# Genome Characterization of *Opuntia ficus-indica*: A Simple and Efficient Micromethod

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## ABSTRACT

A rapid and efficient micromethod for DNA extraction of *Opuntia ficus-indica* plants and subsequent RAPD analysis was established. For extraction, 100 mg of the chlorenchyma from cladodes were used. The DNA quantity extracted was significantly higher than for reported macromethods. Up to 100 µg DNA per g fresh weight were achieved. RAPD analysis was performed with 10 ng template DNA by using a kit that contains two polymerases and reduces the pipetting procedure to three steps. The method is being applied on a *O. ficus-indica* cultivar (Gigante) that we are using for studies on somatic embryogenesis and genetic transformation. Fingerprints obtained demonstrated a very high repeatability of the results and homogeneity of our plant material.

Keywords: *Opuntia ficus-indica,* DNA extraction, RAPD analysis.

## INTRODUCTION

Genome characterization of *O. ficus-indica* is regarded as an important tool in research on genetic diversity and on the breeding potential of this species (Wang *et al.*, 1998; Mondragon-Jacobo and Bordelon, 1996). Isolation of DNA from fresh material is difficult, especially due to the presence of high amounts of mucilage that interfere with DNA extraction. De la Cruz *et al.* (1997) and Wang *et al.* (1998) reported macromethods of DNA isolation from cladodes using a few grams of fresh tissue. The DNA yield ranged between 0.25 µg and 2 µg per gram fresh weight. In a recent report Mondragon-Jacobo et al. (2000) presented a method for DNA extraction, that needs 5 to 8 g of tissue and the DNA amount extracted from *O. ficus-indica* was 29 µg per g fresh material.

RAPD fingerprint analysis was introduced in plant sciences as a putative simple method to characterize genotypes without the need of detailed knowledge about the genomes of interest (Welsh and McClelland, 1990; Williams et al., 1990; Williams et al., 1993). However, after recent years of intensive application of the method, it became obvious that this technique is very sensitive and prone to difficulties with reproducibility. On the one hand, variability of results were reported to be related to technical aspects, such as the thermocycler used, laboratory practice, different persons applying the method, primer design and source of primer as well as DNA purification, Taq polymerase and the PCR program used (Chen et al., 1997; Ellinghaus et al., 1999; Mizukami et al., 1998; Penner et al., 1993; Schweder et al., 1995; Skroch and Nienhuis, 1995; Williams et al., 1993 ) On the other hand, recent results indicate that RAPD fingerprints may also be influenced by differentiation and the physiological state of a tissue (Bitonti et al., 1996; Bogani et al., 1996; Chen et al., 1997; Schaefer et al., 2000; Schaffer and Arnholdt-Schmitt, 2001). Differentiation and physiological conditions may create quantitative polymorphism, i.e., differences in the

intensity of amplified fragments, which even may mask bands. This may erroneously be interpreted as qualitative polymorphism (Schaefer et al., 2000; Schaffer and Arnholdt-Schmitt, 2001).

In this paper, we report on the establishment of a high-yielding micromethod for DNA extraction using only small amounts of a specialized tissue of *O. ficus-indica* cladodes. For RAPD analysis an effective, commercially available kit is used, which eliminates failure during performance and demonstrates a very high repeatability.

## MATERIALS AND METHODS

### Plant Material

Seeds of *O. ficus-indica*, cv. Gigante, obtained from mature fruits collected in May 2000 on a farm in Madalena, Ceará, Brazil, were germinated on a mixture (1:1) of washed river sand and organic matter. The seedlings were transferred to pots and grown in a green house. DNA extraction was performed using chlorenchyma tissues of cladodes.

### DNA Extraction

For DNA extraction, a kit from Amersham Pharmacia Biotech (Nucleon Phytopur for Plant DNA Extraction) was used by introducing some modifications in the protocol provided (the protocol given by Amersham Pharmacia Biotech can be looked up at www.apbiotech.com/technical/technical_index.html). The distal part of cladodes of comparable age was cut and samples were brought from the greenhouse to laboratory. An internal slice of the sample was taken for analysis to avoid contamination and inclusion of lysed cells. Then the cuticle was removed and a piece of about 100 mg of the chlorenchyma was cut using a scalpel and taking care not to include areolar meristems. The tissue was placed into a sterilized microtube, weighed and extraction buffer (reagent 1) was added. For cell lysis the fresh material was homogenized with a micropistill in 600 µl of reagent 1 without using mercaptoethanol. If the mucilage content is especially high, 4% of PVP-40000 may be included in reagent 1. After adding 200 µl of reagent 2, lysis was performed in a water bath for 15 to 20 min at 65°C. The sample was placed on ice for 20 minutes, then centrifuged for 20 min at 10000g. The supernatant was removed to a clean microtube and, for purification, 500 µl of chloroform-isoamylalcohol (24:1, room temperature) were added as well as 100 µl of a resin suspension from the kit to precipitate and/or bind proteins and polysaccharides. After 10 min of mild shaking the suspension was centrifuged for 5 min at about 10000g. The supernatant was carefully mixed with one volume of isopropanol at room temperature and centrifuged to pellet the nucleic acids for 5 to 10 min at 10000g. The pellet was washed twice with 70% ethanol (room temperature), dried, and dissolved in sterile water.

### Estimation of DNA Quality and DNA Quantity

A rough determination of the quality and quantity of nucleic acid extraction was performed by spectral photometric measurements. Subsequently, analysis by electrophoresis was performed in 1% agarose gel, running in parallel known amounts of high-molecular-weight lambda DNA (10, 20, 30, and 40 ng) and the extracted sample. Nucleic acids were visualized by ethidium bromide using a video documentation system (LTF, Wasserburg, Germany). The evaluated concentration of 10 ng DNA of each sample was confirmed in a second electrophoresis.

**RAPD Analysis**

For fingerprint analyses "Ready-to-go RAPD analysis beads" from Amersham Pharmacia Biotech were used. 10mer primers were obtained from Operon Technologies (kit G) and from Amersham Pharmacia Biotech (P1 - P6). Ten ng of template DNA and 25-pmol primer were applied for a 25 µl reaction volume under standard conditions. The thermocycler used for the experiments was supplied by Hybaid (Middlesex, UK, model "PCR SPRINT"). For amplification the following program was performed: 5 min at 95°C, 45 cycles: 1 min 95°C, 1 min 36°C, 2 min 72°C, and 5 min at 72°C. RAPD fragments were analysed in a 1.5% agarose gel.

## RESULTS

Photometric measurements at 260 and 280 nm of the nucleic acid extracts of the various plants of cv. Gigante indicated a quotient (260/280) between 1.72 and 2.16. Subsequent electrophoretic analysis showed a distinct band of high-molecular-weight DNA and a smear of RNA. Considering linearity of staining with ethidium bromide, quantitative evaluation is possible under the selected conditions in the range of 10 to 40 ng of standard DNA. Since 10 ng of DNA had to be applied for RAPD analysis and the amount of template DNA may be critical for the intensity of produced bands (Schäfer et al., 2000; Schaffer and Arnholdt-Schmitt, 2001), the evaluated amount of 10 ng DNA was re-evaluated in a second electrophoresis. Calculation of the yield of DNA was based on this final determination. As a result, DNA yields were achieved typically ranging between 4.6 µg and 27 µg per g of fresh tissue, with the tendency of upper values by increased routine in the procedure. A 3-week-old cladode of an additional accession of *O. ficus-indica* yielded 100 µg DNA per g of fresh weight. Low age and the genotype of the plant material may have influenced DNA yield. This has further to be checked.

Eighteen primers were roughly checked for appropriate pattern formation for *O. ficus-indica* and, subsequently, eight primers (OP-G01, OP-G04, OP-G07, OP-G11, OP-G13, OP-G15, P2, P6) were selected for tests of the repeatability of the method. In Figure 1, results of RAPD analyses of four independent extractions of samples of the same plant using two different primers are given. As can be seen from Figure 1, pattern formation was identical for each primer considering the occurrence as well as the intensity of the bands. The primers tested for repeatability produced seven to ten distinctive bands. All of them were reproducible in different assays without any failure. One primer (G-07) indicated variability in the intensity of bands in repetitive experiments.

Ten plants were randomly chosen for tests of homogeneity and were analysed by use of the eight selected primers. Up to now, no polymorphism was found in our material. Each of the eight primers applied revealed unambiguously homogeneous fingerprints for the individual plants. In Figure 2, RAPD analysis of ten plants by primer P6 is given as an example. Besides indicating genetic homogeneity of the analysed plants, identity of the produced fingerprints is additional evidence for good repeatability of the applied procedures. Figure 3 shows that the same primer P6 was appropriate to indicate polymorphism when applied to a young cladode of a plant of another *O. ficus-indica* accession (B, Figure 3).

## DISCUSSION

The aim of our studies was to develop a rapid and efficient, high yielding micromethod for DNA extraction of fresh material from *O. ficus-indica* that could be used for genome characterization. Standard DNA extraction methods using SDS and potassium acetate gave reliable results in our laboratory for transgenic callus material (see results in Llamoca-Zárate *et al*., 1998) and transgenic cell suspensions of *O. ficus-indica* (unpublished results), but these methods or methods based on CTAB were not useful for

fresh plant material, like cladodes, of this species. Polysaccharides were visible in the extract by their viscous texture, inhibiting pipetting and are supposed to inhibit enzyme activity, or unknown substances that were indicated by analysis of the photometric spectrum contaminated the DNA extract. The extraction method introduced in this paper was carefully checked for repeatability by subsequent RAPD analyses. Four repetitive extraction assays of one plant and extraction of another nine plants with obviously homogeneous genomes confirmed a very strong repeatability of the results. This was true regarding pattern formation as well as the intensity of produced bands. The extraction method is called a micromethod, considering the small amount of plant material used in comparison to existing methods for DNA extraction of cactus plants (De La Cruz et al., 1997; Wang et al., 1998; Mondragon-Jacobo et al., 2000). Additionally, the yield of DNA calculated per g fresh tissue was very high. Extraction of 100 mg of tissue permits up to 1000 RAPD analyses.

Because there is good evidence from other plant species that cell differentiation and the physiological state of cells or tissues may create quantitative polymorphism, which can be revealed by polymorphic RAPD bands (for references see the Introduction), DNA extraction was performed from the chlorenchyma of the distal part of cladodes from *O. ficus-indica* plants at the same age. Recent results on a primary culture system and individual plants from *Daucus carota* indicate an important influence of growth and development as well as age and hormones on DNA organization and, subsequently, on results of RAPD analyses (Arnholdt-Schmitt, 1995; Schaefer et al., 2000; Schaffer and Arnholdt-Schmitt, 2001). Therefore, for comparative studies it is highly recommended to use exclusively the same type of tissues, considering also developmental stage and/or physiological status.

To achieve the highest possible reproducibility for genome characterization, we used a commercially available kit for RAPD analysis. This kit includes all buffer components, nucleotides as well as polymerase activity in a preformed tube. The only components to be added by the experimenter to the reaction mixture are the primer of choice, template DNA, and sterile water to achieve the desired volume. Advantages of this method are: (1) easily achieved complex pattern formation due to the inclusion of two polymerases in the kit and (2) the minimization of technical mistakes while performing the method. Disadvantages, such as the high cost of the kit and dependency on the premixed product, are counterbalanced by very few errors, rapid application of the method, and very good repeatability, even if different persons apply the method, thermocyclers from alternative suppliers are used, or different batches of RAPD analysis beads are taken (see also Arnholdt-Schmitt, 2000; Schaefer *et al.*, 2000; Schaffer and Arnholdt-Schmitt; 2001, Imani *et al.*, 2001).

As shown here, RAPD analyses of our plant material (cv. Gigante, accession Madalena), performed by eight primers, indicated genetic uniformity of this material. These experiments are part of a project which aims to obtain transgenic varieties of *O. ficus-indica* displaying increased levels of proteins in its phylloclads. At this stage, we are working on the induction of somatic embryogenesis in different tissue such as cotyledons, hypocotyls, zygotic embryos, and shoot apex. Because it is well known that the genetic background of plant material can interfere with the effectivity of external inductors for somatic embryogenesis, we are interested in checking our material for homogeneity.

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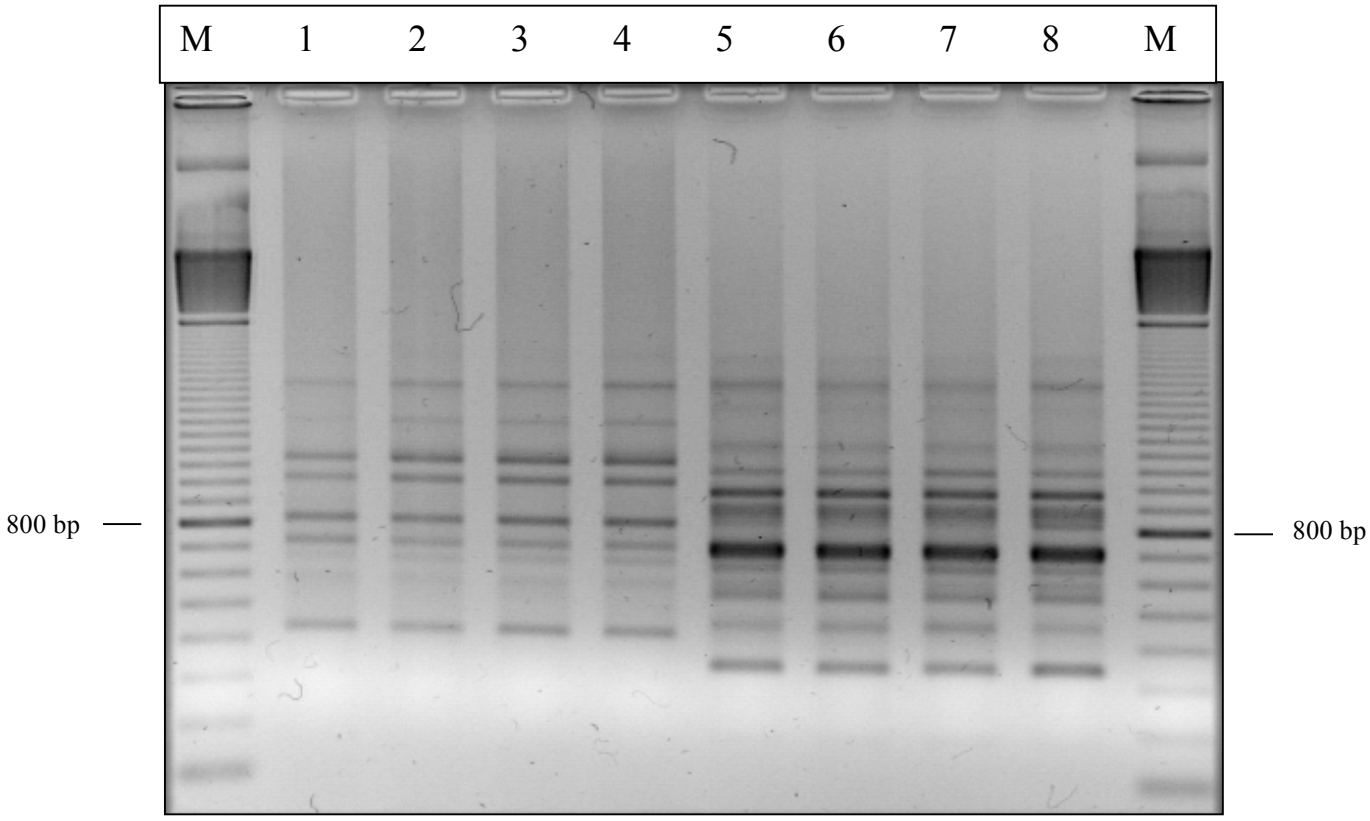


Figure 1. RAPD Analyses by Primer OP-G01 (Lanes 1–4) and OP-G11 (Lane 5–8) of Four DNA Extracts Performed from the Same Plant. M: marker

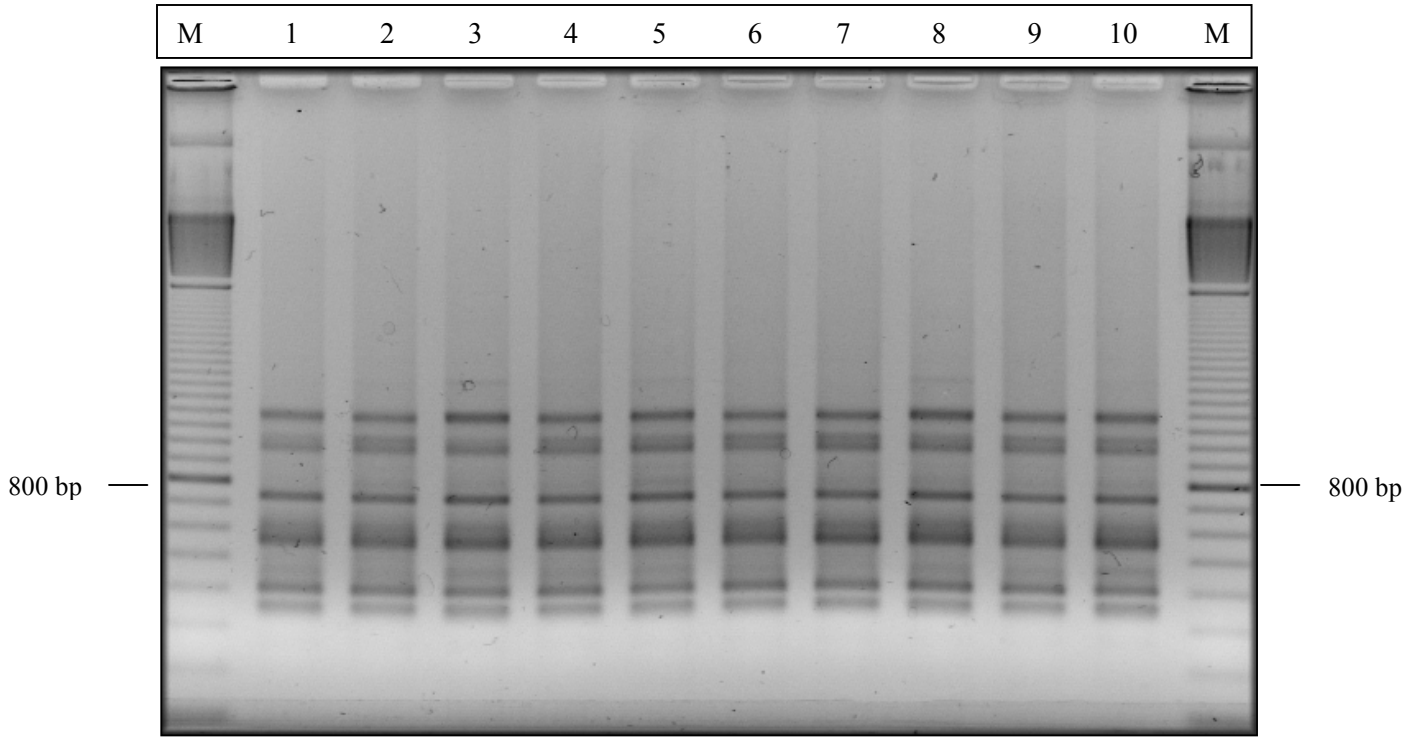


Figure 2. RAPD Fingerprints of Ten Seed-Grown Plants
of cv. Gigante Produced by Primer P6

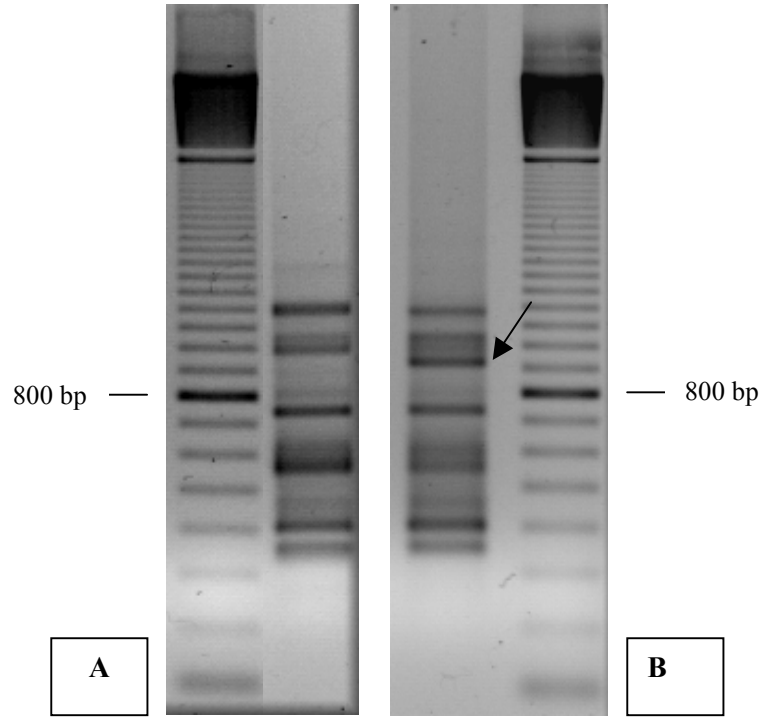


Figure 3. RAPD Fingerprints by Primer P6
of Two *Opuntia ficus-indica* Accessions (A, B)