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MOLECULAR EVALUATIONS OF THIRTY ONE CLONES OF POPLAR BASED ON RAPD AND SSR MOLECULAR MARKERS

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Poplar is an important tree species valued all over the world for its wood importance. Despite limited knowledge of the levels of genetic diversity and relatedness, their cultivation as a source of plywood is widespread. In order to facilitate reasoned scientific decisions on its management and conservation and prepare for selective breeding programme, genetic analysis of 31 genotypes was performed using RAPD and SSR molecular markers. Twenty six RAPD primers and 14 SSR primers amplified a total of 236 and 85 scoreable bands of which 86.44% and 86.02% were polymorphic. The mean coefficient of gene differentiation (Gst) was 0.388 and 0.341 indicating that 61.2% and 65.9% of the genetic variation resided within the populations. Analysis of molecular variance (AMOVA) indicated that majority of genetic variation (94.6% using RAPD and 89% using SSR) occurred among genotypes, while the variation between the three groups (categorized as tall, medium and small plants height) was 5.4% (using RAPD and 11% (using SSR). The dendrogram obtained from NJ and STRUCTURE analysis revealed splitting of genotypes into four clusters with clear distinction between short, medium and tall height genotypes, indicated that genetic differentiations measure with respect to RAPD and SSR. However, both the markers were equally useful in providing some understanding about the genetic relationship of different genotypes of poplar that are important in the conservation and exploitation of poplar genetic resources.

Key words: **:** genetic diversity, poplar clones, RAPD, SSR, RAPD+SSR

INTRODUCTION

The genus *Populus* L. belongs to the family Salicaceae (order Malpighiales), and includes cottonwoods, poplars and aspens, all of which are sometimes termed poplars. They are widely

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distributed in the northern hemisphere and can be found from subtropical to boreal forests (BRAATNE *et al.,* 1992). Although the actual number of poplar species is low, their distinctive morphology and ecology allows classifying them into 6 sections: *Abaso*, *Turanga*, *Leucoides*, *Aigeiros*, *Tacamahaca* and *Populus* (ECKENWALDER, 1996). There is a large consensus in the literature on the characteristics and species composition of the sections, and the major barriers to hybridization in the genus have been observed to mostly lie between sections (ZSUFFA, 1975). Poplars grow naturally in many parts of the world. They grow fast, are easy to propagate and provide an excellent source of different wood products (ECKENWALDER, 1996). *Populus deltoides* is most popular exotic species in India. Wood from poplars is the backbone of vibrant plywood, board, match and paper industrial units. Poplars provide huge cash returns to individuals and communities engaged in their cultivation and management. (KUMAR and SINGH, 2012). These characteristics have made poplars an excellent candidate for short-rotation plantations. In different parts of the world populiculture has seen a major increase in the past decades. In an effort to provide the forest industry with resources that will increase wood production, poplar breeders have set up improvement programs to select superior and well adapted genotypes. Clones that are resistant to common diseases or drought will allow a gain in wood production without the need to increase the area of land used for plantation or the harvesting of natural forest (DIFAZIO *et al*., 1999). The integration of biotechnology into tree improvement programs can accelerate the introduction of desired traits in existing elite clones. However, the development and use of genetically modified trees is presently restricted due to a lack of information regarding their potential environmental impacts. One of the biggest concerns is the possibility of genetic contamination of natural populations by gene flow from transgenic trees (GTLEONARDI *et al.,* 2010). Pollen is capable of traveling long distances and hybrids between natural and genetically modified trees could be created, with potential short- and long-term impacts.

Genetic markers provide a more reliable method for the identification of clones than morphological characters. The genomic organization of various poplar species in India is being tried through DNA molecular markers to map out distances and to find out the correlation between various species for future breeding work. The genetic diversity amongst the collected half sib families and provenances of *P. ciliata* has also been done through RAPD markers to know about the genetic distances of these collections (FALUSH *et al*., 2007). Genetic diversity analysis with the help of AFLP was also performed on 43 of *P. deltoides* accessions in india. The AFLP successfully discriminated all the genotypes and outliers were distinguished very easily from the rest of the *P. deltiodes* clones. Statistical analysis indicated very high genetic similarity within the *P. deltoides* clones grown in India. This study showed that there should not be random mating of different clones as is being done at present, but they must be first screened so that the situation of monoclonal plantations with very narrow genetic base could be avoided (KHURANA, 2012). Allozymes and RAPDs (random amplified polymorphic DNAs,) were the first to be used for the identification of *Populus* clones (CASTIGLIONE *et al*., 1993; CULOT *et al*., 1995). However, both allozymes and RAPDs have some substantial drawbacks. Allozyme loci are often not variable enough for clone identification, while RAPDs are dominant markers, and a homozygote and a heterozygote with the same allele cannot be distinguished (LIN *et al*., 1994). These problems can be avoided by the use of more reliable and variable markers such as microsatellites. These markers have proven to be very successful for clone identification in poplar (SUVANTO and LATVA-KARJANMAA, 2005). Microsatellites (also known as SSR, short sequence repeats) are 1 to 6 bp-long repeat sequences of DNA. They are supposedly selectively neutral, very variable and are

randomly distributed throughout the genome. Aside from clone identification, they have proven to be useful for a variety of population-based analyses and for parentage analyses (KHASA *et al*., 2003). A broad *Populus* species concept produces more readily interpretable distribution, ecological, and evolutionary patterns than narrow one. This information may be helpful in establishing the nature of population genetics for a species. Six populations and 165 clones were examined using 30 SSR markers. However, *F* statistics indicated that 97% of genetic variation is within population and average Nei's genetic distance was low (D = 0.013) (GTLEONARDI *et al*., 2010). The clones developed from open pollinated seeds from natural population of *P. deltoids* by Dr. N.B. Singh, FRI, Dehradune and Dr. D.K.. Khurana, Dr. Y. S. Parmar University of Horticulture and Forestry, have been used to study the genomic relationship among the populations of poplar clones in Nagangi Nursery, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India, with the aim to provide insight to facilitate conservation management of the populations.

MATERIALS AND METHODS

Thirty one genotypes based on height (ten tall, ten medium and eleven small) of poplar clones were collected from Naganji nursery farm of the Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) India, (Table 1). Although these plants showed distinctive taxonomic traits of the different poplar species, they were chosen for their great variability in terms of morphological traits such as height, young and mature leaves, bark colour etc.

Table 1. Details of the one year old poplar clones based on plants height

SN	Poplar Clones Tall (Height Range: 6.46-6.18 metre)	Poplar Clones Medium (Height Range: 4.92-4.34 metre)	Poplar Clones Small (Height Range:2.87-2.09 metre)
1	PAU-I	FRI-AM -16	FRIPD-OP-32
2	H-8	FRI-AM -53	FRIPD-OP-37
3	H-14	FRI-AM -40	FRIPD-OP-10
4	FRI-AM-98	FRI-AM -70	FRIPD-OP-34
5	FRI-AM -21	L-200	FRIPD-OP-08
6	FRI-AM -47	G-3	FRIPD-OP-33
7	FRI-AM -33	5503	H-11
8	FRI-AM -50	200/86	H-18
9	FRI-AM -46	FRIPD-OP-09	G-48
10	FRI-AM -59	FRIPD-OP-30	HYB-2
11	-	-	63-N

The young leaf samples were collected during the period of March to October in sampling bags under aseptic conditions. The leaves were stored at -20°C for DNA extraction. Total genomic DNA was extracted from the frozen leaves (2 g) by the CTAB method (SAGHAI-MAROOF *et al*., 1984) with minor modifications, which included the use of 200 mg of polyvinyl pyrollidone per sample. The extracted DNA was then treated with 20 µl of 10 mg/ml of RNase and incubated at 37°C for 60 min. After incubation with RNase, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently by inverting the microcentrifuge tube followed by

centrifugation at 10,000 rpm for 5 min at room temperature. The supernatant was pipetted out into a fresh tube. The sample was then extracted twice with equal volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated by adding 0.6 volumes of isopropanol and 2.0 M NaCl. To the above, 20 µl of sodium acetate and 1 volume of 80% ethanol were added, incubated for 30 min and centrifuged at 5,000 rpm for 3 min to pelleted the DNA. The pellet was then washed with 70% ethanol twice, air-dried and finally suspended in 40-50 µl of TE buffer. The yield of the extracted DNA and purity was checked by running the sample on 0.8% agarose gel along with standard (non restriction enzyme digested) lamda DNA marker (Biogene, USA). The extracted genomic DNA was tested for purity index (A_{260}/A_{280} absorbance ratio) on Nano drop spectrophotometer. A value of 1.8 of extracted DNA samples indicate high purity, whereas the value <1.8 or >1.8 denotes the contamination of proteins and RNA respectively (SAMBROOK *et al*., 1989).

PCR amplification was carried out in a 25 µl total reaction volume containing 30 ng genomic DNA, 1.5 mM $MgCl_2$, 1 µM of primers and 1 unit of Taq DNA polymerase (Pharmacia) (BARCACCIA *et al*., 1997). Amplification was performed in a 9700 Thermal Cycler (PerkinElmer) under the following temperature profile: initial denaturation for 5 min at 95°C was followed by 3 cycles of 2 min at 95°C, annealing temperature of 35°C for 1 min, 72°C for 2 min to extend, 37 cycles at 94°C for 15 s, 36°C for 30 s, 72°C for 1 min and 72°C for 10 min. The rates of temperature change adopted for heating and cooling were +1°C/2.9 s and -1°C/2.4 s, respectively. Amplification products were electrophoresed on 1.5% agarose gels run at constant voltage and 1X TBE buffer for approximately 2 h, visualized by staining with ethidium bromide (SAMBROOK *et al*., 1989) and photographed under UV light (using DC120 camera, Kodak).

A set of 14 pairs of SSR primers (Table 3) (synthesized by Life Technologies, Inc.) were used in this study. PCR reactions were performed with a protocol reported earlier (BARCACCIA *et al*., 2003) with minor changes. The volume of PCR solution was 25 µl, containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 300 µM each of dCTP, dGTP, dATP and dTTP, 800 µM of primer, 1.5 U of Taq DNA polymerase (Pharmacia Biotech) and 30 ng of genomic DNA. Amplification reactions were performed in a 9700 Thermal Cycler (PerkinElmer) using a touchdown cycling profile. The optimized PCR amplifying conditions used were: initial denaturation at 95°C for 3 min, followed by 2 cycles of 1 min at 95°C, an annealing temperature of 1 min at 63°C and 2 min at 72°C followed by a reduction in annealing temperature by 1°C every two cycles until a final annealing temperature of 56°C was reached. The last cycle was repeated 26 times and was ended by a final step at 72°C for 10 min. The amplified fragments were separated on 2% agarose gels with 1X TBE buffer (SAMBROOK *et al*., 1989) at 150 V for 3 h. Photographs (DC120 camera, Kodak) of the polymerized genomic fragments were taken after staining of the agarose gels with ethidium bromide.

The genetic relationship among the entire genomic DNA under study was assessed by comparing the RAPD and SSR fragments separated according to their size. The banding pattern of each of the primer was scored as present (1) or absent (0), each of which was treated as an independent character. Only the reproducible bands were observed for scoring and the light bands were omitted as they were not reproducible. The Jaccard's dissimilarity coefficient (J) was calculated, subjected to cluster analysis by bootstrapping and neighbor-joining method using the program DARWIN (version 5.0.158). Statistically unbiased clustering of collected genotypes was performed using STRUCTURE (version 2.3.1).

Table 2. List of primers used for RAPD amplification, GC content, total number of loci, the level of polymorphism, resolving power and PIC value.

Primer	Primer sequence	% GC content	Annealing Temperature	Total no. of loci	NPL	PPL	No. of fragments amplified	Rp	PIC
OPT-1	GGGCCACTCA	70	42	8	7	87.5	164	5.96	0.83
OPT-2	GGAGAGACTC	60	41.7	12	11	91.7	207	7.53	0.89
OPT-4	CACAGAGGGA	60	41.7	5	4	80.0	139	5.06	0.77
OPT-5	GGGTTTGGCA	60	38	8	6	75.0	191	6.95	0.85
OPT-6	CAAGGGCAGA	60	45.5	10	10	100.0	151	5.49	0.88
OPT-7	GGCAGGCTGT	70	42	9	7	77.7	127	4.62	0.76
OPT-8	AACGGCGACA	60	41	7	3	42.8	211	7.67	0.85
OPT-11	TTCCCCGCGA	70	41	9	9	100.0	156	5.67	0.99
OPT-13	AGGACTGCCA	60	45.5	5	5	100.0	65	2.36	0.78
OPT-16	GGTGAACGCT	60	43.8	8	6	75.0	130	4.73	0.79
OPT-17	CCAACGTCGT	60	42	8	6	75.0	190	6.91	0.85
OPH-1	GGTCGGAGAA	60	36	15	10	66.7	423	15.38	0.92
OPH-2	TCGGACGTGA	60	36	6	6	100.0	97	3.53	0.79
OPH-3	AGACGTCCAC	60	36	9	9	100.0	134	4.87	0.85
OPH-5	ACGCATCGCA	60	36	15	15	100.0	268	9.75	0.92
OPH-10	CCTACGTCAG	60	36	6	6	100.0	53	1.93	0.80
OPH-15	AATGGCGCAG	60	48.8	13	13	100.0	116	4.22	0.90
OPH-18	GAATCGGCCA	60	48.8	11	8	72.7	226	8.22	0.87
OPBH-1	CCGACTCTGG	70	44.5	12	7	58.3	359	13.06	0.91
OPBH-2	GTAAGCCGAG	60	42.5	9	8	88.0	187	6.80	0.86
OPBH-3	GGAGCAGCAA	60	41.7	9	6	66.7	270	9.82	0.88
OPBH-5	GTAGGTCGCA	60	36.9	11	8	72.7	280	10.18	0.89
OPBH-10	GTGTGCCTGG	70	44	4	1	25.0	152	5.53	0.74
OPBH-12	TCGCCTTGTC	60	43.8	11	11	100.0	161	5.86	0.86
OPBH-15	GAGAACGCTG	60	41	9	9	100.0	170	6.18	0.85
OPBH-16	CTGCGGGTTC	70	36.1	7	4	57.1	180	6.55	0.81
Total				236	207				

NPL, Number of polymorphic loci; PPL, Percentage of polymorphic loci, Rp, Resolving power and PIC, polymorphism information content.

POPGENE software was used to calculate Nei's unbiased genetic distance among different genotypes with all markers. Data for observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H), Shannon's information index (I), number of polymorphic loci (NPL) and percentage of polymorphic loci (PPL) across all the 31 genotypes were analyzed NEI *et al*. (1979). Within group diversity (Hs) and total genetic diversity (Ht) were calculated within the species and within three major groups (based on the male, female and half sib genotypes) by using POPGENE software NEI (1978). The RAPD and SSR data were subjected to a hierarchical analysis of molecular variance (AMOVA) (EXCOFFIER *et al*., 1992), using three hierarchical levels; individual, population and grouping based on their tall, medium and small height genotypes. The non-parametric analysis of molecular variance (AMOVA) was done via

Gen Alex (EXCOFFIER *et al*., 1992), where the variation component was partitioned among individuals within populations, among populations within groups and among groups. The resolving power of the RAPD and SSR primers was calculated according to (PREVOST and WILKINSON, 1999). The resolving power (*Rp*) of a primer is: $Rp = \Sigma\ IB$ where *IB* (band informativeness) takes the value of: 1–[2* (0.5–P)], P being the proportion of the 94 genotypes containing the band.

Table 3. List of primers used for SSR amplification, GC content, total number of loci, the level of polymorphism, resolving power and PIC value.

Primer	Primer sequence	% GC content	Annealing Temp.	Total no. of loci	NPL	PPL	No. of fragments amplified	Rp	PIC
SB-38	CCACTTGAGGAGTGTAAGGAT								
	CTTAAATGTAAAACTGAATCT	47	53	11	5	45.5	325	11.82	0.89
SB-	TGTGAGATAAGATTTGTCGGT								
194	CCATAAATAAAAAACGTGAAC	53	57.6	5	4	80.0	171	6.22	0.80
SB-	CTATTTGGTCTCAATCACCTT								
199	CTTTACCTCAGAAAATCCAGA	53	54.5	9	4	44.4	295	10.73	0.88
SB-85	CTCAGCAACTTAATCCAACTA								
	GTTTGTTAGGGGAGGTAAGAA	47	57.2	6	4	66.7	208	7.56	0.81
SB-80	TAATGGAGTTCACAGTCCTCC								
	ATACAGAGCCCATTTCATCAC	53	58	11	9	81.8	297	10.80	0.89
SB-88	TATTGCTTTGATGGCGACTGC								
	ATGTCATTCAGGTTTGTTTTC	47	53	7	6	85.7	181	6.58	0.83
SB-	ATGTCATTCAGGTTTGTTTTC								
100	ATGGTTTAACTTGTTACTGTA	53	59	10	10	100.0	232	8.44	0.89
SB-	CTGTTTCCTGCCACTATTACC								
196	TATAATCTGTCTCCTTTTGGC	47	58	7	5	71.4	171	6.22	0.82
SB-	CCTCTTTTTCTATTGTGGTCT								
201	GGCATGTATTTTTACTCCAAC	44	54.3	7	3	42.8	243	8.84	0.84
SB-	TATAAAGACAAATACCTGGGG								
210	GACAACGCCATTCACATGACC	44	55.3	9	7	77.8	238	8.66	0.88
SB-	ATTCCTTTCTTCATCAGTAGC								
243	GACAACGCCATTCACATGACC	53	57.7	10	6	60.0	320	11.64	0.89
PHTR	CGCAGGCTCAGAATACTTGA								
-2	GCCCACAGCTCAATAGTCTT	100	55	4	2	50.0	121	4.40	0.71
PHTR	ATTTGCATCCAGTCTTCAGTAATT								
-3	CTCAAAGAAGTGCATAGAGATTTCAT	67	58	8	8	100.0	202	7.35	0.87
PHTR	GGGCTTTAGTCCCATGCATTT								
-4	CCCTGGAGTTTACAAT	100	59	7	7	100.0	141	5.13	0.83

In order to determine the utility of each of the marker systems, diversity index (DI), effective multiple ratio (EMR) and marker index (MI) were calculated according to (POWELL *et al*., 1996). DI for the genetic markers was calculated from the sum of squares of allele frequencies:

$DI_n=1-\sum pi^2$ (where pi is the allele frequency of the ith allele). The arithmetic mean heterozygosity, DI_{av}, was calculated for each marker class: $DI_{av}=\sum Di_{n/n}$, (where n represents the number of the markers (loci) analyzed). The DI for the polymorphic marker is: $(DI_{av})p=\sum Di_n/n_p$ (where n_p is the number of polymorphic loci and n is the total number of loci). EMR (E) is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay EMR (E) = $n_p(n_p/n)$. MI is defined as the product of the average diversity index for polymorphic bands in any assay and the EMR for that assay, $MI=DI_{avp}$ x E.

The frequency of the polymorphism obtained in the genotypes was calculated on the basis of presence (1) and absence (0) of the bands amplified. The PIC was calculated according to ANDERSON *et al*. (1993) based on t[illegible] all the willow genotypes by employing the following formula: $PIC_i=1-\sum_{j=1}^{n} Pij^2$

RESULTS AND DISCUSSION

Molecular markers are a powerful tool with many potential applications in agriculture and forestry. In particular, DNA markers can provide information on the relatedness of various clones or varieties that are difficult to distinguish morphologically, thus helping in the management of plant accessions and in breeding programs. Simple Sequence Repeat DNA markers (SSR), or microsatellite markers has been selected for genotyping using the polymerase chain reaction (PCR). This type of marker is considered the method of choice due to their abundance, polymorphism and reliability compared to other types of DNA markers. Sixteen DNA markers were previously isolated and characterized in aspen (*Populus tremuloides*) and other poplar species (BEKKAOUI *et al*., 2003). Allelic diversity and population structure is important in developing association studies and constructing core collections for tree breeding. Population genetic differentiation in the native *Populus tomentosa* by genotyping 460 unrelated individuals using 20 species-specific microsatellite markers has been examined. Ninety nine alleles with a mean of 4.95 observed alleles per locus, indicating a moderate level of polymorphism across all individuals. These data provide a starting point for conserving valuable natural resources and optimizing breeding programs (QINGZHANG *et al*., 2012). Population substructure has important implications for both basic and applied genetic research. Ten microsatellite markers has been used to characterize population substructure in two ecologically and demographically contrasting populations of the model tree *Populus trichocarpa*. The Marchel site was a continuous stand growing in a mesic habitat in western Oregon, whereas the Vinson site consisted of three disjunct and isolated stands in the high desert of eastern Oregon. It has been reported that pollen-mediated gene flow is extensive in both populations (SLAVOV *et al*., 2010).

The RAPD technique had been successfully used in a variety of taxonomic and genetic diversity studies and it was found suitable for use with *Populus deltiodes* genotypes because of its ability to generate reproducible polymorphic markers. A total of 31 plant samples were fingerprinted using 26 RAPD makers. These primers produced multiple band profiles (Figure 1A) with a number of amplified DNA fragments varying from 5 to 15, with a mean of 9.12 bands per primer. All the amplified fragments varied in size from 100-2000 bp. A representative gel figure for one of the RAPD markers is included in Figure 1A. Out of 236 amplified bands, 207 were found polymorphic (86.44 %) (Table 2). The observed high proportion of polymorphic loci suggests that there is a high degree of genetic variation in the *Populus deltiodes* genotypes. The

resolving power of the 26 RAPD primers ranged from 1.93 for primer OPH-10 to a maximum of 15.38 for primer OPH-1.

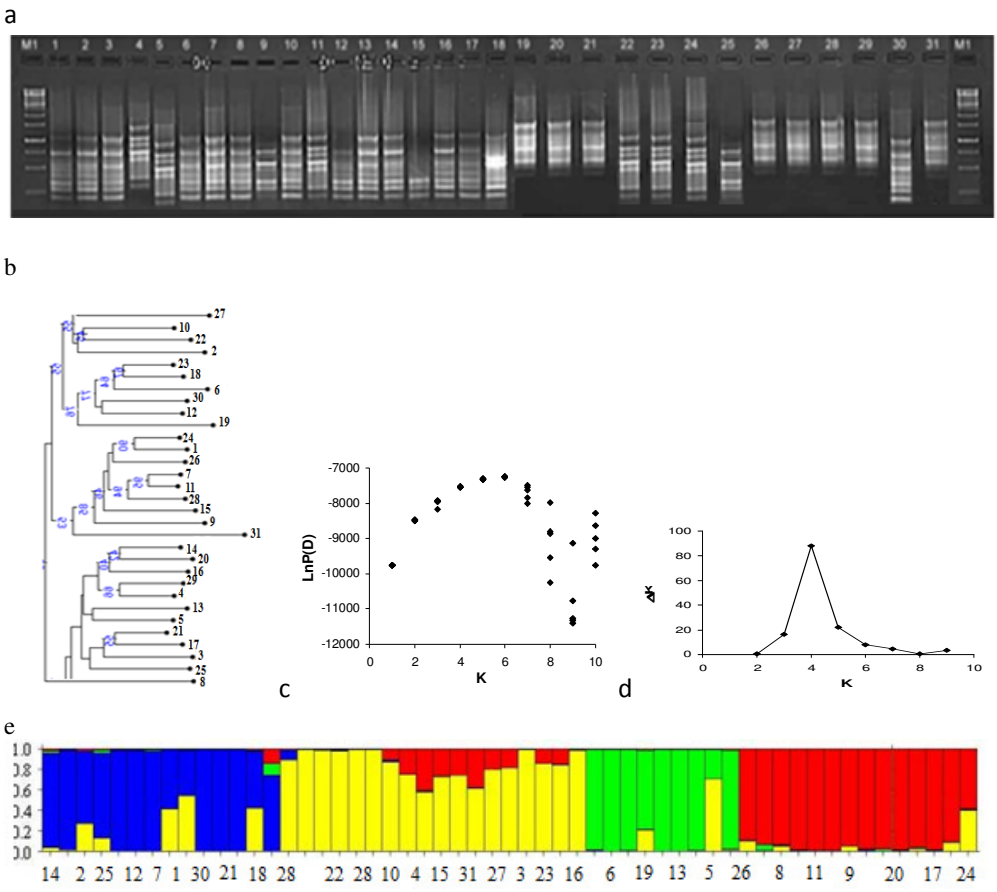

Figure 1. (A) Representative gel picture showing RAPD amplification products obtained from the 31 genotypes of Poplar studied M1 and M2 = the size of molecular markers in base pairs using 100 bp DNA ladder. (B) Dendrogram generated by Neighbor joining (NJ) clustering technique showing relationships between 31 genotypes of Poplar based on RAPD profiling. Number indicates bootstrap support values. (C) The relationship between the number of cluster (K) and the estimated likelihood of data (LnP(D)). A model based clustering of 31 genotypes using STRUCTURE without prior knowledge about the populations and under an admixed model calculated that LnP(D) was greatest when K = 4. (D) The relationship between K and ΔK., i.e. ΔK is reaches its maximum when K =4, suggesting that all genotypes fall into one of the 4 clusters. (E) Grouping of genotypes when K = 4. The genotypes were more likely clustered with respect to one of the 4 clusters. Genotypes from different clusters are represented with different colours: cluster 1(blue), cluster 2 (yellow), cluster 3 (green) and cluster 4 (red).

Table 4. Summary of genetic variation statistics for the combination of (a) RAPD only (b) SSR only and (c) combination of both RAPD and SSR profiling among the genotypes of poplar clones with respect to their distributions into 3 groups as described in table 1.

(a)					RAPD markers			
Plant height	Sample size	Na	Ne	H	I	Ht	NPL	PPL
Tall	10	1.979 (0.144)	1.672 (0.291)	0.380 (0.129)	0.557 (0.161)	0.380 (0.017)	231	97.88
Medium	10	1.945 (0.229)	1.637 (0.298)	0.365 (0.136)	0.537 (0.176)	0.365 (0.018)	223	94.49
Small	11	1.631 (0.483)	1.538 (0.440)	0.286 (0.225)	0.407 (0.316)	0.286 (0.051)	149	63.14
(b)					SSR markers			
Plant height	Sample size	Na	Ne	H	I	Ht	NPL	PPL
Tall	10	2.0 (0.0)	1.755 (0.239)	0.417 (0.099)	0.603 (0.116)	0.417 (0.010)	111	100
Medium	10	1.991 (0.095)	1.729 (0.244)	0.408 (0.099)	0.593 (0.117)	0.408 (0.010)	110	99.10
Small	11	1.658 (0.477)	1.521 (0.411)	0.286 (0.213)	0.411 (0.303)	0.286 (0.046)	73	65.77
(c)					Combination of RAPD and SSR markers			
Plant height	Sample size	Na	Ne	H	I	Ht	NPL	PPL
Tall	10	2.00 (0.00)	1.679 (0.281)	0.385 (0.121)	0.564 (0.145)	0.385 (0.015)	347	100
Medium	10	1.911 (0.286)	1.601 (0.332)	0.344 (0.155)	0.508 (0.205)	0.344 (0.024)	316	91.07
Small	11	1.231 (0.422)	1.164 (0.312)	0.094 (0.175)	0.138 (0.254)	0.094 (0.031)	80	23.05

Na, Observed number of alleles; Ne, Effective number of alleles; H, Nei's gene diversity; I, Shannon's Information index; Ht, Heterozygosity; NPL, Number of polymorphic loci; PPL, Percentage of polymorphic loci

Polymorphism information content (PIC) refers to the value of a marker for detecting polymorphism within a population or set of genotypes by taking into account not only the number of alleles that are expressed but also the relative frequencies of alleles per locus. As evident, RAPD marker 'OPH-1' showed the highest level of polymorphism with PIC value of 0.92, whereas the PIC values for rest of the RAPD markers were in the range of 0.74-0.91. A dendrogram analysis based on bootstrapping and neighbor joining (NJ) method grouped all the 31 genotypes into four main clusters which are further extensively divided into mini clusters: cluster-1 (4 genotypes), cluster-2 (6 genotypes), cluster-3 (9 genotypes) and cluster-4 (12 genotypes) (Figure 1B). An unbiased clustering of genotypes based on STRUCTURE program (PRITCHARD *et. al.,* 2000; FALUSH *et. al.,* 2007), without prior knowledge about the populations, clustered all the

31 genotypes into four major groups. Under the admixed model, STRUCTURE calculated that the estimate of likelihood of the data (LnP(D)) was greatest when K = 4. For K > 4, LnP(D)) increased slightly but more or less plateued, i.e. ΔK reached its maximum value when K = 4 (Fig. 1C,D), suggesting that all the populations fell into one of the 4 clusters albeit small interference (Figure 1E). This result is almost similar to the splitting in the NJ tree. Overall the cluster analysis strongly suggested that the 31 sampled genotypes can be divided into four clusters.

Table 5. Overall genetic variability across all the 31 genotypes of Poplar clones based on RAPD only, SSR only and combination of both RAPD and SSR markers.

	Sample size	Na	Ne	H	I	Ht	Hs	NPL	PPL	Gst	Nm	EMR	MI
RAPD	31	2.00 (0.00)	1.641 (0.299)	0.367 (0.131)	0.543 (0.159)	0.367 (0.017)	0.225 (0.010)	236	100	0.388	0.788	6.49	0.69
SSR	31	2.00 (0.00)	1.768 (0.226)	0.423 (0.092)	0.610 (0.107)	0.423 (0.008)	0.278 (0.009)	111	100	0.341	0.965	3.72	0.55
RAPD +SSR	31	2.00 (0.00)	1.682 (0.283)	0.385 (0.123)	0.564 (0.148)	0.385 (0.015)	0.242 (0.010)	347	100	0.371	0.846		

Gst, Genetic deifferentiation; Nm, Gene flow; EMR= Effective multiplex ratio and MI= Marker Index

The genetic diversity of 31 genotypes was calculated in terms of Na, Ne, H, I, Ht, and PPL with respect to 3 different groups such as tall, medium and small plant height, indicating more variability among the genotypes (Table 4a). Percentage of polymorphic loci using POPGENE was calculated that varied from 97.88% in small genotypes to 63.14% in tall genotypes. Three groups containing genotypes with different plant height showed Nei's genetic diversity (H): 0.380, 0.365 and 0.286 respectively and of Shannon's information index (I): 0.557, 0.537 and 0.407 (Table 4a) respectively showed that there occurred a great genetic differentiation within each of the three groups. The respective values for overall genetic variability for Na, Ne, H, I, Ht, Hs, Gst, NPL, PPL and Gene flow (Nm) across all the 31 genotypes were also given in Table 5. Gst value of 0.388 indicated that 61.2 % of the genetic diversity resided within the population. The rate of gene flow estimated using Gst value was found to be 0.788 which is very low. Analysis of molecular variance among genotypes based on three major groups with respect to plant height indicated that majority of genetic variation (94.6%) occurred among genotypes, while the variation between the three clusters was 5.4% (Table 6).

The fourteen SSR primers selected in the study generated a total of 111 SSR bands (an average of 7.93 bands per primer), out of which 80 were polymorphic (72.07%) (Table 3). Number of bands varied from 4 to 11 with sizes ranged from 807 – 867 bp (Figure 2). Amplification result of 14 primers seems to indicate that microsatellites more frequent in *Populus deltoides* contain the repeated di-nucleotides (AG)n and (GA)n. The number of bands produced with different repeat nucleotide were more with the (AG)n and (GA)n, followed by (CT)n, and (AC)n and for tri-nucleotide were with (CTC)n primers. The primers that were based on the (GA)n, (AG)n and (CT)n motif produced more polymorphism (on average 8.5 bands per primer) than the primers based on any other motifs used in the present investigation. We obtained good amplification products from primers based on (AG)n and (GA)n repeats, despite the fact that (AT)n di-nucleotide repeats are thought to be the most abundant motifs in plant species (MARTÍN and

SANCHEZ-YELAMO, 2000). Similar results were obtained in grapevine (MORENO *et al*., 1998), rice (BLAIR *et al*., 1999), *Vigna* (AJIBADE *et al*., 2000), wheat (NAGAOKA and OGIHARA, 1997) and willow (SINGH *et al*., 2013). A possible explanation of these results is that SSR primers based on AT motifs are self-annealing, due to sequence complementarity, and would form dimers during PCR amplification (BLAIR *et al*., 1999) or it may be due to its-non annealing with template DNA due to its low Tm. The resolving power (Rp) of the 14 ISSR primers ranged from 4.40 to 11.82 (Table 3). Similarly the PIC value ranges from 0.71 to 0.89 demonstrating uniform polymorphism rate among all the 14 ISSR primers.

Table 6. Summary of analysis of molecular variance (AMOVA) based on (a) RAPD only (b) SSR only and (c) combination of both RAPD and SSR markers among the genotypes of Poplar. Levels of significance are based on 1000 iteration steps.

Source of variation	Degree of freedom	Variance component	Percentage of variation	P-value
(a)		Based on RAPD profiling		
Among groups	2.0	2.46	5.4	-
Among genotypes	52.0	43.13	94.6	< 0.001
(b)		Based on SSR profiling		
Among groups	2.0	1.0	4.08	-
Among genotypes	52.0	23.49	95.92	< 0.001
(c)		Based on combination of both RAPD and SSR profiling		
Among groups	2.0	11.0	14.8	-
Among genotypes	52.0	63.48	85.2	< 0.001

The complete data set of 3145 bands was used for cluster analysis based on bootstrapping and NJ method. The genotypes were clustered into four major clusters, well supported by bootstrap value of > 40 (Fig. 2B). The estimated likelihood (LnP(D)) of the clustering of data using STRUCTURE was found to be optimal when K = 4, LnP(D)) increased slightly for K > 4, but more or less plateued (Fig. 2C). ΔK reached its maximum value when K = 4 (Fig. 2D), suggesting that all the populations were distributed with high probability into one of the 4 clusters (Fig. 2E). The clustering pattern of the genotypes were almost similar to the splitting in the NJ tree, however, there is distinct clustering of genotypes based on their tall, medium and small plant genotypes.

A relatively high genetic variation was detected among the genotypes grouped into 3 different groups. Genetic diversity analysis in terms of Na, Ne, H, I, Ht, Hs, and PPL reveals higher value for the group with small plant genotypes in comparison to the group having tall plant genotypes. This disparity may be because of more number of genotypes included in the group with small plant genotypes (Table 4b). Overall genetic variability across all the 31 genotypes in terms of Na, Ne, H, I, Ht, Hs, Gst, NPL, PPL and Gene flow (Nm) were also included in Table 5. The Nei's genetic diversity index was 0.423 and Shannon information index was 0.610 demonstrating high rate of genetic variability. AMOVA for among groups (4.08%) and among genotypes (95.92%) indicated that there are more variations across the genotypes (Table 6). The estimated gene flow was 0.965.

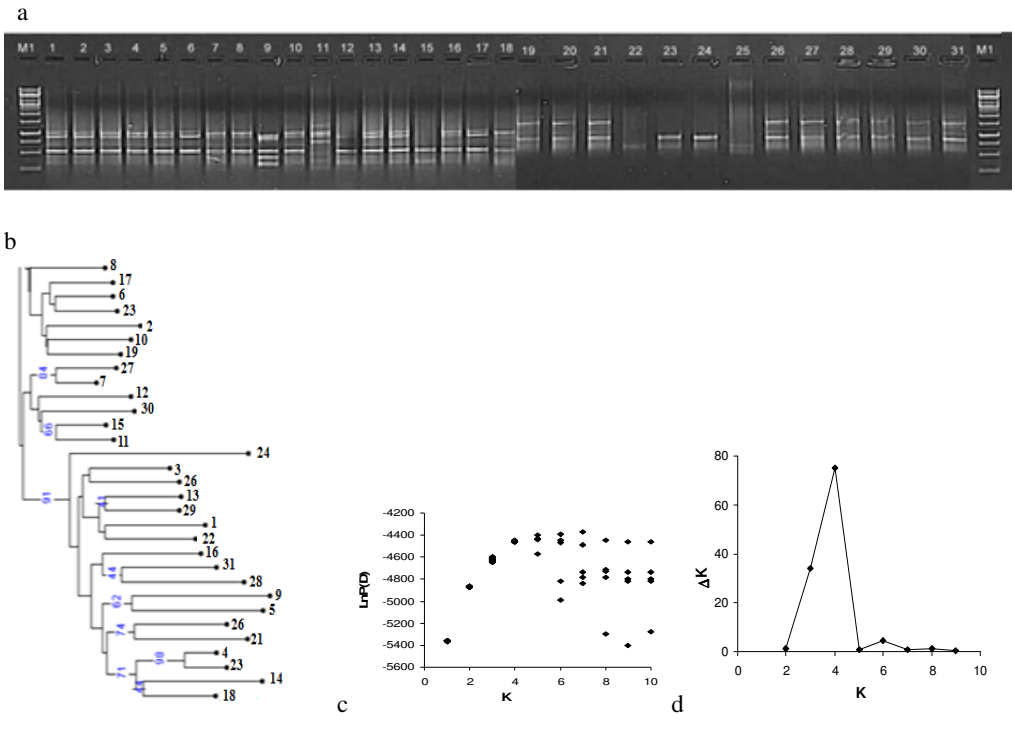

Figure 2. (A) SSR amplification products obtained from the 31 genotypes of Poplar studied M1 and M2 = the size of molecular markers in base pairs using 100 bp DNA ladder. (B) Dendrogram generated by Neighbor joining (NJ) clustering technique showing relationships between 31 genotypes of Poplar based on SSR profiling. Number indicates bootstrap support values. (C) The relationship between the number of cluster (K) and the estimated likelihood of data (LnP(D)). A model based clustering of 31 genotypes using STRUCTURE without prior knowledge about the populations and under an admixed model calculated that LnP(D) was greatest when K = 4. (D) The relationship between K and ΔK., i.e. ΔK is reaches its maximum when K =4, suggesting that all genotypes fall into one of the 4 clusters. (E) Grouping of genotypes when K = 4. The genotypes were more likely clustered with respect to one of the 4 clusters. Genotypes from different clusters are represented with different colours: cluster 1(yellow), cluster 2 (blue), cluster 3 (red) and cluster 4 (green).

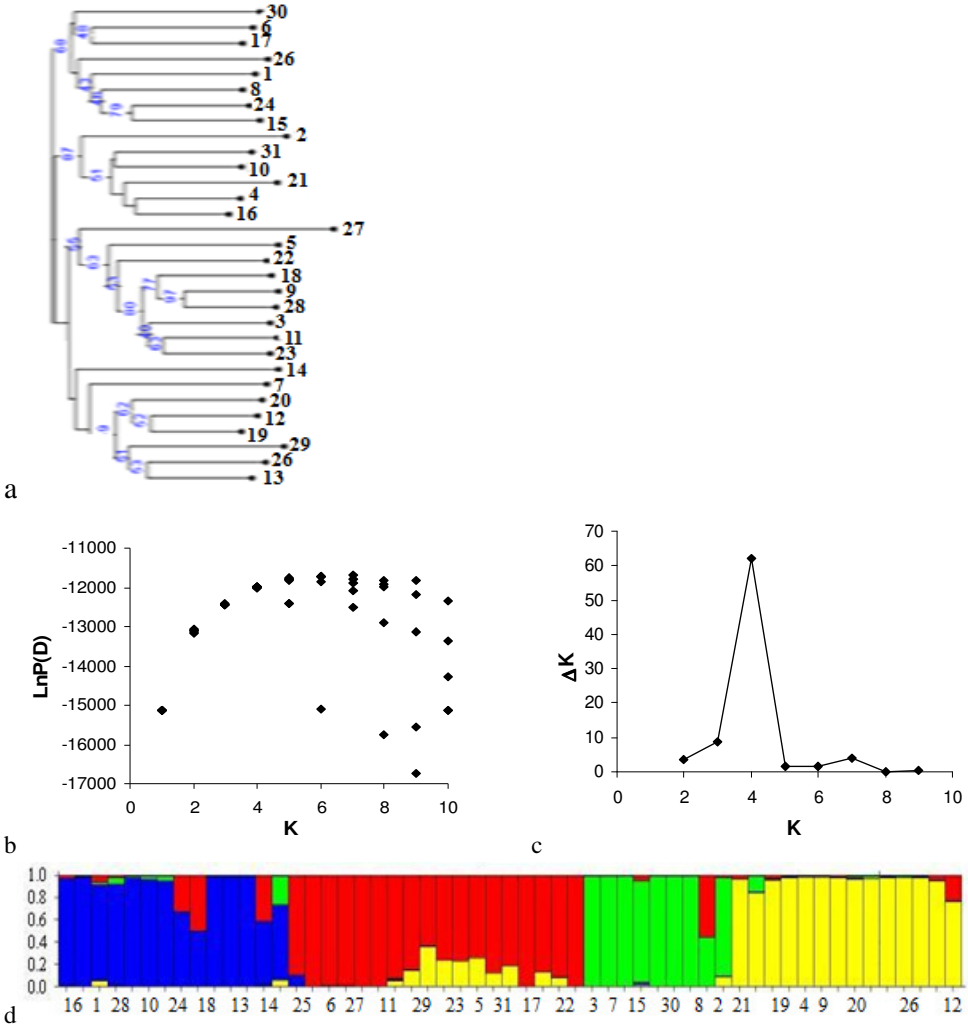

Figure 3. (A) Dendrogram generated by Neighbor joining (NJ) clustering technique showing relationships between 31 genotypes of Poplar based on combination of both RAPD and SSR profiling. Number indicates bootstrap support values. (B) The relationship between the number of cluster (K) and the estimated likelihood of data (LnP(D)). A model based clustering of 31 genotypes using STRUCTURE without prior knowledge about the populations and under an admixed model calculated that LnP(D) was greatest when K = 4. (C) The relationship between K and ΔK., i.e. ΔK is reaches its maximum when K =4, suggesting that all genotypes fall into one of the 4 clusters. (D) Grouping of genotypes when K = 4. The genotypes were more likely clustered with respect to one of the 4 clusters. Genotypes from different clusters are represented with different colours: cluster 1(blue), cluster 2 (red), cluster 3 (green) and cluster 4 (yellow).

Based on combined data set of RAPD and SSR markers, the dendrogram obtained gave similar clustering pattern like RAPD and SSR (Figure 3A). This result is also corroborative with STRUCTURE analysis; the estimated likelihood of distribution (LnP(D)) for all the 31 genotypes was highest when K = 4 (Fig. 3B), ΔK was maximum with K =4 (Fig. 3C), reveals that all the genotypes were clustered better (with high likelihood probability) with 4 clusters (Fig. 3D). Other genetic variation studies were also performed on RAPD and SSR combined data which are represented in different tables (Tables 4, 5 and 6). The differences found among the dendrograms generated by RAPDs and SSRs could be partially explained by the different number of PCR products analyzed (4807 for RAPDs and 3145 for SSRs) reinforcing again the importance of the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships as observed by LOARCE *et al*., (1996) in barley. Another explanation could be the low reproducibility of RAPDs (KARP *et al*., 1997). The genetic similarity of these genotypes is probably associated with their similarity in the genomic and amplified region.

RAPD markers were found more efficient with respect to polymorphism detection (based on average PPL value), as they detected 81.23% polymorphism as compared to 71.82% for SSR markers. This is in contrast to the results obtained for several other plant species like wheat (NAGAOKA and OGIHARA, 1997) and *Vigna* (AJIBADE *et al*., 2000). More polymorphism in case of RAPD than SSR markers might be due to the fact that 14 SSR primers used in the study only amplified 3145 number of fragments (Table 3). While in case of RAPD, all the 26 primers which were used in the investigation amplified 4807 number of fragments (Table 2). Similar polymorphism pattern was also observed in case of *Jatropha* (GUPTA *et al*., 2008) and *Podophyllum* (ALAM *et al*., 2009). This shows that RAPD data is more close to RAPD+SSR combined data. A possible explanation for the difference in resolution of RAPDs and SSRs is that the two-marker techniques target different portions of the genome. The mean effective multiplex ratio and marker index are more for RAPD than for SSR markers (Table 5).

With this study, we can conclude that the molecular analyses of both RAPD and SSR markers were extremely useful for studying the genetic relationships of *Populus deltoides* clonal genotypes. The results indicates the presence of high genetic variability, which should be exploited for the future conservation and breeding of *Populus deltoides*. Since no single, or even a few plants, will represent the whole genetic variability in poplar clones, this appears to be a need to maintain sufficiently large populations in natural habitats to conserve genetic diversity in poplar to avoid genetic erosion.

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MOLEKULARNA EVALUACIJA KLONOVA TOPOLE ZASNOVANA NA KORIŠĆRNJU RAPD I SSR MARKERA

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Izvod

Sa ciljem da se olakšaju naučne odluke u menadžmentu i konzervacije topolr I pripreme za selektivne programe oplemenjivanje izvršena je 31 genetička analiza kod 31 genotipa korišćenjem 26 RAPD i14 SSR markera. Izvršena je amplifikacija ukupno 236 od 83 prebrojana fragmenta od kojih je 86,44 i 86,02 % bili polimorfni. Srednji koeficijent genetičke diferencijacije (Gst) je bio 0.388 i 0.341, ukazujući da 61.2% i 65.9% genetičkog variranja se nalazi u populaciji. Analiza molekularne varianse Analysis of molecular variance (AMOVA) pokazuje da najveći deo gentičkog variranja (94.6% korišćenjem RAPD i 89% korišćenjem SSR) ukazuje da se najveći deo variranja dešava između genotipova, dok je variranje među tri grupe, kategorisane kao visoke, srednje i niske biljke je bilo bilo 5.4% (korišćenjem RAPD) i 11% (korišćenjem SSR). Iz dobijeih dendograma se vidi da su se genotipovi grupisali u četiri klastera sa jasnom razlikom visokih, srednjih i niskih genotipova, što pokazuje da se merenj genetičke diferencijacije poklapaju sa podacima dobijenim korišćebjem molekularnih markera.Utvrđebo je das u korišćeni marker korisni u razumevanju odnosa pojedinih genotipova kao I u konzervaciji i eksploataciji genetičkih resursa topole.

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