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RAPD Fingerprints for identification and for taxonomic studies of elite poplar (*Populus* spp.) clones

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Abstract. RAPD (Random Amplified Polymorphic DNA) fingerprints have recently been used to estimate genetic and taxonomic relationships in plants. In this study RAPD analysis was performed on 32 clones belonging to different species of the genus *Populus*. Of these, 25 clones are registered in several countries for commercial use and, altogether, cover almost 50% of the worlds cultivated poplars. DNA was prepared from leaves and amplified by PCR using random oligonucleotide primers. Amplification products were separated by agarose-gel electrophoresis to reveal band polymorphisms. Four primers out of the 18 tested, were selected on the basis of the number and frequency of the polymorphisms produced. With these a total of 120 different DNA bands were reproducibly obtained, 92% of which were polymorphic. The polymorphisms were scored and used in band-sharing analyses to identify genetic relationships. With a few but interesting exceptions, these are consistent with the present taxonomy of the genus *Populus* and with the known predigrees of cultivated poplars. Moreover, the results show that RAPD analysis allows one to discriminate among all tested clones and can, therefore, be recommended as a convenient tool to defend plant breeders rights.

Key words: Poplar – *Populus* – PCR – RAPD – Arbitrary primers

Introduction

Poplar (*Populus* spp.) is of primary economic importance for the production of wood in temperate regions of the world. It is estimated that this tree covers the majority of all artificial forests (FAO 1980). Its wood is used for a large spectrum of products, such as plywood, particle boards, packages, structural timber, matches, chopsticks and paper. Because of its fast growth and short rotation poplar represents, for many countries, the only solution to the growing wood demand and provides the best alternative to the destruction of forests. The success of interspecific hybridization and the ease of producing asexually propagated clones has made poplars standard material for commercial plantations. Clones have been exchanged among countries making the benefits of breeding available to all users. The case of some Italian clones is a striking example: bred and selected in the Po valley in Northern Italy, they are now cultivated in Europe, South America, Asia and Oceania. At present, the Italian clones make up almost 50% of the entire world poplar culture (Lapietra 1992) and cover about 2 million hectares. The hybrid *Populus* × *euramericana* clone I-214 is considered, by far, the most replicated single genetic unit among forest trees.

The importance of a proper identification of poplar clones was appreciated long ago. However, traditional methods, based on the observation of morphological and phenological characters (FAO 1971; UPOV 1981), are both time consuming and blurred by environmental effects. They are certainly useful in the study of genetic variability and taxonomy expecially if data acquisition is done by machine vision systems (Draper and Keefe 1989) and the data analysed by means of multivariate methods (Hu et al. 1985), but they lack the resolving power that is needed to identify individual genotypes.

Poplar was also the first, among trees, to be investigated for the detection of biochemical markers that could help in identification. Paper chromatography (Bortitz 1962; Boccone 1975) was shown to be useful for the

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discrimination of some species and hybrids but not satisfactory for clones. Gas chromatography was a step forward both for the study of introgression of interfertile poplar species and for the identification of clones (Greenway et al. 1989). Isozyme analysis has also been considered and found to be satisfactory with some specific clones (Rajora 1988, 1989; Rajora and Zsuffa 1989): its resolving power is, however, limited by the number of enzymatic systems that can be conveniently analyzed.

The RFLP (Restriction Fragment Length Polymorphism) methodology, first applied in human genetics (Botstein et al. 1980; Wyman and White 1980), has also been successfully used to identify DNA polymorphisms useful as genetic markers in plants (Helentjaris et al. 1985; Landry et al. 1987; Tanksley et al. 1989). This is a direct approach to the study of genomic DNA, but has not yet been systematically applied to poplars.

More recently the introduction of PCR (Polymerase Chain Reaction) (Erlich et al. 1991) has allowed the development of a new powerful tool to detect DNA polymorphisms. One of its applications, named AP-PCR (Arbitrarily Primed PCR) but better known as the RAPD (Random Amplified Polymorphic DNA) method, is based on the observation that single DNA primers of arbitrary nucleotide sequence can amplify genomic DNA sequences in PCR whenever they find regions of sufficient homology at a favourable distance and in converging orientation on the two DNA strands (Welsh and McClelland 1990; Williams et al. 1990).

RAPDs may be used to detect DNA variability at different levels, from single base changes to deletions and insertions (Williams et al. 1990). The advantages of RAPDs are manifold: they do not depend on the identification of RFLP probes or on sequence information, the use nanogram quantities of DNA and require merely PCR and agarose gel. Genome analysis with RAPDs has already been shown to be possible in animals (Welsh et al. 1991), bacteria (Jayarao et al. 1992), and plants (Hu and Quiros 1991; Welsh and McClelland 1990; Halward et al. 1992). The RAPD approach has also been used, among others, to identify DNA markers linked to specific genes in near-isogenic tomato lines (Martin et al. 1991) and for parentage determination in maize hybrids (Welsh et al. 1991). However, the need is still felt for confirmation of their reproducibility and adequacy, expecially in the case of plants of economic interest.

Variations in the RAPD protocols are possible: for instance Caetano-Anollés et al. (1991) used primers as short as five nucleotides to produce complex patterns. On the other hand, specifically selected reaction conditions can increase the number of annealing sites between longer primers and genomic DNA (Welsh and McClelland 1990) and also produce complex patterns. Thus, a single primer can conveniently amplify up to tens of DNA loci to produce DAF (DNA Amplification Fingerprints), i.e., RAPD fingerprints useful for genotype identification (Caetano-Anollés et al. 1991). These complex patterns are also useful for strain identification, and in the study of phylogenetic relationships, genetic mapping, and germplasm analysis of viruses, bacteria, fungi, plants and animals.

Here we report the application of RAPD markers for the identification of 32 clones of the genus *Populus* selected among those most extensively cultivated in the world. The large number of polymorphic bands produced also made it possible to determine genetic relationships among the different genomes.

Materials and methods

Plant material

The 32 poplar clones used in this study are listed in Fig. 2. They belong to the species: *Populus nigra* L., *P. deltoides* Bartr., *P.* × *euramericana* (Dode) Guinier, *P. fremontii* Wats. and *P. wislizenii* Sarg. (section *Aigeiros* Duby or black poplars); *P. trichocarpa* Torr *et* Gray, *P. simonii* Carr., *P. yunnanensis* Dode *P. maximowiczii* Henry (section *Tacamahaca* Spach or balsam poplars); *P. alba* L. and *P. tremula* L. (section *Leuce* Duby or white poplars and aspens); *P. deltoides* × *maximowiczii*. All of them were from the collection of the Istituto di Sperimentazione per la Pioppicoltura, Casale Monferrato (Alessandria, Italy).

Plant DNA isolation

Two grams of fresh mature poplar leaves were ground to powder in the presence of liquid nitrogen and the DNA extracted as described by Doyle and Doyle (1990). To achieve further purification the resulting DNA was resuspended in 1 ml of urea buffer (8 mM urea, 50 mM Tris-HCl pH 8.0, 350 mM NaCl and 50 mM EDTA), extracted with phenol-chloroform and with chloroform, precipitated with ethanol, resuspended in 1 ml of water, treated for 30 min at 37 °C with 50 µg/ml of DNase-free RNase A (Boehringer Mannheim), purified through a Sephadex G-50 column and treated with 50 µg/ml of Proteinase K (Boehringer Mannheim) for 30 min at 42 °C followed by enzyme inactivation for 10 min at 90 °C. DNA concentration was determined by comparison with serial dilutions of standard lambda DNA.

DNA amplification

Table 1 lists the 18 primers tested in the PCR reaction. This was performed in 10 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP, 0.5 µg of the primer, 0.5 units of *Taq* DNA polymerase (Promega) and 20 ng of poplar DNA. The mixture was assembled at 0 °C, overlaid with one drop of mineral oil and quickly transferred to an MJ Research Inc. thermal controller preheated at 92 °C. After an incubation at 92 °C for 3 min, DNA amplification was performed for 45 cycles. Each cycle was at 92 °C for 30 s (Denaturation), at 30 °C (10-mers) or at 45 °C (longer primers) for 60 s (annealing), at 72 °C for 2 min (extension). Reactions were finally incubated 10 min at 75 °C followed by 10 min at 65 °C. Amplified DNA was stored at −20 °C.

Electrophoresis in agarose gel

The amplification product was analyzed by electrophoresis in 3.0% agarose gel (1.5% LE agarose and 1.5% NuSieve agarose;

Table 1. Primers with arbitrary sequence tested for their effectiveness in the RAPD analysis

No.	Name	Sequence	Number of DNA bands in the gel
1	Deca-1	5′-GATGGCCGGC-3′	0
2	Deca-2	5′-GGGATCCGGC-3′	12–20
3	Deca-3	5′-GGGCCATGGC-3′	0
4	Deca-4	5′-CGTTGGCCCG-3′	14–20
5	Deca-5	5′-CCAAGGGGGC-3′	7–20
6	Deca-6	5′-TAGGGGCCCC-3′	0
7	Deca-7	5′-CCGCCCGGAT-3′	12–20
8	Deca-8	5′-ACGCGGGGGA-3′	0–6
9	Deca-9	5′-TGGCCCCGGT-3′	14–20
10	Deca-10	5′-AGCCGGCCTT-3′	12–17
11	Deca-11	5′-ATCGGCTGGG-3′	18–26
12	Deca-12	5′-CTTGCCCACG-3′	12–17
13	Deca-13	5′-GTGGCAAGCC-3′	20–30
14	Deca-14	5′-CTCCCGGTAG-3′	0–2
15	Chl-1	5′-GAGGCCTACGCCCC-ATAGAA-3′	12–26
16	Chl-2	5′-AATGCGTTGAGGCG-CAGCAG-3′	10–15
17	Chl-4	5′-TTCCCGTGTCTTCCGG-CTTAC-3′	17–33
18	Chl-10	5′-TTCTTCTCCTACCAG-TATCG-3′	17–28

Primer effectiveness was determined by evaluating the gels resulting after PCR amplification of the different poplar accessions under the conditions described in Materials and methods

FMC Bioproducts) in TAE buffer and run in the same buffer for 2–4 h at 70 V. The gels were stained with 0.2 μg/ml of ethidium bromide and photographed under UV light.

Band-sharing analysis

Bands on agarose gels were scored as present (+) or absent (−) and a pairwise similarity matrix was constructed using the Dice similarity (SD) index (Sneath and Sokal 1973). SD values were calculated as the doubled number of shared bands between two patterns divided by the sum of all bands in the same patterns. An UPGMA cluster based on SD values was generated using the NTSYS (Numerical Taxonomy System, Applied Biostatistics, Setauket, New York) computer programme.

Results

DNA extraction with the procedure described in Materials and methods was found to be satisfactory. RNaseA-treatment of the DNA preparation was essential to yield DNA suitable for PCR. Treatment with Proteinase K was introduced because in some cases the use of DNA samples prepared without this treatment resulted in the disappearance of RAPD bands. Possibly, this was due to competition between residual proteins and primers for binding to the template DNA sequences. Furthermore, we have found that low annealing temperatures (30 °C for 10-mer and 45 °C for the 20-mer primers) and a high magnesium concentration are the most satisfactory for the purpose of producing DNA fingerprints.

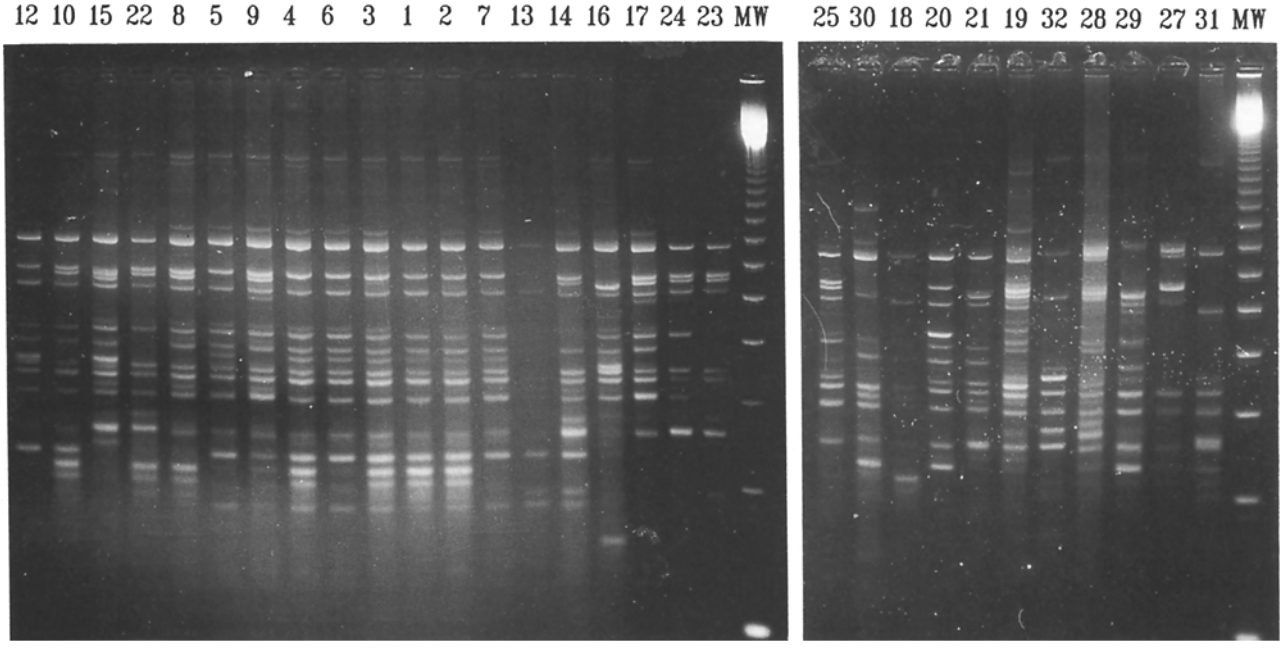

Fig. 1. Agarose-gel electrophoresis of the fragments obtained by PCR amplification of different poplar genomic DNAs with an arbitrary 20-mer oligonucleotide (Chl-10). The nucleotide sequence of Chl-10 is given in Table 1. The numbers on top refer to the poplar accessions listed in Fig. 2. The molecular marker (*MW*) was the 123 bp BRL ladder

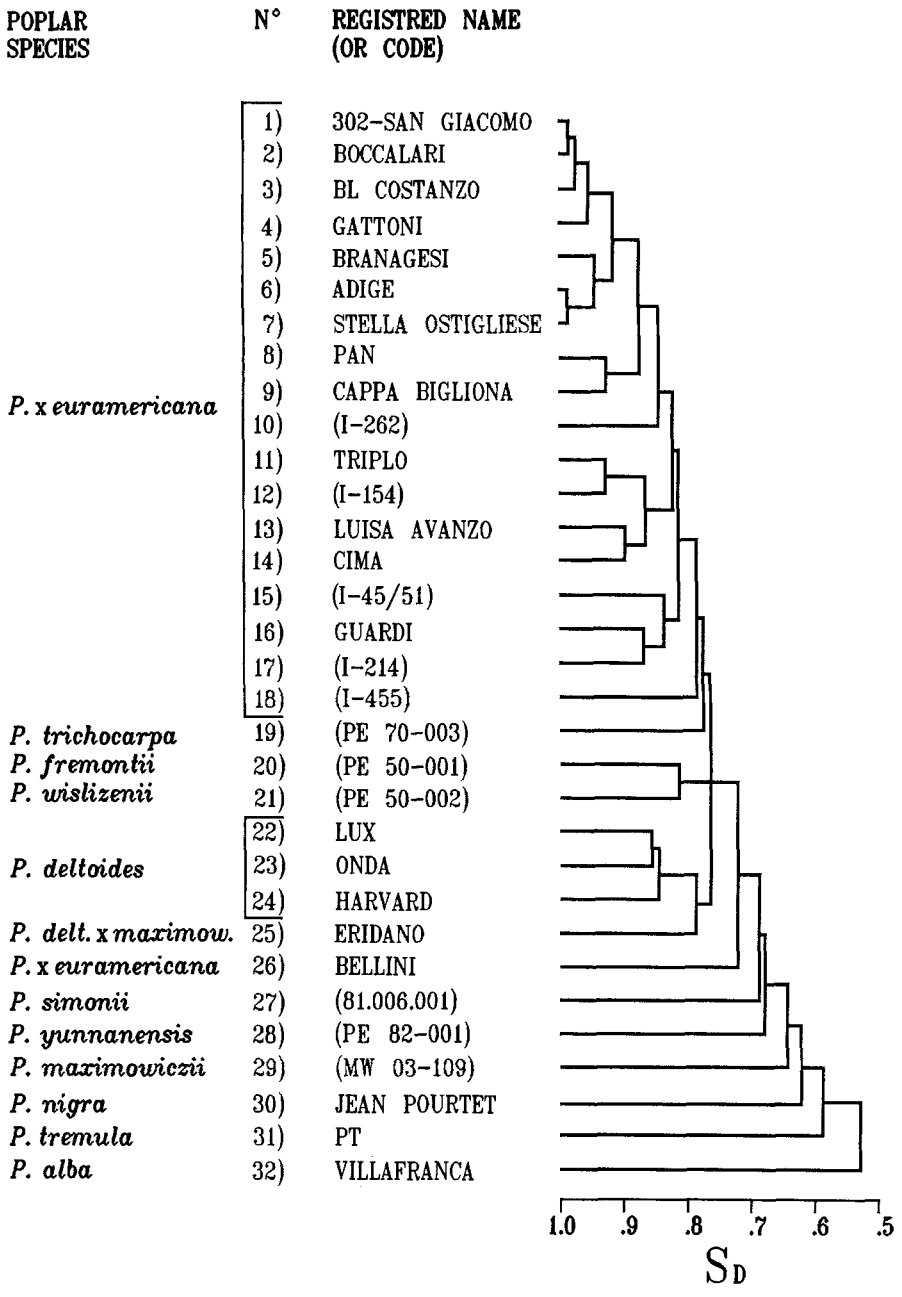

Fig. 2. UPMGA dendrogram based on the Dice similarity index (S_D) illustrating the genetic relationships among the 32 analyzed poplar clones

The sequence of the tested primers and the average number of DNA fragments amplified in their presence are shown in Table 1. Out of 14 10-mers, nine turned out to give satisfactory banding in the gels with all tested poplar genomic DNAs, three did not produce amplification products, and the others produced bands only with some DNA samples.

The 20-mer primers, on the average, identify more DNA polymorphisms then the 10-mer primers. Thus, under our PCR conditions, they can effectively substitute for the shorter primers as suggested by Caetano-Anollés et al. (1991) for DNA fingerprinting.

Reproducibility of results with the same clone but with different DNA preparations was accurately verified. Each RAPD analysis was repeated in separate experiments from two to six times and was always found to be reproducible. The use, in the PCR reaction, of a strictly controlled temperature profile and of a reliable *Taq* DNA polymerase was essential for reproducibility. Furthermore, no variation was found in the patterns obtained with the DNA from different individuals of the same clone. This was expected since poplar clones, including hybrids, are routinely vegetatively reproduced.

Four primers (Deca-11, Chl-1, Chl-4 and Chl-10) were selected, out of the 18 tested, on the basis of the number and frequency of polymorphisms produced among the 32 poplar clones. Figure 1 shows an example of gel analysis of DNAs amplified with the primer Chl-10

(20-mer). Altogether, 26 bands out of 29 were polymorphic. Similar informative patterns were obtained with the other three primers. With the four primers a total of 120 bands were produced. Of these, 110 were polymorphic. Bands on the agarose gels were scored as present or absent. Data obtained with the four primers on the 32 poplar accessions were analyzed as described in Material and methods to produce the dendrogram shown in Fig. 2. Dendrograms obtained by analyzing separately the bands produced with each primer were found to be in satisfactory agreement with Fig. 2.

Discussion

We have shown that the RAPD approach reveals frequent DNA polymorphisms among poplar clones.

Our research was initially devoted to the evaluation of optimum conditions for DNA extraction and to the selection of informative random primers for the PCR reaction. We paid particular attention to the definition of PCR conditions that allow the reproducible production of a number of polymorphic DNA bands sufficient for relationship analysis.

The majority of the 10-mers used in the PCR reaction gave distinctive and satisfactory amplification patterns. However, longer primers produced more polymorphisms. This shows that, as previously suggested (Caetano-Anollés et al. 1991), DNA fingerprinting may not necessarily be linked to the use of very short primers and that longer oligonucleotides can be confidently tested. Furthermore, we found that the number of amplification products was inversely correlated with the increase in the annealing temperature and directly correlated with the increase of Mg^{++} concentration. The combination of long primers with an appropriately low annealing temperature and a high Mg^{++} concentration increase the number of priming sites on the template DNA. Despite the warnings raised by other authors (Devos and Gale 1992; Riedy et al. 1992), the accurate reproduction of all selected parameters in our case assured a satisfactory reproducibility of the results.

The 32 poplar clones analyzed were selected from a collection of poplar commercial cultivars. They were chosen on the basis of relevance as industrial crops, wood quality, disease tolerance, resistance to water stress and to other abiotic stresses. The dendrogram of Fig. 2 essentially matches existing knowledge on the systematics of the genus *Populus* and the genealogical data of clones and hybrids. For example, the accessions numbered 1 to 9 in Fig. 2, known in Italy as "Canadian" poplars, are clustered. Although their origin is lost in the blurred beginnings of modern poplar culture during the early decades of the 19th century, they all have similar characteristics of morphology, wood quality, phenology, and susceptibility to diseases. All of them are female and some were even suspected of being the same genetic entiy under different names (e.g., ADIGE and STELLA OSTIGLIESE). Although no complete identities have been detected in this study, it is quite apparent that the genetic background of the "Canadian" poplars is extremely restricted. Two groups (1–4 and 5–7) are more similar to one another then the two half-sib (or, probably, full-sib) clones LUISA AVANZO and CIMA (13 and 14), which form a small group of their own. Another striking match with genealogical knowledge is that shown in the cases of the clone TRIPLO (a triploid) and I-154 (11 and 12). The male clone used as pollen donor in the crossing that gave rise to TRIPLO was, in fact, clone 438p, a tetraploid obtained by colchicine treatment of the apical meristems of I-154 (Vivani and Sekawin 1953). The three *P. deltoides* clones (22 to 24) also form a single, group, separated from *P. wislizenii* and *P. fremontii* (21 and 20) which some taxonomists have considered as a subspecies of *P. deltoides* (Houtzagers 1950), and also separated from all the specimens of *P.* × *euramericana* that form a single large group (with the notable exception of the clone BELLINI). More difficult to interpret, on the other hand, is the position of the only representative of the other parent species of the euroamerican hybrids, *P. nigra* (30), which is rather far from the other black poplars of the section *Aigeiros*. Also the position of *P. trichocarpa* (19), which appears to be genetically nearer to the *Aigeiros* poplars than to the other balsam poplars of the section *Tacamahaca*, has no apparent logical explanation. The taxonomic position of these plants needs to be investigated further.

Whatever these taxonomic considerations, it is relevant to stress that the RAPD fingerprints produced, although obtained with a limited number of DNA primers, allow us to discriminate among all tested commercial clones, even among those that cannot be distinguished on the basis of morphological and phenological traits.

Three criteria are considered essential for cultivar identification with any methodological appraoch: maximal intervarietal variation, minimal intravarietal variation, and temporal and environmental stability of the markers under study (Bailey 1983). The results presented in this paper show that these criteria are met when RAPD analysis is applied to poplars. It should also be emphasized that no fingerprint variation was ever noticed when different plants of the same clone were assayed independently.

Molecular and biochemical markers are not accepted at present as a satisfactory means for registering new varieties, but may be employed to demonstrate identity. Consequently, they may become the most effective tool to defend a variety once its novelty and distinctness have been tested and accepted by more traditional methods.

A screening for genetic diversity within breeding populations might also help in designing matings with higher efficiency, thus minimizing population size while keeping diversity high.

Other applications of RAPDs are now also possible: the most appealing is the search for linkages between specific amplified DNA sequences and genetic traits of economic relevance, such as resistance or tolerance to biotic and abiotic stresses. Molecular markers of sex might also be generated and these would be of great importance in predicting the sexual expression of these dioecious trees. In some cases, due to uni-directional incompatibilities (as in the case of *P. nigra*, female, × *P. deltoides*, male), it would be of paramount importance to be able to screen breeding material for sexual compatiblity at a very early stage, without waiting for sexual maturity, a process that can take 5–10 years according to the species involved.

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References

Bailey DC (1983) Isozymic variation and plant breeders' rights. In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding, part A. Elsevier, Amsterdam, pp 425–441

Boccone A (1975) Differenze chemiotassonomiche tra specie e cloni di pioppo a livello del contenuto in flavoni. Cellulosa e Carta 27:89–46

Bortitz S (1962) Papierchromatographische Differenzierung einiger Arten und Sorten der Gattung *Populus*. Züchter 32:24–33

Botstein D, White RL, Skolnick M, Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 32:314–331

Caetano-Anollés G, Bassam BJ, Gresshoff PM (1991) DNA amplification fingerprint using very short arbitrary oligonucleotide primers. Biotechnology 9:553–557

Devos KM, Gale MD (1992) The use of random amplified polymorhic DNA markers in wheat. Theor Appl Res 84:567–572

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Draper SR, Keefe PD (1989) Machine vision for the characterization and identification of cultivars. Plant Var Seeds 2:53–62

Erlich HA, Gelfand D, Sninsky JJ (1991) Recent advances in the polymerase chain reaction. Science 252:1643–1650

FAO (1971) Rapport du Sous-Comitè d'enregistrement des noms du peuplier. FAO/CIP/123, Rome

FAO (1980) Poplars and willows. FAO, Rome

Greenway W, Jobling J, Scaysbrook T (1989) Composition of bud exudates of *Populus* × *interamericana* clones as a guide to clonal identification. Silvae Genet 38:29–32

Halward T, Stalker T, LaRue E, Kochert G (1992). Use of single-primer DNA amplifications in genetic studies of peanut (*Arachis hypogaea* L.). Plant Mol Biol 18:315–325

Hamill JD, Rounsley S, Spencer A, Todd G, Rhodes MJC (1991) The use of the polymerase chain reaction in plant transformation studies. Plant Cell Rep 10:221–224

Helentjaris T, King G, Slocum M, Siedenstrang C, Wegman S (1985) Restriction fragment polymorphisms as probes for plant diversity and their development as tools for applied plant breeding. Plant Mol Biol 5:109–118

Houtzagers G (1950) Il genere *Populus* e la sua importanza nella selvicolutra. Biblio Sci Cartiere Burgo Casale Monferrato, Italia

Hu CC, Crovello TJ, Sokall RR (1985) The numerical taxonomy of some species of *Populus* based only on vegetative characters. Taxon 34:197–206

Hu J, Quiros CF (1991) Identification of broccoli and cauliflower cultivars with RAPD markers. Plant Cell Rep 10:505–511

Jayarao BM, Bassam BJ, Caetano-Anollés, Grésshoff PM, Oliver SP (1992) Subtyping of *Streptococcus uberis* by DNA amplification fingerprinting. J Clin Microbiol 30:1347–1350

Landry BS, Kesseli R, Hei Leung, Michelmore RW (1987) Comparison of restriction endonucleases and sources of probes for their efficiency in detecting restriction fragment length polymorphisms in lettuce (*Lactuca sativa* L.). Theor Appl Genet 74:646–653

Lapietra G (1992) Principali caratteristiche dei più noti cloni di pioppo selezionati in Italia. L'Informatore Agrario 18:83–86

Martin GB, Williams JG, Tanksley SD (1991) Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. Proc Natl Acad Sci USA 88:2336–2340

Rajora OP (1988) Allozymes as aids for identification and differentiation of some *Populus maximowiczii* Henry clonal varieties. Biochem Syst Ecol 16:635–640

Rajora OP (1989) Characterization of 43 *Populus nigra* L. clones representing selections, cultivars and botanical varieties based on their allozyme genotypes. Euphytica 43:197–206

Rajora OP, Zsuffa L (1989) Multilocus genetic structure, characterization and relationships of *Populus* × *canadensis* cultivars. Genome 32:99–108

Riedy MF, Hamilton WJ, Aquadro CF (1992) Excess of nonparental bands on offspring from known primate pedigrees assayed using RAPD-PCR. Nucleic Acids Res 20:918

Sneath PHA, Sokal RR (1973) Numerical taxonomy. The principles and practice of numerical classification. W. H. Freeman, San Francisco

Tanksley SD, Young DN, Paterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding: new tools for an old science. Biotechnology 7:257–264

UPOV (1981) Guidelines for the conduct of tests for distinctness, homogeneity and stability: poplar (*Populus* L.). UPOV, TG/21/7

Vivani W, Sekawin M (1953) Esperienze di poliploidia indotta nel genere *Populus* L. FAO/IPC, 2nd Session, Roma, Italy

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18:7213–7218

Welsh J, Honeycutt RJ, McClelland M, Sobral BWS (1991). Parentage determination in maize hybrids using the arbitrarily primed polymerase chain reaction (AP-PCR). Theor Appl Genet 82:473–476

Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Wyman AR and White R (1980) A highly polymorphic locus in human DNA. Proc Natl Acad Sci USA 77:6754–6758