illnesses, and so on [5]. Horticultural values of *Rhododendron* spp. are internationally known, as well [6].

Many *Rhododendron* species, including our chosen species, possess ecological, commercial, aesthetic, sacred, and ethno-medicinal and social importance. Anthropogenic disturbances such as deforestation, unsustainable extraction, over-exploitation, and agricultural activities have made many *Rhododendron* species endangered, rare, and threatened [7], including our chosen species. As a step towards the implementation of ecological preservation measures, determination of genetic diversity is essential. Therefore, it is necessary to know the genetic variability present in a particularly rare species to implement preservation and resource administration [8]. DNA markers are preferred over morpho-anatomical and biochemical ones because of higher specificity and sensitivity, more stability and consistency, unaffected by environmental conditions throughout growth and differentiation, high throughput, and automation of analysis. DNA markers based on polymerase chain reaction (PCR), like random amplified polymorphic DNA (RAPD), have been widely employed to study kinship and variety among plant populations. This technique is very simple and efficient for genomic diversity analysis. Few studies have been published on the use of RAPD molecular markers to examine the genetic variation of *Rhododendron* [9–11]. The goals of this research were to assess the levels of genetic diversity and genome polymorphism of some rare, threatened Darjeeling Himalayan *Rhododendron* spp. Among them, there is no report found for assessing genetic diversity by molecular markers. Therefore, this assessment was initially used to determine the genetic relatedness and diversity of selected Darjeeling Himalayan *Rhododendron* species to utilize effective conservation strategies.

2. MATERIALS AND METHODS

2.1. Collection of Samples

Young and fresh leaf samples (length 1–2.5 cm and breadth 0.5–1 cm) of the 10 *Rhododendron* species (Fig. 1) were collected for the study. *Rhododendron decipiens* Lacaita, *Rhododendron falconeri* Hook.f., *Rhododendron fulgens* Hook.f., *Rhododendron grande* Wight, *Rhododendron maddenii* Hook.f., *Rhododendron niveum* Hook.f.,

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Figure 1. Showing plant and a close-up view of flowers of 10 *Rhododendron* species.

Rhododendron pendulum Hook.f., *Rhododendron setosum* D. Don, *Rhododendron sikkimense* Pradhan & Lachungpa, *Rhododendron triflorum* Hook.f. were gathered from various elevations in the Darjeeling Hills, beginning in Batasia (2,247 masl) and ending in Sandakphu (3,580 masl) (Table 1 and Fig. 2). The differences in phenotypes between the species were observed and noted in Table 1.

2.2. DNA Isolation

Hexadecyl trimethyl ammonium bromide (CTAB) procedure was used to extract DNA from the tender leaves [12]. Initially, 225 mg of leaf sample was crushed using a bowl and stick, and then 60 minutes were spent in an incubation bath (65°C) containing isolation Buffer (600 µl). CTAB (2% wv^{-1}), NaCl (1.40 M), Tris-HCl (100.0 mM, pH 8.0), Polyvinylpyrrolidone (40) (1% wv^{-1}), and 2-mercaptoethanol (1% wv^{-1}) used to make up the isolation buffer. The mixture was extracted using 1:1 chloroformisoamyl alcohol (24:1) after being allowed to cool to room temperature. To separate the phases, the mixture was centrifuged for 10 minutes (15,000 g) at room temperature after being inverted to create an emulsion. After performing an RNase digestion (10 gm l^{-1} RNase A at 37°C for 60 minutes), a second chloroform-isoamyl alcohol extraction was carried out. Later, by adding a 2/3 volume of cold isopropanol, DNA was separated out of the liquid phase. The pellet was rinsed with 76% (vv^{-1}) ethanol and 0.2 M sodium acetate. The DNA was re-suspended in 50 µl of a buffer containing Tris-HCl (10 mM) and TE-EDTA (1 mM) at pH 8.0. For RAPD analysis, DNA was diluted to 50 ng μl^{-1} in TATE pH 8.0. DNA purity and concentration were measured using spectrophotometer (Smart-Spec 3000, UV/Vis spectrophotometer, Bio-Rad Laboratories). The OD260/OD230 of the isolated DNA samples was between 1.80 and 2.80, and the OD280/OD280 ratio was 1.7.

2.3. Amplifying DNA and RAPD Procedure

DNA amplifications were carried out using reaction mixtures (25 µl) containing 50 ng template DNA, 2 mM of dNTPs, 2.5 mM $MgCl_2$, 15 ng of degenerate primer, 2.5 µl of 10X PCR buffer, in addition to 1 unit of AmpliTaq-Gold polymerase (Life Technologies; Grand Island, NY). The MJ Mini™ Gradient Thermal from Bio-RAD Laboratories (India) Pvt. Ltd. (PTC-1148G) was used to conduct the PCR. QIAquick PCR Purification Kit was used to clean the PCR products. In the first step, 10 samples were used to test 19 different oligonucleotide primers (10 base pairs) for their ability to produce clear and consistent band patterns. Out of 19, 6 top-performing oligonucleotide primers were chosen for the examination of the current specimens. Six RAPD primers we selected based on their good data reproducibility and finally used for the characterization of ten *Rhododendron* species. The sequences of 10mer 6 primers are mentioned in Table 2.

2.4. Data Analysis

Data for each band was recorded in Microsoft Excel if either "present" as 1 or "not present" as 0, including monomorphic bands. Genetic data were analyzed by different software. The effective number of alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (Hs), total expected heterozygosity (Ht), inbreeding coefficient (Fst), coefficient of gene differentiation (Gst), gene flow (Nm), Shannon's information index (I), polymorphic percentage (PPL), was calculated by the GenAlEx version 6.502 software [13]. To quantify polymorphic information content (PIC), the 3.25th version of PowerMarker was used [14]. For RAPD-based percentage homology of the samples, pair-wise correlation was performed using the neighbor-joining cluster analysis method produced from Jaccard's estimate [15]. To create the dendrogram, we used Free Tree Software and the neighbor-joining cluster analysis technique utilizing Jaccard's estimate [16].

3. RESULTS

3.1. RAPD Polymorphism and Genetic Diversity

The screening was performed using 19 RAPD primers, and 6 of those primers demonstrated amplification across all the selected species. These six primers exhibited dependable and unambiguous banding patterns, with good repeatability and clear band resolution. PCR, using the six-decamer oligonucleotide primers successfully amplified genomic DNA, and the results are summarized in Table 3. A total of 589 bands were detected using six RAPD primers from ten *Rhododendron* species with an average of 61.10 alleles observed. Using 6 primers among 10 samples, showing a broad range of PPL%, the highest in *R. falconeri* (93.44%) and lowest in *R. setosum* (63.50%) and the mean of 79.43% polymorphism. PIC scores were between 0.180 and 0.227 in *R. decipiens* and *R. triflorum*, respectively, with an average of 0.209. Observed heterozygosity (Ho) ranges from 0.979 (*R. falconeri* and *R. fulgens*) to 0.993 (*R. decipiens*) which was very low in deference. The results of genetic diversity present within the population (Hs) do not show a significant amount of difference among species. The average variation in a population's genes (Hs) of the ten *Rhododendron* species

Table 1. Table showing plant samples, collection site with altitude, abundance status, and the phenotypic differences among the studied species of *Rhododendron*.

Sl. No.	Name of species	Collection sites and altitude (masl)	Geographical coordinate		Abundance (Reference)	Morphological characters
			Latitude (°N)	Longitude (°E)		
1.	*R. fulgens* Hook.f.	Sandakphu 3510	27.062°N	88.019°E	Rare [3]	Medium to tall shrubs (2–3 m) with oval to elliptic-oblong leaves, felted, bright green leaves and dense rusty tomentum beneath. Red, mauve, or rose pink flowers with enormous nectar pouches. This species belongs to subgenus *Hymenanthes*, Section Pontica, and subsection Fulgensia.
2.	*R. pendulum* Hook.f.	Singalila National Park 2570	27.040°N	88.097°E	Rare [3]	Shrub over 1 m tall, growing on trees or growing on rocks, leaves oblong to elliptic; rugose and glabrous above, dense brown woolly hairs below. Flowers single to numerous, white stained with reddish-pink yellow, corolla large and rotate, and seed capsule. This species belongs to subgenus *Rhododendron*, Section Rhododendron, and subsection Edgeworthia.
3.	*R. maddenii* Hook.f.	Neora Valley National Park 2750	27.118N	88.691°E	Endangered [3]	Growing on trees and/or soil, tall up to 1 m, smooth immature shoots, single to many flowers, dry, aromatic, tube-shaped, white stained pink, shape campanulate, funnel-like, many androecia (15 to 20), bald filament, seed capsule. This species belongs to subgenus *Rhododendron,* Section Rhododendron, and subsection Maddenia.
4.	*R. niveum* Hook.f.	Neora Valley National Park 3180	27.121N	88.683°E	Endangered [3]	Short tree (4 to 15 m tall), turbid off-green leaves, smooth shoots, ventral side of leaves with silver-like white, blood red flowers, 2–3 cm sepal length, corolla not inflated, flowers on a lower rounded truss, capsule type seed. This species belongs to subgenus *Hymenanthes*, Section Pontica, and subsection Arborea.
5.	*R. sikkimense* Pradhan and Lachungpa	Singalila National Park 3520	27.064°N	88.029°E	Endangered [3]	Shrubs (2 to 3 m tall), lofty leaves, many shaped under leaf, velvety texture, shiny green to pale yellow, with silver lines. Flowers pink to blood red. This species belongs to subgenus *Hymenanthes*, Section Pontica, and subsection Thomsonia.
6.	*R. decipiens* Lacaita	Singalila National Park 3510	27.067°N	88.032°E	Threatened [31]	Tree (4 to 15 m tall), and large leaves characterize this plant. The pink and purple crimson flowers rose, campanulate with white lobes, gong-like corolla, puffy on one side, ten androecia. This species is a natural hybrid; seed × pollen = *R. hodgsonii* × *R. falconeri* ssp *falconeri*
7.	*R. falconeri* Hook.f.	Senchal Wildlife Sanctuary 2280	27.003°N	88.283°E	Threatened [31]	Moderate tall (4–16 m) tree, huge leaf, rough and dull green on the outside, with rusty wooly thick layer underside, white and pale yellow (unusually pink) blossoms, corolla with an inverted bell form, puffy on the side, upright seed pod. This species belongs to subgenus *Hymenanthes*, Section Pontica, and subsection Falconera.
8.	*R. grande* Wight	Senchal Wildlife Sanctuary 2300	27.004°N	88.285°E	Threatened [31]	Tree habit (4 to 15 m tall), huge leaves, shiny green with both the side whitish, indurated, silvery, white and pale yellow bell-shaped flowers, corolla with an angled form, puffy on the side, upright seed pod. This species belongs to subgenus *Hymenanthes*, Section Pontica, and subsection Grandia.
9.	*R. setosum* D.Don	Senchal Wildlife Sanctuary 2350	26.999°N	88.280°E	Threatened [31]	Very large (60 to 100 m) tree, not scented, bristly and scaly leaves on immature branches, less number of flowers (< 5) in a bunch, purple-pink blooms with wide-open, spreading corolla lobes. This species belongs to subgenus *Rhododendron*, Section Rhododendron, and subsection Lapponica.
10.	*R. triflorum* Hook.f.	Sandakphu3430	27.081°N	88.017°E	Threatened [31]	Small plant (1 to 5m), bald and green leaves with oval to lance-shaped, leaf shape is rounded at the base and pointed at the tip, flower irregular shaped with light yellow with reddish spots on it, 10 androecia, capsule-shaped seeds that are very skinny. This species belongs to subgenus *Rhododendron,* Section Rhododendron, and subsection Triflora.

R. decipiens is a natural hybrid; seed × pollen = *R. hodgsonii* × *R. falconeri* ssp. *falconeri*.

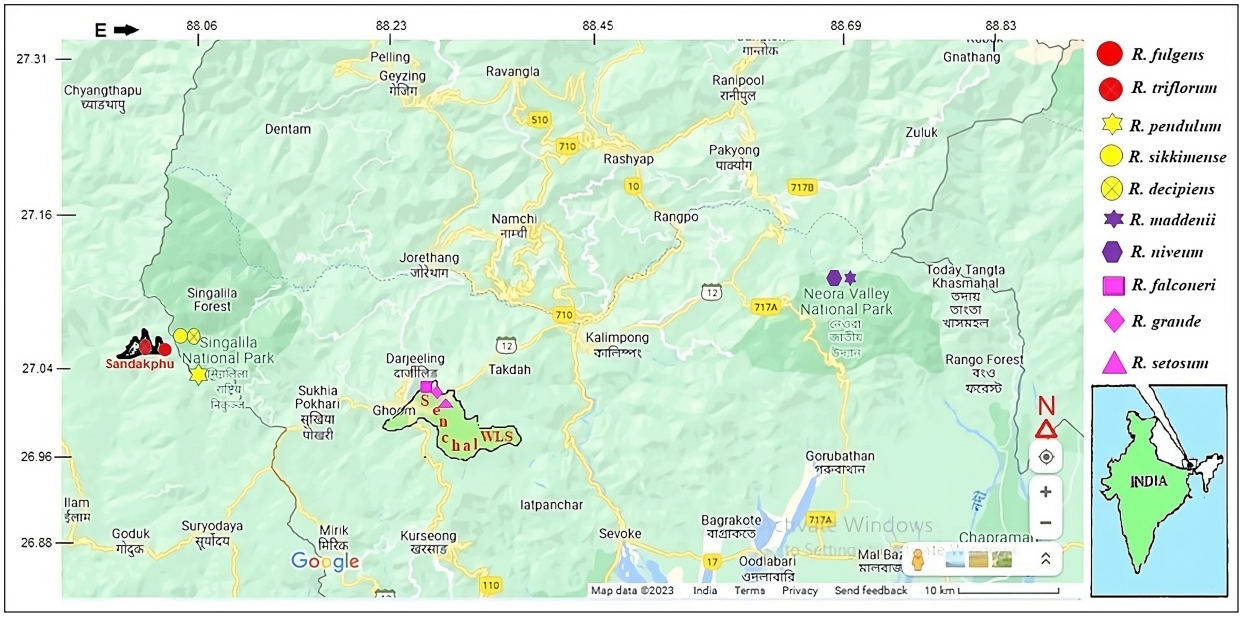

Figure 2. Geographic locations (in Google map) of the 10 *Rhododendron* species taken for study.

Table 2. List of the selected RAPD primers used for study and characterization in 10 *Rhododendron* species.

Primer	Sequence	Remarks
OP*N 18	5′-GGTGAGGTCA-3′	
OPP 07	5′-ACATCGCCCA-3′	
OPP 08	5′-ACATCGCCCA-3′	Primers were selected based on their ability to produce high-quality bands on an agarose gel. All of the selected species' genomic DNA was successfully amplified using these primers.
OPA 02	5′-TGCCGAGCTG-3′	
OPA 03	5′-AGTCAGCCAC-3′	
OPA 17	5′-GACCGCTTGT-3’	
OPA 06	5′-GGTCCCTGAC-3′	
OPA 12	5′-TCGGCGATAG-3′	
OPA 18	5′-AGGTGACCGT-3′	
OPA 20	5′-GTTGCGATCC-3′	
POC 15	5′-GACGGATCAG-3′	
OPD 02	5′-GGACCCAACC-3′	Primers were not selected due to the fact that their banding pattern on the gel was either nonexistent, insufficient, or confusing. All of the selected species' genomic DNA was not successfully amplified using these primers.
OPF 17	5′-AACCCGGGAA-3′	
OPK 15	5′-CTCCTGCCAA-3′	
OPN 16	5′-AAGCGACCTG-3′	
OPN 20	5′-GGTGCTCCGT-3′	
OPS 147	5′-AGCTGCAGCC-3′	
OPS 238	5′-TGGTGGCGTT-3′	
OPS 253	5′-GGCTGGTTCC-3′	

(*OP = Operon technologies and Kits are A, C, D, F, K, N, P, S. Alameda, CA).

is 0.979. The mean of the whole variation in genes (Ht) among ten *Rhododendron* species is 0.985. The inbreeding coefficient (F_{is}) value within individuals was calculated as a negative value except in *R. fulgens* and *R. falconeri.* The two species (*R. fulgens* and *R. falconeri*) shows neutral F_{is} result. The degree of gene flow (Nm) ranges from 34.376 (*R. triflorum*) to 42.080 (*R. fulgens*) and a mean of 38.505. Shannon's information index (I) ranges between the lowest 3.944 (*R. triflorum*) to the highest 4.000 (*R. setosum*) with an average value of 3.973.

3.2. Genetic Differentiation and Phylogenetic Tree

The mean inbreeding coefficient (F_{st}) within subpopulations is 0.006, which is very low. Similarity indices among the ten *Rhododendron* species based on RAPD analysis of genomic DNA made by Jaccard's similarity coefficient computer program, are shown in Table 4. The range of similarity correlations was from 0.93827 to 0.45455.

A dendrogram constructed from Jaccard's estimates in Free Tree through the use of neighbor-joining cluster analysis divided nine (out of ten) *Rhododendron* species into two main sister groups (I and II). In sister group I, *R. niveum, R. fulgens*, *R. setosum,* and *R. sikkimense* are the most primitive of the taxa under study; whereas *R. triflorum* and *R. maddenii* are recent as compared to the previous four taxa, but *R. pendulum* has most recent origin as indicated by high bootstrap value and relatively smaller branch length. However, sister group II containing *R. decipiens* and *R. falconeri* is more recent in origin, than group I due to low bootstrap values (11 and 44) and long branches. *Rhododendron grande* is out-group taxa (Fig. 3).

Table 3. Summary of genetic diversity indices for 10 *Rhododendron* species used for the study.

Species	Na	Ne	Ho	Hs	Ht	Fis	Fst	Gst	Nm	I	PIC	PPL%
R. fulgens	61.000	48.431	0.979	0.979	0.985	0.000	0.006	0.000	42.080	3.987	0.2203	77.05
R. pendulum	61.000	47.605	0.992	0.979	0.985	−0.013	0.006	0.000	39.571	3.977	0.2153	80.33
R. maddenii	62.000	48.639	0.989	0.979	0.985	−0.010	0.006	0.000	41.707	3.991	0.2076	82.26
R. niveum	61.000	48.371	0.986	0.979	0.985	−0.007	0.006	0.000	41.105	3.985	0.2127	77.05
R. sikkimense	61.000	45.956	0.986	0.978	0.985	−0.008	0.007	0.001	34.948	3.954	0.2023	72.13
R. decipiens	60.000	46.085	0.993	0.978	0.985	−0.015	0.007	0.001	35.255	3.951	0.1800	85.00
R. falconeri	61.000	46.750	0.979	0.979	0.985	0.000	0.007	0.000	36.676	3.972	0.2023	93.44
R. grande	61.000	47.002	0.983	0.979	0.985	−0.004	0.007	0.000	37.644	3.966	0.2076	86.90
R. setosum	63.000	48.631	0.989	0.979	0.985	−0.010	0.006	0.000	41.692	4.000	0.2203	63.50
R. triflorum	60.000	45.343	0.983	0.978	0.985	−0.005	0.007	0.001	34.376	3.944	0.2276	76.66
Mean	61.100	47.281	0.986	0.979	0.985	−0.007	0.006	0.000	38.505	3.973	0.2096	79.43
SE	0.277	0.388	0.002	0.000	0.000	0.002	0.000	0.000	0.974	0.004	0.0041	2.62

Na: number of observed alleles; Ne: effective number of alleles; Ho: observed heterozygosity over k pops; Hs: genetic diversity within the population; Ht: total expected Heterozygosity; Fis: inbreeding coefficient within individuals [Fis = (Hs-Ho)/Hs]; Fst: inbreeding coefficient within subpopulations; Gst: coefficient of gene differentiation; Nm: gene flow; I: shannon's Information Index; PIC: polymorphism information content; PPL%: percentage of polymorphic loci; SE: standard error.

Table 4. Similarity index or coefficient for RAPD.

	R. fulgens	*R. pendulum*	*R. maddenii*	*R. niveum*	*R. sikkimense*	*R. decipiens*	*R. falconeri*	*R. grande*	*R. setosum*	*R. triflorum*
R. fulgens										
R. pendulum	0.83019									
R. maddenii	0.67308	0.65909								
R. niveum	0.62745	0.82353	0.66							
R. sikkimense	0.83333	0.90541	0.91358	0.90123						
R. decipiens	0.83333	0.79452	0.85542	0.81481	0.68539					
R. falconeri	0.89286	0.83099	0.88889	0.89024	0.61728	0.45455				
R. grande	0.86585	0.89333	0.91566	0.90361	0.61728	0.49367	0.51948			
R. setosum	0.8642	0.84507	0.84416	0.90244	0.80645	0.79592	0.78495	0.81053		
R. triflorum	0.93827	0.88235	0.87838	0.92308	0.80682	0.7957	0.72619	0.78409	0.78161	

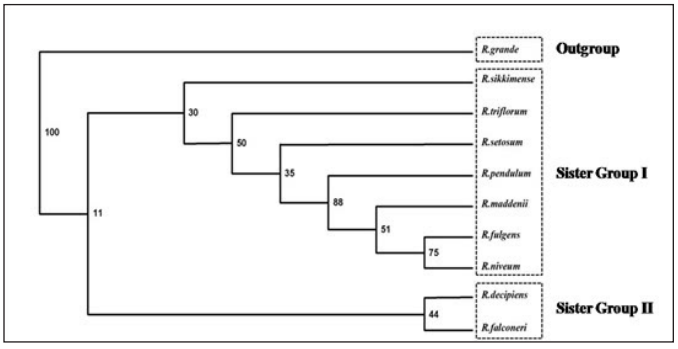

Figure 3. The phylogenetic tree of the 10 *Rhododendron* genotypes based on RAPD markers utilizing neighbor-joining cluster analysis and Jaccards estimations from Free Tree program. Node values are bootstrapped. The evolutionary position of taxa can be determined by correlating theses values with branch lengths.

4. DISCUSSION

Six decamer oligonucleotide random primers were applied for the present investigation. Primers measure moderate to high polymorphism percentage (63.50 to 93.44) with average low Polymorphism Information Content (Mean PIC = 0.2096) of 10 *Rhododendron* species. Small populations have less genetic variety than big populations due to factors including genetic drift and inbreeding [17]. Due to this, genetic diversity is estimated to be lower in rare and endangered species with restricted geographic ranges than in the same species with wider geographic ranges [18]. We found a high average degree of genetic diversity (percentage of polymorphic loci = 79.43%) in selected *Rhododendron* species. In general, present findings backed the theory that some rare and endangered organisms may keep their genetic diversity high though their populations are declining [17,19]. High Shannon's information index (Mean I = 3.973) was calculated among the rare and endangered *Rhododendron* species. The high genetic diversity was found in the current investigation at the species level (Mean, Na = 61.100, Ho = 0.986, and Hs = 0.979, Ht = 0.985). In earlier studies, it was shown that genetic diversity among endangered plant species is surprisingly high to moderate like *Origanum compactum* (He = 0.35) a medicinally important plant, *Paeonia jishanensis* (H_E = 0.340), *Rhododendron protistum* var. *giganteum* [Nei's gene diversity (h) = 0.240], *Paeonia decomposita* (H_E = 0.405), and *Populus wulianensis* (H_E = 0.61) [20,21]. Several factors like mating strategy, biological characteristics, and out-breeding could be considered as significant elements that determine increased levels of genetic variety in *Rhododendron* [17].

The 10 *Rhododendron* species generate average negative inbreeding coefficients ($F_{is} < 0$) (Table 3), illustrating that despite having fragmented habitats, they do not have inbreeding depression [21]. That could be explained by the floral characteristics of the Eastern Himalayan *Rhododendron* species evolved in such a way that bird pollination is common for this population and weather conditions [22]. On the other hand, increased hybridization (natural or artificial) will elevate in risk of extinction of species or populations [23]. The similarity measurement using Jaccard's coefficient values varies from 0.45455 to 0.93827 among selected species (Table 4). The genetic diversity among these species clearly indicates that they must have evolved from genetically divergent parents [24].

The RAPD clustering finding revealed a closer link between the *Hymenanthes* and *Rhododendron* subgenera. In addition, it demonstrated that certain physical qualities could mirror inherited characteristics. In sister group II, *R. decipiens* and *R. falconeri* were gathered together because they shared some common morphological characters (Table 1). Moreover, *R. decipiens* is a natural hybrid between *R. falconeri* and *R. hodgsonii* (Hook. f). In sister group I, *R. sikkimense, R. fulgens,* and *R. niveum* were gathered together because they are morphologically small trees or tall shrubs, semi-deciduous species, and associated with the section Pointicum. *Rhododendron grande* belonging to the same section Pointicum kept as an out-group because it showed little different morphological traits such as trees 4–15 m high, and flowers white to creamy yellow. In Gladiolus plant, the UPGMA cluster analyses method arranged 54 cultivars into four and three primary groups based on their morphological features and RAPD data, while in both cluster studies, "Pusa Lohit" (red-colored flower) branched off from dendrograms, supporting its morphological and genetically uniqueness [25]. Although, *R. triflorum*, *R. setosum*, *R. pendulum,* and *R. maddenii* were in the subgenus *Rhododendron* they had a closer relationship with some species of the subgenus *Hymenanthes*. Therefore, they have been included in the sister group I.

There is a positive correlation between genetic differentiation and F_{st} value [26]. The average F_{st} results for the study revealed that the genetic differentiation between species was negligible (Table 3). Gene flow might inhibit differentiation and mitigate the genetic drift when Nm >1 [27]. The gene flow (Mean Nm = 38.505) between *Rhododendron* species also indicated that gene flow among species is very high. Over gene flow is introgressive and can produce genetic swamping [28]. Through genetic swamping, in which the native organisms have increased the risk of extinction for rare species [29,30].

5. CONCLUSION

The investigation shows that studied *Rhododendron* species are at a high risk of out-crossing depression, which will lead to a population bottleneck. Therefore, immediate action needs to be taken to implement conservation (*in situ* and *ex situ*) measures. Future studies should include more *Rhododendron* species from the hills to strengthen the comprehensive and generalized conclusions.

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7. AUTHOR CONTRIBUTION

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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