НАЦИОНАЛЬНАЯ АКАДЕМИЯ НАУК БЕЛАРУСИ

ЦЕНТРАЛЬНЫЙ БОТАНИЧЕСКИЙ САД

КУЛЬТУРА БРУСНИЧНЫХ ЯГОДНИКОВ: ИТОГИ И ПЕРСПЕКТИВЫ

Материалы Международной научной конференции
Минск, 15-19 августа 2005 года

Минск 2005

УДК 581.522.4:634.739.3:631.5
ББК

Культура Брусничных ягодников: итоги и перспективы: Материалы Международной научной конференции. Минск, 15-19 августа 2005 г. – Минск: 2005. - … с.

Представлены результаты исследований учёных Беларуси, России, Украины, Эстонии, Польши, Словакии, Чехии. В них отражена экологическая проблематика и перспективы развития нетрадиционного ягодоводства, интродукции и селекции, биотехнологии и переработки ягодных растений сем. *Брусничные* в Беларуси и странах ближнего и дальнего зарубежья.

Материалы конференции изданы при финансовой поддержке Белорусского республиканского фонда фундаментальных исследований.

APPLICATION OF DNA AND PROTEIN MARKERS FOR THE ASSESSMENT OF GENETIC POLYMORPHISM AND FOR PASSPORTISATION OF HIGHBUSH BLUEBERRY VARIETIES

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Abstract

The new stage in the selection and genetic studies of highbush blueberry collection of the Central Botanical Gardens is a search for molecular markers allowing to carry out genotyping of the varieties of this culture. The fast and simple method of DNA isolation from leaf tissue was developed, effective primers were selected and conditions for carrying out polymerase chain reaction were optimized. The method of RAPD-analysis for population genetics research and certification of blueberry was adapted. Additional criteria for identification of blueberry varieties were proposed on the basis of the cumulative analysis of spectra of saline- and alcohol-soluble proteins. For the thirty varieties of blueberry introduced to Belarus genetic passports were prepared, with five passports being supplemented with protein markers such as albumins, globulins and gliadins of seeds.

Introduction

Highbush blueberry (*Vaccinium × covillcanum Butcus et Pliszka)* is a valuable food and medicinal plant. This crop has been obtained as a result of hybridization between three North American species of blueberries and it is currently grown on industrial plantations worldwide, including Republic of Belarus. Nowadays there are more than 100 cultivars of blueberry with different agricultural properties, such as plant height, terms of ripening etc. Since 1983 the Central Botanic Gardens (CBG) of NAS of Belarus runs a program for introduction of highbush blueberry to national agriculture. As a result of the long-term investigations it has been proved that cultivation of highbush blueberry in Belarus is economically reasonable and that this culture is more preferred for cultivation than the native species, the bog blueberry (*Vaccinium uliginosum*) (Kurlovich, Bosak, 1998). The blueberry cultivars characterized by stable year-to-year yield and high quality of berries were defined. The climatic zones in Belarus suitable for cultivation of this culture were selected and technology of industrial cultivation was elaborated. The collection of more than 30 varieties of highbush blueberry was founded. The technology of producing planting material from lignified and green cuttings as well as *in vitro* propagation methods were elaborated that will allow to fill national agriculture requirement for planting material. Therefore, identification and certification of the planting material of highbush blueberry has become a crucial task.

Modern analytical methods of analysis of biological macromolecules allow plant genotype identification on principally new basis and to solve problems associated with protection of property rights in the field of selection, to control stability of cultivars and purity of planting material (e. g., from pathogens and viruses). New diagnostic methods using biochemical and molecular markers-proteins and nucleic acids, have successfully complemented traditional approaches for plant

material identification which were based mainly on morphological and physiological characteristics.

The protein markers aimed at being used for cultivar identification have to meet following conditions: 1) markers should be quickly and easily determined, and 2) the given proteins should be expressed constantly and at constant levels irrespective of age of plants and conditions of their cultivation. Due to this only limited number of proteins can serve as markers. Therefore certification of plants nowadays predominantly relies on the DNA-based techniques, with the analysis of protein spectra serving as supplementary to further detail characterization of plant species and varieties.

Present study implemented RAPD (random amplified polymorphic DNA) analysis to assess degree of genetic divergence among varieties of highbush blueberry. RAPD technology has a number of advantages over other methods using molecular markers: it requires small amounts of DNA (5-25 ng per an analysis) meaning that amount of DNA from a single plant leaf may suffice. This method does not require knowledge about DNA to be amplified, it uses arbitrary chosen primers and it circumvent use of radioactivity. Speed of the analysis is also a significant advantage: it takes only 24 hours from the plant material collection to the scoring of results of DNA electrophoresis. Disadvantage of this method is the dominant character of marker inheritance (which is different from the RLFP markers that have co-dominant character of inheritance). In this work we present RAPD analysis-based genetic passports of *Vaccinium covilleanum* cultivars that are maintained in the Central Botanic Garden collection, as well as elecrophoretic spectra of saline-(albumins and globulins) and alcohol-soluble (gliadins) proteins from seeds of 5 *Vaccinium covilleanum* varieties.

Materials and methods

The nine cultivars of *Vaccinium covilleanum* (Tifblue, Atlantic, Weymouth, Concord, Blueray, Aiwengo, Dixi, Rancocas, Erliblue) tha currently are being introduced at Biological Experimental Station «Zhuravinka» of CBG NAS of Belarus. Total DNA was isolated from plant leaves and buds (Doyle J.J. and Doyle J.L., 1990), stem cuttings and from plant material, obtained *in vitro*. Five RAPD primers from twenty commercial primers were selected, conditions of PCR and visualization techniques were as (Westermeier, 2001).

The electrophoretic spectra of saline- (albumins and globulins) and alcohol-(gliadins) soluble proteins from seeds of the five cultivars of *Vaccinium covilleanum* (Tifblue, Atlantic, Weymouth, Concord, Blueray) were obtained. Berries were collected at full ripeness stage (completely pigmented). For extraction of saline-soluble proteins from seeds the phosphate buffer (pH 7.2) with 1M NaCl and for alcohol soluble – 70% ethanol were used. Albumins and globulins from grinded seeds were extracted twice with saline buffer [seeds: buffer ratio 1:3 w/v]. The remaining pellet was extracted once with 70% ethanol [pellet: spirit ratio 1:1 (w/v)] and centrifuged at 4000 g for 30 min to clear supernatant (containing alcohol-soluble proteins fraction). The protein content was determined according to Bredford (1976). Proteins were separated in denaturing polyacrylamide-SDS gel

(Laemmli, 1970) along with protein marker14,4 – 97,0 kD set ("Pharmacia"). The molecular masses of proteins were estimated using the program Sigma Gel (Germany).

Results and discussion

First we have tested different plant tissues of blueberry to select those that are most suitable for the total DNA isolation. We concluded that leaf plates of the green cuttings and the whole young plants obtained via microclonal propagation are most suitable for this purpose and give the highest yield of high-quality DNA. Since the biomorphological parameters of plants are influenced by weather and climatic conditions they can not always serve as easy obtainable and reliable criteria for identification of blueberry varieties. Therefore we have implemented and optimized RAPD-technology to distinguish between the varieties of the collection of CBG. Twenty decanucleotide primers with viriable GC content were tested. Electrophoretic analysis of the RAPD PCR products amplified using 20 primers and DNA templates of different highbush blueberry varieties revealed a wide spectrum of DNA amplicons. Further, five primers were selected that gave sharp polymorphic bands and allow differentiation between varieties. Among DNA amplicons 19 bands were scored from which 11 were polymorphic. On the basis of the received RAPD-spectra for all investigated varieties multi-locus RAPD-passports were prepared (Table). Note, that in the Table 1 only those DNA amplicons are listed that are easily discernable in the agarose gel and are polymorphic. To quantitatively estimate degree of RAPD polymorphism and to define the level of divergence between blueberry varieties the data of RAPD analysis were submitted as a matrix of binary codes: presence of a fragment was assigned as 1, absence – as 0. The designation of zones (amplicons) is given under the name of the corresponding primer with the size of the DNA fragment (in base pairs) given as diacritical mark. For example, a 430-bp zone in the spectrum of PCR products produced with primer with Oligo 3 primer has been designated as Oligo 3^{430} (Fig. 1).

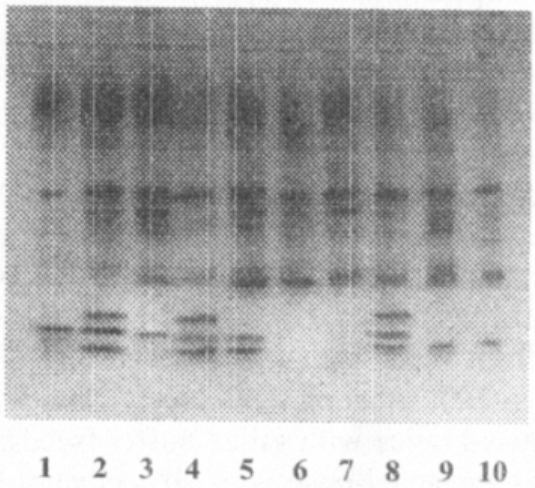

Fig. 1. **RAPD-spectrum of the blueberry varieties by Oligo 3 primer**
(1 –Bluecrop; 2– Tifblue, 3–Atlantic, 4–Weymouth, 5–Concord, 6–Blueray, 7–Aiwengo, 8–Dixi, 9–Rancocas, 10–Earlyblue)

Table. Multi-locus genetic passports of highbush blueberry varieties, based on RAPD-analysis

Locus		highbush blueberry varieties								
		Aiwengo	Tifblue	Weymouth	Concord	Blueray	Dixi	Rancocas	Earlyblue	Atlantic
Oligo 1										
	510	1	0	1	1	1	1	1	0	0
	705	1	1	1	1	1	1	1	1	1
	980	1	1	1	1	1	1	1	1	1
	1140	1	1	1	1	1	1	1	1	1
Oligo 3										
	430	1	0	1	1	0	0	1	1	1
	480	1	1	1	1	0	0	1	0	0
	500	1	0	1	0	0	0	1	0	0
	720	0	1	0	1	0	0	1	1	0
	730	0	1	1	0	1	1	1	1	1
	1800	1	1	1	1	1	1	1	1	1
Oligo 9										
	370	1	1	1	1	1	0	0	1	1
	650	1	1	1	1	1	1	1	1	1
Oligo 10										
	180	1	0	0	1	0	0	0	0	0
	300	1	1	1	1	1	0	0	0	1
	405	0	1	0	1	1	1	1	1	1
	730	1	1	1	1	1	1	1	1	1
	1480	1	1	1	1	1	1	1	1	1
Oligo 11										
	750	1	1	1	1	1	1	1	1	1
	790	1	1	1	0	0	0	0	1	0

Another focus of our research is the search for protein markers suitable for identification of blueberry varieties that may enforce the data of the RAPD-analysis and to expand the list of criteria for plant material certification. For this purpose the electrophoretic analysis of saline- (albumins and globulins) (Fig. 2) and alcohol-soluble (gliadins) proteins of seeds of 5 highbush blueberry varieties (Tifblue, Atlantic, Weymouth, Concord, Blueray) has been carried out.

For convenience the spectra of albumins and globulins of seeds of blueberry have been arbitrary divided into 5 zones (I – proteins from Mm ≥100kDa; II - 75-50 kDa; III – 37-25 kDa; IV – 25-15 kDa; V – 15-10 kDa).

Among the high-molecular proteins (≥100кД) following polypeptides can be used as markers: a polypeptides with Mm of 163 kDa (low expression in var. Tifblue and high – in the other varieties analyzed); 155 kDa (high expression in var. Tifblue, low – in var. Weymouth and Concord; and full absence at all other varieties), 147 and 101,5 kDa (expressed only in var. Tifblue and Atlantic).

Among group II polypeptides: var. Tifblue, Weymouth, Concord are characteristic by presence of polypeptides with Mm 76; 73,5; 70 and 67 kDa. The var. Blueray express only proteins with Mm 70 and 67 kDa, whereas the var. Atlantic has no such protein bands in its spectrum, but possesses a number of bands with Mm 55,5; 53; 50; 48 kDa highly expressed (the latter are also abundant in the var. Blueray). Less abundantly these proteins are represented in seeds of var. Concord, and are hardly distinguishable in the spectra of the var. Tifblue and Weymouth.

The proteins from group III by itself cannot serve as markers of varieties as their presence and high expression are shown for all investigated varieties. Still, there is a proteins with Mm 37-25 kDa which may discerns such varieties as Tifblue, Weymouth and Concord.

The group IV proteins (Mm 25-15 kDa) seem to be good candidates for identification of highbush blueberry varieties. Protein 22 kDa highly expressed in var. Tifblue, Weymouth and Concord but is much less abundant in var. Atlantic and Blueray. Polypeptide of 20 kDa is strongly pronounced in the protein spectrum of the var. Tifblue but is rather poor in the others.

Among the group V proteins (Mm 15-10 kDa) no significant differences between varieties was found.

Apparently, among saline-soluble proteins there is a number of polypeptides which allows to distinguish seeds of 5 varieties of highbush blueberry. However, using only saline-soluble protein markers, it is impossible to distinguish between such varieties as Woodard and Pioneer, possessing identical spectra of albumins and globulins (data are not presented since no RAPD-passports are produced for these varieties yet). Therefore, saline-soluble proteins can serve for identification of varieties of a highbush blueberry only in complex analysis exploiting other protein fractions, for example, alcohol-soluble. The spectra of alcohol-soluble proteins of seeds of all 5 analyzed varieties of blueberry were highly similar. Still, to distinguish var. Concord one may rely on the presence of polypeptides with Mm 45,5; 31; 29 and 4 kDa (high expression), for var. Atlantic and Weymouth - 6 kDa (high expression), and for var. Tifbluel –4,5 kDa (high expression).

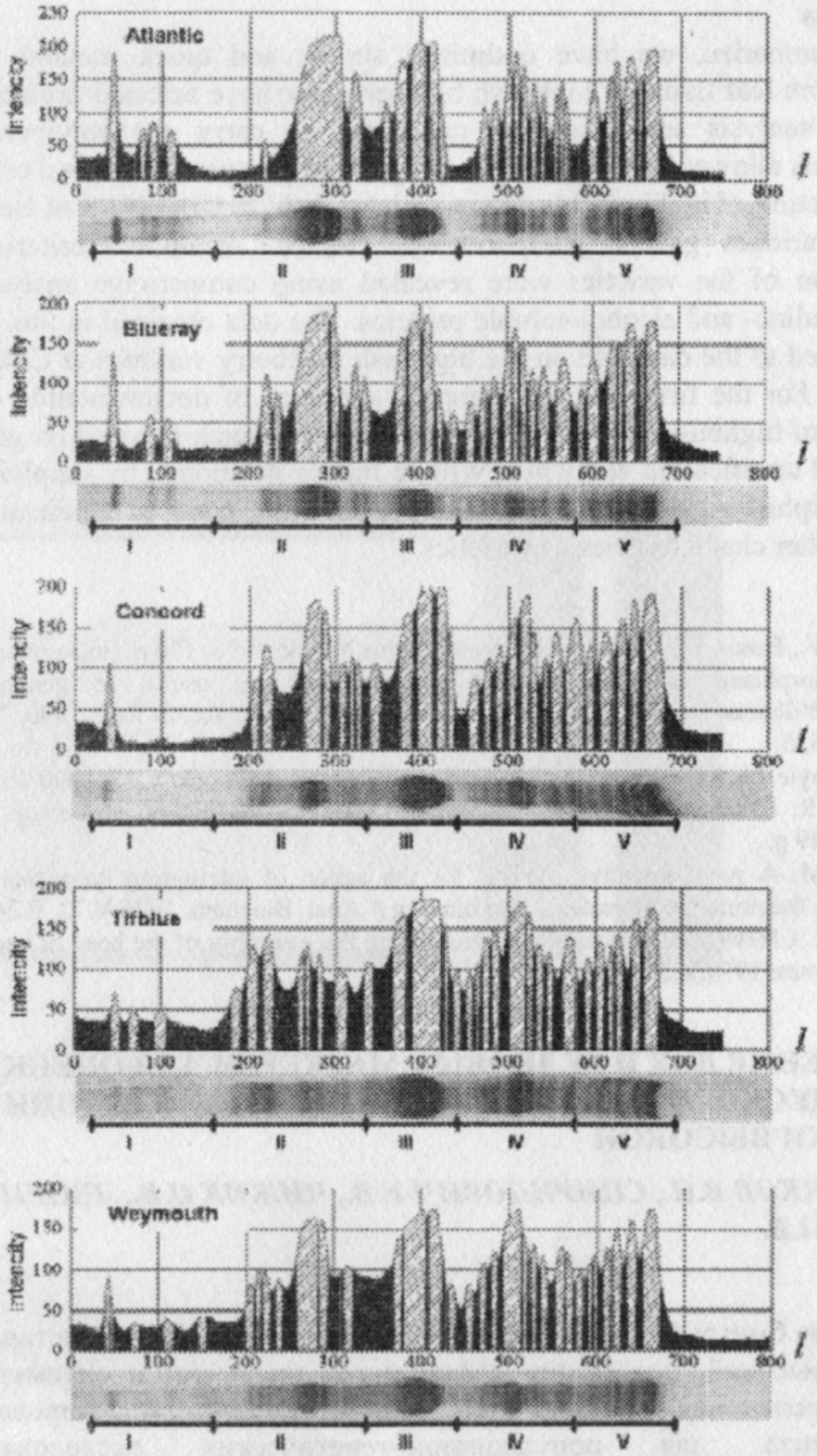

Fig. 2. **Densitometry and electroferetic spectra of saline-soluble proteins of the Blueberry highbush seeds of varieties Tifblue, Atlantic, Weymouth, Concord, Blueray**: I – the group of proteins with Mm ≥100kDa; II - Mm 75-50 kDa; III – Mm 37-25 kDa; IV – Mm 25-15 kDa; V – Mm 15-10 kDa). X- axis – the length of the trek in pixels; Y-axis – light absorption. Densitometry is carried out using SigmaGel–Gel Analysis Software (Sigma, Germany).

Conclusions

To summarize, we have optimized simple and quick method for DNA isolation from leaf tissue of highbush blueberry, we have selected primers suitable for RAPD-analysis and optimized conditions to carry out polymerase chain reaction. This allowed us to initiate the program of passportization and certification of the collection of highbush blueberry varieties held at CBG NAS of Belarus. For the nine varieties genetic passports were issued. Additional criteria for the identification of the varieties were revealed using comparative analysis of the spectra of saline- and alcohol-soluble proteins. The data obtained in this work will be transferred to the database on the highbush blueberry varieties at CBG of NAS of Belarus. For the first time the complex approach of documentation of unique collections of highbush blueberry was implemented which is based on genetic and biochemical certification and which will be further developed by supplying digital photos, morphological and physiological description, areas of application of the plant and other characteristics of varieties.

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ПРИМЕНЕНИЕ ДНК И БЕЛКОВЫХ МАРКЕРОВ ДЛЯ ОЦЕНКИ ГЕНЕТИЧЕСКОГО ПОЛИМОРФИЗМА И ПАСПОРТИЗАЦИИ ГОЛУБИКИ ВЫСОКОЙ

РЕШЕТНИКОВ В.Н., СПИРИДОВИЧ Е.В., ЧИЖИК О.В., ЛЕНЕЦ А.А., ЗУБАРЕВ А.В.

Реферат

Найден быстрый и простой метод выделения ДНК из листовой ткани голубики высокой, подобраны эффективные праймеры и оптимизированы условия проведения полимеразной цепной реакции. Адаптирован метод RAPD-анализа для популяционно-генетических исследований и паспортизации голубики высокой. Выявлены дополнительные критерии идентификации сортов голубики на основе совокупного анализа спектров соле- и спирторастворимых белков. Для 30 сортов-интродуцентов голубики высокой составлены генетические паспорта, для 5 из них – дополнены сведениями о наличии белковых маркеров среди альбуминов, глобулинов и глиадинов семян данных сортов голубики.