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Research Article

Estimation of genetic diversity and population structure of ten scarce Eastern Himalayan *Rhododendron* species

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ABSTRACT

Ten rare, threatened, and endangered *Rhododendron* species were collected from different altitudinal ranges starting from 2247masl to 3580masl. of Eastern Himalaya (particularly Darjeeling Hills) were studied by polymerase chain reaction (PCR) using randomly amplified polymorphic DNA sequences (RAPD) to measure the degree of the genetic diversity and relationship among the species which are urgently needed to promote effective conservation and management activities. Initially, 19 decamer oligonucleotide primers were screened on ten samples for unambiguous and reproducible band patterns. Out of nineteen, six decamer oligonucleotide primers finally yielding the best results were selected and used for the analysis of present samples. Mean 61 alleles were amplified by using six primers, among ten samples. Total 126 were unique bands showing an average of 79.43% polymorphism. The Genetic Similarity Coefficient (GS) was ranging from 0.45455 to 0.93827. The present study shows an average low level of genetic diversity (PIC= 0.2096, Hs= 0.979, Ho= 0.986, Ht= 0.985, I= 3.973). The population of *Rhododendron* shows a negative value of inbreeding coefficients (Fis < 0) indicating heterozygotes produced by the population due to crosses happens in between genetically distant individuals but the value does not support that there is a chance of outbreeding depression and population bottleneck in recent future. Results of AMOVA show that variation among and within populations is 1% and 99% respectively. The mean inbreeding coefficient (Fst) within subpopulations is 0.006. A dendrogram based on RAPD markers using the neighbor-joining cluster analysis method produced from Jaccards estimates using Free Tree software divided nine (out of ten) *Rhododendron* species into two main sister groups this result also supported by Principal Coordinates Analysis (PCoA). The obtained RAPD analysis results also suggested that some rare, threatened, and endangered species of *Rhododendron* in the present study could maintain moderate levels of genetic diversity. STRUCTURE assessment (ΔK= 5) showed the selected species distributed distantly from each other and estimated that the *R. falconeri* genotype is the maximum level of admixture among the ten species. Results of Mantel's test show a positive correlation between geographical distance and genetic similarity or dissimilarity of ten *Rhododendron* species. Whereas the positive regression analysis value (R^2= 0.0809) does not tally for the deep connection between geographical distance and genetic similarity or dissimilarity among the evaluated species. Based on the results, summarized that *Rhododendron* species of Eastern Himalaya are at a high risk of outcrossing depression, which will lead to a population bottleneck. Therefore, immediate conservation (*in-situ* and *ex-situ*) measures have to be taken for these rare, threatened, endangered, economically, and ethno-botanically important *Rhododendron* species.

Key words: *Rhododendron*, Genetic diversity, plant protection and conservation, outbreeding depression, population bottleneck

INTRODUCTION

The genus *Rhododendron* (Ericaceae) comprises 85 species in India, and they are mainly distributed in the Himalayan region (Tiwari & Chauhan, 2006). Out of this, a total of 36 species found in Darjeeling and Sikkim Himalaya alone (Pradhan & Lachungpa, 1990, Gogoi *et al*., 2022). This genus is one of the most neglected groups of plants in terms of scientific inquiry in India (Singh *et al*., 2003). The *Rhododendron* flowers show a wide range of colours, shapes, and sizes in their wild forms (Tiwari & Chauhan, 2006). The major characteristics of this genus are mostly shrub or tree, flowers are actinomorphic, bisexual, pentamerous, hypogynous, corolla gamopetalous, stamens obdiplostemonous and inserted on nectar secreting disc, free and usually not epipetalous, pollen in tetrads, ovary many, small seeds with endosperm and frequently roots are associated with mycorrhiza (Tiwari & Chauhan, 2006). Being among the first to colonize wasteland, the *Rhododendron* plants help prevent soil erosion, allow vegetation regeneration, and maintain an ecological environment (Leach, 1961; Tiwari & Chauhan, 2006; Pandey & Badola, 2018). The decoction of a leaf or dried flower petals of *Rhododendron* spp. has several medicinal uses such as for the treatment of rheumatism, checking diarrhea, blood dysentery, and dissolving fish bones struck in the throat (Laloo *et al*., 2006; Som *et al*., 2019; Swamidasan *et al*., 2020; Balkrishna *et al*., 2022). The horticultural values of *Rhododendron* spp. are internationally known (Sharma *et al*., 2022).

Most *Rhododendron* spp. have been reported to be diploid with 2n = 2X = 26 (Jones *et al*., 2007). The genome size (2C) for diploid *Rhododendron* spp. in general ranges from 1.30pg to 1.90pg and their total number of base pairs ranges from 1274.0 ±98.0 Mbp

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to1862.0 ±78.4 Mbp (De *et al*., 2010). However, some polyploids also occur naturally, including triploids, tetraploids, hexaploids, octaploids, and decaploids (Hu *et al.,* 2023).

Under the present study, we aimed to measure the degree of the genetic diversity and genome polymorphism of ten rare, threatened Eastern Himalayan *Rhododendron* species. Genetic diversity is an essential element of biological diversity for conservation strategies (Kaljund & Jaaska, 2010; Gordon *et al.*, 2012). It is commonly established that protecting endangered species' genetic variety can have a big impact on their long-term survival and evolution in shifting settings (Frankham *et al.*, 2002). Therefore, knowledge of endangered plant species' genetic diversity and population structure is crucial for their conservation and management (Frankham, 2003; Gordon *et al.*, 2012; Lopes *et al*., 2014). Morpho-anatomical markers are routinely used to study the genetic relationship and for genetic diversity analysis. However, in some cases, the relatively narrow range of variation of morpho-anatomical characters limits its resolution and it is vulnerable to environmental conditions (Mohammad *et al.,* 2024). It is well documented that DNA markers have better resolution and many advantages over morpho-anatomical and biochemical markers (Ramesh *et al.,* 2020). Among the DNA markers, polymerase chain reaction (PCR) based markers using arbitrary primers, such as RAPD, have been widely used for investigating genetic relatedness and diversity in plant populations and the environment has no effect on these markers during growth and differentiation. no effect on these markers during growth and differentiation. Since, most *Rhododendron* plants are vegetatively propagated, RAPD profiles must be stable during this propagation. This technique is very simple, and efficient for diversity analysis. There are very few reports on the study of the genetic diversity of *Rhododendron* using molecular markers (Zhao et al., 2021; Cao *et al*., 2022 He *et al.,* 2024; Wang *et al.,* 2025). Among them, there is no report on genetic diversity analysis of Eastern Himalayan Indian *Rhododendron*. Therefore, in the present study, the assessment of genetic diversity and the relationship among ten Eastern Himalayan Indian *Rhododendron* was carried out through RAPD analysis.

MATERIALS AND METHODS

Collection of samples

Young and fresh leaf samples (length 1-2.5cm and breadth 0.5-1cm) of ten *Rhododendron* species (Figure 1) such as *R. decipiens* Lacaita, *R. falconeri* Hook.f., *R. fulgens* Hook.f., *R. grande* Wight, *R. maddenii* Hook.f., *R. niveum* Hook.f., *R. pendulum* Hook.f., *R. setosum* D.Don, *R. sikkimense* Pradhan and Lachungpa, *R. triflorum* Hook.f. were collected from different altitudinal ranges of Darjeeling Hills starting from Batasia (2247masl) to Sandakphu (3580masl) (Table 1 & Figure 2). The phenotypic differences among the studied species were also noted (Table 1).

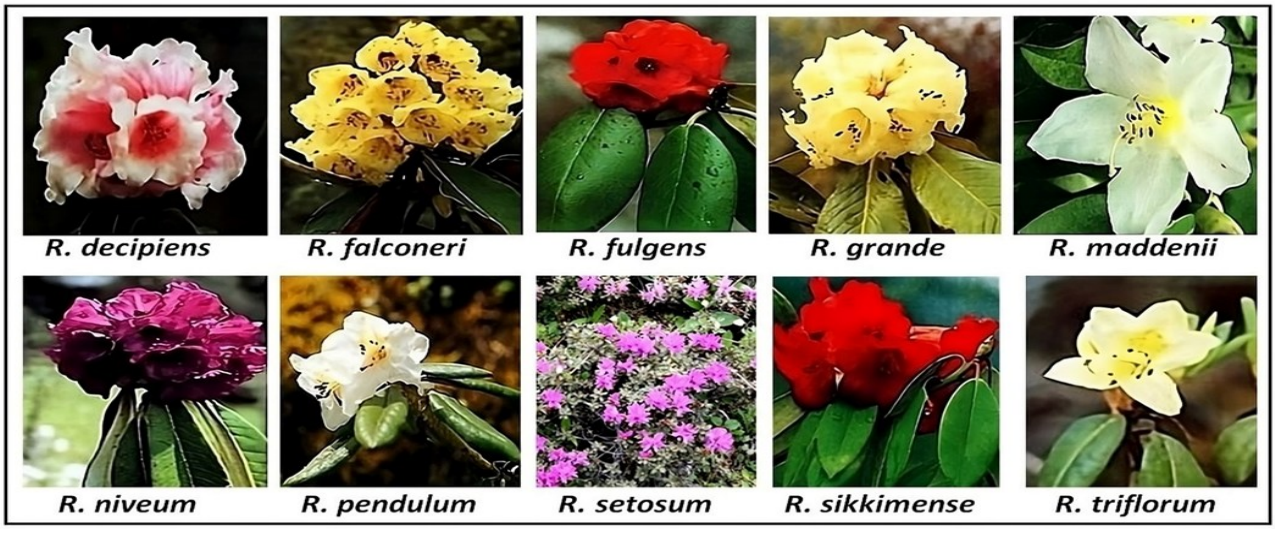

Figure 1. Showcasing a close-up picture of the flowers of ten *Rhododendron* species.

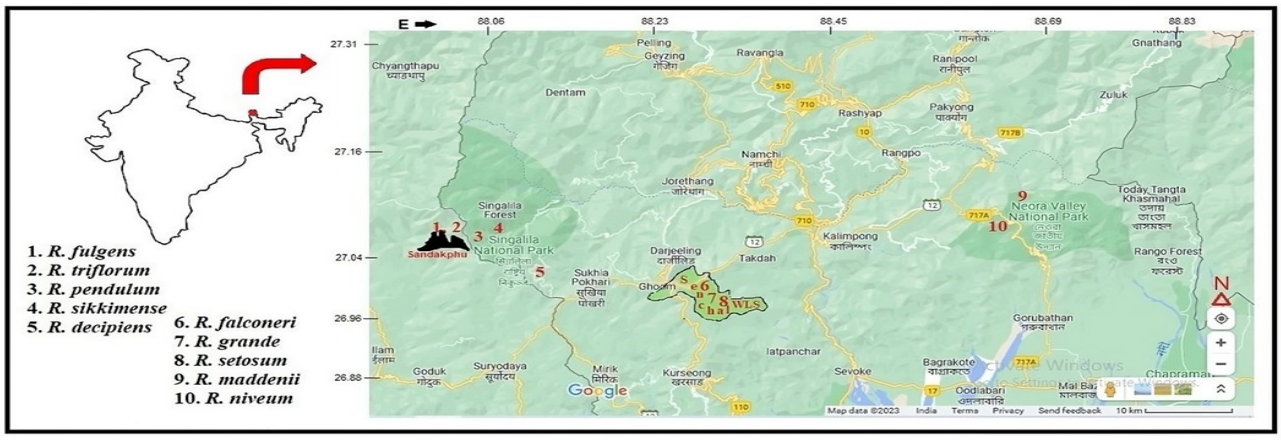

Figure 2. Showing locations where ten species of *Rhododendron* were collected

Table 1. Table displaying the species title, abundance status, collection location, and morphological variations among the studied *Rhododendron* species.

Sl. No.	Name of species	Abundance (Reference)	Collection sites	Morphological characters
1.	***R. fulgens***	Rare Singh *et al*., (2003)	Sandakphu	Medium to tall shrubs usually 2-3m high leaves long, leaves variously shaped, felted beneath, oval to elliptic-oblong, shiny green above, and with dense rusty tomentum beneath. Flowers in the shade of red, mauve, or rose pink. With large blackish nectar pouches at the base. This species belongs to subgenus *Hymenanthes*, Section Pontica, and subsection Fulgensia.
2.	***R. triflorum***	Threatened Singh *et al*., (2009)		Plants usually 1-5cm tall, green and glabrous shrub, presence of minute scaly branches, leaf ovate or sometimes lanceolate, leaf base rounded and apex acute in nature; Raceme type of inflorescence with 2-3 flower, calyx minute, scaly and 5 lobed; Zygomorphic flower with pale yellow color having reddish blotched, open funnel shaped (2-3cm) flower; stamens 10 in numbers, ovary scaly; seed capsule type narrowly cylindrical. This species belongs to subgenus *Rhododendron*, Section Rhododendron and subsection Triflora.
3.	***R. maddenii***	Endangered Singh *et al*., (2003)	Neora Valley National Park	Epiphytic and lithophytic, shrub above 1m high; young shoots not bristly; flowers one to several, not fleshy, tubular-scented, campanulate, white tinged with pink, other than lemon corolla rotate, funnel-shaped; stamens 15-20, filaments glabrous, seed-capsule. Seed capsule ovate, woody, and valves not recurved to their bases. This species belongs to subgenus *Rhododendron*, Section Rhododendron, and subsection Maddenia.
4.	***R. niveum***			Small trees or tall shrubs, trees 4-15m high. Leaves opaque and dull green, leaf petiole and young shoots not bristly. Leaf underside always covered with thin silvery white or fawn tomentum; Flowers blood red or smoky-blue to purple-mauve, flowers smoky-bluer or purple mauve; Calyx 2-3cm long, corolla not swollen flowers in a rounded truss, on the lower side; seeds capsule. This species belongs to subgenus *Hymenanthes*, Section Pontica, and subsection Arborea.
5.	***R. sikkimense***	Endangered Singh *et al*., (2003)		Medium to tall shrubs usually 2-3m high; leaves long, variously shaped beneath. oblong-ovate, leathery and thick matt texture, dull yellowish green above and glaucous green and covered with a thin layer of silvery, yellowish-brown tomentum beneath. Flowers in the shade of red, mauve, or rose pink. Flowers red, flowers blood red, in trusses. This species belongs to subgenus *Hymenanthes*, Section Pontica, and subsection Thomsonia.
6.	***R. decipiens***	Threatened Singh *et al*., (2009)	Singalila National Park	Trees 4-15m high. The leaves are very large. Flowers rose pink to purple crimson. Flowers wide campanulate rose pink fading to almost white lobes; corolla obliquely bell-shaped, swollen on the side; bark not peeling; stamens 10. This species is a natural hybrid; seed x pollen=R. *hodgsonii* x R. *falconeri ssp falconeri*
7.	***R. pendulum***	Rare Singh *et al*., (2003)		Temperate to alpine shrub over 1m in height, epiphytic, lithophytic, or terrestrial; leaves oblong to oblong-elliptic; rugose and glabrous above and covered with dense brown woolly hairs beneath. Or Flowers one to several, white tinged with reddish-pink yellow, not fragrant, rotate; much larger corolla rotate, seed capsule ovate, tubular to funnel-shaped to tubular campanulate; woody and valves not recurved to their bases, young growths covered with thick felt or wooly hairs. Short covered with dense brown wooly hairs. This species belongs to subgenus *Rhododendron*, Section Rhododendron, and subsection Edgeworthia.

8.	***R. falconeri***			Trees 4-15m high, leaves very large, mat green very rugose and covered with dense rusty tomentum beneath. Flowers white to creamy yellow (rarely pink); corolla obliquely bell-shaped, swollen on the side; seed capsule erect. This species belongs to subgenus *Hymenanthes*, Section Pontica, and subsection Falconera.
9.	***R. grande***	Threatened Singh *et al*., (2009)	Senchal Wildlife Sanctuary	Trees 4-15m high, leaves very large, Leaves glossy green above below with their silvery white indumentum; bell Flowers white to creamy yellow; corolla obliquely shaped, swollen on the side; seed capsule curved. This species belongs to subgenus *Hymenanthes*, Section Pontica, and subsection Grandia.
10.	***R. setosum***			Plants usually 60-100cm tall, none aromatic shrublets; alpine and shrublets usually not acceding 1m in height; leaves young branches bristly scaly, flowers few in each cluster, usually 5 or less; flowers bright purple pink with very open and spreading corolla lobes. This species belongs to subgenus *Rhododendron*, Section Rhododendron, and subsection Lapponica.

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

DNA Isolation

DNA was extracted from the young leaves using a hexadecyl trimethyl ammonium bromide (CTAB) protocol adapted from Doyle & Doyle (1990). Initially, 250mg of tissue was ground with mortar and pestle and subsequently incubated at 65^0C for 60 minutes with 600µl of Isolation Buffer. The CTAB ($2wv^{-1}$), 100 mM Tris-HCl buffer (pH 8.0), NaCl (1.4M), Polyvinylpyrrolidone (PVP 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 (15000g) at room temperature after being inverted to create an emulsion. After performing 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 precipitated from the aqueous phase. The pellet was rinsed with 76% (vv^{-1}) ethanol and 0.2M sodium acetate. In 50µl of buffer with 10mM Tris-HCl and 1mM EDTA (TE), pH 8.0, the DNA was once again dissolved. For RAPD analysis, the DNA preparation was diluted in TATE pH 8.0 at 50ngL^{-1}. DNA purity and concentration were measured spectrophotometrically (UV vis-spectrophotometer 1700DC. Simarju, Japan). The DNA sample showed an OD_{280}/OD_{280} ~1.7 and an OD_{260}/OD_{230} ~1.8–2.8.

DNA Amplification and RAPD Procedure

The PCR reaction mixtures had a total volume of 25µl. The mixture contains:
DNA amplifications were carried out using reaction mixtures (25µl) containing 50ng template DNA, 2mM of dNTPs, 2.5mM $MgCl_2$, 15ng of degenerate primer, 2.5µl of 10X PCR buffer, and 1 unit of AmpliTaq-Gold polymerase (Life technologies, Grand Island, NY). The MJ Mini™ Gradient Thermal from Bio-RAD Laboratories (India) Pvt. Ltd. with the catalog number PTC-1148G was used to conduct the PCR. The MinElute PCR Purification Kit (Qiagen, Valencia, CA) was used to clean the PCR products. Initially, 19 (10 base pairs) oligonucleotide primers were screened on ten samples for unambiguous and reproducible band patterns. Out of 19, six oligonucleotide primers yielding the best results were selected and used for the analysis of present samples.

Primer Details

Six RAPD primers we selected based on their good data reproducibility and finally used them for study and characterization in ten *Rhododendron* species. The details of 10mer six primers are mentioned in Table 2.

Table 2. A list of the primers used in the RAPD experiment

Primer	Sequence
OP*A 02	5'- TGCCGAGCTG-3'
OPA 03	5'- AGTCAGCCAC-3'
OPA 17	5'- GACCGCTTGT-3'
OPN 18	5'- GGTGAGGTCA-3'
OPP 07	5'- ACATCGCCCA-3'
OPP 08	5'- ACATCGCCCA-3'

(*OP = Operon technologies and Kits are A, N, P. Ala-

Data Analysis

Band data were collected in binary format as present (1) or absent (0), and values were recorded in Microsoft-Excel, including monomorphic bands. Genetic data were analyzed by a series of software. The number of observed alleles (Na), the effective number of alleles (Ne), observed heterozygosity over k pops (Ho), expected heterozygosity (Hs), total expected heterozygosity (Ht), inbreeding coefficient within individuals (Fis), inbreeding coefficient within subpopulations (Fst), inbreeding coefficient within individuals (Gis), coefficient of gene differentiation (Gst), gene flow (Nm), Shannon's Information Index (I), polymorphic percentage (PPL), and other intermediate results were calculated by using GenAlEx version 6.502 software (Peakall & Smouse, 2012). AMOVA and Principal Coordinates Analysis (PCoA) was also calculated by using GenAlEx version

6.502 software (Peakall & Smouse, 2012). Polymorphic information content (PIC) was measured using PowerMarker version 3.25 software (Liu & Muse, 2005). For RAPD data, the genetic similarity or percentage homology was estimated between pairs of samples using the neighbor-joining cluster analysis method produced from Jaccard's estimate (Legendre & Legendre, 1998). The dendrogram was prepared using the neighbor-joining cluster analysis method of Jaccard's estimate with the help of Free Tree Software (Pavlícek *et al*., 1999). STRUCTURE version 2.3.4 (Pritchard *et al*., 2000) was used to characterize the genetic structure (according to a Bayesian analysis) of the 10 species of *Rhododendron* using different K values ranging from 1 to 10. With a 1,000,000 length burn-in phase and 1,000,000 Markov chain Monte Carlo (MCMC) repeats following the burn-in, ten separate runs per K were run. To figure out the maximum number of clusters in the ten species (the most probable K value), the STRUCTURE calculation outputs (Results.Zip) were uploaded into the web tool Structure Harvester (http://taylor0.biology.ucla.edu/struct_harvest/) (Earl & Vonholdt, 2012). Using GenAlEx 6.502 version software (Peakall & Smouse, 2012), the pairwise geographical distance of ten rhododendron species was calculated. Mantel's (Mantel, 1967) test was performed based on the data of pairwise geographical distance and Fst results of ten rhododendrons using GenAlEx 6.502 version software (Peakall & Smouse, 2012).

RESULTS

RAPD polymorphism and genetic diversity

PCR, using the six-decamer oligonucleotide RAPD primers, yielded distinct, reproducible, different polymorphic banding patterns that were unique to each primer and distinguishable over all samples tested (Figure 3). The results showed that different primers can amplify different bands on the same sample and the different samples can amplify different bands by the primer, which reflect the complexity of their genetic background and genetic diversity is summarized in Table 3. 611 alleles were detected using six RAPD primers from ten *Rhododendron* species with an average of 61.100 alleles observed per locus. Using six primers among ten samples, showing a broad range of polymorphism percentages (PPL%), the highest in *R. falconeri* (93.44%) and lowest in *R. setosum* (63.50%) with an average of 79.43% polymorphism. The PIC value ranges from 0.1800 (*R. decipiens*) to 0.2276 (*R. triflorum)* and a mean of 0.2096. Observed heterozygosity (Ho) ranges from 0.979 (*R. falconeri* and *R. fulgens*) to 0.993 (*R. decipiens*) which is 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 genetic diversity within the population (Hs) of the ten *Rhododendron* species is 0.979. The average total genetic diversity (Ht) is 0.985. The inbreeding coefficient (Fis) value within individuals was calculated as a negative value except in *R. fulgens* and *R. falconeri.* These two species (*R. fulgens* and *R. falconeri*) shows neutral Fis result. The degree of gene flow (Nm) ranges from 34.376 (*R. triflorum*) to 42.080 (*R. fulgens*) with an average of 38.505. Shannon's information index (I) ranges from the lowest 3.944 (*R. triflorum*) to the highest 4.000 (*R. setosum*) with an average value of 3.973.

Table 3. Summary of Genetic Diversity Indices for the selected *Rhododendron* species

Species	Na	Ne	cNe	Ho	Hs	cHs	Ht	cHt	Fis	Fst	Gis	Gst	Nm	I	PIC	PPL%
R. fulgens	61.000	48.431	48.378	0.979	0.979	0.986	0.985	0.986	0.000	0.006	0.008	0.000	42.080	3.987	0.2203	77.05
R. pendulum	61.000	47.605	47.333	0.992	0.979	0.986	0.985	0.986	-0.013	0.006	-0.006	0.000	39.571	3.977	0.2153	80.33
R. maddenii	62.000	48.639	48.588	0.989	0.979	0.986	0.985	0.986	-0.010	0.006	-0.002	0.000	41.707	3.991	0.2076	82.26
R. niveum	61.000	48.371	48.332	0.986	0.979	0.986	0.985	0.986	-0.007	0.006	0.000	0.000	41.105	3.985	0.2127	77.05
R. sikkimense	61.000	45.956	45.869	0.986	0.978	0.985	0.985	0.986	-0.008	0.007	-0.001	0.001	34.948	3.954	0.2023	72.13

R. decipiens	60.000	46.085	46.016	0.993	0.978	0.985	0.985	0.986	-0.015	0.007	-0.008	0.001	35.255	3.951	0.1800	85.00
R. falconeri	61.000	46.750	46.633	0.979	0.979	0.986	0.985	0.986	0.000	0.007	0.007	0.000	36.676	3.972	0.2023	93.44
R. grande	61.000	47.002	46.937	0.983	0.979	0.986	0.985	0.986	-0.004	0.007	0.003	0.000	37.644	3.966	0.2076	86.90
R. setosum	63.000	48.631	48.565	0.989	0.979	0.986	0.985	0.986	-0.010	0.006	-0.002	0.000	41.692	4.000	0.2203	63.50
R. triflorum	60.000	45.343	45.190	0.983	0.978	0.985	0.985	0.986	-0.005	0.007	0.002	0.001	34.376	3.944	0.2276	76.66
Mean	61.100	47.281	47.184	0.986	0.979	0.986	0.985	0.986	-0.007	0.006	0.000	0.000	38.505	3.973	0.2096	79.43
SE	0.277	0.388	0.395	0.002	0.000	0.000	0.000	0.000	0.002	0.000	0.002	0.000	0.974	0.004	0.0041	2.62

Na: Number of observed alleles; Ne: Effective number of alleles; cNe: Mean no of effective alleles over pops; Ho: Observed heterozygosity over k pops; Hs: Genetic diversity within the population; cHs: Corrected Hs; Ht: Total expected Heterozygosity; cHt: Corrected Ht; Fis: Inbreeding coefficient within individuals [Fis = (Hs-Ho)/Hs]; Fst: Inbreeding coefficient within subpopulations; Gis: Inbreeding coefficient within individuals, adjusted for bias [Gis = (cHs-Ho)/cHs]; 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.

The six primers cumulatively produced 126 unique bands for the ten *Rhododendron* species (Table 4). Among the six primers' the OPP 08 produced the highest number of unique bands and the OPA 03 primer produced less number of bands. The number of unique bands is highest in *R. setosum* and lowest in *R. falconeri.*

Table 4. Details of RAPD Unique banding pattern in different species of *Rhododendron*

Sl. No.	**Primer**	**Number of unique bands corresponding to each species**										**Total no of unique band scored**
		R. fulgens	***R. pendulum***	***R. maddenii***	***R. niveum***	***R. sikkimense***	***R. decipiens***	***R. falconeri***	***R. grande***	***R. setosum***	***R. triflorum***	
1	OPN 18	3	3	2	3	4	2	1	1	4	2	25
2	OPP 07	--	3	1	2	3	1	--	1	3	2	16
3	OPP 08	4	3	2	4	2	3	1	3	5	3	30
4	OPA 02	4	1	1	1	2	1	2	2	6	4	24
5	OPA 03	--	1	1	2	3	2	--	1	2	2	14
6	OPA 17	3	1	4	2	3	--	--	--	3	1	17
Total		14	12	11	14	17	9	4	8	23	14	**126**

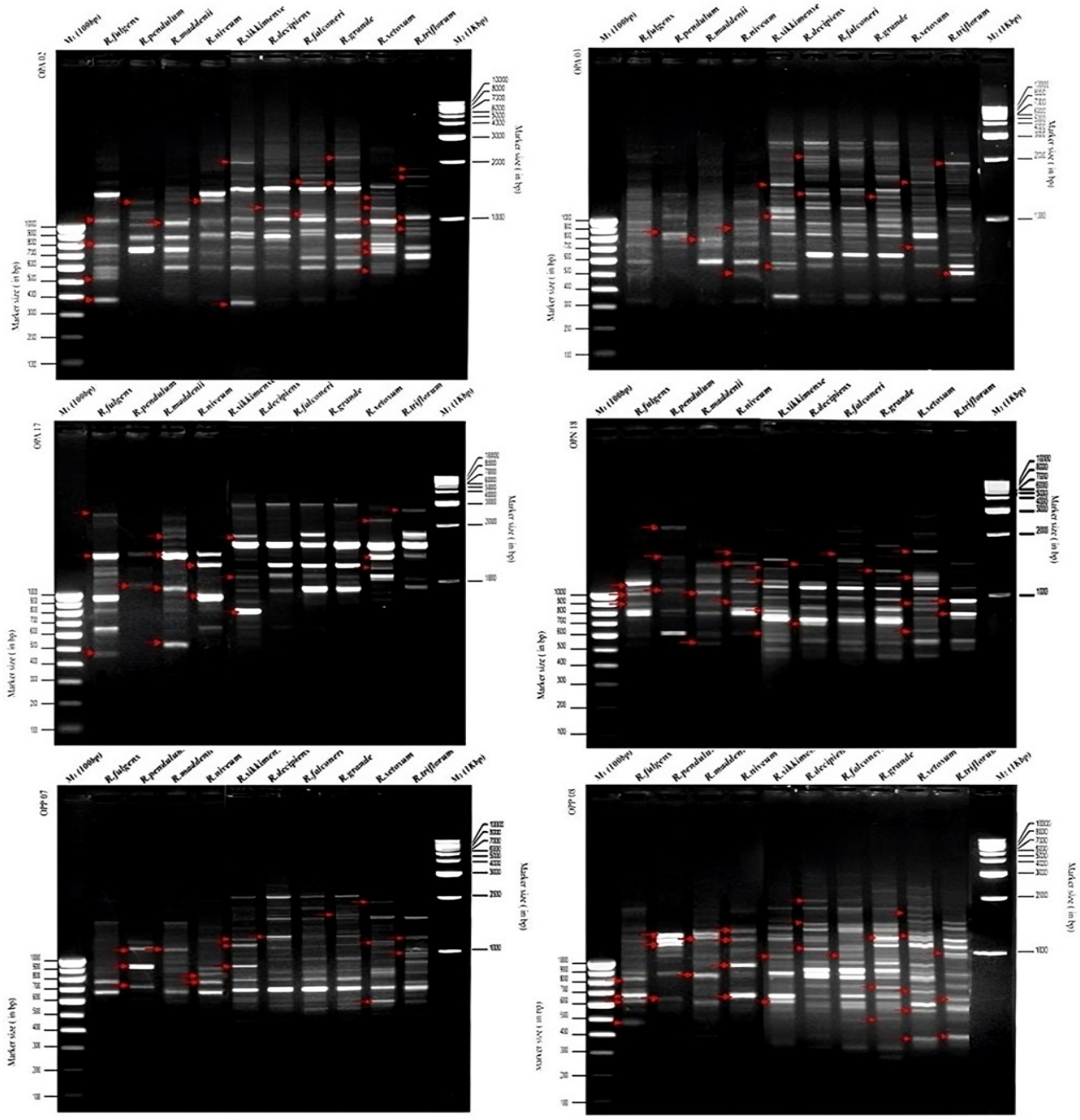

Figure 3. RAPD band profiles of ten *Rhododendron* species

Genetic differentiation

AMOVA-determined genetic differentiation of populations (Table 5) revealed that 1% (P< 0.05) of total variation occurred among populations and 99% (P< 0.05) occurred within populations.

Table 5. Analysis of molecular variance for the *Rhododendron* populations

Source of variance	d.f.	Sum of squares	Variance component	Est. Var.	Percentage (%) of variation	P value
Among populations	3	127.433	42.478	0.284	1%	<0.05
Within populations	6	250.667	41.778	41.778	99%	<0.05
Total	9	378.100		42.062	100%	

The genetic differentiation coefficient (Fst) between the populations was 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 given in Table 6. The similarity relationships ranged from 0.45455 to 0.93827. In RAPD data analysis, the most closely related genotypes with the highest similarity index i.e. above 0.90 were found between *R. fulgens* and *R. triflorum* (0.93827) followed by *R. niveum* and *R. triflorum* (0.92308), *R. maddenii* and *R. grande* (0.91566), *R. sikkimense* and *R. maddenii* (0.91358) then between *R. pendulum* and *R. sikkimense* (0.90541), *R. niveum* and *R. grande* (0.90361), *R. niveum* and *R. setosum* (0.90244), *R. sikkimense* and *R. niveum* (0.90123). The highest genetic similarity indicated their high homology in genetic background. The higher similarity index i.e. above 0.80 but below 0.90 was noted between *R. grande* and *R. pendulum* (0.89333), *R. fulgens* and *R. falconeri* (0.89286), *R. falconeri* and *R. niveum* (0.89024), *R. falconeri* and *R. maddenii* (0.88889), *R. triflorum* and *R. pendulum* (0.88235), *R. triflorum* and *R. maddenii* (0.87838), *R. fulgens* and *R. setosum* (0.8642), *R. fulgens* and *R. grande* (0.86585), *R. maddenii* and *R. decipiens* (0.85542), *R. setosum* and *R. pendulum* (0.84507), *R. setosum* and *R. maddenii* (0.84416) *R. sikkimense* and *R. fulgens* (0.83333), *R. falconeri* and *R. pendulum* (0.83099), *R. pendulum* and *R. fulgens* (0.83019), *R. niveum* and *R. pendulum* (0.82353), *R. decipiens* and *R. niveum* (0.81481), *R. grande* and *R. setosum* (0.81053) *R. sikkimense* and *R. triflorum* (0.80682), *R. sikkimense* and *R. setosum* (0.80645). The high similarity index i.e. above 0.60 but below 0.80 was recorded between *R. fulgens* and *R. niveum* (0.62745), *R. sikkimense* and *R. falconeri* (0.61728) *R. pendulum* and *R. maddenii* (0.65909), *R. maddenii* and *R. niveum* (0.66), *R. fulgens* and *R. maddenii* (0.67308), *R. sikkimense* and *R. decipiens* (0.68539), *R. falconeri* and *R. triflorum* (0.72619), *R. setosum* and *R. triflorum* (0.78161) *R. grande* and *R. triflorum* (0.78409), *R. falconeri* and *R. setosum* (0.78495), *R. decipiens* and *R. triflorum* (0.7957), *R. decipiens* and *R. setosum* (0.79592). The most distantly related species with low similarity index i.e. above 0.40 and below 0.60 were noted between *R. decipiens* and *R. falconeri* (0.45455), *R. decipiens* and *R. grande* (0.49367), *R. falconeri* and *R. grande* (0.51948).

Table 6. Similarity index or coefficient matrix based on RAPD markers

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

R. decipiens	0.83333	0.79452	0.85542	0.81481	0.68539		0.45455	0.49367	0.79592	0.7957
R. falconeri	0.89286	0.83099	0.88889	0.89024	0.61728	0.45455		0.51948	0.78495	0.72619
R. grande	0.86585	0.89333	0.91566	0.90361	0.61728	0.49367	0.51948		0.81053	0.78409
R. setosum	0.8642	0.84507	0.84416	0.90244	0.80645	0.79592	0.78495	0.81053		0.78161
R. triflorum	0.93827	0.88235	0.87838	0.92308	0.80682	0.7957	0.72619	0.78409	0.78161	

A dendrogram based on RAPD markers using the neighbor-joining cluster analysis method produced from Jaccards estimates using Free Tree software divided nine *Rhododendron* species into two main sister groups (I & 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. *R. grande* is out-group taxa (Figure 4).

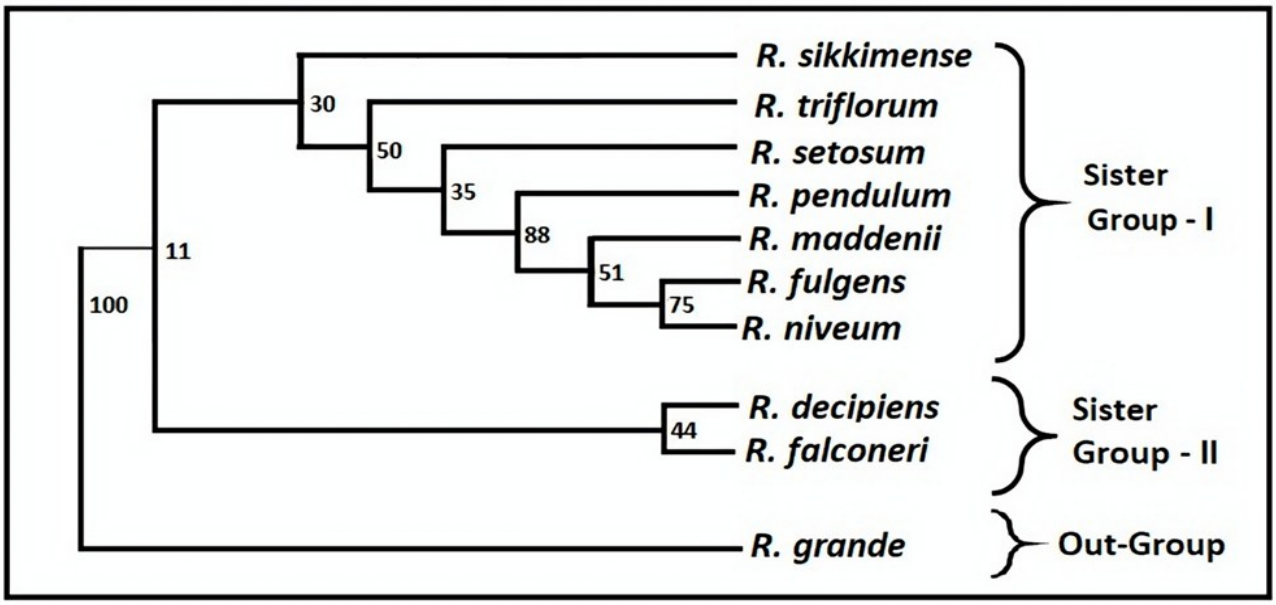

Figure 4. Dendrogram showing the genetic relationship among the studded *Rhododendron* genotypes

Population structure of Rhododendron

Results of the STRUCTURE program of *Rhododendron* species-wise evaluated maximum peak at ΔK= 5 (Figure 5c) and among the ten species, the *R. falconeri* genotype shows a maximum level of admixture whereas the maximum amount of genome is unique in the *R. fulgens* (Figure 5a and b). The ten *Rhododendron* species consist of mainly five genetic clusters. Whereas *R. fulgens*, *R. niveum, R. grande, R. setosum,* and *R. triflorum* show noting or very low amounts of genetic shearing with other *Rhododendron* species. On the other, side other five *Rhododendron* species share their genetic content with other species.

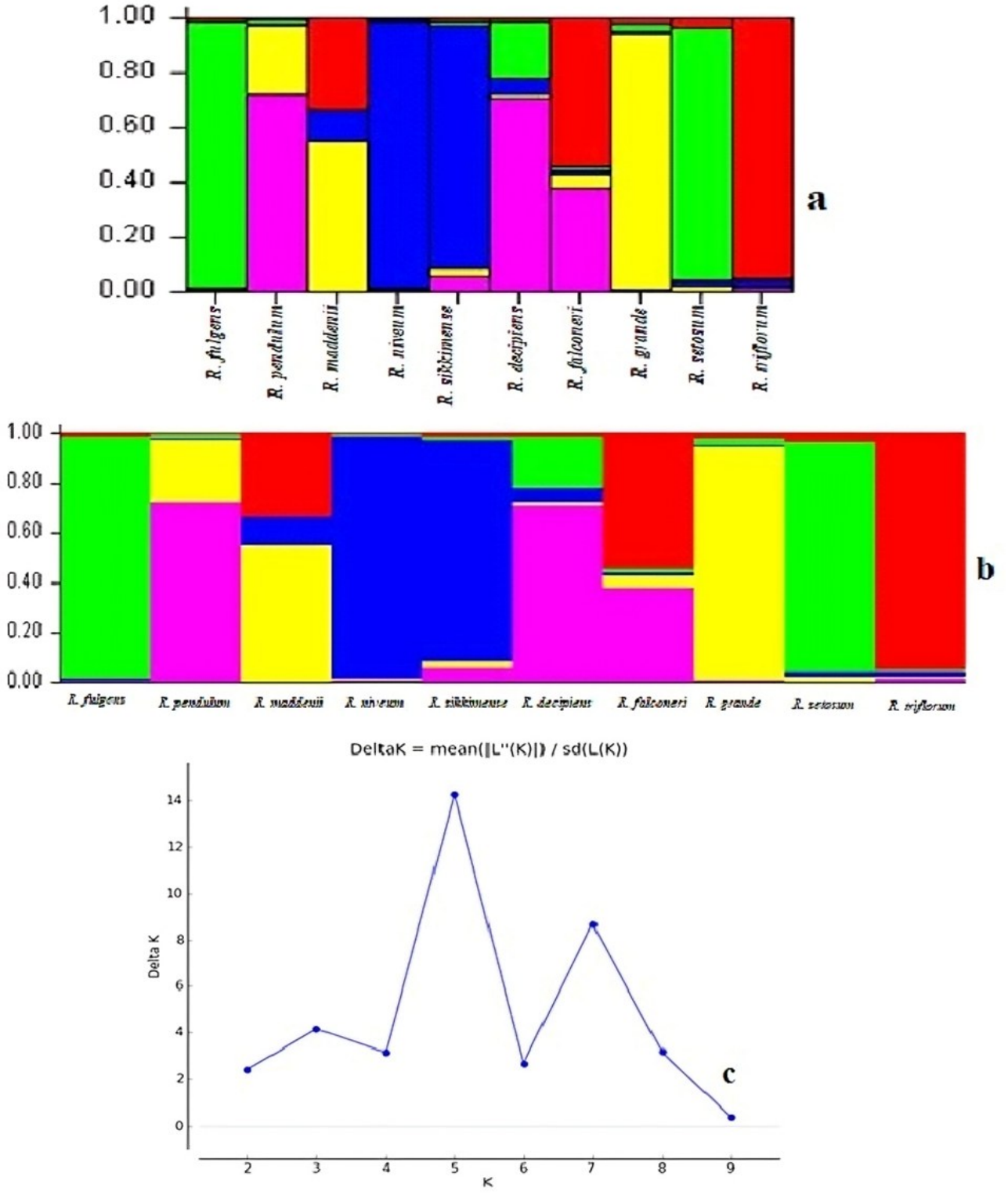

Figure 5. Results of Bayesian clustering using STRUCTURE software of 10 *Rhododendron* species. Each species is marked by a vertical bar colour (a & b) and the membership coefficients of samples based on allele frequencies for subpopulations are represented by the same bars; showing maximum peak at K=5 (c)

Population-wise result of the STRUCTURE program evaluated the maximum peak at ΔK= 6 (Figure 6c) where *Rhododendron* species belonging to Neora Valley National Park (*R. maddenii* and *R. niveum*) and Senchal Wildlife Sanctuary (*R. falconeri, R. grande* and *R. setosum*) maximally shared their genome with them and another group (Figure 6a and b). *Rhododendron* populations of Sandakphu (*R. fulgens* and *R. triflorum*) and Singalila National Park (*R. pendulum*, *R. sikkimense* and *R. decipiens*) genetically shared with other groups are very less amount, which is insignificant. In this structure analysis, a new colour (Cyan) combination was found for genetic clustering which penetrated the minimum amount in Sandakphu and Singalila National Park *Rhododendron* populations and the maximum found in Neora Valley National Park and Senchal Wildlife Sanctuary Rhododendrons.

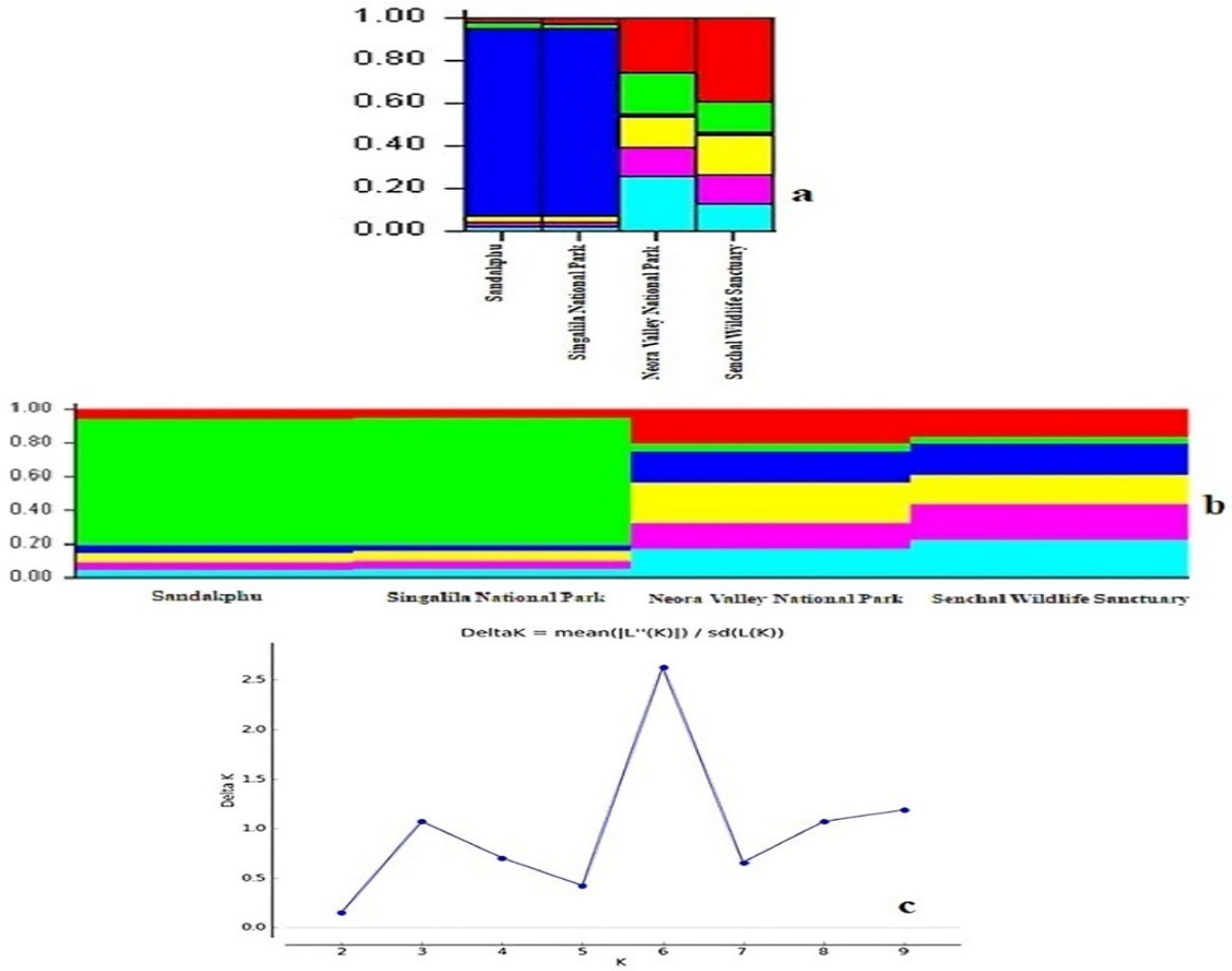

Figure 6. Estimated genetic structure of the four populations (a & b) using STRUCTURE software based bayesian analysis maximum peak at K=6 (c). The membership coefficients of samples based on allele frequencies for subpopulations are represented by the same bars

Principal Coordinate (PCoA) Analysis

The result of PCoA analysis reveals that ten *Rhododendron* species were divided into three main clusters (Figure 7), which are the same as the dendrogram of the phylogenetic tree shown by the Neighbor-Joining cluster analysis method produced from Jaccards estimates using Free Tree software.

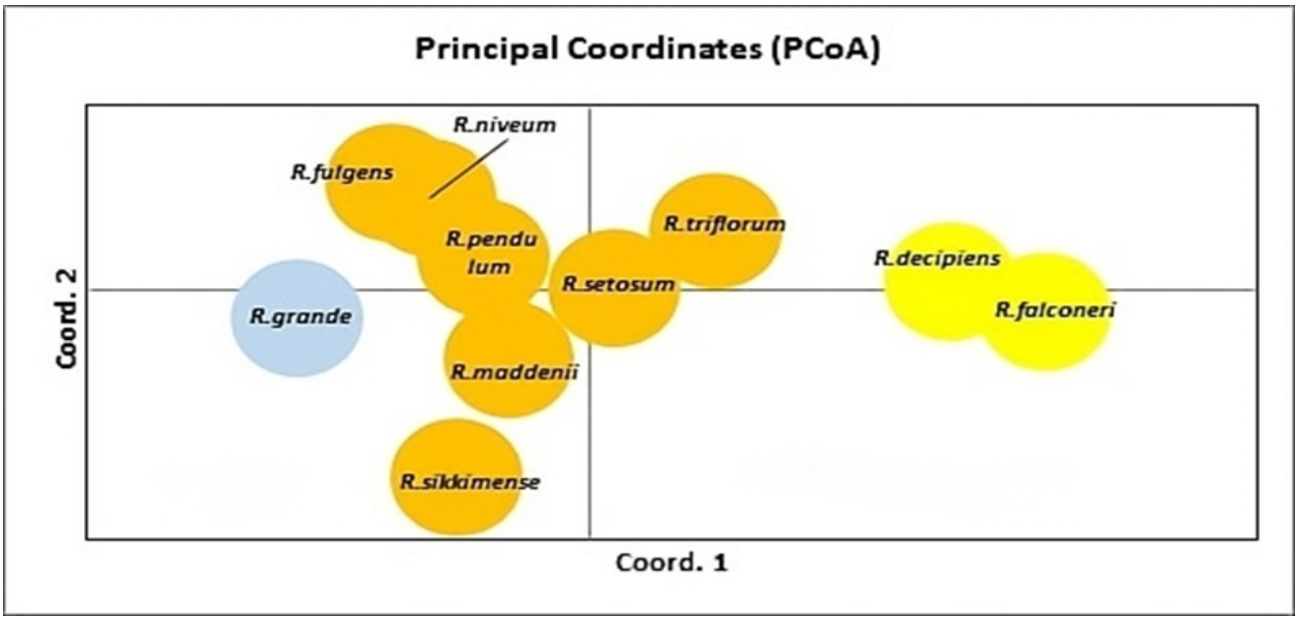

Figure 7. Principal Coordinate Analysis (PCoA) of the ten *Rhododendron* species. Showing group- I (dark gold colour), group -II (yellow colour), and one out-group (sky blue colour)

Geographic Distance

Geographical distance (in Kilometers) among the ten *Rhododendron* species summaries in Table 7. The distance ranged from 7.280km to 116.276km. The geographical distance between *R. triflorum* and *R. niveum* is a minimum (7.280km) and the maximum distance present between *R. falconeri* and *R. pendulum* (116.276km). On the other hand, the genetic distance is minimum between *R. triflorum* and *R. niveum* (0.92308) and a little bit higher between *R. falconeri* and *R. pendulum* (0.83099).

Table 7. Pairwise geographic distance (in Km) index or coefficient

	R. fulgens	*R. pendulum*	*R. maddenii*	*R. niveum*	*R. sikkimense*	*R. decipiens*	*R. falconeri*	*R. grande*	*R. setosum*	*R. triflorum*
R. fulgens	0.00									
R. pendulum	61.07	0.00								
R. maddenii	63.53	33.38	0.00							
R. niveum	78.72	40.03	15.23	0.00						
R. sikkimense	71.51	51.62	18.25	20.81	0.00					
R. decipiens	66.41	24.08	11.40	16.55	28.79	0.00				
R. falconeri	86.31	116.28	89.81	98.11	77.41	100.90	0.00			
R. grande	28.60	51.11	39.62	54.57	43.83	46.04	69.77	0.00		
R. setosum	80.99	73.01	39.85	41.23	21.93	50.70	61.40	52.48	0.00	
R. triflorum	84.17	40.311	21.38	7.28	27.79	19.11	105.19	61.00	47.38	0.00

Mantel tests were performed between Fst and pairwise geographical distance data. We found a slight positive correlation (R^2= 0.0809) between geographic distance and genetic distance (Figure 8). The result showed that regression did not adequately establish a close association between the genetic and geographic pairwise distances.

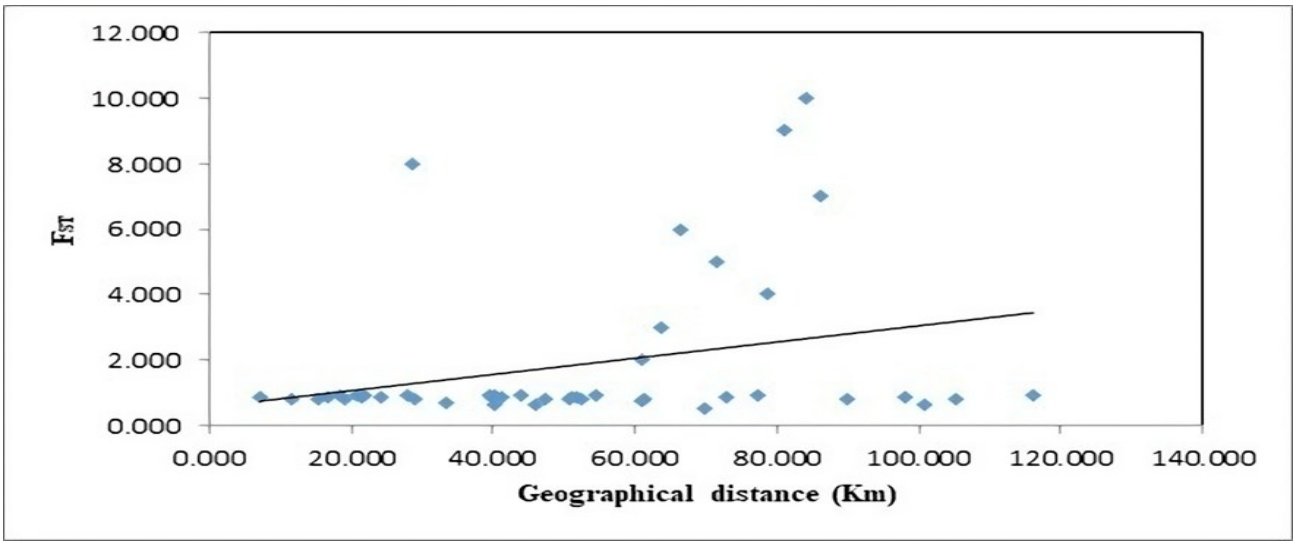

Figure 8. Mantel tests show the relationship between geographical distance and pairwise F_{ST} for ten *Rhododendron* species used for the present study. The fitted line represents the equation, Geographical distance= 0.0246 (F_{ST}) + 0.5769 and the proportion of the variance is R^2= 0.0809.

DISCUSSION

Genetic Diversity among ten Rhododendron species

In order to fingerprint complicated genomes, RAPD uses a single randomly chosen primer and the polymerase chain reaction (PCR). By RAPD a set of primers can generate multiple genetic markers to distinguish closely related species or even varieties. In our study, six decamer oligonucleotide random primers were applied. Primers measure moderate to high polymorphism percentage (63.50 to 93.44) with average low Polymorphism Information Content (Mean, PIC= 0.2096) of ten *Rhododendron* species. Due to genetic drift and inbreeding, species in small populations have reduced genetic diversity than those in large populations (Willi *et al.,* 2006; Li *et al.,* 2012b).

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 (Hamrick & Godt, 1989). Unexpectedly, our study in *Rhododendron* showed an average higher level of genetic diversity [PPL (percentage of polymorphic loci) =79.43%] at the species level. According to the majority (Rossetto *et al.,* 1995; Ci *et al.,* 2008; Gordon *et al.,* 2012; Zhao *et al.,* 2012b; Swarup *et al.,* 2021), our findings in some rare and endangered species can preserve high levels of genetic diversity while having diminishing numbers. Higher genetic diversity was good for the breeding of excellent cultivars (Swarup *et al*., 2021; Salgotra & Chauhan, 2023). 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). Yet, not all endemic and threatened plants have little genetic diversity. Earlier studies show *Origanum compactum* (He= 0.35) a medicinally important plant, *Paeonia jishanensis* (He= 0.340), *Rhododendron protistum* var. *giganteum [*Nei's gene diversity (h)= 0.240], *Paeonia decomposita* (He= 0.405), *Populus wulianensis (He*= 0.611, PPL = 98.6% and species level H_T = 0.741), and *Kelussia odoratissima* (He= 0.72) are endangered plants show moderate to high levels of genetic diversity (Aboukhalid *et al*., 2017; Wang *et al*., 2011; Wu *et al*., 2014; Wang, 2020; Wu *et al*., 2020; Mahdavikia *et al.,* 2024).

The maintenance of high genetic diversity in Rhododendrons can be explained by several possible reasons. In general, biological characteristics, mode of reproduction, and breeding system have frequently been considered to be significant elements that determine levels of genetic variety (Wu *et al*., 2014). Outcrossing species usually have considerably higher levels of genetic diversity than self-crossing species (Hamrick & Godt, 1989; Nybom, 2004; Wu *et al*., 2014**).** Our findings generally agreed with the idea that some rare and endangered species could preserve high levels of genetic diversity while having limited populations (Rossetto *et al*., 1995; Ci *et al*., 2008; Gordon *et al*., 2012; Zhao *et al*., 2012b; Wu *et al*., 2014). This rare and endangered *Rhododendron* species' high level of genetic diversity may be maintained through high levels of gene flow (Neel, 2008). It is suggested that the mating system of *Rhododendron* may be predominantly outcrossed because of pollinators (Hirao *et al*., 2006; Ono *et al*., 2008; Hirao, 2010) like insect vectors and birds (Shen *et al*., 2009), and low rates of self-fertilization (Ma *et al*., 2015) largely promote outcrossing. Pollen grains of *Rhododendron* species connected by sticky viscin threads (Bowers, 1930; Huang *et al*., 2017; Song *et al*., 2019), act as an anchorage for the pollen to the vectors, and the pollen can migrate beyond 10km. This phenomenon is a good justification for outbreeding in Rhododendrons. The taxa is generally long-lived plants and this characteristic is highly advantageous to retain genetic variations (Wu *et al*., 2014). The ten *Rhododendron* species generate average negative inbreeding coefficients ($F_{IS} < 0$) (Table 3), illustrating that despite having fragmented habitats, they do not have inbreeding depression (Wu *et al*., 2020). However, the outcome result shows that *Rhododendron* species used for the study are too much in a heterozygote condition, it may be due to outcrossing. The consequences of outbreeding depression are reduced genetic adaptability of a species, restricted habitat adaptability, decreased reproduction and competitiveness, loss of genetic specificity in small populations, and ecological hazards (Wang & Peng, 2003; Ding *et al*., 2018). A natural hybrid population is common in Rhododendrons. The interspecific boundaries will erase in the near future if threatened and endangered species are pollinated with closely related species in a natural environment (Ellstrand, 1992). The floral characteristics of the East Himalayan *Rhododendron* species evolved in such a way that bird pollination is common for this population and weather conditions supports the evolution (Huang *et al*., 2017). Whereas, increased hybridization (natural or artificial) will elevate in risk of extinction of species or populations (Ding *et al*., 2018).

The Jaccard's similarity coefficient values vary from 0.45455 to 0.93827 among the species (Table 6). This clearly shows that significant genetic diversity exists among the *Rhododendron* spp. Hence, these species are to be preserved as valuable genetic resources for breeding. The high genetic diversity among these species clearly indicates that they must have evolved from genetically divergent parents or have a long history of adaptation to their respective microclimatic regions (Singh *et al*., 2011).

The clustering result obtained from RAPD analysis suggested that subgenus *Hymenanthes* had a closer relationship with subgenus *Rhododendron*. It also showed that some morphological traits could reflect the genetic characters. In sister group II, *R. decipiens* and *R. falconeri* were gathered together because they shared some common morphological characters *viz*. trees 4-15m high, leaves very large & flowers campanulate in shape (Table 1). Moreover, *R. decipiens* is a natural hybrid between *R. falconeri* x *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 with flowers in the shade of blood red, mauve or rose pink in trusses belonging to the section Pointica. *R. grande* belonging to the same section Pointica kept as an outgroup because it showed little different morphological traits such as trees 4-15 m high, underside of the leaf silvery white indumentum and flowers white to creamy yellow. 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. These results suggest that some rare threatened and endangered species *Rhododendron* in the present study are able to maintain high levels of genetic diversity.

Genetic Structure and Genetic differentiation among the species of Rhododendron

In this present study, the AMOVA result shows that 99% of the total variation occurred within populations, while 1% variation was present among populations (Table 5) strongly supporting the variation present within populations. Wright (1978) clearly defined that genetic differentiation is low for Fst < 0.05, moderate for 0.05 <Fst < 0.15, high for 0.15 <Fst <0.25, and very high for Fst> 0.25. The average Fst results revealed that the genetic differentiation between species was negligible (Table 3). Gene flow might inhibit differentiation and mitigate the genetic drift when Nm > 1 (Wright, 1990). The gene flow (Mean Nm= 38.505) result between *Rhododendron* species also indicated that gene flow among species is very high. Over gene flow is introgressive and can produce genetic swamping (Fath, 2018). Through genetic swamping, in which the native genotypes are replaced by hybrids, or by demographic swamping, in which population growth rates are slowed down by outbreeding depression, gene flow from common species has increased the risk of extinction for rare species (Todesco *et al.*, 2016; Buck & Flores-Rentería, 2022). The six primers produced a total of 126 unique bands for the ten *Rhododendron* species. These unique bands are very helpful for managing a germplasm bank since it offers a cheap and trustworthy way to identify many cultivars. However, one risk of this approach is that genetic uniqueness may be highlighted at the expense of genetic diversity, which is necessary for adaptation and may be enhanced by mixing geographically distinct populations (Coleman *et al*., 2013).

STRUCTURE analysis, Principal Coordinate Analysis (PCoA), and NJ-based clustering method carefully examine the genetic makeup of the species of *Rhododendron*. The Dendrogram of the phylogenetic tree shows the genetic relationship among the ten *Rhododendron* species and are divided into three groups that are also supported by the PCoA result (Figure 4 & 7). STRUCTURE analysis of *Rhododendron* species-wise and population-wise evaluated maximum peak at ΔK= 5 and ΔK= 6 respectively (Figure 5c & 6c). The local environmental factors that frequently influence genetic structural patterns, particularly in varied and fragmented habitats, could be to blame for this population clustering (Young *et al*., 1996; Zhu *et al*., 2016; Cao *et al*., 2022). In some plants, excess heterozygote may be related to population regeneration (Stoeckel *et al*., 2010), and in this study; the calculated heterozygosity (Ht= 0.985) is very high. The existence of a high allelic richness in the parent plants or the small number of populations with few individual plants in each population may be the cause of the increased heterozygosity (Pudovkin *et al*., 1996; Huang *et al*., 2021). The amount of gene flow between populations significantly affects their genetic structure and is a crucial indicator of how environmental changes, human meddling and population isolation will affect those (Wu *et al*., 2017). The calculated geographical distance (Median value of geographical distance= 50.69km) among the *Rhododendron* species is higher resulting in difficulty to transmit genes (pollen and seed) from one to another species (Ng & Corlett, 2000; Huang *et al*., 2017; Li *et al*., 2018). As a result, short-distance seed dispersal and distance-limited pollen movement could be responsible for the population divergence. However, Mantel tests results support little but positive correlation in between geographic distance and genetic distance (Figure 8).

Conservation implications

High genetic variation among *Rhododendron* species reflected its strong adaptability and enables species to adapt to changing environments (Zhao *et al*., 2012b). The current endangered status of these species is not due to genetic reasons (e.g. genetic diversity decline, genetic drift, and inbreeding), as evidenced by the moderate to high genetic diversity found in several *Rhododendron* species, a similar type of results found in *Tupistra pingbianensis (*Qiao *et al*., 2010). The main threat to these plant species may be habitat specialization. However, we did not conduct a detailed habitat survey of Rhododendrons. Where these species are still at risk of extinction because of continued habitat disturbance. In vast populations, outbreeding depressions are ephemeral and natural selection acts to eliminate them, but in limited populations, as is the case, it can lead to a population "bottleneck" - decline and even extinction of the species (Frankham *et al.,* 2017; Barmentlo *et al.,* 2018). Thus, genetic data on *Rhododendron* enumerate urgently needed to inform current and future conservation activities. The most efficient way to save rare, endangered species (*Rhododendron* like big trees) is through *in situ* conservation (Shen *et al*., 2009).

CONCLUSION

The present study provided the first molecular level genetic study, AMOVA, genetic structure analysis, geographic distance, and detailed interpretation for the long -term conservation and management of the rare, threatened, and endangered species of Eastern Himalayan *Rhododendron*. Results suggest that rare, threatened, and endangered species are able to maintain high genetic diversity within the population even in a small community. The results emphasize that the chosen species are at a high risk of outcrossing depression, perhaps resulting in a population bottleneck. Therefore, "In situ" conservation is recommended as the most efficient way of protecting genetic diversity of the species.

CONFLICT OF INTEREST STATEMENT (COI)

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This article does not contain any studies with humans or animals performed by the author. This research did not receive any specific grant from funding agencies in the public or commercial sectors.

AUTHORS' CONTRIBUTIONS

Both the authors approved the manuscript for submission. **Animesh Mondal**: conceptualization performed the experiments and initially drafted the research article, revised the manuscript for intellectual content. **Kalyan Kumar De**: supervised the experimental works, validation, reviewing, and proofreading the revised manuscript for intellectual content.

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