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A protocol for large scale genomic DNA isolation for cacao genetics analysis

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Advances in DNA technology, such as marker assisted selection, detection of quantitative trait loci and genomic selection also require the isolation of DNA from a large number of samples and the preservation of tissue samples for future use in cacao genome studies. The present study proposes a method for the preservation of sample tissues for DNA extraction and for manual extraction of large number of samples using spheres. The integrity and concentration of the DNA by these methods were assessed and compared with conventional method using mortar. The best parameters in order to obtain a fine powder using spheres was the use of 4 lyophilized leaf disks (50 mg), a single steel ball of 6 mm in diameter, followed by 30 s of manual maceration. The quantity of DNA obtained was four times higher than the conventional method. The purity of the DNA obtained was satisfactory and proved to be amplifiable by PCR using SSR primers. The present approach is a reliable, rapid, simple and consistent DNA isolation method for cacao, compared to the conventional methods. The protocol greatly increases the efficiency of extraction and suggests an inexpensive and practical way of DNA isolation of cacao for large scale.

Key words: DNA extraction, cacao, spheres, lyophilized.

INTRODUCTION

Cacao (*Theobroma cacao* L.), the chocolate tree, is an important tropical species that provides sustainable economic and environmental benefits to some of the poorest and most ecologically sensitive areas of the world. The emergence of molecular marker analyses in genome studies has greatly enhanced the speed and efficacy of crop improvement and breeding programs. In breeding studies, numerous populations are sampled to detect candidate genes or molecular markers associated with economically important traits (Guiltinan, 2007; Maximova et al., 2007; Micheli et al., 2010). Similarly, a large number of accessions are sampled to determine the genetic diversity present in germplasm collections (Lanaud, 1986; Almeida et al., 1995; Pires et al., 1999; Yamada et al., 2001). Advances in DNA technology such as marker assisted selection (MAS), detection of quantitative trait loci (QTL) and genomic selection also require the isolation of DNA from a large number of samples and the preservation of tissue samples for future use. Widespread application of MAS and molecular characterization often require the collection of tissue samples from sites distant from laboratories (such as germplasm collections or fields) or reference populations. This requires proper methods for the conservation of the samples. The most common method used to preserve samples for subsequent DNA extraction is a rapid dehydration of the leaves mainly

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Table 1. Factors tested to optimize the maceration process.

Type of tissue	Number of spheres	Number of leaf disks	Size of the sphere (mm)
Fresh	1	4	6
Lyophilized	1	3	6
Fresh	1	5	4
Fresh	1	4	3
Fresh	1	6	2
Lyophilized	3	4	2
Lyophilized	2	4	5
Lyophilized	3	4	3
Lyophilized	1	4	6

on silica gel (Chase and Hills, 1991). This method is effective for many species with rapid rates of desiccation that prevents DNA degradation. However, improper use of silica gel (too little for a large amount of tissue or insufficiently dry) may result in poor desiccation of leaf tissue and DNA degradation.

In the present study, we report a methodology for DNA extraction based on lyophilized tissue. The use of lyophilized tissue offers several advantages. Dry tissue can be efficiently disrupted while the DNA is unhydrated and thus less susceptible to shear. Since dry tissue can be stored for several years with little loss in DNA quality (Murray and Thompson, 1980), it can be a perfect association with methods for DNA extraction suitable for genotyping and genetic studies. The optimization of this initial stage of collection and storage of plant material depend on the success of subsequent steps of any molecular study. Another major bottleneck encountered in most genomic and molecular markers laboratories, besides the preservation of sample tissue, is the slowness in the maceration of samples. Much of the slowness is due to the way the leaves are crushed. Usually, the samples are macerated individually and transferred to specific tubes, increasing the time for extraction and the risks of contamination and misidentification in the transference from the mortar to tubes. This step is usually performed using mortar and pestle (conventional method) in the presence of large amounts of liquid nitrogen. Equipments specially designed for the maceration of plant tissues in large scale using commercial beads exist in the market (beadbeaters); however, they are usually quite costly, limiting their use in many laboratories, particularly those in third world countries.

Several DNA extraction protocols already available for cocoa have proven unsuitable because of the presence of high levels of mucilage, polysaccharides and polyphenolic compounds within the tissues. Furthermore, the published protocols developed specifically for cocoa are complex, requiring extensive configuration steps (Figueira et al., 1992; Laurent et al., 1993; Lanaud et al., 1995; Faleiro et al., 2002). The present study proposes a method for the preservation of sample tissues for DNA extraction and for manual extraction of large number of samples using spheres (spheres method). The integrity and concentration of the DNA by these methods were assessed and compared with conventional methods.

MATERIALS AND METHODS

Sample preparation and maceration using spheres method

Leaf disks of 15 mm in diameter (approximately 50 mg of lyophilized tissue) per cacao plant were excised from young, green, fleshy leaves, showing no or minimal damage from microorganisms or insects, previously cleaned with a solution of sodium hypochlorite at 1%. Disks were placed inside 2.0 ml Eppendorf tubes. The leaf discs collected were immediately stored in a freezer at -80°C. Prior the extraction, the leaf disks were lyophilized for 24 h in the microtubes, capped and stored in desiccators for conservation free of moisture until use. The leaf disks were then placed in 2 ml microtubes. On these microtubes it was placed stainless steel balls or spheres of different diameters and quantities (Table 1; Figure 1a). The sets of tubes were then immersed in liquid nitrogen (Figure 1b) and then placed inside a plastic box with lid for storage of the microtubes and posterior maceration. In the maceration process, the box with the microtubes was manually stirred for 30 s (Figure 1c), producing a very fine powder (Figure 1d). Aiming to optimize the maceration process using spheres, the effect of various factors were tested (Table 1) in order to obtain a fine powder, required in the next steps of the DNA extraction.

DNA extraction

DNA extraction was performed according to the procedure described in Risterucci et al. (2000) with some modifications to a set of 20 cacao trees from different progenies used for genetics studies. The fine powder (50 mg of tissue) obtained by manual maceration using mortar (conventional method) or the spheres method described earlier (Figure 1d) was mixed with 850 µL of extraction buffer (1.4 M NaCl, 100 mM Tris HCl pH 8.0, 20 mM EDTA, 10 mM Na_2SO_3, 1% PEG 6000, 2% MATAB) pre-heated to 74°C. The extract was then homogenized for 10 s with a vortex and incubated for 30 min at 74°C; after cooling at 20°C (Figure 2a), an equal volume of chloroform-isoamyl alcohol (24:1 v/v) was added, followed by emulsification. The tube was then centrifuged at 7000 g for 30 min (Figure 2b) and the supernatant was precipitated at - 20°C overnight after the addition of an equal volume of isopropanol (Figure 2c). The DNA was removed with a sterile tip (Figure 2d) and re-suspended in 200 µL of TE buffer (50 mM TRIS-HCl, 10 mM EDTA, pH 7.0) with RNAse at 1%. As a control, the proposed

Figure 1. Proposed cacao leaf maceration protocol: a) tube, steel spheres and 15-mm leaf disks; b) freezing the tubes containing the spheres and the leaf discs lyophilized in liquid nitrogen; c) manual agitation of a set of samples; d) fine powder obtained.

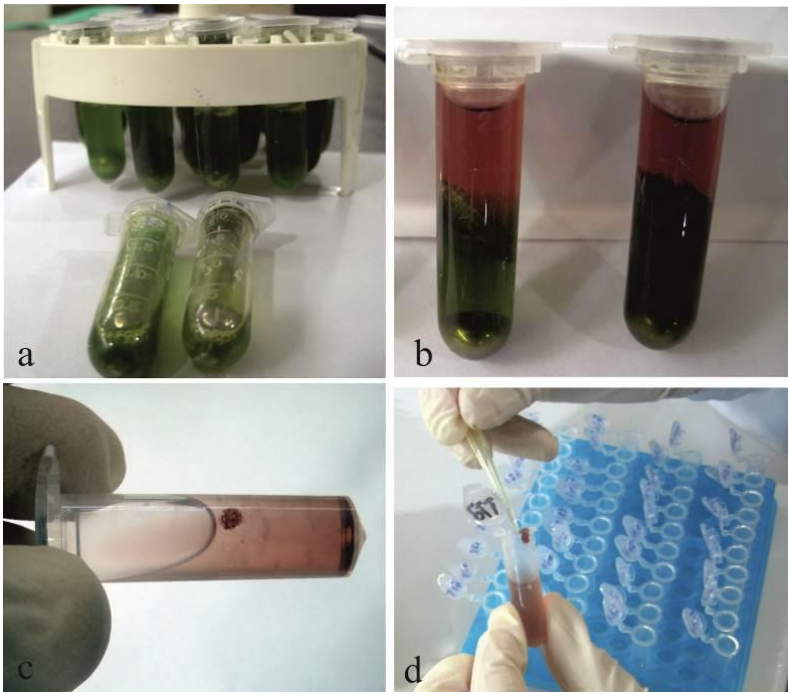

Figure 2. The extraction process: a) leaf disks with the extraction buffer; b) formation of the supernatant after centrifugation; c) formation of the pellet after addition of isopropanol; d) removal of the pellet with sterile tip.

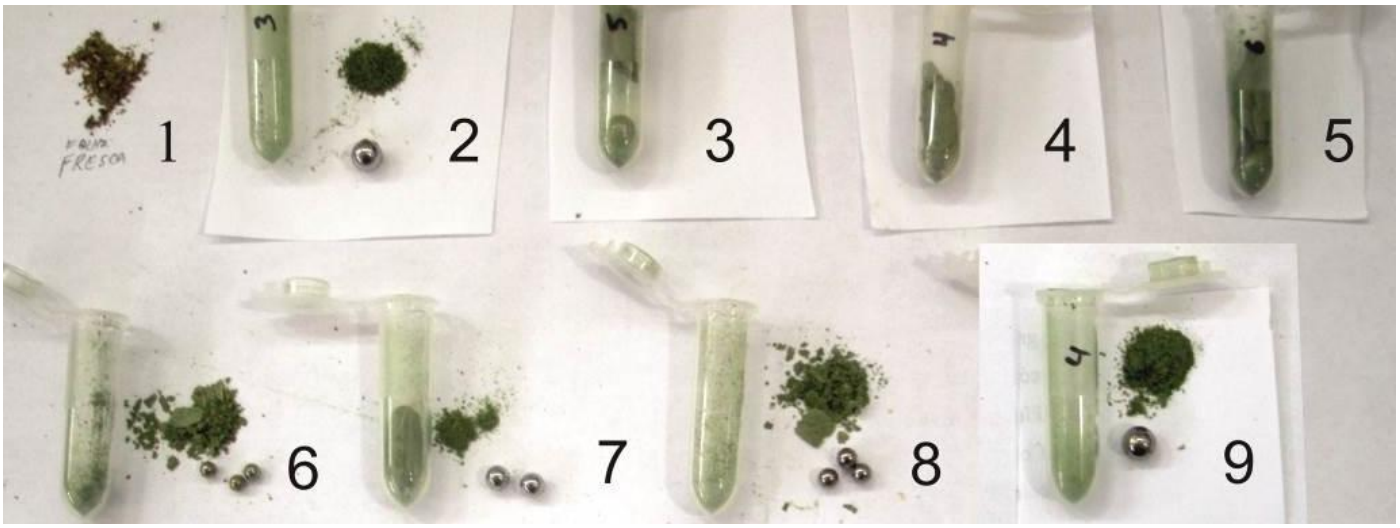

Figure 3. Parameters tested to optimize the maceration process ranging between: type of tissue, quantity of spheres, number of leaf disks and size of sphere (mm), respectively. 1 = fresh, 1, 4 and 6; 2 = lyophilized, 1, 3 and 6; 3 = fresh, 1, 5 and 4; 4 = fresh, 1, 4 and 3; 5 = fresh, 1, 6 and 2; 6 = lyophilized, 3, 4 and 2; 7 = lyophilized, 2, 4 and 5; 8 = lyophilized, 3, 4 and 3; 9 = lyophilized, 1, 4 and 6.

protocol was compared with the standard protocol used for DNA extraction in cacao, using the 20 same samples macerated using mortar, but now using fresh leaf material. After this step the same procedure described earlier was performed.

DNA quantification

Aliquots of 1 µL of extracted genomic DNA were submitted to electrophoresis on 1% agarose gels, stained with GelRed Nucleic Acid Stain (Biotium) and visualized using a transluminator with a system of image capture L-PIX IMAGE 7.1. Images were captured with the software L-PIX IMAGE 1.0.1 (Loccus Biotecnologia, Brazil). Phage lambda DNA (sigma) was used as marker ladder. For each sample, the total DNA extracted was quantified by optical density using a Picodrop spectrophotometer (Picodrop Limited, UK). The ratio A260 /A280 was used to assess the purity of DNA (Sambrook et al., 1989).

PCR amplification using cacao single sequence repeat (SSR) primers

Four samples of isolated DNA were randomly selected and subjected to PCR amplification with two cacao SSR primers [mTcCIR24 and mTcCIR35 (Lanaud et al., 1995)]. The total volume of the PCR reaction was 20 µL, which contained 2 µL of freshly extracted DNA (~ 20 ng), 2 µL of 10x PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 1.2 µL of 25 mM $MgCl_2$, 2 µL of each forward and reverse of 2 pmol primer, 1.6 µL of 2.5 mM dNTPs (dATP, dCTP, dGTP and dTTP), 0.5 µL of 5U of Taq polymerase (Fermentas). Amplifications were carried out in a termocycler Mastercycle (Eppendorf). The PCR cycle consisted of initial denaturing at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 51°C annealing for 1 min and 72°C for 1 min. This was followed by further primer extension at 72°C for 7 min. After PCR samples are subjected to electrophoresis on capillary ABI3100 automated sequencer (applied biosystems).

RESULTS

The best parameters in order to obtain a fine powder using spheres for obtaining a suitable material for DNA extraction is the use of 4 lyophilized leaf disks (50 mg), a single steel ball of 6 mm in diameter, followed by 30 s of manual maceration (Figure 3; picture 9). In a dried state, tissue can be ground or pulverized into a fine powder by mechanical means increasing the efficiency of DNA isolation (Figure 3; pictures 2, 6, 7, 8 and 9). For maceration use, mortar was needed more than 4 leaf discs, because of excessive loss of the material in the walls of mortar. The quantity of DNA obtained using the conventional method (mortar and fresh leaves) ranged from 4.5 to 15.7 µg of total DNA, the average being 10.74 µg. On the other hand, the quantity of DNA obtained with the spheres method and lyophilized tissue ranged from 14.6 to 75.5 µg, with an average of 49.17 µg. The A260/A280 ratio was between 1.7 and 2.0, in 40% of the samples using the conventional method and 90% using the spheres method, indicating low contamination with polyphenols, proteins and polysaccharides according to the A260/A280 ratio (Table 2). The purity of the DNA obtained was satisfactory and visible on agarose gel (Figure 4) compared with the conventional method and proved to be amplifiable by PCR using SSR primers (Figure 5).

DISCUSSION

In this study, a manual method of large scale DNA extraction was standardized to isolate DNA from cacao leaf samples. The protocol was then used to extract DNA from several cacao accessions under study in our laboratory. The sampling of the cacao leaves used in this study allowed for efficient storing and normalization of the plant material obtained from lyophilized cacao tissues. This approach opens the possibility for the creation of leaf banks of lyophilized material for cacao, in order to preserve important genotypes, by storing the leaves

Table 2. DNA quantity total (in µg) and ratio A260/A280 from the samples using the conventional and spheres methods.

Plant	Conventional method		Spheres method	
	Quantity (µg)	Ratio 260/280	Quantity (µg)	Ratio 260/280
1	8.4	1.44	50	1.70
2	15.6	1.33	49.7	1.83
3	8.4	1.42	55.6	1.81
4	7.4	1.52	58.9	1.91
5	8.3	1.80	50.7	1.70
6	12.6	1.91	63.6	1.81
7	9.5	1.90	14.6	1.56
8	4.5	1.73	40.4	1.78
9	7.8	1.41	45.8	1.81
10	9.5	1.57	53.5	1.80
11	14.6	1.60	65.4	1.92
12	5.5	1.40	75.5	2.00
13	14.6	1.72	63.5	2.00
14	8.9	1.80	64.2	1.41
15	13.6	1.43	33.1	1.86
16	15.7	1.54	40	1.84
17	14.6	1.61	40	1.91
18	5.7	1.40	44.8	2.00
19	15.7	1.70	44.5	1.92
20	14	1.73	29.6	1.72
Mean	10.74	1.61	49.17	1.81

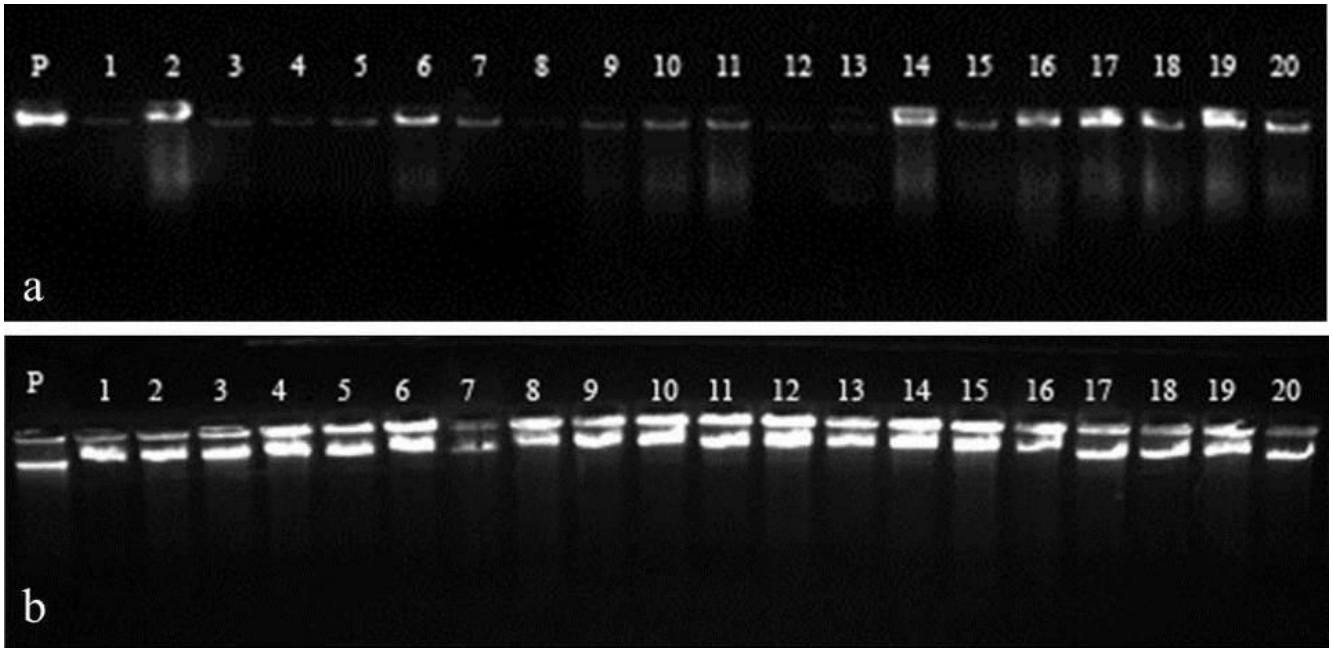

Figure 4. Agarose gel analysis of 20 genomic DNA extracted utilizing conventional method a); 20 same genomic DNA extracted utilizing spheres method b); P, Phage Lambda DNA (100 ng).

before the plant dies. The two major bottlenecks in DNA isolation from large populations of plants are the processing of the tissue to an extractable form and the storage (Tai and Tanksley, 1988). Dry tissue can be stored for

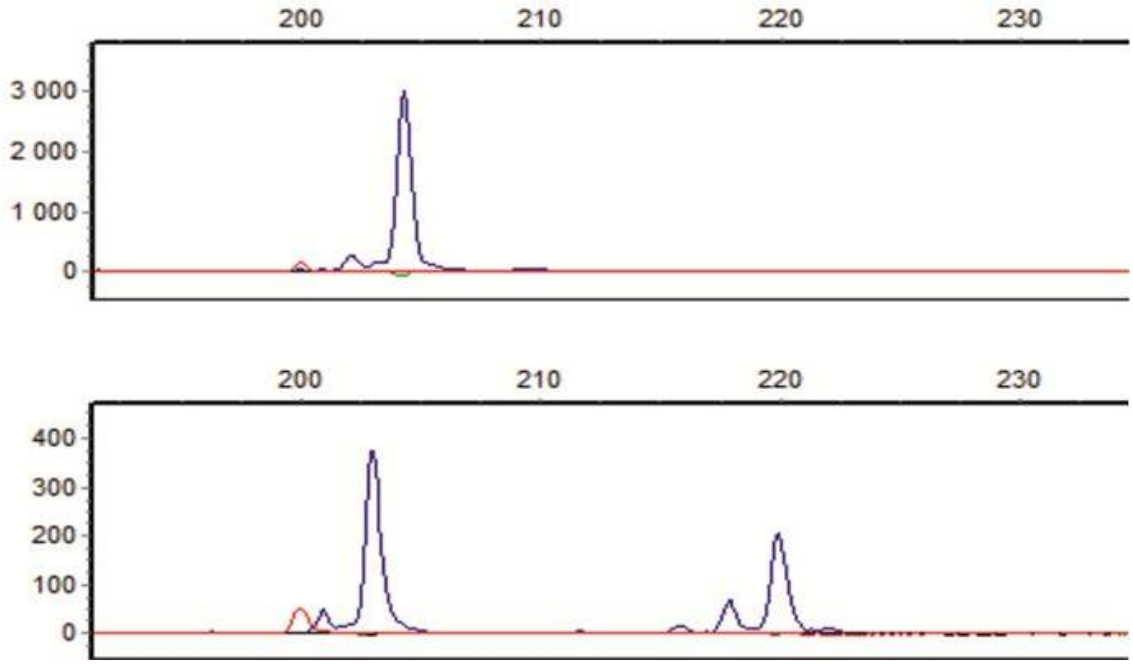

Figure 5. Representative data output for analysis of cacao DNA with marker mTcCIR24. The marker were amplified in a single PCR reaction and separated by capillary electrophoresis. The upper panel depicts the homozygous plants for the alleles, and the lower panel depicts the heterozygous plants. The alleles are represented by the peaks. The sizes in base pairs (bp) are indicated on the top x axis. The values on the y axis indicate fluorescent signal intensity given in relative fluorescent units (RFU) and vary for each sample.

several years with little loss in DNA quality and can be ground or pulverized into a fine powder by mechanical means which increases the efficiency of isolation (Figure 3). Hence, the use of 4 lyophilized leaf disks is sufficient to obtain sufficient amount of DNA in this extraction procedure. Another important point in this procedure also is the fact that it permitted the elimination of the use of liquid nitrogen for maceration, reducing the costs of DNA extraction. According to Tai and Tanksley (1988), the isolation of DNA from plants previously dried proved more efficient than the use of fresh tissue. The major advantage associated with the lyophilization is the decrease in the water content into the tissues and consequently, the reduction of the catabolic process inside the cell, since a less fluid environment slow down the catalytic activity of nucleases and proteases.

Another benefit of the lyophilization is the improvement of the relationship between extraction buffer and dry tissue. With less fluid in the tissue, the dilution of the extraction buffer is smaller and therefore, its activity is better, reflecting a greater amount of DNA extracted (Table 2 and Figure 4b). Unlike other DNA extraction protocols, the modified method presented here was effective to avoid the DNA oxidation in their very beginning. Despite the whole process of lyophilization requires about 24 h for complete dehydration, its applicability is certainly satisfactory, since its inclusion improves the quality and amount of the DNA purified allowing them to be easily removed from the samples before they irreversibly oxidize the DNA molecule. Five minutes was the time required to do the following steps: placing the steel spheres in the 20 tubes, immerging the tubes in liquid nitrogen, placing in the box the tubes, performing the manual agitation, adding the extraction buffer and carrying out the homogenization. In the conventional method, using mortar and pestle, it would take approximately 30 min to perform the same operations. The cost of the beads is extremely low; moreover, they can be reused many times, as long as previous DNA residues are adequately removed between extractions. Decontamination can be done by using the same procedures used to decontaminate the mortar and pestles.

In this study, we preferred the extraction protocol containing MATAB (Risterucci et al., 2000) because it contains fewer steps than the conventional protocol being less laborious and faster. MATAB combined with the use of spheres method for maceration probably reduce the amount of polyphenols and polysaccharides resulting in a viscous substance typically associated with DNA extractions from cacao (Faleiro et al., 2002). These complex molecules interfere with DNA quality, leading to low yields (Tel-Zur et al., 1999), and inhibiting the action of the Taq polymerase (Fang et al., 1992). Hence, their removal is crucial for successful use of DNA in molecular techniques. The quantity of DNA obtained was four times higher than the conventional method utilizing the same quantity of tissue, since it shows greater uniformity, no degradation visible in agarose gels compared with the

conventional method (Figure 4a and 4b). The quality of genomic DNA is evident from high-molecular-weight bands and the absence of RNA along with other polysaccharides that usually affect migration during electrophoresis (Figure 4b) and seen frequently in plants (91%) with ratio A260/A280 between 1.7 and 2.0 (Table 2). The quantity of DNA obtained was high, sufficient for large number of PCR reactions. Amplification products were obtained for all the DNA samples tested with different cacao SSR primers (Figure 5). All samples under investigation amplified the PCR product expected for the SSR primers. Thus, we conclude that the present approach is a reliable, rapid, simple and consistent DNA isolation method for cacao, compared to the conventional methods.

The fact that DNA was successfully extracted indicates that this technique should work on a wide range of plant tissues. This method appears to be very flexible and may be scaled up or down depending upon the need. The spheres method processing of tissue in conjunction with the Risterucci et al. (2000) protocol greatly increases the efficiency of extraction and suggests an inexpensive and practical way of DNA isolation of cacao for large scale.

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