An analysis of apricot cultivars by random amplified polymorphic DNA and microsatellite primers

E. Chroboková, J. Raddová, M. Vachůn, B. Krška, M. Pidra

Faculty of Horticulture, Mendel University in Brno, Lednice, Czech Republic

Abstract

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The random amplified polymorphic DNA (RAPD) technique and microsatellites were used to study the genetic diversity and to identify cultivars within a collection of 95 cultivars of *Prunus armeniaca* L. A dendrogram based on 13 RAPD primers and a dendrogram based on 9 microsatellite primers were prepared using the unweighted pair group method with average (UPGMA) group analysis. In both dendrograms, the cultivars were classified into five groups, according to their geographic origin: hybrids originated by hybridization among cultivars of European and Asian origin, European cultivars, American cultivars, Asian cultivars and interspecific hybrids. Eleven cultivars were not distinguished (9 cultivars with supposed relatedness to Velkopavlovická cv., 2 cvs Vynoslivyj and Vynoslivyj 21/1 that are assumed to be clones) using 9 microsatellite primers. The similarities and the differences revealed among incorporation of cultivars into groups were compared with the literature findings. The results of these analyses have a direct implication on the selection of new breeding progenitors at the Faculty of Horticulture, Mendel University in Brno, Lednice, Czech Republic.

Keywords: *Prunus armeniaca* L.; microsatellites; RAPD primers; cultivar identification

Fast and cost-effective identification of important plant cultivars in agriculture and horticulture as well as for practical breeding purposes and plant proprietary rights protection is very important. The random amplified polymorphic DNA (RAPD) molecular methods and microsatellites based on the DNA study are effective tools often used for these purposes.

The RAPD method was reported by Williams et al. (1990). The greatest advantage of the RAPD approach is its technical simplicity, paired with the independence of any prior DNA sequence information. Molecular RAPD markers facilitate the study of genetic variability among *Prunus* rootstocks (Casas et al. 1999). This polymorphism was evaluated using 7 RAPD primers. Twenty-five apricot cultivars were analysed and 19 were distinguished using the RAPD markers (Mariniello et al. 2002). These markers were also successfully used for identifying and studying of the genetic relationships of grapes (Baránek et al. 2006; Moravcová et al. 2006; Pidra et al. 2006).

Another method used for cultivar identification is the simple sequence repeat (SSR) approach. The existence of repeated simple sequence motives (also called microsatellites) in plant nuclear DNA was demonstrated by Delseny et al. (1983) and Tautz, Renz (1984). Microsatellites are ideal DNA markers for genetic mapping and population-genetic studies because of their abundance, co-dominant character, ease of detection by PCR, extensive genome coverage and requirement of a small amount of starting DNA.

The first application of microsatellites in plants was in cultivar identification, where microsatellites were used to distinguish between cultivars of

grapevine (Thomas, Scott 1993) and soybean (Rongwen et al. 1995).

SSR markers proved to be an efficient tool for fingerprinting apricot cultivars. Messina et al. (2004) reported a set of 99 simple sequence repeats isolated from an apricot genomic library. Twenty SSRs were screened for their polymorphism in 16 apricot cultivars. Seventeen peach SSR markers were used in the molecular characterization of 25 apricot (*Prunus armeniaca* L.) accessions (Sánchez-Pérez et al. 2005). Apricot cultivars were grouped into seven principal groups in accordance with their origin and pedigree. Nine apricot cultivars from Hungary and eleven cultivars from North American and Southern European countries were studied using twelve SSR molecular markers (Romero et al. 2006). These markers made it possible to distinguish all cultivars. Microsatellite polymorphisms in 54 apricot landrace cultivars were identified using 26 *Prunus* microsatellite primers (Krichen et al. 2006). Five microsatellite primers and 28 resulting alleles were sufficient to discriminate among all 54 cultivars.

The objective of this study was to distinguish 95 apricot cultivars maintained by the Department of Fruit Growing at the Faculty of Horticulture in Lednice, Czech Republic. A set of 9 microsatellite primers (Cipriani et al. 1999; Aranzana et al. 2002; Lopes et al. 2002; Hagen et al. 2004; Messina et al. 2004) and the 13 RAPD primers (Operon) were used for this purpose. Finally, the similarity dendrograms of all apricot cultivars were created based on the SSR and RAPD approaches.

MATERIAL AND METHODS

Plant material

A set of 95 apricot accessions was used for this study. Cultivars were obtained from the apricot germplasm collection of the Department of Fruit Growing (Faculty of Horticulture, Lednice, Czech Republic).

DNA extraction

Total genomic DNA was extracted from young frozen leaves (0.1 g) using an DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The DNA concentration was determined by means of electrophoresis on a 1% agarose gel compared with a range of lambda DNA standards containing from 100 to 200 ng/µl DNA.

RAPD analysis

RAPD amplification (Williams et al. 1990) was performed using 13 RAPD primers (OPB-3, OPB-7, OPB-8, OPB-14, OPB-17, OPX-6, OPX-18, OPE-18, OPF-2, OPM-7, OPO-20, R-12, R-3). The primers were selected for a further analysis based on their ability to detect polymorphic amplified products among apricots cultivars.

The RAPD amplification was performed in volumes of 25 µl containing H_2O, 1× PCR buffer for Dynazyme DNA Polymerase (Finnzymes), 100µM each of dATP, dCTP, dGTP, dTTP (Promega), 0.4µM 10-mer primer (Operon), 1 unit of Taq polymerase (Finnzymes) and 20 ng of template DNA. After denaturation at 94°C for 3 min, DNA amplifications were performed for 40 cycles in a Biometra® UNO II thermal cycler (Biometra, Göttingen, Germany), according to the program per Williams et al. (1990) and the program for *Prunus* used in the Mendeleum Research Station (Raddová 2005). Each cycle consisted of: denaturation at 94°C for 20 s, primer annealing at 36°C for 1 min, extension at 72°C for 1 min. At the end of the 40 cycle-long PCR, a further polymerization step was carried out (72°C for 9 min).

The amplified products were separated by electrophoresis in 1.5% agarose gel and visualised by ethidium bromide staining (Sambrook et al. 1996).

RAPD polymorphic bands were scored as 1 for presence of a band and 0 for its absence and were transformed into a binary matrix. The matrix was transferred into a NTSYSpc 2.11T (Sxter Software, Satauket, USA) for dendrogram construction. Similarity among all cultivars was estimated according to the Jaccard coefficient (Jaccard 1901) of unweighted pair group method average (UPGMA). UPGMA is a simple bottom-up data grouping method used in bioinformatics for the creation of phylogenetic trees. The Jaccard coefficient was used to construct a final dendrogram. The dendrogram was displayed using the Tree View 1.6.6 software (Bio-Soft Net, Glasglow, UK).

SSR analysis

Nine different primer combinations originally developed for apricot SSR loci (Cipriani et al. 1999; Aranzana et al. 2002; Lopes et al. 2002; Hagen

et al. 2004; Messina et al. 2004) were used for amplification of DNA from different apricot cultivars. All of the primers were labelled with FAM, NED and JOE fluorescent dyes.

The SSR amplification was performed in volumes of 25 µl containing 1× PCR buffer, 1.5mM $MgCl_2$, 100µM each of dATP, dCTP, dGTP, dTTP (Promega), 0.2µM forward primer and 0.2µM reverse primer, 20 ng of template DNA and 1 unit of Taq polymerase (Finnzymes). The SSR amplification was carried out in 96-well thermal cycler Biometra® UNO II.

The PCR reactions were performed by using the temperature regimes according to Cipriani et al. (1999), Aranzana et al. (2002), Lopes et al. (2002), Hagen et al. (2004), Messina et al. (2004).

The efficiency of the SSR reaction was controlled by electrophoresis in 1.5% agarose gel and visualised by ethidium bromide staining (Sambrook et al. 1996). SSR products were analyzed by an ABI Prism 310 genetic analyzer (Applied Biosystems Inc., Forest City, USA) for the determination of variable size and differences of the alleles. A genetic analysis of fluorescently labelled alleles was carried out using the GeneScan analysis software (Applied Biosystem, Forest City, USA).

The FreeTree software package was used to generate a mean character difference matrix and then to produce a dendrogram using UPGMA analyses.

RESULTS AND DISCUSSION

Polymorphism of RAPD markers

A total of 95 apricot cultivars were analysed. A set of 13 RAPD primers were able to distinguish all the cultivars analysed. The dendrogram of genetic relationships among apricot cultivars based on 13 RAPD primers is presented (Fig. 1). The selected primers generated distinctive products in the size range of 430 bp (primer OPB-8) to 1,550 bp (primer OPO-20). Nei's (1973) gene diversity between cultivars achieved an average of 0.2616 and varied from 0.0208 (loci of O2O-950, E18-1250, M7-950, B14-1000) to 0.4999 (loci of OPB8-720 and OPB3-1350).

Polymorphism of SSR markers

Amplification was successful with 9 apricot SSR primers assayed with a total of 173 polymorphic bands scored. The number of presumed alleles per locus, revealed by the SSR analysis, ranged from fourteen to twenty eight. The mean number of alleles per locus was 19.2. The high number of alleles can be explained by the use of a large set of cultivars and by a high level of polymorphism of the SSRs in previous studies (Cipriani et al. 1999; Aranzana et al. 2002; Lopes et al. 2002; Hagen et al. 2004; Messina et al. 2004). Nei's (1973) gene diversity between cultivars had an average of 0.1305 and varied from 0.0208 to 0.4995.

Out of 95 cultivars analysed, 11 were not distinguished by means of microsatellite primers (a group of 9 cultivars with supposed relatedness to the cv. Velkopavlovická, as well as the Vynoslivyj and Vynoslivyj 21/1_9 cultivars, which are assumed to be clones).

Genetic relationships and clustering of cultivars by means of RAPD and SSR analysis

The RAPD dendrogram classified all the 95 cultivars into several groups. In RAPD dendrogram the first significant group is composed of hybrids between European and Asian cultivars (Fig. 1, parts 1a–c). The Betinka, LE-3241, LE-2927 and LE-2904 cultivars are hybrids of European and Central-Asian cultivars, the Saman Hong genotype is of Chinese origin (Fig. 1, part 1a). The SEO-44 clone was open polinated by an Asian apricot cultivar (Fig. 1, part 1b). In addition, the European Cegledi Bibor cultivar (Fig. 1, part 1c) is localized within this group of hybrids between European and Asian cultivars.

In SSR dendrogram the group including hybrids between European and Asian cultivars (Fig. 2, parts 1a–b) was formed. The cultivars LE-3241, LE-2926, LE-2927 (Fig. 2, part 1a) represent the hybrids coming from the Faculty of Horticulture, Lednice. The following cultivars, Vynoslivyj and Vynoslivyj 21/1_9, are supposed to be clones (Fig. 2, part 1b), moreover cv. Forum is formed by crossing cultivars of European and Irano-Caucasian origin. Zhebentyayeva et al. (2003) confirmed that Vynoslivyj was localized inside the group of European varieties.

In RAPD dendrogram the first significant group is composed by three parts (Fig. 1, parts 1a–c). The molecular RAPD method is casual, moreover, for the identification of genotypes a different information type was used in comparison with the SSR method. In the SSR dendrogram this group is di-

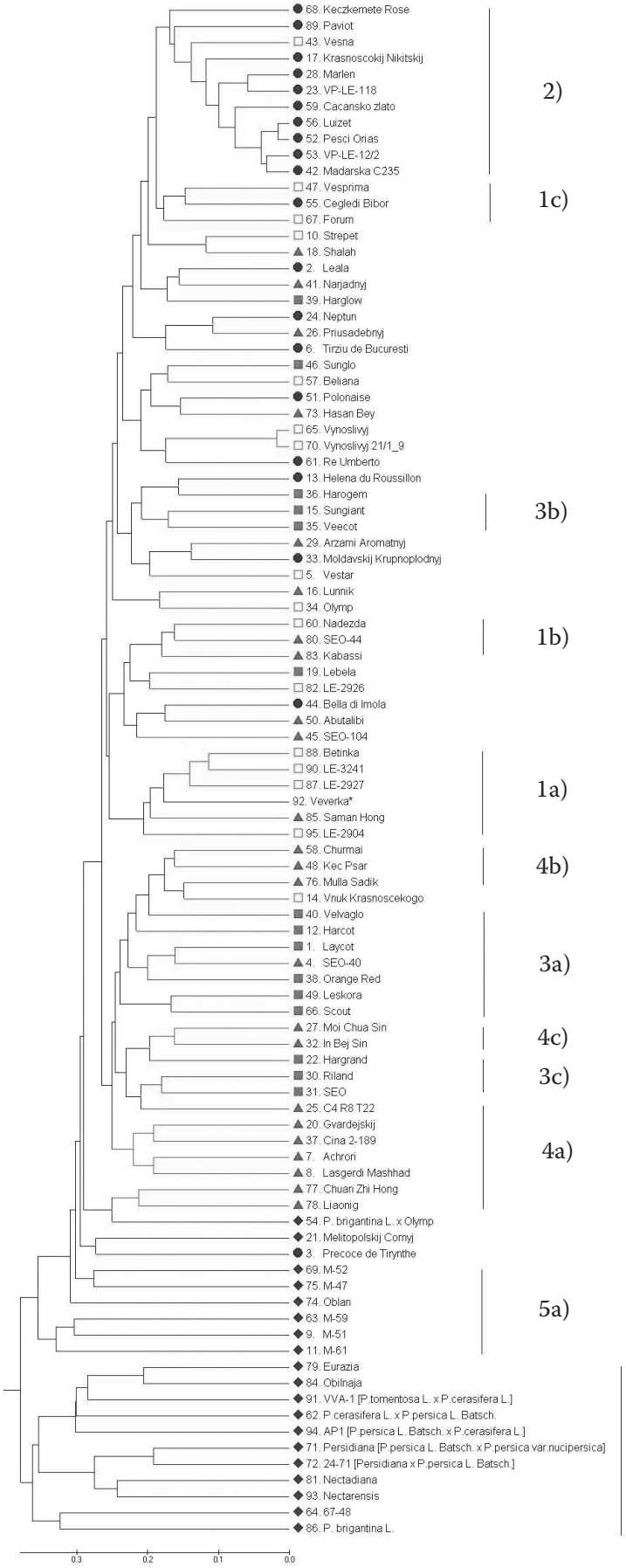

Fig. 1. Dendrogram of genetic relationships between apricot cultivars, constructed by UPGMA based on the Jaccard coefficient, based on 13 RAPD markers

The scale bar represents simple matching distance; symbol meaning: ♦ interspecific hybrid; ● Europe; ■ America; ▲ Asia; □ hybrids

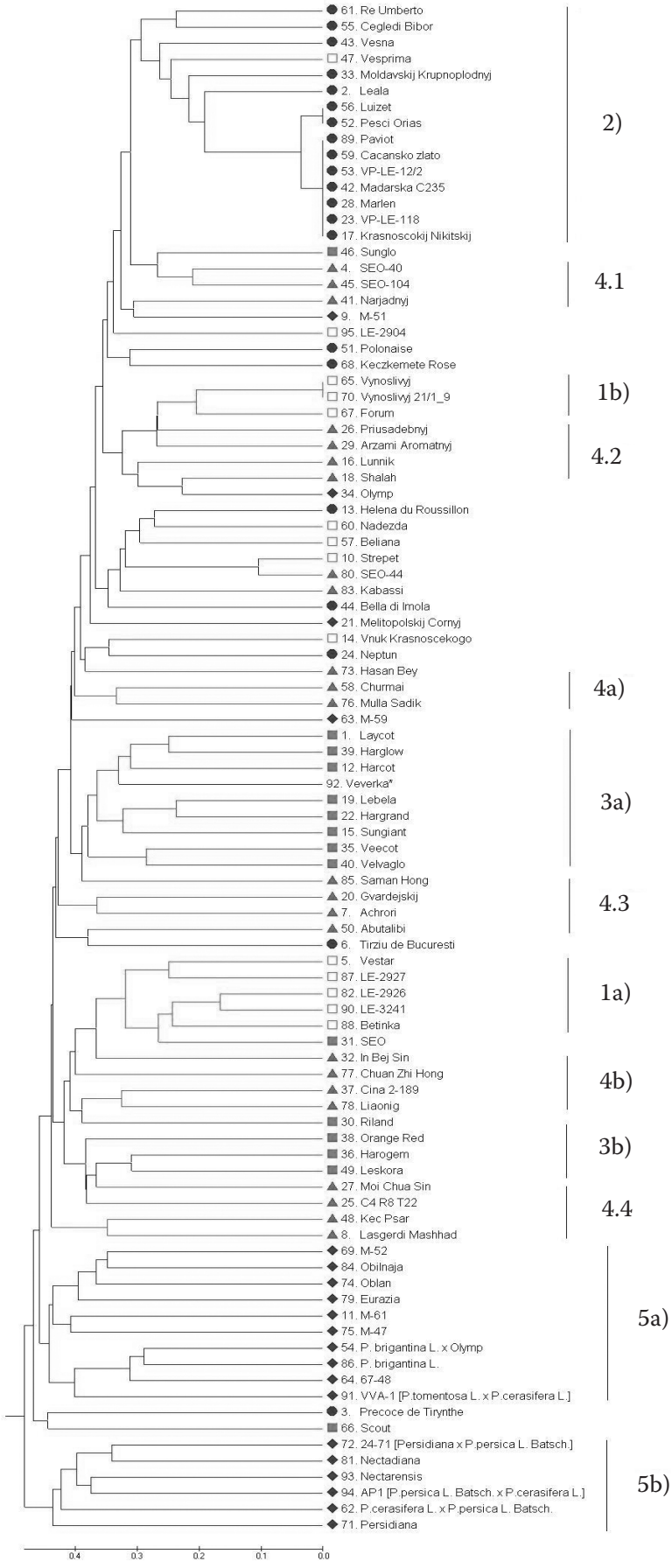

Fig. 2. Dendrogram of genetic relationships between apricot cultivars, constructed by UPGMA based on the Jaccard coefficient, based on 9 SSR markers

The scale bar represents simple matching distance; symbol meaning: ♦ interspecific hybrid; ● Europe; ■ America; ▲ Asia; □ hybrids

vided into two parts (Fig. 2, parts 1a–b). The molecular SSR method offers more accurate genotyping of analysed cultivars.

When comparing RAPD and SSR dendrograms, the genotypes LE-2927, LE-3241 and Betinka (originating from breeding program in Lednice) are common for part 1a in both the RAPD (Fig. 1, part 1a) and SSR dendrograms (Fig. 2, part 1a). Parts 1b and 1c were formed in the RAPD dendrogram for well-arranged assessment of genotypes.

In the RAPD dendrogram the second group contains European cultivars (Fig. 1, part 2). Ten genotypes have European origin, moreover hybrid Vesna is included. Genotype Krasnoscokij Nikitskij and clones of Velkopavlovická (VP-LE-12/2, VP-LE-118) were analysed using isoenzymes and RAPD analysis (Zhebentyayeva, Sivolap 2000). They assumed the Krasnoscokij and Velkopavlovická cultivars were localized within the group of European and Irano-Caucasian varieties.

In the SSR dendrogram the group containing European cultivars (Fig. 2, part 2) was created. Thirteen genotypes are of European origin, moreover 2 hybrids, Vesna and Vesprima, are included. Vesna and Vesprima represent hybrids between European and Asian cultivars, therefore they are located within the European group in the SSR dendrogram (Fig. 2, part 2). In RAPD dendrogram Vesprima is situated near the European genotypes.

The group of genotypes with supposed relatedness to Velkopavlovická was analysed by means of RAPD and SSR methods. In the RAPD dendrogram all the analysed genotypes were differentiated (Fig. 1, part 2). In the SSR dendrogram 9 genotypes with supposed relatedness to Velkopavlovická were not distinguished using SSR primers (Fig. 2, part 2).

In accordance with the SSR analysis, Velkopavlovická was grouped as part of the European cultivars. In the same way Zhebentyayeva et al. (2003) detected that the Velkopavlovická cultivar was grouped as part of the European varieties. Maghuly et al. (2006) supposed that Velkopavlovická was clustered with Ksna Ugarska, near the group of European cultivars.

The Luizet and Pesci Orias cultivars were not distinguished by means of an SSR analysis. They are situated within the group of undistinguished cultivars with supposed relatedness to Velkopavlovická (Fig. 2, part 2). On the other hand, Luizet and Pesci Orias were separated in the special cluster in the RAPD dendrogram (Fig. 1, part 2). Hormaza (2002) suggested Luizet was classed with Gönci-Magyar. In a similar study Maghuly et al. (2006) supposed that the Luizet cultivar was not distinguished with Venus 1414.

According to the SSR results the following cultivars: Cacansko zlato, Cegledi Bibor, Maďarská C-235, Velkopavlovická and Luizet were grouped in the subgroup of Velkopavlovická varieties. This result is in agreement with the study of Maghuly et al. (2005) who reported that Cacansko zlato, Maďarská C-235, Velkopavlovická (Le 1) and Luizet 343 cultivars were localized in one subgroup. The same result was confirmed by Maghuly et al. (2006).

The third group in RAPD dendrogram is composed of American cultivars (Fig. 1, parts 3a–c). In part 3a, 6 genotypes are of American origin, moreover Asian genotype SEO-40 is included (Fig. 1). Other genotypes, SEO-44 and SEO-104, were also analysed. They are coming from open-pollination of Asian genotypes. These genotypes are localized in the vicinity of Asian genotypes or hybrids with partly Asian origin.

According to the RAPD analysis the Harcot cultivar was grouped with the SEO-40 clone (Fig. 1, part 3a). In the same way, Hurtado et al. (1999) used a set of 45 RAPD primers in order to analyse 18 apricot cultivars and the Harcot cultivar was clustered with the Stark Early Orange (SEO) and Sunglo cultivars within the group of North-American cultivars. Badenes et al. (1996) suggested that North-American cultivars originated by hybridization between European and Asian apricots. The following cultivars: SEO, Hargrand and Riland are grouped together (Fig. 1, part 3c). According to Hagen et al. (2002), the SEO cultivar is located in section D with the Russian cultivars Badami and Oranzeno Krasnyj with high chilling requirements.

In the SSR dendrogram the third main group comprises American cultivars (Fig. 2, parts 3a–b).

In the SSR dendrogram (part 3a) 8 genotypes are of American origin, Veverka has unknown origin (Fig. 2, part 3a). Genotypes Velvaglo, Laycot, Harcot are common for part 3a in RAPD dendrogram (Fig. 1, part 3a) and in SSR dendrogram (Fig. 2, part 3a).

According to the SSR analysis, Veecot and Harcot were grouped within the group of American varieties (Fig. 2, part 3a). Hormaza (2002) confirmed that Veecot, Harcot and Henderson were put in one group. This result agrees with the listed varietal registration given by Brooks, Olmo (1997) who indicate that both Veecot and Harcot had cv. Perfection in their pedigree. In a similar study of Romero et

al. (2003) the Veecot, Goldrich and Katy cultivars were grouped in the first smaller group and Harcot, Fergani-apricot, Morden-604, and Tadeo in the second smaller group. In the following study Romero et al. (2006) supported the idea that Veecot and Harcot were grouped together along with the Tadeo, Goldrich and Katy cultivars.

According to the SSR analysis, the Orange Red cultivar is situated in the part 3b of Fig. 2, with Harogem, Leskora and Riland cultivars. Maghuly et al. (2005) formed a group of cultivars of varied origin, among which were Orange Red, Harcot, Shalah, Churmai and Veecot.

The Scout was situated in the RAPD dendrogram within the American group (Fig. 1, part 3a), in the SSR dendrogram Scout was found near the interspecific hybrids (Fig. 2, parts 5a–b). This information is not surprising, because Scout has an unknown origin.

The fourth group is composed of Asian cultivars (Fig. 1, parts 4a–c). The RAPD analysis showed that Churmai was joined with Kec Psar and Mulla Sadik (Fig. 1, part 4b), but the cultivar Arzami Aromatnyj was classed with Moldavskij Krupnoplodnyj. In contrast, according to the results shown by Zhebentyayeva, Sivolap (2000) the Churmai and Arzami cultivars were grouped together. The Chinese Moi Chua Sin and In Bej Sin cultivars are situated within the part 4c of Fig. 1. Zhebentyayeva, Sivolap (2000) confirmed that these cultivars were grouped within one heterogenous group of cultivars of Central Asian, Irano-Caucasian, Chinese and Dzungar-Zailij origin.

The fourth group in the SSR dendrogram consists of Asian cultivars representing Chinese, Irano-Caucasian and Central-Asian areas (Fig. 2, parts 4a, 4b and parts 4.1, 4.2, 4.3, 4.4).

No continuity was found between genotypes situated in additional groups and the RAPD dendrogram. In Fig. 2, part 4a, the Churmai and Mulla Sadik cultivars come from Central Asia. Zhebentyayeva et al. (2003) analysed a similar cultivar Churmai Ranni, which clustered with Kok-pshar and Samyi Rannii cultivars. Genotypes Churmai and Mulla Sadik are common for part 4a in the RAPD (Fig. 1, part 4a) and SSR (Fig. 2, part 4a) dendrograms.

According to the SSR analysis, the In Bej Sin cultivar is localized with Chuan Zhi Hong, Čína (China) 2-189 and Liaonig (Fig. 2, part 4b). In contrast with this result, Zhebentyayeva et al. (2003) suggested In Bej Sin was localized with Moi Chua Sin. Genotypes Chuan Zhi Hong, Liaonig and Čína 2-189 are common for part 4b in the RAPD dendrogram (Fig. 1, part 4b) and the SSR dendrogram (Fig. 2, part 4b).

SSR analysis confirmed that Shalah and Arzami Aromatnyj cultivars are localized in part 4.2 (Fig. 2). In the same way Zhebentyayeva et al. (2003) revealed that the Shalah cultivar and similar Arzami cultivar are clustered together. Both cultivars are similar to Chinese apricot cultivars in anatomical and morphological traits (Kovaljov 1963; Rostova, Sokolova 1992). In addition, Shalah represents an Irano-Caucasian variety. Romero et al. (2003) clustered the Shalah and Hybrid-12 cultivars (it is considered to be open pollination of the Armenian variety Shalah according to the Genetics and Breeding Department, Szent Istvan University, Budapest, Hungary). Shalah showed a very low coefficient of genetic similarity (0.15). Furthermore the Priusadebnyj cultivar was clustered with Shalah and Arzami Aromatnyj (Fig. 2, part 4.2). Maghuly et al. (2005) grouped cv. Priusadebnyj with Samarkandskij Rannij, using 10 polymorphic microsatellite markers. In a similar study Maghuly et al. (2006) used 10 different polymorphic microsatellite markers and the Priusadebnyj and Samarkandskij Rannij cultivars were not distinguished. Moreover Romero et al. (2003) reported that the Shalah and Erevan cultivars are supposed to be synonyms.

The SSR analysis revealed cv. Kec Psar was localized with Lasgerdi Mashhad (Fig. 2, part 4.4). Maghuly et al. (2005) suggested that Kec Psar showed a very high level of genetic distance in the dendrogram. In addition Romero et al. (2003) put the Kec Psar, Vnuk-partizana and Olymp cultivars in the same group.

In the RAPD dendrogram the fifth group includes interspecific hybrids and botanical species (Fig. 1, parts 5a–b).

For example, the M-51 and M-61 hybrids are located in the part 5a (Fig. 1). M-51 originated by crossing [(*P. salicina* L. × *P. armeniaca* L.) × (*P. armeniaca* L.)], however M-61 originated by crossing [(*P. besseyi* L. H. Bailey. × *P. salicina* L.) × (*P. domestica* × *P. armeniaca* L.)] × [*P. armeniaca* L.]. The Eurasia hybrid was localized in part 5b, Fig. 1. Kramarenko (2006) suggested the Eurazia-21 rootstock was an interspecific hybrid from crossing (*P. salicina* L. × *P. americana* L. × *P. cerasifera* L.) species.

The following genotypes Persidiana, 24-71 (Persidiana × *P. persica*), Nectadiana, Nectarensis, *P. cerasifera* × *P. persica*, AP1 (*P. persica* × *P. cerasifera*) are common for the part 5a in RAPD dendro-

gram (Fig. 1, part 5a) and SSR dendrogram (Fig. 2, part 5a). Genotypes Persidiana, 24-71, *P. cerasifera* × *P. persica* and AP1 contain in their origin *P. persica* species.

In the SSR dendrogram the fifth group contains interspecific hybrids and botanical species (Fig. 2, parts 5a–b).

Ten interspecific hybrids are localized in a separate part of the dendrogram (Fig. 2, part 5a). M-52 and M-47 represent hybrids with *P. armeniaca* L. and *P. cerasifera* L. in their pedigree. The complex hybrid M-61 comprises the following species: *P. besseyi* Bailey, *P. salicina* L. and *P. domestica* L. as well as *P. armeniaca* L. In addition, some other hybrids are presented in this group, e.g. *P. brigantina* × Olymp, Obilnaja, Oblan, VVA-1 (*P. tomentosa* L. × *P. cerasifera* L.) and hybrid 67-48.

Six interspecific hybrids are situated in the last part of the SSR dendrogram (Fig. 2, part 5b): *P. cerasifera* L. × *P. persica* L. Batsch., Persidiana × *P. persica* L. Batsch., *P. persica* L. Batsch. × *P. cerasifera* L., Persidiana, Nectadiana and Nectarensis. Localization of all these cultivars together could be explained: *P. persica* is presented as a parent in all three genotypes.

The Precoce de Tirynthe and Scout cultivars are situated between the parts 5a and 5b (Fig. 2). While the parentage of Scout is unknown, it was selected from a Canadian import of seeds from Manchuria that took place in about 1930 (Brooks, Olmo 1972). This cultivar was localized within the group of interspecific hybrids, and considering its unknown parentage this location is logical. On the other hand, the Precoce de Tirynthe cultivar represents a typical Mediterranean variety; therefore its location is not logical within the group of interspecific hybrids. In the RAPD dendrogram the genotype Precoce de Tirynthe is localized in the part 5a, Scout is in the American part 3a.

Both techniques may provide useful information on the level of polymorphism and diversity in apricot. Repeating twice each amplification reaction and using conservative criteria of band selection can lead to generating reliable RAPD data. Nonetheless SSRs is also a marker of choice for an analysis in apricot. High polymorphism, abundance and co-dominant nature of SSRs make these markers more adequate for pedigree analyses and for other marker applications in genetics (segregation studies, genome mapping) or breeding (e.g. importance for a certification program to protect the new releases from breeding programs). The results obtained from this study would be useful for better management and identification of cultivars and also should help to avoid mislabelling of the genotypes studied.

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Corresponding autor:

Ing. Eva Chroboková, Ph.D., Research and Breeding Institute of Pomology Holovousy, Ltd.,
Holovousy 1, 508 01 Hořice, Czech Republic
phone: + 420 493 692 821-3 (line 30), e-mail: chrobokova.vsuo@seznam.cz