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High-throughput DNA extraction from forest trees

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Title: HIGH THROUGHPUT DNA EXTRACTION FOR FOREST TREES

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ABSTRACT

Trees are typically more demanding than other plants to extract pure, high quality DNA for molecular genetics and may not necessarily be amenable to advances in extraction methodology suitable for other plants. A recently available, commercial, high throughput DNA extraction system utilising a silica binding matrix for purification and a multi-sample mixer mill for tissue disruption was evaluated for its suitability with *Eucalyptus* spp., *Pinus* spp. and *Araucaria cunninghamii* (hoop pine). DNA suitable for a range of molecular biology applications was successfully extracted from all genera. The method was highly reliable when tested in over 500 preparations and could be adapted to different tree species with relatively minor modifications to the standard protocols.

INTRODUCTION

Purified genomic DNA, required for many applications in molecular genetics is frequently more difficult to obtain from trees than most other plants. DNA yield and quality often varies amongst species of the same genera and amongst different sources of tissues from the same tree (Henry 2001). The problems with extraction of DNA from trees are generally attributed to impurities, such as terpenes, polyphenolics and polysaccharides that are often abundant in the foliage of perennials and co-extract with DNA. As a consequence, many tree species require more complex extraction methods than annual plants, utilising an initial organelle isolation step under acidic conditions (Graham et al. 1994; Guillemaut and Marechal-Drouard 1992) or special grinding procedures (Scott and Playford 1996).

Application of DNA markers in plant breeding and population genetics is restricted by the cost and time of reliably extracting DNA from large numbers of plant samples. High throughput (HT) DNA extraction methods (>200 samples/day/person) have been developed for marker assays using NaOH digest (Gu et al. 1995) and coupled with a mixer mill (Harvester Tech. Inc. NY) for tissue disruption in 96 well multi-plate formats (McCandless 1998; Zakour 1998). This methodology has been used to successfully extract DNA from large sample numbers in some agricultural and horticultural crops. Another recent advance in high throughput DNA extraction methodology has been the provision of a silica matrix to bind DNA during purification in a multi-sample format in a commercial product (Qiagen P/L). In this study we evaluated the Qiagen DNeasy^{TM} 96 Plant Kit and a multi-sample mixer mill (Retsch MM300) for high throughput DNA extractions from *Pinus* spp., *Eucalyptus* spp. and *A. cunninghamii*.

METHODS

Plant material

Foliage from a set of 29 *Pinus elliottii* var *elliottii* (PEE), *Pinus caribaea* var. *hondurensis* (PCH) and an interspecific F_1 hybrid (PEH) individual were supplied by the Queensland Forest Research Institute (QFRI) and stored frozen at –20°C for this study. Leaf tissue from 180 *A. cunninghamii* individuals from an interprovenance controlled cross (H15 x Gil 24) was collected from a QFRI experiment and stored at –20°C. Foliage from 312 individuals of several *Eucalyptus* and *Corymbia* spp. (*E. cloeziana, E. acmenoides, E. reducta, E. pilularis, E. grandis, C. variegata, C. henryi, C. intermedia, and C. watsonia*) were collected from natural stands or plantations in Queensland and stored frozen at –20°C.

DNA extraction

Genomic DNA was extracted using the DNeasy™ 96 Plant DNA isolation kit (Qiagen P/L, Vic, Aust) with mechanical tissue disruption by a multi-sample mixer mill (MM300 F. Kurt Retsch GmbH & Co. KG, Hann, Germany). The kit uses silica-gel-column matrices for purification in a convenient 96-well plate format. The MM300 mixer mill enables efficient tissue disruption for up to 192 samples in several minutes by oscillating a metal bead in each microtube. In addition to the mixer mill, this procedure requires an eight-channel pipette for volumes of at least one millilitre and a centrifuge capable of attaining 5600 g and a rotor which accommodates DNeasy™ 96 plates.

Basic Extraction Protocol

Tissue was disrupted according to either the fresh tissue or frozen tissue disruption protocol using the MM300 Mixer mill (Qiagen DNeasy™ 96 Plant Kit Handbook) with minor modifications detailed below. The amount of tissue and disruption parameters are given below in the sections with each species. In the fresh tissue protocol, the tissue, a bead, 400 µl of a 400:1:1 mix of lysis Buffer AP1, RNase A and Reagent DX (anti-foaming agent) was added to the each well of the multi-sample disruption plate, the plate was subjected to several disruption sessions at room temperature. In the frozen tissue protocol, tissue was maintained frozen throughout the disruption process by immersion in liquid N_2. Immediately after disruption, 400 µl of a 400:1:1 mix of Buffer AP1 (preheated to 80°C), RNase A and Reagent DX were added, samples were then vigorously mixed for 15 s.

Fresh and frozen protocols were identical from this stage. Sample racks were centrifuged for 10 s at 1500 g. Buffer AP2 (130 µl) was added to each tube, tubes were recapped with fresh caps and racks manually shaken for 15 s. Samples were then centrifuged at 1500 g for 10 s to collect droplets from caps before incubation at –20°C for 10 min. This was immediately followed by centrifugation for five minutes at 5600 g to form a pellet of cell debris. Up to 400 µl of the supernatant for each sample was transferred to a new set of racked collection microtubes and 1.5 x vol of Buffer AP3/E was added to each microtube then racks were manually shaken. Racks were centrifuged for 10 s at 1500 g to collect drops from the caps. One millilitre of each sample was then applied to a well on a DNeasy™ 96 plate (silica columns) mounted on a collection rack. DNeasy™ 96 plates were then sealed with tape and centrifuged for four minutes at 5600 g. After removing the tape, 800 µl of Buffer AW was added to each sample and plates centrifuged for 15 min at 5600 g. DNA was eluted in two stages by adding 50 – 100 µl Buffer AE to each well, sealing the plate with tape, incubating

for one minute at room temperature then centrifuging for two minutes at 5600 g then repeating elution to give total elution volumes between 150 and 200 μl.

Pinus spp.

Optimisation of amount of tissue and disruption protocol

A factorial experiment on four amounts of tissue by three disruption times was carried out in triplicate using the fresh tissue protocol. Starting amounts of foliage were 25, 50, 75 or 100 mg of pine needles (cut into <10 mm lengths). Each starting amount was disrupted for 2 x 1min, 3 x 1 min or 4 x 1 min sessions at full speed (30 1/s). Two 100 μl elutions of purified DNA were collected. Before each disruption, the samples were repeatedly immersed in liquid N_2 for at least one minute to refreeze all samples.

A second factorial experiment was carried out using two amounts of tissue (50 or 100 mg) by three disruption times (2 x 1 min, 4 x 1 min and 6 x 1 min) in triplicate using the frozen tissue protocol as described above. Two-way analysis of variance was carried out using a fixed effects model in ANOVA module of Statistica v4 (Statsoft Tulsa, OK).

A. cunninghamii

A modified version of the frozen tissue protocol was used to extract from *A. cunninghamii*. Duplicate samples of tissue, each of approximately five mg were disrupted in parallel. After disruption, the samples were combined for purification on a single silica matrix column. Two elutions of 100 μl were taken.

Eucalyptus spp.

Approximately 15 to 20 mg tissue was sampled from leaves using a hole punch. Samples were processed according to the fresh or frozen tissue protocol and subject to 2 x 1 min oscillation sessions at 25 l/s for tissue disruption. A further one minute oscillation was used if samples were not fully disrupted and DNA was eluted in 150-200 μl total volume

DNA Quantification

Pinus spp.

Five microlitres of genomic DNA was electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualised under UV fluorescence. DNA quantity was estimated from a regression of band intensity upon standard amounts of DNA (Marker II Roche Diagnostics Aust P/L Vic, Aust)

A. cunninghamii and *Eucalyptus* spp.

Genomic DNA was quantified by spectrophotometry measurement of sample absorbency at 260nm.

DNA Quality

The DNA from the PEH individual was subjected to restriction digestion with *Eco*RI (Pharmacia) and *Mse*I (NEB). In each case, one unit of enzyme was combined with 500 ng of genomic DNA and incubated at 37^{o}C for an hour in the supplied buffer. Both the digested DNA and 500 ng of undigested DNA were electrophoresed on a 1% agarose gel. In addition, *Pinus* spp. samples were assessed for successful amplification at a microsatellite locus, PtTX 3034 (Elsik et al. 2000). PCR products were electrophoresed on a 3% agarose gel. Gels were stained with ethidium bromide and visualised by UV fluorescence.

Araucaria cunninghamii DNA quality was assessed by their suitability for use in AFLP marker development which is dependent on complete digestion with *Eco*R1 and *Mse*I restriction enzymes. AFLP markers were generated according to COSTA *et al*. (2000) using the pre-selective and selective primers specified in REMINGTON *et al*. (1999).

The genomic DNA quality of *Eucalyptus* spp. was assessed for successful amplification of six eucalypt microsatellite loci (BRONDANI *et al*. 1998) according to STOKOE *et al*. (submitted).

RESULTS

Pinus spp.

Analysis of variance on the amount of starting tissue and disruption time on DNA yield within each protocol showed that a starting tissue amount of 50 mg gave maximal yields in both protocols but this amount was not significantly better than other amounts tested in the fresh tissue protocol (frozen protocol, tissue amount treatment F = 19.0 p-value = 0.001) (Tables 1 & 2). A disruption treatment of 4 x 1min gave maximal yields in both extraction protocols but was not significantly better than other times in the frozen protocol (fresh protocol, tissue disruption treatment F = 3.42, p-value = 0.049).

Comparison of the highest yielding treatments from both protocols indicated there was a highly significant difference in the DNA yield. The average yield from the optimal treatment (50 mg tissue and 4 x 1 min disruption sessions) using the frozen tissue protocol was 8.92 ± 2.56 μg (mean ± SD) of DNA which was significantly higher than 2.99 ± 1.32 μg for the fresh protocol (one-tailed t-test assuming equal variances; t-value = -3.56; p-value = 0.01). It was also found that DNA yields from the maximal treatments from the frozen protocol could be increased by a further ~40% (12.4 ± 2.98 μg/column per 100 mg of foliage n = 9) by homogenising 50 mg samples of foliage in duplicate and combining the two homogenates for purification on a single column (see methods for *A. cunninghamii*).

DNA Quality

DNA of the PEH individual was successfully digested with *Eco*RI and *Mse*I (Figure 1). The microsatellite locus PtTX 3034, was successfully amplified from DNA template from each of the 29 *Pinus* spp. individuals extracted using the optimised frozen tissue protocol (data not shown).

Eucalyptus spp.

DNA was prepared from 312 *Eucalyptus* individuals using the fresh tissue protocol. The average yield for a subset of 18 *E. cloeziana* samples was 1.87 ± 0.51 μg in a total elution volume of 200 μl (Table 3). DNA was also quantified from 10 *E. cloeziana* which was extracted using the frozen tissue protocol. This method yielded an average of 1.5 ± 0.2 μg when eluted in a total volume of 150 μl. DNA quality was evaluated by amplification of six single-locus microsatellite loci over the 312 samples prepared using the fresh tissue protocol. One sample amplified inconsistently. This individual had previously given unreliable amplification from DNA prepared by other extraction methods (data not shown) and was most likely the result of tissue quality rather than the extraction protocol.

A. cunninghamii

It was necessary to reduce the amount of tissue disrupted to 5 mg as lysis was ineffective and yield and quality of DNA compromised if more tissue was used. To overcome this limitation, samples were ground and lysed in parallel (2 x 5 mg) and passed through the silica matrix in series. The average yield for 180 samples of *A. cunninghamii* was 2.2 ± 0.66 μg per 10 mg of starting tissue. AFLP markers were successfully generated from every sample on the first attempt (Figure 2).

DISCUSSION

DNA suitable for a range of molecular biology applications was successfully extracted from three major genera of forest trees, *Pinus* spp., *Eucalyptus* spp. and *A. cunninghamii* using a high throughput procedure. The methodology could be adapted to different tree species with relatively minor modifications to the standard protocols. The method produced highly reliable DNA preparations suitable for applications where restriction enzyme digestion or PCR amplification is required.

Maximal yields were achieved by disruption of 50 mg of tissue frozen in liquid N_2 for four, one minute oscillation sessions. Yields of 8.9 μg for 50 mg of starting tissue or 12.4 μg for 100mg are ample for most population genetics and genetic mapping studies based on PCR marker systems. Higher yields with the frozen tissue protocol appeared to be the result of more efficient tissue disruption as tissue was visibly ground to a finer grade with the frozen protocol. Tissue disruption in the fresh tissue protocol was relatively ineffective and no consistent trend was evident. The lack of a significant difference in yields with different starting amounts in the fresh tissue protocol most likely results from the poor performance of this method with Pine foliage that was stored frozen. As disruption treatments used with the frozen tissue protocol were not significantly different, extended disruption was not necessary although it did not seem to significantly effect the average molecular weight size of extracted DNA (data not shown).

The DNA from pines passed critical tests for purity as it was competent for PCR analysis of a single-locus microsatellite and restriction digestion and was of a high molecular weight showing little sign of degradation. Subsequent to these experiments, DNA from *Pinus* spp. prepared by this method has been used successfully for AFLP, a method sensitive to problems with restriction digestion (VOS *et a*l. 1995) (data not shown).

Although largely automated, the extraction methodology was still inherently variable, as DNA yields from replicates of a single tissue sample had a high variation (ie 8.92 ± 2.56 μg mean ± SD). The exact amount of tissue homogenised did not appear critical as small fluctuations in starting amount (ie ± 3 mg) did not significantly affect yield (data not shown). We have not identified any systematic causes of between sample variation but one possible source of variation is differential homogenisation of samples due to their position in the plate. Nonetheless, despite the variability in yields, the current protocol for 50 mg of tissue would only require repeats at the rate of three in 1000 samples (applying a cutoff of two micrograms as a minimum yield) which is an approximately 33 fold improvement to the medium throughput method routinely utilised in the past.

The reliability of the method was tested on *Eucalyptus* spp. Standard protocols were suitable for eucalypts following optimisation of the amount of starting tissue (data not shown). Smaller amounts ie 15 - 20 mg of tissue in comparison to *Pinus* spp. were required to allow adequate tissue disruption. Hence, eucalypt foliage may also benefit from the parallel grinding approach described for *A. cunninghamii* to increase yields if required. The inconsistent amplification of one DNA preparation from 312 extracted indicated the method was highly reliable compared to methodology previously used for DNA extraction of eucalypts in our laboratory. Both the fresh and frozen tissue protocol were trialed on eucalypts. The two protocols produced similar yields and provided DNA of high molecular weight and quality, however, the frozen tissue protocol is now used routinely.

The silica column purification step used in the Dneasy™ method provided DNA of sufficient purity for a range of marker assays from tree species which had previously proven recalcitrant when DNA was prepared by other methods (eg. BOUSQUET *et al*. 1990; GRAHAM *et al*. 1994). In our experience *E. pilularis*, *E. cloeziana* and *E. acmenoides* have been amongst the more difficult species to extract DNA suitable for PCR and restriction digests. Previously, attempts to generate AFLP and amplify microsatellite loci from *A. cunninghamii*, an indigenous conifer had given variable results. Rainforest species are notoriously difficult to extract pure DNA from because of the co-extraction of polysaccharides (Scott and Playford 1996) and have typically required more complex extraction methods than most other tree species (Graham et al. 1996; Graham et al. 1994). However, with relatively minor modifications to the standard Dneasy™ method, DNA of a consistent purity for a range of marker applications could be reliably extracted from a large number of samples.

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Table 1. Differences amongst DNA yields from pine needles due to tissue disruption treatment[1].

Disruption treatment[2]	Mean (μg)	SD	LSD[3]
3 x 1 min	0.81	0.56	a
2 x 1 min	1.18	1.41	a
4 x 1 min	1.89	0.97	b

[1] DNA extracted using the fresh tissue protocol on tissue from a single individual.
[2] See methods for details of disruption treatments.
[3] Least significance difference test (LSD) was applied following a two way ANOVA for the main effects of tissue disruption and amount of tissue (25, 50, 75 or 100mg) using three replicates per factorial combination. Treatments with a different letter were significantly different at $\alpha < 0.05$.

Table 2. Difference in DNA yield from pine needles between two amounts of tissue[1].

Amount of starting tissue (mg)	Mean (μg)	SD	LSD[2]
50	5.35	4.05	a
100	0.44	0.62	b

[1] DNA extracted using the frozen tissue protocol on tissue from a single individual.
[2] Least significance difference test (LSD) was applied following a two way ANOVA for the main effect of tissue disruption (2 x 1 min; 4 x 1 min; and 6 x 1 min) and amount of tissue using three replicates per factorial combination. Treatments with a different letter were significantly different at $\alpha < 0.05$.

Table 3. Comparison of DNA yields between the fresh and frozen protocols for *E. cloeziana*.

Method	n	Average DNA Yield (ug)	SD	Total Elution Volume (µl)
Frozen	18	1.87	0.51	200
Fresh	10	1.47	0.21	150

Figure 1. Restriction digests of genomic DNA from a PEH individual prepared using the frozen tissue protocol. Lane 1 undigested genomic DNA; Lane 2 genomic DNA digested with *Eco*RI; Lane 3 is 100bp molecular weight standard (Roche). The band with the slowest visible mobility is 2642bp. Lane 4 uncut genomic DNA; Lane 5 genomic DNA digested with *Mse*I.

Figure 2 Section of a 5% denaturing PAGE gel of AFLP on 18 Hoop pine individuals with DNA prepared using the HT methodology. Lane 1 is a molecular weight standard GS500 (PE). The highest molecular weight standard band is 300bp and the lowest is 35bp in size. Lanes 2-19 AFLP profiles of 18 full-sib hoop pine individuals amplified using selective primer combination, *Mse*1-CCTG and *Eco*R1-ACG.


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