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Full Length Research Paper

Studies on genetic diversity in poplar (*Populus deltoides* Bartram ex Marsh.) using morphological and random amplified polymorphic DNA (RAPD) marker

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30 popular poplar clones were studied to assess genetic divergence using morphological growth parameters and random amplified polymorphic DNA (RAPD) markers. Based on Mahalanobis D^2 analysis, 15 clusters were formed and clustering pattern indicated presence of substantial genetic diversity among 30 poplar clones. These clones were subjected to RAPD analysis, a total of 374 RAPD loci were detected out of which 20 were monomorphic and 354 were polymorphic. A positive correlation between morphological and RAPD analysis in estimating genetic divergence was found. The present molecular study revealed that genotypes from different geographical region clustered in one group, which signifies occurrence of narrow genetic base in that zone. To promote diversified plantation, a multiculture group comprising of S_7C_1, G-7, 421-2, 82-35-4, PIP-123, D-123, A-194 and 22-N was found to make a broad genetic base for commercial plantations.

Key words: Poplar, clones, D^2, random amplified polymorphic DNA (RAPD), DNA, diversity.

INTRODUCTION

Poplar belongs to the family Salicaeceae, order Salicales and group Amentiflorae. The genus comprises of nearly 35 species classified into five major sections (Dickman and Stuart, 1983; Eckenwalder, 1996). Poplar is of primary economic importance in the production of wood in temperate regions of the world (Licht and Isebrands, 2005). It is estimated that this tree covers the majority of all artificial forests (FAO, 1980). Its wood is used for large spectrum of products, such as plywood, packages, structural timber, matches, chopsticks and paper. Due to its fast growth and short rotation in many countries, the best alternative to the destruction of forests. Many poplar only solution to the growing wood need is provision of the species and their interspecific hybrids are suitable for poplar culture and intensive forest plantations (FAO, 1979). Clones have been exchanged among countries making breeding easy for all users. The importance of a proper identification of poplar clones was appreciated long ago. However, traditional methods based on observation of morphological and phenological characters (FAO, 1971; UPOV, 1981) are both time consuming and environmentally blurred effects.

Poplar was also the first, among trees, to be investigated for detection of biochemical markers that could help in identification. Paper chromatography (Beccone, 1975) was shown to be useful for the discrimination of some species and hybrids but not satisfactory for clones. Isozyme analysis has also been considered and found to be satisfactory with some specific clones (Rajora, 1988; 1989; Rajora and Zsuffa, 1989), and its resolving power

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Table 1. List of 30 poplar clones used in the present study.

S/N	Name of clone	Source
1	S_7 C_1	USA
2	S_7 C_2	USA
3	S_7 C_4	USA
4	S_7 C_8	USA
5	S_7 C_{15}	USA
6	S_7 C_{20}	USA
7	G-3	Australia
8	G-48	Australia
9	3167	FRI, line
10	82-33-3	FRI, line
11	82-35-4	FRI, line
12	82 – 40 – 2	FRI, line
13	421-2	FRI, line
14	S_{13} C_{11}	USA
15	S_4 C_2	USA
16	D -121	USA
17	ST – 124	Germany
18	2502	FRI, line
19	3324	FRI, line
20	111828	FRI, line
21	1113520	FRI, line
22	A – 194	FRI, line
23	PIP – 123	FRI, line
24	PIP – 124	FRI, line
25	L -34/82	Lalkuan selection
26	L – 49 / 82	Lalkuan selection
27	L – 200/84	Lalkuan selection
28	L – 62/84	Lalkuan selection
29	WSL – 45	WIMCO
30	22 - N	FRI, line

is limited by a number of enzymatic systems that can be conventionally analyzed. The restriction fragment length polymorphism (RFLP) has also been successfully used to identify DNA polymorphism which is useful as genetic markers in plants (Helentjaris et al., 1985; Tanksley et al., 1989; Smulders et al., 2008; Fadia and Salah, 2011; Salah, 2011). This is a direct approach to the study of genomic DNA, but has not been systematically applied to poplars. Random amplified polymorphic DNA (RAPD), is a powerful tool 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 eucalyptus (Kell and Griffin, 1994), mango (Schnell et al., 1995), Norway spruce (Scheepers et al., 1997), oil palm (Shah et al., 1994), cacao (Whitkus et al., 1998), cotton (Iqbal et al., 1997) and brassica (Jain et al., 1994). In the genus *Populus*, RAPD analysis has been performed for evaluation of genetic diversity and clonal identification (Castiglione et al., 1993; Chong et al., 1994). The objective of the present study was to assess the genetic diversity among poplar clones using D^2 analysis and RAPD markers, with the ultimate aim of utilizing them for genetic relationship, parent selection, germplasm management and germplasm protection.

MATERIALS AND METHODS

The plant material used in the study consisted of 30 poplar clones (Table 1). Most of the clones were introduced in India from Europe and the United States of America. Plants were grown at Agro-forestry Research Centre, Pathharchatta of G.B. Pant University of Agriculture and Technology, Pantnagar. Pantnagar falls in the subtropical climate zone called Tarai and lies in the foothills of Shivalik range of Himalayas. Geographically it has an altitude of 243.84 m above mean sea level and latitude of 29 °N and 79.30 °E longitude. Average rainfall in this area varies from 1371 to 1626 mm per annum. The experiment was planted in randomized block design with three replications. Four plants of each clone were

Table 2. Clustering pattern of 30 poplar clones on the basis of genetic divergence analysis.

Cluster	I	II	III	IV
Genotype	S_7 C_2, A-194, S_7 C_{15}, S_7 C_4, S_{13} C_{11}, L–62/84, S_7 C_8, S_7 C_{20},22 - N	S_7 C_1, 82-26-5, L–49/82, 3167, PIP-124	G-48, ST – 124	421-2, PIP-123
	V	**VI**	**VII**	**VIII**
Genotype	2502, 3324, L – 200/84	82-33-3, D -121, S_4 C_2	G-3	111828, L-34/82, 113520
	IX	**X**		
Genotype	WSL – 45	82-35-4		

maintained in trial with a distance of 5 m between rows and 4 m between trees. The genetic divergence present in the genotypes was estimated by Mahalanobis D^2 statistics as suggested by Rao (1962). 14 characters viz. tree height, diameter at breast height, clear bole length, crown length, crown width, bark thickness, length of leaf blade, length of leaf petiole, total leaf length, leaf width, leaf area index, leaf fall duration, volume over the bark and volume under the bark were considered for D^2 analysis.

DNA isolation

Total genomic DNA was isolated with the modified cetyl-trimethylammonium bromide (CTAB) method (Saghai-Maroof et al., 1984). Approximately, 5 g leaf material was ground to a fine powder using liquid nitrogen and quickly transferred into 25 ml of prewarmed (60°C) isolation buffer in a capped polypropylene tube, incubated for 1 h at 65°C in a water bath and mixed by gentle swirling after every 10 min. To these tubes, equal volume of chloroform : isoamyl alcohol (CI) was added and the contents were shaken for 10 min by hand. The tubes were centrifuged for 10 min at 8000 rpm; the upper aqueous layer was extracted twice with fresh CI and the final aqueous layer was transferred to a centrifuge tube. To these tubes, 0.6 V of ice-cold isopropanol was added and shaken several times. By using a glass hook, DNA was spooled out in the form of whitish fibers and transferred to washing solution and dried. DNA was dissolved in an appropriate volume of 1X Tris-EDTA (TE) buffer.

For purification, RNase A was added to the tube (50 µg/ml) and the mixture incubated for 1 h at 37°C. DNA was extracted with CI by centrifuging the tubes at 10,000 rpm for 5 min at room temperature. DNA was precipitated with 2 V of ice cold absolute ethanol and was recovered by centrifuging the tubes at 5000 rpm for 10 min; the pellet was washed with 70% ethanol and dissolved in appropriate volume of IX TE.

RAPD analysis

The 10-base oligonucleotide random primers for the PCR were obtained commercially from Operon Technologies, Alameda, California; 30 random primers (OPM-5-19 primers; OPN - 1 to 16) were used in this study. DNA amplification were carried out in 50 µl reaction mixture, each containing 50 ng of template DNA, 2 µM primers, 100 µM each of dNTPs, 2 µl of Taq DNA polymerase 10X buffer, 1 unit Taq polymerase (Perkin Elmer) and 2.5 mM $MgCl_2$. PCR amplification were performed on a PTC-100 Thermal Cycler (MJ Research, Inc. Watertown, MA, USA) under the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturing at 95°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min. The amplification products were resolved on 1.5% agarose gel and visualized under UV light following staining with ethidium bromide.

Data analysis

The frequency of RAPD polymorphism was calculated based on presence (taken as 1) or absence (taken as 0) of common bands (Ghosh et al. 1997). The binary data were used to compute pair-wise similarity coefficient (Jaccard, 1908) on NTSYS-PC. A dendrogram based on similarity coefficient was generated by using the unweighted pair group method of arithmetic means (UPGMA).

RESULTS

Genetic diversity at morphological level

The estimates of genetic divergence were measured using Mohalanobis D^2 statistics as described by Rao (1962). Based on genetic divergence, 30 poplar clones were grouped in 10 clusters (Table 2). Cluster I, the largest comprises of nine genotypes namely: S_7 C_2, A-194, S_7 C_{15}, S_7 C_4, S_{13} C_{11}, L–62/84, S_7 C_8, S_7 C_{20} and 22–N followed by cluster II with five genotypes viz. S_7 C_1, 82-26-5, L–49/82, 3167, PIP-124. Cluster V, VI and VIII comprises of three clones each. Cluster III and IV consist of two clones each, and clusters VII, IX and X each has single clone viz. G-3, WL-45 and 82-35-4, respectively.

Estimates of average intra and inter cluster distances were calculated, the intra cluster distance ranged from 0.00 to 14.55 (Table 3). It was maximum in cluster III (14.55) followed by cluster I (13.13) and VI (12.97). Cluster VII, IX and X each had single genotype, so intra cluster distance of these clusters was zero. The maximum inter-cluster distance was observed between cluster VII and X (59.78), followed by between IV and X (59.10), cluster II and X (56.10) and so on. Cluster nearest to each other were cluster II and IV (16.98), followed by cluster IV and VII (17.51) and so on.

The contribution of different characters was calculated following standard method as suggested by Singh and Chaudhary (1981) where contribution is measured only by the number of times any character was ranked at the top (Table 4). Length of leaf blade was maximum

Table 3. Average intra and inter-cluster D values.

Cluster	1	2	3	4	5	6	7	8	9	10
1	(13.13)	44.64 (11.53)	34.58	48.52	35.80	22.27	49.03	20.11	30.64	18.06
2			21.42 (14.55)	16.98	27.81	54.41	18.93	31.87	50.31	56.59
3				19.66 (11.85)	27.59	45.18	23.82	20.29	44.53	45.16
4					32.78 (12.79)	58.11	17.51	32.83	55.43	59.10
5						35.49 (12.97)	25.44	26.11	28.35	44.99
6							54.55 (0.00)	31.28	18.36	23.51
7								33.88 (10.39)	48.74	59.78
8									44.69 (0.00)	47.21
9										36.69
10										(0.00)

Values in parenthesis are intra cluster distance.

Table 4. Contribution of different characters towards divergence in poplar clones.

S/N	Character	Percent contribution
1	Tree height (m)	8.00
2	Diameter of breast height (cm)	4.02
3	Clear bole length (m)	0.08
4	Crown length (m)	0.08
5	Crown width (m)	0.08
6	Bark thickness (mm	0.12
7	Length of leaf blade (cm)	12.24
8	Length of leaf petiole (cm)	0.27
9	Total leaf length (cm)	1.21
10	Leaf width (cm)	3.62
11	Leaf area index (cm^2)	3.97
12	Volume over the bark (m^3)	6.02
13	Volume under the bark (m^3)	1.30
14	Leaf fall duration (days)	8.50

(12.24%) and crown width was minimum (0.08%) with regards to the expression of genetic divergence.

Genetic diversity at molecular level

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. 30 different RAPD primers were used to evaluate the level of genetic diversity amongst the different clones of poplar. High levels of genetic diversity were observed among the 30 genotypes being analyzed. A total of 374 RAPD loci were consistently resolved by the 30 primers and were scored (Table 5). The number of RAPD loci resolved by one primer ranged from 7 to 20, with an average of 12.46 loci per primer. Of the 374 RAPD loci, 354 were polymorphic. The primers OPN16 and OPN04 were the most informative, whereas the primer OPM18 was the least informative for clone fingerprinting and differentiation.

Table 5. RAPD primers, the number of RAPD loci and percent polymorphism observed in 30 clones.

S/N	Primer	Monomorphic loci		Polymorphic loci		Total no. of RAPD loci
		Number	Percentage	Number	Percentage	
1	OPM-05	1	9.09	10	90.90	11
2	OPM-06	0	0.00	12	100.00	12
3	OPM-07	1	6.25	15	93.75	16
4	OPM-08	1	8.33	11	91.66	12
5	OPM-09	0	0.00	13	100.00	13
6	OPM-10	2	13.33	13	86.66	15
7	OPM-11	1	10.00	9	90.00	10
8	OPM-12	1	10.00	9	90.00	10
9	OPM-13	2	16.66	10	83.33	12
10	OPM-14	0	0.00	11	100.00	11
11	OPM-15	1	7.14	13	92.85	14
12	OPM-16	2	14.28	12	85.71	14
13	OPM-18	1	14.28	6	85.71	7
14	OPM-19	2	13.33	13	86.68	15
15	OPN-01	0	0.00	12	100.00	12
16	OPN-02	1	6.66	14	93.33	15
17	OPN-03	0	0.00	11	100.00	11
18	OPN-04	0	0.00	17	100.00	17
19	OPN-05	0	0.00	16	100.00	16
20	OPN-06	0	0.00	10	100.00	10
21	OPN-07	0	0.00	13	100.00	13
22	OPN-08	0	0.00	7	100.00	7
23	OPN-09	0	0.00	12	100.00	12
24	OPN-10	0	0.00	14	100.00	14
25	OPN-11	0	0.00	10	100.00	10
26	OPN-12	0	0.00	10	100.00	10
27	OPN-13	1	11.00	8	88.88	9
28	OPN-14	1	11.00	11	91.66	12
29	OPN-15	0	0.00	14	100.00	14
30	OPN-16	2	10.00	18	90.00	20
	Total	20.00		354		374
	Average	0.66		11.80		12.46

Association among the 30 genotypes was revealed by UPGMA cluster analysis based on Jaccard coefficient (Figure 1). The dendrogram revealed the presence of two distinct clusters, C1 and C2. The former cluster C1 was found to comprise only three genotypes namely S_7 C_1, S_7 C_{15} and G-3, the latter cluster C2 was comprised of 27 of the 30 genotypes and thus designated as a major cluster. The first main cluster C1 was divided into two major sub-clusters. The first major sub-cluster consisted of two clones S_7C_1 and S_7C_{15} both from USA and the second major sub-cluster consisted of only one clone G-3 from Australia.

The second main cluster comprised of 27 clones, it was divided into two major sub-clusters. The first major sub-cluster comprised of 21 clones namely, S_7C_2, S_7C_4, S_7C_8, G-48, 421-2, 3167, 82-33-3, 82-35-4, 82-40-2, S_4C_2, S_7C_{20}, 113520, 111828, PIP-123, $S_{13}C_{11}$, WSL-45, D-121, ST-124, 2502, 3324 and L-200/84. The second major sub-cluster comprised of six clones viz., A-194, PIP-124, L-49/82, L-62/84, L-34/82 and 22-N out of which A-194, PIP-124 and 22-N were FRI lines and L-49/82, L-62/84 and L-62/84 were from Lalkuan selection. Thus, this sub-cluster comprised of all the clones developed in India.

The first major sub-cluster with 21 clones, separated into two minor sub-clusters. One minor sub-cluster had only a single clone (L-200/84) from Lalkuan selection and the other sub-cluster was left with the rest 20 clones. A detailed study of the second minor sub-cluster revealed seven different groups at different levels. First group comprised of three clones (S_7C_2, S_7C_4 and S_7C_8) from USA. Second group had three genotypes viz., G-48, 421-2 and 3167 out of which clone G-48 was from Australia and clone 421-2 and 3167 were FRI lines. The third group also consisted of three clones (82-33-3, 82-35-4

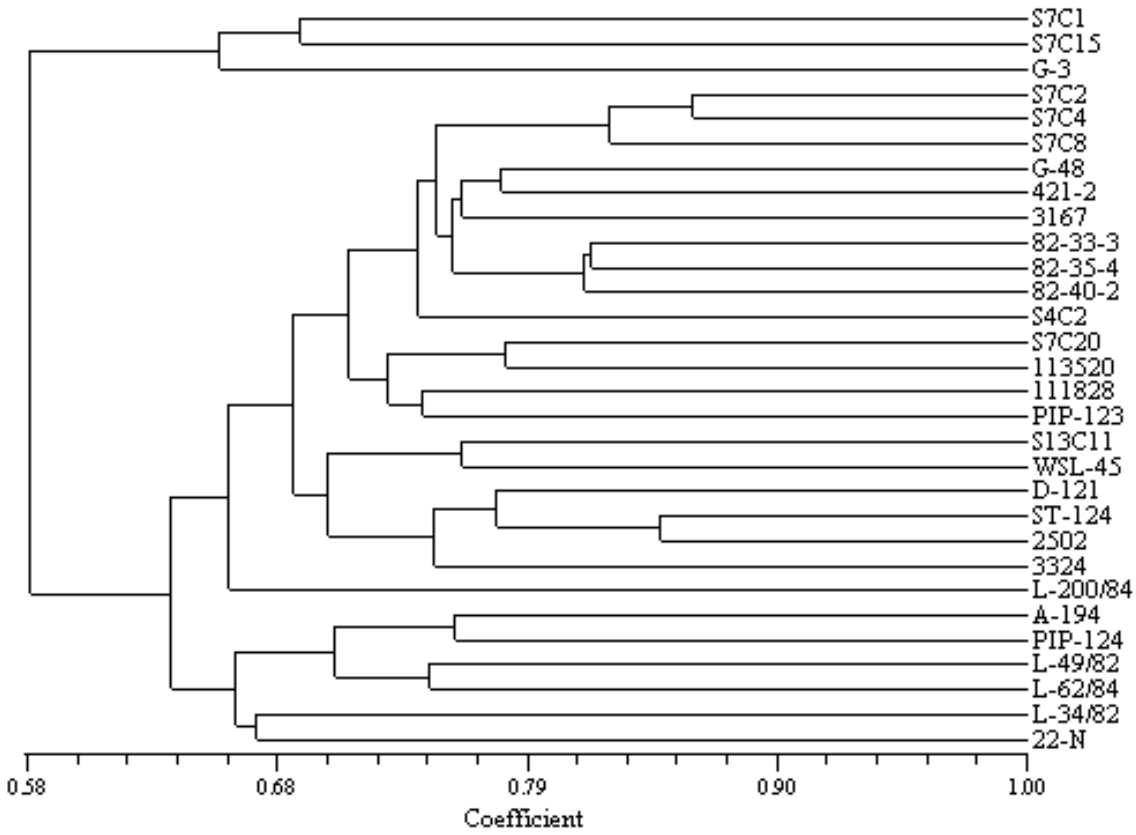

Figure 1. Dendrogram of 30 poplar clones constructed using UPGMA based on Jaccard's similarity coefficients. UPGMA, Unweighted pair group method of arithmetic means.

and 82-40-2), all clones were FRI lines. The fourth group had only one clone S_4C_2 from USA. The fifth group comprised of four clones viz., S_7C_{20}, 113520, 111828 and PIP-123; clone S_7C_{20} was from USA and the other three (113520, 111828 and PIP-123) were FRI lines. The sixth group consisted of two clones $S_{13}C_{11}$ and WSL-45, one clone $S_{13}C_{11}$ was from USA and WSL-45 was WIMCO line. Seventh group consisted of four clones (D-121, ST-124, 2502 and 3324), clone D-121 was from USA, clone ST-124 was from Germany and two clones (2502 and 3324) were FRI lines.

The second major sub-cluster consisted of six genotypes, which bifurcated into two minor sub-clusters. The first minor sub-cluster was bifurcated into two minor sub-clusters again forming two groups, one with two clones (A-194 and PIP-124) which were FRI lines and the other group having two clones (L-49/82 and L-62/84) from Lalkuan selection. The other minor sub-cluster had two clones L-34/82 and 22-N. Out of which, one clone L-34/82 was Lalkuan selection and the other clone 22-N was FRI line.

DISCUSSION

In the present study, the analysis of genetic divergence was carried out following Mahalanobis D^2 statistic in 30 poplar clones using 14 component characters. Morphological data are influenced by age of the tree, geographical area, environment, etc, but molecular data cannot be affected by the environment, this is one of the several advantages of molecular markers over morphological markers.

Clones S_7C_2, S_7C_4, S_7C_{11}, S_7C_8, and S_7C_{20} fell in the same major cluster at morphological and molecular level. In the same way, clones 82-33-3, 82-40-2 and S_4C_2 fell in the common major sub-cluster. Clones 111828 and 113520 were in the same group at both levels. Clones PIP-124 and L-49/82 also shared common pool at morphological as well as molecular levels. Clones G-3 and G-48 were in different main cluster at both morphological and molecular level. For these sets, divergence at molecular level confirms the authenticity of morphological grouping.

Morphologically S_7C_1, L-49/82 and PIP-124 fell in the same cluster, but at molecular level, they were in different main clusters. Clones S_7C_{15} and A-194 were also in the same cluster morphologically, but at molecular level, they were in different main cluster. This may be explained by genotype-environment interaction effects and the different combinations of alleles/genes. Moreover, RAPD

markers provide a better coverage of the whole genome resulting in a better estimate of relationship (Smith and Smith, 1989). In contrast, morphological analysis is based on the limited number of characters that might be highly correlated. These highly correlated characters might dominate the pattern of variation, resulting in the distortion in clustering analysis (Beer et al., 1993). Smith and Smith (1989) suggested that morphological classification is not always the best way to evaluate genetic distance, while molecular markers provided a better estimate of genetic relationship among genotypes. DNA markers can be used to search the cultivars of distinct groups to make heterotic groups, if not to predict actual heterosis for specific combination (Dudley et al., 1991; Melchinges et al., 1992; Stubes et al., 1993). A positive correlation between morphological and RAPD diversity in the present investigation implied that both methods could be useful for genetic distance analysis. In certain cases, agreement between them is of low or moderate magnitude. Use of DNA markers and/or morphological traits for prediction of heterotic performance could maximize opportunities to obtain superior hybrids because there is a higher probability that unrelated genotypes would contribute unique desirable alleles at different loci (Tatineni et al., 1996). However, a major disadvantage of morphological based classification is that addition of new genotypes into cluster would be less accurate because evaluation would have to occur in different environments. In contrast, DNA markers do not suffer the change of environmental condition and give better coverage to the whole genome (Smith and Smith, 1989; Rahman and Rajora, 2002; Yin et al., 2002; Rajora and Rahman, 2003; Zhang and Zhang, 2004). Therefore, DNA marker techniques appear to be a better method in genetic analysis than morphological methods. Due to the fact that RAPD could actively reveal genetic relationship among genotype, all the genotypes should be subjected to RAPD analysis prior to their use for plantations.

The present molecular study was conducted on 30 poplar clones from different sources. Findings reveal that the range of divergences was between 14 and 52%. As depicted by dendrogram, in general, genotypes from an eco-geographical area fell in one group with some exceptions. Grouping of genotypes belonging to an ecological zone indicates prevalence of narrow genetic base. This situation of monoculturing is alarming as outbreak of any disease or pest incidence may surpass this narrow genetic base and wipe out the whole plantation of that ecological zone. On the other hand, diverse genotypes create an aggressive defense line which is relatively tough to break. Therefore, diversified plantations control any epidemic of disease/pest attack more effectively. To promote diversified plantations with the existing genotypes, the selection of genotypes from different groups formed in the dendrogram is recommended. The genotypes should be selected from the groups which are widely apart from each other.

In view of the above discussion, a diversified group comprising of S_7C_1, G-3, 421-2, 82-35-4, PIP-123, D-121, A-194 and 22-N may be formed to make a broad genetic base for the commercial plantation.

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