Extraction of high molecular weight abaca DNA suitable for next-generation sequencing

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Research

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Abstract

Background

The abaca (*Musa textilis* Née) is a fiber crop native to the Philippines with high economic value because of its fiber - the Manila hemp, known to be the strongest of all the natural fibers. DNA extraction in abaca is difficult due to its fibrous nature, high cellulose content and polyphenol compounds. Thus an optimized DNA extraction method is required for extracting high quality abaca DNA for next-generation sequencing applications.

Results

In this study, we have compared five different methods for the extraction of high molecular weight DNA from abaca leaves. The methods are the traditional CTAB method (Protocol 1), the CTAB with PVP method (Protocol 2), the CTAB with 0.3% β-mercaptoethanol method (Protocol 3), SDS-method (Protocol 4) and CTAB with Triton X-100 and PVP method (Protocol 5). Out of the five methods tested, traditional CTAB-method (Protocol 1), CTAB with 0.3% β-mercaptoethanol method (Protocol 3) and SDS-method (Protocol 4) have shown to be the most consistent in giving high molecular weight DNA with good yield and purity based on A260/A280 and A260/A230 absorption values. TissueLyserII was also utilized for homogenization for the three extraction protocols for applications in high-throughput DNA extraction. DNA from two abaca varieties were extracted using the CTAB with 0.3% β-mercaptoethanol method (Protocol 3) and were sent for NGS based on Illumina HiSeq platform having both passed the quality control for library preparation.

Conclusion

The CTAB with 0.3% β-mercaptoethanol method (Protocol 3) was found to be the simplest and most consistent method for extracting average yield DNA with high quality for NGS applications. The SDS-method (Protocol 4) was determined to have the shortest processing time and together with TissueLyserII is the most appropriate method for high-throughput extraction of abaca samples which will be useful for genotyping-by-sequencing (GBS) studies.

Introduction

The advent of next-generation sequencing (NGS) technologies has paved the way for the sequencing of whole genomes of important agricultural crops which is vital in elucidating favourable agronomic traits. Abaca (*Musa textilis* Née) is one of the most important cash crop of the Philippines owing to the world demand for its fibers [1]. Abaca fibers, internationally known as Manila hemp, are being used for various industrial applications such as raw material for the production of specialty paper, monetary notes, fiber composites for automobile parts and aerospace materials [1–3]. Currently, the Philippines supplies 85% of the world market demand for abaca fiber which generates US$111.5M earnings thus serving as an important source of livelihood for millions of Filipinos [4].

Although the abaca industry contributes to the majority of the country's economic growth, there is no study on the most suitable DNA extraction protocol to yield high quality or high molecular weight DNA suitable for NGS applications. Due to the high fiber content of abaca tissue, extraction of high quality DNA has remained a challenge. The abaca plant is a relative of the banana under the family *Musaceae* order *Zingibareles* [5, 6]. The abaca fiber is harvested from the pseudostem and is considered as the strongest natural fiber [1, 7]. It contains 56–63% cellulose, 20–25% hemicellulose, 7–9% lignin and 3% wax [8]. This chemical composition of abaca fiber gives it the characteristics of high tensile strength and high resistance to saltwater damage [1, 7]. It is therefore challenging to extract high quality DNA from the abaca due to the difficulty of disrupting the thick cell wall and the presence of high polysaccharide and polyphenol content.

Currently used DNA extraction protocols for abaca are based on the traditional (cetyltrimethylammonium bromide) CTAB-based method [9–12]. Boguero et al. have supplemented additives such as polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) to contend with the phenolics found in abaca tissues [9]. However, the protocol also requires an RNase A treatment step following DNA precipitation, washing and resuspension. These additional treatment steps prolongs the processing time and in the long run decreases overall DNA yield due to additional handling steps [13, 14]. Protocol by Sta. Cruz et al. [10] was adapted from the CTAB-based method of Su [15] which is optimized for banana leaf tissues. However the protocol requires two rounds of water incubation at 55^{o}C and 65^{o}C which extends the processing time to more than 3 hours. The existing protocol in our laboratory [11, 12] uses a modified CTAB-based protocol optimized for leaf tissues of bananas and sweet potato [16]. This has yielded average DNA quantities suitable for polymerase chain reaction (PCR) experiments [11, 12]; however, the protocol requires half a day to complete and lacks an RNase A treatment step. The long processing time poses a problem when large amounts of high quality abaca DNA must be extracted. Commercial DNA extraction kits are also available but are expensive and will not be cost-effective when processing a large number of abaca samples. Thus, a more simplified, consistent, cost- and time-efficient extraction method is required to be able to extract high quality DNA from abaca. The resulting DNA will not only be suitable for NGS applications but also for other advanced molecular biology techniques such as restriction enzyme fingerprinting, microsatellite marker development and genotyping-by-sequencing (GBS). This study therefore aims to compare the quality and quantity of DNA isolated from abaca leaf sample using five different DNA extraction methods.

Materials And Methods

Plant material and reagents preparation

To ensure availability of fresh leaf samples, mature abaca variety Abuab (NSIC 2017 Mt 001) was obtained from the PhilFIDA Albay Tissue Culture Laboratory. Ihalas, a wild abaca variety, was collected in the Leyte Province. The collected abaca varieties were housed at the National Institute of Molecular Biology and Biotechnology and Bureau of Plant Industry until sample processing. Leaf samples were collected from the second to the youngest leaf of the abaca plant.

To ensure a nuclease-free environment, all reagents were prepared in nuclease-free water (Ambion™, Invitrogen). Mortar and pestles and consumables were sterilized at 121^{o}C at 15 psi for 15 minutes and dried thoroughly at a drying oven (65^{o}C) prior to use. Stock buffers such as 1.0 M Tris-HCl (pH 8.0 and pH 7.5) (Scharlau), 0.5 M EDTA (pH 8.0) (Scharlau), 5M NaCl (Scharlau), 10% (w/v) CTAB (Sigma), 10% (w/v) (sodium dodecyl sulfate) SDS (Merck), 4M guanidine thiocyanate (Sigma), 5 M sodium acetate (pH 5.2) (Scharlau), 5 M potassium acetate (pH 4.8) (Scharlau) and 10% (w/v) PVP (30K) (Sigma) were prepared with nuclease-free water and sterilized at 121^{o}C at 15 psi for 15 min. Ethanol solutions, isopropanol solutions, 10 mg/mL RNase A (Roche) and 1 mg/mL proteinase K (Roche) were diluted with nuclease-free water and filter-sterilized through 0.22 µm filter. All leaf samples were obtained fresh and were ground into a fine powder using liquid nitrogen in the sterile mortar and pestle prior to homogenization with respective protocols' extraction buffers. All centrifugation steps were conducted in a refrigerated microcentrifuge (Hermle Z 32 HK) at 4^{o}C unless temperature was otherwise stated. All extraction buffers were preheated to 65^{o}C in a water bath prior to use to prevent precipitation of contents.

DNA Extraction Protocols

Protocol1: CTAB-method

The protocol was based on the CTAB method of Gawel and Jarret [16] with modifications. Briefly, 100 mg of tissue sample was ground into a fine powder using liquid nitrogen and homogenized in 700 µL of CTAB buffer (0.1 M CTAB, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB and 0.2% β-mercaptoethanol added before use). Six hundred microliters of the homogenate was

transferred to a 1.5 mL tube and incubated at 65 °C for 45 min with inversion every 10 min. Equal volume (600 µL) of 24:1 chloroform:isoamyl alcohol was added and mixed thoroughly. The mixture was centrifuged at 15,000 × g for 2 min at room temperature (RT), and the resulting supernatant (500 µL) was treated with 0.5 µL of 10 mg/mL RNaseA for 15 min at 37ºC in a dry bath (AccuBlock™, Labnet) with constant inversion every 5 min. After RNAse A treatment, chloroform:isoamyl alcohol extraction was repeated. Two volumes (800 µL) of 95% ethanol was added to the recovered supernatant (400 µL) and incubated at − 20 °C for 1 h. The mixture was centrifuged at 15,000 × g for 2 min at 4ºC, and the resulting pellet was washed twice with 75% ethanol. The total recovered DNA pellet was resuspended in 30 µL of nuclease-free water.

Protocol 2: CTAB-method With PVP/

The protocol was based on Hossain et al. [17] with modifications. One hundred milligrams of tissue sample was ground into a fine powder using liquid nitrogen, and 800 µL of CTAB buffer (100 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM EDTA pH 8.0, 2% w/v CTAB, 1% w/v PVP, 0.2% v/v β-mercaptoethanol added before use) was added to the ground tissue and homogenized. The homogenate (700 µL) was transferred to a 1.5 mL tube and was incubated at 65ºC for 15 min with constant inversion every 5 min. Tubes were centrifuged at 15000 × g for 5 min at RT, and the resulting supernatant (600 µL) was transferred into a 1.5 mL tube. Equal volume (600 µL) of 25:24:1 phenol:chloroform:isoamyl alcohol was added to the supernatant and mixed by inversion and vortexing. The mixture was centrifuged at 15000 × g for 5 min at 4ºC and the supernatant was recovered (500 µL) and treated with 0.5 µL of 10 mg/mL RNaseA for 15 min at 37ºC with constant inversion every 5 min. Chloroform:isoamyl alcohol extraction was repeated and 50 µL of 5 M sodium acetate (pH 5.2) was added to the supernatant, followed by subsequent addition of ice cold absolute ethanol (900 µL). Tubes were then incubated at -20ºC for 20 min, and DNA was pelleted through centrifugation at 15000 × g at 4ºC for 5 min. The DNA pellet was recovered through decantation of the absolute ethanol and was further purified through washing twice with 600 µL of 70% ethanol. Finally, the pellet was resuspended in 30 µL of nuclease-free water.

Protocol 3: CTAB-method With 0.3% V/v β-mercaptoethanol

The protocol was based on Healey et al. [14] with minor modifications. One hundred milligram of tissue sample was ground into a fine powder using liquid nitrogen, and 800 µL of CTAB DNA extraction buffer (100 mM Tris-HCl pH 7.5,

25 mM EDTA pH8.0, 1.5 M NaCl, 2% w/v CTAB) supplemented with 0.3% (v/v) β-mercaptoethanol was added. The mixture was further homogenized and was transferred to a 1.5 mL tube. Tubes were incubated at 65^{o}C for 30 min with constant inversion every 10 min. Solid debris were separated from the liquid portion through centrifugation (15000 × g for 5 min at RT). The resulting supernatant (600 µL) was transferred to a new 1.5 mL tube and an equal volume (600 µL) of 24:1 chloroform:isoamyl alcohol was added. The mixture was constantly inverted for 5 min to fully mix the solution and the aqueous phase was separated by centrifugation (15000 × g for 5 min at 4^{o}C). Five hundred microliters of the recovered aqueous phase was transferred to a new 1.5 mL tube and RNA was digested with the addition of 0.5 µL of 10 mg/mL RNAse A. The mixture was incubated at 37^{o}C in a dry bath for 15 min with inversion every 5 min. Chloroform:isoamyl alcohol extraction was repeated, and 400 µL of the resulting supernatant was divided among two 1.5 mL tubes. DNA was precipitated by addition of ½ volume (100 µL) of 5 M NaCl and 3 volumes (600 µL) of ice cold 95% ethanol and incubation at -20^{o}C for 30 min. Tubes were centrifuged at 15000 × g for 5 min at 4^{o}C to pellet the DNA precipitates. Pellets were washed twice with 600 µL of 70% ethanol (centrifuged at 15000 × g for 5 min at 4^{o}C) and were resuspended in 15 µL of nuclease-free water.

Protocol 4: SDS-method

The protocol was based on Ihase et al. [18] with modifications. One hundred milligram of leaf sample was powderized using liquid nitrogen and homogenized in 800 µL of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 5% w/v SDS). The homogenate was transferred into a 1.5 mL tube and was centrifuged at 15000 × g for 5 min at at 4^{o}C. The supernatant (700 µL) was transferred into a 2.0 mL tube and was mixed with 200 µL of 5 M potassium acetate through vortexing. An equal volume (900 µL) of 25:24:1 of phenol:chloroform:isomayl alcohol was added to the mixture, and were altogether mixed thoroughly through vortexing. Mixture was centrifuged at 15000 × g for 5 min at at 4^{o}C. The supernatant (1 mL) was transferred and equally divided between two 1.5 mL tubes and treated with 0.5 µL of 10 mg/mL RNase A for 15 min at 37^{o}C with constant inversion every 5 min. An equal volume (500 µL) of 24:1 chloroform:isoamyl alcohol was added and mixed well by inversion, followed by centrifugation (15000 × g for 5 min at 4^{o}C). The supernatant (400 µL) was transferred to a 1.5 mL tube and was added with 800 µL of absolute ethanol. Tubes were inverted gently and centrifuged at 15000 × g for 5 min at 4^{o}C. The resulting pellet was washed twice with 800 µL of 70% ethanol, air dried and resuspended in 15 µL of nuclease-free water.

Protocol 5: CTAB-method With Triton X-100 And PVP

The protocol was based on Rezadoost et al. [19] with minor modifications. One hundred milligram of leaf sample was powderized using liquid nitrogen and was homogenized with 800 µL of Buffer 1 (200 mM Tris–HCl, 1.4 M NaCl, 0.5% v/v Triton X-100, 3% w/v CTAB). To the homogenate, 0.1% w/v PVP was added and further homogenized. The mixture was transferred to 1.5 mL tube, was vortexed for 20 s and was incubated at 65^{o}C for 30 min. Four hundred microliters of 24:1 chloroform:isoamyl alcohol was added to the mixture, followed by a 2-min inversion of the tubes to mix them thoroughly. Tubes were centrifuged at 15000 × g for 5 min at 4^{o}C. The resulting supernatant (300 µL) was transferred to a 2.0 mL tube, where a half volume (150 µL) of Buffer 2 (50 mM Tris–HCl, 2 M guanidine thiocyanate) with freshly added 0.2% v/v β-mercaptoethanol, 0.2 mg/ml proteinase K and 0.5 µL of 10 mg/mL RNaseA was added and mixed thoroughly by inversion. Mixture was incubated at 40^{o}C for 15 min. After incubation, 2 volumes (600 µL) of 4M NaCl was added and the mixture was placed on ice for 5 min. Two volumes (600 µL) of ice cold isopropanol was added to the mixture and placed on room temperature for 2 min. A pellet was collected by centrifugation at 15000 × g for 5 min at 4^{o}C. The pellet was washed twice with 75% ethanol, air dried and dissolved in 30 µL of nuclease-free water.

Sample Homogenization Through TissueLyserII (Qiagen)

One hundred milligram of cut leaf sample was placed in a 2.0 mL tube together with 2 sterile 7 mm stainless steel beads. Liquid nitrogen was poured and allowed to evaporate until leaf sample was crisp. Frozen samples were ground into fine powder by setting the TissueLyserII (Qiagen) speed at 30 Hz for 1 min. Appropriate amounts of extraction buffer (depending on protocol chosen) were added and samples were further homogenized at 30 Hz for 1 min. Subsequent extraction steps were then followed based on the chosen protocol.

Analysis Of Extracted DNA

DNA (2 µL) was analyzed by agarose gel electrophoresis using 1% (w/v) agarose in 0.5X Tris-Acetate EDTA (TAE) buffer (20 mM Tris base, 10 mM acetic acid, 0.5 mM EDTA). Electrophoresis was performed with 0.5X TAE buffer at a constant voltage of 100 V for 30 min. Gels were stained in a solution of 600X of GelRed™ (Biotium) after the run. The DNA profiles were visualized under ultraviolet (UV) light, and images were acquired with AlphaImager® gel documentation system (ProteinSimple).

The concentration, A260/A280 and A260/A230 ratio were measured with NanoDrop™ 2000c Spectrophotometer (Thermo Scientific) using 2 µL of each sample and with nuclease-free water (Ambion™, Invitrogen) as blank.

Results And Discussion

DNA extraction method comparison

Although abaca is a close relative of banana, their leaves' fibrous nature and higher phenolic content has rendered DNA extraction more difficult. Several methods to isolate DNA from various plant tissues are available, but these methods are time consuming and produce inconsistent yield and quality [20, 21]. Since the first application of CTAB-based extraction method for plants [22], there have been variations depending on the plant species. Although currently published methods on DNA extraction of abaca has been based mostly on the CTAB method of DNA extraction [9–12], several studies on extraction of DNA from the monocot crop palm [18] and RNA from the fiber crop *Cannabis sativa* [23] have shown the effectiveness of using lysis buffers containing SDS. The use of SDS as the main detergent in the lysis buffer of Protocol 4 was also able to extract high molecular weight DNA from abaca leaf samples (Fig. 1). Protocol 4 as compared to the other methods has the shortest processing time due to absence of a 65^{o}C incubation (Table 1). Protocols having CTAB as the main detergent for lysis require an incubation time for at least 15 min at 65^{o}C. This ensures proper lysis of cells and sequestration of proteins and polysaccharides from the DNA [24, 25]. Aside from CTAB, SDS is also an effective detergent in lysing plant tissues [18, 23, 26]. For Protocol 5, Triton X-100 was also added aside from CTAB as lysing reagent which is also an effective detergent for lysing cells [25].

Table 1
Summary of DNA extraction methods used in this study

	Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5
Starting material	100 mg	100 mg	100 mg	100 mg	100 mg
Extraction Buffer	100 mM Tris-HCl pH8.0, 140 mM NaCl, 20 mM EDTA pH 8.0, 2% w/v CTAB, 0.2% v/v β-mercaptoethanol added before use	100 mM Tris-HCl pH8.0, 500 mM NaCl, 20 mM EDTA pH 8.0, 2% w/vCTAB, 1% w/v PVP, 0.2% v/v β-mercaptoethanol added before use	100 mM Tris-HCl pH 7.5, 150 mM NaCl, 25 mM EDTA pH 8.0, 2% w/v CTAB, 0.3% v/v β-mercaptoethanol	100 mM Tris-HCl pH 8.0, 500 mM NaCl, 50 mM EDTA pH 8.0, 5% w/v SDS	Buffer 1: 200 mM Tris–HCl pH 8.0, 1.4 M NaCl, 0.5% v/v Triton X-100, 3% w/v CTAB, 0.1% w/v PVP Buffer 2: 50 mM Tris–HCl, 2 M guanidinethiocyanate, 0.2% (v/v) mercaptoethanol,0.2 mg/ml Proteinase K
Volume of Extraction buffer	700 µL	800 µL	800 µL	800 µL	800 µL
Duration of 65^{o}C incubation	45 min	15 min	30 min	none	30 min
Number of organic extraction step	2	2	2	2	1
Method of precipitation	Absolute ethanol with 1 h incubation at -20^{o}C	3M sodium acetate and absolute ethanol	5M NaCl and absolute ethanol with 30 min incubation at -20^{o}C	Absolute ethanol only	4M NaCl and isopropanol
Number of Centrifugation steps	5	6	6	6	3
Total Duration	3 h	2 h	2 h	1.5 h	2.5 h

Since phenolics have been a major constraint in the extraction of high quality DNA from recalcitrant plants, addition of PVP into CTAB based extraction buffers is done to absorb the phenols released upon grinding and prevent their oxidation [14, 27, 28]. The application of PVP in abaca extraction methods has been explored previously in the study of Boguero et al. [9]. Comparison of the final DNA pellet acquired from the five extraction methods did not show any browning. Comparing protocols with and without addition of PVP did not show any significant difference in the color of the pellets formed. A possible explanation for the lack of phenol oxidation was the source of the abaca leaf samples, the relatively young age of the leaf tissue and the state of health of the plant. Leaf tissue samples were freshly cut from the second to the youngest leaf stalk of the healthy abaca plant prior to start of the DNA extraction process thus phenolic content may be relatively low and oxidation of phenols may not have started yet upon cutting. This highlights the importance of selecting fresh leaf

samples from young healthy plants and its immediate use in DNA extraction experiments to prevent oxidation of phenols [14, 21]. Moreover, the addition of liquid nitrogen allowed rapid disruption of a tissue into powder form at low temperature [29], thus oxidation of available plant phenolic content could have been minimized due to low chemical and biological activity at this temperature. It is a common practice in plant DNA extraction that cell wall disruption to release nuclei and organelles are done in the presence of liquid nitrogen to prevent increased exposure time to air and warm temperature before the ground tissue is completely mixed with the extraction buffer [21].

RNase A treatment during the second chloroform:isoamyl alcohol extraction was adapted from the protocol of Healey et al. [14]. RNase A treatment has traditionally been done after DNA has been precipitated, washed and dissolved in the appropriate buffer [14]. This results in an additional precipitation step to remove the enzyme and is likely to cause overall decrease in DNA yield [13, 14]. Applying this enzymatic step before the second chloroform:isoamyl alcohol extraction step in the other methods proved to be effective in the removal of RNA (Figs. 1 to 3) and eliminates the need for further treatments once the DNA pellet has been resuspended in the appropriate buffer.

The general method of precipitating DNA in most plant DNA extraction methods is through precipitation with alcohols either with ethanol or isopropanol. However, polysaccharides are known to co-precipitate with DNA in the presence of ethanol or isopropanol [14, 28]. Addition of a high salt buffer increases the solubility of polysaccharides in ethanol thereby these polysaccharides can easily be removed from the supernatant once DNA has been precipitated [14, 30].

DNA Quality And Quantity Assessment

Analysis of the A260/A280 values of the five methods have shown almost comparable ratios which are all within the acceptable rage of 1.80 to 2.10 (Table 2). Although the low A260/A230 values show that the methods tested generally have high absorbance at the 230 nm range or less this may be a result of carryover contamination from polysaccharides which are common in plant samples [31–33], from organic solvents such as phenol and β-mercaptoethanol [31], presence of chaotropic agents used for lysis such as guanidine thiocyanate and guanidine hydrochloride [32, 33] and detergents such as CTAB, SDS and Triton X-100 [32]. The presence of protein contamination also causes a shift in the absorbance curve thus overall affecting both A260/A280 and A260/A230 ratios [33]. Protocols 2 to 5 have significantly lower A260/A230 ratio versus Protocol 1 possibly due to higher amounts of one of these organic solvents (Table 1). Protocols 2 and 4 both use phenol extraction which may have carried over to the final DNA pellet. Moreover, Protocol 4 uses the highest concentration of SDS (5% w/v) which may also be a contributing factor. Protocol 5 has adapted 3% w/v of CTAB, Triton X-100 and guanidine thiocyanate which may have carried over to the DNA pellet. Protocol 3 utilized a higher β-mercaptoethanol concentration (0.3% v/v) which may have also contributed to the higher absorbance at 230 nm [31].

Table 2
Summary of abaca genomic DNA extractions using five protocols

	DNA concentration (ng/µL)	A260/A280	A260/A230	Total DNA yield (ng/mg of sample)
Protocol 1	436.50 ± 132.90[a]	2.02 ± 0.06	1.94 ± 0.07	130.95 ± 39.87
Protocol 2	145.33 ± 48.28[a]	1.98 ± 0.03	1.52 ± 0.16	43.60 ± 14.48
Protocol 3	210.72 ± 24.85[a]	2.03 ± 0.03	1.65 ± 0.06	105.36 ± 12.43
Protocol 4	943.44 ± 297.12[a]	1.92 ± 0.03	1.40 ± 0.15	471.72 ± 148.56
Protocol 5	1891.00 ± 231.51[a]	1.81 ± 0.04	0.85 ± 0.07	567.30 ± 69.45

[a] values represent average of four independent DNA extraction experiments

In terms of DNA yield, Protocols 4 and 5 resulted to the highest DNA yield per mg of sample processed, while Protocol 2 resulted to the lowest DNA yield (Table 2). Since Protocol 5 has the least centrifugation and handling steps, it may have minimized DNA loss. The DNA yield between Protocols 1 and 3, on the other hand, are not significantly different (Table 2). Among the other protocols, the A260/A280 and A260/A230 of Protocols 1 and 3 are much more within the acceptable range. Hence, either one of these two protocols can be used for extracting high molecular weight DNA from fresh abaca leaves for NGS applications. The applicability of other protocols for routine analysis of abaca DNA such as for PCR can also be considered when purity is not as crucial as those for NGS applications. Carryover contamination from buffer components such as guanidine thiocyanate, Triton X-100, EDTA and phenol often do not negatively affect downstream applications [32]. Protocol 4 has one of the highest DNA yield and fastest processing time of less than two hours thus DNA acquired from this method may be used for routine PCR applications.

Homogenization With TissueLyserII

Since improving abaca germplasm requires application of genotyping-by-sequencing (GBS) on massive numbers of abaca varieties, a homogenization step that is amenable for high-throughput setup is required. The use of mortar and pestle will be laborious due to constant washing and repeated sterilization to prevent cross-contamination of DNA from different abaca varieties. The use of TissueLyserII will allow for high-throughput DNA extraction [34]. Because DNA extracted via Protocol 1 and Protocol 3 showed successful extraction of high molecular weight DNA and possess acceptable A260/A280 and A260/A230 values for GBS applications (Fig. 1; Table 2), these protocols were subsequently compared using TissueLyserII as the homogenization method instead of manual grinding using mortar and pestle. Moreover, Protocol 4 also resulted to DNA having an acceptable yield and A260/A280 ratios, with an additional advantage of having the shortest processing time. This protocol, hence, was also included in this set-up.

Electrophoresis of extracted DNA from mature abaca leaf samples showed presence of high molecular weight DNA with slight smearing (Fig. 2). Analysis of DNA quality through UV absorption values A260/A230 (Table 3) showed better ratios compared to those in Table 2. This may be caused by less efficient grinding of leaf samples compared to mechanical grinding with a mortar and pestle. Hence, less proteins, phenols and polysaccharides were released when leaf samples were lysed with TissueLyserII. Based on the DNA yield and DNA purity, the three protocols can be used for routine extraction of high molecular weight DNA from abaca leaf samples. Owing to the shorter processing time of Protocol 4 due to the absence of 65^{o}C incubation and – 20^{o}C incubation, this may be an appropriate protocol for rapid and time-efficient extraction of large number of abaca varieties for subsequent GBS experiments.

Table 3
Comparison of genomic DNA extracts using TissueLyserII

	Replicate	DNA concentration (ng/µL)	A260/A280	A260/A230	Total DNA (ng/mg of sample)
Protocol 1	A	149.57	2.02	1.80	44.87
	B	104.63	1.96	1.81	31.39
Protocol 3	A	136.83	2.04	1.96	41.05
	B	146.17	2.04	2.00	43.85
Protocol 4	A	198.87	1.81	2.10	59.66
	B	174.77	1.87	1.93	52.43

Among the five protocols tested, Protocol 3 has the least variation when it comes to DNA yield and DNA purity (Table 2; Table 3). Protocol 3 was subsequently used for extracting high molecular weight abaca DNA for NGS applications. Although the use of TissueLyserII simplifies the homogenization process, the DNA yield acquired with this homogenization

method is not sufficient for NGS applications; hence, for NGS based applications requiring microgram amounts of DNA of very few abaca varieties, mechanical grinding with mortar and pestle is still the method of choice.

NGS Library Submission And Quality Check

Total DNA extracted from two abaca varieties (Abuab and Ihalas) were sent for sequencing and subjected to Macrogen quality check prior to library preparation. Initial electrophoresis showed presence of high molecular weight DNA that passes the quality standards for TruSeq DNA Nano (Illumina) Library platforms (Fig. 3). Moreover, A260/A280 values were within the range of standard set by Macrogen which was around 1.80–2.10 (Fig. 3). Fragment analysis of the extracted DNA for the two varieties showed acceptable fragment lengths (Fig. 4). Library was therefore prepared and sequenced using the Illumina sequencing technology. Quality control of the reads obtained demonstrated that the DNA extracted could be used for NGS purposes (Fig. 5). FastQC version 0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used for quality assessment after standard trimming and quality control using Trimmomatic [35], the data indicate good PHRED quality scores with uniform high quality base calls for both forward and reverse sequenced reads (Fig. 5). This provides confidence in downstream sequence analysis.

Conclusion

Comparison of the five methods showed that the CTAB with 0.3% β-mercaptoethanol method (Protocol 3) of extraction is applicable for extracting high molecular weight DNA from abaca intended for NGS applications. The use of additives such as PVP is not necessary to acquire inhibitor-free DNA from fresh abaca leaf tissues. The SDS-method has proven to have the fastest processing time and combining this extraction method with TissueLyserII mode of homogenization results in high molecular weight DNA that will be useful for high-throughput extraction of abaca DNA for genotyping-by-sequencing applications. The optimized CTAB with 0.3% β-mercaptoethanol method may also be applied to other fiber crops for which high quality DNA have not yet been acquired.

Abbreviations

CTAB
cetyltrimethylammonium bromide
PVP
polyvinylpyrrolidone
SDS
sodium dodecyl sulfate
RNase A
Ribonuclease A
NGS
next-generation sequencing
GBS
genotyping-by-sequencing

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and material

All data generated or analysed during this study are included in this published article.

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Authors' contributions

RBLK, CFCB, VMA and LCG conceived and designed the study. RBLK and CFCB collected and interpreted data. RBLK isolated genomic DNA and drafted the main manuscript. RBLK, CFCB and LCG analysed the NGS sequences. All authors contributed to editing the manuscript. All authors read and approved the final manuscript.

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Figures

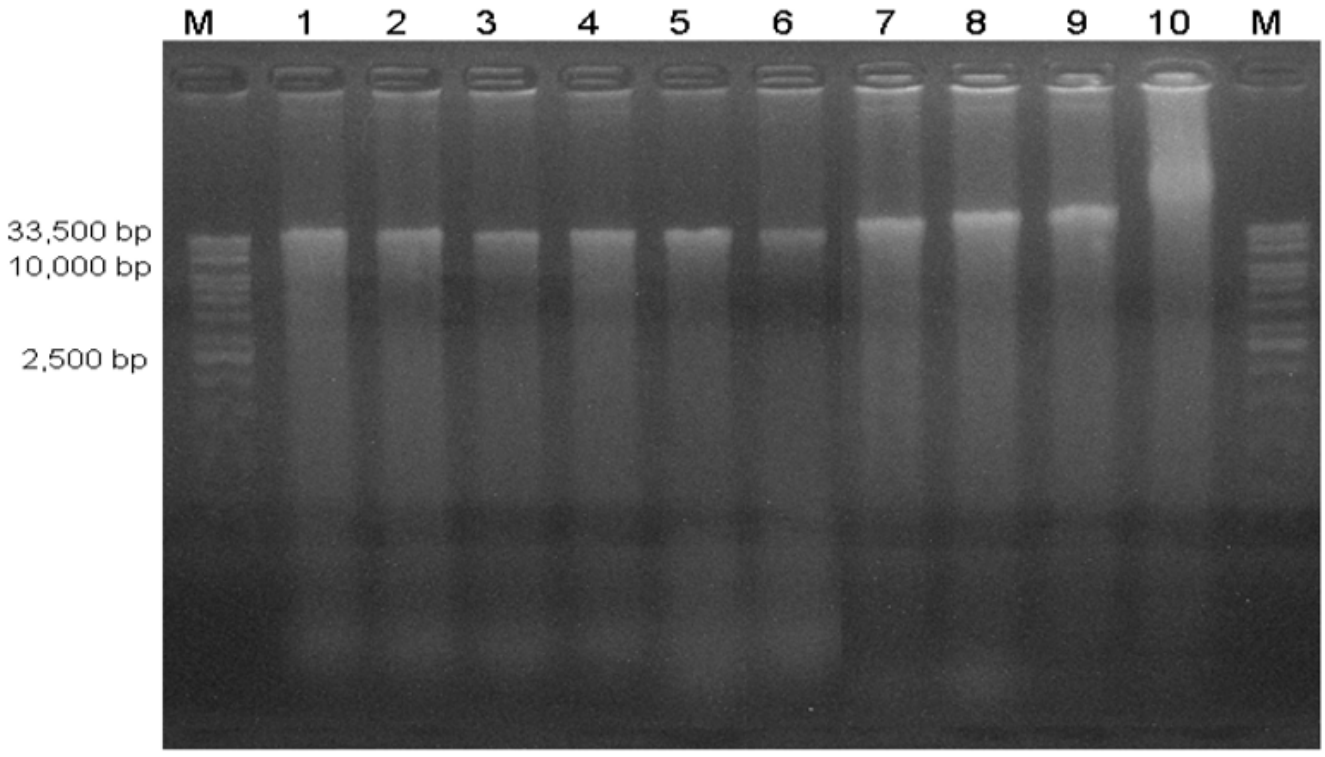

Figure 1

Genomic DNA preparation of abaca resolved by agarose gel electrophoresis. Representative DNA extracted by Protocol 1 (lanes 1 and 2), Protocol 2 (lanes 3 and 4), Protocol 3 (lanes 5 and 6), Protocol 4 (lanes 7 and 8) and Protocol 5 (lanes 9 and 10). Lane M – VC 1kb-Ex ladder (Vivantis).

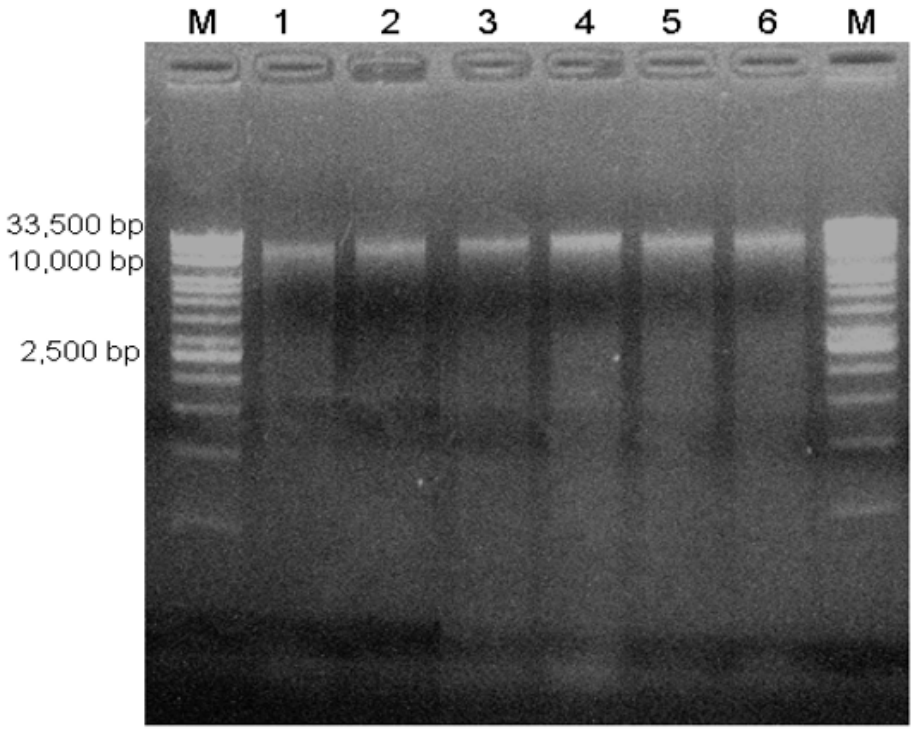

Figure 2

Genomic DNA preparation using TissueLyserII resolved by agarose gel electrophoresis. Lanes 1 to 2 - DNA extracted by Protocol 1 replicates A to B. Lanes 3 to 4 – DNA extracted by Protocol 3 replicates A to B. Lanes 5 to 6 – DNA extracted by Protocol 4 replicates A to B. Lane M – VC 1kb-Ex ladder (Vivantis).

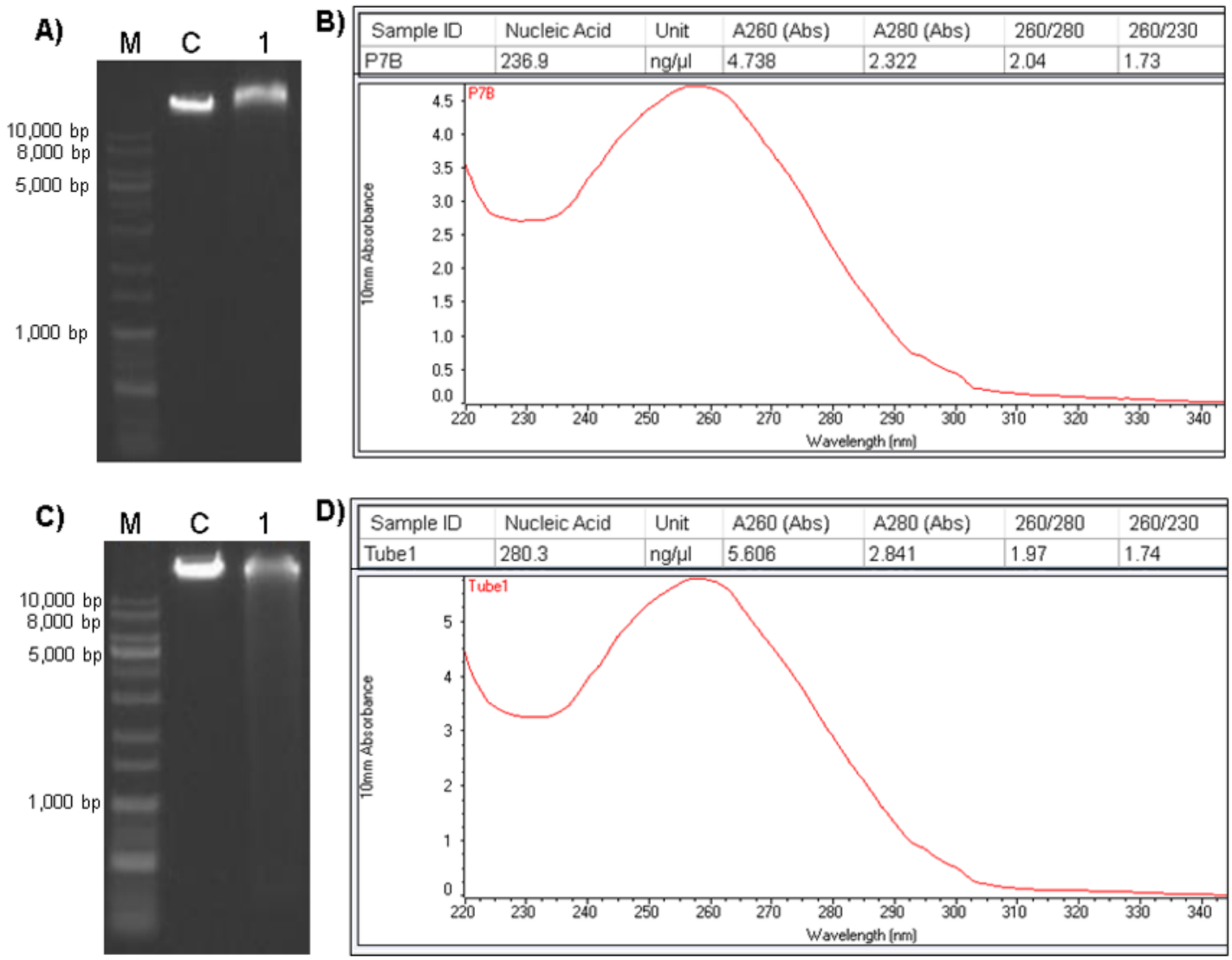

Figure 3

Yield and quality assessment of DNA extracted via the CTAB-method with 0.3% β-mercaptoethanol. A) Genomic DNA preparation for Musa textilis var. Abuab (lane 1). B) Nanodrop measurement profile of Musa textilis var. Abuab. A) Genomic DNA preparation for Musa textilis var. Ihalas (lane 1). B) Nanodrop measurement profile of Musa textilis var. Ihalas. Lane M – 1kb Plus ladder. Lane C - control λ DNA.

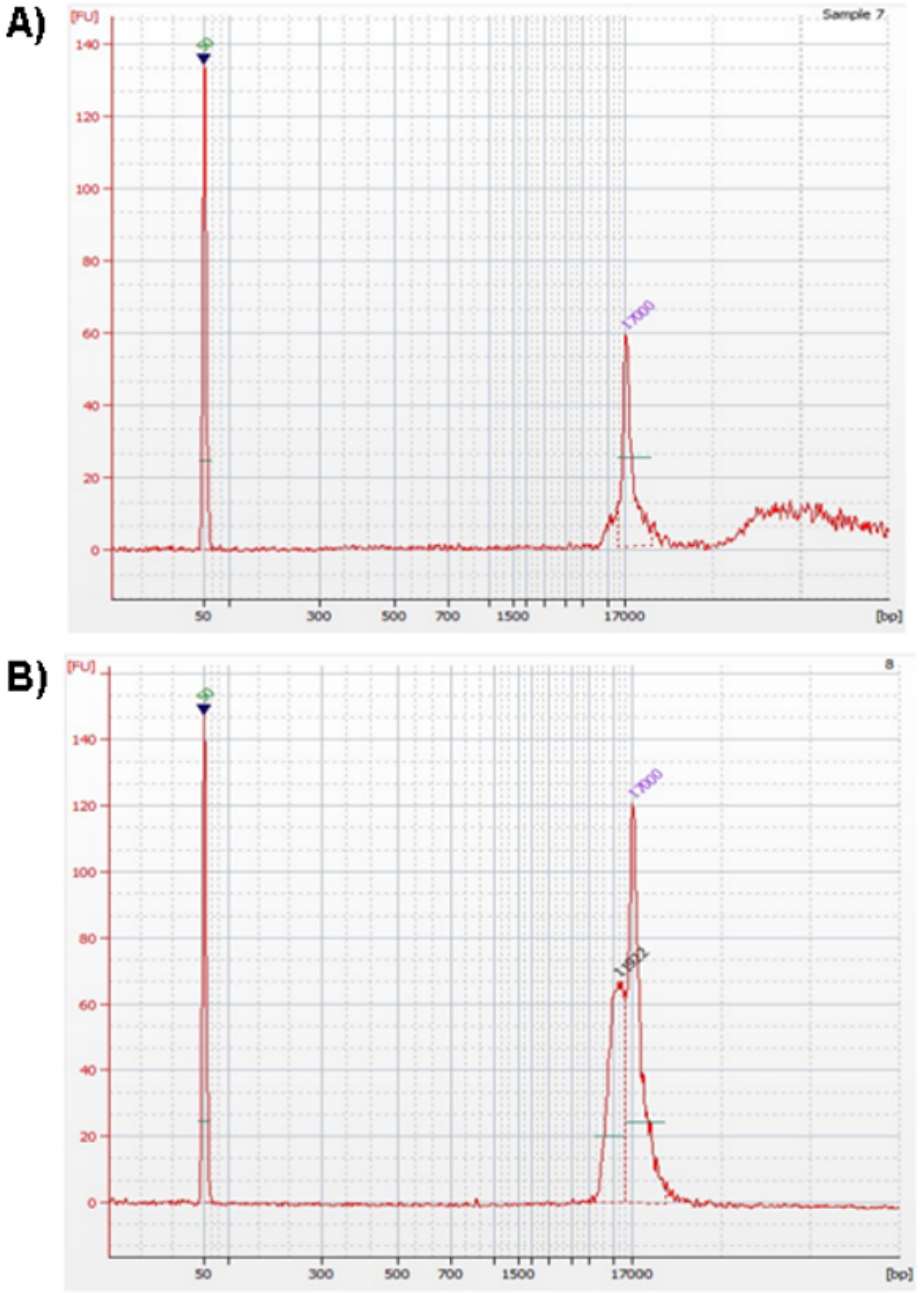

Figure 4

Electropherograms of extracted genomic DNA analysed with Bioanalyzer DNA 12000 chip. DNA extracted from A) Musa textilis var. Abuab and B) Musa textilis var. Ihalas. (Data from Macrogen).

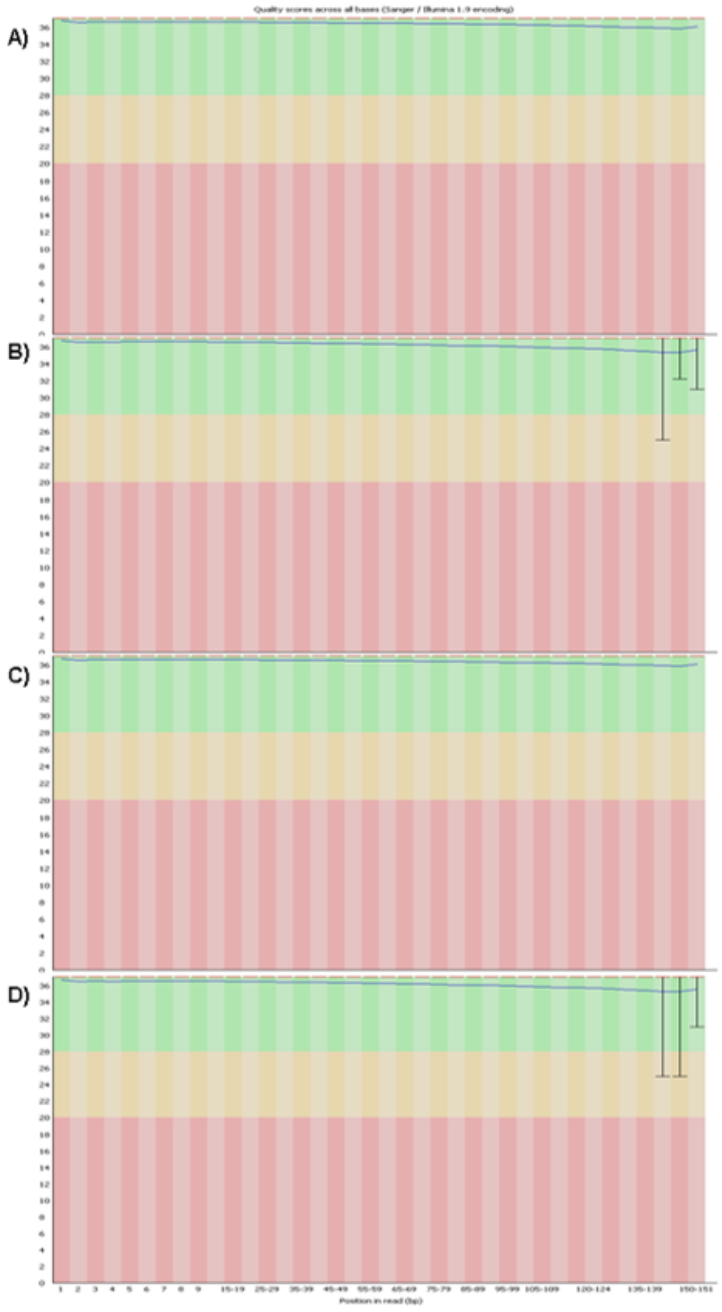

Figure 5

Sequence quality of DNA extracted from abaca varieties using the CTAB with 0.3% β-mercaptoethanol method. Sequence quality assessment for abaca variety Abuab for forward (A) and reverse reads (B) and for abaca variety Ihalas for forward (C) and reverse (D) reads. The DNA sequence libraries were pair-end sequenced (150 bp) on the Illumina 2500 HiSeq Platform. The y-axis shows the PHRED quality scores.