assays. This method can greatly streamline the process of plasmid miniprep analysis and its application in routine molecular cloning. It is possible that entire departments could consolidate diagnostic miniprep work into one facility managed by a well-trained technician who could process at least one thousand clones in part of a working day.

REFERENCES


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Method of Extracting Genomic DNA from Non-Germinated Gymnosperm and Angiosperm Pollen

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Naturally occurring ultraviolet-B (UV-B) radiation (wavelengths between 280 and 320 nm) at the earth’s surface is projected to increase by 30% over the next 15 years because of predicted decreases in stratospheric ozone (3,13). This increase in UV-B radiation is also predicted to have deleterious effects on plant growth and reproduction. Determining the extent of UV-B-induced damage at the genetic level will require an assessment of DNA structural integrity (6). Traditional methods of isolating plant DNA have failed to yield DNA of acceptable quality for use in UV-B damage assays. Typical extraction protocols inherently prejudice results because they involve physical maceration and/or phase separation steps, which may introduce structural damage into the isolated DNA and/or may eliminate small fragments of sheared DNA, respectively. The objective of this study was to develop a simple and efficient method of extracting large quantities of nondegraded genomic DNA from forest tree pollen.

Of all plant reproductive tissues, pollen may be the most UV-B sensitive, with some species exhibiting no germination when damaged by UV-B radiation (18). DNA obtained from a mixture of germinated and non-germinated pollen will predominately originate from germinating, non-damaged pollen, resulting in an underestimation of the extent of damage caused by the UV-B exposure. Thus, an assessment of genetic damage from UV-B-exposed pollen requires a DNA isolation procedure that does not cause structural damage and is able to obtain DNA from both germinated and non-germinated pollen grains. Attempts at lysing non-germinated pollen to obtain high-molecular-weight DNA have been thwarted by the resilient morphology of the pollen coat (2,17). This resilience is mainly attributed to the presence of the pollen coat chemical sporopollenin, which is resistant to numerous chemicals, high temperatures and fungal and bacterial decay (8).

Pollen samples were collected from the following species: Fraser fir [Abies fraseri (Prush.) Poir.], yellow poplar [Liriodendron tulipifera L.], ponderosa pine [Pinus ponderosa Doug]., ex Laws., loblolly pine [Pinus taeda L.], Virginia pine [Pinus virginiana Mill.], eastern cottonwood [Populus deltoides Bartr. ex Marsh.] and European black cottonwood [Populus nigra L.]. All pollen samples originated from local, native individuals, with the exceptions of ponderosa pine for which pollen was collected from a cultivated provenance study in Nebraska and European black cottonwood pollen, which was collected and shipped from Italy. All pollen was dried under desiccant to 10% (wt/vol) moisture content and stored at 4°C before use in the extraction assays.

Several techniques were tested in an effort to determine the combination of steps for extraction and purification that would yield DNA of the highest quality and quantity. Specifically, procedures to liberate DNA from pollen included 4-methylmorpholine-N-oxide (MMNO) treatment (1,2,12), osmotic rupture (7,14,16), snap-freezing (9), and

![Figure 1. Genomic DNA isolated from 50 mg of ponderosa pine pollen using the following procedures (approximate amount of total DNA obtained)](image)

![Figure 2. Genomic DNA isolated from 50 mg pollen from several gymnosperm and angiosperm species. Samples are as follows (approximate-average amount of total DNA obtained; n = 9): lane 2, Eastern cottonwood (19 µg); lane 3, European black cottonwood (17 µg); lane 4, yellow poplar (3.9 µg); lane 5, ponderosa pine (109 µg); lane 6, loblolly pine (44 µg); lane 7, Fraser fir (31 µg); lane 8, red spruce (34 µg); and lane 9, Virginia pine (61 µg). Each electrophoresis well contains 250 ng DNA.](image)

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microwave lysis (10), alkaline/acid hydrolysis/acetone treatments, cellulase/pectinase/hemicellulase cell wall digestion (15), mechanical microbead maceration (4) and hand grinding miniprep (as a control) (9,14). Buffers, used to maintain optimal DNA integrity during concentration and purification, included modified 2× hexadecyl-trimethylammonium bromide (CTAB) buffer (5), sodium lysis buffer (11), lithium lysis buffer (11) and microwave extraction buffer (10). The following reagents were used: (i) 2× CTAB extraction buffer [2% (wt/vol) CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 mM NaCl, 1% (wt/vol) polyvinyl-polypyrrolidone (PVPP), 1% (vol/vol) β-mercaptoethanol]; (ii) sodium lysis buffer [500 mM NaCl, 100 mM Tris-HCl (pH 7.2), 10 mM EDTA, 1% (wt/vol) sodium dodecyl sulfate (SDS), 5 mM dithiothreitol (DTT)]; (iii) lithium lysis buffer [500 mM LiCl, 100 mM Tris-HCl (pH 7.2), 10 mM EDTA, 1% (wt/vol) lithium dodecyl sulfate, 5 mM DTT, 0.05 mg/mL proteinase K]; and (iv) microwave extraction buffer [50 mM Tris-HCl (pH 7.2), 50 mM EDTA, 3% (wt/vol) SDS, 1% (vol/vol) β-mercaptoethanol].

The effectiveness of the lysing techniques was evaluated based on the percent of pollen grains that were ruptured. The effectiveness of the extraction buffers was determined based on genomic DNA integrity and quantity of DNA extracted. Genomic DNA was electrophoresed in a 0.8% (wt/vol) agarose gel under 100 V/cm for 1 h to assess the amount of DNA shearing. Total DNA concentration was determined using a Model TKO 100 Fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA, USA). Ponderosa pine pollen was used to select the optimum DNA extraction procedure; the remaining species were then used to verify the utility of the selected procedure.

Under microscopic examination, it was evident that osmotic rupture, snap-freezing, microwave lysis, acetone treatment and enzymatic digestion treatments did not lyse or eliminate the pollen coat. Pollen coats were degraded using 5 N NaOH, 10 N HCl or MMNO treatments, but the recovered DNA was either degraded or completely eliminated during the extraction procedure (no data shown). Hand grinding using a small pestle under liquid nitrogen in a microcentrifuge tube was successful in lysing the pollen coat, though the recovered DNA was partially sheared and total DNA concentrations were low (Figure 1). The only technique that ruptured the coat and liberated abundant, nondegraded DNA was mechanical microbead maceration.

Table 1. Genomic DNA Extracting Procedure

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Add DNA-free RNase A to a concentration of 25 µg/mL and incubate at 37°C for 30 min. Place the samples at -20°C for storage.</td>
</tr>
<tr>
<td>2</td>
<td>For ponderosa pine, place 50 mg (ca. 9.5 × 10⁶ pollen grains) of pollen in a graduated 1.5-mL microcentrifuge tube, and fill the tube with approximately 1.5 mL of 1-mm (diameter) glass beads (Walter Stern, Fort Washington, NY, USA).</td>
</tr>
<tr>
<td>3</td>
<td>Add the lithium lysis buffer to the top of the glass beads (ca. 750 µL). Agitate each sample for 2 min in a Mini-Beadbeater® (Biospec Products, Bartlesville, OK, USA) and incubate for 30 min at 37°C to activate the proteinase K after mechanical maceration. Then divide the contents of the tube into two aliquots using a small sterile scoopula.</td>
</tr>
<tr>
<td>4</td>
<td>Add an equal volume of chloroform:isoamyl alcohol (24:1) to each aliquot. Gently blend the samples by hand for 10 s, and then place each tube horizontally in an orbital shaker at approximately 100 rpm for 10 min at room temperature. Next, centrifuge each tube for 15 min at 5500× g.</td>
</tr>
<tr>
<td>5</td>
<td>To prevent shearing of the DNA, remove the aqueous phase from the two tubes using a wide-bore disposable transfer pipet, and combine the two aliquots into a single new microcentrifuge tube.</td>
</tr>
<tr>
<td>6</td>
<td>Repeat the chloroform:isoamyl alcohol phase separation step. Place the final aqueous phase into a Microcon®-50 column (Amicon, Beverly, MA, USA) and centrifuge at 14 000× g for 8–10 min or until only approximately 150 µL of the sample remains in the column.</td>
</tr>
<tr>
<td>7</td>
<td>Rinse the DNA in the column with 250 µL of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.0) under centrifugation at 14 000× g for 8–10 min.</td>
</tr>
<tr>
<td>8</td>
<td>Remove the column from the tube and invert the column into a fresh microcentrifuge tube and add 100 µL of TE buffer to the column.</td>
</tr>
<tr>
<td>9</td>
<td>Centrifuge at 1000× g for 5 min to collect the DNA sample. Note that although higher DNA concentrations were obtained using the Microcon-50 columns, a -20°C ethanol or isopropanol (1:1) precipitation was also used successfully.</td>
</tr>
<tr>
<td>10</td>
<td>Incubate the filtered samples at 65°C for 30 min to inactivate any residual proteinase K and allow to cool to room temperature.</td>
</tr>
<tr>
<td>11</td>
<td>Add DNA-free RNase A to a concentration of 25 µg/mL and incubate at 37°C for 30 min. Place the samples at -20°C for storage.</td>
</tr>
</tbody>
</table>

The mechanical microbead maceration with lithium lysis buffer procedure produced the best results for ponderosa pine pollen, thus it was tested further on non-germinated pollen from a variety of forest tree species (Figure 2). This procedure produced nondegraded DNA from all tested gymnosperm species. It was also effective on tested angiosperm species, but resulted in slightly higher DNA degradation, a lower concentration of DNA recovered per mg of pollen and yellow discoloration of the isolated DNA. Part of this
difference may be due to the smaller size of the angiosperm pollen and the inability of the 1-mm (diameter) glass beads to efficiently rupture the pollen cell wall. Smaller beads may be appropriate for small diameter pollen. Total DNA concentration per mg of pollen varied with species, ranging from 3.9 µg per 50 mg of yellow poplar pollen to 109 µg per 50 mg of ponderosa pine pollen.

Based on the above results, the procedure in Table 1 is recommended. The procedure is rapid, requires very few solutions and is effective in isolating genomic DNA from non-germinated pollen grains. Genomic DNA from ponderosa pine, Virginia pine, loblolly pine, red spruce, Fraser fir, yellow poplar, eastern cottonwood and European black cottonwood has been successfully isolated by using the above procedure, with an average yield of 40 µg of DNA from 50 mg of pollen across all samples and all species tested. This procedure provides two additional advantages over traditional plant DNA isolation methods. The first and most beneficial is that the DNA is not degraded as has been seen with other techniques where physical maceration has been used. The second is that the procedure provides high yields of DNA from a small amount of pollen. Although this procedure has at present been tested on a limited number of species, it could be of great help to others working with pollen. The DNA isolated by this procedure has been effectively used in randomly amplified polymorphic DNA polymerase chain reaction (i.e., amplification using four deamer primers yielded 6–10 bands per primer), alkaline gel electrophoresis and alkaline unwinding assays for quantification of genetic damage induced by UV-B radiation.

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Enhanced DNA Extraction and PCR Amplification of Mitochondrial Genes from Formalin-Fixed Museum Specimens

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Enzymatic methods to clone and manipulate DNA using the polymerase chain reaction (PCR) (9,13,19) in conjunction with rapidly advancing DNA sequencing technologies (1) are allowing an increasing number of investigators to work directly with DNA from a wide variety of organisms and tissue samples. Detailed genetic studies are now becoming feasible using fossil amber, bone, leaves, mummies (4,6,8,11), single hair follicles (17), dried blood stains (22), dung (5) and a myriad of archival museum specimens including