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Molecular characterization of the Andean blackberry, *Rubus glaucus*, using SSR markers

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ABSTRACT. The species *Rubus glaucus*, also known as the Andean or "Castilla" blackberry, is one of nine edible species of this genus that grow naturally in Central and South America. In Colombia, this species is the most important of all *Rubus* species for agricultural and commercial purposes. We used 20 SSRs developed for other *Rubus* species to characterize 44 Colombian *R. glaucus* genotypes, collected from eight different departments, and to look for molecular differences between thornless and thorny cultivated blackberries. Eighty-two bands were obtained from 28 loci. The genotypes were classified into eight populations, corresponding to collection sites. The mean number of polymorphic alleles per locus in all populations and genotypes ranged from 1.857 to 2.393. Samples collected from Valle del Cauca, Quindío, Caldas, and Risaralda departments had the highest heterozygosity values. The finding of exclusive bands from *R. glaucus* genotypes from Valle del Cauca, Quindío, and Caldas demonstrates genetic and molecular differentiation between thorny and thornless Andean blackberries.

Key words: Microsatellites; SSRs; *Rubus glaucus*; Colombia; Molecular characterization; Thornless blackberry

INTRODUCTION

One of the most interesting characteristics present in several *Rubus* species is the absence of thorns. Several commercial varieties, for example, "Chester Thornless", "Thornfree", and "Thornless Evergreen", are thornless genotypes from the United States. This one-gene characteristic, controlled by a recessive gene, has been extensively studied in Europe and the United States (Jennings and Ingram, 1983; Hall, 1990; Skirvin et al., 2009).

Among cultivated Colombian blackberry genotypes, several do not have thorns but show the same productivity and fruit size as the thorny genotypes generally cultivated throughout the country. However, given their interesting phenotypic characteristics and much lower production costs, farmers have mass-propagated these genotypes commonly referred to as "thornless" blackberries, using vegetative methods. Marulanda et al. (2007) found two possible origins of thornless *Rubus glaucus* materials: one in the department of Risaralda and the other in the department of Quindío.

However, other sources of thornless *R. glaucus* could exist. Blackberries belong to the family Rosaceae, genus *Rubus*, subgenus *Eubatus* Focke. Commercial *Rubus* species include those with red berries known as "raspberries" (*R. idaeus* L.) and those with black berries known as "blackberries", such as the species *R. occidentalis* L. cultivated in the northern hemisphere, especially Europe and North America. Andean blackberries are regarded as "blackberries" and belong to several species that grow in both Central and South America (Thompson, 1997).

Previous study carried out by Marulanda et al. (2007) and Marulanda and López (2009) on the genetic diversity of Colombian blackberries identified high phenotypic and molecular plasticity in the *R. glaucus* species known as the "Castilla" blackberry in Colombia's central Andean area. Other wild *Rubus* species present in the Andean region are found near farms where the "Castilla" blackberry is commercially grown. These plants were also submitted to morphological, agronomic, and molecular characterizations using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) molecular markers (Marulanda and López, 2009).

This study identified the molecular differences between thorny and thornless *R. glaucus* of wild and cultivated genotypes from eight different blackberry-producing regions of Colombia using 20 microsatellite sequences developed for other *Rubus* species.

MATERIAL AND METHODS

Study material

Forty-four *Rubus* genotypes, both cultivated and non-cultivated, were collected in eight departments of Colombia's Andean region: Cundinamarca, Santander, Valle del Cauca, Antioquia, Huila, Caldas, Quindío, and Risaralda. The materials included thorny and thornless *R. glaucus* genotypes plus the wild species *R. urticifolius* (Table 1).

Table 1. *Rubus* species and genotypes collected in eight different regions of Colombia.

Number	Code	Species	Characteristics	Collection area
1	CVM1	*R. glaucus*	Thorny	Cundinamarca
2	CVM2	*R. glaucus*	Thorny	Cundinamarca
3	CVM3	*R. glaucus*	Thorny	Cundinamarca
4	CVM4	*R. glaucus*	Thorny	Cundinamarca
5	CVM6	*R. glaucus*	Thorny	Santander
6	CVM7	*R. glaucus*	Thornless	Santander
7	CVM8	*R. glaucus*	Thorny	Santander
8	CVM9	*R. glaucus*	Thorny	Santander
9	CVM10	*R. glaucus*	Thorny	Valle del Cauca
10	CVM11	*R. idaeus*	Thornless	Valle del Cauca
11	CVM12	*R. glaucus*	Thorny	Valle del Cauca
12	CVM13	*R. glaucus*	Thorny	Valle del Cauca
13	CVM15	*R. glaucus*	Thorny	Antioquia
14	CVM16	*R. glaucus*	Thorny	Antioquia
15	CVM17	*R. glaucus*	Thorny	Antioquia
16	CVM18	*R. glaucus*	Thornless	Antioquia
17	CVM19	*R. glaucus*	Thorny	Antioquia
18	CVM20	*R. glaucus*	Thorny	Antioquia
19	CVM22	*R. glaucus*	Thorny	Huila
20	CVM23	*R. glaucus*	Thorny	Huila
21	CVM24	*R. glaucus*	Thorny	Huila
22	CVM25	*R. glaucus*	Thorny	Huila
23	CVM26	*R. glaucus*	Thorny	Huila
24	CVM27	*R. glaucus*	Thorny	Huila
25	CVM28	*R. glaucus*	Thorny	Huila
26	CVM	*R. glaucus*	Wild	Caldas
27	CVMA	*R. glaucus*	Thornless	Risaralda
28	CVMB	*R. glaucus*	Thorny	Quindío
29	CVMC	*R. glaucus*	Thornless	Quindío
30	CVMD	*R. glaucus*	Thorny	Risaralda
31	CVME	*R. glaucus*	Thorny	Caldas
32	95	*R. glaucus*	Wild	Quindío
33	107	*R. urticifolius*	Wild	Quindío
34	106	*R. urticifolius*	Wild	Quindío
35	97	*R. glaucus*	Wild	Quindío
36	86	*R. glaucus*	Wild	Quindío
37	22	*R. glaucus*	Thorny	Caldas
38	37	*R. urticifolius*	Wild	Caldas
39	44	*R. urticifolius*	Wild	Risaralda
40	MSA1	*R. glaucus*	Thornless	Risaralda
41	MSA2	*R. glaucus*	Thornless	Risaralda
42	MSA3	*R. glaucus*	Thornless	Quindío
43	MSA4	*R. glaucus*	Thornless	Quindío
44	MSA5	*R. glaucus*	Thornless	Caldas

DNA isolation and fragment analysis

Samples were collected in silica gel, placed in plastic bags, and transported to the Biotechnology Laboratory of the Universidad Tecnológica de Pereira. DNA was isolated using the Plant DNAeasy Miniprep kit (QIAGEN®), following manufacturer instructions. Several samples did not show any DNA after the isolation procedure, so it was necessary to reprocess these samples following the Doyle and Doyle (1990) protocol. In all cases, samples were purified using the protocol described by Castillo (2006).

Twenty microsatellite sequences that belong to other *Rubus* species native to Europe, North America, and Asia were used for DNA amplification (Table 2). These included nine SSR markers developed by Castillo (2006), eight developed by Graham et al. (2002, 2004), and three developed by Amsellem et al. (2001).

Amplification reactions were carried out in a total volume of 12.5 µL, with 20 ng

DNA, 2 µM of each primer, 200 µM of each nucleotide, 1% buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), and 0.5 U Taq DNA polymerase. The amplification profile was 30 cycles at 94°C for 1 min, annealing temperature for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min.

Data processing

The number of loci, the number of alleles per locus (N_A), and the expected (H_E) and observed (H_O) heterozygosities were determined using GenAlEx 6.1 (Peakall and Smouse, 2006). Nei's genetic distance (1978) was calculated and a principal coordinates analysis was performed. The polymorphic information content (PIC) (Cordeiro et al., 2000) and the discrimination power (D) were calculated for each locus to compare the efficiency of markers in varietal identification (Tessier et al., 1999).

RESULTS AND DISCUSSION

A total of 28 loci were detected using the above mentioned microsatellite sequences, with N_A values ranging between 1 and 6 and a total of 82 bands. The 20 SSRs were found to be polymorphic for the 44 genotypes studied (Table 2). The number of alleles per population ranged between 52 and 67, where it was higher in the populations of Quindío, Valle del Cauca and Caldas (Table 3). Rare and low-frequency alleles were detected in the populations of Valle del Cauca, Caldas, Quindío, and Risaralda. Alleles with low frequencies (≤25%) were found in Quindío, Caldas and Risaralda. The existence of *Rubus* genotypes with exclusive or private alleles at several of their loci is evidenced here, and observations indicate that some alleles are shared by all genotypes and populations (Table 4).

The populations of Valle del Cauca, Quindío, Caldas, and Risaralda also showed the highest H_E values (0.501, 0.493, 0.470, and 0.451) (Table 3). The H_O values ranged from 0.728 in Quindío genotypes to 0.893 in the Huila population, with an average H_O of 0.825. On the other hand, H_E values ranged from 0.413 in the Cundinamarca population to 0.501 in the Valle del Cauca population, with the highest values occurring in genotypes from Valle del Cauca, Quindío, Caldas, and Risaralda. The inbreeding coefficient (F) for all loci and populations was -0.823. This parameter represents the variability between individuals, also demonstrating genetic differentiation among them (Table 3).

Both H_E and H_O values of all microsatellites were high. In most cases, the H_O values were higher than the H_E values. The H_O values ranged from 0.056 with the RhM001 marker to 1.00 with the Rubus 259f, mRaCIRRIH3, Rubus 16a, RhM003, and RhM021 markers. Regarding the PIC and D parameters, the highest values were shown by microsatellites Rubus 76b, Rubus 105b, and Rubus 98d, followed by RiM017 and Rubus 259f (Table 2).

Similar results were reported by Castillo (2006), who used 12 SSRs to analyze an extensive collection of North American *Rubus* (raspberry) germplasm. Results indicated from 3 to 16 alleles per locus, with an average of eight alleles per locus and a total of 96 alleles. In the case of the blackberry samples, the same 12 pairs of primers amplified from 6 to 31 alleles per locus, with an average of 15 alleles per locus and a total of 177 alleles. More recently, Flores et al. (2010) isolated 12 microsatellites from SSR-enriched genomic libraries of *Rubus idaeus*. The best SSR loci, based on high H_O and H_E, high PIC, and low F, were RiM019, RhM003 and RhM011. In this evaluation of Colombian *Rubus* materials, RhM003 was found to be one of the most informative.

Table 2. Characteristics of the 20 microsatellite markers used in *Rubus* spp.

No.	Locus	Motif	GenBank accession No.	Primer sequences (5'-3')	Size (bp)	(N)	N_A	PIC	D	H_O	H_E
1	RhM018[3]	$(CTT)_6$	FJ194447	F-CACCAATTGTACACCCAACAAC R-GATTGTGAGCTGGTGTTACCAA	379	52	2	0.3510	0.5094	0.488	0.294
2	RhM036[3]	$(TG)_7$	FJ194455	F-AGCAACCACCACCTCAACTAAT R-CTAGCAGAATCACCTGAGGCTT	315	52	2	0	0		
3	RhM023[3]	$(CAT)_5$	FJ194449	F-CGACAACGACAATTCTCACATT R-GTTATCAAGCGATCCTGCAGTT	196	52	2	0.4864	0.3388	0.824	0.454
4	RhM011[3]	$(TC)_{18}$	FJ194446	F-AAAGACAAGGCGTCCACAAC R-GGTTATGCTTTGATTAGGCTGG	280	58	3	0.5419	0.1448	0.969	0.532
5	RhM001[3]	$(CA)_7$	FJ194444	F-GGTTCGGATAGTTAATCCTCCC R-CCAACTGTTGTAAATGCAGGAA	232	50	3	0.1328	0.2148	0.056	0.090
6	RhM021[3]	$(TC)_6$	FJ194448	F-CAGTCCCTTATAGGATCCAACG R-GAACTCCACCATCTCCTCGTAG	282	50	3	0.5461	0.219	1.000	0.525
7	RhM003[3]	$(TG)_{10}$	FJ194445	F-CCATCTCCAATTCAGTTCTTCC R-AGCAGAATCGGTTCTTACAAGC	200	48	3	0.5151	0.047	1.000	0.512
8	RiM017[3]	$(TG)_6$	FJ194453	F-GAAACAGGTGGAAAGAAACCTG R-CATTGTGCTTATGATGGTTTCG	194	58	3	0.5869	0.2148	0.919	0.536
9	RiM015[3]	$(ATC)_5$	FJ194452	F-CGACACCGATCAGAGCTAATTC R-ATAGTTGCATTGGCAGGCTTAT	350	58	3	0.4462	0.4451	0.632	0.363
10	Rubus 76b[2]	$(CT)_5(CT)_4$	NA	F-CTCACCCGAAATGTTCAACC R-GGCTAGGCCGAATGACTACA	190-210	55	4	0.6004	0.2463	0.919	0.548
11	Rubus 16a[2]	$(AT)_8(GT)_{11}$	NA	F-TGTTGTACGTGTTGGGCTTT R-GGGTGTTTGCCAGTTTCAGT	169	55	4	0.3461	0.0588	1.000	0.515
12	Rubus 116a[2]	$(CT)_{12}(T)_{10}$	NA	F-CCAACCCAAAAACCTTCAAC R-GTTGTGGCATGGCCTTTTAT	299	55	4	0.5297	0.053	0.975	0.518
13	Rubus 105b[2]	$(AG)_8$	NA	F-GAAAATGCAAGGCGAATTGT R-TCCATCACCAACACCACCTA	165-173	55	6	0.5924	0.2171	0.919	0.536
14	Rubus 137a[2]	$(TG)_8(TA)_4$	NA	F-TGTGAGCAGAGTGAAGGAGCTA R-AGCATTATTCGCGCAGTTTT	198	55	3	0.4995	0.0465	0.969	0.496
15	Rubus 259f[2]	$(CT)_4(AG)_8$	NA	F-TGGCACAAGAAGCCTGTAAC R-TCCCATATCCCTCAGCATTC	265	55	5	0.5717	0.1727	1.000	0.548
16	Rubus 263f[2]	$(AT)_{16}(CA)_4$	NA	F-ATTCCGCCCTGCATAAATC R-GGAAATTGGAAACCATTGGA	254	55	3	0	0.0454	0.969	0.496
17	Rubus 98d[2]	$(GAA)_5(GA)_{10}$	NA	F-GGCTTCTCAATTTGCTGTGTC R-TGATTTGAAATCGTGCGGTTA	198	55	2	0.5880	0.2558	0.878	0.534
18	mRaCIRRI1G3[1]	$(GA)_{28}$	AF205116	F-CTCTACAAAAGGATCTGCATGA R-CAGCAAAAGTGAAATGGTTCA	195-265	55	3	0.5473	0.2566	0.888	0.532
19	mRaCIRRIV2A8[1]	$(CA)_{12}(CT)_{11}$	AF261693	F-TAAAAAGGCGCAACAGTCG R-AGACACAGAAACAGGCATCG	191-237	55	3	0.5604	0.23	0.975	0.509
20	mRaCIRRIH3[1]	$(GT)_{15}(GA)_{17}$	AF205117	F-CTGGATGTGTGGGTGTGTATC R-CCTGGATATGTTTACCCTGACC	160-226	55	4	0.5151	0.025	1.000	0.523

[1]Derived from the giant bramble *Rubus alceifolius* (Amsellem et al., 2001). [2]Derived from *Rubus idaeus* (Graham et al., 2002, 2004). [3]Derived from *Rubus idaeus* and blackberry "Marion" (*Rubus* hybrid; Castillo, 2006). N = number of loci; N_A = total number of alleles per locus; PIC = polymorphic information content; D = discrimination power; H_O = observed heterozygosity; H_E = expected heterozygosity; NA = not available.

Table 3. Total values for allelic patterns and diversity parameters per population.

Population	N	N_A	H_O	H_E	F
Cundinamarca	52	1.857	0.813	0.413	-0.969
Santander	52	1.857	0.857	0.429	-1.000
Valle del Cauca	65	2.321	0.780	0.501	-0.567
Antioquia	54	1.929	0.871	0.443	-0.957
Huila	54	1.929	0.893	0.449	-0.989
Caldas	63	2.250	0.771	0.470	-0.654
Quindío	67	2.393	0.728	0.493	-0.511
Risaralda	55	1.964	0.886	0.451	-0.967
Mean values	57.75	2.063	0.825	0.456	-0.823

N = number of loci; N_A = number of alleles per locus; H_E = expected heterozygosity; H_O = observed heterozygosity; F = inbreeding coefficient.

Table 4. Summary of private alleles per population.

Sample	Population	Number of loci with private alleles
CVM11 (*Rubus idaeus*)	Valle del Cauca	9
Wild CVM (*R. glaucus*)	Caldas	3
95 (*R. glaucus*)	Quindío	5
107 (*R. urticifolius*)	Quindío	5
Thornless MSA3 (*R. glaucus*)	Quindío	4
Thornless MSA4 (*R. glaucus*)	Quindío	4

Sixty-one exclusive or rare alleles were obtained in the 28 loci, with the highest number of exclusive alleles (25) observed in genotypes from Valle del Cauca, followed by Quindío (24) and Caldas (12). *Rubus idaeus* genotype CVM11 from Valle del Cauca showed the highest number of exclusive alleles, followed by the wild genotypes *R. glaucus* 95 and *R. urticifolius* 107 from Quindío and the thornless genotypes MSA3 and MSA4, also collected in Quindío, together with CVM from Caldas. The loci in which the private alleles were detected are very important for genotype differentiation, particularly in the case of the thornless genotypes from Quindío because of their outstanding agricultural performance in ongoing field trials in several areas of Risaralda. Furthermore, this demonstrates the genetic differences between these genotypes as compared with the rest of the individuals analyzed (Table 4).

Figure 1 shows the grouping of the 44 genotypes studied. One large group, with 95% similarity, gathers most of the *R. glaucus* genotypes (both cultivated and wild) of the 8 populations. This group is then divided into two subgroups, with close to 98% similarity. The first subgroup gathers different individuals from the Departments of Cundinamarca, Antioquia, Santander, and Valle del Cauca, several with 100% similarity. Genotypes MSA3-Quindío, MSA4-Quindío, CVMA-Risaralda, CVMB-Quindío, CVMC-Quindío, CVMD-Risaralda, 97-Quindío, MSA5-Caldas, and 22-Caldas are in the same subgroup, and based on agronomic evaluations carried out so far, they are considered to be the most promising blackberry materials and are therefore of great interest to this study.

The second subgroup shows 98% similarity and includes individuals from the Departments of Cundinamarca, Santander and Huila. This subgroup gathers individuals of MSA2-Risaralda, CVM8-Santander and MSA1-Risaralda, with few differences between them. MSA1 and MSA2 from Risaralda are thornless cultivated *R. glaucus* genotypes. Individuals of 95-Quindío and 86-Quindío, wild *R. glaucus* species from the Department of Quindío, also belong to this subgroup, but their similarity is lower.

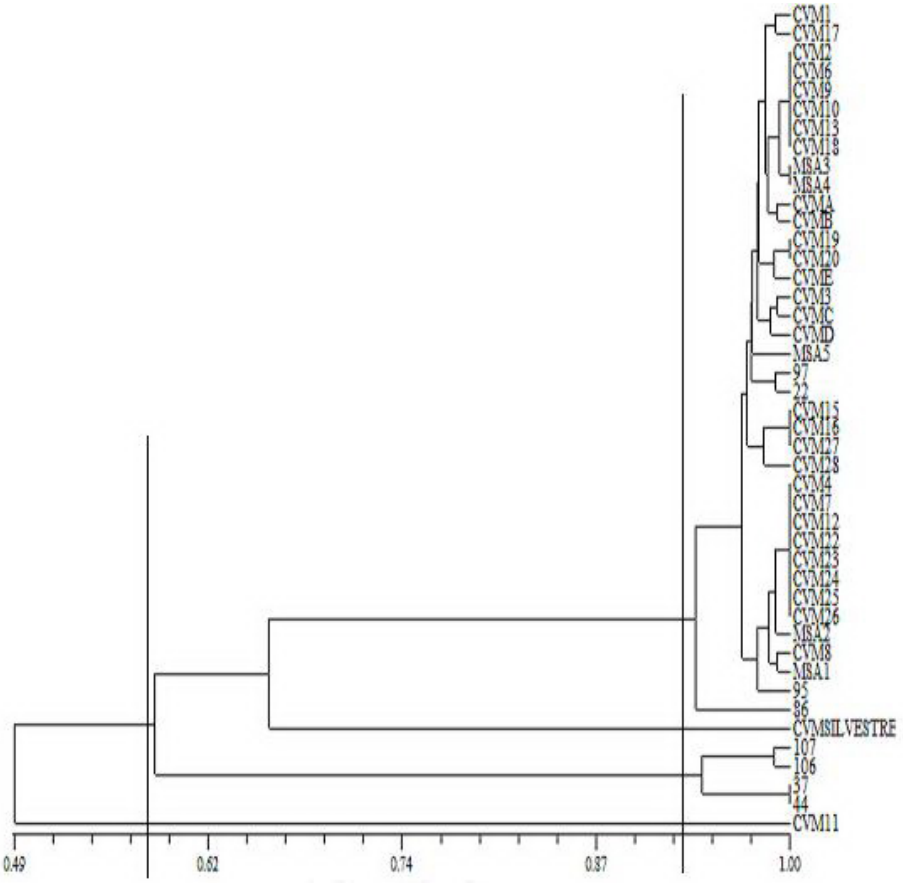

Figure 1. Dendrogram constructed with the Dice (1945) coefficient for Colombian *Rubus* genotypes.

Figure 2 presents the results of the principal coordinates analysis. There is no clear differentiation of genotypes based on collection sites; however, four groups can be distinguished: Group 1, which is characterized by materials broadly differing in origin and includes both cultivated and wild as well as thorny and thornless plants; Group 2, which includes both cultivated and thorny genotypes, mainly from Antioquia; Group 3, which is characterized by genotypes with little genetic distance between them and includes the highest number of genotypes from different sites of origin, most of them being thorny and thornless cultivated plants, and Group 4, located in the lower part of the figure, which includes genotypes, mainly from Quindío, with greater genetic distances, as well as the wild and cultivated thornless genotypes MSA3 and MSA4, collected in Quindío, the wild CVM genotype from Caldas, and finally genotype *R. idaeus* CVM11, a wild material from Valle del Cauca, which was found to differ from most of the other genotypes.

The proximity between thorny and thornless *R. glaucus* genotypes and the wild species *R. urticifolius* (37, 106, 44) should be highlighted. The molecular proximity between the two species had already been described by Aguilar (2006) and Marulanda et al. (2007).

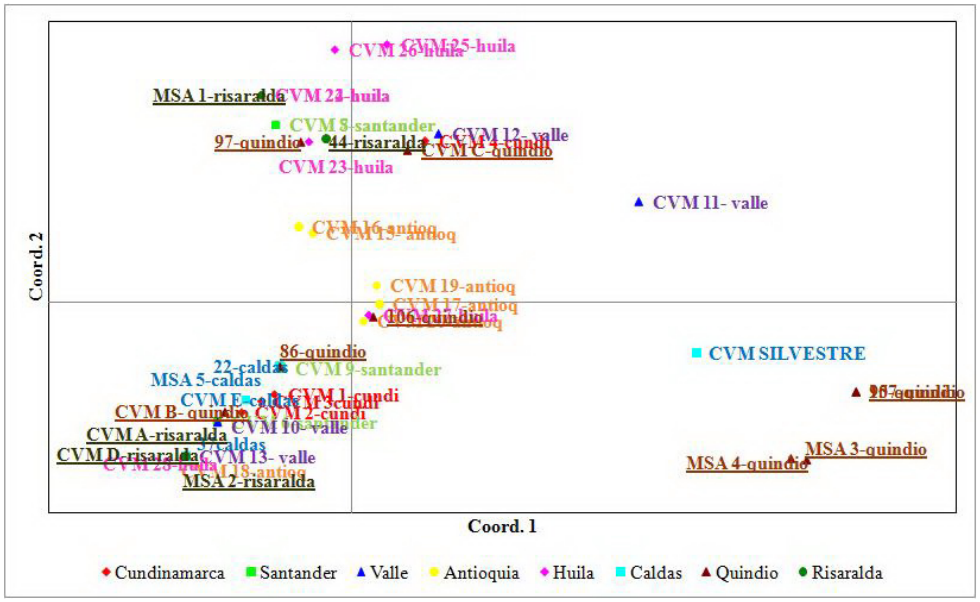

Figure 2. Principal coordinates analysis based on Nei's genetic distance for *Rubus* genotypes.

The highest H_E values occurred in Valle del Cauca, Quindío and Caldas populations. The values obtained in the current study were compared with those obtained by Marulanda et al. (2007). The heterozygosity values were higher in this study because a larger number of microsatellites and loci for blackberries and red raspberries, developed by Graham et al. (2002, 2004), were used; these turned out to be highly polymorphic and informative (Table 2).

Graham et al. (2002) evaluated 50 genotypes belonging to several highly differentiated *Rubus* species, using 10 microsatellite sequences derived from *R. idaeus.* From 7 to 16 alleles were obtained per locus. The H_O values ranged between 0.52 and 0.91, whereas the H_E values were between 0.63 and 0.91. These values are very similar to those obtained in the present study, which evaluated three *Rubus* species and 45 wild and cultivated genotypes, apparently more closely related because of their distribution in a smaller geographical area (Figure 2).

In studies carried out with European accessions of *Rubus*, using the SSRs developed by Graham et al. (2002, 2004), Badjakov (2007) found H_E values that ranged from 0.2916 at the locus Rubus 98d to 0.666 at the locus Rubus 76b, with an average value of 0.4722 for all genotypes and loci. The mean heterozygosity values in Colombian *Rubus* accessions, using the same SSRs as Badjakov (2007), were 0.548 for H_E and 0.919 for H_O at the locus Rubus 76b, and 0.534 for H_E and 0.878 for H_O at the locus Rubus 98d (Table 2). The heterozygosity values for the Bulgarian *Rubus* accessions were lower than those obtained for Colombian *Rubus* accessions, implying a low heterozygosity in the accessions used in Badjakov's study (2007). The opposite occurred with Colombian *Rubus*, with the foregoing evidence of higher heterozygosity in the Colombian *Rubus* germoplasm analyzed. Analysis of molecular variance (AMOVA) showed 90% variability within populations and 10% variation between populations. These data agree with those observed in the principal coordinates analysis, where variation is mostly attributed to individuals.

CONCLUSIONS

Using microsatellites from other *Rubus* species has proven to be a very useful strategy to differentiate between wild and cultivated *R. glaucus* genotypes, as well as between thorny and thornless cultivars.

Based on genetic distances, the grouping of genotypes does not depend on their sites of origin. The thornless genotypes from Quindío were separated from the rest of the groups, whereas the thornless genotypes from Risaralda were also placed in different genetic groups, showing important variability among them.

The dendrogram shows that the similarity among cultivated *R. glaucus* materials is quite high, almost 90%, with some materials even showing 100% similarity.

AMOVA showed a higher variability between genotypes than between populations, which agrees with the results obtained in the principal coordinates analysis.

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