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ASSESSMENT OF GENETIC DIVERSITY BETWEEN *Vaccinium corymbosum* L. CULTIVARS USING RAPD AND ISSR MARKERS

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ABSTRACT

In the present study genetic diversity between 19 blueberry cultivars was evaluated using Randomly Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) markers. In total, nine selected RAPD primers produced 89% polymorphic fragments, whereas eleven ISSR primers – 82%. Resolving power value of the RAPD primers ranged from 2.40 to 7.19, whilst ISSR from 1.90 to 5.78. The similarity coefficients estimated on the basis of the two types of marker systems were very similar, on average amounting to 0.58 for RAPD and 0.60 for ISSR analysis. Cluster analysis based on RAPD markers showed that the 19 accessions can be classified into 6 groups. Taking into account corresponding levels of average genetic similarity (0.59) it is possible to identify three main clusters based on ISSR analysis. The RAPD as well as ISSR markers revealed the existence of genetic differentiation between accessions, which can be exploited in hybridization programs of this species.

Key words: genetic similarity, highbush blueberry, molecular markers, principal component analysis, UPGMA

INTRODUCTION

Commercial cultivation of highbush blueberry in Poland has relatively short history. Plant production of this fruit berry have been developing steadily for about three decades, marking significant progress in the past ten years. Poland is now the European leader in highbush blueberry cultivation, producing approximately 12,000–15,000 tonnes every year, which accounts for 23% of the highbush blueberry market [Kraciński 2014, Smolarz and Pluta 2014]. Cultivars grown in Poland are mainly of American origin [Smolarz and Pluta 2012]. Evaluation of their suitability for cultivation in Poland's conditions requires at least several years of experiments. Nevertheless not all cultivars meet the requirements of the growers concerning yield potential, ripening period and fruit quality, resistance to diseases and pest, as well as adaptation to local environmental conditions [Koziński 2012]. Thus it seems advisable to continue the breeding program of this species, started in 2008 and strictly focused on the expectations of Polish producers, especially that so far only one local cultivar, 'Bonifacy', has been bred, another one still being in an experimental stage [Pluta and Żurawicz 2014, COBORU 2015 http://www.coboru.pl]. It is therefore crucial to have initial breeding material characterized by high variability. The latter may be analysed based on morphological characteristics, which, however, are highly influenced by environmental conditions [Debnath 2014a]. Much more precise assessment of variability may be obtained on DNA level. Various

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DNA-based markers have been used for diversity analysis in *Vaccinium* genus [Debnath 2005]. Restriction Fragment Length Polymorphism (RFLP) was the first DNA marker used by Haghighi and Hancock [1992]. Albert et al. [2003] used RAPD and Amplified Fragment Length Polymorphism (AFLP) markers, Rowland et al. [2003] evaluated suitability Expressed Sequence Tags-derived Polymerase Chain Reaction (EST-PCR) markers, while Wang et al. [1994] and Debnath [2007] applied ISSR markers. Among these techniques, PCR-based technology including RAPD and ISSR markers are relatively easy to apply, inexpensive as compared to others, and they do not require prior knowledge of flanking sequences [Reddy et al. 2002, Debnath 2009]. Therefore they have been widely used for estimation of relatedness, verification of parentage and genetic diversity assessment [Debnath 2014a]. As breeding stock to develop a new cultivar, breeders can use valuable commercially grown cultivars, genotypes with particularly valuable attributes or other related species of *Vaccinium* genus [Prodorutti et al. 2007]. Polish flora, however, except for *V. uliginosum* L. [Mirek et al. 2002] lacks wild blueberry species that could be directly incorporated into the gene pool of this species. Moreover, such intersectional crosses have generally proved to be difficult [Shigyou et al. 2014].

Hence, this study attempts to determine the degree of genetic diversity in cultivars grown in south-east Poland based on the ISSR and RAPD markers in order to use them for practical breeding purposes to obtain improved cultivars with characteristics desired by both growers and consumers.

Table 1. The pedigree of the tested cultivars of highbush blueberry

Name	Code	Pedigree	References
Bluecrop	BC	GM-37 (Jersey × Pioneer) × CU-5 (Stanley × June)	Rejman and Pliszka 1991
Bluejay	BJ	Berkeley × (Pioneer x Taylor)	Boches et al. 2006
Blueray	BR	(Jersey × Pioneer) × (Stanley × June)	Rejman and Pliszka 1991
Bonifacy	BI	Earliblue × Darrow	Pliszka 2004
Bonus	BU	Unknown	Smolarz and Pluta 2012
Brigitta Blue	BB	Open pollination cv. Lateblue	Georgiev at al. 2013
Chandler	CA	Darrow × M -23	Boches et al. 2006
Chanticlear	CC	G-180(G-100 × Collins) × MEUS-6620(E-22 × MEUS-24)	Ehlenfeldt et al. 2000
Croatan	CO	Weymouth × F-6 (Stanley × Crabbe 4)	Rejman and Pliszka 1991
Darrow	DA	(Wareham × Pioneer) × Bluecrop	Rejman and Pliszka 1991
Duke	DU	(Ivanhoe × Earliblue) × 192-8 (E-30 × E-11)	Garriga et al. 2013
Earliblue	EB	Stanley × Weymouth	Rejman and Pliszka 1991
Herbert	HE	Stanley × (Jersey × Pioneer)	Rejman and Pliszka 1991
Jersey	JE	Rubel × Grower	Rejman and Pliszka 1991
Nelson	NE	Bluecrop × G-107 (F-72 × Berkeley)	Garriga et al. 2013
Northland	NO	Berkeley × 19-H	Rejman and Pliszka 1991
Patriot	PA	US3 (Dixi × Mich.LB-1) × Earliblue	Boches et al. 2006
Spartan	SP	Earliblue × US-11-93	Rejman and Pliszka 1991
Toro	TO	Earliblue × Ivanhoe	Garriga et al. 2013

MATERIALS AND METHODS

Plant material and DNA extraction. The research material was made up from 19 cultivars of *Vaccinium corymbosum* L. grown on two experimental farms in Niemce (51°20'N, 22°37'E) and Spiczyn (51°19'N, 22°37'E) in the south-east Poland. The list of cultivars evaluated in the study along with their origin was specified in Table 1. Fresh young leaves from 10 plants of each cultivar were collected in two replications. CTAB procedure described by Doyle and Doyle [1987] was applied to extract DNA.

RAPD analysis. Nine 10-base primers selected from 40 arbitrary primers were used for PCR amplification (tab. 2). DNA amplification for RAPD markers was carried out in a final volume of 15 µl containing 0.5 U of Taq DNA Polymerase (Fermentas), 0.3 µl of oligonucleotide primer (10 µM), 200 µM of dNTPs, 1X PCR Buffer with $MgCl_2$, and 40 ng of genomic DNA as templates. The amplification was performed in a gradient thermal cycler (Biometra GmbH) with reaction conditions programmed as initial predenaturation at 94°C for 4 min, followed by 44 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min. A final extension was done for 7 min at 72°C with a hold temperature of 4°C.

ISSR analysis. Thirty primers (Sigma-Aldrich) were screened, of which 11 primers generated polymorphic and reproducible banding patterns and were selected for final analysis. PCR amplification was carried out in a reaction volume of 15 µL containing 130 µM dNTP mix, 1.5 mM $MgCl_2$, 1 U of Taq polymerase, 1X reaction buffer, 470 pM primer, and 60 ng of genomic DNA. Reactions were performed in a gradient thermal cycler (Biometra GmbH). The profile used was a hot start for 7 min at 94°C and 35 amplification cycles of 30 s (DNA denaturation) at 94°C, annealing at optimum T_m for 30 s, and 2 min at 72°C, followed by a final extension step of 7 min at 72°C. PCR products were electrophoresed in 1.5% agarose gels stained with ethidium bromide at constant voltage (3 V/cm of gel) until bromophenol blue/loading dye migrated to the other end of the gel. The gel was visualized on a UV-transilluminator and photographed using GeneSnap ver. 7.09 (SynGene) a gel documentation system. GeneRuler 100bp DNA Ladder Plus was used to establish molecular weight of the products.

DNA data analysis. RAPD and ISSR products (clearly identifiable and repetitive) were scored as present (1) or absent (0) based on the photographs. The band was assumed to be monomorphic if it was detected in all individuals explored. As a polymorphic profiles considered bands detected in specified genotypes, whereas specific was restricted to a particular individual. The different combinations of bands obtained for each primer were scored as banding patterns. Relative frequency of banding patterns was also calculated. Resolving power of the primer was calculated using the formula: Resolving power (Rp) = $\sum I_b$, where band informativeness is $I_b = 1 - [2(0.5 - p)]$, p is the proportion of occurrence of bands in the genotypes out of the total number of genotypes [Prevost and Wilkinson 1999]. Band informativeness was calculated for each band scored by the primer individually. The data matrix was then used to calculate the genetic similarity index (SI) between pairs of all the cultivars analyzed using Dice formula [Nei and Li 1979]. Using the Mantel test [Mantel 1967], a comparison between both marker systems was performed. Effective number of alleles (Ne), Shannon's information index (I), expected heterozygosity (He), Polymorphism Information Content (PIC) of RAPD and ISSR primers were estimated using the program GenAlEx version 6.5 [Peakall and Smouse 2012]. Analysis of molecular variance (AMOVA) was used to calculate variation among and within genotypes by using the same software.

Genetic relationships among accessions were estimated using Unweighted Pair-Group Method with Arithmetic mean (UPGMA) cluster analysis employing the PAST software package [Hammer et al. 2001]. Using the same software, PCA (Principal Component Analysis) was also carried out to identify any genetic association among the genotypes.

Table 2. Primer sequence, number of products, number and frequency of band profiles and other parameters for each RAPD primer

No.	Sequence 5'–3'	Number of products		Number of banding patterns			Frequency of banding patterns	Primer diversity %	Rp	Ne	I	He	PIC
		polymorphic	monomorphic	specific	total	specific							
1.	CGATTGGACG	8	1	0	19	13	0.68	55.6	2.40	1.52	0.51	0.33	0.22
2.	ATGCCGCGAT	10	1	0	19	17	0.89	90.9	4.74	1.79	0.63	0.44	0.29
3.	TAGCGCCAAT	11	0	0	19	19	1.00	100.0	6.97	1.55	0.53	0.34	0.41
4.	CACCCGATGA	10	0	2	18	18	1.00	100.0	7.19	1.60	0.53	0.35	0.40
5.	ATGTGCCGTA	9	0	3	18	18	1.00	58.3	5.10	1.38	0.42	0.26	0.28
6.	TGGCGCAATA	9	1	0	18	16	0.89	90.0	4.72	1.50	0.49	0.32	0.34
7.	ACAACGCCTC	8	0	1	19	16	0.84	88.9	3.68	1.56	0.52	0.34	0.29
8.	GACCGCTTTG	7	0	0	19	14	0.74	100.0	3.34	1.52	0.50	0.33	0.30
9.	CCTCCTCATC	9	0	1	19	15	0.79	90.0	3.98	1.37	0.42	0.26	0.28
Total		81	3	7	168	146							
Average/primer		9.00	0.33	0.78	18.67	16.22			4.68	1.53	0.51	0.33	0.31
Average/genotype		4.26	0.16	0.37	8.84	7.68							

Rp – resolving power, Ne – effective number of alleles, I – Shannon's information index, He – expected heterozygosity, PIC – Polymorphism Information Content

RESULTS

In the studies presented here, *V. corymbosum* genotypes were analyzed using 40 RAPD primers (Sigma Aldrich), out of which 9 produced high number of polymorphic and repeatable fragments (tab. 2). The same set of RAPD primers was previously used by Wach et al. [2016]. In present study different parameters of this marker system were calculated. Selected primers produced 81 polymorphic fragments. Among the 9 primers no. 1. had the lowest primer diversity (55.6%), and three primers scored 100%. 6 of them did not produce monomorphic bands. The 9 selected primers yielded 168 banding patterns. The number of total banding patterns has averaged 18.67 per primer and 8.84 per genotype. The lowest frequency of banding patterns (0.68) characterized primer no. 1. As shown in Table 2, Rp value of the nine RAPD primers ranged from 2.40 to 7.19. In the presented study, RAPD primers with high resolving power values were able to clearly separate all analyzed genotypes. The effective number of alleles per locus (Ne) ranged from 1.37 for RAPD primer No. 9 to 1.79 for primer No. 2 and the Shannon index ranged from 0.42 for primers No. 5 and 9 to 0.63 for primer No. 2, with an average of 0.51. The expected heterozygosity across loci ranged from 0.26 for RAPD primers No. 5 and 9 to 0.44 for primer No. 2 with an average of 0.33. Polymorphism Information Content for RAPD analysis achieved the average value 0.31.

Table 3 shows characteristics of the 11 selected ISSR primers and their products. In total, the primers produced 102 fragments, out of which 84 (82%) were polymorphic. The total number of ISSR fragments scored per primer varied from 18 (primer ISSR 3) to 19 (other primers). An average of 10.95 banding patterns was obtained per genotype. The resolving power values of 11 most informative ISSR primers varied from 1.90 (primer 1) to 5.78 (primer 4).

The total Rp value for the analyzed primers was 47.34. The effective alleles in ISSR analysis changed from 1.32 to 1.83 with the mean of 1.56. Shannon's information index was on average 0.52 ranging from 0.39 to 0.64. The mean expected heterozygosity calculated on the basis of ISSR markers was 0.34 and the mean PIC reached a value 0.30.

Based on 9 RAPD and 11 ISSR primers the similarity coefficients among cultivars were calculated (tab. 4). Genetic similarities in the case of RAPD varied from 0.36 to 0.75 (mean 0.58) and were more variable than in the case of ISSR, where the level of similarity ranged from 0.44 to 0.76 (mean 0.60). For both marker systems the lowest value of the genetic similarity was noticed in the case of 'Hubert' cultivar ('Hubert' – 'Chanticlear', RAPD and 'Hubert' – 'Earliblue', ISSR). In the RAPD marker system, 'Bluecrop' and 'Patriot' cultivars showed the highest value of similarity, whereas 'Bluejay' and 'Bonus' had the highest value in the ISSR system. The AMOVA results showed that major part of the variation was found within genotypes (tab. 5). For ISSR markers 12% of the total genetic variance occurred among the cultivars, whereas 88% within cultivars. In the case of RAPD percentage of variance reached 16% among the individuals and 84% within cultivars.

Two methods of multivariate molecular analysis UPGMA and PCA were used to group cultivars. Each of them is suitable for data processing designed to sort data in a different way, therefore may be used simultaneously to assess relationship between genotypes. Cluster identification on dendrograms was performed at the level of genetic similarity amounting to 0.59, which was the mean value for both marker systems.

Table 3. Primer sequence, number of products, number and frequency of band profiles and other parameters for each ISSR primer

No.	Sequence 5'–3'	Number of products			Number of banding patterns		Frequency of banding patterns	Primer diversity %	Rp	Ne	I	He	PIC
		polymorphic	monomorphic	specyfic	total	specyfic							
1.	VBV$(AC)_7$	5	0	3	19	7	0.37	62.5	1.90	1.70	0.59	0.40	0.19
2.	BDB$(CA)_7$	7	0	0	19	13	0.68	100.0	4.96	1.64	0.57	0.38	0.43
3.	GCV$(TC)_7$	8	0	2	18	16	0.89	80.0	5.24	1.43	0.45	0.29	0.35
4.	HVH$(TGT)_5$	9	1	0	19	17	0.89	90.0	5.78	1.68	0.57	0.39	0.38
5.	BDB$(CAC)_5$	8	0	3	19	17	0.89	72.7	5.16	1.67	0.58	0.40	0.31
6.	BDV$(CAG)_5$	7	0	1	19	15	0.79	87.5	4.32	1.49	0.49	0.31	0.36
7.	$(GAA)_6$	8	1	3	19	17	0.89	66.7	4.50	1.42	0.46	0.29	0.26
8.	$(ATG)_6$	8	0	1	19	14	0.74	88.9	4.24	1.49	0.49	0.32	0.33
9.	$(GATA)_5$	7	0	3	19	9	0.47	70.0	4.78	1.32	0.39	0.23	0.25
10.	$(GACA)_5$	9	0	0	19	17	0.89	100.0	3.78	1.83	0.64	0.45	0.31
11.	$(AGTG)_4$	8	0	0	19	11	0.58	100.0	2.68	1.50	0.50	0.32	0.24
Total		84	2	16	208	153							
Average/primer		7.64	0.18	1.45	18.91	13.91			4.30	1.56	0.52	0.34	0.30
Average/genotype		4.42	0.11	0.84	10.95	8.05							

Rp – resolving power, Ne – effective number of alleles, I – Shannon's information index, He – expected heterozygosity, PIC – Polymorphism Information Content

Table 4. Genetic similarity estimated among 19 highbush blueberry cultivars, based on RAPD (above a diagonal) and ISSR (below a diagonal) markers calculated by Dice's coefficient

Cultivar	CC	EB	DU	SP	PA	BU	BI	BJ	CA	TO	BC	NE	DA	BB	NO	CO	JE	BR	HE
CC*	1.00	0.58	0.58	0.61	0.63	0.53	0.60	0.44	0.57	0.54	0.58	0.56	0.52	0.41	0.55	0.52	0.57	0.56	0.36
EB	0.66	1.00	0.62	0.61	0.62	0.53	0.64	0.60	0.52	0.70	0.56	0.63	0.44	0.45	0.50	0.43	0.49	0.46	0.40
DU	0.59	0.71	1.00	0.72	0.67	0.61	0.58	0.63	0.58	0.64	0.59	0.69	0.50	0.45	0.58	0.58	0.55	0.51	0.43
SP	0.59	0.72	0.67	1.00	0.68	0.63	0.66	0.54	0.65	0.68	0.68	0.60	0.54	0.40	0.63	0.44	0.65	0.54	0.39
PA	0.54	0.73	0.65	0.72	1.00	0.66	0.71	0.60	0.63	0.71	0.75	0.60	0.57	0.50	0.66	0.59	0.72	0.62	0.53
BU	0.64	0.71	0.62	0.60	0.65	1.00	0.63	0.65	0.60	0.66	0.64	0.62	0.48	0.52	0.58	0.61	0.60	0.57	0.56
BI	0.61	0.69	0.67	0.64	0.70	0.71	1.00	0.59	0.65	0.69	0.66	0.56	0.55	0.47	0.68	0.61	0.59	0.59	0.42
BJ	0.61	0.69	0.67	0.64	0.67	0.76	0.67	1.00	0.58	0.56	0.62	0.58	0.44	0.46	0.51	0.56	0.56	0.49	0.53
CA	0.53	0.68	0.59	0.70	0.69	0.68	0.70	0.66	1.00	0.62	0.63	0.61	0.67	0.48	0.62	0.54	0.67	0.61	0.54
TO	0.63	0.68	0.66	0.73	0.57	0.59	0.68	0.59	0.61	1.00	0.66	0.70	0.58	0.51	0.74	0.58	0.68	0.59	0.52
BC	0.54	0.60	0.64	0.67	0.60	0.64	0.69	0.62	0.66	0.64	1.00	0.73	0.54	0.53	0.73	0.53	0.72	0.62	0.53
NE	0.49	0.59	0.53	0.55	0.58	0.62	0.71	0.64	0.66	0.61	0.67	1.00	0.45	0.50	0.62	0.57	0.64	0.55	0.51
DA	0.59	0.53	0.67	0.53	0.54	0.65	0.65	0.60	0.55	0.64	0.65	0.65	1.00	0.44	0.62	0.49	0.64	0.63	0.49
BB	0.53	0.46	0.55	0.52	0.55	0.55	0.56	0.56	0.54	0.63	0.61	0.64	0.67	1.00	0.48	0.44	0.44	0.44	0.44
NO	0.51	0.60	0.58	0.55	0.58	0.60	0.60	0.68	0.60	0.60	0.60	0.60	0.61	0.59	1.00	0.64	0.68	0.72	0.55
CO	0.45	0.52	0.57	0.53	0.58	0.49	0.55	0.55	0.53	0.59	0.55	0.62	0.58	0.63	0.60	1.00	0.60	0.56	0.52
JE	0.44	0.56	0.65	0.59	0.62	0.55	0.61	0.63	0.63	0.58	0.67	0.63	0.64	0.69	0.66	0.69	1.00	0.63	0.57
BR	0.50	0.58	0.54	0.59	0.54	0.54	0.59	0.54	0.63	0.60	0.64	0.63	0.59	0.53	0.67	0.58	0.62	1.00	0.62
HE	0.46	0.44	0.48	0.50	0.56	0.53	0.55	0.53	0.50	0.55	0.60	0.55	0.61	0.64	0.61	0.55	0.68	0.59	1.00

* Cultivars designated by codes given in Table 1

Table 5. Results of the analysis of molecular variance (AMOVA) based on RAPD and ISSR markers

Source of variation	Degree of freedom		Sum of squares		Mean of squares		Estimated variation		Variation percentage	
	RAPD	ISSR	RAPD	ISSR	RAPD	ISSR	RAPD	ISSR	RAPD	ISSR
Between cultivars	18	18	124.46	120.16	6.91	6.67	0.48	0.35	16	12
Within cultivars	152	190	361.57	486.55	2.37	2.56	2.37	2.56	84	88
Total	170	208	486.03	606.71			2.85	2.91	100	100

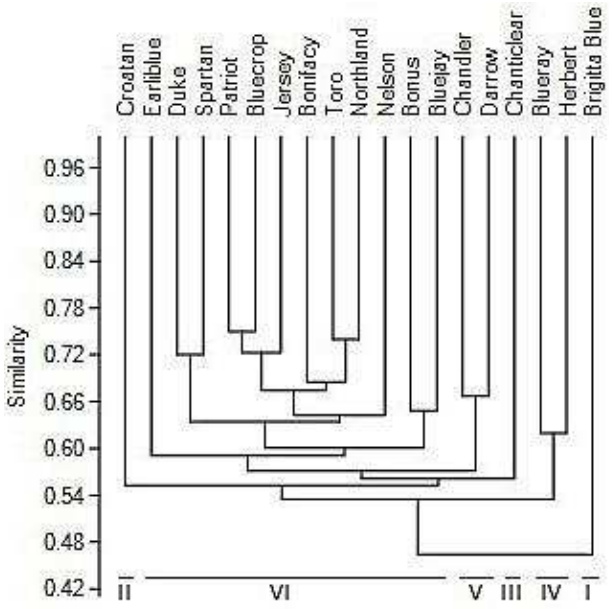

Fig. 1. UPGMA dendrogram of 19 highbush blueberry cultivars based on RAPD markers

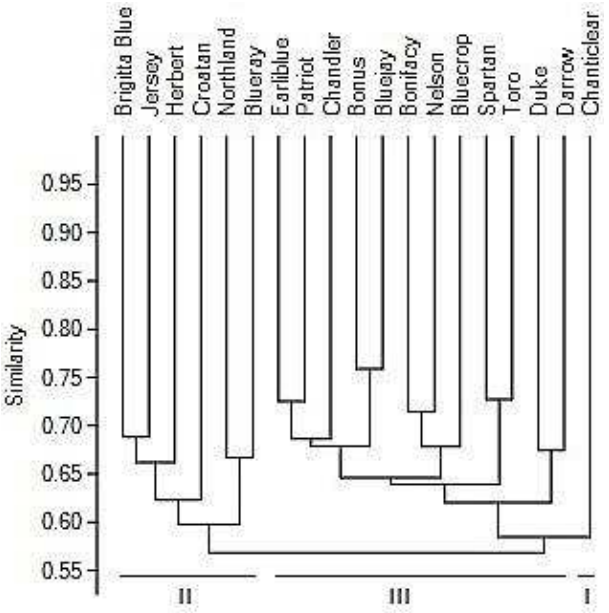

Fig. 2. UPGMA dendrogram of 19 highbush blueberry cultivars based on ISSR markers

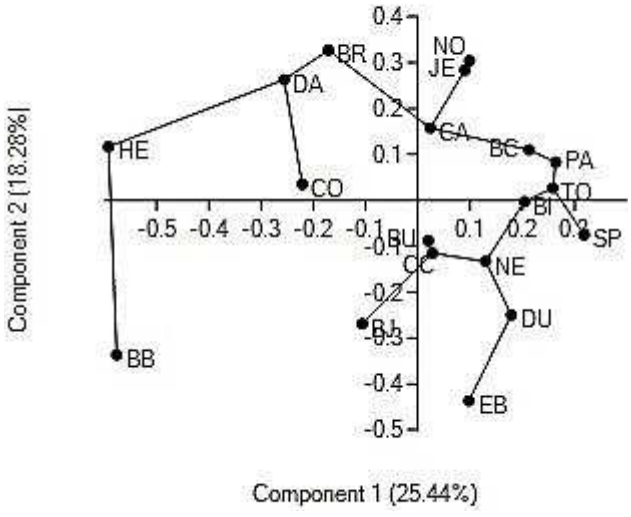

Fig. 3. Dispersion of highbush blueberry cultivars on the biplot of the Principal Components Analysis (PCA) based on RAPD markers (cultivars designated by codes given in Table 1)

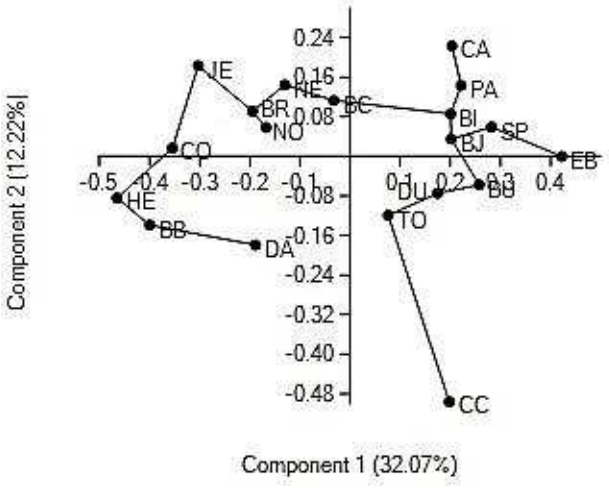

Fig. 4. Dispersion of highbush blueberry cultivars on the biplot of the Principal Component Analysis (PCA) based on ISSR markers (cultivars designated by codes given in Table 1)

The analysis of the dendrogram generated for RAPD markers (fig. 1) indicates separation of 'Brigitta Blue' from other cultivars. The assessment of genetic similarity at the level of 0.59 shows 2 clusters containing only a single cultivar: II – 'Croatan' and III – 'Chanticlear', as well as 2 more clusters each containing 2 cultivars: IV – 'Blueray' and 'Herbert', V – 'Chandler' and 'Darrow' in relation to their pedigree. The remaining 12 cultivars formed a separate cluster VI with similarity range of 0.60–0.75. This group contains 'Earliblue' cultivar as well as all genotypes that have this cultivar in its pedigree ('Bonifacy', 'Duke', 'Patriot', 'Spartan' and 'Toro'). In the case of the dendrogram generated for ISSR markers (fig. 2) a different pattern of clustering was obtained for the analyzed group of genotypes. Taking into account the same level of genetic similarity as for RAPD markers, it is possible to distinguish 3 main clusters. The first contains only 'Chanticlear' cultivar. In the second, which contained 6 cultivars ('Brigitta Blue', 'Jersey', 'Herbert', 'Croatan', 'Northland', 'Blueray'), the similarity ranged from 0.60 to 0.69. In the third cluster, which contained the remaining 12 cultivars, the similarity range was 0.63–0.76. Despite the smaller number of clusters for this type of markers, one of them contained all cultivars that had 'Earliblue' cultivar in its pedigree, as was in the case RAPD markers. Different number of clusters identified for RAPD markers in comparison to ISSR at the same level of similarity, gives an indication of different typology of analyzed dendrograms. It was confirmed by statistically irrelevant correlation (r = 0.44) between genetic similarity matrices, obtained based on polymorphism of both marker systems. Essentially, however, the dendrogram obtained for RAPD markers reflects the similarity of the genotypes to a greater extent than ISSR, taking into account their pedigree. The bi-dimensional dispersion PCA was applied to visualize the genetic distance between cultivars for both marker systems. The first two axes together explained 43.72% of the variance for RAPD and 44.29% for ISSR markers (figs 3 and 4). PCA analysis based on the frequencies of occurrence of the polymorphic ISSR bands among the cultivars led to results similar to those obtained by clustering. Among analysed cultivars, 'Chanticlear' has a separate position, as it was reflected on the dendrogram. Moreover, the first component distinctly separates cluster II (containing 6 cultivars) from the dendrogram, and it should be noted that cultivars 'Bluecrop', 'Nelson' and 'Darrow' also belong to this group. Since on the RAPD dendrogram there were as many as 6 clusters (twice as many as for ISSR), in the PCA analysis a significant dispersion of objects was observed, as expected. The position of the analyzed genotypes in the space of the two components did not correspond to their layout on the dendrogram.

DISCUSSION

Randomly amplified polymorphic DNA is a technique which identified relatively high number of polymorphic products [Burgher et al. 2002, Kizhakkayil and Sasikumar 2010, Carvalho et al. 2014]. Carvalho et al. [2014] evaluated the genetic similarity of ten highbush blueberry cultivars using RAPD and ISSR markers from fruits and leaves and obtained 144 bands in RAPD analysis, of which 112 were polymorphic (77.8%) in fruits and 141 bands of which 118 were polymorphic (83.7%) in leaves, which are slightly lower value than obtain in our research.

In presented studies the number of polymorphic bands generated by single ISSR primer ranged from 5 to 9 with an average of 7.64 per primer and 4.42 per genotype (tab. 3). These values were 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, Yakimowski and Eckert [2008] in *V. stamineum* – 18 polymorphic bands. The differences in the number of polymorphic bands in these studies can be explained by the fact that different species were studied, although they all belong to the *Vaccinium* genus, whereas our study included different cultivars of the same species. Highbush blueberry cultivars were genotyped by Carvalho et al. [2014] with 10 ISSR markers, generating 151 bands in fruits, of which 127 were polymorphic (84.1%) and in leaves it produced 148 bands, 127 being polymorphic (85.8%). All these analyses show that ISSR markers amplified high level of polymorphic bands, even using only a few primers.

Primers with high resolving power are used for the molecular diagnosis of any species from the mixed population [Prevost and Wilkinson 1999, Carvalho et al. 2014, Kaczmarska et al. 2015]. We observed that the RAPD primer no. 4 with the highest Rp value (7.19) was the most effective for distinguishing the cultivars.

In the present study, the average Shannon's information index (0.50 for RAPD and 0.52 for ISSR) was lower than those recorded for *Vaccinium myrtillus* – 0.55 [Albert et al. 2003], *Vaccinium uliginosum* – 0.65 [Albert et al. 2005], but higher than estimated by Zoratti et al. [2015] within 32 wild berry crops (*V. myrtillus*) which ranged between 0.11 and 0.24.

Polymorphism Information Content and expected heterozygosity are commonly used to estimate the degree of polymorphism and variation. A higher PIC value of a marker system means a higher capacity to detect polymorphism. In the present study, the RAPD and ISSR primers showed very similar discriminating capacities on genetic variation (0.31 and 0.30 respectively). A similar capacity to assess the polymorphism (PIC), based on RAPD markers found Bjedov et al. [2015] studying the populations of *V. myrtillus* (PIC = 0.27), *V. uliginosum* (PIC = 0.30), and *V. vitis-idaea* (PIC = 0.30), from the Balkans. In our study, expected heterozygosity reached medium values for both marker systems (from 0.26 to 0.45). However, very high levels of expected heterozygosity (He = 0.86) were observed among the blueberry genotypes by Debnath [2014b]. These high values are probably due to the genetic structure of the studied population which consisted of 28 wild clones, six cultivars and two selections of lowbush, half-high and highbush blueberries. Working with 102 wild cranberry clones collected from four Canadian provinces and five cultivars, An et al. [2015] reported that ISSRs showed the highest mean values of expected heterozygosity (0.97) followed by EST-SSR and EST-PCR.

Unquestionably, two marker systems used in the study showed the existence of significant, but homogeneous genetic diversity among the examined cultivars, due to the relatively distant position of the connections between the clusters compared to the beginning of the coordinate system. The results indicate that RAPD and ISSR markers can be used in genetic identification of blueberry cultivars and in genetic diversity study. Also, as stated Debnath et al. [2008], it can help breeders to select diverse parents with similar phenotypes for breeding purposes.

The 19 highbush blueberry cultivars were previously examined by us [Wach at al. 2016] and the genetic similarity matrix was produced on the basis of RAPD markers using the Jaccard's coefficient. Mean of genetic similarity was calculated at 0.41 and was lower than obtained in current study (0.58) using Dice formula. In contrast, the value of similarity for the RAPD markers varied in the range of 0.40–0.97 in Giongo et al. [2006] and 0.66–0.95 in Carvalho et al. [2014] research. While in a lowbush blueberry study these values reached the range of 0.39–0.99, however high values were obtained in the case when the same cultivars were represented by accessions coming from different geographical areas [Burgher et al. 2002].

We have further expanded our research by conducting ISSR analyzes. The average value of the genetic similarity (0.60) obtained in the present study for ISSR markers was similar to that obtained by Garriga et al. [2013]. The level of genetic similarity results mainly from the set of genotypes tested, and in Garriga et al. [2013] research it ranged between 0.52–0.74, in Zheng et al. [2014] 0.66–0.86, in Carvalho et al. [2014] reached the level of 0.60–0.96 for ISSR markers. ISSR markers used in relationship studies of *Vaccinium* species had higher then in presented research level of molecular variance among genotypes An et al. [2015], Debnath [2009]. Low values may reflect a high gene flow rate among cultivars as can be seen in their pedigree (tab. 1). On the other hand, are high when investigated were genetic lines, clones, cultivars and wild accessions often representing the natural geographic area of their distribution. A high level of genetic variation within individuals for RAPD markers was also observed by Debnath [2005] and Albert et al. [2003] in *V. macrocarpon* and *V. myrtillus* respectively.

In this study, dendrograms generated for RAPD and ISSR markers were analyzed at the same level of similarity. In Burgher et al. [2002] research based on RAPD markers, most clusters reflected known relat-

edness or geographic origin but in some cases closely related selections were not located in the same clades. In the presented study 'Brigitta Blue' cultivar, which was bred in Australia, formed a single cluster according to its geographical origin, whereas the 'Bonifacy' cultivar bred in Poland was grouped among the American cultivars, probably due to the fact that it was obtained from materials originating from cooperation with the US Department of Agriculture (USDA) [Pliszka 2004]. A diverse grouping of cultivars using UPGMA and PCA analysis were also reported by Boches et al. [2006], stating that often the parents of a given cultivar were genetically distant from each other, and the cultivar would cluster closely to only one of its parents on the dendrogram. In PCA, however, it did not group closely to either parent and was equidistant from both. In this work it was the case of 'Bonifacy' cultivar, which was grouped differently in relation to the parental forms 'Earliblue' and 'Darrow', which was shown on the dendrograms as well as in the PCA analysis, both for RAPD and ISSR markers. Moreover, despite the common origin of 'Bluecrop' and 'Blueray' cultivars, the applied set of RAPD markers did not reflect that similarity. However, the 'Blueray' has formed shared cluster with the 'Herbert', which in this case can result from having common ancestors, such as 'Jersey', 'Pioneer' and 'Stanley' cultivars. Even though the use of the analyzed number of markers provides a basis for distinction between cultivars, nevertheless explanation less than 60% of the total variance in PCoA (Principal Coordinate Analysis) analysis suggest add more loci to fully scatter the cultivars in the dispersion using molecular data [Anderson and Willis 2003]. Furthermore, according to Mańkowski et al. [2011], when lower level of the variability was observed in the first two principal components (about 40%) more clusters would be expected to occur which in our study was observed in the case of RAPD markers. Moreover, the cause of the differences in the obtained results between UPGMA and PCA may lie in the specificity of cluster analysis as well as in the principal component method, in which the reduction of dimensions of space data, as stated by Kenkel [2006], is associated with the loss of the part of information on the characteristics of the tested objects. Another factor, explaining why UPGMA derived dendrogram did not reflect known pedigree might be incomplete coverage of the genome [Degani et al. 2001] or insufficient number of fragments scored in the analysis [Oraguzie et al. 2001]. Also, in Yu et al. [2009] research, genetic distance between cultivars did not agree well with known pedigree data and thus it indicated that ISSR data obtained by primers used in this study did not accurately assess the genetic relationship of cultivars within one group. As a way to overcome this problem An et al. [2015] propose combined use of three types of molecular markers in *Vaccinium melanocarpa* L. species to detect sufficient degree of variation allowing for genotype identification in a diverse cranberry germplasm and for more efficient parental choice in the current breeding program.

CONCLUSION

These research identified markers with high capability to distinguishing blueberry cultivars grown in south-east part of Poland. It also indicated the usefulness of molecular markers to assess the genetic diversity which helps breeders select initial materials for breeding purposes. Moreover, it seems that the existing genetic variability between cultivars is sufficient to use these with the highest agronomic value in hybridization programme, which can lead to the improvement of desirable characteristics.

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