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Original article

SSR-based tool for identification and certification of commercial *Populus* clones in Spain

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Abstract – In order to protect the legitimate interests of the poplar breeders, growers and industries, we have developed a reliable and affordable certification tool for commercial poplars in Spain. We have carried out a genetic description of the 28 commercial clones of the current Spanish National Catalogue using 10 microsatellite molecular markers (SSR). The results clearly show that in the Spanish National Catalogue there are labelling mistakes including synonymies, supported by a combined probability of identity (*PI*) of 1.18×10^{-9}. We have also demonstrate that it is possible to identify all the different clones (genotypes) with only three microsatellites, that show a combined probability of identity of 2.4×10^{-4}, and we have optimised the protocols to reduce the time and the economical costs of the analysis. This SSR-based tool could be incorporated in the current certification protocols, to avoid duplication of accessions and identification mistakes.

commercial poplar clone / microsatellite / identification / Spanish national catalogue

Résumé – Développement de marqueurs microsatellites pour l'identification et la certification des clones commerciaux de peuplier en Espagne. Dans le but de protéger les intérêts légitimes des cultivateurs, des sélectionneurs et des industriels de la populiculture, nous avons mis au point un outil de certification fiable et accessible pour les clones de peupliers commerciaux en Espagne. Nous avons fait une description génétique des 28 clones commerciaux de l'actuel catalogue national espagnol en utilisant 10 marqueurs moléculaires microsatellites (SSR). Les résultats obtenus indiquent clairement que ce catalogue contient des erreurs d'identification, dont des clones synonymes, soutenues par une probabilité combinée d'identité (*PI*) de 1.18×10^{-9}. Nous avons également démontré qu'il est possible d'identifier tous les génotypes différents avec 3 microsatellites, qui montrent une probabilité combinée d'identité de 2.4×10^{-4}, et nous avons optimisé les protocoles afin de réduire le temps et le coût de l'analyse. Cet outil fondé sur les SSR pourrait être intégré dans les protocoles actuels de certification afin d'éviter la duplication des matériaux et les erreurs d'identification.

clone commercial de peuplier / microsatellite / identification / catalogue national espagnol

1. INTRODUCTION

The culture of poplar in the Iberian Peninsula has been continuously growing in the last decades, mainly due to its economic profitability. But it is also a response to the demand for quality poplar wood in Spain, and a relatively easy conversion of abandoned agriculture lands to forestry uses, a conversion promoted by the European Community within the CAP (Commune Agricultural Policy). According to the Spanish Poplar National Commission (http:// www.mapa.es/es/agricultura/pags/chopo/cortas.htm) and to the Agriculture Ministry (http://www.mapa.es/es/estadistica/pags/encuestacultivos/resultados.htm), the harvested poplar wood has doubled in the last 25 years, reaching nearly 700 000 m^3 in 2005 with a total planted surface of 132 167 ha. In this context of growing interest for poplar culture, many clones have been tested (see [17] and [19] for reviews), and several management manuals, adapted to the Spanish conditions have been written [2, 12, 17, 25]. As a result of these tests, the National Catalogue including the poplar clones admitted in Spain was expanded in 2003 to 28 clones from only 14 in the previous Catalogue. Spanish laws establish that all the plant material for propagation purposes must be produced in authorised nurseries recorded in the "Registry of Plant Producers", to guarantee plant quality and clonal identity. The choice of a particular clone to be used in a given site combined with soil and climate conditions, and appropriate management techniques, determines the possible uses of the final harvested wood, its technical and industrial characteristics, and thus, its economic yield. In this context, an accurate identification of clones is essential, not only in propagation nurseries, but also in all the phases of poplar propagation and commercial exchanges. Traditionally, the clonal identity control and certification of plant material from nurseries is carried out by regional authorities on the basis of morphological or phenological descriptions and on floral characteristics [18]. Unfortunately, many of these characteristics are variable and depend on the age of the plants and on their sanitary conditions. Some characteristics are often subjective or unstable, as they are affected by environmental factors: soil and climate conditions, spacing and other management

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techniques. In addition, some of the important identification characteristics are not present in the one or two year-old plants coming from nurseries, and others refer to organs, such as leaves and flowers, that can only be observed in particular seasons. Therefore, the use of morphological features as the only criterion for clone identity control and certification is not advisable, because it can lead to mistakes that may not be detected for many years.

The difficulties in visual identification could be bypassed with the use of molecular markers-based tools, which are more reliable, stable and objective than morphological traits. A great amount of useful information for genetic characterization and identification of poplars has accumulated, coming from the use of both allozyme polymorphism [28, 30] or DNA markers: RAPDs [4, 23, 29, 31] AFLPs, [1, 6, 8, 15, 40], microsatellites (SSRs) [3, 6, 10, 14, 30] and ISSRs [20].

Compared to other molecular markers, SSRs are widely used for analysis of relationships between individuals, genetic variability and divergence among natural populations of numerous species (e.g. [13, 16, 32]). They are also the most useful tool for the genetic characterization of plant material, due to their particular genetic traits: high degree of polymorphism, codominance, high reproducibility, transferability between related species and relatively low implementation costs. Also, their resolution capacity and their stability are greater than those of allozymes and RAPDs, and their technical complexity and economic costs are lower than those of high efficiency markers such as AFLPs or SAMPLs. The main restriction for their use is that microsatellite loci have to be detected and sequenced to promote specific amplification, and that their polymorphism has to be tested in each sample set.

In the last decade, several authors have designed specific SSR primers for particular *Populus* species: for *P. tremuloides*, [9, 27]; for *P. nigra,* [33, 37]; and for *P. trichocarpa* [36], most of them available at Poplar Molecular Genetics Cooperative data base (http://www.ornl.gov/sci/ipgc/ssr_resource.htm). As many of them have shown their usefulness on different poplar species or hybrids, it is advisable to use them for the molecular description of commercial poplars, most of which are interspecific hybrids [3, 15, 29, 40].

To protect the legitimate interests of the poplar breeders, growers and industries, we have developed a reliable and affordable certification tool for commercial poplars in Spain. For this purpose, we have carried out a complete genetic description of the 28 commercial clones of the current Spanish Catalogue, to establish their genotypes. In addition, we have optimised the protocols to reduce the time and the economic costs of analysis.

Table I. List of the commercial clones analyzed in this study.

Species	Name of the clone	No. ramets	Provider
P. × *euramericana*	Agathe F.	10	ZA, GU, GE, LE
	Campeador	9	ZA, GU, GE, LE
	Canada Blanco	9	ZA, GU, GE, LE
	Flevo	9	ZA, GU, GE, LE
	I-MC	9	ZA, GU, GE, LE
	I-214	9	ZA, GU, GE, LE
	I-488	6	ZA, GU, GE
	Luisa Avanzo	9	ZA, GU, GE, LE
	Triplo	9	ZA, GU, GE, LE
	2000 Verde	8	ZA, GU, LE, M
	B-1M	7	ZA, GU, LE
	BL-Costanzo	4	ZA, GU
	Branagesi	7	ZA, GU, LE
	Dorskamp	7	ZA, GU, LE
	Guardi	10	ZA, GU, LE, M
	I-454/40	7	ZA, GU, LE
	NNDv	7	ZA, GU, LE
P. deltoides × *P. alba*	I-114/69	6	ZA, GU, GE
Populus deltoides	Lux	6	ZA, GU, GE
	Viriato	6	ZA, GU, LE, M
Populus nigra	Tr 56/75	9	ZA, GU, GE, LE
	Bordils	8	ZA, GU, GE, LE
	Lombardo Leonés	4	GU, LE
P. × *interamericana*	Beaupré	9	ZA, GU, GE, LE
	Raspalje	9	ZA, GU, GE, LE
	Boleare	8	ZA, GU, LE
	Unal	7	ZA, GU, LE
	USA 49-177	5	GU, LE
TOTAL	28	213	

ZA: Servicio de Investigación Agraria de la Diputación General de Aragón (SIA-DGA); GU: Centro Nacional de Mejora Forestal de "El Serranillo" (MIMAM); GE: Estación experimental "Mas Badía" (IRTA-DARP); LE: Vivero de chopo de la Junta de Castilla y León (Villafer, León); M: Vivero del Centro de Investigaciones Forestales del Instituto Nacional de Investigación Agraria (CIFOR-INIA) (Madrid).

2. MATERIALS AND METHODS

2.1. Plant material

The 28 registered poplar clones of the Spanish National Catalogue (Tab. I) used in this study were provided by five nurseries belonging to the "Registry of Plant Producers" of Spain: (i) Servicio de Investigación Agraria de la Diputación General de Aragón (SIA-DGA); (ii) Centro Nacional de Mejora Forestal de "El Serranillo" (MIMAM); (iii) Estación experimental "Mas Badía" (IRTA-DARP); (iv) Vivero de chopo de la Junta de Castilla y León (Villafer, León) (v) Vivero del Centro de Investigaciones Forestales del Instituto Nacional de Investigación Agraria (CIFOR-INIA) (Madrid).

Young leaves and wood from poplar trees were harvested, carefully labelled and lyophilised (24 h, –40 °C) from all five cited nurseries, taking samples from every clone present at each nursery. Lyophilised plant material was stored at –80 °C until DNA extraction.

2.2. DNA extraction and SSR analysis

After crushing lyophilised plant material in a Mixer Mill 301 (Reetsch GmbH & Co, Haan, Germany) (30 Hz, 45 min for wood samples; or 30 Hz, 10 min for leaves), total genomic DNA was isolated following the protocol by Doyle and Doyle [11] modified by Torres et al. [35] for small volumes. Ten nuclear microsatellite regions were selected considering genetic independence between markers and assuming the good synteny already described among *Populus* species [6]. Moreover, five of them are those used in the previous work of Fossati et al. [15], which will allow us to compare the results obtained. The SSR loci used and corresponding linkage groups (LG) were the following: PTR2 (LG IX), PTR4 (LG III) [9] and PTR7 (LG XII) [27] developed for *P. tremuloides*; WPMS9 (LG VI), WPMS14 (LG V), WPMS16 (LG VII), WPMS18 (LG not found), WPMS20 (LG not found) developed for *P. nigra* [33, 37]; PMGC14 (LG XIII) and ORPM127 (LG IV) developed for *P. trichocarpa* [36]. The forward primer for each locus was previously labelled with one of the three fluorochromes: 6-FAM, HEX or NED (Applied Biosystems, Foster City, Ca, USA), and a common fluorochrome label for different SSR markers was chosen for non-overlapping allele ranges.

To reduce the costs of the analysis, several multiplex reactions and lower reaction volumes were assayed with different primer combinations. Initial reaction volumes and amplification conditions were described by the following authors: for PTR2 and PTR4, Dayanandan et al. [9]; for PTR7, Rahman et al. [27]; for WPMS14, WPMS16, WPMS18 and WPMS20, Smulders et al. [33]; for WPMS9, Van der Schoot et al. [37]; for PMGC14, Tabbener et al. [34]; and for ORPM127, Tuskan et al. [36]. In all cases, we used a GeneAmp 9700 thermal cycler (Applied Biosystems) and the amplified fragments were analysed in an ABI-PRISM 310 genetic analyser (Applied Biosystems) using GeneScan ROX-500 as the internal standard. The sizing of the amplified fragments was carried out by GeneScan v.3.7. software (Applied Biosystems), which provides a raw result for the length fragment with two decimals of b.p. These results can vary somewhat (±1 nucleotide) in different machines, gels or capillary electrophoretic runs, while keeping the relative distances of alleles, and thus permitting the absolute identification of the clones.

2.3. Allele binning and data analysis

In order to obtain consistent results, each DNA was extracted, amplified and analysed for each locus, at least, two completely independent times for each sample. The allele binning was carried out using all the fragment sizes obtained in all the samples and all the repetitions analysed. Two approaches were used: frequency histograms for each marker and the plotting technique [22] based in sorted allele sizes.

The parameters calculated in order to evaluate the amount of information given by each locus were: the expected heterozygosity (H_e; [24]), the observed heterozygosity (H_o), the number of alleles for each locus, the probability of identity (*PI*; [26]) and the paternity exclusion probability (*PE*; [39]). We also calculated the probability of identity and paternity exclusion probability combined over all loci. These statistical parameters were calculated with the program IDENTITY 1.0 [38]. Moreover, to estimate the discriminatory power of each locus we considered the number of observed genotypes and the number of clones with unique genotypes. The relationships among the commercial poplar clones in the Spanish National Catalogue were illustrated by a phylogenetic tree based on unrooted neighbor-joining (*NJ*) method using Cavalli-Sforza chord distance D_C [5]. This dendrogram was generated using POPULATIONS software [21].

3. RESULTS

3.1. Genetic description of the 28 commercial clones of the Spanish National Catalogue

Ten SSR markers were used on the 28 commercial clones of Spanish National Catalogue, and their genotypes were determined from their allelic combined constitution. All the markers used yielded consistent amplification products, and, in all cases, the completely independent samples (ramets) analysed from each nursery showed the same results. Only PTR4 on the *P.* × *interamericana* did not amplify. All the analysed loci were polymorphic for this set of samples, ranging from 4 (PTR4) to 18 (WPMS14) alleles with an average of 9.6 alleles per marker (Tab. II). Regarding the observed and unique genotypes, the most informative loci were ORPM127 and WPMS14, with values greatly above the average for these two parameters. In fact, ORPM127 and WPMS14 SSR markers allow for the identification of 16 and 15 out of the 28 clones of the Spanish National Catalogue, respectively. The expected and observed heterozygosity ranged from 0.53 to 0.89 and from 0.04 to 0.93, respectively, with PTR4 giving the lowest and WPMS14 the highest allelic diversity values.

In order to evaluate the identification capacity of each locus, the probability of identical genotypes (*PI*) was calculated. The lowest *PI* value (0.04), corresponding to highest paternity exclusion probability values, was observed for three SSRs: PTR7, WPMS14 and ORPM127. The combined probability for identical genotypes calculated for all the markers together was 1.18×10^{-9} and the total exclusion probability reached a value of 0.99. Among the 28 different clones listed in the Spanish National Catalogue, only 26 different combined genotypes were detected (Tab. III). Two sets of clones [I-214/Campeador] and [I-MC/NNDv/BL-Costanzo] showed identical genotypes for the ten microsatellites assayed. Besides, we also found two different genotypes within the samples labelled as "Bordils" from different nurseries (in eight out of ten loci), both of them unique within the Spanish National Catalogue. They are identified henceforth as "Bordils^{1}" and "Bordils^{2}". In addition, several problems of nursery identification were detected in the following clones: (i) Some of the samples collected under the name "Viriato" in one of the registered nurseries showed the combined genotype specific for "Triplo" (identified henceforth as "Viriato^{2}"). (ii) The samples labelled as "2000 Verde" and "Guardi" from one of the official nurseries showed crossed genotypes based on the results from all the other nurseries.

The neighbor-joining unrooted (*NJ*) phylogenetic tree of Figure 1. show the genetic relations between the clones analysed. All clones appear cluster by species and there is a good correlation to morphological characteristics. Only Flevo clone, which has been reported to have morphological features similar to *P. nigra* comes out among this group of clones (J. Rueda, personal communication).

Table II. Genetic parameters from the microsatellite DNA loci observed for the 28 commercial clones of this study.

Locus	H_e	H_o	No. alleles	No. observed genotypes	No. clones with unique genotype	*PI*	*PE*	Allelic range (p.b.)
PTR 2	0.66	0.67	7	9	3	0.27	0.41	203–221
PTR 4	0.53	0.04	4	3	1	0.53	0.23	195–203
PTR 7	0.89	0.20	11	13	5	0.04	0.78	227–253
WPMS 14	0.89	0.93	18	20	15	0.04	0.78	221–278
WPMS 16	0.76	0.90	7	12	3	0.17	0.55	127–166
WPMS 18	0.62	0.67	8	11	6	0.24	0.41	215–248
WPMS 9	0.81	0.63	10	13	8	0.11	0.11	232–282
PMGC 14	0.82	0.90	9	14	7	0.10	0.66	190–223
WPMS 20	0.62	0.73	6	8	3	0.32	0.37	214–238
ORPM 127	0.89	0.90	16	21	16	0.04	0.79	156–226
Mean	0.75	0.66	9.6	12.4	6.7	0.18	0.51	–

Genetic parameters: expected and observed heterozygosity (H_e and H_o), number of alleles, number of observed genotypes, number of clones with unique genotype, probability of identity (*PI*), paternity exclusion probability (*PE*) and allelic range in base pairs.

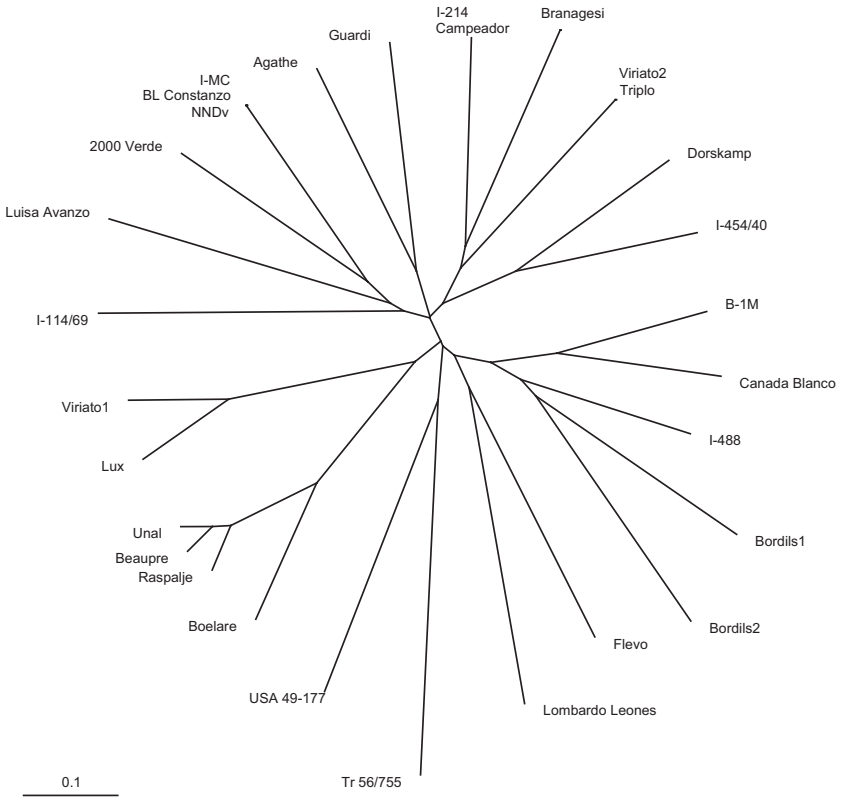

Figure 1. Neighbor-joining unrooted (*NJ*) phylogenetic tree of the 28 commercial poplar clones registered in the Spanish National Catalogue constructed using Cavalli-Sforza chord distance (D_C).

Table III. Size of alleles in base pairs for nine loci in 28 commercial clones registered in the Spanish National Catalogue.

Clones	PTR2		PTR4		PTR7		WPMS14		WPMS16		WPMS18		WPMS9		PMGC14		WPMS20		ORPM_127	
2000 Verde	212	215	201	201	247	247	231	242	133	142	215	230	232	246	193	211	226	232	156	220
Agathe F.	212	212	195	195	247	247	248	251	139	157	215	227	232	248	193	211	220	226	156	212
B-1M	209	212	201	201	235	245	248	248	139	142	215	242	232	246	190	205	220	220	156	190
Beaupré	212	212	–	–	231	231	236	251	127	139	215	215	282	282	190	202	220	232	200	220
BL-Constanzo	212	215	195	195	251	251	231	248	133	142	215	215	232	246	190	223	220	232	156	194
Boelare	212	212	–	–	227	227	254	260	139	166	215	215	282	282	190	202	220	232	198	208
Bordils[1]	209	212	201	201	251	251	245	278	142	142	224	239	250	260	208	211	220	232	164	164
Bordils[2]	209	215	201	201	233	233	221	248	142	148	224	239	250	250	208	223	226	232	158	160
Branagesi	209	212	195	195	237	237	248	272	139	157	215	248	232	246	190	202	220	232	160	190
Campeador	209	215	195	195	249	249	231	248	133	139	215	224	232	246	190	208	220	226	160	190
Canada Blanco	212	212	201	201	235	245	248	272	139	142	215	224	250	250	190	205	220	220	160	194
Dorskamp	209	212	195	195	247	247	251	269	139	142	215	230	234	282	190	202	220	238	156	208
Flevo	209	221	201	201	247	247	251	251	139	148	215	230	234	250	193	202	220	226	156	226
Guardi	212	212	195	195	239	239	260	278	139	142	215	224	232	250	193	211	220	220	158	212
I-114/69	212	212	195	195	253	253	236	263	133	142	215	230	250	250	193	199	214	232	158	222
I-214	209	215	195	195	249	249	231	248	133	139	215	224	232	246	190	208	220	226	160	190
I-454/40	209	215	195	195	247	247	231	272	139	142	215	224	250	250	190	202	220	238	160	190
I-488	209	212	201	201	233	233	248	272	139	142	215	239	232	250	190	202	220	232	160	190
I-MC	212	215	195	195	251	251	231	248	133	142	215	215	232	246	190	223	220	232	156	194
Lombardo L.	209	209	201	201	253	253	245	248	142	157	230	230	246	260	211	211	220	226	160	160
Luisa Avanzo	209	209	195	195	245	245	231	242	133	142	215	221	232	252	193	211	218	232	156	208
Lux	212	218	201	201	237	237	254	263	139	139	215	215	232	232	193	193	220	220	202	220
NNDV	212	215	195	195	251	251	231	248	133	142	215	215	232	246	190	223	220	232	156	194
Raspalje	212	212	–	–	231	231	236	251	139	166	215	215	282	282	190	202	220	232	198	220
Tr 56/75	203	209	197	203	227	251	227	239	142	148	227	230	242	256	199	217	232	232	156	156
Triplo	209	212	195	195	235	245	248	257	133	148	215	230	232	246	190	193	220	220	160	210
Unal	212	212	–	–	231	231	236	251	127	139	215	215	282	282	190	202	220	232	198	220
USA 49-177	206	215	–	–	231	245	266	275	139	142	215	227	242	242	193	205	220	232	202	218
Viriato[1]	212	218	201	201	233	233	254	275	139	139	215	215	232	232	193	193	220	220	218	220
Viriato[2]	209	212	195	195	235	245	248	257	133	148	215	230	232	246	190	193	220	220	160	210

[1] and [2] are two different genotypes with the same name.

3.2. Optimization of the genetic tool

In order to reduce the time and the economic costs of the analysis, two sets of three SSR markers from the ten markers assayed have been multiplexed: ([PMGC14, WPMS20 and WPMS9] and [WPMS14, WPMS16 and WPMS18]). Reduced reaction volumes were used and several amplification programmes were tested for optimization. The final PCRs were carried out in a total volume of 10 μL, containing genomic DNA, 1× GeneAmp PCR Buffer II, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 0.4 μM of each primer and 0.2 u of Amplitaq® DNA polymerase (Applied Biosystems, Ca, USA). The optimized protocol includes one multiplex reaction following a slight modified programme from van der Schoot et al. [37] (reducing the annealing temperature to 55 °C), while the initial protocols have been kept for PTR2, PTR4 and for PTR7.

Stable and clear resolution amplification products were obtained with DNA both from wood and leaves using the same amplification conditions.

4. DISCUSSION

The results of our study clearly prove that microsatellite DNA markers can be an effective tool for genetic identification of commercial poplar clones of the Spanish National Catalogue. All the ten loci analysed were polymorphic and some of them were very informative for identification purposes. Only PTR4 locus, which showed good results in other studies [29], was not informative for the cultivars here considered, due to the lack of amplification on *P.* × *interamericana* clones. PTR4 was first described for *P. tremuloides*, a species reported to be genetically closer to *P. deltoides*, than to either *P. nigra* and *P. trichocarpa*, [7]. However, in our sample set, PTR4 has amplified as well *P. nigra* clones (Bordils[1], Bordils[2], Lombardo Leonés and Tr 56/75) and *P. deltoides* (Lux and Viriato), but not *P. trichocarpa* parental in *P.* × *interamericana* group. This suggests that PTR4 does not amplify or show null alleles in both *P. trichocarpa* and *P. deltoides* species, although this latter could only be demonstrated by segregation analysis.

Of the 28 commercial clones in the Spanish National Catalogue, only 26 different combined genotypes have been found: two groups of clones were identical [I-214/Campeador] and [I-MC/NNDv/BL-Costanzo], and two different genotypes were observed for the same clone name ("Bordils[1]" and "Bordils[2]"). The set of markers used showed a total *PI* of 1.18×10^{-9}, meaning that the probability that two samples are different while showing the same genotypes is 1 out of 85 000 000 000 approximately. This strongly supports the idea that the clones showing identical genotypes are, in fact, synonyms.

The genotypic similarity between [I-214/Campeador] and [I-MC/NNDv] pairs of clones is also supported by the results obtained by Alvárez et al. [1] using AFLP markers and the analogy between genotypes for [I-MC/BL-Costanzo] accessions described in Fossati et al. [15] is also in agreement with our results. For the clone "Bordils" two clearly different genotypes (in eight out of ten loci) have been observed in the samples analysed. Both of them were unique within the Spanish Catalogue. These results are consistent with the origin of the clone, a selection from a natural *P. nigra* population (J. Rueda, personal communication), and a probable subsequent propagation of different individuals in the nurseries.

In this study, errors in labelling and management of plant material from the registered nurseries have been detected: (i) The genetic coincidence detected in one of the nurseries between "Triplo" and "Viriato[2]" clones, seems to be a management mistake affecting the mother trees, and limited to that particular nursery. (ii) The cross genotype between "2000 Verde" and "Guardi" clones from one of the registered nurseries is clearly a recent labelling mistake, because the new samples obtained and analysed from this nursery showed the genotypes found in original samples from the other nurseries. In this latter case, the mistake did not affect the mother trees.

Together, these results emphasize the need for a genetic identification of the poplar clones for authoritative certification and successful commercial management.

Time and costs of the analysis process were greatly reduced from those of the initial protocols, while maintaining stability and quality results (data not shown). Volume reactions and reactive amounts smaller than those used here yielded, in our hands, unstable results, due to difficulties in accurate measuring and handling. Good quality and stable amplification products were obtained from wood material, allowing the genetic analysis to be carried out at any time of the year. The accurate identification of clones of Spanish Catalogue can be achieved by using only three SSR markers: one multiplex reaction [WPMS14, WPMS16] and ORPM127, showing a $PI = 2.4 \times 10^{-4}$.

The need to use molecular methods for the certification of plant material from vegetative propagation has been suggested by numerous institutions and research groups [1, 3, 15, 29, 40]. The European laws permit within the entire community the cultivation of poplar clones listed in the National Catalogue of any member country. Therefore it would be advisable to standardize molecular certification protocols for the genus *Populus* for the entire European Community.

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