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OPTIMIZING GENOMIC DNA ISOLATION IN PINEAPPLE (*ANANAS COMOSUS* L.)

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A B S T R A C T

The isolation of high molecular mass genomic DNA (Deoxyribonucleic acid) is crucial for applications in molecular biology. To this end many protocols were developed for the extraction of plant DNA. However, for pineapple (*Ananas comosus)*, standard protocols are scarce and not always efficient when resources are limited. In this study, we developed a new protocol for nuclear DNA extraction in pineapple. Four existing protocols were tested but none has provided high quality DNA extract. The original laboratory standard protocol based on the use of CTAB (Cetyltrimethylammonium bromide) was successfully modified to optimize the quality of the DNA extract using eighteen pineapple young leaf samples including three parts: the leaf apex, the mid blade, and the leaf base of two cultivars (i.e. Sugarloaf and Smooth Cayenne). The successful extraction of DNA in a sister species, *Ananas bracteatus,* gave evidence that the protocol can be used for others Bromeliaceae. The new protocol yielded 51.76 µg/ml DNA, which is higher than that obtained with previous protocols. The DNA extract was efficiently PCR (Polymerase Chain Reaction) amplified using simple sequence repeat primers. We proposed henceforth the use of this protocol for further DNA isolation in pineapple particularly under limited resources condition when using CTAB.

Keywords: *Ananas bracteatus, Ananas comosus*, Bromeliaceae, CTAB, genomic DNA extraction, MATAB.

INTRODUCTION

Application of genomic techniques, for instance in crop improvement or phylogenetic studies, requires efficient and reliable DNA extraction procedures (Allen *et al.*, 2006; Gawel and Jarret, 1991). DNA extraction and purification are the first steps in molecular genetics and for PCR amplification. Several methods are available for DNA extraction from plant material (Michiels *et al.*, 2003). However, the extraction of pure and sufficient DNA, adequate enough for genomic analyses, has for the last decades been a concern (Chabi Sika *et al.*, 2015; Gawel and Jarret, 1991; Ghosh *et al.*, 2009; Murray and Thompson, 1980). Some of those extraction methods are found lengthy; others are expensive commercial kits though available; and others use very hazardous chemicals (Chabi Sika *et al.*, 2015).

DNA purity is important to assure reproducibility of genomic studies (Stewart and Via, 1993). Using cetyltrimethylammonium bromide (CTAB) DNA extraction protocols, Michiels *et al.* (2003) found that the quantity and purity of DNA varied with the incubation time and the precipitation temperature. In the work of Michiels *et al.* (2003), CTAB extraction protocols, successful on cotton, blackcurrant, ferns, and fruit trees, were unsuccessful for latex-containing plants. For *Musa* and *Ipomoea* genomic research Gawel and Jarret (1991) used another modified CTAB DNA extraction protocol. According to Ghosh *et al.* (2009) DNA isolated by the standard CTAB method either did not amplify in PCR or gave inadequate results due the presence of mucilage compounds. Sometimes, CTAB method results in DNA degradation (Fang *et al.*, 1992). However, CTAB-isolated plant DNA is routinely used for many purposes including mapping, cloning, transgenes detection, transgenic plant identification, marker-assisted plant breeding (Allen *et al.*, 2006).

Although many studies used genomic techniques (e.g. RAPD, AFLP, SSR) to analyse genetic diversity (Duval *et al.*, 2003; Duval *et al.*, 2001; Kato *et al.*, 2004; Machado *et al.*, 2011) or linkage mapping (Carlier *et al.*, 2004) in

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pineapple, only a few of them proposed clear DNA extraction protocols for this genetically variable crop. Moreover, several published DNA extraction protocols tested in pineapple were not successful when applied to our samples. Besides, none of them gave indication on which parts of the pineapple leaf yielded sufficient DNA amount for PCR amplification. The choice of a particular protocol may depend to a large degree on the plant species used (Dellaporta *et al.*, 1983) but also on the leaf part used and this might be true for pineapple.

In this study we compared four DNA extraction protocols on different pineapple leaf parts to suggest an optimized CTAB protocol that consistently yields high-quality amplifiable DNA for genomic studies in the species.

MATERIALS AND METHODS

Plant material: Six samples of young leaves (three samples of Sugarloaf and three of Smooth Cayenne) collected from the pineapple live collection of the Faculty of Agronomic Sciences of the University of Abomey-Calavi in Sékou (South Benin) and two samples of manihot (as check) were used in this study. Each pineapple leaf sample was divided into leaf base, mid blade and leaf apex resulting in 18 sub-samples. These samples were washed with tap water and cleaned before DNA extraction. Six samples of ornamental pineapple *(Ananas bracteatus (Lindl.) Schult. & Schult.f.)* were tested to ascertain the usability of the optimized protocol for Bromeliaceae.

DNA isolation protocol: The extraction buffer used for initial homogenization was made up of 500 mM Tris, 5 mM EDTA and 1.3 M NaCl. The buffer used for the membranes lyse was autoclaved; 0.1% β mercaptoethanol (BME) was added immediately before used. For each protocol the concentrations of CTAB or MATAB, NaCl, Tris, EDTA and BME used are detailed in Table 1. We took 0.6 g of fresh young leaf that was cut into pieces of approximately 1 mm size. We used mortar and pestle to grind leaf samples added with 2 ml of initial buffer extraction, which was centrifuged for 10 min at 10000 rpm and 4°C. The supernatant was transferred and added with 750 µl of the lyses solution for 90 min incubation at 65°C. We added the mix of chloroform isoamyl 24:1 after incubation; the mixture was centrifuged at 10000 rpm for 15 min at 4°C. The aqueous phase was transferred to another tube and the DNA was precipitated by adding ice-cold isopropanol and stored at –20°C for 30 min to 1h. This was further centrifuged at 10000 rpm for 15 min at 4°C. The supernatant was removed carefully and the pellet washed with ethanol (70°) thrice and dried at 37°C. The pellet was finally dissolved using 100 µl of TE buffer. The extract was run on ethidium bromide stained agarose gel (1%).

Table 1. Buffer solutions for membrane lysis and incubation times in five protocols used for pineapple DNA isolation.

Protocol Tested	Chittenden *et al.* (1994)	Gawel and Jarret (1991)	Vanijajiva (2011)	Agbangla *et al.* (2002)	New protocol
CTAB (%)	1	2	1	-	2
MATAB (%)	-	-	-	4	-
Tris (mM), pH=8	100	100	50	100	500
NaCl (M)	0.7	1.4	0.7	1.4	1.3
EDTA (mM)	10	20	10	20	5
βmercapto ethanol (%)	1	0.1	0.1	-	0.1
Incubation (min) (65°)	30	30	30	90	90

DNA quantification: The DNA yield was estimated using a spectrophotometer at UV-VIS 230, 260 and 280 nm. The purity of the DNA was determined by calculating the ratio A260/A280 nm to assess the protein contamination and A260/230 to assess polysaccharide contamination (Wilson and Walker, 2010). The DNA concentration was calculated with the following formula:

$$[DNA] = A_{260} \times DF \times 50\ \mu g/ml,$$

where [DNA] is the DNA concentration, A_{260} is the Absorbance at 260 nm, DF = Dilution Factor; 50 µg/ml is the Concentration of DNA when $A_{260} = 1$.

PCR amplification: PCR amplification was carried out to check the quality of the DNA. Three samples of DNA and four single sequence repeat (SSR) markers of *Ananas comosus* (Table 2) were used to amplify the genome as described by Kinsat and Kumar (2007) and Rodríguez *et al.* (2013). The PCR was performed in the thermal cycler; the volume of the mixture was 25 µL containing 3 µL of genomic DNA. The mixture was subjected to the following PCR program: an initial step of 1 min at 94°C followed by 35 cycles of 30s denaturing step at 94°C, 30s annealing at 55°C and 1 min extension at 72°C, and a final step of 5 min final extension at 72°C. The amplification products were resolved electrophoretically on a 2.5% agarose gel stained with ethidium bromide and visualized using UV light.

Table 2. Microsatellites markers of *Ananas comosus* used for PCR amplification.

Locus	Primer sequence (5'-3')	Repeat motif	Size (bp)
ACPCT651BM	F: GATACATAACAGTGTATTGGAG R: TAACTACTCTATGTTGTGACCA	$(GAA)_{13}$	219–245
ANBR58	F:ATATGATAGGACTTACTTTTGG R: AAGGCTACAGATAGTTAAAGAG	$(CT)_{21}(CA)_{21}$	227–243
ANBR73	F: CATTAGATTAGTTCACAAACAA R: AGAATATTATGGAAAAATTGAG	$(CT)_{17}$	211–227
ANBR75	F: ATGATCTCCTAAAAATCATAAG R: CTTAATTAGGGTTTTATTTGTC	$(GA)_{30}$	210–220

RESULTS

Electrophoresis analysis revealed the presence or absence of DNA in the samples for each DNA extraction protocol (Figure 1a, b, c, d, e). The DNA extraction protocols of Chittenden et al. (1994) and Vanijajiva (2011) showed weak bands for the first six samples which correspond to leaf base and very weak ones for the others samples (e.g. mid blade and apex) (Figure 1a and c). The protocol of Gawel and Jarret (1991) revealed weak bands for all leaf parts (Figure 1b). However, the Mixed Alkyltrimethylammonium bromide-based protocol of Agbangla *et al*. (2002) revealed no bands, which may indicate the absence or the very weak presence of DNA (Figure 1 d). Our new protocol showed bands for all individuals and the intensity of these bands decreased from the first samples to the last (Figure 1E). Compared to the first two protocols, the new protocol revealed the most intense bands. It showed that whatever the cultivar and the leaf part used, the leaf base produced more intense band than the other parts. The new protocol yielded more DNA compared to others (Table 3).

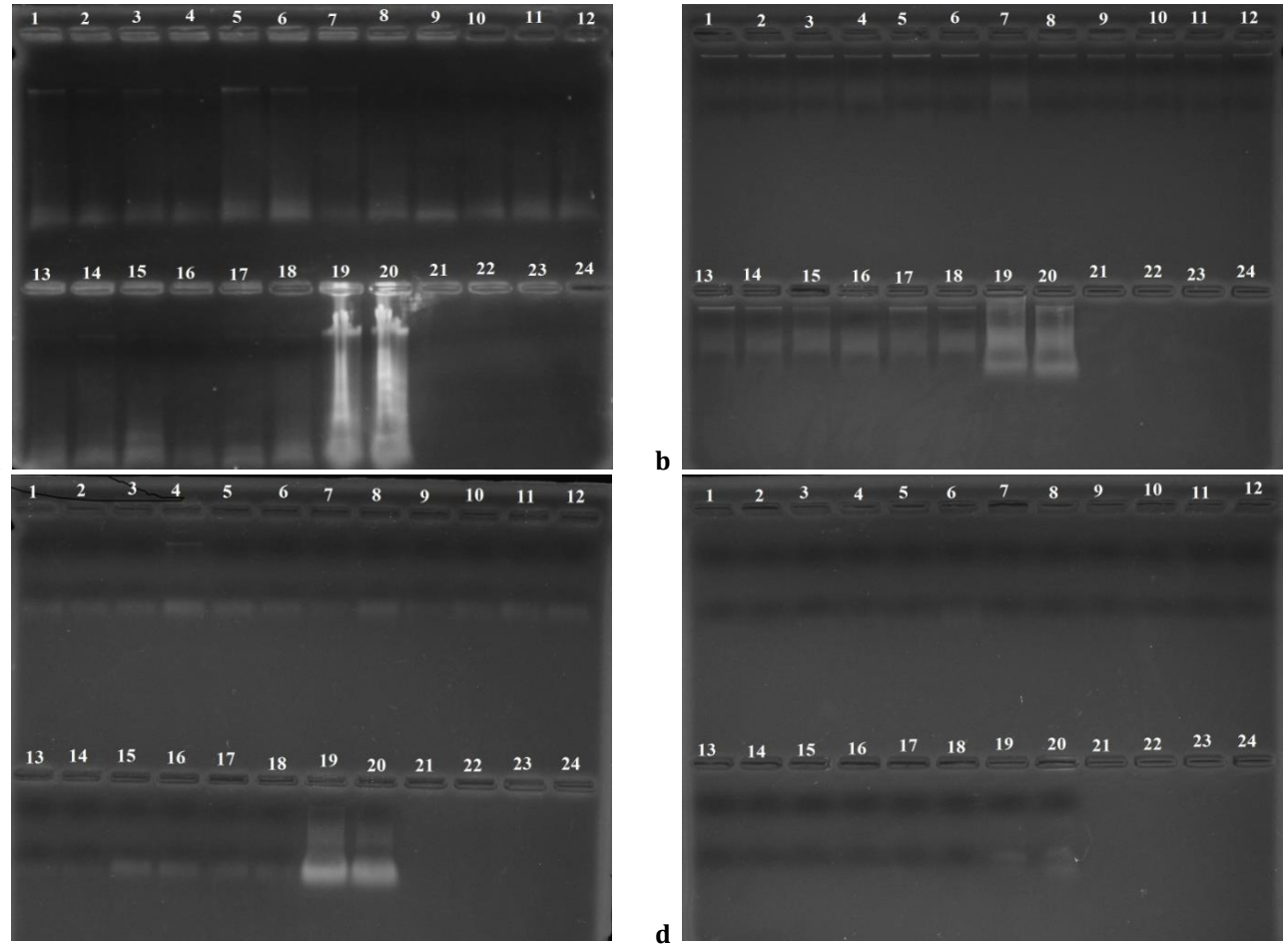
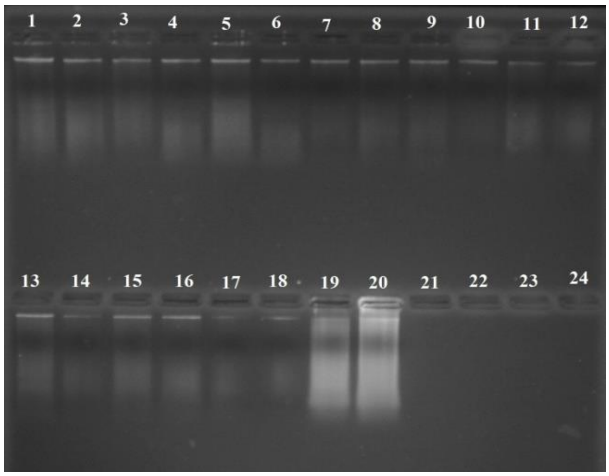


Figure 1 (a, b, c, d). Genomic DNA extracted from Sugarloaf and Smooth Cayenne leaves using five protocols.

Cont…



a. Chittenden *et al*. (1994),
b. Gawel and Jaret (1991),
c. Vanijajiva (2011),
d. Agbangla et al. (2002),
e. new protocol

e

Figure 1(e). Genomic DNA extracted from Sugarloaf and Smooth Cayenne leaves using five protocols.

1, 2, 3, 4, 5, 6 are leaf base lanes; 7, 8, 9, 10, 11, 12 are mid blade lanes, 13, 14, 15, 16, 17, 18 are leaf apex lanes and 19, 20 are control lanes. Sugarloaf DNA was loaded in 1, 2, 3, 7, 8, 9, 13, 14, 15 and the Smooth Cayenne DNA was loaded in 4, 5, 6, 10, 11, 12, 16, 17, 18.

Table 3: DNA yield and purity access by 260/230 and 260/280 ratios resulting from five protocols. The yield was estimated for 1g of fresh leaf.

Protocol	DNA yield µg/ml	Ratio 260/230	Ratio 260/280
Chittenden *et al.* (1994)	32.81 ±12.12	1.32±0.83	1.00±0.96
Gawel and Jarret (1991)	24.85 ±10.82	1.24±1.12	1.06±1.62
Vanijajiva 2011	24.85 ±5.57	1.16±0.92	1.10±0.09
Agbangla *et al.* (2002)	20.13 ±6.98	1.10±0.64	1.04±0.11
New protocol	51.76 ±9.20	2.07±0.17	1.83±0.12

The mean DNA amount of the four old protocols (Agbangla *et al.*, 2002; Chittenden *et al.*, 1994; Gawel and Jarret, 1991; Vanijajiva, 2011) and the new one was 32.81 µg, 24.85 µg, 24.85 µg, 20.13 µg, and 51.76 µg respectively. In the same time, the ratio 260/230 and 260/280 respectively varied from 1 to 1.1 and 1.10 to 1.32 for the four old protocols and was 1.83 and 2.07 for the new protocol. The CTAB protocol yielded more DNA than the MATAB protocol. The variation of the DNA yield indicated that the leaf base yielded higher amount than the others parts whatever the protocol (Table 4). The amount of DNA is 34.5 µg/ml for leaf base with the new protocol.

Table 4: Variation of DNA yield isolated from different parts of pineapple leaf according to the five protocols. Bold figures indicated means values.

Protocols	DNA µg/ml		
	Leaf basal	Leaf blade	Leaf apex
Chittenden *et al.* (1994)	2 – 33.5	0.5 - 22	3.5 - 19
	16.17	12.25	8.08
Gawel and Jarret (1991)	9.5 - 44	0.5 – 27.5	3.5 - 20
	23	10.53	11.16
Vanijajiva (2011)	6 – 21.5	14 - 21	5.5 – 21.5
	14.08	16.33	14.33
Agbangla *et al.* (2002)	2 - 19	5 - 25	5 - 26
	10.25	11.90	12.16
New protocol	22 - 45	18.5 - 40	17.5 – 42.5
	34.5	32.15	31.28

The DNA obtained with the new protocol was very well PCR amplifiable when using simple sequence repeat (SSR) with the four SSR markers (Figure 2). The DNA migration profile of those samples was presented in the Table 5. The protocol was successfully tested on the ornamental pineapple *Ananas bracteatus* which yielded high quality DNA (Figure 3).

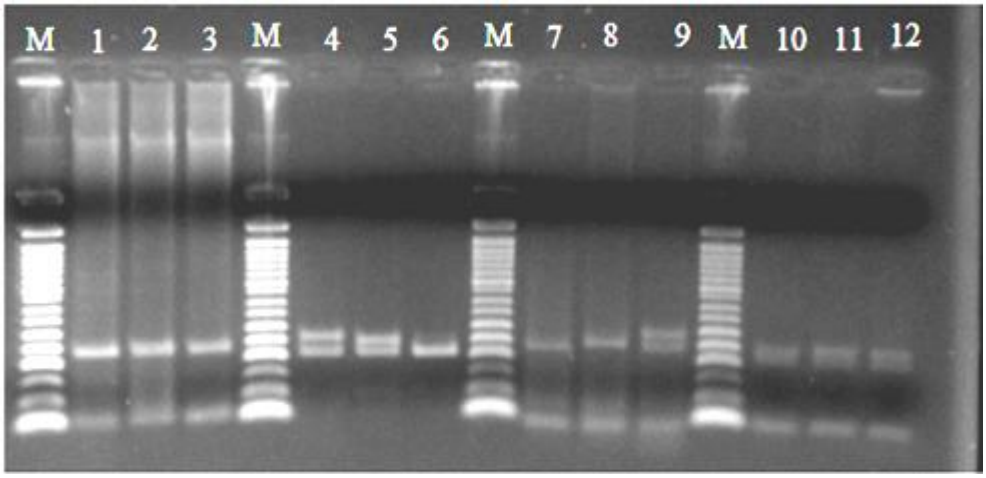


Figure 2: Agarose gel (2.5%) showing PCR amplification of genomic DNA from three samples of pineapple with four SSR markers. Samples 1,2,3 are amplified product of the first SSR marker ACPCT651BM; 4, 5, 6 are amplified product of the marker ANBR58; 7, 8, 9 are amplified product of the marker ANBR73; 10, 11, 12 are amplified product of the marker ANBR75. M is the DNA ladder

Table 5: DNA migration profile of pineapple (*Ananas comosus*) cultivar based on four microsatellite marker gel analysis.

Marker	Cultivar		
	1	2	3
ACPCT651BM	—	—	—
ANBR58	—	—	—
	—	—	
ANBR73	—	—	—
			—
ANBR75	—	—	—
	—	—	—

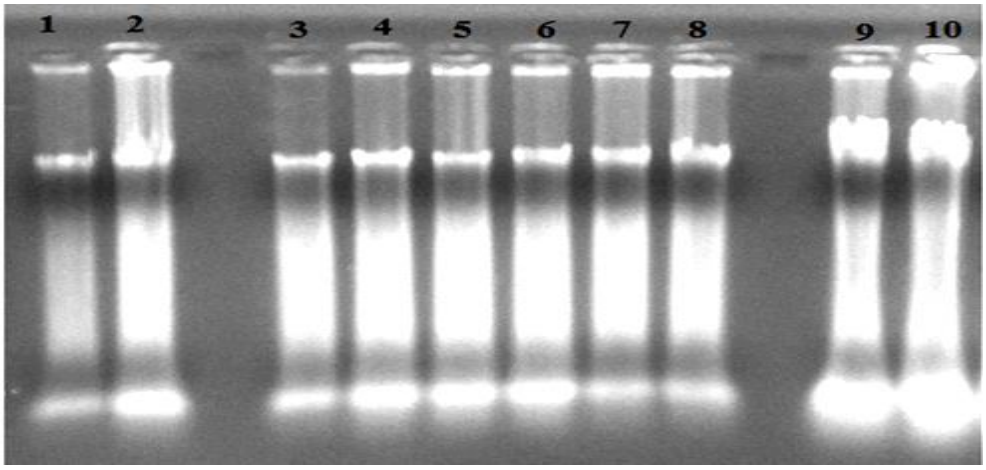


Figure 3: Genomic DNA isolated from ornamental pineapple leaves. Samples 1 and 2 are Sugarloaf and Smooth Cayenne; Samples 3, 4, 5, 6, 7, 8 are the ornamental pineapple; Samples 9 and 10 are the control (Cassava).

DISCUSSION

For the first time a DNA isolation protocol was specifically developed for pineapple. This is a simple modified cetyl trimethylammonium bromide method proposed for isolation of pineapple DNA in order to perform molecular biology applications. The major modifications made in our protocol included the standardization at varying concentration of Tris-HCl, β-mercapto ethanol and NaCl (Table 1). The concentration of those elements in the old protocols certainly explained the low rate and/or absence of DNA extracts.

Several DNA isolation methods were available in the literature (Dellaporta *et al.*, 1983; Doyle, 1987; Murray and Thompson, 1980; Ripoll *et al.*, 2011). Those methods although efficient were rarely applicable with success for all taxa or biological material. In this study, we encountered many difficulties from the very first step of cell lysis to DNA separation in the supernant reaction when we tested the four old methods. Highly viscous pellets were difficult to manipulate and the low A260/A230 and A260/A280 indicated contamination by protein, polysaccharides and phenolic compounds (Moreira and Oliveira, 2011). The ratio A260/230 and A260/280 were lesser than 1.5, which was below the optimal limit of 2 and 1.8 (Sambrook and Green, 2012) and made the extracts no amenable for molecular study.

In this study we have optimized some steps of Gawel and Jarret (1991) methods to suggest a new pineapple DNA isolation protocol with higher DNA amount. The high contaminations of DNA obtained in the previous protocol can be caused by many factors during extraction. For instance, polysaccharides contaminations may be problematic when present in DNA extraction (Bandaranayake, 2002). The contamination by polysaccharides makes the DNA unamplifiable by inhibition of Taq polymerase activity (Fang *et al.*, 1992). The quality and purity of DNA using the new protocol were excellent for all samples as evidenced by A260/A280 and A260/A230 ratios (Poms *et al.*, 2001). In the same time the DNA extract on the basal leaf part was higher and presented free polysaccharide contamination (ratio A260/A230 = 2.07). The utilization of the basal leaf part was ideal to obtain good quality DNA.

The extracts obtained with the four old protocols may contain debris, polysaccharides, proteins and other components, which interfered with DNA, and were difficult to eliminate. Pineapple is rich in protease (Bromelain) (Bitange *et al.*, 2008) and consisted of about 131 amino acid sequence identified for food profiling and also contained ethyl acrylate used in creams, detergents, lotion and soaps (Opdyke, 1975; Reindl *et al.*, 2002). The modifications made in the new protocol contributed to permealize the cell membrane. β mercaptoethanol was often included in plant DNA extraction buffer to break the outer membrane. It was a reducing agent which can remove tannin and polyphenols present in the plant extract (Verduyn *et al.*, 1985). It was also reported that the use of β mercaptoethanol successfully removed the polyphenols (Peterson *et al.*, 1997).

The increase in NaCl and Tris HCl concentration helped solubilize the polysaccharides present in the extract. Khanuja *et al.* (1999) reported that the high concentration of NaCl in plant DNA isolation helped remove polysaccharides, increase DNA yield and prevent DNA and polysaccharides interaction. Fang (1992) also indicated the use of the NaCl above 0.5 M in a CTAB protocol to eliminate polysaccharides. During cell lysis, Tris HCl and EDTA were used to maintain pH stability when removing unwanted cellular components and precipitation. The Tris HCl and EDTA complex also interacted with the lipopolysaccarides present on the outer membrane, serving to destabilize the membranes further (Varma *et al.*, 2007). In our study, the combination of NaCl and CTAB certainly has an effect on the membrane lyses. The variation in NaCl concentration in the isolation buffer also contributed to reduce the viscosity of the mucilage (Ghosh *et al.*, 2009). This NaCl concentration to be used varies according to the plant (Puchoda *et al.*, 2004).

The use of CTAB in pineapple DNA extraction is better than MATAB according to the DNA yield and the electrophoresis results. The same observation was made when it suggested the CTAB procedure for the suppression of polysaccharides and polyphenolic components which affect the DNA quality (Wagner *et al.*, 1987).

DNA fragments size obtained by PCR amplification with this protocol indicated same loci size (200-280 bp) obtained by Rodríguez *et al.* (2013). The DNA profile showed that the marker ACPCT651BM and ANBR75 are monomorphic and non-discriminant while ANBR58 and ANBR73 are polymorphic. This was a confirmation that the DNA extracted with this protocol can be used for genomic studies.

The protocol presented in this study is easy to use and did not contain any dangerous product. It provides high quality DNA usable for efficient genotyping analysis.

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