Original Article

Genetic Diversity of *Pinus Roxburghii* Sarg. Collected from Different Himalayan Regions of India Assessed by Random Amplified Polymorphic DNA Analysis

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ABSTRACT

Present study was aimed at molecular genetic fingerprint profile of 15 genotypes of three populations of *Pinus roxburghii* Sarg. from Himalayan regions of India using random amplified polymorphic DNA (RAPD) based markers. Needles of *Pinus roxburghii* Sarg. were collected from Dharamshala, Himachal Pradesh (HP), Nainital, Uttarakhand (UK) and Darjeeling, West Bengal (WB) regions of India. The samples were subjected to DNA extraction and RAPD analysis using oligonucleotide purification cartridge (OPC) primers. Out of 15 primers tested, nine primers gave scorable bands. Altogether 48 bands were obtained, out of which 43 were found to be polymorphic. Number of amplified fragments with RAPD primers ranged from four to eight with the size of amplicon ranging from 500 to 7,000bp. Investigation of natural diversity at intraspecies level was performed with 15 genotypes. Forty-eight amplification products were scored by RAPD and showed 89.58% polymorphism with a mean intrapopulation genetic diversity (Hpop) of 0.2754. A significant inter- and intrapopulation diversity was observed, with the percentage of polymorphic loci (Pp) ranging from 50.09 to 70.83%, Shannon's information index (I) from 0.3262 to 0.4689 and Nei's gene diversity (h) from 0.2032 to 0.3335 with mean Nei's gene diversity 0.377 and the overall estimate of gene flow being (Nm) 1.3555. Unweighted pair-group method with arithmetic average (UPGMA) analysis based Dendrogram showed single cluster. The variation amongst the samples of the three ecological regions can be attributed to varied climatic conditions and may help in conservation/future cultivation of these species.

Key words: Genetic diversity, Himalayan region, *Pinus roxburghii*, random amplified polymorphic DNA

INTRODUCTION

The genus Pinus is popular as a plant with high medicinal importance. Chir pine or *Pinus roxburghii* is a species commonly found in the Himalayan regions of India and adjoining countries. It finds its distribution in Northwest Frontier province, Punjab and Pakistan occupied Kashmir,[1] Jammu and Kashmir, Himachal Pradesh and Uttarakhand in the Western Himalayan Zones of India, regions of Nepal, Sikkim, West Bengal and parts of Arunachal Pradesh in Eastern Himalayan zones of India, and in Bhutan. In India *Pinus roxburghii* occurs mostly in the tropical regions at the base of the Himalayas dominating the Siwaliks from a height of as low as 450 m in some parts of Himachal Himalayas up to an altitude of 2,300 m in Kumaon region of Uttarakhand. In Sikkim and West Bengal, vegetations of Chir pine occurs along with *Shorea robusta* and other

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tropical plants often restricted to inaccessible lower fringes of river valleys.

In Asian sub-continent, *Pinus roxburghii* Sarg. is also traditionally used as a medicinal plant for the treatment of dermatological and topical diseases, gastrointestinal disorders, and snake bite.[2] Apart from its traditional usage, antidyslipidemic and antioxidant activity of *Pinus roxburghii* Sarg. have also been reported by Puri *et al*.[3] The wide geographical occurrence and in varied climatic conditions has resulted in a wide geophysical adaptability of the plant population. This variation in adaptability of the plants across the whole geographical regions might be a result of variation in their genotype. This variation might also be due to isolation and degradation of population of *Pinus roxburghii* by massive terrace farming and other anthropogenic activities including deforestation along the slopes of mountains. Thus, the gene pools of the natural populations are always dynamic and constantly changing through the interactions of plant characteristics at the site of growth with the forces of evolution such as mutation, gene flow, genetic drift, etc.[4]

The genetic diversity of plants has relevance in conservation. Proper evaluation of genetic diversity is extremely important to prevent depletion of diversity and long-term conservation of these plants.[5] Random amplified polymorphic DNA (RAPD) markers have been used to study the variations in the populations of large number of pine species. Kant *et al*.,[6] used RAPD markers to analyze the genetic variability of *Pinus gerardiana* growing in Himachal Pradesh. DeVerno and Mosseler[7] worked on the genetic variations of red pine (*Pinus resinosa*) using RAPD and restriction fragment length polymorphism (RFLP) analysis.

Presently, a number of studies on the variations of *Pinus roxburghii* Sarg. have also been conducted taking the morphological characteristics such as seedling growth, biomass,[8] and their environmental variations.[9] Genetic similarity studies on natural populations of *Pinus roxburghii* Sarg. of Nepal has also been worked out using nuclear and chloroplast microsatellite loci,[10] but data on genetic diversity of *Pinus roxburghii* from Himalayan regions of India is lacking.

In the present study, genetic variations of *Pinus roxburghii* Sarg. growing along the three geographically different Himalayan regions of India, that is, Himachal Pradesh, Uttarakhand, and West Bengal has been assessed using RAPD analysis. Information obtained from the study might be useful in conserving the populations of these plants in their natural habitat which has continuously suffered degradation due to various anthropogenic activities.

MATERIALS AND METHODS

Chemicals

E.Z.N.A plant DNA midiprep kit for DNA extraction was purchased from Omega Biotek Inc (Georgia). All chemicals and buffers for amplification reactions were procured from Bangalore Genei (Bangalore, India). The oligonucleotide purification cartridge (OPC) primers were purchased from Operon Biotechnologies (Germany).

Plant material and collection site

Needles of *Pinus roxburghii* Sarg were collected from Himalayan tracts of Dharamshala of Himachal Pradesh, Nainital district of Uttarakhand, and Darjeeling district of West Bengal provinces [Figure 1 and Table 1]. Authentication was done on the basis of herbarium of the needles and female cones at the Botany Division, Central Drug Research Institute, India. Authenticated representative of the specimens of Himachal Pradesh, Uttarakhand, and West Bengal were deposited in the Herbarium of Botany Division, Central Drug Research Institute with the Voucher Number of DITRC01, DITRC12, and DITRC15, respectively. Other authenticated specimens were preserved as herbarium.

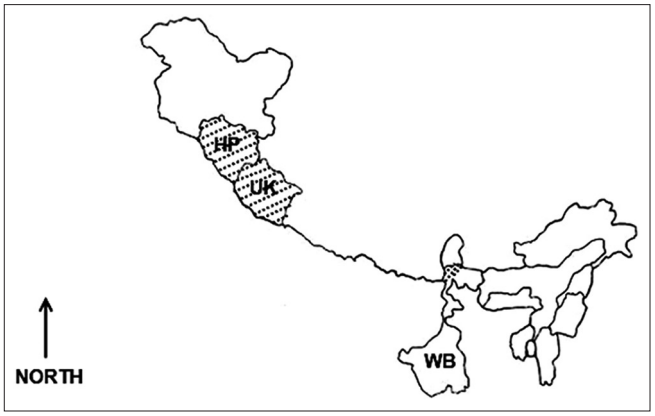

Figure 1: Map of Indian provinces on Himalayan region showing collections sites. WB = West Bengal; UK = Uttarakhand; HP = Himachal Pradesh

Table 1: Details of collection sites of *Pinus roxburghii* Sarg

Genotype code number	Place of collection	Province	Altitude (m)	Latitude	Longitude
HP1, HP2, HP3, HP4, HP5	Palampur	Himachal Pradesh	1,240	76°26′E	32°08′N
UK1, UK2, UK3, UK4, UK5	Nainital	Uttarakhand	1,720	79°26′E	29°22′N
WB1, WB2, WB3, WB4, WB5	Darjeeling	West Bengal	222	88°19′E	26°48′N

HP = Himachal Pradesh, UK = Uttarakhand, WB = West Bengal. Five individual plants (1-5) were collected from each collection site of Himachal Pradesh, Uttarakhand and West Bengal

DNA isolation and RAPD assay

Needles of *Pinus roxburghii* Sarg. were collected, washed with double distilled water, and chopped into fine pieces and stored at −80°C till the time of DNA extraction. A total of 500 mg of chopped frozen needles of the plant were weighed and subjected to DNA extraction using E.Z.N.A. plant DNA midiprep, (Omega Biotek, Georgia) as per the manufacturer's protocol. The DNA obtained was evaluated by electrophoresis in 0.7% agarose gel and examined and photographed in a gel documentation system (Alpha Innotech, USA). The extracted DNA was stored at −80°C till further analysis.

The amplification reaction contained 0.1 μg of DNA, 1.5 units of *Taq* DNA polymerase, 250 μM of dNTP, 25 mM of $MgCl_2$ and 100 ng of OPC primer (Operon Biotechnologies, Germany), and ×1 amplification buffer B (Bangalore Genei, India) in a total volume of 10 μl. A blank was also run without DNA template to check the specificity of the amplification reaction. Amplification reactions were performed in a polymerase chain reaction (PCR) thermal cycler (Eppendorff) with initial denaturation of 4 min at 94°C, followed by 52 cycles (94°C-1 min, 37°C-1 min, and 72°C-1 min) with final elongation of 10 min at 72°C.[11] The amplified products were electrophoresed in a 2% agarose gel and photographed through a gel documentation system (Alpha Innotech, USA).

Data analysis

Banding profiles generated by RAPDs were translated into a data binary matrix based on the presence (1) or absence (0) of the selected band. Only clear and reproducible bands were considered for scoring the data. Smeared and weak bands were excluded. The binary data, thus obtained was used for further analyses of polymorphic loci. The estimation of population genetic structure in an analysis of this data was performed by assuming that the populations are in Hardy-Weinberg equilibrium. Genetic diversity analysis of percentage of polymorphic loci,[12] observed number of alleles, and effective number of alleles.[13] Nei's gene diversity[14] and Shannon index[15] were calculated with POPGENE version 1.31.[16] Gene flow (Nm) was estimated from the Nm = 0.25 × (1- Gst)/Gst along with intra- and interpopulation genetic variations. These statistical significance data were calculated using 1,000 simulated samples. Data were also analyzed using Tree View X to generate Nei's genetic identity and distance coefficient. Dendrogram was prepared for all the individuals using unweighted pair-group method with arithmetic average (UPGMA) clustering for analysis of genetic relationships for all three populations.

RESULTS

RAPD analysis

The genetic diversity among the 15 samples of *Pinus roxburghii* Sarg. collected from three populations of different Himalayan regions of India was evaluated by 15 RAPD primers out of which nine primers yielded 48 amplicons, of which 43 were polymorphic (89.58%). The amplicons ranged between 0.50 and 7 kb with an average of 5.33 amplicons per primer. The primers OPC 9, OPK 16, and OPK 9 exhibited the highest level of polymorphism and the percentage of polymorphic bands was calculated as 90, 88.89, and 77.77%, respectively [Table 2 and Figure 2]. The genetic distance recorded using Nei's unbiased measures (1978) of genetic identity ranged from 0.74 to 0.89 and genetic distance ranged from 0.1122 to 0.2959 [Table 3].

Population structure

The percentage of polymorphic loci (Pp) after analyzing RAPD data ranged from 50.09 to 89.58% [Table 4]. Shannon's diversity values (I) ranged from 0.3262 to 0.5457 and Nei's gene diversity (h) values varied from 0.2038 to 0.3370 with a genetic diversity at inter-population level (Gst) 0.2695 and genetic diversity at intra-population level (Hpop) 0.2754. The estimate gene flow (Nm) was 1.355. The Nei's Unbiased Measures of Genetic Identity and Genetic Distance among the population have also been calculated [Table 4].

Table 2: Sequence of oligonucleotide primers showing scorable bands in random amplified polymorphic DNA analysis and a summary of RAPD markers (kb), banding patterns obtained from RAPD analysis of *Pinus roxburghii* Sarg. DNA

Primer	Nucleotide sequence (5-3′)	Range of molecular weight of bands (kb)	Polymorphic bands	Number of monomorphic bands	Polymorphism (%)
OPC-1	TTCGAGCCAG	0.90-2.40	7	0	100
OPC-3	GGGGGTCTTT	0.70-5.00	5	0	100
OPC-4	CCGCATCTAC	0.75-2.50	5	0	100
OPC-7	GTCCCGACGA	0.50-3.0	7	0	100
OPC-8	TGGACCGGTG	0.75-7.0	6	3	66.66
OPC-9	CTCACCCTCC	0.50-3.0	4	1	75.00
OPC-11	AAAGCTGCGG	3.7-7.0	4	0	100
OPC-12	TGTCATCCCC	0.50-3.0	4	0	100
OPC-15	GACGGATCAG	1.9-2.7	2	1	66.66

OPC = Oligonucleotide purification cartridge. Out of 15 primers tested, nine primers gave scorable bands. RAPD = Random amplified polymorphic DNA

Table 3: Nei's unbiased measures of genetic identity and genetic distance in three wild populations of *Pinus roxburghii* sarg. in Himalayan regions of India

Population ID	HP	UK	WB
HP	-	0.8459	0.7439
UK	0.1673	-	0.8938
WB	0.2959	0.1122	-

Nei's genetic identity (above diagonal) and genetic distance (below diagonal) [Nei, 1978]; HP = Himachal Pradesh, UK = Uttarakhand, WB = West Bengal

Table 4: Genetic diversity and population structure studies of three wild populations of *Pinus roxburghii* Sarg. in Himalayan regions of India

Population	Ss	Na±SD	Ne±SD	h±SD	I±SD	Np	Pp (%)	Hsp	Hpop	Gst	Nm
Himachal Pradesh	5	1.5208±0.5049	1.4773±0.4710	0.2478±0.2419	0.3480±0.3386	25	52.08				
Uttarakhand	5	1.7083±0.4593	1.6429±0.4383	0.3335±0.2222	0.4689±0.3095	34	70.83				
West Bengal	5	1.4902±0.4049	1.4273±0.4211	0.2032±0.1924	0.3262±0.3087	23	50.09				
Overall population	15	1.8958±0.3087	1.6750±0.3041	0.3770±0.1490	0.5457±0.2046	43	89.58	0.3770	0.2754	0.2695	1.3555

Ss = Number of individuals, Na = Observed number of alleles, Ne = Effective number of alleles, h = Nei's (1973) gene diversity, I = Shannon's information index, Np = The number of polymorphic loci is, Pp (%) = The percentage of polymorphic loci, Gst = Diversity among populations, Nm = Gene flow 0.25 (1-Gst)/ Gst, Hpop = Variability within population, Hsp = Total variability, SD = Standard deviation. These statistics were calculated using 1,000 simulated samples

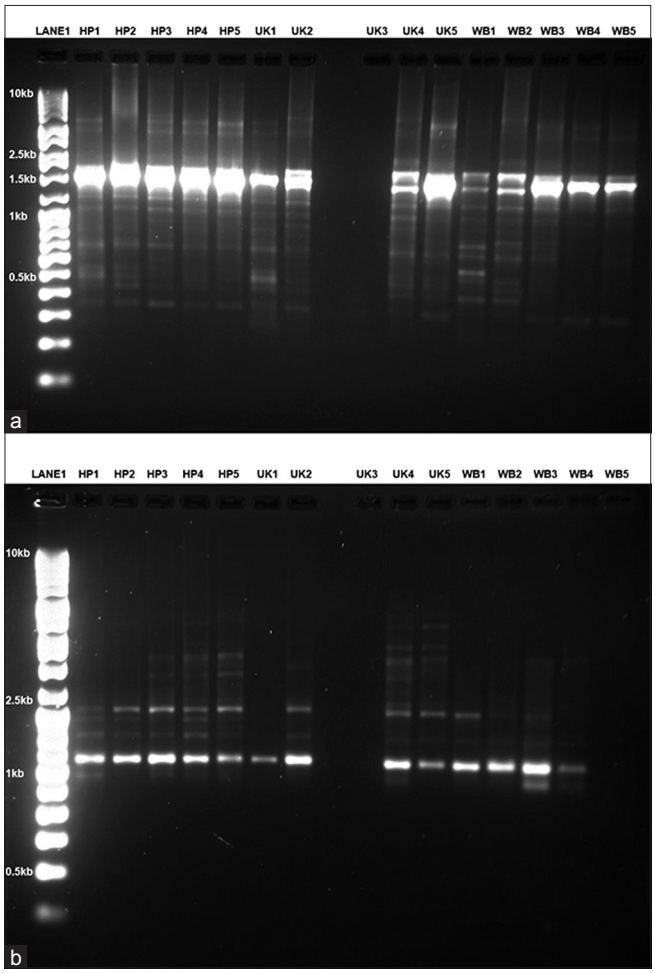

Figure 2: Electrophoresis pattern in agarose gel. (a) Amplification pattern with oligonucleotide purification cartridge (OPC) 8. (b) Amplification pattern with OPC 9. Lane 1 shows banding pattern with DNA ladder. HP1 to HP5 are the samples collected from Himachal Pradesh, UK1 to UK5 shows banding pattern of Uttarakhand samples. WB1 to WB5 shows the banding pattern of the amplification product of samples from West Bengal

Cluster analysis

Based on UPGMA clustering algorithm from RAPD, the populations were grouped into one major cluster [Figure 3]. The cluster consists of populations from West Bengal (WB) and Uttarakhand (UK), while samples of populations from Himachal Pradesh (HP) fall separately.

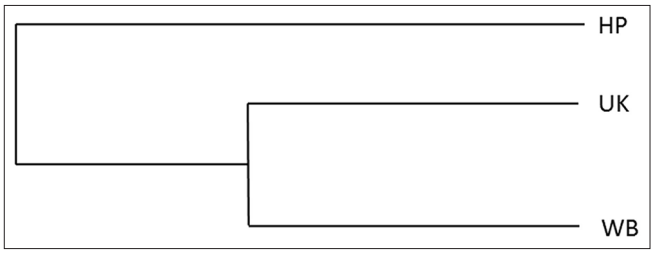

Figure 3: Unweighted pair-group method with arithmetic average dendrograms for the various samples of *Pinus roxburghii*. The dendrogram is based on similarity values obtained from random amplified polymorphic DNA analysis as described in the text

DISCUSSION

An analysis of the extent of diversity within and between populations of a species is vital to carry plantation programs and to provide information for the conservation of the species. Conifers generally express high degree of genetic diversity and are considered to be most variable group among the gymnosperm. They express high degree of variations among population and lesser extent of diversity within the populations.[17] The genetic variation of Pinus may also be due to altitudinal gradients orchestrated with the change in climatic condition with the variation in altitude. In our study, genetic variations were observed amongst the samples studied from different ecological region which varied in altitude. These variations of the population along with altitudinal and environmental gradients would be of great help in conserving the species for future climatic change.[18] Species genetic resource conservation is required as geographically separated populations are expected to have different genetic compositions. Hamrick *et al*.,[19] stated that high genetic variation among the population may be due to woody plants with large geographical ranges with wind-aided seed dispersal.

Pinus roxburghii Sarg. is a gymnosperm grows in the tropical to subtropical regions of Siwalik Himalayas and is used as medicine by the localities of the region. *Pinus roxburghii* Sarg. is found throughout the monsoon belt between 72°E and 95°E longitude and 27°N and 35°N latitude; and is abundant on the hill slopes of both the western and eastern Himalayas between altitudes of 450-2,300 m above sea level.[10] Naturally, it is distributed from Bhutan

(only in drier areas), northern India (Jammu and Kashmir, Punjab, Himachal Pradesh, Uttarakhand, and Sikkim), Nepal, south of Tibet, and Pakistan to Afghanistan.[20] These plants grow in varied climatic zones and presently facing population isolation due to anthropogenic activities. This might be a cause of their genotypic variations which is a matter of importance from conservation point of view and needs to be investigated. The present work was thus initiated to investigate molecular genetic fingerprint profile of 15 genotypes of three populations of *Pinus roxburghii* Sarg. from Himalayan regions of India using RAPD based markers.

In this study, RAPD analysis with nine OPC primers produced (89.58%) polymorphic amplicons. The results suggest that RAPD markers shows high genetic polymorphism in the capacity of producing polymorphic amplicons. Similar results were obtained by Soliman *et al*.,[21] who had worked upon the genetic polymorphism between conifers including *Pinus roxburghii*. Newton *et al*.,[22] also found variations in populations of *Pinus chiapensis* as detected by RAPD and mitochondrial DNA RFLP primers. Their study also justified the importance of genetic variations in both *ex situ* and *in situ* conservations. In the present study, high genetic diversity within population may be attributed to the unique traits of Pinus pollen to float for a long time and distance 10^2-10^3 km[23] in the air being more in downhill direction, and to be carried by storms to very distant localities. The rotation age of *Pinus roxburghii* is 120 years with sexual maturation age from 12 to 14 years for pollen cone and 17-20 years for seed cone production. This helps in maintaining large population numbers while promoting outbreeding and efficient long distance gene flow between populations. Such unique traits are associated with high levels of within population diversity and lower genetic differentiation between populations.[24]

CONCLUSION

This study shows intraspecies genetic variation of *Pinus roxburghii* using RAPD technique. Fifteen genotypes of three populations of *Pinus roxburghii* Sarg. from Himalayan regions of India were assessed. Forty-eight amplification products were scored by RAPD and showed 89.58% polymorphism with a mean intrapopulation genetic diversity (Hpop) of 0.2754. The variation amongst the samples from different ecological regions can be attributed to varied climatic conditions and may be utilized for future cultivation of these species.

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