Botanica SERBICA 38 (1): (2014) 185-189

Original Scientific Paper

A simple and efficient DNA isolation method for *Ornithogalum* L. species (Hyacinthaceae, Asparagales)

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ABSTRACT: We report an efficient, simple and cost-effective protocol for the isolation of genomic DNA from *Ornithogalum* species. Our modification of the standard CTAB protocol includes two polyphenol adsorbents (insoluble PVPP and activated charcoal), high NaCl concentrations (4 M) for removing polysaccharides, and addition of phenol to remove proteins and other contaminants. DNA yield obtained with our protocol was 223 and 312 μg DNA g^{-1} of dry leaf tissue. The absorbance ratio 260/280 nm was 1.879 (*O. refractum)* and 1.753 (*O. sibthorpii),* and the absorbance ratio 260/230 nm was 1.779 (*O. refractum*) and 1.545 (*O. sibthorpii*), revealing lack of contamination. PCR amplifications of one nuclear marker (26S rDNA) indicated that this DNA isolation protocol may be used for *Ornithogalum* plants containing many interfering compounds for further analyses in population genetics and phylogeographic studies.

Keywords: *Ornithogalum*, DNA isolation, CTAB, phenol, PVPP, PCR.

Received: 14 October 2013 Revision accepted 15 February 2014

UDK 581:575.113(497.11)

INTRODUCTION

Investigations of plant population genetics, phylogeographic as well as phylogenetic studies are based on molecular markers from all of the three plant genomes: nuclear, mitochondrial and chloroplast. These molecular markers are PCR-amplifiable in large numbers of individuals and require fast and cost-effective procedures to isolate large quantities of contaminant-free total genomic DNA. A number of protocols for isolation of total genomic DNA from plants have been developed (Murray & Thompson 1980; Doyle & Doyle 1987; Rogers & Bendich 1988; Lodhi *et al.* 1994). Many of them have been widely used with success in many experiments. However, for some plants species (e.g. medicinal or aromatic), a new protocol for DNA isolation needs to be developed or existing protocols modified because of the specific chemical composition of plant tissues. Also, infraspecific chemical polymorphism appears in many plants, suggesting that it may be necessary to tailor DNA isolation protocols according to each plant and sometimes even plant tissues (Varma *et al.* 2007).

According to molecular and morphological studies, the genus *Ornithogalum* L. belongs to the family Hyacinthaceae and ordo Asparagales (Takhtajan 1997; APG II 2002). According to Baker (1872), the genus *Ornithogalum* s. str. consists of one subgenus, while Zahariadi (1980) divided it into five subgenera. Recently, Martinez-Azorin *et al.* (2011) recognized *Ornithogalum* s.str. as a monophyletic genus within

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the family Hyacinthaceae. The monophyletic genus *Ornithogalum* s.str. is separated from *Ornithogalum* s.l., while *Loncomelos* and *Myogalum*, as European genera, were considered separately (Martinez-Azorin *et al.* 2010). *Ornithogalum* includes about 50-60 speces in Euroasia and the Mediterranean region. In the region of the Balkan Peninsula within *Ornithogalum* s. str. ten species are recognized in national floras (Radenkova 1964; Diklić 1975; Zahariadi 1980; Landstrom 1989).

O. refractum Willd. 1814 is distributed in the area of central, east and south Europe, including Turkey and the European part of Russia on stony or cultivated ground (Zahariadi 1980). In Serbia, it is widespread but scattered. The plant is 5-12 cm high, with a short aerial part (up to 2 cm), or with sessile inflorescence on the ground. The bulb is surrounded by numerous bulbils. Leaves are longer then the scapus, with a white stripe on the upper surface. The inflorescence is corymbiform. Pedicels are refracted in fruit, with a pulvinus at the base. Bracts are shorter or longer then pedicels; flower numbers 2-18, flowering March - April (Radenkova 1964; Diklić 1975).

O. sibthorpii Greut. 1967 is distributed in the Balkan peninsula (Zahariadi 1980). In Serbia, it is rarely distributed in the southern part. The plant is 5-15 cm high. The bulb is without bulbils. Leaves are longer then the scapus, with a white stripe on the upper surface. The inflorescence is corymbiform. Pedicels are refracted in fruit, with a pulvinus at the base. Bracts are shorter or longer then pedicels; flower numbers 4-7, flowering February-March (Radenkova 1964; Diklić 1975).

So far, there are a few protocols for DNA isolation from *Ornithogalum* species for diverse studies (Martinez-Azorin *et al.* 2011). They used either the standard CTAB DNA isolation protocol of Doyle & Doyle (1987) with additional purification using CsCl/ethidium bromide gradient or commercially-available DNA isolation kits. However, as *Ornithogalum* species possess many secondary metabolites, dominantly resins, the standard procedure for DNA isolation yielded the smallest amount of poor-quality DNA, or failed to produce PCR amplification products. Flavonoids and sterols have been isolated from plants sourced from all the regions, and homoisoflavanones have been identified from the bulbs (Mulholland *et al.*, 2013).

The aim of our study was to establish an efficient, easy, fast and cost-effective DNA isolation procedure that yields large amounts of pure total genomic DNA from *Ornithogalum* sp. We tested the procedure of Doyle & Doyle (1987) in *Ornithogalum* plants from a natural population, with introduced additional modifications. DNA isolates were used for PCR amplification of one nuclear region (26S rDNA) which can be potentially informative for future phylogenetic, phylogeographic, population genetics, and conservation studies in *Ornithogalum* species.

MATERIALS AND METHODS

Plant material. Plant material was collected during April 2013 in the district of Niš, Serbia, Balkan Peninsula. *Ornithogalum refractum* was collected in Jelasnica (N43 18.116 E22 03.296, 239m), while *O. sibthorpii* was collected in Niška banja (N43 17.580 E22 00.420, 249m). Voucher specimens were identified and deposited in the Herbarium of the University of Novi Sad, Faculty of Sciences - Herbarium BUNS (no. 21704- 21705).

DNA isolation procedure. Plant material-herbarium specimens were used for DNA isolation. One leaf from each plant was frozen in liquid nitrogen, homogenized to a fine powder using a mortar and pestle. The following reagents and chemicals were used in both protocols: CTAB extraction buffer [2 % (w/v) CTAB (Serva, Heidelberg, Germany); 50 mM EDTA (Merck, Darmstadt, Germany), pH 8.0; 100 mM Tris–HCl (Applichem, Darmstadt, Germany), pH 8.0; 1.4 M NaCl (Zorka Pharma, Šabac, Serbia), and 0.2 % β-mercaptoethanol (Sigma, St. Louis, USA) added just before use], insoluble PVPP (Sigma), activated charcoal (Kemika, Zagreb, Croatia), Sevag [24:1 (v/v) chloroform (Lachner, Neratovice, Czech Republic): isoamylalcohol (Zorka Pharma)], isopropanol (Zorka Pharma), phenol (Sigma), 70 % ethanol (Zorka Pharma), RNase A (4 mg/ml, Promega, USA) and sterile deionized water.

Plant material (100 mg) was mixed with insoluble PVPP and activated charcoal, and 1 ml of preheated (65 °C) CTAB/β-mercaptoethanol extraction buffer was added to each sample, vortexed briefly and incubated for 1 h (65 °C), mixed with inversion. An equal volume of phenol was added to each sample and mixed thoroughly by vortexing. An equal volume of SEVAG was added in the mixture, and centrifuged at 13,000 rpm for 10 min at 4 °C. The aqueous phase was mixed with ½ vol of cold 4 M NaCl, mixed and 2/3 vol of cold isopropanol was added, mixed thoroughly by inversions, and incubated at -20 °C for 1 h. After centrifugation at 13,000 rpm for 10 min at 4 °C the supernatant was discarded and the pellet was washed with 70 % cold ethanol, centrifugated at 13,000 rpm for 10 min at 4 °C, and finally the pellet was dried using a vacuum concentrator, and resuspended in 100 µl of deionized water. After adding 0.5 µl of RNase A to the DNA solution, it was incubated at 37 °C for 1 h. Genomic DNA was quantified and assessed for purity utilizing NanoVue (GEHealthcare Europe, Freiburg, Germany), which measures absorbances at 230, 260, and 280 nm.

Table 1. Concentration, yield and absorbance ratio ($A_{260}/_{280}$ and A_{260}/A_{230}) of genomic DNA from *Ornithogalum* sp.

Species	Concentration (ng/µl)	Yield (µg/g tissue)	Absorbance ratio ($A_{260}/_{280}$)/(A_{260}/A_{230})
O. refractum	116.5	223	1.879/1.779
O. sibthorpii	156	312	1.753/1.545

PCR analysis. PCR amplification of one nrDNA (26S rDNA) was performed to assess the utility of DNA isolates for molecular studies involving PCR amplification of nuclear loci. The nuclear locus, which is rather conserved in plant species (Kuzoff *et al.* 1998), was amplified using the F primer 26Sf 5'-ATTCCCAAACAACCCGACTC-3' and R primer 26Sr 5'-GCCGTCCGAATTGTAGTCTG-3', designed by Timotijević *et al.* (2010). The reaction product was used as a template for PCR reaction. The reaction mixture (25 µl) was 25 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.1 µM primers, 1X Taq buffer (Fermentas) and 1U Taq polymerase (Fermentas). PCR reaction was performed in a Biometra Thermocycler, using the following profile after an initial 95 °C for 5 min (initial denaturation): 30 cycles of 95 °C for 30 sec (denaturation), 60 °C 30 sec (annealing), 72 °C 1 min (extension), with final extension for 10 min at 72 °C. The PCR products were separated on a 2 % agarose gel and visualised by UV/EtBr staining.

RESULTS AND DISCUSSION

Ornithogalum is a very interesting genus for phylogenetic studies (Martinez-Azorin *et al.* 2011). Molecular analyses require DNA that could be used in PCR amplification of diverse molecular markers. However, many plants posses secondary metabolites that can interfere with DNA isolation procedures and hamper downstream manipulations at the molecular level (Varma *et al.* 2007). So far, only the standard CTAB procedure of Doyle & Doyle (1987) and a modification utilizing polyphenol adsorbents, such as PVP, PVPP and activated charcoal (Križman *et al.* 2006), as well as rather expensive commercial DNA extraction kits have been used to isolate DNA from plants for various studies (Luciano *et al.* 2007). In addition, some protocols for DNA extraction from *Ornithogalum* used a CsCl/ethidium bromide gradient (Martinez-Azorin *et al.* 2011). The procedure used in this paper is suitable not only for *Ornithogalum*, but also for other plants with high amounts of phenols and other compounds that could interfere with DNA extraction and with further molecular analyses.

Activated charcoal and insoluble PVPP have been components of protocols favoring yield and purity of DNA isolates. PVPP is an adsorbent commonly used to eliminate polyphenols, especially at low pH by forming complexes

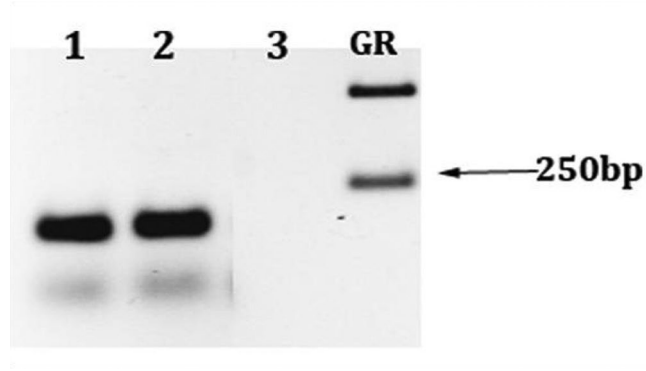

Fig. 1. PCR products obtained after amplification of 26S rDNA from *Ornithogalum* sp. Lanes: 1 - *O. refractum*, 2 - *O. sibthorppi*, 3 - negative control, GR - DNA size marker (1 kb Fermentas).

with polyphenols through hydrogen bonding, allowing them to be separated from DNA, thereby reducing their level in the product (John 1992; Pich & Schubert 1993; Lodhi *et al.* 1994; Porebski *et al.* 1997; Permingeat *et al.* 1998). Activated charcoal also adsorbs polyphenols and has been used alone or in combination with PVP in several DNA isolation procedures (Maliyakal 1992; Bi *et al.* 1996; Porebski *et al.* 1997; Peterson *et al.* 1997; Kim *et al.* 1997; Martellossi *et al.* 2005).

However, using only activated charcoal and insoluble PVPP it was very hard to homogenize tissues from *Ornithogalum* plants. In the first extraction step we added liquid phenol, to remove as many contaminants as possible. Using this modification, we removed not only phenolic compounds that could be cross-linked with DNA and cause browning of the DNA, but also other compounds, predominantly resins and saponins. In this way, we improved DNA yield and purity. Our results indicated that this protocol makes it possible to get high DNA concentrations of high purity. The average DNA concentration was 116.5 ng/µl for *O. refractum* and 156 ng/µl for *O. sibthorpii*. As the final DNA pellet was dissolved in 100 µl of sterile, deionised water, the average yield ranged from 223 µg/g of dried plant material to 312 µg/g. In addition, the purity of isolated DNA was satisfactory - the absorbance ratio A260/A280 was 1.879 and 1.753, and the absorbance ratio A260/A230 was 1.779 and 1.545 for *O. refractum* and *O. sibthorpii*, respectively (Table 1).

The genomic DNA obtained from *Ornithogalum* was consistently amplified with primer pairs for nrDNA 26S rDNA. These primers are not specific for these two species,

but were designed according to the conserved region of the 26S rDNA gene present in all plants, having been tested on *Arabidopsis*, *Nicotiana*, *Fagopyrum*, *Ramonda*, *Pisum* and *Vicia*. After PCR reaction a single band was obtained (Fig 1.).

CONCLUSION

Our results demonstrate that the optimized and modified protocol for DNA extraction from *Ornithogalum* species gave high yields of contaminant-free genomic DNA from *Ornithogalum* species, which would be suitable for future analyses, including population genetics, phylogenetic, phylogeographic and conservation surveys in this species, using nrDNA. Our protocol can be easily implemented in any laboratory in which the standard CTAB method of Doyle & Doyle (1987) is used and requires preparation of a single strong solution of NaCl together with direct addition of insoluble PVPP and activated charcoal to the homogenized plant tissue. In addition, by using phenol in the extraction procedure to remove interfering compounds, additional purification of DNA using a CsCl/ethidium bromide gradient was avoided.

Acknowledgments — This work was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Research Grants 173030, 173005 and the Provincial Secretariat for Science and Technological Development of Vojvodina (grant no. 114-451-2056/2011-01). The authors would like to thank prof. Ivana Bosić for language editing.

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REZIME

Jednostavan i efikasan metod za izolaciju genomske DNK iz vrsta roda *Ornithogalum* L. (Hyacinthaceae, Asparagales)

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Prikazan je jednostavan, efikasan i jeftin protokol za izolaciju genomske DNK iz vrsta roda *Ornithogalum*. Korišćena modifikacija standardnog CTAB protokola uključuje dodatak dva adsorbenta fenolnih jedinjenja (nerastvorni PVPP i aktivni ugalj), rastvora NaCl velike molarnosti (4M) za uklanjanje polisaharida, kao i dodatak fenola u cilju uklanjanja proteina i drugih kontaminanata. Količina dobijene genomske DNK upotrebom navedenog protokola varira od 223 μg DNA g^{-1} suve mase lista (za *O. refractum*) do 312 μg DNA g^{-1} suve mase lista (za *O. sibthorpii*). Spektrofotometrijska merenja su pokazala da je odnos apsorbancija 260/230 nm 1.779 (*O. refractum*) i 1.545 (*O. sibthorpii*), ukazujući na dobru čistoću izolata. PCR umnožavanje jednog nuklearnog markera (26S rDNK) potvrđuje da korišćeni protokol za izolaciju genomske DNK može biti uspešno korišćen kod vrsta roda *Ornithogalum*, bogatih sekundarnim metabolitima, za populaciono-genetičke analize i za filogeografske studije.

Ključne reči: *Ornithogalum*, izolacija DNK, CTAB, fenol, PVPP, PCR.