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Comparison of protocols for genomic DNA extraction from 'velame pimenta' (*Croton linearifolius)*, a native species to the Caatinga, Brazil

M. M. Scaldaferri[12], J. S. Freitas[1], E. S. L. Santos[1,2], J.G. P. Vieira[1], Z. S. Gonçalves[1] and C. B. M. Cerqueira-Silva[1*]

[1]Laboratory of Applied Molecular Genetics, Departamento de Estudos básicos e Instrumentais, Universidade Estadual do Sudoeste da Bahia, Itapetinga - Ba, 45700-000, Brazil.
[2]Laboratory of general biology and botany, Departamento de Estudos básicos e Instrumentais, Universidade Estadual do Sudoeste da Bahia, Itapetinga - Ba, 45700-000, Brazil.

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The Caatinga biome occupies some 12% of the Brazilian territory, which is present in at least nine states. The species that constitute its biodiversity have the potential to be used as natural resources, among them are approximately 700 species of the genus *Croton*. As an example of this potential, the *Croton linearifolius* specie is used by local communities as a natural insecticide. Associated with the economic potential of the Caatinga species, one must stress the risk of extinction or genetic erosion due to the growing deforestation of natural areas of this biome. These factors make it relevant in genetic studies in order to guide conservation strategies. Considering the lack of molecular studies involving *C. linearifolius*, we compared the efficiency of six protocols for genomic DNA extraction previously described in literature. The DNA extraction buffers [based on the use of Cetyl trimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), mannitol and sorbitol] were different in their efficiency to obtaining the genomic DNA of *C. lineariflolus*. In general, protocols using CTAB buffer were more efficient. The use of liquid nitrogen in the maceration process was also evaluated and its use was considered a no necessary factor in obtaining DNA in adequate quantity and quality for PCR platform procedures.

Key words: DNA Isolation, molecular markers, native species of Caatinga.

INTRODUCTION

The Caatinga biome occupies about 12% of the Brazilian territory, including the states of Bahia, Sergipe, Alagoas, Pernambuco, Paraíba, Rio Grande do Norte, Ceará, Piauí and the north of Minas Gerais (Almeida-Cortez et al., 2007). The Caatinga vegetation is considered xerophytic, with diverse aspect and floristics, average rainfall between 240 and 1500 mm, and temperature ranging between 24 and 29°C (Almeida-Cortez et al., 2007).

Faced with the environmental characteristics presented, the Caatinga vegetation is adapted to water deficiency conditions, and produces tree, shrub and moss layers characterized by water-retaining structures, and/or superficial roots (Almeida-Cortez et al., 2007). With such typical environmental characteristics, the Caatinga harbors large quantities of endemic species. Although there is not an authoritative list of Caatinga species, qualitative and quantitative studies about the flora and vegetation of this biome register approximately 600 species, of which at

*Corresponding author. E-mail: csilva@uesb.edu.br. Tel: +55 77 3261-8677. Fax: +55 77 3261-8609.

least 30% are endemic (Tabarelli, 2005).

Among the species that constitute the Caatinga biodiversity are those that belong to the genus *Croton*. Its taxonomic classification is difficult due to the diversity of species, nomenclatures and polymorphisms of its representatives (Webster, 1993). The genus *Croton* belongs to the Crotonoideae sub-family and its approximate 700 species make up one of the most numerous of Euphorbiaceae genera (Berry et al., 2005). Species in the genus *Croton* should be classified as trees, shrubs, subshrubs or less frequently as herbs or vines (Silva et al., 2009), and are very important for the consolidation of an ecosystem, because they form a group of pioneers that are fundamental to recover degraded areas (Lima and Pirani, 2008).

There are some 300 registered *Croton* species in Brazil, many with known chemical and/or pharmacological properties and described as having medicinal potential or as being toxic (Almeida et al., 2003). Having had its extracts used popularly by dwellers of the regions surrounding its area of occurrence, *Croton* spp. is being submitted to chemical analyses, and studies have proved the existence of essential oils and a number of active substances (Randau et al., 2004; Torres, 2008). *Croton linearifolius* is one of such species that according to ethnobotanic studies is largely used as a natural insecticide by the bushmen of Bahia's semiarid region in the Contendas do Sincorá area (Cunha e Silva et al., 2010).

Even when faced with a wealth of biodiversity, with the potential use of its species as natural resources and with the growing risk of genetic erosion caused by the exploitation and degradation of natural environments, many native plant species or species that are endemic to the Caatinga have not been subjected to genetic diversity studies.

It should be stressed that genetic characterizations, especially when associated with the use of molecular markers, are an effective contribution to understanding ecological characteristics, as well as to steer natural resources management and conservation strategies. Among the large number of techniques and methodologies available for molecular studies involving the characterization of genetic diversity, we highlight the use of the RAPD, ISSR, AFLP, SSR and SNP markers.

We highlighted that molecular studies initially lack well-established protocols to obtain DNA, RNA and/or proteins from the targeted species, since the efficiency of standard procedures to extract such molecules can vary among species. Such variations occur, among other factors, as discussed by Moreira and Oliveira (2011) and Porebski et al. (1997), due to the differential presence of secondary compounds such as polyphenols, tannins and polysaccharides, as well as tissue rigidness.

Due to the lack of molecular studies involving *C. linearifolius* and the need for molecular genetic studies, our objective was to compare the efficiency of protocols for genomic DNA extraction from "velame pimenta" (*C. linearifolius*), a species native to the Caatinga.

MATERIALS AND METHODS

Obtaining and storing biological material

Young leaf tissue was collected from 'velame pimenta' (*C. linearifolius*) at a native forest in the municipal area of Contendas do Sincorá, Bahia, and stored in an ultra-cold freezer at the Applied Molecular Genetics Laboratory (LGMA) of Universidade Estadual do Sudoeste da Bahia – UESB, Itapetinga. In order to standardize the extraction tests, 0.2 g of leaf tissue was used for repetition in each protocol.

Description of extraction protocols

Six protocols previously described in literature were assessed for genomic DNA extraction. We performed three repetitions for each extraction protocol (repetition = extraction carried out in a 1.5 mL tube). All protocols used samples macerated in buffer solution (using the descriptions in the articles describing the protocols, cited) (Table 1), which was transferred (800 µL) to 1.5 mL tubes (totaling 18 tubes, three for each of the six protocols), and to which we added 10 µL of proteinase-K (20 $mgmL^{-1}$). Changes made are described in the text which shows each of the protocols used.

Protocol 01: Sunnucks and Hales (1996)

The samples were incubated for 3 h at 55°C in buffer solution containing SDS and inverted every 20 min. By the end of the incubation period, the samples were centrifuged at 12000 x g, for 10 min, at 15°C; the supernatant was removed and 700 µL of cold isopropanol was added. The samples were gently homogenized and again centrifuged at 12000 x g for 10 min at 15°C. The supernatant was removed and 800 µL of cold ethanol 70% was added. The samples were centrifuged at 12000 x g for 5 min at 15°C; the supernatant was removed and 800 µL of cold ethanol 95% was added; they were then centrifuged at 12000 x g, for 5 min, at 15°C; the supernatant was discarded. When fully dried, the pellet was resuspended in 60 µL ultra pure water.

Protocol 02: Modified method by Cerqueira-Silva (2009)

The samples were incubated for 3 h at 55°C in buffer solution containing CTAB and inverted every 20 min. After the end of the incubation period, 700 µL of chloroform isoamyl alcohol 24/1 was added and the samples were homogenized by inversion. They were centrifuged at 12000 x *g* for 10 min at 15°C; the supernatant was removed and placed in a new tube (550 µL) with the addition of 600 µL of chloroform isoamyl alcohol 24/1 and homogenized by inversion. It was once again centrifuged at 12000 x *g* for 10 min at 15°C; the supernatant was removed and placed in a new tube with the addition of cold isopropanol (in the proportion 1/1), gently homogenized, and the samples were incubated overnight at 4°C for precipitation. After this period, they were centrifuged at 10000 x *g* for 10 min at 15°C; the supernatant was discarded and 500 µL of cold alcohol 70% was added to it. They were centrifuged at 10000 x *g* for 5 min at 15°C;, the supernatant was discarded and 500 µL of cold alcohol 95% was added. The samples were centrifuged at 10000 x *g* for 5 min at 15°C and then the supernatant was discarded. Once dried, the pellet was resuspended in 60 µL of Ultra pure water.

Protocol 03: Modified method by Storchova et al. (2000)

The samples were incubated at room temperature for 20 min in extraction buffer containing mannitol. After the end of the incubation period, they were centrifuged for 3 min at 10000 x *g* at 15°C, the

Table 1. Description of the buffer solution used in the protocol available for DNA extraction from *Croton linearifolius*.

Buffer	Reference	Solution
Extraction buffer SDS 10%	Sunnucks and Hales (1996)	0.1 M Tris-HCl, 0.05 M EDTA, 10% sodium sulfate dodecyl, 0.5 M NaCl, 50µ g mL^{-1} Proteinase K and 50µ g mL^{-1}
Extraction buffer CTAB 5%	Modified method by Cerqueira-Silva (2009)	0.1 M Tris-HCl, 0.02 M EDTA, pH 8.0, 5% (w/v) CTAB, pH 8.0, 1.4 M NaCl, 1% (w/v) PVP
Extraction buffer with Manitol	Modified method by Storchova et al. (2000)	0.1 M Tris-HCl, pH 7.5, 0.05 M EDTA, pH 8.0, 0.35 M mannitol and 0.3% b-mercaptoethanol
Lysis buffer		0.2 M Tris-HCl, pH 7.5, 0.05 M EDTA, 2% (w/v) CTAB, pH 8.0, 2 M NaCl,
Extraction buffer with Sorbitol	Modified method by Russell et al. (2010)	0.1 M Tris-HCl, 0.005 M EDTA, 0.35 M sorbitol, 1% PVP
Extraction buffer SDS and NaCl	Mogg and Bond (2003)	0.1 M Tris-HCl, 0.05 M EDTA, 0.7% sodium dodecyl sulfate, 0.5 M NaCl, 50µ g mL^{-1} Proteinase K and 50µ g mL^{-1}
Extraction buffer CTAB 2% and NaCl		0.1 M Tris-HCl, 0.02 M EDTA, 2% (w/v) CTAB, pH 8.0, pH 8.0, 1.4 M NaCl, 1% (w/v) PVP
Precipitation buffer	Barnwell et al. (1998)	0.05 M Tris-HCl, 0.001 M EDTA, CTAB 1%
Saturated TE buffer		10mM Tris-HCl, 1 mM EDTA, 1 M NaCl

supernatant was discarded and 300 µL of lysis buffer was added, containing 2% CTAB and NaCl. Following these stages, the samples were incubated at 65°C for 15 min and 600 µL of chloroform isoamyl alcohol 24/1 was added; the solution was homogenized by inversion during 5 min. The material was subsequently centrifuged for 3 min at 10000 x *g* at 15°C. The supernatant was transferred to a new tube, to which 500 µL of cold isopropanol was added and the samples were incubated during 1 h in a freezer at -20°C. After this period, they were centrifuged for 3 min at 10000 x *g* at 15°C. The supernatant was discarded and the pellet was washed with 400 µL alcohol (70%). The alcohol used for the wash was discarded and the pellet was left to dry completely. Once dried, it was resuspended in 60 µL of ultra pure water.

Protocol 04: Modified method by Russel et al. (2010)

The samples containing sorbitol buffer were centrifuged for 10 min at 5700 x *g* at 15°C. After centrifugation, the supernatant was discarded and the sediment was dissolved with 1 mL sorbitol buffer (procedure repeated until there was no mucilage). Once the sample was dissolved, 1 mL of CTAB was added to it, and it was incubated for 1 h at 60°C. By the end of the incubation period, 700 µL of chloroform isoamyl alcohol 24/1 was added, and the solution was homogenized by inversion during 20 min. In the following stage, it was centrifuged during 10 min at 13000 x *g* and 15°C. The supernatant was transferred to a new tube, to which sodium acetate (1/10 v/v) and cold isopropanol (2/3 v/v) were added, and incubated overnight in a freezer at -20°C. After incubation, it was centrifuged during 30 min at 13000 x *g* and 15°C. Afterwards, the pellet was washed twice with 500 µL of ethanol. Once dry, the pellet was resuspended in 60 µL of ultra pure water.

Protocol 05: Mogg and Bond (2003)

The samples were incubated overnight at 37°C in buffer solution containing SDS and NaCl. Subsequently, 520 µL of NaCl 5 M was added and the samples were centrifuged for 5 min, 3000 x g, at 15°C. The supernatant was transferred to a new tube, to which 800 µL of isopropanol 85% was added and the solution was homogenized by inversion. The samples were incubated at 4°C for 3 h. They were centrifuged at 10000 x *g* for 3 min at 15°C and subsequently, the samples were washed with alcohol (70%). Once dry, the pellet was resuspended in 60 µL of ultra pure water.

Protocol 06: Barnwell et al. (1998)

The samples were incubated at 65°C for 1 h in buffer solution containing 2% CTAB bromide. After the incubation period, they were centrifuged for 5 min at 10000 x *g* at 15°C. The supernatant was transferred to a new tube, to which NaCl 0.7 M (1/10 v/v) was added, as well as 1.25X of the original leaf tissue volume of the precipitation buffer (CTAB 1%). The samples were mixed by inversion and incubated for 30 min at room temperature. They were centrifuged for 15 min at 10000 x *g* at 15°C and subsequently the supernatant was discarded. 3.0 µL of saturated TE buffer and 400 µL of ethanol 100% were added. The material was incubated for 1 h in a freezer at -20°C. It was centrifuged for 10 min at 10000 x *g* at 15°C. The pellet was washed with alcohol 70%. Once dried, the pellet was resuspended in 60 µL of ultra pure water.

Quantifying the DNA

The quality of the DNA samples was assessed in agarose gel 1% (m/v) by electrophoresis (90 min in a 90 V electric current) and visualized with an EZ Vision buffer (according to the manufacturer's specifications) in a Kodak photo documentation system, with incidence of UV light. In order to quantify the DNA concentration (ng μL^{-1}), we adopted an intact Lambda molecular weight marker as standard (undigested Lambda DNA). In order to standardize and improve the reliability of comparing the efficiency of tested protocols, the quantification of DNA samples was also carried out by means of a spectrophotometric reading using 1 µL aliquots of Genomic DNA with a NanoDropTM spectrometer (NanoDrop Technologies), adopting the manufacturer's recommendations. The concentration of DNA was estimated from absorbance at 260 nm. The relationship between nucleic acids and proteins present in the sample was estimated by the relation between absorbance

Table 2. Description of the results observed from the spectrophotometric reading (NanoDrop Technologies) performed with 1 uL of genomic DNA samples of 'velame pimenta' (*Croton linearifol ius*)obtained with the use of different protocols.

Protocols evaluated	Concentration (ng uL^{-1}) (% CV)	260/280 (% CV)	260/230 (% CV)
Protocol 1 (Sunnucks and Hales, 1996)	46.7 (15%)[bc]	2.5 (10%)	0.6 (18%)
Protocol 2 (Cerqueira-Silva, 2009)	873.1 (2%)[a]	2.5 (6%)	1.0 (1%)
Protocol 3 (Storchova et al., 2000)	276.7 (5%)[bc]	3.9 (1%)	0.3 (32%)
Protocol 4 (Russel et al., 2010)	879.3 (6%)[a]	3.5 (2%)	0.6 (21%)
Protocol 5 (Mogg e Bond 2003)	558.6 (20%)[ab]	3.0 (1%)	1.0 (11%)
Protocol 6 (Barnwell et al., 1998)	847.1 (22%)[a]	2.2 (3%)	1.3 (11%)

CV = coefficient of variation; Different letters for presentation of DNA concentrations indicate significant difference ($p < 0.05$) for analysis of variance (Kruskal-Wallis test) and mean comparison test (Student-Newman-Keuls).

rates at 260 and 280 nm (A260/A280) (Sambrook and Russell, 2001).

The variation observed for DNA concentrations estimated with the NanoDrop were submeted Kruskal-Wallis test (ANOVA) and Student-Newman-Keuls test (mean comparison test, SNK), considering significant p-value < 0.05. We also calculated the coefficients of variation observed for each of six protocols. The tests and statistical estimates were made with the aid of the program BioEstat 5.3 (Ayres et al., 2007).

Liquid nitrogen influence assessment test

Tests were carried out to determine liquid nitrogen influence in breaking the physical barriers inherent to the maceration process and initial exposure of the cell contents in each of the six protocols was evaluated. We performed three repetitions for each extraction protocol (repetition = extraction carried out in a 1.5 mL tube).

Amplification tests

Primer tests

Primer tests were done according to the methods used by Santos et al. (2011) (three ISSR primers; DiCA 3G, DiCA 3RG, DiGA 3C) and by Williams et al. (1990) (three RAPD primers; OPD-01, -03, -05) in initial amplification via PCR in three individuals from natural populations of *Contendas do Sincorá* city, Bahia. The amplifications with both primers were conducted in a MJ 96 thermocycler (Biocycler) at a total volume of 15 µL containing 15 ng of the DNA extracted, PCR buffer 1X (20 mM Tris HCl [pH 8.4] and 50 mM Kcl), 1.5 mM $MgCl_2$, 0.2 µM of each dNTP, 1 µM primer and 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, California, USA).

Amplification reactions of PCR

The amplification program adopted for PCR reactions were: 94°C for 5 min, followed by 34 cycles [94°C for 50 s, 48°C (ISSR reaction) or 34°C (RAPD reaction) for 50 s and 72°C for 1 min], with the final extension at 72°C for 5 min, and finalizing with 12°C indefinitely. The amplification products were separated by electrophoresis in 2% agarose gel (m/v) in running buffer TBE 1X, at a constant voltage of 120 V for approximately 2 h.

Gel staining and the acquisition of images were carried out as described for the quantification of nucleic acids, based on the use of EZ Vision buffer and Kodak photodocumentation system. The molecular weight marker of 100 pb DNA Ladder (Invitrogen) was used on all runs.

RESULTS AND DISCUSSION

Although the DNA extraction buffers assessed (based on the use of CTAB, SDS, mannitol and sorbitol) are established for different species (Ojeda, 2012; Souza, 2012; Mogg and Bond, 2003; Štorchová et al., 2000) they differ in their efficiency in obtaining *C. linearifolius* genomic DNA ($p<0.05$; Kruskal-Wallis test). Considering the concentrations estimated using the NanoDrop spectrometer, it is possible to identify at least three efficiency levels ($p<0.05$, SNK test). In this context, DNA concentrations were estimated using protocols 2, 4 and 6 (approximately 800 ng μL^{-1}) and the first protocol (approximately 50 ng μL^{-1}) (Table 2). Although the observed values in 260/280 ratio (Table 2) indicate presence of contaminants, they do not influence the quality of amplifications performed, as can be seen in Figure 1.

The integrity of the DNA extracted with different protocols also presented different results (Figure 1). Protocol 01, which is based on the isolated use of SDS for digestion of the membranes, was not efficient for extraction, in view of the absence of bands detectable in agarose gel (Figure 1). In contrast with the results obtained for protocol 01, the presence of DNA was easily observed for the other protocols (02 to 06). Protocols 02, 04 and 06 used CTAB in common, but although present in protocol 03, reduced quantity of DNA was observed (approximately 270 ng μL^{-1}); was used in the latter in a smaller concentration and for a short period of time. These results is associated with the fact that polysaccharides and nucleic acids possess different solubility in the presence of CTAB detergent, facilitating a subsequent precipitation, while the use of detergent SDS is based on simultaneous precipitation of proteins and polysaccharides (Brazilian and Romano, 1999).

The use and special efficiency of these detergents (CTAB and SDS) with plant species is reported in literature, where it is possible to observe the indication of such detergents for different groups of plants (Romano and Brasileiro, 1999). As an example, we can quote the use of SDS for *Oryza sativa* (rice) (Caverzan, 2008), as well as of CTAB for *Theobroma cacao* (cacao) (Santos et al., 2012).

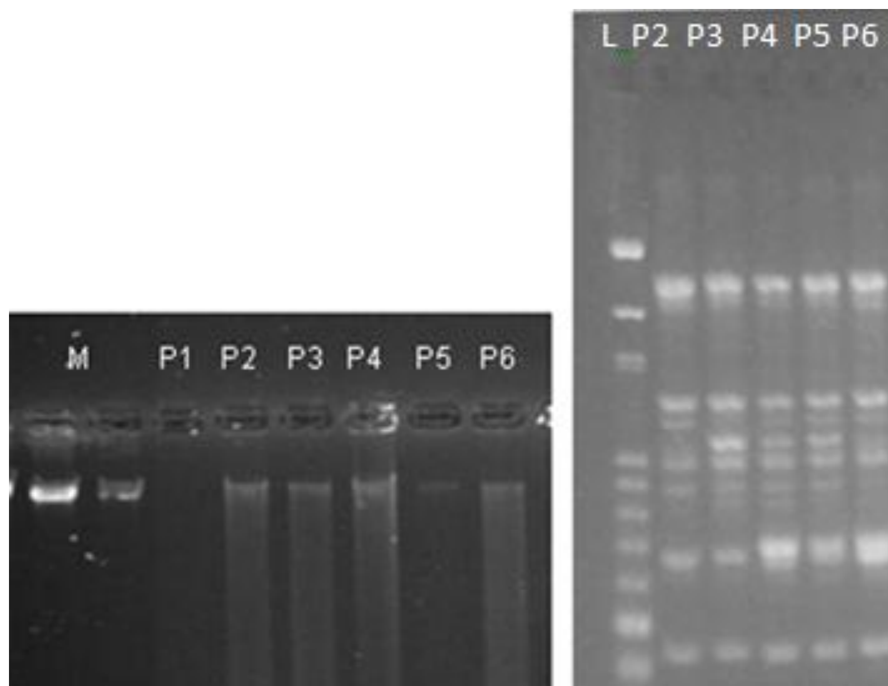

Figure 1. Agarose gel (1%) for the quantification of 'velame pimenta' (*Croton linearifolius*) DNA extracted presenting standard molecular weight (400 and 200 ng / uL), followed by DNA samples obtained from six different extraction protocols [left figure]; Gel Ladder presented, followed by amplification results of RAPD primers used as template DNA obtained from five different extraction protocols [right figure]. The protocol 1 showed no amplification products.

The protocols showed no differences in the tests performed for extractions conducted with and without the presence of liquid nitrogen during the physical barrier breaking phase (maceration of the samples). Therefore, it is possible to infer that in the case of 'velame pimenta', the use of liquid nitrogen does not bring about real benefits for DNA extraction.

With the exception of protocol 1 which showed no amplification products, the DNA amplification tests using ISSR and RAPD primers showed bands for amplifications performed with DNA obtained with the tested protocols (Figure 1). In short, the amplifications performed attest to the quality of the DNA extracted for these protocols, in view that the use of inadequate protocols would result in an absence of amplifications, as observed for tests performed with protocol 1 (data not shown), or still, bands difficult to evaluate, as reported for *Dimorphandra mollies* by Novaes et al. (2009).

Conclusions

The choice of protocols and the use of different detergents interfere in the result of genomic DNA extraction for 'velame pimenta' (*C. linearifolius*). Buffer CTAB was the most efficient, whereas the use of liquid nitrogen proved to be a non-obligatory factor for obtaining DNA in adequate quantity and quality for PCR platform procedures.

The work adds to the actions of the 'BioGen' research group, with the objective of performing molecular studies that enable molecular genetic characterizations of native Caatinga species.

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