Preliminary assessment of AFLP fingerprinting of *Rubus glaucus* Benth. elite genotypes

Evaluación preliminar de la huella genómica de genotipos élite de *Rubus glaucus* Benth. con marcadores AFLP

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ABSTRACT

The Andean blackberry (*Rubus glaucus* Benth.) is a promissory fruit crop for Colombia with potential to become an international commodity due to its high nutritional and nutraceutical value. Farmer genotypes from the national *R. glaucus* collection were selected from eight outstanding accessions according to their nutritional and agronomic value, for distribution among local producers. The goal of this work is to evaluate the genomic fingerprint by AFLP analysis of these elite genotypes using three primer combinations. From 179 total amplified loci produced by the three combinations, 20% resulted polymorphic. The EAGG/MCTT combination was the most informative with a 32% polymorphism and greater discrimination power. The genotypes tested showed a high average similarity (96%) and the accessions San Antonio and ILS-1863 formed independent groups with good statistical support in the clustering analysis. The remaining accessions did not form discrete groups with good support (<50%), probably due to genetic homogeneity among them and/or low resolving power of markers. This study is one of the first attempts to generate a genomic fingerprint of these farmer elite genotypes for protection, seed certification and future support to breeding programs.

Key words: genetic variability, elite genotypes, Andean blackberry.

RESUMEN

La mora de Castilla (*Rubus glaucus* Benth.) es un cultivo promisorio para Colombia por su potencial comercial internacional derivado de su alto valor nutricional y nutracéutico. De la colección nacional de mora se seleccionaron genotipos provenientes de ocho accesiones sobresalientes por sus características nutricionales y agronómicas, para ser distribuidos entre los productores. El objetivo de este trabajo fue evaluar la huella genómica y las relaciones genéticas de estos genotipos élite mediante el análisis de marcadores AFLP generados con el uso de tres combinaciones de cebadores. De un total de 179 loci amplificados por las tres combinaciones, 20% resultaron polimórficos. La combinación EAGG/MCTT fue la más informativa con un 32% de polimorfismo y un mayor poder de diferenciación. Los genotipos presentaron una alta similitud promedio (96%) y las accesiones San Antonio e ILS-1863 formaron grupos independientes con buen soporte estadístico en el análisis de agrupamiento. El resto de accesiones no formaron grupos discretos con buen soporte (<50%), probablemente debido a la homogeneidad genética entre ellas y/o al bajo poder de diferenciación de los marcadores. El estudio representa una primera aproximación al conocimiento de la huella genómica de estos genotipos de agricultor sobresalientes para protección, certificación de semilla y posterior apoyo a programas de mejoramiento.

Palabras clave: variabilidad genética, genotipos élite, mora de Castilla.

Introduction

The Andean blackberry (*Rubus glaucus* Benth.) belongs to the Rosaceae family and is associated with blackberries of subgenus *Rubus* and raspberries of subgenus *Idaeobatus* as it shares morphological characters with both (Ballington *et al*., 1993). Therefore, it is considered that *R. glaucus* is a fertile amphidiploid product of the hybridization between species of these two subgenera (Ballington *et al*., 1993). This fruit is originated in highland intertropical regions of America and is the only native *Rubus* species grown commercially in countries of Central and South America (Ballington *et al*., 1993; Zapata, 2003). Currently, there is an increasing worldwide awareness of its nutraceutical value because of its antioxidant activity given its high ascorbic acid and phenolic contents (Garzón *et al*., 2009).

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In Colombia this species is prioritized for its promotion in export markets, for this reason it is projected a raise of nearly 94% in its production for 2020 (Ministry of Agriculture and Rural Development, 2005; Agronet, 2010). However, this crop has several limitations such as phytosanitary susceptibility, low Brix degree content and low genetic quality of the available sowing material. The propagation is performed using genotypes that local farmers and plant nurseries reproduce without the accomplishment of physiological and sanitary regulations, coupled with their lack of genetic identity.

Accordingly, for certification purposes, genotypes are characterized at the molecular and phenotypical levels to identify promising ones with high added value (Barrero, 2009). Using a sample of 39 accessions of the *Rubus* spp. national collection managed by the Colombian Corporation for Agricultural Research (Corpoica), six *R. glaucus* accessions were identified in collaboration with local farmers which included genotypes with outstanding agronomic, nutraceutical and nutritional characteristics in the Silvania region (Cundinamarca) (Espinosa *et al*., 2009). Two additional accessions with interesting genotypes for Valle del Cauca region are also used for massive *in vitro* propagation and farmer distribution.

In order to contribute to molecular certification of elite *R. glaucus* genotypes, the assessment of genomic fingerprints, i.e. the establishment of a multilocus profile for protection, discrimination and monitoring (Hong and Chuah, 2003; Nazrul, 2007), with the use of informative molecular markers is fundamental. The AFLP (Amplified Fragment Length Polymorphism) markers are highly reliable and reproducible for cultivar differentiation. Because of its multilocus nature, this marker system is useful for detecting polymorphisms and distinguishing genotypes with few pairs of generic primers and without any previous knowledge of the genome (Vos *et al*., 1995; Meudt and Clarke, 2007).

The AFLP technique has been useful in genus *Rubus* in several applications such as the identification of genomic regions in *R. idaeus* related with pathogen resistance (Pattison *et al*., 2007), the recognition of populations of circumpolar species *R. chaemomorus* that remained isolated during the last glaciation (Ehrich *et al*., 2008), the elucidation of the genetic background of interspecific the hybrid "Boysenberry" (Ipek *et al*., 2009) and the detection of genetic instability in *Rubus* introductions subcultured from cryopreserved shoot tips (Castillo *et al*., 2010). *R. glaucus* genetic variability has been assessed in Colombia with natural and cultivated populations using different markers systems including RAPD markers (Random Amplification of Polymorphic DNA) (Marulanda and Márquez, 2001), RAMs (Random Amplified Microsatellites) (Morillo *et al*., 2005), AFLPs and SSRs (Single Sequence Repeats) (Marulanda *et al*. 2007; Marulanda and López, 2009; Espinosa, 2011).

The objective of this study was to assess the molecular profile of elite genotypes coming from eight *R. glaucus* accessions, with the purpose of establishing their genetic relationships performing AFLP analysis. Furthermore, the discrimination power of three primer combinations between the accessions and their respective genotypes was evaluated.

Materials and methods

Plant material

Eight *Rubus glaucus* accessions previously selected in participatory processes with local farmers were used (Tab. 1). Accessions number 4 and 8 were selected in the Valle del Cauca region while the others were selected in Silvania, Cundinamarca (Espinosa *et al*., 2009). Additionally, one individual of *Rubus urticifolius* Poir. from Sandoná (Nariño) was employed as an outgroup for cluster analysis. Valderrama *et al*. (2009) methodology was used for the

TABLE 1. *Rubus glaucus* accessions used in the current study*.

Id. No.	n	ILS code	Accession common name	Departament	Municipality
1	4	ILS 2366	Monterrico Yema	Cundinamarca	Silvania
2	5	ILS 2277	Monteloro	Valle	Tuluá
3	5	ILS 3400	Sin espinas	Risaralda	Santa Rosa de Cabal
4	5	NC	Hartona	Caldas	Manizales
5	5	ILS 1863	ILS 1863	Nariño	La Cocha
6	4	ILS 2282	Riosucio	Caldas	Riosucio
7	5	ILS 2268	Cerezos	Caldas	Manizales
8	4	NC	San Antonio	Antioquia	Medellín

* All the accessions belong to farmer material with the exception of ILS 2268 of wild origin. The accessions 1, 2, 3, 5, 6 y 7 were selected from Álvarez *et al.*, 2009 and Espinosa *et al.*, 2009. **Id. No**: Number that identifies the accessions in the current study; **n**: Number of genotypes of each accession; **ILS code**: Introduction code from the collection belonging to Corpoica, La Selva Research Center; **NC**: Non-coded.

introduction and *in vitro* propagation of shoot tips from accessions grown in a field located at the Monterrico sector in Silvania and for the later greenhouse adaptation of vitroplants. About one month after *ex vitro* adaptation, DNA extractions were performed using young leaves. Currently, these plants can be found in a clonal garden for certification and farmer distribution purposes.

DNA extraction

Plant leaf tissues were ground in liquid nitrogen and preserved at –70°C. DNeasy Plant Mini Kit™ (Qiagen) was used for DNA extractions. Genomic DNA quantity and quality (260/280 and 260/230 ratios) were checked with a NanoDrop-1000™ spectrophotometer (Thermo Scientific). In addition, DNA quality was confirmed by digesting 300 ng with *Eco*RI enzyme (Promega). Digested and non-digested DNA samples were visualized in 1% (w/v) agarose gels in 1X TAE buffer and stained with ethidium bromide (0,5 μg mL^{-1}).

AFLP

Two kits, AFLP Core Reagent Kit™ and AFLP Starter Primer Kit™ (Invitrogen), were used for the procedure and all the steps were carried out in a Programmable Control Thermocycler PTC-100™ (MJ Research). DNA digestion of 300 ng with *Eco*RI/*Mse*I and adapters ligation was performed with the first kit. Complete digestion was verified in 1% (w/v) agarose gels with 11 μL of digestion/ligation products. Preamplification and selective amplification of DNA fragments linked to adapters were carried out with the second kit. The preamplification was made using a 1:5 dilution of digestion/ligation and the amplification was performed with a 1:5 dilution of preamplification product.

Three primers combinations that were polymorphic and reproducible in *R. glaucus*, were used in selective amplification: EAAC/MCTT, EAAC/MCTA and EAGG/MCTT (Espinosa, 2011). The fragments were visualized in 5% (w/v) polyacrylamide and 7 M urea gels, with 0,5X TBE buffer in 38x50 cm gel apparatus (BioRad) using silver nitrate staining (1 mg mL^{-1}). The resulting AFLP bands were compared with a 50 Base Pairs (bp) DNA Ladder™ (Invitrogen).

Analysis of duplicates

The technique reproducibility was tested by comparing the molecular profiles of two DNA independent extractions of the same plant. The error rate was calculated with the quantity of bands that do not match between both profiles and the total amount of comparisons that were done (Bonin *et al*., 2004). Thereby, error rates of four duplicates were estimated and these profiles were also included in the cluster analysis identified with "R".

Data analysis

Markers from 100 bp to 800 bp were scored in EAAC/MCTA and EAAC/MCTT combinations, while markers from 50 bp to 650 bp were scored in EAGG/MCTT as suggested by Espinosa (2011). A binary data matrix was generated scoring presence (1) and absence (0). Discrimination power and information content of primer combinations were assessed establishing the following parameters: 1. Percentage of polymorphic loci taking into account that one locus is polymorphic when the most common allele is present in a frequency below 95% in the sample; 2. Number of unique profiles within the 41 profiles generated with each combination; 3. Resolving power (RP); and, 4. Polymorphic information content (PIC).

The RP, defined as the capacity of each primer combination to discriminate among genotypes, was calculated according to Prevost and Wilkinson (1999), where Ib indicates the discrimination power of each polymorphic band and is defined in terms of p, *i.e*., the proportion of genotypes that harbor the band:

$$RP = \sum Ib$$

$$Ib = 1 - (2|0.5 - p|)$$

PIC was calculated for each band according to Roldan-Ruiz *et al*. (2000) methodology, where f*i* is the frequency of the amplified allele and 1 – f*i* is the frequency of the null allele:

$$PIC_i = 2f_i(1 - f_i)$$

Two average PIC values were calculated for each combination, one including (PIC*t*) and other excluding (PIC*p*) monomorphic bands.

Cluster analysis was performed by constructing a dendrogram with UPGMA (Unweighted Pair Group Method with Arithmetic Mean) hierarchical clustering method (Sneath and Sokal, 1973) and Dice similarity index (Nei and Li, 1979), using NTSYS pc2.02g program (Rohlf, 1998). Confidence level of tree nodes was assessed by bootstrap analysis employing Winboot program with 2000 permutations (Yap and Nelson, 1996). A Principal Coordinate Analysis (PCA) with squared Euclidean distances was carried out with MVSP 3.12d program (Kovach Computing Services, 2001).

Results and discussion

Reproducibility, polymorphism and discrimination power of markers

Data reliability is confirmed since duplicate analysis showed error rates from 0% to 2%, which is an appropriate interval for AFLP reproducibility tests (Bonin *et al*., 2004). Tab. 2 summarizes the results of the three combinations employed. A total amount of 179 bands were scored, from which 20.11% were polymorphic. EAGG/MCTT combination presented the greatest polymorphism with a 32.08% value.

According to Prevost and Wilkinson (1999), an Ib value close to 1 indicates the existence of a highly informative band for genotype differentiation and the summation of values represents the accumulated capability of a primer combination to discriminate them. EAGG/MCTT is the combination that presents the highest value in the summation (8,05) as well as the highest amount of genotypes discriminated with unique profiles (33). The average RP value for the three combinations was 5.66 and three Monteloro genotypes were not discriminated, suggesting that within this accession there are less genotypes and more clones. Nevertheless, it is important to clarify that due to reproducibility inaccuracies or somatic mutations, plants from the same clonal line can differ by a small quantity of markers and present different profiles (Ehrich *et al*., 2008).

Escandón *et al*. (2007) indicate that RP value ability to determine the resolving power of each combination is conditioned by the genotypes assessed. An average RP value of 6,34 with seven combinations, has been reported in the analysis of 46 traditional cultivars of tomato (*Solanum lycopersicum* L.) with a 40% polymorphism and without a unique profile established for each accession (García-Martínez *et al*., 2006). In contrast, with the use of seven primer combinations, a higher average RP value (35,21) and a higher polymorphism (88,3%) was observed in 48 jatropha accessions, in such a way that all the accessions could be identified (Tatikonda *et al*., 2009). Tomato is an autogamous plant associated with bottlenecks, founder effect and selection processes related with its low population polymorphism (Labate *et al*., 2009) while jatropha is a non-domesticated species (Popluechai *et al*., 2009), monoecious with entomophilous pollination in which xenogamy as well as geitonogamy is enabled (Raju and Ezranadam, 2002), in relation with its higher polymorphism. *R. glaucus* traditional propagation in crops is mainly through stakes from mother plants selected by outstanding characters, besides there are clues of material interchange among blackberry growers of different regions (Zapata, 2003). These precedents can explain the low polymorphism detected, which also can be related to a narrow genetic base of the sample analyzed or with the low resolving power of primers.

These results show that discrimination power is also determined by the amount of polymorphic markers generated by each combination. Several authors as Prevost and Wilkinson (1999) and García-Martínez *et al*. (2006) have reported a linear relationship between RP value and the number of unique profiles in combinations, tendency not found in the present study. Tab. 2 demonstrates that EAAC/MCTA combination has a RP value slightly higher than EAAC/MCTT, but differentiates less genotypes with single profiles. This result is due to the fact that EAAC/MCTA combination is the less polymorphic (12.7%); consequently it cannot discriminate more genotypes than a combination with greater polymorphism. The previous agree with Laurentin and Karlovsky (2007) that report a lack of correlation between PIC and RP values with the number of unique profiles, clearing up that these parameters does not always assess unequivocally the information level that provides one combination.

With respect to PIC the highest value for a biallelic locus is 0.5. EAGG/MCTT combination is the most informative in regard to PIC*t* average, because of its highest polymorphism (Tab. 2). However, when observing PIC*p* values, it can be established that EAAC/MCTA combination is the

TABLE 2. Polymorphism and information level for each primer combination.

Combination	No. of bands	No. of polymorphic bands	% Polymorphism	No. of unique profiles	RP	PIC*t*	PIC*p*
EAAC/MCTT	63	11	17.46	21	4.34	0.05	0.26
EAGG/MCTT	53	17	32.08	33	8.05	0.11	0.33
EAAC/MCTA	63	8	12.7	17	4.59	0.05	0.38
Total	179	36	20.11	ND	16.98	ND	ND
Average	59.67	12.00	20.75	23.67	5.66	0.07	0.32
SD	5.77	4.58	10.10	8.33	2.07	0.03	0.06

RP: Resolving power; **PIC*t***: Average polymorphic information content of all bands within a single combination; **PIC*p***: Average polymorphic information content of polymorphic bands within a single combination; **ND**: Non-determined; **SD**: Standard deviation.

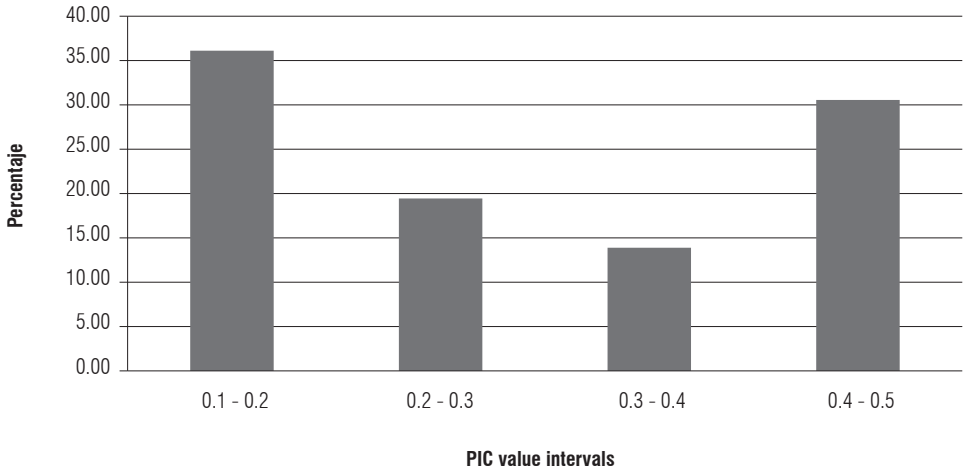

FIGURE 1. Distribution of polymorphic markers within PIC value intervals.

one possessing the most informative markers in spite of its lowest polymorphism. Finally, according to Tatikonda *et al*. (2009), the most informative markers belong to a PIC interval between 0.45 and 0.5; if this interval is extended from 0.4 to 0.5, it can be concluded that 31% of the markers are highly informative (Fig. 1).

Cluster analysis

According to Dice similarity indexes among pairs of genotypes, *R. glaucus* individuals present and average similarity of 96% with a maximum of 100% and a minimum of 91%. When comparing this similarity with the one observed between *R. glaucus* and *R. urticifolius* genotypes (average value of 51%), it turns out that similarity within *R. glaucus* genotypes is quite high. The dendrogram shows that all genotypes tend to group depending on the accession they belong to, with the exception of Monteloro and Sin espinas genotypes (Fig. 2). Nevertheless, these apparent clusters have only statistical support for two accessions, San Antonio (89%) and ILS 1863 (50%), which at the same time seem to separate from the rest. The high bootstrap value of San Antonio cluster is probably linked to its characteristics as it is an outstanding material from a selection made by Corpoica in local farms of San Antonio del Prado region

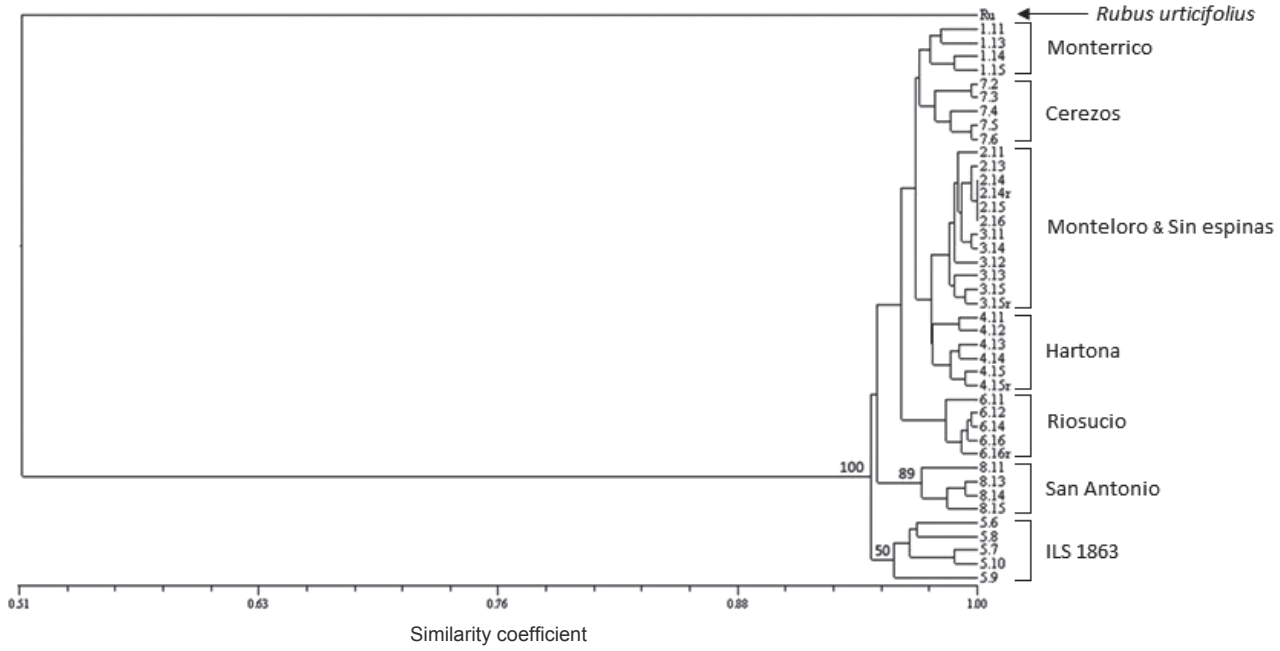

FIGURE 2. Dendrogram of 37 *R. glaucus* genotypes based on Dice similarity index and UPGMA clustering algorithm. *R. urticifolius* was used as an outgroup.

from Medellín (Antioquia), due to its earliest production and highest yield as compared with the traditional cultivated materials (Bernal and Díaz, 2006).

First three coordinates explain nearly 85% of the total variation in PCA. The graphic representation (Fig. 3) confirms that San Antonio and ILS 1863 genotypes belong to discrete groups while the other genotypes do not show evident grouping. ILS 1863 accession, which in the dendrogram is separated from the others (Fig. 2), forms a more dispersed group with an individual that tends to central clustering; something similar happens with Riosucio. The previous indicates that PCA allows the identification of individuals in transition between two groups that cause node instability and in consequence low bootstrap values (García-Martínez *et al*., 2006). PCA confirms the information obtained in dendrogram and bootstrap analysis, therefore AFLP markers reveal precisely the genetic relationships between the accessions, further indicating that the results of the present study are highly reliable (Salamini *et al*., 2004)

The high average similarity of genotypes (96%) and the low bootstrap values in most accessions groups, can be related to the low percentage of informative markers (Fig. 1, Tab. 2) or suggest a narrow genetic base of the analyzed sample, as previously mentioned. These results agree with Espinosa (2011), who observed a high similarity (91%) and a low support in most of the nodes within *R. glaucus* cluster in the AFLP analysis of 34 *R. glaucus* accessions from the national collection managed by Corpoica, which included all accessions of the current work with the exception of San Antonio. In this regard, Zapata (2003) reveals the commercialization of ecotypes from Valle del Cauca to different regions of Colombia from Ginebra and Guacarí municipalities where this crop has a tradition of 40 years, thus clonal propagation and clone distribution through commercial regions of the country can explain the low variability observed (Espinosa, 2011). This situation might be similar to cultivated materials in another Andean fruit namely *Solanum quitoense* L. (commonly known as "lulo" in Colombia) where, narrow genetic variability has been related to a founder effect, which refers to the lost of genetic variability due to the establishment of a "cultivated population" with few selected individuals (Fory *et al*., 2010).

The previous results differs from those of Marulanda *et al*. (2006, 2007) as they report similarities between 20% and 90% using three AFLP combinations in the analysis of 27

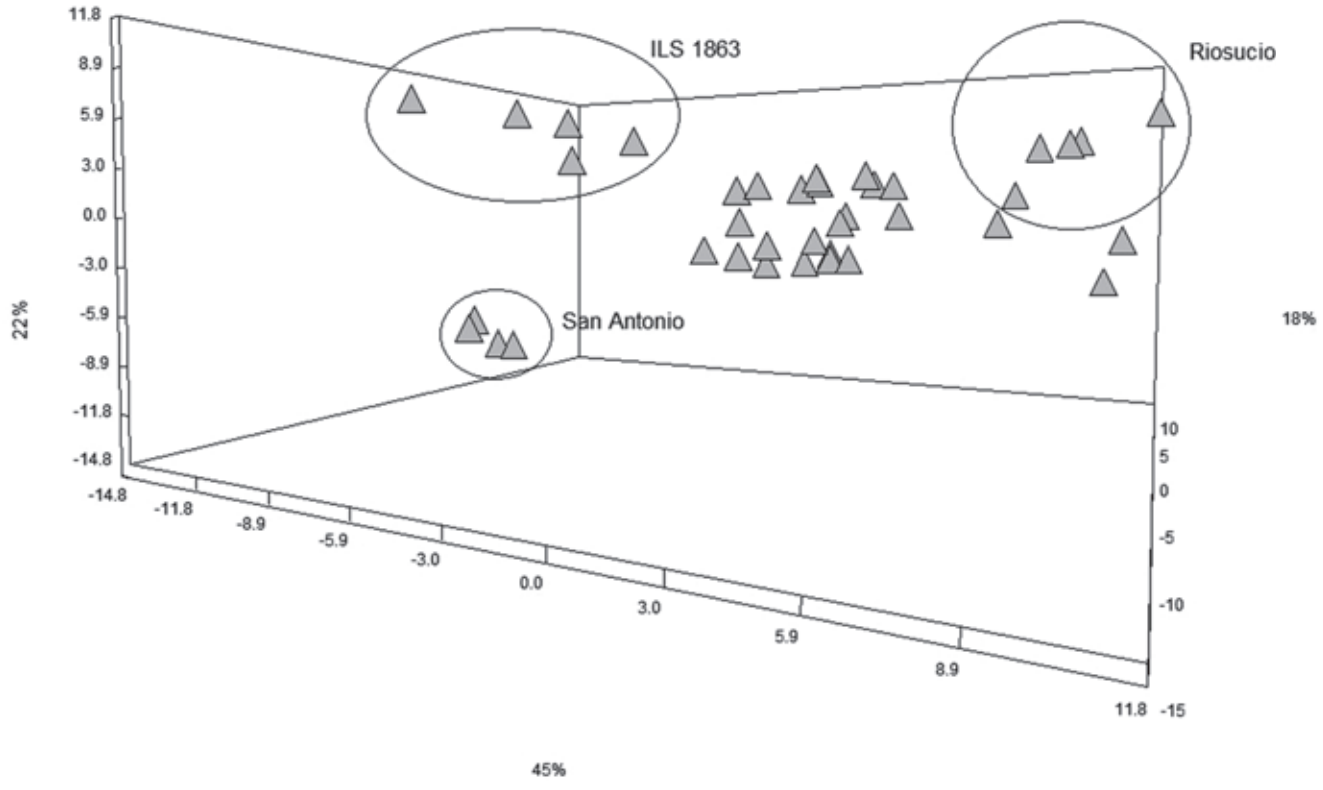

FIGURE 3. Graphic representation of Principal Coordinate Analysis with squared Euclidean distances of 37 *R. glaucus* genotypes.

R. glaucus accessions from Colombian coffee growing area, indicating a broader genetic base. The differences could be due to the inclusion of 13 wild accessions or the use of other combinations; however, when six SSR loci were evaluated in the same accessions, similarities between 60% and 100% were found. Furthermore, *R. glaucus* was not discriminated from other *Rubus* species, as *R. urticifolius*, with neither of the two marker systems.

Subsequently, Marulanda and López (2009) used eight SSR loci in the analysis of 15 wild and cultivated thorny and thornless *R. glaucus* accessions from the Colombian coffee growing region, and their results agreed to a large extent with the current study as it was confirmed that *R. urticifolius* is not genetically close to *R. glaucus* (Fig. 2); it was also evident a homogeneous group of *R. glaucus* with 13 accessions in a similarity level of 90%; in addition, thorny and thornless accessions could not be discriminated as in the present results, where Sin espinas accession groups closely with Monteloro (Fig. 2).

Other studies have shown largest genetic variability within *R. glaucus*. Using RAPD markers, Marulanda and Márquez (2001) observed a 55% similarity in 40 *R. glaucus* and 15 foreign thornless *Rubus* accessions, which did not show defined groups. Using RAMs, Morillo *et al.* (2005) found 31 *R. glaucus* accessions in a similarity level of 60%, which reveals a high variability that can be explained by the inclusion of wild accessions and cultivated accessions propagated by seed, which is a common practice in Juntas (Valle del Cauca) location where some plants were collected for that research.

Finally, in a phenotypical assessment of 32 *R. glaucus* accessions performed in Silvania, from which six accessions were selected for the current study (Tab. 1), three groups with different morphological, agronomical, nutricional and fruit physicochemical advantages were found (Espinosa *et al.*, 2009). The six selected accessions were located in these three groups, for this reason they represent important phenotypic variation within *R. glaucus* species. However, these accessions present low genetic variability (using AFLP technique in the present study), which could suggest that few regions of the genome are responsible for the phenotypic variation or the action of epigenetic mechanisms in the regulation of gene transcription for the expression of different phenotypes (Popluechai *et al.*, 2009). The previous agrees with the results obtained by García-Martínez *et al.* (2006) in traditional cultivars of tomato, where some contrasting phenotypes could not be genetically discriminated using AFLP markers.

Conclusions

The three AFLP combinations used did not generate a statistically significant differentiation between accessions, except for San Antonio and ILS 1863, probably due to genetic homogeneity among them and/or the low polymorphism observed. According to the results, EAGG/MCTT combination is recommended for the analysis of highly related genotypes of the species, because of its higher discrimination power (RP= 8; PIC*p*= 0,33) and the higher percentage of polymorphic bands (32%).

The current study provides important tools for the certification of the clonal garden with elite genotypes for *R. glaucus* breeding in Colombia. It is relevant to continue their morphoagronomic and molecular monitoring using additional AFLP combinations, codominant markers as SSR or others based in DNA sequencing with highest genomic representation, as well as beginning with programs to increase the genetic base of cultivated *R. glaucus*.

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