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DNA fingerprinting of Japanese plum (*Prunus salicina*) cultivars based on microsatellite markers

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Abstract – *Forty-seven Japanese plum (*Prunus salicina*) cultivars were genotyped with eight microsatellite markers, aiming at obtaining the DNA fingerprinting profiling, distinguishing and characterizing a representative set of Japanese plum cultivars. The eight SSR loci amplified 104 alleles (8 to 21 alleles per locus, mean 13). Polymorphism Information Content (PIC) ranged from 0.680 to 0.886 (mean 0.803). The observed heterozigozity (Ho) ranged from 0.529 to 0.915 (mean 0.770). Probability of Identity (I) of each locus ranged from 0.019 to 0.113 (mean 0.054). The combined Probability of Identity was 2.66 x 10^{11}, and the Power of Exclusion of the eight loci was 99.99976%. 57 out of 104 alleles showed frequency lower than 0.05. These low allele frequencies contributed to raise the distinguishability of plum cultivars. These results will contribute, as excellent descriptors, to select parental for crossings, to perform early identification of segregating clones with potential to be cultivars, and to protect the cultivars.*

Key words: *Cultivar protection, cultivar identification, genetic similarity/relationship, SSR markers.*

INTRODUCTION

Japanese plum (*Prunus salicina*) is one of the two predominating species in large-scale commercial plum production. Varieties of this species have a wide range of adaptation from temperate regions to the subtropics and are the predominant fresh market type in America and Asia. The other species, the hexaploid European plum (*Prunus domestica*), is more adapted to cool temperate climates (Okie and Weinberger 1996, Topp et al. 2012).

The term Japanese plum was originally applied to *P. salicina,* but now includes all the freshmarket plums developed by intercrossing various diploid species with the original one. Although it is native to China, this species was initially improved in Japan, and later, to a much greater extent, in the United States (Okie and Hancock 2008).

At the end of the 19th century, Luther Burbank crossed imported *P. salicina* with *P. simonii* and several American plum species. These cultivars formed the base for current cultivars. In 1996, eight of the top ten producing Californian cultivars had Luther Burbank cultivars in their ancestry (Okie and Ramming 1999). A consequence of this poly-specific breeding, associated with the natural outcrossing of Japanese plums, is the great variability among plum cultivars. Byrne (1990) found that the mean inbreeding and coancestry coefficients for plum were one half or less than those for peach. Currently, Japanese plum cultivars are a mixture of *P. salicina* and at least one other plum species.

Plum cultivar identification can be very difficult when relying upon morphological characteristics alone. DNA-based markers, and particularly microsatellites (or SSRs), are very useful tools for distinguishing cultivars since they directly reflect the genotype. In addition, there is a large number of potential polymorphic sequences available for distinct genetic studies. Moreover, microsatellite markers also showed to be a powerful tool for genetic characterization of plum varieties (Ahmad et al. 2004); thus, they can be used to solve cases of taxonomic synonyms, misidentification, and patent or protection issues. Since effective utilization of germplasm resources depends on accurate and unambiguous characterization, microsatellites can also help breeders in their breeding programs.

The objectives of this work were to perform the genetic characterization, and to establish a DNA fingerprinting of commercial plum cultivars. With the use of SSR markers,

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cultivars from the *Prunus* breeding program at EPAGRI-Videira Experimental Station could be discriminated from other cultivars.

MATERIAL AND METHODS

Plant material and DNA isolation

The forty-seven Japanese plum cultivars (Table 1) selected for this study represent a wide genetic spectrum, containing several target genes, particularly to the plum breeding in south Brazil. Currently, some of these cultivars are commercially cultivated in south Brazil, such as Fortune and Laetitia cultivars. Other cultivars represent the genetic pool for the Japanese plum breeding, such as Chatard, Piamontesa and Carazinho, which are highly resistant to leaf scald (Dalbó et al. 2010). The Japanese plum collection is located at Epagri – Videira Experimental Station (Videira, SC, Brazil), lat 27° 00' 30" S, long 51° 09' 06" W, and alt 800 m asl. DNA isolation was carried out with a modified method of Doyle and Doyle (1990), as described in Vieira et al. (2005).

SSR markers

Eight genomic microsatellite markers chosen for the genetic characterization (Table 2) were originally developed by Cipriani et al. (1999), Dirlewanger et al. (2002), Yamamoto et al. (2002) and Mnejja et al. (2004). The choice was based on the fact that these markers are highly polymorphic, and because they amplified microsatellite sequences that are located in distinct linkage groups. CPSCT markers (Mnejja et al. 2004) were designed originally to *Prunus salicina* Lindell. The other microsatellites were designed to peach (*Prunus persica* [L.] Batsch) and apricot (*Prunus armeniaca* L.), and were used for being highly polymorphic in several *Prunus* species, such as almond (*Prunus dulcis*), sweet cherry (*Prunus avium* L.), apricot (*Prunus armeniaca* L.), and others (Mnejja et al. 2010). Each 5′ forward oligo was labelled with a fluorophore in order to enable the automatized genotyping (Table 2).

PCR conditions and genotyping

PCR reactions were performed in a volume of 15 µl containing 10 mMTris – HCl pH 8.8, 50 mMKCl, 1.5 mM of MgCl2, 0.2 mM of each dNTP, 0.3 µM of each primer, 15 ng of genomic DNA, and 1 U of Taq DNA polymerase (AmpliTaq Gold® Invitrogen). A BioRad C1000 Thermal Cycler was used to amplify the eight SSR loci with the following cycling profile: 94 ºC for 3 min, then 30 cycles of 94 ºC for 30 s; annealing temperature specific to each primer (see table 2 for each SSR temperature) for 30 s, and 72 ºC for 30 s; and a final extension step of 5 min at 72 ºC. After the reactions, all PCR products were diluted 20X in ultrapure water in order to be genotyped by capillary electrophoresis in a MegaBACE 1000 DNA Analysis System (GE Healthcare) DNA sequencer. Alleles were genotyped by comparison with ET 400-R size standard (GE Healthcare), using Fragment Profiler software version 1.2 (GE Healthcare).

Table 1. Name and origin of Japanese plum (*Prunus salicina*) cultivars used in this study

No	Cultivar	Origin	No	Cultivar	Origin	No	Cultivar	Origin
1	Harry Pickstone	South Africa	17	Corazon Rojo	Mexico	33	Pluma 7	Brazil
2	Red Beauty	USA	18	Golden King	USA	34	América	Brazil
3	Obilnaja	Russia	19	Gigaglia	Argentina	35	Wade	USA
4	Sempre Amalia	Argentina	20	Sanguinea	Brazil	36	Reubennel	South Africa
5	SA 86-13	South Africa	21	Januária	Brazil	37	Black Ruby	USA
6	Tankiumaru	Japan	22	Gran Cuore	Brazil	38	Blood Plum	Japan
7	Linda Rosa	Argentina	23	Sordum	Japan	39	Fortune	USA
8	Gulf Ruby	USA	24	Gran Sultan	New Zealand	40	Santa Rita	Brazil
9	Rosa Mineira	Brazil	25	Piamontesa	Argentina	41	Coeur de Lion	Italy
10	Camila	Brazil	26	Carazinho	Brazil	42	Robusto	USA
11	Estrela Púrpura	Argentina	27	Songold	South Africa	43	XV de Novembro	Brazil
12	Laroda	USA	28	Santa Rosa	USA	44	Pobeda	Russia
13	Homeside	USA	29	Piuna	Brazil	45	Angeleno	USA
14	Catalina	USA	30	Laetitia	South Africa	46	Black Amber	USA
15	Bruce	USA	31	Methley	South Africa	47	Amarelinha	Brazil
16	Simka	USA	32	Kelsey Paulista	Brazil			

Table 2. Primer sequences, linkage group, annealing temperature, SSR motif and observed size range of SSR *loci* selected for the molecular fingerprinting

Locus	**Primer sequence (5´- 3´)**	**L.G. [5]**	**Annealing temperature** (°C)	**SSR Motif**	**Observed size range (bp)**
BPPCT 02[1]	HEX™ F: TCGACAGCTTGATCTTGACC	2	55	(AG)25	178-210
	R: CAATGCCTACGGAGATAAAAGAC				
CPSCT 06[2]	HEX™ F: ACAAAACCAAGCACCGTCTC	5	62	(CT)15	102-140
	R: GGGCAAATGCTTACCTGTT				
CPSCT 08[2]	6-FAM™ F: TGGATCCAATCCAAGAGTCT	1	62	(GA)17	157-237
	R: GCAGCAAGTTGTTCTTGGTT				
CPSCT 18[2]	6-FAM™ F: AGGACATGTGGTCCAACCT	8	52	(CA)5(CT)20	122-170
	R: GGGTTCCCCGTTACTTTCA				
CPSCT 39[2]	6-FAM™ F: GCCGCAACTCGTAAGGAAT	4	62	(GA)18	96-126
	R: TCCACCGTTGATTACCCTT				
MA 07[3]	6-FAM™ F: GTGCATCGTTAGGAACTGCC	3	55	(TC)4C(CT)27	96-126
	R: GCCCCTGAGATACAACTGCA				
MA 39[3]	6-FAM™ F: AGAAAGGCACTTTATCTAGG	3	50	(GA)23	162-216
	R: TTTGTTTTGGGGATGGTAGT				
UDP97-402[4]	HEX™ F: TCCCATAACCAAAAAAAACACC	4	60	(AG)17	128-144
	R: TGGAGAAGGGTGGGTACTTG				

[1] Dirlewanger et al. (2002); [2] Mnejja et al. (2004); [3] Yamamoto et al. (2002); [4] Cipriani et al. (1999).
[5] L.G. - Linkage group (1-8), according to Lambert et al. (2004).

Data analysis

The Observed heterozygosity (Ho), number of alleles per locus (A), allele frequencies, Polymorphism Information Content (PIC; being $PICi = 1 - \Sigma Pi^2$, where Pi is the frequency of allele I band), Probability of Identity (I; being $I = \Sigma pi^4 + \Sigma (2pipj)^2$), where p_i and *pj* are the frequencies of the *i*th and *j*th alleles and $i \neq j$) and (I unbiased = $n^3(2a^22 - a_4) - 2n^2(a_3 + 2a_2) + n(9a_2 + 2) - 6/(n - 1)(n - 2)(n - 3)$) where *n* is the sample size, a_i equals Σpj^i and p_j is the frequency of the *j*th allele (Paetkau and Strobeck 1994), and Power of Exclusion (Q) (Vandeputte 2012) were calculated using Cervus 3.0 software (Kalinowski et al. 2007). Alleles were considered rare when their frequencies were less than 0.05. A dendrogram was constructed via the unweighted pair-group method with arithmetic means (UPGMA) (Sneath and Sokal 1973), using the Darwin 5.0 software (Perrier et al. 2003), and based on Pearson (r) similarity coefficient and cophenetic correlation coefficient.

RESULTS AND DISCUSSION

The eight SSR markers amplified a total of 104 alleles, showing 8 to 21 alleles per locus, with a mean of 13 alleles (Table 3). The great majority of alleles (54.8%) were considered rare, with frequencies less than 0.05 (Figure 1). Among these alleles, 27 only occurred once. On the other hand, only 11.5% of the alleles showed frequencies higher than 0.2 (Figure 1). This pattern was expected, since a diverse group of cultivars was genotyped (Table 4). Heterozygosity ranged from 0.529 to 0.915 per marker, with mean value of 0.770 (Table 3). UDP 97-402 marker presented the lower heterozygosity value due to the high presence of null alleles (13 of 47 cultivars, Table 4). Non amplified fragments were considered null alleles since the DNA amplified in other markers and the PCR conditions were optimized based on Cipriani et al. (1999). In addition,

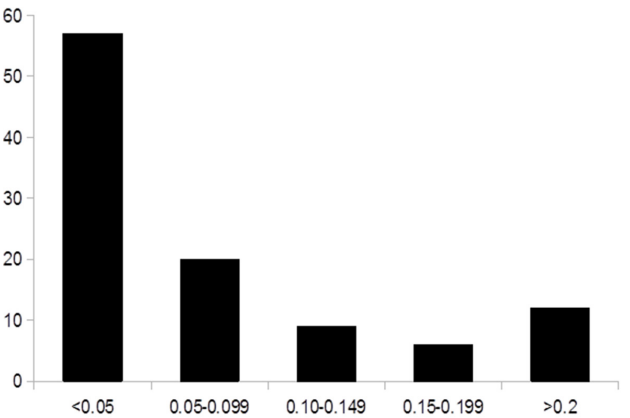

Figure 1. Distribution of 104 detected alleles in five allelic frequency classes for the eight analyzed loci.

Table 3. Number of alleles per locus, Observed heterozigozity (*Ho*), Polymorphism Information Content (PIC), Probability of Identity (I) and Power of Exclusion (Q) of eight SSR loci tested in 47 plum cultivars (Table 4)

Locus	No. of alleles per *locus*	*Ho*	PIC	I	Q
BPPCT 02	10	0.652	0.821	0.045	0.341
CPSCT 06	11	0.681	0.680	0.113	0.424
CPSCT 08	21	0.915	0.886	0.019	0.308
CPSCT 18	14	0.745	0.821	0.045	0.342
CPSCT 39	14	0.870	0.807	0.050	0.349
MA 07	12	0.830	0.829	0.040	0.338
MA 39	14	0.872	0.839	0.037	0.332
UDP 97-402	8	0.529	0.735	0.083	0.390
Mean	13	0.770	0.803	0.054	0.353
All ***loci***	104	-	-	2.66×10^{-11}*	**99.99976%

* Combined probability of identity for the eight analyzed SSR loci.
** Power of exclusion for the eight analyzed SSR loci.

the marker UDP 97-402 was originally developed for *Prunus persica*. In some cases, primers transferability to another species can cause misamplification or nonamplication due to single differences in primer hybridization sites. Null alleles were also found in two other loci, BPPCT02 and CPSCT39 (Table 4). Polymorphism Information Content (PIC) ranged from 0.680 to 0.886, with a mean value of 0.803 (Table 3). Combined Probability of Identity value was 2.66×10^{11}. Power of Exclusion (Q) of the analysis with the eight SSR provided an unambiguous way to discriminate the plum varieties. The four SSR markers transferred from *P. persica* to *P. salicina* showed similar values for the analyzed indices, compared with markers designed for *Prunus salicina*. Mnejja et al. (2010) obtained similar pattern by testing the cross-amplification in five different *Prunus* species and by naming the vast majority of markers as universal within the *Prunus* genus.

The results also allowed validating some cases of parentage reported in the literature. Laetitia (Letícia in Brazil) is reported as being a descendant of the cultivar Golden King by open pollination. The present results are consistent with this hypothesis, since there is always one allele shared by these two cultivars at all analyzed loci. The same occurs to Laroda and Santa Rosa, which is compatible with the information that Laroda results from the cross Gaviota x Santa Rosa. It was also confirmed a case of synonymy, as reported by Okie and Weinberger (1996), between Sordum and Gran Sultan, since both have the same alleles at each tested locus. The cultivar Gran Sultan is originally from New Zealand, and Sordum is an important cultivar in Japan. In this case, the spelling Sordum, which became popular in Japan, is probably a corruption of Sultan.

The number of alleles per locus (8 to 21) can be considered

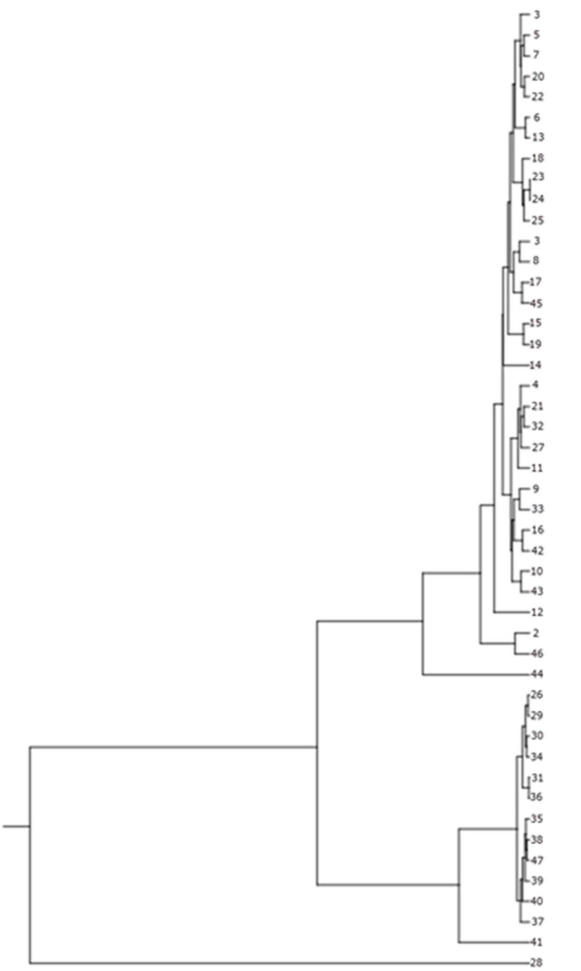

Figure 2. UPGMA dendrogram using Pearson (r) similarity coefficient. Forty-seven japanese plum cultivars genotyped in eight SSR loci, showing a total of 104 alleles (Cophenetic correlation coefficient=0.976). The dissimilarity between Cultivar Santa Rosa (#28) to others is 100.

high, especially if compared with the closest species, such as peach (Aranzana et al. 2003, Aranzana et al. 2010) and apricot (Villanova et al. 2006), and it is slightly lower than that of grape, in which it was found 13 to 23 alleles (This et al. 2004). The observed allele richness was also higher than that reported by Ahmad et al. (2004), who found 2-10

Table 4. Size of the alleles (bp) carried by forty-seven japanese plum cultivars

Cultivar	BPPCT 02	CPSCT 06	CPSCT 08	CPSCT 18	CPSCT 39	MA 07	MA 39	UDP 97-402
Harry Pickstone	192/192	126/134	193/235	148/148	100/120	106/120	172/184	130/144
Red Beauty	178/180	126/138	157/157	142/146	104/116	120/120	168/172	132/132
Obinaja	182/182	112/116	191/209	144/156	112/116	122/122	170/192	138/138
Sempre Amalia	192/194	112/112	193/209	162/164	110/118	118/118	170/188	130/138
SA 86-13	184/184	112/116	193/235	146/150	98/104	106/120	178/188	130/130
Tankiumaru	190/190	128/132	193/235	158/170	98/114	120/120	178/194	130/130
Linda Rosa	184/190	112/132	193/237	152/152	114/118	106/126	170/192	132/132
Gulf Ruby	182/188	112/116	201/237	154/154	104/116	108/126	192/216	130/134
Rosa Mineira	182/184	112/112	187/193	152/154	104/104	106/120	178/178	132/144
Camila	178/182	112/130	195/207	166/170	116/116	106/106	170/192	130/138
Estrela Púrpura	182/190	112/112	187/223	170/170	106/106	106/124	164/178	130/144
Laroda	184/184	112/130	193/193	122/148	114/116	108/116	178/192	128/130
Homeside	180/188	116/134	193/237	152/170	98/116	126/126	178/192	130/130
Catalina	184/190	132/140	195/203	148/170	104/104	106/110	168/192	130/132
Bruce	182/196	116/130	235/235	142/152	106/116	104/112	178/206	134/144
Simka	180/188	112/136	193/195	152/170	104/116	120/126	162/174	130/132
Corazon Rojo	182/196	112/116	201/235	158/166	114/116	104/108	178/192	134/134
Golden King	182/184	112/116	199/235	146/170	96/98	106/122	170/170	130/132
Gigaglia	190/190	126/130	221/221	148/152	100/116	106/120	178/192	144/144
Sanguinea	182/190	112/112	187/221	148/154	100/100	106/124	164/178	130/144
Januária	182/190	112/112	187/223	154/170	100/104	120/124	170/178	130/130
Gran Cuore	178/188	112/126	187/221	148/154	100/116	106/124	164/178	130/142
Sordun	182/190	126/126	209/225	148/170	114/116	118/118	168/176	140/144
Gran Sultan	182/190	126/126	209/225	148/170	114/116	118/118	168/176	140/144
Piamontesa	182/184	112/126	221/235	148/170	100/104	116/120	170/170	130/144
Carazinho	190/190	112/112	221/235	148/170	114/116	104/116	170/178	Null
Songold	180/188	112/112	187/199	152/154	100/106	106/120	162/162	130/130
Santa Rosa	Null	112/112	193/199	122/170	116/120	104/116	172/192	128/128
Piuna	178/184	112/112	209/227	150/170	98/116	106/110	168/170	Null
Laetitia	178/184	112/130	193/235	150/170	98/116	106/110	164/170	Null
Methley	188/210	126/126	191/235	150/154	106/116	106/120	178/184	Null
Kelsey Paulista	184/190	102/112	195/215	154/154	98/104	120/126	170/178	130/130
Pluma 7	188/188	112/126	187/193	154/154	96/114	106/126	178/178	128/130
América	184/184	112/126	199/221	150/150	102/116	106/110	170/172	Null
Wade	180/180	112/112	193/195	148/170	104/116	120/126	172/192	Null
Reubennel	188/210	126/134	193/237	148/148	100/116	106/120	172/184	Null
Black Ruby	184/190	130/138	191/193	150/170	104/104	106/110	168/192	Null
Blood Plum	190/192	112/112	187/193	148/154	104/108	106/124	164/178	Null
Fortune	180/180	112/130	199/221	148/170	104/116	120/126	170/192	Null
Santa Rita	190/190	112/116	167/197	158/170	104/116	124/126	170/192	Null
Coeur de Lion	190/190	112/126	187/193	154/154	Null	106/126	178/178	Null
Robusto	182/190	112/126	193/207	152/170	98/114	120/126	178/188	130/130
XV de Novembro	184/184	112/130	191/193	170/170	114/116	110/126	172/192	132/132
Pobeda	184/184	112/116	193/235	152/152	114/116	96/118	170/174	140/140
Angeleno	178/178	112/130	195/227	148/170	116/126	106/110	168/192	128/130
Black Amber	182/188	130/138	165/175	148/148	104/116	106/120	164/170	132/132
Amarelinha	180/184	112/112	187/199	152/154	100/104	106/120	168/192	Null
Heterozigozity	0.652	0.681	0.915	0.745	0.870	0.830	0.872	0.529

alleles per locus in a study involving 14 cultivars of plum, 7 of apricot, and 7 hybrids (plumcots and pluots). This may be due to the number and diversity of plum cultivars analyzed in this study, or greater variability of loci examined, since they have already been selected for that trait.

The high genetic variability detected allowed cultivar identification by using a relatively small number of loci. Thus, the results of the present study provide the basis for characterization and identification of plum cultivars, and build a database for identification of genetic accessions of this species. A similar situation occurs in grape, with a similar degree of genetic diversity, having one set of six loci proposed as a reference for identification of genotypes of this species (This et al. 2004).

The dendrogram (Figure 2) based on the genetic similarity among the different accessions brought four main groups, being two of them formed solely by one cultivar, Santa Rosa and Coeur de Lion, respectively. The other two groups are divided in several other subgroups, according to genetic proximity. The high genetic similarity is expected between individuals of the same subgroup, once they share a common origin. However, although the detected molecular differences between the accessions were not of great magnitude, it is possible to conclude that the vast majority of analyzed accessions differ among themselves, and this feature is essential in the management of a Plum Active Germplasm Bank and for fingerprinting issues.

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DNA fingerprinting de cultivares de ameixeira japonesa (*Prunus salicina*) por marcadores microssatélites

Resumo – *Quarenta e sete cultivares de ameixeira japonesa (*Prunus salicina*) foram genotipadas com a utilização de oito marcadores microssatélites, objetivando obter o perfil genético (DNA fingerprinting), distinguir e caracterizar o grupo de cultivares possuidores da maior variabilidade genética da espécie. Os oito locos SSR amplificaram 104 alelos (8 a 21 alelos por loco, média 13). O conteúdo de polimorfismo variou de 0,680 a 0,886 (média 0,803). A heterozigosidade observada (Ho) variou de 0,529 a 0,915 (média 0,07). A Probabilidade de Identidade (I) para cada loco variou de 0,019 a 0,113 (média 0,054) e a Probabilidade de Identidade combinada foi de 2,66 x 10^{-11}. O Poder de Exclusão dos oito locos foi 99,99976%. 57 alelos de 104 apresentaram frequência menor que 0,05. As baixas frequências alélicas contribuíram para aumentar a distinguibilidade da análise. Os resultados obtidos irão auxiliar na identificação de parentais para cruzamentos, clones segregantes com potencial de cultivares e proteção de cultivares.*

Palavras-chave: *Proteção de cultivares, identificação de cultivares, marcadores SSR, similaridade/relação genética.*

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