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SHORT COMMUNICATION

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Identification of cultivated and wild *Vaccinium* species grown in Portugal

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Abstract

Vaccinium crops offer a variety of benefits for human health due their high levels of antioxidants. Genetic diversity between two *Vaccinium* species (sixteen cultivars of *Vaccinium corymbosum* and three wild populations of *Vaccinium myrtillus*) were evaluated using Inter Simple Sequence Repeat (ISSR) markers. In *V. corymbosum* 74 polymorphic markers corresponding to 83.2% of polymorphism were obtained while in *V. myrtillus* only four polymorphic markers corresponding to 83.2% and 10.6% of polymorphism were observed. The dendrogram obtained showed a clear division into two distinct groups corresponding to the two analyzed species. *V. corymbosum* group is divided in different sub-clusters based on cultivars pedigree relationships. Twenty-eight specific bands were detected in total; 6 for *V. corymbosum* and 22 for *V. myrtillus*. Results allowed the selection of five primers due it potential to detect specific bands in the two species. These markers could be useful for identifying species and cultivars and consequently help in the management of germplasm collections and in breeding programs.

Additional keywords: blueberry; bilberry; genetic diversity; differentiation; molecular marker.

Abbreviations used: I (intermediate to southern and northern highbush) ISSR (inter simple sequence repeat); MI (marker index); NHB (Nothern highbush); PCR (polymerase chain reaction); PIC (polymorphism information content); Rp (resolution power); SAHN (sequential agglomerative hierarchical nesting algorithm); SHB (Southern highbush); UPGMA (unweighted pair-group method with arithmetic averages).

Authors' contributions: Performed the experiments, data analysis and drafting the manuscript: MC. Design and supervision of the work and critical revision of the manuscript: MM and VC. Supervised and coordinated the research project: VC. All authors read and approved the final manuscript.

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Introduction

In recent years *Vaccinium* crops have emerged internationally as having a high economic value. The consumption of blueberries is increasing worldwide in part due to the numerous reports of their beneficial effects on human health. The major health benefits include risk reduction for cardiovascular and neurodegenerative diseases (Rowland *et al.*, 2012; Zifkin *et al.*, 2012). These positive effects are generally attributed to high level of polyphenolic compounds, in particular flavonoids (Ross & Castillo, 2009; Zifkin *et al.*, 2012).

Vaccinium genus contains more than 400 species and is characterized as shrubs, several of which produce edible fruits, such as blueberry, cranberry, bilberry, and lingonberry. Blueberry (*Vaccinium corymbosum* L.) is native to North America and Europe and is divided into three different types: northern, southern and intermediate highbush blueberry. These types of blueberry vary in the number of chilling hours required for normal floral development and their level of tolerance to winter cold (Hancock, 2009). *Vaccinium myrtillus* L., called bilberry or wild blueberry, is a perennial wild shrub which grows in acidic soils, mountainous mineral heaths, organic forest soils and

old peat bogs in northern to central Europe (Albert *et al.*, 2004).

The identification and classification of blueberries cultivars is mainly based on phenotypic traits and flavor. This type of characterization does not reflect exclusively the genotype and can bring some identification errors for producers. The development of effective and rapid methods for genotype identification is fundamental, especially in vegetative propagated plant species (Debnath, 2009; Carvalho *et al.*, 2014), as blueberry. The arrival and development of molecular markers allowed to overcome the problems associated with phenotype-based classification (including morphologic and flavor characteristics). Molecular markers-assisted breeding could be useful in the efficient use of genetic diversity in crop improvement programs, and consequently helps in germplasm characterization, and in particular breeding purposes (Debnath, 2009; Rowland *et al.*, 2012). Inter Simple Sequence Repeat (ISSR) is one technique easy to perform, allows the identification of multiple loci and the results are easily analyzed. ISSRs are dominant markers, dispersed throughout the plant genome (Zietkiewicz *et al.*, 1994; Kumar *et al.*, 2009) and are very useful in genetic diversity studies and in identification and characterization of cultivars, clones and species of *Vaccinium* (Debnath, 2007a,b; 2009; Garriga *et al.*, 2013; Carvalho *et al.*, 2014). Garriga *et al.* (2013), using leaves of thirteen genotypes of two *Vaccinium* species (*V. corymbosum* and *Vaccinium ashei*), compared the results obtained using ISSRs with those obtained using SSR markers the most used in germplasm characterization. They concluded that the results are very similar and ISSRs provided a greater reliability in cultivar identification. In last years, several studies using molecular markers have been developed in blueberry, but this work is the first using cultivated and wild species. In Portugal, the wild blueberry has been suffering from several genetic erosion events in last decades remaining only few populations in protected areas in the North of Portugal. The present study aimed to assess the genetic relationships between the species *V. corymbosum* and *V. myrtillus* and to analyze the genetic variability using ISSR markers. We also intended to propose some *Vaccinium* species-specific and specific cultivars DNA markers to be applied in cultivar identification and certification that may be useful to the producers.

Material and methods

Biological material

Sixteen highbush blueberry cultivars, from an organic plantation in the central region of Portugal, representative of the most cultivated blueberries in Portugal were used (Table 1). Three wild populations of *V. myrtillus* collected from Natura 2000 network where are included the Marão mountain (*V. myrtillus* 1 and 2) and Alvão Natural Park (*V. myrtillus* 3)

Table 1. Name of cultivars, their pedigree and type according to USDA *Vaccinium* catalogue (http://www.ars.usda.gov).

Cultivar	Pedigree or Origin	Type[1]
Bluecrop	GM-37 (Jersey × Pioneer) × CU-5 (Stanley × June)	NHB
Bluetta	(North Sedgewick × Coville) × Earliblue	NHB
Duke	(Ivanhoe × Earliblue) × 192-8 (E 30 × E 11)	NHB
Earliblue	Stanley × Weymouth	NHB
Georgiagem	(Bluecrop × E-118) × US-75 (Florida 4B × Bluecrop)	SHB
Goldtraube	German variety	NHB
Hardyblue	Virus free clone from 1613[a]	NHB
Jersey	Rubel × Grover	NHB
Legacy	Elizabeth × US-75 (Florida 4B × Bluecrop)	NHB
Olympia	Pioneer × Harding	NHB
O'Neal	Wolcott × Florida 4-15	SHB
Ozarkblue	G-144 × Forida 4-76	I
Patriot	(Dixi × Michigan LB-1) × Earliblue	NHB
Reveille	NC 1171 × NC SF-12-L	SHB
Sierra	US 169 × G-156	NHB
Toro	Earliblue × Ivanhoe	NHB

[1]NHB = Northern highbush. SHB = Southern highbush. I = Intermediate to southern and northern highbush.

mountain were used. *V. myrtillus* is not very common in Portugal but a few small populations of this wild species can be found in the North of Portugal, mainly in Natura 2000 network region. Due to this scarcity, the three genotypes/populations used in this study can be considered representative of *V. myrtillus* species in Portugal. Young leaves of individual plants were collected separately from all nineteen genotypes and frozen at - 80 ºC until DNA extraction.

DNA extraction

DNA extraction was performed with the Nucleo-Spin® Plant II kit (Macherey-Nagel, Düren, Germany) using the Lysis Buffer PL1 and the standard protocol as has been described by Carvalho *et al.* (2014). The concentration and purity of isolated DNA were assured by spectrophotometry (NanoDrop ND1000, Thermo Fisher Scientific, Wilmington, USA) and the integrity on a 1% agarose gel.

ISSR analysis

For ISSR analysis, 30 ISSR primers (UBC#100/9, University of British Columbia, Canada) were tested in all samples and the 10 primers that showed the most polymorphic and discriminative patterns among them were selected (Table 2). Each amplification reaction was repeated at least twice and only the reproducible bands were analyzed. Primers that amplified monomorphic patterns were discarded. PCR amplifications were performed in a final volume of 20 µL with *Taq* polymerase enzyme (Thermo Scientific, Burlington, USA) and the amplification conditions were those described by Matos *et al.* (2001). PCR products were analyzed after electrophoresis on 1.7% agarose gels stained with ethidium bromide. To determine the size of the PCR products the GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific, Burlington, USA) was loaded in each agarose gel.

Data analysis

As ISSRs are dominant markers, it was assumed that each band represented a single bi-allelic locus, and was scored based on presence (1) or absence (0) of each band/marker for genotypes. The number of polymorphic, exclusive and total amplified bands, as well as the polymorphism percentage were determined. The resolution power (Rp) was calculated with the formula $Rp = \sum Ib$ ($Ib = 1- [2 \times (0.5 - p_i)]$ according to Prevost & Wilkinson (1999) being Ib the band informativeness and p_i the proportion of individuals containing the band. The polymorphism information content (PIC) [$PIC_i = 2\ f_i\ (1 - f_i)$] was determined based on Roldán-Ruiz *et al.* (2000) formula being f_i the frequency of

Table 2. Inter simple sequence repeat (ISSR) polymorphism parameters obtained a) from the joint analysis of sixteen *V. corymbosum* cultivars and three *V. myrtillus* populations and b) for each species.

	Sequence	TNB	NPB	NEB		P%	Size range (bp)	Rp	PIC	MI
				VC	VM					
a) Primers										
UBC 807	$(AG)_8T$	14	14	2	2	100	1900-500	15.90	0.37	37.00
UBC 821	$(GT)_8T$	8	8	1	1	100	1900-850	6.00	0.33	33.10
UBC 823	$(TC)_8C$	12	9	0	1	75.00	1800-500	14.00	0.22	16.60
UBC 825	$(AC)_8T$	12	11	1	4	91.70	1900-600	11.90	0.29	26.70
UBC 844	$(CT)_8RC$	12	12	0	2	100	1600-550	11.60	0.33	32.90
UBC 848	$(CA)_8RG$	16	15	0	5	93.80	1900-500	12.30	0.33	30.90
UBC 853	$(TC)_8RT$	12	11	1	2	91.70	1700-650	11.20	0.29	26.90
UBC 856	$(AC)_8YA$	11	9	0	2	81.80	1900-600	9.70	0.26	21.70
UBC 873	(GACAGACAGACAGACA)	12	10	0	1	83.30	1900-600	12.80	0.30	25.20
UBC 880	$(GGAGA)_3$	13	12	1	2	92.30	1600-500	13.80	0.33	30.20
Mean		12.20	11.10	0.60	2.20	90.96	1900-500	11.92	0.31	28.12
b) Species										
V. corymbosum		95	74	6		83.2	1900-550	11.4	0.30	24.6
V. myrtillus		76	8	22		10.6	1900-500	14.5	0.05	0.50
Mean		85.50	41	14		46.90	1900-500	12.95	0.18	12.55

TNB = total number of bands. NPB = number of polymorphic bands. NEB = number of specific bands. VC = *V. corymbosum*. VM = *V. myrtillus*. P% = polymorphism percentage. Rp = resolution power. PIC = polymorphism information content. MI = marker index.

the amplified band. The marker index (MI), defined as the product of polymorphism percentage and PIC, was calculated according to Sorkheh *et al.* (2007). All the analyzed parameters were performed per primer and species (Table 2). Cluster analysis was done applying unweighted pair-group method with arithmetic averages (UPGMA) based on simple matching similarity matrix and sequential agglomerative hierarchical nesting algorithm (SAHN) subroutine through the NTSYS-pc 2.02 software program.

Results and discussion

A precise identification and characterization of different genotypes is fundamental for the cultivar development, certification and protection but it is also important in the propagation, cultivation and marketing of commercial varieties (Garriga *et al.*, 2013). In last years, several molecular markers have been largely used for these purposes.

In this study, sixteen blueberry cultivars and three wild population were analyzed using ten ISSR primers. The ten primers produced a total of 122 markers among the genotypes and the amplified products ranged from 500 bp to 1900 bp (Table 2). An average of 90.96% of polymorphism was determined per primer and ranged from 75% (UBC823) to 100% (UBC807, UBC821 and UBC844). The number of polymorphic bands per primer varied between eight (UBC821) and 15 (UBC848) (Table 2), with an average of 11.10 bands per primer. The high number of polymorphic bands (19.80 per primer) registered in this study is in agreement with those obtained by Debnath (2007a,b; 2009) in *Vaccinium vitis-idaea*, *Rubus idaeus* L. and *Vaccinium angustifolium* (23.70, 18.60 and 17.00 polymorphic bands per primer, respectively) using ISSR markers.

In *V. corymbosum* cultivars 74 polymorphic markers were obtained, while in *V. myrtillus* populations only four, being the percentage of polymorphism 83.20% and 10.60%, respectively (Table 2).

ISSR primers vary in their ability to analyze genetic diversity, and primers with high Rp values are generally more effective in distinguishing genotypes (Prevost & Wilkinson, 1999; Debnath, 2007a). In our study, the primers UBC880 and UBC807 showed to be the most effective (Rp = 13.80 and Rp = 15.90, respectively). The Rp values ranged from 6.00 (UBC821) to 15.90 (UBC807) with an average of 11.92.

PIC values are commonly used in genetic analysis as a measure of polymorphism for a marker locus using linkage analysis, while MI is used to estimate the overall utility of each marker system (Sorkheh *et al.*, 2007). The PIC average was 0.31, being the highest PIC 0.37 (UBC 807) and the lowest 0.22 (UBC823), whereas MI ranged from 16.60 for UBC823 to 37.00 for UBC807 with an average of 28.12. In this set of primers, the UBC807 primer seems to be the most appropriate once it had the highest value of PIC and MI and consequently is the most informative. For the set of *V. corymbosum* cultivars, the PIC was 0.30, the Rp 11.40 and MI was 24.60 while for *V. myrtillus* populations was 0.05, 14.50 and 0.50, respectively. The results obtained clearly demonstrate that ISSR markers are a high efficient marker to characterize *Vaccinium* species (Table 2), as has been referred in other horticultural species.

V. myrtillus and *V. corymbosum* specific-bands could be very useful for species and cultivars identification and could be considered potential markers for species-identification. Bands that are present in all samples of one species and absent in the other can be considered potential species-specific markers. In the same way, bands that are present only in one cultivar can be considered potential cultivar-specific markers. Twenty-eight species-specific bands were observed, six in *V. corymbosum* and twenty-two in *V. myrtillus* (Table 2). The highest number of species-specific bands was detected with UBC825 and UBC848 primers (five bands in each primer) while the lowest number was detected with UBC821, UBC823 and UBC873 primers (one band in each primer). These five ISSR primers can be used as unique fingerprints to identify *V. corymbosum* and *V. myrtillus*. Two cultivar-specific bands were identified in *V. corymbosum* cultivars, one in 'Bluetta' (UBC853) and another in 'Hardyblue' (UBC821). In *V. myrtillus* were identified two specific bands for the Marão mountain population with UBC844 and UBC856 primers. The next step is the conversion of these specific bands into sequence characterized amplification regions (SCARs). A fast and more specific characterization and differentiation of genotypes could be very important for enabling producers to avoid errors in berries identification and commercialization.

Hierarchical analysis, generated by UPGMA method based on Dice coefficient, separated the two *Vaccinium* species in two different clusters (Fig. 1). At same time, gave information about the genetic relationship concerning to the pedigrees of *V. corymbosum* cultivars previously presented by Boches *et al.* (2006) and Garriga *et al.* (2013). The similarity values in cluster I ranged from 0.72 ('Hardyblue' and 'O'Neal') to 0.90 ('Bluecrop' and 'Georgiagem') and could be divided into two sub-clusters, I-A which groups fourteen cultivars and I-B with two cultivars ('Hardyblue' and 'O'Neal'). The sub-cluster I-A includes the 'Bluecrop' and 'Olympia' cultivars that shared in their pedigree 'Pioneer' cultivar. This group also includes 'Georgiagem' and 'Legacy' cultivars which have 'Bluecrop' cultivar

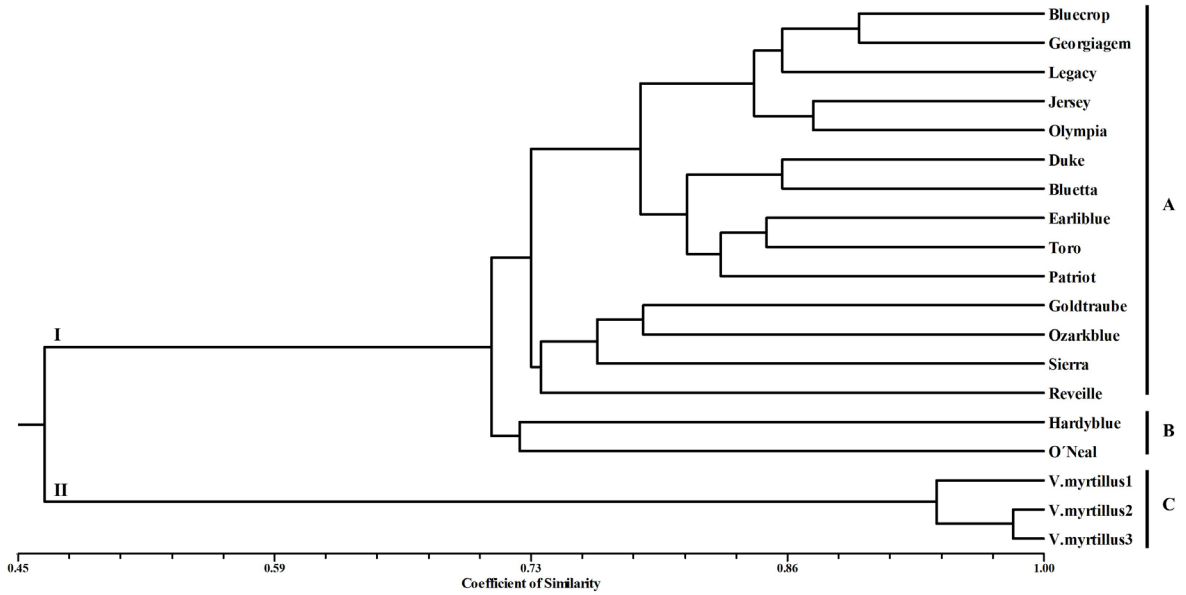

Figure 1. Dendrogram estimating the genetic distance between sixteen *V. corymbosum* cultivars and three populations of *V. myrtillus* based on ten ISSR primers.

as common progenitor. It is also important refer that 'Bluecrop' and 'Georgiagem' cultivars are the closest in cluster I once 'Georgiagem' cultivar has on it pedigree two crosses with 'Bluecrop' cultivar (Table 1). The cultivars 'Bluetta', 'Duke', 'Patriot' and 'Toro' have in common the parental 'Earliblue' cultivar, which could explain the presence of these five cultivars together. The other four cultivars ('Goldtraube', 'Ozarkblue', 'Sierra' and ' Reveille') do not share any progenitor, and do not have any relationship based on the cultivar type (two are northern highbush, one are southern highbush and one is intermediate). However, these cultivars are characterized by early or mid-maturing cultivars except the 'Reveille' cultivar that is a late maturing cultivar. In this first cluster, the similarity coefficient varied from 0.70 and 0.90 among all blueberry highbush cultivars, showing a low genetic diversity among them. This low genetic variability can be explained by the restricted gene pool that has been used in the breeding programs. Inside of *V. corymbosum* group (cluster I), 'Hardyblue' and 'O'Neal' cultivars are the two most distant (sub-cluster I-B). The second cluster includes the three wild populations of *V. myrtillus* and the similarity values ranged from 0.94 to 0.99.

The results obtained confirm the utility of ISSR markers in genetic variability studies in *Vaccinium* species. This molecular marker detected an enough degree of variation among *Vaccinium* genotypes, and proved that could be effective in blueberry genetic diversity studies. The 10 ISSR primers used in this work allowed the differentiation of the two species and the determination of the genetic relationships between *V. corymbosum* cultivars. These results can help in the management of germplasm collection and in breeding programs. The moderate genetic variability found within highbush blueberry cultivars is the result of some inbreeding and underscores the need for including new sources of germplasm in order to increase the genetic diversity and to create variability which will facilitate the obtaining of new cultivars. The ISSR markers could also be used for increase the selection efficiency of parents in crosses for creating diversity for breeding programs. Breeders can maximize the use of wild populations of *V. myrtillus* for new cultivars of *V. corymbosum* development, knowing and understanding the genetic differences among these species.

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