

A ROBUST AND COST-EFFECTIVE METHOD FOR DNA ISOLATION FROM *SATUREJA* SPECIES (LAMIACEAE)

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Abstract - Aromatic species of the genus *Satureja* are rich in secondary metabolites that interfere with DNA isolation procedures. Four protocols based on the standard CTAB DNA extraction protocol of Doyle and Doyle (1987) were tested in six savory taxa. The polyphenol adsorbents activated charcoal and/or polyvinylpyrrolidone 10 were employed in three procedures (B, C and D); for the elimination of polysaccharides, 4M NaCl was applied in the latter two. The highest DNA yield was obtained with Protocol D and averaged 1420.7±398.3 µg DNA/g of dry leaf tissue. Optimal values of the absorbance ratio 260/280 of all DNA solutions revealed the absence or only negligible contamination by proteins. Contamination by polysaccharides inferred from the absorbance ratio 260/230 showed that Protocol C provided the least contaminated material (average of 1.7±0.4). Enzymatic reactions of DNA solutions obtained by Protocol D showed amplification of both loci in all individuals. In conclusion, Protocol D is suitable for the isolation of high quantities of pure DNA from *Satureja* spp.

Key words: DNA isolation protocol, *Satureja*, polyphenols, polysaccharides, activated charcoal, polyvinylpyrrolidone 10, high salt concentration

INTRODUCTION

The genus *Satureja* (savory, family Lamiaceae), is distributed in Europe (Mediterranean area), tropical Africa, Asia and the Americas (Bezić et al., 2009; Cantino et al., 1992; Šilić, 1979). The exact number of species comprising this genus, however, is still questionable due to the considerable taxonomic confusion associated with the generic limits of the so-called "*Satureja* complex". While many taxonomists have split this complex into several genera (*Satureja* L., *Clinopodium* L., *Calamintha* Mill., *Acinos* Mill. and *Micromeria* Benth., (Bentham, 1848; Boissier, 1879; Ball and Getliffe, 1972; Davis, 1982), others have lumped the group into a single genus, *Satureja* s.l. (Briquet, 1896; Brenan, 1954; Greuter et al., 1986;

Seybold, 1988) or *Clinopodium* (Kuntze, 1891). Harley et al. (2004) incorporated much of the most recent morphological and molecular findings and restricted *Satureja* to a comparatively small number of species. Nonetheless, despite such narrow description, this genus appears to be polyphyletic (Bräuchler et al., 2005, 2010).

Many savory species have been well studied to date from various aspects related to their secondary metabolites. These plants, which are annual or perennial herbs and shrubs, have glandular trichomes on the leaf surface that produce and secrete essential oils (Hanlidou et al., 1990; Bezić et al., 2001), like other aromatic plants of the mint family. Essential oils from *Satureja cuneifolia* are comprised mostly

of α -pinene, limonene, linalool and β -cubebene (Miloš et al., 2001), γ -terpinene and carvacrol (Bezić et al., 2009), those of *S. horvatii* contain mostly thymol, carvacrol, γ -terpinene and *p*-cymene (Lakušić et al., 2011), while *p*-cymene and limonene are predominant components of *S. kitaibelii* essential oil. *S. montana ssp. montana* essential oils are rich in *p*-cymene, linalool and borneol (Slavkovska et al., 2001), while those of *S. montana ssp. variegata* and *S. subspicata* have monoterpene phenols thymol and carvacrol as dominant compounds (Dunkić et al., 2010; Čavar et al., 2008). Although the primary role of essential oils is to provide plants chemical defense (Wink, 2003), they display a variety of beneficial activities. Those from *Satureja* spp. have antimicrobial (Skočibušić et al., 2006; Lakušić et al., 2008; Oke et al., 2009), antiviral (Yamasaki et al., 1998), antibacterial (Nedorostova et al., 2011), antifungal (Fraternale et al., 2007; Giordani et al., 2004), anti-inflammatory and antinociceptive effects (Amanlou et al., 2005), etc. Secondary metabolites of savory (and other aromatic plants), however, represent a serious obstacle in molecular biology studies, because along with various cell proteins and plant cell-wall polysaccharides, they interfere with DNA isolation procedures, irreversibly react with nucleic acids and affect enzymatic reactions (Dabo et al., 1993; Pirttilä et al., 2001).

At present, numerous methods are available for the isolation of genomic DNA from plants (e.g. Murray and Thompson, 1980; Dellaporta et al., 1983; Doyle and Doyle, 1987; Rogers and Bendich, 1988; Lodhi et al., 1994). However, due to the variety of chemical compounds present in tissues of diverse plant species, researches frequently need to modify a DNA isolation procedure or to blend two or more different procedures to obtain quality DNA in a particular plant taxon or even to adjust procedures to various plant tissues, as mentioned in Varma et al. (2007). Recently, Bezić et al. (2009) isolated DNA from four south-Croatian savory species to infer their evolutionary relations, and observed that the PCR amplification with genomic DNA (gDNA) isolated by the standard hexadecyltrimethylammonium bromide (CTAB) DNA isolation protocol of Doyle

and Doyle (1987) failed because of the hindering effects of the secondary metabolites that are abundant in these taxa. A similar problem has also been encountered in the phylogenetic and/or biogeographic surveys of the tribe Mentheae in which DNA from several savory taxa was obtained via the Doyle and Doyle (1987) protocol (Kaufmann et al., 1994; Bunsawat et al., 2004), the protocol of Dellaporta et al. (1983) (Alexander, 2007; Drew and Sytsma, 2012) or utilizing commercial DNA isolation kits (Trusty et al., 2004; Bräuchler et al., 2005; 2010).

The above-mentioned phylogenetic and/or biogeographic surveys have not fully incorporated a variety of savory taxa distributed within the Balkans and thus, their evolutionary relations are still unknown (Bezić et al., 2009). In addition, population genetics and phylogeographic surveys of the Balkans' savory species are generally lacking. This strengthens the demand for the improvement of DNA isolation procedures in savory species and the development of such protocols that can be readily applied in diverse molecular genetics studies that involve these taxa and analyze several dozens or hundreds of individuals. In such large-scale surveys, the cost of the initial experimental step, isolation of quality DNA, can be substantial, especially if rather expensive commercial DNA isolation kits are to be employed.

The objective of our study was to establish a robust, simple and cost-effective DNA isolation procedure in savory taxa that can yield large amounts of contaminant-free gDNA suitable for PCR amplification of nuclear (nrDNA) and chloroplast (cpDNA) loci. To accomplish this, four DNA isolation procedures based on the standard CTAB DNA isolation protocol of Doyle and Doyle (1987) in six savory taxa were tested in order to assess whether polyphenol adsorbents activated charcoal and polyvinylpyrrolidone 10 (PVP 10) are efficient in removing polyphenols from DNA solutions of these taxa, and whether the application of high salt concentrations (4 M NaCl) is effective in removing polysaccharides. Enzymatic reactions of all DNA solutions were tested by PCR amplification of one nuclear (ITS intergenic spacer) and one chloroplast (*rpl32-trnL*) locus.

MATERIAL AND METHODS

Plant material

Four savory species were studied, *Satureja cuneifolia* Ten., *S. horvatii* Šilić, *S. kitaibelii* Wierzb., *S. subspicata* Bartl. ex Vis., and two *S. montana* L. subspecies (*S. montana* L. ssp. *montana* and *S. montana* ssp. *variegata* (Host) P. W. Ball. The specimens were collected from two different locations for each of the studied species, except for *S. horvatii* and *S. cuneifolia*, for the samples of which were collected from one location. This is because different savory species as well as individuals from the same species but from different locations might have different chemical compositions, as the chemical profile of plants with aromatic properties is modeled not only by their genetic background but also by environmental factors that may differ throughout the species natural range (Miloš et al., 2001). Therefore, DNA isolation procedures and downstream enzymatic reactions may be differentially affected in individuals from different savory species and in individuals from the same species but from different locations. All samples were collected in late October 2012. One individual per location was sampled. Sampling locations as well as areal type, vegetation, latitude, longitude and altitude of all individuals (10 in total) are presented in Table 1.

In the field, plant material was labeled and packed in sterile tea-filter bags, which were placed in larger PVC bags with silica gel for drying. Dry plant leaves were preferred over fresh material because such source material has been demonstrated to yield more DNA in several plant species (Khanuja et al., 1999). Fresh plant material was dried in silica gel for 5 days and stored at room temperature prior to DNA isolation.

DNA isolation procedures

Four DNA isolation procedures (A, B, C and D) based on the standard CTAB Doyle and Doyle (1987) DNA extraction protocol were used for the isolation of total genomic DNA (gDNA) from six savory taxa.

Dry leaves from each plant were aliquoted (30 mg) in four sterilized 2.0 ml tubes; the tubes were placed in a Tissulyzer II (Qiagen) and homogenized to a fine powder. Each aliquot was used for isolation of DNA by a different protocol and thus, the plant material used for the isolation of gDNA by the four protocols had the same developmental stage and chemical content.

The following reagents and chemicals were used in procedures for isolation of gDNA from savory taxa: CTAB (Serva, Heidelberg, Germany); EDTA (VWR BDH Prolabo, Leicestershire, England); Tris-HCl (VWR BDH Prolabo, Leicestershire, England); NaCl (Alkaloid, Skopje, Macedonia); β -mercaptoethanol (Serva, Heidelberg, Germany), activated charcoal (Centrohema, Stara Pazova, Serbia), polyvinylpyrrolidone 10 (PVP 10) (Sigma Aldrich), SEVAG [24:1 (v/v) chloroform (Centrohema, Stara Pazova, Serbia): isoamyl alcohol (Centrohema, Stara Pazova, Serbia)], isopropanol (VWR BDH Prolabo, Leicestershire, England), 70 % ethanol (Superlab, Belgrade, Serbia), RNase A (Fermentas UAB, Vilnius, Lithuania) and sterile deionized water.

CTAB, which is a frequently used surfactant, was used in extraction buffers of all protocols. It helps in precipitating DNA by forming a complex with it in a low ionic strength environment. At high salt concentration, it forms insoluble complexes with proteins and most acidic polysaccharides, leaving the nucleic acids in the solution, which can then be easily extracted. β -mercaptoethanol is a disulfide group reducing agent that prevents the polymerization of tannins that hinder the isolation process in a manner similar to polysaccharides and destroys the tertiary and quaternary structures of proteins. The CTAB extraction buffer of Doyle and Doyle (1987) with 2% (w/v) CTAB; 20 mM EDTA, pH 8.0; 100 mM Tris-HCl, pH 8.0; 1.4 M NaCl and 0.2% (v/v) β -mercaptoethanol added just before use was applied in three procedures (A, C and D). A slightly modified CTAB extraction buffer was used in Protocol B, with a higher concentration of NaCl (2.0 M NaCl), no β -mercaptoethanol and dissolved 1% (w/v) PVP 10 and 0.5% (w/v) activated charcoal.

Our Protocol A was the standard CTAB DNA extraction procedure of Doyle and Doyle (1987) with the following steps:

1. Add 750 μl of pre-heated (65°C) CTAB/ β -mercaptoethanol extraction buffer to each sample
2. Incubate at 65°C for 1 h with frequent inversions
3. Add 750 μl of SEVAG to each sample and mix thoroughly by inversions
4. Centrifuge at 13,000 rpm for 10 min at 4°C
5. Transfer supernatant to new tubes
6. Add 450 μl of isopropanol, kept in a freezer, mix thoroughly by inversions and stored at 20°C for 1 h
7. Centrifuge at 13 000 rpm for 5 min at 4°C and discard supernatant
8. Add 500 μl of 70 % ethanol, kept in a freezer, mix by inversions
9. Centrifuge at 13 000 rpm for 5 min at 4°C and discard supernatant
10. Dry the DNA pellet at room temperature for 1-2 h
11. Suspend the DNA pellet in 200 μl of sterile deionized water and incubate overnight at 4°C
12. Add 0.5 μl of Rase A to each sample and incubate for 30 min at 37°C

Protocol B was developed by Križman et al. (2006), and is recommended for plant material rich in polyphenols and polysaccharides. This protocol uses activated charcoal (suspended) and PVP 10 (dissolved) in the extraction buffer for binding polyphenols as well as mild extraction and precipitation conditions and higher salt concentrations (2.0 M NaCl)

for the precipitation of polysaccharides. However, it does not use β -mercaptoethanol. Most steps in this protocol were performed at room temperature and its wash solution contained 15 mM ammonium acetate in 75% (v/v) ethanol.

Protocol C was a modified protocol D, because it was comprised of only one polyphenol adsorbent, activated charcoal, which was added directly to each tube with homogenized plant tissue prior to the addition of extraction buffer. One mg of activated charcoal per sample was used.

Protocol D was developed by Aleksić et al. (2012) for DNA isolation from *Salvia officinalis*, which, like *Satureja* spp., is rich in secondary metabolites such as polyphenols and polysaccharides (Slavkovska et al., 2001). It employs the synergetic effect of activated charcoal and polyvinylpyrrolidone (PVP 10) for polyphenol adsorption, like protocol B, but these compounds were applied differently, i.e., activated charcoal (1 mg/sample) and PVP 10 (1 mg/sample) were added to each tube with homogenized plant tissue before the addition of extraction buffer. Also, an additional step in which 300 μl of 4 M NaCl was added to each sample prior to the isopropanol step (step 6), was applied in order to enhance polysaccharide precipitation. The final NaCl concentration upon addition of NaCl to each sample was 1.3 M.

DNA quantification and purity assessment

Genomic DNA was quantified and assessed for purity utilizing NanoVue (GE Healthcare Europe, Freiburg, Germany) that measures absorbance at 230 nm, 260 nm and 280 nm. NanoVue estimates DNA concentrations in ng/ μl , which was used for calculating the DNA yield in μg DNA/g of dry leaf tissue. The purity of DNA isolates was assessed from the absorbance ratios, 260/280 nm and 260/230 nm. A 260/280 absorbance ratio lower than 1.7 indicates contamination by (mostly) proteins, values above 2.0 indicate the presence of RNA, while values in the range 1.7 to 2.0 signify a pure DNA sample (Sambrook et al., 1999; Puchooa and Khoyratty, 2004). A 260/230 absorbance ratio greater than 1.7 indicates DNA prepa-

rations free from contamination by polysaccharides (Peterson et al., 1997; Singh et al. 1999; Chen and Ronald, 1999; Ahmad et al., 2004). The statistical significance of differences in DNA concentrations (ng/ μ l), DNA yield (μ g DNA/g of dry leaf tissue) and absorbance ratios 260/280 nm and 260/230 nm among the protocols was estimated by one-way ANOVA (Hammer and Harper, 2006).

PCR analysis

The DNA isolates were assessed by PCR amplification of the entire nuclear internal transcribed spacer, ITS1-5.8S-ITS2, and one cpDNA region, the *rpl32-trnL* intergenic spacer. The ITS spacer was PCR amplified using two primers designed by G. Sheridan (University of Bath), a forward (F) primer (AB101) annealing in the 18S gene, 5'-ACGAATTCATGGTC-CGGTGAAGTGTTCG-3', and a reverse (R) primer (AB102) annealing in the 26S gene, 5'-TAGAAT-TCCCCGGTTCGCTCGCCGTAC-3' (Douzery et al. 1999). The CpDNA locus was arbitrarily selected out of the 21 cpDNA regions reported by Shaw et al. (2007) as potentially informative for various molecular studies in plants. The *Rpl32-trnL* region was amplified using F primer *rpl32-F*: 5' - CAGT-TCCAAAAAACGTACTT - 3' and R primer *trnL(UAG)*: 5' - CTGCTTCCTAAGAGCAGCGT - 3'.

PCR amplification of both loci was performed in 25 μ l volumes, containing: 100 ng template DNA, 2.5 μ l 10 x Taq Buffer with $(\text{NH}_4)_2\text{SO}_4$ (Fermentas UAB, Vilnius, Lithuania), 2.5 mM MgCl_2 , 0.2 mM dNTPs, 0.1 μ M of each forward and reverse primer, 0.80 % BSA (Bovine Serum Albumin, Fermentas UAB, Vilnius, Lithuania), and 0.025 U/ μ l of Platinum *Taq* DNA polymerase (Fermentas UAB, Vilnius, Lithuania). As a control for PCR amplification of both loci, we used identical mixes of chemicals with corresponding primers but without template DNAs. PCR amplifications were performed using a peqStar 96 Universal thermal cycler (PEQLAB Biotechnologie GmbH, Erlangen, Germany). PCR profile for amplification of the nrDNA locus comprised an initial denaturation at 94°C for 10 min, followed by 35 cycles of denatura-

tion at 94°C for 45 s, annealing at 63°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. The CpDNA locus was amplified using an identical PCR profile that differed from the PCR profile used for amplification of nrDNA locus in annealing temperature only which was set to 53°C.

Amplified products (2.5 μ l of each PCR product) were separated by electrophoresis in a 1% (w/v) agarose gel (PEQLAB Biotechnologie GmbH, Erlangen, Germany) 1 x TAE buffer. The length of PCR products was assessed using a GeneRuler 1-kb ladder (Fermentas UAB, Vilnius, Lithuania). Products were stained with Midori Green DNA Stain (NIPPON Genetics EUROPE GmbH, Dueren, Germany), 2.5 μ l of this stain was added to 2.5 μ l of each PCR product, and they were visualized under a Vilber Lourmat ECX-F20.M transilluminator (Cedex 1, France).

RESULTS AND DISCUSSION

Aromatic plants of the mint family are commonly regarded as recalcitrant targets for the extraction of good-quality DNA because they are exceptionally rich in diverse secondary metabolites that, along with cell proteins and cell wall polysaccharides, may hinder the use of DNA solutions in downstream enzymatic reactions (Pirttilä et al., 2001; Dabo et al., 2003). Although abundant aromatic savory taxa are still genetically understudied, and procedures for isolation of quality DNA from these plants are required for future molecular genetics studies of these taxa. In order to provide a robust, simple and cost-effective procedure for the isolation of high amounts of contaminant-free DNA from savory taxa, four modifications of the most commonly utilized procedure for the isolation of DNA from plants, the CTAB procedure of Doyle and Doyle (1987), were tested. To insure comparability between procedures, aliquots of the same plant for all protocols were used. In that way, plant material at the same developmental stage and chemical content was used in all procedures. To rule out any ecological variations in chemical composition within the same taxon (Miloš et al., 2001), individuals from different locations for four savory taxa were studied.

Table 1 Geographic and ecological characteristics of locations of *Satureja* species used in this study.

No Species	Location	Areal type	Vegetation	Lat (N)	Long (E)	Altitude (m)
1 <i>Satureja cuneifolia</i> Ten.	CRO, Biokovo	Sub-Mediterranean	Mediterranean	43° 17' 55.8"	17° 4' 18.7"	1276
2 <i>Satureja horvatii</i> Šilić	MNG, Orjenske lokve	Sub-Mediterranean	Ostryo-Carpinion orientalis	42° 33' 29.2"	18° 33' 09.0"	1596
3 <i>Satureja kitaibelii</i> Wierzb.	SRB, Manastir Poganovo	Pont-Med	Quercion frainetto	42° 58' 52"	22° 38' 24.5"	514
4 <i>Satureja kitaibelii</i> Wierzb.	SRB, Klisura Temštica	Pont-Med	Quercion frainetto	43° 18' 15.7"	22° 37' 29.0"	584
5 <i>Satureja montana</i> L. ssp. <i>montana</i>	CRO, Jadranovo-Šmrika	Mediterranean	Mediterranean	45° 14' 11.5"	14° 37' 10.9"	147
6 <i>Satureja montana</i> L. ssp. <i>montana</i>	MNG, Mokrine ka Orjenu	Sub-Mediterranean	Ostryo-Carpinion orientalis	42° 31' 01.4"	18° 29' 08.7"	599
7 <i>Satureja montana</i> ssp. <i>variegata</i> (Host) P. W. Ball	CRO, Tunel Lučica, ulaz u Opatiju	Mediterranean	Mediterranean	45° 18' 53.6"	14° 14' 49.2"	486
8 <i>Satureja montana</i> ssp. <i>variegata</i> (Host) P. W. Ball	CRO, Vodnjan-Rovinj	Mediterranean	Mediterranean	44° 58' 57.0"	13° 50' 6.6"	3
9 <i>Satureja subspicata</i> Bartl. ex Vis.	CRO, Jadranovo-Šmrika	Mediterranean	Mediterranean	45° 14' 11.5"	14° 37' 10.9"	147
10 <i>Satureja subspicata</i> Bartl. ex Vis.	MNG, put ka Nikšiću	Sub-Mediterranean	Mediterranean	42° 55' 54.9"	18° 56' 24.9"	1075

(Croatia (CRO), Montenegro (MNG), Serbia (SRB), latitude (Lat), longitude (Long))

Table 2 DNA yield and purity of savory DNA isolates obtained by four protocols for DNA isolation.

No. Species	DNA yield (μg DNA/g of dry leaf tissue)				A260/A280				A260/A230			
	Protocol A	Protocol B	Protocol C	Protocol D	Protocol A	Protocol B	Protocol C	Protocol D	Protocol A	Protocol B	Protocol C	Protocol D
1 <i>S. cuneifolia</i>	963.3	533.3	1173.3	1193.3	1.6	1.6	1.6	1.5	1.4	1.2	1.2	1.2
2 <i>S. horvatii</i>	970.0	406.7	1076.7	1480.0	1.8	1.8	1.9	1.6	1.8	1.4	2.1	1.5
3 <i>S. kitaibelii</i>	990.0	1190.0	1443.3	1786.7	1.9	1.9	2.0	1.9	1.5	1.9	2.1	1.6
4 <i>S. kitaibelii</i>	1303.3	653.3	1910.0	2053.3	1.9	1.8	1.9	1.9	1.8	1.4	1.8	1.7
5 <i>S. montana</i> ssp. <i>montana</i>	750.0	-	786.7	870.0	1.8	-	1.9	1.7	1.2	-	1.3	0.9
6 <i>S. montana</i> ssp. <i>montana</i>	1046.7	240.0	1596.7	1470.0	1.9	1.8	2.0	1.9	1.8	1.1	2.1	2.0
7 <i>S. montana</i> ssp. <i>variegata</i>	1723.3	526.7	1490.0	1570.0	1.9	1.9	1.9	1.9	1.9	1.5	2.1	2.1
8 <i>S. montana</i> ssp. <i>variegata</i>	1100.0	243.3	1520.0	1773.3	1.9	1.7	1.9	1.8	1.8	0.9	2.0	1.7
9 <i>S. subspicata</i>	836.7	316.7	706.7	863.3	1.8	1.3	1.6	1.7	1.6	0.6	1.2	1.4
10 <i>S. subspicata</i>	936.7	586.7	1303.3	1146.7	1.6	1.6	1.6	1.5	1.4	1.2	1.5	1.3
Average	1062±275.8	521.8±291.6	1300.7±372.2	1420.7±398.3	1.8±0.1	1.7±0.2	1.8±0.2	1.7±0.2	1.6±0.2	1.3±0.4	1.7±0.4	1.6±0.4

(sample 5 in Protocol B was lost (-), numbers in boldface represent the highest yield, or optimal values per samples)

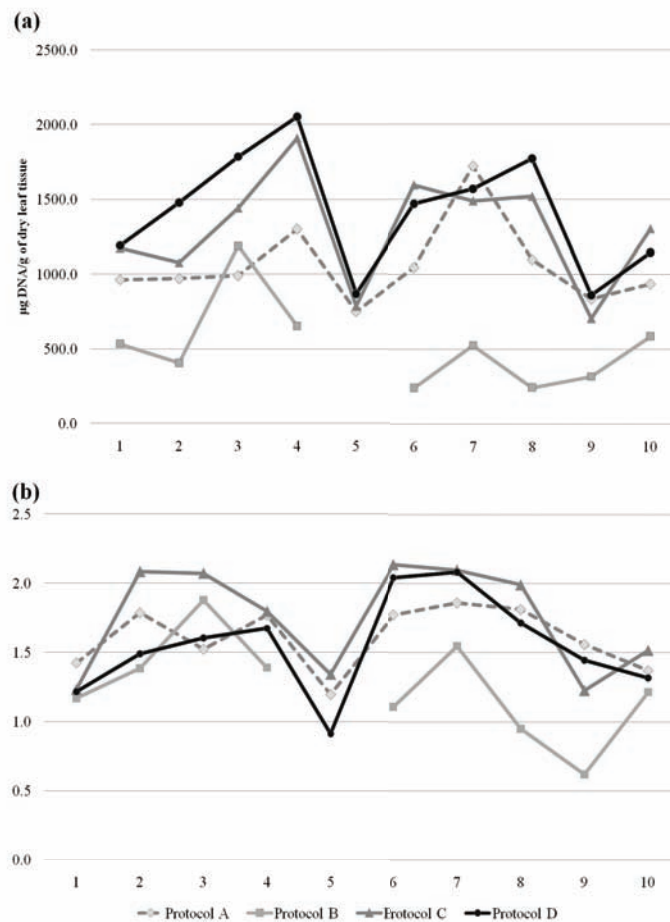


Fig. 1 Graphical representation of DNA yields and absorbance ratio 260/230 of savory taxa obtained by four DNA isolation protocols. The absorbance ratio 260/280, not shown here, was uninformative. DNA yield (a), A260/230 (b), *S. cuneifolia* (1), *S. horvatii* (2), *S. kitaibelii* (3,4), *S. montana* ssp. *montana* (5,6), *S. montana* ssp. *variegata* (7,8), *S. subspicata* (9,10)

The highest DNA yield averaged over 10 individuals representing four savory species and two subspecies was obtained by Protocols D and C, 1420.7 ± 398.3 and 1300.7 ± 372.2 µg DNA/g of dry leaf tissue, respectively (Table 2). Average DNA yields obtained by protocols A and B were 1062 ± 275.8 and 521.9 ± 291.6 µg DNA/g of dry leaf tissue, respectively (Table 2). However, one-way ANOVA revealed statistically significant differences between the protocols ($P < 0.05$), suggesting that with regard to the DNA yield, Protocol D outperformed all other protocols. Protocol A outperformed protocol B for most individuals, except for individual 3 (*S. kitaibelii*), and outperformed

protocols C and D only in individual 7 (*S. montana* ssp. *variegata*). Differences between yields obtained by protocols A, C and D were negligible for individuals 5 (*S. montana* ssp. *montana*) and 9 (*S. subspicata*) (Fig. 1). The biggest difference in DNA yield between protocols was obtained for sample 6 (*S. montana* ssp. *montana*) for which the DNA yield was six times higher with protocol C than with protocol B.

As already mentioned, savory species are rich in diverse secondary metabolites, including polyphenols (Slavkovska et al., 2001). The polyphenol content varies with regard to savory species and differs

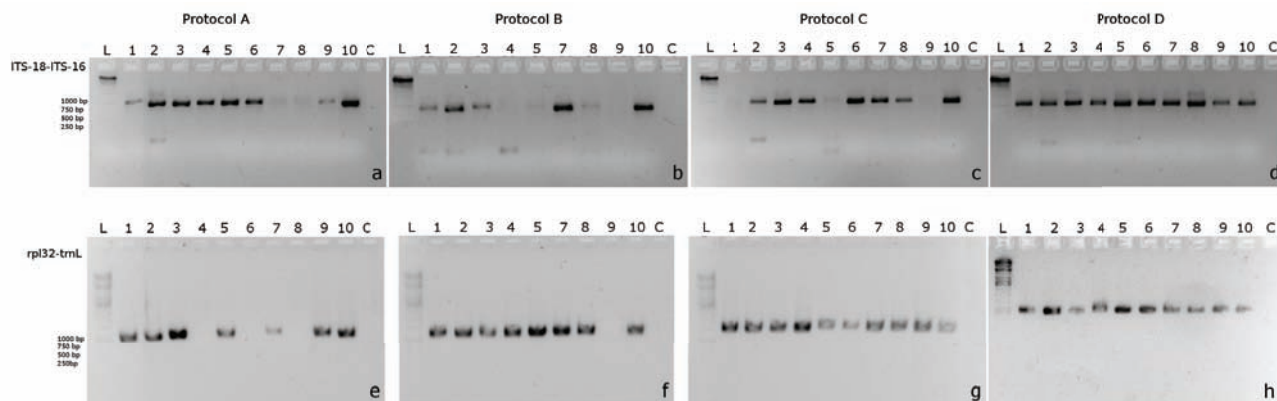


Fig. 2 PCR amplification products of nuclear ITS spacer (first row) and chloroplast *rpl32-trnL* (second row) loci using template DNA obtained by Protocol A (a, e), Protocol B (b, f), Protocol C (c, g) and Protocol D (d, h). L - 1 kb DNA ladder; *S. cuneifolia* (1), *S. horvatii* (2), *S. kitaibelii* (3,4), *S. montana* ssp. *montana* (5,6), *S. montana* ssp. *variegata* (7,8), *S. subspicata* (9,10) individuals; C-control comprising of PCR amplification components except template DNA.

even in individuals from the same species but from different locations characterized by different ecological conditions (Miloš et al., 2001). These compounds are problematic during DNA isolation procedures because they readily oxidize upon release from vacuoles during cell lyses, and undergo irreversible interactions with nucleic acids, causing enzymatic browning of the DNA pellet (Varma et al., 2007). The removal of polyphenols from the DNA solutions of various plant species is usually achieved by the application of polyphenol adsorbents, such as activated charcoal and/or PVP or PVPP (e.g. Maliyakal, 1992; Bi et al., 1996; Peterson and Boehm, 1997; Kim et al., 1997; Porebski and Bailey, 1997; Martellosi et al., 2005; Križman et al., 2006).

Activated charcoal and PVP 10 were applied to remove polyphenols from savory DNA solutions. They were not used only in protocol A (standard CTAB Doyle and Doyle (1987) procedure) and DNA solutions obtained by this procedure were brownish in the majority of samples, especially in *S. cuneifolia* and *S. subspicata*, for which an increased polyphenol content has been reported earlier (Miloš et al., 2001; Skočibušić et al., 2006; Dunkić et al., 2007; Čavar et al., 2008; Bezić et al., 2009). In protocol B, both chemicals, activated charcoal 0.5 % w/v (suspended) and PVP 10 1% w/v (dissolved), were added in the extraction buffer, lacking β -mercaptoethanol, as de-

scribed in Križman et al. (2006). DNA solutions obtained by this protocol were generally contaminated by polyphenols, which caused their browning. This suggested that polyphenol adsorbents as applied in protocol B, characterized also by a modified CTAB extraction buffer and other modifications (see Materials and Methods), failed to remove polyphenols from the majority of savory DNA solutions. In protocol C, only activated charcoal was used to test the effect of one polyphenol adsorbent in removing polyphenols from DNA solutions of savory DNA isolates, while the synergistic actions of the two polyphenol adsorbents, activated charcoal and PVP, were tested in protocol D, which used both chemicals. In both protocols, polyphenol adsorbent(s) were added directly to each tube with the homogenized plant tissue (3% w/w each) prior to the application of extraction buffer. DNA isolates obtained by protocols C and D were clear, suggesting the successful removal of polyphenols by one or both polyphenol adsorbents.

The absorbance ratios 260/280 of DNA isolates from all protocols varied insignificantly in 10 savory taxa in all DNA isolation procedures. They were within the optimal range (1.7 to 2.0), with average values of 1.8 ± 0.1 for protocol A, 1.7 ± 0.2 for Protocol B, 1.8 ± 0.2 for Protocol C and 1.7 ± 0.2 Protocol D (Table 2). These findings revealed that CTAB and β -mercaptoethanol applied as described in the proto-

col of Doyle and Doyle (1987) successfully removed proteins during DNA isolation in protocols A, C and D in the majority of individuals. The removal of proteins in protocol B that lacked β -mercaptoethanol was also successful in most savory taxa. Slightly lower values than optimum obtained for individuals *S. cuneifolia* (1) and *S. subspicata* (10) in all protocols suggested that the protein contamination was present in DNA isolates in these individuals. Higher protein content has already been reported in these species (Bežić et al., 2009), and the application of proteinase K during isolation of DNA may be suitable for these taxa. DNA solutions of *S. horvatii* (2) in protocol D (1.5) and *S. subspicata* (9) in protocols B (1.3) and C (1.6) also had lower values than optimum, suggesting possible human error during the isolation of DNAs, because the absorbance ratios 260/280 of DNA solutions of these individuals obtained by other protocols were within optimal range. Thus, two individuals of *S. subspicata* (9 and 10) displayed different levels of contamination with protein. This corroborates the view that the chemical content of two individuals of the same species from alternative locations can affect the DNA isolation procedures.

DNA isolates of savory taxa displayed rather variable absorbance ratios 260/230, indicating more or less successful removal of polysaccharides from the DNA solutions obtained by different protocols. Polysaccharides represent a persistent contaminant during the isolation of DNA from plant tissues, and they are problematic because they form complexes with DNA recognized as a gelatinous pellet, and in that form, DNA is inaccessible to enzymes such as polymerases, ligases, and restriction endonucleases, etc. (Scott and Playford, 1996; Sharma et al., 2002; Varma et al., 2007). They have commonly been eliminated from plant DNA solutions by high salt concentrations. Fang et al. (1992) observed that the addition of 1 M NaCl increased the solubility of polysaccharides in ethanol and decreased the co-precipitation of polysaccharides and DNA, while Lodhi et al. (1994) indicated that higher concentrations of NaCl (final concentration 2.5 M) removed polysaccharides effectively in *Vitis* species. In our protocols A, C and D, 1.4 M NaCl was used in the extraction buffer, while

the extraction buffer in protocol B had a higher salt concentration of 2.0 M. However, in protocols C and D, an additional step with a final salt concentration of 1.3 M NaCl was applied. The highest average value of absorbance ratios 260/230 was obtained in Protocol C (1.7 ± 0.4) but not in protocol D (1.5 ± 0.4), while, contrary to expectations, the average value for the protocol using 2.0 M NaCl in the extraction buffer (B) was lower than the value obtained for the protocol utilizing 1.4 M NaCl in the extraction buffer (A), 1.2 ± 0.4 and 1.6 ± 0.2 , respectively. The former may be caused by the lack of β -mercaptoethanol in the extraction buffer of protocol B. One-way ANOVA revealed statistically significant differences between protocols ($P = 0.01$) suggesting that the application of a high salt concentration as implemented in protocol C is suitable for removing polysaccharides from savory DNA solutions.

With regard to the polysaccharide contamination of different savory taxa, the obtained values of absorbance ratios 260/230 were near optimal or optimal (1.7 to 2.0) for all of studied taxa, except for *S. cuneifolia* (1), *S. montana* ssp. *montana* (5) and *S. subspicata* (9 and 10) in every protocol (Table 2). These findings would suggest that polysaccharides represent a larger portion of their chemical content as compared to the other studied savory taxa. Interestingly, the second representative of *S. montana* ssp. *montana*, individual 6, displayed increased polysaccharide contamination only in protocol B but not in the other protocols, and this finding would further corroborate the view that even in individuals of the same species but from different locations, different chemical content may be expected due to the effect of environmental factors (Miloš et al., 2001).

Enzymatic reactions of the DNAs obtained by four DNA isolation protocols were tested by PCR amplification of the entire nuclear ITS spacer, which is one of the most commonly used nuclear regions in species-level phylogenetic surveys in plants (Felinier and Rossello, 2007), and chloroplast *rpl32-trnL* region characterized as one of the potentially highly informative regions for future molecular studies in plants (Shaw et al., 2007). The amplification of both

loci using DNA isolates obtained by Protocol A was ambiguous because in some individuals for which low DNA yield and increased contamination was detected, PCR products at both loci were obtained (i.e. *S. cuneifolia* (1) and *S. subspicata* (10)), whereas in individuals for which higher DNA yield and less contaminated DNA was obtained, PCR amplification of a chloroplast locus failed (i.e. *S. kitaibelii* (4), *S. montana* ssp. *montana* (6) and *S. montana* ssp. *variegata* (8)) (Fig. 2). Nonetheless, the amplification of the nuclear ITS region was successful in all individuals. Enzymatic activity of DNA solutions obtained by Protocol B was ambiguous as well, because the cpDNA locus was amplified in all individuals except in *S. subspicata* (9), while the ITS region failed to amplify in *S. kitaibelii* (4) and *S. subspicata* (9). PCR amplifications of both loci were successful when DNA isolates obtained by protocols C and D were used. The lack of PCR amplification of nuclear ITS spacer was observed only for DNA isolates obtained by protocol C in individuals with lower DNA yield and increased contamination (i.e. *S. cuneifolia* (1) and *S. subspicata* (9)). When DNA isolates from protocol D were used, PCR amplification of both loci for all individuals, regardless of the DNA yield and contamination, was obtained.

In conclusion, protocol D is recommended for the isolation of high amounts of good-quality DNA from savory species. This is because the highest average DNA yield was obtained by this protocol despite the finding that a relatively high DNA yield was obtained using protocol C as well. In this case, due to the synergistic effect of activated charcoal and PVP 10 added directly to the homogenized plant tissue prior to the addition of extraction buffer, polyphenols were apparently successfully removed from all DNA solutions of all savory taxa in this protocol, similar to protocol C. In addition, the contamination of DNA solutions of the majority of savory DNA isolates by proteins was resolved in this protocol by the application of 2 % CTAB/0.2% β -mercaptoethanol/ in the extraction buffer. The persistent protein contamination due to the increased protein content of some savory taxa (e.g. *S. cuneifolia* and *S. subspicata*) may be eliminated by additional application of protein-

hydrolyzing enzymes such as proteinase K. Although the lowest polysaccharide contamination of savory DNA isolates was obtained with protocol C, only DNA solutions obtained with protocol D displayed successful PCR amplification of one nuclear and one cpDNA locus. Thus, protocol D is a robust, fast, simple and cost-effective method that can easily be implemented in any laboratory in which the standard CTAB method of Doyle and Doyle (1987) is used; it requires the preparation of just one 4.0 M solution of NaCl along with direct addition of activated charcoal and polyvinylpyrrolidone 10 to homogenized plant tissue. Protocol D is suitable for future taxonomic, phylogenetic, population genetics and phylogeographic studies in cases where nrDNA, cpDNA (and mitochondrial DNA) are to be PCR-amplified in a large number of individuals. It is potentially applicable in other aromatic and medical plants rich in secondary metabolites. Nevertheless, this protocol cannot be used for some molecular biological studies, for example genomic library construction, DNA hybridization studies, etc., where only pure nrDNA is needed.

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