Rapid DNA Extraction from Ferns for PCR-Based Analyses

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Pteridophytes are not as well characterized at the molecular and biochemical level as some higher plants. However, to aid conservation efforts (4), there is growing interest in understanding the population biology of many fern species that are under threat. DNA-based studies offer excellent tools for such work, but are hampered in ferns by difficulties in obtaining good-quality DNA from sufficiently large numbers of samples. Extraction of good-quality DNA from plant material is often problematic due to contamination with polysaccharides and polyphenols (1). These classes of compounds inhibit downstream enzymatic reactions such as polymerase chain reaction (PCR) and restriction enzyme digestions. Ferns in general contain large amounts of secondary plant metabolites, perhaps as a defense against predation, as their life cycle includes a vulnerable gametophytic stage. Common fern secondary metabolites include triterpene hydrocarbons, occasionally cyanogenic glycosides (which are extremely toxic) and, in *Dryopteris*, complex polyketide-derived phenols (7). We are currently studying populations of the maidenhair fern *Adiantum capillus-veneris* in the UK and Southern Europe using anchored microsatellites [inter-simple sequence repeat (SSR) PCR] (8) and, therefore, require a reliable method for extraction of PCR-quality DNA.

A number of rapid DNA extraction procedures for higher plants have appeared in the literature recently (1,6). These include adaptations to improve extraction from recalcitrant higher plants such as some woody species (e.g., fruit trees and conifers) (2). We have tested some of these methods on *A. capillus-veneris* but found clear evidence of PCR inhibition (E.L. Dempster, unpublished results). We have therefore devised a new protocol based around the cetyltrimethylammonium (CTAB) extraction method of Porebski et al. (5), which includes polyvinylpyrrolidone (PVP), 2-mercaptoethanol and a high-salt precipitation. Our protocol is significantly shorter, removing the need for proteinase K digestion and phenol/chloroform extraction steps, to produce a method that within 3 h results in high-quality DNA that can be used reliably in subsequent PCR amplifications. CTAB (1) and high-salt precipitation (5) are included to prevent precipitation of polysaccharides. PVP reduces the ionization of phenolic compounds, and 2-mercaptoethanol prevents their oxidation, thus retaining them in solution (3).

Frond material (0.1 g fresh, dried or frozen) is powdered under liquid nitrogen in a mortar and pestle and transferred into a tube containing 500 µL of extraction buffer [100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% (wt/vol) CTAB, 0.4% (vol/vol) 2-mercaptoethanol, 1% PVP (wt/vol) mol wt 360 000, pH 8.0] preheated to 50°C, ensuring dispersal by mixing with a spatula. Following incubation at 50°C for 15 min, 500 µL of Sevag (24:1, chloroform:isoamyl alcohol) are added, and the tube is shaken for 20 min. Following a 15-min centrifugation at 15 000× *g* in a microcentrifuge, the aqueous layer is transferred to a fresh tube, and the DNA is precipitated with an equal volume of isopropanol and 0.5 vol of 5 M NaCl at -70°C for 1 h. The DNA is recovered by centrifugation at 15 000× *g* for 10 min, washed with 100% ethanol and resuspended in 100 µL TE. One microliter of 1 mg/mL RNase is added, and following a 30-min incubation at 37°C, the DNA is re-precipitated with 2 vol of freezer-cold ethanol and 0.1 vol 3 M sodium acetate, pH 5.0. DNA is again recovered by 10-min centrifugation at 15 000× *g* at 4°C, washed with freezer-cold 70% ethanol, dried for 5 min at 60°C and finally resuspended in 20 µL TE.

We have tested the DNA extracted by this method in several applications. Conserved rRNA primers designed in our laboratory (PUV2 = TTCCATGCTAATGTATTCAGAG and PUV4 = ATGGTGGTGACGGGTGAC) have been used to amplify a region of the 18S rRNA from 9 fern species (Figure 1). PCR was performed in an OmniGene® Thermal Cycler (Hybaid, Franklin, MA, USA) essentially as described previously (6). Twenty-five-microliter reaction mixtures contained 1 µL of extracted DNA (or a 1:10 dilution of the extracted DNA), 2.0 mM $MgCl_2$, 100 ng of primers, 0.5 mM dNTPs, 1.25 U of AmpliTaq® DNA Polymerase (PE Biosystems, Foster City, CA, USA), 10 mM Tris-HCl, pH 8.3 and 50 mM KCl. PCR conditions were the following: 1 cycle of 94°C for 5 min, 50°C for 1 min and 72°C for 1 min followed by 28 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min and then 1 cycle of 94°C for 1 min, 50°C for 1 min and 72°C for 5 min. Good PCR amplification of the expected 459-bp product was obtained from all 9 species tested, demonstrating the applicability of this DNA extraction method across the Pteridophytes.

We have also used DNA extracted from populations of *A. capillus-veneris* to study variability with the anchored microsatellite primer [HVH$(GTC)_5$] between UK populations within individual sites and between UK and Euro-

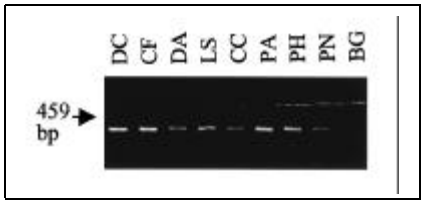

Figure 1. PCR amplification of DNA extracted from 9 fern species. *Dryopteris cycadina* (DC), *Cyrtomium fortunei* (CF), *Doodia aspera* (DA), *Lygodium scadens* (LS), *Cyathea cooperi* (CC), *Polypodium aureum* (PA), *Platycerium hillii* (PH), *Polystichum setiferum* (PS) and *Blechnum gibbum* (BG). Arrow indicates molecular weight of the amplified fragment.

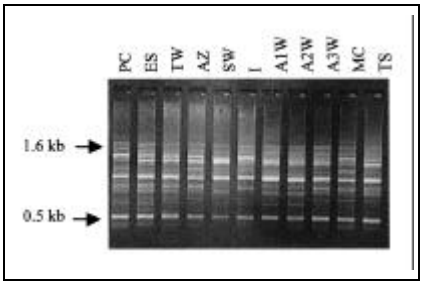

Figure 2. Anchored microsatellite (inter-SSR) amplification of DNA extracted from individuals taken from 11 populations of the fern *A. capillus-veneris*. Portland Y14, Dorset, England (PC); Mohedo del Muerto, Spain (ES); Tresilian 7G, Wales (TW); Ribeira decruz, Flores, Azores (AZ); Stout Point 9G, Wales (SW); Giardini, Naxis, Sicily (I); Aberthaw 97.25, Wales (A1W); Abethaw 97.35, Wales (A2W); Aberthaw 97.40, Wales (A3W); St. Mawes, Cornwall (MC) and Tolex, Spain (TS). Arrows indicate molecular weight markers. Six polymorphic fragments were scorable from this single primer.

pean populations. Frond samples were obtained from three sites in Glamorgan (Wales), one from Cornwall (England), one from Dorset (England) and four from Southern Europe (Spain and Italy). PCR conditions were the following: 1 cycle of 94°C for 5 min, 60°C for 1 min and 72°C for 1 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and then 1 cycle of 94°C for 1 min, 60°C for 1 min and 72°C for 5 min. Clear DNA fragment patterns were obtained (Figure 2), and differences were scorable using this PCR application, which is known to be more sensitive to DNA purity than standard PCR, confirming the high quality of our template DNA.

The extraction technique described is thus applicable to a wide range of fern species and provides sufficient quality DNA for some of the important PCR-based applications needed for population studies. Although we are not using random-amplified polymorphic DNA (RAPD) analysis in our laboratory, the DNA quality required for this procedure is comparable to that needed for inter-SSR PCR, which is a similar technique, and we anticipate that our extraction procedure could also be of use for isolating RAPD markers. The extraction method described could be of use to workers who want to analyze other fern populations, including those from threatened fern species such as *Woodsia alpina* and taxonomically complex genera such as *Dryopteris* and also very variable species such as *Cystopteris fragilis*.

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Rapid, Economical Filter Alternative to Chromosomal DNA Centrifugation in the Alkaline Lysis Plasmid Protocol

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One of the more widely used techniques for preparing plasmid DNA from bacteria is the alkaline lysis method (1,4) in which bacterial cells are treated successively with *(i)* a lysozyme solution to degrade the cell wall, *(ii)* an alkaline sodium dodecyl sulfate (SDS) solution to disrupt the cells and denature chromosomal DNA and *(iii)* a high-salt, low-pH solution to neutralize the sample and selectively precipitate the bacterial chromosomal DNA and proteins, which excludes the plasmid DNA. The chromosomal DNA is removed by two successive high-speed centrifugation steps, and the plasmid is then isolated from the supernatant by one of several techniques (4). With the introduction of DNA affinity resins in column formats for purification, the chromosomal sedimentation step became the most time-consuming (ca. 1.5 h). Further streamlining came with the replacement of the successive centrifugations with a simple filter step (QIAfilter™ Cartridges; Qiagen, Valencia, CA, USA). While convenient, the filters may represent a 15% or more increase in cost per purification, and occasionally there may be fewer filters than samples, necessitating that some samples be centrifuged.

We have found that an efficient filter can be simply and inexpensively assembled from common laboratory items: a syringe and glass wool. We assessed the quality of plasmid DNA obtained by removal of chromosomal DNA with the original centrifugation method, a commercial filter or an in-laboratory syringe filter, by the criterion of restriction enzyme digestion and transfection efficiency. Bacteria (XL1-Blue; Stratagene, La Jolla, CA, USA) harboring a eukaryotic expression plasmid for green fluorescent protein (pEGFP-N1; Invitrogen, Carlsbad, CA, USA) were grown overnight in 100 mL