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Analysis of peonies cultivars and their parental forms using RAPD primers

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It is very important to select genetically and morphologically different individuals creating new, physiologically different peony cultivars. Genetic differences of morphologically similar species cannot be assessed only by comparing the external features. Investigated peonies varied morphologically by the height, type of blossom, shape and color, aromatic features, strength and number of stems, leaf density and color, flowering time and duration. Genetic differences between 13 different peony cultivars, which are growing in the Kaunas Botanical Garden of Vytautas Magnus University, were compared using a random amplified polymorphic DNA (RAPD) method. 9 RAPD primers were chosen for a genetic analysis. The number of DNA fragments varied from 1 to 15, and their length from 100 to 1 800 base pairs (bp). Most of polymorphic DNA fragments were amplified with four chosen primers: MP-8, OPB-7, OPA-18 and OPB-8. A total number of different-sized DNA fragments ranged from 1 to 15, and their length ranged from 100 to 1 800 bp. The genetically most distant peony cultivars were 'Skeivienės vėlyvasis' and 'Garbė Motinai'. The genetically closest peony cultivars were 'General Mac Mahon' and 'Germaine Burgos'. The genetically closest cultivar to its parental forms was 'Freda', and the most distant cultivars were 'Darius–Girėnas' and 'Skeivienės vėlyvasis'.

Key words: *Paeonia lactiflora*, cultivar, Lithuanian, RAPD

INTRODUCTION

The gene pool of plants, animals and other useful living organisms is an important national estate of every country. Numerous plant collections are stored in science and study institutes of Lithuania.

According to plant groups, storage and research of national plant genetic diversity is coordinated in these science and study institutes: Lithuanian Institute of Agriculture, Lithuanian Forest Research Institute, Lithuanian Institute of Horticulture and Floriculture, Vilnius University Botanical Garden and the Ornamental Plants Sector of Vytautas Magnus University Kaunas Botanical Garden (Varkulevičienė et al., 2006; Dapkūnienė et al., 2007). Peonies were cultivated firstly in China and Japan because warm full winter over there is perfect for growth of herbaceous and tree peonies. Intensive genetic researches of peonies have been carried out in China and Japan since 1996 (Cheng et al., 2011).

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The first molecular researches of tree peonies were carried out using the RAPD method. This method was successfully used for identifying genetic kin as well as for new cultivar identification. Most of the studies focused on the establishment of phylogenetic relationships among wild species or interspecies. A few molecular marker techniques have been applied to analyze tree peony cultivars such as RAPD (Chen et al., 2001; SU et al., 2006), AFLP (Liu et al., 2006) and ISSR (Suo et al., 2004; Suo et al., 2005). Big attention is focused on biochemical researches of peonies (Bomi et al., 2008).

A DNA polymorphism analysis was made on herbaceous peonies species and cultivars in the Lithuanian Institute of Horticulture and Floriculture (LIH) (Mažeikienė et al., 2007). Peonies are widely used in selection because their genes change slowly and / or do not change for many years (Halevy et al., 2002). Comparison of peonies external characteristics lack capabilities of evaluating how peony species or cultivars are genetically different (Mažeikienė et al., 2008).

After a morphological trait evaluation 10 peony species and 7 cultivars were rated and genetically evaluated in the ornamental plant collections in LIH (Mažeikienė et al., 2008). Researches were conducted on these peonies: 2 examples of *Paeonia lactiflora*, 2 seedling forms of *P. officinalis* (alba plena and rose plena), *P. tenuifolia*, *P. tenuifolia* var. plena, *P. peregrina*, *P. daurica*, *P. anomala*, *P. veitchii* and five *P. lactiflora* cultivars and hybrids created by O. Skeivienė – 'Garbė Motinai', 'Virgilijus', 'Prof. K. Grybauskas', 'Maironis', 'Darius–Girėnas', and 2 worldwide cultivars created by French plant breeders: 'Sarah Bernhard' and 'Festiva Maxima'. Herbaceous peonies in the ornamental plants collection in LIH show a large variety of morphological features. Peony species and cultivars were polymorphic in different ways according to the basis of morphological and genetic characteristics (Mažeikienė et al., 2008). Search of genes and their expression researches allow accessing the peony plant cell genetic response to environmental conditions (Mažeikienė et al., 2010). Peony genetic alterations in low temperatures were also studied (Mažeikienė et al., 2010).

Peony introduction and selection were performed by Ona Skeivienė in the Kaunas Botanical Garden of Vytautas Magnus University since 1947. During 1953–1958 a comparable rating of created hybrids was made. There were selected 6 promising hybrids: 'Skeivienės vėlyvasis', 'Virgilijus', 'Darius–Girėnas', 'Garbė Motinai', 'Freda', 'Prof. K. Grybauskas' (Skevienė, 1964). Three hybrids – 'Garbė Motinai', 'Prof. K. Grybauskas' and 'Virgilijus' – were created locally in 1976. Copyright was granted to the originator Ona Skeivienė in 1979. Ona Skeivienė is an author of other 16 hybrids, that are candidates for the cultivars. The peonies collection was mentored by Ona Skeivienė till 1976 (Varkulevičienė et al., 2005, 2006; Varkulevičienė et al., 2013). Genetic relatedness of cultivars and hybrids of Lithuania and introduced species and cultivars has not been studied extensively.

This study evaluated the genetic difference of a total of 13 samples including Lithuanian 6 peony cultivars and their parents.

MATERIALS AND METHODS

DNA extraction from peonies. Thirteen peony cultivars (6 Lithuanian cultivars and 7 parental forms) were chosen from the field collection in the Kaunas Botanical Garden of Vytautas Magnus University (Table 1).

Fresh young leaf material was sampled in spring. DNA extraction was made using a CTAB (Murray and Thompson, 1980) method modified by Areškevičienė (2009).

DNA analysis by RAPD method. Nine primers were used for the RAPD-PCR analysis (Table 2). DNA amplification reaction was performed in the 25 µl reaction mix made of 12.5 µl 2 × PCR Master Mix (0.05 U/µl Tag DNA polymerase; 4 mm $MgCl_2$; 0.4 mM each dNTP) (Thermo Scientific, Lithuania), 2 µl primer (concentration 10 pmol/µl) (Roth, Germany), 6.5 µl twice distilled water and 4 µl (25 ng/µl) DNA. PCR reaction was performed in a thermo cycler (Master Cycler®, Eppendorf,

Table 1. Pedigrees of the Lithuanian peony cultivars and their parental forms in this study

Parental forms	Peony cultivars					
	'Virgilijus'	'Garbė Motinai'	'Prof. K. Grybauskas'	'Freda'	'Darius–Girėnas'	'Skeivienės vėlyvasis'
'Auguste Dessert'	x			x		
'Germaine Burgos'		x			x	
'General Mac Mahon'			x			
'Perette'			x	x		
Madame Calot'					x	
'Eugene Verdier'						x
'Germaine Bigot'						x

Germany) under the following conditions: initial denaturation for 3 min at 94 °C, 45 cycles of denaturation for 45 s at 94 °C, primers annealing for 45 s at 32–52 °C (depending on the primer), extension for 45 s at 72 °C followed by a final extension for 5 min at 72 °C.

The amplified DNA fragments were separated according to their molecular weights using electrophoresis on 1.5% agarose gel prepared in a 1XTBE buffer. A standard molecular marker – Gene Rulers™ DNA Ladder Plus (Thermo Scientific, Lithuania) – was used to determine the molecular weight of the amplified bands. Gels were examined visually under UV light using ethidium bromide. The gels were photographed and documented with a Hero Lab transilluminator and a Win32 system (Hero Lab, Germany).

Statistical analysis. Relationships among peonies cultivars and parental forms were evaluated using a dendrogram based on Nei and Li's (1979) genetic distances. It was generated by the UPGMA (unweight pair group method) cluster analysis method. Calculation of genetic distances and the UPGMA cluster analysis were performed with the TREECON program for Windows V 1.3b (Nei, Li, 1979). Statistical analysis was performed using the Gen Alex 6 program (Peakal, Smouse, 2006). Calculation of the observed number of alleles, Nei's gene diversity (H), Shannon's Information Index (I), Wright's Fixation Index (F = 1-Ho/He) and generation of a Nei's genetic distance based dendrogram were carried out with the POPGENE V 1.31 software.

RESULTS AND DISCUSSION

We investigated the phylogenetic relationship among Lithuanian parental forms and cultivars of peony. That kind of research has been done for the first time.

The nine RAPD primers detected a total of 87 fragments ranging from 100–1 800 bp in 13 peonies cultivars. Most fragments were detected with the OPA-18 primer. The primer OPB-19, however, generated only one fragment (Table 2).

Parental forms and cultivars are described in Table 1. The most common fragments were detected among the cultivar 'Skeivienės vėlyvasis' and its parental forms 'Germaine Bigot' and 'Eugene Verdier'. Numbers of common fragments of 'Skeivienės vėlyvasis' with its two parental forms are 20 and 10, respectively. 'Garbė Motinai' has the least common fragment with the parental form 'Germaine Burgos'. They have only one common fragment (Table 3).

The maximum quantity of fragments was amplified using the primers OPA-18, OPB-8 and MP-8. 'Skeivienės vėlyvasis' and its parent 'Germane Bigot' have the most common fragments. They have four common fragments. 'Garbė Motinai' and 'Germaine Burgos' have no common fragments.

With the primer OPA-18 15 fragments were detected. 'Skeivienės vėlyvasis' and its parents remain the lineages with the most common fragments, i. e. 4 fragments between 'Skeivienės vėlyvasis' and 'Germaine Bigot', and 3 between 'Skeivienės vėlyvasis' and 'Eugene Verdier'.

Table 2. Primers and their sequences used for RAPD analysis

Primers	Primers sequences	Length of fragments, bp	Number of fragments
OPB-7	5'-AGG TGA CCG T-3'	390–1 350	10
OPB-8	5'-GGT GAC GCA G-3'	480–1 800	13
OPB-17	5'-GTC CAC AGG G-3'	340–1 600	11
OPB-19	5'-AGG GAA CGA G-3'	400	1
OPA-7	5'-ACC CCC GAA G-3'	620–1 480	6
OPA-8	5'-GTC ATG CCT GGA-3'	450–1 100	9
OPA-18	5'-GTA AAA CGA CGG CCA TG-3'	350–1 500	15
MP-5	5'-GAA ACG GGT G-3'	350–1 700	8
MP-8	5'-GTG ACG TAG G-3'	100–1 400	13
Total:		100–1 800	86

'Garbė Motinai' and 'Germaine Burgos', 'Freda' and 'Darius–Girėnas' and their parental forms have no common fragments.

With the primer OPB-8 13 fragments were identified (Fig. 1). It is one of the best-performing primers. The most common fragments come from 'Skeivienės vėlyvasis' and two of its parents 'Germaine Bigot' (4 common fragments) and 'Eugene Verdier' (3 fragments). 'Garbė Motinai' and 'Germaine Burgos', 'Darius–Girėnas' and 'Germaine Burgos', 'Prof. K. Grybauskas', 'Freda' and their parental forms had no common fragments detected by OPB-8.

There are 13 fragments identified with the primer MP-8. The most common fragments come from 'Skeivienės vėlyvasis' and its parent 'Germaine Bigot' (5 common fragments) (Fig. 2). Common fragments come from 'Garbė Motinai' and 'Germaine Burgos'. Lithuanian cultivars and their parental forms have no common fragments with this primer.

There are no common fragments with the primer OPA-7. With primers OPA-8 one common fragment was detected from the species 'Skeivienės vėlyvasis' and 'Germaine Bigot'. With the MP-5 primer common fragments were detected from 'Freda' and two of its parents 'Auguste Dessert' (1 common fragment) and 'Perette' (2 common fragments), 'Skeivienės vėlyvasis' and one of its parents 'Germaine Bigot'. Other cultivars had no common fragments detected with their parental

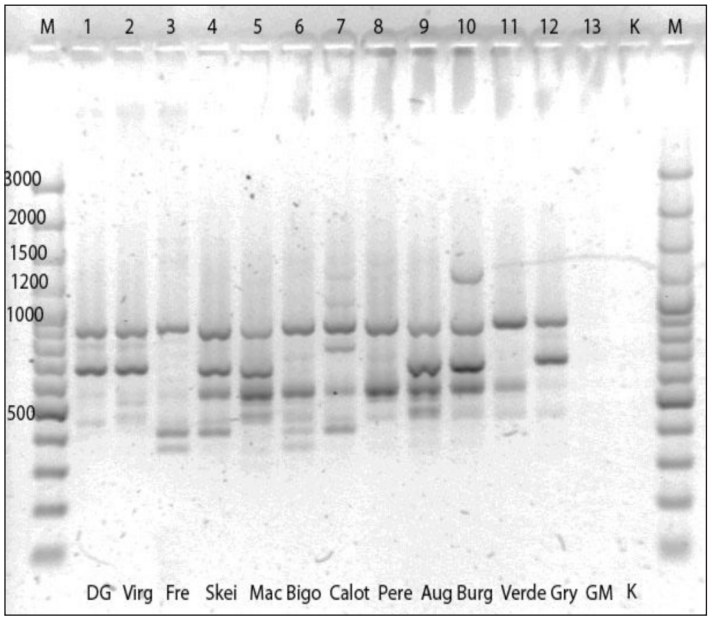

Fig. 1. PCR spectrum of the OPB-8 primer. Cultivars: track 1 – 'Darius–Girėnas'; track 2 – 'Virgilijus'; track 3 – 'Freda'; track 4 – 'Skeivienės vėlyvasis'; track 5 – 'General Mac Mahon'; track 6 – 'Germane Bigot'; track 7 – 'Madame Calot'; track 8 – 'Perette'; track 9 – 'Auguste Dessert'; track 10 – 'Germaine Burgos'; track 11 – 'Eugene Verdier'; track 12 – 'Prof. K. Grybauskas'; track 13 – 'Garbė Motinai'

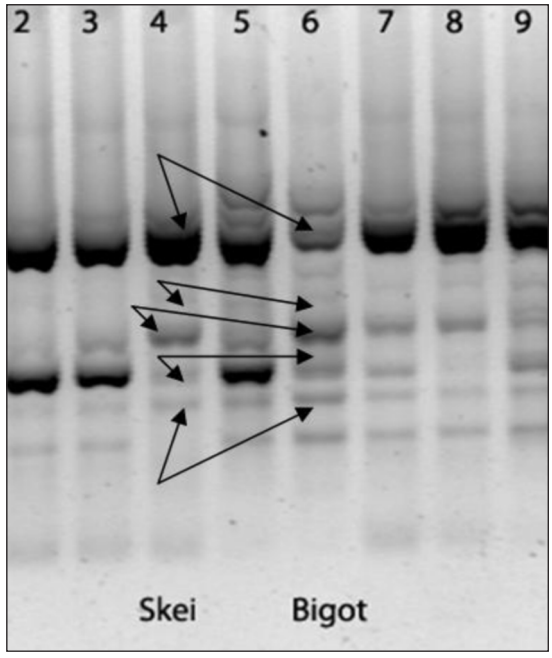

Fig. 2. MP-8 primer part of the spectrum. Common fragments of 'Skeivienės vėlyvasis' (track 4) and its parental form 'Germane Bigot' (track 6)

forms by this primer. There were no common fragments detected between Lithuanian peony cultivars and parental forms had no common fragments detected by the OPB-17. The dendrogram analysis (Fig. 3) showed that the most distant peony cultivars were 'Skeivienės vėlyvasis' and 'Garbė Motinai'. The genetically closest cultivars are 'General Mac Mahon' and 'Germaine Burgos'.

According to the number of common fragments among parental forms and cultivars (Table 3), the dendrogram data showed that the most common parental form was 'Freda', and the most distant ones were 'Darius–Girėnas' and 'Skeivienės vėlyvasis'. There were no researches on 'Pierre Reignoux' and the second parental form of 'Virgilijus' and 'Garbė Motinai'.

The NJ tree, however, can give us instructive information. Increasing the knowledge of the molecular diversity of a crop is essential for extending its genetic base, identifying cultivars, and selecting parental varieties for breeding programs. In the sense, tree peony cultivars are poorly characterized. Although many classification methods including many molecular marker systems have been applied to these species, the cultivars are still classified mainly based on traditional phenotypic characteristics such as flower color, flower form, and geographic distribution (WANG and STRECH, 2001).

A UPGMA tree is constructed using the informative fragments as an agent of genetic distance for these peony cultivars (Fig. 3). All branches are statistically supported with the bootstrap value above 25%. Peonies are divided into two clusters according to this dendrogram. The cultivar 'Garbė Motinai' stands separately from these clusters. The parental form 'Germaine Burgos' of this cultivar is in

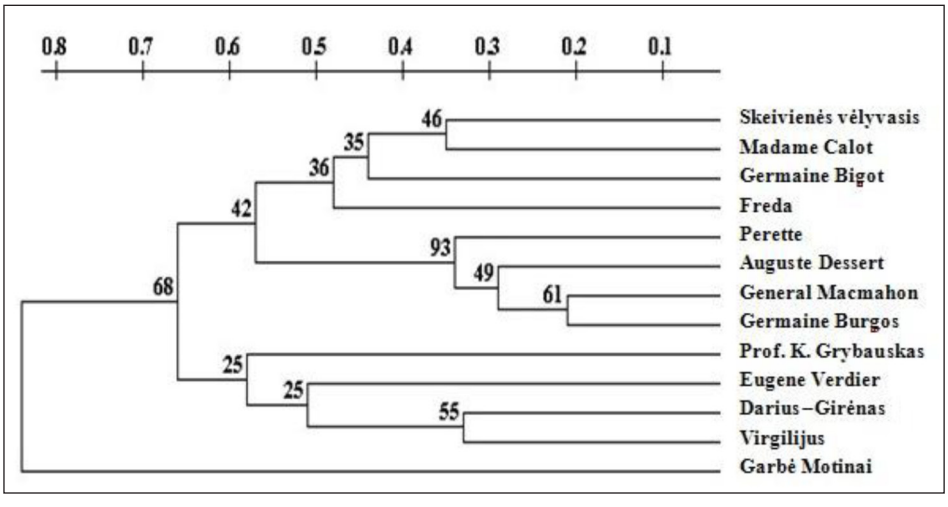

Fig. 3. Dendrogram of the investigated peony cultivars

Table 3. Comparison of common fragments of the Lithuanian peony cultivars and their parental forms identified using RAPD primers

Cultivar	Parental form	Common fragment number
'Virgilijus'	'Auguste Dessert'	8
'Garbė Motinai'	'Germaine Burgos'	1
'Prof. K. Grybauskas'	'General Mac Mahon'	7
'Prof. K. Grybauskas'	'Perette'	5
'Freda'	'Auguste Dessert'	7
'Freda'	'Perette'	7
'Darius–Girėnas'	'Germaine Burgos'	5
'Darius–Girėnas'	'Madame Calot'	5
'Skeivienės vėlyvasis'	'Germaine Bigot'	20
'Skeivienės vėlyvasis'	'Eugene Verdier'	10

different cluster of the dendrogram. The cultivars 'Virgilijus', 'Prof. K. Grybauskas' and 'Darius–Girėnas' are located in different clusters than their parental forms. One parental form of the cultivar 'Skeivienės vėlyvasis' is in different cluster 'Germane Bigot' and other parental form of 'Eugene Verdier' is in next cluster. 'Freda' and its parental forms are in the same cluster. According to peony distribution in the dendrogram we can conclude that Lithuanian peony cultivars and their parental forms in clusters are located independently of their genetic relationships. Parental forms can be in the first, second or both first and second clusters, regardless of which cluster cultivar belongs to.

In our research, the Lithuanian hybrid 'Garbė Motinai' occupied a separate branch in the dendrogram. That is why it should attract the interest of peony breeders. Other Lithuanian researches separated Lithuanian cultivars such as 'Garbė Motinai', 'Virgilijus', 'Profesorius K. Grybauskas' and hybrids 'Maironis' and 'Darius Girėnas' genetically closer to French cultivars, but highly genetically distant from both investigated genotypes of wild *Paeonia lactiflora* species (Mažeikienė et al., 2007). In our research, genetic similarity of Lithuanian clones to other countries has not been tested, but some of cultivars that are in the field of interest of other Lithuanian researches are separated in one cluster. This could be because of their origin. Lithuanian peony cultivars and their parental forms in clusters are located independently of their genetic relationships.

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BIJŪNŲ VEISLIŲ IR JŲ TĖVINIŲ FORMŲ ANALIZĖ NAUDOJANT RAPD ŽYMENIS

Santrauka

Kuriant naujas augalų veisles labai svarbu pasirinkti genetiškai ir morfologiškai besiskiriančius augalus. Genetiniai skirtumai morfologiškai panašių rūšių negali būti vertinami tik pagal išorines savybes. Vytauto Didžiojo universiteto (toliau – VDU) Kauno botanikos sodo bijūnų kolekcijoje bijūnų veislės turi labai skirtingas morfologines savybes: skiriasi pagal aukštį, žiedų tipą, formas ir spalvas, aromatines funkcijas, jų stiprumą, o taip pat stiebų skaičių, lapų tankį ir spalvą, žydėjimo laiką ir trukmę. VDU Kauno botanikos sodo bijūnų kolekcijoje pavasarį buvo atlikti DNR tyrimai. Tirta puikiojo bijūno (*Paeonia lactiflora*) 6 lietuviškos veislės ir 7 tėvinės formos. Parinktos naujai sukurtos lietuviškos bijūnų veislės, kurios skyrėsi genetiškai, morfologiškai ir fiziologiškai. Genetiniai skirtumai tarp 13 skirtingų bijūnų veislių buvo palyginti naudojant atsitiktinai pagausintos polimorfinės DNR (APPD, angl. – *RAPD*) metodą. Daugiausiai polimorfinių DNR fragmentų pagausinta su keturiais pasirinktais pradmenimis MP-8, OPB-7, OPA-18 ir OPB-8. Bendras DNR fragmentų skaičius svyravo nuo 1 iki 15, o jų dydis – 100–1 800 bp. Genetiškai labiausiai viena nuo kitos nutolusios bijūnų veislės – 'Skeivienės vėlyvasis' ir 'Garbė Motinai'; genetiškai panašiausios veislės – 'General MacMahon' ir 'Germaine Burgos'; genetiškai panašiausia tėvinėms formoms veislė – 'Freda', o labiausiai nutolusios – 'Darius-Girėnas' ir 'Skeivienės vėlyvasis'.

Raktažodžiai: puikusis bijūnas, veislės, lietuviškos, APPD