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High yield and high quality DNA from vegetative and sexual tissues of Mexican white pine (*Pinus ayacahuite*)

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Pines are considered to be difficult for DNA extraction. However, from one species to the other there is variation in phenolic profiles and seed size that might affect final DNA yields and quality. Two DNA extraction protocols (CTAB and SDS based) were compared for their ability to produce DNA on leaves, gametophyte and embryo from *Pinus ayacahuite*, a pine species with a large seed (8 - 18 mm). The DNA obtained from both procedures was quantified and tested by PCR. The CTAB protocol provided higher DNA yields from vegetative tissue and embryo than the SDS method. Embryos (2n) and gametophytes (n) proved to be very good sources of DNA and the DNA isolated was suitable for PCR-RAPD and SSR markers. This paper reports the results and describes the modified CTAB protocol.

Key words: CTAB, DNA isolation, *Pinus ayacahuite*, SDS, seed tissue, vegetative tissue.

INTRODUCTION

The Mexican White Pine (*Pinus ayacahuite* Ehrenb. ex Schlttdl) is an important source of timber, pulp and nuts. It is also the fastest growing soft pine in Mexico (Farjon and Styles, 1997). Mexican forests are threatened by an alarming rate of deforestation which leads to a decline of genetic diversity within species. Although Mexican pines have been planted outside of México (CAMCORE, 2000), very little is known about the genetics of Mexican pine species in their native habitats (Sáenz-Romero et al., 2003). Experimental plantations with *P. ayacahuite* and *P. chiapensis* in South-Africa have yielded good results although nowadays these species are not widely planted (Din, 1958; Vásquez and Dvorak, 1996). The Central America and Mexico Coniferous Resources Cooperative (CAMCORE, North Caroline State University) is attempting to reintroduce *P. chiapensis* into Southern Mexico using seeds from field conservation banks in South-Africa. Plantation establishment and gene conservation

of pines would benefit if species were genetically characterized and genetic diversity values were well known.

Genetic analysis of plants relies on high yield of high quality DNA samples. Pines are considered to be recalcitrant plants for DNA extraction since leaves and seeds contain high levels of polysaccharides which co-precipitate with the DNA and thus interfere with DNA purification, quantification, amplification by PCR and restriction enzyme digestion (Demerek and Adams, 1992; Guillemaut and Maréchal-Drouard, 1992; Crowley et al., 2003). Leaves are also rich in phenolic compounds, which bind to DNA after cell lysis, interfering with the isolation of high quality DNA (Kim et al., 1997). However, DNA yields and quality from pines often differ depending on the extraction methods and tissues used (Bousquet et al., 1990).

Although several protocols of DNA extraction have been successfully employed in pines (Bousquet et al., 1990; Sperisen et al., 2000; Azevedo et al., 2003; Crowley et al., 2003), species show variations in phenolic profiles and gametophyte size, which may affect DNA extraction results (Price et al., 1998; Padmalatha and Prasad, 2006). The embryo might be a valuable source of DNA amongst species like *P. ayacahuite* which produces a large size seed (8-18 mm). Due to the small seed size of many pine species, the option of using embryo as DNA source has been scarcely explored (Farjon and Styles,

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Abbreviations: CTAB, hexadecyltrimethylammonium bromide; SDS, sodium dodecyl sulfate; RAPD, random amplified polymorphic DNA; SSR, simple sequence repeats; RFLP, restriction fragment length polymorphism; PVP, polyvinylpyrrolidone.

1997; Bousquet, et al., 1990).

We tested and compared modified CTAB and SDS based DNA extraction protocols (Doyle and Doyle, 1987; Dellaporta et al., 1983) on leaves, gametophytes and embryos of *P. ayacahuite*. The CTAB protocol provided higher yields of high quality DNA from vegetative tissue and embryos than the SDS method. The DNA isolated was suitable for RAPD and SSR markers. We present here our results and describe the optimized CTAB based DNA extraction protocol on vegetative tissue and seeds from this economically important pine species.

MATERIALS AND METHODS

Plant material

Forty leaves samples and twenty seeds from different individuals of *P. ayacahuite* were used for DNA extraction. Leaf tissue was collected from natural populations in Mexico. After collection, leaves were kept on ice until their arrival to the laboratory and then maintained at -20°C . Seeds were rehydrated with distilled water two days at room temperature and later germinated in 1% hydrogen peroxide during 30 min. Germinated seeds were dissected with a scalpel and the outer brown scale covering the megagametophyte was removed. Gametophytes and embryos were then isolated and maintained at -80°C in microtubes until DNA extraction.

Chemicals and solutions

Extraction buffer consisted of 2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% PVP (MW 10,000, Sigma). In addition, chloroform/isoamyl alcohol (24:1), phenol/chloroform/isoamyl alcohol (24:24:1), washing buffer (70% ethanol, 10 mM ammonium acetate), TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), 70% ethanol, and 7.5 M ammonium acetate, were prepared. Absolute ethanol and isopropanol were also needed.

Protocols

DNA extraction from leaves

One gram of leaf tissue was pulverized in liquid nitrogen using a mortar and pestle pre-chilled to -20°C . Ground tissue was immediately transferred to 50 ml Nalgene tube containing 8 ml of pre-heated (65°C) CTAB buffer. The tube was shaken and incubated at 65°C for 60 min while gentle shaking from time to time. Furthermore 7.5 ml of chloroform-isoamyl were added; the tube was gently but thoroughly mixed and centrifuged at 10,000 g for 20 min to room temperature. The upper aqueous phase was pulled out with a wide-bore pipette and transferred to a clean 50 ml Nalgene tube. The supernatant was precipitated using 2/3 volume of -20°C isopropanol. The tube was gently mixed by inversion and centrifuged at low speed for 5 min (or the fibrous DNA was spooled out with a pipette). Then, the DNA was transferred to a 50 ml tube and after 20 min washed out with 10 ml of the washing buffer, the tube was centrifuged at low speed (or DNA was spool out). The pellet was air-dried and re-suspended in 1 ml of TE buffer, 2 ml of distilled water and 1.5 ml of ammonium acetate (7.5 M) and mixed by inversion. Next, 10 ml of cold absolute ethanol were added. The tube was gently mixed to precipitate DNA and centrifuged at low speed (or DNA was spool out). The pellet was washed with 5 ml of 70% ethanol and centrifuged at low speed. Finally, the pellet was air-dried and re-suspended in 500 - 750 μl of TE buffer.

DNA extraction from gametophyte and embryo

The gametophyte or embryo was weighed and transferred to a 2 ml eppendorf tube containing 200 μl of CTAB extraction buffer. The tissue was ground with a pestle. Next, 800 μl of pre-heated (65°C) extraction buffer was added and the tube was incubated at 65°C for 60 min while gently shaking from time to time. Then, 1 ml of phenol-chloroform-isoamyl alcohol was added to the gametophyte extract or 1 ml of chloroform-isoamyl alcohol was added to the embryo preparation. The tubes were gently but thoroughly mixed and centrifuged at 10,000 g for 20 min at room temperature. The subsequent steps followed the same protocol as with leaf tissue but reducing the volumes to a 1/3.

DNA quantification

DNA yields were estimated by using a UV Spectrophotometer (JENWAY 6405 UV/VIS) at 260 nm. The nucleic acid concentration was calculated following Sambrook et al. (1989).

The purity of DNA was determined according to the ratio of absorbance at 260 nm to that of 280 nm. DNA purity was also determined by running samples on a 0.8% agarose gel stained with ethidium bromide based on the intensity of the high molecular weight band.

RAPD and SSR amplifications

RAPDs reactions followed the protocols described by Williams et al. (1990) with minor modifications and SSR reactions were performed according to Rajora et al. (2000).

RESULTS AND DISCUSSION

Isolation of large amounts of high quality genomic DNA from pines is frequently a difficult issue (Kim et al., 1997). Therefore, existing extraction procedures have been modified or new methods have been developed to overcome isolation problems (Bousquet et al., 1990; Guillemaut and Maréchal-Drouard, 1992; Sperisen et al., 2000). Both the CTAB and the SDS based methods tested in this work led to the isolation of high molecular weight DNA from *P. ayacahuite* (Figure 1). Nevertheless, the CTAB protocol showed higher DNA yields on leaves and embryos than the SDS protocol (Table 1). The average yields with the CTAB procedure was 340 ± 28 ng/mg, 960 ± 100 ng/mg, 4950 ± 450 ng/mg with regard to leaves, gametophytes and the embryos respectively. The purity (A_{260} nm/ A_{280} nm ratio) was within the range of 1.6 to 1.7, indicating minimal levels of contaminating metabolites.

Most of the reported DNA extraction procedures from conifers use vegetative tissue and gametophytes as DNA source but alternative sources have been seldom explored. Bousquet et al. (1990), using a CTAB method without RNase treatment, report DNA yields between 100 and 500 ng/mg from vegetative and sexual tissues of several tree species, amongst which *P. banksiana* and *P. strobus* were included. DNA yields between 95 and 929 ng/mg from leaves and between 330 and 10,000 ng/mg

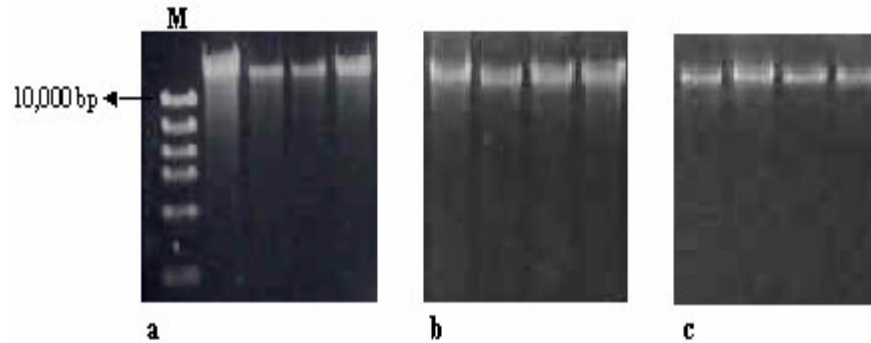


Figure 1. DNA samples extracted from (a) leaves, (b) gametophytes, and (c) embryos of *Pinus ayacahuite* with the modified CTAB protocol. M, DNA size marker (High DNA mass ladder, Invitrogen-Life Technologies).

Table 1. Average yields and quality of DNA extracted from *Pinus ayacahuite* tissues using the CTAB or SDS protocol.

Protocol	Plant material	n	DNA yield (mean \pm SE)	A ₂₆₀ /A ₂₈₀
CTAB	Leaves	40	340 \pm 28 ng/mg	1.70 \pm 0.02
	Gametophyte	20	960 \pm 100 ng/mg	1.72 \pm 0.02
	Embryo	20	4950 \pm 450 ng/mg	1.61 \pm 0.03
SDS	Leaves	20	169 \pm 20 ng/mg	1.57 \pm 0.04
	Gametophyte	20	1430 \pm 150 ng/mg	1.65 \pm 0.05
	Embryo	20	2800 \pm 300 ng/mg	1.68 \pm 0.04

from sexual tissue of *P. ayacahuite*, were obtained. Sperisen et al. (2000) describe a rapid SDS method for DNA isolation from fresh leaves of gymnosperms and the average DNA yield from *P. sylvestris* (366 ng/mg) is similar to our results.

Due to the small seed size of many conifers, the total amount of DNA isolated from embryo is generally too low for RFLP analysis (Newton et al., 2002). Interestingly, large embryos of *P. ayacahuite* proved to be valuable source of DNA; one embryo yielded about 100 μ g of DNA, which is enough for several RFLP's reactions (Dowling et al., 1996). Thus embryos might be excellent source of DNA for conifers with large seed.

Differences in DNA quality and yields may correlate with the relative levels of the secondary products which might interfere with the extraction. It has been reported that the inclusion of PVP to the DNA extraction buffer helps eliminating polyphenol compounds (Kim et al., 1997). The addition of PVP to the extraction buffer used in our modified CTAB protocol was crucial to reduce DNA degradation. Although we did not observe any marked variation concerning DNA yields between leaves samples treated with PVP and samples without PVP, the effect on DNA quality was noticeable. It is also worth mentioning that the inclusion of phenol to the washing solution used on the gametophyte was helpful to improve both DNA quality and yields. Frequently, DNA extraction protocols

do not consider some side effects of the chemical compounds such as PVP and phenol. It is important to observe that DNA extraction from the embryo using the CTAB protocol does not require either PVP or phenol.

In almost all DNA samples, RNA smears were visualized on agarose gels, however the DNAs were suitable for PCR-RAPD and SSR reactions (Figure 2). No differences were observed in banding profiles between leaves, the gametophyte and the embryo. Even though RNase treatment was not necessary for the molecular analysis, it is important to keep in mind that the addition of 5 μ l of RNase A (30 mg/ml) to the DNA solution obtained from leaves led to about half of the initial DNA concentrations. The yield was similar to the one obtained from *P. radiata* using a modified CTAB method that included RNase treatment (Stange et al., 1998).

In conclusion, the CTAB based DNA extraction method produced high yields of high quality DNA from tissues of *P. ayacahuite*. In addition, seeds provided higher DNA yields than leaves and the embryo proved to be the best source of DNA.

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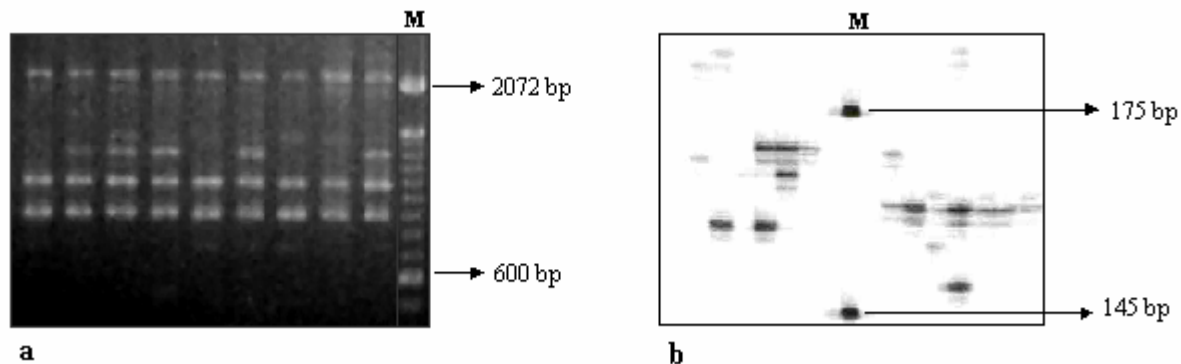


Figure 2. Amplification of DNA from leaves, gametophytes and embryos of *Pinus ayacahuite*, (a) RAPDs (OPA-06), (b) SSR (RPS 50). M, DNA size marker.

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