diverse population so that the genetically broad based productive clones are maintained (GURUMURTHI *et al.,* 1994). The approach would help in channelising the productivity efforts of the population towards higher value without significantly affecting the genetic variation.

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Evaluation of Genetic Diversity in the Himalayan Poplar Using RAPD Markers

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Summary

The current investigation reports the evaluation and identification of genetic diversity in *P. ciliata* using the RAPD assay. Eighteen random decamer primers were used to assess variation within twenty five different clones, representing various provenances from the Himalayan region. A total of 159 amplification products were obtained of which 111 were polymorphic while the remaining were monomorphic in nature. Informative primers producing high multiplex ratio were identified from the study. The potential utility of these primers for large scale screening of germplasm and designing conservation strategies in the species has been discussed. The JACCARD's similarity coefficient and the UPGMA clustering method were employed to construct the phylogenetic tree. The dendrogram revealed a high level of variation between the clones which was found to lie in accordance to the diversity observed using morphological data. Two distinct clusters namely C1 and C2 were identified. The cluster C1 comprised of twenty three of the twenty five accessions and was thus designated to be the major cluster while C2 consisted of only two clones and was thus considered to be a minor cluster. The major cluster C1 was comprised of distinct sub-clusters which were found to be in concordance to their geographical distribution. Highest similarity within the major cluster was detected between the clones Katrain and Karain Bihal while the clone Lidder was found to be the most distinct. Bootstrap analysis and principal coordinate (PCO) analysis was performed which supported the pattern of clustering in the dendrogram. The clustering of the other clones in relation to their geographical location has been discussed.

Key words: Populus ciliata, Salicaceae, RAPD, conservation strategies, genetic diversity, intrapopulation variation.

Introduction

Poplars, members of the willow family, are known to comprise nearly 35 species, classified into five sections to which two more sections have been added (FAO, 1979; KHOSLA and KHURANA, 1982). Of the different species, *P. ciliata,* belonging to the section, Ciliata is one of the lesser known species being endemic to the temperate Himalayan belt of the Indian sub-

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continent and China. Improvement programmes in any species by conventional breeding methods primarily involves widening its genetic base by collection of available germplasm or developing intra- and interspecific hybrids which are better performers. The development of such diverse planting material often circumvents the shortcomings encountered when monocultures are established within a genus. The interspecific hybrids are not always successful. Therefore, many programmes are geared towards the development of intraspecific hybrids which will, to a great extent, depend upon the genetic diversity prevalent in the existing population.

In order to identify the level of diversity, marker systems have almost become indispensable. The use of morphological markers for identification of elite genotypes have not been of much success in trees as visual inspection may not really be a reliable method. Isozyme based marker systems also have a limited scope as these are known to be influenced by environmental factors and alter with developmental stages. DNA based markers, on the other hand, are highly reliable and thus are the most preferred choice for germplasm characterization. RAPD (*R*andom *A*mplification of *P*olymorphic *D*NA), is a powerful technique which has been used to discriminate and identify genetically diverse genotypes in many plant and animal systems. RAPD, as the name implies involves the use of primers of arbitrary sequence in a polymerase chain reaction (WILLIAMS *et al.,* 1990). The technique has been successfully used to study genetic diversity in many plant genera such as mahogonies (CHALMERS *et al.,* 1994), eucalyptus (KEIL and GRIFFIN, 1994), mango (SCHNELL *et al.,* 1995), *Populus* spp. (CASTIGLIONE *et al.,* 1993), oil palm (SHAH *et al.,* 1994), Norway spruce (SCHEEPERS *et al.,* 1997), cacao (WHITKUS *et al.,* 1998), amaranthus (CHAN and SUN, 1997), cotton (IQBAL *et al.,* 1997) and brassicas (JAIN *et al.,* 1994). Varietal mis-classification has also been identified in eucalyptus (KEIL and GRIFFIN, 1994) using RAPD. In addition to their use as potential marker systems for PCR based fingerprinting, RAPD markers have also been used to tag genes of agronomic importance (MICHELMORE *et al.,* 1991; HORMAZA *et al.,* 1994) and to develop genetic linkage maps in Norway spruce (BINELLI and BUCCI, 1994) and *Populus* sp. (BRADSHAW *et al.,* 1994).

In the genus *Populus,* studies in terms of the germplasm characterization have been performed employing the different marker systems. Isozyme markers (RAJORA, 1988; RAJORA and ZSUFFA, 1989) have been used for clonal identification in the genus. RFLP analysis of chloroplast (RAJORA and DANICK, 1995a and b) and mitochondrial DNA (BARRETT *et al.,* 1993) have been used to examine inter- and intraspecific variation. Nuclear DNA RFLP was employed to obtain characteristic intra- and interspecific variability in the genus (FAIVRE-RAMPANT *et al.,* 1992). RAPD analysis has been performed for evaluation of genetic diversity and clonal identification in the genus (CASTIGLIONE *et al.,* 1993; CHONG *et al.,* 1994). The objectives of the present study were to assess the level of genetic diversity in the natural population of the Himalayan poplar and to correlate the edaphic variation to molecular markers, with the ultimate aim of utilizing them for characterization of clones for specific planting sites.

Material and Methods

The plant material for this study included different accessions of *P. ciliata* collected from natural populations from different geographical regions of the Himalayas (*Figure 1*). These accessions are currently maintained at the germplasm bank of Dr. Y.S. Parmar University of Horticulture and Forestry, Solan, India.

DNA Extraction

Nuclear DNA was isolated from young emerging leaves using the protocol described by OTTO *et al.,* (Personal communication). Lyophilized leaf material (250 mg) was ground to fine powder and suspended in 600 µl of nuclei buffer (0.3 M sucrose, 10 mM Tris-HCl pH-7.9, 1 mM EDTA). To the nuclear pellet, 750 µl of DNA extraction buffer (10 mM Tris-HCl pH 7.9, 500 mM NaCl, 1% SDS, 0.1% β-mercaptoethanol with freshly added diethyldithiocarbamate) and Proteinase K solution was added. This was incubated at 65 °C for 1 h to 3 h. Equal volume of phenol: chloroform was added and centrifuged briefly. DNA was precipitated by addition of isopropanol. RNAse treatment was given to the DNA solution followed by extraction with phenol: chloroform. To the aqueous phase an equal volume of 5 M ammonium acetate was added and DNA was precipitated with isopropanol. The DNA pellet was washed with 70% ethanol, dried and dissolved in appropriate volume of sterile water.

PCR Amplification

Nuclear DNA was isolated from young emerging leaves as described previously. The amplification reactions were performed in a total volume of 25 µl containing 10 mM Tris HCl pH 9.0, 50 mM KCl, 0.1% Triton-X 100 (Bangalore Genei), 2.5 mM $MgCl_2$, 10 mM each of dATP, dCTP, dGTP and dTTP, 15 ng of random primers (Operon Technologies, USA) and 0.5 units of *Taq* DNA Polymerase, (Bangalore Genei). Twenty nanograms of template DNA was used for each amplification reaction in a Perkin Elmer Thermal Cycler programmed for the following parameters: 94 °C for 60 sec, 36 °C for 30 sec and 72 °C for 60 sec for one cycle and then 35 cycles at 94 °C for 5 sec, 36 °C for 15 sec, and 72 °C for 60 sec. An additional extension at 72 °C for

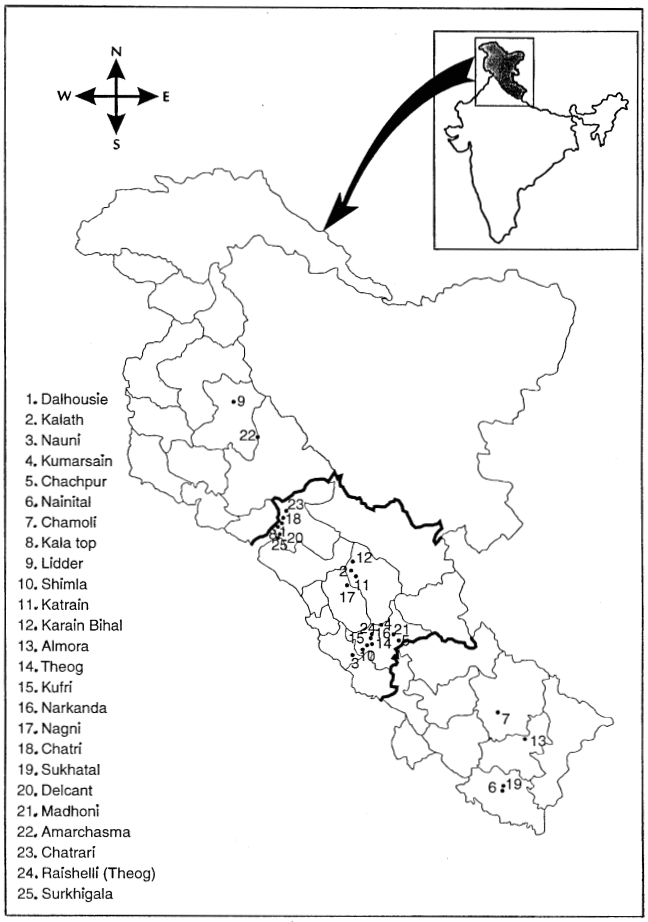

Figure 1. – Map depicting geographical regions where *P. ciliata* accessions were sampled.

7 min was performed to facilitate complete extension. The products were separated on 1.5% agarose gels using 0.5 X TBE buffer. Amplification with each primer was repeated at least twice in order to ensure reproductibility and only those bands which occurred consistently were considered for the analysis.

Scoring of Bands and Data Analysis

Each amplified product was scored across all the accessions of *P. ciliata* for its presence or absence. Co-migrating bands were considered to represent the same locus and thus treated as the same band while scoring. Presence of an amplified product was designated as "1" and absence was marked as "0". Amplified products ranging from 300 bp to 2000 bp were considered for analysis. Intensity of the amplified products was not taken into account while scoring. Polymorphic bands obtained were assigned to different categories based on their degree of occurrence. Fragments detected in a maximum of six of the twenty five genotypes were considered to be rare, while those present across all the accessions except a few (extending upto a maximum of three genotypes) were designated as frequent bands. Pairwise comparison of the accessions based on both unique and shared amplification products were employed to obtain the similarity coefficient. The JACCARD coefficient (JACCARD, 1908) was used since it omits out the negative matches while constructing the similarity matrix. The matrix was then used to derive a preliminary phenetic cluster using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA). The final dendrogram was then constructed using the NTSYS-pc software (ROHLF, 1989). Principal Coordinate Analysis was also done using the same software. Bootstrap analysis was performed using the Winboot software.

Results

Variation and Diversity

The geographical distribution of the different *P. ciliata* clones taken for this study is shown in *figure 1.* It is evident from *figure 1* that the clonal material was collected from a latitudinal range of 29°N to 33°N. The germplasm collection ranged between 75°E to 79.5°E longitudes within an altitudinal range of 1500 m to 2500 m.

In order to assess optimal reaction parameters which contribute effectively to the reproducibility of the RAPD assay, concentrations of the various reaction components yielding consistent banding patterns were standardized. To facilitate analysis of larger number of samples, the cycling conditions were also standardized. Earlier in our laboratory RAPD assays were optimized with the long cycling conditions (JAIN *et al.,* 1994). A comparison of the amplification profiles under the long and short cycling conditions (as in materials and methods) revealed identical banding pattern (Figure not shown). Hence, for this study the short cycling conditions were employed. Independent preparations of the same template DNA when subjected to amplification generated identical banding pattern (Figure not shown).

Eighteen different RAPD primers were used to evaluate the level of genetic diversity amongst the different clones of *P. ciliata.* High levels of genetic diversity was observed amongst

Table 1. – List of primers used for the study alongwith their percent polymorphism.

Primer	Sequence	Number of amplification products	Number of polymorphic bands	Percentage polymorphism
OPA-10	GTGATCGCAG	6	6	100
OPC-19	GTTGCCAGCC	10	9	90
OPE-01	CCCAAGGTCC	12	6	50
OPE-04	AAGACCCCTC	7	5	71.4
OPG-02	GGCACTGAGG	7	6	85.6
OPG-04	AGCGTGTCTC	14	11	78.5
OPG-05	CTGACACGGA	10	8	80
OPG-13	CTCTCCGCCA	6	0	0
OPG-16	AGCGTCCTCC	14	8	57
OPG-17	ACGACCGACA	9	8	88
OPH-03	AGACGTCCAC	13	9	69
OPH-04	GGAAGTCGCC	6	3	50
OPH-05	AGTCGTCCCC	4	4	100
OPA-07	GAAACGGGTG	7	3	42.8
OPK-20	GTGTCGCGAG	4	4	100
OPC-11	AAAGCTGCGG	10	8	80
OPE-06	AAGACCCCTC	10	6	60
OPG-06	GTGCCTAACC	10	7	70

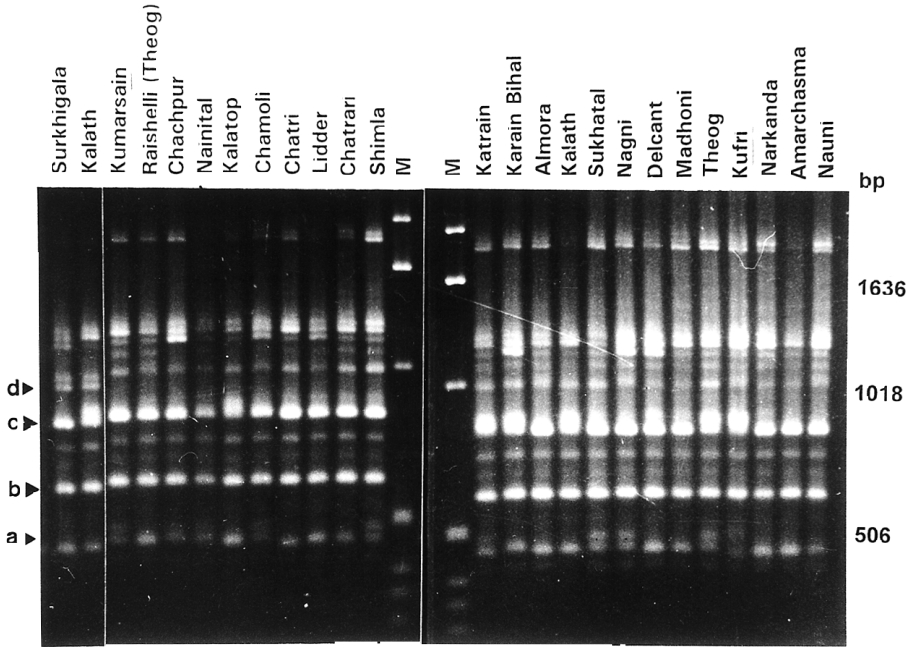

Figure 2. – RAPD profiles generated by the primer OPG-16. Lane M represents the molecular size marker used (1kb ladder, BRL). The sizes of the marker are indicated on the right side. Bands highlighted as a to d are some of the monomorphic and the polymorphic amplification products.

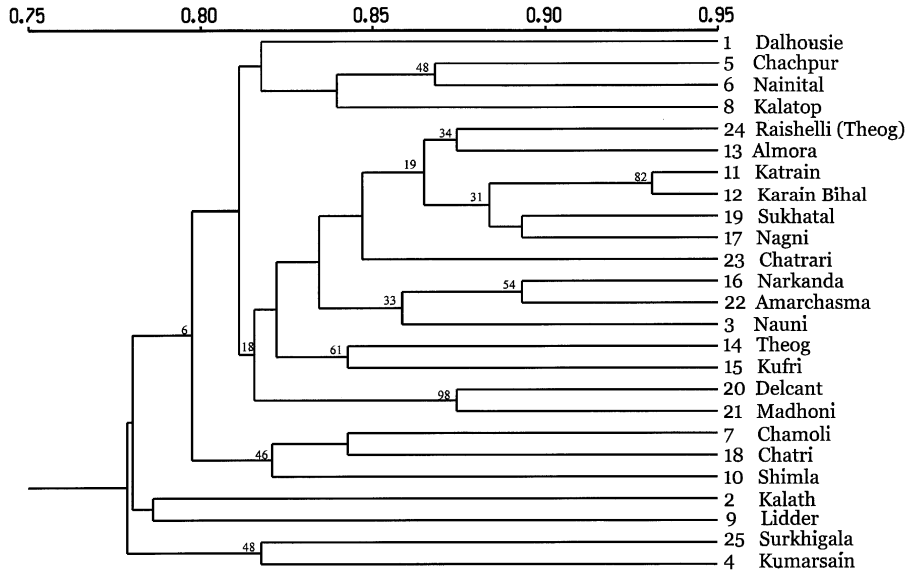

Figure 3. – Associations among the twenty five different *P. ciliata* accessions obtained by employing the UPGMA clustering method.

the 25 genotypes being analysed. Even the percent polymorphism ranged from 0% (OPG-13) to 100% (OPA-10, OPH-05 and OPK-20), the average was 70%. A total of 159 amplification products were obtained of which 111 were polymorphic in nature. *Table 1* gives a list of the primers used for the study alongwith the percent polymorphisms. The number of amplification products obtained per primer varied from as low as 4 to a maximum of 14 with an average of 9.0 bands per primer.

The RAPD amplification profile obtained using the decamer primer OPG-16 is shown in *figure 2.* With this primer as many as fourteen bands were amplified of which eight were polymorphic. Most of the amplified fragments ranged from 500 bp to 1500 bp in size. A number of intense amplification products were obtained with this primer which were uniformly present across all the accessions (represented as b,c). The primer also amplified a number of rare bands. One such rare band (marked d) at ca. 900 bp was found to be present only in the clones Surkhigala and Kalath. Similarly, another rare band (marked a) at ca. 550 bp was present in six of the twenty-five accessions.

Cluster Analysis Based on RAPD Data

Figure 3 represents the preliminary clustering of different genotypes based on the JACCARD's coefficient. Highest value of similarity coefficient of 0.93 was found between the clones Katrain and Karain Bihal thus suggesting their similar nature. This was followed by similarity values of 0.89 between the clones Sukhatal and Nagni which are almost 2° latitudinally apart but are known to share common edaphic conditions. The clones Surkhigala and Kumarsain on the other hand, displayed low levels of similarity index to most of the clones thus

suggesting their distinct nature. Similarly, the clones Kalath and Lidder also exhibited a low level of genetic similarity.

The dendrogram using the Jaccard's coefficient revealed the presence of two distinct clusters, C1 and C2 (*Figure 3*). The former cluster C1 was found to comprise twenty three of the twenty five genotypes and was thus designated to be a major cluster while the latter cluster C2 had only two genotypes namely Surkhigala and Kumarsain. Six distinct sub-clusters were identified within the major cluster C1 and these have been named SC1 to SC6. The clones namely Dalhousie, Chachpur, Nainital and Kalatop represent the sub-cluster SC1. In the sub-cluster SC2, of the seven genotypes present, the highest similarity was found to be between Katrain and Karain Bihal followed by the similarity between Sukhatal and Nagni. The clones namely Narkanda, Amarchasma and Nauni were found to represent the third sub-cluster SC3. The sub-cluster SC4 and SC5 comprised of four (Theog, Kufri, Delcant and Madhoni) and three (Chamoli, Chatri and Shimla) accessions, respectively. Only two genotypes namely Lidder and Kalath were found to represent the sub-cluster SC6. Of the two clones, Lidder was found to be the most distant from other members of the major cluster.

Using the dataset with 159 amplification products similarity matrices were obtained based on SM and DICE coefficients. Dendrograms constructed with these revealed that the major groupings in all dendrograms (including that based on JACCARD coefficient) remained the same (data not shown). Bootstrap analysis was performed in order to provide support for the clustering pattern observed in the dendrograms. Sub-clusters containing clones with high genetic similarity were supported by high bootstrap values.

PCO analysis was carried out using the JACCARD's similarity matrix (*Figure 4*). The groupings were in accordance with those obtained in the dendrogram. Genotypes such as Surkhigala and Kumarsain or Kalath and Lidder which formed distinct sub-clusters in the dendrogram were well separated in the PCO. Clones which showed high levels of genetic similarity, such as Katrain and Karain Bihal, grouped very close in the PCO plot.

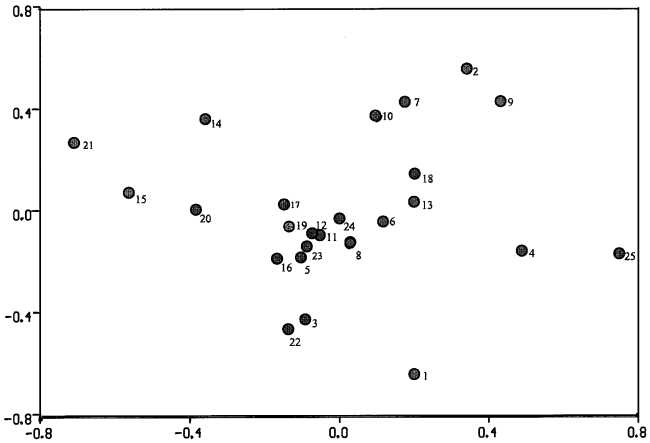

Figure 4. – Principal coordinate (PCO) plot of the twenty five genotypes of *P. ciliata.*

Discussion

This is the first report describing the evaluation of genetic diversity in the Himalayan poplar using RAPD markers. The use of RAPD assay to identify genetic variation is preferred over the conventional morphological and biochemical markers since these are completely devoid of effects of environment and the stage of the experimental material thus making them highly reliable. Although RAPD assays are known to be cost effective, user friendly and easy to perform, the reproducibility of RAPD profiles often pose a major limitation. Standardization of RAPD conditions is thus an essential pre-requisite for obtaining reliable amplification profiles. Our data indicates that the quality of DNA, played a key role in determining the consistency of RAPD profiles. The presence of RNA in the DNA preparation was found to be inhibitory for the RAPD assay. Besides, the concentration of Mg ions was also proved to be important in determining the reproducibility of RAPD profiles.

The present investigation revealed a high level of genetic variation within the population of the Himalayan poplar and led to the establishment of genetic relationships between the various provenances. Establishment of such provenance relationships has been performed in *Salix* (BARKER et al., 1999), Norway spruce (SCHEEPERS *et al.,* 1997) and *Mangifera indica* (SCHNELL *et al.,* 1995). The dendrogram obtained also suggested that no two clones are similar within the given population taken for the study. Considering the level of diversity prevalent, an effort was also made to identify rare bands which could distinguish between clones from various provenances. The current investigation led to the identification of primers producing rare bands. The forty rare bands that were obtained could distinguish 24 of the 25 genotypes used for the study. The informative primers namely OPG-16, OPC-19, OPE-06 either singly or in combination may be of great potential use in the establishment of clonal identity of unknown genotypes, fidelity of tissue cultured raised plantlets and also to monitor designed crosses involving these genotypes. Similar strategy has been adopted to resolve varietal mis-labelling / mis-classification which is of common occurrence in forest tree species. KEIL and GRIFFIN (1994) have used RAPD assay to establish the identity of a number of unknown samples of *Eucalyptus.* The development of a fingerprint key which can differentiate between 96 different individuals of Norway spruce by SCHEEPERS *et al.* (1997) has led to the creation of a fingerprint library which could potentially distinguish between clones as close as half-sibs thus suggesting RAPD as a powerful tool to establish the clonal identity of unknown genotypes. The present investigation has led to the identification of primers such as OPK-20, OPC-19, OPG-02 and OPG-17 which give rise to amplification products with most of them being polymorphic in nature. These primers with a high multiplex ratio may be of potential utility in large scale screening of *P. ciliata* germplasm and elimination of duplicate samples (which at present may be a result of simultaneous efforts towards collection of germplasm by different organizations) thereby conserving diverse germplasm with minimal population size. In the genus *Populus,* earlier reports using RAPD markers have led to the estimation of taxonomic relationships between 32 clones belonging to 10 different Populus species (CASTIGLIONE *et al.,* 1993). The RAPD fingerprints generated by CASTIGLIONE *et al.,* (1993) led to the clear discrimination between the 32 clones of commercial utility which might otherwise have appeared similar on the basis of morphological traits.

Cluster analysis revealed the presence of a wide genetic base in the species. Two major clusters (C1 and C2) were identified with most of the clones falling in the former cluster. The existence of variation in this species has been previously reported based on phenotypic characters by KHOSLA *et al.* (1979, 1980) and KHURANA and KHOSLA, (1982). Their study focussed on identification of variants which could be exploited in selective breeding programmes in the species either for high productivity or for establishment on harsh sites. Natural populations of the species were classified into four distinct eco-

logical groups and the clones within each group were analyzed for wood variation, wood chemical variation and other features which remain unaffected with age (KHURANA and KHOSLA, 1982). The investigation also revealed that genotypes present in the ravines and bihals (such as Katrain, Kalath) had reached an equilibrium to the edaphic sites. Thus clones found in these areas were found to perform better in comparison to the other genotypes. Variations on the basis of morphological traits may be difficult to interpret under certain edaphic conditions as these might have an overriding influence on the genotype of a clone. Thus, the use of molecular systematics for the evaluation of genetic diversity becomes almost essential for breeding programmes. Our next focus would thus be to evaluate the level of diversity within these clones and also to design strategies to conserve the variation observed within these environmental blocks.

The clustering of genotypes in the dendrogram revealed the clones Katrain and Karain Bihal to be most similar. This observation was confirmed by the PCO and bootstrap values. These two clones are known to be geographically located very close to each other and are present on the right and the left bank of the river Beas. It is possible that the two clones represent progenies of the same parent as most of the natural regeneration on the banks of the river systems are a result of seed fall of a seeding mother tree upstream. However, the genotype Kalath which was geographically in close proximity to Katrain and Karain Bihal clustered separately. This can be explained by the fact that the Kalath collection was from a plantation and not from a naturally occurring tree. In addition, Kalath has an outcrossing nature. The distinct nature of the clone Lidder as revealed by cluster analysis and PCO may be due to the distant geographical location of this clone from the remaining genotypes of the major cluster C1. The close clustering of the two genotypes namely Narkanda and Amarchasma was due to the high level conifer associations that are characterised by high altitude, low temprature and short photoperiod. The clone Nauni that clustered with the above mentioned clones is a planted accession and also represents a high level association. The occurrence of the clones Surkhigala and Kumarsain into one cluster may be attributed to the fact that these clones represent low conifer associations at nearly 1500 m above sea level and longer photoperiod. Even though the clones Sukhatal and Nagni were distantly located geographically, yet they revealed a high value of genetic similarity. This may be due to the fact that the individual tree selected from Sukhatal region was from a plantation.

Identification of intrapopulation diversity also forms a very essential pre-requisite for promising conservation strategies. Considering the fact that a number of genetic traits in tree species still need to be elucidated, preservation of the existing genepool becomes highly imperative. Such an evaluation can be used to maintain diverse germplasm by ex-situ methods and also to design crosses giving rise to superior progeny. In the genus *Phyllanthus,* evaluation of intrapopulation diversity has led to the development of conservation strategies (SHAANKER and GANESHAIAH, 1997) thereby facilitating the preservation of the available genepool with least population size. The extent of intrapopulation diversity in most species has been directly correlated to the rate of outbreeding. Evaluation of variation in these plant species thus play a major role in germplasm enhancement as inbreeding depression is a common phenomenon (SHAH *et al.,* 1994). Being an outbreeder, by virtue of the dioceious nature, our future work would aim to identify and evaluate the level of intrapopulation diversity prevalent within the different clones in order to design future conservation and breeding strategies.

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Genetic Characterisation of *Populus tremula* Regions of Origin in Spain Using RAPD Fingerprints

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Abstract

Random Amplified Polymophic DNA (RAPD) was used to identify 89 *Populus tremula* trees sampled from different Spanish origins. The combination of four primers provided a set of four banding patterns that permitted the identification of all genotypes with a high discrimination capacity. Some trees located in the same stand or in near places showed the same fingerprint, probably due to clonal propagation.

Multidimensional scaling analysis of the amplification bands showed low genetic variability between different Spanish origins, but two different groups could be distinguished. The first one formed by individuals from the Northwest (León, Lugo, Orense and Zamora) and Huesca; and the second one by Huesca, Segovia and Navarra. Huesca province (near the Pyrenees, in Northeast Spain) contained both groups, showing high variability between individuals of the same origin. Analysis of the RAPD fingerprints from different origins showed that Huesca had the greatest number of banding patterns in common with others origins, suggesting a genetic flow from the Northeast to the rest of the Iberian Peninsula.

Key words: DNA fingerprinting, PCR, RAPD markers, *Populus* Aspen.

Introduction

Interest in the culture of fast growing trees such as *Populus* species for wood, fuel and fiber plantations has been developed in the last few decades (ZSUFFA *et al.,* 1984). Furthermore, European aspen (*P. tremula*) may also be used for restoration or creation of permanent forests. This species does not form extended forests in Spain but appears in small groups dispersed in other main species forests (HUNTLEY and BIRKS, 1983). It has been suspected by foresters that these groups are not formed by seedlings but by ramets vegetatively propagated by root suckers. Thus, it is important to find a tool for identification at the genetic level, especially if large scale plant production by tissue culture is going to be used for reforestation (BUENO *et al.,* 1993).

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Progress in Molecular Biology has permitted the development of new analytical tools for taxonomic classification and identification. RFLP have been used for clone differentiation in poplars (KEIM *et al.,* 1989; D`OVIDIO *et al.,* 1991; FAIVRE-RAMPANT *et al.,* 1992). Nevertheless RFLP is time consuming and expensive (CASTIGLIONE *et al.,* 1993). Polymerase Chain Reaction (PCR) (SAIKI *et al.,* 1988) can be applied to different techniques, for instance in PCR-RFLPs (AKOPYANZ *et al.,* 1992). This technique has been applied to *Populus nigra* (HEINZ, 1998). SSRs (HEARNE *et al.,* 1992), have been isolated recently in *P. tremuloides* (DAYANANDAN *et al.,* 1998; RAHMAN *et al.* 1999). AFLPs (ZABEAU and VOS, 1992) were used to study the genetic diversity in *P. nigra* (WINFIELD *et al.,* 1998). RAPD screening (WILLIAMS *et al.,* 1990; ERLICH *et al.,* 1991) has some advantages, e.g., only DNA nanograms are needed, it is rapid, less expensive and there is no need DNA sequencing. RAPD markers have been used for DNA fingerprinting and for the study of genetic and taxonomic relationships among poplar clones (CASTIGLIONE *et al.,* 1993; LIN *et al.,* 1994; SIGURDSSON *et al.,* 1997; RANI *et al.,* 1995).

In our study, DNA fingerprints by RAPD markers have been used for identification of trees from European aspen populations of Spain. Sample size has been chosen proportionally to the abundance of European aspen in each region. Collected individuals have been micropropagated for reforestation purposes.

A statistical multidimensional analysis of the banding pattern obtained by PCR amplification of four selected primers was performed. Also, a study of the genetic relationship between populations was based on the presence of the different fingerprints found. Fingerprinting with molecular markers will be applied to conservation of genetic resources and breeding programs.

Materials and Methods

Plant material

Plant material collection in the field was performed in eight Spanish provinces. Two located in the Central Mountain Range: Madrid (M) and Segovia (S); two near the Pyrenees (Northeast): Huesca (H) and Navarra (N); and four in the