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PCR-based molecular markers for assessment of somaclonal variation in *Pinus pinea* clones micropropagated *in vitro*

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Abstract

Four different markers [random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP), and selective amplified microsatellite polymorphism length (SAMPL)] were applied for evaluating somaclonal variation of micropropagated genotypes of stone pine (*Pinus pinea* L.). The total number of primers tested was 130, with 223 combinations assayed. A high number of them amplified successfully (178), representing 79.82 % of the total, and the average number of amplified fragments ranged from 2.47 (ISSR) to 65.76 (SAMPL). Based on internal controls, no problem of reproducibility was detected. Almost no somaclonal variation was detected within the clones. Of the tested markers, ISSR, AFLP, and SAMPL showed monomorphic amplification profiles, with only RAPD markers showing some interclonal variation.

Additional key words: AFLP, caulogenesis, clonal stability, conifers, ISSR, RAPD, SAMPL, stone pine.

Introduction

In vitro propagation of valuable genotypes requires an evaluation of genetic stability, especially in forest trees and other woody plants with long rotation times (Ryynänen and Aronen 2005). Vegetative propagation of conifers based on tissue culture is a suitable method of clonal production of selected individuals, but the use of high concentrations of growth regulators (often used to enhance the rate of shoot multiplication) has been found to cause somaclonal variation in micropropagated plantlets (Venkatachalam *et al.* 2007). However, genetic fidelity of *in vitro*-propagated pines has been scarcely studied, *e.g.* in *Pinus thunbergii* (Goto *et al.* 1998) and *P. taeda* (Tang 2001).

Many approaches have been proposed to evaluate somaclonal variation. A first approach used to be morphological and physiological traits, but these methods require extensive observations of the plants until maturity, and differences may disappeared after a few growing seasons. Furthermore, some changes induced by *in vitro* culture cannot be observed *ex vitro* (Palombi and Damiano 2002). More efficient tools have now been developed using techniques related to DNA-based markers like random amplified polymorphic DNA (RAPD; Williams *et al.* 1990), inter simple sequence repeat (ISSR; Zietkiewicz *et al.* 1994), simple sequence repeat or microsatellite (SSR; Tautz 1989), amplified fragment length polymorphism (AFLP; Vos *et al.* 1995) and selective amplified microsatellite polymorphism length (SAMPL; Witsenboer *et al.* 1997). The cited techniques have their own specifications, as well as some limitations, that must be taken into account, *i.e.* selecting the marker system and technique used constitute two of the most important decisions in the experimental design (McGregor *et al.* 2000). The potential for polymorphism detection, even between closely related genotypes or in species characterized by low genetic diversity, indicates their usefulness (Witsenboer *et al.* 1997, Chandrika *et al.* 2008, Yao *et al.* 2008, Beharav *et al.* 2010).

Conifers are characterized by a high genetic diversity (González-Martínez *et al.* 2004), but previous studies on

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Abbreviations: AFLP - amplified fragment length polymorphism; ISSR - inter simple sequence repeat; PCR - polymerase chain reaction; RAPD - random amplified polymorphic DNA; SAMPL - selective amplified microsatellite polymorphism length; SSR - simple sequence repeat or microsatellite; UPGMA - unweighted pair-group method with arithmetic mean.

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several *Pinus* spp. have shown a low level of variability, regardless of the markers used, such as in *P. squamata* (Zhang *et al.* 2005) and *P. resinosa* (Mosseler *et al.* 1992). Stone pine (*Pinus pinea* L.) is a conifer principally found in the Iberian Peninsula, and the exceptionally low genetic polymorphism of this species has been confirmed in studies applying different markers, such as isozymes (Fallour *et al.* 1997), chloroplast and nuclear microsatellites (González-Martínez *et al.* 2004, Vendramin *et al.* 2008) and RAPD (Evaristo *et al.* 2002).

A micropropagation technique has successfully been developed for stone pine (Alonso *et al.* 2006), but there is just one published report on the genetic fidelity of the micropropagated plants (Cuesta *et al.* 2008). Therefore, we have undertaken a study of somaclonal variation analysis for stone pine, selecting those markers based on random genome amplification, such as RAPD, ISSR, AFLP and SAMPL, which have hitherto not been applied in this species. We have excluded SSR markers, because as González-Martínez *et al.* (2004) mention, no polymorphism was detected in the transfer of SSR motifs from *P. taeda* to *P. pinea*. Moreover, SSR markers have not been successfully used to examine clonal variation in plants, as they do not always detect major forms of genomic instability (Leroy and Leon 2000).

Materials and methods

Plants and DNA extraction: Plant material comprised needles from two-year old plants growing in greenhouse. These plants were regenerated clones, which were obtained after micropropagation (*via* adventitious organogenesis) of 6 selected half-sibling families, belonging to two different provenances (Cuesta *et al.* 2008). This micropropagation procedure consisted of cotyledon excision, a shoot induction on medium with 44.4 μM benzyladenine (BA) followed by shoot elongation, root induction and acclimatization of the plantlets. Some clones represented by 8 - 11 plantlets, belonging to different cotyledons of the same seed, enabled the assay of intraclonal variation while comparison of seeds from the same family permitted evaluation of the interclonal variation. Moreover, even those clones from different families represented by only 1 - 2 plantlets allowed comparison between families (Fig. 1*B*).

DNA was extracted by method using CTAB buffer (Doyle and Doyle 1987) with slight modifications. Parallel DNA extractions on 10 random selected samples were developed to test reproducibility. DNA concentration was measured by a *Beckman-Coulter DU800*® spectrophotometer (Fullerton, CA, USA).

Sixty primers were used for the RAPD analysis (*Operon Technologies*, Alameda, CA, USA), of which 20 had previously been tested (Cuesta *et al.* 2008). For the ISSR analysis, 21 primers were assayed. The UBC set was based on *Pinus squamata* (Zhang *et al.* 2005; UBC 807, 808, 811, 812, 813, 818, 820, 825, 828, 834, 840, 842, 844, 855, 857, 864, 886) and the LL set (LL1-LL4, was described by Leroy and Leon 2000) (Table 1*A*); all of them purchased from MWG Laboratories (*MWG-Biotech AG*, Ebersberg, Germany). DNA amplification reactions and PCR conditions were performed as detailed in Tables 1*B*,*C*. Amplification products were resolved by electrophoresis on a 2 % agarose gel stained with ethidium bromide, and then photographed on a *GelLogic 100 Kodak* UV transilluminator (*Eastman Kodak*, Rochester, NY, USA). In all cases lambda phage DNA digested with *Eco*RI (*Biolabs*®, New England, USA) and *Hind*III (*Takara Bio Europe SAS*, Saint-German-en-Laye, France) was used as size marker.

For AFLP and SAMPL analyses, genomic DNA (200 ng) was submitted to enzymatic digestion using 3 U each of *EcoRI* and *MseI* in their corresponding restriction/ligation (RL) buffer, in a final volume of 35 mm^3 at 37 ºC for 2 h. A volume of 10 mm^3 of ligation mixture was made (5 μM of *MseI* adapter, 0.5 μM of *EcoRI* adapter, 1.2 mM ATP, 1 U of T4 DNA ligase and RL buffer), added to the restriction reaction, and incubated for 5 h at 37 ºC. A template of 5 mm^3 of restriction-ligation reaction was used for the preamplification, and then verified by electrophoresis in 1.5 % agarose. Both amplification reactions and PCR conditions were carried out as indicated in Table 1*B*,*C*. Primers used were described in Table 1*A* and 2; in the case of SAMPL markers, the R-SP-SMPL set was based on Gupta *et al.* (2005), and CATA and GATA primers were based on GA and CA motifs, these being the most abundant motifs in conifer genomes (Schmidt *et al.* 2000). The primers were labelled with an infrared dye (IRD 700 and 800), sensitive to the automated sequencer *Licor Global IR2* DNA analyzer (*Licor*, Lincoln, NE, USA). The run was performed in 8 % polyacrylamide gel, and this automated *Licor* system generated digitized fingerprints, which were used in analysis with compatible analysis software.

In order to assure the fidelity of the results obtained, reactions were performed at least twice, including internal controls (replicates of the same sample in each combination, and different DNA extractions of the same individual). Only the consistently reproduced and distinguished bands were considered. The amplified fragments for each genotype and primer combination were scored manually as present or absent. A summary of a comparison between markers were carried out, quantifying the number of primers used, the number of assayed combinations and which of them were amplified, the total number of bands generated, and the average number of bands per molecular marker type. In instances where polymorphism was detected, a dendrogram was generated using the unweighted pair-group method with arithmetic mean (UPGMA) as well as statistical analysis (*AMOVA*), performed with *PAST* and *ARLEQUIN* ver. *3.0* software (Hammer *et al*. 2001, Excoffier *et al*. 2005, respectively).

Table 1. PCR conditions for the different molecular markers assayed. *A* - Primer sequences of ISSR and SAMPL assays. *B* - PCR reaction compounds. *C* - PCR programmes for each marker (SAMPL conditions are similar to AFLP.) Thermocycler used was *Gene Amp PCR System 9700*. R/L - restricted/ligated DNA; [a] - concentration of IRD-labelled primer [b] Ta - Annealing temperature 54 ºC (LL1); 62 ºC (LL2); 42 ºC (LL3); 52 ºC (LL4); [e] - touchdown of 1 ºC for each cycle.

A

ISSR: UBC-set	Sequence (5'-3')	SAMPL: CATA/GATA	Sequence (5'-3')
UBC-807	AGA GAG AGA GAG AGA GT	CATA	CAC ACA CAC ACA CAC ATA T
UBC-808	AGA GAG AGA GAG AGA GC	GATA	GAG AGA GAG AGA GAG ATA TA
UBC-811	GAG AGA GAG AGA GAG AC	SAMPL: R-SP-SMPL set	
UBC-812	GAG AGA GAG AGA GAG AA	R-SP-SMPL01	CTC TCT CTA ATA TAT ATA TA
UBC-813	CTC TCT CTC TCT CTC TT	R-SP-SMPL02	CAT CAT CAT CAT CGT CAT CAT
UBC-818	CAC ACA CAC ACA CAC AG	R-SP-SMPL03	CAT CAT CGT CCT CCT CAT AT
UBC-820	GTG TGT GTG TGT GTG TC	R-SP-SMPL04	ATC ATC ATC ATA TCA TCA TC
UBC-825	ACA CAC ACA CAC ACA CT	R-SP-SMPL05	ATC ATC ATC ATC AAT ATC ATC
UBC-828	TGT GTG TGT GTG TGT GA	R-SP-SMPL06	TCT CTC TCG TAC ACA CAC ACA C
UBC-834	AGA GAG AGA GAG AGA G(CT) T	R-SP-SMPL07	TTG TTG TTA TTC TTC TTC TTA
UBC-840	GAG AGA GAG AGA GAG A(CT) T	R-SP-SMPL08	ACA TAT ATG TAT GTA TGT ATG TAT
UBC-842	GAG AGA GAG AGA GAG A(CT) G	R-SP-SMPL09	ACA CAC ACA CAT ACA CAC AC
UBC-844	CTC TCT CTC TCT CTC T(AG) C	R-SP-SMPL10	CTC TCT CTT TTC TCC TTC TC
UBC-855	ACA CAC ACA CAC ACA C(CT) T	R-SP-SMPL11	GAA GAA GGA AGA ATG TGT GTG
UBC-857	ACA CAC ACA CAC ACA C(CT) G		
UBC-864	ATG ATG ATG ATG ATG ATG		
UBC-886	(AGC) (AGT) (AGC) CTC TCT CTC TCT CT		
ISSR: LL-set			
LL1	CAA CAA CAA CAA CAA		
LL2	CAG CAG CAG CAG CAG		
LL3	GAT AGA TAG ATA GAT A		
LL4	GAC AGA CAG ACA GAC A		

B

Molecular marker		DNA	$MgCl_2$	10× buffer	dNTPs	Primer	Taq polymerase	Total volume
RAPD		10 ng	2.5 mM	2.5 mm^3	0.2 mM	0.2 µM	1.00 U	25 mm^3
ISSR	UBC	20 ng	2.5 mM	2.0 mm^3	0.5 mM	2.5 µM	1.50 U	20 mm^3
	LL	10 ng	2.5 mM	2.5 mm^3	2.5 mM	10.0 µM	1.25 U	25 mm^3
AFLP	preamplification	5 mm^3 R/L	2.5 mM	5.0 mm^3	0.2 mM	0.3 µM	1.25 U	50 mm^3
	selective	2 mm^3 Preamp	2.5 mM	1.0 mm^3	0.2 mM	0.2 µM[a]/0.3 µM	0.75 U	10 mm^3

C

Molecular marker		PCR programme	Detection system
RAPD		1 min 95 ºC, [1 min 95 ºC/1 min 40 ºC/1 min 72 ºC] × 44 cycles, 5 min72 ºC	agarose 2 %
ISSR	UBC	5 min 94 ºC, [30 s 94 ºC/45 s 49 ºC/1.5 min 72 ºC] × 35 cycles, 7 min 72 ºC	agarose 2 %
	LL	1 min 94 ºC, [1 min 94 ºC/1 min Ta[b]/4 min 72 ºC] × 27 cycles, 7 min 72 ºC	agarose 2 %
AFLP	preamplification	5 min 92 ºC, [1 min 92 ºC/45s 60 ºC/1 min 72 ºC] × 26 cycles, 5 min 72 ºC	agarose 1.5 %
	selective	5 min 92 ºC, [1 min 92 ºC/45s 65 ºC/1 min 72 ºC][c] × 9 cycles, 1 min 72 ºC, [1 min 92 ºC/45s 56 ºC/1 min 72 ºC] × 24 cycles, 5 min 72 ºC	polyacrylamide 8 %

Results

Different molecular methods were used to evaluate the putative variability of *in vitro* clones of stone pine (Fig. 1*A,B,C,D*). There was no lack of reproducibility, and though markers applied are sensitive and reliable, there was no somaclonal, *i.e.*, intraclonal variation, but some interclonal variation was identified.

Of the 60 primers from three different Operon sets tested, 41 amplified (Fig. 1*A*). The total number of scored

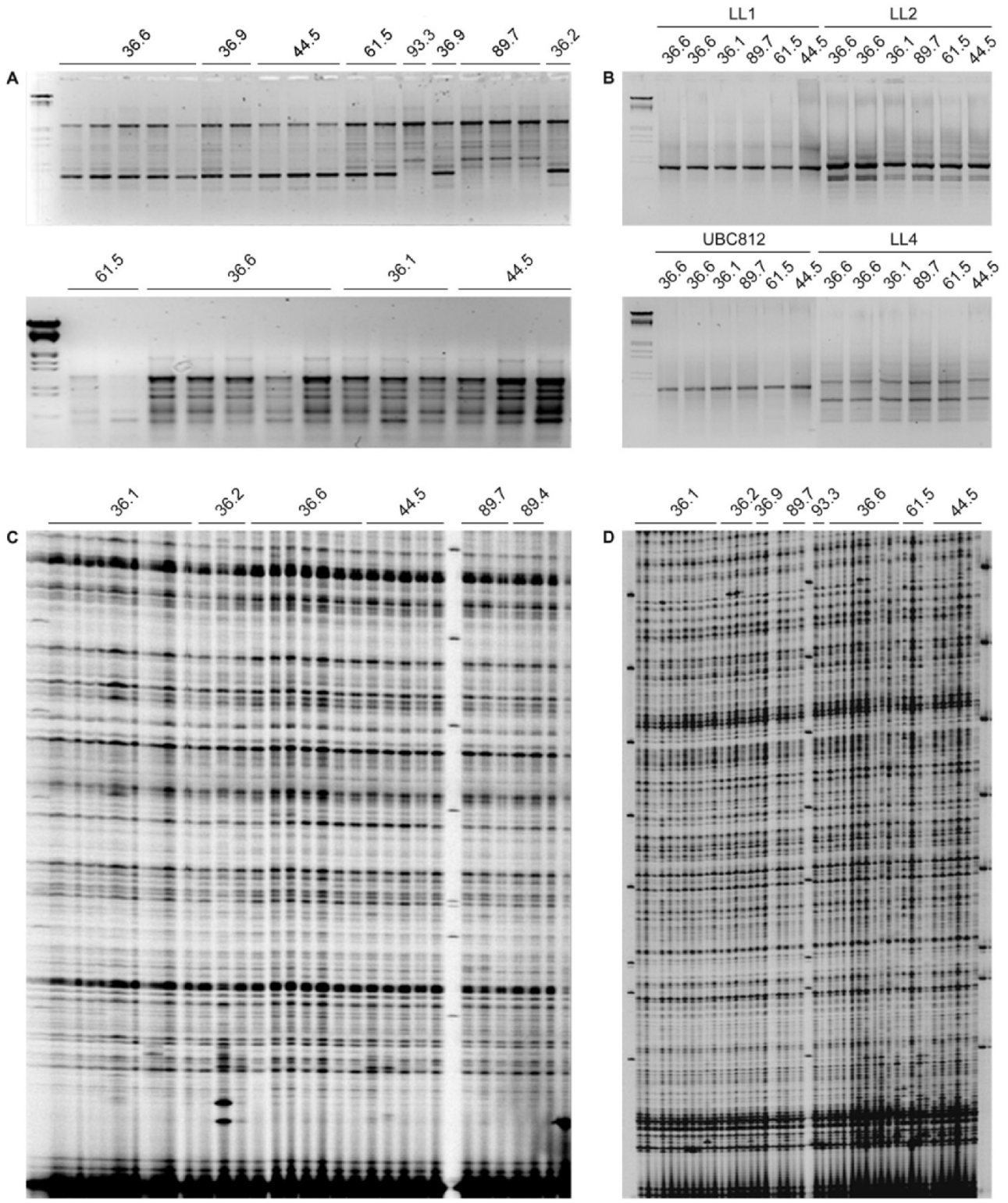

Fig. 1. Gel profiles of the different PCR-based markers assayed in micropropagated plantlets of selected families of stone pine. *A* - RAPD markers. The *upper part* shows one of the polymorphic primers (OPA11). The *lower part* presents a typical monomorphic pattern (OPH13). *B* - ISSR markers. Screening of 4 primers (LL1, LL2, UBC812, LL4). *C* - AFLP profile of a monomorphic combination (Eco+AGCG/Mse+CGTA). *D* - SAMPL monomorphic pattern (GATA/Eco+ACA).

Table 2. Combinations tested and number of bands obtained in AFLP and SAMPL assays of somaclonal variation in selected half-sibling families of stone pine. *A* - AFLP results. *B* - SAMPL results. Average only takes into account the amplified combinations; n/t: non tested; "-": absence of amplification.

A

Primer 1	Primer 2: Mse+ CAC	CAG	CAT	CCA	CCT	CGA	CCAC	CCAG	CCCG	CCGC	CCGT	CCTA	CCTG	CGAC	CGAG	CGTA	CGTC	CTTC
Eco+AGCG	19	28	43	24	50	31	-	35	20	50	46	68	18	36	45	61	36	-
Eco+AGCT	67	39	60	79	-	51	24	56	40	72	45	59	33	48	24	27	56	32
Eco+AAACA	n/t	62	-	38	n/t	42	n/t	n/t	n/t	32	29	n/t	n/t	65	-	32	n/t	-
Eco+AAACT	10	51	16	-	22	-	53	22	11	-	34	61	14	-	16	-	-	30

B

Primer 1	Primer 2: Eco + AAC	AAG	AAT	ACA	ACC	ACG	ACT	AGA	AGC	AGG	AGT	ATC	M+CTT
CATA	88	90	78	87	66	72	69	72	53	71	70	84	44
GATA	102	128	97	114	57	73	97	84	58	70	81	77	42

Primer 1	01	02	03	04	05	06	07	08	09
Eco+AGA	-	50	44	56	66	16	70	-	76
Eco+AGC	-	42	55	67	67	50	34	-	70
Eco+AGG	-	44	82	65	-	-	-	-	60
Mse+CTT	-	37	39	66	58	18	50	-	65
Mse+CCTA	21	-	-	n/t	n/t	n/t	57	-	71

Table 3. Summary and comparison of results obtained with the four molecular markers assayed in evaluating the genetic stability of micropropagated selected families of stone pine. [a] Number of primers used does not coincide with the general value, because some primers are common to different combinations. [b] +3/4/5 indicate the number of selective nucleotides of the corresponding primer.

Molecular marker[b]		Number of primers used[a]	Number of assayed combinations	Number of amplified combinations	Number of total bands	Average bands
RAPD	Operon A	20	20	17	102	6.00
	Operon C	20	20	12	42	3.50
	Operon H	20	20	12	43	3.58
ISSR	UBC	17	17	17	33	1.94
	LL	4	4	4	12	3.00
AFLP	Mse+3/Eco+4	8	12	11	491	40.90
	Mse+3/Eco+5	8	10	7	241	24.10
	Mse+4/Eco+4	14	24	22	931	38.80
	Mse+4/Eco+5	14	18	12	399	22.20
SAMPL	CATA,GATA/Eco+3	14	24	24	1938	80.75
	R-SP-SMPL/Eco+3	14	33	24	1421	59.20
	R-SP-SMPL/Mse+3/4	13	19	14	764	54.57

bands was 187, with an average of 4.36 bands per primer (Table 3). Only one set (Operon A) presented polymorphism, with a 0.29 % of polymorphic combinations. Though RAPD marker profiles cannot completely distinguish genotypes, *AMOVA* results established that 59.74 % of variation was due to intrapopulation variability, which might be due to within-family variation, although some interclonal variability was detected. Of the 21 arbitrary ISSR primers initially screened, all produced clear and scorable bands (Fig. 1*B*). The average bands per primer were 2.47 (Table 3), fewer than in RAPD markers, and every primer tested was monomorphic. Bands generated by both RAPD and ISSR primers ranged in size from 500 to 3 000 bp, similar to the findings of Evaristo *et al.* (2002).

In AFLP analysis, 64 combinations of primers were tested of which 52 showed amplification, but no polymorphism was observed. Results can be classified according to the number of selective nucleotides of the primers used (Table 3); the more selective nucleotides assayed, the lower the number of average bands observed (from 40.90 bands for Mse+3/Eco+4 to 22.20 bands for

Mse+4/Eco+5). The total number of scorable bands was 2062, their size ranging from 75 to 400 bp.

SAMPL assays tested 78 combinations, 64 of them being successfully amplified. No polymorphism was observed, although a total number of 4209 bands were scored. The CATA/GATA primers set presented the highest number of average bands (80.75), meanwhile the R-SP-SMPL set varied between 59.20 and 54.57 (Table 3); both sets ranged from 75-100 to 350-400 bp. The average number of bands for this molecular marker was 65.76, the highest value observed among the 4 PCR-based markers tested. Typical profiles generated by the employment of different markers assayed are shown (Fig. 1*A*,*B*,*C*,*D*).

Discussion

Tissue culture techniques may induce stress in regenerated or micropropagated plants. Such stress conditions could also be responsible for the DNA changes observed in these plants, consequently true-to-type clonal fidelity is one of the most important prerequisites in the micropropagation of any crop species (Lakshmanan *et al.* 2007). In this way, PCR-based techniques would be required to ascertain the genetic fidelity of plants regenerated, testing the specific protocol developed, particularly when high levels of cytokinins are used (Venkatachalam *et al.* 2007). Some previous reports have observed that the use of cytokinins, especially benzyladenine, combined with the continuous availability of high levels of nutrients induce morphological alterations (*e.g*. hyperhydricity), although such changes were not associated with genetic modifications in *P. thunbergii* (Goto *et al.* 1998) and *Musa acuminata* (Lakshmanan *et al.* 2007). As the micropropagation system applied here for the clonal multiplication of selected families of *P. pinea* requires a high concentration of benzyladenine (44.4 μM) in order to produce large numbers of shoots, this evaluation of the clonal fidelity of plantlets was essential. In the above mentioned assay (Cuesta *et al.* 2008), neither morphological alterations nor genetic changes were observed during the whole *in vitro* process. This fact however may be the result of using only one marker (RAPD) and it may be the case that undetected changes may have occurred as a consequence of point mutations occurring outside the priming sites (Lakshmanan *et al.* 2007).

A better analysis of genetic stability of plantlets could be achieved by using more than one DNA amplification technique, allowing increased possibilities for the identification of genetic variation, as different regions of the genome would be targeted (Palombi and Damiano 2002, Lakshmanan *et al.* 2007). The fact that RAPD markers quickly scan the whole genome, whereas AFLP markers check large portions of it (Arcade *et al.* 2000), and microsatellites (and PCR-markers based on them) detect variation at pre-determined sites, such as DNA repetitive regions lends support to the validity of this area of investigation.

The present study analyzes the possible variability of micropropagated plantlets of stone pine, testing four different molecular markers, and applying a high number of combinations (223). Almost 80 % of the assayed combinations amplified successfully with 6503 scored bands, thus we can be assured that an extensive screening of the genome was carried out. Results obtained highlighted the effectiveness of RAPD markers, as this was the only technique to detect interclonal variation, although not all assayed families were uniquely identified. However, it must be acknowledged that some authors have hypothesized that the absence of intraclonal RAPD polymorphism cannot guarantee genetic stability, because important variations like genomic mutations could be missed (Palombi and Damiano 2002). Conversely, several reports have demonstrated the ability of RAPD markers to detect genetic variations in different species (Tang 2001, Zhang *et al.* 2005, Hussain *et al.* 2008), even in closely related organisms (Lakshmanan *et al.* 2007, Feyissa *et al.* 2007). In contrast, ISSR scorable bands (45) did not reveal any polymorphism, with fewer average bands than RAPD. In relation to the AFLP assay, and in order to facilitate the readability of gels, an increasing number of selective nucleotides were screened, obtaining fewer bands per gel, as is recommended in cases of high complex genomes such as conifers (Arcade *et al.* 2000). Results from the frequently recommended technique SAMPL also showed no variation in amplification profile. This SAMPL technique has been designed to enhance the number of scorable characteristics to enable unequivocal identification of clones (Giménez *et al.* 2005), and to generate more amplified fragments than other techniques, owing to its ability to survey the hypervariable microsatellite region in the genome. One advantage of this marker is its less complex banding pattern compared to that of AFLP (Gupta *et al.* 2005). However, our data does not confirm the improvement of gel readability in conifers (Arcade *et al.* 2000); indeed, we have tested all the primers proposed by Gupta *et al.* (2005), and though 9 of them amplified correctly, none presented a clearer pattern than AFLP.

An extensive problem associated with molecular markers is related to the reproducibility of banding patterns (Leroy and Leon 2000, McGregor *et al.* 2000), caused, for instance, by heteroduplex formation of homologous sequences or by competition among different DNA fragments for amplification. In our case, ISSRs presented high reproducibility, principally because of using longer primers and higher annealing temperatures than those for RAPD (Zhang *et al.* 2005). AFLP and SAMPL techniques are undoubtedly reliable (Jones *et al.* 1997), and in our study, RAPD assays showed no lack of reproducibility, probably because a higher

annealing temperature than usual (40 ºC) was used.
The presence or absence of somaclonal variation depends on the source of explant and the method of regeneration; plantlets derived from tissue-cultured shoots being more resistant to genetic changes than those obtained from unorganized callus (Varshney *et al.* 2001). Indeed, most of the organized cultures, especially the shoot tips, maintain strict genotypic and phenotypic stability under tissue culture conditions (Goto *et al.* 1998). Our results, which shared a monomorphic pattern, are in accordance with this finding. Therefore we suggest that the present data indicates that no genetic variation is induced during *in vitro* growth culture of stone pine. If a molecular marker had to be recommended for somaclonal assessment in this species, it would be the RAPD marker, as, despite its limitations, it was the only one able to detect variation at interclonal level.

To the best of our knowledge, no extensive study on molecular markers in stone pine has been made before. Powerful techniques have been applied, showing successful amplifications with no intraclonal polymorphism detected. Moreover, after testing 4 different techniques, some of which are widely recognized as the most powerful (*e.g*. AFLP and SAMPL), we confirmed an absence of somaclonal variation in the micropropagation system of the stone pine, *i.e*. can be carried out without much risk of genetic instability.

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