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RESEARCH ARTICLE

Validation of a minimal panel of microsatellite markers for blueberry cultivar identification and frequency of spontaneous mutations

Hélder Miteca[1], María Herminia Castro[2], Marco Meneses[2], Loreto Prat[3], Carlos Muñoz[3], and Patricio Hinrichsen[2*]

[1]Mozambique Agriculture Research Institute, Nampula, Mozambique.
[2]Instituto de Investigaciones Agropecuarias, INIA La Platina, Santa Rosa 11610, Santiago, Chile.
[3]Universidad de Chile, Facultad de Ciencias Agronómicas, Santa Rosa 11315, Santiago, Chile.
[*]Corresponding author (phinrichsen@inia.cl).
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ABSTRACT

Global blueberry (*Vaccinium* spp.) growing has increased exponentially in the last two decades, fueled by a very dynamic offer of new cultivars. In this scenario, misclassification of genotypes is a matter of concern, despite that good nursery management practices are in place in most countries and new molecular fingerprinting tools have become available elsewhere. In this framework, here we report the use of three highly informative microsatellite (SSR) markers, selected after evaluating the performance of 12 SSRs on 30 commonly planted blueberry cultivars. We present here the validation of this set, comprising markers CA344, CA421 and NA1040 tested on a set of 136 cultivars and lines, all of which can be differentiated by these markers. A dendrogram built with the generated data set grouped the rabbiteye genotypes in a separate clade. The whole polymorphic information content of this set of SSRs was 0.878 and the observed and expected heterozygosity index averaged 0.890 and 0.886, respectively. Additionally, we detected 14 genotypes that showed minor allelic variants, exhibiting mostly single changes in their patterns with respect to the reference cultivar. Overall, the frequency of mutations was 0.6%, considering the 18 allelic variants identified in approximately 3000 analyzed samples. Marker CA421 exhibited the largest frequency of mutations, with 16 out of the 18 variants identified. Plants carrying these variants corresponded to the most planted cultivars introduced to Chile, which have been extensively micropropagated. The implications of these variations for the traceability of plants based on their fingerprinting are discussed.

Key words: Allelic variants, fingerprinting, SSR, polymorphism, *Vaccinium* spp.

INTRODUCTION

Blueberries (*Vaccinium* spp.) were introduced to Chile for experimental purposes by the Instituto de Investigaciones Agropecuarias (INIA) that imported rooted cutting of several Northern Highbush (*Vaccinium corymbosum* L.) cultivars to be planted at three locations, from latitude 36º to 42º S (Muñoz et al., 1989). The first introduction was done in 1979 and included five cultivars. Later on, in 1982 and 1984, 12 additional cultivars were introduced. Rabbiteye blueberries (*V. virgatum* Aiton) were introduced in 1985 and included a collection of 16 cultivars (Muñoz et al., 1989). Starting in 1990, several nurseries offered rooted cutting ready to be planted, including cultivars of the Southern Highbush type released by the University of Florida Breeding Program. According to Retamales et al. (2014) by 1992 an estimated 280 ha were already planted with this species in Chile. It is important to notice that commercial plantings were done mostly based on micropropagated rooted cuttings, and that most of the nurseries established in the country also used micropropagation, at least as starting material.

Today, Chile is the second main exporter of blueberries from the Southern Hemisphere (Ormazábal et al., 2020), due to the good adaptation of the crop to the different edaphic and climatic conditions of the country. This explains the high yields records obtained with some cultivars at specific locations, along with an excellent quality of the harvested fruit (Morales, 2017). One major factor that promoted the rapid and safe expansion of the blueberry industry in Chile, was that most of the cultivars were introduced using in vitro cultured plantlets. This fact guaranteed that most plants were free of pests and diseases present in their country of origin. Additionally, micropropagation enabled the rapid multiplication of the new introduced cultivars.

In recent years, however, a significant increase in off-type blueberry plants has been observed, particularly in both morphological and physiological traits, such as changes in the duration of the harvest period, number of flowers per cluster, color and size of the fruits, among other important traits. In most cases, these variations turned out to be just a misclassification or confusion in the cultivar name, a situation that can be solved using molecular fingerprinting and comparison with reference materials. In other cases, variations were true changes, which could be the result of mutations occurring during the propagation process (Bidabadi and Jain, 2020).

Mutations have been defined as heritable changes in the genetic material of an individual, not derived from genetic segregation or recombination (Lamo et al., 2017). Mutations can be spontaneous or can be induced by physical, chemical, or biotechnological procedures. Spontaneous mutations are rare and occur at random along the genome (Lamo et al., 2017). Some cultivars can present mutated phenotypes that affect a portion of the plant, sometimes spreading to entire branches that acquire a different characteristic; when these branches (shoots or sports) are vegetatively propagated, they can give rise to a new cultivar, often exhibiting only one character (or a few) that differentiate the new cultivar from the original one (Prasanna and Jain, 2017). Spontaneous mutations have been reported in most cultivated plant species, including annual, fruit and ornamental crops (Lamo et al., 2017). The type and frequency of occurrence of spontaneous mutations vary, depending on the species and on the environment where they are grown. Furthermore, mutations can also be induced to augment the genetic variability present in a given species, therefore they have potential for breeding purposes (Ahloowalia, 1998). However, under a productive context, such as in the case of plant propagation, they are undesirable, since they can affect the uniformity of the propagated material especially if traits of agronomic importance or with economic value are affected. This is the case in plantain (*Musa* spp.), where undesirable mutations have been commonly reported affecting the phenotypic stability of the propagules (Lamo et al., 2017).

In this context, the precise, irrefutable identification of true-to-type genotypes (cultivars) appears as critical to provide support to the industry and different molecular tools have been used to abord this problem. In the case of *Vaccinium* species and cultivars, simple sequence repeats (SSR), also known as microsatellites, have been the most relevant fingerprinting tools (Boches et al., 2005; 2006; Bidani et al., 2017; Bassil et al., 2020). For instance, SSRs have been used for different purposes in *Vaccinium*, ranging from the characterization of genetic diversity and population structure in wild species (Bian et al., 2014), to the construction of linkage maps (McCallum et al., 2016), as well as to differentiate species (Bassil et al., 2010), cultivars (Hinrichsen et al., 2009; Bidani et al., 2017; Bassil et al., 2020) and, to some extent, to detect intra-cultivar variants (Martínez et al., 2007). The first SSRs available for *Vaccinium* spp. were based on dinucleotidic units (Boches et al., 2005), which were soon used for fingerprinting (Hinrichsen et al., 2009). More recently, however, following what has been proposed for human forensic studies, five trinucleotidic SSRs were proposed as a standard set for the genetic identity confirmation in blueberry (Bidani et al., 2017). However, the identification of a single case of tie between two different cultivars out of over 150, forced the expansion of this set to 10 trinucleotidic SSRs, which evaluated on a collection of 164 cultivars and accessions performed adequately (Bassil et al., 2020).

At the intra-cultivar level, mutations can theoretically be detected by molecular genetic analyses. In this sense, RAPD markers have been used to characterize off-type genotypes observed in ‘O’Neal’, which presented a banding pattern clearly different from that of the reference sample (Martínez et al., 2007). No other marker types have been used to study spontaneous mutations in blueberries, despite the increase in off-type plants, detected both in nurseries and at the field.

In this work, we present the validation of a minimal and confident panel of SSR marker for blueberry fingerprinting, based on the smallest possible number of markers. Following this idea, successfully developed in other fruit crops (Rojas et al., 2008; Guajardo et al., 2021), we identified a set of three SSR markers that were able to differentiate all tested genotypes. We have validated this new fingerprinting platform on thousands of samples, covering 136 cultivars and lines; among them, there were samples that could correspond to off-type plants (possible mutations), or that could be misclassified material (cultivar confusion). Based on these markers and dataset, we present here (i) a database of genetic patterns of these genotypes that include the most planted cultivars and new releases available in Chile, and (ii) an estimation of the frequency of appearance of allelic variants (mutations) occurring in the SSRs used for fingerprinting. Based on these results, we discuss the strength of using a reduced panel of SSR markers for fingerprinting, in comparison to the larger set of markers recently proposed by Bassil et al. (2020). To our knowledge, this is the first report of SSR allelic variants in blueberries.

MATERIALS AND METHODS

Source of plant material

Leaves and young shoots from 136 blueberry (*Vaccinium* spp.) genotypes collected from orchards and nurseries in Central and Southern Chile were used. These samples were transported under refrigerated conditions to the laboratory, where they were stored at -70 °C until DNA extraction. A minor fraction of the samples come from in vitro-grown plants.

DNA extraction from blueberry leaves

The protocol described for grapevine by Lodhi et al. (1994) adapted to blueberry (Hinrichsen et al., 2009), was used for DNA extraction. Approximately 0.1 g leaf tissue was used. Grinding was done in a mortar and pestle containing the sample and liquid nitrogen. Next, 700 µL cetyltrimethylammonium bromide (CTAB) hot extraction buffer (50 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB and 1% β-mercaptoethanol) was added. The mixture was incubated at 60 ºC for 30 min, followed by two extractions with chloroform:isoamyl alcohol (24:1). Isopropanol was used to precipitate nucleic acids and the pellet obtained was dissolved in distilled water and homogenized for 3 min. This homogenate was incubated at 60 °C for 25 min and cooled to room temperature. Subsequently, 600 µL chloroform:isoamyl alcohol (24:1) was added and mixed gently by immersion. After centrifugation at 8000 g for 15 min, the supernatant was transferred to another tube and its volume was measured (450 µL). Then ½ volume of 5 M NaCl (225 µL) was added. Subsequently, two volumes of absolute ethanol (900 µL) were added, mixed, and left to stand for, at least, 30 min at 4 °C. Later, the mixture was centrifuged at 6000 g for 6 min and the supernatant was discarded. Ethanol 76% (500 µL) was added, centrifuged at 6000 g for 5 min and the supernatant discarded. Finally, the pellet was dried at room temperature on a hot plate. The dried pellet was resuspended in 100 µL sterile distilled water containing RNase (0.1 mg μL^{-1}) and incubated at 37 °C for 15 min in a water bath. The DNA was stored at 4 °C for short-term use or at -20 °C for long-term storage.

Microsatellite amplification by PCR

The reaction mixture (16 µL) contained 3 µL DNA, 1.2 µL 10X PCR buffer, 0.36 µL 50 mM $MgCl_2$, 0.6 µL solution 10 mM dNTPs (2.5 mM each), 5 pmoles μL^{-1} (mix) of each primer (direct and reverse), 0.5 U Taq DNA polymerase and 5.9 µL sterile distilled water. In the present study, 12 markers were initially tested (Table 1) based on the studies of Boches et al. (2006). The PCR amplifications were performed under the following conditions: Initial denaturation at 95 °C for 5 min; 35 cycles of 30 s at 94 °C, annealing temperature specific to each primer pair for 30 s, extension at 72 °C for 30 s, followed by 8 cycles of 30 s at 94 °C, annealing at 56 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 5 min (Boches et al., 2005; Hinrichsen et al., 2009).

Table 1. SSR markers used in our study. T[a]: Annealing temperature. Source: Boches et al. (2005).

Nr	Marker	Sequence (Forward) 5'	Sequence (Reverse) 3'	T[a]	Expected size
				ºC	bp
1	CA344	TTACCAAAACGCCTCTCCAC	GTTTCTTCCTTACGCCCCTGAAAT	60	164
2	CA421	TCAAATTCAAAGCTCAAAATCAA	GTTTAAGGATGATCCCGAAGCTCT	60	201
3	CA794	CGGTTGTCCCACTTCATCTT	GTTTGAAITI'GGCTTCGGATTC	60	227
4	CA855	CGCGTGAAAAACGACCTAAT	GTTTACTCGATCCCTCCACCTG	64	253
5	NA1040	GCAACTCCCAGACTTTCTCC	GTTTAGTCAGCAGGGTGCACAA	60	194
6	NA41	TTCCTTTAGTCGCGTCATCA	GTTTAAGGTCGCTACGAGACTCCA	62	203
7	NA741	GCCGTCGCCTAGTTGTTG	GTTTGATTTTGGGGGTTAAGTTTGC	58	248
8	VCC_I2	AGGCGTTTTTGAGGCTAACA	TAAAAGTTCGGCTCGTTTGC	62	220
9	VCC_J5	CCCCAACGGTCTTGATCTTA	GTTTCCTCTCTCTCCAACCCCAGT	54	275
10	VCC_K4	CCTCCACCCCACTTTCATTA	GCACACAGGTCCAGTTTTTG	62	234
11	VCC_H9	TCCGAGCCATTTAGTGTCAA	GTTTACAAAAACCAAAAGCCATGC	62	211
12	VCC_J9	GCGAAGAACTTCCGTCAAAA	GTGAGGGCACAAAGCTCTC	62	173

Polyacrylamide gel electrophoresis

Sequencing-type electrophoresis gels were prepared, mounted and run as described by Narváez et al. (2001). The polyacrylamide gel was prepared by mixing 65 mL of a 6% acrylamide-urea solution, 180 µL 10% ammonium persulfate and 95 µL TEMED. For polymerization a 0.5X TBE buffer was placed on the supports and electrophoresis was run for 2 h at a power of 90 W, 50 mA, with a constant temperature of 50 °C. Prior to sample loading, the gel was preheated at 50 °C for approximately 5 min. The PCR product was denatured by adding 1 volume of loading buffer (95% formamide, xylene cyanole 1 mg mL^{-1} and EDTA 10 mM pH 8.0), heated for 10 min at 95 °C and immediately placed on ice. Then 2.5 µL each sample was loaded into each well. With the electrophoresis run completed, DNA fragments were stained with silver nitrate following the procedures published by Hinrichsen et al. (2009). The size of the microsatellite alleles was determined by comparison with DNA amplicons of known size loaded in the same gel (obtained from the database of the Instituto de Investigaciones Agropecuarias [INIA], Chile). Finally, the gels were scanned for subsequent reading and analysis.

Annotation of microsatellite alleles (SSRs)

Microsatellite alleles were scored according to the observed sizes and a codominant data matrix was produced, with unknown allelic dosages. The obtained patterns were used to determine the different genotypes and the allele and genotypic frequency were calculated.

Statistical analyses

Following references for polyploid species, the observed heterozygosity (Ho) was calculated for each marker according to Hardy (2016), which is the frequency of heterozygous gametes over the total number of individuals studied. Expected heterozygosity (He) was calculated according to Meirmans et al. (2018) for tetraploid individuals, as He is an estimate of genetic diversity and not heterozygosity per se: $H_e = 1 - \sum_i^k p_i^k$, where pi is frequency of allele i for the locus under study and k is number of the ploidy of the individual under study. The "effective alleles" (A_e) were calculated according to $A_e = \frac{1}{1-H_e}$. Polymorphic information content (PIC), which determines how informative in terms of polymorphism a marker is in the population according to allele frequencies (Botstein et al., 1980), was calculated according to the following formula: $PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 * p_i^2 * p_j^2$, where pi is frequency of allele i for the locus under study and pj is frequency of allele (i + 1).

Considering the polyploid nature of *Vaccinium* genotypes, we also analyzed the SSR-derived polymorphisms using polysat, an ad-hoc R-based software (Clark and Jasieniuk, 2011) already applied to studies on *Vaccinium* spp. (Schlautman et al., 2018; Vega-Polo et al., 2020).

Genetic distances and dendrograms

Euclidean distances and Bruvo distances among individuals were calculated using the stats package (R-Core Team, 2020), and polysat packages (Clark and Jasieniuk, 2011), respectively.

The Euclidean distance matrix, calculated from the microsatellite data, was used to perform a hierarchical clustering, using unweighted pair-group method with arithmetical averages (UPGMA) algorithm from the stats package. Then, the dendrogram was constructed to represent genetic distances among individuals with the help of the R program (R-Core Team, 2020) and the ggtree package (Yu, 2022).

RESULTS

In a preliminary approach using a group of 25 blueberry cultivars, out of 12 SSR markers evaluated just three (CA344, CA421 and NA1040) were sufficient for the identification and differentiation of each one, due to their high discrimination capacity and clear electrophoretic pattern that resulted in an easy scoring (Hinrichsen et al., 2009). In this work we validated the ability of these three SSRs on a total of 136 genotypes, including 93 cultivars, 43 breeding lines and 11 non-identified genotypes (Table 2), considering a total of over 3000 samples obtained from 20 nurseries and commercial orchards distributed throughout the Chilean Central Valley. Currently, these three highly informative SSRs are routinely used for fingerprinting of blueberry cultivars. Table 3 summarizes some genetic descriptors for the three markers evaluated on the 136 genotypes. The total number of alleles per marker (nine for CA344, 31 for CA421 and 30 for NA-1030) was directly related to the discriminating capacity of each one. Concomitantly, the effective alleles determined were 6.29, 11.11 and 10.87 for CA344, CA421 and NA1040, respectively. The allele size for each marker ranged 147-168 for CA344, 166-220 for CA421, and 180-256 for NA1040. The average heterozygosity values, Ho and He, were 0.890 and 0.886, respectively. The Ho, in increasing order, was 0.849 for CA344, 0.898 for NA1040 and 0.924 for CA421. On the other hand, the He was 0.841 for CA344, 0.908 for NA1040 and 0.910 for CA421. Polymorphic information content, on the other hand, was rather high among these markers, ranging from 0.822 to 0.906, with an average of 0.878 (Table 3). Overall, these results illustrate the high discriminatory power of these markers, as well as the high genetic diversity of this polyploid species evidenced by the large number of alleles observed among the genotypes analyzed.

Differentiation ability of the SSR markers

The effectiveness of a marker is defined here as its ability to differentiate a genotype from any other tested. In our case, the ability of each SSR to differentiate each of the 136 genotypes under evaluation was quite different (Table 4): Marker CA421 led this score because it was able to differentiate 105 genotypes, closely followed by NA1040, that differentiated 98 genotypes, and then quite below, marker CA344, that only differentiated 25 genotypes. This is consistent with the heterozygosity of the species and the PIC values of each marker (Table 3). By the opposite, marker CA344 exhibited the largest number of groups of non-differentiated genotypes, including four groups with 6 to 9 genotypes each. Marker NA1040 showed no groups of non-differentiated genotypes with more than three members, which is very promising for use in combination with other markers to define alternative fingerprinting schemes. The combination of two of any of these SSR markers was able to differentiate all genotypes analyzed, making the identification of blueberry genotypes a highly assertive process. In addition, the electrophoretic patterns of each one of these three SSRs is almost free of stuttering, making the score of the patterns very easy and reproducible for any genotype-marker pair analyzed.

Genetic diversity of blueberry germplasm revealed by the SSR markers

The dendrogram obtained from the dissimilarity matrix of the three SSR markers data for the 136 cultivars and lines by the UPGMA clustering method (Figure 1), allowed the differentiation of all these genotypes. In addition to showing a series of clades, the most striking result was the inclusion of the complete set of cultivars of the rabbiteye blueberry (REB) species considered in this work (n = 16; labeled with a blue dot) in the most distant clade. This clade was in turn separated into three branches, and included only a single genotype of unknown type or species (NN-15, purple dot), most probably a REB genotype. The largest number of varieties and lines, belonging to Southern (SHB) and Northern Highbush (NHB) types, were distributed in several additional less-evident clades, not exhibiting a clear structuring between them, except for small groups of one or the other type (Figure 1).

Table 2. Genotypes studied and allelic patterns for each SSR marker considered in this study. *Genotypes harboring an SSR allelic variant (mutation) for one or more analyzed samples. SHB: Southern Highbush blueberry; REB: rabbiteye blueberry; NHB: Northern Highbush blueberry.

Nr	Cultivar or Selection	CA344	CA421	NA1040	Cultivar type
1	Abundance	156-159-162	170-186-192-222	190-192-216	SHB
2	Alapaha	150-153-156-159-162	168-170-178-182-192	180-194-196-208-220	REB
3	Aliceblue	153-162	168-172-174-182-188-192	180-196	REB
4	Apolo	147-159-162-165	166-174-180-186	182-190-192	SHB
5	Aurora	156-159-168	170-180-182	190-192-256	NHB
6	Beckyblue	153-156-162	168-172-188-190-212	180-184-190-206-220	REB
7	Bella	153-156	170-182-190-192	182-192-200-216	SHB
8	Berkeley	156-159-162-165	186-190-192-198	186-192-208-216	NHB
9	Biloxi	147-153-159-168	170-182-192-224	192-216	SHB
10	Bliss	147-156-159-162	166-168-222	184-192-196	SHB
11	Bluecrisp	147-156-162	186-192-198-224	186-192-206	SHB
12	Bluebelle	147-162	170-182-186-188-190	184-194-208-214-240	REB
13	Bluechip	153-156-162	166-182-190-192	184-192-200-210	NHB
14	Bluecrop*	153-156-162-168	166-170-182-198	184-192-210-216	NHB
15	Bluehaven	153-156-162-165	180-182-190-198	188-190-192-216	NHB
16	Bluejay	162-165-174	186-192-198	184-196-208-216	NHB
17	Blueray	153-156	166-170-180-198	184-188-216-256	NHB
18	Bluetta	156-162-165	166-180-184-222	184-186-192	NHB
19	Bobolink	147-156-162	172-186-192-222	186-188-218-228	SHB
20	Bonita*	153-156-165	166-168-172-188-190-198	180-184-190-204	REB
21	Brigitta*	153-156-165-168	166-170-180-198	188-190-192	NHB
22	Brigthwell*	156-159	170-180-(190)-192	180-196-208-220	REB
23	Camellia	147-156-165-168	166-186-192	182-192	SHB
24	Cape Fear	156-159-162	166-170-182-198	182-190-192-210	SHB
25	Centurion	147-150-153-159-162	168-170-174-178-182	180-194-220-240-252	REB
26	Choice	147-156-162	182-190-192-198-202	180-182-204-208-216	REB
27	Climax	162-165	168-170-180-190-198	184-204-220-240	REB
28	Clockwork	156-162	166-170-182-206	182-184-192	NHB
29	Collins	153-156-165	166-190-192-210	188-192-200-208	NHB
30	Corona*	147-159-162	168-186-192-198	182-188-192-216	SHB
31	Coville	153-156-165-168	166-182-190-198	184-192-216-256	NHB
32	Darrow	156-162	176-190-192-198	190-192-196	NHB
33	Daybreak	147-153-162	182-190-224	190-192-224-232	SHB
34	Draper*	153-156-165	166-186-198-206	184-188-196	NHB
35	Duke*	156-162-165	166-186	184-186-190-192	NHB
36	EarliBlue	156-162-165	166-192-198-222	184-188-192	NHB
37	Elliott*	153-156-159-162	182-190	188-192-256	NHB
38	Emerald	153-156-159-168	170-186	182-184-192-210	SHB
39	Farthing*	147-156-159-168	162-186-192-198	182-188-192	SHB
40	Georgia Gem	156-159-162	170-192-198	182-184-188-210	SHB
41	Herbert	153-156-159-165	166-182-186-190	184-192-216	NHB
42	Jersey	156-162-168	176-182-186-198	192-196-216	NHB
43	Jewel	147-159-165	168-172-186-222	182-192	SHB
44	Julia	156-159-168	182-192-198	192-200-216	SHB
45	Julieta*	147-156-165	186-190-192-198	192-210	SHB
46	Keepsake	147-162-165	166-198-224	210-216	NHB
47	Kestrel	156-165-168	190-192-198	188-206-216-228	SHB
48	Last Call	156-159-162	170-182-190	188-192-216	NHB
49	Legacy*	156-159-162-168	168-182-190-192	182-190-196-210	SHB
50	Liberty	153-156-159-165	170-182-198	188-190-192	NHB
51	Magnifica	156-159-168	190-192-196-198	190-192-216	SHB
52	Marimba	153-156-162	186-198-222	186-192-206-216	SHB
53	Meadowlark	147-156-168	180-182-186-198	182-200-216	SHB
54	Millennia	153-156-168	170-186	184-200-210-216	SHB
55	Misty	153-156-162-165	186-196-198-222	186-208-216-234	SHB
56	Nelson	153-156-162	180-182-190-198	184-186-192-216	NHB
57	O'Neal	156-159-162	166-198-222	188-190-192-228	SHB
58	Ochlockonee	147-153-156-165	170-180-182-188-192	180-182-196-208-220	REB
59	Osorno	156-162-165-168	182-186-190-206	184-190	NHB
60	Ozarkblue	159-162-168	166-170-182-192	192-210-216-256	SHB
61	Palmetto	159-162-165	166-180-192-220	182-192-216	SHB
62	Patriot	156-162	166-182-192-198	184-190-192	NHB
63	Powderblue	147-156	180-182-190-192-212	180-196-208	REB
64	Premier	147-156-162	168-184-188-198-212	180-182-194-208-220	REB
65	Presto	147-153-159-162	166-198-222	190-206-210-228	SHB
66	Primadonna	159-162	166-172-198-204	192-206-228	SHB
67	Primoblue	147-153-156-159	166-168-186-198	182-184-192-196	SHB
68	Raven	147-156-159-168	196-198-222	190-208-216-228	SHB
69	Reveille	156-168	184-192-198-222	184-192-206-228	NHB

70	Rocío*	159-168	168-192-198-224	188-190-192-194	SHB
71	Santa Fe	153-156-159-168	170-172-186-192	188-192-206-216	SHB
72	Sapphire	147-162	186-190-192-224	190-192-208-216	SHB
73	Scintilla	153-159-168	162-186-198	192-228-234	SHB
74	Sensation	156-159	180-182	186-188-192	SHB
75	Sierra*	156-162-165	166-170-190-194	184-188-192-256	NHB
76	Snow Chaser	147-153-159-165	166-198	206-210-216-228	SHB
77	Southland	147-153-156	174-182-186-188-192-198	180-184-196-240	REB
78	Southmoon	156-159-168	186-192	182-192-216-236	SHB
79	Spartan	156-162-168	166-180-198	184-192-216	NHB
80	Springhigh	153-156-168	182-186-192	182-190-192-234	SHB
81	Star	153-156-159-162	166-190-196-198	186-190-228-256	SHB
82	Stella	159-162-165	168-186-218-224	192-216	SHB
83	Sunrise	156-159-162	178-180-184-192	188-192-202	NHB
84	Suzieblue	153-159-165	166-190-192-210	182-184-190-228	SHB
85	Temptation	147-156-159-165	172-186-206-224	188-192-196	SHB
86	Tifblue	147-153-156-165	182-190-192-198-216	180-182-196-208-214	REB
87	Toro	156-159-162-168	166-186-198	184-192-216	NHB
88	Ventura	156-159-168	186-190-192-198	188-190-192	SHB
89	Vernon	147-150-159-162	168-174-184-186-192-198	180-190-198-208-220-232	REB
90	Victoria	153-162-168	180-186-224	182-210-216	SHB
91	Windsor	147-156-159	192-198-222	188-190-192-228	SHB
92	Woodard	147-162	168-174-182-186-192-198	180-204-216	REB
93	Zilla	156-159-162	166-190-192-198	188-210-228-256	SHB
94	62-3	159-162-168	172-190-192	192-206	
95	748	159-162-168	190-192-196-198-204	192-228	
96	751	156-159	166-172-186-222	182-184-192-216	
97	752	147-156-159	182-198-224	192-216-234	
98	753	153-159-162	166-168-198-220	186-192-228	
99	754	159-162-168	190-192-196-198-204	192-206-228	
100	755	147-153-156-159	170-182-222	182-188-192-228	
101	759	156-159-162	186-192-198-222	190-192-216-228	
102	XN-1	156	166-186-198-206	184-190-192-196	NHB
103	XN-2	147-156-159-162	166-186-198	186-190-208	SHB
104	XN-3	162-165-168	166-186-198	182-190-192-214	NHB
105	XN-4	156-162-165	166-186-198	182-192-216	NHB
106	CV 8-10	147-159-168	170-184-192-198	184-192-228	SHB
107	CV 8-11	153-156-162	170-186-198-210	182-188	SHB
108	CV 8-16	162-168	170-190-198	192-212	SHB
109	CV 8-22	156-159-162-168	166-170-182-186	182-192-226	SHB
110	CV 8-35	153-156-162-165	166-180-182	182-206-212-216	SHB
111	CV 8-45	147-153-159-162	166-182-186-192	188-192-216-228	SHB
112	NN-1	147-153-156-162	170-186-198-224	182-192-212-228	
113	NN-2	147-153-156	170-186-198	182-184-190-192	
114	NN-3	147-156	186-222	184-192-228	
115	NN-4	147-159-162	166-170-186-224	192-206-212-228	
116	NN-5	153-156-159-168	170-172-204-224	182-192-228	
117	NN-6	147-159-162	166-192-198	188-192-216	
118	NN-7	147-153-156-168	186-198	184-188-190-192	
119	NN-8	156-159-162-165	166-170-190	182-210	
120	NN-9	147-156-168	172-224	192-206	
121	NN-10	156-159-168	168-170-224	188-192-228	
122	NN-11	153-159-162	166-170-186-198	192-212-228	
123	NN-12	147-159-165	166-182-186-192	182-184-192-214	
124	NN-13	147-156	186-198	184-188-192	
125	NN-14	153-156-168	186-190-192-198	188-196-210	
126	NN-15	147-153-159-162	170-174-178-182-186-188	180-184-196-220-240	
127	NN-16	153-156-162-168	166-170-182-198-202	184-192-210-216	
128	NN-17	153-156-162	182-190-198	186-192-210-216	
129	NN-18	153-156-162	182-190-194-198	186-192-210-216	
130	NN-19	147-156-165-168	170-180-192-222	182-192-210-216	
131	NN-20	153-156	166-186-198	182-188-192	
132	NN-21*	147-156-168	166-170-192-198	184-190-192-210	
133	NN-22	156-159-162	168-178-182-186	180-194-216	
134	NN-23	147-156-165	166-190-192-222	186-192-228	
135	VCDC-001	153-162-168	166-170-186-192	182-190-192-216	
136	VCCB-002	156-162-165	166-170-190-192	182-210	

Table 3. Diversity figures for the SSR markers used in blueberry fingerprinting. A: Absolute alleles per marker; Ae: effective alleles; ASR: allele size range; Ho: observed heterozygosity; He: expected heterozygosity; PIC: polymorphic information content.

Marker	A	Ae	ASR (bp)	Ho	He	PIC
CA344	9	6.29	147-168	0.849	0.841	0.822
CA421	31	11.11	166-220	0.924	0.910	0.906
NA1040	30	10.87	180-256	0.898	0.908	0.905
Average			-	0.890	0.886	0.878

Table 4. Number of individualized genotypes and non-differentiated groups for each SSR marker. *The column headed as "≥ 5x" includes groups of five or more non-differentiated genotypes with the corresponding SSR; for example, CA344 has four groups in this category: Two of six non-differentiated genotypes, plus one of seven and one of nine. Bolded numbers correspond to the total number of genotypes per category.

	Individualized genotypes	Groups of non-differentiated genotypes for each SSR			
		Pairs	Trios	Tetrads (4x)	≥ 5x*
CA344	25	18	13	2	4 (6x*2 + 7x + 9x)
Genotypes per group	**25**	**36**	**39**	**8**	**28**
CA421	105	11	0	1	1 (5x)
Genotypes per group	**105**	**22**	**0**	**4**	**5**
NA1040	98	16	2	0	0
Genotypes per group	**98**	**32**	**6**	**0**	**0**

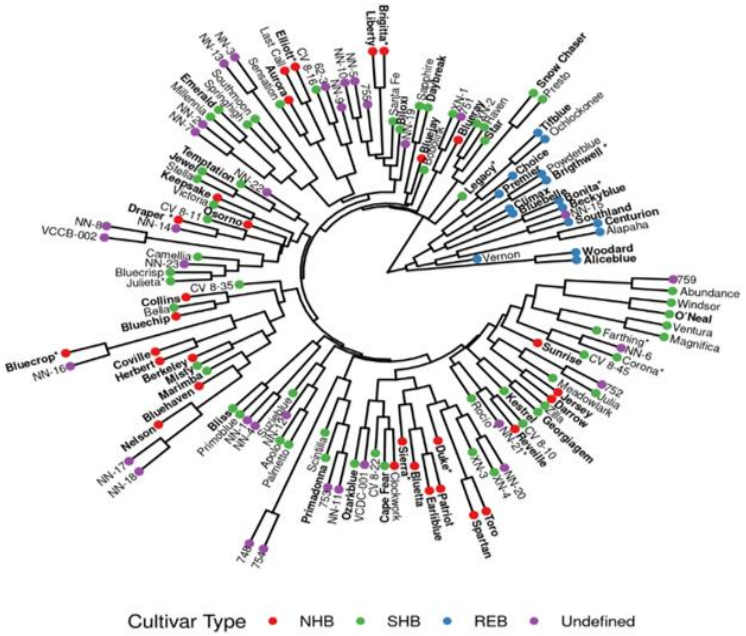

Figure 1. Dendrogram generated by UPGMA cluster analysis from the similarity matrix obtained by Euclidean distance of SSR polymorphisms for the 136 genotypes analyzed. Key for botanical types (dot colors): Red for Northern Highbush blueberry (NHB), green for Southern Highbush blueberry (SHB), blue for rabbiteye blueberry (REB), purple for non-determined blueberry species or types.

The germplasm considered in this study presented different ploidy levels among individuals, as could be expected when considering REB varieties (*V. virgatum*, 6X) and NHB or SHB varieties (*V. corymbosum*, 4X) (Song and Hancock, 2011). The analysis of the data using polysat, a software specially designed to study polyploids, rendered very similar results, including the separation of all the REB varieties in a separate, more distant clade (data available upon request from the corresponding author).

Allelic variants

In 14 out of the 136 analyzed genotypes we found individual plants exhibiting minor variations in their allelic pattern. Three possibilities exist to explain this situation: Lost (null allele; n = 7) or gain (n = 5) of an allele, and size change (n = 7) of a particular allele. In two cases, 'Brigitta' and 'Duke', we found three variants in each. In total, we observed 18 variants (Table 5). In general, only one allelic change was identified per plant, the exception being a sample of 'Duke' that harbored two mutations at loci CA344 (lost of allele "165") and NA1040 (change 188:190) (Table 5). Most of the variants were detected with the marker CA421 (15 out of 18); NA1040 showed two cases and CA344 just one. If we consider that these variants were found after analyzing approximately 3000 samples, the percentage of mutated plants was *ca*. 0.6%. This value is relevant if we consider that only three markers were evaluated in the whole population. As an example, Figure 2 shows the banding patterns for 20 samples analyzed with marker CA421. Of these samples, 11 were labeled as 'Duke'; however, the second allele of the sample loaded on line 13 showed the presence of an additional allele of 182 bp, representing an extra band respect of the standard pattern for this cultivar/SSR (Table 5). This new band could correspond, for instance, to a deletion of two dinucleotidic units in the fourth allele, presumably the allele "186". No one of the samples exhibiting SSR allelic variants presented off-type phenotypes respect to the corresponding variety.

Table 5. SSR allelic variants detected in some blueberry genotypes. Alleles that would have changed molecular size are indicated in red; "Null" is a lost band compared to the original pattern of the indicated genotype.

	Original pattern			Allelic variations		
Genotype	CA344	CA421	NA1040	CA344	CA421	NA1040
Bluecrop	153-156-162-168	166-170-182-198	184-192-210-216	153-156-162-168	Null-170-182-198	184-192-210-216
Bonita	156-159-162	180-186-192-198	188-200-216-228	156-159-162	170-180-186-192-198	188-200-216-228
Brigitta	153-156-165-168	166-170-180-198	188-190-192-256	153-156-165-168	166-170-180-198	188-190-192-Null
Brigitta	153-156-165-168	166-170-180-198	188-190-192-256	153-156-165-168	166-170-180-200	188-190-192-256
Brigitta	153-156-165-168	166-170-180-198	188-190-192-256	153-156-165-168	166-170-180-194	188-190-192-256
Brigthwell	156-159	170-180-190-192	180-196-208-220	156-159	170-180-Null-192	180-196-208-220
Corona	147-159-162	168-186-192-198	182-188-192-216	147-159-162	168-186-192-Null	182-188-192-216
Draper	153-156-165	166-186-198-208	184-188-196	153-156-165	166-186-198-206	184-188-196
Duke	156-162-165	166-186-198	184-186-188-192	156-162-Null	166-186-198	184-186-190-192
Duke	156-162-165	166-186-198	184-186-188-192	156-162-165	166-186-Null	184-186-188-192
Duke	156-162-165	166-186-198	184-186-188-192	156-162-165	166-182-186-198	184-186-188-192
Elliot	153-156-159-162	182-190	188-192-256	153-156-159-162	182-190-192	188-192-256
Farthing	147-156-159-168	162-186-192-200	182-188-192	147-156-159-168	162-186-192-198	182-188-192
Julieta	147-156-165	186-190-192-198	192-210	147-156-165	186-190-192-Null	192-210
Legacy	156-159-162-168	170-182-190-192	182-190-196-210	156-159-162-168	168-182-190-192	182-190-196-210
NN-21	153-156-162	182-190-198	186-192-210-216	153-156-162	182-190-194-198	186-192-210-216
Rocío	159-168	168-192-198-224	188-190-192-194	159-168	168-192-198-214	188-190-192-194
Sierra	156-162-165	166-170-190	184-188-192-256	156-162-165	166-170-190-194	184-188-192-256

DISCUSSION

The use of SSRs for genetic diversity, population structure (Bian et el., 2014) and comparative genetic mapping in a polyploid as *Vaccinium* spp. (Schlautman et al., 2018) has been shown as very assertive using markers from the same species, or based on their cross-transferability demonstrated in wild species of this and other genera (Tomczyk et al., 2020). In this study, we have scored the data as presence/absence of the alleles for each marker, assuming the eventual loss of allelic frequency, following the approach known as "allelic phenotyping" (Urrestarazu et al., 2018). Based on this approach, the fingerprinting of different fruit crops using SSR markers, including blueberries and other polyploid species, has been recently revised (Testolin et al., 2023).

Out of the 12 SSR markers initially tested to evaluate the genetic diversity of the species (Table 2), three were chosen (CA344, CA421 and NA1040) due to their high capacity to discriminate between genotypes, which has allowed the correct differentiation and identification of more than 3000 samples of cultivars and breeding lines analyzed at our laboratory. These markers, originally developed from expressed sequence tag (EST) libraries (Boches et al., 2005), were synthesized from *Vaccinium corymbosum* 'Bluecrop' using floral buds, either cold-acclimated (CA) or not (NA). To our knowledge, the identification of the *loci* to which the primers are associated with, is not straightforward since *Vaccinium* species are polyploid. *Vaccinium corymbosum* is tetraploid and *V. virgatum* is hexaploid. *Vaccinium darrowii*, instead, is diploid ($2n = 2x = 24$) and it is the most important source of low chill requirement for the hybrids with *V. corymbosum*, the so called Southern highbush blueberries. As high collinearity exists between these two genomes (Yu et al., 2021), the blast of the primer sequences on the available genome for *V. darrowii* suggest that CA344 is located on chromosome (chr) 3, NA1040 is located on chr 6 and CA421 is located on chr 9, e.g., well distributed in the blueberry genome, but up to now not associated to any trait. Therefore, this set of markers serve to assign a particular allele combination for each one of the 136 genotypes considered. The allelic patterns found here (Table 2) showed full agreement when compared with one of the most comprehensive databases for the species, available online at the USDA-ARS genetic repository in Corvallis, Oregon, USA (Global-GRIN database at https://www.ars.usda.gov/pacific-west-area/corvallis-or/national-clonal-germplasm-repository/). In this study, a total of 1581 amplicons from the 136 genotypes evaluated were obtained (Table 3), which is the sum of 485, 559 and 537 amplicons identified with markers CA344, CA421 and NA1040, respectively. Likewise, the number of alleles detected for CA344, CA421 and NA1040 were 9, 31 and 30, respectively, with an average of 23.3 and with an overall size ranging from 147 to 256 bp (Table 4). The polymorphic index of these SSRs, represented by the average number of alleles per marker, exceeds largely the value observed recently by Bassil et al. (2020) using a different set of SSRs, which averaged 9.0 alleles per marker, and contrasting to a previous study of the same group (Boches et al., 2005), where the average number of alleles was like the value presented here. The average PIC value of these markers (0.878) was quite high and similar to the value presented in previous studies that had considered a much smaller number of genotypes (n = 25; Hinrichsen et al., 2009). Moreover, the discriminating capacity of the markers proposed here for fingerprinting of blueberries was such that even using two of them it was still possible to differentiate the total set of cultivars and breeding lines analyzed. Also, the average values for Ho and He obtained in this study (0.890 and 0.886, respectively) were slightly higher than those reported by Bassil et al. (2020). This heterozygosity is linked to the high genetic diversity present in *Vaccinium* spp., which can be associated to the polyploid nature of these species and by the fact that interspecific hybridization has been frequently used in breeding modern cultivars (Muñoz, 1989; Morales, 2017). This can also be deduced from the dendrogram based on the present data set, where the only well separated clade was the one conformed mostly by cultivars of the rabbiteye (*V. virgatum* Aiton) species (Figure 1). This in part could result from the low number of markers used in the study (n = 3), which, nevertheless, did not interfere with the fingerprinting application, main objective of this work.

Most of the genotypes considered in this study have complex genetic backgrounds (Brevis et al., 2008), composed of several *Vaccinium* species, such as *V. corymbosum* L., *V. angustifolium* Aiton, *V. darrowii* Camp and *V. virgatum* Aiton. Thus, about 75% of the current blueberry cultivars and lines are derived from crosses of *V. corymbosum* and *V. angustifolium*, e.g., 'Bluecrop', 'Jersey', 'Weymouth', 'Blueray' and

'Berkeley' (Mainland and Coville, 2014). Other examples are the hybrids 'Legacy', which is 25% *V. darrowii* and 2% *V. angustifolium*, and 'Sierra', with 20% *V. darrowii*, 15% *V. virgatum*, 13% *V. constablei* A. Gray and 2% *V. angustifolium* in its genome (Hancock et al., 2008). The complexity of the blueberry pedigrees, including a combination of SHB and NHB cultivars, could in part explain the lack of groupings among the *Vaccinium* cultivars studied (Hancock, 2006).

Studies on the detection and analysis of spontaneous mutations in blueberries are scarce. In our case, we found intra-cultivar allelic variations in 14 genotypes (*ca*. 10%), with minor variants that ranged from 2 to 10 nucleotides, in relation to the standard cultivar. Considering that these SSRs are dinucleotidic, their mutations could be associated with the disruption of a coding sequence (Predieri, 2001), if the repetitive sequence are located in a coding sequence. These variations, which totaled 18 events (including one double mutation), can be classified in two types: (i) Change of the size of a particular band or allele (37% of the variants observed) and (ii) lost (37%) or gaining of a band (26%) with respect to the standard pattern of the genotype. Changes in type (i) case are the simplest to identify, since they are easily readable by electrophoresis, so they are the most valuable and trustable from an analytical perspective. Changes in type (ii) may correspond to the disappearance of a band (null allele) derived from a point mutation and consequent loss of the primer binding site(s), or the loss of a segment of the chromatid that contained the complete or part of the amplified fragment and, therefore, no amplification was possible, and no amplicon was obtained. Another case of "disappearance" of a band occurs when a change is produced in the amplified fragment (in the repetitive units or not), which produce a new band that coincidentally has the same size of another amplicon already existing. Alternatively, this latter change could originate a totally new amplicon of different size, and so it generates a new, modified allelic pattern for the cultivar. An example of these allelic pattern changes is shown in Figure 2 for SSR CA421.

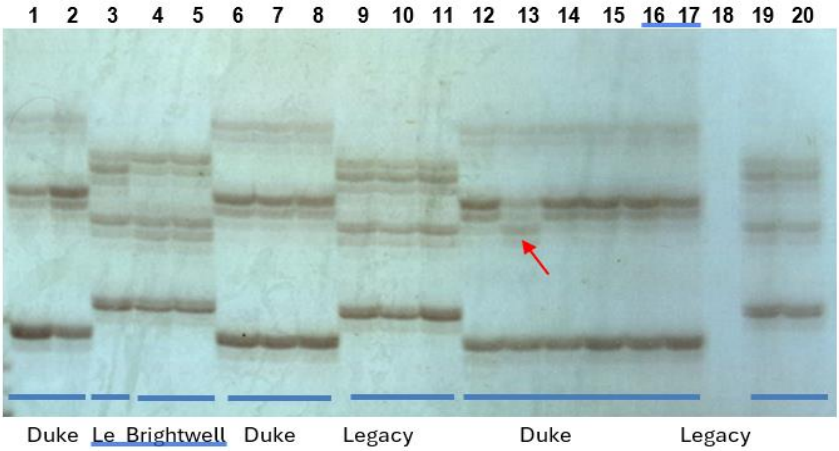

Figure 2. Polyacrylamide gel electrophoresis of 19 samples of *Vaccinium corymbosum* analyzed with SSR marker CA421. Lanes correspond to the indicated cultivar ("Le" is for 'Legacy'). Lane 18 was not loaded (blank). Red arrow shows the allele from a 'Duke' sample that had a difference (size variant) respect of the standard pattern (182 bp *vs*. 186 bp; compare to other 'Duke' samples).

Among the SSRs included in this study, CA421 stands out since it detected most of the intra-cultivar variations (mutations), including the case of 'Bonita', where a supernumerary allele, i.e., a fifth allele, could correspond to an artifact of the technique (e.g., a band that appears as an amplified fragment, but which may be a PCR "stuttering"). Another possible explanation for this supernumerary allele is the presence of a histological chimera, which has been described in other vegetatively propagated woody species, such as grapevine. In this case, L1 and L2 cell layers coexists and each one contributes with different alleles because they carry independent mutations (Moncada et al., 2006), resulting in a higher number of alleles than the

ones expected according to the ploidy of the species. In the case of *V. corymbosum* and its tetraploid relative species, this means over four alleles, as in the case of 'Bonita'. Whatever the cause of the large number of variants detected with CA421, it is comparatively more unstable that the other two SSRs assayed (CA344 and NA1040). This difference is not associated with the number of alleles, because NA1040 had 30 alleles but just two allelic variants. This variability is undesirable for a marker used for fingerprinting, where reproducibility is highly valued. But it could become valuable if these allelic variations were associated to phenotypic changes, which was not the case. There was no evidence of any relationship between these allelic variants and the phenotypic variations occasionally found in relevant agronomic traits such as yield, fruit quality, harvest time and plant architecture. In addition, variability could become useful when closely related genotypes should be differentiated, as is the case in clones or lines essentially derived, obtained spontaneously or by artificially induced methods. Since the frequency of allelic variants detected was quite low, marker CA421 can still be considered useful, because it is informative and trustable at the same time.

The mutations at the microsatellite loci detected here could reflect more massive changes occurring at a large scale within the genome of the propagated materials. The occurrence of such mutations can be associated with the length of time a single explant is propagated in vitro. Some cultivars have been micropropagated for almost 40 yr (Lamo et al., 2017), where many opportunities may have occurred for the accumulation of genomic variations that, at some point, could affect traits related to phenology, yield or fruit quality. Explanations for the increase in somaclonal variations during *in vitro* culture are diverse, and include the wounds induced in tissues during plantlet manipulations, the chemicals used for the surface sterilization of explants, the imbalance of certain components of the culture media like phytohormones, the source of carbohydrates, and the stressing environmental conditions during plantlet incubation, such as the light and humidity (Bidabadi and Jain, 2020). Different culture conditions may have various effects at the molecular level: They can result in the generation of point mutations at the DNA level; changes in the number of copies of DNA segments; chromosomal rearrangements and recombinations; and the jumping of transposable elements (Jelenić et al., 2001). Also, micropropagated plants may exhibit other types of heritable changes in gene expression and function that cannot be explained only by changes in the DNA sequence, such as epigenetic variations, which include histone modification in the chromosomes through acetylation and methylation processes or through micro and small RNAs interference (Richards, 2006).

Our results are equivalent to those presented by Martínez et al. (2007), who studied the genetic patterns in different *Vaccinium* spp. using random amplified polymorphic DNA (RAPD) markers. They found that these markers detect the existence of off-type plants, although they did not confirm whether the appearance of these polymorphisms would be associated with spontaneous mutations. In addition, blueberry mutant cultivars have been described, such as the ornamental pink-fruited 'Pink Lemonade' that exhibit a mutation in its anthocyanin pathway (Die et al., 2020), as well as the wild bog bilberry (*V. uliginosum* L.) that produce white berries (Primetta et al., 2015). On the other hand, this study can be compared to that conducted by Aranzana et al. (2003), who working with microsatellite markers in peach (*Prunus persica* (L.) Batsch) obtained the mutant genotype that presented new alleles with ± 2 bp in three cases and loss of alleles in two other cases, presumably null allele mutants. A similar case of mutations due to allele size changes and presence of supernumerary alleles has been described in grapevines (Moncada et al., 2006; Pelsy, 2010). Also, Martins-Lopes et al. (2001), working with single strand conformation polymorphism (SSCP), detected allelic variations of the single nucleotide polymorphism (SNP) type in wheat, another polyploid species. Furthermore, the present study coincides with that described by Gisbert et al. (2016), that working with SSR markers were able to identify spontaneous mutations in 'Black Beauty' eggplant (*Solanum melongena* L.)

The results presented here are pioneering in the study of mutations in blueberry based on co-dominant SSR markers. Further research is needed to accurately characterize each allelic variant, by sequencing the amplified fragments of variable size to confirm the mutations detected and determine the changes at the nucleotidic level. This would be expected to confirm an increase or reduction in the number of repeats of the microsatellites exhibiting variants.

All this information would be the basis for the correct identification of the different blueberry genotypes, both for nurserymen and growers, but also to rule out any suspicion of mutations, thus guaranteeing the uniformity, productivity, and genetic quality of the propagated materials. On the other hand, this information

based on SSR analysis can be incorporated into a quality control scheme of a nursery to support both the cultivar identity as well as the homogeneity of the planting material.

Finally, the mutations identified in the present study, as well as others that could be identified in the future, could become useful for blueberry breeding, because even when mutations are intrinsically undesirable, they are a source of genetic variation that can also be beneficial in the development of new cultivars.

CONCLUSIONS

Three microsatellite markers evaluated on 136 blueberry genotypes showed a high level of polymorphism and the ability to differentiate each one in a perfectly reproducible way. Rabbiteye cultivars formed a separate clade respect of the other two blueberry types considered in the study, Southern Highbush and Northern Highbush, which did not form separate clades. In addition, allelic variants or intracultivar mutations were detected in 18 cases (14 genotypes) for these informative markers, demonstrating the presence of mutations in 0.6% of the samples obtained from nurseries and orchards of Central Chile. These mutations would correspond to insertions or deletions of SSR units, with variants ranging from ± 2 to 10 nucleotides, plus six genotypes also showing null alleles. By far, the marker with the largest number of variants detected was CA421.

Author contribution

Conceptualization: P.H., C.M. Methodology: M.H.C. Software: M.M. Validation: M.H.C. Formal analysis: H.M., M.M. Resources: P.H. Writing-original draft: H.M., M.M. Writing-review & editing: P.H., C.M., M.L.P. All co-authors reviewed the final version and approved the manuscript before submission.

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