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The Applicability of Three DNA Isolation Methods in SSR Analysis of Hexaploid Plum (*Prunus domestica* L.) Cultivars

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Abstract

The main goal of any DNA extraction procedure is to ensure reliable and reproducible results in a simple, fast and inexpensive manner. When it comes to plant tissues, this goal is challenging to achieve due to the presence of a variety of metabolites that interfere with DNA during isolation and downstream analysis. In this study, we compared the efficiency of three methods for DNA extraction from plum kernels: 1) the standard CTAB Soltis method which is the most common protocol for DNA extraction from various plant tissues (seeds, young leafs, mature leafs, root); 2) CTAB-based method originally described for DNA isolation from medicinal plants with high levels of secondary metabolites; 3) and one of various commercially available kits. The usefulness of the obtained DNA was evaluated by SSR analysis with seven microsatellite markers. Although the latter two extraction protocols retrieved genomic DNA that gave positive PCR results, only DNA isolated by kit produced full SSR profile.

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Introduction

It is estimated that around 70% of all plant taxa have evolved through the process of polyploidization (Masterson, 1994). Numerous plants, considered

diploid today, are actually paleopolyploids, whose genomes had duplicated but subsequently undergone massive gene loss and genomic reorganization (Wolfe, 2001). Many of important contemporary crop plants are polyploids. One such species is *Prunus domestica*, European plum, with most of the commercially grown cultivars being hexaploids (Okie & Hancock, 2008). Population genetic studies on polyploid species have long been hindered by the challenge of deciding on a correct number of allele copies at particular molecular marker (Cuenca et al.,

2013). This is particularly true when single-locus markers, such as allozymes and microsatellites, are used. In order to accurately estimate population diversity and dynamics, it is of utmost importance to correctly assign genotypes for all analyzed markers. Different approaches have been designed in order to overcome this problem, such as MAC-PR (microsatellite DNA allele counting—peak ratios) method, based on the quantitative values for microsatellite allele peak areas (Esselink et al., 2004). However, successful application of MAC-PR is completely dependent on the quality of the scoring data, allowing for no or very few stutter bands. In the process of getting unambiguous genotyping data, the critical steps are the quality of PCR product and, more importantly, the quality and concentration of isolated DNA.

Although numerous generic protocols and commercial kits are available, extraction of high quality DNA remains the limiting factor in molecular analysis of plant species. The main reason is the presence of diverse compounds, mostly secondary metabolites, polysaccharides and proteins, in high concentrations in plant tissue. During extraction, phenols, alkaloids, polyphenols and polysaccharides tend to precipitate along with DNA, therefore affecting the quality and quantity of isolated DNA and inhibiting the subsequent PCR or restriction reactions (Porebski et al., 1997; Sarwat, 2006; Barra et al., 2012). Precipitated polysaccharides make DNA pellets viscous and difficult to manipulate (Abdel-Latif & Osman, 2017). Yellow, brown or greenish color of DNA pellet is an indicator of a contamination with polysaccharides and polyphenols (Lade et al., 2014).

Secondary metabolites are of immense importance for the fitness of plants (Wink, 1999). They can function as signal molecules in various interactions (within plant, plant-plant, plant-herbivore, etc.), but more often they serve as chemical defense compounds against animals, microbes or competing plants. Numerous plum varieties are rich in secondary metabolites, mostly anthocyanins, caffeoylquinic acid and procyanidins as well as phenolic compounds (Crozier et al., 2008; Venter et al., 2013). Total phenolic and flavonoid contents and antioxidant capacity of various plum cultivars have been already

determined (Kim et al., 2003; Venter et al., 2013; Abaci et al., 2014).

The aim of this study was to test the effects of three different methods for DNA isolation on the quality of the microsatellite scoring data in European plum, a polyploid species rich in secondary metabolites.

Materials and methods

Fresh plum kernels were grounded using mortar and pestle. Approximately 20 mg of tissue per sample was transferred into 1.5 ml microcentrifuge tubes and used for the testing of three different DNA extraction protocols. The quality of isolated genomic DNA (gDNA) was analyzed by gel electrophoresis. Five microliters of gDNA were run in 1.5% (w/v) agarose gel in 1x SB (sodium borate) buffer, pH 8 (Brody & Kern, 2005) and visualized under UV light after staining with Midori Green (Nippon Genetics Europe).

For standard CTAB (cetyltrimethylammonium bromide) method, DNA was extracted according to the Soltis Lab protocol (Doyle & Doyle, 1987; Cullings, 1992) with incubation times as follows: overnight for tissue lysis, 30 min on ice after adding isopropanol and overnight for resuspension. Pellet was dried at a room temperature for 1-2 hours and resuspended in 30-40 µl of ddH₂O, depending on the size of the pellet.

The second CTAB-based extraction procedure was used as described in Padmalatha et al., 2008. After washing DNA pellet with 70% ethanol for the first time, it was air-dried, resuspended in TE buffer and left in refrigerator at 4°C overnight. This protocol included adding RNaseA and incubation at 37°C for 1 hour. Depending on the size of of pellet, DNA was resuspended in 30-40 µl of ddH₂O.

DNA extraction using commercial spin-column-based method, DNeasy Plant Mini Kit (Qiagen) was performed according to the manufacturer's instructions. Samples were eluted in 50 µl of elution buffer, twice. The second elution was used for PCR amplification.

In order to assess the impact of a given DNA isolation method on an amplification success, seven microsatellite markers were used: BPPCT 014,

BPPCT 034, BPPCT 039, BPPCT 040 (Dirlewanger et al., 2002), UDP 96-005, UDP 98-407 (Cipriani et al., 1999) and PacA33 (Decroocq et al., 2003). For each marker forward primer was fluorescently labeled at 5' end. PCR reactions were performed in a total volume of 10 µl containing 1 µl of template DNA, 2 mM Tris-HCl (pH 8.0), 10 mM KCl, 0.2 µM of each primer, 0.2 mM dNTPs, 2 mM MgCl₂ and 1 unit TaqNovaHS DNA polymerase (Gdansk). Amplification was carried out in 35 cycles: 45s denaturation at 94°C, 45s annealing at 57°C, 2 min extension at 72°C and 4 min final extension at 72°C in Agilent SureCycler 8800. Amplified PCR products were run on ABI PRISM™ 3500 Genetic Analyzer (Applied Biosystems) and scored using GeneMapper Software ID v5 (Applied Biosystems).

Results and Discussion

The overall success and the average yield of isolated genomic DNA varied based on the employed extraction method (Figure 1). DNA extraction using Soltis Lab protocol gave the lowest yield of DNA with bands that were hardly visible in agarose gel (Figure 1-1). Although successful PCR technically requires only a small amount of DNA, no amplification was detected when DNA recovered by Soltis protocol was used as a PCR template (Figure 2, bottom plot). Gel electrophoresis showed higher yields of DNA obtained using CTAB-based extraction protocol modified by Padmalatha et al. (2008). However, the concentrations of simultaneously precipitated contaminants were higher as well, visible as smears in the gel (Figure 1-2). The highest yield and purity of extracted DNA was obtained by DNeasy Plant Mini Kit (Figures 1-3a and 1-3b). DNA was eluted twice in 50 µl of elution buffer, with average concentration of 350 ng/µl for the first elution and 100 ng/µl for the second. DNA concentrations from the second elution appeared to be more suitable for SSR analysis as they produced more balanced peaks with minimal stuttering (Figure 2, top plot).

Although DNA isolated according to the protocol by Padmalatha et al. (2008) gave positive amplification, allelic profiles were partial, with longer fragments missing and the fluorescent signal threefold weaker compared to the profiles obtained using DNA extracted by the commercial kit (Figure 2).

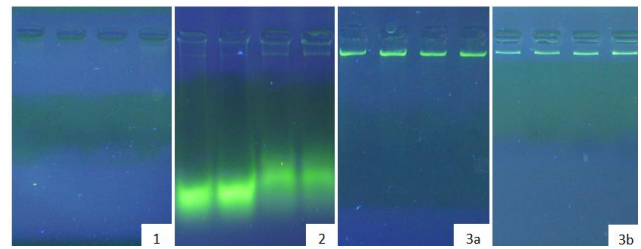


Figure 1. Gel electrophoresis of DNA isolated by: (1) Soltis Lab protocol (Doyle & Doyle, 1987; Cullings, 1992); (2) CTAB-based extraction procedure described by Padmalatha et al., 2008; (3) DNeasy Plant Mini Kit (Qiagen; 3a- first elution; 3b- second elution)

The differential success of DNA isolation and downstream amplification could be due to the type of material used for extraction coupled with applied extraction protocol. While most of the plant studies use leaves as a DNA source, in this experimental work kernels were used. Since seed has to provide all needed nutrients for the growth of a young plant, the most important storage materials in endosperm are carbohydrates, proteins, fats and oils (Bhatnagar & Sawhney, 1981). Plum seeds contain 22.4% carbohydrates, 3% proteins, 11.6% fat and 62.4% fiber (Kamel & Kakuda, 1992). Of the two CTAB-based isolation procedures evaluated, the one according to Padmalatha et al. (2008) contains most of the extraction buffer components in higher final concentrations than the protocol by Doyle & Doyle (1987): 2M vs. 1.4M NaCl, 1% vs. 0.2% β-mercaptoethanol and 25mM vs. 20mM EDTA, respectively. NaCl is responsible for the removal of carbohydrates and proteins (Fang et al., 1992; Abdel-Latif & Osman, 2017). The antioxidant β-mercaptoethanol destroys the structural organization of proteins hence aiding in their removal (Varma et al., 2007).

Polyvinyl pyrrolidone (PVP) is used to eliminate polyphenols during DNA purification (Maliyakal, 1992). Both methods use 2% CTAB as a detergent that lyses all membranes and releases the genetic material into the extraction buffer. CTAB is a cationic surfactant which dissociates and selectively precipitates DNA from the histone proteins (Irfan et al., 2013; Abdel-Latif & Osman, 2017). Addition of pre-warmed CTAB buffer (65°C) to the pulverized plant tissue in Padmalatha et al. (2008) protocol possibly further enhances the recovery of a high-quality DNA. This protocol also includes more

precipitation steps and phenol:chloroform washes than the Soltis procedure. Generally, these additional manipulations reduce total yield, but in cases where larger amounts of DNA are not imperative, they will improve the purity of DNA, as evidenced by the results of SSR analysis. Taking into account the extraction buffer composition and multiple precipitation steps, the protocol by Padmalatha et al. (2008) appears to have greater capacity to successfully remove the contaminants and to recover DNA of higher purity in concentrations suitable for downstream analytical applications than the Soltis Lab protocol. This feature is beneficial since, more often than not, the absence of contaminants in DNA sample is more crucial for the successful amplification than high concentration of isolated DNA.

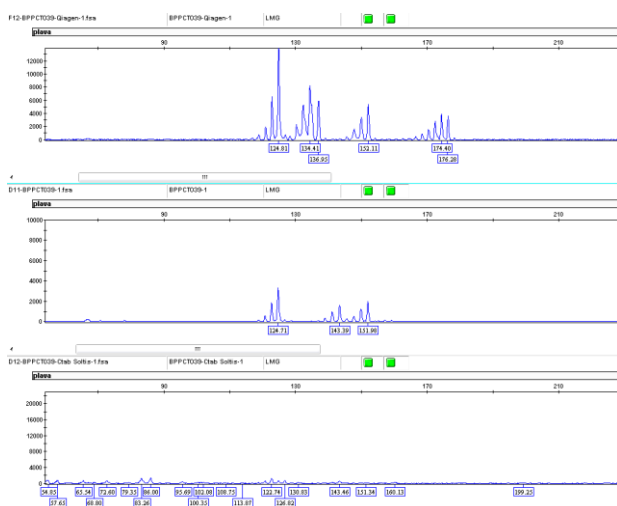


Figure 2. Example of electropherogram traces for BPPCT039 locus obtained by DNA extracted with three methods; Top plot: DNeasy Plant Mini Kit; Middle plot: Protocol described by Padmalatha et al., 2008; Bottom plot: CTAB Soltis Lab protocol (Doyle & Doyle, 1987; Cullings, 1992)

Out of the three isolation methods tested, the best results in terms of quantity, quality (Figures 1-3a, 1-3b) and suitability of isolated DNA for SSR analysis (Figure 2, top plot) were obtained with DNeasy Plant Mini Kit (Qiagen). DNA yields of both elutions were in range advertised by the manufacturer. Instead of using phenol or chloroform extraction or alcohol precipitation for the purification of DNA, proteins and polysaccharides are salt-precipitated and further removed by centrifugation, along with cell debris. During this process, DNA preferentially binds to the column membrane, therefore avoiding any co-

precipitation of contaminants. This protocol requires minimal handling, thus facilitating the analysis of multiple samples. When DNA isolated by DNeasy Plant Mini Kit was used for PCR, resulting SSR profiles for all tested markers generated all the expected alleles, with little or no baseline noise and stuttering.

Although CTAB based protocols are broadly used and seen as a benchmark method for genomic DNA extraction from plant material (Blatter et al., 2014), its constant modifications and optimizations for the use in different plant species support the fact that there is no universally applicable isolation protocol. Therefore, the rule of a thumb remains to use “whatever delivers” for a particular plant species since sometimes certain protocol does not perform equally successfully even for the different tissues of the same species, due to differential chemical composition. Even commercial kits can sometimes be inefficient in plants with high polysaccharide or high polyphenolic content like peanut (*Arachis hypogea*) and members of Asteraceae (Cichorioideae) family, e.g., *Cichorium intybus*, *Taraxacum officinale* and *Lactuca sativa* (Michiels et al., 2002; Sharma et al., 2000). The experiences from our laboratory were similar. When commercial kits were ineffective, the original Soltis Lab method was successfully used for DNA extraction in the analysis of European chestnut and apple (Skender et al., 2017; Gaši et al., 2016), while protocol described by Padmalatha et al. (2008) enabled isolation of high-quality genomic DNA from five endemic plant species of Lamiaceae family (Lasić et al., 2016).

Apart from considering the requirements of the experiment, when deciding on the isolation protocol one also has to take into account the cost-benefit ratio in terms of time consumption, cost and health safety (Table 1). Although generally less costly, most in-house DNA isolation protocols for plant material rely on the use of hazardous chemicals and are rather lengthy and laborious for high-throughput application. On the other hand, the commercial kits do not require the use of toxic reagents or intense manual labor. They are more amenable to automation which reduces the contamination risk and workload, making them more suitable for the manipulation of multiple samples, but also more expensive. Though the cost

reduction of commercial kits is anticipated with future technical advancement, it is also expected that the development of alternative methods and protocols for DNA extraction from various materials would continue due to the limited financing of the scientific research in less-research-intensive countries worldwide.

Table 1. A comparison of attributes of the three evaluated DNA extraction methods

Reference	CTAB Soltis Lab	Padmalatha et al., 2008	DNeasy Plant Mini Kit
Cost	Low	Moderate	High
Time consuming	Moderate	High	Low
Health hazard	Moderate	Moderate/ high	None

Conclusions

Although certain number of in-house protocols and commercial kits for the isolation of DNA from plant material are widely followed, there is no broad-spectrum procedure that can be applied to an extensive range of plant species and tissue types. It is a common practice for researchers to combine two or more procedures to adjust the isolation protocol according to the requirements of the experiment. When opting for an appropriate extraction approach, one needs to account for the purity and concentration of recovered DNA, time consumption, workload, cost and safety aspect. This is particularly true when dealing with polyploid species or those rich in secondary metabolites, such as medicinal plants, which pose additional challenge in obtaining good quality DNA necessary for ensuring precise, reliable and reproducible results in downstream applications.

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