CTAB Method to Isolate DNA from Silica-Dried Frond Tissues of Several Tree Fern Species from Peninsular Malaysia

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Abstract

Extraction from field specimens of high-quality DNA often involves processing under liquid nitrogen that sometimes is not readily available in some laboratories. In this paper, a method of extracting DNA from silica-dried frond tissues of several tree fern species is described as existing protocols for genomic DNA isolation have their restrictions due to the existence of high content of tacky polysaccharides and polyphenols. Such protocols contaminate the DNA extracted with a viscous matrix, preventing for further use especially PCR performance. Polysaccharides and polyphenols were extracted in our adapted CTAB method before DNA precipitation and isopropanol was used to precipitate the genomic DNA. It was then amplified for testing with the non-coding primer trnL intron and trnL-F intergenic spacer. The highest concentration of DNA was from 0.03 g of frond tissues stored at -20 °C (205.80 ng/µL) for 24 hours, and even the lowest concentration obtained by this procedure was capable of generating enough PCR product for sequencing. The results showed the efficiency and reliability of the modified method as opposed to the unmodified method, suggesting that the combination of silica drying increases the quality and quantity of the DNA extracted.

Keywords DNA isolation, CTAB, Silica-Gel Drying, Genomic DNA, Pteridophytes

Introduction

A comprehensive study of biological activity for the tree ferns has not yet been explored thoroughly. Several scientific assessments in the field have been carried out for this family as it is necessary to allow the observation of field conditions and the assessment of any imminent threats to their habitat, as well as to provide insight into the natural variation of species. However, in the current years, molecular studies on the plants had been used to confirm the traditional findings of the plant species thus collecting the specimen and preserved it in the silica gel has been the most practical and inexpensive method for DNA preservation. The aptitude to sequence DNA has divulged a prodigious insight on where an organism fit in taxonomically and helps identify new species. DNA is now used in conjunction with morphology and ecology to authenticate an organism's individuality in the biological field. The extraction of high-molecular-weight DNA from pteridophytes has been difficult due to high levels of secondary compounds, such as polysaccharides and polyphenols. These groups of compounds inhibit enzymatic downstream responses, such as polymerase chain reaction (PCR) and restrictions enzymes digestion [1, 2]. In general, pteridophytes produce significant quantities of secondary plant metabolites, perhaps as a defense against predation, as a weak gametophytic stage is included in their life cycle and common compounds include triterpene hydrocarbons and cyanogenic glycosides [1]. Superior quality DNA is a must for most applications in phylogenetics thus having a high level of secondary compounds has provided a difficult obstacle for pteridologist to indulge in studying pteridophytes in general [3].

A tree ferns DNA can be extracted from fresh tissues, freeze-dried specimens and dried tissues stored in silica gel [4-7]. Between them, drying preservation using silica gel [4] is the best method in molecular systematic studies, as specimens are collected from diverse geographical areas. Attempts to reduce treacherous chemicals for high-throughput DNA has involved costly kits, facilities, and labor-intensive methods. Further, drawbacks like short shelf life, impurities, reduced recoveries and poor amplification [8] demand for innovative protocols to isolate DNA from dried leaves. With an objective to develop a simple method for isolating DNA, which removes the need to use liquid nitrogen that sometimes is not readily available in some laboratories. The resulting optimized CTAB (Cetyl Trimethyl Ammonium Bromide) protocol allows, cost effective while also producing high-quality genomic DNA that is appropriate for restriction digestion and later amplification process of tree ferns genes[9-11].

Methodology

Sample collections

A sampling was undertaken in several districts in Peninsular Malaysia to search for several species of tree ferns. The specimens were sampled according to accessibility. Parts of the frond were collected and placed in a zip-lock bag containing silica-gel. The fronds were detached using machete and secateurs.

DNA Isolation

Tree ferns DNA was isolated using CTAB method developed by [12, 13] with some modification. Polyvinylpyrrolidone (PVP) was added to the CTAB buffer a day prior

extraction and was dissolved by placing it in a water bath at 65 °C. β-mercaptoethanol was added at this point. Meanwhile, 0.03 g of silica-dried tree fern fronds are grounded using mortar and pestle with an added fine sterilized sand of 62.5–125 µm in size until turned into a fine powder, then transferred into a 2 ml Eppendorf tube before adding 800 µl of warm CTAB buffer. The mixtures were left to incubate for 1 hour at 65 °C but periodic mixing was done by overturning the tubes for every 5-10 minutes. The tubes were then centrifuged at 13000 rpm for 3 minutes and following the centrifugation, the debris should be seen on the bottom. Using a pipette, the supernatant is transferred into a 1.5 ml Eppendorf tube without the colloidal debris. Chloroform and iso-amyl alcohol are added in the respective ration of 24:1 and mixed well to obtain an emulsion. The emulsion step was continued by overturning for a further 1 minute before centrifuged at 13000 rpm for 5 minutes. Now, the constituents are divided into 3 layers and the next phase was done quickly so the layers do not remix. A pipette is used to transfer the aqueous phase (top layer) into a 1.5 ml Eppendorf tube. The chloroform extraction step was repeated but this time 1.5 ml screw-cap tube was used. 2/3 volume of chilled isopropanol was added and mixed well before placing it in the freezer for 1 hour or longer. After that, the tubes were centrifuged at 3000 rpm for 5 minutes until pellet was formed and proceeded to carefully discarded the liquid. 1 ml of wash buffer was added afterwards before it was left to stand for 5 minutes, then centrifuged again at 3000 rpm for 5 minutes. Carefully discarded the liquid and left the pellets to dry. The pellets were resuspended in 90 µl of resuspension buffer. There is worth to note if a pellet does not dissolve, it needs to be placed in a water bath for at least 10 minutes at this stage, undissolved constituents (if present) indicate DNA contamination with polysaccharide or protein. The pellet needs to be centrifuge at 3000 rpm for 1 minute and transfer the supernatant into a new screw cap tube before adding 180 µl of reverse osmosis water, 135 µl of 7.5 ammonium acetate and 1000 µl of ice-cold Ethanol. Leave the tubes in the freezer for 1 hour to precipitate before centrifuged it for 10 minutes at 13000 rpm to pellet and discarded the liquid. Add 700 µl of cold 70 % Ethanol and mixed well. Left the tubes to stand until the pellet detached from the tube before centrifuged it at 13000 rpm for 1 minute and discarded the liquid afterwards. The pellets were then left to airdry before resuspended in 100 µl of TE buffer. The samples were stored in the fridge up to 24 hours to resuspend the pellet and just before running the gel, the tubes need to be gently flicked and pulse down. The samples were stored at -20 °C for future usage [1, 3, 9, 13-17].

DNA Amplification

The tree ferns DNA was then amplified for testing with the non-coding primer *trnL* intron and *trnL-F* intergenic spacer. The primers used for these purposes were trnF (ATTTGAACTGGTGACACGAG) from Taberlet et al. (1991) and FernLr1 (GGCAGCCCCCAGATTCAGGGGAACC) from Li et al. (2011). Standard Polymerase Chain Reaction (PCR) was performed in 50 µl reaction mixtures containing 25 µl of EconoTaq Plus (Sigma-Aldrich, Germany), 1 µl of BSA, 1.75 µl of 10 uM of primer forward and reverse, 18.5 µl of Nano water and 2 µl of 50 to 100 ng/µl template DNA. Templates amplifications were run on a T100 BioRad (USA) thermal cycler with the following protocol: initial denaturation for 5 minutes at 95 °C; followed by 35 cycles of denaturation for 50 seconds at 95 °C, annealing for 50 seconds at 57 °C, and extension for 1 minutes at 72 °C; then continued in a final extension step for 10 minutes at 72 °C[1, 3, 13, 14].

Result and Discussion

The DNA quality of isolated tree ferns were resolute by loading 3 µl samples in 1 % agarose gel and running it at a persistent voltage at 5 V/cm for 1 hour and followed with visualizing and archiving in gel documentation system (Syngene, UK). The suspension containing DNA of the tree ferns was diluted 30 times and underwent quality evaluation by taking A260 and A280 absorbance by using light Spectrophotometer (Quawell, USA; Table 1). Isolated DNA from eleven tree ferns species with one *Salvia* sp. acting as a positive control was then used as templates for PCR and the amplified products were separated on 2 % agarose gels followed by GelRed (Sigma-Aldrich, Germany) staining and visualization under UV light before being documented using a gel documentation system (Syngene, UK) (Figure 1).

Table 1

Quantification of DNA using modified method

No.	Species	Maximum DNA yield (ng/µL)	A260/A280 ratio
1	Cyathea contaminans	112.6	1.98
2	Cyathea borneensis	212.9	1.90
3	Cyathea latebrosa	201.0	1.98
4	Cyathea glabra	190.2	1.99
5	Cyathea obscura	132.6	1.96
6	Blank	-	-
7	Cibotum barometz	121.9	1.99
8	Cyathea recommutata	126.5	1.99
9	Cyathea hymenodes	129.1	1.89
10	Cyathea moluccana	180.9	1.98
11	Cyathea altenans	205.8	1.92
12	Positive Control	161.8	1.97
13	Negative Control	-	-

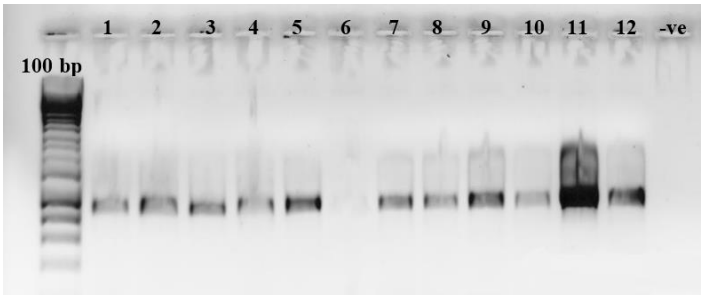

Figure 1 Amplification of *trnL-F* region (344 to 364 bp). The lanes marked as 100 bp contain 100 bp DNA Ladder

The original CTAB protocol from [12, 13] requires significant DNA isolation costs and time from a large number of samples. A modified version of the original CTAB method for isolating and amplifying DNA from silica gel-dried specimens has been identified in the current report. The method is simple, inexpensive, and effective to yield high molecular weight DNA in the range 112.6-205.8 µg for every 30 mg sample. The quality

of DNA from the specimens was revealed based on the absorbance ratio (A260/A280) which extended from 1.89-1.99 that indicate a high-quality DNA. The isolated DNA was effectively used to produce a good profile (Figure 1). In this modified CTAB method, prior to DNA precipitation, the impurities were successfully extracted and these changes greatly reduced the time and use of laboratory materials.

Conclusion

In conclusion, this modified version of the CTAB extraction method using 30 mg specimen materials were excellent conditions to extract the DNA from the tree ferns species in an efficient and low-cost setup. It is hoped that this method will able to reduce the DNA extraction cost in laboratory works for molecular analysis, not only for the tree ferns species but for other pteridophytes that are rich in polysaccharides and polyphenols.

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