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A high-throughput protocol for extracting high-purity genomic DNA from plants and animals

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Abstract

DNA extraction techniques that employ the reversible binding of DNA to silica via chaotropic salts can deliver high-quality genomic DNA from plant and animal tissues, while avoiding the use of toxic organic solvents. Existing techniques that use this method are either prohibitively expensive, or are applicable to only a restricted set of taxa. Here we describe a cost-effective DNA extraction technique suitable for a wide range of plant and animal taxa that yields microgram quantities of high-molecular-weight genomic DNA at a throughput of 192 samples per day. Our technique is particularly robust for tissue samples that are insoluble or are rapidly discoloured or oxidized in standard DNA extraction buffers. We demonstrate the quality of DNA extracted using this method by applying the amplified fragment length polymorphism technique to plant species.

Keywords: automation, chaotropic salt, DNA extraction, high-throughput, microtitre-plate, nucleic acid extraction, recalcitrant tissue samples, silica

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In molecular ecology, there is an increasing need for the rapid extraction and analysis of high-quality DNA from large numbers of individuals. This need is particularly acute for systematic surveys of genetic diversity for conservation purposes (Pearse & Crandall 2004) and for studies of adaptive diversity within natural populations (Luikart et al. 2003). Extraction methods that employ the reversible binding of DNA to silica are particularly well suited to fulfil this demand because they can yield contamination-free DNA of high molecular weight (Elphinstone et al. 2003; Ivanova et al. 2006; Hoarau et al. 2007). This advantage facilitates the rapid generation of genetic data, for example by allowing the efficient multiplexing of panels of single-locus markers, or the use of multilocus markers such as amplified fragment length polymorphisms (AFLPs). Several commercial kits and ‘home-made’ methods have already been developed to improve the throughput of this approach by enabling the simultaneous extraction of DNA from 96 individuals within silica-containing filter plates. These methods, however, are either prohibitively expensive, applicable only to restricted groups of organisms, or deliver low yields of DNA (e.g. Hoarau et al. 2007).

Following the homogenization of tissue to release DNA from cells, there are two main challenges to developing a general DNA extraction technique that is cheap, yields nucleic acids of a high quality and is amenable to automation. The first is the minimization of secondary chemical reactions (including oxidation) in the initial crude tissue extract that might otherwise lead to loss of DNA yield (see below). The second is the efficient separation of any insoluble cell fractions including plant cell walls or insect exoskeletons from samples that otherwise inhibit automation.

Defensive and antinutritive factors that protect plants from herbivores (Green & Ryan 1972; Felton et al. 1989; Constabel et al. 1995; Chen et al. 2005) provide a good example of compounds that interfere with DNA extraction and downstream polymerase chain reaction (PCR) applications when leaf tissues are homogenized. Plant phenol-oxidase enzymes oxidize phenolic compounds to form reactive quinones that can then bind covalently to proteins (Pierpoint 1969a, b; Felton et al. 1989), affecting their solubility. These

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reactions result in the browning or discolouration often observed in leaf tissue extracts (Pierpoint 1966). Polyphenol-bound proteins created by phenol-oxidase enzymes are also present in insect cuticles (Arakane et al. 2005). When copurified with DNA, polyphenolic compounds can be potent inhibitors of PCR (Singh et al. 1998, 2002; Koonjul et al. 1999). The mechanism of this inhibition is not understood, although it has been widely reported (while not proven) that polyphenols can also bind to the DNA itself and inhibit PCR directly (John 1992; Jobes et al. 1995; Koonjul et al. 1999).

We present a high-throughput method for extracting DNA from desiccated plant and animal tissue samples that is cost-effective, rapid and free from toxic organic solvents. Our method is particularly well suited to extracting nucleic acids from plants and insects because it minimizes oxidation of the extraction mixture (including that affecting phenolic compounds). In our method, insoluble cell fractions are removed via a combination of centrifugation, salt precipitation and filtration, all carried out in 96-well microtitre plates. We show that our method enables extraction of highly purified DNA from a wide range of nonmodel insects, plants and other animal taxa.

Materials and methods

Tissue samples were collected as dried vouchers, either dried in self-indicating silica gel (plants, Chase & Hills 1991) or desiccated in 70–100% ethanol (insects) and stored at room temperature. Excess ethanol was removed from insect specimens prior to DNA extraction. Each sample, of approximately 3 mg of plant tissue (dry weight) or 0.3–3.5 mg of insect tissue, was homogenized using a mixer mill fitted with a 96-well plate adaptor and racked sets of 96 microtubes (800 μL volume) with caps, each containing a tungsten carbide bead (all supplied by QIAGEN). The mixer mill was run for 5 minutes at a speed of 25 Hz, or until the tissue samples used across all trials = 2.8 mg). The full set of DNA tissue samples was homogenized completely.

The dry-homogenized tissue samples were resuspended in an extraction buffer (225 μL) containing 100 mM Tris (pH 7.4), 500 mM NaCl, 50 mM EDTA, 0.7% sodium dodecyl sulphate, 52 mM sodium sulphite, 3.6 μg RNase A (Roche Diagnostics, Germany) and 36 μg Proteinase K (Singh et al. 2002; Baranwal et al. 2003; Mogg & Bond 2003), and incubated at 55 °C for 1 h. The extraction buffer included sodium sulphite because sulphur-containing reducing agents (including sulphite salts) have been shown to be inhibitors of phenol-oxidase enzymes or reducing agents of their reactive quinone products (Pierpoint 1966; Anderson & Rowan 1967; Loomis 1974).

A precipitation buffer (225 μL; 3.6 mM potassium acetate, 2.4 mM acetic acid) was added to the samples, which were then mixed by inversion and incubated on ice for 15 min to facilitate precipitation of cellular debris and the SDS detergent. Samples were centrifuged in a bench-top plate centrifuge for 10 min at 3220 × g to pellet the precipitates.

Four hundred microlitre of supernatant was recovered by any residual ethanol.

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One and a half volumes (650 μL) of chaotropic DNA binding buffer (Elphinstone et al. 2003) containing 6 mM sodium iodide was added to each sample.

DNA was bound to and eluted from the glass-fibre-containing filters of a microtitre filter plate (1.0 μm plus 0.65 μm glass fibre/hydrophilic membrane, Millipore):

1. We used a Biomek 2000 automated workstation (Beckman Coulter) to transfer five aliquots of 200 μL of each sample to the silica-containing filter plate, which was drained simultaneously using a vacuum-manifold system (Beckman Coulter). Automation of this step was necessary to reduce pipetting error due to the limitation of sample volume in the filter plate wells.
2. DNA bound in the filter plate wells was washed twice with 200 μL of washing buffer [50% ethanol, 10 mM Tris, 0.5 mM EDTA and 50 mM NaCl (Elphinstone et al. 2003)]. During each wash, the liquid was removed by applying a vacuum to the microtitre plate.
3. The filters of the DNA-containing microtitre plate were dried to remove excess ethanol by applying a vacuum to the plate for at least 20 min. Centrifugation of the plates at 3220 × g for 5 min or drying overnight on the desktop was found to be equally effective.
4. DNA was eluted from the filter-plate by adding 40 μL preheated low TE buffer (80 °C; 10 mM tris-HCl pH 7.4, 0.1 mM EDTA) to each well and collecting the eluate in a fresh 96-well plate positioned underneath the filter plate. This elution was repeated (for a total elution volume of 80 μL) to circumvent the inhibition of elution by any residual ethanol.

Steps 1–4 described above may alternatively be carried out through the use of a plate centrifuge. Two critical factors in obtaining DNA in sufficient yields appeared to be ensuring that DNA-containing samples passed through the silica-containing microtitre filter plate at a slow speed (50 × g or ≤ 50 μL/s, to allow time for DNA to bind to the filter), and ensuring that filters were ethanol-free prior to DNA elution.

To investigate the effects of different steps of our DNA extraction protocol on the total yield of resulting DNA, we conducted trials where individual steps were modified or omitted. Each trial was conducted on four replicate leaf tissue vouchers from the plant Cirsium eriophorum (woolly thistle) of known mass (mean mass for all leaf tissue samples used across all trials = 2.8 mg). The full extraction protocol (control) was compared to reduced

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protocols that omitted: (i) the sodium sulphite from the extraction buffer, (ii) the precipitation step, and (iii) the filtering step. DNA yields for the trials were compared by analysis of covariance (ancova) using initial tissue mass as a covariate.

**AFLP analysis**

We tested the amenability of DNA extracted using our protocol to PCR analysis by applying a modified AFLP technique (Vos et al. 1995) to DNA extracted from C. eriophorum. In brief, 100 ng of DNA was digested for 3 h at 37 °C with 1 U EcoRI (Roche Diagnostics) and 1 U MseI (New England BioLabs) in 1× TA Buffer (10 mM Tris-acetate pH 7.9, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol), and 3 μg of bovine serum albumin (BSA, Sigma-Aldrich). Following digestion, 2.7 μM of each of two DNA adaptors (EcoRI: 5′-CTCGTAGACTGTCAT-3′, 5′-AATTTGAGCTGAGTCTAC-3′) and (MseI: 5′-GACGATGTA GTCTGAG-3′, 5′-TACTCAGGACTCAT-3′) were added to each sample along with 1 μL T4 DNA ligase buffer (Invitrogen) and 0.5 U of T4 DNA ligase (Invitrogen). The mixture was incubated at 16 °C overnight and then diluted by addition of four volumes of ultrapurified water.

Preselective PCR was carried out using 1 μL diluted, ligated DNA in a total volume of 5 μL containing 1× reaction buffer (75 mM Tris-HCl pH 8.8, 20 mM (NH₄)₂SO₄, 0.01% Tween-20), 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.125 U Taq polymerase (ABgene), 2% formamide, 15 μg BSA and 0.25 μM of each primer (EcoRI primer E.TC 5′-GACTGCG-TACCAATTCTC-3′ and MseI primer M.C 5′-GATGAGTCCT-GAGTAAC-3′). PCR profiles followed Hayashi et al. (2005) after Kim et al. (2004) with minor modifications. Preselective PCR consisted of 72 °C for 2 min; 20 cycles of 94 °C for 20 s, 66 °C for 30 s and 72 °C for 2 min; with a final extension step of 72 °C for 10 min. The resulting PCR products were diluted by adding nine volumes of ultrapurified water.

The template for selective amplification was 1 μL of diluted preselective PCR product. Selective amplification took place in a total volume of 5 μL containing 1× reaction buffer (75 mM Tris-HCl pH 8.8, 20 mM (NH₄)₂SO₄, 0.01% Tween-20), 2 mM MgCl₂, 0.2 mM dNTPs, 0.05 U Taq polymerase (ABgene), 2% formamide, and 0.5 μM of each primer (E.TC (fluorescently labelled) and M.CTA). The selective PCR profile was 94 °C for 2 min; six cycles of 94 °C for 20 s, 66 °C for 30 s and 72 °C for 2 min, with a decrease in annealing temperature of 1 °C per cycle; 19 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 3 min; then 72 °C for 10 min. Selective amplification products were diluted 10 times using purified water. PCR products were separated and detected using an ABI 3730 Genetic Analyser and sized using an internal ROX-labelled size standard (Applied Biosystems) according to the manufacturer’s instructions. AFLP fingerprints were visualized using the software GENEMAPPER version 3.7 (Applied Biosystems).

**Results and discussion**

Inclusion of sodium sulphite in the extraction buffer prevented samples from discolouring (Fig. 1). This discolouration was not removed by the precipitation step, and in larger-scale trials (e.g. Figure 2), we have observed staining of silica filters which can potentially be carried over into the eluted DNA solution. Such discolouration is best attributed to polyphenol contamination (Pierpoint et al. 1995). We have found that, for some plant species, the presence of sodium sulphite also affects the efficiency of the precipitation step, influencing the reliability with which multiple samples may be filtered evenly (results not shown).

There was a significant difference in yield among the four DNA extraction trials (ancova, P < 0.001). This difference was attributable to a severe reduction in DNA yield.
plate. Total DNA yield varied linearly with tissue mass on to the membrane of the silica-containing microtitre (including polysaccharides and proteins, e.g. DNases) step was caused by contaminants being carried over that loss of yield in the trial that omitted the precipitation step. We suggest for the trial that omitted the precipitation step. We suggest that loss of yield in the trial that omitted the precipitation step was caused by contaminants being carried over (including polysaccharides and proteins, e.g. DNases) on to the membrane of the silica-containing microtitre plate. Total DNA yield varied linearly with tissue mass ($P < 0.001$) after statistical removal of the effects of the extraction modifications, indicating that, over the range of tissue mass investigated here (1.6–4.7 mg), the wells of the silica filter plate had not been saturated with DNA. The mean total yield for the full extraction protocol was $1.97 \mu g$ DNA, corresponding to 0.63 $\mu g$ DNA per milligram of dried tissue. Agarose gels indicated that, for the trials resulting in successful nucleic acid extraction, the purified DNA was of high molecular weight, nondegraded and free from RNA contamination (Figs 2 and 3).

Our extraction protocol has so far proved useful in many plant species, including Arabis glabra, Arabis petraea and Iberis amara (Brassicaceae), Anthoxanthum odoratum (Poaceae), Carex cryophylla (Cyperaceae), Cirsium heterophyllum and Cirsium eriophorum (Asteraceae), Gentianella campestris (Gentianaceae), Pinguicula vulgaris (Lentibulariaceae), Trollius europaeus (Ranunculaceae), and Dianthus deltoides (Caryophyllaceae) (results not shown); nomenclature follows Stace (2001). Our protocol also works well for animal taxa (Fig. 3) including insects Psyllodes marcidus, Coelorhynchos minutus (Coleoptera, whole insects, 0.3–3.5 mg), Polyommatus icarus, Coenonympha pamphilus, Aricia artaxerxes (Lepidoptera, head tissue, 1.2–1.7 mg), amphibians (Triturus cristatus, single toe-clips), birds (Passer domesticus, blood, 10 $\mu L$), and mammals (Myotis nattereri, 3-mm diameter wing punch biopsies). Agarose gel analysis of the DNA resulting from these extractions (Fig. 3) showed that it was not degraded and was of high molecular weight. DNA yield varied with the size of the tissue samples extracted, with the lowest yields occurring for Coelorhynchos minutus (0.3-mg tissue sample) and Myotis nattereri (3-mm wing punch biopsy). The yields observed for these species using our DNA extraction method approximately matched yields observed in phenol–chloroform DNA extractions (results not shown). Despite the low yields for these species, the extracted DNA was amenable to PCR analysis (results not shown).

To test the suitability of DNA extracted using our method for PCR analysis, we carried out the AFLP procedure on DNA extracted from C. eriophorum. AFLP demands DNA of high molecular weight that is free from contamination. Our C. eriophorum DNA samples were digested completely by the restriction endonucleases used for AFLP (results not shown). AFLP fingerprints resulting from selective amplification are shown (Fig. 4). AFLP fingerprints derived from DNA purified using our extraction protocol were of sufficient quality to allow semi-automated collection of genotypes. These genotypes have also been shown to be repeatable across independent genotyping trials (Whitlock et al. 2007).

Since we developed our DNA extraction technique, it has been used successfully in studies of mitochondrial DNA sequence variation (J. Craven, personal communication), genetic variability within multiple genes assayed by single nucleotide polymorphism multiplexes (K. Tanaka, personal communication), microsatellite marker-based wildlife forensics applications (A. C. Frantz, personal communication), as well as in our own studies on the conservation genetics of British plant species. In general, our technique will be particularly useful in situations where homogenized tissue does not digest or dissolve completely in DNA extraction buffer, or carries a significant load of potential contaminants. The cost of employing this protocol is approximately a quarter of the cost per sample of commercially available kits, although an initial investment is required to obtain the necessary laboratory hardware. We note that related DNA extraction techniques (e.g. Elphinstone et al. 2003) may prove more economical (in time and cost) where tissue samples are easily broken down in extraction buffers and contain few potential contaminant compounds. This choice of complementary methodological approaches gives flexibility to silica-based DNA extraction systems and should allow laboratories to tackle nucleic acid extraction from a wide range of taxa.
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