ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume LX 25 Number 8, 2012

GENETIC ANALYSIS OF THE GENUS *DIOSPYROS* SSP. USING RAPD AND I-PBS METHODS

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Received: September 13, 2012

Abstract

RADDOVÁ, J., PTÁČKOVÁ, H., ČECHOVÁ, J., ONDRÁŠEK, I.: *Genetic analysis of the genus Diospyros ssp. using RAPD and i-PBS methods.* Acta univ. agric. et silvic. Mendel. Brun., 2012, LX, No. 8, pp. 205–216

Molecular techniques (RAPD and i-PBS) were used to study genetic diversity within persimmon collection at Horticulture Faculty of Mendel University in Lednice. The aim of the work was to distinguish 14 known and 6 of unknown origin persimmon cultivars. The basic screening of 20 OPT primers was applied to 4 cultivars differring in the place of origin. Within the group of screened primers there were chosen those, which gave polymorphic repeatable strong and middle strong bands. Selected primers were used for the RAPD reactions within the whole persimmon collection. Three OPA primers previously described in the literature were also used for the RAPD reactions within the whole persimmon collection. Additional 16 i-PBS primers previously described in the literature were also used for i-PBS analysis of the whole group of cultivars. Amplification was successful with 12 i-PBS primers. The FreeTree software package was used to generate a similarity matrix and then to produce a dendrogram using UPGMA analyses. The similarity dendrograms of all persimmon cultivars were created based on both approaches and also on combination of both analyses by program Tree View. All the dendrograms clearly separated the assessed cultivars into 4 clusters. There are cluster of American persimmons – Meader' (1), 'Garretson' (2) and 'Early Golden' (3). They are representatives of *D. virginiana*. Further part of dendrogram includes single *D. lotus* (5), which is also clearly separated from other cultivars of the genus *Diospyros*. The third cluster includes interspecific hybrids 'Rossiyanka' (10) and 'Nikitskaiya Bordovaiya' (13), which arised from crosses of *D. virginiana* and *D. kaki*. The last cluster is formed by cultivars of Japanese persimmon – 'Mikatani Gosho', 'Zenjimaru', 'Tone Wase', 'Hiratanenashi', 'Fuyu', Chinese cultivar – 'Sansi' and two Italian cultivars 'Vaniglia' and 'Tipo'. They are clustered without significant distinction. The similarities and the differences revealed among incorporation of cultivars into groups were compared with the literature findings.

persimmon, RAPD, i-PBS, primer, dendrogram

Persimmon belongs to important fruit cultures. Persimmon is mainly cultivated in the subtropical areas nevertheless some very early ripenig cultivars and interspecific cultivars can be grown under climate conditions in the Czech Republic (Ondrášek and Krška, 2009). The largest distributions are in the countries of its origin such as China, Japan and Korea. China is the biggest world producer of persimmon and Italy is major European producer of persimmon (Faostat, 2009).

There are three the most important species of *Diospyros*, namely, *Diospyros kaki* Thunb. (Japanese persimmon), *Diospyros virginiana* L. (American persimmon) and botanical species *Diospyros lotus* L. *Diospyros kaki* is the most important species from commercial point of view (Yonemori *et al.*, 2000; Giordani, 2002). Japanese persimmon can be classified into four types based on the relationship among astringency in the fruit at harvest, presence of seed, and flesh colour, *i.e.*, pollination constant and non-astringent (PCNA), pollination variant and non-astringent (PVNA), pollination variant and astringent (PVA), pollination constant and astringent (PCA) (Sugiura, 2005).

More than 900 cultivars are known nowadays. Identification of persimmon cultivars is one of the main problems in the present due to misleading transliterations, local names, synonyms and homonyms. Cases of synonymies and homonyms do exist accessions collected in different sites, and possibly the names of a given genotype do not correspond to the denomination of the true to type accession held by reference germplasm collections of the countries of origin (namely Japan). At the European level 37% of accessions are positively identified, while the status of identification of the remaining 63% is probable (55%) and totally uncertain (8%) (Bellini and Giordani, 2003).

For characterization of germplasm it is essential to identify individual genotypes. Therefore one of the important aspects of fruit growing is the reliable determination of grown cultivars (Giordani, 2002; Sugiura, 2005; Yakushiji and Nakatsuka, 2007). Authenticating the identity of germplasm resources of persimmon would be a great value for breeding (Badenes *et al*., 2003).

There are various methods for cultivar identification and distinctness. First, there are methods based on morphological traits, and biochemical markers as analysis of isozymes (Mondini *et al*., 2009). Limiting factors of these techniques for cultivar identification led to the development of methods based on polymerase chain reaction (PCR) (Akbulut *et al*., 2008).

Therefore to identify different varieties of persimmon there are nowadays applied techniques based on *restriction fragment length polymorphisms* (RFLP) (Nakamura and Kobayashi, 1994), *random amplification of polymorphic DNA* (RAPD) (Thaipong *et al*., 2003; Yamagishi *et al*., 2005; Yýldýz, *et al*., 2007; Akbulut *et al*., 2008; Zahng *et al*., 2009), *amplified fragment length polymorphism* (AFLP) (Yonemori *et al*., 2008) or *single sequence repeat* (SSR) (Guo and Luo, 2011).

RAPD markers have been used successfully for estimating genetic relationships in several fruit crops such as apple (Koller *et al*., 1993), peach (Badenes *et al*., 1998), almond (Bartolozzi *et al*., 1998), plum (Boonprakob *et al*., 2001), guava (Prakash *et al*., 2002), grapevine (Ulanovsky *et al*., 2002), apricot (Ercisli *et al*., 2009), citrus fruit (Ji *et al*., 2011) and also persimmon (Thaipong *et al*., 2003; Bellini *et al*., 2003). The RAPD method seems to be a very effective indicator of different persimmon cultivars (Nakamura and Kobayashi, 1994).

One recently described retrotransposon-based molecular marker technique was used to identify the varieties (Mondini *et al*., 2009). There are several retrotransposon-based molecular methods which require sequence information to design element-specific primers (Kalendar, 2011; Kalendar *et al*., 2011). Retrotransposons are kind of elements which can be found in the genome of many eukaryotic organisms (Kumar and Bennetzen, 1999). One retrotransposon-based molecular method does not require previous sequence knowledge, it's i-PBS (*inter-primer binding site*). I-PBS primers were designed according the highly conservative sequences called Primer Binding Site (PBS) to which tRNA binds, acting as a primer for reverse transcriptase during the replication cycle of retroviruses and LTR retrotransposons. I-PBS is a universal and efficient method from identification of polymorphism (Kalendar *et al*, 2010; Kalendar, 2011; Kalendar *et al*., 2011).

The aim of this paper was to differentiate persimmon collection by means of RAPD and i-PBS methods, study genetic diversity within persimmon collection and to incorporate a unknown persimmon cultivars in the dendrogram.

MATERIAL AND METHODS

Plant materials

Twenty cultivars of persimmon (14 known cultivars and 6 unknown cultivars, see Tab. I) was included in this study. All plant material was obtained from a local persimmon collection situated in Lednice in the premises of Horticulture Faculty, Mendel University, Czech Republic.

DNA Extraction

Total genomic DNA was extracted from young frozen leaves (0.1 g) using DNeasy Plant Mini Kit (Qiagen). The DNA quality and concentration was determined by means of electrophoresis on a 0.8% agarose gel compared with lambda DNA standards containing 200 and 400 ng/µl DNA. The DNA concentration was also determined by fluorometr Modulus (Turner BioSystems).

RAPD Analysis

The RAPD amplification was performed in volume of 25 µl containing H_2O, 1× PCR buffer (Finnzymes), 100 µM each of dNTPs (Promega), 0.4 µM 10-mer primer (Operon), 1 unit of Taq polymerase (Finnzymes) and 20 ng of template DNA. DNA amplifications were performed in a thermal cycler UNO II (Biometra), according to the program of Williams *et al*. (1990) and the program used in the Mendeleum Research Station (Raddová, 2005). The program consisted of: denaturation at 94 °C for 3 min, followed by 40 cycles (denaturation at 94 °C for 20 s, annealing at 36 °C for 1 min, extension at 72 °C for 1 min), finishing at 72 °C for 9 min. The amplified products were separated by horizontal electrophoresis Agagel Maxi (Biometra©) in 1.5% agarose gel and visualised by GelRed.

At first, screening of 4 cultivars different in the place of origin ('Early Golden', 'Mikatani Gosho', 'Sansi', 1115 KOK) by using 20 OPT primers (OPT-1 to OPT-20) was performed. RAPD polymorphic bands were scored as 1 for presence of a band and 0 for its absence and were transformed into a binary matrix. Data from screening were transferred to software Popgen (Yeh and Boyle, 1997) and then the primers were evaluated with the highest genetic

I: *Persimmon cultivars used in this study*

Number	Cultivars	Species	Origin
1.	'Meader'	*D. virginiana*	USA
2.	'Garretson'	*D. virginiana*	USA
3.	'Early Golden'	*D. virginiana*	USA
4.	'Mikatani Gosho'	*D. kaki*	Japan
5.	*Diospyros lotus*	*D. lotus*	Southwest Asia
6.	'Čokoladnyj' = 'Zenjimaru'	*D. kaki*	Japan
7.	'Vaniglia'	*D. kaki*	Italy
8.	Hybrid 1921 SVK	*unknown origin*	
9.	Hybrid 2014 TNK	*unknown origin*	
10.	'Rossiyanka'	*D. kaki x D. virginiana*	Ukraine
11.	'Tone Wase'	*D. kaki*	Japan
12.	'Hiratanenashi'	*D. kaki*	Japan
13.	'Nikitskaiya Bordovaiya'	*D. kaki x D. virginiana*	Ukraine
14.	Hybrid 0723 CVK	*unknown origin*	
15.	Hybrid 1015	*unknown origin*	
16.	'Sansi'	*D. kaki*	China
17.	Hybrid 1115 KOK	*unknown origin*	
18.	Hybrid 0803 HCK	*unknown origin*	
19.	'Fuyu'	*D. kaki*	Japan
20.	'Tipo'	*D. kaki*	Italy

diversity (Nei, 1972). The OPT primers which produced polymorphic repetable strong bands were afterwards used in RAPD analysis of whole group of cultivars. At the same time analysis of whole group of cultivars was performed by using 3 primers from kit OPA (OPA-8, OPA-18, OPA-19) which successfully used Badenes *et al.* (2003).

Similarity among all cultivars was estimated according to unweighted pair group method average (UPGMA). The FreeTree software package (Hampl *et al.*, 2001) was used to generate a mean character difference matrix/similarity matrix and then to produce a dendrogram using UPGMA analyses. Final dendrogram was constructed by program Tree View (Page, 1996).

I-PBS technique

The i-PBS amplification according to work of Kalendar *et al.* (2010) was performed in volume of 25 µl containing H_2O, 1× PCR buffer, 1 µM 12-mer primer (i-PBS primers), 0.2 mM dNTPs (Promega), 1.25 unit of Taq polymerase (Finnzymes) and 20 ng of template DNA. After denaturation at 95 °C for 3 min, DNA amplifications were performed for 29 cycles in a UNO II thermal cycler (Biometra), according to the program of Kalendar *et al.* (2010). The PCR program consisted of: 1 cycle at 95 °C for 3 min; 28–30 cycles of 95 °C for 15 s, 50–60 °C (Kalendar *et al.*, 2010) for 60 s, and 68 °C for 60 s; a final extension step of 72 °C for 5 min. Also 16 i-PBS primers (2074, 2076, 2077, 2078, 2270, 2271, 2272, 2273, 2374, 2376, 2377, 2378, 2385, 2386, 2389, 2394) were used for analysis of whole group of cultivars.

The amplified products were separated by horizontal electrophoresis Agagel Maxi (Biometra©) in 1.5% agarose gel and visualised by GelRed. I-PBS polymorphic bands were scored as 1 for presence of a band and 0 for its absence and were transformed into a binary matrix. The FreeTree software package (Hampl *et al.*, 2001) was used to generate a mean character difference matrix/similarity matrix and then to produce a dendrogram using UPGMA analyses. Final dendrogram was constructed by program Tree View (Page, 1996).

RESULTS

OPT primer screening

A set of 20 OPT primers was used for screening of 4 cultivars. Primers generating a higher genetic polymorphism were chosen. Nei's (1972) gene diversity between cultivars achieved an average of 0.325. Out of 20 primers tested, 5 of them were not possible to evaluable, 1 of them amplified non-polymorphic product and 14 primers amplified from 2 to 12 polymorphic bands ranged from 200 to 2200 bp. The number of polymorphic markers was 83 (82.93%) out of the total number of 328 bands.

Consequently, 10 OPT primers (OPT-01, OPT-03, OPT-09, OPT-11, OPT-12, OPT-16, OPT-17, OPT-18, OPT-19, OPT-20) were selected for further work after screening, visual and statistical evaluation. These 10 OPT primers were used for analysis of the whole collection of 20 persimmon cultivars.

RAPD analysis used for all cultivars

The group of RAPD primers selected during the basic screening distinguished all the analysed cultivars. The OPT primers created a total of 1140 polymorphic bands scored in 57 markers. Selected primers generated from 3 to 11 polymorphic products in the size range of 220 bp to 2100 bp. The OPA primers made a total of 620 polymorphic bands scored in 31 markers. Each primer generated from 7 to 15 polymorphic bands. The size of the amplified fragments ranged from 150 bp to 1400 bp.

In total, 1760 polymorphic bands in 88 markers were generated by RAPD primers (OPT and OPA) and the size of the amplified fragments ranged from 150 bp to 2100 bp. The OPA primers reached an average yield of 10.3 markers/primer and the OPT primers had an average yield of 5.7 markers/primer.

The example of image – Fig. 1 shows electrophoretic spectrum of analysed cultivars. Numbers 1–20 mean numbers of cultivars. The letter "M" indicates the 100 bp DNA ladder (Life Technologies). Cultivars like: 'Meader' (1), 'Garretson' (2), 'Early Golden '(3), botanical species *Diospyros lotus* (5), 'Rossiyanka' (10) and 'Nikitskaiya Bordovaiya' (13) have similar spectrum of products and were significantly distinguished from others, in the Fig. 1. Further cultivars 'Tone Wase' (11) and 'Hiratanenashi' (12) have similar spectrum of products and were significantly distinguished from other cultivars too.

The dendrogram of genetic relationships among persimmon cultivars based on RAPD primers is presented in Fig. 2. The RAPD dendrogram classified all the 20 cultivars into four groups. Cluster analysis grouped the cultivars as follows.

First is a cluster of American persimmons, where 3 cultivars of American persimmon are included: 'Meader' (1), 'Garretson' (2) and 'Early Golden' (3). They are representatives of *D. virginiana*. This cluster was clearly separated from the rest of cultivars, while cultivars 'Meader' (1) and 'Early Golden' (3) were not distinguished using the RAPD markers.

The second part of dendrogram includes single botanical species *Diospyros lotus* (5), which is also clearly separated from other cultivars of the genus *Diospyros*.

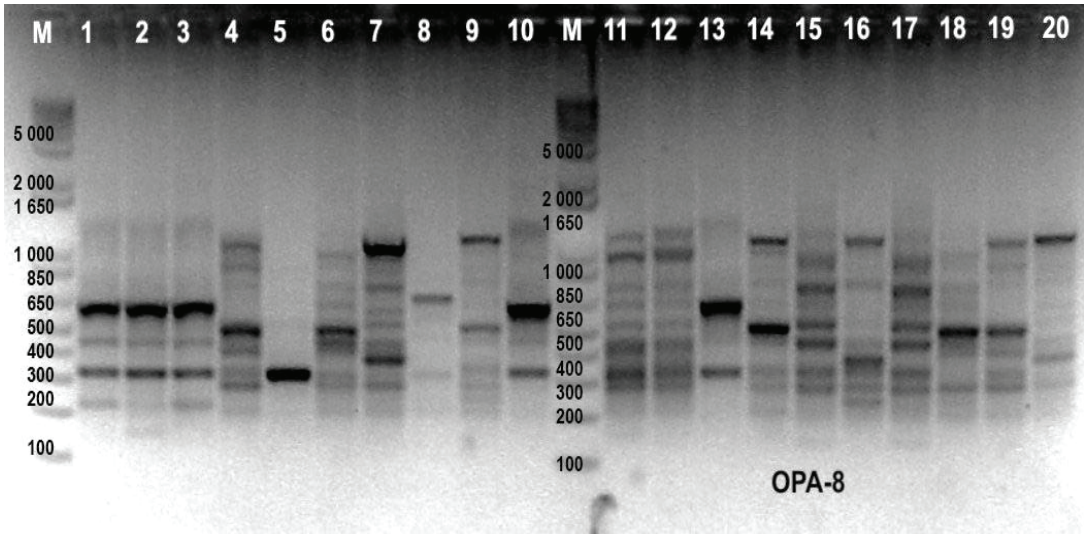
The third cluster includes interspecific hybrids 'Rossiyanka' (10) and 'Nikitskaiya Bordovaiya' (13), which arised from crosses of *D. virginiana* and *D. kaki*. The remaining and largest part creates a cluster of cultivars without significant distinction. This cluster includes fourteen cultivars belonging to the species *D. kaki*. Cultivars of unknown origin 1115 KOK (17), 1015 (15), 1921 SVK (8), 2014, TNK (9), 0723 CVK (14) and 0803 HCK (18) are in the same cluster like known cultivars 'Zenjimaru' (6), 'Vaniglia' (7), 'Sansi' (16), 'Tipo' (20), 'Hiratanenashi' (12),'Tone Wase' (11), 'Mikatani Gosho' (4) and 'Fuyu' (19) and therefore these cultivars share similar genetic background.

I-PBS analysis

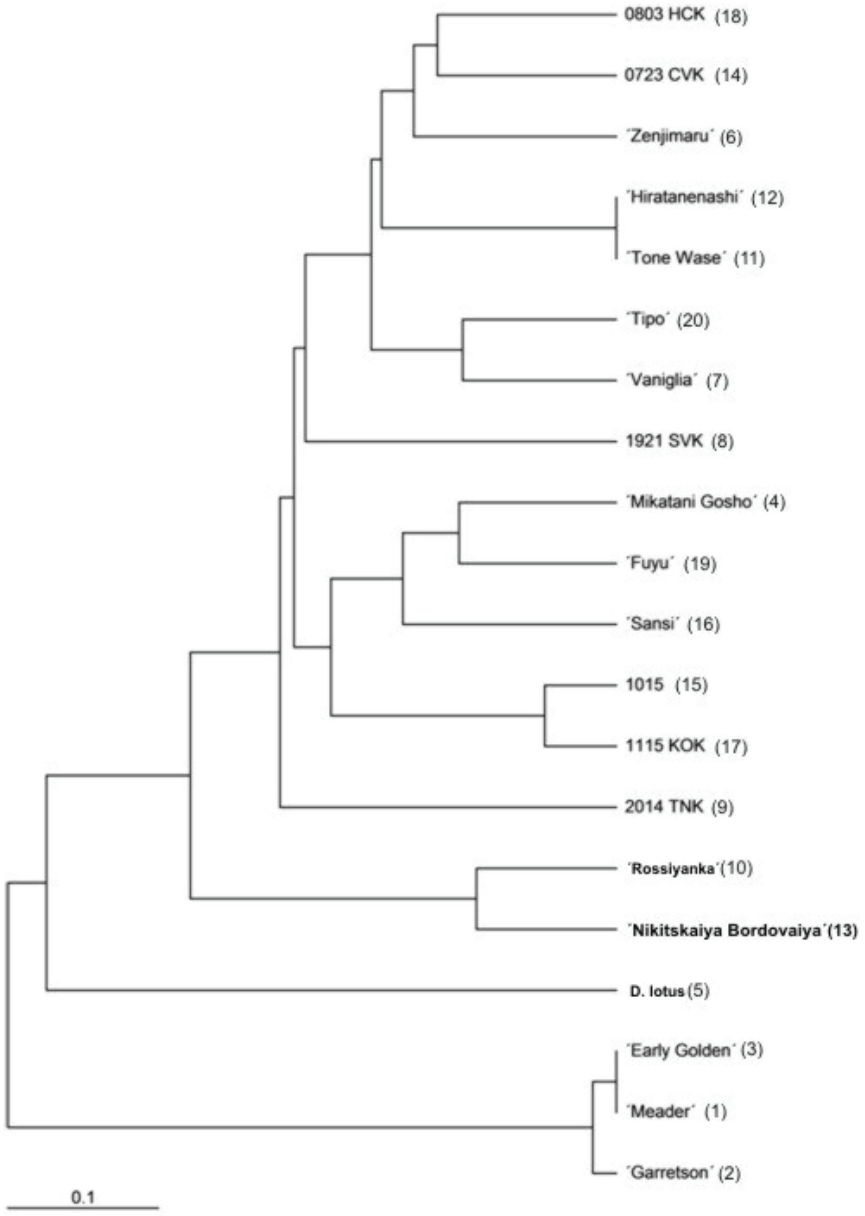
Sixteen i-PBS primers were used for analysis of the whole group of cultivars. The amplification was successful with twelve i-PBS primers (primers: 2076, 2077, 2078, 2272, 2273, 2374, 2376, 2377, 2378, 2386, 2389, 2394). Four i-PBS primers (primers: 2074, 2270, 2271, 2385) it was not possible to evaluate, even after repeating.

Twelve i-PBS primers created a total of 1760 polymorphic bands scored in 88 markers and generated distinctive 4 to 12 polymorphic bands products in the size range of 100 bp to 2000 bp. Primers i-PBS reached an average yield of 7.3 markers/primer.

The example of image – Fig. 3 shows electrophoretic spectrum of analysed cultivars. Numbers 1–20 mean numbers of cultivars. The letter "M" indicates the 100 bp DNA ladder (Life Technologies). There is the name of the primer and annealing temperature in the right corner of



1: *RAPD analysis with OPA-8 primer, 1–20 – numbers of cultivars, "M" – 100 bp DNA ladder (Life Technologies)*

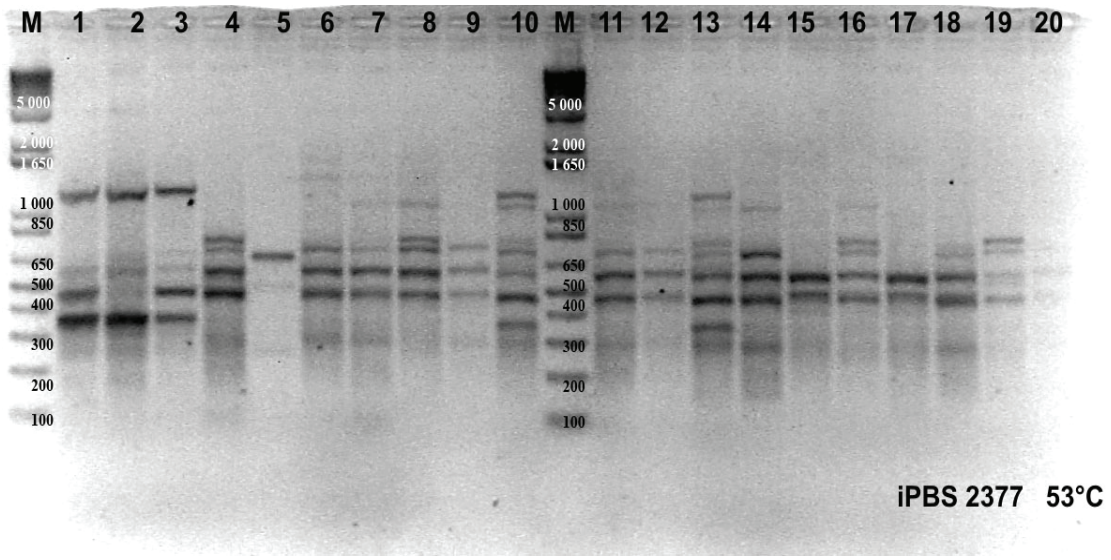


2: *Dendrogram of genetic relationships of 20 persimmon cultivars (RAPD analysis); on the x-axis is the similarity coefficient*

the picture. Analyses were carried out in order to accurately determine the individual cultivars and their assignment to the standard. The primer in Fig. 3 significantly distinguished cultivars like: 'Meader' (1), 'Garretson' (2), 'Early Golden' (3), botanical species *Diospyros lotus* (5), 'Rossiyanka' (10) and 'Nikitskaiya Bordovaiya' (13) from other cultivars.

The dendrogram of genetic relationships among persimmon cultivars based on 12 i-PBS primers is presented in Fig. 4.

The i-PBS dendrogram classified all the 20 cultivars into four groups. The first group includes single *D. lotus* (5), which is also clearly separated from other cultivars of the genus *Diospyros*. The second cluster is composed of American persimmons like 'Meader' (1), 'Garretson' (2), 'Early Golden '(3). The third cluster includes interspecific hybrids 'Rossiyanka' (10) and 'Nikitskaiya Bordovaiya' (13), which arised from crosses of *D. virginiana* and *D. kaki*. The rest of 14 cultivars which were clustered in the last cluster belongs to group of Japanese, Italian and Chinesese persimmons. Cultivars of unknown origin 1115 KOK (17), 1015 (15), 1921 SVK (8), 2014 TNK (9), 0723 CVK (14) and 0803 HCK (18) are in the same cluster like known cultivars 'Zenjimaru' (6), 'Vaniglia' (7), 'Sansi' (16), 'Tipo' (20), 'Hiratanenashi'(12), 'Tone Wase' (11), 'Mikatani Gosho' (4), and 'Fuyu' (19) and therefore these cultivars share similar genetic background.



3: *I-PBS analysis of all cultivars with i-PBS primer 2076; 1–20 – numbers of cultivars, "M" – 100 bp DNA ladder (Life Technologies)*

Results of combination of both methods (RAPD and i-PBS)

Genetic polymorphism was evaluated in 14 known cultivars and 6 cultivars of unknown origin of the genus *Diospyros* by using 12 i-PBS primers, 10 OPT primers and 3 OPA primers. A total of 3520 polymorphic bands were evaluated and polymorphism was assessed in 176 markers. The similarity dendrogram is presented in Fig. 5 and Fig. 6.

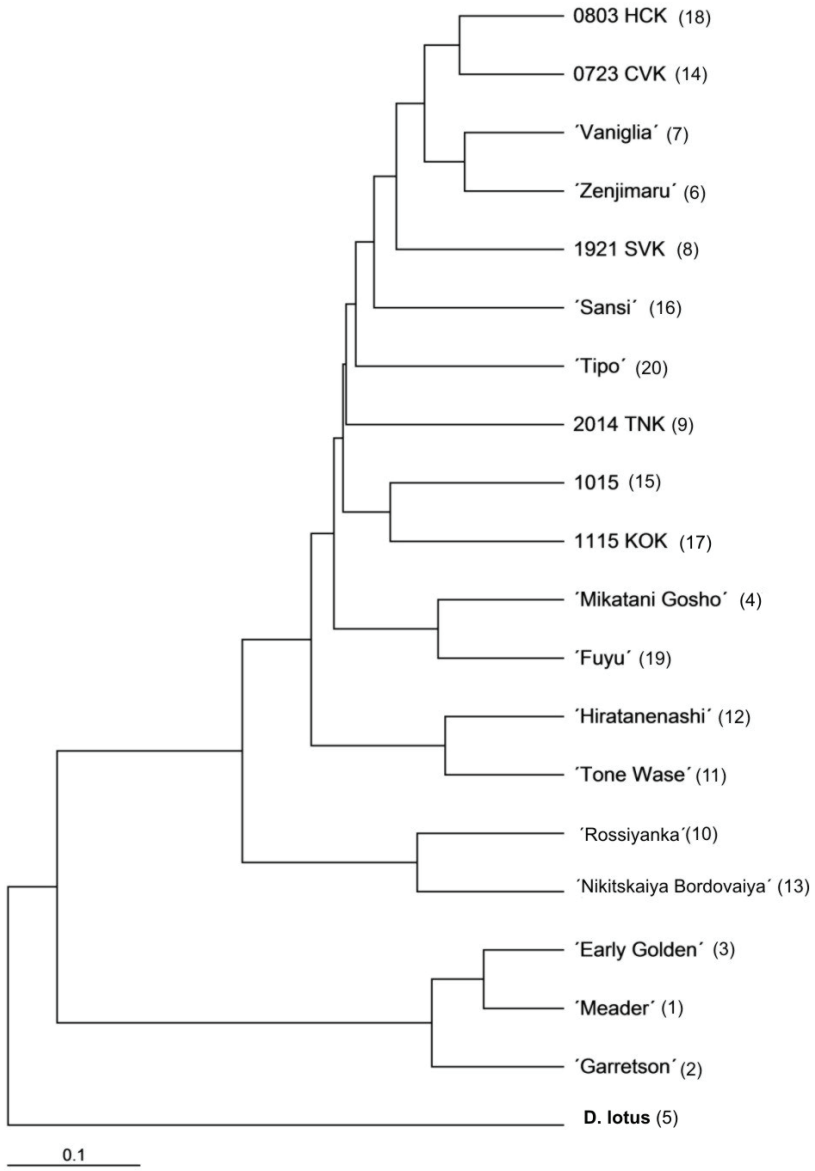
Distribution of cultivars in the dendrogram was very similar to previous dendrograms. The first part of dendrogram includes single botanical species *Diospyros lotus* (5), which is also clearly separated from other cultivars of the genus *Diospyros*. The second is cluster of American persimmons, where 3 cultivars of American persimmon are included: 'Meader' (1), 'Garretson' (2) and 'Early Golden' (3). This cluster was clearly separated from the rest of cultivars. The third cluster includes two interspecific hybrids 'Rossiyanka' (10) and 'Nikitskaiya Bordovaiya' (13), which originated from crosses of *D. virginiana* and *D. kaki*. The fourth cluster includes cultivars without significant distinction. There are fourteen cultivars belonging to Japanese, Italian and Chinesese persimmon in this cluster. Cultivars of unknown origin 1115 KOK (17), 1015 (15), 1921 SVK (8), 2014 TNK (9), 0723 CVK (14) and 0803 HCK (18) are in the same cluster like known cultivars 'Zenjimaru' (6), 'Vaniglia' (7), 'Sansi' (16), 'Tipo' (20), 'Hiratanenashi '(12),' Tone Wase' (11), 'Mikatani Gosho' (4) and 'Fuyu' (19) and therefore these cultivars share similar genetic background.

The fourth cluster is divided into several subclusters. The cultivar of unknown origin TNK 2014 (9) is separated from the other cultivars. This cultivar is the closest to pair of other cultivars of unknown origin 1115 KOK (17) and 1015 (15) and it means they are closely related. These three cultivars are genetically closest to cultivars like 'Mikatani Gosho' (4), 'Fuyu' (19) and 'Sansi' (16), which create another subcluster. Pairs of cultivars like 'Hiratanenashi' (12) and 'Tone Wase', 'Vaniglia '(7) and 'Tipo '(20) and triplet of cultivars of unknown origin like 0723 CVK (14), 0803 HCK (18) and 'Zenjimaru' (6) create last subcluster. The cultivar 1921 SVK (8) is separated from the others, which belong to this subcluster.

DISCUSSION

In the previous literature sources it is shown that the RAPD method is suitable for the study of persimmon cultivars (Nakamura and Kobayashi, 1994; Badenes *et al.*, 2003). Badenes *et al.* (2003) and Yýldýz *et al.* (2007) used a set of primers OPA to distinguish different cultivars of persimmon. Badanes *et al.* (2003) used the OPA-8, OPA-18 and OPA-19. Yýldýz *et al.* (2007) used primer OPA-18 and OPA-19. Primers OPA-8, OPA-18 and OPA-19 were used also in this work. The OPA primers amplified from 7 to 15 polymorphic products in size from 150 to 1400 bp. In this work, an average yield of OPA primers reached 10.3 markers/primer, which is three times more than average of 2.4 markers/primer in Badenes *et al.* (2003), but less than 12.6 markers/primer in Yýldýz *et al.* (2007). Primers OPT reached an average yield of 5.7 markers/primer and primers i-PBS reached an average yield of 7.3 markers/primer. It follows, that the primers OPA gave the most polymorphic bands.

The i-PBS technique was tested in work of Kalendar *et al.* (2010) in several plant and animal species. The effectiveness of this method was confirmed also by Gailite *et al.* (2011). According to recent literature sources, no one has tested the i-PBS method for distinguishing cultivars of persimmon. On the basis of these results it can be said that the



4: *Dendrogram of genetic relationships of 20 persimmon cultivars (i-PBS analysis); on the x-axis is the similarity coefficient*

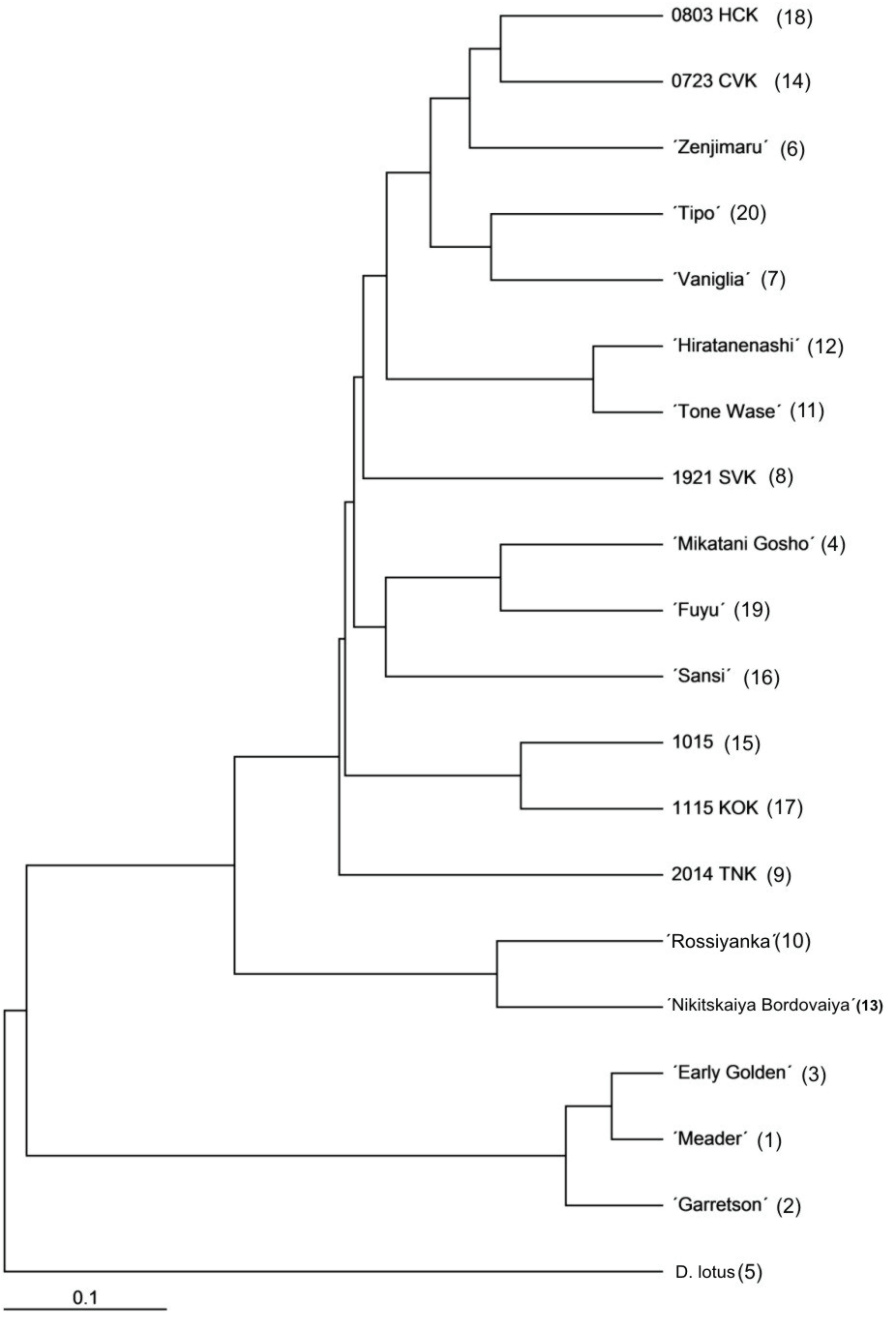
persimmon cultivars have a relatively high level of genetic diversity.

Earlier studies using RAPD in Badenes *et al.* (2003); Akbulut *et al.* (2008) showed large genetic variation between different cultivars of D. kaki. In the papers of Thaipong *et al.* (2003) and Guo and Luo (2011) cultivars of Japanese and American persimmon were significantly separated. It was also confirmed in this work. Cultivars were assigned to the cluster according to the place of origin.

The largest group is formed by cultivars of Japanese persimmon (*D. kaki*). Japanese cultivars ('Mikatani Gosho', 'Zenjimaru', 'Tone Wase', 'Hiratanenashi', 'Fuyu'), Chinese cultivar 'Sansi' and two Italian cultivars 'Vaniglia' and 'Tipo'. They are clustered without significant distinction. One explanation may be that the Italian cultivars have their origin in the Japanese cultivars. Yonemori *et al.* (2008) using the UPGMA analysis ranked cultivar 'Vaniglia' into the first group of European cultivars, compared with the Neighbor Joining analysis clustered cultivar 'Vaniglia' in the second group of European cultivars. Yonemori *et al.* (2008) came to the conclusion that groups of Japanese and European cultivars share a similar genetic background.

In the work of Giordani (2002) it is written that cultivar 'Tonewase' is a bud mutation of the cultivar 'Hiratanenashi', which is confirmed by the placement of these two cultivars in the dendrogram in this work.

The dendrograms created by two different methods (RAPD and i-PBS) are very similar. Both methods are "random"



5: *Dendrogram of genetic relationships of 20 persimmon cultivars (combinaton of both analyses RAPD and i-PBS); on the x-axis is the similarity coefficient*

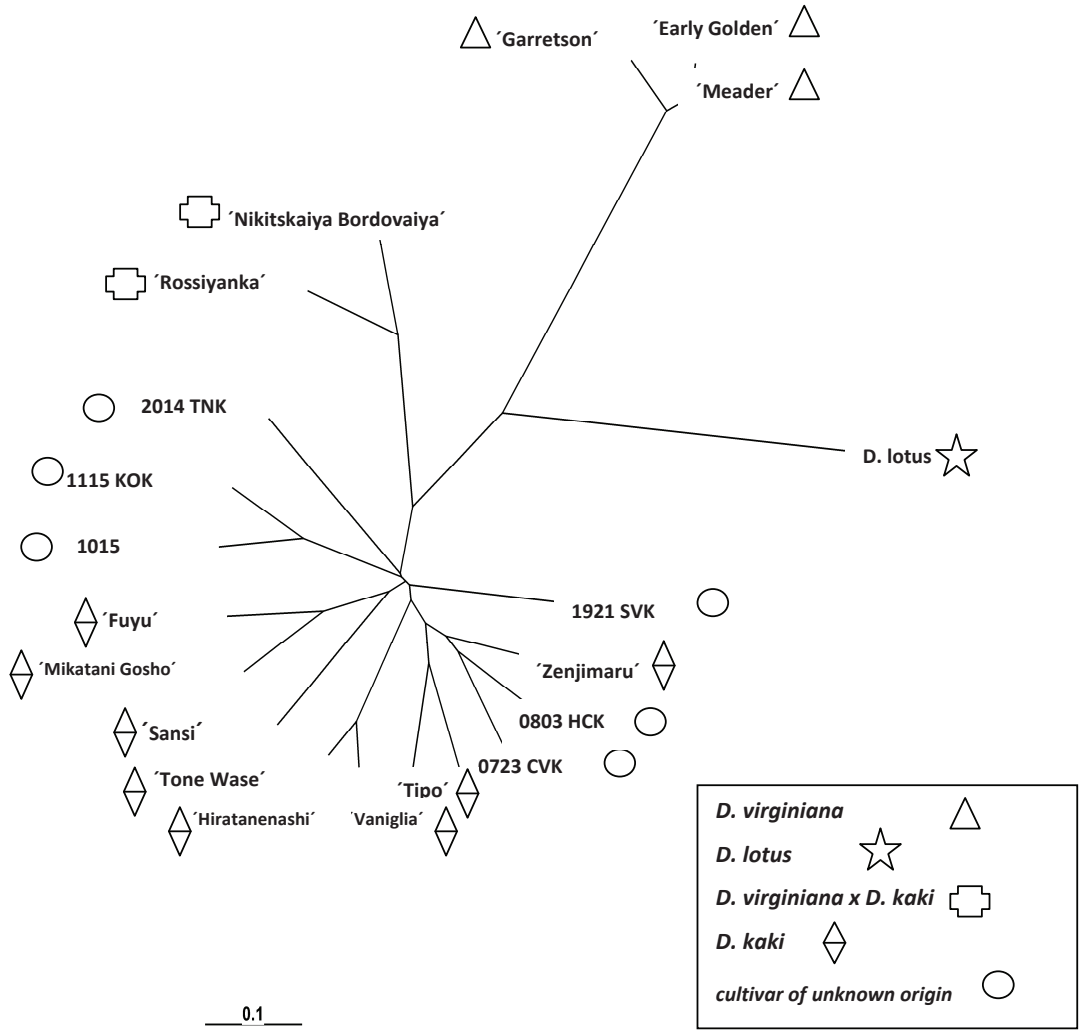
when random primers bind the target DNA. Better distinction of persimmon cultivars could be identified by using other amplification methods, such as the SSR method. Therefore, the analysis could follow by SSR analysis, which is more specific and reliable than RAPD. According to Guo and Luo (2011) the SSR analysis presents new opportunities for studying genetic diversity of Japanese persimmon.

Molecular methods present unlimited access to the source of genetic variation. In addition, we can discover some genotypic differences of which distinction by other methods is difficult or impossible.

CONCLUSION

Fourteen known cultivars and six cultivars of unknown origin of the genus *Diospyros* were subjected to RAPD and i-PBS analyses.

Basic screening of OPT primers was performed and subsequently 10 selected primers OPT and 3 primers OPA previously described in the literature were used to evaluate polymorphism in 88 markers. Altogether 1760 polymorphic bands were evaluated



6: *Unrooted dendrogram of 20 persimmon cultivars (combinaton of both analyses RAPD and i-PBS); on the x-axis is the similarity coefficient*

and the size of the amplified fragments ranged from 150 bp to 2100 bp.

Twelve i-PBS primers determined 1760 polymorphic bands scored in 88 markers and primers generated from 4 to 12 polymorphic bands. The size of the amplified fragments ranged from 100 bp to 2000 bp. A total of 3520 polymorphic bands were generated and polymorphism was assessed in 176 markers.

All the dendrograms clearly separated the assessed cultivars into 4 clusters. It is obvious, that the unknown cultivars belonging to the cluster of Japanese persimmon (*D. kaki)*. According to this study the RAPD and i-PBS were reliable enough to detect differences between the genetically close cultivars of persimmon.

SUMMARY

Identification of persimmon cultivars is one of the main problems in the present due to misleading transliterations, local names, synonyms and homonyms. The RAPD and i-PBS techniques were used to study genetic diversity within the persimmon collection at Horticulture Faculty of Mendel University in Lednice. The aim of the work was to distinguish between 14 known and 6 persimmon cultivars of unknown origin. The basic screening of 20 OPT primers was applied to 4 cultivars differring in the place of origin. Those of screened primers within the group there were chosen, which gave polymorphic repeatable strong and middle strong bands. The total 10 OPT primers (OPT-01, OPT-03, OPT-09, OPT-11, OPT-12, OPT-16, OPT-17, OPT-18, OPT-19, OPT-20) were selected for further work after screening, visual and statistical evaluation for the RAPD analysis within the whole persimmon

collection. Three OPA (OPA-8, OPA-18, OPA-19) primers previously described in the literature were also used for the RAPD reactions within the whole persimmon collection. Further 16 i-PBS, primers previously described in the literature, were also used for i-PBS analysis of the whole group of cultivars. The amplification was successful with 12 i-PBS primers. The FreeTree software package was used to generate similarity matrix and then to produce a dendrogram using UPGMA analysis.

The group of RAPD primers selected during the basic screening distinguished all the analysed cultivars. The OPT primers created a total of 1140 polymorphic bands scored in 57 markers. Selected primers generated from 3 to 11 polymorphic products in the size range of 220 bp to 2100 bp. The OPA primers made a total of 620 polymorphic bands scored in 31 markers. Each primer generated from 7 to 15 polymorphic bands. The size of the amplified fragments ranged from 150 bp to 1400 bp. In total, 1760 polymorphic bands in 88 markers were generated by RAPD primers (OPT and OPA) and the size of the amplified fragments ranged from 150 bp to 2100 bp. The OPA primers reached an average yield of 10.3 markers/primer and the OPT primers had an average yield of 5.7 markers/primer. The dendrogram of genetic relationships among persimmon cultivars based on RAPD primers is presented in Fig. 2. The RAPD dendrogram classified all the 20 cultivars into four groups. First is a cluster of American persimmons, where 3 cultivars of American persimmon are included: 'Meader' (1), 'Garretson' (2) and 'Early Golden' (3). They are representatives of *D. virginiana*. This cluster was clearly separated from the rest of cultivars, while cultivars 'Meader' (1) and 'Early Golden' (3) were not distinguished using the RAPD markers. The second part of dendrogram includes single botanical species *Diospyros lotus* (5), which is also clearly separated from other cultivars of the genus *Diospyros*. The third cluster includes interspecific hybrids 'Rossiyanka' (10) and 'Nikitskaiya Bordovaiya' (13), which arised from crosses of *D. virginiana* and *D. kaki*. The remaining and largest part creates a cluster of cultivars without significant distinction. This cluster includes fourteen cultivars belonging to the species *D. kaki*. Cultivars of unknown origin 1115 KOK (17), 1015 (15), 1921 SVK (8), 2014, TNK (9), 0723 CVK (14) and 0803 HCK (18) are in the same cluster like known cultivars 'Zenjimaru' (6), 'Vaniglia' (7), 'Sansi' (16), 'Tipo' (20), 'Hiratanenashi' (12),'Tone Wase' (11), 'Mikatani Gosho' (4) and 'Fuyu' (19) and therefore these cultivars share similar genetic background.

Twelve i-PBS primers created a total of 1760 polymorphic bands scored in 88 markers and generated distinctive 4 to 12 polymorphic bands products in the size range of 100 bp to 2000 bp. Primers i-PBS reached an average yield of 7.3 markers/primer. The i-PBS dendrogram classified all the 20 cultivars into four groups. The first group includes single *D. lotus* (5), which is also clearly separated from other cultivars of the genus *Diospyros*. The second cluster is composed of American persimmons like 'Meader' (1), 'Garretson' (2), 'Early Golden '(3). The third cluster includes interspecific hybrids 'Rossiyanka' (10) and 'Nikitskaiya Bordovaiya' (13), which arised from crosses of *D. virginiana* and *D. kaki*. The rest of 14 cultivars which were clustered in the last cluster belongs to group of Japanese, Italian and Chinesese persimmons. Cultivars of unknown origin 1115 KOK (17), 1015 (15), 1921 SVK (8), 2014 TNK (9), 0723 CVK (14) and 0803 HCK (18) are in the same cluster like known cultivars 'Zenjimaru' (6), 'Vaniglia' (7), 'Sansi' (16), 'Tipo' (20), 'Hiratanenashi'(12), 'Tone Wase' (11), 'Mikatani Gosho' (4), and 'Fuyu' (19) and therefore these cultivars share similar genetic background.

Further genetic polymorphism was evaluated in 14 known cultivars and 6 cultivars of unknown origin of the genus *Diospyros* by using 12 i-PBS primers, 10 OPT primers and 3 OPA primers. A total of 3520 polymorphic bands were evaluated and polymorphism was assessed in 176 markers. Distribution of cultivars in the dendrogram was very similar to previous dendrograms. First part of dendrogram includes single botanical species *Diospyros lotus* (5), which is also clearly separated from other cultivars of the genus *Diospyros*. The second is cluster of American persimmons, where 3 cultivars of American persimmon are included: 'Meader' (1), 'Garretson' (2) and 'Early Golden' (3). This cluster was clearly separated from the rest of cultivars. The third cluster includes two interspecific hybrids 'Rossiyanka' (10) and 'Nikitskaiya Bordovaiya' (13), which originated from crosses of *D. virginiana* and *D. kaki*. The fourth cluster includes cultivars without significant distinction. There are fourteen cultivars belonging to Japanese, Italian and Chinesese persimmon in this cluster. Cultivars of unknown origin 1115 KOK (17), 1015 (15), 1921 SVK (8), 2014 TNK (9), 0723 CVK (14) and 0803 HCK (18) are in the same cluster like known cultivars 'Zenjimaru' (6), 'Vaniglia' (7), 'Sansi' (16), 'Tipo' (20), 'Hiratanenashi '(12),' Tone Wase' (11), 'Mikatani Gosho' (4) and 'Fuyu' (19) and therefore these cultivars share similar genetic background.

According to this study the RAPD and i-PBS were reliable enough to detect differences between the genetically close cultivars of persimmon. The similarities and the differences revealed among incorporation of cultivars into groups were compared with the literature findings.

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