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Genetic relationships and clonal identity in a collection of commercially relevant poplar cultivars assessed by AFLP and SSR

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Abstract A collection of 66 poplar commercial clones widely cultivated in Italy, China and in other countries of southern Europe and belonging to various poplar species and hybrids, have been fingerprinted using both amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) techniques. Three AFLP primer combinations and six SSRs unambiguously genotyped the analysed poplar collection, with the exception of three groups of six, four and two individuals, which turned out to be indistinguishable even if they met the standards currently applied for distinctness, uniformity and stability (DUS) testing when registered. High levels of variation were detected with both molecular techniques; a total of 201 AFLP bands were amplified of which 96% turned out to be polymorphic and up to 15 SSR alleles were identified at a single locus, with a mean of 9.3 alleles per locus in the case of *Populus* × *canadensis*. The probability of matching fortuitously any two genotypes at all the SSR loci in the case of *P.* × *canadensis* was less then 7.5×10^{-9}. The AFLP-derived dendrogram and principal coordinate analysis (PCOORDA) clustered the clones with respect to their taxonomic classification, and allowed their genetic interrelationships to be established. Correct identification of poplar varieties is essential for ensuring the effective correspondence between the real and the declared identity of a clone, to avoid commercial frauds, and to establish breeding programmes. Molecular markers may play a major role to satisfy all these needs.

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Introduction

Trees of the genus *Populus* L. (*Salicaceae*), i.e. poplars, cottonwoods and aspens, are major suppliers of industrial wood and fiber in many countries of temperate areas of the world. Their fast growth makes them attractive to farmers, who can obtain a quick return from their investments, through short rotations ranging between 5 and 20 years. Easy and cheap vegetative propagation of poplars was exploited by breeders in creating clonal cultivars. The homogeneity of size and wood quality of clonal plantations is highly appreciated by the industry in that minimal adjustments to processing operations are required, even for quality-demanding productions such as thin-layer plywood.

A proper identification of cultivars is therefore of paramount importance in commerce, in order to protect the legitimate interests of poplar breeders, growers and industry. The European Union requires that propagation materials should be accompanied by a certificate of origin or of clonal identity. Control and certification are demanded by nationally appointed authorities. However, controls are mainly based on the regularity of documents: certificates, registers, labels, invoices, etc. A control of the correspondence between the identity reported on documents and the real identity of clones is extremely difficult for a number of reasons:

(i) Observable traits are often affected by environmental factors.
(ii) Distinctive characteristics may be observable for a limited period of the year, while on site controls in nurseries can only be occasional.
(iii) Individual descriptions are of limited use if two or more similar clones cannot be observed simultaneously.

The most comprehensive description system based on phenotypic traits is that adopted by UPOV (1981). UPOV requires clones proposed for protection in countries adhering to the UPOV Convention, to be accompanied by a de-

tailed description according to 64 morphological traits. However, the only use made of the description is to help authorities in charge of the technical examination (the so-called DUS tests, for 'distinction', 'uniformity' and 'stability') to appropriately select similar varieties to be compared with the new one. DUS tests in fact do not produce a "one and for all" description of clones, but only state if the new clone can be distinguished from those already known and having similar declared features, when they have been observed simultaneously in the same environment. Overall this method for clonal identification is difficult, ambiguous, time consuming and subjective.

Since the late 1980s, allozymes (Rajora 1988, 1989a–c; Rajora and Zsuffa 1989, 1991) and random amplified length polymorphism (RAPD—Castiglione et al. 1993; Lin et al. 1994; Sigurdsonn et al. 1995; Rajora and Rahman 2003) markers have been used for poplar cultivar identification. However allozyme markers are limited due to the restricted number of polymorphisms they can detect; as a consequence they are sometimes unable to unambiguously discriminate the analysed cultivars (Rajora and Zsuffa 1989).

The problem of characterisation of poplar cultivars through allozyme markers can now be overcome with the use of modern molecular techniques such as microsatellite and RAPD markers (Rahman et al. 2000; Rahman and Rajora 2002; Rajora and Rahman 2003). RAPD use however has been questioned many times because of the lack of reproducibility (Riedy et al. 1992; Ellsworth et al. 1993; Jones et al. 1997; Pérez et al. 1998). In comparison, AFLP and SSR markers are highly polymorphic and reproducible (Jones et al. 1997; Mueller and Wolfenbarger 1999). Both techniques have already demonstrated their effectiveness in identifying duplicated individuals in poplar (Arens et al. 1998; Winfield et al. 1998; Fossati et al. 2003; Storme et al. 2004), in estimating genetic diversity and in genotyping cultivars of commercial relevance in various plant species (Powell et al. 1996; Mueller and Wolfenbarger 1999; Fossati et al. 2001; Steiger et al. 2002; Esselink et al. 2003), including a limited set of *P.* × *canadensis* clones (Rajora and Rahman 2003). In the latest case SSRs proved capable of distinguishing the poplar clones Canada—Blanc and Ostia, which had shown identical allozyme pattern in a previous study (Rajora and Zsuffa 1989). Moreover SSR genotyping has already been proposed for construction of databases where new varieties or new markers can be easily added (Bredemeijer et al. 2002; Röder et al. 2002; Esselink et al. 2003).

For all these reasons molecular methods can be considered the most suited to distinguish identical genotypes from seedling-derived plants (in an obligate out-breeding species such as poplar). In this aspect, they are superior to studies based on morphology as those used for the DUS tests. However, molecular methods (based on neutral markers as SSRs) cannot predict whether different genotypes will be distinguishable in morphology or in relevant agricultural traits. A useful distinction for the application of molecular markers is, therefore, between identifying character states useful for granting plant breeders' rights and those useful for recognition of a variety. As suggested by Cooke et al. (2003) to help in solving this problem an alternative would be to pay attention to those markers which are linked to expressed parts of the genome, preferably those with known functions, and ideally those linked to the phenotypic characteristics that are currently assessed. Since such characteristics are mostly under active selection pressure, fewer difficulties with uniformity and/or stability would be expected.

In the present study, the AFLP technique was used to fingerprint 66 of the most relevant poplar commercial clones from different poplar species and hybrids registered in Italy and/or in other European countries (Castiglione et al. 1993) and to determine their genetic interrelationships. A subset of these clones (*P.* × *canadensis*; *P. deltoides* and *P. nigra*) was also fingerprinted at six SSR *loci* to verify the presence of duplication and redundancy.

Materials and methods

Plant material and DNA extraction

The 66 registered poplar clones (Table 1) used in this study and belonging to various poplar species and hybrids were kindly provided by the Istituto Sperimentale per la Pioppicoltura (I. S. P.-Casale Monferrato, Italy).

DNA was extracted from leaf tissues using the DNeasy Plant Extraction kit by Qiagen, following the supplier's instructions. DNA concentration was determined by agarose gel electrophoresis and ethidium bromide staining.

AFLP analysis

AFLP markers were generated as described by Vos et al. (1995). Three hundred nanograms of DNA were used for the restriction reaction. The second amplification reactions were performed with primers carrying three selective bases at the 3′ end (primer E32=E+AAC; primer E33=E+AAG; primer E44=E+AGC; primer M36=M+ACC; primer M40=M+AGC). The *Eco*RI primer was radiolabelled with γ-^{33}P. Reactions were separated on 6% polyacrylamide gels, then gels were dried and then exposed to X-ray films.

SSR analysis

Six SSR *loci*, developed from *Populus trichocarpa* (PMGC014; primer sequence available at the web site http://www.cfr.washington.edu/research.poplar/pmgc.ssr/) and *P. nigra* (WPMS09, WPMS14, WPMS16, WPMS18 and WPMS20; Van der Schoot et al. 2000; Smulders et al. 2001) were used for DNA fingerprinting of the commercial *P. deltoides* (6), *P. x canadensis* (49) and *P. nigra* (2) clones. Amplification conditions, thermal profiles and gel electrophoresis were as described by Van der Schoot et al. (2000) and Smulders et al. (2001, 2002) with the exception that the forward primer of each SSR *locus* was γ-^{33}P labelled. A recently published genetic linkage map of *P. nigra* (Cervera

Table 1 List of the commercial clones analysed in this study. (a), (b), (c) Groups of clones which turned out to be duplicated genotypes under the molecular analysis

Number	Name of the clone	Species	Sex
1	A4A	*P.* × *canadensis*	F
2	302 S. Giacomo (a)	*P.* × *canadensis*	F
3	Adda	*P.* × *canadensis*	F
4	Adige (a)	*P.* × *canadensis*	F
5	Altichiero	*P.* × *canadensis*	M
6	Ballottino	*P.* × *canadensis*	?
7	Blom	*P. trichocarpa*	M
8	BL Costanzo (b)	*P.* × *canadensis*	F
9	Boccalari (a)	*P.* × *canadensis*	F
10	Branagesi (a)	*P.* × *canadensis*	F
11	Brenta	*P.* × *canadensis*	F
12	Cappa Bigliona (b)	*P.* × *canadensis*	F
13	Carolina di Santena[a]	*P.* × *canadensis*	F
14	Carpaccio	*P.* × *canadensis*	F
15	Cima	*P.* × *canadensis*	F
16	Dora[a]	*P.* × *canadensis*	F
17	Dorschkamp	*P.* × *canadensis*	M
18	Heidemij	*P.* × *canadensis*	M
19	Gattoni (a)	*P.* × *canadensis*	F
20	Gerlica	*P.* × *canadensis*	M
21	Ghoy	*P.* × *canadensis*	F
22	Guardi	*P.* × *canadensis*	F
23	I-154	*P.* × *canadensis*	M
24	I-214	*P.* × *canadensis*	F
25	I-262	*P.* × *canadensis*	M
26	I45/51	*P.* × *canadensis*	M
27	I-455	*P.* × *canadensis*	F
28	I-476	*P.* × *canadensis*	M
29	I-488	*P.* × *canadensis*	F
30	Lambro[a]	*P.* × *canadensis*	M
31	Luisa Avanzo	*P.* × *canadensis*	F
32	Marilandica	*P.* × *canadensis*	F
33	MC (b)	*P.* × *canadensis*	F
34	Mella	*P.* × *canadensis*	F
35	Neva	*P.* × *canadensis*	F
36	NND	*P.* × *canadensis*	F
37	PAL S39	*P.* × *canadensis*	F
38	Pan (b)	*P.* × *canadensis*	F
39	Panaro	*P.* × *canadensis*	M
40	Patrizia Invernizzi	*P.* × *canadensis*	F
41	Pegaso	*P. generosa* × *P. nigra*	M
42	Robusta	*P.* × *canadensis*	M
43	Rochester	*P. nigra* × *P. maximowiczii*	F
44	S. Martino[a]	*P.* × *canadensis*	F
45	Serotina	*P.* × *canadensis*	M
46	Sesia	*P.* × *canadensis*	F
47	Soligo[a]	*P.* × *canadensis*	M
48	Stella Ostigliese (a)	*P.* × *canadensis*	F
49	Stura[a]	*P.* × *canadensis*	F
50	Tardif de Champagne	*P.* × *canadensis*	?
51	Taro[a]	*P.* × *canadensis* × *P.* × *interamericano*	M
52	Timavo	*P.* × *canadensis*	M
53	Triplo	*P.* × *canadensis*	M
54	Jean Pourtet	*P. nigra*	M
55	Vereeken	*P. nigra*	M
56	Dvina	*P. deltoides*	M
57	Harvard (c)	*P. deltoides*	M
58	Lena	*P. deltoides*	M
59	Lux (c)	*P. deltoides*	F
60	Oglio	*P. deltoides*	M
61	Onda	*P. deltoides*	M
62	Eridano	*P. deltoides* × *P. maximowiczii*	M
63	Sile	*P. deltoides* × *P. ciliata*	F
64	Beuprè	*P.* × *generosa*	F
65	Fritzi Pauley	*P. trichocarpa*	F
66	Villafranca	*P. alba*	F

[a]Clones which have been traditionally reported as *P.* × *canadensis*, which turned out to be *P. deltoides* under the molecular analysis

et al. 2001) indicated that most of the investigated loci (WPMS09, WPMS16, WPMS20 and PMGC014) belong to separate linkage groups, therefore we considered these markers as independent. For WPMS18 and for WPMS14 data were not available, in fact the two microsatellites could not be mapped by means of this pedigree.

Data analysis

AFLP fingerprints were evaluated by visual inspection of autoradiograms. DNA bands were scored as binary characters for their presence (1) or absence (0). The AFLP data were used to estimate similarities between the genotypes, using the statistical package NT-SYS PC, version 2.1 (Exeter Software Co., New York). Similarity–dissimilarity matrices were computed with Jaccard's coefficient for qualitative data and then processed by principal coordinate analysis (PCOORDA). A dendrogram was constructed by cluster analysis based upon the unweighted pair-group method with arithmetical averages (UPGMA, Sneath and Sokal 1973) algorithm. "Goodness" of the dendrogram was verified using the MXCOMP program and the correlation coefficient was calculated. The MXCOMP program allows direct comparison of the original similarity matrix and the cophenetic value matrix derived from the dendrogram, as described by Rohlf (2000). A correlation coefficient r higher than 0.9 is considered indicative of high confidence of the produced dendrogram. Bootstrap analysis (Felsenstein 1985), with 1,000 bootstrap re-samples, was computed using Win boot (Yap and Nelson 1996) to determine the confidence limits of the UPGMA dendrogram. The NT-SYS PC and Win boot programs produced the same main clusters,

however further sub-groupings were not unequivocal and are not discussed in this work.

The number of clones with a unique fingerprint at each primer combination was also recorded.

In the case of SSR analysis, to facilitate the scoring and recording of the alleles we attributed an alphabetical letter to each allele, starting with the uppercase A letter for the largest one (Smulders et al. 2002). This procedure avoids the need to estimate the exact size in terms of base pairs of each allele; moreover the data recorded can be directly used for population studies when a software package such as POPGENE 1.32 is employed. Allele number and the number of observed genotypes were recorded; the polymorphic information content (PIC) of an SSR *locus* was calculated as described by Saal and Wricke (1999):

$$\mathrm{PIC} = 1 - \sum_{i=1}^{k} pi^2$$

where p_I is the frequency of the *I*th allele out of the total number of alleles at an SSR *locus* and k is the total number of different alleles for that *locus*.

Genotypes of individual commercial clones were determined from their allelic constitution. The number of clones with a unique fingerprint at each SSR *locus* was also calculated and an assignment test was run to verify the doubtful classification of several clones; this analysis was facilitated by the use of the freeware software GeneClass, available at the web site http://www.ensam.inra.fr/CBGP.

The matching probability (M) for two individuals of the investigated collection was also estimated in the case of SSR loci.

The algorithm used to calculate M is the following:

$$M = \left[\prod_{i=1}^{k} \frac{ni(ni+1)}{2}\right]^{-1}$$

where k is the number of observed alleles at locus i. M attributes to each allele of the assessed loci the same chance to be found in the collection and it doesn't take into account the frequency of each revealed allele because no information is available relatively to the population size employed during the breeding programme, or to the natural population from which the poplar clone was harvested and selected.

Results

AFLP analysis

In this study the AFLP technique was adopted to fingerprint a total of 66 poplar commercial cultivars (listed in Table 1), registered as original genotypes of different poplar species and hybrids. The three used primer combinations (E32–M36; E33–M36; E44–M40) produced 53, 91 and 57 scorable fragments respectively, of which 48, 88 and 57 were polymorphic over all the species. Under the analysis of the 201 total scored fragments, 54 out of the 66 accessions showed an unique AFLP profile, while the remaining 12 cultivars clustered into three groups composed by individuals with identical AFLP profiles. Of the polymorphic fragments, 16 (7.9%) were only present, and 10 (4.9%) only absent in the clone Villafranca (*P. alba*). A single fragment was observed only in the two *P. trichocarpa* clones (Blom and Fritzi Pauley); 11 DNA frag were always absent from the *P. deltoides*, *P. nigra* and *P.* × *canadensis* clones, while present in all the other poplar species. Within the clones belonging to the species *P. deltoides*, 95 fragments were recorded, of which 42 (44%) turned out to be polymorphic. Of the 168 bands present in the *P.* × *canadensis* clones, 137 (80%) were polymorphic. Finally, the two *P. nigra* genotypes analysed (Jean Pourtet and Vereeken) displayed a total of 87 bands, of which 13 (15%) could distinguish the two clones.

The complete data set of 201 fragments was used to investigate the differences among the commercial poplar cultivars at the DNA level. A similarity matrix based on Jaccard's coefficient was calculated and subsequent UPG MA cluster analysis (Fig. 1a) and PCOORDA (Fig. 1b) were performed. In both cases distinct clusters of *Populus* species and hybrids were observed. In particular, the statistical analysis run from the AFLP similarity matrix separated the 66 clones in three major clusters corresponding to *P.* × *canadensis*, *P. nigra* and *P. deltoides* (Fig. 1a, b) with bootstrap values of 100% (distinguishing the *P. deltoides* cluster from that composed by *P. nigra* and *P.* × *canadensis*) and of 65% (further dividing *P.nigra* from *P.* × *canadensis*). Moreover the dendrogram showed that the clones Beauprè, and Eridano, were linked with the *P. deltoides* group (Fig. 1a) with a bootstrap value of 50%. The *P. deltoides* group consisted of 13 instead of only 6 individuals as expected on the bases of their passports, suggesting a possible case of misclassification of poplar clones Carolina di Santena, Dora, Lambro, San Martino, Soligo and Stura which have been previously registered as *P.* × *canadensis* (Fig. 1a, b). The two clones Sile (*P. deltoides* × *P. ciliata*) and Taro (*P. deltoides* × *P. interamericana*) were also associated to the *P. deltoides* group by the statistical analysis.

The dendrogram of Fig. 1a also showed that three groups of six (302 San Giacomo, Adige, Boccalari, Branagesi, Gattoni and Stella Ostigliese), four (BL Costanzo, Pan, Cappa Bigliona and MC) and two (Lux and Harvard) individuals were indistinguishable based on their similarity value of Jaccard's index, which was always below the error limit of the AFLP technique supposed in the case of poplar by Arens et al. (1998) and Winfield et al. (1998). Finally, two groups of two individuals (I-262/I-455 and Robusta/Hejdemij) showed a similarity of 95% (Fig. 1a), a value which is within the bounds of the scoring errors for AFLPs (Winfield et al. 1998). As a consequence, on the basis of the AFLP analysis it was not possible to ascertain if the

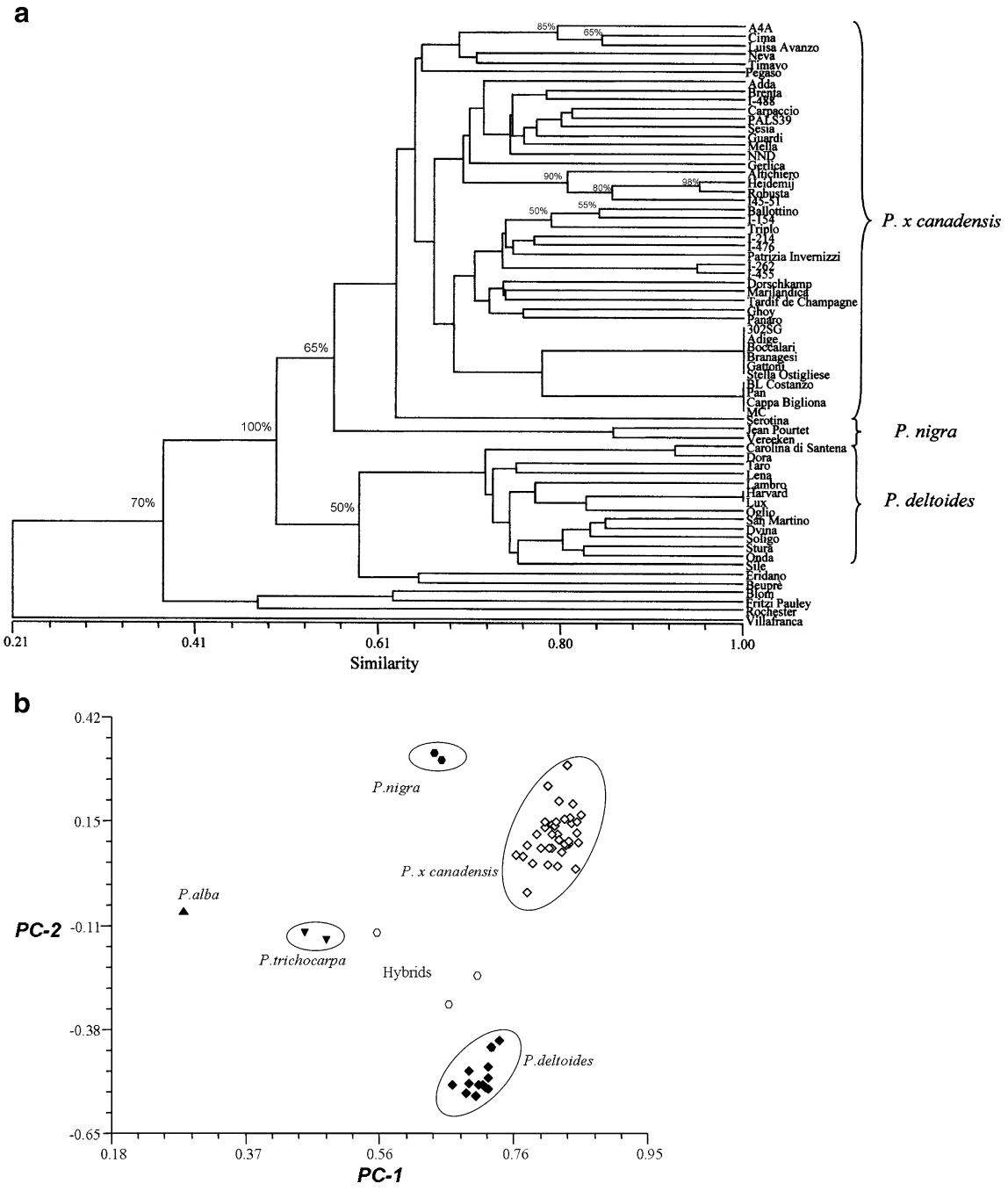

Fig. 1 UPGMA cluster analysis with bootstrap values (**a**) and PCOORDA analysis (**b**) of 66 poplar cultivars based on 201 AFLP fragments. *Filled triangle* sample 66, *open circle* samples 43, 62, 64, *filled circle* samples 54, 55, *filled inverted triangle* samples 7, 65, *filled diamond* samples 13, 16, 30, 44, 47, 49, 51, 56, 57, 58, 59, 60, 61, 63, *open diamond* samples 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 14, 15, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 45, 46, 48, 50, 52, 53. Numbers correspond to samples as in Table 1

individuals within the two groups had to be regarded as distinct genotypes or as clones.

Robustness of the dendrogram was confirmed by its high correlation coefficient (r=0.94) between the original similarity matrix and the cophenetic value matrix derived from the dendrogram.

SSR analysis

A subset of 57 poplar clones composed by *P. deltoides*, *P.* × *canadensis* and *P. nigra* as described in Materials and methods, was fingerprinted at six SSR *loci* (Table 2) and the allelic composition of each clone at each SSR

Table 2 SSR DNA *loci*, number of alleles, number of genotypes and polymorphic information content (PIC) calculated for the *P.* × *canadensis* (49), *P. nigra* (2) and *P. deltoides* (6) commercial clones

Locus	No. of alleles			No. of genotypes			No. of cultivar with unique fingerprint			PIC	
	P. deltoides	*P. nigra*	*P.* × *canadensis*	*P. deltoides*	*P. nigra*	*P.* × *canadensis*	*P. deltoides*	*P. nigra*	*P.* × *canadensis*	*P. deltoides*	*P.* × *canadensis*
PMGC014	2	3	8	3	2	12	1	2	5	0.480	0.810
WPMS09	1	2	11	1	2	11	0	2	2	0	0.642
WPMS14	6	3	15	5	2	27	2	2	15	0.720	0.880
WPMS16	5	3	7	3	2	12	1	2	4	0.823	0.720
WPMS18	3	1	9	1	2	9	1	2	4	0	0.560
WPMS20	3	2	6	2	2	7	0	2	2	0.210	0.547
mean	3.3	2.3	9.3	2.5	2.0	13	0.83	2.0	5.3	0.372	0.693

locus was established as described in Materials and methods (table available on request). All the analysed loci were highly polymorphic in *P.* × *canadensis* with allele number ranging from 6 to 15 and quite polymorphic in the *P. deltoides* clones, ranging from 1 to 6. The number of different microsatellite genotypes and the number of the cultivars with unique genotype at a locus were also calculated: as shown in Table 2 SSR locus WPMS14 was the most effective in cultivar identification both in *P.* × *canadensis* and in *P. deltoides*. The average PIC was 0.693 in the case of *P.* × *canadensis* and 0.372 for *P. deltoides*. PIC was not calculated in the case of *P. nigra*, due to the limited number of analysed clones (two).

An interesting observation is relative to the number of alleles recorded in the hybrid group where a larger number of alleles was present. In fact the (true) hybrids harboured more genetic variation (e.g., number of alleles) than the parent species, because they combine the polymorphism in the SSR loci from both parent species.

Based on the analysis of the six SSR loci, all the commercial clones were identified with a unique allelic phenotype (including the two couples I-262/I-455 and Robusta/Heidemij which gave ambiguous results under the AFLP analysis), except for the three groups already mentioned above (AFLP analysis). Furthermore, based on the analysis of SSR loci WPMS09, WPMS18 and PMGC014, displaying species-specific alleles useful to distinguish *P. deltoides*, *P. nigra* and *P.* × *canadensis* (Fossati et al. 2003), some cultivars, previously reported as *P.* × *canadensis*, only showed the *P. deltoides* diagnostic alleles (Table 1). To determine if these clones belong to *P. deltoides* or to *P.* × *canadensis* species an assignment test based on the GeneClass computer software was run. To this purpose only those clones, which showed a characteristic F1 hybrid SSR profile (Fossati et al. 2003) were considered representative of the *P.* × *canadensis* hybrids, while the group of *P. deltoides* reference trees was formed by the six poplar clones listed in Materials and methods and considered as such on the basis of their passport and genealogy. The likelihood of each hybrid clone of doubtful origin (Carolina di Santena, Dora, Lambro, San Martino, Soligo Stura and Taro) was estimated and all the mentioned clones turned out to be pure *P. deltoides* genotypes.

Discussion

Many authors have published in the past about the molecular identification of poplar clones (e.g. isozymes, RAPD, microsatellite), but in all these cases only one single molecular technique was adopted (Rajora 1989a–c; Rajora and Zsuffa 1989; Castiglione et al. 1993; Heinze 1998a,b; Thakur and von Wuelisch 2001; Lakshmi and Tewari 2003), or, when two molecular tools were jointly employed, one of the two was not free of artefacts, or lacked of reproducibility (Castiglione et al. 1993; Rajora and Rahman 2003). The results of this work clearly demonstrate that both AFLP and SSR markers can be used for successful cultivar identification of commercially relevant

poplar clones. In fact, the majority of the analysed cultivars were uniquely identified both by their AFLP fingerprints and by their multilocus SSR profiles. The only exceptions were represented by three groups of six, four and two clones, which couldn't be resolved by both techniques. The first group included *P.* × *canadensis* Adige, Boccalari, Branagesi, Gattoni, Stella Ostigliese and 302-San Giacomo; the second one was composed of *P.* × *canadensis* BL Costanzo, Cappa Bigliona, MC and Pan; the third one was formed by *P. deltoides* Lux and Harvard. Both molecular markers techniques used to discriminate the 66 poplar clones showed that the individuals within the groups are duplicated genotypes. In fact, the estimated probability that two distinct poplar individuals of the analyzed collection could exactly match at the six SSR loci is so low (7.5×10^{-9} in the case of *P.* × *canadensis*) that, probably, the total number of living poplar trees on earth is smaller than that calculated in our study. A possible explanation for the presence of duplicated genotypes among registered cultivars is given by the fact that in DUS tests cultivars are evaluated for their performance with respect to controls that are not necessarily the most similar varieties, but rather those in wide use in the area where tests are conducted. Therefore the most likely conclusion is that several people, independently, had recognized the merits of the same varieties and taken the initiative to introduce them (Bisoffi, personal communication).

Moreover, in the case of the two groups of hybrids analysed in this study, failure to distinguish individual clones within the groups by observable characteristics had already been noted (Bisoffi and Cagelli 1996), when they were grown "side by side" in the field at I.S.P. and also phenological traits, gender and resistance to diseases were unable to provide clues to identity (Bisoffi, personal communication). The same subset of hybrids was previously analysed by Castiglione et al. (1993) and cluster analysis based on RAPD data showed that these clones were tightly linked, but the presence of artifactual "polymorphisms" (they can reach up to 60%, see Pérez et al. 1998), had perhaps disguised the real situation, which could be disclosed only when AFLP and SSR markers were used.

In this sense molecular tools would play a major role in: (1) pre-screening of cultivars to be compared with a new one, by selecting the more closely related one; (2) identification of duplicates among previously registered clones.

In the case of *P. deltoides* clones Lux and Harvard we can argue that the two clones were confused during vegetative propagation. In fact, Harvard and Lux, selected at I.S.P. during the 1970s with different gender, show, at present, the same (female) sex in the repository of I.S.P. Misidentification and mislabelling of the clones species identity could easily happen in the poplar and cultivation process (Rajora and Zsuffa 1991).

Keeping in mind that poplar is a dioecious plant, its reproductive biology may be of two sorts: vegetative [this is the less common situation (Fossati et al. 2003)], or strictly heterogamous, it is extremely unlikely that three groups of poplar trees of our collection could show a perfect genome identity and a very low matching probability (7.5×10^{-9} in the case of *P.* × *canadensis*) as we demonstrated. Consequently they have to be treated as events of vegetative propagation instead of sexual reproduction as previously supposed.

Therefore, the two adopted molecular markers offer: (1) the opportunity to verify the identity of any clone or cultivar during any step of vegetative or seed propagation; (2) the possibility to assess the correspondence between the real and the declared identity of cultivars even in cases of suspected frauds.

The AFLP-derived dendrogram (Fig. 1) as well as the PCOORDA allowed a clear distinction among species and hybrid groups: three main clusters corresponding to *P.* × *canadensis*, *P. nigra* (2 clones) and *P. deltoides* (12 clones) were identified and supported by bootstrap analysis. Within the *P.* × *canadensis* group, some clones of known co-ancestry were confirmed to share high genetic similarity: clones I-154 and Triplo clustered in the same group, as expected. In fact, Triplo is a triploid genotype obtained by crossing a *P. deltoides* female from the Mississippi delta, with a now lost tetraploid genotype (458p) derived from I-154 by colchicine treatment. (Vivani and Sekawin 1953). Clone Ballottino, appearing in the dendrogram even closer than Triplo to I-154 is a cultivar of uncertain origin selected by a private poplar grower that might well be traced back to the same genetic stock that gave rise to the other two. Luisa Avanzo and Cima were obtained by open pollination of the same *P. deltoides* female tree from the area of Stoneville (Mississippi, USA) grown near Rome; the father is probably a local *P. nigra* and the possibility that the same father was shared by both clones cannot be excluded. The unrelated clone A4A has a mother from the same area of origin.

Some inconsistency appeared in the bottom part of the dendrogram where balsam poplars (Section *Tacamahaca*) were located: clone Beaupré (*P. trichocarpa* Torr.et Gray × *P. deltoides*) clustered with Eridano (*P. deltoides* × *Populus maximowiczii* Henry) rather far from its own mother, *P. trichocarpa* Fritzi Pauley. The latter clone was closer to *P. deltoides* × *P. trichocarpa* Blom and to *P. maximowiczii* × *P. nigra* Rochester than to its own daughter.

These inconsistencies could be explained considering that all these hybrid poplar clones were obtained through a selection process in the framework of a specific breeding programme, and, at present, it is not known whether the selective pressure could favor the hybrid progeny the presence of particular traits of one of the two mated genomes due to aneuploidy phenomena as described by Cervera et al. (2001), or to other chromosome rearrangements such as deletions, insertions and translocations. The same consideration can be applied to the two clones Sile and Taro, which have been clustered with the *P. deltoides* group upon the molecular analysis. Moreover, strict similarity of Sile to *P. deltoides* genotypes had already been observed when a morphometrical analysis on leaf shape was performed (Bisoffi, personal communication).

Quite consistently with current botanical classification, *P. alba* L. Villafranca (Section *Leuce*) was distinct from all the other cultivars.

Several cultivars which have traditionally been reported as *P.* × *canadensis* (Table 1) turned out to share more ge-

netic similarity to *P. deltoides* upon the AFLP analysis and PCOORDA (Fig. 1a, b), and were homozygous for the *P. deltoides* alleles at the three diagnostic SSR *loci* PMG C014, WPMS09 and WPMS18, clearly suggesting that they are not F1 hybrids, but pure *P. deltoides* as demonstrated also by the results of the assignment test. Since the genealogy of the majority of these clones is unknown, it is possible that they were generated by open pollination of *P. deltoides*. In fact the morphological characters recorded were consistent with those representative of *P. deltoides* species.

At present, molecular and biochemical markers are not still accepted as satisfactory means for registering new varieties, but, up to now, AFLP and SSR are the most reliable and reproducible molecular tools to assess genetic information in plant and animal populations and collections (Powell et al. 1996; Mueller and Wolfenbarger 1999). Our data provide support for using them in all aspects required by UPOV (distinctness, uniformity, and stability). To take the maximum advantage from the use of molecular markers it should be useful to establish, even in the case of poplar collections and genebanks, a consensus database continually fed with data from different laboratories, as already established for tomato (Bredemeijer et al. 2002) and wheat (Röder et al. 2002).

To this purpose AFLP are not the most suitable marker system, in fact they are sometimes difficult:

(i) To set up in different laboratories when the same material as to be analysed (Jones et al. 1997)
(ii) To compare from lab to lab (Smulders et al. 2002)
(iii) To include their huge amount of information in a data base.

Moreover, as can be concluded from the results of Winfield et al. (1998), they may display up to 5% of variability, which does not allow an unambiguous definition of "clone." Such a situation was also observed in our work in the case of I-262/I-455 and Heidemij/Robusta (Fig. 1a). These ambiguous results have been easily resolved by SSR analysis, which clearly demonstrated the distinctness of the four genotypes.

Base on all these considerations in our opinion SSRs are, at present, the most useful molecular system for establishing databases, even if it is tricky to determine the correct size of the alleles across laboratories. However, this drawback can be easily overcome including in any analysis a set of reference samples with different allele sizes, or a core collection representative of the species considered, or better, producing diagnostic molecular kits certified for their ability to discriminate unambiguously any uncertain situation for a particular plant or animal species.

On the basis of the data presented in this paper we suggest that the two techniques should be employed for different purposes. AFLP can be appropriate for species and hybrid discrimination, for a quick evaluation of similarity degree among cultivars or when SSR are not still available. SSR may be exploited to recognise identities, to detect duplications among previously registered poplar clones and to contribute to a more exact definition of "clone." Consequently they may become the most effective tool to defend a variety, once its novelty and distinctness have been verified and accepted by more traditional methods. In fact morphological and molecular information does not cancel each other out, but they support each other and one of the two cannot, for sure, fully substitute the other.

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