Original Paper

Genetic variability of commercially important apple varieties (*Malus* × *domestica* Borkh.) assessed by CDDP markers

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Apple stand on the top of the most desirable and most produced fruit species in the world. Despite enormously wide genetic diversity among existing apple varieties, the current market is mostly oriented on several cultivars of commercially attractive traits. For the preservation of genetic resources and for breeding programmes, the evaluation of genetic diversity is fundamental. Number of marker systems have been developed and adopted in the assessment apple germplasm. In the present study, CDDP marker technique was used to analyse polymorphism within the genomes of fifteen apple varieties which are of large commercial use. Five primer combinations were used in the PCR amplification: WRKY-F1/WRKY-R1, WRKY-F1/WRKY-R2, WRKY-F1/WRKY-R2B, WRKY-F1/WRKY-R3 and WRKY-F1/WRKY-R3B. All primer combinations produced polymorphic amplification patterns. In some primer combinations, identical amplification profiles were observed in few varieties, but amplification patterns of all combinations merged were specific for each apple variety. Identical profiles were only seen in Red Delicious and Granny Smith in F1/R1 primer combination, May Gold and Paula Red, and Selena and Melodie in F1/R2 primer combination and May Gold and Paula Red when F1/R3B primers were used. Based on the CDDP markers, UPGMA algorithm assessed cultivars Paula Red and May Gold as the most similar (with similarity value of 0.909), whereas Gloster and Ambrosia showed to be the least similar within the analyzed set (similarity value of 0.200). The study proved CDDP markers to be suitable tool to produce polymorphic amplification patterns in apple genotypes.

Keywords: conserved DNA-derived polymorphism, genetic variability, WRKY, DNA markers

1 Introduction

Apple (*Malus* × *domestica* Borkh.) is considered one of the most desirable fruits in the world and the most economically important fruit crop in the temperate zone (Sansavini et al., 2004). The crop is perennial and relatively old, being originally domesticated before at least 4 000 years in central Asia (Cornille et al., 2014). During the history of its cultivation, more than 10 000 apple cultivars have been breeded but many of them already perished (Way et al., 1990; Velasco et al., 2010). Still, approximately 7,000 varieties are cultivated in temperate areas of the northern and southern hemisphere and provide huge genetic diversity. Apple can be perceived somewhat unique in terms of employing new alongside with older cultivars in cultivation practice and marketplace, in contrast to other perennials such as grapes, where new cultivars are not easily adopted (Myles et al., 2011; Myles, 2013). Thus, apple has both long history and a continued history of crop improvement up to present time (Gross et al., 2014). However, despite great genetic diversity of apple cultivars, the contemporary production tends to be restricted to only a few, leading to dramatic loss of diversity. For instance, as many as 70% of European apple production in 2018 comprised only 10 cultivars (WAPA, 2019).

The assessment of genetic diversity is fundamental for the identification and preservation of genetic resources (*in situ* or *ex situ*). To date, numerous molecular markers and DNA fingerprinting techniques have been developed for identification and polymorphism analysis of many plants. Molecular markers may serve to generate genotype-specific DNA profiles or to tag genes to select suitable genotypes and are therefore a useful tool for enhancing the efficacy of breeding. Genetic diversity of apple cultivars has been evaluated using various types of DNA markers (Goulao and

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Oliveira, 2001; Oraguzie et al., 2005; Pereira-Lorenzo et al., 2007). The diversity of subsets of apple genotypes has been assessed e. g. by SSRs (simple sequence repeats) (Goulao and Oliveira, 2001; Royo and Itoiz, 2004; Oraguzie et al., 2005; Pereira-Lorenzo et al., 2007;), ISSRs (inter-simple sequence repeats), RAPD (random amplified polymorphic DNA) (Goulao and Oliveira, 2001; Oraguzie et al., 2001; Royo and Itoiz, 2004; Dar et al., 2019), AFLP (amplified fragment length polymorphism) (Goulao et al., 2001; Tignon et al., 2001) and RFLP markers (restriction fragment length polymorphism) (Gardiner et al., 1996; Tignon et al., 2001; Žiarovská et al., 2019).

Within the trend toward gene-targeted functional markers rather than markers of non-coding regions, different marker systems were developed. Conserved DNA-derived polymorphism technique (CDDP) targets candidate plant genes (Collard and MacKill, 2009). Similarly to RAPD and ISSR techniques, the method is based on the single primer amplified region principle as a single primer is used as a forward and reverse primer. Gene-specific primers used in the amplification target conserved DNA regions within genes, or, ideally, sequences of gene families present in multiple copies within plant genome. Specific sequences anneal to conserved parts of common functional genes playing role in responses to biotic and abiotic stress stimuli or development. The technique uses conserved gene sequences present at multiple sites within plant genome which provide multiple primer binding sites and produce polymorphic banding pattern easily separated by agarose electrophoresis. Regarding a relatively big number of conserved gene regions and families, any region of plant genome can be tagged using this technique. The reproducibility of the technique is believed to be improved over RAPD method by using longer primers and applying higher annealing temperature (Collard and Mackill, 2009). In the evaluation of genetic diversity, the CDDP technique has already shown its usefulness for various plant species including wheat (Kyseľ et al., 2019), *Oryza sativa* (Collard and Mackill 2009), date palm (Atia et al., 2017), chrysanthemum (Li et al., 2013), *Paeonia suffruticosa* (Wang et al., 2014), chickpea (Hajibarat et al., 2015), *Pistacia vera* (Aouadi et al., 2019) and *Rosa rugosa* (Jiang and Zang, 2018). To our knowledge, this is the first report aiming to examine genetic diversity in apple germplasm by CDDP markers.

2 Material and methods

2.1 Biological material and DNA extraction

Apple fruit of different varieties was purchased from commercially available trade networks. Our analyzed set included 15 varieties: Ambrosia, Gala, Gloster, Granny Smith, Jonaprince, May Gold, Melodie, Modi, Paula Red, Pink Lady, Pinova, Red Delicious, Renoire, Sampion and Selena. After the transport to the laboratory, the fruit was surface sterilized with ethanol, rinsed with distilled water and kept frozen at -20 °C until next processing steps. Total genomic DNA was isolated from apple pulp randomly extracted from fruit using GeneJET Plant Genomic DNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. Total genomic DNA was extracted as a pool from individual processed pulp extraction for every apple variety. The assessment of the quantity and quality of extracted DNA was carried out spectrophotometrically by Nanophotomoter P360 (Implen).

2.2 CDDP markers

For the evaluation of genetic diversity among studied apple varieties CDDP marker system was chosen. These markers are gene specific and are based on conserved regions of DNA or gene families. The primers are derived from protein sequences of well characterized genes. In our study, primers designed by Collard and Mackill (2009) were used for PCR amplifications of functional regions of WRKY gene (Table 1). The PCR reaction was performed by TProfessional Basic gradient XL (Biometra) and followed the steps: initial denaturation at 95 °C for 15 min; 40 cycles consisting of

Table 1 CDDP primers used in the study

Primer	Sequence (5'–3')	Length (bp)
WRKY-F1	TGGCGSAAGTACGGCCAG	18
WRKY-R1	GTGGTTGTGCTTGCC	15
WRKY-R2	GCCCTCGTASGTSGT	15
WRKY-R3	GCASGTGTGCTCGCC	15
WRKY-R2B	TGSTGSATGCTCCCG	15
WRKY-R3B	CCGCTCGTGTGSACG	15

Source: Collard and MacKill, 2009

95 °C for 45 s, 54 °C for 45 s, 72 °C for 90 s; and the final extension was held at 72 °C for 10 min. The PCR amplicons were separated on 1.5% agarose gels prepared with 1× TBE buffer and DNA fragments were stained using GelRed® Nucleic Acid Gel Stain (Biotinum). BDAdigital system 30 (Analytik Jena) was used to visualize amplicons on agarose gels.

2.3 Data analysis

Amplified and separated fragments visualized on gels were scored for the presence (1) or absence (0), generating the binary 0/1 matrix. Based on the binary matrix, similarity matrix using Dice coefficient was calculated and UPGMA dendrogram was constructed using online accessible software (http://genomes.urv.cat/UPGMA/). POPGENE 1.31 (Yeh et al., 1999) was used to calculate the following measures of genetic diversity: Nei's (1973) gene diversity and Shannon's Information indices.

3 Results and discussion

CCDP marker system has been developed by Collard and MacKill (2009) to exploit conserved DNA regions of well-characterized plant genes. The authors examined the genetic polymorphism of 10 rice varieties and breeding lines and propose the marker system to be simple and suitable for most laboratories, especially for those with preference for agarose gel electrophoresis. Since then, these markers were applied in multiple studies to evaluate the genetic diversity of numerous plant species as this mehod is conservative enough to generate repeatable fingerprints for individual varieties but polymorphic enough to find polymorphism among different varieties of the same specie (Li et al., 2013; Wang et al., 2014; Hajibarat et al., 2015; Atia et al., 2017; Jiang and Zang, 2018; Kyseľ et al., 2019). In the present study, we applied CDDP marker system to evaluate the genetic diversity within apple cultivars. To our knowledge it is the first time these markers have been used in this fruit species. Our analyzed set comprised mainly cultivars of commercial importance such as Gala, Granny Smith, Red Delicious a. o. Using five primer pair combinations (Table 1) we observed large scale of polymorphic patterns.

Five primer combinations generated in total 79 fragments. Ambrosia was determined to be the variety with the lowest number of amplicons, whereby Gloster showed amplification patterns with the highest number of amplified products. Using primer combination F1/R1 we observed altogether 14 fragments in the set of studied genotypes. The length of acquired fragments ranged from 49 bp to 982 bp and none of the observed amplicons was generated in all 15 varieties. Only Red Delicious and Granny Smith shared the same amplification pattern, whereas the remaining varieties produced specific profile present only in the one variety (Figure 1A). PCR amplification with F1/R2 primer combination resulted in 15 amplicons of various length (41–994 bp), whereby May Gold with Paula Red and Selena with Melodie shared the same profile (Figure 1B, C). The combination of primers F1 and R2B in the PCR reactions gave rise to overall 16 different amplicons (75–980 bp) which created specific profile for each apple genotype. Similarly, 16 products of various length (55–1,060 bp) were recorded after amplification with F1/R3 primer combinations,

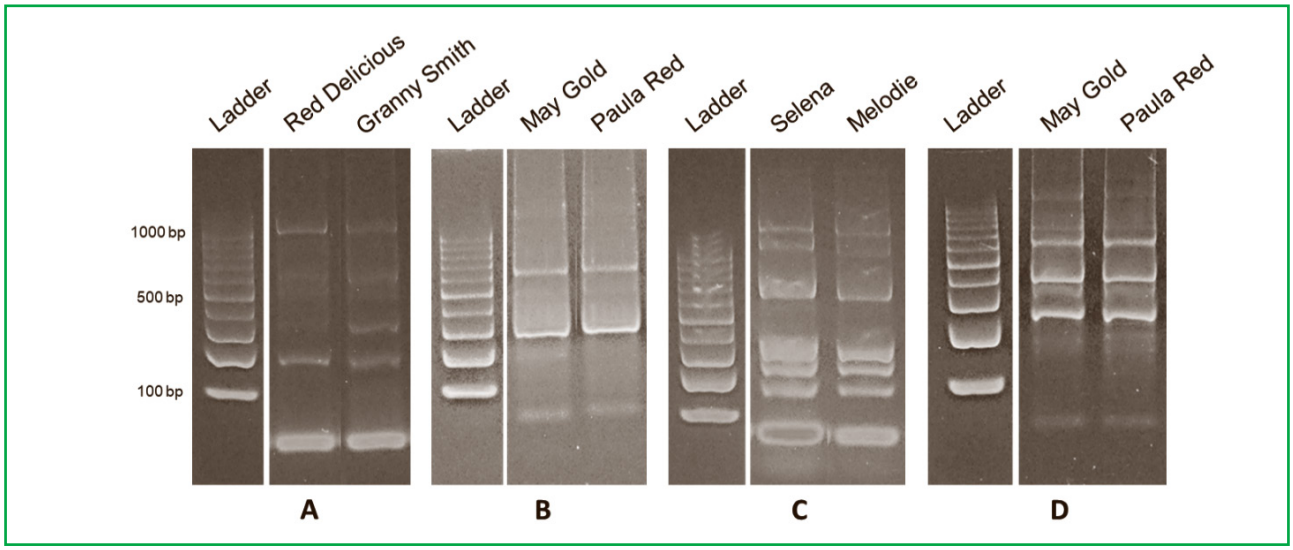

Figure 1 Identical amplification profiles acquired by CDDP markers in the analysed set of apple varieties
A – primer combination F1/R1; B–C – primer combination F1/R2; D – primer combination F1/R3B

creating specific profiles for each variety. Only 600 bp long amplicon was produced in these PCR reactions in all apple varieties. Overall, 18 amplicons with lengths varying from 40 bp to 1,112 bp were produced using F1/R3B primers. All varieties except for Renoire had a 700 bp amplicon. May Gold and Paula Red shared the same profile for this primer combination (Figure 1D), whereas unique profiles were recognized in the remaining 13 apple genotypes. Similarly to F1/R1 primer combination, none of the amplicons generated by F1/R2, F1/R2B and F1/R3B was present in the CDDP profile of the whole set of studied varieties.

Figure 2 illustrates the resulting dendrogram constructed based on 0/1 matrix of CDDP amplification profiles. The UPGMA clustering algorithm grouped the apple genotypes into several clusters. Gala and Pink Lady were assessed as the most similar (similarity value of 0.921) and were therefore clustered together as their marker profiles were the most similar within the analyzed sample collection. In each primer combination, the two varieties differed in the presence/absence of one amplicon. As for F1/R1 primers, a 210 bp amplicon was present in the variety Pink Lady but absent in Gala. On the other hand, Gala variety had additional amplicons in F1/R2 (41 bp), F1/R2B (192 bp), F1/R3 (913 bp) and F1/R3B (280 bp) primer combinations.

Similar amplification patterns within the profiles of Paula Red and May Gold (0.909) led to clustering these varieties together (Figure 2). In all five primer combinations, the amplification resulted in generation of 33 fragments in both varieties. As mentioned, Paula Red and May Gold had the same amplification patterns when primers F1/R2 and F1/R3B were applied in PCR reactions. Yet, these genotypes were polymorphic for amplification patterns of F1/R1, F1/R2B and F1/R3 primer combinations. Compared to Paula Red, May Gold had extra 3 amplicons in case of F1/R1 – 210 bp, F1/R2B – 277 bp and F1/R3 – 707 bp but was absent for amplicons F1/R2B – 104 bp and 129 bp and F1/R3 – 707 bp.

In the Ambrosia genotype, the lowest number of amplicons was recognized contrarily to the Gloster variety which showed the highest number of amplicons. This led to the lowest level of similarity between these two genotypes (0.200) among the analyzed set. Ambrosia and Gloster both had only 6 shared amplicons: for F1/R1 – 549 bp, for F1/R2B – 605 bp, for F1/R3 – 187 bp, 518 bp and 600 bp and for F1/R3B – 700 bp. Low degree of similarity was also determined for Ambrosia and Jonaprince (0.286) and Granny Smith and Paula Red (0.303).

Analysis of individual generated CDDP loci levels for all of the analysed apple varieties was performed by Nei's (1973) gene diversity and Shannon's Information index. When comparing the gene diversity among individual primer

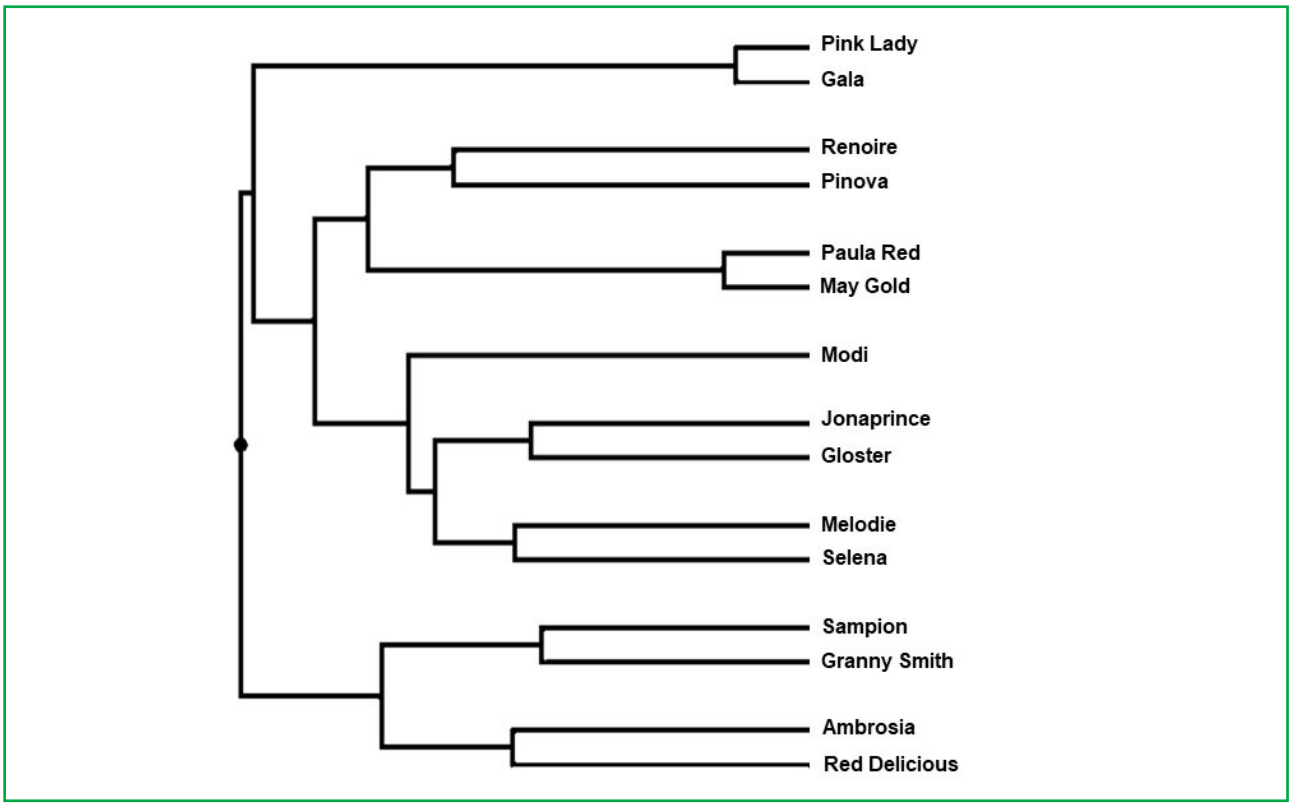

Figure 2 Dendrogram constructed by UPGMA clustering algorithm

combinations, the lowest value of 0.337 was obtained for F1/R2 and the highest of 0.407 for F1/R2B. This pointed to average values relevant to heterozygocity of this marker technique for the variety analyses of apples. Shannon's Information index values has ranged from 0.514 (F1/R2) up to the 0.596 (F1/R2B) what confirms CDDP to be a very usable in apple varieties distinguishing based on the length polymorphism based on fingerprints generated by it.

The assessment of genetic diversity and evaluation of the genetic relationships are a valuable source of information for conservation strategies and breeding programs. The strategies have a particular role in characterization of individual cultivars, in revealing the duplications of genetic material in germplasm collections and as a useful guide for selection of parents for breeding (Davila et al., 1998). Various types of molecular markers have been applied for the assessment of fruit species but no single technique can be universally ideal. The choice of a technique is dependent upon the objective, skills and financial or technical possibilities and constraints (Kafkas et al., 2008; Pavlovic et al., 2012). During the past few years, number of molecular markers have been developed including conserved DNA-derived polymorphism (Collard and Mackill, 2009). This marker system was chosen in our study to assess genetic diversity in 15 commercially important apple varieties. The system is lower-cost PCR amplification based technique. CDDP tends to generate candidate markers, where the gene markers can be a part of or closely linked to known genes. The marker system has many advantages such as convenience and rich polymorphism which can effectively serve to produce markers related to target traits (Collard and MacKill, 2009). CDDP markers proved its usefulness in the analysis of several plant species such as *Chrysanthemum* and *Paeonia suffruticosa* (Li and Zheng, 2013; Li et al., 2014). In our study, we observed relatively high level of polymorphism in amplification patterns across the studied genotypes. None of the apple varieties shared the same amplification pattern in all primer combinations taken together. The only concordant amplification profiles were generated for Red Delicious and Granny Smith (F1/R1), May Gold and Paula Red (F1/R2 and F1R3B), Selena and Melodie (F1/R2) but these cultivars differed in the remaining profiles generated by other primer combinations.

4 Conclusions

The study was aimed at verifying CDDP marker system as a useful tool to evaluate genetic diversity in apple genotypes. Variability of CDDP profiles in 15 apple varieties commonly used in commercial practice and trade were described. CDDP marker technique proved to be convenient and suitable to generate polymorphic patterns in apple genotypes as none of the primer combination produced monomorphic profile. Contrarily, polymorphic profiles were observed, whereby primer combinations F1/R2B and F1/R3 were recognized to produce specific profile for every of the studied varieties.

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