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Molecular characterization of the plum collection [*Prunus domestica* (L.) Borkh] of the Pedagogical and Technological University of Colombia

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Eight Random Amplified Microsatellite markers (RAMs) were used to characterize the genetic diversity found in 14 Prunus materials belonging to the deciduous collection of the Pedagogical and Technological University of Colombia. A total of 121 bands were generated: they range from nine for the GT primer to 26 for the ACA primer, and have molecular weights between 100 and 2050 Kb. At a Nei-Li similarity level of 0.75, four groups were formed, according to the characteristics of the fruit. The number of polymorphic loci varied between 8 (GT and AG) and 21 (ACA); the higher levels of heterozygosity were CA (0.43) and CT (0.41). The average value of heterozigosity for the total population was 0.35, much lower than those found in other *Prunus* species, but higher than other fruit species where RAM markers were used. Therefore, strategies must be deployed for the collections in order to increase genetic variability, such as the introduction of wild or hybrid materials. The RAM technique proved useful as a method for assessing genetic diversity in species of the *Prunus* genus.

Key words: *Prunus domestica*, genetic diversity, Random Amplified Microsatellite markers (RAMs), deciduous.

INTRODUCTION

In the last decades of the twentieth century, fruit market has been on the increase, so one can say the century is exotic fruits century, which are mostly of tropical origin. Colombia is a mega diverse country with great potential to increase fruit production. This is due to its large edaphoclimate that makes it produce fruits of different species throughout the year, from sea level of 2,800 m altitude (Toro and Tafur, 2007).

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The country (Colombia) produces plum (*Prunus domestica*), which belongs to the Rosaceae family and is a native of Europe and Asia (Fábregas, 1995). It is one of the tropical fruits in the last years, with increased market. The main producing countries are Spain, the United States, Italy, Japan, Greece and China. It is distributed in temperate regions around the world and in the mountainous tropical areas of Latin America and Africa. The main component of its fruit is water, followed by carbohydrates, where sorbitol is present. The fruits with sweet taste are consumed fresh, those with sour taste are used for the production of jams; they are also popular as ornamental garden tree due to their early flowering and the color of their flowers (INEGI, 2003).

Boyacá is one of the richest in natural resources departments, which focuses its economic activity on traditional agriculture and is a pioneer in the national production of deciduous plant. This indicates its vocation and tradition on this type of cold weather crop, over the years. The rise of the deciduous tree is due largely to development which promoted the Colombian Institute of Agrarian Reform, Incora, at the Experimental Center established in New Colon, which included the training and technology transfer to the region (Plan National Fruit, 2006).

Considering the importance of deciduous plant for agriculture region of Boyacá, GTZ program, the Faculty of Agronomy of the Pedagogical University of Colombia (UPTC) and the Technical University of Berlin developed the Colombo-German project, which established in the Experimental Farm Tunguavita in Paipa, a collection of deciduous materials imported from different thermal floors (Fisher and Torres, 1990). Subsequently, Puentes (2008) conducted an analysis of the deciduous plant from business perspective, emphasizing the peach and plum as an alternative to the Department of Boyacá.

In Colombia, the supply of new planting materials for these deciduous crops is sparse; therefore, breeding work designed to find elite materials that adapt to our edaphoclimatic conditions has not been performed. Complete information about the pedigree of these materials is not available and their morphological characteristics are not always suitable, because the cultivars and varieties closely related may show the same morphological characteristics (Stanys et al., 2012). In the case of fruit species, molecular markers are very useful because the assessment of morphological characters consumes much time and the variety expressions must be evaluated in several years. Markers can identify quickly and efficiently polymorphisms for genetic studies by increasing their efficiency in the processes of selection (Stanys et al., 2012).

In the evaluation of the characteristic of genetic and molecular level, early studies were performed on peach (*Prunus persica* L.) (Messeguer et al., 1987) and almond (Cerezo et al*.,* 1989). Subsequently, De Vicente et al. (1998), using RFLPs and Hurtado et al. (2002), using AFLPs, were able to distinguish apricot materials (*Prunus armeniaca* L.). RAPD markers have also been used for fingerprinting of *Prunus* species, such as peach (Warburton and Bliss, 1996) and almond (Bartolozzi et al., 1998). DNA profiles based on patterns of polymorphic bands, such as RAPD (Shimada et al., 1999) and AFLP (Goulao et al., 2001) have been used to analyze the genetic variability of Japanese plum cultivars.

However, Microsatellite markers are the preferred technique used for genetic relationships studies between species and for assays of genetic diversity among cultivated species (Gupta et al*.,* 1996). This is due to high polymorphism and abundant and co-dominant inheritance. The majority of SSRs used for fingerprinting have been developed in Peach and sweet cherry (Clarke and Tobutt, 2003) and used for molecular characterization and genetic similarity of genotypes in several species of *Prunus* including peach (Aranzana et al., 2010). Microsatellite markers are also used for genetic map of peach (Aranzana et al., 2003), almond (Bliss et al., 2002), and apricot (Hurtado et al., 2002). Another application for these sequences of microsatellites is in the study of markers associated with characteristic of interest that may be included within the strategies of Marker Assisted Selection (MAS) (Testolin, 2000).

Among the Simple Sequence Repeat markers, we find that Random Amplified Microsatellite (RAMs) are very useful for measuring genetic diversity in plants and animals, difference between families, between and within species (Muñoz et al., 2008), show the basis of variation of individuals, allow you to select specific regions within the DNA molecule for determined studies, the number of polymorphisms detectable is theoretically unlimited and it is possible to analyze both information that is expressed (Henríquez, 2000). This methodology is feasible for small laboratories in terms of equipment and facilities cost, does not require prior knowledge of sequences and the use of radioactive isotopes (Hantula et al., 1996).

Markers achieved by RAMs can be used for population studies (Hantula et al., 1996). This technique is useful in identifying duplicates in banks or collections of germplasm, used for the establishment of phylogenetic relationships in different fruit species (Bonilla et al., 2008; Sanabria et al., 2006). In this context, this research aims to identify the genetic variability present in the collection of plum (*Prunus domestica*) of the University Pedagogical and Technology of Colombia, to establish genetic relationship that exists between the materials and thereby provide a tool that can be used as breeding strategies on the species and to identify elite material that may be a new productive alternative for our farmers.

MATERIALS AND METHODS

A total of 11 accessions of plum (2 of apricot- *Prunus armeniaca* and 1 of almond- *Prunus dulcis*), belonging to deciduous genebank

Table 1. *Prunus* materials of the deciduous collection from the UPTC used for the assessment of genetic diversity with Random Amplified Microsatellites (RAMs).

S/N	Material	Classification**
1	Horvin	Red Plum Var.
2	Chileno	
3	Methey	Red Plum Var.
4	Morado	
5	Reina Claudia	
6	Gold Fruly	.
7	Early	
8	Real Beauty	Red Plum Var.
9	Beauty	Red Plum Var.
10	Ecuatoriano	Yellow Plum Var.
11	Chileno-2	
12	Albaricoque Bulida	
13	Albaricoque Canino	
14	Almendro.	

*Source: Martínez (2013).

Table 2. Primers used in the RAM Microsatellite technique.

Markers	Sequence (5´to 3´)
CCA	$DDB(CCA)_5$
CGA	$DHB(CGA)_5$
ACA	$BDB(ACA)_5$
GT	$VHV(GT)_5G$
AG	$HBH(AG)_7A$
CT	$DYD(CT)_7C$
TG	$HVH(TG)_7T$
CA	$DBDA(CA)_7$

of the UPTC and established on the experimental farm at Tunguavita, Paipa were evaluated (Table 1).

Molecular characterization

Molecular characterization was done in Molecular Biology Research Laboratories, Gebimol and Bioplasma, of UPTC, Colombia, Tunja. For DNA extraction, Dellaporta et al. (1983)'s protocol was used. The total DNA was visualized with 0.8% agarose gels, stained with Z-Vision, in a Maxicell EC-340 Primo Gel Electrophoresis System chamber. In order to determine the DNA concentration of each accession, a dilution curve of DNA from bacteriophage Lambda with an initial concentration of 20 ng/µL was made. For analysis, eight RAM primers synthesized by Technologies Inc. were used (Table 2). The amplification reaction RAMs was prepared in a sterile microcentrifuge tube (1.5 ml) to a final volume of 25 µL. The reaction mixture was prepared with 1X buffer, 1.5 mM $MgCl_2$, 0.2 M dNTPs, 1U Taq Polymerase, 2 uM primer and 10 ng genomic DNA (Sanabria et al., 2006).

The following designations are used for degenerated sites: H (A/T/C); B (G/T/C); V (G/A/C) and D (G/A/T).

The amplification was carried out in a thermocycler PTC 100 Programmable Thermal Controller (MJ. Research, Inc). Initial denaturation was at 95°C for 5 min; denaturation at 95°C for 30 s, annealing temperature of 50°C (AG and CA primers), 55°C (CCA, TG and CT primers) and 58°C (GT and CGA primers) for 45 s, an extension of 72°C for 2 min, 37 cycles of denaturation, and, finally, extension at 72°C for 7 min. Amplified products were separated by electrophoresis in polyacrylamide gels in a ratio of 37:1 (acrylamide: bisacrylamide) at 7% and 150 V for 1 h in a small DNA Sequencing System chamber, Fisher Biotech FB-SEQ-3545. The staining was carried out using silver salts (Sanabria et al*.,* 2006).

Statistical analysis

An absent (zero) and present (one) binary matrix was generated. The genetic similarity between individuals was calculated using the similarity coefficient of Nei and Li (1979), also known as DICE (1945) (Sneath and Sokal, 1973). The cluster analysis was conducted by the UPGMA method and a dendrogram was generated using the statistical package NTSYS (Numerical Taxonomy System for Personal Computer, PC version 2.02). To evaluate the genetic diversity, unbiased heterozygosity and percentage of polymorphic loci were estimated using the statistical package TFPGA (Tools For Population Genetic analysis, version 1.3, 1997). Unbiased statistical f with a confidence interval of 95% was determined.

RESULTS AND DISCUSSION

In the analysis with the Nei-Li coefficient, at a similarity level of 0.75, the population was distinguished into four groups based mainly on the characteristics of the fruit (Figure 1). At a similarity level of 0.65, we found Horvin and Methey plums in group I, according to the classification of Campos (1989); they belong to the red varieties, which are characterized by a high pruina content, rounded shape, small size and average weight of 35 g. Horvin differs from Methey, basically, at the plant level (Campos, 2013).

In the second group, we found Red Beauty and Beauty sharing a similarity of 0.80 and a litter further away (0.75) was the Early plum. Red Beauty is the earliest in the Spanish market; Beauty has a red skin with slight tips, pruina, an insipid, slightly acidic taste (Carrera, 2002), and a heart-shaped medium size with an average weight of 70 g. Within this group, the Ecuadorian and Chilean-2 materials share a similarity of 0.80; they are farther away from the rest of the plums in this group. The Ecuadorian variety is characterized by having yellow skin, with reddish dyes at maturation and low contents of pruina (Campos, 2013). In group II, a similarity of 0.85 is seen for Gold Fruly and Queen Claudia. The last one, with a European origin, has yellowish-green skin; it is heart-shaped and large in size; its production is very prolific. The yellow Japanese variety (Ogden) has yellow flesh; its juicy, pruina, has a heart-shape, large size, and also known by the common name, egg yolk. It is wrongly called Queen Claudia because its characteristics correspond to a yellowish-green skin, yellow stoneless pulp (Campos, 2013).

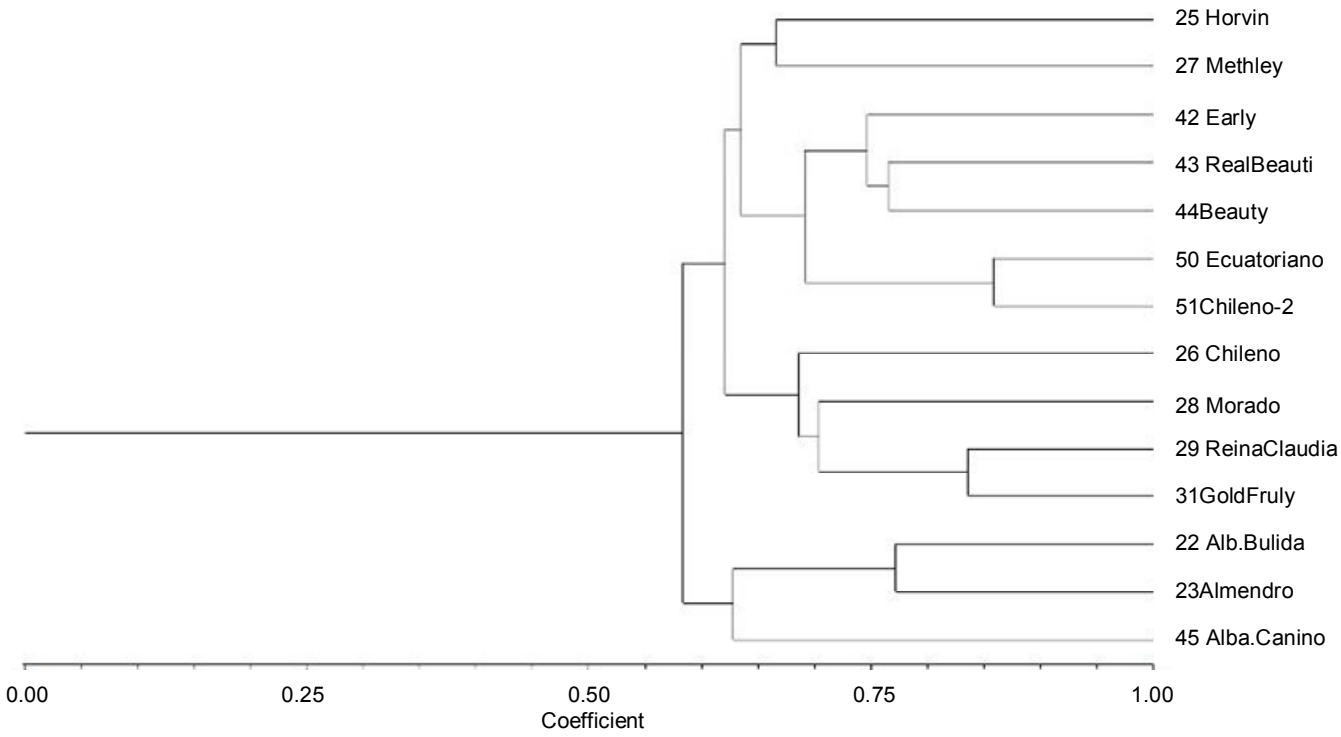

Figure 1. Dendrogram of the 11 *Prunus domestica* materials, based on the Nei-Li similarity coefficient and calculated with eight RAM markers with the UPGMA classification method, SAHN and TREE of NTSYS-pc version 1.8 (Exeter Software, NY, USA).

In the fourth group, we found the apricot (*Prunus armeniaca* L.). Among the most important varieties are Bulida (of Spanish origin, adapts to all kinds of soils and very juicy) and Canina, which has good quality fruit, marketing (Martínez et al., 2004) and presents the lowest similarity with the rest of the evaluated materials. The almond tree is a rustic species that adapts to extreme conditions, such as dry climates. It is supported by temperate climates; therefore, flowering is early and requires little cold. Its affinity with the apricot is very high (Gallego, 2010), which can be visualized in the dendrogram.

The analysis of the similarity between the materials from the evaluated deciduous collection showed high homogeneity, with associations responding to morphological characteristics of varieties, specially related to fruit (Stanys et al., 2008). In addition, it was established that the materials that are believed to be the same (Chileno and Chileno-2) were found to be two different materials from Chile. We cannot establish whether the groups are due to the geographical site where the materials were collected because there is no such information. Finally, compatibility between the plum and almond and other species related to the almond or apricot can be observed.

The eight RAM Microsatellite markers used for the assessment of the genetic diversity in the plum generated a total of 121 bands, which ranged from 9 for the ACA primer to 26 for the GT primer, with molecular weights between 100 and 1000 kb. The number of polymorphic loci ranged from 5 to 16 for GT and CGA, respectively (Table 3). The number of bands was considered adequate for estimating genetic parameters (Stanys et al*.,* 2012). The CCA primer made the highest contribution to the observed genetic variation, Fst 0.85. This means they can be useful for achieving greater differentiation between materials of the *Prunus* genus.

Heterozygosity values ranged between 0.28 (CGA) and 0.43(CA). The average value of heterozygosity for the general population of 0.35 was much lower than that reported in other SSR studies for *Prunus* species; they include work done on peaches (*Prunus persica* L.) by Aranzana et al. (2010) (0.46); almond by Turkoglu et al. (2010) (0.61) and Fernández et al. (2009) (0.72); sweet cherry by Fathi et al. (2008) (0.79) and Wünsch et al. (2002) (0.49); apricot by Schueler et al. (2003) (0.66); guava using RAM markers by Martín et al. (2011) (0.70-0.58) and (Sanabria et al*.,* 2006). In other fruit species where genetic diversity was studied with RAM markers, there were expected average heterozygosity values lower than those found in this study (Bonilla et al. (2008); they were Cape gooseberry (0.25) and mandarin (0.31) (Mora et al., 2013).

In the plum, Carrasco et al. (2012) molecularly

Table 3. Estimated average heterozygosity (He) and percentage of polymorphic loci for the eight RAM primers evaluated in 11 plum materials, 2 apricot materials and 1 almond material.

Primer	N° Polimorphic loci	He estimated	% Loci Polimorphic (95%)	Fst	SD
ACA	21	0.33	80.8	0.48	0.06
TG	11	0.35	100	0.60	0.09
CGA	18	0.28	85.7	0.61	0.09
CT	13	0.41	92.8	0.51	0.06
CCA	11	0.34	84.61	0.61	0.07
CA	14	0.43	93.33	0.37	0.06
AG	8	0.34	66.67	0.46	0.09
GT	8	0.37	88.8	0.42	0.09
Population total		**0.35**	**86.4**	**0.51**	**0.03**

characterized cultivars of the Japanese plum (*Prunus salicina*), using the molecular markers SSR and ISSR. The mean values of observed and expected heterozygosity for the SSRs were 0.9 and 0.8. These results suggest that the high level of genetic variability can be explained by self-incompatibility mechanisms favoring exchanges between genetically distant cultivars of *Prunus* strategies and intra- and interspecific hybridization, frequently used in breeding programs for plums. Ahmad et al. (2004) studied genetic diversity in 20 cultivars of plums using simple sequence repeats. They obtained heterozygosity values of 0.70. Mnejja et al. (2004), in eight plum cultivars, found heterozygosity values of 0.73; thereby, showing great variability in the material being evaluated. This does not agree with the results obtained in this study. it can be due to the selected number and class, such as the marker type materials used. Shimada et al. (1999) studied 42 cultivars of Japanese plum using RAPD markers and found low levels of polymorphism (24%). Goulao et al. (2001) analyzed 28 plum cultivars using ISSR and AFLP, and again, the genetic variability (polymorphic ISSR = 87.4% and 62.8% polymorphic AFLP) was less than that found in this study.

Aran et al. (2012) morphologically and molecularly differentiated selected plum seedlings with 22 RAPD primers to improve patterns. They found 195 polymorphic bands and a similarity between 0.27 and 0.77. Studying the genetic diversity in 27 plum cultivars, using 10 RAPD primers, Hend et al. (2009) obtained 97.3% polymorphism and a genetic similarity between 0.18 and 0.80. Shimada et al., (1999) also studied the genetic variation of plum cultivars with RAPD markers and reported 24% polymorphism. It should be noted that Shimada studied commercial genotypes while the samples used in Aran et al. (2012)'s and Hend et al. (2009)'s studies were wild materials. The diversity among wild genotypes compared with a commercial one can be addressed by the narrow genetic diversity due to selection within the cultivated materials. Many studies have been conducted on plum cultivars with important results obtained using RAPDs (Liu et al., 2007, Lisek et al., 2007).

However, the value of average heterozygosity found in this study (0.35) was higher than that reported in studies characterizing the genetic diversity of natural populations of *Prunus davidiana* (0.17) (Cheng et al., 2011) and peach (0.22) (Martinez et al. 2003). Furthermore, the values were greater than the average estimated genetic variability of allogamous plants (He = 0.22 for dominant markers; Nybom, 2004). A low level of polymorphism in the sweet cherry has also been detected by Gerlach and Stösser (1997), using RAPD markers. This probably reflects a narrow genetic base in the germplasm of the sweet cherry and peach, but is consistent with the assertions of Imbert and Lefévre (2003), who believe that an excess of heterozygosity is not very usual in tree species and generally this is favored by self-incompatibility and interspecific crosses. The narrow genetic base used to develop cultivars and the existence of common ancestors in the pedigree of peaches and plums could explain the results found (Okie and Hancock, 2008).

The coefficient of genetic differentiation (Fst) obtained in evaluating 11 plum materials with eight RAM markers was 0.51 with a standard deviation of 0.03 (Table 3). According to Wright (1978), values of 0.25 show high genetic differentiation, which may be reflected in the high degree of domestication that these materials have suffered, since most of them are commercial varieties. Carrasco et al., 2012 found an index fixation of F = -0.127, higher than those in other allogamous species such as the almond (F = 0.15; Fathi et al., 2008), and wild apple (F = 0.10; Coart et al., 2003). Excess heterozygosity could be explained by the negative selective mating related to the self-incompatibility system, such that the parental lines carry different favored alleles than the interspecific cross usually used in plum breeding

programs.

The low values of heterozygosity that were seen corroborate with high values of identity and Nei-li genetic distances (1978, 1979) (0-65-0.79). The analysis of molecular variance showed that 73% of the observed genetic variability is located within the formed groups and only 27% is due to differences between the groups; which suggests lower and finer levels of hierarchy than those used in this study. Casas et al. (1999) also reported high values of similarity using RAPD markers (0.71). In the last decade, molecular markers have become a valuable tool that provides a huge amount of genetic information on fruit crops, allowing its use for the identification of genotypes and the evaluation of the genetic diversity of this species group. Simple sequence repeats (SSRs) and random amplified (RAMs) microsatellites can be valuable tools for the study of genetic diversity and conservation, especially for cultivars or genotypes of interest before they disappear. On the other hand, in the case of cultivated species of this genus, some genetic limitation has been described due to the use of a limited number of varieties in breeding programs. The introduction of genes from these wild Prunus species through interspecific breeding programs could be an interesting addition to improve existing programs by widening the genetic basis and increasing the efficiency in the selection process (Scorza et al., 1996).

Conclusion

Molecular characterization of the plum collection of the Pedagogical University of Colombia with RAM markers showed low genetic diversity. It needs to be developed through the incorporation of wild materials or interspecific hybrids that would contribute new allelic variants to the collection. The RAM technique proved to be a useful tool for the differentiation of species materials of the *Prunus* genus.

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