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Short Communucation

Genetic relationship between three Himalayan pines of Indian occurrence

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The genus *Pinus* is one of the most widely distributed genera of conifers in the Northern Hemisphere. In India seven species of pines *viz*., *Pinus roxburghii, P. wallichiana, P. armandi, P. gerardiana, P. kesiya, P. bhutanica* and *P. 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. The positions of several endemic species as well as relationships among and between Asian pines are still not well settled [1-4].

In order to establish a better understanding about the genetic relationship among the main species of Himalayan pines *viz*., *P. roxburghii, P. wallichiana* and *P. kesiya* and *P. gerardiana* a hyper-variable ISSR marker technique was employed. The published information has shown that ISSR markers have great potential for such studies related to natural population [5].

Two populations each of *P. wallichiana* (from Badrinath, Ranikhet and Kilbury, Nainital), *P. kesiya* (from Assam and Meghalaya) and *P. roxburgii* (from Gangolihat and Thalisian, Uttarakhand) were taken for the study. In each population five trees were included. Genomic DNA was isolated from needles of individual trees using the cetyltrimethylammonium bromide (CTAB) method of Stange *et al.* [6]. The isolated DNA was quantified and all the samples were brought down to a uniform concentration of 50 ng/µl to be used as template DNA for Polymerase Chain Reaction (PCR). The isolated DNA from five trees of each population was bulked uniformly and a composite sample representing the population was made. Tests were performed for standardizing PCR conditions and finally PCR amplification was carried out at 94°C for 5 min for initial denaturation, followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing for 30 sec at temperatures given in Table 1, extension at 72°C for 60 sec, and termination by 5 min at 72°C.

The PCR mixture contained 10 ng DNA template, 200 mmol of each deoxyribonucleotide (dNTP, GibcoBRL, Life Technologies, USA), 0.5 mmol of each of the primer pair, and 1.5 units of *Taq* DNA polymerase (GibcoBRL) in a total volume of 50 ml. Subsequently amplification products were electrophoresed using 1.5% agarose gels (Fig. 1) with 1x TBE buffer at pH 8.0 for 3 hours. Gels were visualized by ethidium bromide staining and photographed under UV light using Gel Documentation system.

All the amplified bands were scored as present or absent for each DNA sample and further the ISSR profiles were analyzed using the software NTSYS. In order to analyze the relatedness among the species, a dendrogram based on Unweighted Pair Group Method with Arithmetic Averages (UPGMA) and NEI and Li genetic distance matrix [7] value using Gene Profiler Software was obtained.

Out of twenty ISSR primers screened, six gave reproducible and consistent amplification and were used for scoring and genetic analysis. The details and nucleotide sequence of the ISSR primers is given in Table 1. The number of amplification products produced ranged from as low as 2 to a maximum of 11, with an average of 6 bands per primer. In case of *P. gerardiana*

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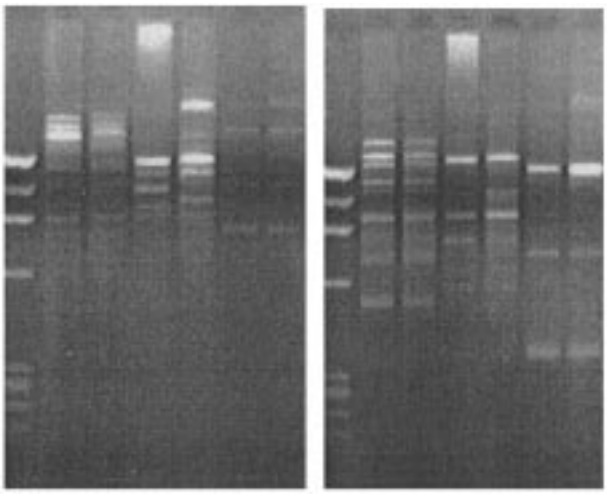

Fig. 1a&b. DNA bands as amplified by primers - ISSR-8 (1a) and ISSR-11 (1b)

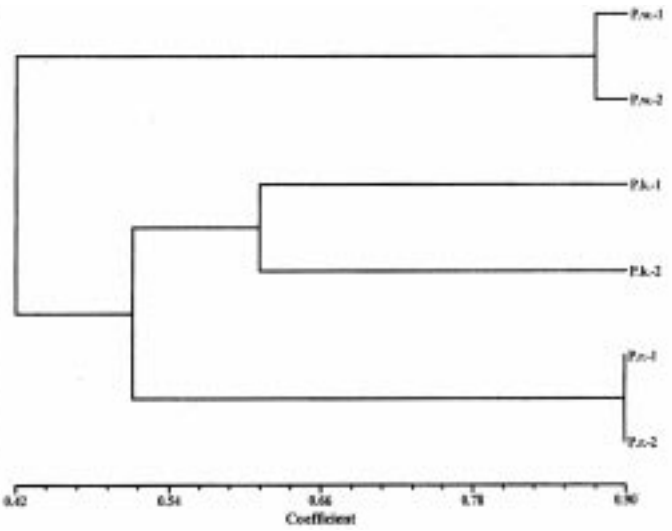

Fig. 2.Dendrogram showing divergence among 3 species of *Pinus*

no amplification with ISSR primers was achieved, hence only three pine species were used for comparison.

The UPGMA based dendrogram (Fig . 2) clearly differentiate *P. roxburghii, P. kesiya* and *P. wallichiana* from each other. The dendrogram revealed that the variation between population of *P. roxburghii, P. wallichiana* and *P. kesiya* is less in comparison to the variation between the species, as indicated by higher value of similarity coefficient 0.900, 0.875 and 0.612 respectively. Across the species the highest value of similarity coefficient was found between *P. roxburghii* and *P. kesiya.* The lowest similarity coefficient value was observed between *P. wallichiana* and *P. kesiya.* Results indicated that *P. roxburghii* is more closely related to *P. kesiya* than *P. wallichiana.* The UPGMA cluster analysis differentiated *P. roxburghii, P. kesiya* and *P. wallichiana* clearly from each other and a highly reproducible genetic relationship was obtained (Fig. 2).

The Himalayan *P. roxburghii* is known to show considerable divergence from all the other hard pines [8]. *P. roxburghii* and *P. wallichiana have* been considered as close relatives of *P. canariensis* of Canary Islands [9, 10]. However recent analysis of chloroplast (cp) DNA restriction site data and ITS sequences have suggested high levels of divergence among them [11, 3]. The present relationship is in support to the findings of Wang *et al.* [8] who established phylogenetic relationships of thirty-two Eurasian pines based on chloroplast *rbcl, math, rpl20-rps18* spacer, and *trnv* intron sequences.

Klaus [10] suggested that *P. roxburghii* originated from Mediterranean ancestors of *P. canariensis* that followed the Tethys coast to the east and reached the Himalayan region in the Upper Cretaceous-Lower Tertiary and led to the rise of *P. roxburghii.* The divergent character of *P. roxburghii* as revealed by our present

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

Name of primer	Base sequence (5'-3')	Annealing temperature (^{o}C)
ISSR-3	GAGAGAGAGAGAGAGAC	43
ISSR-4	GAGAGAGAGAGAGAGAA	44
ISSR-5	GTGTGTGTGTGTGTGTYG	54
ISSR-8	ACACACACACACACACYT	51
ISSR-10	ACACACACACACACACYG	53
ISSR- 11	TGTGTGTGTGTGTGTGRT	54

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

	P.w-1	P.w-2	P.k-1	P.k-2	P.r-1	P.r-2
P.w-1	1					
P.w-2	0.8750	1				
P.k-1	0.2875	0.2875	1			
P.k-2	0.4750	0.5750	0.6125	1		
P.r-1	0.4500	0.4250	0.5125	0.5500	1	
P.r-2	0.4500	0.4250	0.4375	0.5500	0.9000	1

and other [3] molecular evidence suggests an early split of *P. roxburghii* lineage from *P. wallichiana* than *P. kesiya.* Alternatively, *P. roxburghii* might represent an ancestral stock to the Asian hard pines [8].

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