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Study on population genetics of *Rhododendron mechukae*, an endemic species of the Eastern Himalayas

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Abstract

This study presents the first molecular analysis of *Rhododendron mechukae*, a critically endangered and narrowly distributed species endemic to Arunachal Pradesh in the Eastern Himalayas. Population genetic diversity was assessed using simple sequence repeat (SSR) markers across 50 genotypes sampled from natural populations in Mechuka, Shi Yomi district. Out of 20 SSR primer pairs screened, nine exhibited successful amplification with polymorphic information content (PIC) values ranging from 0.231 to 0.540. The effective number of alleles (Ne) averaged 1.38 ± 0.11, Shannon's information index (I) was 0.35 ± 0.07, and observed heterozygosity (Ho) was extremely low at 0.032 ± 0.01. STRUCTURE analysis revealed two distinct genetic clusters (K = 2), with Population 1 showing higher genetic diversity (Na = 2.333, He = 0.333) than Population 2 (Na = 1.556, He = 0.103). Analysis of molecular variance (AMOVA) indicated that 63% of the total genetic variance occurred among populations, confirming strong genetic structuring. The mean fixation index (F = 0.731 ± 0.09) indicated severe heterozygote deficiency and a high prevalence of homozygosity. These findings show that although *R. mechukae* retains moderate genetic diversity at the species level, it faces pronounced vulnerability due to limited gene flow and population isolation, highlighting the need for urgent conservation interventions.

1 Introduction

The Eastern Himalayas are a globally important biodiversity hotspot that supports exceptional floristic diversity but is increasingly threatened by anthropogenic pressures and climate change (Nautiyal and Kaechele, 2007).The genus *Rhododendron* (Ericaceae), with more than 1,200 species worldwide, reaches its highest diversity in this region, and Asian mountains harbour approximately 90% of wild *Rhododendron* populations (Pradhan et al., 2015).These species often function as keystone taxa in fragile high-altitude ecosystems, stabilizing slopes, providing habitat, and supporting complex ecological interactions (Bharali et al., 2013).

Rhododendron mechukae, recently described from Arunachal Pradesh, India, is a narrowly distributed endemic species restricted to temperate forests in the Mechuka region (Mao et al., 2013).It is characterized by rough brownish peeling bark, leaves with rufous-brown indumentum on the abaxial surface, short pedicels up to 1.8 cm long, and early flowering from February to March (Mao et al., 2013).Its distribution is highly localized, and populations are under strong pressure from habitat degradation and timber extraction (Mao et al., 2013; Bharali et al., 2013).

Conserving endangered plant species is essential for maintaining biological diversity and ecosystem stability (Torres-Díaz et al., 2021). Genetic diversity within and among populations strongly influences long-term survival, adaptability, and resilience to environmental change (Jiajia et al., 2023).

Populations with reduced genetic variation are more vulnerable to extinction because of inbreeding depression, genetic drift, and limited adaptive potential (Reed and Frankham, 2003; Frankham, 2005; Johnson and Molano-Flores, 2023). Characterizing genetic variation within and between populations is therefore crucial for designing effective conservation strategies for small and fragmented populations (Frankham, 2005; Johnson and Molano-Flores, 2023).

Despite its conservation importance, no molecular or genomic data were previously available for *R. mechukae*, leaving a major gap in evidence-based conservation planning.

The present study addresses this gap by providing the first population-level genetic assessment of *R. mechukae* using microsatellite markers, with the aims of quantifying genetic diversity, resolving population structure, and generating baseline information for conservation management.

2 Materials and methods

2.1 Study area and sample collection

Field surveys were conducted in the natural distribution range of *R. mechukae* in Mechuka, Shi Yomi district, Arunachal Pradesh, India (28°39'53.70" N, 93°59'08.70" E) at elevations of 2,000–3,500 m. A total of 50 individual genotypes were randomly sampled from five populations (Segong, Hanuman Camp, Yorlung, Pogor, and Lamang) using linear transects, and the sampling locations are presented in Fig. 4. GPS coordinates were recorded for each sampled plant, and three healthy leaves per genotype were collected during September-March 2024 following the original taxonomic description. Leaf samples were rapidly desiccated in silica gel and stored at − 80°C until DNA extraction.

Figure 4 Sampling locations *of R. mechukae* populations in Mechuka region, Arunachal Pradesh, India

2.2 DNA extraction and quantification

Genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method following Doyle and Doyle (1993). Approximately 2 g of leaf tissue was ground to a fine powder in liquid nitrogen using a pre-cooled mortar and pestle. The powder was mixed with CTAB extraction buffer (100 mM Tris–HCl, pH 8.0; 20 mM EDTA; 1.42 M NaCl; 5 mM ascorbic acid; 3% CTAB; 3% polyvinylpyrrolidone) and incubated at 55°C for 45 min. After extraction with chloroform:isoamyl alcohol (24:1) and precipitation with isopropanol, DNA pellets were dissolved in sterile water and treated with RNase. DNA concentration and purity were measured spectrophotometrically using A260/A280 ratios, with values around 1.8 indicating acceptable purity. DNA quality was further checked on 0.8% agarose gels, which showed intact high-molecular-weight bands with minimal degradation.

2.3 SSR marker analysis

Twenty SSR primer pairs previously developed for related *Rhododendron* species were initially screened (Table 1). PCR amplification was performed in 15 µl reactions containing 1.9 µl PCR buffer with MgCl2, 1.5 µl dNTPs, 0.45 µl of each primer, 0.5 µl Taq DNA polymerase, 8.7 µl molecular-grade water, and 1.5 µl template DNA. Thermal cycling consisted of an initial denaturation at 94°C for 3 min; 35 cycles of 94°C for 1 min, locus-specific annealing temperature for 1 min, and 72°C for 1 min; followed by a final extension at 72°C for 8 min. PCR products were separated on 2% agarose gels, visualized under UV light, and scored as present (1) or absent (0). Polymorphic information content (PIC) was calculated as $PIC = 1 - \sum P_i^2$, where P_i is the frequency of the ith allele at a locus (De Riek et al., 2001).

Table 1
Primer pairs used in the study

S.no.	Locus	Code	Motif/SSR	Forward Primer (5'-3')	Reverse Primer (5'-3')	References
1	RHM_MS13	SSR-1	(CTT)5	F: TCGATCTCCACCTACAAACTA	R: ACAAAGGCCTTGAACATCT	Sharma et al. 2020
2	R394	SSR-2	(TC)16	F: GGAAAGTGTGGGTGTTAGTGC	R: TTGAGAGATGGCGAGAGAGAG	Choudhary et al. 2014
3	RE101	SSR-3	(AG)16	F: GACGGGAATGAGCAAGGTTG	R: CTTCAATTCTGCAAGCCCGA	Choudhary et al. 2014
4	RA430	SSR-4	(TC)10(CT)6	F: GCGTAAATCGAGTTCGGAAG	R: CTCTCTCTAATCGAATTCCCG	Choudhary et al. 2014
5	RF87A	SSR-5	(AG)10(AG)10	F: TGGGTCATGTTCTGGAAGGT	R: TGAACTAACCCTAGCCACACT	Choudhary et al. 2014
6	RA324	SSR-6	(AG)8(GA)8	F: GCGTACAACATGCCCAAATA	R: CCCTGTTCTCATTGCTCACA	Choudhary et al. 2014
7	RF304	SSR-7	(AG)13(GT)9	F: TCCTAGGGTTTGTTCGCAAT	R: TGCTAGCGATTTCCTAGGGT	Choudhary et al. 2014
8	RA272A	SSR-8	(CT)8(CT)11	F: GCCCCGGTGACTCATAAAAT	R: TGGTACAAGTGGGACACGA	Choudhary et al. 2014
9	R460	SSR-9	(GA)13(AG)12	F: CCCTACTTCTTTCATCACATACAA	R: CAACTCCGGTCATTTTTGGT	Choudhary et al. 2014
10	RA267	SSR-10	(GA)11(AG)10	F: ACGGAGAAGCAGTGAGCATT	R: TGCACAGGAACACCCAATAA	Choudhary et al. 2014
11	RA346	SSR-11	(TC)9	F: CGGAGCAAGCTCTCTTATCG	R: CCTCTCCTGTGTAGCAAGTCG	Choudhary et al. 2014
12	RM1D1	SSR-12	(CT)17	F: ATCTGGAGGCCATTGGTAGT	R: TATTGGGTCCGATGACAGAC	Kameyama et al. 2002
13	RM2D2	SSR-13	(CT)16	F: ATGTGTTTCGTTGCTACTGT	R: ATGGTTGGTTTGTTTTCCTA	Naito *et al*.1998
14	RM3D4	SSR-14	(CT)7(CT)19	F: CTCCCAACAAACAAATCCAT	R: CACCGAACGAAGACACTCAG	Naito *et al*.1998
15	RM9D6	SSR-15	(GA)16	F: CTCGCCTCCCAAAAGCAAT	R: CGTGTCCTCACCCCCGTAAC	Naito *et al*.1998
16	RA470	SSR-16	(GA)14(AG)10	F: AGGGACAAGAAGAAGCCACA	R: TCGCGCTTATTACAGCTCTTC	Choudhary et al. 2014
17	RA254	SSR-17	(CT)16(CT)10	F: AGTAGCAACACCCACACACT	R: GGAGGGGCTGTAGTCTGATT	Choudhary et al. 2014
18	RA321	SSR-18	(GA)9	F: AGAGATGGGTTTGTGTAAAGTCTG	R: TATTTCGCTGCCACCCTAAC	Choudhary et al. 2014
19	RA351	SSR-19	(AG)12(AG)11	F: TGTCGCTCTCTCACTGATCG	R: TTTGTAGTTTTCCCGTGTCCTT	Choudhary et al. 2014
20	R97	SSR-20	(AG)10(AG)13	F: AGCAGCAACAATGGTGTCC	R: TCTAGAAGGCCTCCCATTCC	Choudhary et al. 2014

2.4 Population genetic analysis

Genetic diversity parameters, including number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I), observed heterozygosity (Ho), expected heterozygosity (He), and fixation index (F), were estimated using GenAlEx v6.5 (Peakall and Smouse, 2012).Population structure was analysed using STRUCTURE v2.3.4 with a Bayesian Markov chain Monte Carlo (MCMC) approach (Pritchard et al., 2000).Ten independent runs were performed for K values from 1 to 10, each with a burn-in of 100,000 iterations and 200,000 MCMC repetitions.The most likely number of clusters (K) was determined using Structure Harvester based on the ΔK method (Earl and VonHoldt, 2012; Evanno et al., 2005).

AMOVA was conducted to partition genetic variance among populations, among individuals within populations, and within individuals (Excoffier et al., 1992).Wright's F-statistics (Fis, Fit, Fst) and gene flow (Nm) were calculated to assess population differentiation and

migration. Genetic relationships among populations were visualized using UPGMA/Neighbour-Joining trees constructed in DARwin v6.0 and principal coordinate analysis (PCoA) in GenAlEx (Perrier and Jacquemoud-Collet, 2006; Peakall and Smouse, 2012).

3 Results

3.1 SSR marker polymorphism

Of the 20 SSR primer pairs tested, nine (45%) produced clear and reproducible amplification across all samples and were polymorphic. These nine markers (SSR1, SSR3, SSR9, SSR10, SSR11, SSR13, SSR15, SSR17, SSR18) yielded alleles in the size range 150–310 bp, and representative amplification profiles for SSR11 and SSR15 are shown in Figs. 5 and 6, respectively. PIC values ranged from 0.231 (SSR13) to 0.540 (SSR11), with an average of 0.390, indicating moderate to high informativeness for population genetic analysis (Table 2).

3.2 Genetic diversity assessment

Genetic diversity estimates revealed moderate allelic richness but very low heterozygosity. The mean number of different alleles (Na) was 1.94 ± 0.18, ranging from 1.50 (SSR3, SSR10) to 3.00 (SSR9). The effective number of alleles (Ne) varied between 1.10 ± 0.10 (SSR10) and 1.92 ± 0.92 (SSR11), with a mean of 1.38 ± 0.11. Shannon's information index (I) ranged from 0.12 ± 0.06 (SSR18) to 0.59 ± 0.06 (SSR15), with an overall mean of 0.35 ± 0.07. Observed heterozygosity (Ho) was extremely low, ranging from 0.00 for five loci (SSR10, SSR11, SSR13, SSR15, SSR17) to 0.12 ± 0.06 (SSR18), with a grand mean of 0.032 ± 0.01. In contrast, expected heterozygosity (He) ranged from 0.00 (SSR17) to 0.40 ± 0.06 (SSR15), with a mean of 0.218 ± 0.04. Fixation indices (F) were high and positive for most loci, ranging from 0.13 ± 0.15 (SSR18) to 1.00 ± 0.00, with a mean of 0.731 ± 0.09, indicating strong heterozygote deficiency and a predominance of homozygotes (Table 3).

Table 2
List of successfully amplified primers with allele size and PIC

Locus	Code	Motif/SSR	Forward Primer (5'-3')	Reverse Primer (5'-3')	Allele size (bp)	Tm°C	PIC
RHM_MS13	SSR-1	(CTT)5	F: TCGATCTCCACCTACAAACTA	R: ACAAAGGCCTTGAACATCT	235–270	55.7	0.371
RE101	SSR-3	(AG)16	F: GACGGGAATGAGCAAGGTTG	R: CTTCAATTCTGCAAGCCCGA	165–181	50.5	0.373
R460	SSR-9	(GA)13(AG)12	F: CCCTACTTCTTTCATCACATACAA	R: CAACTCCGGTCATTTTTGGT	245–275	59.4	0.431
RA267	SSR-10	(GA)11(AG)10	F: ACGGAGAAGCAGTGAGCATT	R: TGCACAGGAACACCCAATAA	205–225	58.2	0.366
RA346	SSR-11	(TC)9	F: CGGAGCAAGCTCTCTTATCG	R: CCTCTCCTGTGTAGCAAGTCG	290–310	60.1	0.540
RM2D2	SSR-13	(CT)16	F: ATGTGTTTCGTTGCTACTGT	R: ATGGTTGGTTTGTTTTCCTA	165–181	58.7	0.231
RM9D6	SSR-15	(GA)16	F: CTCGCCTCCCAAAAGCAAT	R: CGTGTCCTCACCCCCGTAAC	250–310	55.7	0.471
RA254	SSR-17	(CT)16(CT)10	F: AGTAGCAACACCCACACACT	R: GGAGGGGCTGTAGTCTGATT	150–190	57.3	0.356
RA321	SSR-18	(GA)9	F:AGAGATGGGTTTGTGTAAAGTCTG	R: TATTTCGCTGCCACCCTAAC	175–190	59.9	0.372

Table 3
Assessment of genetic diversity characters for 9 SSR molecular markers on 50 genotypes of *R. mechukae*

Primer code	N	*Na*	*Ne*	*I*	*Ho*	*He*	u*He*	F
SSR- 1	24.50 ± 7.50	2.00 ± 0.00	1.60 ± 0.03	0.56 ± 0.01	0.07 ± 0.04	0.37 ± 0.01	0.38 ± 0.01	0.79 ± 0.12
SSR- 3	24.50 ± 6.50	1.50 ± 0.50	1.16 ± 0.16	0.20 ± 0.20	0.01 ± 0.01	0.12 ± 0.12	0.12 ± 0.12	0.87 ± 0.12
SSR- 9	24.00 ± 8.00	3.00 ± 1.00	1.51 ± 0.45	0.48 ± 0.34	0.07 ± 0.01	0.27 ± 0.21	0.28 ± 0.21	0.38 ± 0.42
SSR- 10	24.50 ± 7.50	1.50 ± 0.50	1.10 ± 0.10	0.15 ± 0.15	0.00 ± 0.00	0.08 ± 0.08	0.08 ± 0.08	1.00 ± 0.08
SSR- 11	25.00 ± 7.00	2.00 ± 0.00	1.92 ± 0.92	0.53 ± 0.53	0.00 ± 0.00	0.32 ± 0.32	0.32 ± 0.32	1.00 ± 0.32
SSR- 13	25.00 ± 7.00	2.00 ± 0.00	1.28 ± 0.17	0.39 ± 0.17	0.00 ± 0.00	0.21 ± 0.10	0.21 ± 0.10	1.00 ± 0.00
SSR- 15	25.00 ± 7.00	2.00 ± 0.00	1.70 ± 0.17	0.59 ± 0.06	0.00 ± 0.00	0.40 ± 0.06	0.41 ± 0.06	1.00 ± 0.00
SSR- 17	24.50 ± 6.50	2.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	NA
SSR- 18	25.00 ± 7.00	2.00 ± 0.00	1.20 ± 0.15	0.12 ± 0.06	0.12 ± 0.06	0.15 ± 0.10	0.16 ± 0.10	0.13 ± 0.15
Grand mean	24.66 ± 1.73	1.94 ± 0.18	1.38 ± 0.11	0.35 ± 0.07	0.032 ± 0.01	0.218 ± 0.04	0.222 ± 0.04	0.731 ± 0.09

N = No. of alleles, *Na* = No. of different alleles, *Ne* = No. of effective alleles, *I* = Information Index, *Ho*= Observed heterozygosity, *He*= Expected heterozygosity, u*He*= Unbiased heterozygosity, PIC= Polymorphic Information Content, F=Fixation Index

3.3 Population structure

STRUCTURE analysis supported K = 2 as the most probable number of genetic clusters among the 50 genotypes. Population 1 comprised 31 genotypes, Population 2 comprised 18 genotypes, and one genotype (CHFRM28) showed admixed ancestry between the two clusters (Table 3) and the STRUCTURE bar plot illustrating membership coefficients is presented in Fig. 8.

The Neighbour-Joining dendrogram based on Nei's genetic distance clearly separated the two main groups (Fig. 9), and PCoA also placed genotypes into two clusters in multivariate space (Fig. 10). AMOVA showed that 63% of the genetic variation occurred among populations, 32% among individuals within populations, and only 4% within individuals. This distribution indicates that most genetic variation is structured at the population level, consistent with strong subdivision and limited gene exchange. Estimates of population differentiation and gene flow further supported this pattern. The overall Fst value was 0.517, indicating very high genetic differentiation among populations, while gene flow (Nm = 0.815) was lower than the threshold generally required to counteract genetic drift. Population 1 harboured six private alleles, whereas Population 2 had three, suggesting that Population 1 may be an important reservoir of unique genetic variants.

Figure 10 Principal coordinate analysis (PCoA) of 50 *R. mechukae* genotypes based on SSR markers

4 Discussion

4.1 Genetic diversity and population structure

This study provides the first molecular evidence of population genetic structure in *R. mechukae*, revealing a combination of moderate species-level diversity and strong population subdivision.

The presence of two well-defined genetic clusters with limited gene flow reflects the narrow distribution and patchy habitat of *R. mechukae* in the Mechuka landscape.

The overall diversity parameters observed here are lower than those reported for several other *Rhododendron* species, including *R. longipedicellatum*, *R. simsii*, *R. pudingense*, and *R. rex* subsp. *rex*, where higher heterozygosity or greater within-population variation has been documented (Cao et al., 2022; Wang et al., 2019; He et al., 2024; Zhang et al., 2020).

4.2 Inbreeding and heterozygote deficiency

The combination of very low observed heterozygosity and moderate expected heterozygosity indicates pronounced deviations from Hardy-Weinberg equilibrium.

The high fixation index (mean F = 0.731) suggests strong inbreeding, likely driven by small effective population sizes, geographic isolation, and limited pollen and seed dispersal (Frankham, 2005).The absence of heterozygotes at several loci may reflect founder effects or strong drift in small, isolated populations (Frankham, 2005). Such homozygosity excess can reduce fitness and adaptive potential, increasing the risk of

local extinction (Reed and Frankham, 2003; Frankham, 2005).Compared with congeners for which moderate differentiation and higher within-population variation have been reported, *R. mechukae* shows much stronger spatial structuring, emphasizing its vulnerability in a restricted and fragmented habitat (He et al., 2024; Cao et al., 2022; Frankham, 2005)

4.3 Conservation implications

The strong genetic differentiation and low gene flow among populations of *R. mechukae* imply that each population should be treated as a distinct management unit.

Population 1, which harbours higher diversity and more private alleles, should be prioritized as a key reservoir of adaptive genetic variation.Restoring or maintaining habitat connectivity could promote gene flow and help counteract drift, but the current levels of inbreeding suggest that some populations may already be experiencing reduced viability (Frankham, 2005). Compared with other *Rhododendron* species that show higher within-population variation and lower Fst, *R. mechukae* appears to be particularly affected by habitat fragmentation and narrow ecological amplitude, underscoring the urgency of targeted conservation measures (Torres-Díaz et al., 2021; Johnson and Molano-Flores, 2023; Cao et al., 2022; He et al., 2024).

5 Conclusions

This study demonstrates that *R. mechukae* faces serious genetic challenges despite retaining moderate overall diversity. Extreme population fragmentation, high inbreeding, and restricted gene flow have produced strong genetic structuring and severe heterozygote deficiency. The existence of two distinct genetic lineages may reflect adaptation to local environmental conditions, but continued erosion of genetic variation threatens the long-term viability of the species. Effective conservation of *R. mechukae* will require integrated strategies that include strict protection of existing habitats, improvement of landscape connectivity, and active genetic management where necessary. The baseline information generated here provides a foundation for designing such interventions and highlights the importance of incorporating genetic data into conservation planning for endemic Himalayan species.

Future work should focus on high-resolution genomic approaches such as single nucleotide polymorphism (SNP) genotyping to identify adaptive variation and detect fine-scale population structure. Studies on reproductive biology, including pollination ecology and seed dispersal, are needed to clarify the mechanisms limiting gene flow. Ecological niche modelling under current and projected climate scenarios could help predict future habitat suitability and guide potential assisted migration. Comparative studies with other co-occurring *Rhododendron* species would also help place the genetic status of *R. mechukae* in a broader phylogeographic and conservation context.

Declarations

Data statement

The data that support the findings of this study are available from the corresponding author upon reasonable request

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Figures



Figure 1

Populations of *Rhododendron mechukae* in natural stands at Mechuka.

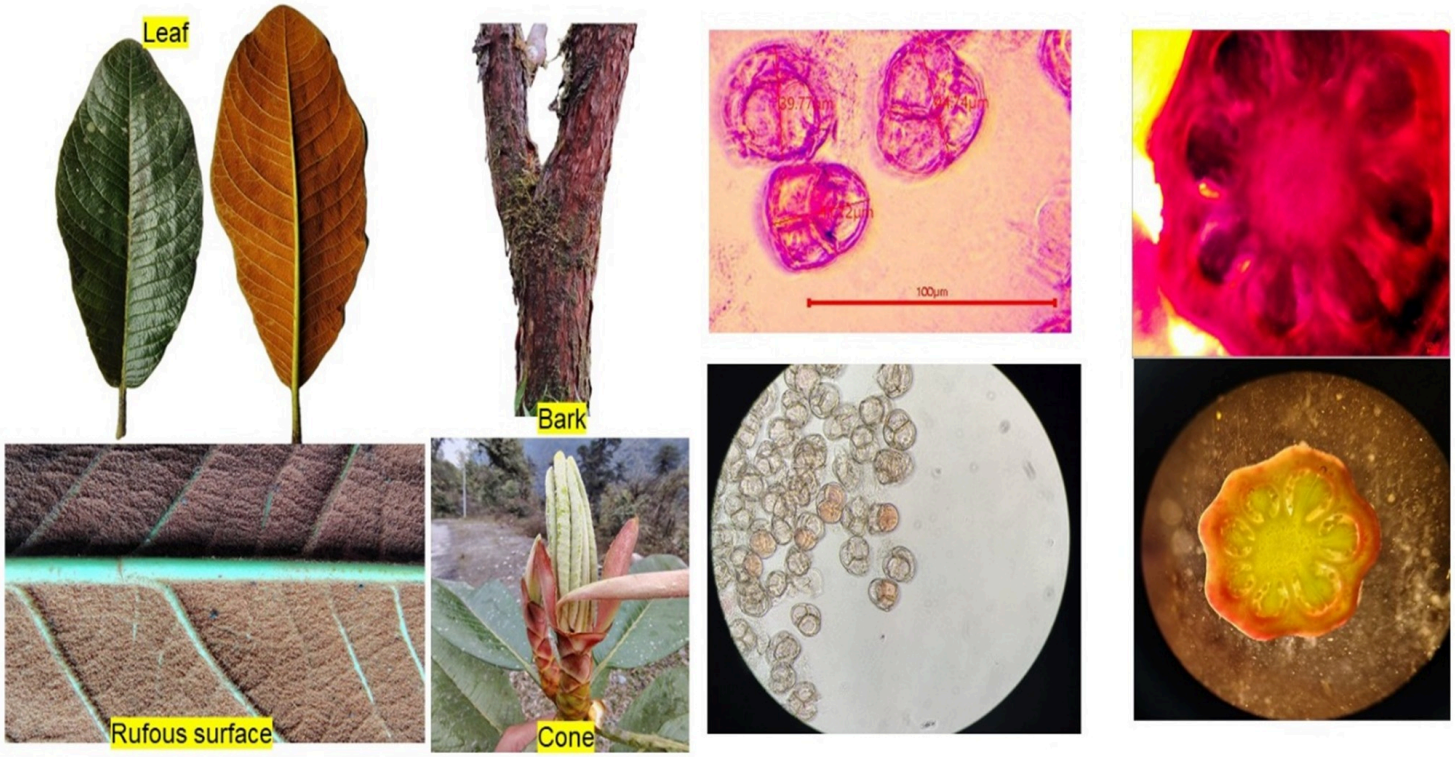


Figure 2

Taxonomic characters of *R. mechukae*: (A) leaf; (B) rufous abaxial leaf surface; (C) bark; (D) cone; (E) pollen under high magnification; (F) pollen grains in microscopic field; (G) transverse section of gynoecium under high magnification; (H) transverse section of gynoecium in microscopic field.

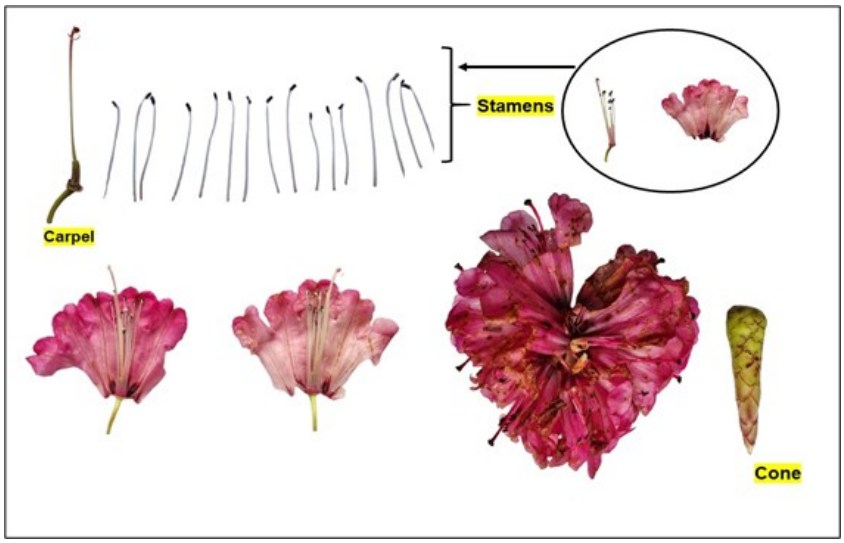


Figure 3

Flowers of *R. mechukae*: (A) carpel and numerous stamens; (B) sepals dissected from main cluster; (C) flower with cone.

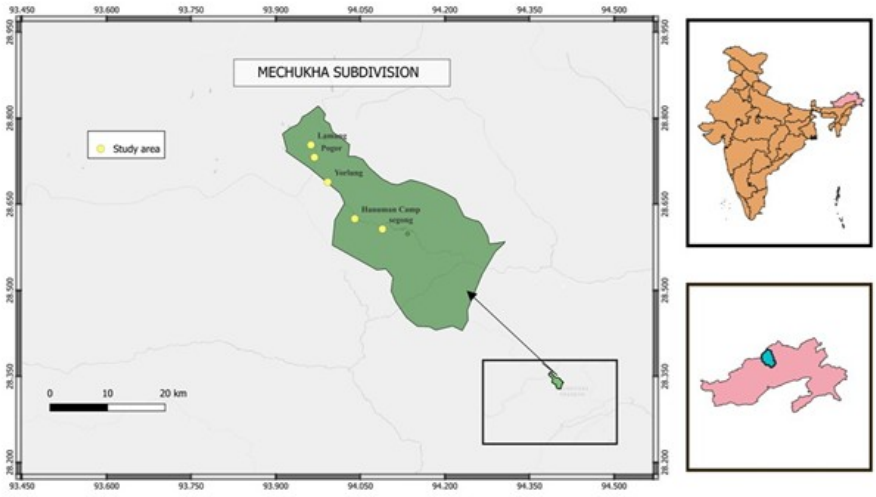


Figure 4

Sampling locations *of R. mechukae* populations in Mechuka region, Arunachal Pradesh, India

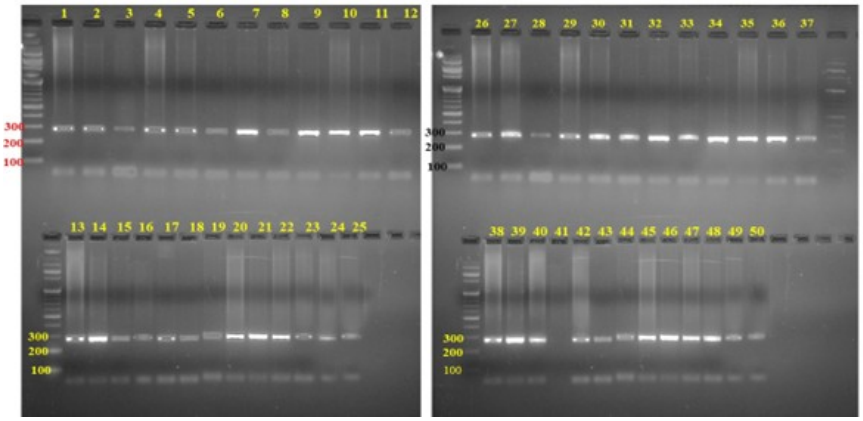


Figure 5

SSR11 amplification profile in *R. mechukae* genotypes on 2% agarose gel.

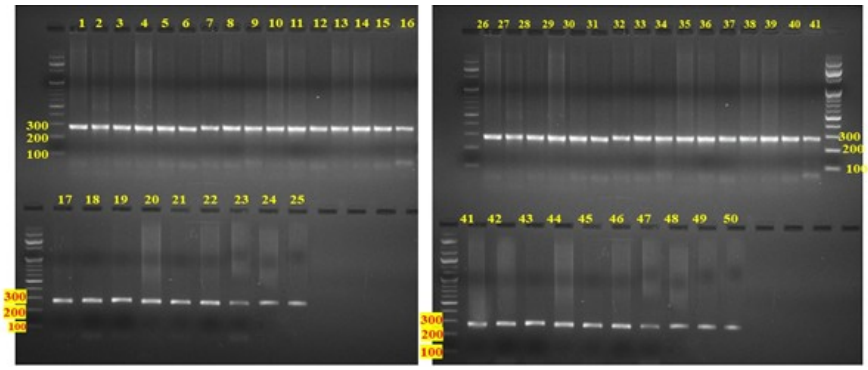


Figure 6

SSR15 amplification profile in *R. mechukae* genotypes on 2% agarose gel.

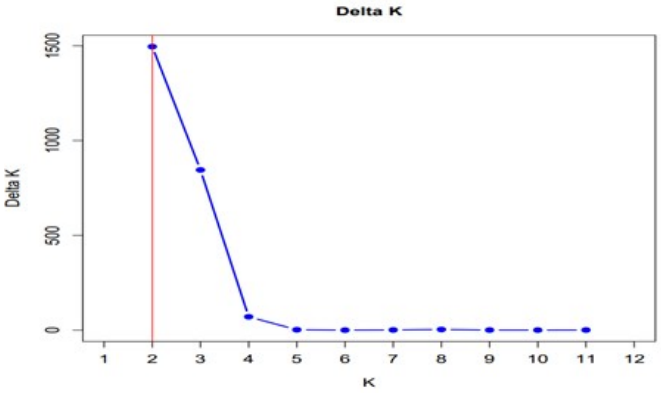


Figure 7

ΔK values used to infer the most likely number of genetic clusters (K) in 50 *R. mechukae* genotypes.

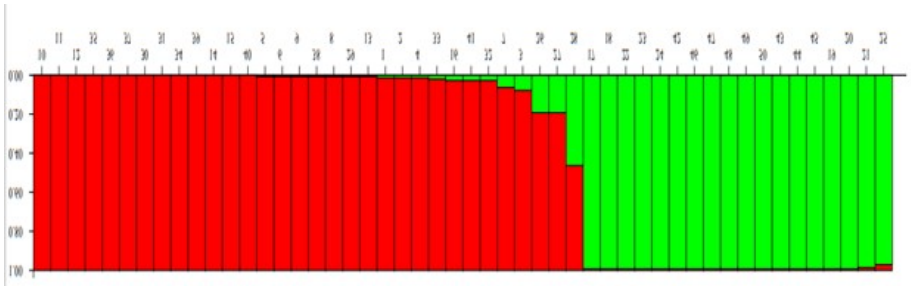


Figure 8

STRUCTURE bar plot showing individual membership coefficients for K = 2 clusters: (A) progenies in original order; (B) progenies sorted by inferred ancestry (Q).

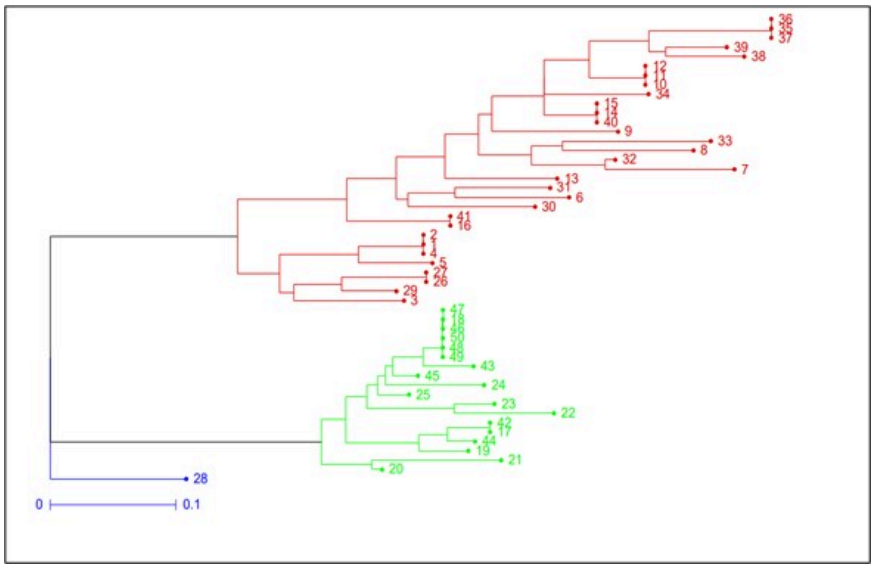


Figure 9

Neighbour-Joining dendrogram of 50 *R. mechukae* genotypes based on SSR data.

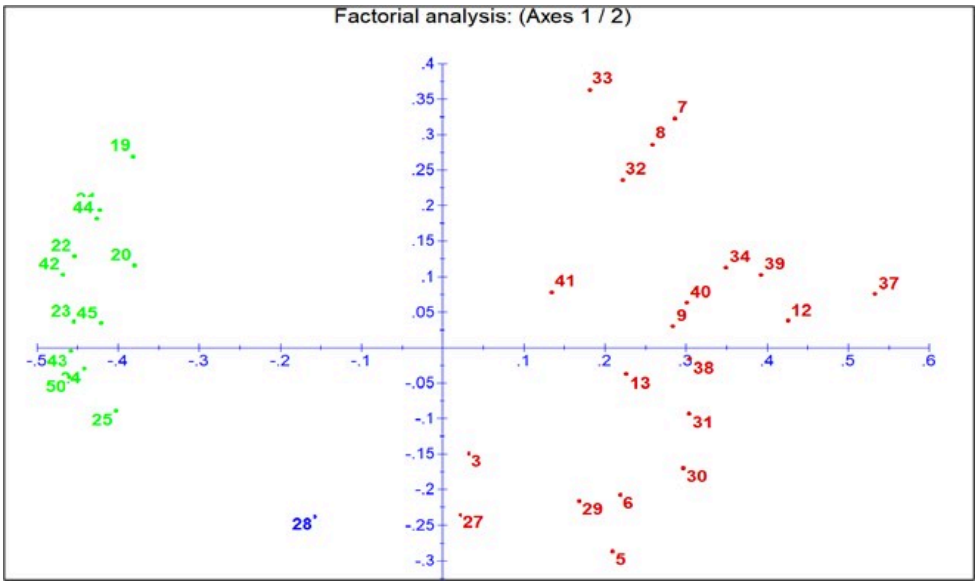


Figure 10

Principal coordinate analysis (PCoA) of 50 *R. mechukae* genotypes based on SSR markers