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Genetics and plant breeding

Evaluation of SSR and SNP markers in *Rubus glaucus* Benth progenitors selection

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Abstract -*Rubus glaucus* Benth (known as "mora de castilla") is a Colombian agricultural product, with probably, the major potential. This fruit combines features of *Idaeobatus* and *Rubus* subgenera. Despite its recognized importance in the economy of small producers, this crop has received little technological development; as a result, sowing procedures of this specie is done by using local cultivars asexually propagated by producers. Associated *Rubus* producers in Colombia has noticed the necessity to formalize the offer of planting material, starting with plant breeding programs tending to the obtention of more productive varieties with morphological features that facilitates cultural activities. This study presents the results of the evaluation of SSR and SNPs markers obtained in previous experimental works from a RNA-Seq transcriptome analysis. It was evaluated 15 promissory *R. glaucus* cultivars, which could be potential progenitors in future breeding schemes. Genetic characterization was accomplished by testing 22 SSR microsatellite and 78 single nucleotide polymorphisms (SNPs) markers. From evaluated SSR markers, 15 yielded positive PCR amplification generating 29 *loci* and 58 alleles. From evaluated SNPs markers, 36 yielded positive PCR amplification. Obtained sequences from amplified products with SNPs showed high homology with species belonging to Rosaceae family. Selection criteria of progenitors were based on the results of molecular characterizations and useful morphological features in the culture management. This research demonstrates the utility of molecular markers to assess genetic diversity of potential progenitors susceptible to plant breeding processes.
This process, highly known as development of parental populations, determines in a great manner the success of plant breeding processes.
Index terms: Plant breeding, andean blackberry, genetic diversity.

Avaliação dos marcadores SSR e SNP na seleção de progenitores em *Rubus glaucus* Benth

Resumo-Amora de castilla (*Rubus glaucus* Benth) é um dos produtos com maior potencial de desenvolvimento agrícola Colômbiano, que combina características dos subgéneros *Idaeobatus* e *Rubus*. Apesar da sua reconhecida importância na geração de rendas para os pequenos produtores, este cultivo tem recebido pouco desenvolvimento tecnológico. Como resultado a semeadura desta espécie é feita mediante cultivares locais propagados asexualmente pelos agricultores. Os produtores associados de amora na Colômbia têm ressaltado a necessidade de formalizar a oferta de cultivares para semente, começando com processos de fito-melhoramento para obter variedades mais produtivas, com características morfológicas que ajudem nas atividades culturais. O presente artigo, mostra os resultados da avaliação com marcadores tipo SSR e SNP obtidos em trabalhos prévios desde uma análise do transcriptoma por ARN-Seq. Quinze cultivares promissórios de *R. glaucus* foram avaliados para ser possivelmente utilizados num futuro processo de cruzamento. Na caracterização, 22 marcadores tipo SSR e 78 marcadores polimórficos tipo SNP foram selecionados. Dos 22 marcadores SSR usados, 15 rendeu amplificação positiva gerando um total de 29 loci e 58 alelos. Similarmente, dos 78 marcadores tipo SNP avaliados, 36 mostraram amplificação positiva. As sequencias obtidas dos produtos amplificados com SNP mostraram uma alta homologia com espécies da família Rosaceae. Os critérios de seleção dos progenitores foram realizados com base em os resultados das caracterizações moleculares junto com os critérios morfológicos úteis no manejo do cultivo. Este trabalho prova a utilidade dos marcadores moleculares para estimar a diversidade genética dos possíveis progenitores para ser usados num processo de fito-melhoramento. Um aspecto conhecido como o desenvolvimento da população parental, e que determina de uma forma grande o sucesso dos processos de fito-melhoramento.
Termos para indexação: Fito-melhoramento, amora de castilla, diversidade genética.

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Introduction

Rosaceae family comprises nearly 90 genera and 300 species, among them fruit trees with economic importance are included such as apples (*Malus pumila* Mill.) and pears (*Pyrus* spp.); stone fruits or drupes like peaches (*Prunus persica*); several ornamental species including the rose (*Rosa* spp.), and soft fruits as strawberries, raspberries, blackberries, among others. Different taxonomic classifications of the family has been proposed based upon morphology, whilst Schulze-Menz (1964) suggested a new family classification into subfamilies: *Maloideae, Amygdaloideae, Rosoideae* y *Spiraeoideae* based on chromosome number and fruit type (LONGHI, et al. 2014).

Genetic variability of *Rubus* genus is known over the world and has been widely studied over the phenotypical, morphological, chromosomal and molecular aspects (DOSSETT, et al. 2012; ALICE, et al. 1999; GRAHAM AND MCNICOL, 1995; GRAHAM et al., 1997). One of the most interesting features of the genus is the variability in the number of chromosomes, polyploidy and hybridization; in contrast, only *Idaeobatus, Dalibarda*, and *Anoplobatus* subgenera are predominantly diploid, whilst *Dalibardastrum, Malachobatus*, and *Orobatus* are exclusively polyploid (THOMPSON, 1995, 1997). Hybridization in *Rubus* occurs mainly between closely related species (NARUHASHI, N., 1990; KRAFT, 1995) and, in some cases, between subgenera (JENNINGS, 1979; WEBER, 1996; ALICE, et al. 1997), thus, some intersubgeneric hybrids possess commercial importance (WAUGH, et al. 1990).

Rubus glaucus or Andean blackberry is distributed over the main Colombian mountain and combines *Idaeobatus* and *Rubus* features. This specie is a fertile amphidiploid or allotetraploid, probably originated by genome fusion of two species (JENNINGS, 1988). (DELGADO, et al. 2010) found 28 chromosomes in *R. glaucus* cultivars, assuming a basic number n=7 for *Rubus* genus, it confirms its tetraploidy (4x).

Despite its well-known importance in the income generation for small producers, this cultivar has received little technological development, as a result, cultivar quality and productivity shown high variability, mainly due to the lack of formal varieties and the scarcity of planting material with good genetic and phytosanitary quality. Nowadays, planting of this specie is still done by the usage of local cultivars asexually propagated by growers (LOBO et al., 2002). This specie shows low yielding rates, mainly caused by anthracnose caused by por *Glomerella cingulata* (Stoneman) Spauld & H. Schrenk (teleomorph state of *Colletotrichum gloeosporioides*). This disease is considered the most devastating affecting *R. glaucus*, creating losses above the 50%. In addition, chemical treatment of this agent increases production costs (SALDARRIAGA-CARDONA, et al. 2008).

Associated Colombian blackberry producers, has highlighted the necessity to formalize the offer of planting material, starting by plant breeding schemes that allow the obtention of more productive varieties with morphological features that facilitates cultural activities and certain tolerance to fungal attack, especially those related to anthracnose. It is well known that the first step in plant breeding programs is the selection and characterization of promising cultivars.

In this regards, (BERTRAND, et al. 2008) has stated that with help of polymerase chain reaction (PCR), public institutions and commercial organizations in charge of plant breeding programs has implemented molecular markers, including SSR and progenitor genotyping to make more efficient those processes. In addition, the evolution of molecular techniques developed the polymorphisms of a single nucleotide (SNP). Bertrand et al. (2008), classified selection schemes assisted by markers onto 5 areas: *1)* Development of parental population for its selection and hybridization, *2)* construction of ligation maps for its evaluation over phenotypical features, *3)* QTL (Quantitative trait loci) validation, confirming the position and effects of QTL, *4)* Selection assisted by markers, and *5)* marker validation (BERTRAND, et al. 2008).

In Colombia, some studies regarding genetic diversity of *Rubus* genus has been carried out: Zamorano et al. (2004) conducted a molecular and morphological characterization of species belonging this genus using Random Amplified Microsatellite (RAMS). Duarte et al. (2011) evaluated genetic relations of elite Colombian *Rubus glaucus* cultivars through AFLP analysis obtained by the employment of three primer combinations. (MARULANDA, et al. 2007) assessed genetic diversity of wild and cultivated species of *R. robustus*, *R. urticifolius*, *R. glaucus* and *R. rosifolius* through AFLP and SSR markers developed for *R. alceifolius* (heterologous markers, when applied to *R. glaucus*). Marulanda and López (2009), performed molecular (SSR markers) and morpho-agricultural charcaterization for cultivated and wild varieties of *Rubus glaucus* with and without thorn, paying special attention to fruit size. (MARULANDA, et al. 2012) developed especific SSR markers for *Rubus glaucus*, aiming to obtain higher discrimination power. They concluded the necessity to develop more discriminatory molecular markers associated to morphological desired features. (LÓPEZ-VÁSQUEZ, et al. 2013), found differential responses in blackberry cultivars against anthracnose attack.

In recent years, it has been carried out the differential expression of blackberry cultivars against to anthracnose (*Colletotrichum gloeosporioides*) through transcriptome analysis (RNA-Seq) where two cultivars (UTP-1, tolerant & UTP-4, susceptible) were inoculated with a highly pathogenic strain of *C. gloeosporioides*,

together with a control treatment (cultivar inoculated with sterile water). Afterwards, RNA was extracted 72 hours later and the genetic material sequencing were compared between treatments (unpublished results). This study allowed the design of new molecular markers (SSR and SNPs) which were finally used in this project.

In order to start with plant breeding processes for *Rubus glaucus*, evaluations with SSR and SNPs were conducted over promissory cultivars that potentially could be used in future breeding schemes. Genetic distance and other features such as thorn presence/absence and fruit size were considered at the time of selecting cultivars.

Materials and methods

Plant material and DNA extraction - Fifteen Andean blackberry cultivars with agricultural interesting features coming from participative selections made with producers in different regions of the country were selected. These cultivars were previously characterized with heterologous (transferred from other *Rubus* specie) and homologous SSR markers (developed for *R. glaucus*) (MARULANDA, et al. 2012). Selected cultivars shown differential response against *C. gloeosporioides* attack (LÓPEZ-VÁSQUEZ, et al. 2013). Table 1, gather all data related to the sampled material (name, place of collection, thorn presence/absence and response against *C. gloeosporioides* (MORALES, et al. 2010).

DNA extraction of healthy foliar tissue was accomplished using the commercial *Plant DNeasy Mini Kit* (QIAGEN) following manufacturer instructions.

Molecular marker development - The development of the SSR and SNPs markers from a previous RNA-Seq analysis of the *R. glaucus* interaction against *C. gloeosporioides*, and its further use in this study is described.

SSR molecular markers - Detection of the simple sequence repeats (SSR) from the transcriptome analysis was completed using the MIcroSAtellite (MISA) software. From these sequences, 22 primers were designed. *Rubus glaucus* genome possess several microsatellite with different repetitions and lengths, as well as the majority of plant genomes analyzed so far. Thus, it was decided to select sequences with longer repetition than *tri-* nucleotides given that they has demonstrated to be more polymorphic and reproducible than microsatellite with *di*-nucleotide repetitions (VUKOSAVLJEV, et al. 2015; FAN, et al. 2013). Primer design was limited to sequences with a high number of repetitions of the base unit (> 5 for *tri-* nucleotide and > 4 for repetitions bigger than *tetra-* nucleotides). Another criterion were to select primers with annealing temperatures between 58°C and 61°C and expected PCR product sizes between 100 and 200 base pairs (bp) (see Table 2). In addition, Table 3 shows homology of generated primer sequences with other Rosaceae family species.

Table 1. Description of promissory *R. glaucus* cultivars.

Code	Latitude (N)	Longitude (W)	Height (m. a. s. l.)	Thorn Presence/ Absence	Collect Place	Response against *Colletotrichum gloeosporioides** attack
UTP1	4°52’15.0”	75°37’32.4”	2000	Absence	Risaralda	Tolerant
UTP2	4° 39´7´´	75° 35´26.3´´	2014	Presence	Quindío	Tolerant
UTP3**	4°38’36”	75°28’41,5”	2300	Absence	Quindío	Moderately tolerant
UTP4**	4°48´99.2´´	75°41´86´´	1950	Presence	Risaralda	Very susceptible
UTP5	5° 2´2.7´´	75° 27´10.5´´	1800	Presence	Caldas	Very susceptible
UTP6	4° 44´45.1´´	75° 36´39.6´´	1850	Absence	Risaralda	Moderately tolerant
UTP7	4°11’36.1”	75°48’14.6”	2000	Absence	Quindío	Moderately tolerant
UTP11	4°79’33”	74°42’68”	2288	Absence	Cundinamarca	Moderately tolerant
UTP15	6°99’44”	72°98’80”	2157	Absence	Santander	Tolerant
UTP16	6°59’39”.1	72°59’13”	2176	Presence	Santander	Very susceptible
UTP20**	4°13’23.8”	76°25’35.9”	2380	Presence	Valle del Cauca	Very susceptible
UTP21	4°13’23.8”	76°25’35.9”	2380	Presence	Valle del Cauca	Very susceptible
UTP26	6°09’15.4”	75°23’00.1”	2000	Absence	Antioquia	Moderately tolerant
UTP27	6°09’15.4”	75°23’00.1”	2000	Presence	Antioquia	Very susceptible
UTP28**	6°09’15.4”	75°23’00.1”	2000	Presence	Antioquia	Very susceptible

* (Morales, Y. M., Marulanda, M. L., ; Isaza, L., 2010).
** Cultivars with outstanding fruit size

Amplification reactions for this type of markers was accomplished following described conditions by (MARULANDA, et al. 2012). The *"touchdown"* amplification profile consisted of 32 denaturing cycles at 95°C by 1 minute; annealing for 1 minute with decrease of 1°C every two cycles from 63°C to 58°C; 10 cycles at 59°C and 10 cycles at 58°C; elongation at 72°C for 1 minute.

Afterwards, amplicon visualization was conducted over denaturing 6% polyacrylamide electrophoresis gels. Obtained results was analyzed through GenAlex v6.2 (PEAKALL AND SMOUSE, 2006) and PAST (*Paleontological statistics software package for education and data analysis*) (HAMMER, et al. 2001). Hardy-Weinberg Equilibrium (HWE) analysis was evaluated employing the Markov chain in GenAlex v6.2 (PEAKALL AND SMOUSE, 2006). In the SSR analysis, it was also incorporated another specie belonging to *Rubus* genus as external group.

Table 2. SSR primer markers for *Rubus glaucus*.

Identification code	SSR motif	F Primer	R Primer	Annealing Temperature	Expected Product Size (Bp)
CL1004.Contig2_All_94_1	$(GAA)_7$	CAGATTTGAATTATGGTGGGTGT	TCCTTTCCTTCTCACCCTTTAAC	60,0	141
CL1101.Contig1_ll_110_1	$(TCACCC)_4$	GACCCAACTATGCTTGTCGTTAC	GATTGGAACACGAGACCTACAAC	59,9	113
CL1366.Contig2_All_134_1	$(GAA)_6$	AAGGATGATTGTCACGTATGAGG	ACTCGGCAATCCATTCTCTATTT	60,3	129
CL1491.Contig11_All_143_1	$(CTT)_6$	CTTGGCTTTAGAAACTTGGGAGT	CTTCAAAGAAGAAAGTTGTTGGC	59,5	100
CL150.Contig2_All_12_1	$(TTG)_6$	CCATCAAGATTGAGTTTGCTTCT	ATTGAAGAATGCAACGAGATGAT	60,0	124
CL1916.Contig1_All_167_1	$(AAAGTG)_4$	ACAGCCAAGAATGACCTACAAAA	ACGTGAAAAACTGAGTTGGAAGAG	59,8	129
CL1891.Contig3_All_166_1	$(GCA)_7$	GAGGGAGAGATTTGGAGATGAAT	GTGCCATAAGCTTACAGGTTCAG	60,2	139
CL2218.Contig3_All_177_1	$(GAA)_6$	AAGCTTTCAAGTGCAACCTACTG	TTTGGGATTTTGGAATTTTTCTT	60,0	149
CL2322.Contig2_All_181_1	$(CAT)_6$	CTGTTTGCGAAGGATCTGTAAC	TGACGCAATGATATTACGATGAG	60,0	146
CL2455.Contig1_All_192_1	$(AGC)_6$	AGCTTGGACTGTGAACAAGGAT	CAACAATCACCAACCCAAGAC	60,3	138
CL2455.Contig1_All_193_1	$(AAG)_7$	CAGATTTCAGCCAAGAAGAGGTT	CGATCTCCTTCTTCTTCCTCTTT	59,5	148
CL2364.Contig3_All_186_1	$(GTGGTA)_4$	CCAAACATGAAATCAGTAGGGAA	TCATAAGAGGGCCATAAGAATGA	59,9	159
CL274.Contig3_All_22_1	$(TGT)_6$	CTGTTGTTATCGCTGTTGTTGAT	AGAGACCTTGTGAAGGAGTGGTT	60,6	160
CL2958.Contig1_All_219_1	$(TTC)_7$	TTATTTCTCTCCAAAATGCAACG	AAAAAGGAACAAACACCTGAACC	60,6	112
CL2556.Contig1_All_201_1	$(AGAGGG)_4$	AGAGGTGTGGTGTTGTTTGTGT	AAAATGCCACTTTTCCTATTGAA	59,1	156
CL3540.Contig2_All_254_1	$(TCT)_7$	CAACTCCAATCTCAGCTTTCTGT	CGATAITGACGACTCTACCTTCG	60,2	151
CL2787.Contig1_All_210_1	$(GGAAA)_4$	TAGATCTTAGGCCTCGTTTGGTT	CCAAACACTTGAAAGGAAAGCTA	59,8	116
CL3301.Contig1_All_237_1	$(AGAA)_5$	TGTGTATGGATATAGGGAGGGTG	TGTTCCTTCTTCCTTCCTTCTTT	59,8	85
CL3840.Contig1_All_265_1	$(GAA)_7$	GAAGTCAAAGTCCTGGAGGAGAG	CTCACTCTCCGTAAACCCATCAC	62,1	156
CL4175.Contig2_All_292_1	$(CTT)_7$	CTGTGATCATCTTCTTCCTGCTT	ACCAAAGCTTTTACCTTGGTGTT	60,3	160

Table 3. Accession number and homologous sequences for developed SSR markers.

		Homologous sequences in other rosaceae species	
Identification code	**Genbank Accession Number**	**Reported accession number in other Rosaceae species**	**Specie**
CL1004.Contig2_All_94_1	MH516338	XM_024331960.1	*Rosa chinensis*
CL1101.Contig1_All_110_1	MH516339	XM_008365700.2	*Malus x domestica*
CL1366.Contig2_All_134_1	MH516340	XM_024327521.1	*Rosa chinensis*
CL1491.Contig11_All_143_1	MH516341	***	
CL150.Contig2_All_12_1	MH516342	XM_004293478.2	*Fragaria vesca* subsp. *vesca*
CL1916.Contig1_All_167_1	MH516343	XM_024315090.1	*Rosa chinensis*
CL1891.Contig3_All_166_1	MH516344	XM_024315199.1	*Rosa chinensis*
CL2218.Contig3_All_177_1	MH516345	XM_021953392.1	*Prunus avium*
CL2322.Contig2_All_181_1	MH516346	XM_024325000.1	*Rosa chinensis*
CL2455.Contig1_All_192_1	MH516347	XM_020567229.1	*Prunus persica*
CL2455.Contig1_All_193_1	MH516348	XM_021972419.1	*Prunus avium*
CL2364.Contig3_All_186_1	***	XM_024325669.1	*Rosa chinensis*
CL274.Contig3_All_22_1	***	XM_024304460.1	*Rosa chinensis*
CL2958.Contig1_All_219_1	***	XM_009353829.2	*Pyrus x bretschneideri*
CL2556.Contig1_All_201_1	***	XR_002271838.1	*Prunus persica*
CL3540.Contig2_All_254_1	***	XM_024301335.1	*Rosa chinensis*
CL2787.Contig1_All_210_1	***	XR_907125.1	*Fragaria vesca* subsp. *vesca*
CL3301.Contig1_All_237_1	***	XM_007217879.2	*Prunus persica*
CL3840.Contig1_All_265_1	***	XM_021945224.1	*Prunus avium*
CL4175.Contig2_All_292_1	***	XM_024301524.1	*Rosa chinensis*

SNP molecular markers - Bowtie2 v2.2.4 (LANGMEAD, et al. 2012) and samtools v0.1.19 (LI AND DURBIN, 2009) software were used in the SNP marker identification. Given that whole genome sequencing of *Rubus glaucus* had not been carried out so far, the *Fragaria vesca* genome was employed as reference genome, as well as the comparison between tolerant and susceptible samples. Finally, SNPs were identified in 200 genes from susceptible and tolerant *R. glaucus* against *C. gloeosporioides*, allowing the design of 78 primers. In addition, homology of generated primer sequences with Rosaceae family was evaluated. Table 4, present in detail primer sequences of the SNP markers (UNIGENE primers).

Amplification reactions for SNP markers was accomplished following described conditions by (MARULANDA, et al. 2012). The *"touchdown"* amplification profile consisted of 32 denaturing cycles at 95°C by 1 minute; annealing for 1 minute with decrease of 1°C every two cycles from 64°C to 59°C; 10 cycles at 58°C and 10 cycles at 57°C; elongation at 72°C for 1 minute.

SNP´s fragment visualization was accomplished through gel electrophoresis and amplicons were sequenced by extension using the ABI PRISM® *BigDyeTM Terminator Cycle Sequencing* kit in a capillary ABI PRISM® 3730XL (96 capillary type) sequencer.

To analyze SNP sequences and to corroborate homology of obtained data in the sequence, BLAST (*Basic Local Alignment Search Tool* – NCBI) tool was employed using an E- cutoff value of 0.000001. Then, an individual analysis of each UNIGENE consisting of a multiple sequence alignment with Clustal Omega (EMBL – EBI), online version (https://www.ebi.ac.uk/Tools/msa/clustalo/). Finally, for the alignment of obtained sequences for all samples it was employed the MAFFT software, online version (www.ebi.ac.uk/Tools/mafft). A dendogram was obtained through the clustering method Neighbor Joining with UPGMA (*Unweighted Pair Group Method with Arithmetic Mean*), the substitution model proposed by (JUKES AND CANTOR, 1969) and a replacing number of 100. Genetic diversity parameters were estimated for haploid data with GenAlex 6.5b4 software (PEAKALL AND SMOUSE, 2006).

Results and discussion

Microsatellite marker analysis - It was found 4799 simple sequence repeats consisting mainly of *di*-nucleotide repetitions, followed by *tri*- and *tetra*-nucleotide repetitions. From the 22 evaluated SSR markers, 15 yielded positive amplification generating 29 loci and 58 alleles. Thirteen of them amplified 2 loci and the allelic number was about 15. Informative alleles were approximately 3 (see Table 5). In that regards, (DOSSETT, et al. 2012) showed that when assessing genetic diversity in *R. occidentalis* cultivars using SSR markers, observed allelic diversity was low with 3 or least alleles in 15 of the 21 evaluated loci, similarly to the observed in this study where allelic number was set around 3.

Expected heterozygosity (He) ranged between 0,607 and 0,7575; whilst observed heterozygosity (Ho) varied among 0,5665 and 1. Consequently, (GRAHAM, et al. 2004) explains that *Rubus* genus comprises highly heterozygous species. In that study *Rubus idaeus* varieties with thorn (e.g. Latham) are compared with glabrous ones (e.g. Glen Moy) demonstrating that thorn-possessing varieties showed higher heterozygosity levels than thorn-absent varieties. These differences associated to a morphological feature could support obtained values for *R. glaucus*, values that would be corroborated once progenies are established.

(DOSSETT, et al. 2012) argues that *R. occidentalis* cultivars show a noticeable heterozygosity level. For this specie in every evaluated locus (SSR), Ho was higher than He. Parallel, for *Rubus glaucus* this behavior was the same for the majority of markers (higher Ho values), excepting the marker CL2322. Additionally, (DOSSETT, et al. 2012) explains that this phenomena could be attributed to selection process and clonal propagation, similar situation to *R. glaucus* in Colombia where local selections made by producers are asexually propagated. Respect to variability parameters, (CLARK, et al. 2013) detected for *R. fruticosus*, a diploid specie with polyploidy ancestors and invasive behavior in United States, very low allele numbers, ranging between zero and 2,56 alleles per locus. That reported values are lower than obtained in the present study where the average value for polymorphic alleles was 5,448. This behavior is supported considering that polyploidy species is expected to obtain higher values, such as *R. glaucus*.

In the HWE analysis, five markers were in equilibrium while the rest (10) showed significant or highly significant disequilibrium (Table 5). (FU, et al. 2016) reported that a loss of the HWE for the specie *Ziziphus jujube* is explained because there did not existed a random selection of the samples, similar to this case of study, where samples corresponded to selected and asexually propagated cultivars.

Genetic diversity estimation through Dice index allowed the construction of a dendogram, depicted in Figure 1. Detachment of cultivar UTP1 is explained considering its recognized tolerance to *C. gloeosporioides* attack in the RNA-Seq analysis. The presence of groups in the distance analysis evidence a geographical tendency, corroborating that the interchange of planting material in Colombia is apparent. The fact of thorn present/absent cultivar clustering contributes to the design of future breeding schemes.

(DOSSETT, et al. 2012) assessed the genetic diversity of cultivated and wild plants of *R. occidentalis*, a berry from temperate regions from North America and Europe, through the usage of 21 SSR markers aiming to stablish a plant breeding process over a germplasm bank that was thought to possess low diversity levels. This study raised the probability to perform the breeding process with higher levels of Ho in cultivated samples rather than wild ones, similar situation observed in the present study, where Ho in a general trend were higher than He. McCallum et al. (2016) carried out the construction of a ligation map for the auto-tetraploid specie, *Vaccinium corymbosum*, through SNPs and SSR markers obtained from a Genotyping by Sequencing (GBS) analysis, a technique that combines DNA fragmentation with restriction enzymes and its further sequencing with high performance tools. This work yielded 207 codominant primer pairs. Obtained SSR primers have made genetic characterizations more efficient by covering larger portions of the genome.

Previous works published by (MARULANDA, et al. 2007; 2012), have characterized *R. glaucus* cultivars transferring SSR markers from other *Rubus* species to *R. glaucus*, with positive polymorphic amplification for some markers and no amplification or monomorphic results for others, similarly to this study. With the use of the new SSR markers polymorphic amplification of the samples was achieved.

(LONGHI, et al. 2014; SALAZAR, et al. 2015) has reported that after the emergence of the Next Generation Sequencing (NGS) techniques, the Rosaceae specie *Fragaria vesca* has received the major SSR marker design derived from analysis using those techniques, with more than 4000 markers reported to the date. Other species including *Malus* spp, *Prunus* spp, *Pyrus* spp, *Rosa* spp. and *Rubus* spp., have also had significant developments (LONGHI, et al. 2014). The massive SSR development derived from high performance sequencing have triggered the use of these type of markers and have diminished costs associated to genetic characterizations at the time that new regions of the genome are covered.

Table 4. Primer sequences for employed SNP markers.

Primers				Homologous sequences in other Rosaceae species	
Sequence identification	***Forward***	***Reverse***	**Genbank accession number**	**Reported accession number in other Rosaceae species**	**Specie**
Unigene11151	TATGTGGGGGTGAAGAAAGC	ACAGGACCCAATCATCCAAC	MH479026	XM_008223370.1	*Prunus mume*
Unigene11157	CCAAGGAAACTTGCTCCAAC	AGCCTTAAACTTGCCAGCAC	MH479027	XM_024322668.1	*Rosa chinensis*
Unigene11255	TGATGGCGCAGATAAGAAGA	AGACTCAACAGCGCCAACTT	MH479028	XM_024331931.1	*Rosa chinensis*
Unigene12343	TGGATCCAGATGAGTCCAGA	CGGACGTTTTCCCAAATCTA	MH479029	XM_021960956.1	*Prunus avium*
Unigene12924	GGACCAATTCCTTGTGTGCT	TGCCGTGACTGTATCCTTGA	MH479030	XM_004287281.2	*Fragaria vesca* subsp. vesca
Unigene13090	GGCTCAGAACTGTGGGGTTA	CACATTGTAGGCATCCCAGA	MH479031	XM_024300962.1	*Rosa chinensis*
Unigene1465	TCGTCTGTTTTGGCTCTTGA	TACTCCCCTTGCTTGAGTCG	MH479032	XM_024340632.1	*Rosa chinensis*
Unigene14681	ATCAGGAATGGGCTGAGCTA	AGCAGCCTTCAAACTCTCCA	MH479033	XM_024324523.1	*Rosa chinensis*
Unigene14822	TACTGGATCGCTCAGCTCCT	TGTGTACACCAACCCGAATG	MH479034	XM_021968382.1	*Prunus avium*
Unigene14951	ATGGCAGTACCCAAATCAGC	TGGGTAATTGATGGTGGTGA	MH479035	XM_008377277.2	*Malus x domestica*
Unigene15095	TTCCTGCTGATGAATGCAGA	GAACCTGTCCTTGGAGCTTG	MH479036	XM_021964662.1	*Prunus avium*
Unigene15115	CCATTCATGGGGTAATTTGC	AAGCTTTCCCAATTGCCTCT	MH479037	XM_011465001.1	*Fragaria vesca* subsp. Vesca
Unigene15294	GCCAGGAGTTTGCTGAGTTC	ATGGGCAAGTAGCTCTCCAA	MH479038	XM_024315181.1	*Rosa chinensis*
Unigene15456	ACAAGCTTCTGGTGGAAGGA	AAGAAGAGCCCCGTCAAACT	MH479039	XM_024302866.1	*Rosa chinensis*
Unigene15499	TGCACCAACAGACCATAAGC	ATAATTCCCACAGGCTGTCC	MH479040	XM_024307824.1	*Rosa chinensis*
Unigene15574	CCTGCTGAGGTGGAATCAGT	CGATCCAACAAACATGCCTA	MH479041	XM_020565943.1	*Prunus persica*
Unigene16239	AGAGGGAGGATCAAGGAGGA	GGGCATTGTATCATGTACGG	MH479042	XM_007221893.2	*Prunus persica*
Unigene16323	AAAGACGGTGGAGAGGAACC	TTTATGTAGGAGGCCGCAAG	MH479043	XM_024317156.1	*Rosa chinensis*
Unigene16368	GGTTGCCAAGATCAAAGAGG	CCGGTGTGCTTAGTTCCTTG	MH479044	XM_024304514.1	*Rosa chinensis*
Unigene16415	GCGGGTGCAGATAAGAAAAG	TTCTTCTTGCGCTCCATAGC	MH479045	XM_024307003.1	*Rosa chinensis*
Unigene16433	AGCAGGAGAGGAAACTCCAA	TGGCATAAAGCTCAAGATGC	MH479046	XM_021957543.1	*Prunus avium*
Unigene16551	CTTGTTTCCCCTTCATCCAA	TTGCAGCATTTCCCTCTCTT	MH479047	XM_024324543.1	*Rosa chinensis*
Unigene2064	AGTACACGGATGCCTTGCTT	GAGGCGCTACAGGGATGTTA			
Unigene2247	GTGTCCGGAGATCAAGCAAC	CTTTGATAGCCTGCCCAATC	MH479048	XM_008234124.2	*Prunus mume*
Unigene2361	AGCAGTTGCTGCACTTTCAA	AACGCCTGTTCACTTTTTGC	MH479049	XM_021957567.1	*Prunus avium*
Unigene2373	TGGCCTCACCAACACTTGTA	GAGTCGCCACAGCGATAGAT	MH479050	XM_021946702.1	*Prunus avium*
Unigene242	GGAGAAGAAGCTGCTGGAGA	TCGCTCTTGACCCTCTCAAT	MH479051	XM_024332998.1	*Rosa chinensis*
Unigene31842	CTGGCCAGAAGAAGGATTGA	TCTCCAAGAAGAATGTTTGAAGG	MH479052	XM_004289533.2	*Fragaria vesca* subsp. Vesca
Unigene33255	TGATGGCTTGAAGCTTTTGA	AGCGCTTGAAACAAATTTCC	MH479053	XM_024315336.1	*Rosa chinensis*

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Unigene34846	GTCAGGACCTCAGTGCTGCT	GGTGGGTGAGTACCAAATATGTC			
Unigene34996	ATCAGTGCTGGGGTGAATG	CTCCCCTGATGCGATCTTAG	MH479054	XM_004295163.2	*Fragaria vesca* subsp. Vesca
Unigene351	TTGCTGATGACACGAATGGT	TTGTTGGCAAATGTCCGATA	MH479055	XM_024307531.1	*Rosa chinensis*
Unigene36231	AAGGGAGATGTGGTGTTGGA	TAAAACCAACACCCCCAAGA			
Unigene3673	GGGCTGCACCTCTTTGTATC	ACATGCTCCCACAAACGAAT			
Unigene37334	GTCCACAAGGCTTCCTTCAG	GATTCTGTTGCCCTTGCACT			
Unigene42870	TAGAGGGCTCGAAGAAGGTG	AGGTCCATCTTGCTGGGTAG	MH479056	XM_018645776.1	*Pyrus x bretschneideri*

Table 5. Genetic variability of SSR markers for *R. glaucus*.

SSR marker	Number of Loci	N	Na	Ne	Ho	He	HWE
CL150.Contig2_All_12_1	2	14,5	5,5	3,6495	1	0,703	ns
CL1916.Contig1_All_167_1	2	15,5	5,5	2,6845	1	0,6275	***
CL1891.Contig3_All_166_1	2	16	6	3,886	1	0,735	ns
CL1101.Contig1_All_110_1	2	16	4,5	2,34	1	0,6975	***
CL1366.Contig2_All_134_1	2	15	6	4,3335	1	0,7245	ns
CL1491.Contig11_All_143_1	2	16	8	4,168	0,7815	0,7575	*
CL2455.Contig1_All_192_1	2	14	4,5	3,829	0,9645	0,7375	*
CL2556.Contig1_All_201_1	2	16	6	3,1315	0,844	0,681	*
CL1004.Contig2_All_94_1	1	15	4	2,542	0,733	0,607	*
CL2322.Contig2_All_181_1	2	16	6,5	3,527	0,5665	0,71	**
CL2455.Contig1_All_193_1	2	15,5	6,5	4,063	0,969	0,7315	*
CL3840.Contig1_All_265_1	2	16	5	2,9505	1	0,658	*
CL2364.Contig3_All_186_1	2	15	4,5	3,426	1	0,697	ns
CL274.Contig3_All_22_1	2	16	4	3,2315	0,7815	0,6525	ns
CL2958.Contig1_All_219_1	1	16	4,5	2,906	0,938	0,638	**
All SSR (Average)		15,448	5,448	3,407	0,911	0,685	
Standard Deviation		0,127	0,283	0,182	0,035	0,015	

[1] **N:** Allele number; **Na:** Polymorphic allele number; **Ne:** Informative allele number; **Ho:** Observed heterozygosity; **He:** Expected heterozygosity; **HWE:** Hardy-Weinberg equilibrium (Detection of significant differences through Chi^2 test); **ns:** Non-significant differences, *P<0.05; **P<0.01, ***P<0.001.

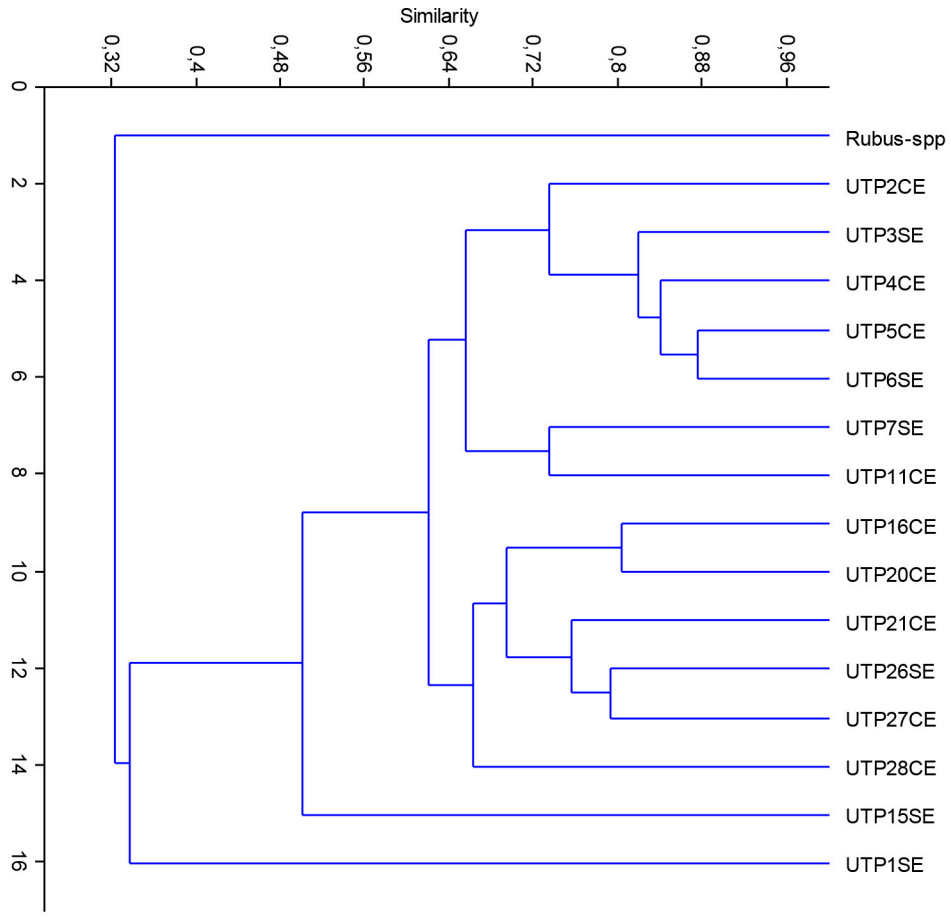

Figure 1. Dendogram obtained from SSR markers employing the Dice Index.

SNP marker analysis - From the 78 evaluated SNP-containing DNA fragments, 36-yielded positive amplification. Obtained amplicon sequences showed high homology with Rosaceae species: *Prunus* spp. (29%); *Fragaria vesca* (23%); *Pyrus* spp. (5%); *Malus* spp. (2%) (Table 4). Other homologies were established with species of other families (9%) and another corresponded to sequences with non-reported homologies in public data bases (32%). Using sequence alignments (Figure 2), a dendogram was constructed with the clustering method of Figure 3. This dendogram showed 4 clusters with any clustering tendency by morphologic features (thorn presence/absence) nor geographical origin. The first group comprises cultivars UTP1, UTP11, UTP6, UTP2; the second consisted of the UTP16, UTP21, UTP4, UTP3 cultivars; the third clustered UTP15, UTP26, UTP5 and UTP27; and the fourth possessed the most distant cultivars, UTP7 and UTP28. Surprisingly, cultivars with desirables features (UTP1, UTP4 y UTP7) were located in different groups, an important annotation to guide the progenitor selection in breeding processes. Both SSR and SNP-derived dendograms allowed the progenitor selection with noticeable differences in their genomes.

Sequence homology result were consistent with other Rosaceae species. It is expected that *R. glaucus* shows high homology within its family to species that have complete or partially sequenced genomes. In addition, is important to state that the development of SNP markers from transcriptome analysis has been widely used in *Prunus* spp. (Rosaceae) to evaluate segregant populations of pears in Europe. The employment of SNP markers in the evaluation of germplasm banks of peaches and the construction of microarrays from transcriptome analysis-derived SNPs in apples, have been also reported. Based in that evidence, the development of such methodologies have improve the selection processes in breeding programs (YAMAMOTO AND TERAKAMI, 2016).

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170502-085_B10_UTP6_SE_Unigene_37334.ab1      TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
170502-085_P08_UTP5_CE_Unigene_37334.ab1      TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
170502-085_L08_UTP3_SE_Unigene_37334.ab1      TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
170502-085_H08_UTP1_SE_Unigene_37334.ab1      TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
170502-085_F10_UTP11_CE_Unigene_37334.ab1     TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
170502-085_D12_UTP28_CE_Unigene_37334.ab1     TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
170502-085_B12_UTP27_CE_Unigene_37334.ab1     TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCA
170502-085_N08_UTP4_CE_Unigene_37334.ab1      TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
170502-085_N10_UTP21_CE_Unigene_37334.ab1     TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
170502-085_L10_UTP20_CE_Unigene_37334.ab1     TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
170502-085_H10_UTP15_SE_Unigene_37334.ab1     TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
170502-085_J10_UTP16_CE_Unigene_37334.ab1     TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
170502-085_D10_UTP7_SE_Unigene_37334.ab1      TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCA
170502-085_J08_UTP2_CE_Unigene_37334.ab1      TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
170502-085_P10_UTP26_SE_Unigene_37334.ab1     TAAAATGTTTGAAGCACTAACGAGTTCTACTCAAGCTTTAGTGCAAGGGCAACAGAATCC
                                              ************************************************************.
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Figure 2. Segment of the sequence alignment with UNIGENE37334 with Clustal Omega software (EMBL–EBI), online version (https://www.ebi.ac.uk/Tools/msa/clustalo/).

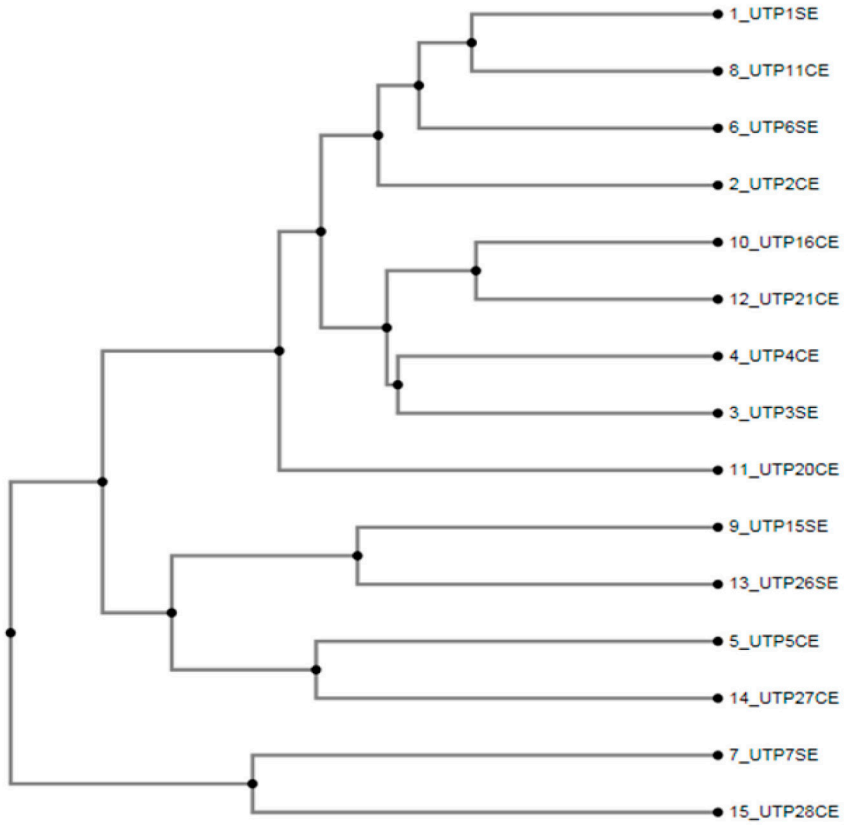

Figure 3. Dendogram obtained from SNP-containing DNA fragment alignments employing the Neighbor Joining clustering method.

Genetic diversity parameters for haplotypical data are presented in Table 6. It was found 1162 SNP-containing fragments, corresponding to 1082 effective SNPs and a polymorphism of 12,49%. In regards to the specific nature of each SNP and SSR marker, biallelic nature of SNP markers makes their discrimination power lower than SSR. In that sense, the greater variability observed in SSR compared to SNPs allows better possibilities in the identification of cultivars and its genetic variability assessment (SÁNCHEZ-PÉREZ, et al. 2006), making them leading markers for genotyping, fine mapping or to increasing QTL resolution.

Table 6. Genetic diversity parameters for haplotypical data obtained with SNP markers in *R. glaucus*.

Parameter	Average value	Standard deviation
Number of SNP-containing regions	1162	0.005
Number of SNP-containing effective regions	1082	0.003
Percentage of polymorphic SNPs	12.49%	------

Progenitor selection - Genetic diversity analysis between previously selected cultivars are used to recommend progenitors susceptible to be used in future breeding processes (Table 7). In order to make those recommendations, the morphologic features related to thorn presence/absence and *C. gloeosporioides* tolerance (very desirable features in new cultivars) were also considered; in that sense, tolerant or moderately tolerant without thorn material was privileged. Respect to cluster analysis, there were selected samples from different clustering groups. Moreover, cultivars UTP5, UTP20 and UTP28 possess, according to producers, fruits with greater size, making them very popular in Colombia, despite its thorn presence and significant susceptibility to *C. gloeosporioides.*

Table 7. Progenitor selection for future breeding schemes.

Selected cultivar	Thorn presence/ absence	Response against *C. gloeosporioides* attack	Cluster number with SNPs	Cluster number with SSR
UTP1	Absence (SE)	Tolerant	1	4
UTP5	Presence (CE)	Very susceptible	3	1
UTP7	Absence (SE)	Moderately tolerant	4	2
UTP11	Absence (SE)	Moderately tolerant	1	2
UTP20	Presence (CE)	Very susceptible	2	3
UTP28	Presence (CE)	Very susceptible	4	3

(HE, et al. 2014), states that plant breeding can be performed through two main strategies, classic and molecular approaches. The classic process employs closely related varieties that could interbreed, whilst the molecular breeding consist in the application of molecular biology and biotechnology approaches to accomplish the development of new cultivars through the Marker-assisted Selection (MAS) and the genetic transformation (MOOSE AND MUMM, 2008). This work employed SSR and SNP markers in the development of a progenitor population aiming to move towards hybridization processes that permit an increase in the genetic gain.

Conclusions

This work evidence the utility of molecular markers to assess the genetic diversity of possible progenitors susceptible to be employed in future breeding processes. This aspect, widely known as the development of a parental population, determines in great manner the success of breeding schemes. The SSR and SNP markers employed in this study, allowed the characterization of such population studying different genome regions. Morphological and fungal tolerance selection criteria, previously evaluated, were also considered in the selection of six progenitors.

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