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Full Length Research Paper

Molecular investigation between four Himalayan pines of India through random amplified polymorphic DNA markers

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Studies were undertaken to identify genetic relationship in four different species of *Pinus* L. through randomly amplified polymorphic DNA (RAPD) markers. A total of 500 DNA fragments ranging from 234 to 1353 bp were amplified using 5 selected primers. The number of amplification products produced by a primer ranged from as low as 4 to a maximum of 13, with an average of 8 bands per primer. The cluster analysis revealed one major cluster and one outlier. In the major cluster, *Pinus roxburghii* from Malithi, *Pinus wallichiana* from Malithi, *P. wallichiana* from Taradevi H.P, *Pinus kesiya* from Taradevi H.P, *Pinus gerardiana* from Chamba and *P. roxburghii* from Chamba falls into subcluster 1 and *P. kesiya* from Jubbal (east) and *P. kesiya* from Jubbal (west) falls into subcluster 2. The similarity coefficient value varied from 0.54 to 0.88. The highest similarity coefficient (0.88) was detected between samples collected from *P. wallichiana* (Malithi) and *P. roxburghii* (Malithi) as well as between *P. roxburghii* (Malithi) & *P. wallichiana* (Taradevi, H.P) and the lowest (0.54) was detected between the *P. gerardiana* (Raspa) and *P. kesiya* (South Vietnam). The level of polymorphism in our study was not so much which showed that samples used for the analysis could have close relationship.

Key words: Randomly amplified polymorphic DNA (RAPD), similarity coefficient, polymorphism, *Pinus*, primer.

INTRODUCTION

The genus *Pinus* (Pinaceae) comprises about 120 species and sub species. It has a cosmopolitan distribution mainly found in the Northern hemisphere (Maheshwari and Konar, 1976). In India, seven species of pines *viz*. *Pinus roxburghii*, *Pinus wallichiana*, *Pinus armandi, Pinus gerardiana, Pinus kesiya, Pinus bhutanica* and *Pinus merkusii* are known to occur in the Himalayas. However, only four species *viz P. roxburghii, P. wallichiana, P. gerardiana* and *P. kesiya* contribute significantly to the economy of the country and protect the watersheds (Ginwal et al., 2009). In forest trees, the genetic variation are in an unstable equilibrium and their dynamism has an important bearing on the ecosystem evolution since continuous changes are required to ensure productivity and maintenance of forest ecosystems.

Forest tree species often show large variations, within and between populations, along their natural range of distribution; higher levels of variations are related to mating preferences and selection pressures (Vij et al., 2002). In order to establish a better understanding of the genetic relationship among the main species of Himalayan pines, molecular techniques were employed. Randomly amplified polymorphic DNA (RAPD) amplified

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Table 1. List of RAPD primers sequences used for the study.

S/N	Name of primer base sequence (5'-3')
1	Mosseler-119 ATTGGGCGAT
2	Mosseler-147 GTGCGTCCTC
3	Mosseler-156 GCCTGGTTGC
4	Mosseler-184 CAAACGGCAC
5	Mosseler-186 GTGCGTCGCT

by arbitrary primers, could be very useful genetic markers (Williams et al., 1990). RAPD is a powerful technique, which has been used to differentiate and identify diverse genotypes in many plant and animal systems.

Insights into the relative genetic diversity within populations of Indian pines would be useful for the development of strategies for *ex situ* conservation of plant genetic resources, designing strategies for gene introgression and breeding programs to produce desired recombinant hybrid genotypes (Giri et al., 2012). The present study reports the extent of diversity between different populations of Indian pines.

MATERIALS AND METHOD

The populations of *P. kesiya* from South Vietnam (P.k1), Jubbal east (P.k2), Jubbal west (P.k3), Taradevi H.P (P.k4), *P. roxburghii* from Malithi (P.r1), Chamba (P.r2), *P. wallichiana* from Malithi (P.w1), Taradevi H.P (P.w2), ad *P. gerardiana* from Chamba (P.g1) and Raspa (P.g2) were collected from the respective places and maintained in the greenhouse at the Genetics and Tree Propagation Division, Forest Research Institute, Dehradun, Uttarakhand, India.

Their average mean sea level is as follows: South Vietnam (1650 mts), Jubbal (2600 mts), Taradevi (2397 mts), Malithi (1500 mts) Chamba (6400 mts) and Raspa (300 mts). The fresh leaf materials were collected from five individuals of each population of six month old and DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method of modified Stange et al. (1998). The quality of DNA was checked on 1.0% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml) at 90 V for 1 to 2 h and quantification was done by using biophotometer (Eppendrof, Germany) and all the samples were brought down to a uniform concentration of 5 ng/µl to be used as template DNA for polymerase chain reaction (PCR).

RAPD-PCR

The isolated DNA from five individuals of each population was bulked uniformly and a composite sample representing the population was made. Five decamer primers Mosseler (Mosseler et al., 1992) were used as primers (Table 1). Tests were performed for standardizing PCR conditions and finally, PCR amplification was carried out at 94°C for 3.5 min for initial denaturation, followed by 44 cycles of denaturation at 94°C for 1 min, primer annealing for 1 min at 37°C, extension at 72°C for 2 min and final extension at 72°C for 7 min. Reaction mixture (25 µl) contained 5 ng genomic DNA, 1X reaction buffer (100 mM Tris pH 9.0; 500 mM KCl; 0.1% gelatin, 2.0 mM $MgCl_2$, 200 µM (0.2 mM) of each dNTPs (Bangalore Genei, Bangalore, India), 0.4 µM of each primer, 1 unit of Taq DNA polymerase (Bangalore Genei, Bangalore, India). The amplified products was separated by electrophoresis in 1.5% (w/v) agarose (Genei, Bangalore) gel with 1X TBE buffer, stained with ethidium bromide (0.5 µg/ml) at 90 V for 3.0 to 3.5 h and photographed using Gel Documentation system (UVP, USA). The sizes of the amplification product were estimated using 100 bp to 3.0 kb ladder (Φ × 174 DNA/ BsuRI (Hae III), fermentas. All the reactions were repeated in at least three independent experiments.

Data analysis

All the amplified bands were scored as present or absent for each DNA sample and further, the RAPD reaction results were analyzed using software Gene Profiler. In order to analyze the relatedness among the species, a dendrogram based on unweighted pair group method with arithmetic average (UPGMA) and NEI and Li genetic distance matrix (Nei and Li, 1979) value was obtained.

RESULTS AND DISCUSSION

Out of the ten RAPD primers screened, five gave reproducible and consistent amplification and out of these five decamer primers, three: M-156, M-184 and M-186 (Figure 1a, b and c) showed polymorphism, while the rest two showed monomorphism within the total ten populations of *Pinus*. The details and nucleotide sequence of the RAPD primers is given in Table 1. The number of amplification products produced by a primer range from as low as 4 to a maximum of 13, with an average of 8 bands per primer. The size of amplification products varied from 234 to 1353 bp. The maximum and minimum numbers of bands were produced by the primers M-184 (13) and M-147 (4), respectively. A total of 500 amplified fragments were obtained across ten populations for the 5 selected primers and were used to estimate relationship among them.

The patterns of RAPD fragments produced by the decamer primer M-156 showed 1 distinct extra band in P.g2, 4 extra bands in P.k1. One band was missing in both P.k2 and P.k3. Primer M-184 showed 1 distinct extra band in P.g2. In P.k1, 2 different bands were present. In P.k2 and P.k4, 1 same band was absent. In P.k2, P.k3 ad P.k4, 1 same band was missing. In P.r2, 1 band was absent, while P.w1 and P.w2 had similar banding pattern. Primer M-186 showed 1 different extra band in P.k3 and 1 different band missing in P.k1. In P.r2, 1 band was missing.

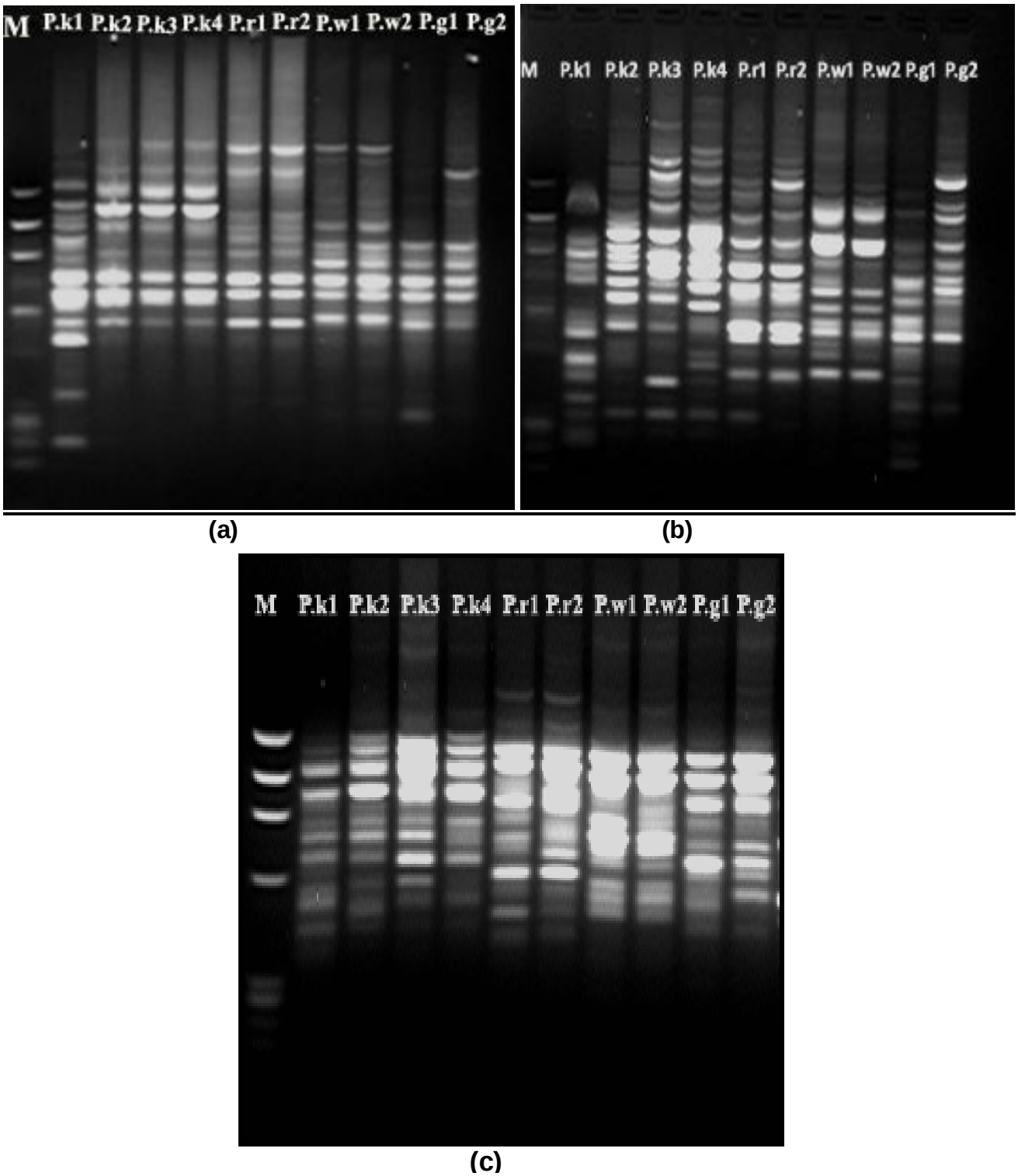

Figure 1. Gel image showing the RAPD amplified products using a) primer M-156, b) primer M-184 and c) primer M-186, in 1.5% agarose gel.

The unweighted pair group method with arithmetic average (UPGMA) based dendrogram (Figure 2) revealed one major cluster and one outlier. The major cluster consists of P.r1, P.w1, P .w2, P.k4, P.g1, P.r2,

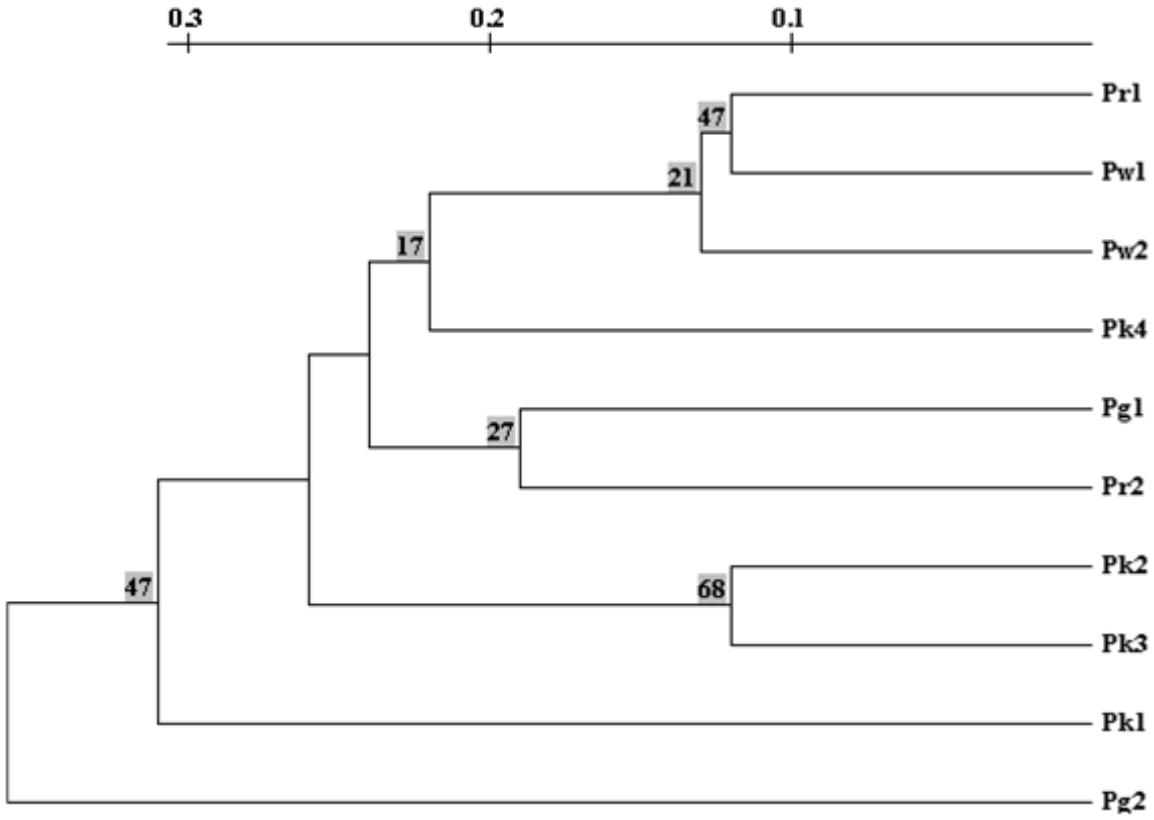

Figure 2. Genetic divergence between four species of *Pinus* based on UPGMA cluster analysis.

Table 2. Similarity index for simple matching coefficient of four different species of *Pinus*.

Parameter	P.g1	P.g2	P.k1	P.k2	P.k3	P.k4	P.r1	P.r2	P.w1	P.w2
P.g1	1									
P.g2	0.72	1								
P.k1	0.7	**0.54**	1							
P.k2	0.67	0.58	0.73	1						
P.k3	0.67	0.59	0.64	0.87	1					
P.k4	0.72	0.64	0.69	0.67	0.81	1				
P.r1	0.67	0.67	0.64	0.7	0.77	0.74	1			
P.r2	0.78	0.62	0.67	0.73	0.72	0.69	0.8	1		
P.w1	0.7	0.69	0.67	0.64	0.72	0.77	**0.88**	0.75	1	
P.w2	0.78	0.69	0.58	0.73	0.8	0.85	**0.88**	0.83	0.83	1

P.k2, P.k3, P.k1 and the outlier P.g2. In the major cluster, P.r1, P.w1, P.w2, P.k4, and P.g1, P.r2 falls in subcluster 1 and P.k2 and P.k3 in subcluster 2. These groupings relate closely to their geographical distribution, as P.r1, P.w1, P.w2 and P.k4 belong to different species but still they are in a single cluster, which is similarly with P.g1 and P.r2. The similarity coefficient value varied from 0.54 to 0.88. The highest similarity coefficient 0.88 was detected between samples collected from *P. wallichiana* (Malithi) and *P. roxburghii* (Malithi) as well as between *P. roxburghii* (Malithi) and *P. wallichiana* (Taradevi, H.P) and the lowest 0.54 was detected between *P. gerardiana* (Raspa) and *P. kesiya* (South Vietnam) (Table 2). P.w1 and P.r1 both belongs to Malithi. The most geographically distant population P.k1 and P.g2 also grouped distantly according to UPGMA.

Several studies have been made where molecular polymorphism was studied by using RAPD in plants. A RAPD analysis was made in eight species of *Pinus* to identify genetic relationship and to evaluate the genetic

variance within population of eight different species (Nkongolo et al., 2002). Similarly polymorphic studies were done among ten populations from five locations of *Pinus massoniana* (Peng et al., 2003). The level of polymorphism in our study was low which showed that samples used for analysis would have close relationship. Although, there are some levels of polymorphism among the given population, an elaborate work is needed by using more number of primers and large number of populations from different regions to draw a suitable conclusion.

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