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Emanuel Araya / Olman Murillo / Gabriel Aguilar / Oscar Rocha
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A DNA EXTRACTION PROTOCOL AND INITIAL PRIMERS SCREENING IN *Hyeronima alchorneoides* Fr. All. FOR AFLP APPLICATIONS

Emanuel Araya[1*], Olman Murillo[1], Gabriel Aguilar[2] y Oscar Rocha[2]

Resumen

Un método de extracción de ADN simple, rápido y de alto rendimiento fue desarrollado para *Hyeronima alchorneoides*. Este método está basado en el uso de CTAB y es una modificación de un protocolo reportado previamente. Para determinar la calidad del ADN extraído se utilizaron tres métodos: electroforesis en un gel de agarosa al 0.8%, digestión del ADN con las enzimas de restricción EcoR I y Hind III y cuantificación del ADN en un fluorómetro. Con el procedimiento desarrollado se obtuvo ADN de alta calidad, con valores entre los 30 y 40 ng/µL. Además, con el método descrito en este estudio, el ADN extraído fue completamente digerido con EcoR I y Hind III. Se realizaron reacciones de AFLP para evaluar la idoneidad del ADN extraído. Cincuenta y seis combinaciones de imprimadores selectivos de AFLP fueron evaluados para la amplificación del ADN, pero solamente nueve combinaciones de imprimadores selectivos amplificaron para el ADN extraído de *Hyeronima alchorneoides*. Veinticinco combinaciones de imprimadores mostraron una un alto número de bandas y una alta resolución de las mismas.

Abstract

A simple, fast and high yielding DNA extraction method for *Hyeronima alchorneoides* was developed. This method is based on a modified CTAB protocol. Three methods were used for DNA quality determination: an agarose gel (0.8%) electrophoresis, DNA digestion with restriction enzymes EcoR I and Hind III, and quantification in a fluorometer. The procedure showed to yield good quality DNA, with values between 30-40 ng/µL. With the method described in this study, DNA extracted was completely digested with EcoR I and Hind III. AFLP reactions were performed in order to evaluate the suitability of the extracted DNA. Fifty six primer combinations were evaluated for amplifying DNA, but only forty nine combinations amplified for the extracted DNA from *Hyeronima alchorneoides*. Twenty five primer combinations showed a high resolution banding and a large number of bands.

Key words: AFLP*,* CTAB, DNA extraction, *Hyeronima alchorneoides,* primer combinations.

Introduction

Hieronyma alchorneoides is a dioecious native tree species from low and humid lands in Central America. It has been planted commercially in Costa Rica, and recently subjected to breeding programs since 1999 (Badilla *et al.*, 2002). Clonal propagation and breeding has been directed by Technological Institute of Costa Rica and local forest companies (Murillo *et al*., 2003). Breeding strategy for this tree species has been based on the forest development model of the country: intensive forest production in small land units. In this type of programs becomes clearly relevant all possible early selection initiatives, like gene markers utilization.

New molecular markers and tools has been developed in last years. More and more become of key importance in breeding and specially, in gene conservation programs. Previous works based on RAPD's was developed with *H. alchorneoides* in Costa Rica, aimed to measure variation among natural populations and a protocol development of markers for early male and female assessment (Villalobos, 2000).

AFLP's markers are one of the PCR-based technology and has been utilized not only in trees population genetics studies, but also for tree selection (Grattapaglia *et al.*, 1997; Coart *et al.*, 2002). This type of applications normally require the analysis of large population samples. Therefore, a suitable, fast and low cost DNA extraction procedures are mandatory in these cases (Chen and Ronald, 1999). However, there is a major consideration associated with the isolation of DNA

[1] Escuela de Ingeniería Forestal, Instituto Tecnológico de Costa Rica.
[2] Escuela de Biología, Universidad de Costa Rica.
[*] Author for correspondece. Current address: Escuela de Ingeniería Forestal, Instituto Tecnológico de Costa Rica, Cartago, Costa Rica. E-mail: evalverde@itcr.ac.cr; Fax: (506) 591 4182; Phone: (506) 550 2511.

from higher plants. Enzyme-inhibiting polysaccharides are often present in the "purified" DNA (Rogers and Bendich, 1988). Also, enzyme-degrading DNA is a problem on a DNA isolation protocol (Valadez and Kahl, 2000). Most extraction methods have employed the expensive and time-consuming cesium chloride density technique to get rid off polysaccharides (Rogers and Bendich, 1988). Alternatively, there is an extraction method based on CTAB (cetyltrimethylammonium bromide) that makes possible to extract purified high molecular weight DNA, without using expensive equipment and time-consuming procedures (Rogers and Bendich, 1988). CTAB method has been used on tree species with high levels of polysaccharides such as *Pinus radiata* (Stange *et al.*, 1998), *Pinus hartwegii* (Iglesias *et al.*, 2003) and with grape (*Vitis vinifera*) (Muhammad *et al.*, 1994).

In order to isolate high quality DNA from *Hieronyma alchorneoides*, a protocol described by Muhammad *et al.* (1994) has been modified. It was determined a suitable protocol for further AFLP analysis with this tree species.

Materials and methods

DNA extraction. *Hyeronima alchorneoides* young leaves were collected from 8-years-old trees in a progeny test established at a Research Station from the Instituto Tecnológico de Costa Rica in northern Costa Rica (San Carlos, Alajuela). Approximately 50-100 mg of tissue were put in a 1.5 mL Eppendorf. The tissue were ground with 500 µL of the buffer extraction (20 mM sodium EDTA and 100 mM Tris-HCl, adjust pH to 8.0 with HCl, 1.4 M NaCl, 2.0% (w/v) CTAB, 2% (w/v) PVP and 0.2% of beta-mercaptoethanol) and additional 250 µL were added later. The tubes were incubated at 60 °C for 20 minutes. After incubation 700 µL of chloroform:octanol (24:1) were added and tubes mixed gently by inverting 20 times. The mixture was centrifuged at 6000 rpm for 15 minutes at 4 °C and 400 µL of the aqueous phase was transferred into a new 1.5 mL tube. A second chloroform:octanol extraction was performed when the aqueous phase was brown-colored or turbid. In order to precipitate DNA, 0.5 volume of 5M NaCl and two volumes of cold 95% ethanol were added and refrigerate for 1 hour at -20°. Alternatively, DNA can be precipitated at 4-6 °C overnight. The solution was centrifuged at 10 000 rpm for 10 minutes at 4 °C. The supernatant was pour off and the pellet kept at the bottom of the tube. The pellet was washed with 70% ethanol and the ethanol removed without drying the DNA. The DNA was dried at 37 °C for 1 hour and then dissolved in 100 µL TE. For RNA treatment 1 µL RNase was added to each tube and incubated at 37 °C for 1 hour. DNA can be kept at -20 °C until its use.

DNA quality determination. Extracted DNA quality was determinated through running samples in an agarose gel (0.8%). Also, DNA quantity was determinated using a fluorometer (Hoefer®). In addition, extracted DNA was subjected to two restriction enzyme digestions (EcoR I and Hind III) for 1 hour and subjected to agarose gel (1%) electrophoresis with undigested DNA as control.

AFLP reactions. AFLP was performed according to Vos *et al.* (1995) with available kits from Applied Biosystems (AFLP™ Plant Mapping). Pre-amplification step was performed with primers complementary to the EcoR I and MseI adaptors. The PCR reactions were performed in Perkin Elmer GeneAmp PCR system 9600 using the following PCR conditions: 72 ºC for 2 minutes, 94 ºC for 20 seconds *(step 2),* 56 ºC for 30 seconds, 72 ºC for 2 minutes, go to step 2 and repeat 19 more times, 60 ºC for 30 minutes and 4 ºC forever. After pre-amplification, fifty six primer combinations were evaluated by combining seven EcoR I selective primers (AAC, ACC, AGC, AAG, ACG, AGG and ACT) with eight MseI selective primers (CAA, CAC, CAG, CAT, CTC, CTG, CTT and CTA). PCR reactions were performed in a Perkin Elmer GeneAmp PCR system 9600 with the steps as follows: 94 ºC for 2 min, 94 ºC for 20 sec, 66 ºC for 30 sec, 72 ºC for 2 min, 94 ºC for 20 sec, 65 ºC for 30 sec, 72 ºC for 2 min, 94 ºC for 20 sec, 64 ºC for 30 sec, 72 ºC for 2 min, 94 ºC for 20 sec, 63 ºC for 30 sec, 72 ºC for 2 min, 94 ºC for 20 sec, 62 ºC for 30 sec, 72 ºC for 2 min, 94 ºC for 20 sec, 61 ºC for 30 sec, 72 ºC for 2 min, 94 ºC for 20 sec, 60 ºC for 30 sec, 72 ºC for 2 min, 94 ºC for 20 sec, 59 ºC for 30 sec, 72 ºC for 2 min, 94 ºC for 20 sec, 58 ºC for 30 sec, 72 ºC for 2 min, 94 ºC for 20 sec, 57 ºC for 30 sec, 72 ºC for 2 min, 94 ºC for 20 sec *(step 32),* 56 ºC for 30 sec, 72 ºC for 2 min, go to step 32 for 19 more times, 60 ºC for 30 min, 4 ºC forever. After amplification, samples were denatured adding 1 µL of formamide and heating for 5 minutes at 95 ºC; then, tubes were immediately placed on ice. AFLP fragments were separated by PAGE using 5% denaturing polyacrylamide gels. Fragments were separated using constant power (55 W) for approximately 1 hour. After electrophoresis, gels were revealed using silver stain reagents from Promega (SILVER SEQUENCE™ DNA Sequencing Systems).

Results and discussion

DNA extraction and quality determination. The CTAB based method for DNA extraction evaluated yields high quality DNA. DNA degradation was not observed when samples were

run in a 0.8% agarose gel (figure 1). Also, quantification in the fluorometer showed DNA concentrations between 30-40 ng/µL. The basis for the separation of polysaccharides from nucleic acid is their differential solubility in the presence of CTAB (Rogers and Bendich, 1988). CTAB binds strongly to DNA, removes proteins and prevents DNA degradation (Valadez and Kahl, 2000). In addition, the methodology evaluated was low time consuming. DNA from fifty samples can be extracted in a working-day. However, we have observed this methodology can be more time consuming in tree species with harder leaves than *H. alchorneoides.*

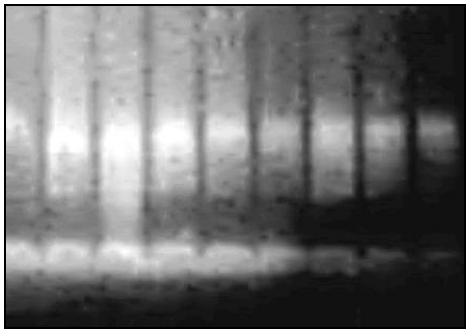

Figure 1. Agarose gel (0.8%) of DNA extracted from *Hyeronima alchorneoides* leaves.

Results of DNA restrictions with EcoR I and Hind III showed complete digestion (figure 2) and it was also observed that non-digested DNA exhibits high quality. DNA was extracted with a modified protocol described for Muhammad *et al.* (1994). These authors report a complete digestion with three endonucleases (EcoR I, Eco RV and Hind III) working with *Vitis* and *Ampelopsis* species.

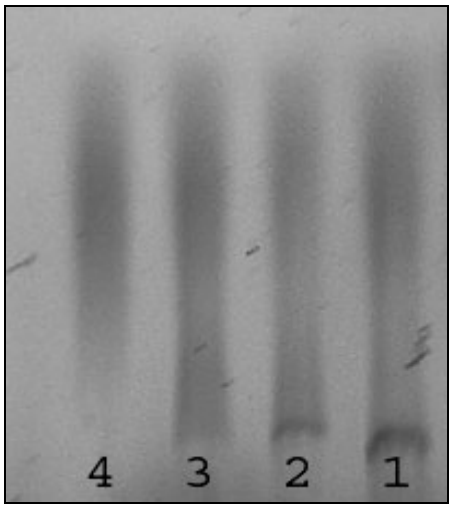

Figure 2. Agarose gel (1%) of non-digested DNA as control (lane 1). DNA was cut with EcoR I (lane 2), Hind III (lane 3) and EcoR I+Hind III (lane 4).

AFLP reactions. In order to evaluate the suitability of *H. alchorneoides* DNA for its use in AFLP analysis, reactions were performed as described before. Amplification of DNA with the primer combinations was revealed by PAGE electrophoresis and classified as: non-amplification (N), regular amplification (R) and good amplification (G) (table 1).

Table 1. Classification of primer combinations evaluated on *H. alchorneoides* DNA for its suitability on AFLP analysis. . (N = non-amplification ; G = good amplification; R = regular amplification).

EcoR I / Mse I	CAA	CAC	CAG	CAT	CTC	CTG	CTT	CTA
AAC	N	N	N	G	R	N	G	G
ACC	N	N	N	N	G	N	N	R
AGC	G	N	N	G	G	N	G	N
AAG	G	G	N	R	N	N	G	G
ACG	G	G	N	G	R	G	G	G
AGG	N	G	N	R	R	N	G	G
ACT	N	G	N	R	G	N	G	G

Twenty four primer combinations did not amplify *H. alchorneoides* DNA. However, other twenty five primer combinations showed a high resolution banding and a large number of bands. In figure 3, banding resolution for three primer combinations classified as “good amplification” is presented.

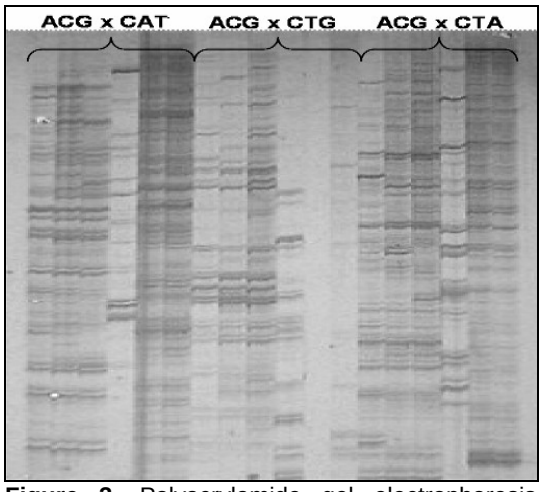

Figure 3. Polyacrylamide gel electrophoresis showing AFLP amplifications with three different primer combination.

Seven combinations were observed with a regular amplification exhibiting a low band resolution and number of bands. Using a similar method for DNA extraction, Muhammad *et al.* (1994) obtained high quality DNA from *Vitis* species. It was suitable for Southern hybridization and also amplifiable in PCR using the RAPD technique.

Conclusions

A suitable DNA extraction and amplification method for *H. alchorneoides* has been obtained. A number of primer combinations with a large number of bands exhibiting high resolution has been developed. Twenty five primer combinations exhibited the best resolution and high banding patterns.

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