PhD Thesis

GENETIC CHARACTERIZATION OF APRICOT CULTIVARS USING DNA-BASED MOLECULAR MARKERS

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1   INTRODUCTION

Apricot cultivation has a long tradition in Hungary. It has been cultivated for centuries. The remarkable characters of Hungarian apricot are recognized worldwide. However, good reputation alone is not enough to be successful on the market. We need to produce good quality fruits in order to do so. We have suitable lands, traditional varieties with good quality parameters and experts to achieve the above-mentioned goals. We only can fulfil the varying consumer demands if we continuously improve our production. The innovation should cover all areas including the selection of the cultivation areas, cultivation techniques and marketing. The usage of appropriate cultivars also has an important role to improve the competitiveness of apricot production. Unfortunately the variety selection accessible for Hungarian growers is only slowly increasing despite the fact that there is a high demand for new marketable varieties with PPV resistance.

The majority of apricot cultivars produced worldwide originates from the European eco-geographical group. Most of the varieties belonging to this group are self-compatible therefore their genetic variability is rather low. In order to meet the consumer demands the breeding programs have to use apricot accessions with even wider genetic backgrounds.

One of the tools to enhance plant breeding is the usage of DNA-based molecular markers for variety selection. The molecular markers are highly polymorphic inherited by Mendelian fashion, relatively easy to identify them and suitable for the examination of genetic origin and relation of varieties and for identification of accessions. Molecular makers also have an increasing role for the protection of intellectual property rights. The techniques can help the breeders collect royalty, which is the basis of the future breeding programs.

2 OBJECTIVES

The experimental design covered the followings:

- Adaptation of DNA-isolation procedures for apricot and available laboratory conditions.

- Identification and DNA fingerprinting of the most widely cultivated apricot cultivars in Hungary using RAPD and SSR markers.

- Comparison of different SSR markers isolated from peach and apricot in the examination of genetic diversity of apricot.

- Analysis of the genetic relation of different eco-geographical groups of apricot using microsatellites.

- Examination of the variability of Central European group in comparison to the polymorphisms of other main eco-geographical groups.

3 MATERIALS AND METHODS

3.1 Plant material

Apricot cultivars and hybrids of diverse type and origin were used in the experiments obtained from the orchard of the Corvinus University of Budapest, Department of Genetics and Plant Breeding in Szigetcsép, from the apricot germplasm collection of the Hungarian National Institute for Agricultural Quality Control in Tordas, from the orchard of the Fruit Culture Research Institute in Cegléd, from gene bank of BOKU University (Vienna) and from Laimburg and Ferrara (Italy).

3.2 DNA-isolation

Total genomic DNA was extracted from 0.1 g young leaves using three different protocols adapted to apricot. Leaf samples were harvested only in April and May. For the RAPD analysis we used the improved CTAB method while for SSR studies we used Qiagen's Dneasy Plant Mini Kit. The DNA was quantified by spectrophotometry (GeneQuant II RNA/DNA) and diluted to the appropriate concentration.

3.3 RAPD analysis

Sixteen apricot cultivars were selected for the study. The selection contained 14 Hungarian and two widely known ('Harcot', 'Bergeron') varieties. Sixty decamer oligonucleotides (Kits OPB, OPO and OPC from Operon Technologies) were used for PCR reaction. To ensure the amplified band reproducibility every reaction was repeated at least 4 times. Amplified products were size fractionated by electrophoresis in 1% agarose gels stained by ethidium-bromide and photographed.

3.4 Variety identification using SSR markers

Same 16 varieties were used for this study as in the case of RAPD analysis. We used 8 apricot specific fluorescently labelled (Cy5) primers with the published PCR programs. The amplified products were initially checked by electrophoresis in 3% BMA Metaphor agarose gel. Molecular sizes of the amplified fragments were estimated using 25 bp ladder (Promega). The precise fragment size was determined by using Pharmacia/ALF gene sequencer and Fragment Analyser 1.03 (Amersham-Pharmacia) software.

3.5 SSR analysis using peach specific microsatellite primers

Forty-five apricot accessions were selected which represent the cultivars grown in Central Europe. Extracted apricot genomic DNA was PCR amplified using 19 primer pairs, previously cloned and sequenced in peach (18) and apricot (1). Amplified PCR products were separated by using electrophoresis using 3% Metaphore agarose (Biowittaker, Maine, USA) gel, 1 x TBE (89 mM Tris, 89 mM boric acid, and 2 mM 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).

The genetic distance matrix was calculated based on the proportion of similar alleles method using MICROSAT software. The cluster analysis was conducted UPGMA algorithm using the NEIGHBOR program in PHYLIP version 3.5c and a dendrogram was constructed using the program TREEVIEW. Direct-count heterozygosity was calculated as the number of heterozygous genotype at a given locus divided by the total number of genotypes.

3.6 SSR analysis using apricot specific microsatellite primers

One hundred and thirty-three apricot accessions and one sample of *P. x dasycarpa, P. brigantiaca* and Plumcot were chosen to represent the European, Irano-Caucasian, Central Asian and North American cultivars with different origin. Ten different primer combinations originally developed for apricot SSR loci and representing different regions of the apricot genome were used for amplification of DNA from different apricot cultivars. PCR amplifications were conducted according to the required conditions of the primers. Fluorescently labelled microsatellite fragments were analyzed on an ABI 3100 capillary sequencer. Fragment sizing was performed using the ABI Genotyper software.

For each of the defined loci, SSR allelic composition was determined in 136 accessions. Putative alleles were indicated in alphabetical order (A for smallest fragment, etc.).

The program POPGENE was used to calculate allele frequencies at each locus, number of alleles, inbreeding coefficient (F_{ST}), gene flow (N_m) estimated from the formula $N_m=0.25(1/F_{ST}-1)$; observed (H_o) and expected (H_e) heterozygosity were measured. Based on the microsatellite data, genetic identity (I) and genetic distance (D) were calculated and values of D were used to conduct cluster analysis with an unweighted pair group method with arithmetic mean (UPGMA) algorithm and construct a dendrogram; a further dendrogram was constructed using the program TREEVIEW. Pair wise genetic distances among the 133 apricot accessions were computed using the program PAUP version 4.

4   RESULTS AND DISCUSSION

4.1   Adaptation of DNA extraction method for apricot

The first step of our work was to select the best plant material suitable for DNA isolation. We tested leaves of different development stages and we found fresh and young leaves just after budding the most applicable to the extraction. Fresh leaves contain less polysaccharides, phenols and other organic compounds that would make the DNA isolation even more difficult.

In our study we compared several DNA isolation techniques in order to find an appropriate method that fulfils certain criteria. Our aim was to find a method that enables us to extract high quality DNA suitable for PCR reactions able to handle large number of samples and relatively harmless to human health and natural environment.

At first we tried the (American) bean method. With increasing the incubation times and repeating the precipitation steps we could enhance the efficiency of the method. The method initially was elaborated to bean plant and not to apricot whose harsher tissues contains certain materials, which make the DNA isolation difficult. Eventually we managed to obtain high quality DNA but because of the above-mentioned difficulties we didn't use any longer.

The next method we studied was a CTAB protocol elaborated for woody species. In the beginning we obtained only very fragmented poor quality DNA, which was inappropriate for PCR reaction. After reducing the rotation speeds and incubation temperatures and avoiding those steps when DNA is a subject to rough impacts, the result was satisfactory. This method was proven to be fast and rather cheap. The main reason that we didn't use it further was the hazardous compounds in vast quantity necessary for the execution.

Finally, we chose the DNeasy Plant Mini Kit produced by Qiagen. This method can be performed in totally closed system, the used solutions are rather safe, and it can easily handle a large number of samples and means no threat for the users and environment. The only disadvantage of the method is the relatively high cost of the used chemicals. However the numerous beneficial features can easily compensate for its costliness.

4.2   Variety identification and DNA fingerprinting of the most popular apricot cultivars grown in Hungary using RAPD and SSR markers

The Operon primers were widely and efficiently used for genetic characterization studies within the *Prunus* species. Forty-five of the 60 primers tested were able to produce well detectable fragments but only 8 of them amplified highly reproducible polymorphic bands. These primers resulted 14 (53%) polymorphic amplification fragments. Several unique markers were detected which were present in one or two cultivars.

We have managed to differentiate among almost all of the varieties and to create a unique DNA fingerprint. In case of two 'Hungarian Best' clones 'Gönci magyar kajszi' and 'Magyar kajszi C.235' got same patterns. These results confirm our previous expectation that clones share almost the same genetic background and it is impossible to reveal the slight genetic differences using RAPD markers.

We did not manage to differentiate among the so called giant apricots ('Szegedi mamut', 'Ligeti óriás', 'Ceglédi óriás'). This confirms the hypothesis that cultivars 'Szegedi mamut' and 'Ceglédi óriás' emerged as a result of an admixture of varieties. Between cultivars 'Ligeti óriás' and 'Ceglédi óriás' there are no morphological differences only slight pomological ones (ripening time) presumably their genetic background are rather similar.

The results obtained in this research showed that relatively small numbers of RAPD primers can be usefully applied to identify apricot cultivars. RAPD markers are not only a perfect tool

for research purposes but they might be appropriate for commercial identification. Based on an agreement the National Quality Control Authority has the intention to use our elaborated method for variety identification purposes.

We have found five highly polymorphic SSR loci, which were able to differentiate among the cultivars examined. These five loci revealed SSR 35 alleles altogether in the 16 varieties, which were enough to set a unique DNA profile for most of the varieties
Since one of the examined varieties ('Gönci magyar kajszi') had reported allele sizes for these five loci we could use it as an inner standard to determine all fragment sizes within one base pair accuracy. Hereby our results are comparable with other labs data using the same SSR markers.
Our preceding hypothesis, which were set after examination with morphological and RAPD markers, that 'Szegedi mamut', 'Ceglédi óriás', and 'Ligeti óriás' varieties have the same genetic background, was confirmed after this microsatellite analysis. We could not find any differences between two Hungarian Best clones ('Gönci magyar kajszi' and 'Magyar kajszi C.235') either.

4.3 Molecular characterization of 45 apricot cultivars using cross species SSR amplification with peach primers

The flanking sequences of microsatellites are conserved in *Prunus* species therefore primers isolated from peach SSR loci is possible to use for genetic diversity studies in apricot.

10 primers produced clearly detectable polymorphisms and showed banding patterns consistent with their inheritance as single loci. 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 to as high as 0.98 for individual loci, with an average value of 0.58 for all the loci studied. There were several rare alleles detected only in few cultivars.

The dendrogram generated from the UPGMA cluster analysis based on Reynold's (F_{ST}) similarity index classified of the 45 cultivars included in this study into two major groups several subgroups. The two major groups divided into cultivars with non-Asian and Asian origin. The non-Asian group divided into several subgroups. The biggest and well confinable subgroup consists of 9 accession represent the so called 'Hungarian Best' containing cultivars grown in Hungary and in the neighbouring countries. Other big subgroup contains the giant apricot variety group along with other cultivars from Cegléd.

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. The high value of the average alleles detected per locus confirms that microsatellites are a very useful tool for apricot cultivar identification compare to other molecular marker systems.

MetaPhore agarose gel electrophoresis has been used for screening of microsatellites since, compared to PAGE electrophoresis or automated analysis, this is far more the cheapest and easiest. Rather good resolution can be obtained especially in the 100-200 bp range. One of the main advantages of the used method is, that it could be transferred and adopted for routine analysis in the commercial 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.

4.4 Microsatellite variability in 136 accessions using apricot SSRs to reflect their geographical origin and breeding history

<u>Microsatellite polymorphisms</u>

136 apricot accessions and 3 related species were analysed with 10 apricot SSRs. Polymorphic bands were obtained with all primers. Altogether, 133 alleles were identified in the set of 133 apricot accessions, with an average of 13.30 alleles per locus. The observed heterozygosity for individual loci ranged from 0.8636 in locus UDAp-410 to 0.3182 in locus ssrPaCITA 27, with an average of 0.6281. In all individuals, the observed heterozygosity (H_o) was clearly lower than the expected heterozygosity (H_e). Out of the 133 different alleles detected in all individuals, 32 alleles occurred only once in the investigated samples.

The results obtained in this study show that highly polymorphic homologous microsatellite markers could be effectively used for fingerprinting purposes in apricot. The higher number of alleles in our study is due to the use of 10 SSRs developed in apricot and selected for their high level of polymorphism and due to the high number of analysed cultivars.

F_{ST} values describe the proportion of variance within a species that is due to the populations' subdivision. According to Wright, F_{ST} values above 0.25 indicate very great genetic differentiation. As the average of F_{ST} values were 0.5768, genetic differentiation is relatively high among accessions. A high level of F_{ST} value indicates a low level of gene flow. Indeed, the gene flow estimated in this study was low, as described by N_m. The N_m value was 0,1834 in our study. If the N_m value lower than 1, the gene flow is considered low.

<u>Apricot genetic diversity and relationships among accessions</u>

To elucidate the genetic relationship among apricot cultivars, a dendrogram was produced using UPGMA analysis of pairwise genetic distances over 10 SSR loci. The dendrogram divided the cultivars into two major clusters. In cluster 1, there are two subclusters (1.1 and 1.2) that include most of the East European cultivars. Subcluster 1.1 includes six European cultivars and the American cultivar 'Goldrich'. Subcluster 1.2 comprises most of the European cultivars that are divided into five East European groups, with pedigree relatedness based on morphological characteristics: Magyar kajszi hybrids, 'Ceglédi óriás' hybrids, 'Luizet' hybrids, crossed with 'Ananas' or 'Umberto'; 'Kecskemét' apricots and 'Shalakh' hybrids. The rest of the apricot cultivars originating from different eco-geographical areas were located in the remaining subclusters of cluster 1 (1.3). Cluster 2 includes only Asian accessions (seedlings from Pakistan) with a high variability of different alleles. As a rule, cultivated varieties of fruit trees are maintained vegetatively by cutting, rooting of twigs, suckers or by the seed. This is in sharp contrast with their wild relatives, which reproduce from seed. Wild populations maintain themselves through sexual reproduction and, as a rule, are distinctly allogamous.

The dendrogram generated from the UPGMA cluster analysis produced several significant groups related to the pedigree and/or geographical origin of the genotypes.

Several hybrids with large fruits and sweet kernels belonging to the self-compatible Magyar kajszi or Hungarian Best group are present in Central Europe, particularly in the Pannonia region, with different designations suspected to be synonyms. In fact, we found that 15 out of 18 accessions are closely related, five of them ('Albena', 'Andornaktályai magyar kajszi', 'Crvena ungarska', 'Gönci magyar kajszi' and 'Nagygyümölcsű magyar kajszi') could even be synonyms.

Cultivars including the hybrids of 'Ceglédi óriás' are divided in two groups, one representing self-incompatible cultivars, also known as the "giant group" in Hungary, which confirm previous morphological characteristics and RAPD analysis, and the second self-compatible group with 'Ceglédi kedves' and 'Ceglédi arany'. This fertility trait could be the reason for the separation. The first group clustered with 'Ceglédi bíbor', which might be a hybrid or an ancestor of 'Ceglédi óriás'.

Subcluster 1.1 and 1.3 includes the West and East European and most of the North American cultivars. The presence of 'Harcot', haveecot', 'Bahrt' ('Orange red') and 'Morden-604' in subcluster 1.3 could be explained by the fact that most of the American cultivars carry European germ plasm in their pedigree. The vicinity of Irano-Caucasian accessions including 'Erevan', 'Shalakh' and 'Khurmai' supports the view that most American cultivars, besides having European germ plasm in their pedigree, have also been enriched with germ plasm of Asian origin.

Species comparison

In comparison with the related species, the common apricots were clustered, indicating a common genetic basis. The most distant from the common apricots is *P. brigantiaca*. It has several morphological differences from the common apricot, such as its fruit resembles a golden cherry tomato, plum taste and botanists class it as a little apricot. In addition, other authors using RAPD and AFLP markers, found *P. brigantiaca* as the most distantly related species of the common apricot cultivars. *P. x dasycarpa* and Plumcot, hybrids of *P. armeniaca* x *P.cerasifera* and *P. armeniaca* x *P. salicinia*, respectively, are intermediate between *P. brigantiaca* and other apricot species, also confirmed by others (Figure 1.).
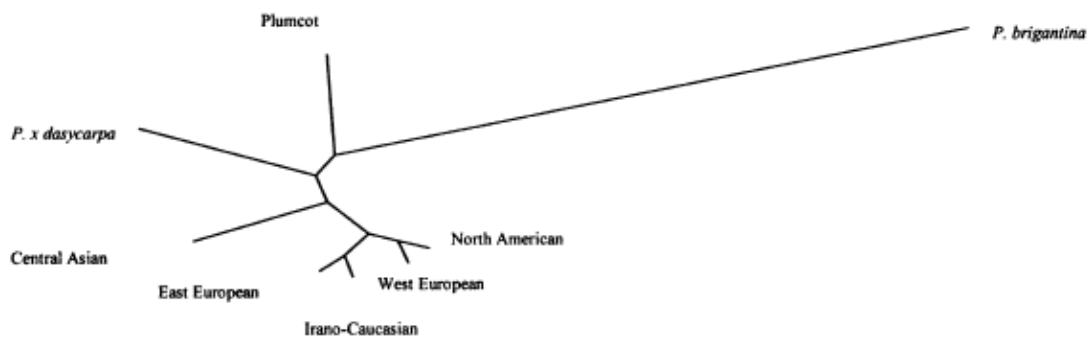
Figure 1 Dendrogram of apricot cultivars and related species by UPGMA based on Nei's genetic distance (1972)

Variation between eco-geographical groups

Traditional classification of apricot based on physiological and morphological differences mention four major eco-geographical groups. In this study, three groups were represented: the European group including West and East European and North American cultivars, the Irano-Caucasian group and the Central Asian group. The UPGMA cluster analysis of genetic distance between the groups highlighted the relationship between them. The explanation for the presence of North American group and the West European group in one cluster could be the fact that all the American cultivars carry some European germ plasm. According to

literature, some North American cultivars originated from hybridisation between European and Asian apricots. It is possible that with our nine selected North American cultivars, we have chosen some cultivars that are derived from European cultivars, which could explain the presence of this group close to the West European cultivars. The presence of the East European group with the Irano-Caucasian group in one subgroup would support the hypothesis that most of the European cultivars have originated by hybridisation with genotypes from the Irano-Caucasian group.

The use of molecular markers for genotyping of the accessions, the classification and management of apricot collections, germ plasm management or breeding purpose can be also tools to potentially promising paternal genotype advantageous in fruit tree species. Cultivars of most fruit tree species are maintained by vegetative propagation, and selection has operated during a limited number of generations. To achieve certain breeding goals in apricot, particularly for wider ecological adaptation, disease resistance and novel fruit quality traits, the use of germ plasm from different groups and eco-geographical regions will be necessary. Results from this study indicate that SSRs are excellent co-dominant markers for pedigree analysis that can be used in plant breeding programmes and to distinguish synonyms and hybrids.

5   New Scientific Results

- We identified such RAPD and SSR markers, which were able to set a unique DNA-profile for the 16 most widely cultivated apricot cultivars in Hungary.

- We observed that highly polymorphic homologous apricot microsatellite markers could be effectively used for fingerprinting purposes in apricot. They were proven to be more effective than the usage of heterologous peach primers were extensively used for cross-amplification in stone fruits.

- Our results clearly demonstrated that comparing the genetic distances and the presence of unique and shared alleles among the Irano-Caucasian, East European, West European Central Asian and North American cultivars, the Central European group - containing mainly Hungarian cultivars - and the Irano-Caucasian group showed the highest genetic identity. We confirmed the genetic relatedness of two French cultivars 'Bergeron' and 'Luizet' to the Hungarian cultivars. We have managed to prove that both European and Asian gene pools played an important role in the origin of North American cultivars. We identified unique alleles in every group except the Central European cultivar group. The Central Asian group contained the highest number of unique alleles.

- Our results revealed that genetic diversity was the lowest in the Central European group comprises of mainly Hungarian cultivars.

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