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A productive DNA separation technique for tropical plants

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Due to interfering components such as polysacharrides, polyphenols, etc, DNA isolation from tropical plants had been challenging. We developed a safe, universal and efficient DNA extraction method, which yielded high-quality DNA from 10 tropical plants including cassava, rubber tree, banana, etc. In the extraction buffer, 2 M NaCl was used to provide a high ionic strength reaction environment, ethylenediaminetetraacetic acid (EDTA), lauroyl sarcosine (LSS) and cetyl trimethyl ammonium bromide (CTAB) could inhibit DNase activity effectively, polyvinylpolypyrrolidone (PVPP) produced a deoxidized reaction environment, and borax enhanced the precipitation of interfering compounds. Ordinary reagents like β-mercaptoethanol, chloroform and phenol were unnecessary in this protocol, which made it safe and friendly to use. PCR and EcoR I enzyme restriction digestion results show that the obtained DNA is good enough for downstream analysis. In conclusion, this protocol is expected to be a preferable DNA extraction protocol for tropical plants.

Key words: DNA extraction, tropical plants, cetyl trimethyl ammonium bromide (CTAB).

INTRODUCTION

Preparation of high-quality DNA is a prerequisite for succeeding in subsequent molecular biology research. There are many specialized DNA extraction methods including both solution-based and column-based ones (Tan and Yiap, 2009). However, different DNA extraction methods are often specifically designed for different plant tissues, especially for the scarce and tricky tissues (Hasan et al., 2008; Rogers and Bendich, 1985; Tang et al., 2009). Tropical plants are often rich in cellulose, polysaccharides, polyphenols, proteins and lipids, which complicate the nucleic acid separation and purification (de la Cruz et al., 1997; John, 1992; Porebski et al., 1997; Wang et al., 2008).

A wide variety of DNA extraction techniques have been developed for isolation of DNA from containing high polysaccharide and polyphenol components and applied these methods in Malvaceae plants (John, 1992), strawberry (Porebski et al., 1997), cacti (de la Cruz et al.,

1997), etc. In recent years, methods for relatively high-throughput extraction of high-quality DNA from tropical trees (Mace et al., 2003), tropical grass species (Chandra and Saxena, 2007) and large amount of fresh and herbarium-stored plant samples (Chen et al., 2010; Chiou et al., 2007; Li et al., 2011) were reported. However, these methods have tended to use β-mercaptoethanol, chloroform and/or phenol extractions from detergent containing buffers which are very toxic and so substitutes should be considered wherever possible. One alternative is to incorporate polyvinylpyrrolidone or polyvinylpolypyrrolidone into a detergent containing buffer in place of mercaptoethanol (Chandra and Saxena, 2007; Cubero et al., 1999; John, 1992; Wang et al., 2008). Furthermore, protocols with ethanol precipitations to eliminate chloroform and/or phenol require additional centrifugation steps which can result in decreased yields (Cubero et al., 1999). Although there are several methods

Table 1. Purity and yields of total DNA extracted from leaves of different plants by the DNA^{simp} protocol.

Family	Genus	Plant species	Concentration (µg/ml)	Purity (A260/230)	Purity (A260/280)	Yield (µg/g)
Asteraceae	*Coreopsis*	*Coreopsis tinctoria* Nutt.	362.50 ± 3.28	0.93 ± 0.02	1.28 ± 0.00	319.85 ± 2.89
Arecaceae	*Roystonea*	*Roystonea regia* Kunth	512.50 ± 97.77	1.72 ± 0.12	1.84 ± 0.04	384.38 ± 73.33
	Archontophoenix	*Archontophoenix alexandrae* (F.Muell.) H.Wendl. & Drude	313.00 ± 23.57	1.65 ± 0.28	2.12 ± 0.67	260.83 ± 19.65
Caricaceae	*Carica*	*Carica papaya* L.	446.67 ± 138.19	1.73 ± 0.07	1.87 ± 0.10	558.33 ± 172.73
Moraceae	*Ficus*	*Ficus carica* L.	181.83 ± 30.22	1.46 ± 0.44	1.72 ± 0.20	227.29 ± 37.77
Aizoaceae	*Sesuvium*	*Sesuvium portulacastrum* L.	238.50 ± 17.97	0.56 ± 0.02	2.00 ± 0.30	137.60 ± 10.36
Euphorbiaceae	*Manihot*	*Manihot esculenta* Crantz	794.33 ± 18.25	1.83 ± 0.02	1.89 ± 0.04	700.88 ± 16.10
	Hevea	*Hevea brasiliensis* Mull.	608.00 ± 4.36	2.18 ± 0.05	1.97 ± 0.05	456.00 ± 3.27
Solanaceae	*Nicotiana*	*Nicotiana tabacum* L.	742.67 ± 80.65	1.87 ± 0.12	1.87 ± 0.12	655.29 ± 71.16
Musaceae	Musa	*Musa nana* Lour.	237.67 ± 41.86	0.98 ± 0.04	1.99 ± 0.08	254.64 ± 44.85

Values given are the average of three replications.

devised for isolating DNA from tropical plants such as tropical tuber crops (Sharma et al., 2008), banana (Shankar et al., 2011), rubber trees (An and Huang, 2005), coconut (Angeles et al., 2005), etc., the use of these methods to other tropical plants is somehow limited. Thus, a friendly and efficient DNA isolation method for tropical plants has become increasingly necessary, especially when many samples need to be analyzed.

We presented here an optimised cetyl trimethyl ammonium bromide (CTAB) extraction protocol (named 'DNA^{simp,} hereafter) for tropical plants. The method is similar to those of Cubero et al. (1999) and Wang et al. (2008), but it has been optimized for tropical plants by the increase of CTAB reagents, and the inclusion of polyvinylpolypy-rrolidone, sodium lauroyl sarcosine and disodium tetraborate decahydrate to provide a strong reducing environment and stimulate cell lysis. This protocol is universally applicable to a variety of common tropical plants regardless of the complexity of their biology. The ground tropical plant tissue samples were first dissolved in our

newly developed DNA lysing buffer. After the cell lysis stage, the pure DNA was extracted following several simple steps with centrifugation and precipitation. No chloroform, phenol or other toxic chemicals were used, which makes the protocol safe and friendly to use. The quality of DNA was further determined by the subsequent common molecular analysis including PCR and enzyme digestion. The results show that it is a friendly and efficient protocol for DNA extraction from common tropical plants.

MATERIALS AND METHODS

Plant materials

Some common tropical plant species in Hainan Island (China) were used in order to test the usefulness of the protocol (Table 1). These were of both herbaceous plants and woody plants in tropical areas. Fresh samples of young leaves, mature leaves and old leaves were collected from outdoor fields in Chinese Academy of Tropical Agricultural Sciences, Haikou. The collected leaves from one plant were mixed into one sample. All the samples were frozen

immediately and subsequently ground into fine powders in liquid nitrogen using sterilized mortars and pestles. Approximately, 100 mg tissue powders were used to isolate total DNA. The resulting tissue powders were stored at -80°C until use.

Reagents and chemicals

DNA^{simp} lysing buffer: 2% (w/v) CTAB (Amresco, USA), 200 mM Tris (Beijing Solarbio S&T Co., Ltd, China), 2 M NaCl (Guangdong Guanghua Chemical Factory Co., Ltd, China), 2% (w/v) polyvinylpolypyrrolidone (PVPP) (Beijing Solarbio S&T Co., Ltd, China), 25 mM disodium salt of ethylenediaminetetraacetic acid(Na$_2$EDTA) (Beijing Solarbio S&T Co., Ltd, China); 1% (w/v) sodium lauroyl sarcosine (LSS) (Beijing Solarbio S&T Co., Ltd, China); 20 mM disodium tetraborate decahydrate (borax) (Guangzhou Chemical Reagent Factory, China); Total pH 8.0. Phenol: chroloform: isoamyl alcohol (Guangzhou Chemical Reagent Factory, China), 25:24:1; chroloform: isoamyl alcohol (Guangzhou Chemical Reagent Factory, China), 24:1; isopropyl alcohol (Guangzhou Chemical Reagent Factory, China); 75 and 100% ethanol (Guangdong Guanghua Chemical Factory Co., Ltd, China); TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and RNase A (Takara, Dalian, China) were all obtained for the study .

Table 2. Reagents used in both DNA*simp* and standard CTAB lysing buffer.

Reagent	CTAB	DNA*simp*
NaCl	1.4 M	2 M
EDTA	20 mM	25 mM
Tris	100 mM	200 mM
CTAB	2%	2%
β-Mercaptoethanol	0.2%	--
PVPP	--	2%
LSS	--	1%
Borax	--	20 mM

DNA isolation protocol

1. Preheating the lysing buffer to 65°C.
2. Grinding fresh plant material with liquid nitrogen.
3. Adding ~100 mg tissue powders, 1.0 ml lysing buffer in 2.0 ml Eppendorf tube.
4. Incubating at 65°C for 30 min. Mixed several times.
5. Centrifuge for 10 min at 14,000 ×g and 4°C. For DNA*simp* protocol, skip to step 8. For DNA*simp* protocol with chloroform used, skip to step 7.
6. This step is only limited to DNA*simp* protocol using both chloroform and phenol. Transfer 800 μl of supernatant to a new Eppendorf tube; add 800 μl of phenol : chroloform : isoamyl alcohol (25:24:1) and mix. Centrifuge for 5 min at 14,000 ×g at 4°C. Then, go to step 7.
7. Transfer 600 μl of supernatant to a new Eppendorf tube; add 600 μl of chroloform : isoamyl alcohol (24:1) and mix. Then, centrifuge for 5 min at 14,000 ×g at 4°C.
8. Removing 500 μl of the supernatant and adding 500 μl of isopropyl alcohol. Mixing the solution and store it at -20°C for at least 15 min.
9. Centrifuging for 5 min at 14,000 ×g and 4°C and removing the supernatant.
10. Washing the pellet, adding 1 ml 75% ethanol (4°C) and centrifuging for 2 min at 14,000 ×g at 4°C. Then the supernatant is removed.
11. Washing of the pellet, adding of 1 ml 100% ethanol and centrifuged for 2 min at 14,000 ×g at 4°C. Then the supernatant is removed and the pellet was dried at room temperature.
12. Dissolving the pellet with 50 μl TE buffer containing 10 mg/ml of RNase. Stored at -20°C.

Technical hints

CTAB, LSS and PVPP cannot be dissolved together in DNA*simp* reagent. For CTAB and LSS, they can be dissolved when the reagent is heated at 65°C in water and stirred. However, PVPP is insoluble. When PVPP is added to the reagent, the reagent needs to be evenly mixed before use (Wang et al., 2008). Another solution is to add 2% (w/w) PVPP to the ground tropical plant tissue samples before using the DNA*simp* reagent without PVPP.

DNA quality analysis

The quality, purity and quantity of the extracted DNA were assessed by PGENERAL T6 Spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., China). The A260/280 and A260/230 absor-

bance ratios were used to determine the contamination of protein and polyphenolic/polysaccharide compounds, respectively. DNA extracted from all samples were dissolved in 50 μl TE buffer respectively. To evaluate the integrity and compare the productivity of the DNA, gel electrophoresis was carried out by loading 10 μl DNA solution on 1% agarose gel (Invitrogen, California, USA), stained with GoldView (Beijing SBS Genetech Co., Ltd., China), and visualized under UV light with UVP BioImaging system (EC3 system, USA).

PCR and restriction digestion

To measure the quality of extracted DNA for downstream analysis, PCR and restriction digestion were done. PCR was performed in a 50 μl volume, consisting of: 25 μl 2× taq PCR Master Mix (TransGene), 1 μl 10 pm/μl of each primer, 5 μl 10 ng/μl genomic DNA and 18 μl sterilized H_2O. Primers for Rubisco large subunit gene (rbcL) were set as rbcL-F (5′-AATCTTCTACTGGTAC-ATGGAC-3′) and rbcL-R (5′-TCATCATCTTTGGTAAAATCAAG-3′) (Angeles et al., 2005). The expected amplified PCR products for rbcL genes were ~433 bp. The amplification program consisted of one initial denaturation at 94°C for 5 min followed by 40 cycles of 30 s at 94°C for denaturation, 40 s at 54°C for primer annealing, 60 s at 72°C for extension and DNA synthesis and final extension at 72°C for 7 min. Digestion of restriction enzymes were set to a total volume of 20 μl (4 h at 37°C) containing: 1 μg DNA, 2 μl 10×H buffer (TakaRa, Dalian, China), and 1 μl of *Eco*R I (15 unit/μl) (TakaRa, Dalian, China).

RESULTS

Comparison of DNA extraction efficiency with different methods

The reagents for DNA*simp* lysing buffer are listed in Table 2. To compare the DNA isolation efficiency of DNA*simp* with the traditional CTAB protocol, we chose two tropical plants namely *Maninot esculenta* (cassava) and *Hevea brasiliensis* (rubber tree) as materials to evaluate the DNA yield and purity. Fresh leaves from cassava and rubber trees were collected with liquid nitrogen and used for total DNA extraction immediately. Four different treatments were done as follows: the traditional CTAB method (Sambrook and Russell, 2001), DNA*simp* (described in 'Materials and methods'), DNA*simp* with chloroform used in precipitation of impurities, and DNA*simp* with both chloroform and phenol used in precipitation of impurities.

Total DNA from cassava and rubber tree leaves were obtained by both DNA*simp* and CTAB protocols. Although each of the obtained DNA was dissolved in 50 μl sterilized H_2O, the 10 μl volume-equal gel electrophoresis results show obvious different concentrations of DNA from each treatment (Figure 1). Table 3 lists the purity and yield of total DNA. Samples treated with DNA*simp* resulted in higher DNA concentration than the others, which was extraordinarily obvious in rubber trees (~1.7 times higher). DNA purity and yield determined by spectrophotometry measurement showed that DNA produced by the DNA*simp* protocol had a somewhat high purity (A260/280≈1.8) as
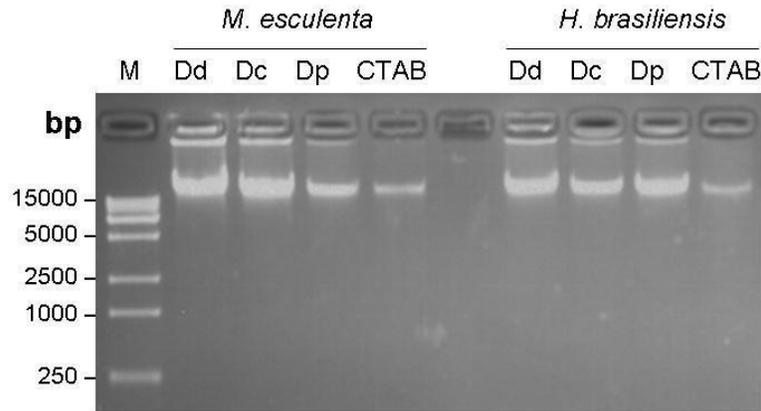
Figure 1. Gel electrophoresis of total DNA extracted by different protocols from leaves of cassava and rubber tree. M, DNA marker (Trans15k, Trans); Dd, DNA^{simp}; Dc, DNA^{simp} with chloroform used; Dp, DNA^{simp} with phenol and chloroform used. CTAB, CTAB method.

Table 3. Purity and yields of total DNA extracted from leaves of cassava and rubber tree by different protocols.

Group[a]	Concentration (µg/ml)	Purity (A260/230)	Purity (A260/280)	Yield (µg/g)
Dd-C	1015.50 ± 12.62	1.23 ± 0.01	1.83 ± 0.10	725.36 ± 9.01
Dc-C	976.83 ± 48.50	1.35 ± 0.20	1.99 ± 0.01	610.52 ± 30.31
Dp-C	960.33 ± 36.10	1.51 ± 0.04	2.10 ± 0.02	685.95 ± 25.78
CTAB-C	989.67 ± 12.96	1.45 ± 0.08	1.94 ± 0.06	494.83 ± 6.48
Dd-R	912.83 ± 38.15	1.72 ± 0.06	1.82 ± 0.08	570.52 ± 23.84
Dc-R	613.33 ± 36.35	1.63 ± 0.21	1.74 ± 0.14	438.10 ± 25.97
Dp-R	668.83 ± 59.05	1.73 ± 0.04	2.03 ± 0.10	371.57 ± 32.80
CTAB-R	538.67 ± 55.85	1.69 ± 0.05	1.96 ± 0.17	299.26 ± 31.03

Values given are the average of three replications. [a]Dd, DNA^{simp}; Dc, DNA^{simp} with chloroform used; Dp, DNA^{simp} with phenol and chloroform used; CTAB, CTAB method. '-C', Cassava; '-R', rubber tree.

well as a high yield (~1.5-2.0 fold higher than CTAB). Furthermore, the DNA purity of DNA^{simp} was almost the same as those produced by the DNA^{simp} method containing chloroform and phenol (Dc and Dp). In the other hand, both DNA concentration and yield were reduced in groups Dc and Dp (Figure 1 and Table 3), indicating chloroform and phenol precipitation steps normally applied in CTAB method could be skipped when the DNA^{simp} lysing buffer was used for isolating DNA from common plant tissues. In general, our experiments showed that DNA^{simp} lysing buffer had predominant advantages on CTAB method for total DNA extraction: it is more productive and safe.

DNA^{simp} lysing buffer is efficient for DNA extraction in 10 common tropical plants

To further test its universal utility, we applied DNA^{simp} to DNA extraction from leaves of 10 common tropical plants (Table 1). It turned out that DNA^{simp} lysing buffer was successful in the extraction of tropical plant total DNA.

Equal-volume-load gel electrophoresis showed obvious different productivity of each plant material (Figure 2). DNA quality measured by spectrophotometry indicated that DNA purity was good (A260/280≈1.8) (Table 1). The differences list in Table 1 refers to DNA concentration and yields which are in correspondence with the pattern depicted in Figure 2.

Interestingly, we found that samples with higher A260/230 value might lead to higher DNA yields and vice versa. For example, *M. esculenta* produced the highest DNA yield and the value of A260/230 was up to 1.83, whereas, *S. portulacastrum* had the lowest yield and the value of A260/230 was only 0.56 (Table 1). In general, the DNA purity and yields (>> 100 µg/g) could fulfill the needs of most molecular studies.

PCR and restriction digestion analysis

DNA extraction is the very first step in ordinary molecular study and the feasibility of the DNA extraction method needs to be tested by downstream experiments. Here, we
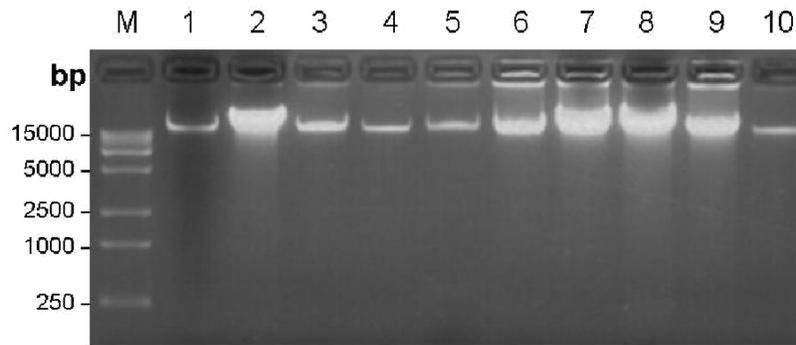
Figure 2. Gel electrophoresis of total DNA extracted from 10 tropical plants by DNA^{simp}. M, DNA marker (Trans15k, Trans); 1, *C. tinctoria*; 2, *R. regia*; 3, *C. papaya*; 4, *F. carica*; 5, *S. portulacastrum*; 6, *M. esculenta*; 7, *H. brasiliensis*; 8, *N. tabacum*; 9, *A. alexandrae*; 10, *M. nana*.
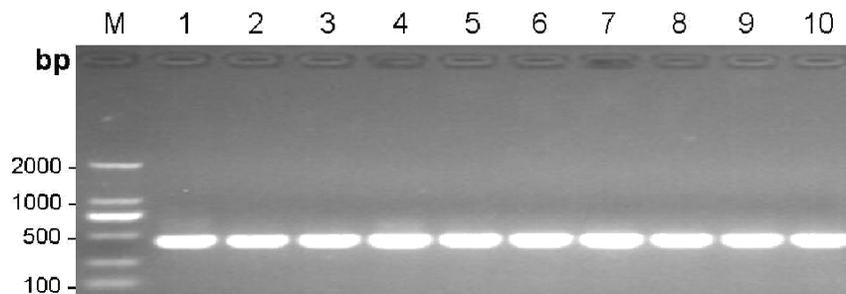

Figure 3. PCR products of rbcL gene using total DNA extracted by DNA^{simp} method from 10 tropical plants. M, DNA marker (DL2000, Takara); 1, *C. tinctoria*; 2, *R. regia*; 3, *C. papaya*; 4, *F. carica*; 5, *S. portulacastrum*; 6, *M. esculenta*; 7, *H. brasiliensis*; 8, *N. tabacum*; 9, *A. alexandrae*; 10, *M. nana*.
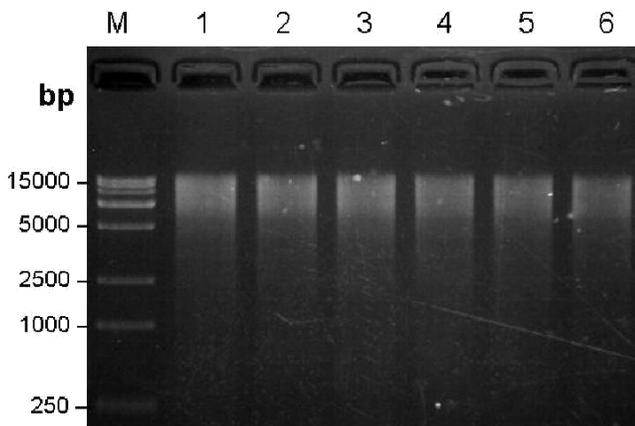

Figure 4. EcoR I restriction of total DNA extracted by DNA^{simp} from tropical plants. M, DNA marker (Trans15k, Trans); 1, *M. nana*; 2, *M. esculenta*; 3, *H. brasiliensis*; 4, *C. papaya*; 5, *R. regia*; 6, *A. alexandrae*.

applied polymerase chain reaction (PCR) and restriction digestion analysis to further test the efficiency of the

DNA^{simp} protocol. We applied a pair of universal primers to amplify the Rubisco large subunit gene (rbcL) from total DNA produced by the DNA^{simp} protocol from the 10 tropical plants mentioned above. Clear and clean target products of rbcL gene (~433 bp in length) were successfully amplified in all these samples (Figure 3).

EcoR I restriction digestion of total DNA is shown in Figure 4. The resulting distribution of digested DNA fragments on agarose gel implies high purity of the extracted DNA, thus indicating fairly high quality of DNA extracted by DNA^{simp} to enzymatic digestion based study (Figure 4). Both PCR and enzyme digestion experiments suggest that our method is unlikely to pose an inhibitory impact on downstream molecular studies.

DISCUSSION

The protocol described here permits the extraction of DNA from many common tropical plants. Up to 10 samples can be treated in an hour with this protocol. However, as proposed by Cubero et al. (1999), larger sample numbers can be processed by dividing the extrac-tion procedure into several steps which can give rise to an

increase in yield. It would permit the extraction of more than 100 samples in a single day with this protocol.

In comparison with traditional CTAB method (Sambrook and Russell, 2001) and other DNA isolation protocols designed for tropical plants (Chandra and Saxena, 2007; Cruz et al., 1995), more NaCl and EDTA were used in DNA^{simp} lysing buffer which resulted in a higher ionic strength to facilitate cell lysis, separation and precipitation. Furthermore, β-mercaptoethanol, a harmful and foul-smelling reagent, was not included in the DNA^{simp} lysing buffer. Instead, PVPP, a safe and easy-to-use reagent (Chandra and Saxena, 2007; Cubero et al., 1999) was introduced to provide a strong reducing environment and prevent polyphenols from oxidation. In addition, 1% LSS, an anionic surfactant, was used to promote plant cell cracking and improve the release of cell contents. Furthermore, the use of 20 mM borax which could bind polysaccharide and polyphenols has made the precipitation of the secondary metabolites and other interfering compounds much easier (Wang et al., 2008).

Combining the aforementioned findings, we conclude that the newly developed DNA^{simp} lysing buffer was efficient for DNA extraction from common tropical plants. Considering that no harmful and dangerous reagents were used in this method, it was a safe and friendly DNA extraction protocol. Furthermore, as reported by Mace et al. (2003), the clarified lysate can be purified on glass fiber plates for increased throughput or alternatively precipitated in 96-well plates following the original protocol which would broaden the range of potential applications of the newly developed DNA^{simp} lysing buffer. In conclusion, the method we introduced in this study might be an attractive approach for DNA extraction from common tropical plants.

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