Fingerprinting of *Vaccinium corymbosum* cultivars using DNA of fruits

M. Carvalho[1], M. Matos[1,2], V. Carnide[1,2]

[1]*Institute for Biotechnology and Bioengineering, Centre of Genomics and Biotechnology. University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*
[2]*Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*

Abstract

Carvalho M., Matos M., Carnide V., 2014. **Fingerprinting of *Vaccinium corymbosum* cultivars using DNA of fruits**. Hort. Sci. (Prague), *41*: 175–184.

In recent years the production and consumption *Vaccinium corymbosum* has increased. Highbush blueberry cultivars are divided into three types, northern, intermediate and southern. The traditional methods for classification of highbush blueberry cultivars using morphological and flavour traits are largely unsuccessful, due to environmental influences. The genetic similarity of ten highbush blueberry cultivars was evaluated using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers from fruits and leaves. The DNA concentrations obtained in fruits and leaves were very similar and the band profiles observed in the two tissues were analogous with both molecular markers. RAPD analysis generated 144 bands, of which 112 were polymorphic (77.8%) in fruits and 141 bands of which 118 were polymorphic (83.7%) in leaves. In fruits, ISSR analysis produced 151 bands of which 127 were polymorphic (84.1%) and in leaves it produced 148 bands with 127 being polymorphic (85.8%). Dendrogram and principal coordinates analysis (PCO) analysis using the both markers results were concordant and a clear division of the types of highbush blueberry cultivars (northern and southern) into two distinct groups was verified.

Keywords: molecular markers; blueberry; fruits and leaves; characterization

Blueberries belong to the Ericaceae family, Vaccinoideae subfamily, *Vaccinium* genus, *Cyanococcus* subgenus and are an important fruit crop. This genus contains about 400 species that grow around the world and exhibit a high level of morphological diversity (Luby et al. 1991; Ratnaparkhe 2007). Blueberries are mostly found in the tropics at high elevations but also appear in temperate and boreal regions (Ratnaparkhe 2007). The three blueberry species responsible for the largest production worldwide are *Vaccinium corymbosum* L. (highbush blueberry), *V. angustifolium* Ait. (lowbush blueberry) and *V. ashei* (rabbiteye blueberry) (Ratnaparkhe 2007; Song, Hancock 2011). Recently increased production and consumption of blueberries can be attributed to the health benefits ascribed to this fruit related to their rich quantities of polyphenolic compounds, in particular flavonoids. Some researchers consider blueberries as having one of the highest *in vitro* antioxidant capacities among fresh fruits (Prior, Gu 2005; Zifkin et al. 2012).

Blueberry fruits and leaves are characterized by a high level of polyphenolic compounds (Prior, Gu 2005; Zifkin et al. 2012). The composition and content of polyphenolic compounds in blueberries are influenced by the cultivar, the growing season and location (Howard et al. 2003; Dragovic-Uzelac

et al. 2010). According to Dragovic-Uzelac et al. (2010) the cv. Sierra contains higher amounts of total phenols (331.34 galic acid equivalents/100 mg of fresh mass (f.m.)) and cvs Bluecrop and Duke have lower amounts of total phenols (291.56 and 279.21 galic acid equivalents/100 mg f.m., respectively).

The highbush blueberry group can be divided into three types: the northern, southern and intermediate highbush blueberry (Hancock 2009). These types vary in the number of chilling hours required for normal floral development and their levels of winter cold tolerance. Northern highbush blueberries cultivars are the most widely planted blueberries in the world and are adapted to quite cold mid-winter temperatures, whereas the southern highbush cultivars do not tolerate winter temperatures much below freezing (Hancock 2009; Sterne et al. 2011). The classification of the genus *Vaccinium* and blueberry cultivars is mainly based on phenotypic characteristics and flavour, and the development of methods for accurate and rapid genotype identification is very important to producers, especially in vegetatively propagated plant species (Debnath 2009) such as blueberry.

Molecular markers were successfully developed during recent decades and have largely overcome problems associated with phenotype-based classification. Their use is the most effective way for characterization and identification of species and cultivars with the advantage that they allow for a direct comparison of genetic material independently of environmental influences (Weising et al. 1995; Debnath 2007). Moreover, it could be useful (i) to promote the efficient use of genetic variations in crop improvement programs, (ii) to help in germplasm characterization and (iii) field genebank management, and (iv) to guarantee proprietary-right protection (Paterson et al. 1991; Debnath 2009).

Random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) are two dominant molecular markers dispersed throughout plant genomes and the techniques are easy to perform and to analyse (Awasthi et al. 2004; Kumar et al. 2009). So far only a few attempts have been made to identify and characterize blueberry cultivars by molecular markers and to determine genetic diversity in the highbush blueberry or other species of *Vaccinium* genus (Levi, Rowland 1997; Albert et al. 2003; Garkava-Gustavsson et al. 2005; Debnath 2007, 2009; Yakimowski, Eckert 2008).

Genotype identification using molecular markers for quality control or protection of breeders' rights may be performed in different situations. The usual tests are based on leaves but there are circumstances that may require the use of other tissues, such as analysis of fruits collected from a storage facility or a supermarket shelf. Leaves and fruits have not been used to assess genetic similarity between highbush blueberry cultivars yet. The main objective of this work was to assess genetic similarity, using RAPD and ISSR markers from the leaves and fruits of ten highbush blueberry cultivars of different types.

MATERIAL AND METHODS

Plant Material. Ripe fruits and young leaves from the same plants were collected from ten highbush blueberry cultivars (Bluecrop, Duke, Goldtraube, Ozarkblue, Earlyblue, Hardyblue, O'Neal, Patriot, Reveille and Sierra). Three samples of each tissue were collected from three different plants. The tissues were frozen at –80°C until DNA extraction. Table 1 describes the highbush blueberry cultivars studied and their type (northern or southern).

DNA extraction. Total genomic DNA was extracted from 50 mg of each tissue (fruits and leaves) with the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) using the Lysis Buffer PL1 (based on the CTAB method) and the standard protocol according to the manufacturer's instructions with minor modifications (the incubation time was increased to 75 min and an extra centrifugation of the crude lisate was done).

The quantity and quality of the DNA obtained was assessed by spectrophotometry at 260 nm and

Table 1. Highbush blueberry cultivars and their classification

Cultivar	Type
Bluecrop, Duke, Earliblue, Goldtraube, Hardyblue, Ozarkblue, Patriot, Sierra	northern
O'Neal, Reveille	southern

280 nm. The A_{260} was used to calculate the DNA concentration and the A_{260}/A_{280} ratio was used to assess contamination with proteins and/or RNA. The concentration of the DNA samples was measured in triplicate using a spectrophotometer (NanoDrop 1000, Invitrogen, Carlsbad, USA). In order to verify DNA integrity, 3 µl DNA were subjected to gel electrophoresis on 1.0% (w/v) agarose gel (SeaKemRLE Agarose; Lonza, Basel, Switzerland), stained with ethidium bromide.

RAPD PCR. A total of 10 RAPD primers (Operon Technologies, Alameda, USA; VH Bio, Newcastle upon Tyne, UK) were selected from a set of 35 primers initially tested, based on reproducibility and polymorphic patterns. PCR amplifications were performed in a final volume of 25 µl containing 2 µl of DNA template (~20 ng), 2.5 µl of Taq buffer with $(NH_4)_2SO_4$ (10×), 2.5 µl of $MgCl_2$ (25 mM), 1.5 µl of RAPD primer (10 µM), 0.5 µl of dNTPs mix (10 mM each), 0.4 µl of Taq polymerase (5 U/µl) (Thermo Scientific, Waltham, USA). Amplification reactions were carried out using the following cycle profile: initial denaturation at 94°C for 5 min followed by 45 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min and a final extension at 72°C for 6 minutes. DNA fragments were separated by electrophoresis on 1.6% agarose gels in 1× Tris Boric Acid Buffer (TBE) running at 100 V for 270 min and stained in an ethidium bromide solution.

ISSR PCR. From a set of 38 ISSR primers (UBC#100/9) initially screened, 10 primers were selected according to their clear and reproducible band patterns and the polymorphism revealed. PCR amplifications were performed in a final volume of 20 µl containing 2 µl of DNA template (~20 ng), 2 µl of Taq buffer with $(NH_4)_2SO_4$ (10×), 1.6 µl of $MgCl_2$ (25 mM), 2 µl of ISSR primer (5 µM), 0.5 µl of dNTPs mix (10 mM each), 0.4 µl of Taq polymerase (5 U/µl) (Thermo Scientific, Waltham, USA). The amplification programme was described by Matos et al. (2001). The PCR products were analysed on 1.7% agarose gels in 1× TBE running at 100 V for 210 min and stained in an ethidium bromide solution.

Data Analysis. DNA banding patterns generated by RAPD and ISSR markers were obtained using the Molecular Image Gel-DocTM XR^+ with Image Lab™ Software (BioRad, Hercules, USA). As these markers are dominant it was assumed that each band represented a single bi-allelic locus and each amplified band/marker was scored as present (1) or absent (0). Each primer-sample combination was repeated at least twice, and only reproducible bands were used for analysis.

The number of polymorphic, exclusive and total bands and the polymorphism percentage were determined. The resolution power (*Rp*) was calculated following Prevost and Wilkinson (1999) where $Rp = \sum Ib$ ($Ib = 1 - [2 \times (0.5 - p_i)]$) and *Ib* is band informativeness and p_i is the proportion of the accessions containing band; the polymorphism information content (PIC) according to Roldán-Ruiz et al. (2000) where $PIC_i = 2f_i(1 - f_i)$ (f_i is the frequency of amplified allele and $1 - f_i$ is the frequency of null allele of marker *i*); the effective multiplex ratio (EMR) following Powell et al. (1996) where EMR is number of polymorphic products from a single amplification reaction; and the marker index (MI) following Sorkheh et al. (2007) defined as the product of polymorphism percentage and PIC. Cluster analysis was done, applying an unweighted pairgroup method with arithmetic averages (UPGMA) based on a simple matching similarity matrix and SAHN subroutine to compute a principal coordinate (PCO) analysis through the NTSYS-pc Ver. 2.02 (Exeter Software, New York, USA).

The robustness of the dendrogram was evaluated by estimating the cophenetic correlation for the dendrogram and comparing it with the similarity matrix using Mantel's matrix correspondence test (9999 permutations; Mantel 1967) in a NTSYS-pc software package, Ver. 2.02. The result of this test is a cophenetic correlation coefficient, *r*, indicating how well the dendrogram represents similarity data. The Mantel test (Mantel 1967) was performed for estimating correlation between similarity index matrices produced by any two-marker systems.

RESULTS AND DISCUSSION

The use of two tissues had the main objective of showing whether tissue (fruits and leaves) could be used in these kinds of studies. High-quality genomic DNA was extracted from fruits and leaves of highbush blueberry cultivars using a commercial kit. Agarose gel and a spectrophotometer were used to compare the quality and quantity of DNA from both tissues. DNA runs at a very high molecular weight showing clear, thick bands, and the intensities were very similar in both tissues. Nei-

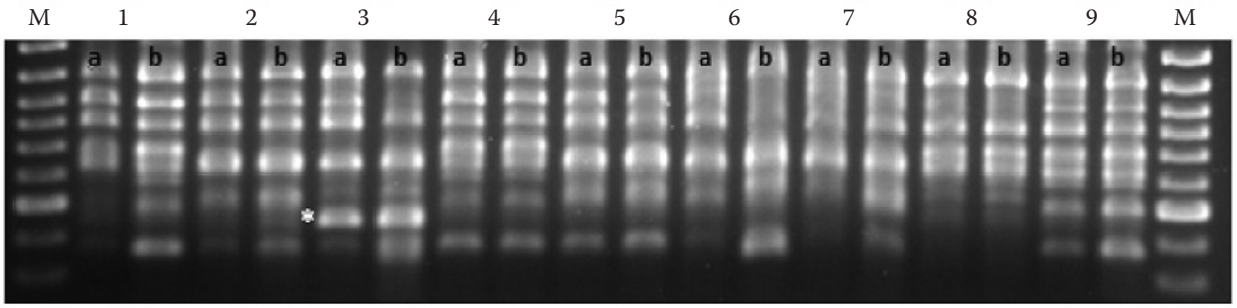

Fig. 1. Random amplified polymorphism DNA (RAPD) banding pattern of fruits (a) and leaves (b) in highbush blueberry cultivars using OPI07 primer

1 – Bluecrop; 2 – Duke; 3 – Goldtraube; 4 – Ozarkblue; 5 – Earlyblue; 6 – Hardyblue; 7 – O'Neal; 8 – Patriot; 9 – Reveille; M – DNA marker GeneRuler 100 bp

ther RNA contamination nor degradation was observed. The A_{260}/A_{280} ratio was in the range of 1.7 to 1.9 for DNA samples from fruits and leaves, respectively. DNA extraction could be prejudiced due to contaminating agents that precipitate with DNA and interfere with DNA polymerase activity (Pandey et al. 1996). Molecular marker analyses using fruits and leaves as biological material were done in other species including citrus (Sugawara et al. 2002), sweet cherry (Struss et al. 2003) and olive (Doveri et al. 2006), but have not been reported in highbush blueberry, yet. Our results demonstrate that the protocol used is suitable for extraction of high-quality genomic DNA from fruits and leaves of blueberry cultivars and this DNA could be used for RAPD and ISSR analyses.

The values of DNA concentration obtained by spectrophotometric analysis were very similar in both tissues; they varied from 59.8 ng/µl in fruits and 56.7 ng/µl in leaves (obtained in cv. Hardyblue) to 33.8 ng/µl in fruits and 35.2 ng/µl in leaves (obtained in cv. Goldtraube). This similarity of DNA concentrations in both tissues is in accordance with the values obtained by Struss et al. (2003) in sweet cherry. In olive trees, Doveri et al. (2006) determined that the amount of DNA was variable depending on the tissue type and that more DNA was extracted from leaves than fruits.

The combined use of RAPD and ISSR markers was successfully used in a considerable number of horticultural species such as potato (Moulin et al. 2012), strawberry (Morales et al. 2011) and mulberry (Awasthi et al. 2004; Kalpana et al. 2012).

Ten RAPD primers were used to characterize ten highbush blueberry cultivars, and the banding patterns obtained in fruits and leaves with the OPI07 primer are shown in Fig. 1. The values obtained in the different parameters analysed (Table 2) presented minor differences in fruits and leaves. In fruits a total of 144 bands were produced; 112 polymorphic (77.8 polymorphism) and band size ranged from 380 to 2,700 bp. In leaves, 10 primers produced 141 bands in total; 118 polymorphic (83.7 polymorphism), and band size ranged from 380 to 2,700 bp. In both tissues the OPE01 primer produced the highest number of bands and showed

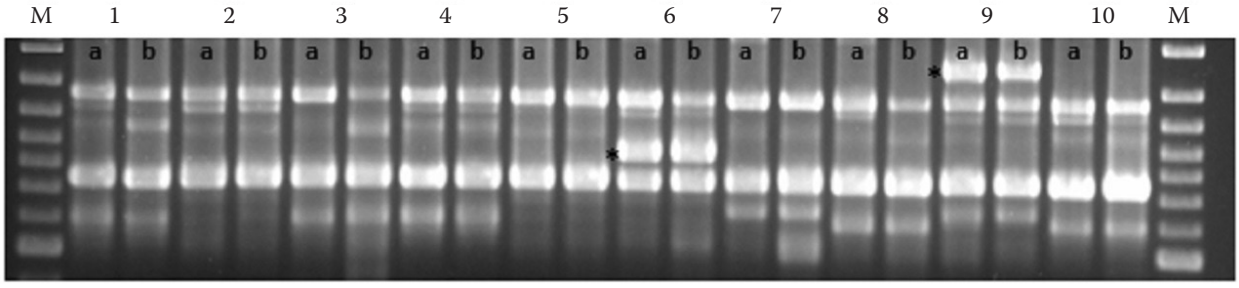

Fig. 2. Inter simple sequence repeat (ISSR) banding pattern of fruits (a) and leaves (b) in highbush blueberry cultivars using UBC856 primer

1 – Bluecrop; 2 – Duke; 3 – Goldtraube; 4 – Ozarkblue; 5 – Earlyblue; 6 – Hardyblue; 7 – O'Neal; 8 – Patriot; 9 – Reveille; 10 – Sierra; M – DNA marker GeneRuler 100 bp

Table 2. Results obtained in two tissues of *V. corymbosum* cultivars with RAPDs and ISSRs

Molecular marker	Primer	TNB		NPB		NEB	P (%)		Rp		PIC		MI		EMR	
		fruits	leaves	fruits	leaves		fruits	leaves	fruits	leaves	fruits	leaves	fruits	leaves	fruits	leaves
RAPD	OPC06	16	16	13	14	2	81	88	16.8	15.6	0.30	0.25	24.3	22.0	12.3	10.6
	OPC12	9	9	7	8	0	78	89	12.4	11.6	0.23	0.30	18.0	26.9	7.1	5.4
	OPE01	21	21	19	20	3	90	95	20.8	20	0.31	0.35	28.4	33.4	19.1	17.2
	OPE05	11	11	8	10	2	73	90	13.6	12.6	0.25	0.26	18.5	23.6	9.1	5.8
	OPI07	18	18	14	15	4	78	83	20	18	0.26	0.26	20.6	21.7	12.5	10.9
	OPN04	11	11	6	7	0	55	64	14.8	14.4	0.23	0.24	12.5	15.5	4.5	3.3
	OPO04	16	15	14	13	1	88	87	16.6	15	0.35	0.35	30.7	30.2	11.3	12.3
	OPO10	14	13	8	9	0	57.1	69.2	22.8	17.2	0.19	0.23	10.8	16.2	6.2	4.6
	OPR16	15	15	13	13	0	86.7	86.7	14.2	14.2	0.29	0.28	25.4	23.9	11.3	11.3
	OPR19	13	12	10	9	1	76.9	75	15.4	15.6	0.20	0.23	15.1	17.3	6.8	7.7
ISSR	UBC807	14	14	13	12	0	93	86	17	15.2	0.34	0.35	32.0	29.9	10.29	12.07
	UBC821	12	12	9	10	2	75	83	13	11.4	0.29	0.33	21.9	27.4	8.33	6.75
	UBC823	14	13	11	10	1	79	77	18	15.4	0.27	0.29	21.1	22.4	7.69	8.64
	UBC825	13	13	10	11	1	77	85	17.2	14.6	0.30	0.30	23.0	25.1	9.31	7.69
	UBC844	18	17	17	16	0	94	94	19.8	18	0.41	0.41	38.7	38.5	15.06	16.06
	UBC848	17	17	15	15	0	88	88	19.4	16.6	0.36	0.37	31.9	32.7	13.24	13.24
	UBC853	11	11	8	9	0	73	81	17.2	16.4	0.27	0.30	19.3	24.4	7.36	5.82
	UBC856	12	12	10	10	3	83	83	11.6	10.8	0.29	0.30	23.9	25.3	8.33	8.33
	UBC873	19	19	16	17	1	84	89	18.2	16.8	0.30	0.33	25.4	30.0	15.21	13.47
	UBC880	21	20	18	17	1	86	85	25	23	0.34	0.33	29.3	27.97	14.45	15.43
Total		295	289	239	245	22	79.8	83.9	17.2	15.6	0.29	0.30	23.5	25.7	10.4	9.8

TNB – total No. of bands; NPB – No. of polymorphic bands; NEB – No. of exclusive bands; P – polymorphism; Rp – resolution power; PIC – polymorphism information content; M – marker index; EMR – effective multiplex ratio; RAPD – random amplified polymorfic DNA; ISSR – inter simple sequence repeat

the highest percentage of polymorphism (> 90). In contrast, the OPC12 primer revealed the lowest number of bands and the OPN04 primer had the lowest percentage of polymorphism (< 65).

Ten ISSR primers were used to characterize the highbush blueberry cultivars, each primer showing a distinct polymorphic banding pattern. The pattern obtained with the UBC856 primer in leaves and fruits from the ten cultivars is shown in Fig. 2. In fruits, a total of 151 bands were produced, of which 127 were polymorphic (84 polymorphism), while in leaves a total of 148 bands were produced among which 127 were polymorphic (85.8 polymorphism) and band size ranged from 350 to 2,900 bp for both tissues (Table 2). In both tissues the UBC880 primer revealed the highest number of bands (21 in fruits and 20 in leaves) while the highest percentage of polymorphism was revealed by the UBC844 primer (94 in fruits and leaves). The UBC853 primer showed the lowest number of bands (11 in fruits and in leaves) and the lowest percentage of polymorphism was obtained with the UBC821 primer in fruits (75) and the UBC823 primer in leaves (77).

The dendrogram based on UPGMA, simple matching similarity matrix and SAHN subroutine analysis (not shown) grouped the fruits and leaves of each cultivar in the same cluster. The coefficient of similarity ranged from 0.66 to 0.95 with RAPDs and from 0.60 to 0.97 with ISSRs. The high genetic diversity between *V. corymbosum* cultivars could be hypothetically explained by the high ploidy level (Boches et al. 2006) and the origin of each cultivar that consequently makes the blueberry a highly heterozygote genetic structure. High levels of genetic variation were also observed with these molecular markers in *V. myrtillus* (Albert et al. 2005), *Vaccinium vitis-idaea* (Debnath 2007) and *V. angustifolium* (Debnath 2009).

With the assembled primers (10 of RAPDs and 10 of ISSRs) it was possible to differentiate and to disclose the genetic similarity between highbush blueberry cultivars in contrast with the results obtained by Levi and Rowland (1997) who used fifteen RAPD primers and seven SSRs *loci* and did not accurately assess the genetic relationships of cultivars within *V. corymbosum* cultivars (Bluecrop, Duke, Patriot and Sierra were included in the study). Garkava-Gustavsson et al. (2005), using seven RAPD primers and DNA from leaves to assess relatedness and genetic diversity from fifteen *V. vitis-idaea* populations, obtained 59 polymorphic bands, while with ten primers we observed 129 polymorphic bands of which 118 were in leaves and 112 in fruits.

The number of polymorphic bands (12.7 per primer in the two tissues) obtained with ISSR markers in *V. corymbosum* cultivars was slightly lower compared to other studies in other *Vaccinium* species [Debnath (2007) in *V. vitis-idaea* (23.7 polymorphic bands), Debnath (2009) in *V. angustifolium* (17 polymorphic bands) and Yakimowski and Eckert (2008) in *V. stamineum* (18 polymorphic bands)]. The differences in the number of polymorphic bands in these studies can be explained because different species were studied, although they all belong to the *Vaccinium* genus.

The polymorphism generated by the RAPD and ISSR primers is very close (87.2% and 88.2%, respectively) and is concordant with the results obtained by Moulin et al. (2012) in potato, where the RAPD and ISSR markers generated 96.7% and 92.4% polymorphism, respectively. However, Morales et al. (2011) and Kalpana et al. (2012) verified that ISSR markers were more polymorphic than the RAPD markers in strawberry and mulberry, respectively.

The RAPD primer OPO10 showed the highest Rp value (22.8), and was obtained in fruits. Also in fruits, the lowest PIC and MI (0.19 and 10.8, respectively) were recorded with this primer. The highest PIC and MI (0.35 and 33.4, respectively) were obtained in leaves with the OPE01 primers. The highest EMR (19.1) was obtained with this primer in fruits (Table 2).

For ISSR markers the greatest value of Rp was recorded in fruits with the UBC880 primer (25) and the lowest in leaves with the UBC856 primer (10.8). The highest PIC and MI were observed with the UBC844 primer in fruits (0.41 and 0.27, respectively) and the lowest with the UBC853 primer in leaves (38.7 and 19.3, respectively). These two primers also showed the highest and lowest values for EMR (16.06 and 5.82, respectively), both of which were in leaves (Table 2).

Primers with high Rp values are generally more effective for distinguishing genotypes (Gilbert et al. 1999; Prevost, Wilkinson 1999). We observed that the RAPD OPI07 primer for leaves, OPO10 for fruits and the ISSR UBC880 primer in both tissues were the most powerful/effective for distinguishing the cultivars. However, in a study developed by Debnath (2007) in wild lingonberry (*V. vitis-idaea*) with fifteen ISSR primers (which included three of the ISSR primers used in our study) the UBC880 primer was the least effective.

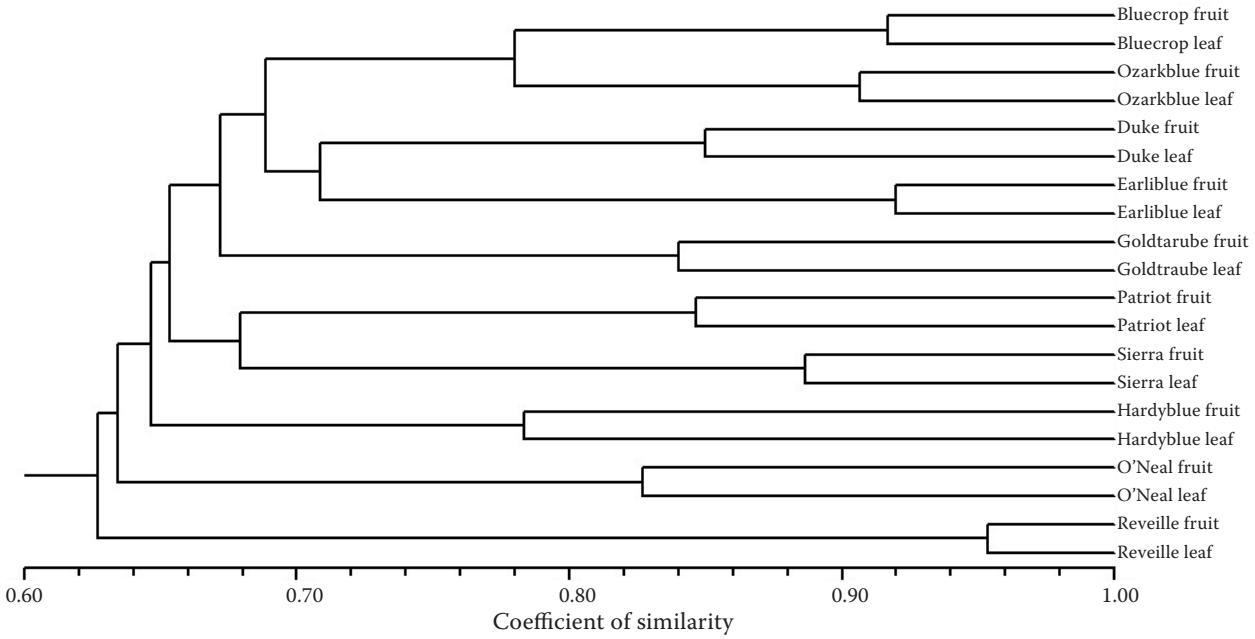

Fig. 3. Dendrogram showing the genetic relationships among *V. corymbosum* (fruits and leaves) cultivars using RAPD and ISSR markers

The PIC parameter is used in genetics as a measure of polymorphism for a marker locus (Roldán-Luiz et al. 2000), the MI parameter is used to estimate the overall utility of each marker system (Sorkheh et al. 2007) and EMR is used to evaluate whether a primer is effective (Powell et al. 1996). The most appropriate primers to differentiate species/cultivars were the OPE01 in RAPDs and UBC844 in ISSRs since both presented the highest value of PIC, MI and EMR in both tissues (fruits and leaves).

The bands present exclusively in one cultivar were considered exclusive (positive specific markers). In this study thirteen RAPDs and nine ISSRs exclusive bands, present in both tissues, were obtained. Two exclusive bands in cv. Reveille and one in cv. Patriot were produced by the OPE01 primer and two exclusive bands in the cv. O'Neal and one exclusive band in the cvs Goldtraube and Reveille were registered with the OPI07 primer. The ISSR UBC821 and UBC856 primers were those that produced more exclusive bands. One exclusive band was obtained in cvs Hardyblue and Patriot with the UBC821 primer and one in cv. Hardyblue and two in cv. Reveille with the UBC856 primer. These bands could be very useful for accurate cultivar identification, allowing producers to avoid possible errors during production since they are considered potential markers. Further, putative genotype specific bands could be converted to sequence characterized amplification regions (SCARs) after sequencing and designing specific primer pairs to develop robust genotype specific markers. SCARs are high fidelity DNA markers (Michaelmore et al. 1991) and have certain advantages compared with RAPD and ISSR markers. Using longer primers, SCARs have high reproducibility and are locus-specific.

Polymorphic profiles, where a band is absent only in one cultivar (negative specific marker), were detected with both molecular markers. Three RAPDs and four ISSRs bands absent in both tissues in cvs Bluecrop, O'Neal, Patriot, Reveille and Sierra were detected. The cvs Bluecrop and O'Neal presented the highest number of negative specific markers, two with RAPDs (OPE05 and OPO10) and two with ISSRs (UBC844 and UBC880) primers, respectively.

Comparing the different parameters used to analyse the results obtained in two tissues and with the two marker systems (Figs 1 and 2) and the results obtained in the dendrograms (where fruits and leaves of each cultivar were grouped in the same cluster; Fig. 3), it is possible to see that either fruits or leaves can be used to identify, distinguish and characterize this collection of *Vaccinium corymbosum* cultivars. As genomic DNA from fruits is suitable for evaluating genetic diversity and studies using blueberry fruits as biological material were not found, only the fruit results were used to analyse the variability between the highbush blueberry cultivars through PCO analysis (Fig. 4).

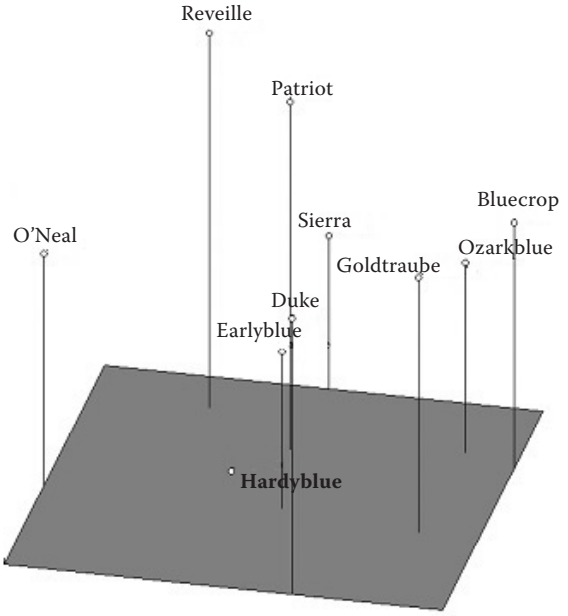

Fig. 4. Three-dimensional plot of the principal coordinate (PCO) analysis of genetic distance among fruits of ten *V. corymbosum* cultivars

The similarity matrices resulting from the combined DNA markers data were performed to generate correct relationships based on large and different genome regions. The dendrogram based on UPGMA analysis generated in order to determine the genetic relationship between the ten highbush blueberry cultivars clearly separates the northern and southern highbush blueberry types of *V. corymbosum* (Fig. 3). The eight northern highbush blueberry cultivars are all in one cluster (cluster I) while the two southern highbush blueberry cultivars are in another cluster (cluster II). Cluster I was further divided into two sub-clusters (I.1 and I.2). Sub-cluster I.1 consists of six northern highbush blueberry cultivars where the cvs Bluecrop and Ozarkblue, that are the more closely related cultivars having a coefficient of similarity of 0.81, are included. Probably the proximity between those two cultivars should be due to the fact that both are midseason maturing varieties. This can explain why these are the two most cultivated cultivars in this region. Sub-cluster I.2 grouped the cvs Patriot and Sierra, the two northern highbush blueberry cultivars. Cluster II included the cvs O'Neal and Reveille, both southern highbush blueberry cultivars. The cv. Reveille is the most distant cultivar phylogenetically, with a coefficient of similarity of 0.66 and is the only cultivar that has late season maturing. The maturing season did not have any interference with the clusters and sub-clusters formed, since the cultivars with early-maturing are distributed randomly in the different clusters obtained in the dendrogram. The coefficient of similarity ranged from 0.64 to 0.81. PCO analysis (Fig. 4) is in complete accord with the phylogenetic analysis (dendrogram) presenting two southern highbush blueberry cultivars isolated from the northern highbush blueberry cultivars. The observed genetic variability could be explained by the restricted gene pool that has been used in the breeding programs as the cultivars share genetic material from a distant common ancestor.

Close correspondence between the similarity matrices of RAPD and ISSR, RAPD and combined RAPD and ISSR, and ISSR and combined RAPD and ISSR with the two tissues (fruits and leaves) was established by means of high matrix correlation values of 0.805, 0.921 and 0.942, respectively. Hence, the utilization of individually or combined RAPD and ISSR markers allows and helps the determination of genetic relationships among highbush cultivars.

In conclusion, the use of RAPD and ISSR could be a powerful tool to assess genetic diversity in *Vaccinium corymbosum*. This study shows that it is possible to extract high-quality genomic DNA from fruits of highbush blueberry cultivars using a commercial kit. New information about genetic variability, cultivars identification and the general phylogenetic relationship among cultivars in highbush blueberry was provided. The separation between northern and southern types is possible with these molecular markers. The data obtained can be used in crop improvement programs in order to develop new cultivars with new agronomic and/or nutritional characteristics.

References

Albert T., Raspé O., Jacquemart A.L., 2003. Clonal structure in *Vaccinium myrtillus* L. revealed by RAPD and AFLP markers. International Journal of Plant Sciences, *164*: 649–655.

Albert T., Raspé O., Jacquemart A.L., 2005. Clonal diversity and genetic structure in *Vaccinium myrtillus* populations from different habitats. Belgian Journal of Botany, *137*: 155–162.

Awasthi A.K., Nagaraja G.M., Naik G.V., Kanginakudru S., Thangavelu K., Nagaraju J., 2004. Genetic diversity and relationship in mulberry (genus *Morus*) as revealed by RAPD and ISSR marker assays. BMC Genetics, *5*: 1–9.

Boches P., Bassil N.V., Rowland L., 2006. Genetic diversity in the highbush blueberry evaluated with microsatellites markers. Journal of the American Society for Horticultural Science, *131*: 674–686.

Debnath S.C., 2007. Inter simple sequence repeat (ISSR) to assess genetic diversity within a collection of wild lingonberry (*Vaccinium vitis-idaea* L.) clones. Canadian Journal of Plant Science, *87*: 337–344.

Debnath S.C., 2009. Development of ISSR markers for genetic diversity studies in *Vaccinium angustifolium*. Nordic Journal of Botany, *27*: 141–148.

Doveri S., O'Sullivan D.M., Lee D., 2006. Non-concordance between genetic profiles of olive oil and fruit: a cautionary Note to the use of DNA markers for provenance testing. Journal of Agricultural and Food Chemistry, *54*: 9221–9226.

Dragovic-Uzelac V., Savic Z., Brala A., Levaj B., Kovacevic D.B., Bisko A., 2010. Phenolics and antioxidant capacity of blueberry. Food Technology and Biotechnology, *48*: 214–221.

Garkava-Gustavsson L., Persson H.A., Nybom H., Rumpunen K., Gustavsson B.A., Bartish I.V., 2005. RAPD-based analysis of genetic diversity and selection of lingonberry (*Vaccinium vitis-idaea* L.) material for ex situ conservation. Genetic Resources and Crop Evolution, *52*: 723–735.

Gilbert J.E., Lewis R.V., Wilkinson M.J., Caligari P.D., 1999. Developing an appropriate strategy to assess genetic variability in plant germplasm collections. Theoretical and Applied Genetics, *98*: 1125–1131

Hancock J., 2009. Highbush blueberry breeding. Latvian Journal of Agronomy, *12*: 35–38.

Howard L.R., Clark J.R., Brownmiller C., 2003. Antioxidant capacity and phenolic content blueberries as affected by genotype and growing season. Journal of the Science of Food and Agriculture, *83*: 1238–1247.

Kalpana D., Choi S.H., Choi T.K., Senthil K., Lee Y.S., 2012. Assessment of genetic diversity among varieties of mulberry using RAPD and ISSR fingerprinting. Scientia Horticulturae, *134*: 79–87.

Kumar P., Gupta V.K., Misra A.K., Modi D.R., Pandey B.K., 2009. Potential of molecular markers in plant biotechnology. Plants Omics Journal, *2*: 141–162.

Levi A., Rowland M.J., 1997. Identifying blueberry cultivars and evaluating their genetic relationships using randomly amplified polymorphism DNA (RAPD) and simple sequence repeat (SSR) anchored primes. Journal of the American Society for Horticultural Science, *122*: 74–78.

Luby J., Ballington J.R., Draper A.D., Pliszka K., Austin M., 1991. Blueberries and cranberries (*Vaccinium*). Acta Horticulturae (ISHS), 290: 393–458.

Mantel N., 1967. The detection of disease clustering and a generalized regression approach. Cancer Research, *27*: 209–220.

Matos M., Pinto-Carnide O., Benito C., 2001. Phylogenetic relationships among portuguese rye based on isozyme, RAPD and ISSR markers. Hereditas, *134*: 229–236.

Michaelmore R.W., Paran I., Kesseli R.V., 1991. Identification of markers linked to disease resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions using segregating populations. Proceedings of the National Academy of Sciences of the USA, *88*: 9828–9832.

Morales R., Resende J., Faria M., Andrade M., Resende L., Delatorre C., Silva P., 2011. Genetic similarity among strawberry cultivars assessed by RAPD and ISSR markers. Scientia Agricola, *68*: 665–670.

Moulin M.M., Rodrigues R., Gonçalves L.S.A., Sudré C.P., Pereira M.G., 2012. A comparison of RAPD and ISSR markers reveals genetic diversity among sweet potato landraces (*Ipomoea batatas* (L.) Lam.). Acta Scientiarum (Agronomy), *34*: 139–147.

Pandey R.N., Adams R.P., Flournoy L.E., 1996. Inhibition of random amplified polymorphic DNAs (RAPDs) by plant polysaccharides. Plant Molecular Biology Reporter, *14*: 17–22.

Paterson A., Tanksley S.D., Sorrells M.E., 1991. DNA markers in plant improvement. Advances in Agronomy, *46*: 39–90.

Powel W., Morgante M., Andre C., Hanfey M., Vogel J., Tingey S., Rafalski A., 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Molecular Breeding, *2*: 225–238.

Prevost A., Wilkinson M.J., 1999. A new system of comparing PCR primers applied to ISSR fingerprint of potato cultivars. Theoretical and Applied Genetics, *98*: 107–112.

Prior R.L., Gu L., 2005. Occurrence and biological significance of proanthocyanidins in the American diet. Phytochemistry, *66*: 2264–2280.

Ratnaparkhe M.B., 2007. Blueberry. In: Kole C. (ed.), Genome Mapping and Molecular Breeding in Plants. Berlin, Spring-Verlag: 217–227.

Roldán-Ruiz I., Dendauw J., Van Bockstaele E., Depicker A., De Loose M., 2000. AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). Molecular Breeding, *6*: 125–134.

Song G.Q., Hancock J.F., 2011. *Vaccinium*. Wild crop relatives. In: Kole C. (ed.), Genomic and Breeding Resources. Temperate Fruits. Berlin, Spring-Verlag: 197–221.

Sorkheh K., Shiran B., Gradziel T.M., Epperson B.K., Martinez-Gomez P., Asadi E., 2007. Amplified fragment length polymorphism as a tool for molecular charac-

terization of almond germplasm: genetic diversity among cultivated genotypes and related wild species of almond, and its relationships with agronomic traits. Euphytica, *156*: 327–344.

Sterne D., Liepniece M., Sausserde R, Abolin M., 2011. Influence of temperature on the productivity of highbush blueberry cultivars. Sodininkystė ir Daržininkystė, *30*: 77–84.

Struss D., Ahmad R., Southwick S.M., Boritzki M., 2003. Analysis of sweet cherry (*Prunus avium* L.) cultivars using SSR and AFLP markers. Journal of the American Society for Horticultural Science, *128*: 904–909.

Sugawara K., Wakizuka T., Oowada A., 2002. Histogenic identification by RAPD analysis of leaves and fruit of newly synthesized chimeric *Citrus*. Journal of the American Society for Horticultural Science, *127*: 104–107.

Weising K., Nybom H., Wolff K., Meyer W., 1995. DNA Fingerprinting in Plants and Fungi. London, CRC Press: 1–3.

Yakimowski S.B., Eckert C.G., 2008. Populations do not become less genetically diverse or more differentiated towards the northern limit of the geographical range in clonal *Vaccinium stamineum* (Ericaceae). New Phytology, *180*: 534–544.

Zifkin M., Jin A., Ozga J.A., Zaharia I., Schemthaner J.P., Gesells A., Abrams S.R., Kennedy J.A., Constabel C.P., 2012. Gene expression and metabolite profiling of developing highbush blueberry fruit indicates transcriptional regulation of flavonoid metabolism and activation of abscisic acid metabolism. Plant Physiology, *158*: 200–224.

Received for publication January 22, 2014
Accepted after corrections June 19, 2014

Corresponding author:

Manuela Matos, PhD., University of Trás-os-Montes and Alto Douro, Institute for Biotechnology and Bioengineering, Centre of Genomics and Biotechnology, Quinta dos Prados, 5000-801 Vila Real, Portugal
phone: + 351 350 735, e-mail: mmatos@utad.pt