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RESEARCH ARTICLE

PCR-BASED MOLECULAR MARKERS FOR ASSESSMENT OF SOMACLONAL VARIATION AND FINGERPRINT VARIETIES IN *IN-VITRO* CULTURED *PRUNUS SPP.*

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ABSTRACT

Tissue culture was used to preserve interesting agronomic traits present in old cultivars of different *Prunus spp.*, maintained in collection at Fruit Trees Research Centre in Rome. Assessment of somaclonal variation and fingerprinting of varieties, using RAPD and SSR analysis, were performed on one-year old plants of *Prunus persica* (L. Batsch), *Prunus armeniaca* (L.) and *Prunus domestica* (L.), in comparison to the mother plant, *in vivo* maintained. For both of the molecular markers, only reproducible fragments were scored and used to measure genetic similarity by Dice similarity index; similarity estimates were analysed by the UPGMA and the resulting clusters were expressed as dendrograms. The results disclosed genetic variability among the clones analysed from each varieties. Furthermore, while RAPD primers could amplify all DNA samples and can be useful applied to assess genetic variability and fingerprint peach, plum and apricot varieties, SSR primers used, obtained from peach, furnished results which were ambiguous for plum varieties. This last result is also discussed in this report.

INTRODUCTION

In situ germplasm preservation plays an important role in the maintenance of biodiversity and avoidance of genetic erosion, but preservation of woody plants in field gene banks requires huge land areas and it is expensive (Panis and Lambardi, 2005). For this, at Fruit Tree Reseach Centre in Rome, which is the seat of the Centre for the National Fruit Tree Germplasm Conservation, new methodologies included cryopreservation and *in vitro*-propagation, are applied (Engelmann and Engels, 2002) to preserve old varieties of different fruit trees, included *Prunus spp*. A major problem associated with *in vitro* techniques is the occurrence of somaclonal variation amongst sub-clones of one parental line, arising as direct consequence of *in vitro* culture of plant cells, tissue and organs (Larkin and Scowcroft, 1981). Thus, periodic monitoring of the degree of genetic stability of *in vitro* conserved plants is of utmost importance, especially in woody plants with long rotation time. The assessment of the genetic integrity of *in vitro* maintained genotypes in regular intervals can significantly reduce or eliminate the chance of occurrence of somaclonal variation. Several strategies are available to detect genetic variation (Bairu *et al.*, 2011), including phenotypic identification, flow cytometry and DNA analysis techniques. Flow cytometry, performed to study DNA content stability of regenerated plants, offers the possibility of fast and large scale analysis of the DNA content of cells for a genotype of purposes, e.g. determination of species specific DNA amount, analysis of the cell cycle activity in different tissues and measurement of endopolyploidization levels (Nassour *et al.*, 2003; Guo *et al*., 2005; Borchert *et al*., 2007). Phenotypic identification based the description of the morphological and physiological traits can be used (Seliskar and Gallagher, 2000; Podwyszynska, 2005), although this method requires extensive observation of plants until maturity. Nevertheless, some changes induced by *in vitro* culture, cannot be observed because the structural difference in the gene product does not always alter its biological activity, to such extent to be observed also in the phenotype. When this occurs, somaclonal variability can be evaluated by DNA analysis techniques.

Different DNA markers have been developed such as AFLP (Amplified Fragment Length Polymorphism,), RAPD (Random Amplified Polymorphic DNA) ISSR (inter simple sequence repeat) and SSR (simple sequence repeat or microsatellites); a few of them have been applied to assess genetic variation induced by tissue culture in plants (Rodrıguez Lopez *et al*., 2004; Prado *et al*., 2007; Cuesta *et al*., 2010; Bhatia *et al*., 2011; Dann and Wilson, 2011), including fruit trees (Palombi and Damiano 2002; Carvalho *et al*., 2004; Feuser *et al*., 2003; Orbovic *et al*., 2008). In order to evaluate the effects of *in vitro* culture on genetic integrity of micropropagated *Prunus spp*., we used RAPDs and SSRs molecular markers. Both these markers are visualized by PCR, the first with 10-mer primers and the second with a pair of specific primers designed on microsatellite flanking region. These kinds of DNA markers offer the advantage to being simple, less expensive and quicker to perform than RFLP (Powell *et al*., 1996) or AFLP (Lanham and Brennam, 1999), they can be conveniently used to rapidly evaluate somaclonal variability in micropropagated plants (Leva and Petruccelli, 2010; Mohanty *et al*., 2011) and

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also to fingerprint *Prunus* genotypes (Warburton and Bliss, 1996, Testolin *et al*., 2000; Quarta *et al*., 2001). 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) and could constitute a powerful tool to monitoring somaclonal variation due to tissue culture. In order to preserve *Prunus spp* germplasm, in this study RAPD and SSR markers were applied to monitoring genetic integrity of *in vitro*-maintained varieties of *P. persica* (L. Batsch), *P. domestica* (L.) and *P. armeniaca* (L.). We also discussed some evidences due to the cross-transferability of SSRs in *P. domestica.*

MATERIALS AND METHODS

Plant material and DNA extraction

Plant material comprised needles from one-year old plants of *Prunus persica* (L. Batsch) cultivars San Giorgio, Poppa di Venere and Charles Ingouf; *Prunus domestica* (L.) cultivars Favorita del Sultano and Zucchella and *Prunus armeniaca* (L.) cultivars Boccuccia Spinosa and San Castrese, previously obtained as reported in Damiano *et al*., (2008; 2011).

Somaclonal variation was evaluated on a variable number of rooted plants for each cultivar, in comparison to the mother plant *in vivo*-maintained. In particular, we analyzed 19 and 14 clones respectively for plum and apricot varieties, while in peach we analyzed 16 clones of the cultivar Charles Ingouf, 15 clones of the cultivar Poppa di Venere and 13 clones of the cultivar San Giorgio. DNA for RAPD and SSR analysis was extract from one gram of leaves by using the "5 Prime" DNA extraction kit (Eppendorf) followed by an RNAse treatment and two phenol-chloroform purifications. The Nano-Drop 1000 Spectrophotometer (Thermo Scientifica) was used to measure DNA concentration, ranged from 80 to 300 ng/µl and each sample was diluted to 10 ng/µl in sterile distilled water and used to perform SSR and RAPD amplifications.

PCR amplification and data analysis

PCR amplifications for RAPD and SSR were carried out in a Biometra Thermal Cycler. RAPD amplification was done in a total volume of 30 µl containing 20 ng DNA template, 10mM Tris HCl pH=9, 50 mM KCl, 2 mM $MgCl_2$, 400 µM each nucleotide, 0.2 µM primer and 0.5 U Taq Polymerase (Pharmacia Biotech.), using the amplification program reported in Williams *et al*., (1990). RAPD fragments were separated by electrophoresis on a 1.5% agarose gel in TBE 1x (90 mM Tris-borate, 2 mM EDTA, pH 8.0) stained in ethidium bromide and photographed under UV light.

Table 1. RAPD primers used

RAPD primers sequences			
70.1 [b]	CCGCCCAAAC	70.2 [b]	GCGCGTACGC
70.3 [b]	CCCAGCTGTG	70.4 [a,b,c]	CCCGCTACAC
70.5 [c]	CAAAGGGCGG	70.6 [a*]	ACCGCATGGG
70.9 [a]	AACGGGCGTC	79.10 [a]	ACCGCGAAGG
70.11 [c]	GTCTCGTCGG	70.12 [a]	GGCCTACTCG
70.15 [b]	GCCCTCTTCG	70.17 [b]	GAGACCTCCG
70.18 [b]	GGCCTTCAGG	70.20 [c]	TGCACGGACG
70.21 [a*,b]	GGACCCAACC	70.22 [b]	GTCGCCGTCA
70.23 [b]	TTGGCACGGG	70.24 [a*]	GTGTGCCCCA
70.27 [c]	CCTTCGGAGG'	70.29[c]	GGCAAGGCCT
80.21 [b, c]	ACGCGCCAGG	80.22 [c*]	ACTCGGCCCC
80.23 [a]	GGCCCCATGC	80.24 [a,b*]	CGCGAGGTGC
80.25 [b*]	CGATCCGCGC	80.26 [a*,b*,c]	CCCGACTGCC
80.27 [a,c]	GGCAAGCGGG	80.28 [b]	AGCGCGGACC

a= peach; b= plum; c= apricot. The RAPD primers (*) labeled were polymorphics and were necessary to fingerprint peach, plum and apricot varieties.

Table 2. SSR pair primers used

SSR	*Reference*	*SSR*	*Reference*
UDP96- 001 [a*]	Cipriani et al., 1999	CPPCT 006 [b]	Aranzana et al., 2002
UDP96- 008 [a]	"	CPPCT 017 [b]	"
UDP96- 010 [a]	"	CPPCT 022 [b]	"
UDP96- 013 [a]	"	BPPCT 006 [c]	Dirlewanger et al., 2002
UDP96- 015 [a*]	"	BPPCT 007 [c*]	"
UDP96- 018 [a]	"	BPPCT 017 [c]	"
UDP98- 022 [a]	"	BPPCT 023 [c]	"
UDP96- 401 [a*]	"	BPPCT 025 [c]	"
UDP98- 406 [a]	"	BPPCT 030 [c]	"
UDP98- 409 [a]	"	BPPCT 031 [c]	"
UDP98- 410 [a]	Testolin et al., 2000	BPPCT 035 [c]	"
UDP98- 411 [a]	"	BPPCT 037 [c]	"
UDP98- 412 [a]	"	BPPCT 039 [c]	"
pchgms 3 [b]	Sosinski et al., 2000	pchgms 27 [b]	Sosinski et al., 2000

a= peach; b= plum; c= apricot
*= polymorphic fragments

Table 3. Effectiveness of RAPD markers in detecting polymorphism of *Prunus* genotypes.

	P. persica	*P.domestica*	*P.armeniaca*
Total bands scored	39	47	44
Polymorphic fragments scored	4	3	2
Percentage of polymorphism	10.25	6.38	4.54
Number of primers used	11	13	10
Minimum polymorphism scored	1	1	1
Maximum polymorphism scored	2	2	1

Table 4. Effectiveness of SSR markers in detecting polymorphism of *Prunus* genotypes.

	P. persica	*P. domestica*	*P. armeniaca*
Total bands scored	75	10	48
Polymorphic fragments scored	3	0	1
Percentage of polymorphism	4.0	0	2.08
Number of primers used	13	5(*)	10
Minimum polymorphism scored	1	0	1
Maximum polymorphism scored	1	0	1

(*) only SSR CPPCT and pchgms labelled were able to amplified in this species

Reactions for microsatellites amplification were done in a total volume of 20 µl whit 60 ng DNA template, 10 mM Tris HCl pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 1 µM of both specific primers, 200 µM of each nucleotide and 1 U Taq polymerase (Pharmacia Biotech.). The amplifications were performed according to Testolin *et al*., (2002) for primer pairs UDP-labeled; Dirlewanger *et al*., (2002) for primer pairs BPPCT-labeled, Aranzana *et al*., (2002) for primer pairs CPPCT-labeled and Sosinski *et al*.(2000) for primer pairs pchcm-labeled. Amplification products were resolved by electrophoresis on a 3% Metaphor (FMC Bio Products) agarose gel in TBE 1x (90 mM Tris-borate, 2 mM EDTA, pH 8.0), stained in ethidium bromide and photographed under UV light. RAPD and SSR primers analyzed are reported in Tables 1 and 2 respectively. In order to assure the fidelity of the results obtained, reactions were performed at least twice and only the consistently reproduced and distinguished bands were considered. The amplified fragments for each genotype of the same species, were scored manually as present (1) or absent (0) and those readings were entered in a computer file as binary matrix, one for each molecular marker. Matrices were then analyzed by NTSYS pc-version 1.8 (Numerical Taxonomy System and Multivariate Analysis. Exeter Software New York, USA). Similarity for qualitative data was computed by using Dice similarity index (Sneath and Sokal, 1973) and similarity estimates were analysed by the UPGMA (unweighted pair-group method arithmetic averages), the resulting clusters were expressed as dendrograms.

RESULTS

In RAPD analysis, in order to increase confidence in the fragments included in the matrices (one for each markers), we scored only those were very conservatively, excluding weak bands or bands were ambiguous for some genotypes. A total of 35 ten-mer primers were tested and 28 (Table. 1) were selected to analyzed *Prunus* species. In peach 11 primers, which showing reproducible and well resolved bands, were finally analyzed. RAPD primers produced fragments were ranged from about 3500 to 250 base pairs size, but only bands from 500 to 2500 base pairs were judged reproducible and were scored to be analyzed. These primers furnished 39 fragments, 4 of which were polymorphics. In plum, amplification from13 primers were judged reproducible and from these we scored 47 fragments, 3 of which were polymorphics. In apricot RAPD analysis was obtained using 10 primers, which showing 44 fragments and 2 of these were polymorphics. Nevertheless, RAPD analysis produced polymorphics fragments, we never observed somaclonal variation among clones of the same cultivar (for each *Prunus spp*.), but a reduced set of these primers (Tab. 1) was sufficient to fingerprint varieties for peach (Fig. 1), plum (fig. 2) and apricot (Fig. 3), as showed in the dendrograms. A summary of the effectiveness of RAPD markers is given in Table 3; it includes the polymorphic scored fragments, effectively used in the data analysis. In SSR analysis we tested 34 primers, using DNA from two samples for each cultivar, in comparison of the mother plant. The preliminary results showed as only 21 (Tab. 2) of these primer pairs are able to amplified samples from the *Prunus spp* and were used to detect genetic variability.

Microsatellites primers produced fragments ranged from 50 to 250 base pairs. The number of SSR fragments scorable depend on the species: in peach we analyzed 75 SSR markers, the highest number of fragments obtained among the *Prunus spp* analyzed, and among these 3 were polymorphics (Tab. 4). In apricot we obtained only 1 polymorphic fragment for 48 scorable bands (Tab. 4). In both of the species polymorphics fragments are able to fingerprint cultivars (Table. 2) within the species but not to detect somaclonal variation among plantlets of the same variety. The similarity matrices obtained from SSR markers, analyzed as above reported, furnished, for peach and apricot varieties the same dendrogram show in RAPD analysis. In plum the results for SSR analysis were ambiguous. Microsatellites primer pairs UDP and BPPCT-labeled amplified fragments very faint and not always reproducible and for this they were discarded. For the others SSR primers pairs the results are reported in Tab. 4. The results indicate as also using SSR analysis we did not detect somaclonal variation but, while in *Prunus armeniaca* and in *Prunus persica* we could univocally characterized varietires, in *P. domestica* (cultivar Zucchella and Favorita del Sultano) these microsatellites did not produced polymorphic amplification patterns, neither within the same variety nor between the two varieties. Tissue culture techniques may induce stress in *in vitro*-maintained plants. Such stress conditions could also be responsible for the DNA changes observed in these plants,

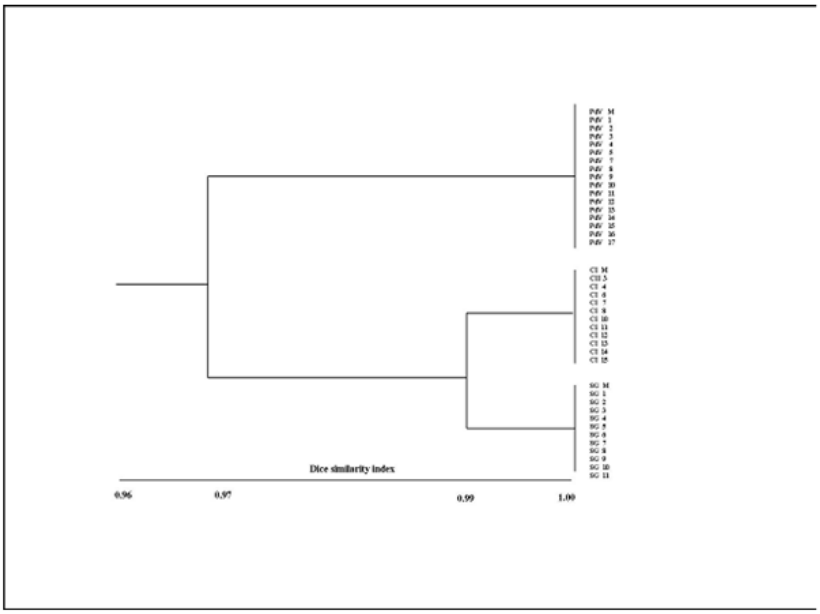

Fig.1 Dendrogram of peach varieties, based on RAPD markers (pdV, poppa di Venere; CI, Charles Ingouf; SG, San Giorgio).

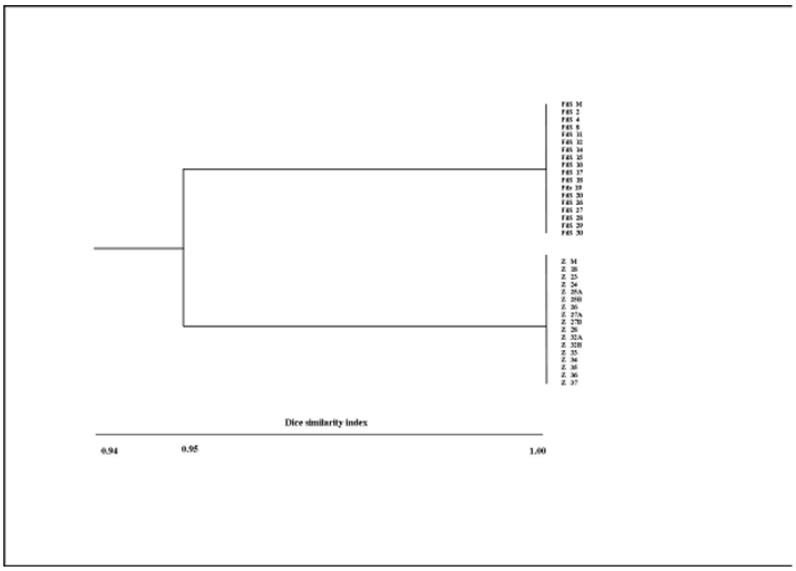

Fig.2 Dendrogram of plum varieties, bases on RAPD markers (FdS, Favorita del Sultano; Z, Zucchella)

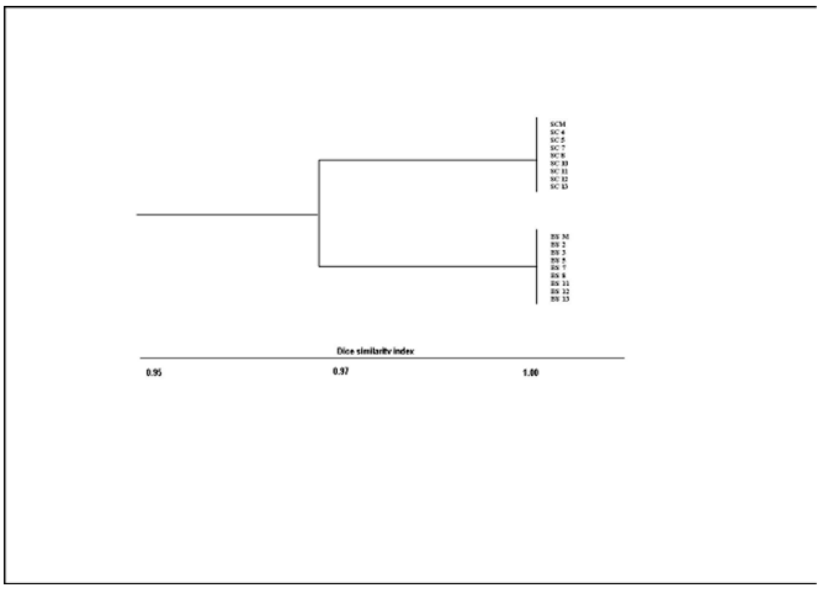

Fig. 3 Dedrogram of apricot varieties, based on RAPD markers (SC, San Castrese; BS, Boccuccia spinosa)

consequently true-to- type clonal fidelity is one of the most important prerequisites for *in vitro* culture techniques, such as micropropagation or cryopreservetion of any crop species (Lakshmanan *et al.*, 2007). Thus, periodic monitoring of the degree of genetic stability of *in vitro* conserved plants is of utmost importance. The assessment of the genetic integrity of *in vitro*-maintained genotypes in regular intervals can significantly reduce or eliminate the chance of occurrence of somaclonal variation. In this way, PCR-based techniques would be required to ascertain the genetic fidelity of plants regenerated, testing the specific protocol developed particularly when, how in this case, we use *in vitro* propagation to preserve germplasm. 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). For this the present study analyzes the possible variability of micropropagated plantlets of different *Prunus spp.*, testing two different molecular markers, using also SSR because they are both more polymorphic and codominant markers than RAPD.

In our case we did not detect somaclonal variation neither using RAPDs nor SSRs. In RAPD analysis, nevertheless we found polymorphic fragments we never observed somaclonal variation within the varieties analyzed for each species. The RAPD primers tested are unable to detect somaclonal variation but they are useful to characterize (fingerprinting) varieties among the *Prunus spp*, as showed in the dendrograms. The number of RAPD tested seems sufficient to analyze the effects of *in vitro* culture on genetic integrity, as reported in red ginger (Mohanty *et al*., 2011), in *Anuthum graveolens* (Jana and Shekhawat, 2011), in *Populus deltoids* (Rani *et al*., 1995) were the authors analyzed, respectively, 18, 10 and 11 RAPD primers, without found somaclonal variation due to tissue culture. On the other hand, many reports analyze a major number of RAPD primers to evaluate genetic variation in tissue culture: in almond plantlets genetic stability was evaluate using 64 RAPD primers (Martins *et al*., 2004); in micropropagated *Pinus thumbergii* 30 RAPD primers were used and in both of the case without detect somaclonal variation. The results obtained from RAPDs analysis indicate that the specific protocol used to micro-propragate the different varieties does not induce somaclonal variation.

To confirm these results we also used SSR markers, because, the use of more than one DNA analysis technique could detect somaclonal variation due to tissue culture, as reported in kiwifruit (Palombi and Damiano, 2002). In fact, different markers analyzed DNA at different levels: RAPD markers quickly scan the whole genome (Milbourne *et al*., 1997), whereas AFLP markers check large portions of it (Arcade *et al.*, 2000), and microsatellites detect variation at pre-determined sites, these sites are hypervariable with respect to other regions of genome and this hypervariability is due to a particular mechanism named slippage, that can occurs more frequently that point-mutation or insertion-delection events, responsible for generating polymorphism detectable by RAPD (Milbourne *et al*., 1997). In our case, nevertheless we used SSR primer pairs which are placed in all the eight linkage groups in *P. persica*, (Aranzana *et al*., 2003) and which were polymorphics in a wide sample of *Prunus* species (Cipriani *et al*., 1999; Testolin *et al*., 2000; Aranzana *et al*., 2002; Dirlewanger *et al*., 2002; Zhebentyayeva *et al*., 2003) and which were reported to be conserved in *Prunus* genome (Wünsch, 2009), we could not detect somaclonal variability. On the other hand, our result also confirm the conservation of SSR loci in different species of the genus *Prunus*, in fact SSR markers developed in *P. persica* are useful to detect genetic variability in different *Prunus spp*. (Wünsch and Hormaza, 2002, Vendramin *et al*., 2007) included *P. armeniaca* (Romero *et al*., 2003; Zhebentyayeva *et al*., 2003; Wünsch, 2009). Regarding the species *P. domestica* the SSR UDP and BPPCT -labeled amplified fragments very faint and not always reproducible. The other SSR markers utilized are able to amplified DNA for the two plum varieties, but without discrimination power between the varieties Favorita del Sultano and Zucchella and also without detect somaclonal variation within the two varieties. These results confirm that the specific protocol used for *in vitro*-propagated the different *Prunus* varieties does not induce somaclonal variation and can be used also to preserve germplasm. The results obtained from SSR also furnished others information about the possibility to use SSR markers developed in peach to analyzed genetic variability in a different species.

The European plum *P. domestica* is an hexaploid ($2n = 6x = 48$) and its biological origin is still unclear. It was generally thought that the species results from the cross between the diploid *P. cerasifera* (Ehrh.) and the tetraploid *P. spinosa* (L.), (Crane and Lawrence, 1952; Horvath *et al*., 2011). Furthermore, cytogenetics and comparative morphology seem indicate that plum may result from polyploidy forms arising from cherry plums, forming a "P. cerasifera–P domestica polyploid crop complex" (Horvath *et al*., 2011; Zohary and Hopf, 2000) also if the possibility of secondary hybridisation with other species, including sloe, cannot be excluded. These evidences and the fact that, in this study, we used SSR markers developed in peach could explain because SSR primer pairs used are not all able to amplified DNA from European plum, probably because SSR loci are not well conserved in this species. In fact, our results seem to be not in agreement with respect to previously report (Wünsch, 2009; Horvath *et al*., 2011), where SSR markers developed in peach amplified DNA from European plum varieties. Is a fact that *P. domestica* is a less-investigated species of *Prunoideae* subfamily; furthermore, the mentioned reports analyzed respectively only two genotypes or few SSR markers, for this our results could contribute to increase knowledge for this species and gave further information respect on the useful of SSR markers developed in peach for cross-species amplifications in *Prunus.*

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REFERENCES

Aranzana, M.J., Garcia-Mas, J., Carbo, J., Arus, P. 2002, Development and variability analysis of microsatellite markers in peach. Plant Breeding, 121: 87–92.

Aranzana, M.J., Pineda, A., Cosson, P., Dirlewanger, E., Ascasibar, J., Cipriani, G., Ryder, C.D., Testolin, R., Abbott, A., King, G.J., Iezzoni, A., Arus, P. 2003. A set of simple-sequence repeat (SSR) markers covering the Prunus genome Theor. Appl. Genet., 106: 819–825.

Arcade, A., Anselin, F., Faivre Rampant, P., Lesage, M.C., Pâques, L.E., Prat, P. 2000. Application of AFLP, RAPD and ISSR markers to genetic mapping of European and Japanese larch. Theor. Appl. Genet., 100: 299-307.

Bairu, M.W., Adeyemi, O.A., Van Staden, J. 2011. Somaclonal variation in plants: causes and detection methods. Plant Growth Reg., 63: 147-173.

Beharav, A., Maras, M., Kitner, M., Šuštar-Vozlič, J., Sun, G.L., Doležalová, I., Lebeda,. A, Meglič, V. 2000. Comparison of three genetic similarity coefficients based on dominant markers from predominantly self-pollinating species. Biol. Plant., 54: 54-60.

Bhatia, R., Singh, K.P., Sharma, T.R., Jhang, T.,, 2011. Evaluation of the genetic fidelity of in vitro-propagated gerbera (Gerbera jamesonii Bolus) using DNA-based markers. Plant Cell Tiss. Org. Cult., 104: 131–135.

Borchert, T., Fuchs, J., Winkelmann, T., Hohe, A. 2007. Variable DNA content of *Cyclamen persicum* regenerated *via* somatic embryogenesis: rethinking the concept of long-term callus and suspension cultures. Plant Cell Tiss. Org. Cult., 90: 225-263.

Carvalho, L., Goulão, L., Oliveira, C., Gonçalves, J.C., Amâncio, S. 2004. RAPD assessment for identification of clonal identity and genetic stability of *in vitro* propagated chestnut hybrids. Plant Cell Tiss. Org. Cult., 77: 23-27.

Chandrika, M., Thoyajaksha, V., Rai, R., Kini, R. 2008. Assessment of genetic stability of *in vitro* growth *Dictyospermum ovalifolium.* Biol. Plant., 52: 735-739.

Cipriani, G., Lot, G., Huang, W.G., Marrazzo, M.T., Peterlunger, E., Testolin, R. 1999. AC/GT and AG/CT microsatellite repeats in peach [Prunus persica (L) Batsch]: isolation, characterisation and cross-species amplification in *Prunus*. Theor. Appl. Genet., 99: 65–72.

Crane, M.B., Lawrence, W.J.C. 1952. The Genetics of Garden Plants. Macmillan, London.

Cuesta, C., Ordás, R.J., Rodríguez, A., Fernández, B. 2010. PCR-based molecular markers for assessment of somaclonal variation in Pinus pinea clones micropropagated in vitro. Biol. Plant., 54: 435-442.

Damiano, C., Monticelli, S., Frattarelli, A. 2011. La micropropagazione dell'albicocco: recenti progressi e ricerche su nuovi terreni di coltura per un efficiente protocollo di propagazione In: La micropropagazione in Italia: stato attuale e prospettive future. Pp13-14.

Damiano, C., Ponticelli, S., Frattarelli, A. 2008. Allestimento di una collezione in vitro di cultivar di pesco di interesse storico. VI Convegno Nazionale sulla Peschicoltura Meridionale p. 60.

Dann, A.L., Wilson, C.R. 2011. Comparative assessment of genetic and epigenetic variation among regenerants of potato (Solanum tuberosum) derived from long-term nodal tissue-culture and cell selection. Plant Cell. Rep., 30: 631-639.

Dirlewanger, E., Cosson, P., Tavaud, M., Aranzana, M.J., Poizat, C., Zanetto, A., Arus, P., Laigret, F. 2002. Development of microsatellite markers in peach [Prunus persica (L.) Batsch] and their use in genetic diversity analysis in peach and sweet cherry (Prunus avium L.). Theor. Appl. Genet., 105: 127-138.

Engelmann, F., Engels, J.M.M. 2002. Technologies and strategies for *ex situ* conservation. In Engels, JMM, Ramanatha Rao, V, Brown, AHD and Jackson, MT (eds) Managing Plant Genetic Diversity. Wallingford and Rome, CAB International and IPGRI, pp 89-104.

Feuser, S., Meler, K., Daquinta, M., Guerra, M.P., Nodari, R.O. 2003. Genotypic fidelity of micropropagated pineapple *(Ananas comosus)* plantlets assessed by isozymes and RAPD markers. Plant Cell Tiss. Org. Cult., 72: 221-227.

Guo, Y.D., Mizukami, Y., Yamada, T. 2005. Genetic characterization of androgenic progeny derived from *Lolium perenne* × *Festuca pratensis* cultivars. New Phytol., 166: 455-464.

Horvath, A., Balsemin, E., Barbot, J.C., Christmann, H., Manzano, G., Reynet, P., Laigret, F., Mariette, S. 2011. Phenotypic variability and genetic structure in plum (Prunus domestica L.), cherry plum (P. cerasifera Ehrh.) and sloe (P. spinosa L.). Scientia Hort., 129: 283–293.

Jana, S., Shekhawat, G.S. 2011. In vitro regeneration of Anethum graveolens, antioxidative enzymes during organogenesis and RAPD analysis for clonal fidelity. Biol. Plant., 56: 373-376.

Lakshmanan, V., Venkataramareddy, S.R., Neelwarne, B. 2007. Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers Electron. J. Biotechnol., 10: 106-113.

Lanham, P.G., Brenner, R.M. 1999. Genetic characterisation of goosberry (*Ribes grossularia* subgenus *Grossularia*) germplasm using RAPD, ISSR and AFLP markers. J. Hort. Sci. Biot., 74: 361-366.

Larkin, P.J., Scowcroft, W.R. 1981. Somaclonal variation. A novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet., 60: 197-214.

Leva, A.R., Petruccelli, R. 2010. Monitoring of cultivar identity in micropropagated olive plants using RAPD and ISSR markers. Biol. Plant., 56: 373-376.

Martins, M., Sarmento, D., Oliveira, M.M. 2004. Genetic stability of micropropagated almond plantlets as assessed by RAPD and ISSR markers. Plant Cell Rep., 23: 492–496.

McGregor, C.E., Lambert, C.A., Greyling, M.M., Louw, J.H., Warnich, L. 2000. A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tetraploid potato (*Solanum tuberosum* L.) germplasm. Euphytica. 113: 135-144.

Milbourne, D., Meyer, R., Bradshaw, J.E., Baird, E., Bonar, N., Provan, J., Powellm W,, Waugh, R. 1997. Comparison of PCR-based marker system for the analysis of genetic relationships in cultivated potato. Mol. Breed., 3: 127-136.

Mohanty, S., Panda, M.K., Sahaoo, S., Nayak, S. 2011. Micropropagation of *Zingiber rubens* and assessment of genetic stability through RAPD and ISSR markers. Biol. Plant., 55: 6-20.

Nassour, M., Chasseriaux, G., Dorion, N. 2003. Optimization of protoplast-to-plant system for P*elargonium* × *hortorum* Alian and genetic stability of the regenerated plants. Plant Sci., 165: 121-128.

Orbović, V., 0alović, M., Viloria, Z., Nielsen, B., Gmitter, J.F.G., Castle, W.S., Grosser, J.W. 2008. Analysis of genetic variability in various tissue culture-derived lemon

plant populations using RAPD and flow cytometry. Euphytica. 161: 329–335.

Palombi, M.A., Damiano, C. 2002. Comparison between RAPD and SSR molecular markers detecting genetic variation in kiwifruit (*Actinidia deliciosa* A. Chev). Plant Cell Rep., 20:1061-1066.

Panis, B., Lambardi, M. 2005. Status of cryopreservation technologies in plants (crops and forest trees). International Workshop "The Role of Biotechnology for the Characterisation and Conservation of Crop Forestry Animal and Fishery Genetic Resources". Turin March 5-7 pp. 43-54.

Podwyszynska, M. 2005. Somaclonal variation in micropropagated tulips based on phenotype observation. J. Fruit Ornam. Plant Res., 13: 109-122.

Powell, W., Morgante, R., Andre, C., Hanafey, M., Vogel, J., Tingey, S., Rafalsky, A. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breed., 2: 225-238.

Prado, M.J., Gonzales, M.V., Roma, S., Herrera, M.T. 2007. Adventitious plant regeneration of leaf explants from adult male kiwifruit and AFLP analysis of genetic variation. Plant Cell Tiss. Org. Cult., 88: 1-10.

Quarta, R., Dettori M.T., Verde, I., Marchesi, U., Palombi, M.A. 2000. Characterization and evaluation of genetic diversity in peach germplasm using RAPD and RFLP. Acta Hort., 546: 489-498.

Rani, V., Parida, A., Raina, S.N. 1995. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh. Plant Cell Rep., 14: 459-462.

Rodrıguez Lopez, C.M., Wetten, A.C., Wilkinson, M.J. 2004. Detection and quantification of in vitro-culture induced chimerism using simple sequence repeat (SSR) analysis in Theobroma cacao (L.). Theo.r Appl. Genet., 110: 157–166.

Romero, C., Pedryc, A., Muñoz, V., Llácer, G., Badenes, M.L. 2003. Genetic diversity of different apricot geographical groups determined by SSR markers. Genome, 46: 244–252

Seliskar, D.M., Gallagher, J.L. 2000. Exploiting wild population diversity and somaclonal variation in the salt marsh grass *Distichlis spicata* (poaceae) for marsh creation and restoration. Am. J. Bot., 87: 141–146.

Sneath, P.H.A., Sokal, R.R. 1973. Numerical Taxonomy. The Principles and Practice of Numerical Classification. Freeman, San Francisco.

Sosinski, B., Gannavarapu, M., Hager, L.D., Beck, L.E., King, G.J., Ryder, C.D., Rajapakse, S., Baird, W.V., Ballard, R.E., Abbott, A.G. 2000. Characterization of microsatellite markers in peach [Prunus persica (L.) Batsch]. Theor. Appl. Genet., 101: 421–428.

Testolin, R., Marrazzo, T., Cipriani, G., Quarta, R., Verde, I., Dettori, M.T., Pancaldi, M., Sansavini, S. 2000. Microsatellite DNA in peach (Prunus persica (L.) Batsch) and its use in fingerprinting and testing the genetic origin of cultivars. Genome, 43: 512–520.

Vendramin, E., Dettori, M.T., Giovinazzi, J., Micali, S., Quarta, R., Verde, I. 2007. A set of EST-SSRs isolated from the peach fruit transcriptome and their transportability across Prunus species. Mol. Ecol. Notes, 7: 307–310.

Warburton, M.L., Bliss, F.A. 1996. Genetic diversity in peach (Prunus persica L. Batch) revealed by random amplifed polymorphic DNA (RAPD) markers and compared to inbreeding coeffcients. J. Am. Soc. Hort. Scie., 121: 1012-1019.

Witsenboer, H., Vogel, J., Michelmore, R.W. 1997. Identification, genetic localization, and allelic diversity of selectively amplified microsatellite polymorphic loci in lettuce and wild relatives (*Lactuca* spp.). Genome, 40: 923-936.

Wünsch, A. 2009. Cross-transferable polymorphic SSR loci in Prunus species. Science Hort., 120: 348–352.

Wünsch, A., Hormaza, J.I. 2002. Molecular characterisation of sweet cherry (Prunus avium L.) genotypes using peach Prunus persica (L.) Batsch SSR sequences. Heredity, 89: 56–63.

Yao, H., Zhao, Y., Chen, D.F., Chen, J.K., Zhou, T.S. 2008. ISSR primer screening and preliminary evaluation of genetic diversity in wild populations of *Glycyrrhiza uralensis*. Biol. Plant., 52: 117-120.

Zhebentyayeva, T.N., Reighard, G.L., Gorina, V.M., Abbott, A.G. 2003. Simple sequence repeat (SSR) analysis for assessment of genetic variability in apricot germplasm. Theor. Appl. Genet., 106: 435–444.

Zohary, D., Hopf, M. 2000. Domestication of plants in the old world. Oxford University Press, Oxford, United Kingdom.

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