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Molecular characterization of apricot (*Prunus armeniaca* L.) cultivars using cross species SSR amplification with peach primers

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Summary: Apricot takes an important place in Hungarian fruit production. Considering morphological characteristics of apricots it was concluded that the genetics background of European cultivars is very limited. Molecular markers and their use for genotyping have revolutionized the identification of cultivars. In a classic apricot breeding program, it is important to be able to establish unique DNA profiles of selections to identify them unambiguously and to determine their genetic relationship. Presently SSR is far the most frequently performed technique for genetic diversity studies. In this study there were used peach and apricot primer pairs from four different sources in order to examine microsatellite polymorphism among cultivars and investigate relationships among them. The possibility of cross species amplification among different *Prunus* species using SSR primers allowed us to use primers developed in peach to study genetic diversity in apricot. In this work, 90% of the primers used were able to amplify SSRs in apricot and more than half of them were polymorphic. With the 10 primer pairs utilized were proven to be sufficient to set unique fingerprint for several cultivars studied. The obtained dendrogram classified of the 45 cultivars included in this study into two major groups and several subgroups

Key words: *Prunus armeniaca* L., apricot cultivar, SSR marker, UPGMA

Introduction

Apricot is an economically important member of the family *Rosaceae* and takes an important place in Hungarian fruit production. Apricot (*Prunus armeniaca* L.) originally native to Manchuria China but has spread across the world over the past few centuries. The transfer to Europe occurred via Armenia. Due to the favorable climate of this region this place served as a second gene centre for apricots and produced a rich selection of varieties. Finally the Greeks brought them to Europe and the Romans planted them all over Hungary. From the morphology of apricots it was concluded that European cultivars are genetically close together.

The main production is located in central Hungary not because of the special characters of the conditions here, but for the long tradition and the expertise of the cultivation. The yield fluctuates due to the early blooming and the frequently resulting freezing damage in spring. The volume of yearly harvest varies between 10 and 20 tones per hectare (commercial) and a very significant (domestic) production takes place in home gardens.

Molecular markers and their use for genotyping have revolutionized the identification of cultivars. During the past few decades, isozyme markers have been used to assess genetic variability in apricots (*Battistini & Sansavini*, 1991; *Badenes* et al., 1996). However isozyme analysis does not detect enough polymorphism to discriminate Hungarian cultivars and reveal relationships among them (*Major* et al., 1999).

In a classic apricot breeding program, it is important to be able to establish unique DNA profiles of selections to identify them unambiguously and to determine their genetic relation. The development of DNA markers has provided the possibility for the rapid identification of cultivars. Different types of DNA based markers were used on apricot in the past few years: restriction fragment length polymorphism (RFLPs) (*de Vicente* et al., 1998) and randomly amplified DNA (RAPDs) (*Gogorcena* et al., 1994; *Hurtado* et al., 1999) analysis. More recently new studies have been performed by using amplified fragment length polymorphisms (AFLPs) (*Hagen* et al., 2001; *Hurtado* et al., 2002) and using sequence characterized amplified regions (SCARs) (*Mariniello* et al., 2002).

Simple sequence repeats (SSR and microsatellites) have become the tool for identification in many plant species, because they are PCR-based, highly reproducible, polymorphic and codominant in plant genomes (*Powell* et al., 1996). In many cases, microsatellites are conserved between taxa and they make it transferable across related species

(Huang et al., 1998). Primers designed for peach SSR loci have been used to amplify loci in other related crops (almond, apricot cherry, plum) and are recommended for use in comparative mapping within the family (*Cipriani* et al., 1999; *Sosinski* et al., 2000). Comparing the DNA-based marker techniques SSR is far the most frequently performed technique for genetic diversity studies showing the usefulness and effectiveness of this method (*Zhebentyayeva* et al., 2002; *Hormanza* et al., 2002; *Romero* et al., 2003; *Regner* et al., 2004).

In this study, we used peach and apricot primer pairs from four different sources for cross species amplification in apricot in order to examine microsatellite polymorphism among cultivars and investigate relationships among them. Our intention was to try newly isolated peach primers, which had only little reference to apricot. When the research was carried out, the sequences of three new sets of peach primers were released (*Dirlewanger* et al., 2002; *Wang* et al., 2002; *Yamamoto* et al., 2002) which were chosen for this study.

Materials and methods

45 apricot accessions were selected that represent the cultivars grown in Central Europe. The selection contained the 14 most frequently planted Hungarian apricot cultivars, and six other cultivars which presumably are closely related to the so called Hungarian Best cultivar group (*Table 1*). The rest of the cultivars have different origins the common point is that they all play an important role in Hungarian apricot breeding and they are part of the breeding program of the Department. Finally some well-known cultivars were included such as Bergeron and Moniqui as reference. All plant material was collected from the reference collection of the Institute of National Quality Control Hungary (Tordas).

Total genomic DNA was extracted from leaves with Qiagen™ Kit following the producer's protocol. Only young leaves resulted in high quality DNA with good performance for PCR. Leaf samples were harvested only in April and May. The DNA was quantified by spectrophotometry and 60 ng/l DNA was used as template for PCR.

Extracted apricot genomic DNA was PCR amplified using 19 primer pairs, previously cloned and sequenced in peach and apricot. PCR reaction were performed in 25 µl volume containing 20mM Tris-HCl, 50 mM KCl, 2,5 mM $MgCl_2$ 5% DMSO, 25 pmol of each primer, 0,2 mM of each dNTP, and 1U Taq polymerase (Sigma). The PCR reactions were carried out in a 96-well thermal cycler (model MJ research, PTC-200) with the following program: an initial step of 94 for 3 min, 35 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 1 min 15 s, and finishing with 72 °C for 10 min. Following the amplification the samples were stored at 4 °C prior to electrophoresis

Amplified PCR products were separated by using electrophoresis using 3% Metaphore® agarose (Biowittaker, Maine, USA) gel, 1 x TBE (89mM Tris, 89 mM boric acid, and 2mM EDTA (pH 8.0)) buffer, stained by ethidium bromide 0,7 µg/ml, and visualized under UV light. Molecular sizes of amplified products were estimated using a 100-bp ladder (Promega).

Table 1 The examined 45 cultivars and their origin

Cultivar	Origin	Cultivar group
Ceglédi óriás	Hungary	"Giant" group
Ligeti óriás	Hungary	"Giant" group
Szegedi mamut	Hungary	"Giant" group
Rózsa C.1406	Hungary	"Rose" group
Borsi-féle kései rózsa	Hungary	"Rose" group
Mamaia	Romania	(Ananas × Ananas) × (Tarzii de Bukuresti × Ananas)
Kalasek	Czech R.	Local cultivar
Rana Dokucana		Local cultivar
Sabinovska	Slovakia	Local cultivar
Ceglédi Piroska	Hungary	Ceglédi óriás × Magyar kajszi C.1789
Ceglédi kedves	Hungary	Ceglédi óriás × open pollination
Ceglédi arany	Hungary	Ceglédi óriás × Rózsabarack C.1668
Ksna ugarska	Bulgaria	Hungarian Best
Silistrenka compotna	Bulgaria	Hungarian Best
Andornaktájai magyar kajszi	Hungary	Hungarian Best
Magyar kajszi C.235	Hungary	Hungarian Best
Paksi magyar kajszi	Hungary	Hungarian Best
Crvena ungarska	Macedonia	Hungarian Best
Gönci magyar kajszi	Hungary	Hungarian Best
Harmat	Middle-Asia	Hybrid
Korai zamatos	Middle-Asia	Jubilar (Salah open pollination) open pollination
Borjana	Moldavia	Magyar kajszi × Jerevani
Vesna	Slovakia	Magyar kajszi × pollen mixture
Marculesti 18/6	Romania	Marculesti 17/52 × Marculesti 43/1
Jerevan (syn.: Salah)	Armenia	Local cultivar
Salah	Armenia	Local cultivar
Krimskij amur	Ukraine	Vinoslivij x Aromatnij
Bergeron	France	Unknown seedling
Ceglédi bíbor	Hungary	Local cultivar
Korai piros	Hungary	Local cultivar
Pisana	Italy	ICAPI 26/5 open pollinated
Kech-psar	Middle Asia	Local cultivar
Cacansko zlato	Serbia	Local cultivar
Moniqui	Spain	Local cultivar
Ananasnij ciurpinskij	Ukraine	Local cultivar
Darunek malahojev	Ukraine	Local cultivar
Kijevskij aromatij	Ukraine	Unknown
Zard	Middle Asia	Local cultivar
Nyikitskij	Ukraine	Krasnoschekij's clone
Gvardejskij	Ukraine	?
Oranjevo krasnij	Ukraine	?
Krupnoplodnaja	Ukraine	Local cultivar
Dolgocutna	?	?
Rakvice	Czech R.	Local cultivar

The genetic distance matrix was calculated based on the proportion of similar alleles method (*Reynolds* et al., 1983) using MICROSAT software (*Minch* et al., 1997). The cluster analysis was conducted UGMA algorithm using the NEIGHBOR program in PHYLIP version 3.5c (*Felstein* et al., 1989) and a dendrogram was constructed using the program TREEVIEW (*Page* et al., 1996). Direct-count heterozygosities were calculated as the number of heterozygous genotype at a given locus divided by the total number of genotypes.

Table 2 List of microsatellites that did not result repeatable or polymorphic amplification in the cultivars tested

SSR	Reference	origin	Predicted length	Size range (bp)	Cause of exclusion
BPPCT011	Dirlewanger et al. 2002	peach	172	170–180	Monomorphic
BPPCT029	Dirlewanger et al. 2002	peach	159	150–180	Complex band pattern
Pchgms12	Wang et al. 2002	peach	433	460	Monomorphic
Pchgms14	Wang et al. 2002	peach	500	500	Monomorphic
MA006b	Yamamoto et al 2002	peach	295	–	No amplification
MA009b	Yamamoto et al 2002	peach	132	–	No amplification
MA010a	Yamamoto et al 2002	peach	122	100–130	Monomorphic
MA019a	Yamamoto et al 2002	peach	108	90–110	Monomorphic
MA030a	Yamamoto et al 2002	peach	237	230–260	Monomorphic

Table 3 List of microsatellites that produced polymorphic repeatable amplification among the cultivars examined

SSR	Reference	origin	Predicted length (bp)	Size range (bp)	No of alleles	Heterozygosity
BPPCT007	Dirlewanger et al. 2002	peach	149	110–180	5	0.87
BPPCT009	Dirlewanger et al. 2002	peach	171	170–210	4	0.19
BPPCT017	Dirlewanger et al. 2002	peach	174	190–210	3	0.54
BPPCT030	Dirlewanger et al. 2002	peach	175	150–180	3	0.64
BPPCT037	Dirlewanger et al. 2002	peach	155	120–150	3	0.68
Pchgms10	Wang et al. 2002	peach	198	170–210	3	0.98
Pchgms20	Wang et al. 2002	peach	252	250–270	2	0.33
MA013a	Yamamoto et al 2002	peach	211	190–220	3	0.60
MA027a	Yamamoto et al 2002	peach	153	120–150	4	0.31
Ac7b	Apricot SSR has not published yet	apricot	–	160–200	5	0.70

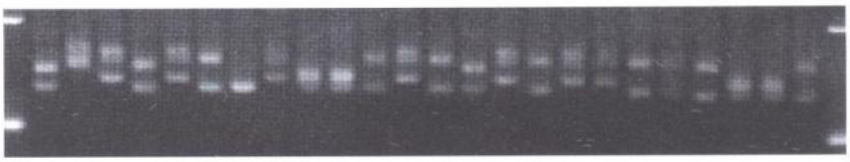

Figure 1 An example for the easy scoring and detection system using 3% Metaphore agarose in case of primer BPPCT007

Results

Total nineteen primer pairs were isolated, 18 from peach (*Dirlewanger* et al., 2002; *Wang* et al., 2002; *Yamamoto* et al., 2002) and 1 from apricot (not yet published) were used in this study. Seventeen of them were able to amplify distinct SSR products in the group of apricot cultivars tested. In the cases of only two primers (MA006b, MA009b) we did not obtain any amplification. Five primers resulted monomorphic patterns (BPPCT011, pchgms12, pchgms14, MA010a, MA019a, MA030a). Primer BPPCT029 showed complex band pattern (more than two bands/genotype) that made accurate scoring impossible therefore this marker was also discarded from the variability study (*Table 2*). This is probably implying the duplication of the locus in question in apricot. The remaining 10 primers (*Table 3*) produced clearly detectable polymorphisms and showed banding patterns consistent with their inheritance as single loci. An example of the easy scoring and detection system is presented in *Figure 1*.

The total number of polymorphic alleles detected was 35 ranging from 2-5 per locus with a mean value of 3.5 alleles/locus. The observed heterozygosity was relatively high ranged from 0.19 in BPPCT009 to as high as 0.98 in pchgms10 for individual loci, with an average value of 0.58 for all the loci studied.

There were several rare alleles detected in only a few cultivars. For example 'Ananasnij-cjurpinskij' and 'Kechpshar' or 'Ananasnij cjurpinskij and Korai piros' were sharing a common band, which is not present in the other cultivar. With the 10 primer pairs utilized were proven to be sufficient to set unique fingerprint for several cultivars studied.

The dendrogram generated from the UPGMA cluster analysis based on Reynold's (FST) similarity index classified of the 45 cultivars included in this study into two major groups and several subgroups. The two major groups (I. and II. contains cultivars with non Asian and Asian origin) The non Asian group was divided into several subgroups. The biggest and well confinable subgroup (I.a) consists of 9 accessions represent the so called 'Hungarian Best' containing cultivars grown in Hungary and in the neighbouring countries. Other big subgroups (I.b.) contains the 'óriás' variety group along with other cultivars from Cegléd (*Figure 2*).

Discussion

The possibility of cross species amplification among different '*Prunus*' species using SSR primers allowed us to use primers developed in peach to study genetic diversity in apricot. In this work, 90% of the primers used were able to amplify SSRs in apricot and more than half of them were polymorphic

Agarose-gel electrophoresis has been used for screening of microsatellites since, compared to PAGE electrophoresis or automated analysis, this is by far the cheapest and easiest.

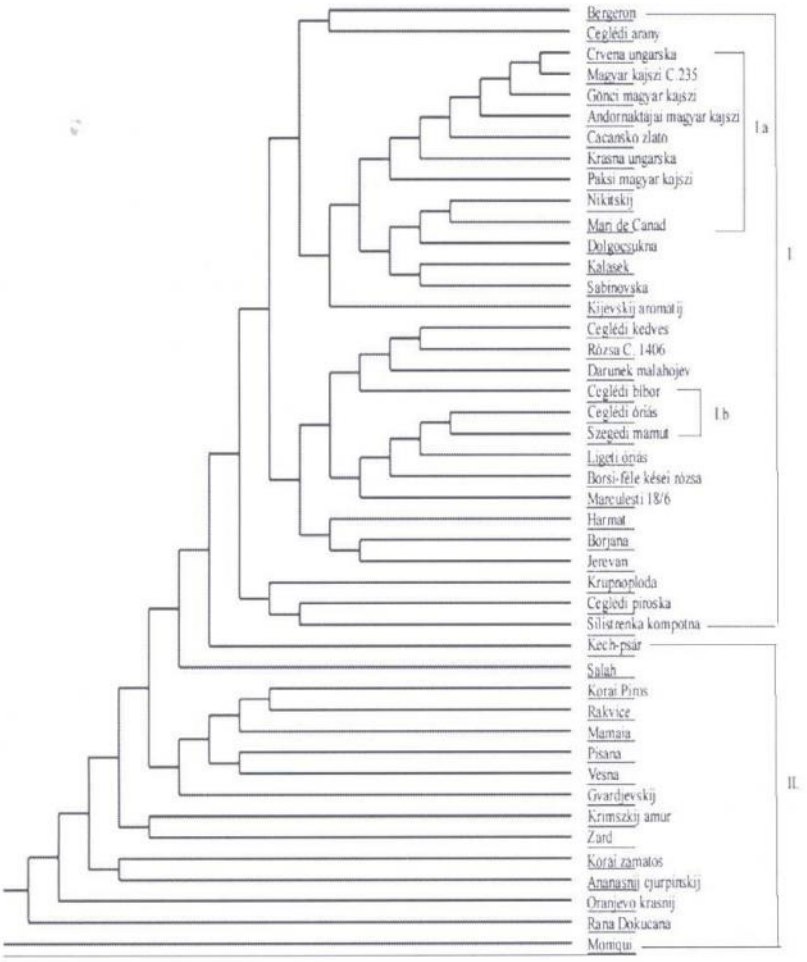

Figure 2 Dendrogram of 48 apricot cultivars based on UPGMA analysis using the similarity matrix generated the proportion of similar alleles method (*Reynolds* et al. 1983) after amplification with 10 SSR primers

Rather good resolution can be obtained especially in the 100-200 bp range (*Figure 1*). One of the main advantages of the method used is that it could be transferred and adopted for routine analysis in the productive sector such as nurseries or control agencies.

However it is possible that an automated detection system would be able to resolve allelic variation at a finer scale than electrophoresis analysis and, consequently the number of alleles obtained would be even higher than that reported in this work. On a higher resolution the six other primers showed monomorphic patterns could be polymorphic and their inclusion in the study would raise the level of differentiation.

The polymorphic loci showed an average 3.5 alleles/locus similar to that found by (*Badenes* et al., 2002) (3.1), (*Sanchez-Perez* et al., 2004) (3.9) and (*Hormanza* et al., 2002) (4.1) in apricot but definitely lower than that observed by (*Zhebentyayeva* et al., 2002) (7.6). The size ranges of most of the amplification fragments obtained in this work were similar to those reported for the same microsatellites in peach by (*Dirlewanger* et al., 2002; *Wang* et al., 2002; *Yamamoto* et al., 2002). From the 19 primers have been tried only 4 have reference on apricot. The size ranges of amplified fragments of all 4 primers were similar to those observed in apricot by (*Zhebentyayeva* et al., 2002) (pchgms 10, pchgms 20) and (*Sanchez-Perez* et al., 2004) (BPPCT007, BPPCT017).

The high value of the average alleles detected per locus (3.5) confirm that microsatellites are a very useful tool for apricot cultivar identification compare to other molecular marker systems such as isozymes (*Badenes* et al., 1996), RFLPs (*de Vicente* et al., 1998) or even RAPDs, with an average of only 2 alleles/primer (*Hurtado* et al., 1999). The fact that from the 19 only one primer used in this study have been developed in apricot, demonstrates the possibility of cross species transportability of high number of SSRs and consequently the availability of a number of primers developed in other species of *Prunus*. This means that it would not be required to develop new microsatellite primers in every Prunus species which is a rather long and costly process.

The obtained fingerprint and dendrogram presumably due to the low resolution of SSR screening method is far from complete. However, in spite of its incompleteness some very interesting observations can be made. The 'Hungarian Best' variety group contains many cultivars with different denomination. On one hand just in Hungary we know quite a few cultivars which belong to this group. On the other hand basically every neighbouring country has its own 'Hungarian Best' cultivar. These cultivars are not always the results of deliberate breeding work therefore their genetic origin is in question. This recent study was not able to find any variation among these cultivars confirming the assumption that they share a similar genetic background. One of the closest cultivars to this group was Bergeron. This observation corresponds to the findings of (*Hormanza* et al., 2002) that placed 'Gönci magyar kajszi' into a group of French cultivars containing Bergeron. It is quite possible and it has already been suggested by (*Faust* et al., 1998) that the presence of 'Gönci magyar kajszi' cultivar occurs in the pedigree of some French cultivars.

The 'óriás' variety group 'Ceglédi óriás' 'Szegedi mammut' and 'Ligeti óriás' are very similar according to pomological characteristics but considered as different genotypes. Previous studies carried out using RAPD and two different SSR markers (*Ruthner* et al., 2003), (*Romero* et al., 2003) have pointed out that they are very closely related and expectedly having the same genetic background. With this present work using a fourth set of SSR markers no differences have been found among the three cultivars in question.

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