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Full Length Research Paper

Inter population genetic diversity analysis using ISSR markers in *Pinus roxburghii* (Sarg.) from Indian provenances

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Inter population genetic diversity analysis was undertaken between four *Pinus roxburghii* populations of India using ISSR markers. A total of 72 accessions of genomic DNA from mature needles from four distantly located populations (two native/natural from sub-tropical forests and two provenance trial plantations in plains) were isolated and subjected to ISSR analysis. The percent band polymorphism was 100%, observed number of alleles (na*-2.00), effective number of allele (ne*-1.30), Nei's Gene diversity/Heterozygosity (h*-0.19) and Shanon's information index (I*- 0.31). For these 4 populations upon inter-population genetic diversity analysis, Gene flow (Nm*-1.39) was obtained. Unweighted paired group method with arithmetic mean for similarity index and genetic distances were calculated using Jaccard's coefficient. The highest genetic distance of 0.0904 was obtained between native and provenance trial plantation. Likewise, for similarity index the clusters in the dendrogram were found separated at 0.3 to 0.4 similarity values, indicating robust separations. Such type of diversity analysis will be helpful in raising sustainable plantation forestry programs in plains of India for this species. Diversity analysis data using distinctly located populations of *P. roxburghii* has been done for the first time in Indian provenances.

Key words: Inter simple sequence repeat (ISSR), genetic diversity, inter-population diversity analysis, Nei's gene diversity, Shannon's information index.

INTRODUCTION

Pinus roxburghii Sargent, commonly known as 'chir pine' is the most important pine among the six indigenous pine species of Indian provenances and amongst the dominant species of the sub-tropical pine forests of India (Tiwari, 1994). The species has wide ecological and economic value for its timber and oleoresin (Anonymous, 1968; http://www.frienvis.nic.in/chir pine.htm). They occur in the monsoon belt of outer Himalaya, from North-eastern part of Pakistan to Arunachal Pradesh in India at elevations varying from 450 to 2300 MSL. The forest type of this species comes under the Himalayan subtropical pine forests which are largest in the Indo: Pacific regions (www.worldwildlife.org). These pines are found distributed over a long strip of 3,200 km between Latitudes 26 to 36°N and Longitudes 71 to 93°E. The species is economically very important and is used for variety of purposes viz., timber for house construction, fuel wood extraction, charcoal formation, resin tapping, needles for fuel briquetting cattle bedding, manufacturing organic

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Abbreviations: PA, Palampur; **DJ,** darjeeling; **GG,** gureghar; **RJ,** rajahmundry.

manures and 'Pine oil' (essential aromatic oil) used along with eucalyptus oil for medicinal purpose. Seventy percent of turpentine production in India comes from this species (Ghidiyal et al., 2009).

During phytosociological studies of this species in lesser Himalayan and Hindu Kush ranges it was observed that forests are unstable, degraded and would vanish if not maintained properly (Siddique et al., 2009). In Nepal this species comprises 17% of total forest area and is widely planted by individual farmers, government managed forests and community forests (Jackson, 1994). In that country, *P. roxburghii* has outnumbered all other species in afforestation programs in its natural zone of occurrence (Gauli et al., 2009).

Genetic diversity is a key parameter in evaluating and planning the sustainable management of forest ecosystems. An understanding of the degree of diversity within and between the populations of a species is required to support the continuously rising plantation programs and to provide information for the conservation of the species' genetic resources since geographically separated populations are expected to have different genetic compositions. Within each tree species, amount and distribution of genetic variation is crucial for long-term stability of forest ecosystems (Sharma et al., 2002). Genetic diversity is of prime importance for the species' persistence (Wang and Szmidt, 2001), since the evolutionary adaptive potential of populations depends on genetic variation patterns (Siregar, 2000; Finkeldey and Hattemer, 2007).

Polymerase chain reaction (PCR) amplification of Inter simple sequence repeat (ISSRs) has been widely used for genetic analysis of plants. The method uses a single oligonucleotide primer composed of 4 to 10 tri or di nucleotide repeats and ending with 3'- or 5'- anchor sequence. ISSR targets the highly variable microsetellite regions of the nuclear genome providing a large number of polymorphic fragents (Gupta et al., 1994). It provides more reproducibility than similar method viz., RAPD, AFLP and SSRs (Awasthi et al., 2004).This technique has been used extensively to evaluate genetic diversity both within and between plant populations in angiosperms and gymnosperms including *Pinus* and *Picea* species (Osborn et al., 2005).

Even though the species has been existing in such a large area of Indian provenances, to our knowledge no study has been conducted for measuring inter-population diversity of this species. Purpose of this study was to determine levels of inter population genetic diversity using ISSR markers. The four populations selected were from four distant corners of India and almost 1000 to 2000 Km away from each other (Figure 1). Palampur (PA) in Himachal Pradesh state was a native population with Altitude of 1153 MSL, Longitude 76° 30'E and Latitude 32° 10'N, Darjeeling (DJ) being a natural population in West Bengal state with Altitude of 531 MSL, Longitude 88° 19' E, Latitude 27° 03'N. These populations were atleast 1700 km from each other. Two provenance trial plantations were Gureghar (GG) in Maharashtra state with Altitude of 1193, Longitude 73° 49'E and Latitude 17° 01'N and Rajahmundry (RJ) - Meridumalli provenance trial in Andhra Pradesh state with Altitude of 431 MSL, Longitude 81° 48'E and Latitude 17° 01'N, with almost 1000 km distance between them. Native population was from Palampur (PA) and Natural population was Darjeeling (DJ). Remaining two populations were the systematic provenance trial locations from east and west sides of southern states of India. Gureghar (GG) provenance trial plot was situated in state of Maharashtra 13 Km away from Panchgani a hill station, while Rajahmundry (RJ) provenance trial plot was in state of Andhra Pradesh where more than 1000 trees of different tropical Pines were planted during tropical pine trials conducted during 1970's.

MATERIALS AND METHODS

Sample collections

Fresh chir pine mature needles from individual trees of each population were collected. Individuals within each population were chosen so as to try to cover the whole population transversely. From PA population total 13 accessions were selected. From DJ, 20 accessions and from GG, 20 accessions were selected while from RJ, 19 accessions of needles along with shoots were collected. Around 10 gram of fresh needle tissue from each tree were sampled and labeled properly and subsequently stored in ice/cool packs. Labeled samples were stored at -70°C upon returning to the laboratory, till the extraction and isolation of DNA.

DNA isolation

One gram of the needle tissue was ground to fine powder in liquid nitrogen contained in a porcelain mortar with pestle. Genomic DNA was isolated using Doyle and Doyle (1987) method with some modifications. Total 72 samples of DNA were quantified using UV spectrophotometer (Perkin Elmer, Germany) at 220/280 nm wavelength and also by visual comparison to standards on 0.8% Agarose gels with a 100 bp DNA ladder (GeNei, Merck, India). For each sample, 200 µL of stock was prepared then each sample was diluted to a concentration of 5 µg/µl.

DNA amplification

This standard concentration (10 µg/ 2 µl) of DNA sample was used as template DNA for all the genetic diversity analysis. PCR amplification was performed in a 25 µL reaction volume. The mixture contained 10 µg of template DNA (2 µL PCR stock), 3.5 µL of 10 mM Tris-HCl Buffer ; 3.5 µL $MgCl_2$, 200 µM of each dNTP's (5 µL stock), 0.625U of Taq DNA polymerase (GeNei, Merck India), 8 µL of 0.5 M primer (IDT,USA). To makeup the volume to 25 µL, 2.5 µL of sterile distilled water (Milli Q) was added in each reaction mixture. For each primer, a negative control reaction with sterile water 2.0 µL (Milli Q) was included instead of DNA template.

Total 9 ISSR primers were finally used for the analysis. Out of these five motifs were UBC#9 primers namely UBC 807, 809,

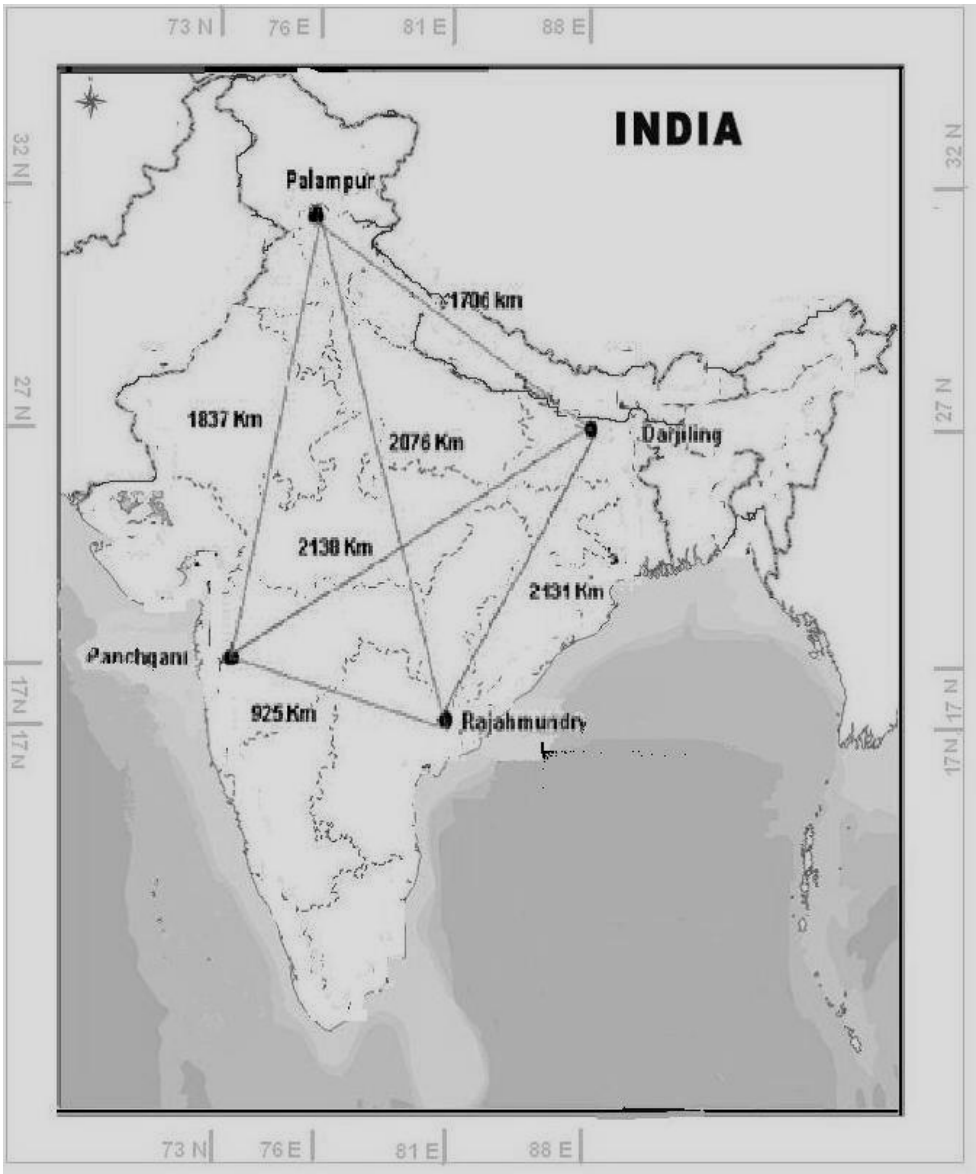


Figure 1. India map with locations of different populations of *Pinus roxburghii* Serg. Showing the interpopulation geographic distance.

810,818 and 834. Remaining 4 motifs were Andrea Wolfe (2001) developed primers namely, HB12, HB15, 17901 and 17899A. Amplification was carried out on a Stratagene Robocycler 9600. The Thermal profile standardized for getting optimum bands was the modification of Mehe's (2007) PCR profile, having one cycle of denaturation step at 95°C for 5 minutes then 42 cycles comprising of (a) Denaturation at 95°C for 1.3 min, (b) 2.0 min at various annealing temperature specific for each primer (the primers and their respective annealing temperatures are given in Table 1 which ranged from 45 to 58°C , and (C) extension at 72°C for 1.0 min with final one cycle at 72°C for 1.0 min and final extension step at 6°C.

PCR products were subjected to electrophoresis on Bio Era submarine gel electrophoresis unit on 1.5% Agarose gel (SRL, India) which was prepared in 0.5 × Tris borate EDTA (TBE) buffer. Ethidium bromide (2 µL / 200 mL gel) was added to the gel for visualizing DNA bands. Thirty wells comb was used for each gel slab. One lane was run for 100 bp ladder byloading 10 µL(Gei Nei, Meark) in that well as a size standard followed by Control lane (without DNA template). The running buffer comprised of 0.5 X TBE. PCR product (25 µL) was loaded in each well after adding 1 µL Bromophinol blue as running dye. The Gel was run at 70 mA Current for 3 to 4 h depending upon the voltage for minimum 10 cm distance (3/4 gel slab). Each digital image was captured and analyzed using SYNGENT G:BOX, UK Gel documentation system Software.

Band scoring and data analysis

In order to accredit the polymorphism to a given ISSR marker, locus absence of an amplified band in at least one individual tree sample

Table 1. Screened primer IDs, their sequences, standardized annealing temperatures and their respective molecular weight range for the inter- population diversity analysis of *Pinus roxburghii*.

S/N	Primer ID	Primer Sequence	Annel*	Mol. Wt. range for primers#			
		5'→3'	Temperature (°C)	PA	DJ	GG	RJ
1	HB 12	CAC CAC CAC GC	47	100-800	100-700	200-1000	100-1300
2	HB 15	GTG GTG GTG GC	49	70-700	100-700	100-800	50-1400
3	17899 A	GTG TGT GTG TGT CA	47	100-400	100-400	100-400	100-1000
4	17901	CAC ACA CAC ACA AG	45	200-700	200-600	100-500	100-500
5	UBC 807	AGA GAG AGA GAG AGA GT	47	100-800	100-700	100-600	100-700
6	UBC 809	AGA GAG AGA GAG AGA GG	51	100-600	100-700	100-600	500-1000
7	UBC 811	GAG AGA GAG AGA GAG AC	47	100-800	100-800	100-1000	100-700
8	UBC 818	CAC ACA CAC ACA CAC AG	51	400-600	100-700	100-600	100-800
9	UBC 834	AGA GAG AGA GAG AGA GYT	49	100-500	100-300	100-800	400-600

*Annealing temperature was standardized for each primer before the analysis. # Range of Band size was calculated from Excel sheet data before converting to Binary data. Total of 307 loci were counted with the 72 accessions with these 9 ISSR primers.

Table 2. Percent Band Polymorphism (PBP) for four populations of *Pinus roxburghii* used during inter population diversity analysis.

Primer No.	Primer ID	PA	DJ	GG	RJ
1	HB 12	100	65	52	79
2	HB 15	85	85	75	82
3	17901	100	60	64	52
4	17899 A	100	80	60	64
5	UBC 807	91	100	78	72
6	UBC 809	100	100	76	80
7	UBC 811	50	100	100	72
8	UBC 818	100	100	100	85
9	UBC 834	100	53	52	86
Average PBP		**86**	**82**	**72**	**74**

was taken as a minimum criterion. Percent Band Polymorphism (PBP) was calculated, which is the No. of Polymorphic Bands × 100 / Total No. of Bands obtained for each primer. Amplified DNA banding patterns generated by ISSR primers in the lanes were scored as (1) for the presence or (0) for the absence. All amplifications were repeated twice and only reproducible bands were scored. Binary format data was analyzed using MVSP (Multiple variances Statistical Package) (www.MVSP.com software) for similarity Index calculation and Jaccard's coefficient values between four populations. Using Popgene 32 software, Percentage of polymorphic loci, percent band polymorphism (PBP), Observed number of alleles (na *), Effective number of allele (ne*), Nei's unbiased gene diversity (h*) which is equivalent to Expected Heterozygosity (H_E*) of a population were obtained. Another genetic diversity parameter Shannon's Information Index (I*) and Gene flow (*Nm) between four populations and genetic distances were also obtained for all these four populations.

RESULTS

Nine of the tested primers which were screened produced clear and distinct patterns across all the 72 accessions and gave reproducible and consistent amplification and were used for band scoring and genetic analysis. During the present interpopulation diversity studies the primers which were used for the analysis are given in the Table 1. They generated total 307 loci (bands) ranging from molecular wt 50 to 1300 bp.

Over all, the level of inter-population polymorphism that is, Percent Band Polymorphism (PBP) detected by these nine ISSR primers ranged between 72 to 86 (Table 2). Maximum band polymorphism for PA was 86% which was a Native population followed by DJ population (82%) which was a natural population. For both provenance trial locations, values of band polymorphism were less, 72% for GG and 74% for RJ. This clearly shows that both Natural and Native populations had high genetic polymorphism compared to provenance trial plantations of GG and RJ.

Inter-population analysis revealed that, observed numbers of alleles (na*) and expected no. of alleles (ne*) were 1.3073 ± 0.3224 and 1.1942 ± 0.1683 respectively.

Table 3. Nei's Genetic distance (below diagonal).

POP ID	RJ	PA	GG	DJ
RJ	****	****	****	****
PA	**0.0623**	****	****	****
GG	0.0855	**0.0904**	****	****
DJ	0.0849	0.0875	0.0845	****

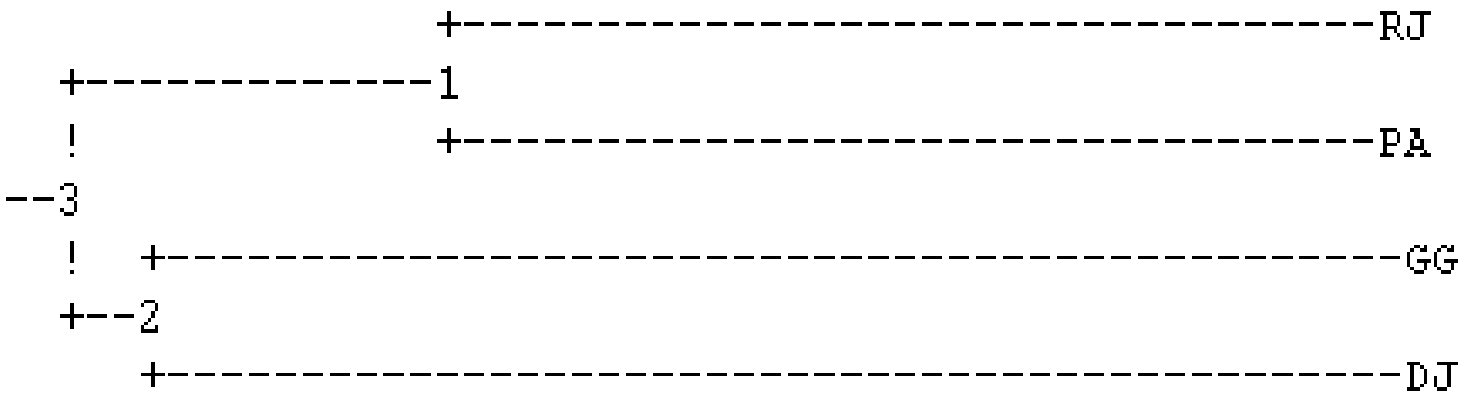


Figure 2. UPGMA Dendrogram (Based on Nei's 1972 and 1978 - Genetic distance-Modified from NEIGHBOR procedure of PHYLIP Version 3) for genetic distance between all the four studied populations after interpopulation genetic diversity analysis.

Nei's Gene Diversity (*h) which is equivalent to the Average Heterozygosity (H_E*) in the above study was less than two (0.1942 ± 0.1683). Another genetic diversity parameter which was obtained was Shanon's Information Index (*I) with value of (0.3141 ± 0.2265). These results also indicate that populations harbor moderate interpopulation diversity amongst themselves, thereby, enhancing their chances to survive under variable climatic conditions. Gene flow value obtained was 1.395 for above four populations. Since the populations are geographically located far away, this gene flow value suggests that provenance plantations must have been raised from the seeds of the native populations. It is presumed that the genetic source for RJ provenance plantations might be from seeds of PA native population.

UPGMA based dendrogram showed genetic distance between the four populations. Four populations get differentiated from each other. Table 3 Shows that the highest Nei's genetic distance between the populations was 0.0904 which was between PA and GG, whereas the lowest was between populations PA and RJ (0.0623). These results indicate that the RJ which is the provenance trail plantation is genetically similar to the PA population which is a native population and also probably the source of the seeds for the RJ was from the PA population. Figure 2 shows the UPGMA based dendrogram of Jaccard's coefficient.

The binary band scoring data of the 9 primers was subjected to MVSP software for deriving the UPGMA dendrogram based on the Jaccards coefficient for similarity indexing and presented in Figure 3. The dendrogram distinctly differentiates the four studied populations of *Pinus roxburghii*. The UPGMA dendrogram shows four main clusters. Each of these clusters contained plants from a single location, revealing high inter-population differentiation, which was also evident from Figure 3. The clusters in the dendrogram were separated at 0.3 to 0.4 similarity values, indicating robust separations. In addition, the dendrogram also highlights the intra-population diversity. The lowest similarity index between two samples was about 0.75 in cluster 2. This indicates that majority of the sampled trees were genetically different from each other, resulting in a high intra-population diversity. The dendrogram revealed that the higher values of genetic similarity coefficient was 0.7 for GG and lowest 0f 0.28 for RJ population. Figures 4 and 5 show the agarose gel electrophoresis patterns of amplification of the four populations with four different primers.

DISCUSSION

The assessment of genetic diversity among tree populations is essential in population genetics in order to obtain information on the phylogeny of coniferous species, the characterization of the present gene pools, the evaluation of human impacts on the natural level of genetic diversity

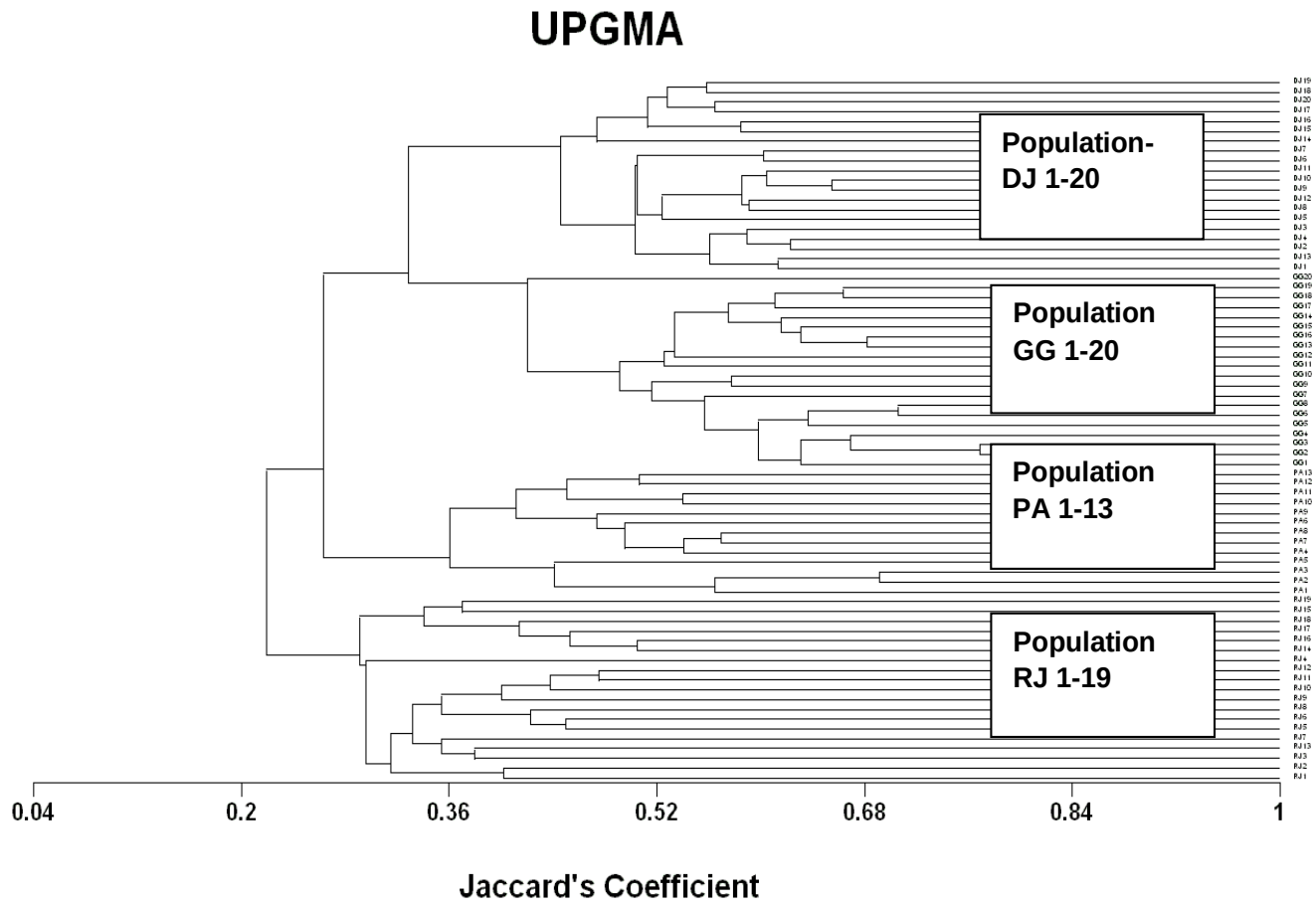
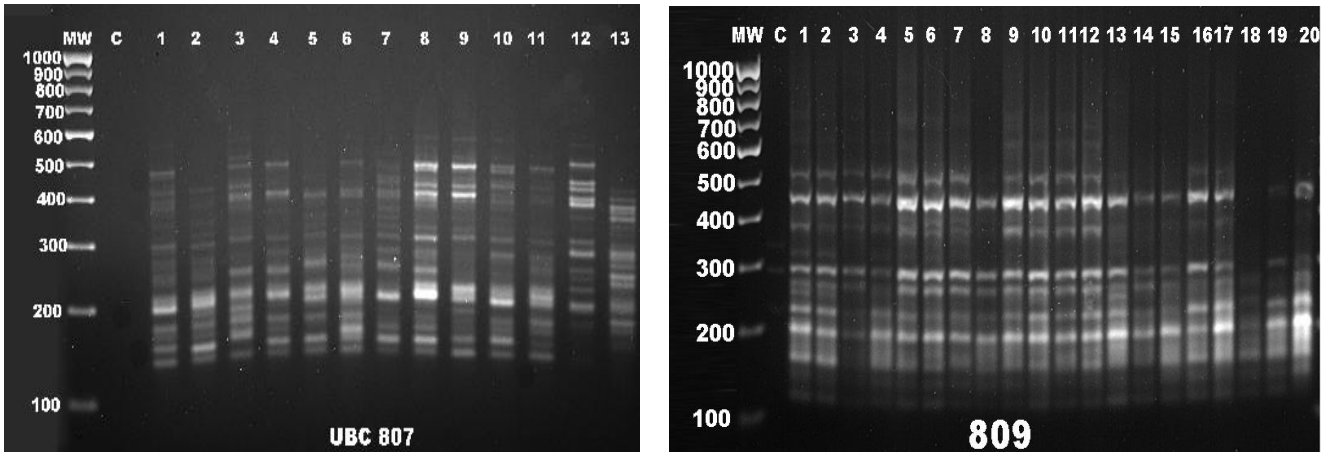
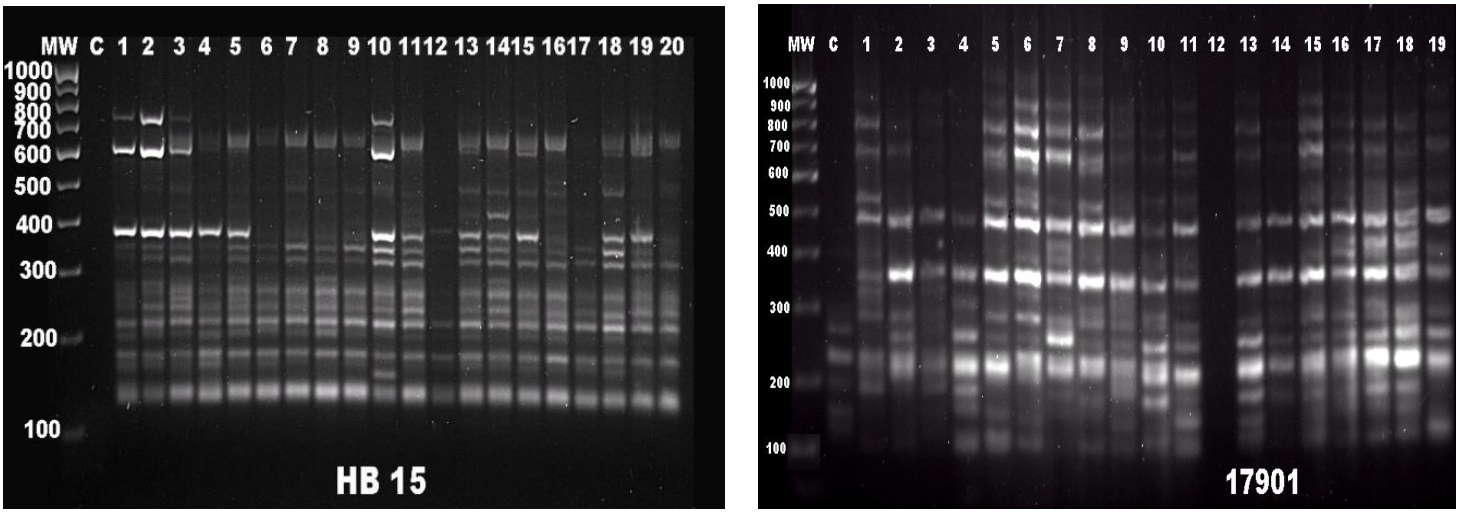


Figure 3. Dendrogram based on Jaccard's coefficient similarity Index for four populations of *P. roxburghii* (Bitmap Image below the dendrogram can be double clicked to see the details of populations in the above dendrogram).



A: PA Population **B: DJ Population**

Figure 4. Representative agarose gel electrophoresis pattern of amplification. A: PA Native Population 13 trees using the primer # UBC 807 at annealing temperature 47°C. Lane MW-100 bp Mol. Wt. Ladder, Lane C-Control, lane 1-13 Trees in order. B: DJ Natural Population with 20 trees using the primer #UBC 809 at annealing temperature 51ºC. Lane MW 1-100 bp ladder Lane C-Control, lane 1-20 trees in order.



A: GG Population **B: RJ Population**

Figure 5. Representative agarose gel electrophoresis pattern of amplification from provenance trial Plantations. A: GG Plantation 20 trees using the primer # HB 15 at annealing temperature 49°C. Lane MW 100 bp Mol. Wt. Ladder, Lane C-Control, Lane 1 - 20 trees in order. B: RJ plantation 19 trees using the primer 17901 at annealing temperature 45°C. Lane MW 1 - 100 bp Mol. Wt. ladder Lane C- Control, Lane 1 - 19 trees in order.

and the utilization with respect to tree breeding. Therefore, multiallelic markers such as ISSR primers, confirming the high genetic variation usually associated with tree species are most desirable tools for such studies (Schubert and Muller-Starck, 2002).

Gymnosperms/conifers are one of the most important forest tree species which includes Larch, *Picea* and *Pinus* spp. There are numerous reports for assessment of genetic diversity from different eco regions in this group, which has been carried out since last 2-3 decades. Genus *Pinus* is most widely distributed, and includes most of the economically valuable species of forest trees (Strauss and Doerkesen, 1990). Genetic diversity analysis in this genus has also been initiated way back in 1996, using RAPD, SSRs, and ISSR markers.

Marker based study was reported in *P. roxburghii* using the allozyme variation in eight natural populations of *P. roxburghii* (Sarg.) from India, (Sharma et al., 2002). Ginwal et al. (2009) in a small scale study compared genetic relationship between 3 pinus species including *P. roxburghi* from India using six ISSR markers and concluded that *P. roxburghii* was more closely related to *P. kesiya* than *P. wallichana*. Gauli et al., (2009) have studied diversity using three SSR primers from Nepal in three natural populations and two plantations of *P. roxburghii.*

In the present study, genetic diversity analysis is undertaken between four *Pinus roxburghii* populations that are distantly located in Indian provenances. Locations selected for the present study were based on literature survey from Indian forester “Special Issue on Tropical Pines in India” (1982). This was based upon the trials conducted in India, in 1970’s for the mass propagation of tropical pines in “plains of India” (Southern states of India). Absence of the tropical pines was observed by foresters in these states and hence it was decided during 1970’s that a mass scale planting of the Pines would need to be undertaken (especially tropical pine species *e.g. P. occupera*, *P. caribaea*, *P. patula* etc) throughout Indian provenances. If they will perform well, tropical pine plantations could be tried for plantation programs throughout the plains in India (Pande, 1982). For the above study the states were selected which must be located on different corners of the country. Finally the selection was finalized for 4 provenances covering 4 corners of the country.

The annealing temperature of primers which were used for final diversity analysis ranged mostly between 45 - 51°C. The unique temperature variation played an important role in hybridization. The above pattern variation was most likely caused by the greater efficiency of primer hybridization and not to nonspecific hybridization. Each primer has variability in its annealing temperature. Modification in annealing temperature was known to have a great impact on the richness and legibility of fingerprints.

All four populations showed PBP between 72 to 86% as shown in results. However, Popgene software analysis has revealed 100% polymorphism as stated earlier. Nei’s genetic diversity value h* for these four populations was 0.19, it is equivalent to average heterozyosity H_E of the other studies. For different *Pinus* species during different diversity analyses this heterozygosity values varied. For

P. taeda H_E value was 0.513 (Elsik et al., 2000). For reginosa Pine (*P. reginosa*) H_E value was 0.375 (Walter and Epperson, 2001), for *P. taeda* H_E value 0.358 (Elsik et al., 2000), For *P. occarpa* H_E value of 0.358 (Diaz et al., 2001), for *P. banksiana* and *P. contorta* H_E value of 0.43 and 0.44 respectively (Dong and Wagner, 1994) and for *P. penderosa* HE value was 0.395 (Osborn et al., 2005) was obtained. Compared to all these pines for *P.roxburghii* H_E value was lower 0.194 during present inter-population genetic diversity study. But this value was higher as compared to red pine *P. resinosa* H_E value was 0.034. For jack pine *P. banksiana* it was 0.046 - 0.153, with an average of 0.100 H_E (Ranger et al., 2008; Vandeligt et al., 2011). Hence, compared to these two pines *P. roxburghii* HE value was higher.

Another genetic diversity parameter the Shannon's information index (Shannon and Weinner, 1963) range for red pine populations was 0.07 to 0.250 with an average of 0. 153 (Zhang et al., 2005; Ranger et al., 2008), hence they concluded that the level of genetic variation was much lower in the red pine population. For *P. roxburghii* populations, Shannon's information index value (I*) was 0.31 which was more than red pine and jack pine populations. Hence it shows higher genetic variation compared to red pine and jack pine. In another genetic analysis study with *P. stroubus* and *P. monticola* populations from Canada using ISSR and RAPD markers (Mehes et al., 2007, 2009) the genetic variation values among these two populations was much lower for *P. monticola* than *P. strobus*. The populations from *P. monticola* were genetically more closely related than populations from *P. strobus* based on ISSR and RAPD analysis. The SSRs (Simple sequence repeats) which are the basis for primer sites of ISSR are known to have a high rate of gaining and loosing repeat units due to DNA slippage (Schlotterer et al., 1991) and may account for the ISSR variation detected. Mutations and chromosomal structure rearrangements have also been suggested source of ISSR variation (Wolfe et al., 1998).

Considering the inter population variation, the populations also diverged significantly, forming robust clusters in the UPGMA dendrogram. These results indicate that the populations harbor high intra as well as inter population diversity and as a result, they might survive harsh and varied climatic conditions in long term. The present study is in agreement with the genetic similarity carried out for the same species from Nepal populations using three SSRs (Gauli et al., 2009). The high inter population genetic diversity indicated low genetic exchange or gene flow among the four distantly located populations. This is expected as the populations have been collected from different provinces of India, which are separated by large geographic distances. To the best of our knowledge, very few studies have been conducted using accessions of four populations in conifers for diversity analysis.

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REFERENCES

Anonymous (1968) Wealth of India. *Pinus.* Council of Scientific and Industrial Research New Delhi, India, 8: 69-82.

Awasthi AK, Nagaraj GM, Naik GV, Kanginakudru S, Thangavelu K, Nagaraju J (2004). Genetic diversity and relationships in Mulberry genus *Morus* as revealed by RAPD and ISSR markers assays. BMC Genet., 5:1-9.

Diaz V, Muniz LM, Ferrer E (2001). Random amplified polymorphic DNA and amplified fragment length polymorphism assessment of *Pinus oocarpa.* Mol. Ecol., 10: 2593–2604

Dong J, Wagner DB (1994). Paternal Inherited Chloroplast Polymorphism in Pine: Estimation of Diversity and Population Subdivision, and Tests of Disequilibrium with a Maternally Inherited Mitochondrial Polymorphism. Genet., 136: 1187-1194.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf material. Phytochem. Bull., 19: 11-15.

Elsik CG, Minihan VT, Hall SE, Scarpa AM, Williams CG (2000). Low-copy microsatellite markers for *Pinus taeda* L. Genome, 43: 550-555.

Finkeldey R, Hattemer HH (2007). Genetics of Tropical Forests. In Finkeldey R, Hattemer HH (eds) Tropical Forest. Genetics, Springer, Berlin, Heidelberg, pp. 315.

Gauli A, Ailing O, Stefenon VM, Finkledley R (2009). Genetic similarity of natural populations and plantations of *P roxburghii* Sarg in Nepal. Ann. For. Sci., 66:703-10.

Ghidiyal SK, Sharma CM, Gairola S (2009). Environmental variation in seed and seedling characteristics of *Pinus roxburghii* Sarg. from Uttarakhand, India. Appl. Ecol. Environ. Res., 7:121-129.

Ginwal HS, Bisht P, Chauhan P, Maurya SS, Barthwal S (2009). Genetic relationship between three Himalayan pines of Indian occurrence. Ind. J. Genet., 69: 81-83.

Gupta M, Chyi YS, Romero-Severson J, Owen S (1994). Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple sequence repeats. Theory Appl. Genet., 89: 998-1006.

http://www.frienvis.nic.in/chir pine.htm, http://www.mapxl.com/path-finder/map_routing.phtml

Jackson JK (1994). Manual of Afforestation in Nepal. Kathmandu Forest Research and Survey Centre. Minist. For. Soil Conserv., 1:1-25.

Mehes MS, Nkongolo KK, Michael P (2009). Assessing genetic diversity and structure of fragmented populations of eastern white pine (*Pinus strobus*) and western white pine (*P. monticola*) for conservation management. J. Plant Ecol., 2: 143-151.

Mehes MS, Nkongolo KK, Michael P (2007). Genetic analysis of *Pinus strobus* and *Pinus monticola* populations from Canada using ISSR and RAPD markers: development of genome-specific SCAR markers. Plant Syst. Evol., 267:47-63.

Nei M (1972). Genetic Distance between populations. Am. Nat., 106: 283-292.

Nei M (1978). Estimation of average heterozygosity and genetic distance from small number of individuals. Genet., 89: 583-590. Osborn B, Lightfoot DR, Hill A (2005). Assessment of genetic polymorphism of *Pinus ponderosa* by Inter Simple Sequence Repeats (ISSR). J. Sci., 1:46-51.

Pande GC (1982). Tropical Pines in India-An Overview. Ind. For., 108:1-28.

Ranger M, Vandeligt K, Michael P, Nkongolo KK, Beckett P (2008). Microsatellite and ISSR analysis of *Pinus banksiana* and *Pinus resinosa* from metal contaminated areas in the greater Sudbury region. In: Joint Annual Meeting of the Canadian Genetic Society and

Canadian Flu Society in Montreal (QC). June 18-22.
Schlotterer C, Amos B, Tautz D (1991). Conservation of polymorphic simple sequence loci in *cetacean* species. Nat., 354:63-65.
Schubert R, Muller-Starck G (2002). Development and application of molecular markers in conifers. In: Jain SM, Brar DS, Ahloowalia BS (eds). Molecular techniques in crop improvement, pp. 139-160.
Sharma K, Degan B, Wuehlisch GV, Singh NB (2002). Allozyme variation in eight natural population of *Pinus roxburghii Sarg.* in India. Silvae Genet., 51:246-253.
Siddique MF, Ahmed M, Wahab M, Khan N, Khan MU, Nazim K, Hussain SS (2009). Phytosociology of *Pinus roxburghii* Sarg. (Chir pine) in lesser Himalayan and Hindu Kush range of Pakistan. Pak. J. Bot., 41: 2357-2369.
Siregar IZ (2000). Genetic aspects of the reproduction system of *Pinus merkusii* Jungh. et de Vriese in Indonesia. Ph.D thesis in Forestry Science at the faculty of Forestry Sciences and Forest Ecology, George-August University of Gottingen. Germany Cuvillier Verlag Gottingen, p. 147.
Strauss SH and Doerksen AH (1990) Restriction fragment analysis of Pine phylogeny. Evolution, 44:1081-1096.
Tiwari DN (1994). A monograph on (Chir pine) *Pinus roxburghii Sarg.* International Book Distributors, Dehradun, India. p. 311.
Vandeligt KK, Nkongolo KK, Mehes M, Beckett P (2011). Genetic analysis of *Pinus banksiana* and *Pinus resinosa* populations from stressed sites contaminated with metals in Northern Ontario (Canada). Chem. Ecol., 27: 369-380.
Walter R, Epperson K (2001). Geographic pattern of genetic variation in *Pinus resinosa:* area of greatest diversity is not the origin of postglacial populations. Mol. Ecol., 10: 103:111.
Wang XR, Szmidt AE (2001). Molecular markers in population genetics of forest trees. Scand. J. For. Res., 16:199-220.
Wolfe AD (2001). Protocols for inter simple sequence repeats (ISSR) approach. www.oak.cats.ohiou.eud/ ballardh/molsyst/issr.doc
Wolfe AD, Xiang Q-Y, Kephart SR (1998). Assessing hybridization in natural populations of Penstemon (*Scrophulariaceae)* using hypervariable inter-simple sequence repeat (ISSR) bands. Mol. Ecol., 7:1107-1125.
www.worldwildlife.org
Zhang ZY, Chen YY, Li DZ (2005). Detection of low genetic variations in a critically endangered Chinese Pine, *Pinus squamata*, using RAPD and ISSR markers. Biochem. Genet., 43: 239-249.