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Screening interspecific hybrids of *Populus* (*P. ciliata* × *maximowiczii*) using AFLP markers

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Abstract Hybrids of *Populus ciliata* × *maximowiczii* are very vigorous and outperform both the parents in growth performance and yield. Genetic evaluation of 24 of these interspecific hybrids along with the two mother trees (*Populus ciliata*), and five male-parent (*Populus maximowiczii)* genotypes was carried out using the AFLP marker assay. Eight AFLP primer combinations detected 428 markers, of which 280 (66%) were polymorphic. Genetic relationships within the samples were evaluated by generating the similarity matrix based on Jaccard's coefficient. The phenetic dendrograms, as well as the PCO plots, separated the hybrids and the two parent species into three distinct clusters. The hybrids grouped closer to the *P. ciliata* (female parent) cluster as compared to the *P. maximowiczii* (male parent) cluster. The hybrid cluster contained internal groupings, which correlated to some extent with growth performance. The four best performing hybrids (42m1, 65m1, 23m2, Cm2-5-20/91) formed a distinct sub-cluster. Data from a single primer combination was sufficient for distinguishing the hybrids from the parents and assigning paternity. The hybrids showed 22 markers that were absent in *P. ciliata* but were monomorphically present in all the hybrids, suggesting outcrossing and common paternity. Further, these 22 markers were found in all the *P. maximowiczii* genotypes confirming it as the male parent. These male-specific markers can be converted to SCAR markers and used for rapid screening of the *P.ciliata* × *maximowiczii* hybrids. The primer combination E-AAC × M-CAA was identified as most suitable for ascertaining true hybridity. AFLP proves to be a useful tool for screening of *P. ciliata* × *maximowiczii* hybrids at the early stages of development.

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Introduction

The genus *Populus* L.(Salicaceae) comprises nearly 30 species showing considerable variation in the adaptive traits (FAO 1979; Khosla and Khurana 1982). *Populus ciliata* Royle, *Populus glauca* Haines, and *Populus gamblei* Haines are the three species endemic to the Himalayan region (Khurana 2002). The breeding of poplars and their hybrids to produce fast-growing, pest-resistant select clones has been very successfully practiced by plant breeders all over the world. Moreover fast-growing hybrid clones of poplars are developed because, in the due course of time, existing clones tend to decline in vigor or become susceptible to pests and diseases (Khurana and Thakur 1995). Poplar interspecific hybrids are the most important *Populus* being cultivated commercially in many parts of the world. (Zsuffa 1975).

In India, interspecific hybrids between *P. ciliata* (the species endemic to the Himalayan region) of section Ciliata and *Populus maximowiczii* Henry (the cold-hardy species from Japan) of section Tacamahaca were developed recently (Khurana and Thakur 1995). These hybrids were raised either for the sites not amenable to *P. ciliata*, or where *Populus deltoides* Bartr. ex Marsh clones were not found suitable. The *Populus ciliata* × *maximowiczii* F1 hybrids are continuously being evaluated and monitored for their growth characteristics. Based on their nursery and field performance amongst these F1 hybrids, many clones have been selected and the best performing clones are being multiplied. There are many more potential fast-growing hybrids which need to be screened every breeding season, but are not screened owing to limited space in the nurseries. These hybrids need to be properly and precisely analyzed for their true hybrid

nature, variation and heterosis, in order to recommend them for future clonal developmental programmes and breeding work. Initial screening of hybrids for at least some outcrossing or male-parent specific characters (true hybridity) would help in speeding up the tree improvement program.

In a recent study by Rahman and Rajora (2002) it has been shown that DNA based markers could differentiate clones that cannot be discriminated on the basis of their morphological, phenological and allozyme markers. Eleven *P. maximowiczii* clones could be uniquely fingerprinted using few SSR markers (Rahman and Rajora 2002), which was not possible even by using 35 allozyme markers (Rajora 1988). Similar results are shown for a hybrid poplar (*P.* × *canadensis*) where microsatellite markers were found to be nine times more informative than allozyme markers (Rajora and Rahman 2003).

AFLP markers have been utilized in evaluating hybrids and in parentage assignment in many species (VanToai et al. 1996; Krauss 2000; Lima et al. 2002). AFLP studies carried out in soybean revealed that it is possible to assess, with adequate precision and reasonable cost, the parental contributions to subsequent progeny generations (VanToai et al. 1996). Accurate estimation of selfing rates can also be determined by AFLP markers because all outcrossing events can be detected (VanToai et al. 1996). Unambiguous paternity assignment to progeny, and powerful estimates of genetic similarity, has also been obtained in *Persoonia mollis* using AFLP markers (Krauss 2000). The present study was undertaken to screen for hybridity of the recently developed *P. ciliata* × *maximowiczii* hybrids by AFLP assay and to screen for genetic variation within these hybrids.

Materials and methods

Plant material and AFLP analysis

The study was carried out on 24 selected hybrid clones of *P. ciliata* × *maximowiczii,* which were developed using *P. maximowiczii* pollen from Japan and the two *P. ciliata* mother-trees, in the nursery of Dr. Y.S. Parmar University of Horticulture and Forestry, Solan, India. The pollen were received from the Oji paper Company, Japan, but exact location of the source tree could not be traced out; hence, *P. maximowiczii* germplasm of known Japanese origin were included in the study. The growth characteristic of the various hybrids was recorded. The Japanese *P. maximowiczii* leaf material was kindly provided by Dr.Stefano Bisoffi of the Poplar Research Institute, Italy, and Dr. Pierre Perinet of Ministère des Ressources naturelles du Québec, Sainte-Foy (Québec). The list of the genotypes used in the study is given in Table 1.

Genomic DNA was extracted from the freeze-dried leaf material using the modified CTAB method of Doyle and Doyle

Table 1 List of genotypes analyzed

	Code	Species	Clone no.	Geographical location	Height at 9 months (cm)
1	Pc-1	*P. ciliata*	Pc-1	India	*
2	Pc-2	*P. ciliata*	Pc-2	India	*
3	A	Pc × Pm hybrid	220m2	India	NA[a]
4	B	Pc × Pm hybrid	65m1	India	360
5	C	Pc × Pm hybrid	Cm2-5-20/91	India	360
6	D	Pc × Pm hybrid	165m1	India	357
7	E	Pc × Pm hybrid	96m1	India	350
8	F	Pc × Pm hybrid	52m2	India	350
9	G	Pc × Pm hybrid	23m2	India	350
10	H	Pc × Pm hybrid	42m1	India	340
11	I	Pc × Pm hybrid	84m1	India	340
12	J	Pc × Pm hybrid	212m2	India	328
13	K	Pc × Pm hybrid	37m1	India	320
14	L	Pc × Pm hybrid	281m2	India	320
15	M	Pc × Pm hybrid	Cm2-54	India	315
16	N	Pc × Pm hybrid	228m2	India	315
17	O	Pc × Pm hybrid	54M1	India	315
18	P	Pc × Pm hybrid	Cm2-62	India	300
19	Q	Pc × Pm hybrid	6M1	India	300
20	R	Pc × Pm hybrid	95M2	India	285
21	S	Pc × Pm hybrid	207M2	India	275
22	T	Pc × Pm hybrid	4M1	India	270
23	U	Pc × Pm hybrid	84M2	India	240
24	V	Pc × Pm hybrid	60M2	India	238
25	W	Pc × Pm hybrid	227m1	India	217
26	X	Pc × Pm hybrid	46M2	India	200
27	m-1	*P. maximowiczii*	MW05-244	Japan	+
28	m-2	*P. maximowiczii*	MW07-222	Japan	+
29	m-3	*P. maximowiczii*	MW07-232	Japan	+
30	m-4	*P. maximowiczii*	MW12-105	Japan	+
31	m-5	*P. maximowiczii*	93-2	Japan	+

* *P. ciliata* height at 12 months is 87.05Â±10.04
+ *P. maximowiczii* height at 12 months is 139Â±4.3
[a] NA, data not available

(1990). AFLP analysis was performed using the protocol of Zabeau and Vos (1993) with some modifications as given by Singh et al. (1999, 2002). Amplified fragments were resolved on the 6% polyacrylaminde gel and autoradiographed.

Data analysis

All unambiguous AFLP fragments were scored manually for presence (1) or absence (0) across the 31 samples for the eight primer combinations analyzed. The 1/0 data matrix was used to calculate the genetic similarity matrix using Jaccard's coefficient (Jaccard 1908) with the help of NTSYSpc software, version 2.02 (Rohlf 1998). The similarity matrix was subjected to UPGMA clustering (unweighted pair group method analysis; Sokal and Michener 1958) and the dendrogram was constructed. The cophenetic correlation coefficient was calculated to test the goodness of fit between the similarity matrix and the cophenetic matrices (Sneath and Sokal 1973). Principal co-ordinate analysis (PCO) was also carried out. The robustness and reliability of the phenetic dendrogram was tested by bootstrap analysis (Felsenstein 1985) with 1,000 replications, using the WINBOOT software (Yap and Nelson 1995). All the groupings at a branch above 50% confidence limits were considered as statistically significant (Highton 1993).

Results

Analysis of 24 *P. ciliata* × *maximowiczii* hybrids along with its female parental clones (two *P. ciliata* trees) and five *P. maximowiczii* (male parent species) genotypes was carried out. Morphological data was recorded for the hybrids showing extremely vigorous growth. The initial results showed that rooting was profuse in the hybrids in comparison to *P. ciliata*; the leaf size was nearly double that of *P. ciliata* and four times that of *P. maximowiczii*. The *P. ciliata* × *maximowiczii* siblings showed a range of variation in branching pattern from the rosette shape of branching in *P. maximowiczii*, to the simple alternate type in *P. ciliata*. Data on height growth of the hybrids was recorded at the end of the first growing season. The height growth in the hybrids ranged from 200 cm to 360 cm, where as the height growth in a 1 year-old *P. ciliata* was 87.05±10.04 cm and of *P. maximowiczii* was 139±4.3 cm; thereby confirming the induction of vigour in the hybrids by way of fast growth rates as compared to both the parents (Table 1).

AFLP analysis

The AFLP technique was employed to analyze the 31 samples (2 *P. ciliata*, 5 *P. maximowiczii* and 24 *P. ciliata* × *maximowiczii* hybrids; Table 1). Figure 1 shows the AFLP profile generated by using primer combination E-AGC × M-CAT. This primer combination detected 55 bands of which 36 were polymorphic and 19 were monomorphic (Table 2), revealing 65.45% polymorphism. The monomorphic bands may be assigned as *Populus* specific bands as they were present in *P. ciliata* and *P. maximowiczii*, as well as all the hybrids. The primer combination revealed differences between *P. ciliata* and *P. maximowiczii* species based on their banding pattern. In addition, this primer combination clearly indicated that the hybrids showed bands specific to both the *P. ciliata* and the *P. maximowiczii* genotypes, indicating the presence of *P. ciliata* and *P. maximowiczii* genomes in the hybrids. Among the bands which were monomorphic for the hybrids, some were absent in *P. ciliata*, but present in the *P. maximowiczii* lanes, such as '**a1**' and '**a2**' (Fig. 1). On the other hand, bands absent in *P. maximowiczii* genotypes but present in the mother and the hybrids were also detected, such as band marked '**b** (Fig. 1). Bands such as '**c**', '**d**' and '**e**' (Fig. 1), showing polymorphic banding pattern among the hybrids, were also detected.

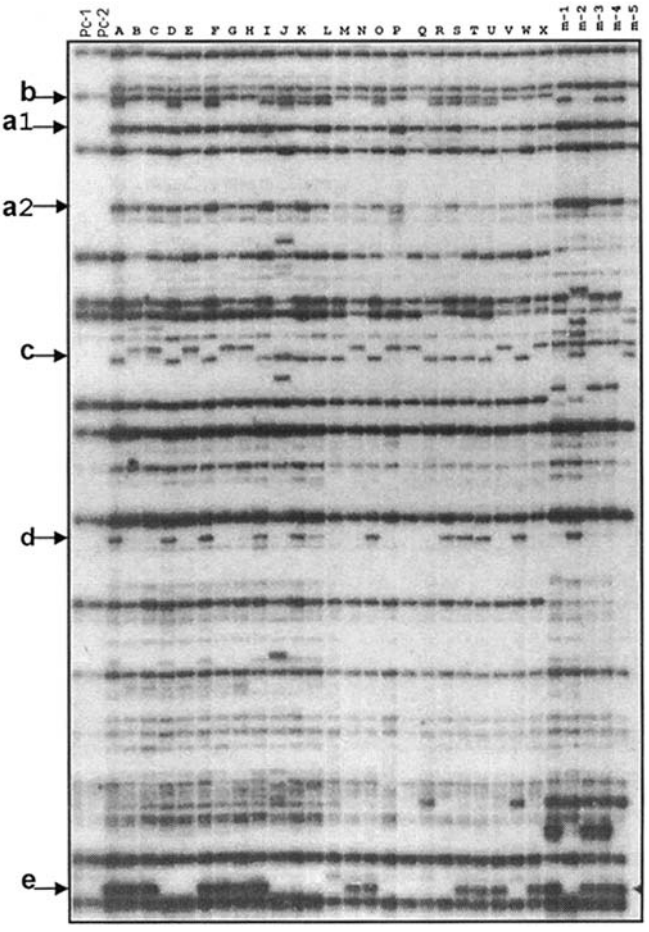

Fig. 1 Autoradiograph of an AFLP fingerprint revealed by the primer combination E-AGC × M-CAT. The first *two lanes* are of the two *P. ciliata* mother trees (labelled Pc-1 and Pc-2), followed by the 24 hybrids (*lanes A to X*). The last five lanes are of the five *P. maximowiczii* genotypes (*labelled m-1 to m-5*). Bands marked '*a*' and '*b*' indicate the *P. maximowiczii* and *P. ciliata* species-specific loci respectively. Bands marked '*c*', '*d*' and '*e*' show the polymorphic banding pattern among the hybrids

The 31 genotypes were further analyzed using eight different AFLP primer combinations which revealed a total of 428 fragments of which 280 were polymorphic between two or more genotypes (Table 2). The number of polymorphic fragments detected per primer combination

Table 2 Number of bands and polymorphism detected by eight AFLP primer combinations in the 31 genotypes

Primer combination	Total bands	Polymorphic bands	Monomorphic bands	Percent polymorphism
E-AAC × M-CAA	57	45	12	78.95
E-AAG × M-ACA	49	32	17	65.31
E-AAG × M-CTC	47	34	13	72.34
E-ACC × M-CAA	48	39	9	81.25
E-AGC × M-CAT	55	36	19	65.45
E-AGC × M-CTC	57	37	20	64.91
E-ACG × M-CAG	64	30	34	46.88
E-ACG × M-CTA	51	27	24	52.94
Total	428	280	148	65.40
Average	53.5	35	18.5	66.00

Table 3 Number of bands and polymorphism detected by different data sets

Item	All 31 genotypes	Only hybrids	Hybrids+Mothers	Hybrids+*P. maximowiczii*
Total no. of bands	428	401	401	428
Monomorphic bands	280	246	219	195
Polymorphic bands	148	155	182	195
Percent polymorphism	65.4	38.65	45.39	54.44

ranged from 27 to 45, and the percentage of polymorphic fragments ranged from 46.88% to 81.25% (Table 2).

The complete AFLP dataset of 31 samples was divided into three different subsets to study the band-sharing pattern between the hybrids and the parents:

1. A 24 sample data subset comprised of only the hybrids,
2. A 26 sample data subset comprised of the 24 hybrids and two *P. ciliata,* and
3. A 29 sample data subset comprised of the 24 hybrids and the five *P. maximowiczii* genotypes (Table 3).

In the complete data of 31 samples 428 AFLP markers were generated, of which 401 markers were present in the hybrids (Table 3). All the 401 bands present in the hybrids were detected in either the *P. ciliata* or *P. maximowiczii* genomes, or in both. Analyzing only the hybrids, 38.65% polymorphism was found which increased to 45.59% when the two mother trees were also included in the analysis (Table 3). The percent polymorphism was 54.44, when the five *P. maximowiczii* genotypes were also included in the study (Table 3). The data subset consisting of hybrids and *P. maximowiczii* samples was analyzed in which all the 428 bands were found (Table 3). There were 27 bands exclusive to the *P. maximowiczii* genome. The data subset comprising only the hybrids and mother trees revealed a total of 401 bands (Table 3). Out of these 401 bands, 119 bands were found to be absent in *P. ciliata* but present in the hybrids, indicating that they were outcross progenies. Further, out of the 119 markers, 22 were found to be monomorphic among the hybrids and were also detected in the *P. maximowiczii* lines, hence confirming it to be the male donor.

Determination of genetic similarity and cluster analysis

The AFLP 1/0 data matrices were used to determine the genetic similarity between the 31 genotypes. Initially Jaccard's similarity matrices were calculated on two data sets, 'total scorable bands' (428 bands) and 'polymorphic bands only' (280 bands) (data not shown). As both the data sets revealed similar interpretation of the genetic similarity among the samples, for further analysis only the total-band data set was used. The cophenetic correlation was very high for the two similarity matrices (r=0.99). Data from eight individual primer combinations were analyzed separately, as well as after the successive addition of data from different primer combinations. The Jaccard's similarity coefficient (GS_J) for the 465 possible pairs of 31 genotypes ranged from 0.522 (between genotypes PM-3 and PC-1) to 0.971 (between genotypes 96m1 and 95M2); data not shown. *P. ciliata* and *P. maximowiczii* shared very low genetic similarity with the GS_J value ranging from 0.5222 to 0.586 (between PM-3 and PC-2, and PM-5 and PC-1, respectively), which is an indication of their being diverse germplasm ideal for hybridization.

Cluster analysis of the 31 samples showed a clear separation of the two different species and the hybrids (Fig. 2). Three major clusters were obtained which contained the *P. ciliata* genotypes (cluster PC), the *P. maximowiczii* genotypes (cluster PM) and the hybrid genotypes (cluster PH), as three distinct groups (Fig. 2). The five *P. maximowiczii* genotypes separated as a distinct cluster at a genetic similarity value of 0.68 from the hybrids and the female parents. The *P. ciliata* genotypes grouped out at the 0.72 similarity coefficient from the hybrids. All the hybrids grouped very close to each other in the PH cluster. To describe the relationship among the hybrids, the cluster PH was subdivided into

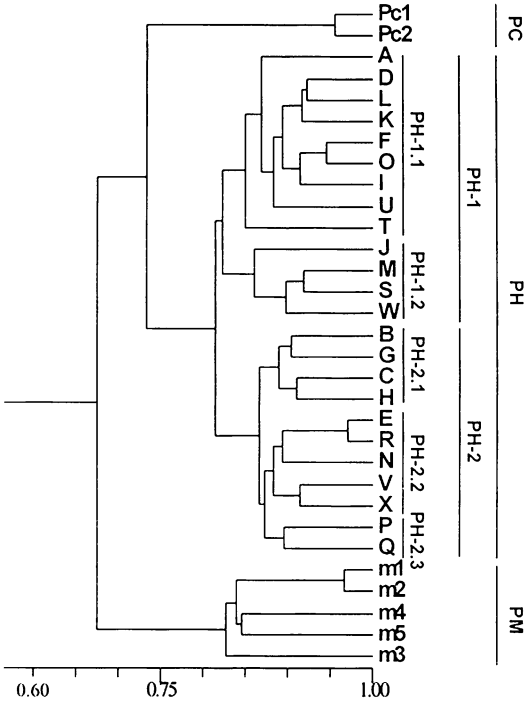

Fig. 2 UPGMA dendrogram based on Jaccard's similarity coefficient. Major clusters are marked as PC, PH, and PM indicating the *P. ciliata*, the hybrids and the *P. maximowiczii* groupings, respectively. See Table 1 for details

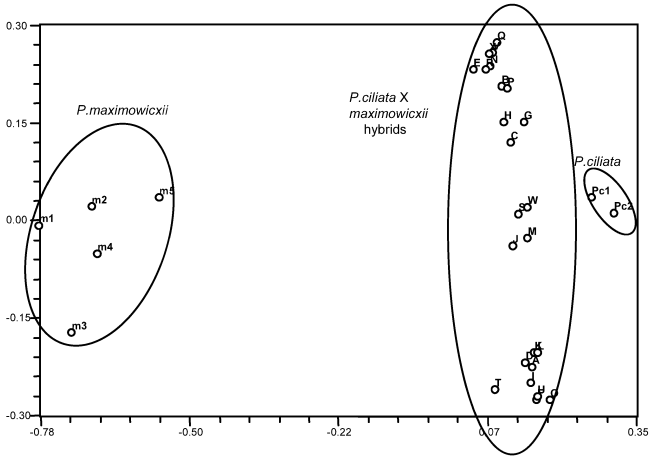

Fig. 3 PCO scatter plot showing the distinct distribution of *P. ciliata*, hybrids and *P. maximowiczii* into different groups in the two dimensions

smaller groupings. Two sub-clusters were assigned within the hybrid main cluster, which are marked as PH-1 and PH-2 in Fig. 2. These two sub-clusters were identified at the 0.81 similarity coefficient. The PH-1 sub-cluster was further discriminated into two groups: PH-1.1 (containing the hybrids A, D, L K, F, O, I, U and T) and PH-1.2 (containing hybrids J, M, S and W). Similarly, the hybrids in sub-cluster PH-2 were grouped into three sub-groups, PH-2.1 (containing samples B, C, G and H), PH-2.2 (containing samples E, R, N, V and X) and PH-2.3 (containing samples P and Q). Most of the groupings showed significant confidence levels on bootstrap analysis with the nodes being supported by 99% to 40.8% confidence limits (data not shown). Principal co-ordinate analysis (PCO) was carried out and the two dimensional scatter-plot (Fig. 3) also showed a clear-cut separation of the hybrids, *P. maximowiczii* and *P. ciliata* genotypes, in relation to the first two principal axes of variation.

Comparison to height-growth

The molecular data was also utilized to check for any correlation with the growth performance in terms of the height-growth of the hybrids (Table 1). In the dendrogram (Fig. 2), the hybrid T (270 cm) separates out first in sub-cluster PH-1.1, leading to two smaller groupings in the remaining cluster. One of the group comprises of D, L and K, which are the hybrids with a very fast growth nature (height at 9 months = 320–360 cm), and the hybrid A whose growth data is not available. In the second group (Fig. 2), the hybrid U with a height of 240 cm separates from the remaining hybrids (F, O and I) whose heights are more than 300 cm. Thus, there may be a correlation between height-growth and the clustering pattern. Similarly, the sub-cluster PH-2.3 comprises P and Q, which have very similar growth (310 and 300 cm respectively). The sub-cluster PH-2.1 includes the best performing hybrids among those analyzed. This cluster includes hybrids B, C, G and H which not only outperform the parents in having a fast growth rate (height at nine months = 360, 350, 340, and 360 cm respectively), but also have a high petiole to nerve ratio (P/N ratio = 24.75, 22, 61.9 and 20.3; data not shown). The hybrids E, N, R, V and X group together (sub-cluster PH-2.2), though they show varying growth pattern ranging from 200 to 350 cm height at 9 months (Table 1). A perfect correlation between the height-growth and clustering pattern is not obtained. The association of markers with the trait of interest would help in establishing a good correlation, and would form the next part of the study.

Discussion

The hybrids between *P. ciliata* and *P. maximowiczii* are vigorous and easy to root and are currently undergoing field performance trials. Continuous efforts are being made to develop this hybrid, and initial screening is the most important step towards rejecting the progeny which is not a true hybrid (Khurana and Thakur 1995). Molecular markers find wide utility in tree breeding programs including that of poplars (Bradshaw et al. 1994; Cervera et al. 1997). The usefulness of AFLP markers for its utility to screen the *P. ciliata* × *maximowiczii* hybrids has been shown in the present study.

The polymorphism detected by AFLP depends on the species under investigation and the different primer combinations being utilized (Ellis et al. 1997). Therefore, data from eight individual primer combinations were analyzed separately, as well as after successive addition of the data from different primer combinations. The primer combinations tested could uniquely fingerprint all the 31 genotypes. The best primer combinations identified (E-AAC × M-CAA) showed 78.95% polymorphism and was able to detect the highest number of outcrossing events.

The AFLP profiles produced very informative banding patterns with some species-specific bands being amplified. AFLP fragments, present exclusively in only one species or landrace, have been amplified for *Isatis* spp. (Gilbert et al. 2002) also. Species-specific alleles have also been detected in poplars using microsatellite markers (Rahman and Rajora 2002). The detection of species-specific bands can be very useful for easy and early detection of any outcrossing event as well as assigning parentage. The *P. maximowiczii* specific bands can thus be easily utilized for ascertaining paternity to the hybrids. The results detected 22 such fragments which may be converted to SCAR markers and can be used to screen the newly raised hybrids of *P. ciliata* × *maximowiczii* for testing the hybrid nature. This study also helped in identifying genetic variation among the hybrids, which may be useful in further breeding programs.

The species-specific bands may have contributed towards the differentiation of genotypes into the three main clusters seen in the dendrogram. This species-specific clustering pattern of the hybrids has also been observed in papaya (Van Droogenbroeck et al. 2002) and coffee (Steiger et al. 2002). Our results show the clustering of the *P. ciliata* × *maximowiczii* hybrids with the two parent species, with the hybrid cluster being closer to the *P. ciliata* cluster. The maternal bias detected in the present study can be attributed to the inherent ability of the hybrid genome to preserve the female genome. This bias may also be due to the maternal inheritance of the organellar genomes, and the fact that the AFLP technique targets nuclear as well as organellar DNA. In the PM cluster the five *P. maximowiczii* genotypes showed some variation among themselves which is consistent with earlier reports on the chloroplast DNA studies of *Populus* that had revealed intraspecific variation within *P. maximowiczii* species (Rajora and Dancik 1995).

An interesting finding was that the internal groupings of the hybrids in the dendrogram were related with the growth performance. The sub cluster PH-2.1 was especially interesting as it included the hybrids 65m1, 23m2, 42m1 and Cm2-5-20/91, which not only outperform the parents exceedingly in having a fast growth rate, but also have a high petiole to nerve ratio. Field data indicates that 65m1 and 42m1 are among the best-performing hybrids based on morphological screening. Kopp et al. (2002) showed the utility of AFLP in selecting parents whose progeny will be highly variable. AFLP studies in *Salix* for predicting variability in juvenile height-growth, though not completely successful, detected a strong negative correlation between mean female-parent similarity-indices and the standard deviation of height of the half-sib progeny (Kopp et al. 2002). It would be very useful if a good correlation between the growth performance and AFLP markers can be established in *P. ciliata* × *maximowiczii* hybrids. The field performance of hybrid poplars cannot be accurately predicted based on the measurement of morphological variables of greenhouse-grown trees (Promnitz and Wray 1976). The identification of molecular markers associated with the growth performance will be very useful in screening at an early stage of development.

In summary, AFLP markers discriminate successfully both *P. ciliata* and *P. maximowiczii* species and their hybrids into distinct groups. Primer combination E-AAC × M-CAA is very useful in easy and early screening of the putative hybrids. The results indicate the usefulness of the AFLP technique, in not only identifying true hybrids and detecting variation among them, but also in identifying better-performing hybrids. If AFLP fragments exclusive to the required characteristic can be converted to SCAR marker, it will help in easy detection of useful hybrids at an earlier stage. This would save time and resources in maintaining plants prior to morphological screening and, therefore, significantly improve plant selection for breeding of the *P. ciliata* × *maximowiczii* hybrids.

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