Agronomy Research **5**(1), 13–20, 2007

Determining of genetic diversity and genetic relationships among Lithuanian selections of *Actinidia kolomikta*

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Abstract. The assessing of Lithuanian cultivars and clones of *Actinidia kolomikta* DNA by RAPD method corroborated the significant genetic diversity and defined the level of their relationship. Genomic DNA was isolated from fresh leaves following CTAB method. Six decamer oligonucleotide primers Akt–1, Akt–2, Akt–3, 2B, OPA–02 and OPC–02 were used for PCR and a total of 42 scorable fragments yielded. The genetic distances fall into interval 0.059–0.914. The lowest genetic distances were calculated for the clones F2 and F4, as well for the clones F2M2 and F3M3: 0.059 and 0.097, respectively. Clones specific markers for the cultivar 'Laiba' and clone F4M4 were identified with the primers OPC–02 and 2B. The dendrogram grouped the selections by UPGMA method and revealed two main clusters at the level of 0.500 genetic distance. The cultivar 'Laiba' could be characterized as the most genetically separate accession in the *Actinidia kolomikta* germplasm collection of the Kaunas Botanical Garden of Vytautas Magnus University.

Key words: accession, dendrogram, fragment, genetic distance, RAPD primer

INTRODUCTION

The under-utilized crops which have typical valuable economic peculiarities and fruit accumulating significant amounts of biologically active substances are common objects of investigation in recent decades. There is interest in the species of *Actinidia* Lindl. genus in Lithuania, Russia as well as in the USA. The berries of the *Actinidia* are characteristic of chemical compounds with antioxidative properties (Ferguson & MacRae, 1992; Moskvina et al. 1998; Chesoniene et al., 2004). The kiwifruit is a 20th century new crop widely cultivated nowadays in temperate climates.

Kolomikta actinidia, *Actinidia kolomikta* (Maxim.) Maxim., is the mostly widely grown species in Lithuania. It possesses exceptionally decorative properties, valuable berries and therefore can supplement the assortment of berry plants cultivated in Lithuania. The selection work resulted in breeding four Lithuanian *A. kolomikta* cultivars at the Lithuanian University of Agriculture in 1972–1996 (Pranckietis, 1998; Pranckietis & Pranckietienė, 2000). Amateur horticulturists continually carried out selection of *kolomikta actinidia* seedlings along with scientific investigations conducted at the Lithuanian University of Agriculture during several decades. Currently, the clones selected by amateur breeders with the valuable economic properties are grown, spread and further propagated vegetatively in different regions of Lithuania.

The investigations of *Actinidia kolomikta* have ascertained that this species is characteristic of widely diverse morphological peculiarities such as, colour of buds, shoots and leaves, size and shape of berries etc (Ferguson, 1984; Kostyrko, 1989; Osipova, 1989; Plekhanova, 1990). Phenotypic diversity of *A. kolomikta* cultivars and clones has been investigated and informative phenotypic peculiarities singled out (Chesoniene, 2000). Molecular markers such as isozymes, have been used in assessing genetic diversity as well (Mesina et al., 1991). However, effectiveness of the isozyme method is limited. In recent years techniques based on DNA markers have been used to detect variation at DNA level and have proven to be very effective for distinguishing between closely related genotypes (Williams et al., 1990; Shirkot et al., 2002, Xiao et al., 2003).

The aim of this study was to assess the genetic variation of Lithuanian cultivars and clones of *kolomikta actinidia* and detect their genetic relationships.

MATERIALS AND METHODS

The samples for investigations were obtained from the collection of *A. kolomikta* at the Kaunas Botanical Garden of Vytautas Magnus University in 2004 (Table 1). The cultivars and clones for this collection were gathered in consideration of fundamental principles of genetically diverse plant material selection (Guarino et al., 1995).

Table 1. The list of female *Actinidia kolomikta* cultivars and clones investigated.

Accession	Type of accession	Place of origin
'Paukštės Šakarva'	cultivar	Lithuanian University of Agriculture
'Landė'	– „ –	– „ –
'Lankė'	– „ –	– „ –
'Laiba'	– „ –	– „ –
F1	clone	Kaunas distr., Babtai
F1M1	– „ –	Elektrėnai
'Anykšta'	cultivar	Anykščiai
Felė	clone	Elektrėnai
F2	– „ –	Kėdainiai distr., Dotnuva–Akademija
F4	– „ –	Kaunas
F2M2	– „ –	Kaunas distr., Ringaudai
F3M3	– „ –	Kėdainiai distr., Dotnuva–Akademija
F4M4	– „ –	Kėdainiai distr., Dotnuva–Akademija
La3	– „ –	Kaunas distr., Ringaudai

Genomic DNA was isolated from leaf tissue following the cetyltrimethylammonium (CTAB) method (Doyle & Doyle, 1990). Young leaves were collected and quickly frozen in liquid nitrogen. 0.2g of leaves were finely ground and mixed with 1 ml buffer CTAB: 100 mM TRIS-HCL, pH 8.0; 20 mM EDTA; 1.4 M NaCl; 1% PVP; 0.2% ß–mercapthoetanol. The prepared leaves were placed in Eppendorf tubes and incubated at 65°C for 40 min. An equal volume of chloroform/isoamyl alcohol was added after incubation and centrifuged for 10 min at 9500 g. The supernatant was transferred to a new Eppendorf tube, the equal amount of isopropanol was added and centrifuged at 7800 g for 5 min. DNA was dissolved in

0.150 ml TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The quality of the DNA was checked by electrophoresis on 1% agarose gel.

The primers used for RAPD reactions were chosen in order to generate scorable fragments of *kolomikta actinidia* accessions DNA and were established empirically. Finally, six decamer oligonucleotides were used for polymerase chain reaction (PCR): Akt–1, Akt–2, Akt–3, 2B, OPA–02, OPC–02 (Table 2). DNA amplification was carried out in 20 μl volumes containing: PCR buffer (10 mMTris-HCl, pH 8.0; 50 mM KCl; 3.0 mM $MgCl_2$), 20 μM of each dNTP, 0.3 μM primer, 1 unit Taq polymerase and 10 ng template DNA. The tubes were placed in a thermal cycler (Eppendorf Master Gradient) programmed as follows: 5 min at 94°C, 35 cycles of 80 s at 94°C, 60 s at 33°C, 90 s at 72°C. The final step consisted in 6 min incubation at 94°C. The amplified products were separated on 1% agarose gel in TAE buffer, pH 8.0 (40 mM Tris-acetate, 1 mM EDTA). All reagents used for DNA extraction and PCR were purchased from ROTH, Germany.

The amplified RAPD fragments of each accession were scored independently only for reproducible and clear bands. The presence or absence of fragments was recorded as 1 or 0, respectively. Pair-wise comparison was accomplished according to the formula (Link et al., 1995):

$$GD_{xy} = N_x + N_y / N_x + N_y + N_{xy},$$

where N_x is the number of fragments in line x and not in line y; N_y is the number of fragments in line y and not in line x; N_{xy} is the number of fragments shared in lines x and y. The data were presented as a dendrogram of genetic distances using UPGMA (unweighted pair-group method of arithmetic averages) and TREECON programme for Windows. The bootstrap method was employed to evaluate the reliability of tree topology. Numbers of dendrogram branches indicate bootstrap values (%) generated after 1000 permutations.

RESULTS AND DISCUSSION

Amplification products of 14 clones and cultivars of *A. kolomikta* with 6 decamer primers yielded a total of 42 scorable fragments, 29 of which were polymorphic. The size of the amplification products ranged from 250 to 3000 bp (Table 2).

A range of 5 to 9 amplified fragments per primer was observed, with an average 7 fragments. The largest number of fragments (9) was observed with the primer OPA–02, whereas the primer 2B generated the largest number of polymorphic fragments (6).

Different primers showed variation in their ability to detect polymorphism. The lowest number of fragments was obtained with the primer OPC–02 (5), however, 4 of them were polymorphic. The percentage of polymorphism ranged from 55.6% (primer OPA–02) to 80.0% (primer OPC–02). The differences in the number of amplified fragments between accessions were ascertained as well. The female clone F2 was distinguished for the largest total number (36) as well as for the number of polymorphic fragments (21).

Table 2. The results of amplification of *Actinidia kolomikta* clones and cultivars.

Primer code	Sequences, 5' – 3'	Fragment size (bp)	Number of fragments observed		Percentage of polymorphic fragments
			total	polymorphic	
Akt–1*	TCGGCACGCA	300–1500	7	5	71
Akt–2 •	TCCCTGTGCC	250–1500	7	5	71
Akt–3 •	GAGACGTCCC	450–1160	6	4	67
2B •	CAAACGTCGG	320–1350	8	6	75
OPA–02 •	TGCCGAGCTG	600–3000	9	5	56
OPC–02 •	GTGAGGCBTC	250–780	5	4	80

* – primer synthesized by ROTH, Germany
• – primer synthesized by JSC 'Fermentas', Lithuania

The primers Akt–1, Akt–2, 2B and OPA–02 did not generate any fragments for the cultivar 'Laiba'. The primer Akt–3 and OPC–02 generated 6 fragments for this cultivar. Only the cultivar 'Laiba' was characteristic for the 620 bp polymorphic fragment (primer OPC–02). The clone F4M4 distinguished for the 320 bp fragment with the primer 2B. The 750 bp and 450 bp fragments (primer Akt–3) and 780 bp fragment (primer OPC–02) were typical for all accessions investigated.

The genetic distance matrix was compiled by accomplishing 91 pair-wise comparisons (Table 3).

The lowest genetic distances were calculated for the clones F2 and F4 (0.059) as well as for the clones F2M2 and F3M3 (0.097). The cultivar 'Laiba' revealed the highest genetic distances with other accessions of Lithuanian origin: from 0.700 with the cultivar 'Landė' to 0.914 with the clone F4.

The dendrogram grouped the clones and cultivars on the basis of genetic distances by the UPGMA method (unweighted pair–group method using arithmetic average) (Fig. 1).

The dendrogram revealed two main clusters at a level of 0.500 genetic distance. The first cluster recognized two accessions (cultivar 'Landė' and female clone F1M1) joined at a level of 0.333 genetic distance with the high bootstrap value of 82%. The second cluster comprised two subclusters at the 0.350 genetic distance. The first subcluster contained four clones (F2, F4, F1 and Felė). The second cluster included other six accessions, namely the clones La3, F4M4, F3M3 and F2M2 as well as the cultivars 'Lankė' and 'Paukštės Šakarva'. The cultivar 'Anykšta' joined to this subclusters separate at a level of 0.400 genetic distance with the high bootstrap value of 70%. 'Laiba' was the most divergent cultivar and joined to accessions investigated at the 0.824.

Table 3. Distance matrix values based on RAPD data between 14 Lithuanian selections of *A. kolomikta.*

Accession	'Lankė'	'Paukštės Šakarva'	'Landė'	'Laiba'	'Anykšta'	Felė	F1	F4	F2	F1M1	F2M2	F3M3	F4M4	La3
'Lankė'	0.000													
'Paukštės Šakarva'	0.258	0.000												
'Landė'	0.433	0.531	0.000											
'Laiba'	0.778	0.821	0.700	0.000										
'Anykšta'	0.485	0.438	0.464	0.792	0.000									
Felė	0.429	0.333	0.545	0.828	0.355	0.000								
F1	0.525	0.447	0.555	0.882	0.382	0.286	0.000							
F4	0.487	0.361	0.555	0.914	0.382	0.333	0.171	0.000						
F2	0.436	0.351	0.541	0.889	0.371	0.324	0.167	0.059	0.000					
F1M1	0.576	0.576	0.333	0.818	0.517	0.500	0.471	0.471	0.457	0.000				
F2M2	0.273	0.324	0.438	0.800	0.344	0.294	0.324	0.324	0.316	0.438	0.000			
F3M3	0.303	0.250	0.469	0.833	0.375	0.273	0.306	0.306	0.297	0.469	0.097	0.000		
F4M4	0.417	0.324	0.529	0.875	0.394	0.294	0.278	0.229	0.270	0.438	0.181	0.156	0.000	
La3	0.226	0.333	0.505	0.786	0.505	0.303	0.378	0.421	0.410	0.500	0.187	0.219	0.294	0.000

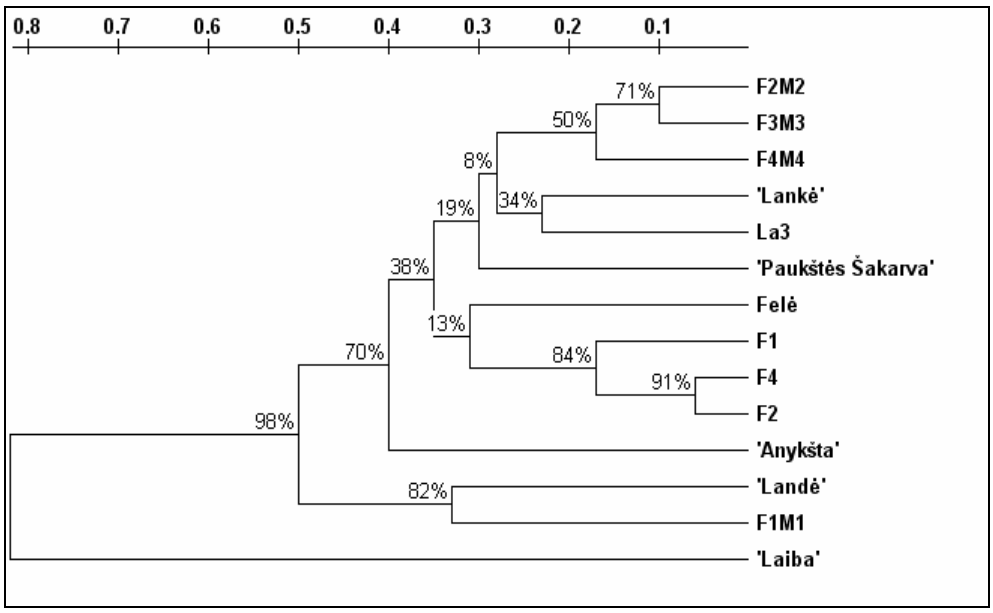

Fig. 1. UPGMA dendrogram of 14 cultivars and clones of *A. kolomikta.*

The bootstrap values for this dendrogram ranged from 8% to 98%. The genotype with bootstrap values below 50% indicate that the positions of these genotypes may change if other primers systems are applied or other genotypes are involved in the analysis.

The investigations of phenotypic diversity of cultivars and clones determined separation of the main criteria for purposive collection of *A. kolomikta* genetic resources (Chesoniene L., 2000; Daubaras et al., 2002). However, morphological peculiarities are unreliable when positive identification of an accession is desired (Nuel et al., 2001). Morever, interaction between genotype and environment complicate the evaluation of clone and cultivars. Molecular markers could provide a procedure for determining distinctness as well as increase reliability of decisions and save experiments and time (Novy & Vorsa, 1995; Hodkin et al., 2001, Huang et al., 2003). Investigations by RAPD markers revealed phylogenetic relationships in *Actinidia* (Cipriani et al., 1997; Huang et al., 2002; Marsh et al., 2003) as well.

Genetic information should play a significant part by noting or misindentification of clones and cultivars of *A. kolomikta* as well as reduce duplication in germplasm collection. RAPD markers identified in this investigation could be used to assist the breeding programme by establishment of relationships among *kolomikta actinidia* cultivars and clones.

CONCLUSIONS

1. Heterogeneous genetic material of *A. kolomikta* collected at the Kaunas Botanical Garden was a main precondition for the comprehensive investigations of

DNA. The results of this study corroborated significant genetic diversity of Lithuanian accessions and defined the level of their relationships.

2. The genetic distances fall into the interval 0.059–0.914 while calculated according to formula (Link et al., 1995). The lowest pair–wise GD_{xy} values were calculated between female clones F2M2 and F3M3 (0.097) and between clones F4 and F2 (0.059). These clones appeared to be closely related to one another.

3. The cultivar 'Laiba' exhibited the highest GD_{xy} values in pair–wise comparisons (GD_{xy} ranged from 0.700 to 0.914) and could be characterized as the most genetically separate Lithuanian accession.

4. Clone specific fragments were identified with the primers OPC–02 and 2B. The cultivar 'Laiba' and female clone F4M4 could be distinguished from other Lithuanian accessions by the presence of unique fragments 620 bp and 320 bp, respectively.

ACKNOWLEDGEMENTS. This work was supported by the Lithuanian Ministry of Education and Science programme 'Genefund'.

The authors wish to thank dr. B. Gelvonauskis (Plant Gene Bank) for rendering assistance in the conduct of investigations.

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