SHORT COMMUNICATION

COMPARISON OF TWO RAPID DNA EXTRACTION PROTOCOLS FOR GYMNOSPERMS FOR APPLICATION IN POPULATION GENETIC AND PHYLOGENETIC STUDIES

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ABSTRACT

Population genetic and phylogenetic analyses of plant species are often limited by time-consuming DNA extraction and purification procedures. We developed a rapid protocol for the isolation of genomic DNA from gymnosperms using silica-based membranes in a 96-well plate format. This protocol yields high-quality DNA as evidenced by PCR-amplification of chloroplast DNA and RAPD-PCR analyses. The DNA quality was higher than that observed for DNAs isolated using a one-tube extraction process.

Key words: chloroplast DNA, conifers, DNA extraction, gymnosperms, *trnL* intron, RAPD-PCR.

Rapid development of new genetic markers and the wide use of polymerase chain reaction (PCR) provide the basis for many population genetic, phylogeographic, and phylogenetic investigations. In many cases, DNA extraction has nowadays become the time limiting step in such studies, because many samples need to be analyzed for sufficient statistical power. It is therefore desirable to reduce the time as well as number of handling steps in DNA extraction protocols. However, high quality of DNA extracts is essential in order to successfully type genetic markers. Plants, unlike animals, often contain polysaccharides and secondary metabolites in their tissues, which may inhibit PCR-amplifications. DNA isolation of gymnosperms has shown to be especially difficult because of large amounts of, e.g., polyphenols, resins, and terpenoids (ZIEGENHAGEN *et al.* 1993). Here, we present a rapid DNA extraction protocol for gymnosperms using DNeasy silica-gel membranes in a 96-well plate format (QIAGEN, Hilden, Germany). The quality of the DNA extracts is verified by PCR-amplification of a chloroplast DNA marker and, since known to be sensitive to DNA quality, by random amplified polymorphic DNA (RAPD)-PCR using a primer known to amplify across a wide range of taxa (FRITSCH *et al.* 1993; unpubl. data). We compare the PCR-reliability of these DNAs with DNA obtained from one-tube extractions using rapid one-step extraction (ROSE) buffer (STEINER *et al.* 1995).

We selected 32 species of as many different gymnosperm genera that covered a wide taxonomic range (Table 1). For the DNeasy extraction, we prepared three replicates of fresh needle tissue (100 mg) per species in 2-mL Eppendorf tubes that contained two 5-mm stainless steel beads. After lyophilizing the samples, they were ground to a fine powder using a shaking mill MM2000 (Retsch, Haan, Germany) at full speed for 2 min. We added 800 µL extraction buffer (100 mM sodiumacetate, 50 mM EDTA, 500 mM NaCl, 2 % (w/v) PVP, 1.4 % (w/v) SDS; pH 5.5; ZIEGENHAGEN *et al.* 1993), including 1 % (w/v) sodium bisulfite, to each sample and incubated the samples in a shaking water bath at 65 °C for 30 min. After adding 200 µL of 3 M potassium acetate (pH 5.2), we incubated the samples on ice for 30 min, and centrifuged them at 20000 × g for 30 min. For each sample, we added 420 µL of the supernatant to 210 µL of Buffer AP3 (QIAGEN) in a collection-microtube rack of 96 (1.1 ml tubes; QIAGEN), mixed the solution thoroughly, and added 440 µL of pure ethanol. Each sample was then loaded onto the DNeasy 96-well plate (DNeasy 96 Tissue Kit), which was placed on a square-well block, and sealed it with adhesive tape permeable to air. We centrifuged the samples at 6000 × g (Sigma 4K15 Centrifuge, QIAGEN Plate Rotor 2 × 96) for 20 min[1] and washed the filters twice with 500 µL Buffer AW (QIAGEN), centrifuging at 6000 × g for 4 min both times. Ethanol residues were

[1] In two species, remaining solution had to be removed

Table 1. DNA was extracted from 32 gymnosperm species using either the DNeasy or ROSE buffer protocol. For each species, the table indicates average DNA yields in µg (± SD) per 100 mg fresh tissue obtained from three replicate samples using the DNeasy protocol, and compares the DNA quality resulting from both methods as indicated by the number of replicate samples (*) leading to successful amplification in PCR and RAPD-PCR. Plant material originated from the arboretum of WSL, Birmensdorf, and the Botanic Garden, Zürich.

Species	Family	Yield [µg] (mean±SD)	CpDNA-PCR		RAPD-PCR	
			DNeasy	ROSE	DNeasy	ROSE
Cycas circinalis L.[1)]	Cycadaceae	28.6 ± 29.8	***	***	***	
Ginkgo biloba L.	Ginkgoaceae	19.6 ± 1.3	***	***	***	
Gnetum gnemon L.[1)]	Gnetaceae	33.7 ± 1.1	***	***	***	
Ephedra distachya ssp. *helvetica* L.[1)]	Ephedraceae	36.8 ± 4.1	***	**	***	
Abies alba Mill.	Pinaceae	23.1 ± 2.2	***	**	***	
Cedrus atlantica (Endl.) Manetti ex Carr.	Pinaceae	19.8 ± 2.2	***	***	***	
Larix decidua Mill.	Pinaceae	31.1 ± 5.1	***	**	***	
Picea abies (L.) Karst.	Pinaceae	18.4 ± 2.5	***	***	***	**
Pinus sylvestris L.	Pinaceae	36.6 ± 4.2	***	***	***	**
Pseudotsuga menziesii (Mirb.) Franco	Pinaceae	15.2 ± 3.4	***	**	***	***
Tsuga canadensis (L.) Carr.	Pinaceae	20.8 ± 0.6	***	***	***	
Podocarpus lawrencei Hook. f.[1)]	Podocarpaceae	17.2 ± 2.8	***	*	***	
Agathis brownii L. H. Bailey [1)]	Araucariaceae	11.1 ± 3.2	***		***	
Araucaria angustifolia (Bertol.) Kuntze [1)]	Araucariaceae	21.4 ± 5.3	***		***	
Sciadopitys verticillata (Thunb.) Schinz & Zucc.[1)]	Sciadopitaceae	13.4 ± 1.6	***	***	***	
Taxus baccata L.	Taxaceae	25.1 ± 2.7	***	***	***	
Torreya nucifera (L.) Schinz & Zucc.[1)]	Taxaceae	35.1 ± 2.7	***	***	***	***
Cephalotaxus harringtonia var. *drupacea* (Forbes) K.Koch[1)]	Cephalotaxaceae	39.5 ± 2.2	***	***	***	***
Cryptomeria japonica (L. f.) D. Don	Taxodiaceae	9.1 ± 1.7	***			
Cunninghamia lanceolata (Lamb.) Hook [1)]	Taxodiaceae	22.1 ± 3.7	***		***	
Metasequoia glyptostroboides Hu & Cheng	Taxodiaceae	13.2 ± 2.2	***	*	***	
Sequoia sempervirens (D. Don) Endl.[1)]	Taxodiaceae	34.5 ± 9.5	***	*	***	
Sequoiadendron giganteum (Lindl.) Buchh.	Taxodiaceae	17.7 ± 2.0	***	**	***	
Taxodium distichum (L.) A. Rich.[1)]	Taxodiaceae	33.5 ± 1.9	***		***	
Callitris preissii Miq.[1)]	Cupressaceae	33.7 ± 6.1	***	***	***	
Calocedrus decurrens (Torrey) Florin	Cupressaceae	23.7 ± 2.6	***	***	***	
Cupressocyparis × *leylandii* Dallimore & A. B. Jackson [1)]	Cupressaceae	16.1 ± 1.1	***	**	***	
Cupressus arizonica Greene [1)]	Cupressaceae	36.5 ± 7.9	***	***	***	
Tetraclinis articulata (Vahl) Masters	Cupressaceae	23.1 ± 4.8	***		***	
Thuja plicata Donn ex D. Don	Cupressaceae	19.7 ± 2.1	***	*	***	
Thujopsis dolabrata Schinz & Zucc.[1)]	Cupressaceae	8.3 ± 2.5	***		***	
Widdringtonia cedarbergensis J. A. Marsh [1)]	Cupressaceae	13.8 ± 7.5	***		***	

[1)] Plant material received from Botanic Garden, Zürich

allowed to evaporate at 70 °C for 5 min before we eluted the DNAs into a sterilized collection-microtube rack (QIAGEN) by twice adding 100 µL TE buffer (10 mM Tris-HCl, pH 9.0; 1 mM EDTA) and centrifuging at 6000 × g for 2 min. TE buffer was preheated at 70 °C, and samples were left to dissolve off the filters at 70 °C for 5 min before centrifugation.

We used an expandable multichannel pipette (Matrix Impact2; Integra Biosciences, Wallisellen, Switzerland) which allowed transfer of solutions from widely spaced 2-mL Eppendorf tubes in racks to microtiter format. The time taken for the extraction protocol can be reduced by pulverizing lyophilized samples directly in racked microtubes (see Steiner *et al.* 1995). Furthermore, incubation time on ice and, depending on the species, centrifugation times may be significantly shorter. On the other hand, the purity of the extracts was improved by adding dichloromethane (or chloroform) to the samples before DNA precipitation (unpublished data).

Visual inspection of genomic DNA on agarose gels revealed no evidence of degraded DNA (except in *Gnetum gnemon*) or high amounts of RNA (Fig. 1). We determined DNA concentration fluorometrically (DyNA Quant 200; Hoefer Pharmacia Biotec, Düben

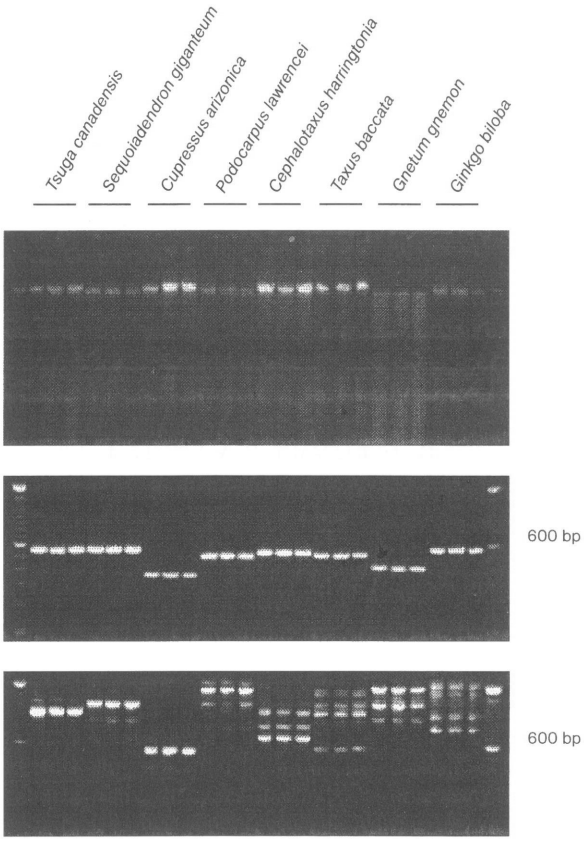

Figure 1. 0.9 µL genomic DNA (top), *trnL* (UUA) intron in the large single-copy region of cpDNA (center), and RAPD-PCR banding patterns using primer B07 (bottom) of DNeasy extracts from eight gymnosperm species of as many families, with three replicates per species placed side-by-side. 15-µL PCR mixtures for amplification of the chloroplast *trnL* (UUA) intron contained 1× *Taq* DNA polymerase buffer (SIGMA, Buchs, Switzerland), 1.6 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer, 1.5 U *Taq* DNA polymerase (SIGMA), and 2.5 µL of DNA extract, diluted to 4 $ng \cdot \mu L^{-1}$ in TE buffer (10 mM Tris-HCl, pH 8.4; 1 mM EDTA). A PTC-100 thermo-cycler (MJ-Research, Watertown, USA) was programmed with the following steps: initial denaturing for 2 min at 94 °C, followed by 41 cycles of 1 min at 94 °C, 1 min at 57 °C, and 2 min at 72 °C. RAPD-PCRs were performed with Operon primer B07 (5'-GGTGACGCAG; Microsynth, Balgach, Switzerland) as described in GUGERLI *et al.* (1999), but with 0.6 U *Taq* DNA polymerase and 2.5 µL of diluted DNA extract (4 $ng \cdot \mu L^{-1}$) in a 15-µL reaction mixture. Agarose gels were 0.8 % for genomic DNA analysis and 1.5 % for PCR-product analysis in 1× TBE buffer (SAMBROOK *et al.* 1989; 0.0005 % ethidium bromide added for visualzing under UV light). Lanes 1 and 26: 15 ng of λ DNA marker or 100-bp ladder, respectively (Gibco-BRL, Gaithersburg, USA).

dorf, Switzerland) according to manufacturer's instructions. Yields varied between 7 and 60 µg per sample (Table 1) and differed significantly among species (ANOVA: $F = 5.9$, d.f.= 31, 64; $P < 0.001$).

Amplification of the *trnL* (UUA) intron in the large single-copy region of cpDNA using a pair of universal primers (TABERLET *et al.* 1991) gave PCR-products in all replicates of the 32 investigated gymnosperm species (Table 1, Fig. 1). RAPD-PCRs gave consistent banding patterns for replicated extractions within each of the species except for *Cryptomeria japonica*, which showed no amplification products at all (Table 1). These results indicate the generally high quality of DNA extracts in taxonomically very distant gymnosperm species.

ROSE buffer DNA extractions of the same tissue samples followed the protocol given in STEINER *et al.* (1995) with minor changes: We pulverized lyophilized tissue as described above (originally in racked microtubes). In addition to the original protocol, we centrifuged the samples at 20000 × g for 10 min to optimize settlement of tissue and PVPP. For PCR, we diluted the supernatants 170-fold with water. Concentrations in the reaction mix for cpDNA-fragment amplification and RAPD-PCR followed STEINER *et al.* (1995), adjusted to our standard PCR protocol.

Compared with the DNeasy extraction, fewer DNA extracts led to amplification of the cpDNA fragment (Table 1): All replicates amplified in 14 taxa, eight taxa did not show any amplification, and in the remaining ten taxa, only one or two of the three replicate samples were successfully amplified. In RAPD-PCR, the DNA extracts clearly showed reduced amplification success compared to the DNeasy extracts: In only three species were amplification products consistent in all three replicates, and in only two more species were they consistent between two of the three replicates.

We have shown that the above described DNeasy DNA extraction protocol can be applied to many gymnosperm species and that it results in high-quality DNA for PCR techniques. The protocol provides a basis for both phylogenetic studies that include various taxonomic groups, as well as for population genetic studies with large sample numbers and thus the need for high throughput. In the latter case, the protocol can be adjusted in order to minimize the time required, depending on the species under study (e.g., *Picea abies* and *Pinus cembra*; unpublished data). Costs for DNA extraction may be equilibrated by subsequently reduced expenses in terms of time and money since the high DNA quality minimizes the number of extraction and PCR repetitions. The faster protocol of STEINER *et al.* (1995) may be preferred due to an even larger throughput and lower costs, but PCR-reliability is reduced, and an additional effort may be needed for species-specific optimization.

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